The Epidemiology of Norovirus in England: Diagnostics, Incidence and Transmission

By

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Abstract

Existing estimates of the incidence of infectious intestinal disease (IID) caused by norovirus in the community are based on electron microscopy or reverse transcription-polymerase chain reaction (RT-PCR). Neither method accurately represents norovirus disease burden: electron microscopy has poor diagnostic sensitivity, RT-PCR has poor diagnostic specificity.

In this thesis, for the first time, viral load measurements were used to identify cases of norovirus-associated IID, to update the existing, electron microscopy-based estimates of norovirus disease burden in the community in England and to conduct a comprehensive analysis of risk factors for sporadic norovirus-associated IID. The prevalence and characteristics of asymptomatic norovirus infection in England were also described. Data for this work were taken from the Study of Infectious Intestinal Disease faecal specimen archive, which had been subject to semi-quantitative real-time RT-PCR norovirus testing. Finally, routine surveillance data and time-series regression modelling were used to produce an additional and independent estimate of the incidence of general practice consultations for norovirus-associated IID in England and Wales.

Using viral load for norovirus diagnosis, the age-adjusted community incidence of norovirus-associated IID in England was 4.5 per 100 person-years (95% credibility interval: 3.8, 5.2), equating to 2 million episodes per year between 1993 and 1996. Amongst children aged less than five years, the community incidence was 21.4 per 100 person-years (95% credibility interval: 15.9, 27.7) and the incidence of consultations to general practitioners for norovirus-associated IID was 3.2 per 100 person-years (95% credibility interval: 2.6, 3.8), with 100 000 children visiting their GP for norovirus-associated IID each year.

The main risk factor for sporadic, community-acquired norovirus-associated IID was contact with a person with IID symptoms. This result indicates that reduction of person-to-person transmission would substantially decrease the burden of norovirus-associated IID in the community in England, e.g. through good hand hygiene and appropriate cleaning of environmental surfaces.

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AIC	Akaike's information criterion	Kb	Kilobases
AR	Autoregressive	LAMP	Loop-mediated isothermal amplification
AUC	Area under the curve	LRT	Likelihood ratio test
cDNA	Copy DNA	NASBA	Nucleic acid sequence based amplification
Ct	Cycle threshold	NHS	National Health Service
CI	Confidence interval/Credibility interval	OR	Odds ratio
DALY	Disability adjusted life year	ORF	Open reading frame
DNA	Deoxyribonucleic acid	P domain	Protrusion domain (of norovirus capsid protein)
ELISA	Enzyme-linked immunosorbent assay	PAF	Population attributable fraction
EM	Electron microscopy	PCR	Polymerase chain reaction
GP	General Practice	RCGP	Royal College of General Practitioners
HBGA	Human blood group antigen	RdRp	RNA dependent RNA polymerase
HPA	Health Protection Agency	RNA	Ribonucleic acid
IEM	Immune electron microscopy	ROC	Receiver-operating characteristic
IgA	Immunoglobulin A	RT-PCR	Reverse transcriptase polymerase chain reaction
IgG	Immunoglobulin G	S domain	Shell domain (of norovirus capsid protein)
IgM	Immunoglobulin M	SPIEM	Solid phase immune electron microscopy
IID	Infectious intestinal disease	UK	United Kingdom
IQR	Interquartile range	VLP	Virus-like particle

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Chapter 1: Introduction

Norovirus is the most common cause of both sporadic cases and outbreaks of infectious intestinal disease (IID) in high income countries[1-9]. In the past decade, electron microscopy, which was used to first identify norovirus as a pathogen in the 1970s[10], has been replaced by reverse transcription-polymerase chain reaction (RT-PCR) as the method of choice for routine diagnosis of norovirus-associated IID[11-13]. However, the only existing estimates of the incidence of sporadic norovirus-associated IID in the community in England are based on electron microscopy diagnosis[8]. These estimates therefore need to be updated, to reflect the burden of norovirus disease identified by the more sensitive RT-PCR diagnostic methods.

Accurate estimates of norovirus-associated IID incidence in the community are essential for understanding the epidemiology and transmission of norovirus, especially in relation to the introduction of norovirus into hospitals and other healthcare environments, where norovirus outbreaks cause substantial disruption to service provision and can worsen patient prognosis[14-19]. If norovirus vaccines are further developed, beyond the phase I trials already completed[20], estimates of norovirus-associated IID incidence in the community will also be essential for planning and assessing the effects of vaccination, and indeed of any other public health interventions designed to reduce the burden of norovirus infection and disease.

RT-PCR methods have much higher diagnostic sensitivity than electron microscopy: RT-PCR detects many more norovirus infections amongst exposed individuals than electron microscopy[11-13]. However RT-PCR has much poorer diagnostic specificity for norovirus-associated IID than electron microscopy: a substantial proportion of healthy individuals without IID symptoms, who therefore do not have norovirus-associated IID, are positive for norovirus by RT-PCR in population-based surveys[5, 21-24]. If healthy individuals are frequently infected with norovirus and positive by RT-PCR, it is possible that some individuals with IID, who are norovirus RT-PCR positive at the time of their illness, do not actually have disease caused by norovirus; another pathogen, detected or undetected, may be the true underlying cause of their illness. Estimates of norovirus disease burden should only include individuals with disease caused by norovirus.

This thesis therefore focuses on the appropriate interpretation of the diagnostic methods available for norovirus, to facilitate the production of updated, accurate

estimates of norovirus disease burden in England. Specifically, a quantitative approach to diagnosing norovirus-associated IID is developed, to replace the current qualitative classification of norovirus aetiology using RT-PCR. In this new method, norovirus diagnosis will involve consideration of the amount of norovirus being shed by individuals with IID, rather than assigning norovirus aetiology based only on detection of norovirus in clinical specimens. The new estimates of norovirus-associated IID incidence will have important uses in public health action against norovirus infection in England, as described above, but the quantitative methods developed for diagnosing norovirus-associated IID will have wider relevance to the diagnosis of sporadic disease caused by other enteric viruses, respiratory viruses and any other pathogens that are found at notable prevalence in healthy individuals in the general population.

1.1. Aims and objectives

The aims of the research presented in this thesis were to obtain new estimates of the incidence of IID caused by norovirus in England, based on current RT-PCR diagnostic methods, and to examine the transmission of norovirus in the community setting.

Specific objectives of the work were to:

i. Develop a method for interpreting the results of a semi-quantitative RT-PCR assay to diagnose norovirus aetiology in episodes of IID;
ii. Estimate the incidence of norovirus-associated IID in the community and of consultations to general practitioners, through application of this new method for interpreting semi-quantitative RT-PCR diagnostic results;
iii. Estimate the incidence of general practice consultations for norovirus-associated IID in England and Wales, using routine surveillance data on laboratory diagnoses of gastrointestinal pathogens and general practice consultations for IID;
iv. Investigate risk factors for norovirus-associated IID and asymptomatic norovirus infection occurring in the community.

1.2. Thesis outline

This thesis contains eight further chapters in which the background to the work is described, each of the research objectives is addressed and the results and outputs from the work are discussed.

Chapter 2 provides a review of diagnostic methods used for norovirus, current estimates of the incidence of disease caused by norovirus globally and in high income countries, and the current evidence on routes of norovirus transmission. In addition, an overview of other important information about norovirus is presented, including the pathogenesis, symptoms, viral shedding and immune response during human norovirus infection and a description of norovirus virology, genetic diversity and molecular evolution.

Chapter 3 provides a description of the datasets used for the analyses presented in subsequent chapters. The majority of the work is based on the Study of Infectious Intestinal Disease in England. Additional datasets from routine surveillance of gastrointestinal pathogen laboratory diagnoses and of general practice consultations for IID in England and Wales are also described, which are used in Chapter 7.

In Chapter 4, norovirus faecal viral load measurements from individuals with IID and healthy individuals are compared, to develop a quantitative method for accurately diagnosing norovirus-associated IID.

In Chapter 5, cases of norovirus-associated IID are identified using the new quantitative diagnostic method developed in Chapter 4. The characteristics of norovirus-associated IID cases and healthy individuals with norovirus infection are described, the prevalence of mixed pathogen infections in these two groups is presented and the significance of these mixed infections is investigated.

In Chapter 6, risk factors for norovirus-associated IID (defined using the quantitative diagnostic method developed in Chapter 4) and for asymptomatic norovirus infection are investigated, using multivariable logistic regression.

In Chapter 7, time-series adapted Poisson regression is used to estimate the incidence of general practice consultations for norovirus-associated IID in England and Wales, based on routine surveillance data on gastrointestinal pathogen laboratory diagnoses and general practice consultations for IID. This statistical modelling provides a completely independent estimate of general practice consultations caused by norovirus, for comparison to the direct estimate provided in Chapter 8.

In Chapter 8, the quantitative approach to norovirus-associated IID diagnosis is used to generate updated and accurate estimates of the incidence of IID caused by norovirus in the community and leading to general practice consultations in England, based on data from the Study of Infectious Intestinal Disease.

In the final chapter, the results from all of the presented analyses are summarised and discussed, with consideration of the limitations of the work and recommendations for future research and practice in the diagnosis of norovirus-associated IID and estimation of norovirus disease burden.

Chapter 2: Review of methods for norovirus diagnosis, estimates of norovirus disease burden and current evidence on norovirus transmission routes

2.1. The global burden of infectious intestinal disease

Infectious intestinal disease (IID) is a major cause of morbidity and mortality worldwide[25]. In low and middle income countries IID is among the ten most important causes of mortality and disability adjusted life years (DALYs)[25]. The great majority of IID deaths in these countries occur in children aged less than five years, with current estimates of between 1.87 and 2.5 million deaths per year globally in this age group[26, 27]. Whilst the large loss of DALYs is mostly due to the high infant and child mortality attributable to IID[28], there is also evidence that frequent IID episodes in early life can lead to impaired growth and development[27].

In high income countries, IID is not a major cause of mortality or DALYs[29], but still represents a significant disease burden, with incidence estimates ranging from 194 to 800 episodes per 1000 person years in prospective cohort studies[5, 6, 8], and up to 1400 episodes per 1000 person years in retrospective surveys[30-33]. This disease burden is associated with substantial economic costs from healthcare service utilisation and loss of economic productivity[30, 34-36]. In addition to the burden of illness in the general population, particular gastrointestinal pathogens, such as *Listeria* spp., *Clostridium difficile* and norovirus, also have a substantial impact on populations in health and long-term care facilities[14, 16, 37-42].

Population-based studies show that enteric viruses are amongst the most common causes of sporadic IID across all ages in the community in high income countries[5-8, 16], and they are also associated with a significant proportion of more severe IID cases presenting to primary care services[8, 22, 43] and hospital emergency services[44-46]. Rotavirus in particular is associated with severe childhood gastroenteritis and is the leading cause of general practice and hospital visits for IID amongst children aged less than five years in high income countries[43, 47-52]. Astrovirus, Sapovirus and Adenovirus types 40 and 41 cause milder, predominantly paediatric IID[53]. In contrast to these other viruses, humans do not develop long-lasting protective immunity after norovirus infection[53-57], meaning that older children and adults experience repeated symptomatic infections throughout life. Norovirus is therefore the most common viral cause of sporadic community-acquired IID in high income countries, in addition to being the

most frequently reported causative pathogen in IID outbreaks in a range of settings[2, 14, 38, 40].

2.2. Norovirus-associated IID characteristics and immunity

2.2.1. Pathogenesis

Knowledge of the pathogenesis of norovirus infection comes mainly from adult volunteer studies[58-61]. These volunteer studies demonstrated cellular and structural abnormalities in the proximal small intestine (the jejunum) during symptomatic norovirus infection[58-61]. These histological changes were predominantly present in symptomatic volunteers, although a small number of volunteers who remained asymptomatic after inoculation developed similar jejunal lesions[59, 60]. The lesions were present during incubation, acute illness and for a limited number of days after symptom resolution[59, 60], but convalescent biopsies taken at two weeks post-inoculation showed normal histology[58, 61, 62]. Studies of experimentally inoculated gnotobiotic calves and pigs found similar histopathological changes and, importantly, demonstrated localisation of norovirus antigen to the affected areas of the jejunum and duodenum, but with very little norovirus detected in more distal parts of the intestine (ileum and colon)[63, 64]. A more recent study of individuals presenting to hospital with IID, and diagnosed with norovirus infection, indicated that similar damage occurs in the epithelium of the duodenum (which joins the stomach and jejunum), with increased cell apoptosis in the areas of the epithelium covered by these lesions[65]. However, a major disadvantage of studying natural norovirus infection is the lack of documentation of pre-infection intestinal physiology in participants and uncertainty over the microbiological aetiology of the IID.

Whilst delayed gastric emptying, which has been documented in norovirus-inoculated volunteers who develop symptomatic infection, may contribute to the vomiting mechanism in norovirus-associated IID[66], the biochemical pathways leading to diarrhoea and vomiting have not been identified[67]. However, the existing histopathological evidence from the volunteer and animal studies, and the lack of any readily identifiable secretory toxin genes in the norovirus genome[68], indicate that viral replication probably plays a significant role in the pathogenesis of norovirus disease.

2.2.2. Symptom profile

Norovirus-associated IID was first described as 'winter vomiting disease' by Zahorsky, in 1929[69]. When outbreaks of norovirus-associated IID were first investigated in the late

1960s and 1970s[70], the high prevalence of vomiting amongst cases was a key epidemiological characteristic used to attribute outbreaks to norovirus, in the absence of any routinely available microbiological diagnostic tests (the Kaplan criteria)[62]. Subsequently, identification and isolation of norovirus from stool specimens collected during outbreaks facilitated a number of volunteer inoculation studies, which examined the infectiousness, pathogenesis and disease syndrome of norovirus infection[58-60, 71], in addition to many more volunteer studies investigating the immune response to norovirus and the degree of immunological cross-protection between norovirus isolates [56, 72-79].

The volunteer studies, during which otherwise healthy adults were experimentally inoculated with norovirus isolated from outbreak stool specimens, confirmed the symptom profile reported in the early outbreak investigations. Volunteers who became infected and symptomatic after inoculation developed a range of symptoms, predominantly diarrhoea and vomiting, or vomiting without diarrhoea, and, in addition, headache, nausea, muscle ache, fever and chills, abdominal pain, and loss of appetite[58-60, 75, 80, 81]. The incubation period of experimental norovirus infection ranged from 10 to 48 hours and symptoms lasted between 16 and 48 hours[58-60, 75, 79]. A number of the volunteers did not develop diarrhoea or vomiting after inoculation, although some were shown to be infected by the detection of norovirus in their stools and of a norovirus-specific serum antibody response [56, 75, 77-80, 82]..These norovirus-infected volunteers with no diarrhoea or vomiting did report other symptoms, including abdominal pain, nausea and some non-gastrointestinal symptoms such as headache, fever and muscle ache.

The characteristics of natural norovirus infection have been studied in both sporadic community cases, ascertained during prospective research studies, and also in cases identified during norovirus outbreak investigations. The reported prevalence of diarrhoea amongst sporadic community cases is high, at 70% or more, with some indication that diarrhoea might be slightly more common in adult cases compared to child cases[7, 83, 84]. The converse pattern has been reported for vomiting during norovirus-associated IID; whilst the prevalence is high, at 60% or more[7, 83, 84], vomiting is more frequently reported in child cases compared to adult cases[83, 84]. The average symptom duration in adult community cases matches that observed in the volunteer studies (two days) but longer symptom duration has been reported in child community cases (median up to six days) [83, 84]. The prevalence of fever in community cases ranges from 30 to 70%, although differences in the definition of fever and the way in

which information about this symptom is captured in epidemiological questionnaires may account for some of the variability between studies[7, 83-85]. Muscle ache, headache, abdominal pain and nausea have also been reported by community cases. A number of studies have reported disease severity comparable to rotavirus-associated IID amongst children presenting to hospital emergency services with norovirus-associated IID[86-88], with a few reports of more severe manifestations of infection such as encephalopathy, seizures and viraemia[89-91]. Descriptions of symptoms in the elderly come mainly from norovirus outbreaks in hospitals and nursing homes. In the elderly, symptoms tend to last longer than amongst younger adults, with the median duration at 3 days[92, 93], and the prevalence of vomiting is lower. Hospitalisations (amongst nursing home residents) and deaths are occasionally reported from norovirus outbreaks in elderly populations[15-19].

The incubation period of norovirus after natural infection has been estimated in a small number of point-source foodborne outbreaks, with estimates mostly towards the upper range of that reported in the volunteer studies (median incubation approximately 36 hours, ranging from 2 to 61 hours)[85, 94-96]. However, it is possible that the biology of experimental inoculation and foodborne outbreaks may differ from sporadic infection acquired by direct person-to-person transmission, if the size of the inoculum is significantly different[97]; a recent volunteer study has demonstrated that the probability of developing illness after norovirus infection may increase with the size of the infecting dose[98].

Current evidence indicates that norovirus-associated IID is a largely mild and self-limiting illness in otherwise healthy adults in the community, with some slight differences in clinical manifestation in children and the elderly. However, amongst children and the elderly, the duration of symptoms is longer and disease may be more severe, with some serious clinical outcomes reported.

2.2.3. Viral shedding

Faecal norovirus shedding peaks at or just prior to the onset of symptoms in experimentally inoculated volunteers[75, 80, 99]. Peak viral loads of between 10^{10} and 10^{11} viral genomes per gram of stool, measured by quantitative real-time RT-PCR, have been reported in both experimentally inoculated and naturally infected individuals[80, 100-102]. Faecal norovirus load remains at this high level throughout the duration of symptomatic illness and then begins to decrease at, or just after, resolution of symptoms[80, 99, 103], returning to levels seen in asymptomatically infected individuals by

five to 12 days after symptom onset[80] (Figure 2.1). In the one volunteer study that has systematically measured the duration of norovirus shedding detectable by RT-PCR, volunteers shed norovirus for between 7 and 54 days after symptom resolution. Although this volunteer study was not specifically designed to examine the relationship between the inoculum dose and the duration of viral shedding, there was no apparent correlation between them[80].

In studies of norovirus shedding during and after naturally acquired norovirus-associated IID, specimen collection is not as regular or comprehensive as in volunteer studies, with study participants dropping out or sampling finishing before norovirus shedding has stopped. Despite these limitations, the studies of natural norovirus infections indicate that shedding frequently lasts for at least one or two weeks after symptom resolution, in norovirus cases of all ages[84, 104-107], with a tendency for longer shedding in young children (both in the community and those presenting to hospital emergency services) and the elderly[84, 104, 106-108]. One study has provided limited evidence that symptoms may last longer in individuals who have higher viral loads during acute illness[102]. A single study has reported differences in viral load between natural infections with norovirus genogroup I and genogroup II [100], but this is not consistent with other published comparisons of viral load between the genogroups[109, 110], and may be due to differences between the genogroups in the efficiency of amplification in the real-time reverse transcription-polymerase chain reaction assay, rather than a difference in actual underlying faecal norovirus load (Gray, J. personal communication, 2009).

A substantial prevalence of asymptomatic norovirus shedding has been reported amongst otherwise healthy individuals in a number of population-based studies, ranging from 5% to 16%[5, 21-24]. Although one study, conducted in Australia, failed to identify any asymptomatic norovirus infections in a sample of healthy controls, the number of samples tested was substantially smaller than in the other population-based studies that reported asymptomatic norovirus infection prevalence and samples were only tested during two months that are known to be a time of low norovirus activity in Australia[111-113].

Only a few studies have systematically compared viral load in asymptomatic norovirus infections and cases of norovirus-associated IID: one volunteer study and one study of paediatric IID cases with a healthy control group[24, 80]. Whilst the case definition for norovirus-associated IID in the volunteer study was particularly strict and the doses of norovirus ingested by some of the volunteers much higher than might normally be

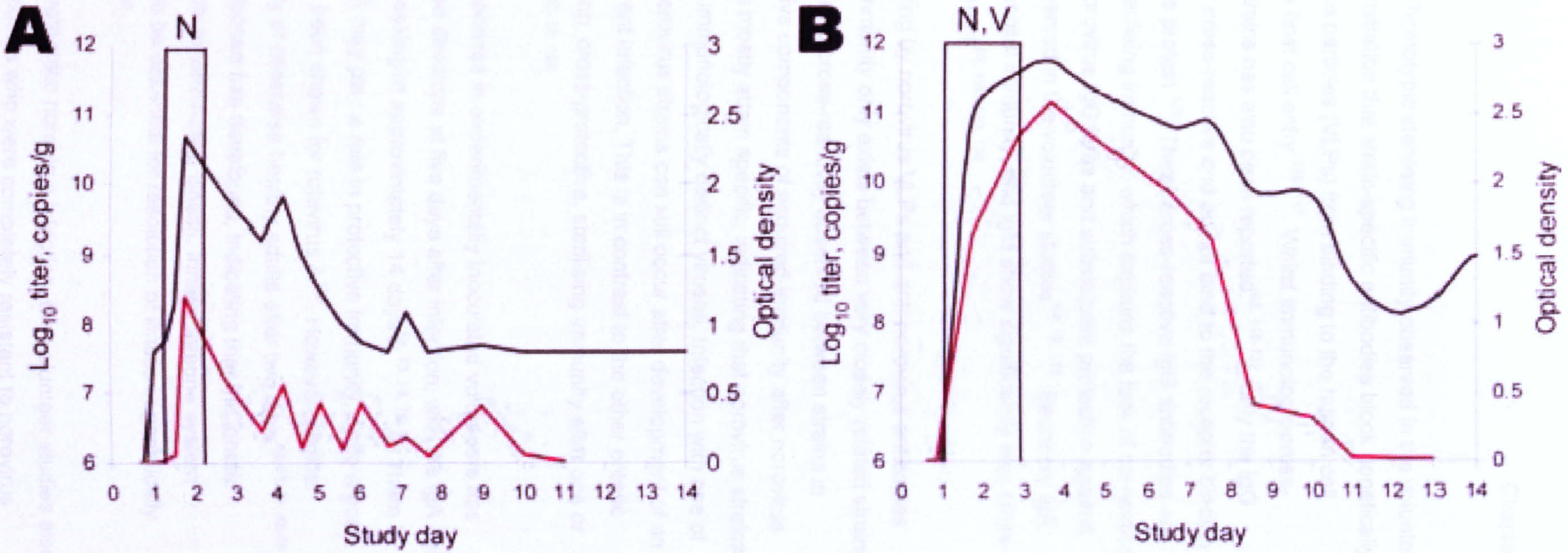
expected to be encountered during natural transmission, the study did demonstrate that volunteers who did not meet the case definition generally shed norovirus at lower concentrations than those with clinical gastroenteritis and for slightly shorter duration (Figure 2.1), although shedding lasted up to four weeks[80]. The study of norovirus infection in paediatric IID cases and healthy age-matched controls also showed that the viral load was significantly higher in children with IID symptoms and norovirus infection; compared to the healthy controls with norovirus infection. Interestingly, the norovirus viral load was much lower in IID cases with a concurrent rotavirus infection compared to those with only norovirus infection[24]. A recent analysis of viral loads detected in food handlers who provided stool specimens during investigations of foodborne norovirus outbreaks, indicated that viral loads may be similar in symptomatic and asymptomatic food handlers[114], although the study compared mean viral loads rather than median viral loads and it did not take account of delays in specimen collection from symptomatic food handlers.

Current evidence indicates that asymptomatic norovirus infection is relatively common in the general population in high income countries and that higher concentrations of norovirus are shed during symptomatic norovirus infection. However, viral shedding in symptomatically infected individuals decreases after symptom resolution and returns to levels found in asymptomatic individuals within a week of symptom onset in most cases.

2.2.4. Immunity and resistance to norovirus infection and disease

Early studies of norovirus outbreaks and volunteer inoculation experiments demonstrated a lack of correlation between the occurrence of clinical symptoms after exposure to norovirus and pre-existing total serum and IgG anti-norovirus antibody levels[54, 72, 115]. The volunteer studies showed that short-term immunity to the same norovirus strain was generated after initial infection and protected against clinical illness. However, volunteers became ill again when re-challenged with the same inoculum two or more years after their initial infection[54, 56]. Subsequent volunteer studies showed that short-term, strain-specific, protective immunity against symptomatic norovirus infection probably only lasts up to six months[77]. Although repeated experimental challenge and infection with the same strain led to longer-lasting protective immunity in inoculated volunteers[77], it is unknown whether the required frequency of homotypic re-exposure occurs naturally in the general population.

Figure 2.1 Changes in viral load during (A) asymptomatic and (B) symptomatic norovirus infection after experimental inoculation with norovirus (strain GI.1), reproduced from Atmar et al 2009[80]. The black line continuing throughout the study period (up to day 14) shows the norovirus viral load (genomes per gram of stool); the red line shows quantity of norovirus antigen measured as the optical density of the specimen in a strain specific antigen ELISA; the black line at the beginning of the study period shows the duration of reported symptoms (N is nausea, V is vomiting); volunteer A experienced no diarrhoea or vomiting (inoculum dose 4800 RT-PCR units), volunteer B reported only vomiting and nausea and no diarrhoea (inoculum dose 48 RT-PCR units).



A potential mechanism for the homotypic sterilising immunity observed in the volunteer studies comes from the demonstration that strain-specific antibodies block genetically homologous norovirus virus-like particles (VLPs) from binding to the human cell receptors involved in norovirus host cell entry [116, 117]. Whilst immunologic cross-reactivity between norovirus strains has also been reported[82, 118-122], only the IgG antibody subclass are strongly cross-reactive and do not bind to the receptor binding domain in the norovirus surface protein [120]. These cross-reactive IgG antibodies are therefore unlikely to provide sterilising immunity, which explains the lack of correlation between existing serum anti-norovirus IgG titres and subsequent protection against symptomatic infection also observed in the volunteer studies[54, 72, 115]. Secretory IgA, which is directly involved in mucosal immunity, and IgM show significantly less cross-reactivity between norovirus strains[82, 120, 123, 124].

In vitro studies of receptor binding by norovirus VLPs and anti-norovirus antibodies indicate that cross-protective immunity only exists between very closely related strains in genogroup I, with no neutralising cross-reactivity observed between strains in genogroup II[125-127]. The protective components of acquired-immunity after norovirus infection therefore appear to be mostly strain specific, indicating that norovirus strains represent a large population of immunologically distinct viruses. Infection with one of the many other co-circulating norovirus strains can still occur after development of an immune response during a current infection. This is in contrast to the other enteric viruses, which induce long-lasting, cross-protective, sterilising immunity after one or two initial infections in early life[53, 55, 128].

Characterisation of immune responses in experimentally inoculated volunteers has shown that an antibody response develops at five days after infection, with the IgA and IgM antibody subcomponents peaking at approximately 14 days[54, 73, 74, 76, 82]. There is some evidence that memory IgA may play a role in protective immunity, or be a good correlate of protection[82], as has been shown for rotavirus A[129]. However, clinical symptoms resolve in the majority of otherwise healthy adults after two days[58-60, 80, 83-85], before the adaptive immune response has developed, indicating that the innate immune response may play a role in terminating illness. Innate immune system components have been shown to be essential for resolution of illness in genetically engineered laboratory animals[130].

In addition to documenting asymptomatic norovirus infection, the volunteer studies also identified a further group of individuals who were completely resistant to norovirus

infection, remaining free of clinical symptoms after repeated inoculation over the course of several years; these resistant individuals did not mount any immune response against the inoculated norovirus strain, indicating a complete lack of infection[54, 56, 72, 73, 75, 77]. The investigation of innate resistance to norovirus infection has focused on the receptors used by norovirus to enter host cells. Initial studies showed that norovirus binds to ligands that are ubiquitous on human cells and subsequent work on the haemagglutination of red blood cells by norovirus VLPs indicated that the ligands may be from the human blood group antigen (HBGA) complex[131, 132]. X-ray crystallography of norovirus VLPs has been used to show that the distal, surface–exposed P2 domain of the norovirus capsid protein binds to synthetic HBGA peptides, providing evidence that these ligands act directly as receptors for norovirus binding and cell entry[133]. The HBGAs are highly polymorphic carbohydrate ligands, expressed on the surface of all human cells, including those in the mucosal lining of the gut, and binding experiments using human tissue biopsies from the intestine have shown that norovirus binds to antigens present at the gut mucosal surface[134].

The combination of HBGA ligands and the variants of each that are expressed by an individual are genetically determined by multiple gene loci. Three groups of HBGAs appear to be important for norovirus binding: the H type antigens, the Lewis antigens and the A and B antigens[135]. The specificity of different norovirus strains for particular combinations of ligands has been extensively investigated using volunteer studies[136-138], outbreak and other epidemiological investigations[139-147], seroepidemiological studies[148], *in vitro* VLP-binding studies[117, 149-153] and animal studies[154]. Each norovirus strain appears to have a unique HBGA binding specificity[150, 154, 155], meaning that the risk of a particular individual becoming ill after exposure to norovirus is dependent on the match between the norovirus strain ligand specificity and the host HBGA phenotype.

Norovirus vaccines, based on nasal or oral administration of VLPs have been shown to be immunogenic and safe in human and animal studies[20, 156-162], but currently there has been no further development of the vaccines for commercial use.

Current evidence indicates that the innate immune system probably plays a major role in the clearance of norovirus infection, but that secretory IgA formed during the adaptive immune response provides protection against the infecting norovirus strain for a limited time after infection (approximately six months). The rapid waning of this strain-specific immunity and the absence of cross-protective immune responses between the large number of norovirus strains are mechanisms by which repeated symptomatic

norovirus infections occur throughout life. There also appears to be a genetic mechanism that determines the occurrence of infection in an individual after exposure to norovirus, based on the match of the norovirus strain receptor specificity and the HBGA phenotype of the exposed individual.

2.3. Norovirus virology

2.3.1. Virus structure and molecular biology

Norovirus is part of the family *Caliciviridae* in which there are four virus genera: norovirus and sapovirus, which infect humans and a few other mammals[163, 164]; lagovirus, which infects rabbits and hares; and vesivirus, which infects a range of marine and terrestrial mammals[68]. The norovirus genome is a positive sense single stranded RNA molecule with three open reading frames (approximately 7.7kb) (Figure 2.2)[68, 163-167]. The first open reading frame (ORF) of the norovirus genome codes for several non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) involved in genome replication; the second ORF codes the single capsid protein; and the third ORF codes for a minor additional structural protein of unknown function, which has been shown to interact with the capsid protein *in vitro*[68, 168, 169]. Different levels of genetic variability can be functionally tolerated across the norovirus genome and the relative genetic conservation or variation in different genome regions has been exploited in molecular diagnosis and molecular epidemiological studies. For example, the junction region between ORF1 and ORF2 is highly conserved and the most widely used primers for polymerase chain reaction diagnostic assays target this region[170].

Norovirus virions are comprised of a single capsid protein with two principle domains: a shell domain (S domain), which forms the main virion capsid and is highly conserved across genera, and a protrusion domain (P domain) involved in binding to host cell receptors for initiation of cell entry[171-176]. The highly variable P2 sub-domain, which is the most exposed part of the capsid protein, is the site of both the key receptor binding sites and the major immunodominant epitopes[133, 173, 177-180] and shows both sequence and structural diversity between norovirus strains[172].

2.3.2. Genetic diversity and molecular typing schemes

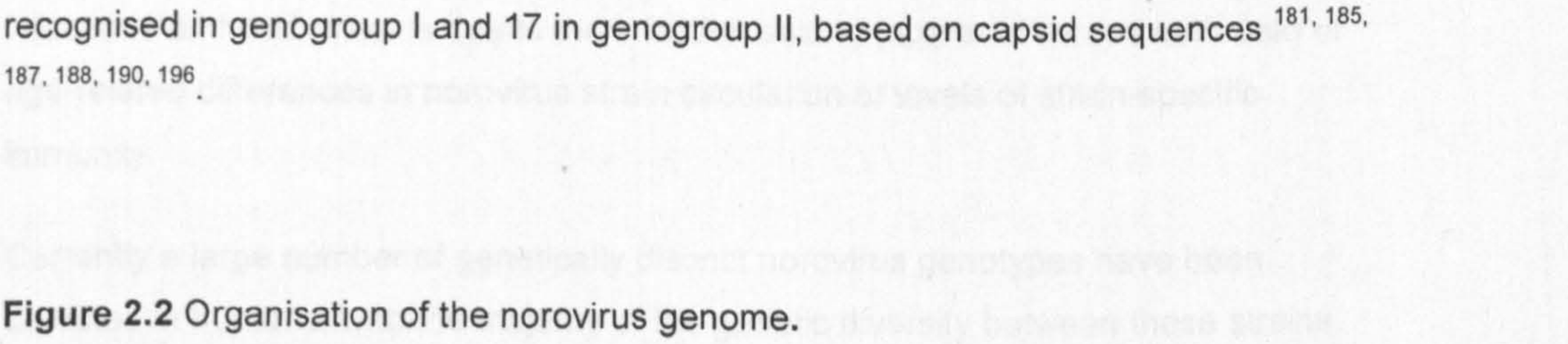
The degree of genetic diversity between human norovirus strains is much higher than for other positive sense single stranded RNA viruses that infect humans[181, 182]. The genetic variability is concentrated in the P domain, probably facilitated by structural flexibility between the S domain and P1 and P2 sub-domains, which allows amino acid

sequence diversity without compromising the functional properties of the capsid protein[172]. However, genetic variation has been detected in other parts of the norovirus genome; early genetic typing was based on the relatively conserved RdRp gene in ORF1 and nucleotide sequence diversity in this region was shown to correlate with putative serotypes defined by solid phase immune electron microscopy[183]. Two genetically distinct groups were identified within the human noroviruses based on RdRp gene diversity, subsequently designated genogroup I and genogroup II[183-186]. Three further genogroups are now recognised: genogroup III contains bovine noroviruses; genogroup IV contains strains that also infect humans; and genogroup V which contains murine noroviruses[181].

A number of distinct genetic clusters, or genotypes, exist within each of the genogroups, and are most extensively characterised for the human genogroups I and II[181, 187]. There have been difficulties in developing a comprehensive typing scheme, because of the high degree of genetic diversity amongst these putative norovirus strains and differences in this variability across the norovirus genome: different genome regions produce different phylogenetic classifications[188-190]. Regions of the RdRp gene or the capsid gene are typically used for phylogenetic analysis, although it is unclear what degree of genetic difference in either region represents an antigenically or serotypically distinct strain[82, 118, 121, 185, 191-195]. Currently there are up to nine genotypes recognised in genogroup I and 17 in genogroup II, based on capsid sequences [181, 185, 187, 188, 190, 196].

Figure 2.2 Organisation of the norovirus genome.

Norovirus genome organisation based on Southampton virus from genogroup I[68]. Genes p48, p22 and ORF 3 have unknown function; Helicase is a 2C-like helicase, VPg is the viral genome linked protein; Pro is a 3C-like protease; RdRp is the RNA dependent RNA polymerase; capsid is the capsid protein.



The majority of molecular epidemiological studies have described the distribution of genotypes detected in norovirus outbreaks or in paediatric cases presenting to health care services. The global predominance of genogroup II genotype 4 (GII.4) noroviruses in outbreaks in healthcare and other institutional settings is widely recognised, but poorly understood, with some researchers suggesting that it may have higher transmissibility in such semi-closed settings[97, 197-204]. In other settings, especially those linked to food and catering, a broader range of genotypes is detected in norovirus outbreaks[205-207]. In Japan, where a large proportion of norovirus outbreaks are linked to consumption of oysters contaminated with human sewage, the strain diversity is particularly high[101, 114, 208].

Strain diversity in sporadic norovirus infections in the community is poorly characterised, because these cases are rarely seen by health care services[83], so specimens are not routinely available for typing. Studies of norovirus infection amongst paediatric general practice patients with norovirus-associated IID in the UK and Canada indicate that the spectrum of strains is very different to that in the outbreaks captured by national surveillance systems in these countries, with a much broader diversity, although genogroup II noroviruses were still at high prevalence[209, 210]. However, the majority of outbreaks reported in the literature involved adults, so it is difficult to determine whether the difference in strain diversity between sporadic infections and outbreaks is due to the infection setting (sporadic versus outbreak) or age-related differences in norovirus strain circulation or levels of strain-specific immunity.

Currently a large number of genetically distinct norovirus genotypes have been detected in humans, with the majority of the genetic diversity between these strains concentrated in the P domain of the capsid protein, which is involved in receptor binding and is the site of the immunodominant epitopes. In spite of this broad range of circulating norovirus genotypes, viruses in the GII.4 genetic cluster predominate in causing norovirus outbreaks, although other genotypes are often reported in foodborne outbreaks. A greater diversity of genotypes is detected in sporadic paediatric cases of norovirus-associated IID, although further detailed characterisation of norovirus molecular epidemiology is required at the community level.

2.3.3. Norovirus evolution and emergence of novel variants

There is now widespread evidence that both genetic recombination and antigenic drift generate genetic diversity in the norovirus genus[177, 179, 211-216]. The highly conserved

sequence at the ORF1-ORF2 junction promotes genetic recombination between different norovirus strains through homologous RNA interaction[211, 214] and recombinant norovirus strains have been widely reported, although no recombinants of genogroup I and genogroup II strains have been detected, probably because of insufficient sequence homology between the genogroups[215]. The recombinant strains commonly possess the RdRp gene (and therefore probably the entire ORF1) of one established genotype and the capsid gene (and therefore probably ORF2 and ORF3) of another genotype[188, 211-213], although recombination within the capsid gene has also been reported, with breakpoints at the junctions between the capsid domains and sub-domains[215]. Whilst the majority of recombinant strains have been detected during retrospective analysis of archived strains, some have spread to sufficient prevalence to be detected in routine molecular epidemiological surveillance[217-220]. However, it is unclear what evolutionary advantage, if any, these recombinant viruses may have, especially because the immunodominant epitopes lie within the P2 subdomain of the capsid, which is transferred completely intact from the parent strain.

In contrast to the characterisation of genetic recombination, which has examined norovirus genotypes in both genogroups, the study of antigenic drift has focused on the most common genotype, GII.4. During the last 15 years there have been periodic global epidemics of norovirus-associated IID, identified by marked increases in the incidence of outbreak and/or sporadic cases above the endemic seasonal incidence[221-223]. Molecular epidemiological studies revealed that the majority of the infections during these epidemics were caused by GII.4 noroviruses and that each of the recent epidemics in 1995, 2002, 2004 and 2006 coincided with the emergence of a genetically novel GII.4 variant that replaced the previously circulating GII.4 viruses[200, 201, 221, 222, 224-234]. Subsequent genetic characterisation of the capsid gene in these sequential GII.4 variants has revealed amino acid substitutions at sites in the S-domain and P1 and P2 sub-domains[177, 179, 216, 235, 236]. The major structural changes are concentrated in the P2 sub-domain, with computer modelling suggesting the occurrence of substantial alterations in tertiary protein structure and electrostatic surface charge, which would affect binding to host antibodies[177, 235, 236], although there is evidence of negative selection acting to preserve specific amino acid residues within the HBGA binding site[180, 236-238]. Retrospective serological studies using paired sera from historical norovirus outbreaks have confirmed the occurrence of the immune-driven selection suggested by these virological studies, demonstrating that these novel GII.4 variants are antibody escape mutants[179, 223]. With GII.4 being the predominant strain detected in cases of norovirus-associated IID, this immune-driven virus evolution, with periodic

emergence of antibody escape mutants, provides a third mechanism by which repeated norovirus infections occur throughout life, in addition to waning immunity and the existence of multiple other immunologically distinct genotypes.

2.4. Diagnostic methods

2.4.1. Electron microscopy

Immune electron microscopy was first used to identify norovirus as a cause of IID in the 1970s[10]; norovirus particles in a stool specimen were allowed to react with antibody in convalescent phase sera from the infected individual, prior to microscopic examination, to enhance the visibility of the virus and to demonstrate that the observed particles were immunogenic and therefore likely to be a pathogen. Direct electron microscopy, using only a staining technique, without coating the virus particles in antibody, was used for routine norovirus diagnosis in the UK until the end of the 1990s[12, 239], because it was the only method that could detect a broad range of norovirus genotypes until reverse transcription-polymerase chain reaction assays became more widely available. The detection limit of electron microscopy is approximately 10^6 virus particles per gram of stool, viral loads which are only present during the acute symptomatic phase of norovirus infection[240-243]. Norovirus is no longer detectable by electron microscopy after symptom resolution, when viral loads decrease substantially[99, 103].

The high detection limit of electron microscopy translates into a high diagnostic specificity[244] for norovirus-associated IID, i.e. the ability to correctly exclude norovirus as a cause of illness in individuals with IID caused by another pathogen. Individuals will only be positive by electron microscopy if they have high viral loads present during norovirus disease; individuals with low viral loads, present in asymptomatic norovirus infection, will be negative by electron microscopy[80]. However, the analytical specificity of electron microscopy (the ability to produce a negative test result for specimens with other pathogens present in the absence of norovirus[244]) may be suboptimal if false positive results are produced because other gastroenteritis viruses are wrongly identified as norovirus during inspection of the specimen[240]. Other practical difficulties in the use of electron microscopy for norovirus diagnosis are the dependence on specimen quality and preparation, which gives low diagnostic sensitivity[13, 244-246], the single specimen throughput and the requirement for highly specialised and experienced technicians [11, 12, 240, 241, 243].

2.4.2. Norovirus detection by nucleic acid amplification

After the full genome sequences of two prototype norovirus strains were determined in the mid 1990s[165, 166, 247], the design of primers for a range of nucleic acid amplification diagnostic tests became possible. Conserved regions of the RNA polymerase gene were targeted in diagnostic assays[170, 186, 248-255], for broad detection of all norovirus genotypes, whereas more variable regions of the capsid gene were used to characterise genetic diversity[190, 198, 201]. All nucleic acid amplification assays require extraction of norovirus RNA from the faecal specimen, to free the RNA from the norovirus virions and to exclude substances that inhibit the nucleic acid amplification reaction[256, 257]. A variety of techniques have been developed, although the commercialised method most commonly used for norovirus RNA extraction employs guanidinium thiocyanate to lyse the norovirus virions and silica particles to bind the viral RNA for separation from the faecal suspension[256-259]. Whilst this has been shown to be the optimal method for extraction of norovirus RNA, the exact efficiency of the extraction step is unknown [260, 261].

2.4.2.1. Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) [262, 263] is the most widely used nucleic acid amplification method for norovirus detection in stool specimens[186, 248-250, 264-267]. In the reverse transcription step, extracted norovirus RNA is converted into a DNA copy (cDNA), which is then subject to multiple rounds of DNA amplification in the PCR step, with cyclic changes in the temperature of the reaction mixture used to initiate the different stages of the DNA replication process. Random primer sequences are commonly used for the reverse transcription step, to ensure that all RNA is converted to DNA[268], but the primers for the PCR are matched to a unique norovirus genomic sequence, to provide specific amplification[110, 248, 249]. Originally, the RT-PCR assays were developed with a separate amplicon identification step, where the DNA products of the PCR were separated on agarose gel, by electrophoresis, and stained to demonstrate the presence of a DNA amplicon of the size expected after amplification of the target sequence in the norovirus genome. The faecal specimen would be classified positive or negative for the presence of norovirus based on the DNA bands identified in the gel. The gel-based RT-PCR assay therefore provides a qualitative (binary) norovirus diagnosis.

More recently, real-time RT-PCR assays have been developed, which closely monitor the kinetics in the early stages of the PCR and provide a method of quantification,

based on the number of PCR cycles required to raise the target sequence copy number above a pre-set threshold[110, 261, 269-277]. The number of PCR cycles completed before crossing the threshold is referred to as the cycle threshold (Ct) value (further explanation of real-time RT-PCR is provided in Appendix 1.1). The Ct value provides a relative indication of the amount of norovirus in different stool specimens tested with the same real-time RT-PCR protocol and is inversely related to the faecal viral load: a low Ct value indicates a high viral load. Full quantification of the actual faecal viral load requires the generation of standard curves, from known dilutions of the target sequence, to translate the Ct value into a number of norovirus genomic copies per gram of stool[278]. The major advantages of real-time RT-PCR over gel-based RT-PCR are the possibility of quantification and the shorter assay time; the test result is produced directly from the PCR without the need for a separate identification step[273]. The real-time RT-PCR assays for norovirus may also have slightly higher analytical sensitivity than the gel-based assays, although inclusion of a second set of nested primers can increase the sensitivity of gel-based RT-PCR[110, 279, 280]. Current practice in most diagnostic laboratories is to treat any Ct value from the real-time RT-PCR as a positive result, effectively making this quantitative assay qualitative, like the gel-based RT-PCR.

Gel-based and real-time RT-PCR both have much lower detection limits than electron microscopy; approximately 10^4 genome copies per gram of stool are required for detection by the PCR methods, compared to 10^6 genome copies for electron microscopy[80, 99, 110, 280]. PCR-based methods will therefore detect norovirus in individuals with norovirus-associated IID for many more days after symptom resolution, when viral loads have decreased below the detection limit of electron microscopy. This is generally viewed as a major advantage of PCR over electron microscopy[12, 110, 279, 281]. However, this high diagnostic sensitivity compromises the diagnostic specificity for norovirus-associated IID: individuals who remain asymptomatic after norovirus infection, and therefore by definition do not have disease caused by norovirus, are frequently positive by RT-PCR[5, 21-23, 80, 99, 114].

The poor diagnostic specificity of RT-PCR has greater significance when diagnosing norovirus as the cause of sporadic IID compared to identifying norovirus outbreaks. During microbiological investigation of an IID outbreak, specimens are collected from multiple cases and if two or more are positive for norovirus after testing, this is taken as an indication that norovirus is the likely cause of the outbreak. Even if some cases are shedding norovirus at low viral loads at the time of testing, the detection of norovirus in

multiple individuals involved in the outbreak provides strong evidence that norovirus caused the outbreak. However, when diagnosing norovirus in individual sporadic IID cases, this reasoning cannot be used and the low diagnostic specificity of the RT-PCR produces uncertainty as to whether a positive result really indicates that norovirus is the cause of illness, especially when concurrent infection with other pathogens is detected simultaneously.

2.4.2.2. Nucleic acid sequence-based amplification and loop-mediated isothermal amplification

Nucleic acid sequence based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) assays have recently been developed for norovirus, to provide an alternative to RT-PCR diagnosis[251-255, 282, 283]. NASBA, which requires reverse transcription of the norovirus RNA genome into cDNA, and LAMP, which amplifies RNA directly, are carried out at a constant reaction temperature, removing the need for the thermal cycling machines that are used for PCR[252, 253, 255, 282]. The sensitivity and specificity of NASBA and LAMP are comparable to RT-PCR, although the lower amplification temperature used for NASBA (40°C) means that it may have slightly poorer analytical specificity than LAMP (carried out at 60°C) and RT-PCR, with some studies reporting poor reproducibility of results from NASBA assays[251, 279, 282]. The use of simpler laboratory equipment and the quicker turnaround time are major advantages of NASBA and LAMP over RT-PCR, especially for investigation of norovirus outbreaks, although the lack of automation, quantitation or production of nucleic acid sequences for genotyping, mean that these methods are unlikely to replace RT-PCR diagnosis for norovirus[252, 253, 255, 282].

2.4.3. Enzyme-linked immunosorbent assays

Antigen and antibody detection enzyme-linked immunosorbent assays (ELISA) for norovirus were initially developed using stool and serum reagents from volunteer studies and outbreaks[284-289]. However the limited reagent supplies meant that the first generation antigen ELISAs were not suitable for general diagnostic purposes. After sequencing of the norovirus genome[165, 166, 247] and subsequently the capsid gene of many different norovirus genotypes[164, 182, 290, 291], the capsid protein was expressed *in vitro* using baculoviruses[119, 192, 247, 291-294]; the capsid protein monomers self-assembled into virus-like particles (VLPs), with similar morphology and immunogenicity to norovirus virions [175, 295]. The baculovirus expression system can produce large quantities of norovirus VLPs, which are used to inoculate laboratory animals to

generate antibodies against the norovirus capsid protein[296]. The hyperimmune animal sera are then used as reagents in the diagnostic antigen detection ELISAs[245, 297-301]. The VLPs are also used directly in antibody ELISAs for seroepidemiological studies of norovirus antibody prevalence[302-314].

Whilst the diagnostic antigen ELISAs are cheaper and use simpler technology than RT-PCR [245, 315, 316], they have very narrow reactivity, only detecting the norovirus genotype against which the hyperimmune sera were raised and sometimes a few closely related genotypes[296]. The commercially available antigen ELISAs are targeted against several common genotypes, but still cannot provide the broad and reliable detection demonstrated by RT-PCR and they are therefore only recommended for screening large numbers of specimens for common genotypes before the application of RT-PCR [11, 315-318].

Immunochromatography assays have recently been developed for norovirus, with the same advantages, limitations and recommended applications as the existing antigen ELISAs[319-323].

2.4.4. Cell culture

Whilst other enteric viruses such as rotavirus and adenovirus (types 40 and 41) can be propagated in monolayer cell cultures[324, 325], the only successful *in vitro* propagation of norovirus has been in a highly differentiated three-dimensional cell culture, which reproduces the structure and function of the human small intestine epithelium[326, 327]. However, feline calicivirus, a vesivirus, grows readily in monolayer cell culture and has been used for studies of receptor binding and cell entry[171] and calicivirus survival and inactivation[328-332].

2.5. Incidence of norovirus-associated IID

It is widely recognised that norovirus is the most common cause of sporadic, community-acquired IID and of IID outbreaks in high-income countries. A number of studies have also shown a notable prevalence of norovirus infection amongst paediatric IID cases in low and middle income countries[49, 67, 333]. A range of study designs have been used to evaluate the burden of disease caused by norovirus: a small number of prospective community-based studies have directly estimated the incidence of norovirus-associated IID in high-income countries[5-8, 22, 43, 334], but the majority of studies consider IID cases presenting to healthcare services and seek to determine the prevalence of norovirus infection amongst these individuals[49]. A number

of seroepidemiological studies have examined the general population prevalence of antibodies to norovirus, as a proxy for the incidence of infection in childhood[302, 304, 306, 307, 309-314, 335-337]. Finally, a few studies have used statistical modelling to examine correlations between syndromic disease data and unlinked laboratory diagnoses e.g. correlating hospitalisations for IID in England with nationally collated reports of norovirus laboratory diagnoses[338, 339].

2.5.1. Global estimates of norovirus disease burden

A recent systematic review and meta-analysis of studies reporting the prevalence of norovirus infection amongst cases of IID in high, middle and low income countries estimated that 12% of IID cases ascertained in the community or after presentation to primary care or outpatient clinics were caused by norovirus, based on RT-PCR diagnosis; 12% of hospitalisations for IID amongst children aged less than five years were also attributed to norovirus in both high income and in low and middle income country settings[49]. From these prevalences it was estimated that norovirus may cause up to 900 000 primary care or outpatient clinic visits and 64 000 hospitalisations annually amongst children aged less than five years in high income countries, and 1.1 million hospitalisations and 218 000 deaths amongst children aged less than five years in low and middle income countries. However there are a number of significant limitations to this review: (1) norovirus is likely to be at higher relative prevalence in the community than amongst IID cases presenting to primary care services because it generally causes mild self-limiting disease, but these two groups were combined in the review; (2) most studies included in the review recruited only young children, meaning that the community/primary care prevalence of 12% is unlikely to be representative of the prevalence of norovirus in older children and adults; (3) high, middle and low income countries were considered together in the pooled prevalence in the community, but are likely to have significantly different prevalences of norovirus, although there were only two studies from low or middle income countries that considered community or primary care cases; (4) the estimated number of deaths requires the significant assumption that the distribution of pathogens amongst IID-related fatalities is the same as amongst hospitalised IID cases; (5) all RT-PCR positive cases have been treated as cases of norovirus-associated IID, which is unlikely to give an accurate estimate of norovirus disease burden because of the poor diagnostic specificity of RT-PCR, as discussed above in section 2.4.2; and (6) the prevalence of norovirus amongst IID cases will be influenced by the incidence of other pathogens in a population, which is likely to vary substantially between high, middle and low income country settings,

between different age groups and over time. However, the review does demonstrate that norovirus infection is highly prevalent amongst IID cases around the world, and that it therefore must be a cause of IID in all countries, although its relative importance compared to other pathogens is likely to vary between high income and low and middle income settings.

2.5.2. Norovirus-associated IID incidence in high income countries

Whilst the review by Patel et al.[49] highlighted the large number of studies that have been conducted in high income countries to determine the prevalence of norovirus infection amongst paediatric patients presenting to healthcare services [44, 49, 109, 203, 340-342], only a small number of prospective research studies have estimated the incidence of norovirus-associated IID in the community and amongst patients presenting to general practitioners or equivalent primary health care services (Table 2.1)[5-8, 22, 43, 334, 343]. These studies have all shown that norovirus is the most common cause of IID at the community level, with incidence estimates ranging from 12 to 100 episodes of norovirus-associated IID per 1000 person years in the different studies (Table 2.1)[5-8, 334]. The Study of Infectious Intestinal Disease in England, which reported the lowest norovirus incidence, used electron microscopy for norovirus diagnosis[51], so is likely to have substantially underestimated the burden of disease caused by norovirus, because of the low diagnostic sensitivity of electron microscopy. The other community-based studies in the Netherlands, Australia and the United States used RT-PCR diagnosis, so it is possible that they may overestimate the burden of norovirus disease to some extent, because of the sub-optimal diagnostic specificity of RT-PCR.

The prevalence of norovirus was higher in the study in the Netherlands compared to the Study of Infectious Intestinal Disease in England, but the difference may be less than might be expected given the difference in the sensitivity of electron microscopy and RT-PCR. A new variant of norovirus GII.4 emerged in 1995[201, 222, 223, 233], which may have caused a periodic increase in norovirus incidence during the study in England, whereas the study in the Netherlands was conducted during a time when no new GII.4 variants emerged and would therefore have recorded normal levels of norovirus transmission[179, 223]. In addition, the case definition for IID was less inclusive in the study in the Netherlands (at least three diarrhoea and/or vomiting episodes in 24 hours or two diarrhoea episodes with additional IID symptoms) than in the Study of Infectious Intestinal Disease in England, which included anyone who had a diarrhoea episode or two or more vomiting episodes in 24 hours; given the milder manifestation of norovirus disease in the community, the case definition in the study in the

Netherlands may have failed to identify all cases of norovirus-associated IID. In contrast, the incidence of norovirus-associated IID was much higher in the Australian study compared to both the Study of Infectious Intestinal Disease in England and the study in the Netherlands. However, this is because of the comparative over-representation of young children in the Australian cohort, which led to a substantially higher incidence of all-cause IID than in the other studies.

Norovirus was also identified as a significant cause of general practice consultations for IID, with incidence varying from 0.3 to 6.3 consultations per 1000 person years, although the majority of consultations were from young children (Table 2.1)[8, 22, 43, 343]. The low incidence of consultations in the study in the Netherlands probably reflects the much lower incidence of consultations for all-cause IID; in the Netherlands, general practice guidelines recommend that mild or uncomplicated cases of IID are given self-care advice via the telephone, substantially reducing the number of clinic visits[22].

Seroepidemiological studies have demonstrated a high prevalence of specific IgG antibody against a number of genogroup I and genogroup II noroviruses in the general population of high income countries[302, 304, 309, 311, 312, 335, 337, 344]. A high prevalence of maternal antibody was identified in infants aged up to six months[304, 309, 311, 312, 337], but after this age the prevalence of antibody dropped and gradually increased with age up to a seroprevalence of 90% or more in adults[302, 304, 309, 311, 312, 335, 337]. Seroprevalence increases more rapidly with age for genogroup II norovirus genotypes compared to those in genogroup I, reflecting the higher incidence of genogroup II infections reported in studies of norovirus-associated IID.

A study of seroconversion to GI.1 norovirus amongst Finnish infants and children, aged up to four years at recruitment, showed that half of the children experienced infection during the two year follow-up period[307]. A similar study in the general population in Canada showed that, across all ages, one third of individuals experienced infection with GI.1 norovirus during the 18 month follow-up period. These studies demonstrate the high incidence of infection, even with this single genogroup I norovirus strain that is recognised to be at much lower prevalence than other strains in genogroup II.

2.5.3. Norovirus incidence in the United Kingdom

The only estimates of the community incidence of norovirus-associated IID and of general practice consultations for norovirus in the United Kingdom (UK) are those from the Study of Infectious Intestinal Disease in England, described above[8, 83]. Three recent

studies have examined the prevalence of norovirus amongst young children presenting to general practitioners or hospital emergency services[13, 209, 345]. In studies conducted between 1999 and 2003, norovirus was identified in approximately 14% of children presenting to general practitioners and in 9% to 14% of children presenting to hospital emergency services[13, 345], both substantially lower than the prevalence of rotavirus in these study populations. The highest prevalence of norovirus infection was amongst patients aged less than three years. In the study conducted during 2006 and 2007, norovirus infection was identified in 25% of children presenting to general practitioners with IID, which was slightly higher than the prevalence of rotavirus in this study[209]; as discussed above, the unusually high prevalence of norovirus in this study could be due to the emergence of another novel GII.4 variant during 2006[177, 179, 223, 224].

Norovirus is also a major cause of IID outbreaks in the UK, with routine public health surveillance systems capturing information on outbreaks in a range of settings[1-3, 38, 40, 346, 347]. Outbreaks of norovirus occurring in hospitals in England and Wales are now voluntarily reported, in real-time, to the Health Protection Agency by infection control teams, using a web-based surveillance system dedicated specifically to norovirus hospital outbreaks[348]. This new surveillance system has confirmed the large burden of norovirus outbreaks, and related service disruption from ward closures, which was demonstrated in a recent prospective research study in the UK and other previous sources of surveillance data[14, 40]. There is a substantial incidence of norovirus outbreaks in other semi-closed settings such as nursing homes, schools, prisons and cruise ships[1-3, 38, 346, 347], as well as foodborne outbreaks in public food outlets and at catered events, which are predominantly due to food contamination by food handlers[346].

Laboratory diagnoses of norovirus are also reported to the Health Protection Agency by public health microbiology laboratories in England and Wales, providing further information about trends in norovirus incidence. However, the majority of these diagnoses are likely to come from norovirus outbreaks, rather than sporadic cases in the community, and changes in the sensitivity of diagnostic methods (moving from electron microscopy to RT-PCR) and a lack of denominator data (number of tests carried out) make it difficult to interpret trends in the number of norovirus reports over time, or to extrapolate these trends to the incidence of disease in the community.

Table 2.1 Summary of studies measuring the incidence of norovirus-associated IID and primary care consultations in high income countries.

Study	Study period	Country	Case ascertainment	Diagnostic method	Norovirus prevalence in IID cases	Incidence of norovirus-associated IID per 1000 person years[b]
Community						
Wheeler et al 1999[a]	1993-1996	England	Prospective cohort (age representative of general population)	Electron microscopy	<5 years: 11% ≥5 years: 6% All ages: 7%	<5 years: 80.4 ≥5 years: 9.5 All ages: 12.5
de Wit et al 2001	1996-1999	The Netherlands	Prospective cohort (age representative of general population)	Gel-based RT-PCR	<5 years: 18% ≥5 years: 13% All ages: 16%	All ages: 75.4
Hellard et al. 2001 & Marshall et al. 2003	1997-1999	Australia	Prospective cohort (families with 2 or more children aged 1-15 years)	Gel-based RT-PCR	All ages: 13%	All ages: 103.2
Vernacchio et a. 2007	2001-2002	U.S.A	Prospective cohort (children aged 3 months to 6 years)	Gel-based RT-PCR	3 months to 6 years: 2%	3 months to 6 years: 40.0
General Practice						
Wheeler et al 1999[a]	1993-1996	England	Patients with IID	Electron microscopy	<5 years:10% ≥5 years: 5% All ages:7%	All ages: 1.99
de Wit et al 2001	1996-1999	The Netherlands	Patients with IID	Gel-based RT-PCR	<5 years:10% ≥5 years: 4% All ages: 5%	All ages: 0.3
Karsten et al. 2009	2004	Germany	Patients with IID	Gel-based RT-PCR	All ages: 16%	All ages: 6.3

[a] The Study of Infectious Intestinal Disease in England
[b] Only Wheeler et al. and Karsten et al. reported pathogen-specific incidence; for other studies, the norovirus prevalence was multiplied by the all-cause IID incidence.
Abbreviations: IID, infectious intestinal disease; RT-PCR, reverse transcription-polymerase chain reaction

Further information about national surveillance of norovirus laboratory reports in England and Wales is provided in Chapter 3.

Whilst there have been no studies directly enumerating hospitalisations and deaths due to norovirus-associated IID in the UK, two studies have used statistical modelling techniques to estimate the frequency of these events[338, 339]. Data on all-cause outcomes, i.e. hospitalisations or deaths due to IID, are modelled using unlinked data on norovirus infections (the norovirus laboratory diagnoses from the Health Protection Agency) and a proportion of the all-cause outcomes attributable to norovirus is extracted from the model. These studies estimated that there may be more than 3000 hospital admissions for norovirus-associated IID amongst individuals aged 65 years and older each year in England and Wales[339], and that there are at least 200 deaths from norovirus-associated IID in this age group annually[338].

Data on outbreaks of norovirus have been collected in the UK since the mid 1990s and the large burden of outbreaks in hospitals and care homes is well documented, although there is less knowledge about the frequency of outbreaks in other community settings. A new surveillance system has now been set up to routinely capture information on the occurrence of norovirus outbreaks in hospitals in England and Wales. Whilst a limited number of studies have examined the importance of norovirus as a cause of healthcare consultations for paediatric IID, the only existing estimates of the amount of sporadic disease caused by norovirus at the community level comes from the Study of Infectious Intestinal Disease in England, which used electron microscopy for norovirus diagnosis and is therefore likely to have underestimated norovirus-associated IID incidence in the community.

2.6. Norovirus transmission

2.6.1. Infectious dose and attack rates

The predominant route of norovirus transmission is faeco-oral, which has been demonstrated in a large number of volunteer studies where individuals were inoculated with suspensions of stool filtrates from cases of norovirus-associated IID and subsequently became ill[56, 58-61, 72-80]. Whilst putative vomitus-oral transmission of norovirus has been reported from outbreak investigations[349-351], the infectiousness of vomit from cases of norovirus-associated IID has never been examined in a volunteer study.

Recently, a comprehensive dose-response volunteer study has confirmed previous assumptions about the extremely low infectious dose of norovirus[98]. This study showed that norovirus infection may occur after ingestion of as few as 100 norovirus virions in individuals who are genetically susceptible to the infecting strain. However, even when very large infecting doses were given to genetically susceptible individuals the infection rate was not 100%, indicating that pre-existing immunity may be preventing infection in some individuals, or that further uncharacterised mechanisms of genetic resistance exist. Whilst the probability of infection increased only very little with inoculating doses greater than 1000 virus particles, the probability of developing disease after infection increased almost linearly with the infecting dose, even up to very high inocula of 10^{10} particles. However, the precision of the infectious dose measured is limited by the use of real-time RT-PCR to quantify norovirus virions in the inocula; it is highly likely that not all genome copies detected by real-time RT-PCR were from viable, infectious virions, meaning that the number of infectious particles in the inocula may be different to the number of genome copies reported as the inocula concentrations in the study. In spite of this limitation, the study provides the first direct evidence of the high infectiousness of norovirus and provides a useful estimate of the infectious dose for risk analyses of environmental and food contamination.

The infectiousness of norovirus is reflected in the high attack rates reported from both food- and water-borne outbreaks and those caused by direct person-to-person transmission. The attack rate reported in published investigations of foodborne outbreaks is typically close to 60%, ranging from 33% to 67%[94, 352-365]. In person-to-person transmitted outbreaks, the attack rates reported are more variable, ranging from 13% to 62% in hospital settings[14, 366-369], from 10% to 74% in residential care home settings[19, 370-376], and from 23% to 85% in other community settings such as day care centres and hotels[377-379].

2.6.2. Risk factors for sporadic norovirus infection and disease

A small number of case-control studies have examined risk factors for sporadic, community-acquired norovirus-associated IID[22, 83, 140, 380]. Contact with individuals with IID symptoms, either within or outside the household, was identified as a major risk factor for norovirus-associated IID in these studies[22, 83, 140, 380] and the risk increased as the number of household infectious contacts increased[380]. Two studies of household norovirus transmission have shown that there is a greater risk of norovirus infection in household members from child index cases, compared to adult index cases[85], and that children are at higher risk of becoming infected by a household contact[365].

Recent foreign travel was strongly associated with norovirus-associated IID in one case-control study[83], possibly reflecting exposure to a broader or different range of norovirus strains, or transient behavioural changes that may increase the risk of norovirus infection. However, the majority of cases in the study were recruited after presentation to a general practitioner with norovirus-associated IID and were compared to population-based control subjects; recent foreign travel strongly increases the probability of consulting a general practitioner after the development of IID, regardless of the causative pathogen[381], meaning that the association between norovirus-associated IID and foreign travel may be due to non-comparability of the case and control groups for this exposure. Individuals in lower social classes (based on occupation) were also shown to be at a three-fold higher risk of norovirus-associated IID in the same study[83].

The norovirus strains circulating in animal populations are genetically distinct from those causing illness in humans and there is no evidence that zoonotic transmission contributes to the burden of norovirus-associated IID in humans [163, 164, 181, 382-384]. In fact two studies have shown that pet-ownership and recent contact with animals is associated with a decreased risk of norovirus-associated IID[22, 380]. Whilst it is possible that frequent exposure to norovirus, through contact with infected animals, could boost immunity to the virus and therefore decrease the risk of norovirus-associated IID, strains that commonly infect animals are genetically distinct from those causing disease in humans[163, 164, 181, 382-384], and are therefore unlikely to stimulate cross-protective immunity[125, 126]. It is likely that these animal exposures are correlated with other life style factors that are protective against norovirus-associated IID.

Two seroepidemiological studies have investigated risk factors for norovirus infection. One study in rural Mexico examined seroconversion during a one year period, across all ages, and showed that poor hand hygiene practices amongst household members was strongly associated with an increased risk of norovirus seroconversion in young children[336]. In older children and adults, variables that correlate with low socioeconomic status (infrequent meat consumption, agricultural work, dogs near the house) were associated with an increased risk of seroresponse in the Mexican study. In a cross-sectional seroprevalence study in Chile, individuals in lower social classes (based on occupation, education and housing conditions) were more likely to be norovirus antibody positive[310]. Factors such as child care attendance and seafood consumption were also associated with norovirus seropositivity, but only in subsets of the study population, and it is difficult to assess their potential correlations with social class.

Whilst these seroepidemiological studies did not distinguish between norovirus infection and disease, there are currently no published studies that specifically examined risk factors for asymptomatic norovirus infection.

A cohort study of infants and young children attending day care centres in the United States demonstrated a high incidence of IID amongst cohort participants, with more than two episodes per child each year[385, 386]. A case-control study conducted amongst children who attended paediatric healthcare clinics with IID, showed that attendance at day care centres increased the risk of presenting with IID[387]. These two studies focused on the role of rotavirus in causing IID, but more than half of the cases remained undiagnosed after testing for rotavirus and other common bacterial and protozoan gastrointestinal pathogens; it is therefore very probable that other enteric viruses, including norovirus, were important aetiological agents of IID in these day care settings. Environmental testing has also revealed widespread rotavirus contamination on fomites and surfaces in day care centres[388], and again it is likely that there will be concomitant norovirus contamination, because of the high norovirus infection prevalence amongst young children and the common faeco-oral transmission route of norovirus and rotavirus[389].

2.6.3. Norovirus outbreak investigations

Norovirus transmission has been most extensively studied in the context of outbreaks investigated by public or environmental health authorities. A number of high income countries conduct national surveillance of IID outbreaks, including those caused by norovirus[1, 2, 4, 346, 390, 391]. Direct person-to-person transmission is the most commonly reported route of norovirus infection in the outbreaks captured in these national surveillance systems. However, all of the surveillance systems rely on passive reporting, with some focusing on foodborne outbreaks, whilst others may be more biased towards norovirus outbreaks occurring in healthcare settings, and cannot therefore be used to conclusively demonstrate the relative importance of person-to-person and foodborne transmission[38, 392]. However, outbreak investigations do provide the most direct evidence of the transmission routes of norovirus infection. The spatio-temporal spread of infection can be recorded in detail during outbreaks involving person-to-person transmission[96, 350, 352, 393-395] and modern molecular epidemiological methods can be used to link IID cases and particular food items, or water sources, in food- and waterborne outbreaks[396, 397]. This detailed investigation is not possible in studies of community-acquired sporadic norovirus-associated IID, where inference of

the route of transmission relies on cases retrospectively reporting exposures in standard epidemiological questionnaires[22, 83, 140, 380].

In addition to direct faeco-oral transmission, a number of outbreaks have reported airborne transmission, following a vomiting episode, with infection occurring after inhalation of aerosolised virus particles[350, 393-395]. However, faeco-oral and vomitus-oral transmission may also occur through contamination of environmental surfaces and objects, rather than through direct transfer between infected and susceptible individuals. Environmental norovirus contamination has been demonstrated using sensitive RT-PCR testing during outbreak investigations[18, 398-401], and a number of investigations have implemented environmental contamination, with norovirus being transferred to the hands of individuals and subsequently ingested, as the most likely route of norovirus infection[402, 403, 403-408]. A small number of outbreak investigations have examined hand hygiene practices amongst exposed individuals; those who practiced effective hand-washing were at a lower risk of norovirus-associated IID during a cruise ship outbreak where person-to-person transmission was predominant[406] and a mathematical modelling study showed that implementation of hygiene measures in a large and prolonged person-to-person outbreak at a campsite reduced the transmission of norovirus, although not sufficiently to completely stop the outbreak[409].

The majority of foodborne norovirus outbreaks that occur in commercial catering settings are attributed to contamination of food (or drink) during preparation by an infected food handler, using either epidemiological information and/or molecular testing[38, 349, 351, 360, 397, 400, 410-418]. Norovirus is the most common cause of foodborne outbreaks where food handlers, rather than foodstuffs, are implicated as the source of contamination[419], highlighting their greater direct transmissibility compared to other gastrointestinal pathogens[98, 420].

Contamination of food at the source, and during industrial processing, has also lead to norovirus outbreaks; this is especially a problem for products such as oysters and other molluscan shellfish that are consumed raw or after minimal cooking[95, 354, 364, 421-430]. Marine mollusc beds are frequently contaminated with human sewage and norovirus becomes concentrated on the animals during filter feeding, meaning that large doses are ingested when they are eaten[362, 431-440].There is also some evidence that the internal temperatures reached during steam-cooking of molluscs may not high enough to inactivate norovirus[95, 441-443]. Outbreaks have also been linked to contamination of raw fruit and vegetables, contaminated during production, with inadequate heat-processing prior to consumption[444, 445].

A number of norovirus outbreak investigations have implicated private[446-450] and public/municipal[451-457] drinking water supplies after specific, documented contamination events. Norovirus outbreaks have also been reported after contact with sewage contaminated recreational water[96, 458-462].

2.6.4. Experimental virus transfer and survival studies

Experimental studies of norovirus and other related caliciviruses that are used as surrogates for norovirus (murine norovirus and feline calicivirus)[463-465], have shown that they are readily transferable between human hands and environmental surfaces and foods[329, 466]. The viruses can survive for many hours or days on surfaces such as computer keyboards and kitchen utensils[328, 329] and on refrigerated pre-prepared foods[331, 332], supporting the epidemiological evidence of these transmission routes from outbreak investigations. Survival studies also indicate that some industrial food processes designed to decontaminate pre-prepared foods may not be effective against norovirus[467-470].

Whilst a number of studies have shown that alcohol-based hand sanitizers reduce the concentration of infectious feline calicivirus and murine norovirus, using cell culture and *in vivo* pathology assays[471-476], a study of human norovirus inactivation, using real-time RT-PCR quantitation of norovirus RNA, has shown that mechanical removal with water rinsing and antibacterial soap is more effective at reducing the norovirus RNA load than alcohol[477]. Whilst feline calicivirus and murine norovirus may be poor surrogates for norovirus in inactivation studies, evidenced by differing resistance to heat and pH changes[331, 463-465, 478], a major limitation of using RNA quantitation of human norovirus is that studies comparing RNA load and the presence of infectious feline calicivirus have shown very poor correlation between the two[471, 478]. Therefore, the efficacy of commonly used hand sanitizers against norovirus remains unknown, but mechanical removal of virus using prolonged washing with soap and water may be effective[330].

2.7. Summary

Norovirus is recognised as the most common cause of both sporadic cases and outbreaks of IID, across all age groups, in high income countries. Norovirus is highly infectious and is predominantly transmitted directly through contact between infected and susceptible individuals, or through the contamination of food, drink or environmental surfaces by infected individuals. Disease caused by norovirus has rapid onset, characterised by vomiting and/or diarrhoea, but is generally self-limiting and of

short duration, except in young children and the elderly, who may experience longer-lasting and more severe symptoms. Immunity to norovirus is short-lived and strain-specific, with a large number of antigenically distinct circulating norovirus strains and antigenic drift documented in the predominant genotype, meaning that individuals remain susceptible to both infection and disease throughout life. Symptoms are probably attributable to norovirus replication in the proximal small intestine and a correlation between viral load and the occurrence of disease has been documented. However, RT-PCR, which is now the main method of norovirus diagnosis, readily detects norovirus in asymptomatic individuals. RT-PCR therefore provides poor predictive value for diagnosing sporadic norovirus-associated IID, because of the high prevalence of asymptomatic infection in the general population in high income countries and the poor diagnostic specificity of the assay. A new method for diagnosing norovirus-associated IID in sporadic community cases is therefore required, to produce accurate estimates of the incidence of norovirus-associated IID. Current estimates of norovirus disease burden in high income countries are based on either electron microscopy (in England) or RT-PCR (in the Netherlands, Germany, Australia and the United States) and neither method can be considered satisfactory for accurately enumerating cases of norovirus-associated IID in the community.

Chapter 3: Description of datasets

The majority of the work presented in this thesis was based on analysis of data from the Study of Infectious Intestinal Disease in England. The study methods and the data collected are described in detail in this chapter, to aid understanding and interpretation of analyses presented in subsequent chapters. In addition, two sources of routinely-collected surveillance data are described, which provide information on general practice consultations for IID and norovirus laboratory diagnoses in England and Wales. These surveillance datasets were used to estimate the incidence of general practice consultations for norovirus-associated IID, presented in Chapter 7.

3.1. The Study of Infectious Intestinal Disease in England

3.1.1. Aims and objectives

The Study of Infectious Intestinal Disease was conducted in England between 1993 and 1996, to address the recommendations of the Richmond Report on the microbiological safety of food[479]. The main aim of this multi-component study was to:

"estimate the incidence and aetiology of infectious intestinal diseases occurring in the population and presenting to [general practitioners] in England."[83]

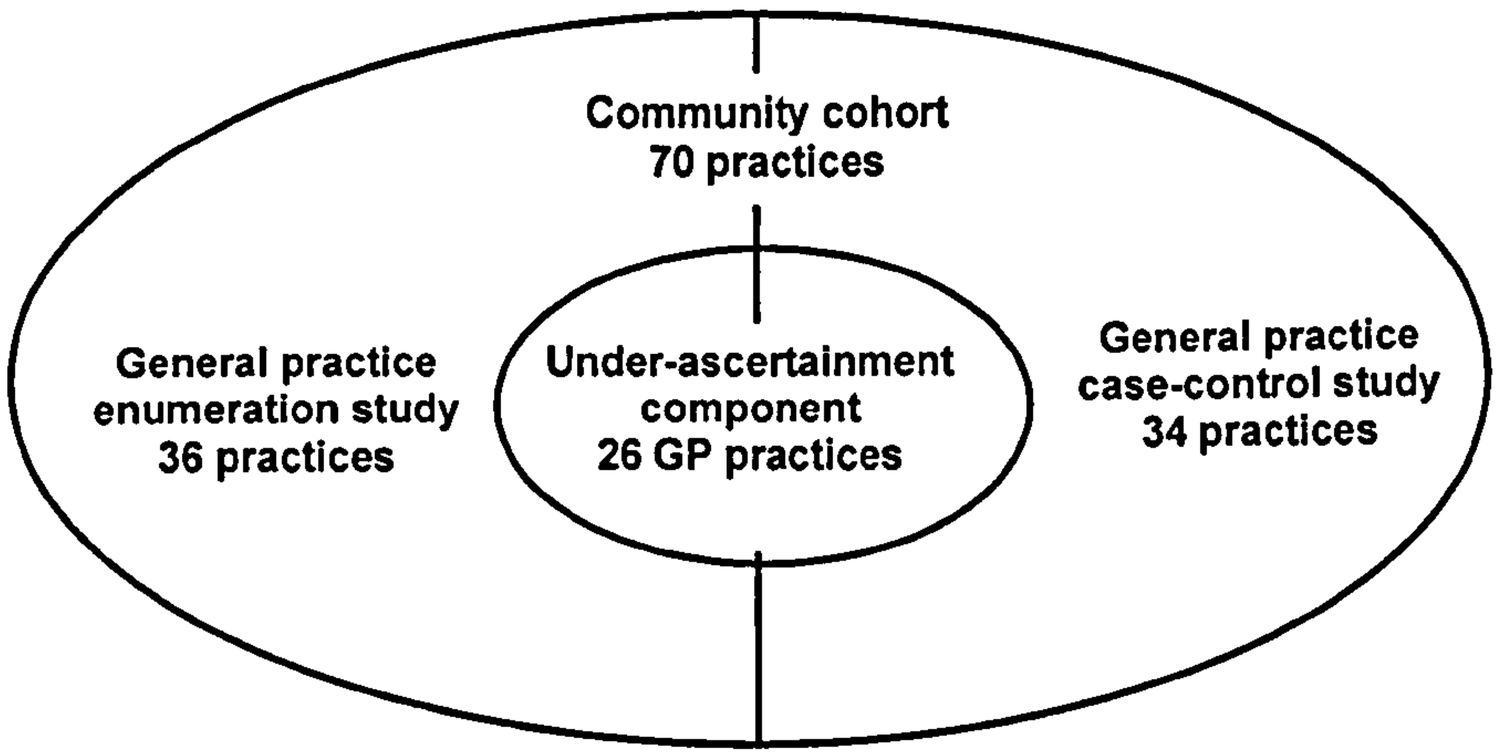
In the original study, the data used in this thesis were collected to address the following research objectives:

i. Estimate the number and aetiology of cases of IID in the population, presenting to general practitioners and having stool specimens routinely sent for laboratory examination;

ii. Estimate the prevalence of asymptomatic infection with agents associated with IID;

iii. Document differences between cases of IID (in the population and presenting to general practitioners) and similar but well people (controls)[83].

Figure 3.1 Structure of the community and general practice study components in the Study of Infectious Intestinal Disease.



The data used in this thesis are taken from the study components based in the community and in general practices, shown in Figure 3.1. There were 70 general practices included in the study, all of which recruited participants for the community-based cohort. Thirty-four general practices were then randomly allocated to the general practice case-control study component and 36 to the general practice enumeration study. Twenty-six practices also provided data for the under-ascertainment study, 13 each in the enumeration study and the general practice case-control study[83, 480].

3.1.2. Case and control definitions

Cases of IID were defined as: individuals with diarrhoea (any loose stools) or significant vomiting (2 or more vomiting episodes per 24 hours), lasting less than two weeks, without an identified non-infectious cause, preceded by a symptom-free period of at least three weeks[83, 480].

Controls were defined as: individuals with no recent history of diarrhoea (any loose stools) or vomiting prior to the onset of illness in the matched case.

The exclusion question in the control questionnaire asked: "In the last 10 days did you have any diarrhoea or vomiting?"[83] If controls had experienced these symptoms in the previous 10 days before completing the questionnaire, they were asked to return the questionnaire to the study team, without completing any further questions. However, in the report and related peer-reviewed papers from the Study of Infectious Intestinal

Disease the control definition states that controls had to have been free of diarrhoea and vomiting for at least three weeks prior to recruitment[83, 481, 482]. The analyses presented in this thesis include all controls who were free of diarrhoea and vomiting for 10 days prior to questionnaire completion and/or stool submission.

3.1.3. Community cohort and nested case-control study

Each general practice recruited two successive cohorts, which were followed-up for six months each. A total of 4026 person-years of follow-up were completed[8, 83]. At recruitment, all cohort members were asked to complete a baseline questionnaire, providing basic demographic details and information on employment, accommodation and food storage and preparation practices (Table 3.1) [83]. The cohort was broadly representative of the population of England in terms of age, gender and ethnicity, although the following groups were slightly under-represented: adults aged between 15 and 24 years; individuals in manual occupational social classes. Married individuals and property-owners were slightly over-represented in the cohort[83]. Individuals living in institutions, such as care homes or prisons, were not eligible for inclusion in the cohort.

Active case ascertainment was used to ensure that all cohort members experiencing IID symptoms were identified; cohort members returned diary cards to the practice nurses in each week of the study to confirm whether they had experienced any IID symptoms[83, 480]. Cohort members who were ascertained as IID cases were ineligible to be a case again for four weeks after the date of symptom onset in their first episode. Those cohort members matching the case definition were asked to join the nested case-control study. An age- and sex- matched control was recruited concurrently for each case, from within the same general practice. The matching criteria are summarised in Appendix A1.2.

All IID cases and controls were asked to provide a stool specimen for diagnostic testing and to complete a risk factor questionnaire, in which they provided information on symptoms, illness in other household members and specific exposures related to IID (Table 3.1) [83, 480]. IID cases were also asked to complete an additional questionnaire three months after their IID episode, examining the socio-economic costs of their illness. Information on long-term symptoms and hospital visits from the socio-economic cost questionnaire were used in this thesis (Table 3.1).

Table 3.1 Summary of information collected in the questionnaires in the Study of Infectious Intestinal Disease.

Questionnaire	Characteristic	IID case exposure period	Control exposure period
Baseline	Demographic details	-	-
	Employment & education status/details	-	-
	Accommodation details	-	-
	Day care/school attendance (children <16 years)	-	-
	Food storage and preparation facilities, food hygiene	General behaviours	General behaviours
Risk factor	Symptoms – IID and non-specific (severity & duration for IID cases)	During initial illness	3 weeks before questionnaire
	Illness in other household members and contacts outside of the household	10 days before onset of illness	10 days before questionnaire
	Travel abroad and water sports activities	10 days before onset of illness	10 days before questionnaire
	Contact with animals	10 days before onset of illness	10 days before questionnaire
	Food and water consumption	10 days before onset of illness	10 days before questionnaire
Socio-economic cost (IID cases, 3 months after illness)	Continuing long-term symptoms	Symptoms beginning with IID episode and still present	n/a
	Admission to hospital	At time of IID episode	n/a

Abbreviations: IID, infectious intestinal disease.

Adults completed the questionnaires themselves; a parent or guardian completed the questionnaires on behalf of children aged less than 16 years.

3.1.4. General practice case-control study

Patients presenting to one of the 34 participating general practices, with IID symptoms matching the case definition, were invited to participate in the general practice case-control study[83, 480]. Age- and sex- matched controls were recruited concurrently to each case from within the same general practice. IID cases and controls were asked to provide a stool specimen for diagnostic testing and to complete a single questionnaire, comprised of the baseline and risk factor questionnaires used in the community cohort (Table 3.1). IID cases were asked to complete the socio-economic cost questionnaire at three months after their illness.

3.1.5. General practice enumeration study

General practices in the enumeration study were asked to complete a single case-report form for each patient presenting with IID symptoms matching the case definition[83, 480]. Diagnostic testing was carried out as per routine practice.

3.1.6. General practice under-ascertainment study

General practices with electronic patient records were included in the under-ascertainment study. Patient records were searched, using Read codes related to IID, to identify all patients who presented to the general practices during the study and met the case definition[83, 480]. Under-ascertainment was calculated as the percent of eligible IID cases not recruited into the study. Percent under-ascertainment was translated into a practice-level adjustment factor, applied to the numerator in the incidence of general practice consultations for IID, to improve the accuracy of the incidence estimate. Separate under-ascertainment adjustment factors were calculated for practices in the case-control study and the enumeration study, and for those in urban and rural locations.

3.1.7. General practice list inflation

The patient registers from the general practices were used to generate denominators for the incidence of general practice consultations for IID. The number of registered patients in each practice who were no longer actively using the practice, because they had died or

moved away, was estimated from the proportion of patients who could not be contacted during recruitment of the community cohort[83, 480]. The estimated proportion of patients no longer actively using each practice was used to adjust the number of registered patients used in the denominator for the calculation of IID consultation incidence.

3.1.8. Diagnostic testing

Stool specimens from IID cases and controls were tested for the presence of 19 bacterial, viral and protozoal gastrointestinal pathogens, shown in Table 3.2[51]. In the original study, no polymerase chain reaction (PCR) methods were used; pathogens were detected using bacterial culture, light microscopy, electron microscopy and enzyme-linked immunosorbent assay (ELISA). All specimens with sufficient volume remaining after testing were archived in frozen storage[51]; 2819 controls provided stool specimens in the original study, with 78% being archived, and 3654 IID cases provided specimens, with 66% archived (Table 3.3).

All archived specimens from IID cases and controls, including those previously positive for one or more pathogens in the original testing, were subsequently retested for eight of the most common pathogens, using PCR methods (Table 3.2)[21].

Two RT-PCR assays were used to diagnose norovirus, a separate assay for genogroup I noroviruses and genogroup II noroviruses. The RT-PCR testing therefore allowed identification of the genogroup of norovirus detected, but no further genotyping was carried out. The prevalence of norovirus greatly increased in both IID cases and controls with the application of RT-PCR testing: in IID cases, the prevalence of norovirus diagnosed by electron microscopy was 6%, which increased to 34% by RT-PCR, and amongst controls the prevalence increased from 0.2% by electron microscopy to 16% by RT-PCR.

Table 3.2 Pathogens targeted in diagnostic testing, diagnostic methods and prevalence in IID cases and controls from the Study of Infectious Intestinal Disease specimen archive.

Pathogen	Diagnostic method	Percentage prevalence	
		IID cases (n=2422)	Controls (n=2205)
Norovirus	EM & RT-PCR	34.4	16.4
Rotavirus A	EM/ELISA & RT-PCR	31.1	14.1
Campylobacter spp.	Bacterial culture & PCR	22.6	5.0
Diarrhoeagenic *Escherichia coli*	Bacterial culture & PCR	15.4	9.1
Salmonella spp.	Bacterial culture & PCR	5.8	0.7
Aeromonas	Bacterial culture	5.1	4.5
Clostridium perfringens	Bacterial culture & toxin test	4.1	0.8
Sapovirus	EM & RT-PCR	3.9	2.0
Adenovirus	EM & ELISA	2.9	0.2
Cryptosporidium spp.	Light microscopy & PCR	2.5	0.5
Astrovirus	EM & ELISA	2.6	0.2
Yersinia spp.	Bacterial culture	2.2	2.6
Giardia spp.	Light microscopy & PCR	2.1	1.5
Clostridium difficile	Bacterial culture & toxin test	1.5	1.8
Shigella spp.	Bacterial culture	0.8	-
Rotavirus C	EM	0.3	-
Staphylococcus aureus	Bacterial culture & toxin test	0.3	0.2
Bacillus spp.	Bacterial culture & toxin test	0.2	0.5
Vibrio spp.	Bacterial culture	0.04	-

Abbreviations: EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; IID, infectious intestinal disease; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

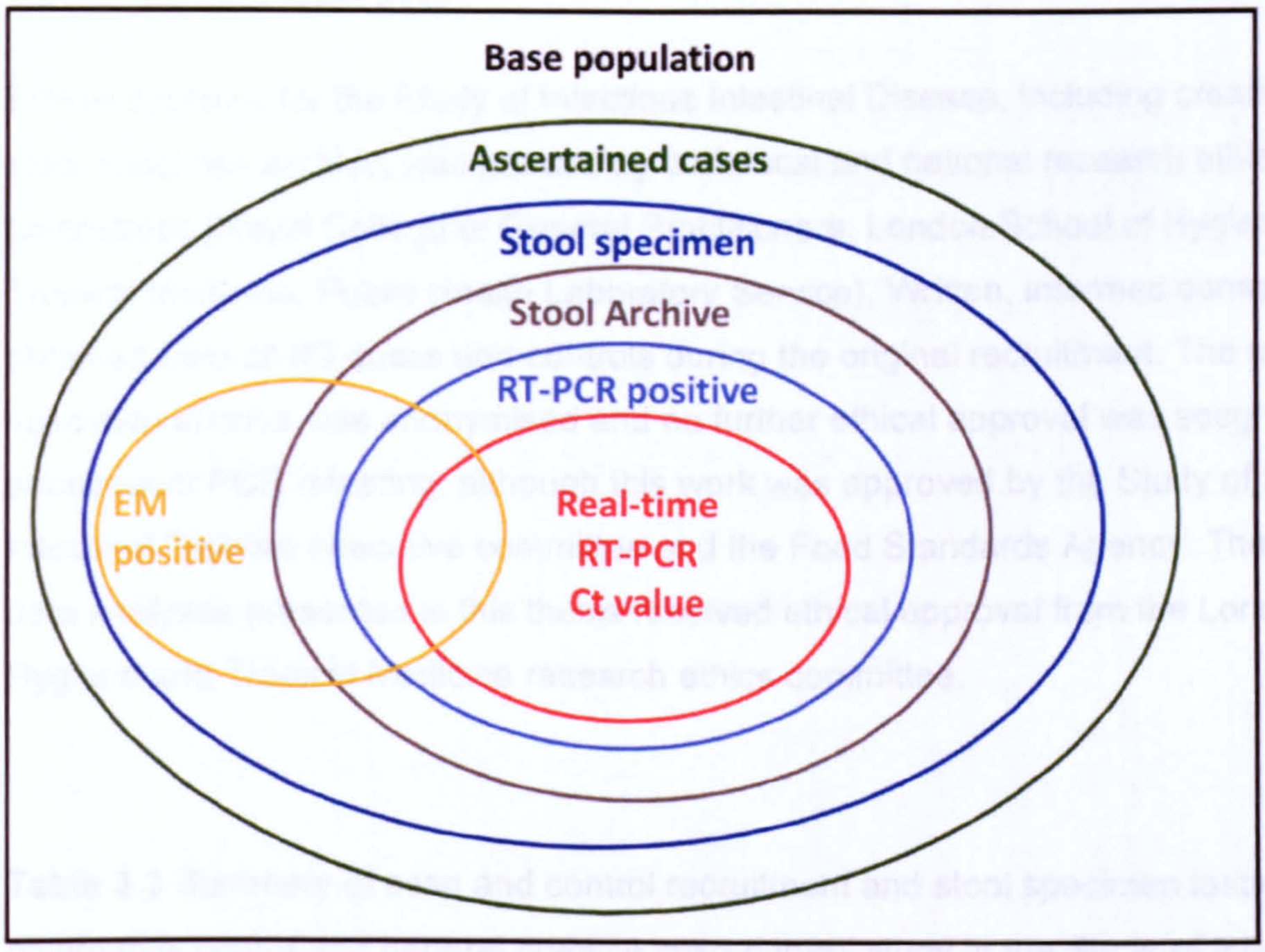
3.1.9. Quantitative real-time RT-PCR testing for enteric viruses

All the norovirus positive specimens were retested using quantitative real-time RT-PCR[110] to determine the viral load. The viral load was determined for the majority of norovirus positive specimens (86% of norovirus positive IID cases and 55% of norovirus positive controls). The ascertainment of IID cases into the study, archiving and norovirus testing are summarised in Figure 3.2; the numbers of IID cases and controls recruited, archived and positive for norovirus are provided in Table 3.3.

The cycle threshold (Ct) value from the real time RT-PCR assay was used as a proxy measure of faecal viral load; it is inversely proportional to the amount of virus present in the specimen, so the lower the Ct value the higher the faecal viral load. The Ct value represents the number of rounds of PCR replication required to raise the number of copies of the target DNA sequence in the reaction mixture above a pre-determined threshold. The real time RT-PCR assay was run for 40 cycles, so the maximum possible Ct value for positive specimens was 39. Appendix 1.1 provides further explanation of the real-time RT-PCR reaction and interpretation of the Ct value.

Characterisation of the norovirus real-time RT-PCR assay indicates that there is variability in the efficiency of the PCR reaction between norovirus genotypes (J. Gray, personal communication). This is indicated by the detection limit of the assays, which is determined by serial dilution of standard solutions of DNA plasmids containing the norovirus genetic sequence targeted in the assay. The majority of the genotypes in genogroup II show the same assay efficiency, except two less common genotypes (Appendix A1.3).The assay efficiency is generally lower in genogroup I, with more variation between the genotypes (Appendix A1.3). Differences in assay efficiency between norovirus genotypes means that the same Ct value is likely to represent a different viral load in the original stool specimens; it is therefore not necessarily valid to analyse the Ct value data from different genotypes together. The problems with the norovirus real-time RT-PCR assays are discussed further in Chapter 4.

Figure 3.2 Summary of case recruitment and stool specimen testing for norovirus in the Study of Infectious Intestinal Disease. Applicable to both the community cohort and the general practice case-control study.



Abbreviations: Ct, cycle threshold; EM, electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction.

3.1.10. Representativeness of the IID cases and controls in the specimen archive

The analyses presented in this thesis used diagnostic and epidemiological data only from the IID cases and controls who provided a stool specimen for diagnostic testing that was included in the specimen archive. The IID cases and controls in the specimen archive were very similar, in terms of age (Appendix A1.4) and sex (Appendix A1.5), to all the IID cases and controls originally recruited in the Study of Infectious Intestinal Disease.

The proportion of archived IID cases remaining undiagnosed (with no pathogen identified) after the original testing, but before application of the PCR retesting, was similar to the proportion of all recruited IID cases remaining undiagnosed: 45% of IID cases recruited in the general practice case-control study were undiagnosed compared to 42% in the archive; and 63% of IID cases recruited in the community cohort were undiagnosed compared to 62% in the archive. Amongst both the recruited and archived controls 80% had no pathogens detected after the original testing.

3.1.11. Ethical approval

Ethical approval for the Study of Infectious Intestinal Disease, including creation of the stool specimen archive, was granted by both local and national research ethics committees (Royal College of General Practitioners, London School of Hygiene and Tropical Medicine, Public Health Laboratory Service). Written, informed consent was obtained from all IID cases and controls during the original recruitment. The stool specimen archive was anonymised and no further ethical approval was sought for the subsequent PCR retesting, although this work was approved by the Study of Infectious Intestinal Disease executive committee and the Food Standards Agency. The secondary data analyses presented in this thesis received ethical approval from the London School of Hygiene and Tropical Medicine research ethics committee.

Table 3.3 Summary of case and control recruitment and stool specimen testing in the community cohort and general practice case-control study in the Study of Infectious Intestinal Disease..

	Community cohort	General practice case-control study	Controls
Ascertained IID cases	781	4011	-
Stool specimen	761	2893	2819
EM positive for norovirus	50	169	9
Stool specimen archived	517	1905	2205
RT-PCR positive for norovirus[a]	211	623	361
Viral load determined with real-time RT-PCR for norovirus	174	544	199

[a] Includes those previously positive by EM

Abbreviations: EM, electron microscopy; IID, infectious intestinal disease; RT-PCR, reverse transcription-polymerase chain reaction.

3.1.12. Use of data from the Study of Infectious Intestinal Disease in this thesis

The use of data from the Study of Infectious Intestinal Disease in this thesis is summarised in Table 3.4. In Chapters 4, 5 and 6 data from IID cases in the community cohort and the general practice case-control study were analysed together and the advantages and limitations of this approach are discussed in the individual chapters. In Chapter 8 the two groups of IID cases were analysed separately, to estimate the incidence of norovirus-associated IID at the community and general practice levels.

Table 3.4 Use of data from the Study of Infectious Intestinal Disease in this thesis.

Thesis chapter	Analysis objective	Study component / data						
		Community cohort	Nested case-control	GP case-control	GP enumeration	GP under-ascertainment	GP list inflation estimate	Diagnostic results	Norovirus viral load
4	Select viral load cut-off to attribute disease to norovirus in IID cases	✓	✓					✓	✓
5	Describe symptoms and mixed infections in norovirus IID cases and asymptomatic norovirus infections	✓	✓					✓	
6	Identify risk factors for norovirus-associated IID and asymptomatic norovirus infection	✓	✓						
8	Estimate the incidence of norovirus-associated IID in the community and the incidence of general practice consultations for norovirus IID	✓		✓	✓	✓	✓	✓	✓

Abbreviations: GP, general practice.

3.2. Royal College of General Practitioners Surveillance Scheme

The Royal College of General Practitioners (RCGP) Research and Surveillance Centre was established in 1957 to monitor trends in diseases managed by general practitioners in England and Wales[483, 484]. Aggregated data on consultations for a range of infectious and non-communicable diseases are collected from participating general practices on a weekly basis. Prior to 1994, data were submitted in paper format, but between 1994 and 1998 participating practices were transferred to the current electronic reporting system[484]. Consultations for specific diseases are extracted from the practice patient records using Read codes, which have been mapped to International Classification of Disease (ICD) codes.

Between 1993 and 2006, approximately 70 practices participated in the surveillance scheme, covering a registered patient population of approximately 600 000 (Figure 3.3). After additional practices were recruited in 2006, data are now collected from approximately 100 general practices across England and Wales, with a registered patient population of 900 000 (Figure 3.3). The participating practices are broadly representative of all practices in England and Wales in terms of geographical location and socio-demographic characteristics[485].

Data on IID consultations have been collected since 1967[485]. Consultations for IID are defined as those assigned International Classification of Disease version 9 (ICD9) codes between 001 and 009 inclusive. No information on pathogen diagnoses is collected. The incidence of general practice consultations for IID has declined significantly in the last two decades, particularly amongst children aged less than five years (Figure 3.4)[485]. The reasons for this decline in consultations are unknown, although a concurrent increase in the incidence of IID consultations to secondary care services has been reported[485].

Data on general practice consultations for IID, occurring between 1993 and 2007, provided by the RCGP Research and Surveillance Centre, were used in the generalised linear regression analysis, presented in Chapter 7.

Figure 3.3 Size of registered patient population covered by the Royal College of General Practitioners surveillance scheme,1993 to 2007.

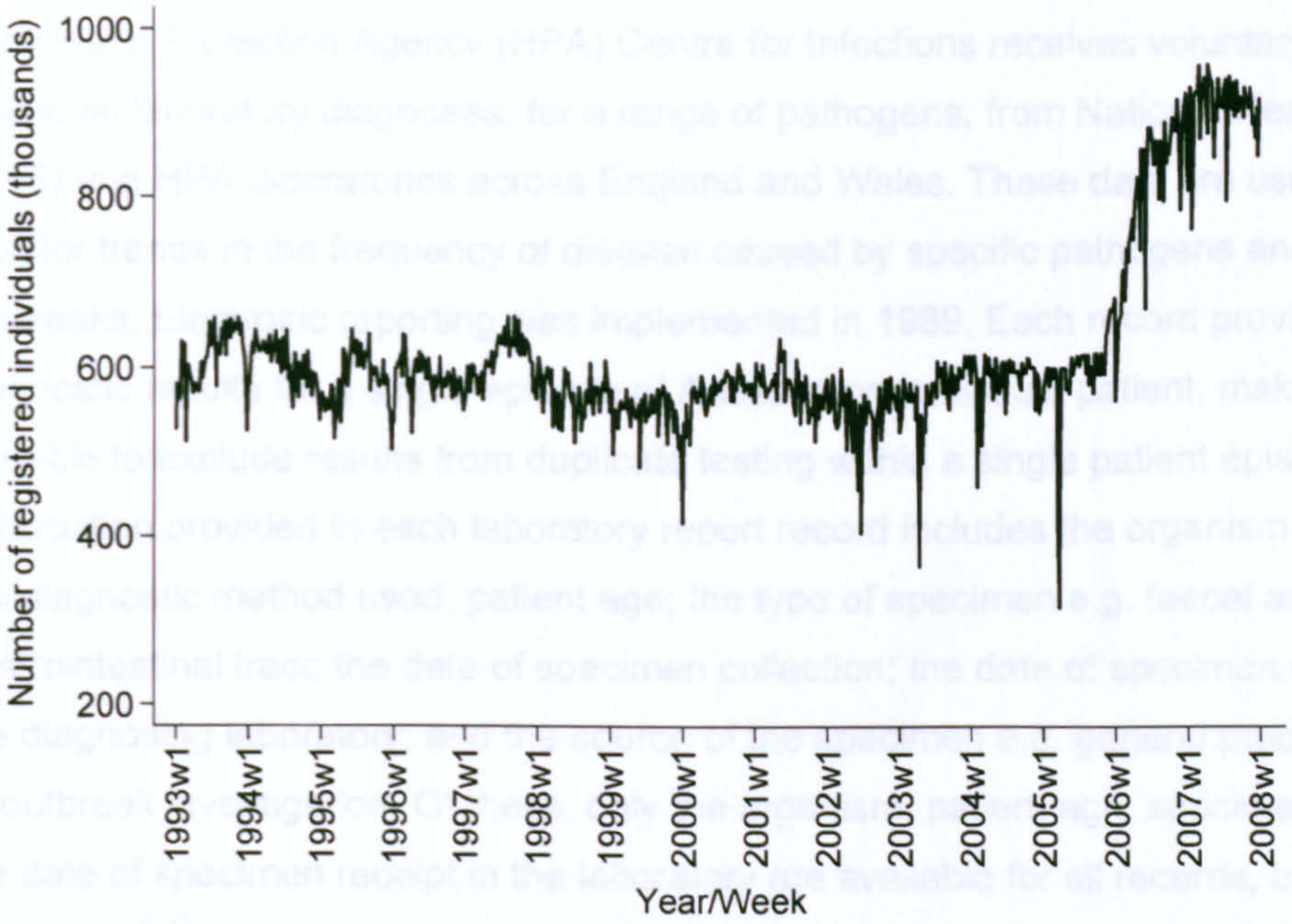
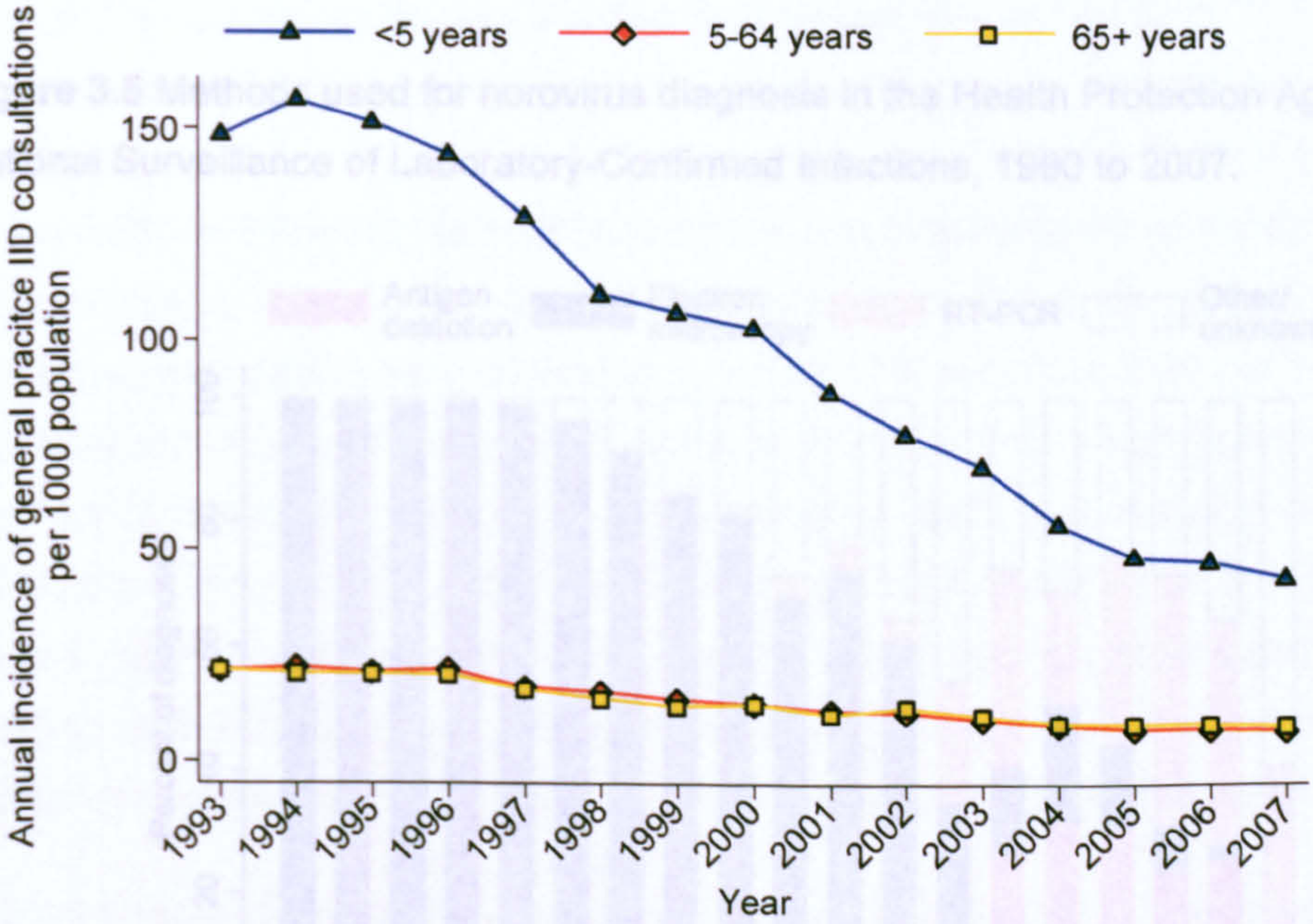


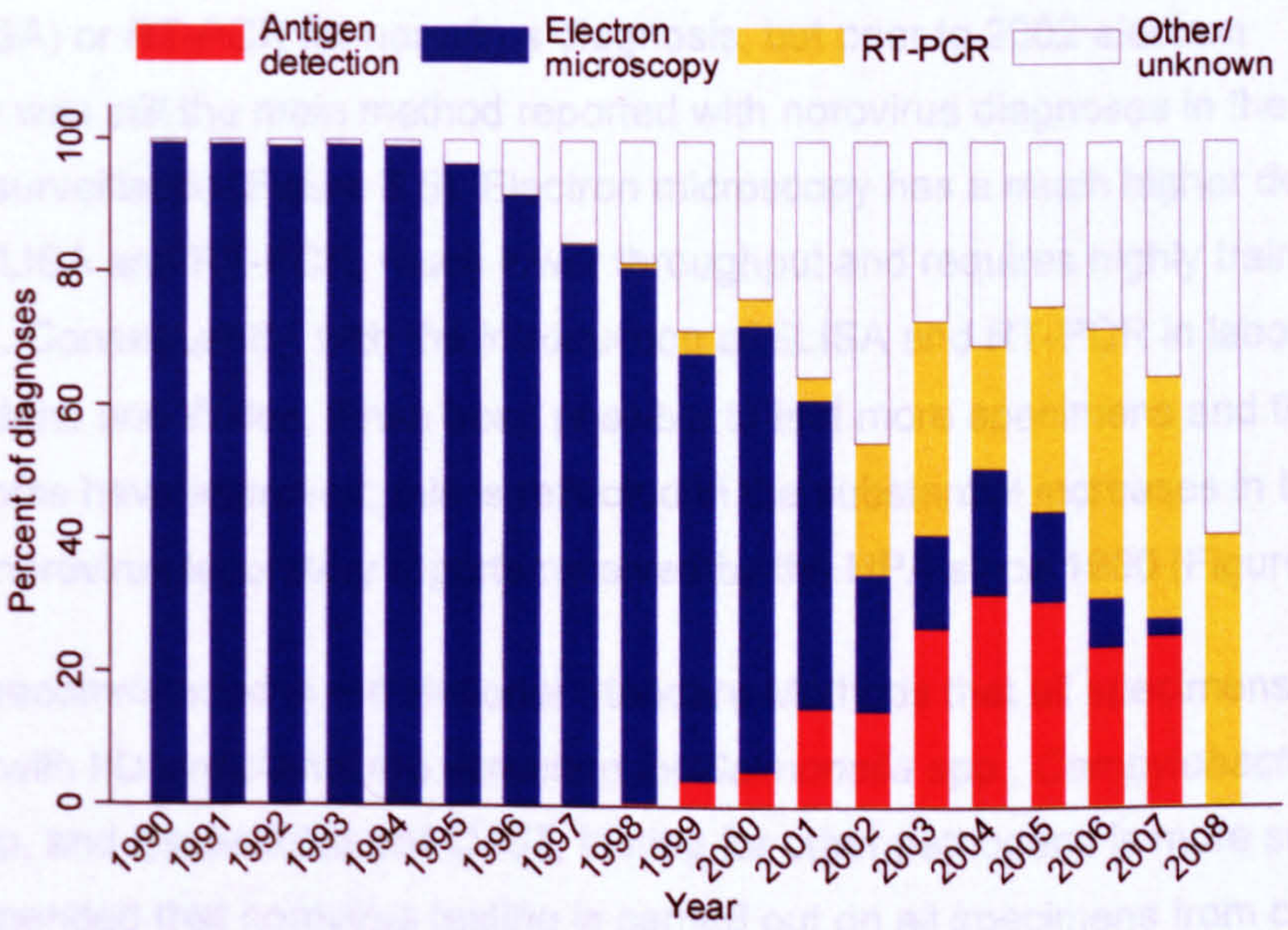
Figure 3.4 Annual incidence of general practice consultations for IID in the Royal College of General Practitioners surveillance scheme, 1993 to 2007.



3.3. Health Protection Agency National Surveillance of Laboratory-Confirmed Infections

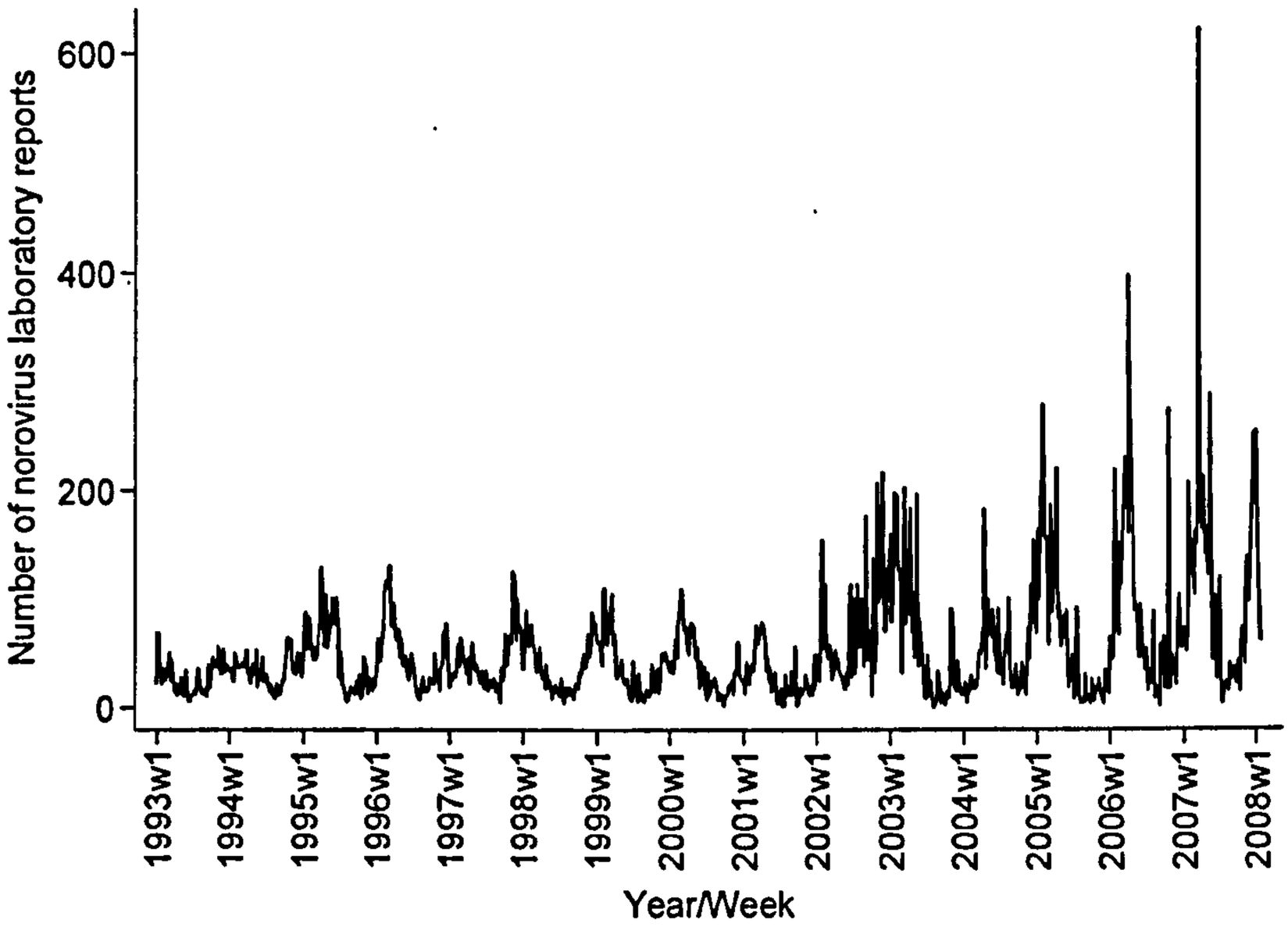
The Health Protection Agency (HPA) Centre for Infections receives voluntary reports of individual laboratory diagnoses, for a range of pathogens, from National Health Service (NHS) and HPA laboratories across England and Wales. These data are used to monitor trends in the frequency of disease caused by specific pathogens and to detect outbreaks. Electronic reporting was implemented in 1989. Each record provides diagnostic results for a single episode of illness in an individual patient, making it possible to exclude results from duplicate testing within a single patient episode. Information provided in each laboratory report record includes the organism identified; the diagnostic method used; patient age; the type of specimen e.g. faecal and lower gastrointestinal tract; the date of specimen collection; the date of specimen receipt at the diagnosing laboratory; and the source of the specimen e.g. general practice patient or outbreak investigation. Of these, only the organism, patient age, specimen type and the date of specimen receipt in the laboratory are available for all records, because of incomplete data entry by the laboratories submitting the reports. Reporting of all gastrointestinal pathogen diagnoses to the HPA national surveillance is non-mandatory, meaning that diagnoses are under-reported, with the degree of under-reporting varying between pathogens[83].

Figure 3.5 Methods used for norovirus diagnosis in the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections, 1990 to 2007.



Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction.

Figure 3.6 Weekly laboratory reports for norovirus in the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections, 1990 to 2007.



The HPA publishes recommended diagnostic methods and testing policies in the National Standard Methods, for HPA and NHS laboratories in the UK[486, 487]. The recommended methods and testing policies for the 19 common gastrointestinal pathogens included in the analysis in Chapter 7 are summarised in Table 3.5.The current National Standard Methods recommend use of enzyme-linked immunosorbent assay (ELISA) or RT-PCR for norovirus diagnosis, but prior to 2002 electron microscopy was still the main method reported with norovirus diagnoses in the HPA laboratory surveillance (Figure 3.5). Electron microscopy has a much higher detection limit than ELISA and RT-PCR, much lower throughput and requires highly trained technicians. Consequently, with the introduction of ELISA and RT-PCR in laboratories across England and Wales, it has been possible to test more specimens and the detection rates have improved; this is reflected in the substantial increases in the number of norovirus laboratory reports received by the HPA since 1990 (Figure 3.6).

Whilst it is recommended in the National Standard Methods that all specimens from individuals with IID symptoms are screened for *Salmonella* spp., *Campylobacter* spp,, *Shigella* spp, and *Escherichia coli* O157, testing for other pathogens is more selective. It is recommended that norovirus testing is carried out on all specimens from children

aged less than five years and adults aged 60 years and older, but only on specimens from children and adults aged between five and 64 years if they are part of a recognised IID outbreak (Table 3.5). In addition, the large number of norovirus outbreaks investigated in hospitals and other care settings each year in England and Wales, mean that the norovirus laboratory report data are dominated by specimens from outbreak investigations, rather than from patients with sporadic illness presenting to healthcare services. The problems arising from the under-representation of sporadic illness in the norovirus laboratory reports when using them to estimate the incidence of norovirus-associated IID are discussed further in Chapter 7.

Table 3.5 Health Protection Agency National Standard Methods[486, 487] – recommended diagnostic tests and testing policies for National Health Service and Health Protection Agency laboratories.

Pathogen	Recommended diagnostic method	Recommended testing policy
Campylobacter spp. *Salmonella* spp. *Shigella* spp.	Bacterial culture	All specimens
Escherichia coli O157	Bacterial culture	All specimens / outbreaks
Escherichia coli (other)	Bacterial culture	None specified
Cryptosporidium spp.	Microscopy	Clinician request / indicated by clinical details / Outbreak
Giardia spp.	Microscopy	Clinician request / indicated by clinical details
Adenovirus	Enzyme immunoassay or PCR (EM if negative)	Specimens from children < 5 years, adults >60 years, immuno-compromised, food handlers / Nosocomial outbreak
Astrovirus	Electron microscopy	
Sapovirus	Electron microscopy	
Rotavirus	Enzyme immunoassay or RT-PCR (EM if negative)	Specimens from children < 5 years, adults >60 years, immuno-compromised, food handlers / Nosocomial outbreak / Norovirus negative outbreaks in children <2 years and adults >60 years
Norovirus	Enzyme immunoassay or RT-PCR (EM if negative)	Specimens from children < 5 years, adults >60 years, immuno-compromised, food handlers / Outbreak
Clostridium perfringens *Bacillus* spp.	Bacterial culture, vegetative cell count, spore count (*Clostridium perfringens* only)	Outbreak specimen / Food poisoning (from clinical details)
Vibrio cholerae & Vibrio parahaemolyticus	Bacterial culture	Cholera symptoms / Seafood consumption / Recent travel to cholera area / Outbreak specimens
Staphylococcus aureus	Culture (vegetative cell count)	Food poisoning (clinical details)

Abbreviations: EM, electron microscopy; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; spp., species

Diagnostics

Chapter 4: Diagnosing norovirus-associated infectious intestinal disease using viral load

4.1. Background

The only existing estimates of the incidence of norovirus-associated IID in the community in England are based on electron microscopy testing of IID cases in the Study of Infectious Intestinal Disease. Whilst electron microscopy was widely used for norovirus diagnosis in microbiology laboratories across England and Wales in the mid-1990s[2], RT-PCR is now the method of choice for detecting norovirus in clinical specimens. RT-PCR detects norovirus at lower concentrations and is less affected by specimen quality and preparation than electron microscopy[110, 241, 243, 279, 280]; larger numbers of specimens can be tested simultaneously by RT-PCR, compared to the single throughput for electron microscopy; and electron microscopy requires highly trained and experienced technicians for optimal specimen preparation and virus identification[240, 242, 243], whereas PCR is a now a commonly used technique in diagnostic laboratories and is largely automated after the assays are developed[488, 489].

However, whilst the use of RT-PCR has improved the detection rate of norovirus amongst IID cases, it is now widely documented that a substantial proportion of healthy individuals are also positive for norovirus when tested by RT-PCR[5, 22-24, 43, 490], including 16% of the controls from the Study of Infectious Intestinal Disease specimen archive[21]. It is therefore unclear whether norovirus is actually causing illness in all IID cases who are positive by RT-PCR. It is possible that the norovirus infection is 'asymptomatic' in some IID cases, with another pathogen, detected or undetected, actually causing their symptoms. If RT-PCR positivity does not correlate well with norovirus-associated IID, it cannot be used alone to attribute illness to norovirus in IID cases. The poor diagnostic specificity of PCR, and the associated difficulties for clinical interpretation of test results, have been highlighted for other viral pathogens[491-493].

Volunteer studies and histopathological investigations of norovirus infection have demonstrated differences in faecal viral load between symptomatically and asymptomatically infected individuals[80] and that damage to the intestinal epithelium, caused by norovirus replication, may contribute to the mechanism of pathogenesis in norovirus infection[58-60]. It is therefore biologically plausible that IID symptoms may be a result of high viral loads and that it may be possible to use viral load to indicate where norovirus is causing illness in naturally occurring infections. The aim of this analysis

was to use norovirus faecal viral load measurements to determine when illness is attributable to norovirus in IID cases. Specific objectives were to: (i) describe and compare the distribution of norovirus viral load in IID cases, diagnosed by electron microscopy and RT-PCR, and in asymptomatic controls; (ii) compare norovirus viral load between community and general practice IID cases, and between children and adults, and examine the seasonality of norovirus viral load; (iii) use receiver-operating characteristic (ROC) analysis to select an appropriate cut-off in viral load to identify where norovirus is the cause of illness in IID cases; and (iv) examine the effects of patient age on selection of the cut-off.

4.2. Methods

4.2.1. Specimens and testing

Full details of recruitment and testing during the Study of Infectious Intestinal Disease were provided in Chapter 3. During the PCR retesting of the specimen archive, 2422 IID cases and 2205 controls were tested for norovirus by RT-PCR and of these 834 IID cases and 361 controls were positive for norovirus by RT-PCR. The analyses in this chapter were based on the 718 norovirus positive IID cases and 199 norovirus positive controls for whom a Ct value was determined using real-time RT-PCR. Information about other pathogens diagnosed in these IID cases and controls during the original study (by bacterial culture, ELISA or light microscopy) was also used in the ROC analysis. Cases from the community cohort and the general practice case-control study were analysed together, except in the descriptive analysis, where Ct values in cases from the two study components were compared. The genogroup of norovirus was identified from the genogroup-specific RT-PCR results, as described in Chapter 3.

The cycle threshold (Ct) value from the real-time RT-PCR was used as a proxy measure of faecal viral load. The Ct value is inversely proportional to the amount of virus present in the specimen, so the lower the Ct value the higher the faecal viral load. The real-time RT-PCR assay was run for 40 cycles, so the maximum possible Ct value for positive specimens was 39. Further description of the real-time RT-PCR process is provided in Appendix 1.1.

4.2.2. Descriptive analysis

The median Ct value and inter-quartile range were calculated for IID cases and controls and comparisons were made between the following groups using the rank-sum test in Stata 10[494]:

i. Electron microscopy positive cases, RT-PCR positive cases and controls;

ii. IID cases ascertained in the community cohort and in the general practice case-control study;

iii. Cases aged less than five years and cases aged five years or older.

The seasonality of norovirus Ct values was examined by comparing the median Ct value in IID cases and in controls across the months of the year.

4.2.3. Receiver-operating characteristic analysis

Receiver-operating characteristic (ROC) analysis was used to select a cut-off in the Ct values, to attribute disease to norovirus in IID cases. There is no gold standard test for diagnosing norovirus-associated IID. Microbiological and clinical characteristics were used to select suitable reference groups for the ROC analysis. The reference positive group must have Ct values representative of where norovirus is causing illness and the reference negative group must have Ct values representative of where norovirus is not causing any illness.

4.2.3.1. Reference positive groups

Three reference positive groups were selected and the ROC analysis was carried out separately using each group (Table 4.1). Reference Positive Group 1 included only IID cases who were norovirus positive by electron microscopy. The high viral loads required for detection by electron microscopy correspond to the levels of norovirus shedding during acute infection of experimentally inoculated volunteers and in individuals infected in point-source norovirus outbreaks[80, 99, 103, 106, 240]. The electron microscopy positive IID cases are therefore very likely to have IID caused by norovirus.

Table 4.1 Inclusion criteria for the ROC analysis reference groups.

Reference group	Inclusion Criteria
Reference Positive 1	1. IID 2. Norovirus detected by electron microscopy 3. Norovirus infection confirmed by RT-PCR
Reference Positive 2	1. IID 2. Norovirus detected by electron microscopy 3. Norovirus infection confirmed by RT-PCR Or 1. IID 2. Electron microscopy negative 3. Norovirus detected by RT-PCR 4. No other pathogen detected 5. Specimen collected within 3 days of symptom onset
Reference Positive 3	1. IID 2. Norovirus detected by electron microscopy and/or RT-PCR 3. Negative for *Campylobacter* spp., *Salmonella* spp. and *Shigella* spp. by bacterial culture and *Cryptosporidium* spp. by light microscopy (and rotavirus A by ELISA in children aged <5 years only)
Reference Negative 1	1. No history of IID in previous 3 weeks 2. Norovirus detected by RT-PCR
Reference Negative 2	1. IID 2. Norovirus detected by RT-PCR 3. Infection with *Salmonella* spp., *Campylobacter* spp. or *Shigella* spp. detected by bacterial culture or *Cryptosporidium* spp. detected by light microscopy (or rotavirus A by ELISA in children aged <5 years only)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IID, infectious intestinal disease; RT-PCR, reverse transcription-polymerase chain reaction.

In Reference Positive Group 2, IID cases who were electron microscopy negative and subsequently RT-PCR positive were included in addition to electron microscopy positive IID cases, providing that they had no other pathogens identified in their stool, by either the original diagnostic methods (bacterial culture, ELISA, light and electron microscopy) or PCR, and that they had collected a specimen early in their illness (less than three days after symptom onset) (Table 4.1). These restrictions on the presence additional infections and specimen collection timing were used to ensure that norovirus was the most likely cause of illness in these IID cases, so that their faecal viral loads were representative of acute symptomatic norovirus infection. This second reference positive group was selected to assess whether using only electron microscopy positive cases in Reference Positive Group 1 may have biased the cut-off to lower Ct values (higher viral loads); if the same cut-off was selected using Reference Positive Group 1 and Reference Positive Group 2, this suggests that using only electron microscopy positive cases in Reference Positive Group 1 did not bias the cut-off.

A third reference positive group was selected that does not use electron microscopy diagnosis as an inclusion criterion (Table 4.1). Electron microscopy is no longer used for routine norovirus diagnosis in clinical laboratories in the UK, so cannot be used to select a reference positive group in future work to validate the cut-offs developed here. However, it is important that clinical virology laboratories across the UK independently select Ct value cut-offs for their norovirus real-time RT-PCR assays, because there is substantial variability between UK virology reference laboratories in the Ct values produced from standard reference specimens[495]; the same cut-off may not be appropriate for all laboratories because of these differences in assay performance. In addition, if the cut-off based approach to diagnosing norovirus-associated IID is to be applied more widely in routine clinical diagnosis in other countries and with other real-time RT-PCR protocols, it is important for laboratories to be able to independently validate the cut-off selected here. Reference Positive Group 3 included IID cases who were RT-PCR positive for norovirus (including those previously positive by EM) and were negative for other bacterial, protozoal and viral pathogens that are routinely detected in clinical diagnostic algorithms for sporadic IID in National Health Service and Health Protection Agency laboratories in the UK[486, 496] (Table 4.1). This restriction was used to make norovirus the most likely cause of illness in these IID cases, so that their Ct values were representative of symptomatic norovirus infection. Furthermore, because this third reference group is not reliant on electron microscopy diagnosis, it serves as another check of the sensitivity of the cut-off to the use of electron microscopy positive cases in Reference Positive Group 1.

4.2.3.2. Reference negative groups

Two reference negative groups were selected to have Ct values representative of where norovirus is not causing illness (Table 4.1). Reference Negative Group 1 included norovirus-infected healthy controls (infection was defined as any RT-PCR positive result). Reference Negative Group 2 included norovirus infected IID cases with a bacterial infection diagnosed by culture, or rotavirus infection diagnosed by ELISA (for children aged less than five years only). Bacterial culture without enrichment may indicate the presence of high concentrations of viable bacterial cells, meaning that the bacteria detected are likely to be causing illness. Similarly, rotavirus ELISA has a high detection limit that correlates well with disease[497, 498], meaning that rotavirus is probably the cause of illness in ELISA-positive individuals. Therefore IID cases positive for these other pathogens by bacterial culture or ELISA are likely to have norovirus Ct values representative of where norovirus is not causing any illness. This second reference negative group was selected to explore whether it is suitable for identifying a cut-off in conjunction with Reference Positive Group 3, because specimens from healthy controls are not routinely received in clinical laboratories, so cannot be used as the reference negative group if clinical virology laboratories are to use this method to develop a cut-off for their real-time norovirus RT-PCR assays.

4.2.4. Cut-off selection

The diagnostic sensitivity and specificity were calculated for each potential cut-off in the range of Ct values, by comparison to the reference group classification, and an empirical ROC plot created using Stata 10[494]. Further explanation of the production and interpretation of a ROC curve is provided in Appendix A2.1.The Youden index (sensitivity + specificity-1) was calculated at each Ct value and the maximum Youden index was used to identify the optimal Ct value cut-off[499-501].

It may not be valid to directly compare the Ct values between the two norovirus genogroups because of differences in the efficiency of the genogroup specific real-time RT-PCR assays. A particular Ct value may represent a different underlying norovirus concentration in the stool specimen for the two genogroups, meaning that the same cut-off may not be appropriate. Therefore, the ROC analysis was carried out separately for norovirus genogroup I and genogroup II.

Due to the smaller numbers of genogroup I noroviruses, the ROC analysis was carried out only using Reference Positive Group 1 and Reference Negative Group 1, for all age

groups together. For genogroup II noroviruses, the ROC analysis was carried out using all of the reference groups shown in Table 4.1. The genogroup II ROC analysis using Reference Positive Group 1 and Reference Negative Group 1 was repeated separately for children aged less than five years and for children and adults aged five years or older.

4.3. Results

4.3.1. Descriptive analysis

Ct values were generated for 718 IID cases and 199 healthy controls; 119 (17%) of the IID cases were previously positive by electron microscopy and 597 (83%) were negative by electron microscopy but subsequently positive by RT-PCR. IID cases were aged up to 94 years and controls up to 84 years; 40% of IID cases and 60% of controls were aged less than five years. Approximately 80% of both IID cases and controls were infected with norovirus genogroup II (Table 4.2).

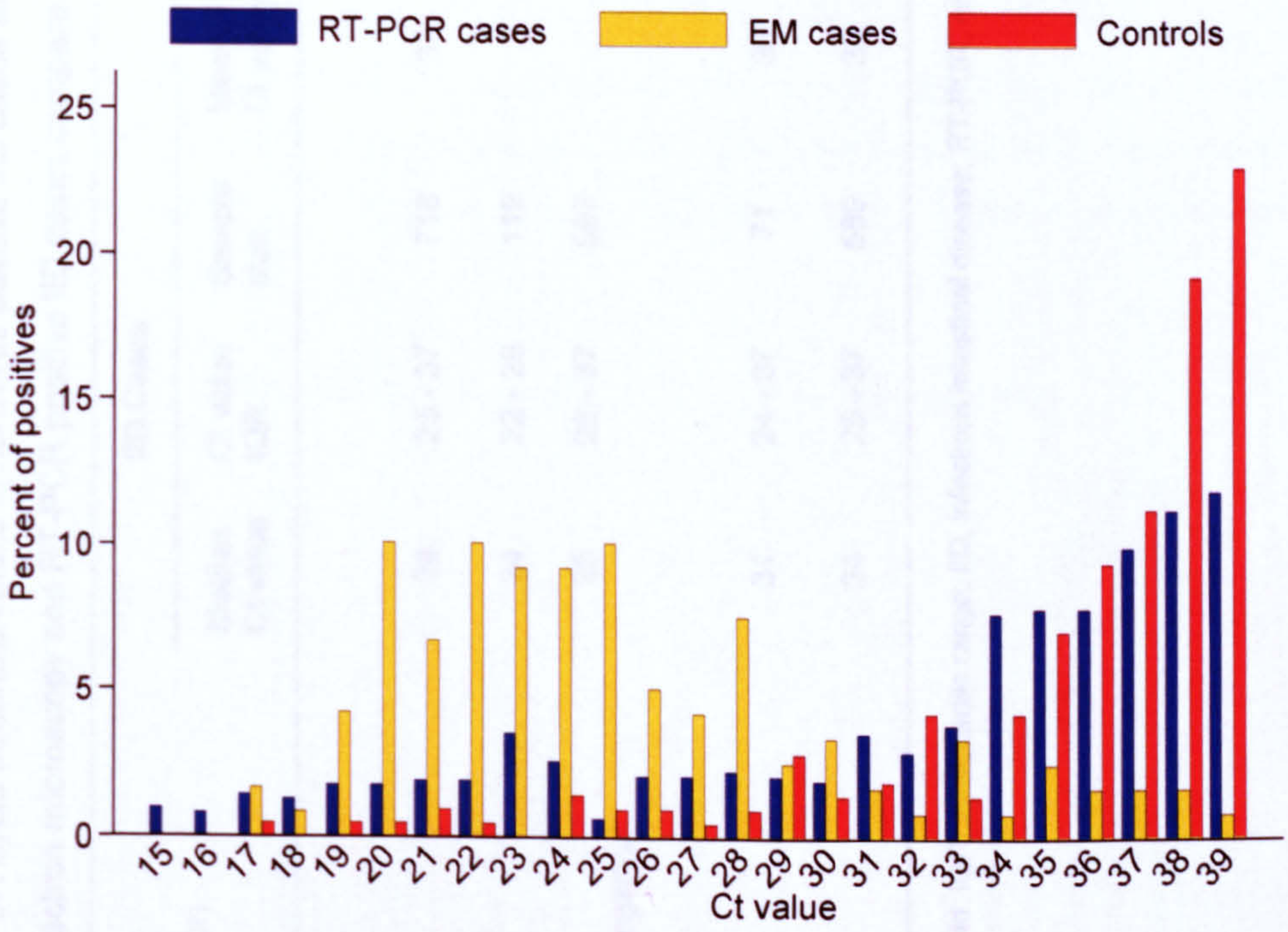
Table 4.2 Norovirus genogroup detected in IID cases and controls with real-time RT-PCR cycle threshold values determined.

		Number[i] (percent)			Total
		Genogroup I	Genogroup II	Mixed genogroup I & genogroup II	
IID cases	All	71 (10)	589 (82)	56 (8)	718
	<5 years	19 (6.5)	255 (88.5)	14 (5)	288
	≥5 years	52 (12)	334 (78)	42 (10)	430
Controls	All	18 (9)	159 (80)	22 (11)	199
	<5 years	14 (11.5)	92 (77)	14 (11.5)	120
	≥5 years	4 (5)	67 (85)	8 (10)	79

[i]The norovirus genogroup was not recorded for two IID cases.

The median Ct value was lower in IID cases (median 34) than in controls (median 37) (Table 4.3). The difference compared to controls was greatest for IID cases positive by electron microscopy (median 24) (Table 4.3); there was very little overlap in the distribution of Ct values in electron microscopy positive IID cases and controls (Figure 4.1). The distribution of Ct values for the IID cases who were negative by electron microscopy and subsequently RT-PCR positive (median 35) overlapped substantially with the controls, although a small proportion had the higher viral loads seen in the electron microscopy positive IID cases (Figure 4.1, Table 4.3). The pattern was the same in children and adults (Appendix A2.2) and in genogroup I and genogroup II infections (Table 4.3, Appendix A2.3).

Figure 4.1 Real-time RT-PCR cycle threshold values in IID cases and controls. 'EM cases' are IID cases positive by electron microscopy, 'RT-PCR cases' are IID cases negative by electron microscopy and subsequently positive by RT-PCR. Sample sizes: EM cases = 119, RT-PCR cases = 597, controls = 199.



Abbreviations: Ct, cycle threshold; EM, electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction.

Table 4.3 Real-time RT-PCR cycle threshold values in norovirus positive IID cases and controls.

The rank-sum tests for electron microscopy and RT-PCR positive IID cases compare them to all controls.

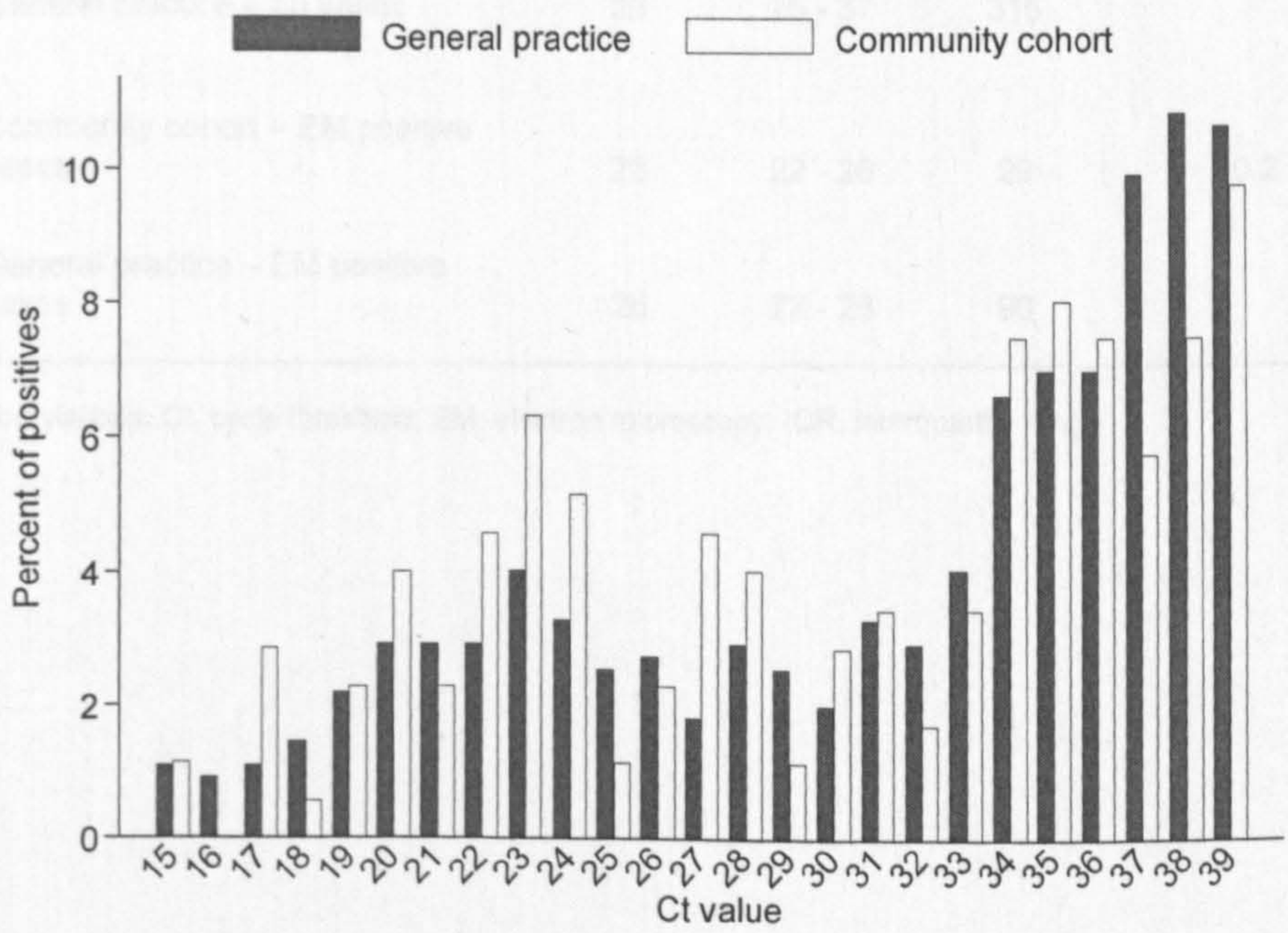
Method of norovirus detection	IID Cases			Controls			Rank-sum test *P* value comparing cases to controls
	Median Ct value	Ct value IQR	Sample size	Median Ct value	Ct value IQR	Sample size	
All ages & genogroups							
All	34	25 - 37	718	37	34 - 39	199	<0.001
Electron microscopy	24	22 - 28	119				<0.001
RT-PCR (Electron microscopy negative)	35	28 - 37	597				<0.001
Genogroup I	31	24 - 37	71	36	31 - 38	18	<0.001
Genogroup II	34	25 - 37	589	38	35 - 39	159	<0.001

Abbreviations: Ct, cycle threshold; IQR, interquartile range; IID, infectious intestinal disease; RT-PCR, reverse transcription-polymerase chain reaction.

Whilst the median Ct values for both IID cases and controls were slightly lower in genogroup I compared to genogroup II there was no strong statistical evidence of a difference in the distribution of the Ct values (rank-sum *P*: IID cases=0.2; controls=0.07). Similarly, there was no strong evidence of a difference in Ct value distribution between IID cases aged less than five years and IID cases aged five years and older (Table 4.4, Appendix A2.4), or between IID cases ascertained in the community cohort and those from the general practice case-control study (Table 4.4, Figure 4.2). However, the median Ct value was lower in children aged less than five years in the community cohort compared to those in the general practice case-control study (Table 4.4).

The median Ct value in IID cases ranged from 30, in September, to 36 in February, although there was no clear seasonal pattern (Appendix A2.5). Similarly, the median Ct value in controls varied between 36 and 39 throughout the year, but there was no seasonal pattern (Appendix A2.5).

Figure 4.2 Real-time RT-PCR cycle threshold values in IID cases from the community cohort and general practice case-control study. Dark grey bars show IID cases from the community cohort (n=174); white bars show IID cases from the general practice case-control study (n=544).



Abbreviations: Ct, cycle threshold.

Table 4.4 Comparison of real-time RT-PCR cycle threshold values in IID cases (children vs. adults, community cohort vs. general practice case-control study.) The rank-sum tests compare child cases (<5 years) to older children and adults (aged 5 years and older) and compare community cohort cases to general practice cases.

Group of cases	Median Ct value	Ct value IQR	Sample size	Rank-sum test *P* value comparing case groups
Children < 5 years	34	25 - 37	288	0.8
Children & adults ≥5 years	33	25 - 37	430	
Community cohort – all cases	32	24 - 36	174	0.08
General practice – all cases	34	25 - 37	544	
Community cohort – <5 years	31	22 - 35	59	0.02
General practice – <5 years	34	26 - 37	229	
Community cohort – ≥5 years	33	24 - 37	115	0.6
General practice – ≥5 years	33	25 - 37	315	
Community cohort – EM positive cases	23	22 - 26	29	0.2
General practice – EM positive cases	25	22 - 28	90	

Abbreviations: Ct, cycle threshold; EM, electron microscopy; IQR, interquartile range

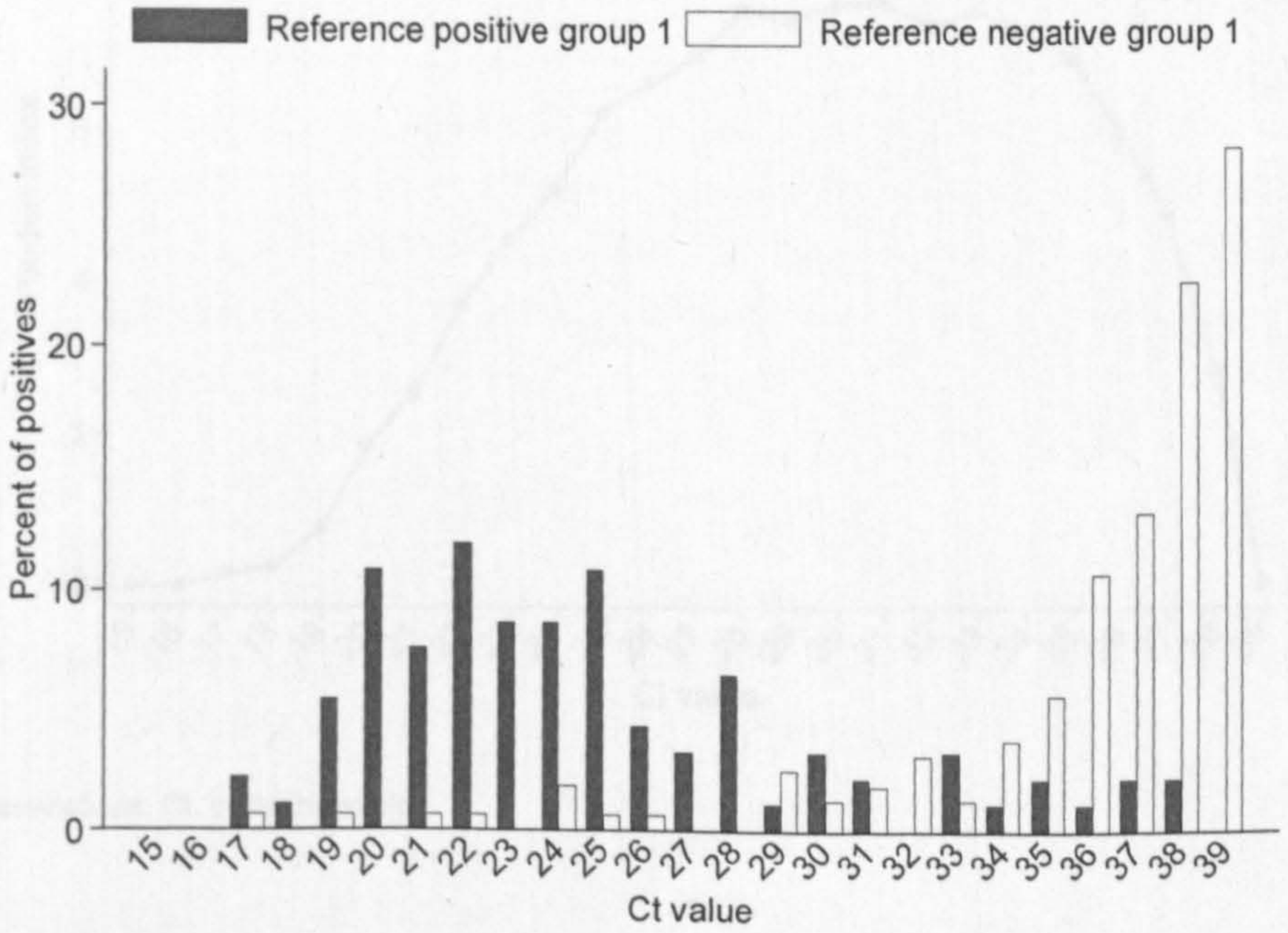
4.3.2. ROC analysis

The numbers of IID cases and controls meeting the inclusion criteria for each of the reference groups are shown in Table 4.5. The Ct value distributions for each of the reference groups, the Youden index values and the ROC curves for each ROC analysis are provided in Appendices A2.6 to A2.10.

4.3.2.1. Optimal reference groups - Reference Positive Group 1 and Reference Negative Group 1

The optimal cut-off for attributing illness to genogroup II noroviruses in IID cases was at Ct value 31 (Table 4.5), corresponding to the maximum Youden index for the ROC analysis with Reference Positive Group 1 and Reference Negative Group 1 (Figure 4.4 and Figure 4.5). Using this cut-off, IID cases with norovirus genogroup II Ct values of 31 or below are classified as 'positive' for norovirus-associated IID: they have disease caused by norovirus. IID cases with Ct values above 31 are classified as 'negative' for norovirus-associated IID: they have IID but their norovirus infection was not the cause of their symptoms.

Figure 4.3 Real-time RT-PCR cycle threshold values in reference positive group 1 and reference negative group 1 for genogroup II ROC analysis, all ages. Grey bars show reference positive group 1 (n=92); white bars show reference negative group 1 (n=159).



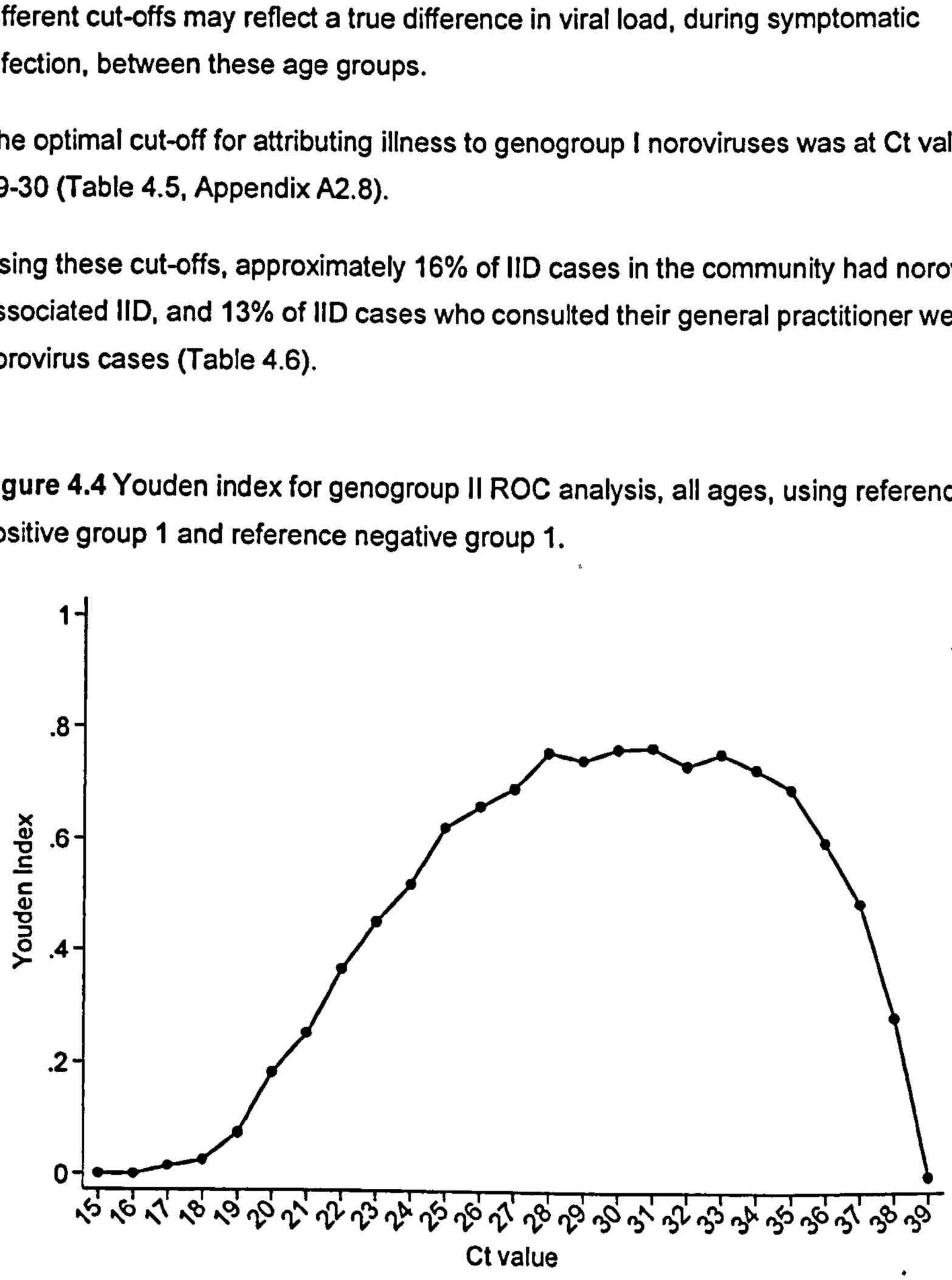
Abbreviations: Ct, cycle threshold.

The optimal cut-off for genogroup II in children aged less than five years was at Ct value 30, whereas for older children and adults it was at Ct value 33 (Table 4.5, Appendices A2.6 & A2.7). There was some evidence of a difference in Ct value distribution between electron microscopy positive IID cases in these two age groups (rank-sum test p=0.036), with the median in children aged less than five years at Ct value 23 and at Ct value 25 for older children and adults. This indicates that the different cut-offs may reflect a true difference in viral load, during symptomatic infection, between these age groups.

The optimal cut-off for attributing illness to genogroup I noroviruses was at Ct values 29-30 (Table 4.5, Appendix A2.8).

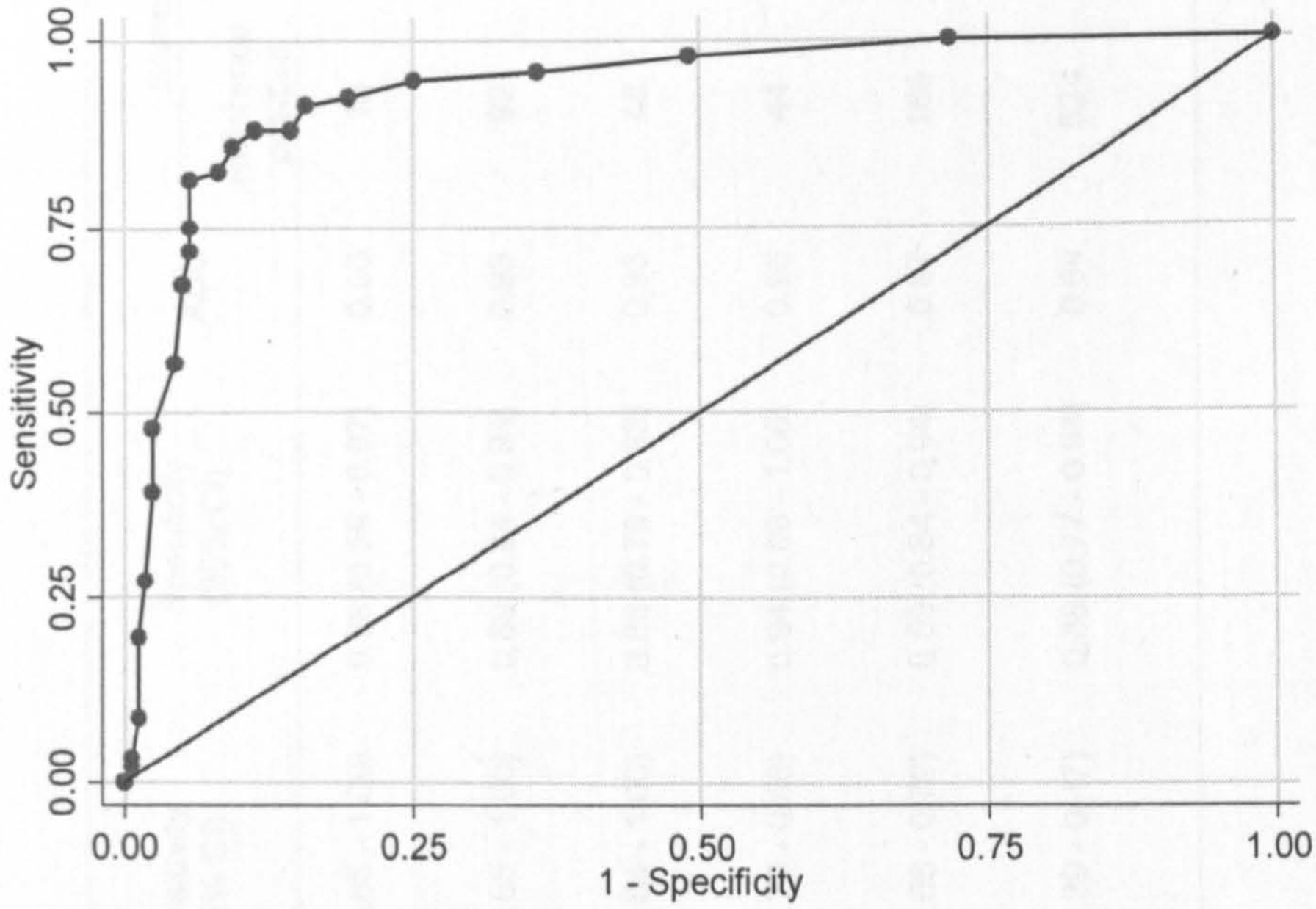
Using these cut-offs, approximately 16% of IID cases in the community had norovirus-associated IID, and 13% of IID cases who consulted their general practitioner were norovirus cases (Table 4.6).

Figure 4.4 Youden index for genogroup II ROC analysis, all ages, using reference positive group 1 and reference negative group 1.



Abbreviations: Ct, cycle threshold.

Figure 4.5 ROC plot for genogroup II ROC analysis, all ages, using reference positive group 1 and reference negative group 1.



4.3.2.2. Alternative reference groups

The optimal genogroup II cut-off (all ages) was also at Ct value 31 when RT-PCR positive cases with no other pathogen detected and early specimen collection were included in the reference positive group (Reference Positive Group 2) (Table 4.5, Appendix A2.9). The optimal genogroup II cut-off was also at Ct value 31 when norovirus positive IID cases who were negative for other commonly tested enteric pathogens were used as the reference positive group (Reference Positive Group 3), and the bacterial culture positive IID cases were used as the reference negative group (Reference Negative Group 2) (Table 4.5, Appendix A2.10).

4.3.2.3. Discriminatory power of Ct values

In the analysis of both genogroup I and genogroup II norovirus infections, the Ct values discriminated well between Reference Positive Group 1 and Reference Negative Group 1, because the area under the ROC curve was close to the maximum value of one (Table 4.5, Figure 4.5). This is consistent with the highly separated distributions of Ct values in these reference groups (Figure 4.3).The discriminatory power of the Ct values was poorer for the ROC analysis where RT-PCR positive cases with no other pathogen

Table 4.5 ROC analysis results.

The reference groups are described in Table 4.1.

Norovirus genogroup	Reference groups	Age group	Optimal Ct cut-off	Youden Index	Sensitivity (95% CI)	Specificity (95% CI)	AUC	Sample size	
								Reference positive	Reference negative
Genogroup I	Ref positive 1 Ref negative 1	All	29-30	0.62	0.85 (0.65 - 1.00)	0.78 (0.59 - 0.97)	0.90	13	18
Genogroup II	Ref positive 1 Ref negative 1	All	31	0.77	0.88 (0.65 - 1.00)	0.89 (0.84 - 0.94)	0.93	92	159
		<5 years	30	0.80	0.94 (0.84 - 1.00)	0.86 (0.79 - 0.93)	0.93	48	92
		≥5 years	33	0.83	0.89 (0.79 - 0.98)	0.94 (0.88 - 1.00)	0.96	44	67
Genogroup II	Ref positive 2 Ref negative 1	All	31	0.61	0.72 (0.66 - 0.79)	0.89 (0.84 - 0.94)	0.87	169	159
Genogroup II	Ref positive 3 Ref negative 2	All	31	0.29	0.43 (0.39 - 0.47)	0.86 (0.77 - 0.94)	0.64	524	64

Abbreviations: AUC, area under the curve; Ct, cycle threshold; CI, confidence interval

detected and early specimen collection were included in the reference positive group (Reference Positive Group 2, genogroup II only) because the AUC was reduced to 0.87 (Table 4.5, Appendix A2.9). The discriminatory power was very low for distinguishing between Reference Positive Group 3 and Reference Negative Group 2 (genogroup II only) because the area under the curve was close to 0.5 (Table 4.5, Appendix A2.10), which is indicative of a test with no discriminatory power.

Table 4.6 Percent of IID cases classified as norovirus cases using different diagnostic methods: (1) all electron microscopy positive IID cases; (2) all electron microscopy or RT-PCR positive IID cases; (3) IID cases with a Ct value at or below the age group specific cut-offs.

	Community cohort			General practice			All
	Children aged <5 years (n=120)	Children & adults aged ≥5 years (n=397)	All (n=517)	Children aged <5 years (n=522)	Children & adults aged ≥5 years (n=1383)	All (n=1905)	(n=2422)
(1) EM	10.8	6.0	7.2	10.3	4.6	6.1	6.4
(2) EM and/or RT-PCR	55.8	36.3	40.8	45.8	27.8	32.7	34.4
(3) Ct value at or below cut-off[ii]	20.0	15.4	16.4	14.2	11.3	12.1	13.0

Abbreviations: EM, electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction.

[i] Ct values were available for 86% of IID cases positive for norovirus by gel-based RT-PCR; these proportions are not adjusted for these additional cases who could not be classified using the Ct value cut-off

[ii] At Ct value 30 for children aged <5 years and Ct value 33 for older children and adults aged ≥5 years with genogroup II norovirus infection and at Ct value 30 for IID cases of all ages with genogroup I infection.

4.4. Discussion

In this analysis, the difference in viral load between naturally occurring symptomatic and asymptomatic norovirus infection was described. Whilst healthy controls tended to have lower norovirus viral loads than IID cases, a substantial proportion of IID cases who were only norovirus positive by RT-PCR had viral loads equivalent to those in healthy controls. This supports the hypothesis that norovirus is probably not always the cause of illness where it is detected by RT-PCR. ROC analysis was used to select an appropriate cut-off in viral load (real-time RT-PCR Ct values) for attributing disease to norovirus in IID cases. The optimal cut-off for genogroup II noroviruses was at Ct value 31, with a slightly lower cut-off (Ct value 30) selected in young children and a slightly higher cut-off (Ct value 33) selected in older children and adults. The optimal cut-off for genogroup I noroviruses was at Ct value 29 to 30.

Using these cut-offs, approximately one third (30%) of the IID cases originally negative by electron microscopy, but positive by RT-PCR, would be classified as cases of norovirus disease, indicating that electron microscopy significantly under-diagnosed norovirus cases in the Study of Infectious Intestinal Disease. This is in contrast to the results of a similar analysis conducted for rotavirus[497] (Appendix 7.1). ELISA was used for rotavirus diagnosis during the Study of Infectious Intestinal Disease, and indeed is still widely used for diagnosing rotavirus-associated IID; the WHO recommend the use of ELISA diagnosis in baseline burden-of-disease studies prior to introduction of rotavirus vaccines[502]. The analysis of rotavirus viral load showed that ELISA positivity correlates extremely well with rotavirus disease, based on the relative distribution of Ct values in IID cases and controls; no IID cases with rotavirus infection diagnosed only by RT-PCR had the high viral loads seen in ELISA positive IID cases. The difference in correlation with viral IID between electron microscopy and ELISA probably reflects the greater dependence of a positive electron microscopy diagnosis on specimen quality and preparation, in addition to viral load, which affects diagnosis by either method[13, 240, 241, 243, 245, 246].

A major strength of the data generated from the Study of Infectious Intestinal Disease specimen archive retesting is the availability of specimens from healthy controls. There are few community studies of IID with large control groups available, but they are essential for interpreting the RT-PCR results in IID cases. Norovirus is detected at such high prevalence in healthy individuals by RT-PCR that simple detection in IID cases may not be sufficient to give a confident diagnosis of norovirus-associated IID. It is essential that the results of norovirus RT-PCR assays can be appropriately interpreted,

to increase the specificity of diagnosis, both in clinical diagnostic services for individual patients and for generating accurate population-level estimates of norovirus disease burden. The Ct value cut-off provides a major improvement in specificity compared to the current qualitative use of RT-PCR in norovirus diagnosis because by using the Ct value cut-off it is possible to exclude IID cases who are unlikely to have disease caused by norovirus.

It is inevitable that there will be some misclassification of individual IID cases who have norovirus Ct values close to the cut-off, especially because the Youden index did not show a distinct peak in the ROC analyses, with similar index values across a range of Ct values around the selected cut-offs (Figure 4.4, Appendices A2.6 to A2.10). However, in population-level studies of norovirus-associated IID incidence, a small amount of misclassification will have little impact on the overall estimates because the number of misclassified cases on either side of the cut-off is likely to be similar. It may also be possible to incorporate the uncertainty in the cut-off into the incidence estimate. In a clinical setting, where the focus is on diagnosing the cause of illness to guide individual patient care, it is important that other clinical and epidemiological information is also considered in the diagnosis of disease aetiology, especially for patients with norovirus Ct values close to the cut-off, to prevent misdiagnosis based solely on viral load.

The specimens from the Study of Infectious Intestinal Disease archive were originally collected during the mid-1990s and the viral RNA may have degraded during the prolonged storage and repeated freeze-thaw cycles for retesting. Therefore the cut-off developed here may not be directly applicable to real-time RT-PCR results from fresh specimens without further validation. Similarly, the cut-off should not be applied to assays with different protocols, because the Ct values may not equate to the same viral load per gram of stool. It is unlikely, however, that there will have been differential degradation of RNA between specimens during storage, so it is still valid to compare the viral load between specimens in this collection, and to assume that the relative differences observed between IID cases and controls are a true reflection of symptomatic and asymptomatic infection. Indeed, the differences in viral load between the natural symptomatic and asymptomatic norovirus infections described in this analysis confirm observations from experimental volunteer inoculation studies[80].

Converting the Ct values into actual viral loads per gram of stool would provide cut-offs that can be used with different real-time RT-PCR protocols, but there are a number of limitations to this approach: (1) generation of standard curves for the conversion of Ct

values into actual viral loads is time-consuming and resource-intensive and needs to be regularly repeated in case of any changes in assay protocol or conditions; (2) whilst the efficiency of the real-time PCR has been characterised (Gray, J. personal communication), the exact efficiency of the extraction and reverse transcription steps are more difficult to quantify[260, 261], but will greatly affect the relationship between Ct values and viral loads; (3) conversion to viral loads will not remove the need for independent verification of the cut-offs because of the possibility of partial RNA degradation during storage, as described above. Therefore, the cut-offs have been specified using Ct values, which is sufficient for further analysis of the data from the Study of Infectious Intestinal Disease in this thesis, and other clinical laboratories are encouraged to independently develop cut-offs for their own assays.

In order to facilitate independent validation of the cut-offs by clinical diagnostic laboratories, alternative reference groups were selected that did not use specimens from healthy controls or electron microscopy diagnosis. The same all-age genogroup II cut-off was selected using both the optimal reference groups (electron microscopy positive IID cases and healthy controls) and these alternative reference groups. The area under the ROC curve for the alternative reference groups was very low, possibly because the viral loads in many of the IID cases in Reference Positive Group 3 were not representative of symptomatic norovirus infection; this is reflected in the low sensitivity for the cut-off using Reference Positive Group 3 (Table 4.5). Despite this low discriminatory power, the same cut-off was selected using the alternative reference groups, indicating that these are appropriate selection criteria for future studies without a control group or electron microscopy testing.

The cut-off in viral load should only be applied to specimens collected from IID patients during acute symptoms, when the viral load is representative of disease aetiology. After symptoms resolve in norovirus-associated IID the viral load quickly drops to levels seen in asymptomatic infection[80]. At the population level, the predictive value of the cut-off will decrease as the proportion of specimens collected after symptom resolution increases, because the false-negative rate will increase. However, this problem is not specific to the norovirus Ct value cut-off; the predictive value of any diagnostic test that directly detects the target pathogen in a specimen will be affected by the timing of specimen collection[503].

Whilst genotype-specific differences in the efficiency of the norovirus real-time RT-PCR assay have been identified (see Chapter 3), the norovirus specimens from the Study of Infectious Intestinal Disease archive have not been genotyped. It was therefore only

possible to take account of broad differences between the genogroups in the underlying viral load represented by specific Ct values. However, there is substantial variation in assay efficiency between genotypes within norovirus genogroup I (Appendix A1.3), meaning that the degree of misclassification in genogroup I may be high and it may not be appropriate to use the cut-off identified here for classification of disease aetiology in individual IID cases. There were also a very small number of genogroup I specimens meeting the inclusion criteria for the ROC analysis reference groups, meaning that there is greater uncertainty in the cut-off selected for genogroup I. The real-time RT-PCR assay also has poorer efficiency (higher detection limit) for two of the rarer genotypes in genogroup II (GII-7 and GII-8) (Appendix A1.3); however, at a population level, the degree of misclassification is likely to be small because of the low prevalence of GII-7 and GII-8[206, 504, 505] and the misclassification would be conservative, i.e. IID cases with disease caused by these two genogroups of norovirus would be misclassified as having disease caused by another pathogen. The distribution of norovirus genogroups was very similar in IID cases and healthy controls, meaning that the difference in assay efficiency between the genogroups is not responsible for the differences in viral load between IID cases and controls.

The causal relationship between disease symptoms and norovirus viral load has not been established. However, if the relationship between the occurrence of disease and viral load is consistent, regardless of whether high viral loads are a cause or a consequence of disease, viral load will be a good marker of norovirus-associated IID and the approach developed here is valid. Viral load is routinely used to predict outcome and guide clinical management for a number of viruses that cause chronic infections, such as Epstein-Barr virus[506] and cytomegalovirus[507] in transplant patients, HIV[508], hepatitis C[509] and Human T-Lymphotrophic Virus [510]. However this is the first time that viral load has been used to diagnose enteric viruses as the cause of acute IID. The method may also be useful for viral pathogens linked to other disease syndromes, such as acute respiratory infections, for which the same problems with the interpretability of PCR have been described[492].

4.5. Summary

Examination of norovirus viral load in IID cases and controls from the Study of Infectious Intestinal Disease has demonstrated that it is unlikely that all IID cases with norovirus detected by RT-PCR actually have disease caused by norovirus. The difference in viral load between IID cases and controls has been used to select an appropriate cut-off for attributing disease to norovirus in IID cases. Using the viral load

measurements and the cut-off, there was a substantial increase in the number of IID cases with norovirus-associated IID compared to electron microscopy diagnosis in the original study. It is therefore important to update the estimates of norovirus-associated IID incidence from the Study of Infectious Intestinal Disease, using this new diagnostic method and to re-examine risk factors for norovirus-associated IID using the larger and more representative group of cases. This work is described in subsequent chapters. In the next chapter, the characteristics of both the norovirus-associated IID cases and the asymptomatic norovirus infections in healthy controls are described.

Chapter 5: Characteristics of symptomatic and asymptomatic norovirus infection

In Chapter 4, norovirus viral load measurements were compared between IID cases and healthy controls, to select a cut-off for identifying IID cases with disease caused by norovirus. In this chapter the characteristics of the norovirus cases identified using the Ct value cut-off are described and the prevalence and significance of co-infection with other pathogens is examined. The prevalence and characteristics of asymptomatic norovirus infections are also described.

5.1. Background

5.1.1. Characteristics of norovirus-associated IID

The predominant symptoms of norovirus-associated IID are diarrhoea and vomiting. A high prevalence of vomiting amongst cases is a distinguishing characteristic of norovirus outbreaks[62, 511] and a higher frequency of vomiting is reported amongst young children with norovirus-associated IID compared to older children and adults[84, 85, 87]. Other frequently reported symptoms include nausea, abdominal pain, muscle ache, headache and fever[60, 62, 84, 92, 94, 104, 108, 414, 512]. Symptoms last for 24 to 48 hours in otherwise healthy adults[60, 84, 85, 92, 94, 414], but many studies report longer duration of symptoms in young children and the elderly[92, 93, 104, 108, 512, 513]. One study has suggested that viral loads may be correlated with the duration of symptoms[102].

Occasionally, more severe symptoms and some deaths may occur in very young children and the elderly with norovirus-associated IID[15, 16, 16-18, 89, 90, 338]. A recent systematic review indicated that up to 30% of children presenting to hospital emergency services with acute IID around the World may be infected with norovirus[49], and it is estimated that norovirus may cause up to 3000 hospitalizations amongst the elderly in the UK each year[339]. Several studies have reported that children presenting to hospital with norovirus-associated IID suffer symptoms of comparable severity to children with rotavirus-associated IID[86, 88].

5.1.2. Characteristics of asymptomatic norovirus infections

Norovirus infection has been identified in a substantial proportion of individuals with no IID symptoms in several community-based studies, with crude prevalences of up to 16% reported in high income countries[5, 21, 22, 24]. Volunteer studies have also

demonstrated the occurrence of norovirus infection with no concurrent IID after experimental inoculation[56, 75, 77-80, 82]. Whilst these volunteer individuals experienced no diarrhoea or vomiting, some reported other symptoms such as headache, fever, muscle ache, abdominal pain and nausea.

5.1.3. Gastrointestinal pathogen co-infections

Co-infection with two or more gastrointestinal pathogens is frequently reported from studies of IID, in both high and low income countries[345, 514-532]. Whilst some studies have reported potentially poorer clinical outcomes in individuals infected with multiple pathogens[533, 534], the epidemiological relevance of these co-infections remain undetermined. Co-infections may be caused by epidemiological mechanisms, such as common transmission routes, or by host-level biological factors leading to increased susceptibility to infections. However, many gastrointestinal pathogens are commonly found infecting healthy individuals as well as individuals with IID[21]; with such high population prevalence, co-infections may occur by chance alone. It is therefore important to use statistical methods to examine whether particular pairs of pathogens are found co-infecting more often than expected by chance alone, although few studies have used such an approach[535]. The null hypothesis in such statistical analyses is that the pathogens are independently distributed across individuals in the study population; evidence of deviations from this null distribution may indicate the existence of one or more epidemiological and biological mechanisms of co-infection.

5.1.4. Aims and objectives

The aims of this analysis were to describe the characteristics of symptomatic and asymptomatic norovirus infection in the Study of Infectious Intestinal Disease specimen archive and to examine the prevalence and significance of co-infections in norovirus cases and asymptomatic norovirus infections. Specific research objectives were to:

i. Describe the age- and season-specific prevalence of asymptomatic norovirus infection in the population in England;

ii. Describe the symptoms experienced by norovirus cases and asymptomatic norovirus infections, with comparison to norovirus negative healthy controls;

iii. Describe the prevalence of additional pathogen co-infections in norovirus cases and asymptomatic norovirus infections;

iv. Investigate whether the prevalence of additional pathogens in norovirus cases and asymptomatic norovirus infections is greater than that expected from the population prevalence of these pathogens, i.e. whether particular pathogens are detected in norovirus cases or asymptomatic norovirus infections more often than expected by chance.

5.2. Methods

5.2.1. Specimens and testing

Recruitment of participants into the Study of Infectious Intestinal Disease was described in Chapter 3. All 2205 controls and 2422 IID cases from the specimen archive were included in this analysis. For this analysis, controls who had been free of diarrhoea and vomiting for at least 10 days prior to recruitment were considered asymptomatic with respect to IID, although they may have experienced other symptoms during that period and may also have experienced diarrhoea or vomiting prior to this 10-day exclusion period. IID cases from the community cohort and general practice case-control study were analysed together, except where disease severity was compared between cases ascertained through these different routes. Full diagnostic results for all of the pathogens targeted in the original and PCR retesting were used in the analysis of co-infections.

5.2.2. Case and control definitions

Cases of norovirus-associated IID ('norovirus cases') were:

i. IID cases infected with genogroup II noroviruses, who had a Ct value determined by real-time RT-PCR testing that was equal to or less than the age-specific Ct value cut-offs described in Chapter 4 (Ct value 30 for children aged less than five years, Ct value 33 for children and adults aged five years and older);

Or

ii. IID cases infected with genogroup I noroviruses, detected by electron microscopy and confirmed by RT-PCR.

'Asymptomatic norovirus infections' were:

Controls who tested positive for norovirus by electron microscopy and/or RT-PCR.

IID cases with disease caused by a pathogen other than norovirus were:

IID cases who tested negative for norovirus by electron microscopy and RT-PCR.

'Norovirus negative controls' were:

Controls who tested negative for norovirus by electron microscopy and RT-PCR.

The genogroup I cut-off was not used because it is unlikely that it would accurately identify individual IID cases with disease caused by genogroup I noroviruses, due to the small sample size for the genogroup I ROC analysis and the problems with variable efficiency of the real-time RT-PCR assay between genotypes in genogroup I. Only those genogroup I infected IID cases who had been diagnosed by electron microscopy were included in the analysis because the high viral loads required for detection of norovirus by electron microscopy correlate with norovirus shedding during acute norovirus-associated IID in experimentally inoculated volunteers[80, 240].

5.2.3. Community prevalence of asymptomatic norovirus infections

The age-specific prevalence of norovirus infection (determined by RT-PCR testing) amongst controls from the Study of Infectious Intestinal Disease specimen archive was calculated. The age-adjusted prevalence of asymptomatic norovirus infection in the community in England was calculated by standardising[a] the prevalence in the study population against the mid-1994 population estimate for England, obtained from the Office for National Statistics, UK. The age-adjusted, monthly prevalence of asymptomatic norovirus infection was calculated from the prevalence amongst children aged less than five years and amongst older children and adults (aged five years or older) in each month, by standardizing against the mid-1994 population estimate for England.

[a] Weighted sum of the age specific prevalence (age groups shown in Figure 5.1); weights are the proportion of the population of England (1994 mid-year estimate) in each age group.

5.2.4. Description of symptoms in norovirus cases and asymptomatic norovirus infections

Norovirus cases, asymptomatic norovirus infections and norovirus negative controls provided details of gastrointestinal and non-specific symptoms in the epidemiological questionnaire (Table 3.1), although details of fever and nausea were not collected from controls. Appendix A3.1 shows the symptoms that both IID cases and controls were asked to report. IID cases were asked to report symptoms experienced at the time of their illness and controls were asked to report symptoms occurring in the previous three weeks before questionnaire completion (Table 3.1). In addition to reporting the presence of symptoms, IID cases were also asked to report the duration (in days) of any symptoms experienced and the maximum number of diarrhoeal and vomiting episodes in a given 24-hour period during their illness.

An adapted version of the Vesikari severity score[536] was used to summarise the severity of symptoms in norovirus cases. Four components of the Vesikari score were used: duration of diarrhoea; duration of vomiting; maximum number of diarrhoeal episodes per 24 hours; and maximum number of vomiting episodes per 24 hours. Each component had up to three points, with a minimum possible score of three and a maximum score of 12 (Table 5.1).

The prevalence, severity and duration of gastrointestinal symptoms were compared between norovirus cases and IID cases with disease caused by other pathogens. This comparison was used to determine whether vomiting, which is recognised as a characteristic of norovirus outbreaks, is a distinguishing feature of norovirus-associated IID in the community and to investigate whether sporadic, community-acquired norovirus-associated IID is mild and self-limiting in comparison to IID caused by other pathogens. Symptom prevalence, severity and duration were also compared between norovirus cases who were positive by electron microscopy and those identified only by the Ct value cut-off, to ascertain whether any clinical features correlate with electron microscopy positivity and therefore whether the electron microscopy positive cases, which were used to define the Ct value cut-off, differ systematically from those norovirus cases only identified by RT-PCR.

Given previous reports of differences in symptom prevalence, duration and severity in norovirus cases of different ages, the following comparisons were made. The prevalence of vomiting was compared between young children and older children and

Table 5.1 Adapted version of the Vesikari severity score used to describe symptom severity in norovirus cases.

Symptom	Value	Score
Diarrhoea duration in days	1 - 4	1
	5	2
	6 or more	3
Maximum number of diarrhoeal stools per 24 hours	1 - 3	1
	4 - 5	2
	6 or more	3
Vomiting duration in days	1	1
	2	2
	3 or more	3
Maximum number of vomiting episodes per 24 hours	0	0
	1	1
	2 - 4	2
	5 or more	3
Total possible score		12

adults. The severity and duration of gastrointestinal symptoms in norovirus cases was compared between:

i. Young children (aged less than five years) and older children and adults (aged between five and 64 years);

ii. The elderly (aged 65 years and older) and older children and adults (aged between five and 64 years).

The severity of disease was also compared between norovirus cases in the community cohort and the general practice case-control study, to investigate whether symptom severity increases the likelihood of healthcare consultation during norovirus-associated IID. Symptom prevalences were compared using 95% confidence intervals for prevalence differences and the rank-sum test was used to examine differences in symptom duration and severity.

The relationship between norovirus viral load and the severity and duration of symptoms was examined using norovirus cases who collected a stool specimen within two days of symptom onset. This restriction was used to ensure that the norovirus viral load was representative of norovirus shedding during acute illness in these IID cases.

A number of volunteer studies have reported non-gastrointestinal symptoms amongst norovirus-infected volunteers who do not develop diarrhoea and vomiting. Therefore, non-gastrointestinal symptoms reported by norovirus cases and asymptomatic norovirus infections were compared to those reported by norovirus negative controls, to investigate the association of these particular symptoms with norovirus infection.

5.2.5. Prevalence and significance of co-infections

To investigate whether additional pathogens were co-infecting norovirus cases and asymptomatic norovirus infections more often than expected by chance, the prevalence of additional pathogens was compared to the prevalence amongst norovirus negative controls. The additional pathogens included in the analysis were those at a prevalence of 1% or more in IID cases in the Study of Infectious Intestinal Disease specimen archive (Table 3.2); 14 pathogens, including norovirus, were used in the analysis. The prevalence of each additional pathogen detected in norovirus cases and asymptomatic norovirus infections was compared to the prevalence of that specific pathogen in norovirus negative controls, using a prevalence ratio with a 95% confidence interval and a Z-test *P* value. The analysis was carried out separately for children ages less than five years and for older children and adults (aged five years and older) because of age-specific differences in pathogen prevalence.

The prevalence of additional pathogens was also compared between norovirus cases with disease caused by genogroup II noroviruses and those IID cases in whom genogroup II norovirus infection was detected, but who were not classified as norovirus cases using the Ct value cut-off. These comparisons were made to qualitatively assess the appropriateness of the Ct value cut-off selected for genogroup II.

All statistical analyses (95% confidence intervals for prevalence ratios and prevalence differences, rank-sum tests) were carried out using Stata 10[494].

5.3. Results

There were 274 IID cases who met the norovirus case definition, 361 asymptomatic norovirus infections and 1844 norovirus negative controls in the specimen archive.

Epidemiological questionnaires, with details of symptoms, were returned by 237 norovirus cases (86%), 344 asymptomatic norovirus infections (95%) and 1721 norovirus negative controls (93%). Of the 1553 IID cases in the specimen archive with disease caused by another pathogen, 1301 (84%) returned the epidemiological questionnaire.

5.3.1. Community prevalence of asymptomatic norovirus infection

The age-adjusted, community prevalence of asymptomatic norovirus infection was 12% (95% confidence interval (CI): 11 – 14), with the highest prevalence in children aged less than five years (29%), although more than 5% of individuals in older age groups were infected (Figure 5.1). The prevalence of asymptomatic infection showed a wintertime peak of 20% during November, December and January (Figure 5.2); the seasonal pattern was less distinct for children aged less than five years compared to older children and adults (Appendix A3.2).

Figure 5.1 Age-specific prevalence of asymptomatic norovirus infection during the Study of Infectious Intestinal Disease. Numbers above the bars show the number of participants tested in each age group. Black T-bars show the 95% confidence intervals.

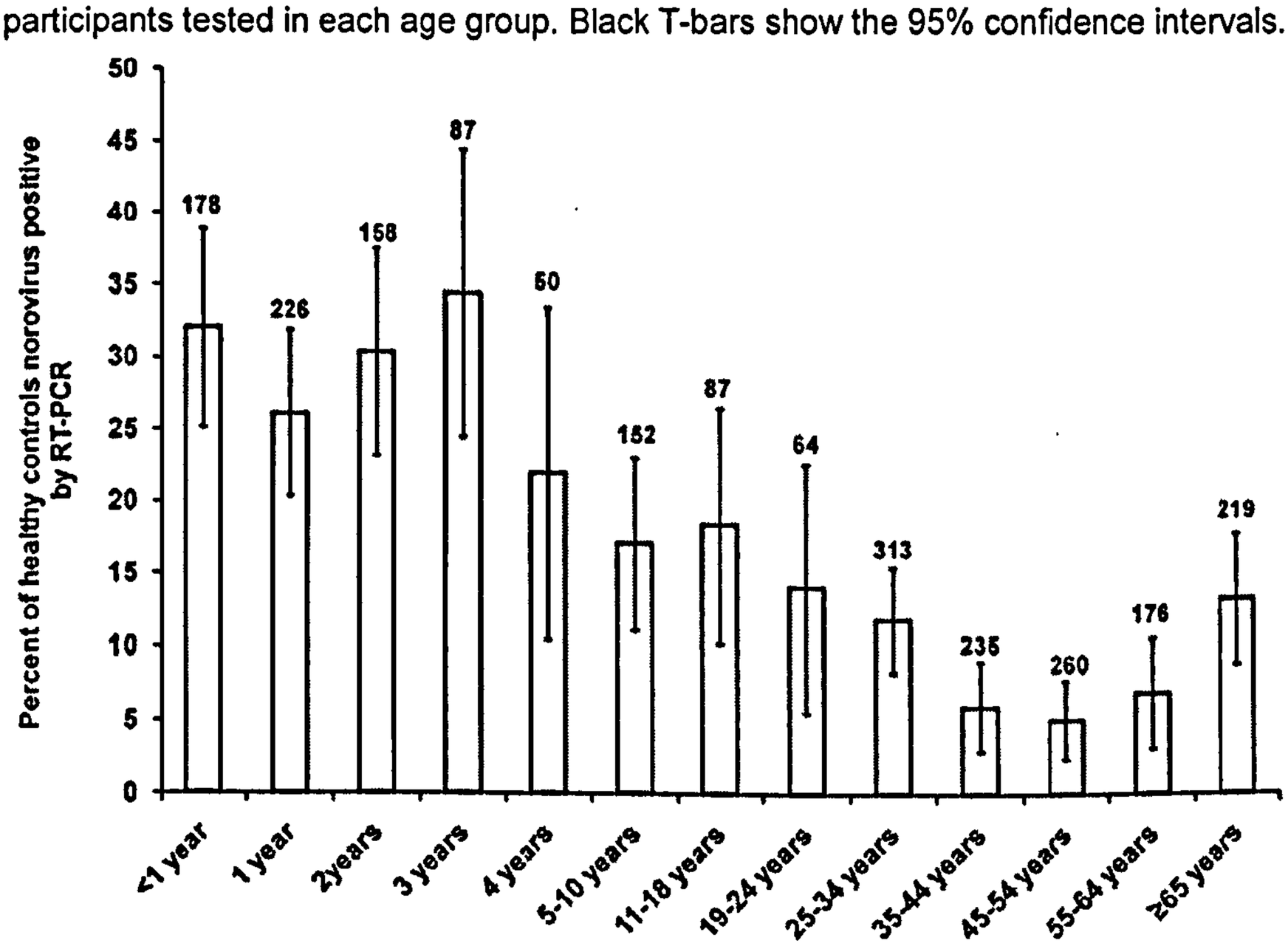
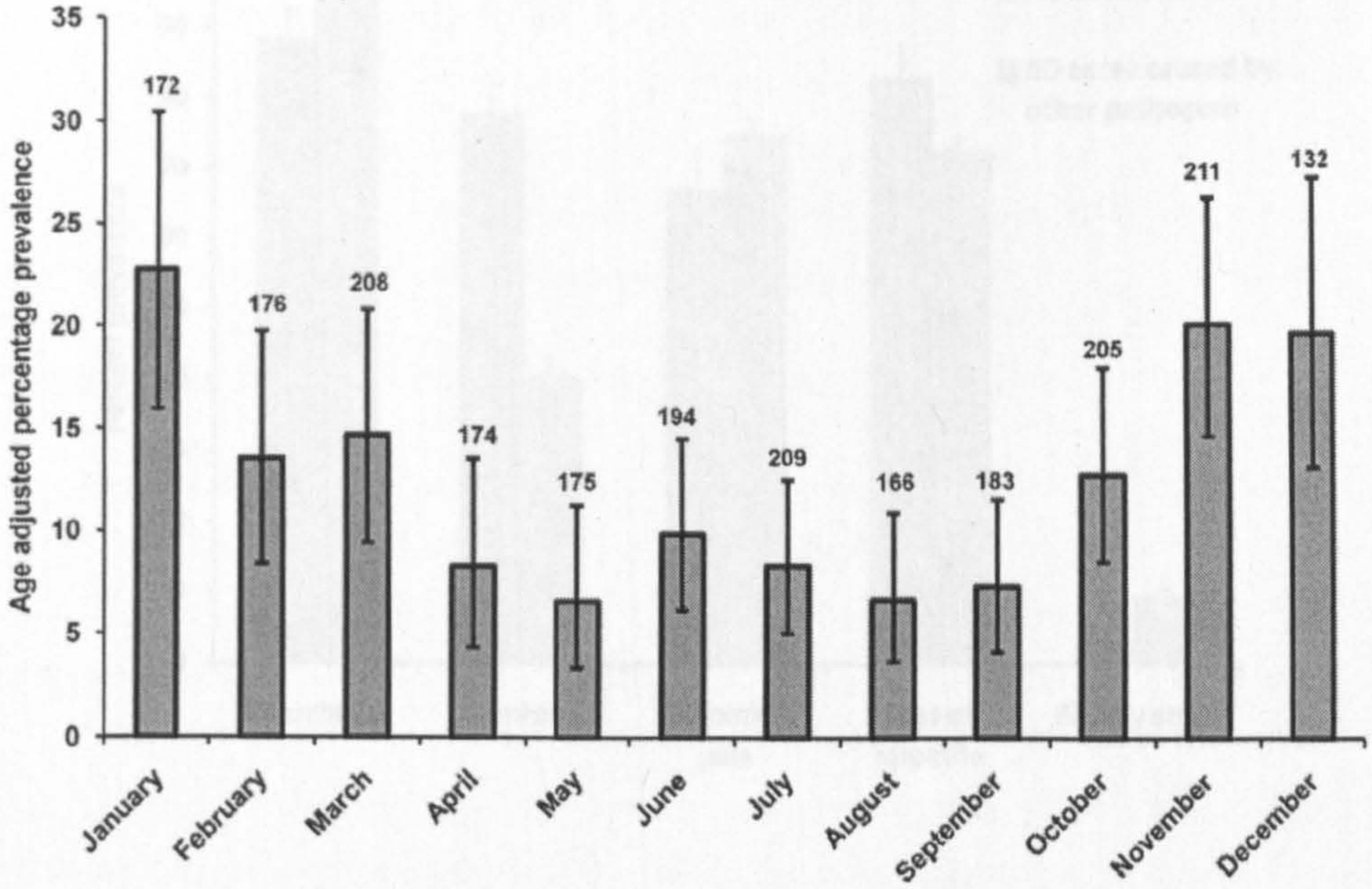


Figure 5.2 Age-adjusted monthly prevalence of asymptomatic norovirus infection (detected by RT-PCR) during the Study of Infectious Intestinal Disease. Numbers above the bars show the number of participants tested in each month. Black T-bars show the 95% confidence intervals.



5.3.2. Symptoms in norovirus cases and asymptomatic norovirus infections

5.3.2.1. Comparison of symptoms reported by norovirus cases, other IID cases and norovirus negative controls

The majority of norovirus cases reported having diarrhoea (88%) and vomiting (78%) and a substantial number reported experiencing abdominal pain (67%) and loss of appetite (82%) (Figure 5.3). Other symptoms that were reported substantially more often by norovirus cases compared to norovirus negative controls were headache (44%) and aching muscles (37%) (Figure 5.4). The prevalence of vomiting was substantially higher in norovirus cases compared to IID cases with disease caused by another pathogen (prevalence difference: 34% [95% CI: 28 – 41]) (Figure 5.3), which was true for both children aged less than five years and for older children and adults (aged five years and older), although the difference in vomiting prevalence between the two groups of cases was greatest in older children and adults (Appendix A3.3).

Figure 5.3 Prevalence of gastrointestinal symptoms in norovirus cases (n=237) and IID cases with disease caused by another pathogen (n=1301).

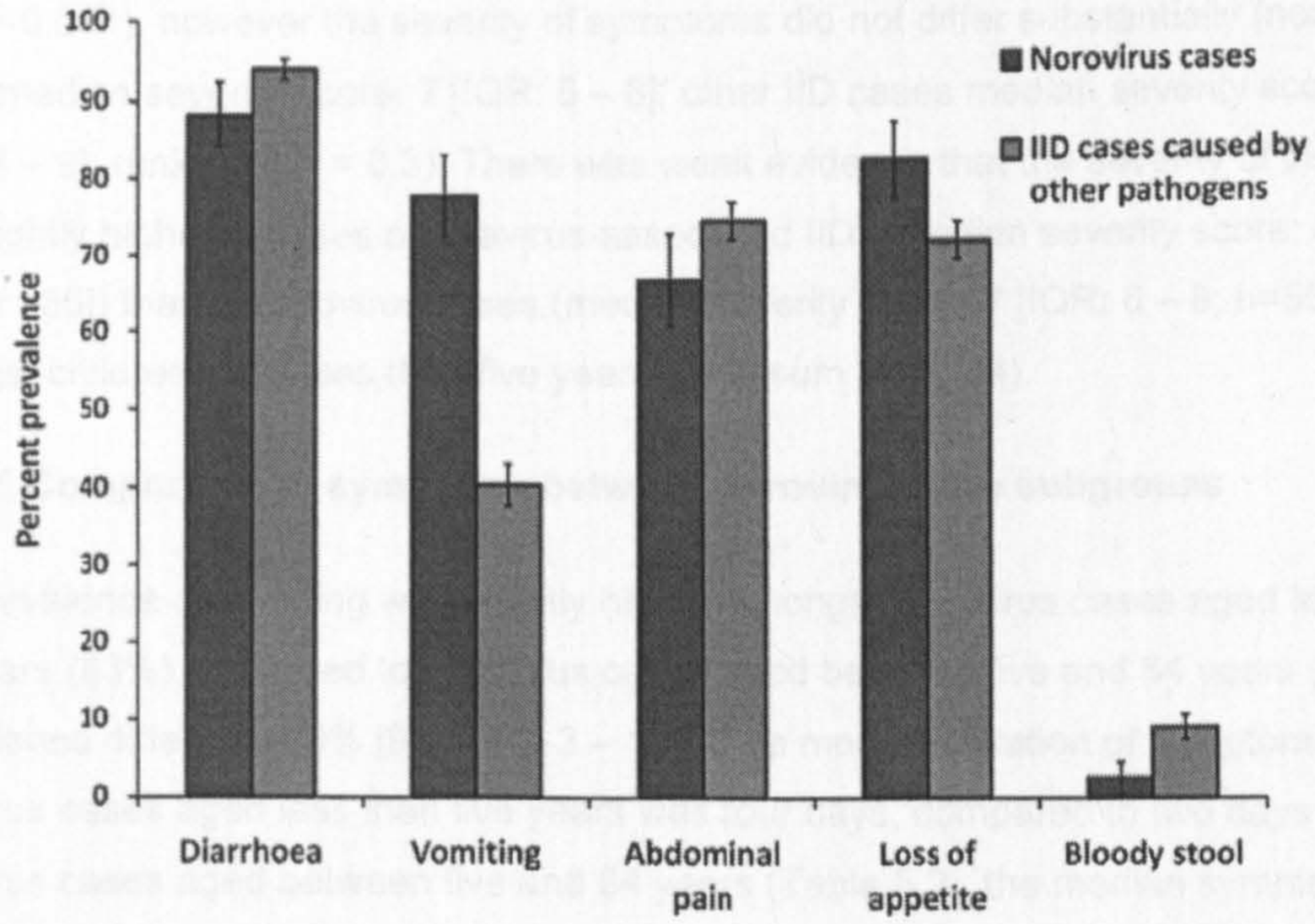
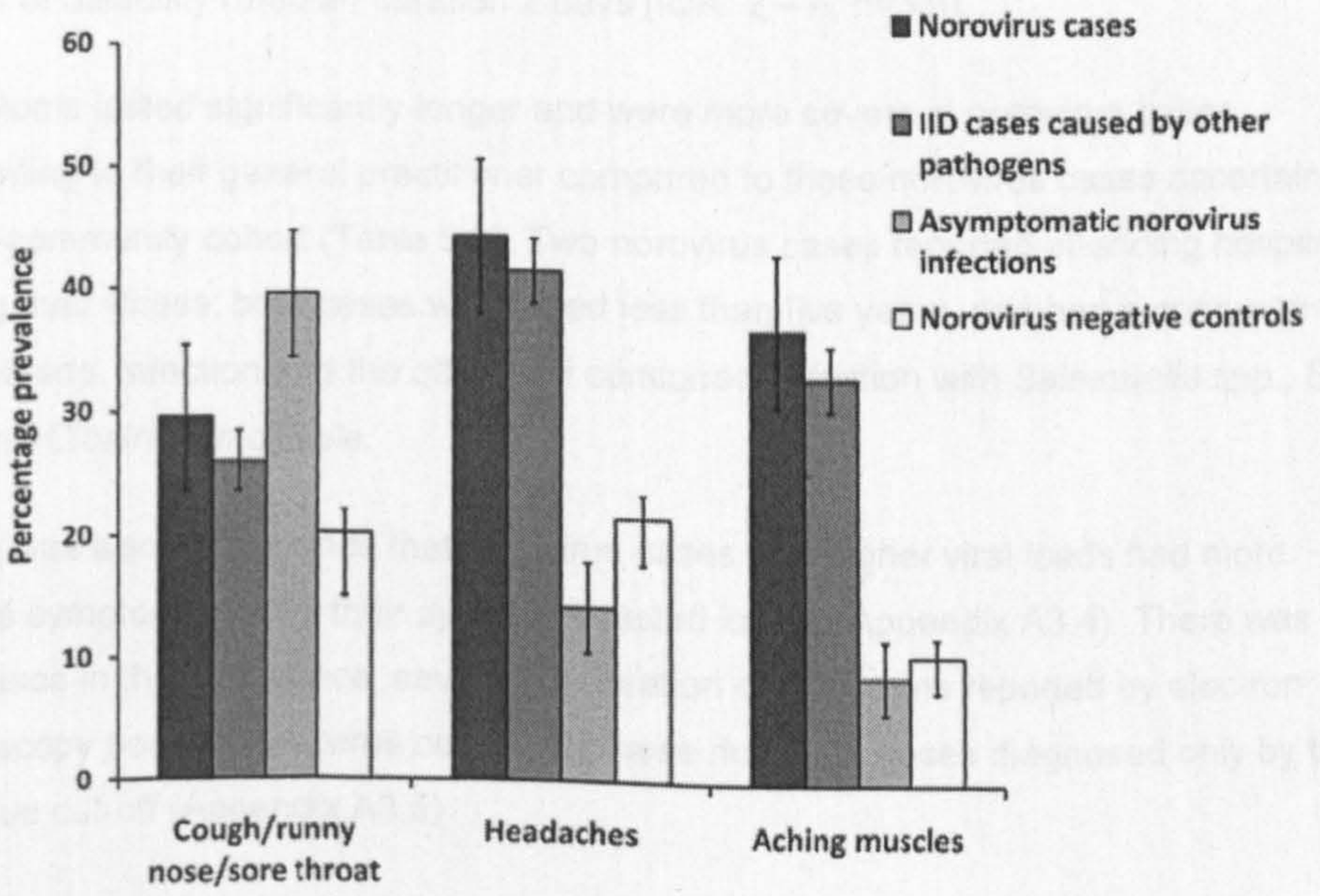


Figure 5.4 Prevalence of non-gastrointestinal symptoms in norovirus cases (n=237), IID cases with disease caused by another pathogen (n=1301), asymptomatic norovirus infections (n=344) and norovirus negative controls (n=1721).



There was evidence that the duration of symptoms was shorter in norovirus cases (median duration: 3 days [IQR: 2 – 4; n=178]) compared to IID cases with disease caused by other pathogens (median duration: 4 days (IQR: 2 – 6; n=1045]) (rank-sum test P <0.001); however the severity of symptoms did not differ substantially (norovirus cases median severity score: 7 [IQR: 6 – 8]; other IID cases median severity score: 7.5 [IQR: 6 – 9]; rank-sum P = 0.3). There was weak evidence that the severity of disease was slightly higher in cases of rotavirus-associated IID[b] (median severity score: 8 [IQR: 7 – 9; n=55]) than in norovirus cases (median severity score: 7 [IQR: 6 – 8; n=55]) amongst children aged less than five years (rank-sum P = 0.04).

5.3.2.2. Comparison of symptoms between norovirus case subgroups

The prevalence of vomiting was slightly higher amongst norovirus cases aged less than five years (83%) compared to norovirus cases aged between five and 64 years (75%) (prevalence difference: 8% [95% CI: -3 – 18]). The median duration of symptoms in norovirus cases aged less than five years was four days, compared to two days in norovirus cases aged between five and 64 years (Table 5.2); the median symptom duration in norovirus cases aged 65 years or older was 3 days. There was no evidence that norovirus symptoms were more severe in young children or adults aged 65 years and older (Table 5.2), compared to children and adults aged between five and 64 years, nor in individuals with pre-existing long-term illness or disability (median severity 8 [IQR: 7 – 9; n=18]) compared to norovirus cases who did not report any long-term illness or disability (median duration 2 days [IQR: 2 – 4, n=33]).

Symptoms lasted significantly longer and were more severe in norovirus cases presenting to their general practitioner compared to those norovirus cases ascertained in the community cohort (Table 5.2). Two norovirus cases reported attending hospital during their illness; both cases were aged less than five years, one had a concurrent *Giardia* spp. infection and the other had concurrent infection with *Salmonella* spp., *E. coli* and *Clostridium difficile*.

There was also no evidence that norovirus cases with higher viral loads had more severe symptoms or that their symptoms lasted longer (Appendix A3.4). There was no difference in the prevalence, severity or duration of symptoms reported by electron microscopy positive norovirus cases and those norovirus cases diagnosed only by the Ct value cut-off (Appendix A3.5)

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[b] Defined as IID cases positive for rotavirus by ELISA.

5.3.2.3. Recent history of gastrointestinal symptoms in asymptomatic norovirus infections

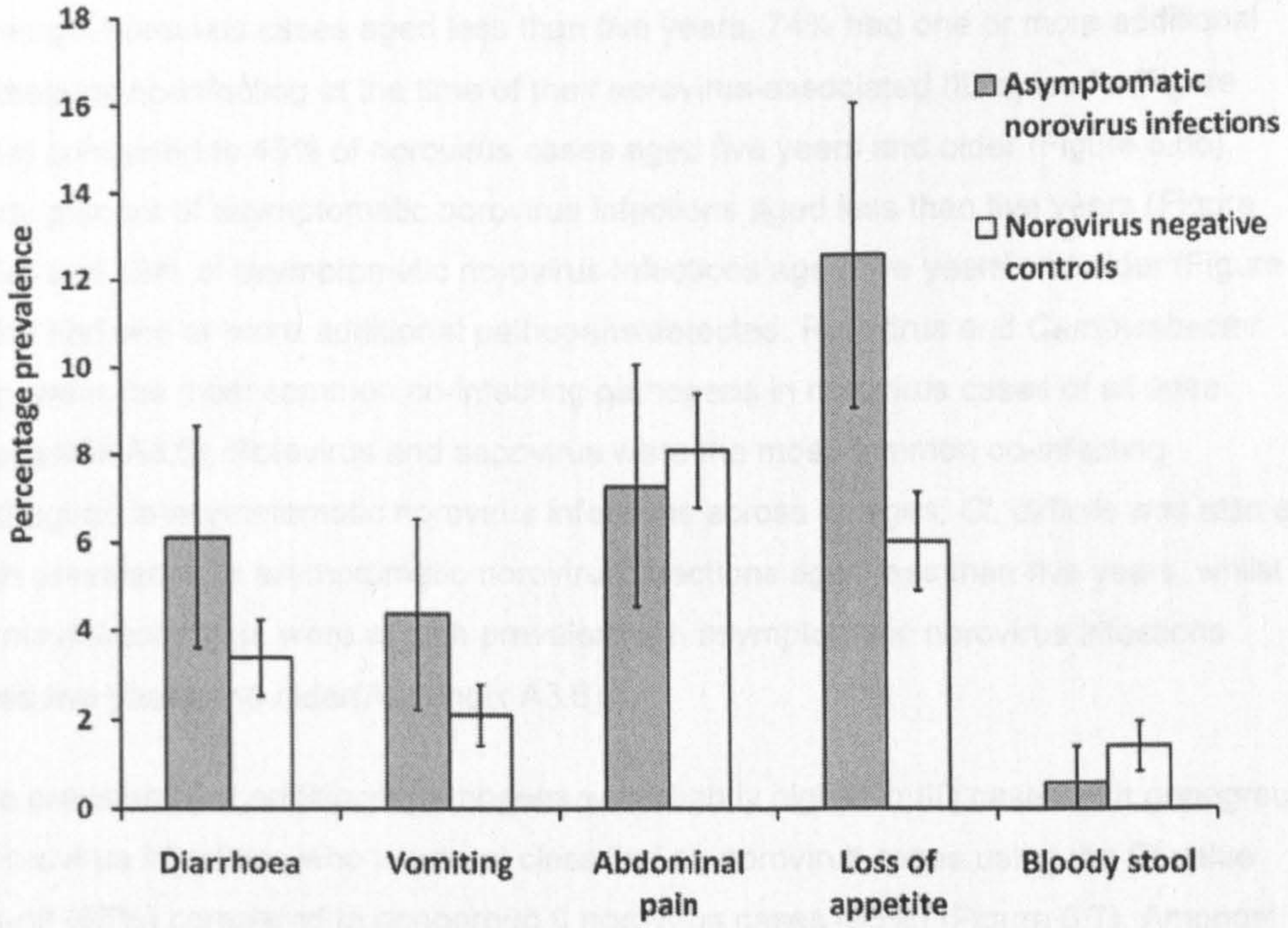
Nine percent of asymptomatic norovirus infections experienced diarrhoea and/or vomiting prior to the 10-day exclusion period, but within three weeks of questionnaire completion (Figure 5.5). The prevalence of diarrhoea and/or vomiting was higher amongst asymptomatic norovirus infections compared to norovirus negative controls, for both children aged less than five years (asymptomatic norovirus infection: 10% [95% CI: 6 – 15]; norovirus negative: 7% [95% CI: 5 – 10]; prevalence difference: 3% [95% CI: -2 – 8]), and older children and adults aged five years and older (asymptomatic norovirus infection: 8% [95% CI: 4 – 12]; norovirus negative: 4% [95% CI: 3 – 5]; prevalence difference: 4% [95% CI: -0.5 – 8]). Older children and adults (aged five years and older) with asymptomatic norovirus infection also reported loss of appetite more often than norovirus negative controls in this age group (Figure 5.5) (asymptomatic norovirus infection: 9% [95% CI: 4 – 13]; norovirus negative: 3% [95% CI: 2 – 4]; prevalence difference: 6% [95% CI: 1 – 11]).

Table 5.2 Symptom severity and duration in norovirus cases by age and route of recruitment.

	Symptom severity			Symptom duration		
	Median severity score (IQR)	Rank-sum test *P* value[ii]	Total cases reporting severity components	Median duration of diarrhoea and vomiting in days (IQR)	Rank-sum test *P* value[ii]	Total cases reporting duration
Children aged <5 years	7 (6 - 9)	0.3	46	4 (3 - 6)	<0.001	65
Elderly (≥65 years)	8 (6 - 8)	0.7	6	3 (4 - 8)	0.1	11
Older children and adults (5-64 years)	7 (6 - 8)		62	2 (1 - 3)	.	102
Community cohort	6 (6 - 7)	<0.001	31	2 (1 - 3)	<0.001	45
General practice case-control study	8 (7 - 9)		83	3 (2 - 5)		133

[ii] Rank-sum test is comparing symptom severity/duration in children aged < 5 years to older children and adults, in the elderly to older children and adults and in the community cohort to the general practice case-control study.
Abbreviations: IQR, interquartile range..

Figure 5.5 History of gastrointestinal symptoms in asymptomatic norovirus infections (n=344) and norovirus negative controls (n=1721) (during the period between 10 days and 3 weeks prior to recruitment).



5.3.2.4. Non-gastrointestinal symptoms in norovirus cases and asymptomatic norovirus infections

During the three weeks preceding questionnaire completion, a cough, sore throat and other cold-like symptoms were reported by 61% of children aged less than five years with asymptomatic norovirus infection (95% CI: 54 – 68), compared to 52% (95% CI: 47 – 56) of norovirus negative controls in this age group (Figure 5.4) (prevalence difference: 9% [95% CI: 0.7 – 17]; prevalence ratio adjusted for month of the year: 1.2 [95% CI: 1.0 – 1.4]). There was a smaller excess of cold-like symptoms in older children and adults with asymptomatic norovirus infection; the prevalence in asymptomatic norovirus infections was 12% (95% CI: 7 – 17) compared to 9% in norovirus negative controls (95% CI: 7 – 10) (prevalence difference: 3% [95% CI: -2 – 8]; prevalence ratio adjusted for month of the year 1.3 [95% CI: 0.8 – 2.0]). Cold-like symptoms were also at higher prevalence in both norovirus cases and IID cases with disease caused by other pathogens, compared to norovirus negative controls (Figure 5.4). However after adjusting for month of the year, the prevalence was only higher in

those norovirus cases (prevalence ratio: 2.7 [95% CI: 1.9 – 3.7]) and other IID cases (prevalence ratio: 2.7 [95% CI: 2.2 – 3.4]) who were aged five years and older.

5.3.3. Prevalence and significance of co-infections

Amongst norovirus cases aged less than five years, 74% had one or more additional pathogens co-infecting at the time of their norovirus-associated IID episode (Figure 5.6a) compared to 45% of norovirus cases aged five years and older (Figure 5.6b). Forty percent of asymptomatic norovirus infections aged less than five years (Figure 5.6a) and 19% of asymptomatic norovirus infections aged five years and older (Figure 5.6b) had one or more additional pathogens detected. Rotavirus and *Campylobacter* spp. were the most common co-infecting pathogens in norovirus cases of all ages (Appendix A3.6). Rotavirus and sapovirus were the most common co-infecting pathogens in asymptomatic norovirus infections across all ages; *Cl. difficile* was also at high prevalence in asymptomatic norovirus infections aged less than five years, whilst *Campylobacter* spp. were at high prevalence in asymptomatic norovirus infections aged five years and older(Appendix A3.6).

The prevalence of additional pathogens was slightly higher in IID cases with genogroup II norovirus infections who were not classified as norovirus cases using the Ct value cut-off (65%) compared to genogroup II norovirus cases (55%) (Figure 5.7). Amongst IID cases with genogroup II norovirus infections who were not classified as norovirus cases using the Ct value cut-off, there was no difference in the prevalence of additional pathogens in those with timely specimen collection (within five days of symptom onset) and those with late specimen collection (5 days or more after specimen onset) (Figure 5.7). Amongst those with timely specimen collection, 36% had no other pathogen detected, whilst 34% of those with late samples had no other pathogen detected. Since norovirus has been excluded as a possible cause of illness in these individuals, based on their norovirus Ct value, they effectively remain undiagnosed, because no other pathogens have been detected after both the original and PCR testing. This undiagnosed fraction is similar to the 36% of norovirus negative IID cases in the Study of Infectious Intestinal Disease specimen archive in whom no pathogens were detected after all diagnostic testing was completed.

Figure 5.6 Distribution of co-infection with additional pathogens in norovirus cases, asymptomatic norovirus infections and norovirus negative controls. Number of pathogens includes norovirus in norovirus cases and asymptomatic norovirus infections.

Figure 5.6a Children aged less than five years.

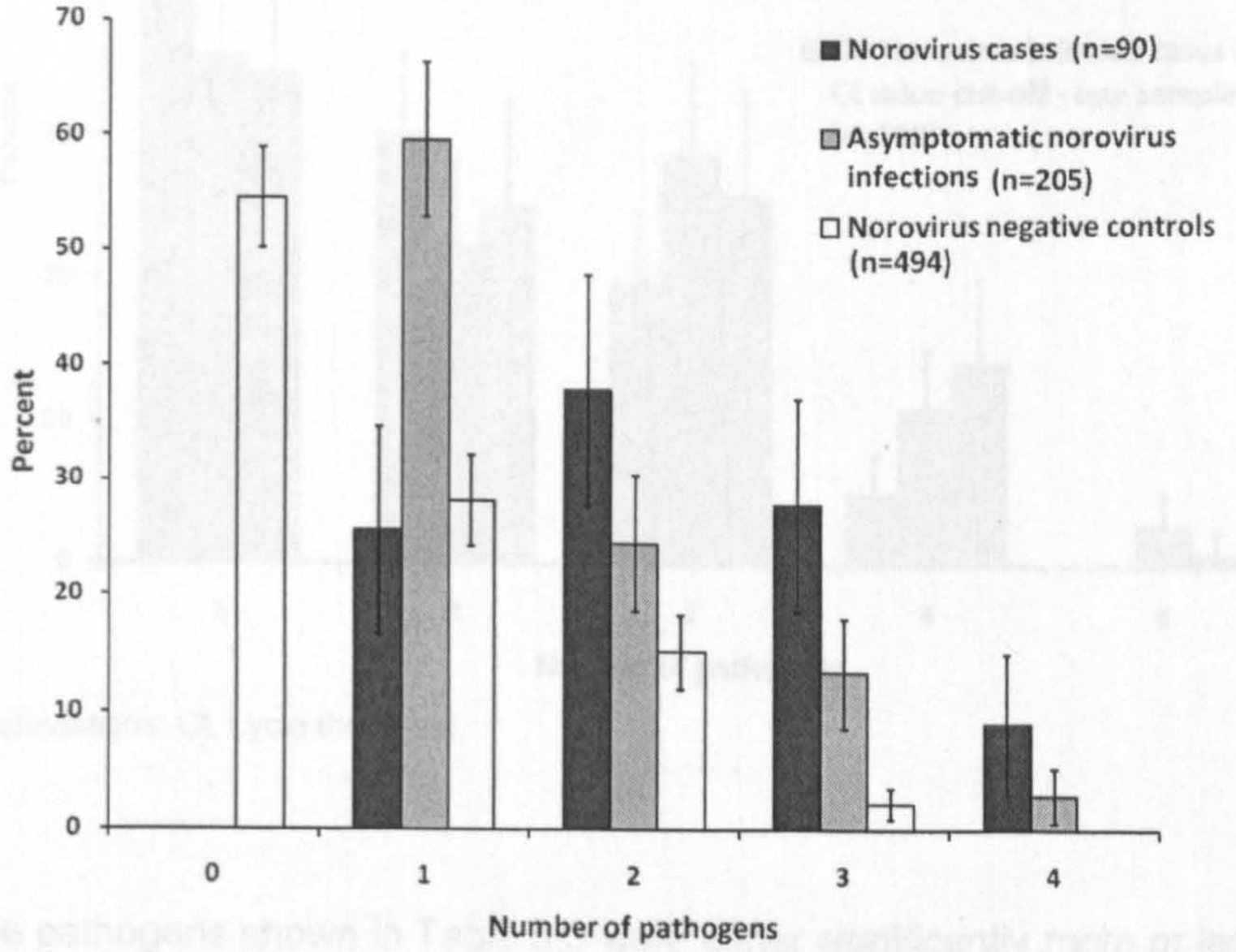


Figure 5.6b Children and adults aged five years and older.

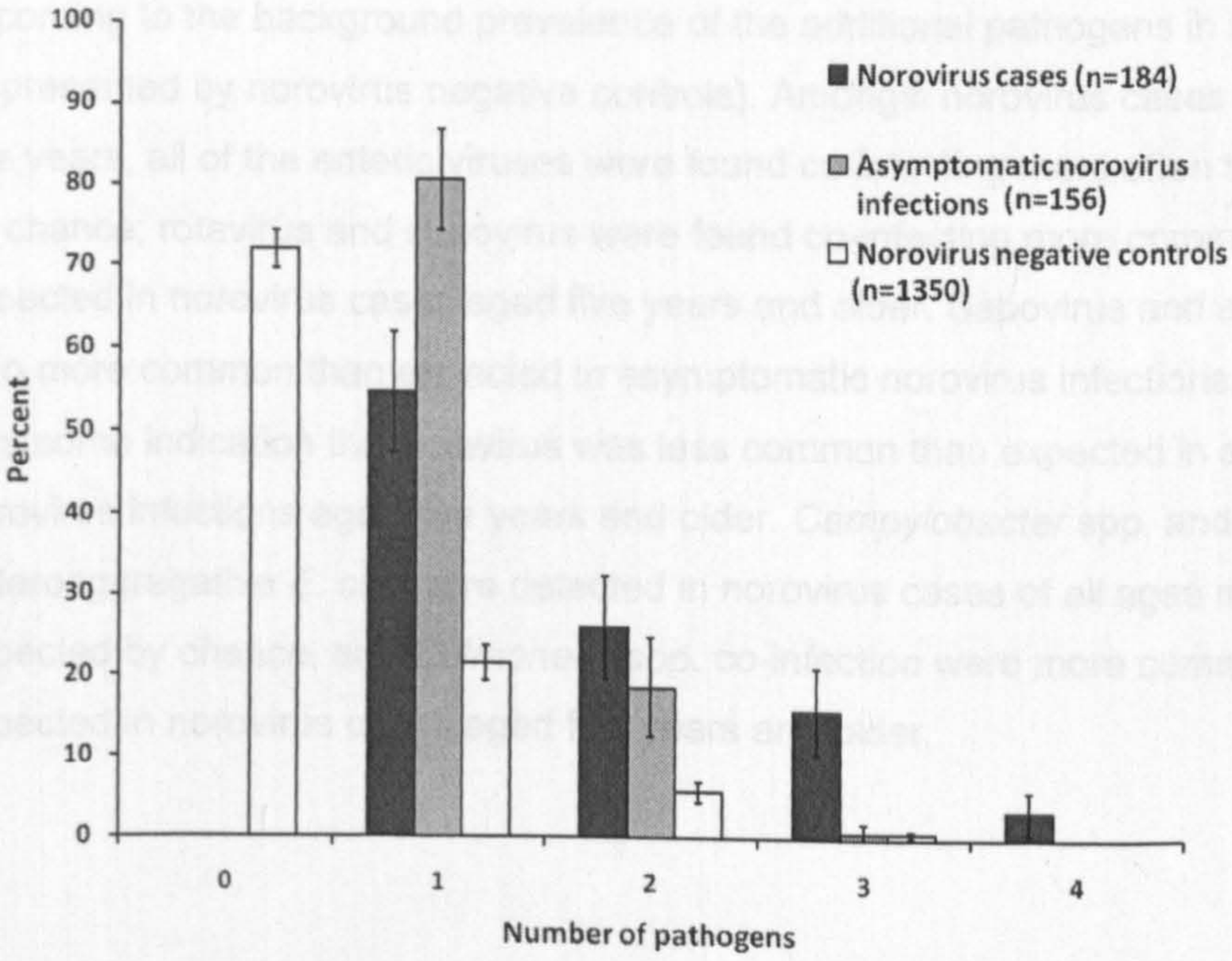
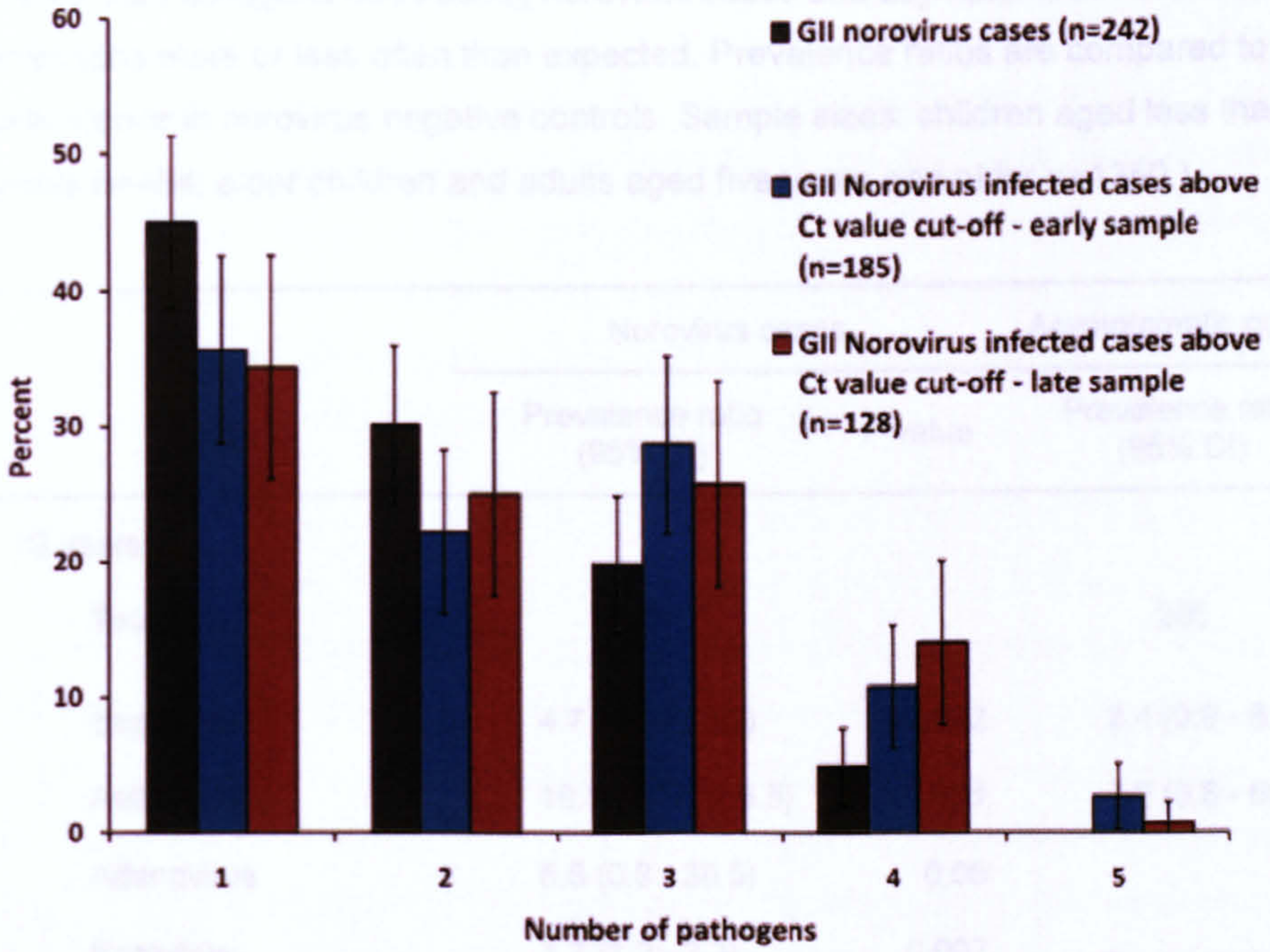


Figure 5.7 Number of pathogens (including norovirus) detected in genogroup II infected norovirus cases and IID cases who were not classified as norovirus cases using the Ct value cut-off.



Abbreviations: Ct, cycle threshold.

The pathogens shown in Table 5.3 were either significantly more or less common in norovirus cases and asymptomatic norovirus infections compared to norovirus negative controls; the prevalence of these co-infections differed from that expected by chance, according to the background prevalence of the additional pathogens in the population (represented by norovirus negative controls). Amongst norovirus cases aged less than five years, all of the enteric viruses were found co-infecting more often than expected by chance; rotavirus and sapovirus were found co-infecting more commonly than expected in norovirus cases aged five years and older. Sapovirus and astrovirus were also more common than expected in asymptomatic norovirus infections, although there was some indication that rotavirus was less common than expected in asymptomatic norovirus infections aged five years and older. *Campylobacter* spp. and enteroaggregative *E. coli* were detected in norovirus cases of all ages more often than expected by chance, and *Salmonella* spp. co-infection were more common than expected in norovirus cases aged five years and older.

Table 5.3 Pathogens co-infecting norovirus cases and asymptomatic norovirus infections more or less often than expected. Prevalence ratios are compared to prevalence in norovirus negative controls. Sample sizes: children aged less than five years n=494; older children and adults aged five years and older n=1350.)

	Norovirus cases		Asymptomatic norovirus infections	
	Prevalence ratio (95% CI)	*P* value	Prevalence ratio (95% CI)	*P* value
<5 years				
Total	90		205	
Sapovirus	4.7 (1.6 - 13.7)	0.002	2.4 (0.9 - 6.8)	0.09
Astrovirus	16.5 (1.7 - 156.5)	<0.001	7.2 (0.8 - 69.1)	0.04
Adenovirus	5.5 (0.8 - 38.5)	0.05	-	
Rotavirus	1.7 (1.2 - 2.3)	0.002	-	
Campylobacter spp.	4.7 (2.7 - 8.4)	<0.001	-	
Enteroaggregative *E. coli*	4.2 (2.1 - 8.3)	<0.001	-	
≥5 years				
Total	184		156	
Rotavirus	2.0 (1.5 - 2.8)	<0.001	0.4 (0.2 - 0.9)	0.02
Campylobacter spp.	3.8 (2.6 - 5.5)	<0.001	1.7 (1.0 - 3.1)	0.06
Salmonella spp.	5.7 (2.2 - 15.1)	<0.001	-	
Enteroaggregative *E. coli*	3.4 (1.9 - 6.3)	<0.001	-	
Sapovirus	-		3.9 (1.7 - 8.7)	<0.001
Aeromonas spp.	-		0.2 (0.02 - 1.2)	0.04

Abbreviations: CI, confidence interval; spp., species.

5.4. Discussion

In this analysis, the community prevalence of asymptomatic norovirus infection in England was presented and the characteristics of norovirus cases and asymptomatic norovirus infections were described and compared to IID cases with disease caused by other pathogens and to norovirus negative controls. Statistical methods were used to examine the epidemiological significance of co-infection with other pathogens in norovirus cases and asymptomatic norovirus infections.

The community prevalence of asymptomatic norovirus infection in England was 12%, which is higher than that reported in two previous studies, conducted in Germany and the Netherlands, which recruited comparable samples of asymptomatic individuals[5, 22]. Real-time RT-PCR is known to have slightly higher sensitivity than gel-based RT-PCR[279] which was used for norovirus diagnosis in these two previous studies. However, this is unlikely to account for the difference of 7% between the prevalence of asymptomatic norovirus infection described in this analysis and the prevalence in the previous study conducted in the Netherlands (5%)[5]. Nested gel-based RT-PCR was used in the study conducted in Germany[22]; the use of nested PCR primers increases the sensitivity of the gel-based assay[280], meaning that the assay used in the study in Germany is likely to have comparable sensitivity to the real-time RT-PCR used in this study. It is possible that the differences in asymptomatic norovirus prevalence between the studies are due to differences in the genetic strains of norovirus circulating at the time that the studies were carried out. Periodic emergence of new norovirus strains has been associated with increases in the incidence of disease, but it is plausible that there may be a concomitant increase in the prevalence of infection as well; a new strain emerged in 1995 to 1996, during recruitment of participants into the Study of Infectious Intestinal Disease[201, 537]. Asymptomatic norovirus infection showed winter-time seasonality, matching the seasonality of norovirus-associated IID community incidence presented in Chapter 8.

Previous studies of both community and outbreak cases have described norovirus-associated IID as a mostly mild, self-limiting illness in healthy adults, but have highlighted a high prevalence of vomiting compared to IID caused by other pathogens. The characteristics of the norovirus cases in this study reflected these previous findings: symptoms lasted fewer days in norovirus cases compared to IID cases with illness caused by other pathogens and vomiting was twice as common amongst norovirus cases. The median duration of symptoms in norovirus cases aged less than five years was double the duration in norovirus cases aged between five and 64

years[84, 108, 513], although there was little indication that the severity of symptoms or the prevalence of vomiting was higher in young children, which have also been reported in previous studies[84, 85]. It was difficult to assess the relative difference in the manifestation of norovirus-associated IID in individuals aged 65 years and older because there were very few norovirus cases in this age group who returned the epidemiological questionnaire. There was no evidence that symptom duration was related to viral load in norovirus cases, which has been reported in one previous study[102]. There was no evidence that symptom severity in norovirus cases varied substantially with age or viral load, but norovirus cases presenting to a general practitioner had more severe and longer-lasting symptoms compared to those norovirus cases ascertained in the community cohort.

There was an excess of cold-like symptoms in both norovirus cases and asymptomatic norovirus infections, compared to norovirus negative controls. After adjustment for season, there was only evidence of a significant excess in norovirus cases aged five years and older and in asymptomatic norovirus infections aged less than five years. Any excess of cold-like symptoms may be due to a co-infection with a respiratory virus, because viruses causing the common cold and influenza are transmitted via similar routes to norovirus, e.g. through direct person-to-person contact or from contaminated environmental surfaces[538, 539]. In previous studies, experimentally-inoculated volunteers have reported non-specific symptoms such as headache, fever and muscle ache during norovirus infection[80]; details of fever were not collected from asymptomatic participants in the Study of Infectious Intestinal Disease, so it is also possible that the excess of cold-like symptoms may represent non-specific symptoms associated with norovirus infection.

Diarrhoea, vomiting and loss of appetite were reported more frequently in asymptomatic norovirus infections than norovirus negative controls in the three weeks prior to recruitment. Healthy controls were recruited into the Study of Infectious Intestinal Disease because they had been free of diarrhoea and/or vomiting for at least 10 days; the aetiology of any recent IID symptoms prior to this 10-day exclusion period was not established. Post-symptomatic shedding after experimental inoculation has been demonstrated, lasting up to eight weeks[80]. Therefore it is possible that some of the asymptomatic norovirus infections were caused by post-symptomatic shedding, rather than being truly asymptomatic infections; this is consistent with the small excess of recent diarrhoea and vomiting symptoms in asymptomatic norovirus infections. It is also possible that some asymptomatic norovirus infections were due to pre-

symptomatic shedding, although the short incubation period of 24 to 48 hours for norovirus disease[85] means that only a small number of the asymptomatic norovirus infections are likely to be due to pre-symptomatic shedding. Pre-existing levels of anti-norovirus antibody were not measured in the study, so the role of host immunity in the occurrence of these asymptomatic norovirus infections cannot be assessed.

The highest prevalence of co-infections was in children aged less than five years, for both norovirus cases and asymptomatic norovirus infections, reflecting the higher prevalence of infection with a range of pathogens in this age group compared to older children and adults. Of those co-infecting pathogens, only a small number were found in norovirus cases and asymptomatic norovirus infections more often than expected from their prevalence in the population (represented by the prevalence in norovirus negative controls). Enteric viruses were found co-infecting in both norovirus cases and asymptomatic norovirus infections more often than expected, which may reflect the common transmission route through person-to-person contact or contact with contaminated environmental surfaces[389, 540]. A notable exception to this pattern is the lower than expected prevalence of rotavirus in asymptomatic norovirus infections aged five years and older. However, this negative association and a number of the other associations had only reasonable statistical support from the Z-tests, and given the large number of comparisons made, it is possible that some of these were spurious associations. Enteric bacteria (*Campylobacter* spp., *Salmonella* spp. and enteroaggregative *E. coli*) were detected in norovirus cases more often than expected; *Campylobacter* spp. were also in excess in asymptomatic controls aged five years and older. Whilst *Campylobacter* spp. and *Salmonella* spp. are commonly reported to be predominantly acquired through zoonotic foodborne transmission, a number of studies have highlighted the possibility that other modes of transmission, in particular environmental contamination, may be of greater importance outside of outbreak settings[80, 481, 541, 542]. It is therefore plausible that the association with norovirus-associated IID and asymptomatic norovirus infection reflects a common transmission route. However, no information has been collected on host-level factors, meaning that increased susceptibility to infections may also contribute to these associations, although further biological studies of the interaction between host cells and norovirus and these enteric bacteria would be required to assess the relevance of this hypothesis.

There was no indication that norovirus cases diagnosed only by real-time RT-PCR had different symptoms or disease severity to those also positive by electron microscopy,

who were used to define the Ct value cut-off. Most importantly, after applying the Ct value cut-off, the proportion of norovirus-infected IID cases above the cut-off who had no other pathogens detected, and were therefore effectively undiagnosed, was the same as the proportion of all norovirus negative IID cases who were undiagnosed at the end of testing. This indicates that the Ct value cut-off selected is unlikely to be grossly under-diagnosing norovirus-associated IID in this study population.

5.5. Summary

The norovirus cases identified in the Study of Infectious Intestinal Disease specimen archive, using real-time RT-PCR testing and the Ct value cut-off, had similar disease characteristics to those reported in previous studies, in terms of symptom prevalence, duration and severity. There was no difference in disease characteristics between norovirus cases previously diagnosed by electron microscopy and those diagnosed only by real-time RT-PCR with the Ct value cut-off, providing further support for the conclusion in Chapter 4 that it was appropriate to use only electron microscopy positive cases in the ROC analysis reference positive group. Further validation of the Ct value cut-off is provided by the similar undiagnosed fraction amongst norovirus-infected IID cases who were not classified as norovirus cases using the cut-off and other norovirus-negative IID cases; if a much larger proportion of the norovirus-infected IID cases had remained undiagnosed, this may have indicated that the cut-off was under-diagnosing norovirus-associated IID. There was a high population prevalence of asymptomatic norovirus infection in England at the time of the Study of Infectious Intestinal Disease (1993-1996), although the relative importance of pre- and post-symptomatic shedding and true asymptomatic infection could not be determined. Norovirus cases and asymptomatic norovirus infections both experienced cold-like symptoms and additional enteric virus infections more often than healthy, norovirus-negative individuals in the study population, which may be attributable to the common person-to-person transmission route for these enteric and respiratory pathogens. In the next chapter, the importance of contact with potentially infectious individuals, and other exposures, to the acquisition of norovirus-associated IID and asymptomatic norovirus infection will be investigated.

Transmission

Chapter 6: Risk factors for symptomatic and asymptomatic norovirus infection

In Chapter 4, norovirus viral load was compared between IID cases and healthy controls, to select a cut-off in viral load for attributing disease to norovirus in IID cases. In the previous chapter, the characteristics of norovirus cases identified using this viral load cut-off, and asymptomatic norovirus infections, were described. In the analysis presented in this chapter, risk factors for the acquisition of these symptomatic and asymptomatic norovirus infections were investigated.

6.1. Background

Norovirus outbreak investigations[4, 378, 390, 391, 402, 406, 543, 544] and case-control studies of sporadic norovirus-associated IID[22, 83, 380] indicate that the predominant mode of norovirus transmission is faeco-oral. Norovirus transmission occurs through direct physical contact with an infected individual[352], and also via food[360, 380, 397, 403, 410, 412, 413, 545], environmental surfaces or other objects[328, 329, 404-407, 546], which have been contaminated by an infected person, with susceptible individuals ingesting norovirus from their hands or food[329, 331, 332, 466]. Airborne norovirus transmission, following a vomiting episode, has also been documented during outbreaks, either through direct inhalation of aerosolised virus or through contact with vomit contaminated surfaces and foods[349-351, 393-395, 545, 547, 548]. Accordingly, outbreak, experimental and seroepidemiological studies have shown that hand-washing is protective against norovirus-associated IID[336, 406, 409, 466].

Outbreaks of norovirus are often associated with consumption of oysters and other shellfish[423-426, 443] and occasionally with contamination of raw fruit and vegetables during production[444, 445]. A number of outbreaks have been reported after contamination of private[446-448, 450] and mains[452] water supplies, and also after contamination of recreational waters[458-461]. Foreign travel has been identified as a risk factor for norovirus-associated IID[140, 549-551] and outbreaks are frequently reported on cruise ships[552-554].

Whilst there is a large body of epidemiological evidence on the modes of transmission and risk factors for norovirus-associated IID during norovirus outbreaks, there have been only a few studies examining risk factors for sporadic norovirus-associated IID acquired in the community, outside of recognised outbreaks[22, 140, 380]. None of these

previous risk factor studies, all which used RT-PCR testing, took account of norovirus viral load in the selection of norovirus cases; the analysis presented in Chapter 4 indicates that inclusion of all norovirus RT-PCR positive IID cases, regardless of viral load, may lead to outcome misclassification. The only investigation of risk factors for norovirus-associated IID in England is the analysis of electron microscopy diagnosed norovirus cases from the Study of Infectious Intestinal disease, which excluded children aged less than five years[83]. Risk factors for asymptomatic norovirus infection have never been investigated.

The aim of this analysis was to identify risk factors for both symptomatic and asymptomatic norovirus infection in the community, across all ages. The analysis made use of the larger group of norovirus cases now identified in the Study of Infectious Intestinal Disease specimen archive, through real-time RT-PCR testing and application of the viral load cut-off from Chapter 4, and the substantial number of asymptomatic norovirus infections identified in the specimen archive retesting. The analysis was informed by the large number of studies of norovirus epidemiology that have been published since the Study of Infectious Intestinal Disease was carried out, and used recently-developed multiple imputation techniques to account for missing responses in the dataset[555-560].

6.2. Methods

6.2.1. Inclusion criteria and testing

Full details of recruitment and testing during the Study of Infectious Intestinal Disease were provided in Chapter 3.

IID cases in the specimen archive, from either the community cohort or the general practice case-control study, were eligible for inclusion in this analysis if a norovirus Ct value had been determined by real-time RT-PCR. The genogroup II Ct value cut-offs, selected in Chapter 4, were used to identify those IID cases who had disease caused by genogroup II noroviruses. However, the genogroup I cut-off was not used, because it is unlikely that it would accurately identify IID cases with disease caused by genogroup I noroviruses, due to the small sample size for the genogroup I ROC analysis, and the problems with variable efficiency of the real-time RT-PCR assay between genotypes in genogroup I. Therefore, only those IID cases infected with genogroup I noroviruses who were previously positive for norovirus by electron microscopy were included in this analysis. Use of electron microscopy positive

genogroup I-infected IID cases will minimise outcome misclassification because the detection limit of electron microscopy is at high viral loads that correlate well with norovirus disease[80, 99, 103, 240]. Mixed genogroup infections were classified according to their genogroup II Ct value using the genogroup II cut-offs.

All healthy controls from the Study of Infectious Intestinal Disease specimen archive, who had been free of diarrhoea and vomiting for at least 10 days, were eligible for inclusion in this analysis. These controls were considered asymptomatic with respect to IID, although they may have experienced other symptoms during that time, as described in Chapter 5. Norovirus-infected individuals who were recruited as controls in the original study were used to examine risk factors for asymptomatic norovirus infection. Individuals who were recruited as controls in the original study who tested negative for norovirus were used as the comparison group in the analyses of risk factors for norovirus infection and disease.

Selection of IID cases and controls for inclusion in this analysis is summarised in Figure 6.1.

6.2.2. Case and control definitions

Cases of norovirus-associated IID ('norovirus cases') were:

i. IID cases infected with genogroup II noroviruses, who had a Ct value determined by real-time RT-PCR testing that was equal to or less than the age-specific Ct value cut-offs described in Chapter 4 (Ct value 30 for children aged less than five years, Ct value 33 for children and adults aged five years and older);

   Or

ii. IID cases infected with genogroup I noroviruses, detected by electron microscopy and confirmed by RT-PCR.

'Asymptomatic norovirus infections' were:

Controls recruited in the original study who tested positive for norovirus by electron microscopy and/or RT-PCR.

'Norovirus negative controls' were:

Controls from the original study who tested negative for norovirus by electron microscopy and RT-PCR.

6.2.3. Epidemiological data

Norovirus cases, asymptomatic norovirus infections and norovirus negative controls provided information on socio-demographic characteristics and risk factors relating to IID in the baseline and epidemiological questionnaires, as described in Chapter 3. The specific questionnaire items used in this analysis are shown in Appendix A4.1 and the variables created from these items are shown in Table 6.1.

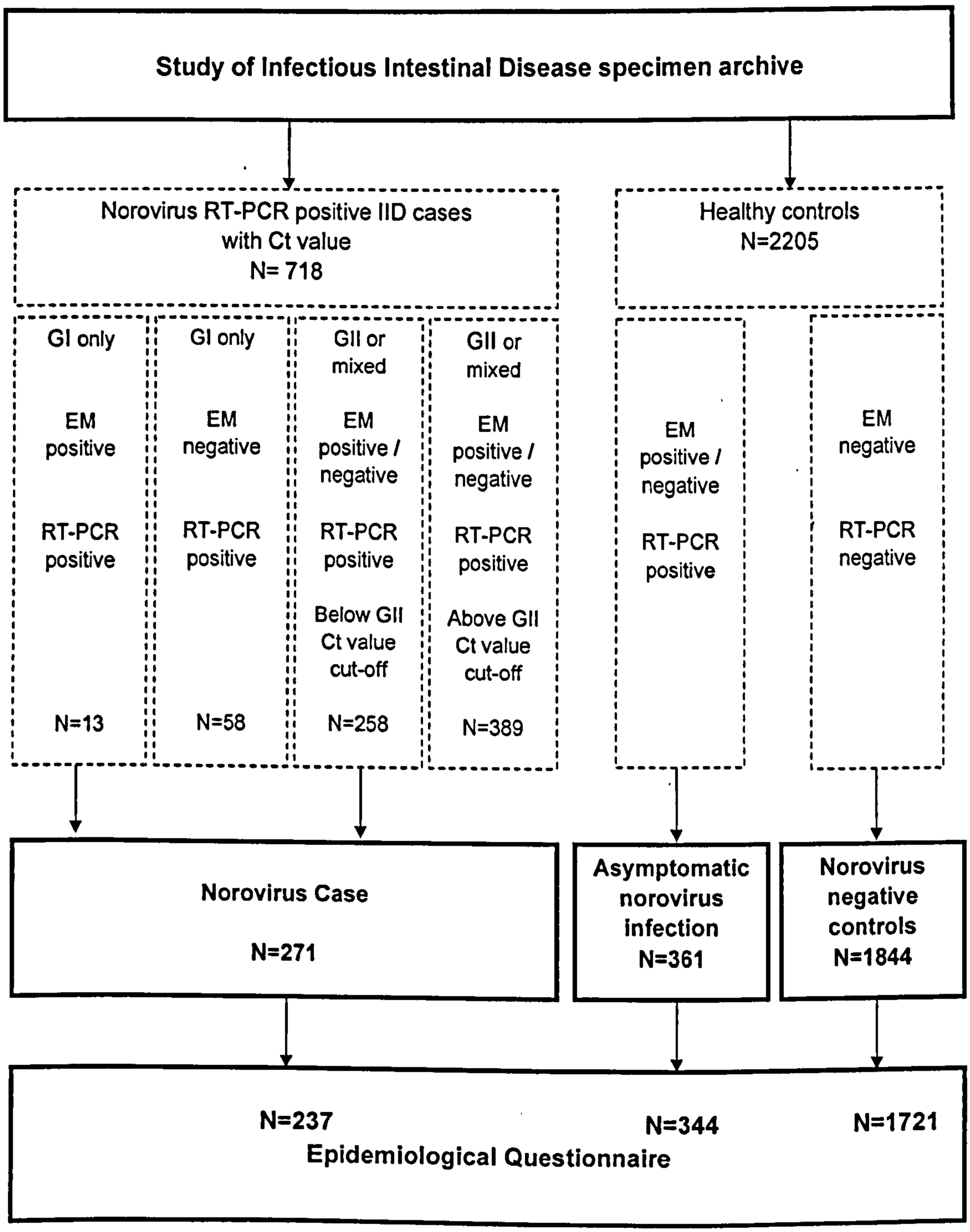
6.2.4. Conceptual framework

Risk factors for norovirus-associated IID were investigated by comparing norovirus cases to norovirus negative controls. Risk factors for asymptomatic norovirus infection were investigated by comparing asymptomatic norovirus infections to norovirus negative controls. All norovirus cases were analysed together, i.e. community cohort and general practice cases and genogroup I and genogroup II cases.

A hierarchical conceptual framework[561] was used to investigate risk factors, separately, for norovirus-associated IID and asymptomatic norovirus infection (Table 6.1). The conceptual framework had three levels: (i) distal factors, which are general characteristics and long-term behaviours e.g. socioeconomic and demographic information; (ii) intermediate factors, which are specific behaviours that may increase the risk of exposure for a short time but are not necessarily always a direct source of infection ; and (iii) proximal factors which are a direct source of infection. Reporting of intermediate and proximal risk factors was limited to the previous 10 days before symptom onset for norovirus cases, and 10 days before questionnaire completion for asymptomatic norovirus infections and norovirus negative controls (Table 3.1).

The intermediate and proximal risk factor models were adjusted for higher level variables in the conceptual framework. Indicator variables for the general practice through which each individual was recruited and month since the beginning of the study were added to the proximal risk factor models that included infectious contacts, to account for potential geographical and temporal variation in norovirus transmission. Norovirus cases were allocated to the month in which their symptoms started and asymptomatic norovirus infections and norovirus negative controls were allocated to the month in which they provided a stool specimen and/or were recruited.

Figure 6.1 Summary of testing, case and control selection and sample size for the analysis of risk factors for norovirus-associated IID and asymptomatic norovirus infection. Abbreviations: GI, genogroup I; GII, genogroup II; EM, electron microscopy.



During the Study of Infectious Intestinal Disease, controls in the nested case-control study and general practice case-control study were recruited concurrently to IID cases, from within the same general practice, and were matched on age and sex (Appendix A1.2). There were insufficient numbers of norovirus cases with matched controls from the original recruitment, who were norovirus negative by RT-PCR, to allow a matched analysis of risk factors for norovirus-associated IID based on the original matching (18 pairs aged less than five years, 49 aged five years and older). Therefore, an unmatched analysis was performed, but, in addition to including indicator variables for time (month since beginning of the study) and recruiting general practice, the regression models were also adjusted for the other matching factors (sex and age) to account for similarities between norovirus cases and norovirus negative controls introduced during recruitment. The age bands used for matching controls to IID cases during recruitment were used for age adjustment in the regression models (Appendix A1.2).

Risk factors for norovirus infection and disease were analysed separately in children aged less than five years and older children and adults (aged five years or older).

6.2.5. Dealing with missing values

Two separate analyses were carried out using: (i) all participants, creating a categorical indicator for missing responses (missing indicator); and (ii) all participants with missing responses imputed (multiple imputation).

Missing responses in the explanatory variables included in the conceptual framework were imputed, using imputation by chained equations, in Stata 10.1[494, 559]. The imputation prediction model, used to select the most likely value for each missing response, included all variables in the conceptual framework. In addition, indicator variables for the following characteristics were included in the prediction model: registered general practice; month since the beginning of the study; the route of recruitment into the study (community cohort or general practice case-control study); norovirus infection/disease status; and the norovirus season in England and Wales during 1993 to 1996 (defined using Health Protection Agency norovirus laboratory reports, as described in Appendix A4.2). There were no missing data in these indicator variables, they only informed the imputation of missing responses in the explanatory variables from the conceptual framework. Twenty imputed datasets were created and analysed together.

Table 6.1 Conceptual framework for analysis of risk factors for norovirus-associated IID and asymptomatic norovirus infection.

Level	Variable	References
Distal factors	Age	
	Sex	
	Social class[a]	83
	Household size (number of people)	
	Household age structure (number of children <5 years)	
	Household crowding (number of people per room)	
	Baby in nappies in the household[b]	
	Pet ownership	163
	Sharing a bathroom or toilet with another household	
	Nursery/day care attendance[c]	562
	Breast feeding[d]	
	Hand hygiene[e]	336, 406, 409, 466
Intermediate factors	Water sports	458-461
	Foreign travel	140, 549, 551-554
	Animal contact	22, 163, 380
Proximal factors	Food (raw fruit/vegetables/shellfish/meals prepared outside home)	349, 360, 397, 410, 412, 413, 423, 425, 426, 443-445, 545, 563
	Household infectious contact	22, 83, 85, 365, 380, 406
	Infectious contact outside the household	22, 83, 380, 402, 543

[a] Social class was based on occupation of the wage earner in the household[564].
[b] Investigated as a risk factor only for children and adults aged five years and older.
[c] Investigated as a risk factor only for children aged less than five years.
[d] Investigated as a risk factor only for infants aged less than one year.
[e] Measured as the response of the person in the household responsible for food shopping and preparation to the statement "it doesn't matter whether you wash your hands or not before handling food" – response options were agree / disagree / don't know.

6.2.6. Regression modelling

The entire model selection process was carried out separately on the missing indicator and multiple imputation datasets.

Standard logistic regression models were fitted using Stata 10.1[494]. The imputed datasets were analysed using the *ice* suite of commands[494, 555-560], in which the logistic regression model is fitted separately to each of the 20 imputed datasets. The results of the 20 regression models are then combined, to give one point estimate for each odds ratio, with standard errors that take account of uncertainty in both the multiple imputation process and the standard regression.

For each analysis, the distal risk factor model was fitted first, with all variables included together; any variables with a *P* value below 0.1 were selected for inclusion in the final model, for further investigation of their effects. This variable selection process was repeated for the intermediate and proximal risk factor models. The results presented include variables with a *P* value less than 0.1 in the final model.

6.2.7. Population attributable fractions

Population attributable fractions (PAFs) were calculated in Stata 10.1 from the final multiple imputation regression models, using the *aflogit* programme[565, 566] within the *ice* programme, with user-defined code (I. White personal communication).

6.3. Results

There were 237 norovirus cases, 344 asymptomatic norovirus infections and 1721 norovirus negative controls available for the analysis (Figure 6.1). Results from the multiple imputation models are presented in Tables 6.2 to 6.4. The final model for the missing indicator analysis was identical to that from the multiple imputation analysis, with very similar effect estimates. Results from the missing indicator analysis are provided in Appendices A4.3 and A4.4.

6.3.1. Risk factors for norovirus-associated IID in children aged less than five years

Children from households in which the main wage earner had a manual or unskilled occupation had more than twice the odds of norovirus-associated IID compared to

Table 6.2 Risk factors for norovirus-associated IID in children aged less than five years (multiple imputation model).

	Exposure prevalence				
	Norovirus cases	Norovirus negative controls	Odds Ratio[a]	95% CI	*P* value[b]
Total	81	461			
Social class					
Non-manual	35.8	56.8	1.0		
Manual/Unskilled	50.6	35.6	2.3	1.4 - 3.9	0.002
Military	1.2	0.9	2.3	0.2 - 22.1	0.46
Housewife/student/carer	6.2	2.6	4.1	1.4, 12.3	0.01
Missing	6.2	4.1			
Foreign travel	2.5	0.9	6.6	0.9 - 47.3	0.06
Missing	1.2	1.3			
Animal Contact	32.1	44.5	0.6	0.3 - 1.0	0.06
Not sure	1.2	2.0	0.5	0.1 - 4.8	0.57
Missing	1.2	3.0			
Fruit eaten	60.5	75.9	0.5	0.3 - 0.8	0.007
Pre-prepared raw salad or /vegetables eaten	1.2	7.2	0.2	0.0 - 1.3	0.08
Household infectious contact	39.5	9.3	5.7	2.0 - 16.2	0.001
Not sure	2.5	1.3	3.6	0.3 - 47.9	0.33
Missing	9.9	3.3			
Infectious contact outside the household	34.6	6.7	33.9	9.5 - 121.1	<0.001
Not sure	16.0	13.9	4.4	1.5 - 13.3	0.009
Missing	1.2	0.7			

[a] All odds ratios are from multiple imputation models and are adjusted for age and sex; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model.
[b] *P* values are from a Wald test of regression coefficients.
Abbreviations: CI, confidence interval.

those from non-manual occupational social classes (Table 6.2). Recent foreign travel greatly increased the odds of norovirus-associated IID (Table 6.2). Norovirus-associated IID was strongly associated with contact with individuals with IID symptoms (Table 6.2). Whilst the odds ratio was much higher for contacts outside the household (Table 6.2), they accounted for a similar proportion of norovirus-associated IID episodes as infectious contacts inside the household (household infectious contacts: PAF 33% [95% confidence interval (CI): 19, 48]; infectious contacts outside the household: PAF 32% [95% CI: 20, 44]). Together, infectious contacts inside or outside the household accounted for 54% of norovirus-associated IID episodes in children aged less than five years (95% CI: 42, 66). For infectious contacts inside the household, the odds of norovirus-associated IID were higher when the infectious contact was another young child, compared to infectious contacts aged five years or older, and the odds increased slightly with the number of infectious household contacts (Table 6.4).

Eating fruit and raw vegetables was associated with lower odds of norovirus-associated IID in children aged less than five years, as was contact with animals during this time (Table 6.2).

6.3.2. Risk factors for norovirus-associated IID in older children and adults

Older children and adults (aged five years or older) living in a household with a baby in nappies had three times the odds of norovirus-associated IID (Table 6.3). The odds were also increased for older children and adults living in households where at least one member was a child aged less than five years (Table 6.3) and these two exposures accounted for similar proportions of norovirus-associated IID episodes (living in a household with children aged less than five years: PAF 20% [95% CI: 10, 30]; living with a baby: PAF 16% [95% CI: 8, 25]).

Individuals reporting recent contact with a person with IID symptoms were at increased odds of norovirus-associated IID, but the risk was similar for household contacts and contacts outside the household (Table 6.3). Infectious contacts accounted for almost half of norovirus-associated IID in older children and adults (household infectious contacts: PAF 24% [95% CI: 13, 33]; infectious contacts outside the household: PAF 22% [95% CI: 13, 30]; combined PAF 39% [95% CI: 29, 49]). For infectious contacts inside the household, the odds of norovirus-associated IID were much higher if the infectious contact was aged less than five years and the odds increased with the number of infectious contacts (Table 6.4).

Table 6.3 Risk factors for norovirus-associated IID in older children and adults (aged five years and Older) (multiple imputation model).

	Exposure prevalence				
	Norovirus cases	Norovirus negative controls	Odds Ratio[a]	95% CI	*P* value[b]
Total	156	1260			
Household structure					
Single person household	4.5	6.9	1.1	0.5 - 2.3	0.86
Adults and children aged ≥5 years only	53.8	71.0	1.0		
≥1 children aged <5 years	30.1	14.1	2.5	1.6 - 4.0	<0.001
Missing	11.5	8.0			
Baby wearing nappies in the household	25.0	9.0	2.9	1.8 - 4.6	<0.001
Missing	1.9	2.9			
Water sports in last 10 days	10.3	17.9	0.4	0.2 - 0.8	0.009
Missing	7.7	6.1			
Foreign travel	7.1	2.5	3.3	1.5 - 7.3	0.004
Missing	1.9	2.4			
Animal Contact	19.2	36.3	0.4	0.3 - 0.7	<0.001
Not sure	3.8	1.0	3.3	1.0 - 10.7	0.05
Missing	5.8	3.2			
Oysters eaten	1.9	0.1	18.3	1.5 - 226.6	0.02
Whelks/winkles eaten	1.9	0.1	20.5	1.6 - 265.7	0.02
Fruit eaten	71.8	82.5	0.6	0.4 - 0.8	0.006
Household infectious contact	26.9	6.7	4.9	2.7 - 8.8	<0.001
Not sure	4.5	2.5	2.2	0.7 - 6.9	0.16
Missing	12.8	9.8			
Infectious contact outside the household	26.9	9.2	4.5	2.5 - 8.0	<0.001
Not sure	20.5	14.5	2.1	1.1 - 3.9	0.01
Missing	2.6	1.7			

[a] All odds ratios are from multiple imputation models and are adjusted for age, sex and social class; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model, except the presence of a baby in the household.
[b] *P* values are from a Wald test of regression coefficients. Abbreviations: CI, confidence interval.

Foreign travel and eating shellfish increased the odds of norovirus-associated IID in older children and adults (Table 6.3). Shellfish consumption accounted for a small proportion of norovirus-associated IID episodes in older children and adults (oysters and whelks or winkles: PAF 2% [95% CI: 0, 4]).

Consumption of fruit, recent participation in water sports and contact with animals were associated with lower odds of norovirus-associated IID (Table 6.3).

6.3.3. Risk factors for asymptomatic norovirus infection

Females had slightly increased odds of asymptomatic norovirus infection (children <5 years odds ratio (OR): 1.4 [95% CI: 1.0, 1.9; *P* = 0.07]; older children and adults OR: 1.4 [95% CI: 1.0, 2.0; *P* = 0.09]). After adjusting for age and sex, both eating salad (OR: 0.6 [95% CI: 0.4, 0.8; *P* = 0.004]) and participation in water sports (OR: 0.6 [95% CI: 0.4, 1.0; *P* = 0.06]) were associated with lower odds of asymptomatic norovirus infection in older children and adults. No other variables were associated with asymptomatic norovirus infection. Appendix A4.5 shows the asymptomatic norovirus infection odds ratios for the variables that were associated with norovirus-associated IID.

Table 6.4 Risk of norovirus-associated IID due to the number and age of household infectious contacts (multiple imputation model).

	Children aged < 5 years			Older children and adults aged ≥5 years		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Number of household infectious contacts						
0	1.0			1.0		
1	1.6	0.8 - 3.1	0.15	1.4	0.8 - 2.4	0.26
≥2	2.9	0.8 - 10.3	0.10	5.8	1.7 - 19.3	0.005
Age of household infectious contact						
No infectious contacts	1.0			1.0		
Children aged <5 years	2.6	1.0 - 6.8	0.06	4.3	1.9 - 9.6	<0.001
Older children & adults only (≥5 years)	1.6	0.8 - 3.3	0.16	1.5	0.8 - 1.6	0.172

[a] Odds ratios are from multiple imputation models and are adjusted for age, sex, social class and all other risk factors included in the final models shown in Table 6.2 and Table 6.3, except a baby in the household for older children and adults and infectious contact variable.
[b] *P* values are from a Wald test of regression coefficients.
Abbreviations: OR, odds ratio; CI, confidence interval.

6.4. Discussion

The major risk factors for norovirus-associated IID were related to contact with an infectious person. Foreign travel and consumption of shellfish increased the risk of norovirus-associated IID, whilst consumption of raw fruit and vegetables, participation in water sports and contact with animals were associated with a decreased risk of norovirus-associated IID. There was no evidence that any exposure greatly increased the risk of asymptomatic norovirus infection, although eating salad and water sports participation were associated with a lower risk of asymptomatic infection in older children and adults.

Infectious contacts accounted for more than half of norovirus-associated IID in children aged less than five years and almost half in older children and adults. The importance of contact with individuals with IID for transmission of norovirus has been reported in previous case-control studies[22, 83, 380] and there is substantial evidence of person-to-person transmission from outbreak investigations[4, 140, 352, 378, 391, 402, 406, 543, 544]. Whilst neither household size, nor crowding (the number of people per room), affected the risk of norovirus-associated IID, the age of household members was a risk factor in older children and adults. Living in a household with a baby or young children accounted for a third of norovirus-associated IID in this age group. The highest incidence of norovirus-associated IID is in young children (see Chapter 8), so this association may reflect the fact that they are more likely to introduce norovirus into a household than older individuals. Furthermore, when a household contact with IID symptoms was reported, the risk of norovirus-associated IID was greatest when this contact was a young child. This pattern of transmission, from young children to adults, was also observed in a large household transmission study following cases from a point-source norovirus outbreak[85].

In young children, the risk of norovirus-associated IID from infectious contacts outside the household was much greater than that associated with infectious contacts inside the household, although there was no risk specifically associated with attendance at day care. Norovirus causes symptomatic infection, with high viral loads, in individuals of all ages, so there is potential for transmission to children in a wide variety of settings, not just through contact with other young children in day care settings, which has been shown to be important for acquiring rotavirus-associated IID[386, 482, 567]. Breast-feeding was not protective against norovirus-associated IID in infants. Norovirus-specific immunoglobulin A has been recovered from breast milk[568], but the antigenic variation of

noroviruses is extensive, with very little cross-protection between strains[123, 125, 179, 191], and even strain-specific immunity is believed to last no longer than a year[54, 56].

Individuals of all ages who had recently travelled outside the UK had an increased risk of norovirus-associated IID. This risk has been demonstrated in previous studies[549], and may be attributable to changes in risk behaviours whilst travelling, or exposure to a different spectrum of norovirus strains[569]. Recent foreign travel also increases the likelihood that an individual with IID, due to any pathogen, will present to a GP[381]; the majority of the norovirus cases in this analysis (73%) were from the general practice case-control study, rather than from the community cohort nested case-control study, but the prevalence of recent foreign travel was very similar in general practice and community norovirus cases (6% and 5% respectively), so it is unlikely that this association is due to the predominance of general practice cases in this analysis. However, it is possible that using cases recruited at general practitioners may have caused the association between social class and norovirus-associated IID in young children, because substantially more community cases were from non-manual occupational social classes (community cases 70% vs. general practice cases 41%).

There was no evidence that hand washing was protective against norovirus-associated IID, despite many experimental, intervention and observational studies showing that hand washing with soap and water is an effective method for reducing the incidence of infection with directly-transmitted viruses[389, 570, 571], including norovirus[336, 406, 409, 466]. Hand hygiene was not measured directly in the Study of Infectious Intestinal Disease; it was collected only in relation to food preparation, by self-report from the person in the household responsible for food shopping and preparation (not always the study participant). In addition, normal hand-washing practices used by study participants may not be as rigorous as those used in experimental studies and participants may also have falsely reported good hand hygiene because this is a socially desirable response[572]. It is therefore unlikely that this variable accurately captured the general hand hygiene behaviour of study participants.

A large number of published outbreak investigations have attributed norovirus-associated IID to contamination of food during preparation in restaurant and catering settings[349, 351, 360, 397, 410, 412, 413, 573], as well as to raw fruit and vegetables contaminated during wholesale production[444, 445], consumption of oysters and other shellfish[423-426, 443], and to contamination of both drinking[446-448, 450, 452, 574] and recreational water[458-461]. Information on drinking water exposures was not collected in a suitable format for reliable analysis. Water sports participation and raw fruit and vegetable consumption

were actually protective against norovirus-associated IID, as was contact with animals. There was no evidence of increased risk associated with eating at restaurants or catered events. The reduced risk associated with animal contact has been reported in two previous case-control studies of community-acquired norovirus-associated IID in high income countries[22, 380] and water sports were associated with a reduced risk of IID due to other pathogens in this Study of Infectious Intestinal Disease[83]. There are a number of potential explanations for these exposures reducing the risk of norovirus-associated IID: (i) they are correlated with other lifestyle factors that are protective against norovirus-associated IID; (ii) they do lead to norovirus transmission but are repetitive, long-term behaviours, so exposed individuals have higher levels of norovirus immunity, due to regular immune boosting; or (iii) specifically for consumption of fruit and vegetables, they have positive effects on gut immunity or the balance of intestinal bacterial flora , increasing resistance to IID. In contrast, oysters and other shellfish, which may be consumed less frequently than fruit and vegetables, and in which high-level norovirus contamination is common[431-435, 440], did increase the risk of norovirus-associated IID, although they accounted for only a small proportion of norovirus disease in this study population. Finally, although foods prepared in restaurants are commonly reported as vehicles of infection in outbreaks, it is possible that breakdowns in food hygiene are relatively infrequent and therefore contribute little to the overall population burden of sporadic norovirus-associated IID.

None of the exposures that increased the risk of norovirus-associated IID were associated with asymptomatic norovirus infection, although eating salad and water sports participation reduced the risk of asymptomatic infection in older children and adults. The asymptomatic norovirus infections detected in this study were prevalent, not incident, infections. Asymptomatic individuals were recruited at random from the general population and prior to determination of their norovirus infection status. It is possible that the transmission event leading to many of these asymptomatic infections occurred outside of the 10-day retrospective exposure period that was measured in the risk factor questionnaire, and was therefore not captured in the responses. Norovirus has been detected by RT-PCR for at least two weeks after experimental inoculation in otherwise healthy adult volunteers, who did not develop diarrhoea or vomiting[80]. Prolonged post-symptomatic shedding has also been demonstrated, lasting from one to eight weeks[80, 84, 104, 107, 108, 504, 575]. Even for those individuals who were infected during the questionnaire exposure period, if they did not collect their specimen concurrently to questionnaire completion, their norovirus infection status may not correspond to the exposures reported. Only studies with frequent and regular stool specimen collection

and testing, irrespective of disease status, could ensure that proximal risk factors reported by individuals with asymptomatic norovirus infection relate to the norovirus transmission event. However, such studies are resource intensive and may be difficult to justify, in terms of the benefits to patients, or improvements in epidemiological knowledge, without first demonstrating the importance of asymptomatic infections in transmission.

A recent volunteer study examining the relationship between inoculum size and the development of norovirus infection and disease has shown that whilst norovirus infection occurred even at very small doses, the development of symptoms after infection was linearly related to the concentration of the inoculum: the higher the infecting dose the greater the probability of a volunteer developing symptoms once norovirus infection has occurred[98]. It is therefore also possible that the lack of association between asymptomatic norovirus infection and symptomatic infectious contacts in this analysis reflects a real difference in the route of transmission between symptomatic and asymptomatic norovirus infection, i.e. the high viral loads shed by norovirus cases tend to lead to further symptomatic norovirus infection, whereas asymptomatic norovirus infections tend to arise from the lower-level shedding from other asymptomatic norovirus infections. However, given the documented importance of environmental contamination for norovirus transmission, and the likelihood that the norovirus concentration encountered on contaminated surfaces is much lower than that encountered through direct contact with a norovirus case, the transmission pattern between symptomatic and asymptomatic norovirus infections is unlikely to be that simple.

Cases from the community cohort and the general practice case-control study were analysed together and the matching used for recruitment in the Study of Infectious Intestinal Disease was relaxed, to increase statistical power. However, to prevent bias, due to relaxing the matching, the regression models were adjusted for the original matching factors (age, sex, registered general practice, time)[576]. Multiple imputation was used to account for missing responses in the dataset, so it was possible to fit a multivariable regression model without limiting the inclusion criteria to those individuals who answered all of the questionnaire items used in the analysis ('complete case analysis'), an approach, which is widely recognised to bias results if data are not missing completely at random[577, 578]. The analysis of the multiply imputed dataset and the analysis using a categorical indicator for missing values, produced very similar results, supporting the use of multiple imputation in future work, which should be the

preferred method because it provides standard errors that also take account of uncertainty in the results due to the missing data.

This is the first study of risk factors for norovirus-associated IID to use viral load to identify cases of norovirus-associated IID, rather than a positive RT-PCR result or electron microscopy detection. The analysis presented in Chapter 4 showed that many IID cases in the Study of Infectious Intestinal Disease who were norovirus RT-PCR positive had the same viral loads seen in healthy controls, indicating that their norovirus infection may not actually be the cause of their illness. Including cases whose IID is not caused by norovirus in the analysis would introduce misclassification with respect to the outcome; it is likely that the IID cases with low viral loads will have disease caused by a range of other gastrointestinal pathogens, so it is difficult to predict the consequences of incorrectly including these cases in an analysis of risk factors for norovirus-associated IID. However, using viral load to diagnose norovirus-associated IID substantially reduces the problem of outcome misclassification, although it may not eliminate it completely.

The genogroup I Ct value cut-off was not used because it was likely that there would be substantial outcome misclassification, because of the small sample size for the genogroup I ROC analysis and because each Ct value does not represent the same underlying viral load for all the genotypes within norovirus genogroup I (see Chapter 3). Therefore only those genogroup I norovirus infected cases who were previously positive by electron microscopy were included in the analysis. This does mean that genogroup I norovirus cases were underrepresented in the analysis, but comparison of risk factors between the genogroups was not the aim of the analysis, and even if the cut-off had been used, it is unlikely that there would have been sufficient numbers of genogroup I cases for such a comparison. There have been no studies comparing risk factors between norovirus genogroups or genotypes, so it is unclear what effect, if any, grouping them together in the analysis might have had on the results. However, evidence from outbreaks indicates that genogroup I and genogroup II noroviruses are both directly transmissible, via person-to-person spread, and both genogroups have been detected in food- and water-borne outbreaks[4, 354, 390, 450]. The only notable, currently recognised difference between norovirus genotypes is the HBGA binding specificity required for host cell infection[150, 154, 155], which was not determined in the individuals included in this study. A study of risk factors for individual norovirus genogroups or genotype, or one that also considers host susceptibility in terms of pre-

existing immunity and genetic resistance, would be enormously resource intensive and perhaps logistically impossible.

6.5. Summary

The results of this updated analysis of risk factors for norovirus-associated IID broadly agree with the results from the previous analysis using only electron microscopy positive IID cases from the Study of Infectious Intestinal Disease. However, in this analysis, with a larger sample size, and with more variables investigated, it has been possible to provide a more detailed understanding of the transmission of norovirus in the community. In particular characteristics such as household age structure and the differing risk associated with child and adult infectious contacts clearly indicate that transmission from children to other household members is very important, as has been shown for influenza[579, 580]; this information is important in the design of any potential public health interventions to reduce norovirus transmission. Similarly the association of particular lifestyle and dietary factors with reduced risk of norovirus infection may allow generation of hypotheses about the mechanisms of norovirus pathogenesis and the factors that determine whether infection is symptomatic, which could have potential for informing efforts to develop therapeutic or prophylactic treatments.

Incidence

Chapter 7: Incidence of general practice consultations for norovirus-associated infectious intestinal disease in England and Wales based on routine surveillance data

In the previous two sections a method was developed for interpreting real-time RT-PCR test results to identify individuals with norovirus-associated IID, the clinical and epidemiological characteristics of these norovirus cases, and of individuals with asymptomatic norovirus infection, were then examined and compared. In this final section, two separate and independent methods will be used to estimate the incidence of IID caused by norovirus in the community, and to estimate the frequency of consultations to general practitioners for norovirus-associated IID. In this chapter, routine surveillance data from England and Wales will be used to estimate the incidence of general practice consultations. In Chapter 8, the incidence of community disease and general practice consultations for norovirus will be calculated using the viral load data from the Study of Infectious Intestinal Disease.

7.1. Background

In the UK, the major public health impacts of norovirus-associated IID occur in healthcare settings, where norovirus outbreaks disrupt service provision, increase care costs, and may worsen patient outcomes[14, 92, 338], and at the primary-care level, where norovirus causes a substantial frequency of consultations for IID[8]. Whilst the frequency of norovirus outbreaks in hospitals in England and Wales is now measured in a routine surveillance system[348], there is no ongoing surveillance of general practice consultations for norovirus-associated IID.

Prospective research studies, such as the Study of Infectious Intestinal Disease[83], can provide very accurate estimates of the incidence of general practice consultations for norovirus-associated IID. However, norovirus has particularly complex epidemiology [97, 581], making it important to have up-to-date estimates for setting healthcare service priorities, and for predicting and assessing the impact of any future vaccination programmes, or other public health interventions, against norovirus. Cohort studies are particularly expensive, resource intensive and time-consuming, so it is useful to have cheaper and more rapid methods for continual assessment of the burden of disease caused by norovirus and other pathogens.

7.1.1. Routinely available information on general practice consultations for norovirus-associated IID in England and Wales

In England and Wales, general practices record microbiological diagnostic results in patients' records. However, only a small proportion of patients presenting to their general practitioner with IID are asked to provide stool specimens for diagnostic testing, because it is not always necessary for optimal patient care[8, 582]. The microbiological cause of illness remains undiagnosed in approximately three quarters of patients presenting with IID[8, 582]. Physicians may also be more likely to request stool specimens from patients with bacterial IID compared to those with viral infections[8], meaning that norovirus is probably particularly under-diagnosed. Diagnostic results from general practice patient records will therefore not provide an accurate estimate of the frequency of consultations caused by norovirus.

National Health Service (NHS) and Health Protection Agency (HPA) laboratories voluntarily report norovirus diagnoses to the HPA national surveillance of laboratory-confirmed infections. However, diagnostic results for all gastrointestinal pathogens are underreported, with the degree of underreporting varying by pathogen[8]. A large amount of norovirus testing in NHS and HPA laboratories is conducted as part of IID outbreak investigations, rather than to diagnose microbial aetiology in general practice patients with IID. The source of the specimen is reported for very few of the norovirus laboratory reports in the national surveillance, so it is not possible to distinguish the diagnoses arising from outbreaks versus general practice patients. Due to underreporting and the bias towards outbreak investigation in the norovirus reports, national laboratory surveillance data cannot directly provide estimates of the frequency of general practice consultations for norovirus-associated IID.

7.1.2. Use of generalised linear regression modelling to attribute syndromic disease burden to individual pathogens

Several previous studies have used generalised linear regression modelling to overcome the lack of accurate information on microbial aetiology amongst patients presenting with infectious diseases[47, 48, 338, 339, 583-585]. Time series of healthcare consultations for a particular syndrome (e.g. IID or acute respiratory infections), are modelled as the dependent variable, with time series of un-linked laboratory diagnoses for selected pathogens as the independent variables. The pathogen-specific regression coefficients are then used to attribute a proportion of the overall disease burden to particular pathogens.

In the analysis presented below, generalised linear modelling is used to estimate the incidence of general practice consultations for norovirus-associated IID in England and Wales. The limitations of using the norovirus laboratory report data in these models are discussed further and a novel adaptation of the method is presented, to account for these limitations.

7.2. Methods

7.2.1. Datasets

7.2.1.1. Royal College of General Practitioners Surveillance Scheme

Weekly counts of general practice consultations for IID in England and Wales, between 1993 and 2007, were provided by the Royal College of General Practitioners (RCGP) Research and Surveillance Centre[483, 484]. Consultations for IID were defined as those assigned International Classification of Disease version 9 (ICD9) codes 001 to 009. Repeat consultations by a patient for the same IID episode were excluded. The RCGP Surveillance Scheme included approximately 600 000 patients between 1993 and 2005, and approximately 900 000 from 2006 onwards, when additional general practices were recruited (Figure 3.3). Further details about the RCGP Research and Surveillance Centre data are provided in Chapter 3.

Whilst there are other available datasets in the UK that capture information on general practice consultations, these other data sources provide individual level data, rather than aggregate level data, and therefore also charge a licence fee for access to the data, to cover the costs of the more extensive data cleaning and management required. Aggregated data was sufficient for the purposes of this analysis, so the RCGP Surveillance Scheme data were considered most suitable.

Table 7.1 Description of the Royal College of General Practitioners IID consultations and pathogen laboratory diagnoses reported to the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections, across all ages, 1993 to 2007.

	Median	IQR	Min	Max	% zero weeks
RCGP IID episodes	196	147 - 296	71	456	0
Campylobacter spp.	896	718 - 1165	97	1865	0
Salmonella spp.	312	195 - 497	0	1279	0.5
Rotavirus	136	54 - 464	14	1289	0
Shigella spp.	29	21 - 44	3	444	0
Giardia lamblia	78	60 - 102	6	217	0
Cryptosporidium spp.	72	49 - 107	5	558	0
Norovirus	36	20 - 66	1	621	0
Adenovirus	25	18 - 32	3	102	0
Escherichia coli	18	9 - 30	1	155	0
Astrovirus	2	1 - 6	0	35	24.7
Staphylococcus aureus	1	0 - 2	0	424	42.9
Sapovirus	1	0 - 2	0	10	43.8
Vibrio spp.	1	0 - 2	0	9	29.9
Clostridium perfringens	0	0 - 1	0	65	59.3
Bacillus spp.	0	0	0	4	94.9

Abbreviations: IID, infectious intestinal disease; IQR, interquartile range; RCGP, Royal College of General Practitioners; spp., species.

7.2.1.2. Health Protection Agency National Surveillance of Laboratory-Confirmed Infections

Weekly counts of laboratory diagnoses of 15 common gastrointestinal pathogens, made in England and Wales between 1993 and 2007, were provided by the HPA. Electronic reports of laboratory diagnoses are submitted voluntarily by NHS and HPA microbiology laboratories. Diagnoses included in this analysis were those made on faecal or lower gastrointestinal tract specimens only. Repeat specimens from the same patient during investigation of a single disease episode were excluded. The pathogens included in the analysis, and the median weekly counts of diagnoses are shown in Table 7.1.The diagnostic methods and recommended testing policies for the pathogens included in this analysis are summarised in Table 3.5. Further details about the HPA national surveillance of laboratory-confirmed infections are provided in Chapter 3.

7.2.2. Limitations of the norovirus laboratory report data

When using generalised linear modelling to attribute healthcare consultations to a particular pathogen, it is essential that the time series of laboratory diagnoses, used as the explanatory variables, are representative of the overall trends in pathogen incidence at the level of healthcare contact under consideration, e.g. community disease, primary or secondary care consultations. Given the testing policies recommended in the HPA National Standard Methods (summarised in Table 3.5 [486, 487]) and the epidemiology of the pathogens considered in this analysis, it is likely that the laboratory reports for the majority of pathogens arise from sporadic cases of IID, who have presented to primary or secondary healthcare services, rather than cases identified during investigations of IID outbreaks[2]. Individuals tested during IID outbreak investigations may not necessarily consult a general practitioner, and outbreaks caused by a particular gastrointestinal pathogen may have different epidemiology to sporadic cases caused by that pathogen[83, 481, 586-589].

In contrast to the other common gastrointestinal pathogens in the UK, norovirus is a major cause of both healthcare-associated and community outbreaks of IID in England and Wales; therefore it is likely that a substantial proportion of laboratory reports for norovirus arise from outbreak investigations, rather than from individuals consulting healthcare services. In the microbiological investigation of IID, the HPA National Standard Methods[486, 487] recommend norovirus testing for all specimens collected from children aged less than five years and adults aged 60 years or older; application of norovirus testing to specimens from children and adults aged between five and 59 years is only recommended if they are part of a recognised IID outbreak (Table 3.5). However, within inpatient healthcare and other semi-closed residential settings, norovirus outbreaks are much more common amongst the elderly than younger age groups[14, 38], meaning that the majority of norovirus laboratory reports in older adults are likely to arise from outbreak investigations as well. The age distribution of the patients represented in the norovirus laboratory reports indicates that outbreak specimens constitute a large majority of the records: 67% of specimens were submitted from patients aged 65 years or older (Table 7.2). In contrast, only 9% of patients presenting to general practitioners with norovirus-associated IID were aged 65 years or

Table 7.2: Age distribution of norovirus positive patients with IID from the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections and general practice patients included in the Study of Infectious Intestinal Disease.

Age	National Surveillance of Laboratory-Confirmed Infections	The Study of Infectious Intestinal Disease	
	1993-2007 (n=37186)	1993-1996 (n=6153)	1993-1996 (n=232)
0 – 4 years	11%	26%	32%
15 – 64 years	22%	21%	59%
≥65 years	67%	53%	9%

older in the Study of Infectious Intestinal Disease, during which comprehensive microbiological testing was carried out in all patients (Table 7.2) [8, 51]. Children and adults aged between five and 64 years are particularly underrepresented in the norovirus laboratory report data compared to general practice patients in the Study of Infectious Intestinal Disease (Table 7.2).

The epidemiology of healthcare-associated norovirus outbreaks differs from community outbreaks[590]; there is a distinct winter seasonality in the incidence of outbreaks in healthcare settings, whereas outbreaks in community settings show no seasonality. This indicates that healthcare-associated norovirus infections, which predominate in the norovirus laboratory report data, may have different epidemiology to norovirus disease in the community. It is therefore unlikely that norovirus laboratory reports for children and adults aged five years or older will be representative of changes in the frequency of general practice consultations for norovirus-associated IID, because the majority of these reports come from norovirus outbreaks, rather than sporadic cases presenting to healthcare services, as described above. Only the norovirus laboratory reports for children aged less than five years are likely to be representative of healthcare consultations for norovirus-associated IID because comparatively few come from norovirus outbreaks.

7.2.3. Generalised Linear Regression Model

7.2.3.1. Overview

In light of the limitations of the norovirus laboratory report data, the frequency of general practice consultations for norovirus-associated IID was estimated using two approaches:

1. The 'direct method' - the norovirus laboratory report data were used as an explanatory variable in a generalised linear model of the general practice consultations for IID and the norovirus regression coefficient was used to estimate the frequency of norovirus consultations;

2. The 'indirect method' – norovirus was excluded from the explanatory variables and laboratory report data for all other gastrointestinal pathogens in Table 7.1 were used to model variation in general practice consultations for IID; the remaining variation in the consultations, after fitting the model, was used to estimate the frequency of norovirus consultations.

The direct method applies the commonly used approach for attributing health outcomes to particular pathogens in the absence of data on microbial aetiology, including studies that have estimated the incidence of hospitalisations[339] and deaths[338] due to norovirus in England and Wales. These direct estimates are subject to the limitations of the norovirus laboratory data described above. The estimates from the indirect method will not be affected by these problems because the norovirus reports are not used in the model. However, the indirect method does require the assumption that after modelling variation due to all other common gastrointestinal pathogens, any remaining variation in the general practice consultations must be due to norovirus infections.

The same basic model structure and confounder model was used in both the direct and indirect methods. The model components were fitted in the order:

1. Confounder variables;
2. Pathogen explanatory variables;
3. Investigate interactions between norovirus laboratory reports and time (direct model only);
4. Autocorrelation adjustment;
5. Sensitivity analyses.

7.2.3.2. Model structure

Multivariable time series-adapted Poisson regression was used to model variation in consultations to general practitioners for IID in England and Wales. The weekly counts of pathogen laboratory reports were used as the primary explanatory variables. Additional explanatory variables were created to model the effects of confounders present in time series data. An additive model, on the normal scale, was used because changes in general practice consultations are most likely to be proportional to changes in the incidence of individual pathogens, rather than the relationship being multiplicative; in the additive model, the general practice consultations were modelled directly on the normal scale, rather than using the log of the consultation counts as the response variable, which is the default link-function used in a Poisson regression model. The model residuals were adjusted for Poisson overdispersion in the general practice consultations.

The general practice consultations were modelled separately in three age groups: children aged less than five years; children and adults aged between five and 64 years; and adults aged 65 years or older.

The basic structure of the model was:

$$Y_x = \alpha + \sum_{i=1}^{n} \beta_{ix} P_{ix} + \gamma_{tx} T_x + \gamma_{sx} S_x + \sum_{y=1}^{9} \theta_{yx} D_y + \gamma_{ax} A_x + \varepsilon_x \qquad \text{Equation 7.1;}$$

where Y_x is the weekly count of general practice consultations for IID in age group x; α is a constant term; P_{ix} is the weekly count of laboratory reports for pathogen i in age group x, β_{ix} is the corresponding regression coefficient and n is the number of pathogen variables included in the model; T_x is a trend adjustment term, specific to age group x, with coefficient γ_t; S_x is a seasonal adjustment term, specific to age group x, with coefficient γ_s; D_y is a series of nine indicator variables for events such as bank holidays and other reporting artefacts, the same in all age groups, and θ_y is the corresponding regression coefficient for age group x; A_x is an autocorrelation adjustment term, specific to age group x, with coefficient γ_a; and ε_x is a model-specific error term.

7.2.3.3. Confounder model

Seasonality: Fourier terms were used to adjust for seasonality in the general practice consultations (S in Equation 7.1). Fourier terms are linear combinations of pairs of sine and cosine functions of time, used to recreate regular temporal patterns in time series data. The more Fourier terms added to the model, the more complex the seasonal

pattern recreated. The optimal number of Fourier terms was determined using the likelihood ratio test to compare the log-likelihood of successive models with increasing numbers of Fourier terms; Fourier terms were added until there was no further improvement in model fit, evidenced by a change in the log likelihood ratio (likelihood ratio test P value >0.1).

Long-term trends: Optimal adjustment for long term trends in the general practice consultations (*T* in Equation 7.1) was achieved by successively adding a linear, quadratic, then cubic function of time into the model, until there was no further improvement in model fit, evidenced by a change in the log likelihood ratio (likelihood ratio test P value >0.1).

Bank holidays and other reporting artefacts: Binary indicator variables (*D* in Equation 7.1) were used to adjust for bank holidays in England and Wales and RCGP data extraction dates, which cause reductions in the number of general practice consultations for IID that are unrelated to changes in disease incidence (see Figure 3.3). These events were identified a priori and were therefore included in all models, without assessing their effect on model fit.

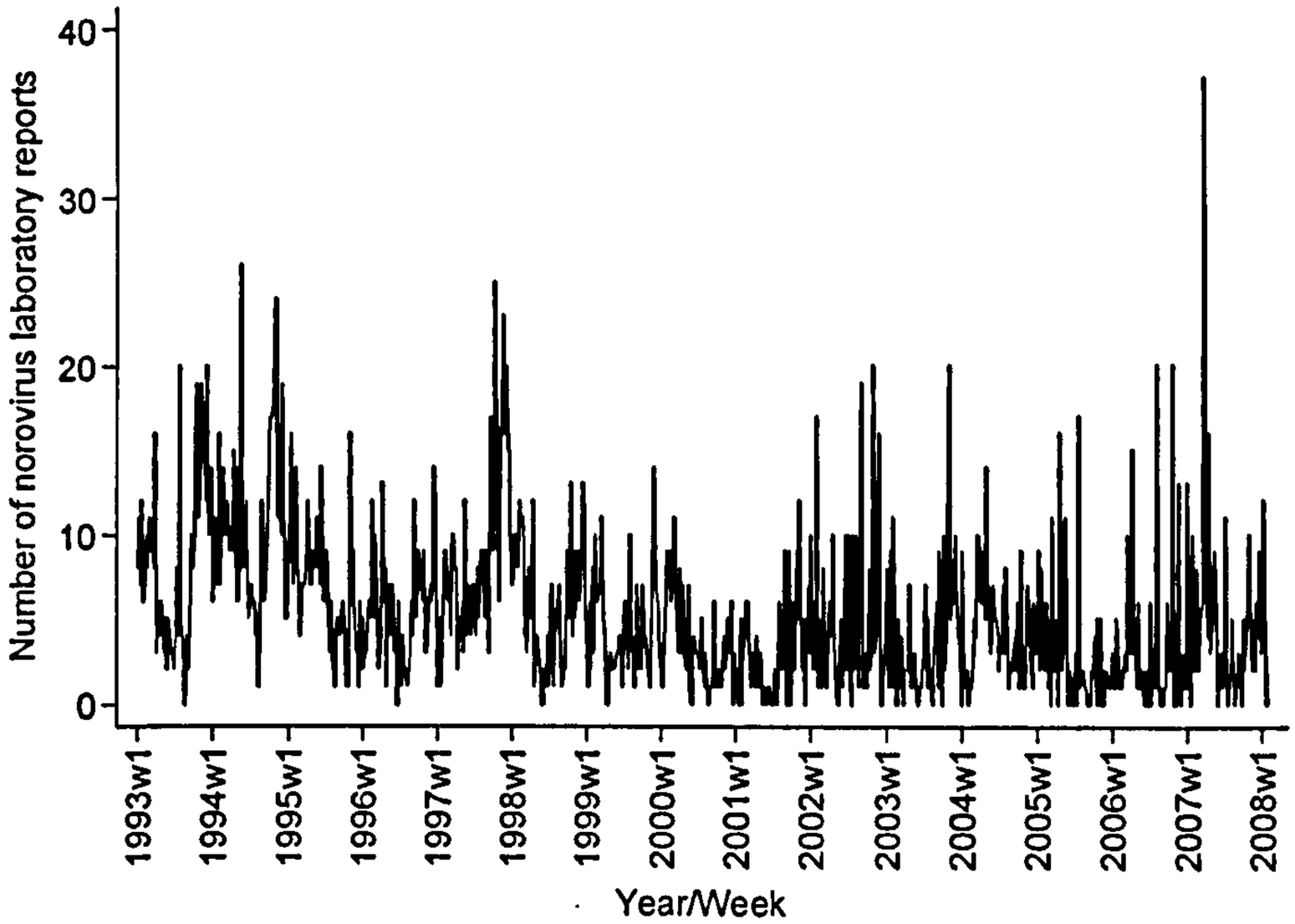
Appendix A5.1, demonstrates the effects on model fit of adding each of the confounder variables (data are shown only for children aged less than five years, to illustrate the process). Inclusion of these confounder variables prevents spurious correlations between the pathogen laboratory reports and general practice consultations due to non-causal similarities in seasonality or long term trends, ensuring that the pathogen regression coefficients represent the short term effects of changes in pathogen incidence on general practice consultations.

7.2.3.4. Pathogen explanatory variables

Model selection: The pathogen laboratory reports were added to the optimal confounder model. Any pathogens with a positive regression coefficient that had a Wald test *P* value less than 0.1, were included in the final model, which was used to estimate incidence. Norovirus laboratory report data were only included as an explanatory variable in the direct models.

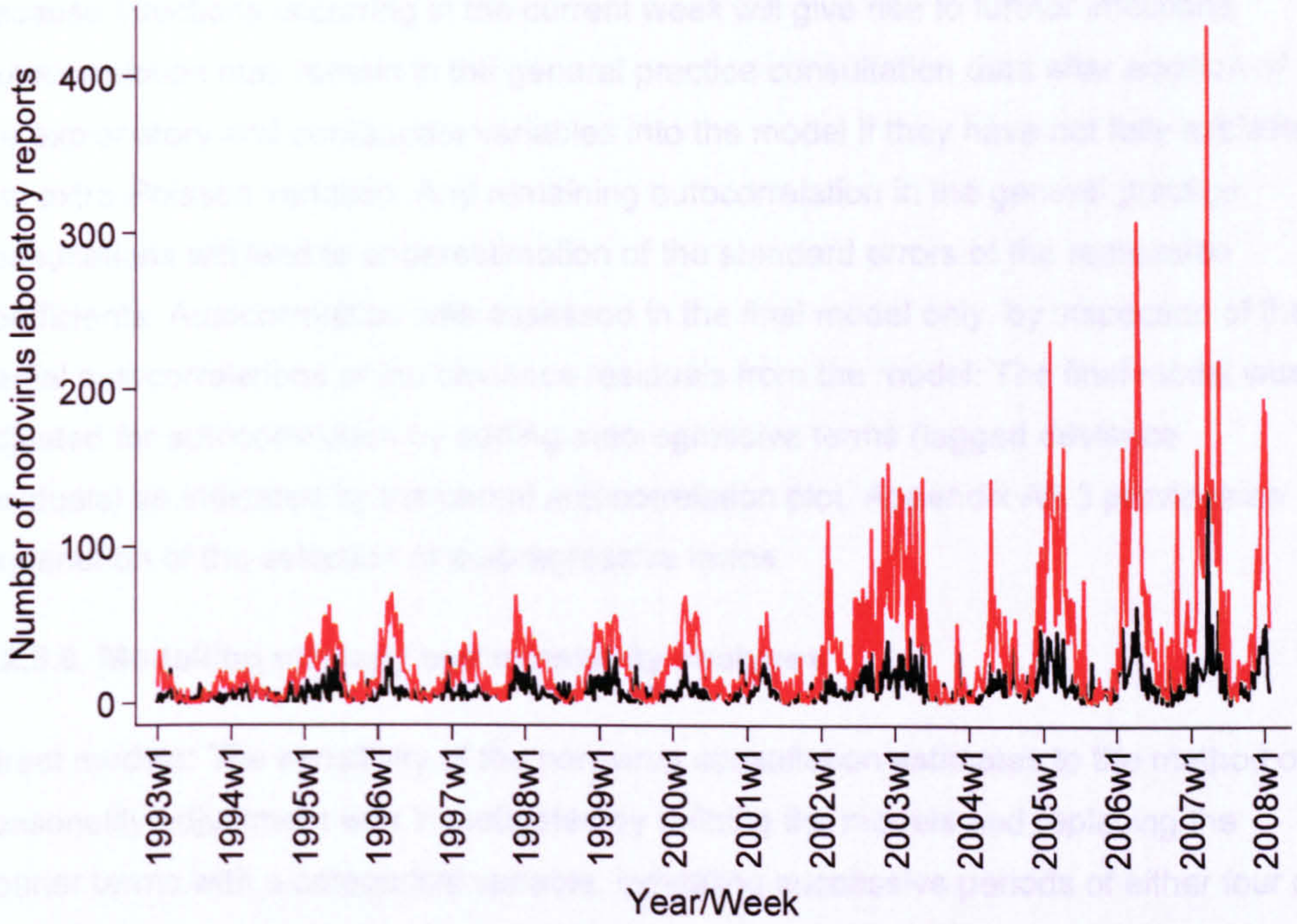
Time lags: The date of specimen receipt in the laboratory was used to temporally relate the pathogen laboratory reports to the general practice consultations, because it was the only date available for all laboratory report records. The date of specimen collection

Figure 7.1 Weekly counts of norovirus laboratory diagnoses for children aged less than five years, reported to the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections, 1993 to 2007.



was not available for laboratory reports prior to 2002, but was reported for the majority of specimens tested between 2002 and 2007 (99.6%). If we assume that the laboratory reports have arisen from patients consulting their general practitioner, then general practice consultations in a given week will correspond to laboratory reports in a subsequent week, because of the delay between presentation, specimen collection and receipt in the laboratory. Lags between events in the dependent variable and the explanatory variables can be explicitly incorporated in time series-adapted regression, by modelling the dependent variable against values of the explanatory variable in a different week. The median delay between specimen collection and receipt in the laboratory was estimated using records between 2002 and 2007 and was used as the forward lag applied to the pathogen laboratory report data. General practice consultations in week 'n' were therefore fitted against pathogen laboratory reports in week 'n+m' where m is the median number of weeks between specimen collection and laboratory receipt. The lags used for each pathogen are shown in Appendix A5.2.

Figure 7.2 Weekly counts of norovirus laboratory diagnoses for children and adults aged five years or older, reported to the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections, 1993 to 2007. The black line shows children and adults aged between five and 64 years; the red line shows adults aged 65 years and older.



7.2.3.5. Other time series components

Interactions with time: In the direct models only, changes in the relationship between norovirus laboratory reports and general practice consultations during the study period were investigated by fitting an interaction between the laboratory reports and a linear time variable. The relationship between the norovirus laboratory reports and the general practice consultations may change over time due to improvements in the sensitivity of diagnostic methods or changes in clinician behaviour with regards to requesting stool specimens for testing. If there was evidence of an interaction, it was included in the final direct model only if it resulted in positive coefficients throughout the study period, i.e. if the interaction term was negative, the coefficient for the norovirus in 2007 must still be positive.

Autocorrelation: Autocorrelation is present in any time series data with regular patterns, particularly in seasonal infectious disease incidence data. Two adjacent or proximal data-points in the time series will have more similar values than if they had occurred

through a truly random, Poisson process because there is a deterministic underlying mechanism giving rise to the data: pathogen transmission. In weeks when there is high incidence, there is very likely to be similar high incidence in subsequent weeks because infections occurring in the current week will give rise to further infections. Autocorrelation may remain in the general practice consultation data after addition of the explanatory and confounder variables into the model if they have not fully explained any extra-Poisson variation. Any remaining autocorrelation in the general practice consultations will lead to underestimation of the standard errors of the regression coefficients. Autocorrelation was assessed in the final model only, by inspection of the partial autocorrelations of the deviance residuals from the model. The final model was adjusted for autocorrelation by adding autoregressive terms (lagged deviance residuals) as indicated by the partial autocorrelation plot. Appendix A5.3 provides an explanation of the selection of autoregressive terms.

7.2.3.6. Modelling strategy and sensitivity analyses

Direct models: The sensitivity of the norovirus consultation estimates to the method of seasonality adjustment was investigated by refitting the models and replacing the Fourier terms with a categorical variable, indicating successive periods of either four or 13 weeks across the study period. Sensitivity to long-term trend adjustment was examined by replacing the continuous (linear, quadratic or cubic) trend term with a categorical term for each year of the study period.

Indirect models: The Pearson's residuals were used to estimate the frequency of consultations due to norovirus. It was expected that some seasonality should be evident in the residuals for the indirect models if they are to be considered representative of norovirus consultations. However, after fitting the models as described, the residuals showed no seasonality. Preliminary work applying the direct and indirect modelling methods to estimate rotavirus consultations in children aged less than five years indicated that the lack of seasonality in the residuals from the indirect models was due to the inclusion of seasonal and autocorrelation adjustment terms (See Appendix A5.15). The indirect models for norovirus consultations were refitted without autocorrelation and seasonality adjustment and further sensitivity analyses were carried out. The sensitivity of the results to long-term trend adjustment was assessed by replacing the continuous trend term with a categorical year term, and sensitivity to the lags used for the pathogen laboratory reports, by fitting general practice consultations against laboratory reports from the same week.

7.2.4. Estimating the incidence of general practice consultations for norovirus-associated IID

Direct estimates: The norovirus regression coefficient was multiplied by the norovirus laboratory reports, to generate weekly counts of expected general practice consultations for norovirus. Where an interaction between norovirus laboratory reports and time was included in the final model, the incidence was calculated as the product of the norovirus coefficient, the interaction coefficient, the value of the linear term used to model time in the interaction and the norovirus laboratory reports in a given week. The weekly incidence of norovirus consultations was estimated using the size of the patient population registered in the RCGP Surveillance Scheme in each week. To account for temporal changes in the size of the RCGP registered patient population, which are not reflected in the laboratory reports, an offset was used for 2006 and 2007 when there was a large increase in the registered population; the incidence in these years was multiplied by:

$$1 + \left(\frac{P_n - P_{1993}}{P_{1993}}\right) \qquad \text{Equation 7.2}$$

where P_n is the mean weekly registered population in 2006 or 2007 and P_{1993} is the mean weekly registered population in the first year of the study period. In weeks when RCGP Surveillance Scheme data extraction events reduced the registered population, the mean of the registered population in the previous and subsequent week was used as the denominator for the incidence. Annual incidence was calculated by summing the estimated norovirus consultation counts over each year of the study period and using the mean registered patient population for each year as the denominator, with the offset for 2006 and 2007.

Indirect estimates: The Pearson's residuals from the indirect models were used to estimate of the number of consultations due to norovirus in each week. When fitting a generalised linear model the median of the Pearson's residuals should be approximately zero, meaning that half of the weekly residuals from the indirect models were negative. The most conservative estimate of the weekly count of norovirus general practice consultations was based only on the residuals with positive values. A plausible upper estimate for the weekly count of norovirus consultations was generated by adding the absolute value of the largest negative residual to all other residuals within each norovirus year (defined as 1st July in calendar year 'n' to 30th June in year 'n+1'); this standardised the residual series within each norovirus year so that the

minimum value was zero. Residuals were standardised within each norovirus year, rather than across the whole time series, because norovirus activity varies between years. An intermediate estimate was generated by standardising the residual series to the 25th percentile within each norovirus year and discarding any remaining negative residual values when calculating incidence. The weekly and annual incidence of consultations due to norovirus was calculated from these weekly estimated counts of norovirus consultations in the same way as for the direct estimates, but without the denominator offset.

This approach, using standardisation of the residual series, was developed in preference to the alternative of adding the weekly residual value to the model constant term for a number of reasons:

i. A priori, using the constant term would lead to overestimation of norovirus consultations because this is equivalent to assuming that all consultations not accounted for by the explanatory variables are due to norovirus, which cannot be true because many minor gastrointestinal pathogens were not included as explanatory variables in the final indirect models; even for the common gastrointestinal pathogens included in the final indirect models, their contribution to the general practice consultations is unlikely to be completely captured in such a model; using only the residual series therefore provides a more conservative estimate of consultations due to norovirus;
ii. Interactions with time were included in some of the final models, making it difficult to interpret the constant term across the whole time series because in an interaction model it represents the baseline consultations in the first time period considered, e.g. if there was an interaction with year, the constant represents the baseline of consultations in 1993;
iii. The direct and indirect rotavirus models in children aged less than five years were used to validate the use of the indirect modelling approach for norovirus, but the constant term in the rotavirus indirect models was negative, whereas the constant term was large and positive in the norovirus models, making it difficult to develop an indirect method appropriate to both pathogens that included using the constant term;
iv. The marked decrease in the weekly counts of general practice consultations for IID across the study period meant that the constant term was actually larger than the weekly observed counts of consultations in some weeks

during the final years of the study period; norovirus incidence estimates based on use of the constant would be larger than the total incidence of consultations caused by all pathogens, and therefore biologically implausible;

v. Estimation of general practice consultations due to rotavirus, via both the direct and indirect methods in children aged less than five years, allowed validation of the indirect method based on the residual series only; the laboratory reports for rotavirus are not subject to the limitations described for norovirus, so that the direct estimates should provide a reliable estimate of the number of general practice consultations due to rotavirus in children aged less than five years; the indirect rotavirus consultation estimates were very similar to these direct estimates, indicating that the indirect method was appropriate.

All analyses were carried out using Stata v10.1[494].

7.3. Results

7.3.1. Descriptive analysis

During the study period, the incidence of general practice consultations for IID in the RCGP Surveillance Scheme declined substantially, although the greatest decrease was amongst children aged less than five years (Chapter 3, Figure 3.4). The weekly number of laboratory reports for norovirus remained reasonably constant in children aged less than five years (Figure 7.1), but the number of reports in older age groups increased substantially (Figure 7.2).

Figure 7.3 shows the weekly counts of general practice consultations for IID, which were used as the dependent variables in the models. Descriptions of the pathogen laboratory report data used in this analysis are provided in Appendices A5.4 and A5.6. Appendix A5.7 shows the crude correlation between laboratory reports for each pathogen and the general practice consultations. In children aged less than five years, weekly norovirus laboratory reports and general practice consultations for IID were positively correlated across the time series, whereas the correlation was negative in children and adults aged five years and older (Appendix A5.7g).

Figure 7.3 Weekly counts of general practice consultations for IID, from the Royal College of General Practitioners Surveillance Scheme, 1993 to 2007.

Figure 7.3a Children aged less than five years.

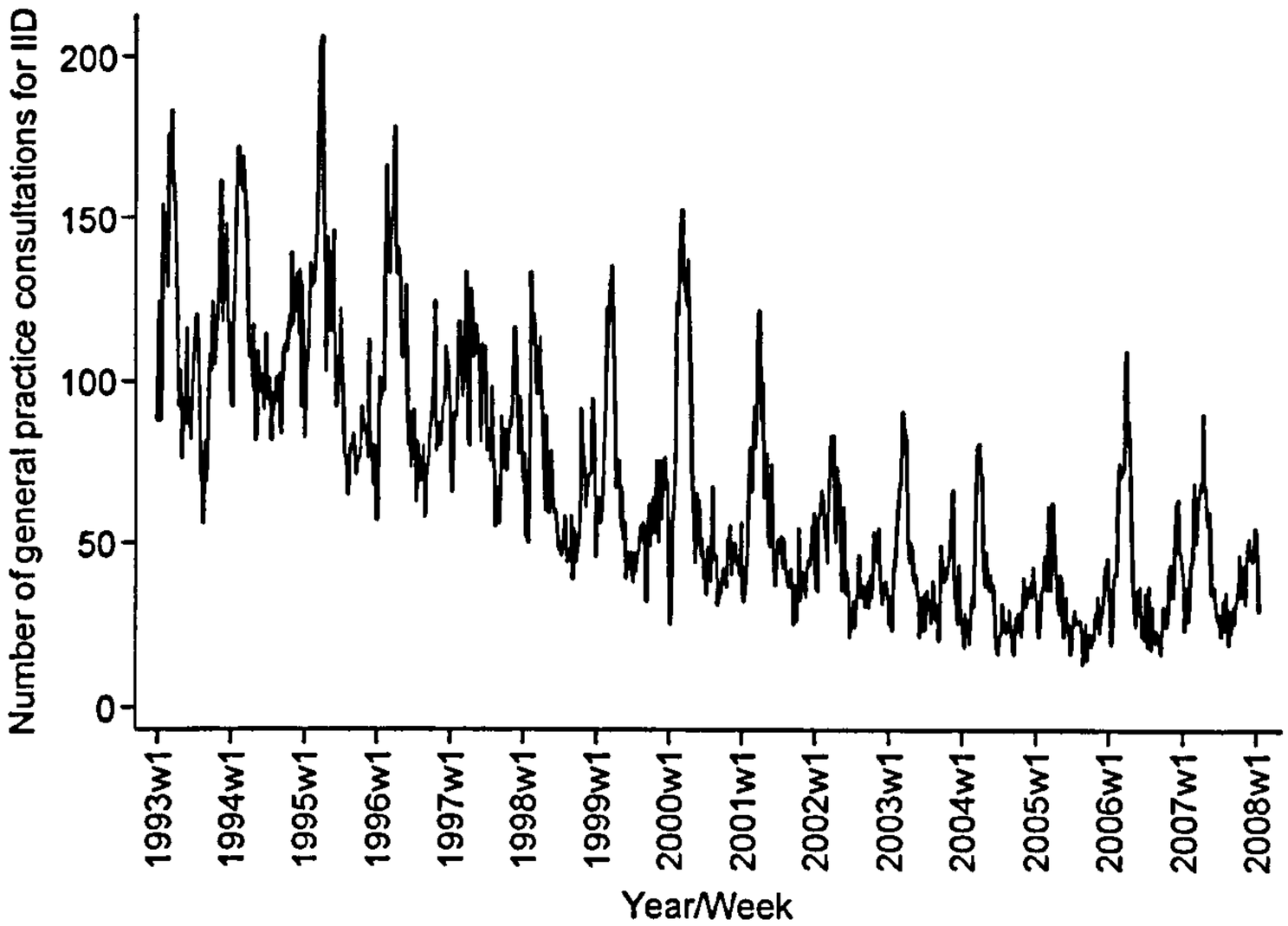


Figure7.3b Children and adults aged between five and 64 years

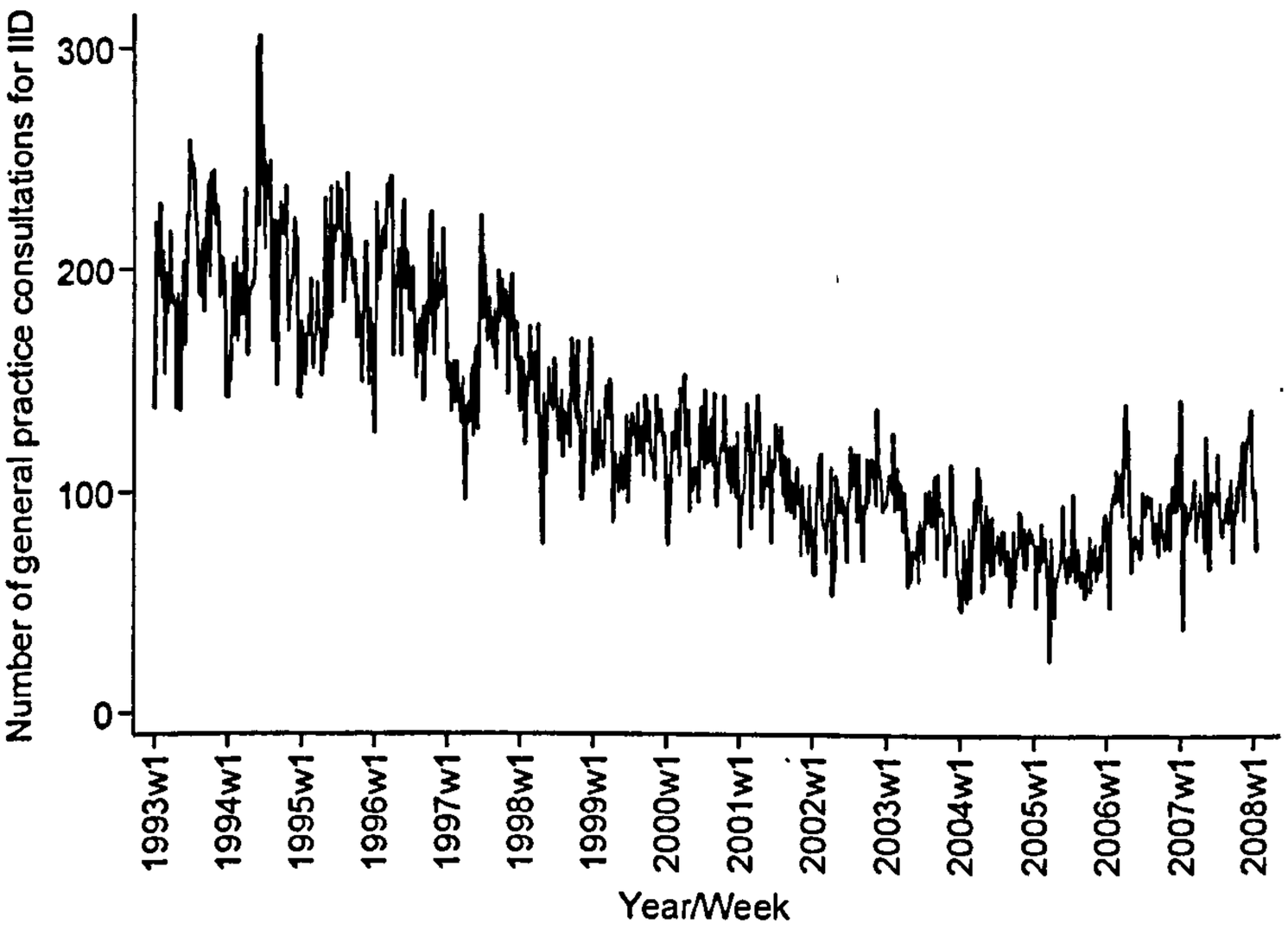
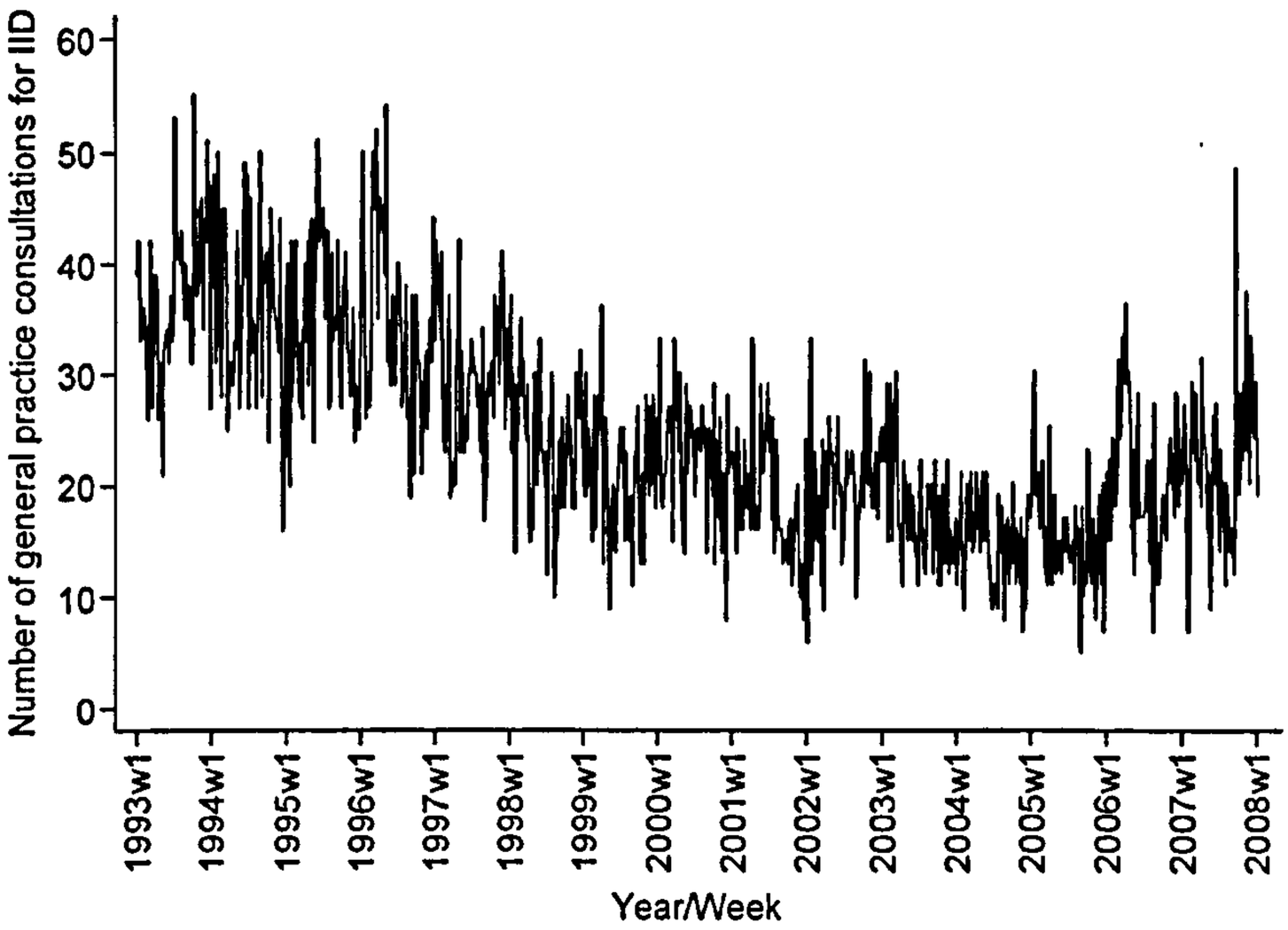


Figure7.3c Adults aged 65 years and older



7.3.2. Model fit

Tables 7.3 to 7.5 show the components included in each of the final models. Appendices A5.8 to A5.11 show the results for each stage of the model fitting process.

The direct and indirect models displayed heterogeneity of variance, with larger Pearson's residuals at the beginning of the study period, indicating poorer model fit prior to the year 2000 (Appendix 5.13 and Appendix 5.14). The model fit was particularly poor for adults aged 65 years and older; inspection of the fitted values (Appendix 5.14, Figure 5.14m to 5.14q) indicates that very little of the variation in general practice consultations is predicted by the pathogen laboratory reports and confounder variables in either the direct or indirect models in adults aged 65 years and older.

The norovirus general practice consultation incidence estimates from the direct models were sensitive to the method of seasonal adjustment used, but not to the method of trend adjustment (Tables 7.3 to 7.5, Appendix A5.12). The estimates from the indirect models showed little sensitivity to the method of long-term trend adjustment or inclusion of the forward lags on the pathogen laboratory reports (Tables 7.3 to 7.5), although there were some changes in the pathogen laboratory report explanatory

variables included in the final models when the method of seasonality adjustment was changed and the lags were removed.

In the indirect models, removal of the seasonal and autocorrelation adjustment variables slightly increased the seasonality of the estimated norovirus consultations in children aged less than five years only (Figure 7.4a and Figure 7.4b). There was no seasonality in the estimated norovirus consultations in children and adults aged between five and 64 years (Figure 7.5) or adults aged 65 years or older (Figure 7.6), regardless of the presence of the seasonality and autocorrelation adjustment variables.

An interaction between the rotavirus laboratory report explanatory variable and time (year of the study period) was introduced into the indirect model for norovirus incidence in children aged less than five years, because the higher peak in incidence at week 40 of the norovirus year, estimated from indirect model 2 (Figure 7.4b), corresponds to the single annual peak of rotavirus incidence (Appendix 5.15) and may be due to underestimation of the number of consultations attributable to rotavirus in certain parts of the study period. Introducing this interaction improved the model fit (AIC reduced from 8.89 to 8.57, Table 7.3) and reduced the size of the later peak in norovirus incidence (Figure 7.4c), which now matches the seasonality of the norovirus laboratory reports in children aged less than five years (Appendix A5.5).

Table 7.3 Details of regression models fitted to estimate the incidence of general practice consultations for children aged less than five years in England and Wales. Indicator variables were included in all models. The highlighted model was used for final incidence estimation.

Model	Pathogens	Season	Trend	Autocorrelation adjustment	Pathogen time lags	Interaction pathogen*time	AIC	Pearson's χ2	Mean annual norovirus incidence per 1000 population, 1993-2007[ii, iii]
Direct 1	Norovirus Rotavirus *Shigella*	8 Fourier	Cubic	Yes (2 AR terms)	Yes	No	7.67	1.77	4.9 (3.8, 6.0)
Direct 2	Norovirus Rotavirus *Shigella* Sapovirus	13 week	None	Yes (1 AR term)	Yes	No	7.85	1.93	2.9 (2.3, 3.6)
Indirect 1	Rotavirus *Shigella*	8 Fourier	Cubic	Yes	Yes	No	7.72	1.83	6.4
Indirect 2	Rotavirus *Shigella* *Cryptosporidium* *Giardia* Astrovirus	No	Cubic	No	Yes	No	8.89	3.02	8.3
Indirect 3	Rotavirus *Shigella* *Giardia* Astrovirus Adenovirus	No	Cubic	No	No	No	9.13	3.26	8.6
Indirect 4	**Indirect 2**	**No**	**Cubic**	**No**	**Yes**	**Rotavirus *time**	**8.57**	**2.70**	**7.7**
Indirect 5	Indirect 2	No	Categorical year term	No	Yes	No	8.71	2.86	7.9

[i] Partial autocorrelation plots are shown in Appendix A5.11
[ii] Norovirus regression coefficients used to calculated the direct estimates are shown in Appendix A5.12
[iii] Indirect estimates are based on positive residuals only
Abbreviations: AIC, Akaike's information criterion; AR, autoregressive.

Table 7.4 Details of regression models fitted to estimate the incidence of general practice consultations for children and adults aged between five and 64 years in England and Wales. Indicator variables were included in all models. The highlighted model was used for final incidence estimation.

Model	Pathogens	Season	Trend	Autocorrelation adjustment	Pathogen time lags	Interaction norovirus*time	AIC	Pearson's χ2	Mean annual norovirus incidence per 1000 population, 1993-2007[ii, iii]
Direct 1	Norovirus Rotavirus *Salmonella* *Shigella*	8 Fourier	Cubic	Yes (3 AR terms)	Yes	Yes	8.69	2.08	0.7 (0.6, 0.8)
Direct 2	Norovirus Rotavirus *Salmonella* *Shigella*	13 week	None	Yes (1 AR term)	Yes	No	8.60	1.97	0.2 (0.2, 0.3)
Indirect 1	Rotavirus	8 Fourier	Cubic	Yes	Yes	No	8.74	2.12	0.71
Indirect 2	**Rotavirus** ***Shigella*** ***Salmonella*** ***Giardia*** **Astrovirus**	**No**	**Cubic**	**No**	**Yes**	**No**	**9.43**	**2.84**	**0.85**
Indirect 3	Rotavirus *Salmonella* *Shigella*	No	Cubic	No	No	No	9.42	2.83	0.85
Indirect 4	Indirect 2	No	Categorical year term	No	Yes	No	9.19	2.56	0.79

[i] Partial autocorrelation plots are shown in Appendix A5.11
[ii] Norovirus regression coefficients used to calculated the direct estimates are shown in Appendix A5.12
[iii] Indirect estimates are based on positive residuals only
Abbreviations: AIC, Akaike's information criterion; AR, autoregressive

Table 7.5 Details of regression models fitted to estimate the incidence of general practice consultations for adults aged 65 years and older in England and Wales. Indicator variables were included in all models. The highlighted model was used for final incidence estimation.

Model	Pathogens	Season	Trend	Autocorrelation adjustment	Pathogen time lags	Interaction norovirus*time	AIC	Pearson's χ2	Mean annual norovirus incidence per 1000 population, 1993-2007[ii, iii]
Direct 1	Norovirus Rotavirus	1 Fourier	Cubic	Yes (1 AR term)	Yes	Yes	6.39	1.38	0.2 (0.2, 0.3)
Direct 2	Norovirus Rotavirus	13 week	None	Yes (1 AR term)	Yes	No	6.33	1.26	0.1 (0.05, 0.1)
Indirect 1	Rotavirus Astrovirus *Salmonella*	8 Fourier	Cubic	Yes	Yes	No	6.41	1.40	1.4
Indirect 2	**Rotavirus** **Astrovirus** ***Salmonella***	**No**	**Cubic**	**No**	**Yes**	**No**	**6.50**	**1.50**	**1.4**
Indirect 3	Rotavirus	No	Cubic	No	No	No	6.54	1.54	1.4
Indirect 4	Indirect 2	No	Categorical year term	No	Yes	No	6.46	1.45	1.4

[i] Partial autocorrelation plots are shown in Appendix A5.11
[ii] Norovirus regression coefficients used to calculated the direct estimates are shown in Appendix A5.12
[iii] Indirect estimates are based on positive residuals only
Abbreviations: AIC, Akaike's information criterion; AR, autoregressive.

7.3.3. Norovirus incidence

The annual incidence of norovirus consultations amongst children aged less than five years, estimated from the optimal indirect model (Indirect Model 4), was 16 per 1000 population for the period 1993 to 2007, with a plausible range between 8 and 38 per 1000 (Table 7.6). The annual incidence of consultations was much lower in children and adults aged between five and 64 years, and in adults aged 65 years and older (based on Indirect Model 2), at 2 per 1000 (range 1-4) and 3 per 1000 (range 1-7) respectively (Table 7.6). The incidence for all age groups decreased over the study period (Table 7.6). The indirect estimates were substantially higher than the direct estimates in all age groups.

Table 7.6 Incidence of general practice consultations for norovirus-associated IID in England and Wales.
Incidence estimates are presented using only the positive residuals ('positive only'), by standardising to the 25th percentile value of the residuals ('25th percentile') and by standardising to the value of the lowest residual ('lowest residual').

	Mean annual incidence of general practice consultations for norovirus-associated IID per 1000 population								
	1993-2007			1993-1996			2002-2007		
	25th percentile	Positive only	Lowest residual	25th percentile	Positive only	Lowest residual	25th percentile	Positive only	Lowest residual
< 5years[i]	15.5	7.7	37.7	17.8	11.3	43.1	11.7	5.4	24.7
5-64 years[ii]	1.6	0.85	4.0	2.5	1.6	5.8	0.9	0.5	2.7
≥65 years[ii]	2.8	1.4	6.7	4.0	2.3	9.0	1.9	0.9	4.7

[i] Based on indirect model 4 (long-term trend adjustment and interaction between rotavirus laboratory reports and time)
[ii] Based on indirect model 2 (long-term trend adjustment only)

Figure 7.4 Seasonality of the incidence of general practice consultations for norovirus-associated IID in children aged less than five years.

Figure 7.4a Indirect model 1 with adjustment for seasonality and autocorrelation.

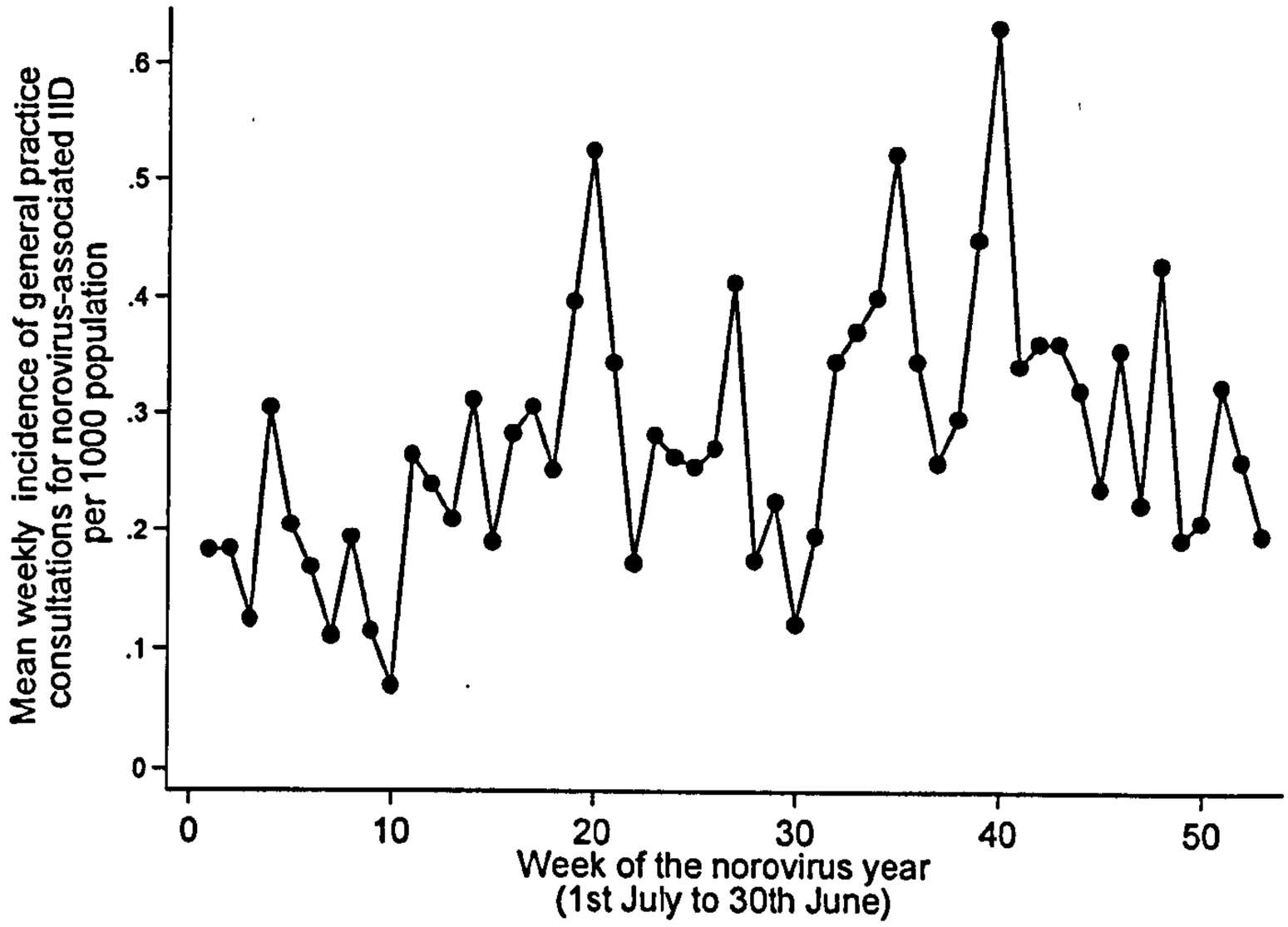


Figure 7.4b Indirect model 2 with adjustment only for long-term trends.

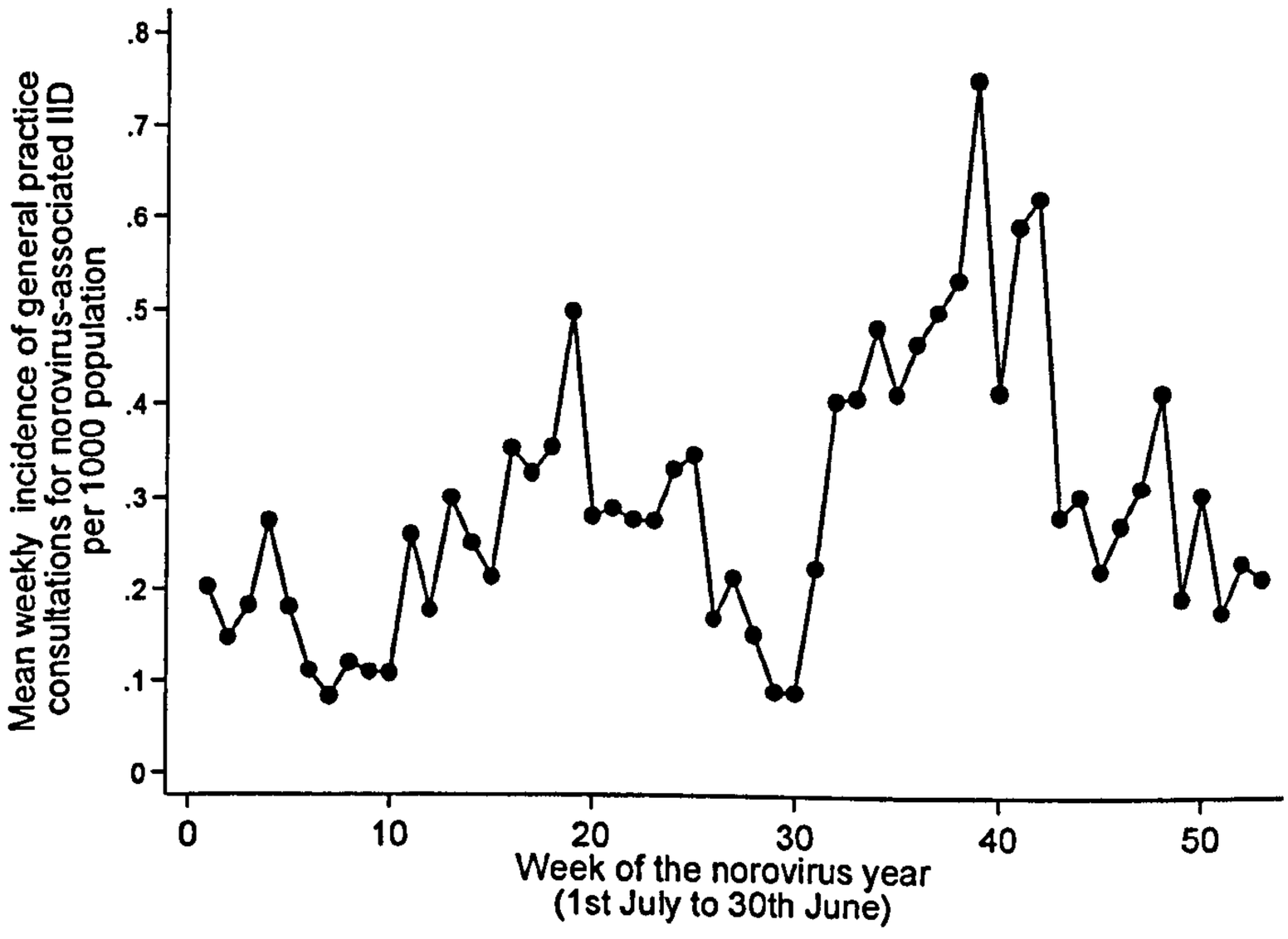


Figure 7.4c Indirect model 4 with adjustment for long-term trends and an interaction between rotavirus laboratory reports and time.

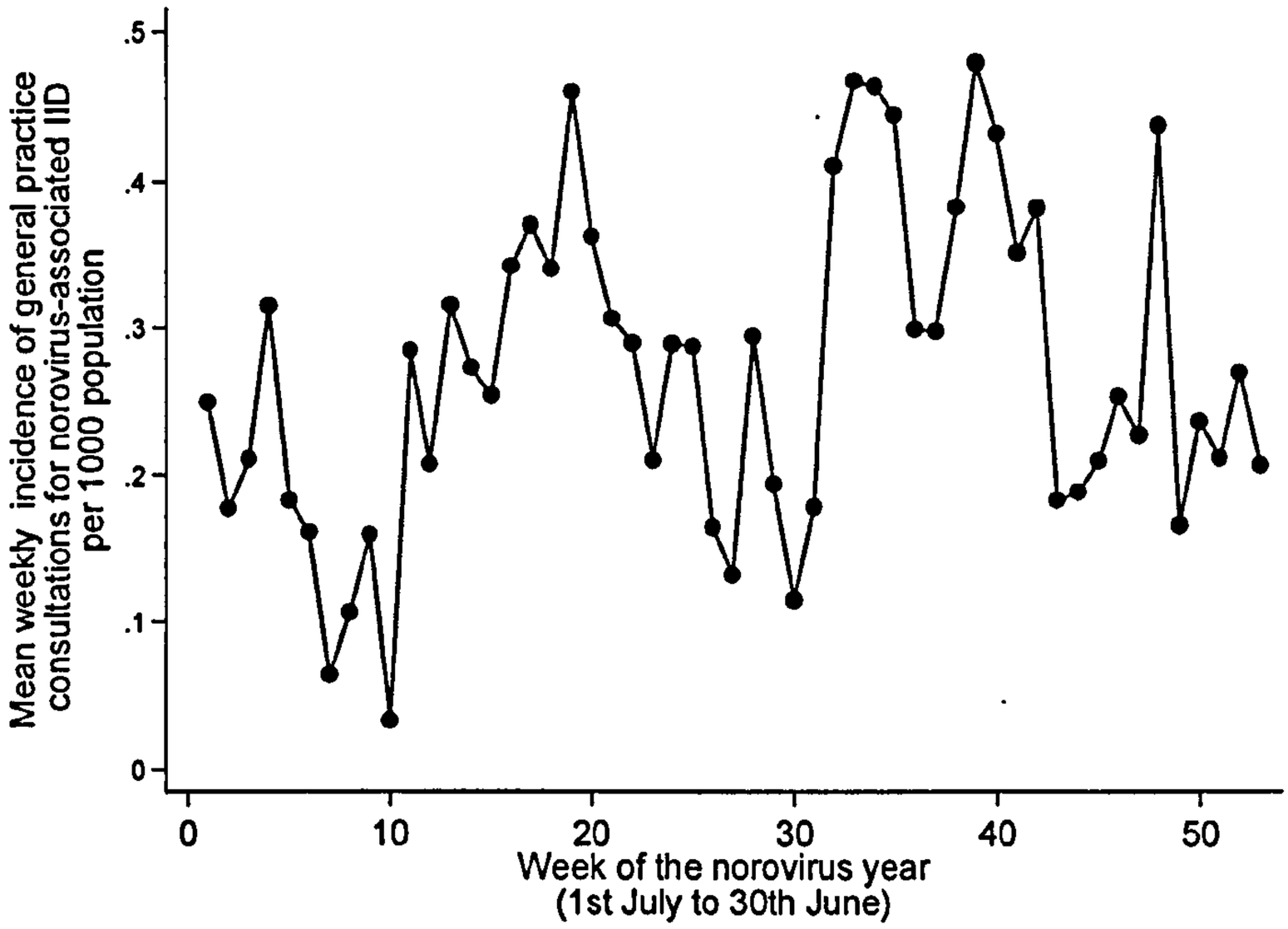


Figure 7.5 Seasonality of the incidence of general practice consultations for norovirus-associated IID in children and adults aged between five and 64 years.

Figure 7.5a Indirect model 1 with adjustment for seasonality and autocorrelation.

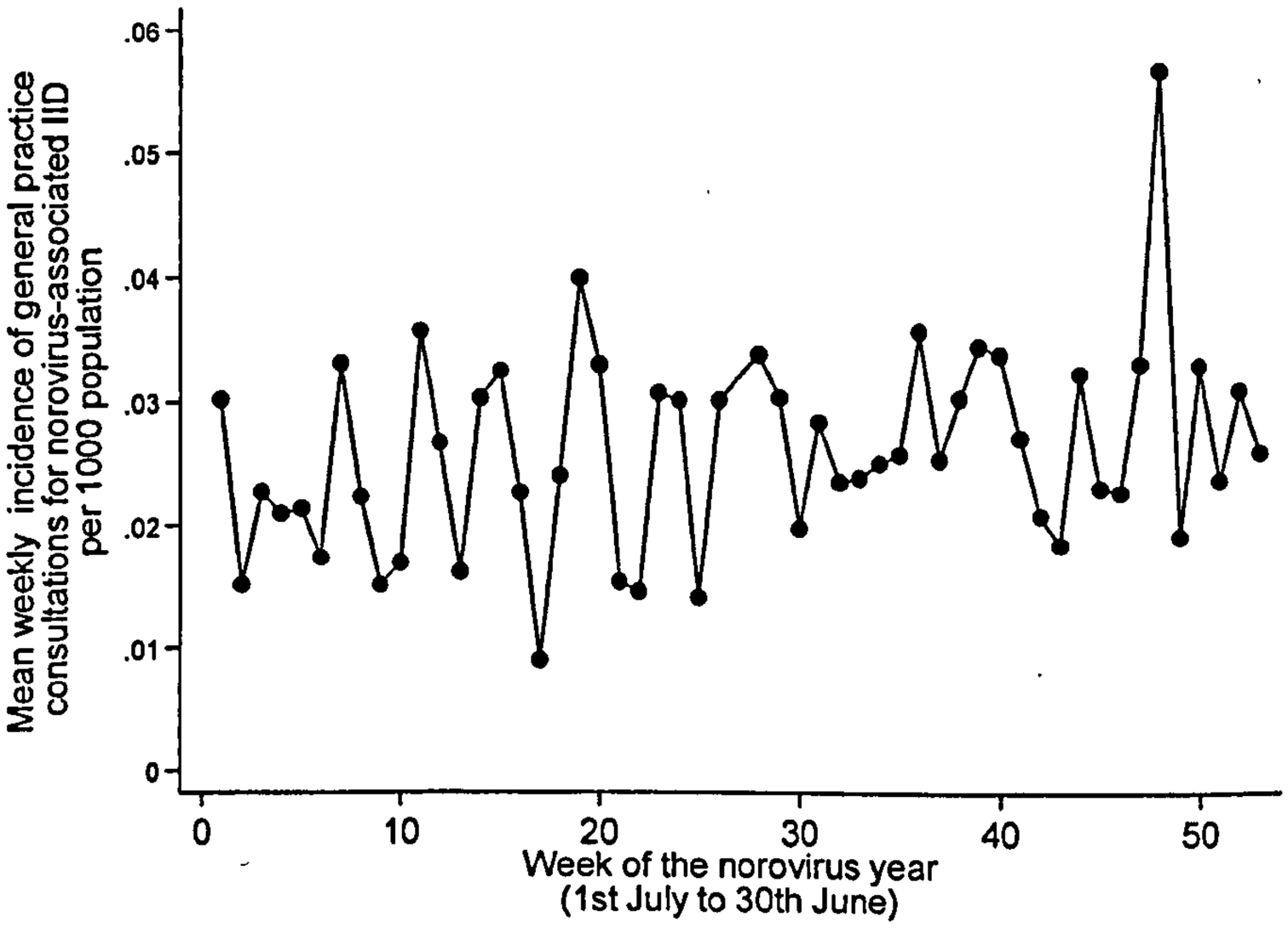


Figure 7.5b Indirect model 2 with adjustment only for long-term trends.

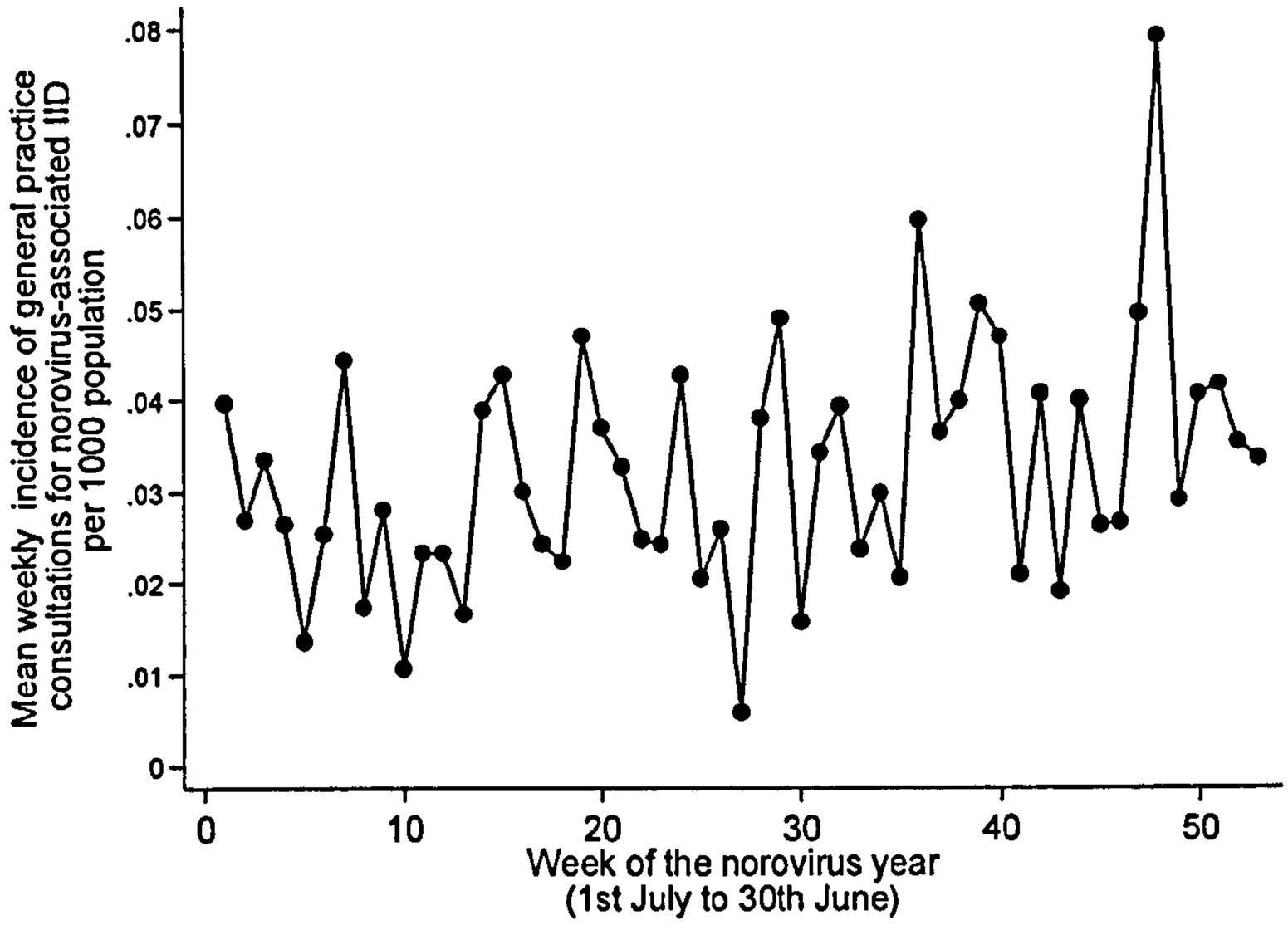


Figure 7.6: Seasonality of the incidence of general practice consultations for norovirus-associated IID in adults aged 65 years and older.

Figure 7.6a Indirect model 1 with adjustment for seasonality and autocorrelation.

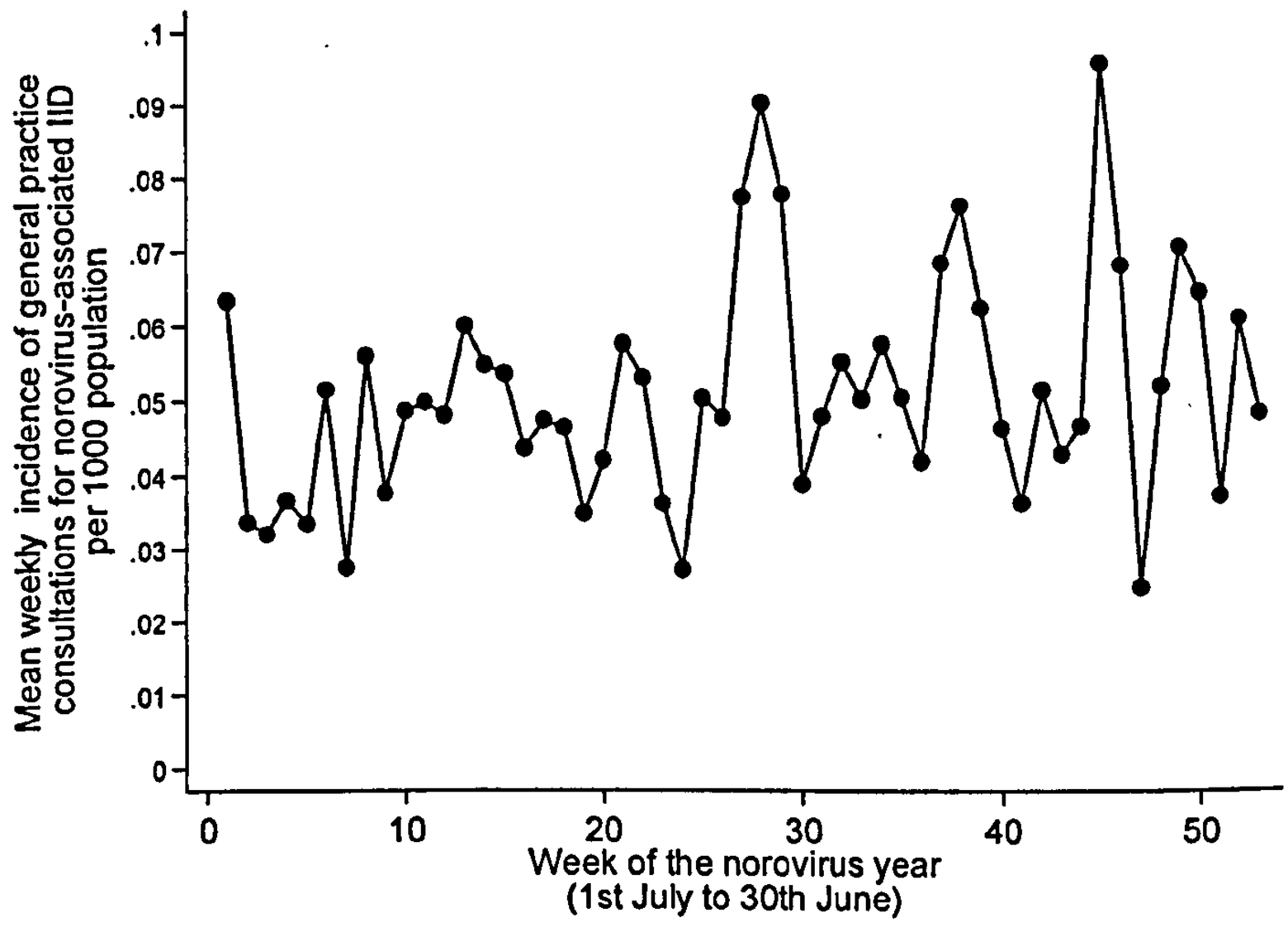
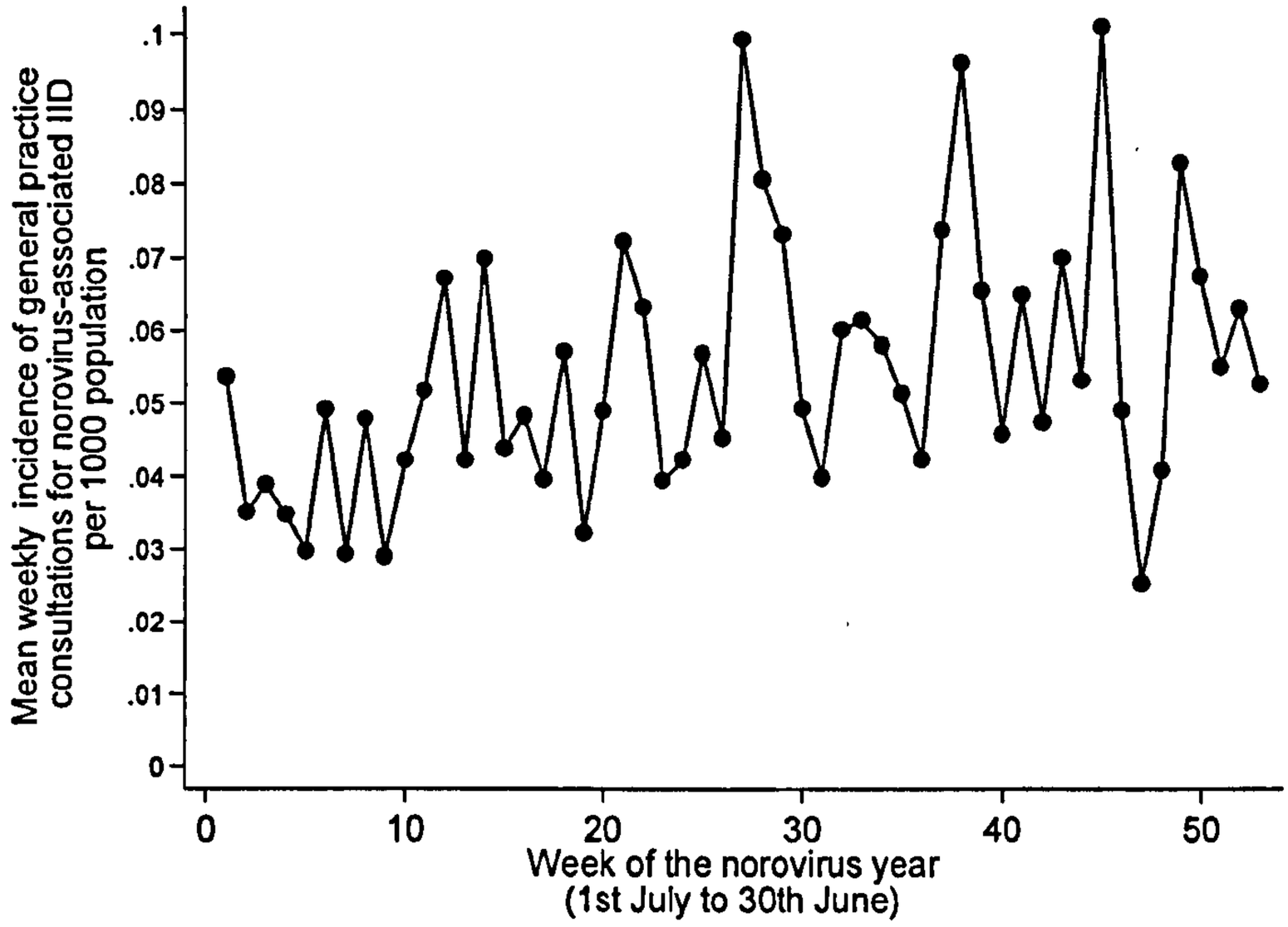


Figure 7.6b Indirect model 2 with adjustment only for long-term trends.



7.4. Discussion

In this analysis, time series-adapted Poisson regression was used to estimate the incidence of general practice consultations for norovirus-associated IID in England and Wales. The limitations of using the norovirus laboratory reports from the HPA national surveillance with this widely applied method were described and the modelling approach was modified to account for these problems. Using the adapted method, it was estimated that approximately 1.5% of children aged less than five years in England and Wales presented to their general practitioner with norovirus-associated IID each year between 1993 and 2007. The overall incidence of general practice consultations for IID declined during this period, so that approximately 1% of children in this age group presented with norovirus-associated IID in the last five years of the study (2002 to 2007). In older age groups, only 0.1% to 0.2% of individuals presented each year between 2002 and 2007, although the fit of the regression models was much poorer in these age groups, especially in adults aged 65 years or older, so the results may be less reliable.

A major advantage of using generalised linear modelling to estimate pathogen-specific healthcare consultations is that it does not directly rely on complete reporting of pathogen diagnoses or on knowledge of the degree of underreporting. The method relies only on using the trends and patterns in the pathogen laboratory report data. However, when the laboratory report data are not representative of healthcare consultations due to the pathogens considered, which is probably the case for norovirus reports from individuals aged five years and older, the reliability of the results is greatly reduced. In addition, when the pathogen laboratory counts are very small, statistical power is reduced and there is increased probability of type II errors (failing to find or underestimating the size of a real association between the consultations and a pathogen contributing to them.) The weekly norovirus report counts from children aged less than five years were very small and the direct estimates of norovirus consultations in this age group were sensitive to the method of seasonal adjustment, reducing confidence in the reliability of these direct estimates. In contrast, the direct estimates of the incidence of consultations for rotavirus-associated IID in the same age group, for which a much larger number of laboratory reports are received, were robust to changes in the seasonal terms used (Appendix A5.15).

Whilst not subject to the limitations of the norovirus laboratory reports, the indirect modelling method does require the significant assumption that, after selecting the best fitting model of general practice consultations for IID, all remaining variation in the consultation data is attributable to norovirus. The strength of this assumption is reduced by the inclusion of a constant term in the models, but this constant term was not used to estimate consultations attributable to norovirus, so that a number of consultations in each week are attributed neither to norovirus nor to causes explicitly represented in the explanatory variables. However, it is still essential that the laboratory report data for the pathogens used as explanatory variables in the indirect models are of good quality and are representative of consultations made for these pathogens. This was assumed to be the case, based on the application of testing recommended by the HPA National Standard Methods[486, 487] and knowledge of the epidemiology of these pathogens in England and Wales. Data on *Clostridium difficile* were not used because of the large amount of testing associated with hospital-acquired infections and hospital patient screening in England and Wales, meaning that these data are unlikely to be representative of the incidence of general practice consultations for *C. difficile*. However, *C. difficile* caused only a small number of general practice consultations in the Study of Infections Intestinal Disease[8], so it is unlikely that exclusion of *C. difficile* laboratory reports in this analysis will have greatly affected the results. There are likely

to be a number of other uncommon gastrointestinal pathogens contributing to the burden of general practice consultations for IID, which were not included in the models, but again the effect of their omission in these particular models is likely to be minimal. The inclusion of the constant term further reduces the problem of excluding laboratory reports from pathogens that cause a low incidence of general practice consultations.

Development of the indirect method relied on the existence of good quality laboratory report data for rotavirus in children aged less than five years, with which reliable direct estimates of general practice consultations due to rotavirus could be made using the regression models. It was therefore possible to fit both direct and indirect models for rotavirus in children aged less than five years and to compare the rotavirus consultation estimates to provide "proof of principle" for the application of the indirect method to estimate norovirus consultations.

The indirect models provided a good fit to the general practice consultations in children aged less than five years, evident from inspection of the fitted values. Fitting of the Fourier terms indicated two clear annual peaks in general practice consultations for IID in children aged less than five years, which correspond to the recognised seasonality of norovirus and rotavirus in young children[209] and matched the seasonality of the norovirus laboratory reports in this age group. This increases confidence in the indirect estimate of norovirus consultations because the method independently reproduced the expected annual pattern of norovirus incidence. In addition, with such good model fit, the assumption that the remaining variation in consultations is due to norovirus is justifiable.

However, the incidence of general practice consultations in older age groups was much less seasonal and both the direct and indirect models showed much poorer fit to the consultation data. In fact, given the very small amount of overdispersion indicated by the Pearson's χ^2 test, it seems that the consultations for IID in adults aged 65 years and older are essentially random. This lack of seasonality in general practice consultations for IID in adults contrasts with the distinct and regular seasonality of the laboratory reports for pathogens such as *Salmonella* spp. and *Campylobacter* spp. (Appendix 5.4), which are known to be major causes of IID in adults[8]. The lack of seasonality in adult consultations may indicate that a larger range of pathogens, with different and overlapping seasonality, contribute equally to the aetiology of IID in adults consulting general practitioners. In contrast, the model fit was very good for children aged less than five years, in which enteric viruses are the predominant cause of IID consultations and are recognised to have distinct seasonal patterns, with rotavirus, the

most common pathogen, having the most consistent seasonality (Appendix 5.4). Whatever the underlying reasons for the poorer model fit in adults, with such poor explanatory power in the fitted model, it is difficult to justify using the residual series to represent norovirus consultations. In addition, the seasonality of norovirus incidence at the community level in adults, rather than outbreaks, is poorly described, so it is unclear whether the lack of seasonality in the residual series from the indirect models in adults should be expected, again making it more difficult to justify the assumption that they represent norovirus consultations. Until the expected seasonal pattern of norovirus incidence in adults is better described, it remains impossible to determine whether the lack of winter-time seasonality in the residuals and the overall general practice consultations for IID in adults is due to a difference in norovirus epidemiology compared to young children, or because norovirus is not a significant cause of consultations in adults.

The direct estimates were much lower than the indirect estimates, across all age groups. In children aged less than five years this may have been expected because the weekly counts of norovirus laboratory reports were very small, which may prevent accurate estimation of the relationship between norovirus reports and general practice consultations. Indeed, the indirect estimates for 1993 to1996 in this age group are much closer to the estimates from the Study of Infectious Intestinal Disease, presented in Chapter 8, although the estimates from the Study of Infectious Intestinal Disease lie towards the upper limit of the range presented for the indirect estimate in this analysis. The 25th percentile from the rotavirus indirect model in children aged less than five years gave a reasonable approximation to the direct estimate for rotavirus, but this may not necessarily be appropriate for the indirect norovirus estimates. However, it is difficult to objectively select a way to present the indirect estimates and the 25th percentile provides a mid-point between the lower and upper limits represented by the positive residuals and standardisation to the lowest negative residual. The purpose of this estimate of norovirus consultation incidence, which can never be as accurate as those from prospective research studies, is to provide a plausible range in which the number of consultations lies, rather than to produce a single point estimate, so it is important not to place too much emphasis on the mid-point presented, because it does not represent a point-estimate in the common statistical sense.

The indirect modelling approach developed here provides a novel way of estimating general practice consultations for norovirus-associated IID, in the absence of any robust routinely collected data on this outcome. However, it is reliant on the use of

good quality laboratory report data for other gastrointestinal pathogens responsible for general practice consultations, in order to remove variation from the RCGP consultation data attributable to these other pathogens. In children aged less than five years, the laboratory report data provided a good fit to the general practice consultation data, which may be because of the predominance of rotavirus as a cause of consultations in this age group; rotavirus has a distinct annual peak that was correlated well to the annual peak in the general practice consultation data. Therefore, the indirect estimates of norovirus consultation incidence in children aged less than five years may be reasonably reliable. However, the laboratory report data for other age groups did not provide as good a fit to the consultation data, meaning that a large amount of variation remained unexplained in the model and it was difficult to justify using the residuals as a representation of norovirus consultations. It may be that this indirect approach is most useful for healthcare consultations that show distinct seasonality, well-matched to the seasonality of recognised causative pathogens, but that for less seasonal phenomena and in the absence of a priori reasons to believe that the residual variation is all attributable to the omitted pathogen, the method is less appropriate.

Chapter 8: Community incidence of norovirus-associated infectious intestinal disease in England based on viral load

In Chapter 4 norovirus viral load measurements in IID cases and controls were used to select a cut-off in viral load for identifying individual cases of norovirus-associated IID. In this final analysis, the same viral load data were used to estimate the incidence of norovirus-associated IID in the community in England and the incidence of general practice consultations for norovirus-associated IID. However, rather than classifying the norovirus aetiology of individual IID cases, exclusion of RT-PCR positive IID cases whose illness is unlikely to have been caused by norovirus was carried out at the population-level, using a probability-based method. This method was chosen because it allows explicit incorporation of uncertainty in the viral load data into the confidence bounds of the incidence estimates.

8.1. Background

Norovirus is recognised as the most common cause of IID in the community in high income countries[5, 7] and a substantial prevalence of norovirus infection has been reported amongst IID cases seeking primary and secondary medical care [49]. Existing estimates of norovirus-associated IID incidence in the community and of general practice consultations caused by norovirus in England are based on electron microscopy testing of IID cases in the Study of Infectious Intestinal Disease. The poor diagnostic sensitivity of electron microscopy for identifying norovirus-associated IID was demonstrated in the analysis of viral load data in Chapter 4, where a substantial proportion of those IID cases who were negative by electron microscopy were subsequently classified as norovirus cases using the real-time RT-PCR testing and the Ct value cut-off.

It is therefore very likely that the estimates based on electron microscopy under-represent the burden of disease caused by norovirus in England and it is important that they are updated in light of the RT-PCR retesting of the Study of Infectious Intestinal Disease specimen archive. However, it is essential that those IID cases shedding norovirus at the low concentrations seen in the healthy controls are excluded from the incidence estimate, because it is very unlikely that they actually have disease caused by norovirus, despite being infected at the time of their illness. Only individuals with IID caused by norovirus should be included in estimates of norovirus disease burden.

8.2. Methods

Full details of recruitment and testing during the Study of Infectious Intestinal Disease were provided in Chapter 3.

8.2.1. Calculating the incidence of all-cause IID in the community and of IID consultations to general practitioners

Poisson regression was used to calculate the incidence of all-cause IID in the community, from the number of IID cases ascertained in the community cohort and the number of person-years of follow up completed (Table 8.1). Poisson regression was also used to calculate the all-cause incidence of general practice consultations for IID. The numerator for the incidence of general practice consultations for IID was the number of IID cases ascertained in the general practice case-control study and the general practice enumeration study, adjusted for the level of case under-ascertainment, which was estimated in the under-ascertainment study (see Chapter 3) (Table 8.1). The denominator for the incidence of general practice consultations for IID was the number of patients registered across all the general practices participating in the study, adjusted for list inflation (the number patients who were no longer actively using the practices, described Chapter 3), multiplied by the duration of practice participation in the study (Table 8.1).

8.2.2. Norovirus diagnostic testing

The results from both gel-based RT-PCR and real-time RT-PCR norovirus testing of the archived specimens were used in this analysis. The number of IID cases positive for norovirus by RT-PCR (Table 8.1) was used directly in calculation of the incidence of norovirus-associated IID. The real-time RT-PCR viral load measurements, from both IID cases and controls, were used to calculate an adjustment factor, to remove IID cases whose disease was not caused by norovirus from the incidence estimate, as described below. For those IID cases positive by real-time RT-PCR who had mixed genogroup I and genogroup II norovirus infections, the lowest Ct value was used in the analysis.

Table 8.1 Summary of case recruitment and stool specimen testing in the community cohort and general practice case-control and enumeration studies in the Study of Infectious Intestinal Disease.

	Community Cohort	General practice case-control and enumeration studies
Person-years of follow-up	4026	409878[a]
Ascertained cases	781	13619[b]
Stool specimen	761	2893[c]
Stool specimen archived	517	1905
RT-PCR positive for norovirus[d]	211	623
Ct value determined with real-time RT-PCR	174	544

[a] Adjusted for registered patients no longer actively using participating GP practices
[b] Adjusted for under-ascertainment
[c] Stool specimens were only collected from patients in 34 practices participating in the general practice case-control study
[d] Includes those previously positive by EM
Abbreviations: Ct, cycle threshold; EM, electron microscopy; GP, general practitioner; RT-PCR, reverse transcription-polymerase chain reaction.

8.2.3. Calculating the incidence of norovirus-associated IID

The incidence of norovirus-associated IID (*INV*) was calculated as (Formula 1):

$$INV = I \times p(NV) \times A$$

where *I* is the incidence of all-cause IID per 100 person-years, *p(NV)* is the proportion of IID cases positive for norovirus by RT-PCR in the specimen archive, and *A* is a factor used to adjust for those IID cases with norovirus infection who have low viral loads and are therefore unlikely to have disease caused by norovirus.

Rather than using the Ct value cut-off from Chapter 4 to classify norovirus aetiology for individual IID cases, the Ct value distributions from the reference groups in the ROC analysis were used to calculate Adjustment Factor *A*. The ROC analysis did not provide confidence limits around the selected Ct value cut-off, whereas calculation of Adjustment Factor *A*, from the same viral load data, allows uncertainty due to sampling error in these Ct value distributions to be incorporated into the incidence estimate. The reference positive group used to calculated Adjustment Factor *A* included IID cases with norovirus detected by electron microscopy, from both the community cohort and

the general practice case-control study (Reference Positive Group 1 from the ROC analysis in Chapter 4). IID cases from both study components were analysed together to increase the sample size of the reference positive group; there was no evidence from the analysis in Chapter 4 that there was a difference in Ct value distribution between electron microscopy norovirus positive IID cases in the two study components (Table 4.4), so it is appropriate to combine Ct values from IID cases in the two study components to calculate the adjustment factor. The reference negative group included healthy controls (Reference Negative Group 1 from the ROC analysis in Chapter 4).

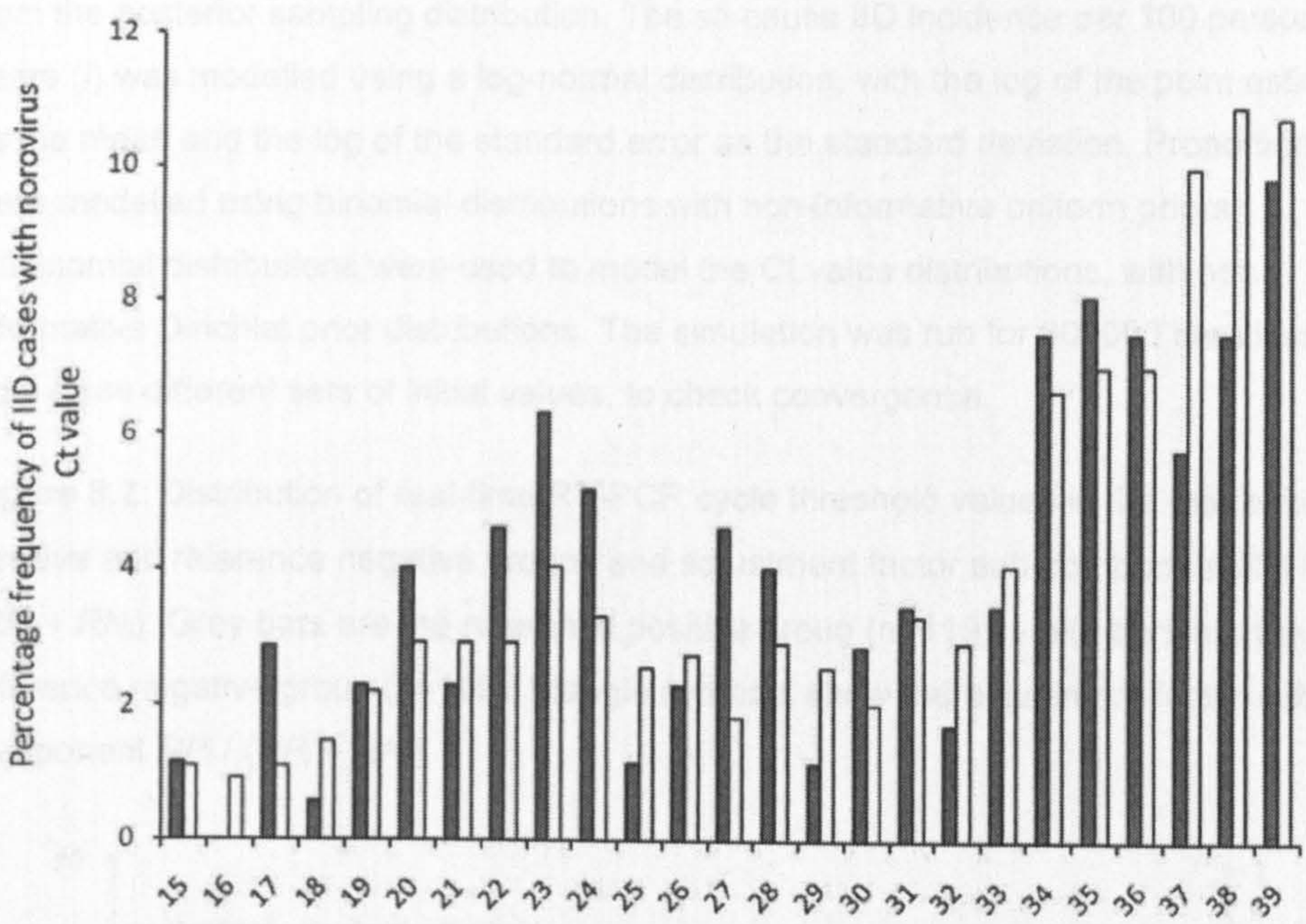
Adjustment factor *A* was calculated as (Formula 2):

$$A = \sum_{i=15}^{i=39} Ct_i \times \frac{RP_i}{RP_i + RN_i}$$

where RP_i is the moving average of the proportion of the reference positive group at Ct value *i* (over *i*-2 to *i*+2); RN_i is the moving average of the proportion of the reference negative group at Ct value *i* (over *i*-2 to *i*+2); Ct_i is the proportion of IID cases positive by norovirus real-time RT-PCR with Ct value *i*. Adjustment factor *A* varies between zero and one. Adjustment factor *A* is therefore a weighted average of the relative frequency of the reference positive and reference negative groups at each Ct value, weighted by the proportion of all norovirus infected IID cases at each Ct value (shown in Figure 8.1).

Figure 8.2 shows the distribution of Ct values in the reference groups and the value of the sub-component $[RP_i + (RP_i + RN_i)]$, which represents the relative frequency of the reference groups. At low Ct values, where viral loads are high and there are few individuals from the reference negative group, sub-component $[RP_i + (RP_i + RN_i)]$ is close to one, indicating that the majority of IID cases with norovirus infection at these concentrations have disease caused by norovirus. In contrast, at the high Ct values (low viral loads) found in the majority of the disease-free reference negative group, sub-component $[RP_i + (RP_i + RN_i)]$ is close to zero, indicating that very few IID cases with norovirus infection at these concentrations have disease caused by norovirus; these IID cases are likely to have an 'asymptomatic' norovirus infection concurrent to illness caused by another pathogen.

Figure 8.1 Distribution of real-time RT-PCR cycle threshold values in IID cases from the community cohort and the general practice case-control study in the Study of Infectious Intestinal Disease. Dark grey bars are IID cases from the community cohort (n=174); white bars are IID cases from the GP study (n=544).



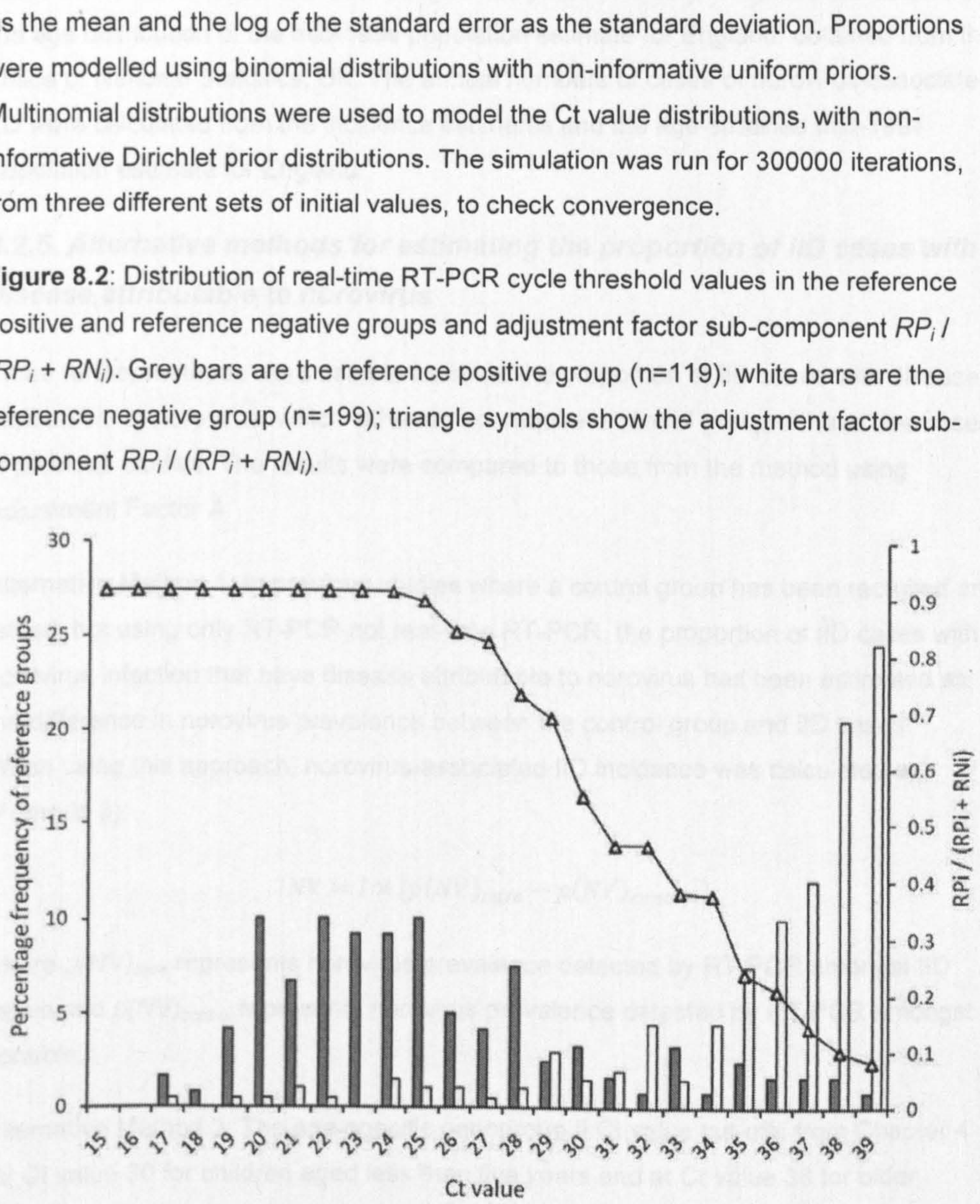
Abbreviations: Ct, cycle threshold; IID, infectious intestinal disease.

When calculating age-stratified and age-adjusted incidence, Adjustment Factor *A* was calculated separately for children aged less than five years and for older children and adults (aged five years or older), using the corresponding reference groups from the ROC analysis in Chapter 4. The same reference groups were used to calculate sub-component [$RP_i \div (RP_i + RN_i)$] of Adjustment Factor *A* for both the community and general practice incidence of norovirus-associated IID. Sub-component [$RP_i \div (RP_i + RN_i)$] was then applied separately to the Ct value distribution from IID cases in each study component (Ct_i in Formula 2, shown in Figure 8.1), to create separate adjustment factors specific to the community and general practice norovirus-associated IID incidence.

8.2.4. Incidence estimation by Monte Carlo simulation in WinBUGS

The incidence of norovirus-associated IID was estimated using Monte Carlo simulation in WinBUGS v1.4[591], to incorporate statistical uncertainty due to sampling error in each of the component proportions in Formula 1 and Formula 2. Confidence limits for the incidence of norovirus-associated IID were based on the Bayesian credibility intervals from the posterior sampling distribution. The all-cause IID incidence per 100 person-years (I) was modelled using a log-normal distribution, with the log of the point estimate as the mean and the log of the standard error as the standard deviation. Proportions were modelled using binomial distributions with non-informative uniform priors. Multinomial distributions were used to model the Ct value distributions, with non-informative Dirichlet prior distributions. The simulation was run for 300000 iterations, from three different sets of initial values, to check convergence.

Figure 8.2: Distribution of real-time RT-PCR cycle threshold values in the reference positive and reference negative groups and adjustment factor sub-component $RP_i / (RP_i + RN_i)$. Grey bars are the reference positive group (n=119); white bars are the reference negative group (n=199); triangle symbols show the adjustment factor sub-component $RP_i / (RP_i + RN_i)$.



Abbreviations: Ct, cycle threshold; RP_i, moving average of the proportion of the reference positive group at Ct value i; RN_i, moving average of the proportion of the reference negative group at Ct value i.

Separate simulations were run to estimate the incidence of illness caused by norovirus in the community and the incidence of general practice consultations for norovirus-associated IID. The numbers of IID cases with norovirus Ct values limited the number of age groups in which the community incidence could be presented. Age- and season-stratified incidence was calculated by applying the simulation procedure separately to data from each age group or season. Age-adjusted incidence was calculated (also in WinBUGS) as a weighted average of the incidence in children aged less than five years and older children and adults (aged five years or older); weights were taken from the age distribution of the mid-1994 population estimate for England, obtained from the Office of National Statistics, UK. The annual numbers of cases of norovirus-associated IID were calculated from the incidence estimates and the age-stratified mid-1994 population estimate for England.

8.2.5. Alternative methods for estimating the proportion of IID cases with disease attributable to norovirus

Three further methods were used to estimate the proportion of IID cases with disease attributable to norovirus, which either do not require a control group or have been used in previous studies. The results were compared to those from the method using Adjustment Factor *A*.

Alternative Method 1: In previous studies where a control group has been recruited and tested, but using only RT-PCR not real-time RT-PCR, the proportion of IID cases with norovirus infection that have disease attributable to norovirus has been estimated as the difference in norovirus prevalence between the control group and IID cases[49]. When using this approach, norovirus-associated IID incidence was calculated as (Formula 3):

$$INV = I \times [p(NV)_{case} - p(NV)_{control}]$$

where $p(NV)_{case}$ represents norovirus prevalence detected by RT-PCR amongst IID cases and $p(NV)_{control}$ represents norovirus prevalence detected by RT-PCR amongst controls.

Alternative Method 2: The age-specific genogroup II Ct value cut-offs from Chapter 4 (at Ct value 30 for children aged less than five years and at Ct value 33 for older children and adults) were applied to all IID cases with a norovirus Ct value, from both genogroup I and genogroup II. The proportion of IID cases with a norovirus Ct value at or below the Ct value cut-off was substituted for Adjustment Factor *A* in Formula 1. In

addition, to explore the effect of late specimen collection on norovirus incidence, probable cases of norovirus-associated IID were defined as those IID cases with a Ct value above the cut-off, a specimen collected five or more days after symptom onset and no other pathogen detected. These probable cases were added to the IID cases with a norovirus Ct value at or below the cut-off and incidence recalculated.

Alternative Method 3: Mixture modelling was used to estimate the proportion of IID cases with a norovirus Ct value who have disease attributable to norovirus, using only the viral load data from IID cases. This proportion was substituted for Adjustment Factor *A* in Formula 1 and uncertainty around the proportion was represented using a beta distribution, based on the 95% confidence interval provided from the mixture model. Details of the mixture model are provided in Appendix A6.1.

8.2.6. Incidence based on electron microscopy and RT-PCR testing

Incidence of norovirus-associated IID based on electron microscopy testing and based on classifying any IID case positive for norovirus by RT-PCR as a case of norovirus-associated IID were also calculated, to demonstrate the effect of using the new method based on Adjustment Factor *A*. The estimates based on electron microscopy testing were calculated using Formula 4:

$$INV = I \times P$$

where *P* is the proportion of IID cases positive by electron microscopy. When calculating the incidence of norovirus-associated IID based on classifying any norovirus RT-PCR positive IID case as a case of norovirus-associated IID, *P* in Formula 4 was the proportion of IID cases positive by gel-based RT-PCR.

The incidence of rotavirus-associated IID based on ELISA diagnosis (in children aged less than five years only), was calculated in the same way.

8.3. Results

The crude community incidence of norovirus-associated IID was 4.1 per 100 person-years (Table 8.2); after adjusting for the age distribution of the cohort, the community incidence was 4.5 episodes per 100 person-years (Table 8.2). The incidence was highest in children aged less than five years, with 20% experiencing norovirus-associated IID every year, but incidence was substantial in all age groups (Table 8.2).

Table 8.2 Incidence of norovirus-associated IID in England, 1993 to 1996.

	Community		GP consultation		Ratio of Community to GP cases
	Incidence per 100 person-years	95% Credibility interval	Incidence per 100 person-years	95% Credibility interval	
Crude	4.1	3.4 - 4.8	0.49	0.43 - 0.55	8.4
Age-adjusted	4.5	3.8 - 5.2	0.54	0.48 - 0.60	8.3
Age-stratified					
< 5 years	21.4	15.9 - 27.7	3.2	2.6 - 3.8	6.7
≥5 years	3.3	2.6 - 3.9	0.35	0.30 - 0.39	9.7
0 – 1 years	27.2	17.9 - 38.6	6.4	5.2 - 7.7	4.3
2 – 4 years	16.7	11.4 - 23.3	1.5	1.2 - 2.0	11.1
5 – 14 years	6.5	4.5 - 8.9	0.44	0.31 - 0.59	14.8
15 – 44 years	4.1	3.1 - 5.3	0.38	0.32 - 0.45	10.8
≥45 years	1.7	1.1 - 2.3	0.29	0.24 - 0.35	5.9
45 – 64 years	-	-	0.26	0.20 - 0.32	-
≥65 years	-	-	0.37	0.27 - 0.47	-
Season-stratified					
January - March	4.7	3.4 - 6.3	0.46	0.37 - 0.57	-
April - June	3.8	2.7 - 5.1	0.52	0.43 - 0.62	-
July - September	3.3	2.4 - 4.5	0.43	0.35 - 0.51	-
October - December	4.8	3.6 - 6.3	0.56	0.46 - 0.66	-
Rotavirus-associated IID					
0 – 1 years	13.7	5.6 - 25.1	6.4	5.2 - 7.7	2.1
2 – 4 years	6.2	2.5 - 11.5	1.5	1.2 - 2.0	4.1
<5 years	8.5	4.6 - 13.6	3.2	2.6 - 3.8	2.7

Table 8.3 Estimated annual numbers of norovirus-associated IID cases in the community and consulting a general practitioner in England, 1993 to 1996.

	Community		General Practice	
	Thousands of cases	95% Credibility interval	Thousands of cases	95% Credibility interval
All ages (age-adjusted)	2175.8	1836.8 - 2543.0	261.5	233.4 - 290.6
0 – 1 years	-		81.0	65.4 - 97.8
2 – 4 years	-		30.4	22.7 - 38.8
< 5 years	691.4	513.4 - 897.1	103.7	85.2 - 123.2
5 – 14 years	403.1	279.0 - 550.3	27.1	18.9 - 36.6
15 – 44 years	854.3	635.6 - 1104.9	78.6	65.1 - 93.0
≥45 years	308.4	211.4 - 426.8	54.9	45.0 - 65.6
45 – 64 years	-		28.2	21.9 - 35.4
≥65 years	-		28.1	21.0 - 36.2

Norovirus–associated IID incidence in the community was highest between October and March (Table 8.2).

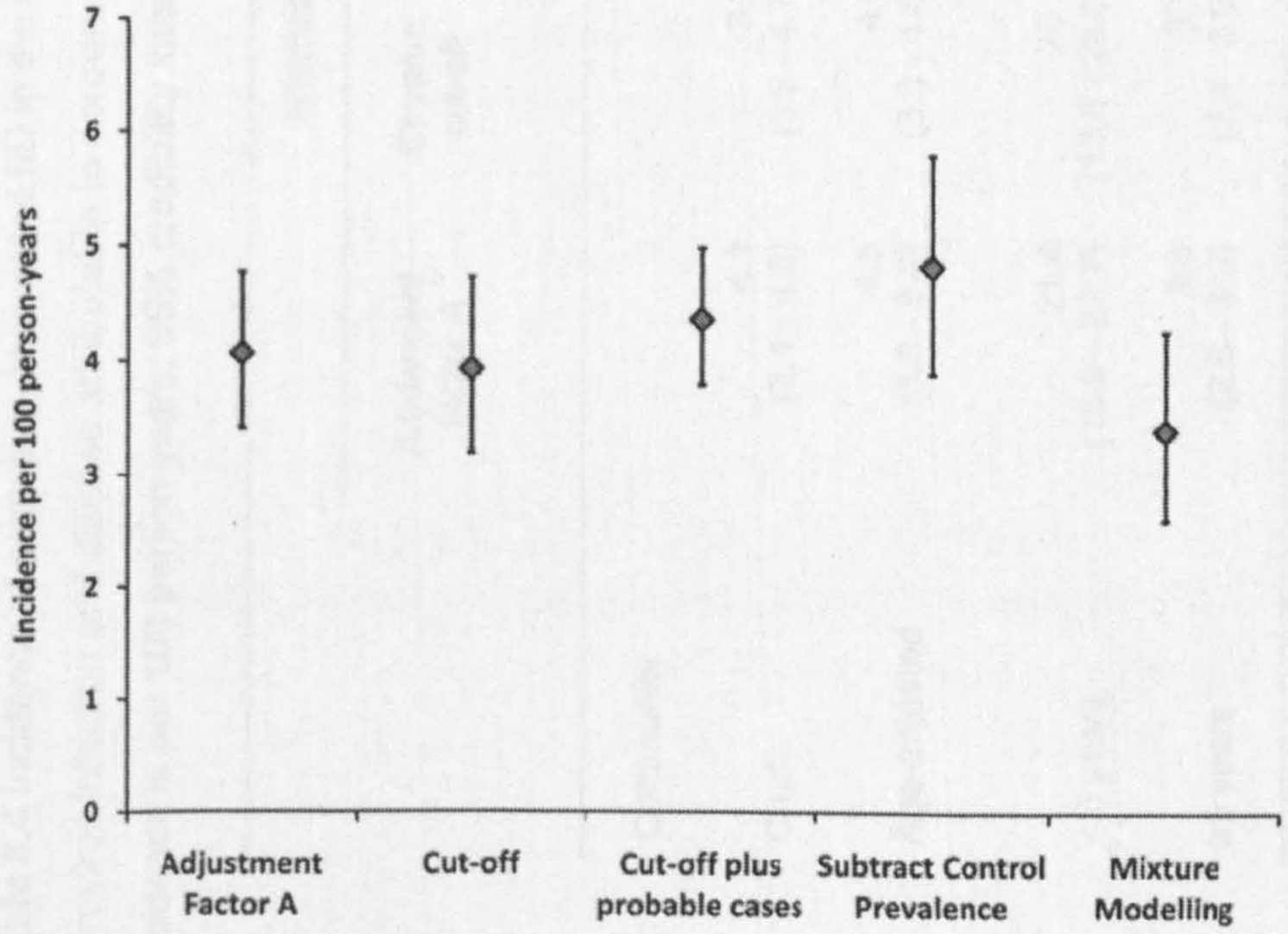
There were 0.5 general practice consultations for norovirus-associated IID per 100 person-years (Table 8.2). The incidence of general practice consultations was highest amongst children aged less than two years, at 6.4 per 100 person-years (Table 8.2). Approximately one in seven children aged less than five years who experienced norovirus-associated IID presented to a general practitioner (Table 8.2), compared to one in three of those with rotavirus-associated IID in the Study of Infectious Intestinal Disease (Table 8.2).The incidence of general practice consultations for norovirus-associated IID was similar for all individuals aged five years and older (Table 8.2). The seasonality of general practice consultations for norovirus-associated IID was less pronounced than the community incidence (Table 8.2). The incidence translates into over two million episodes of norovirus-associated IID in the community each year during 1993 to 1996 and more than 200 000 general practice consultations (Table 8.3). Whilst the highest absolute burden of norovirus-associated IID occurred in adults, the

greatest number of general practice consultations occurred in children aged less than five years, who accounted for almost half of the consultations (approximately 100 000) (Table 8.3).

The results of the alternative estimation methods and the community incidence based on electron microscopy and all norovirus RT-PCR positive IID cases are shown in Table 8.4 and Figure 8.3, general practice consultation incidence based on these alternative methods is provided in Appendix A6.2. The incidence estimates using the Ct value cut-off were slightly lower than those using Adjustment Factor *A* and the confidence intervals were narrower. Subtracting the control norovirus prevalence from that in IID cases produced higher incidence estimates in young children, but lower estimates in older children and adults. Mixture modelling produced the lowest estimates. The age-adjusted incidence in the community based on Adjustment Factor *A* was three times that based on electron microscopy and half the incidence based on all RT-PCR positive cases. The age-adjusted incidence of general practice consultations based on Adjustment Factor *A* was two and a half times higher than the incidence based on electron microscopy and half that based on all RT-PCR positive IID cases.

The values of Adjustment Factor A calculated in the Monte Carlo simulations are shown in Appendix A6.3.

Figure 8.3 Crude incidence of norovirus-associated IID in the community in England based on alternative methods for estimating the proportion of IID cases with norovirus infection and disease attributable to norovirus.



Black T-bars show the 95% credibility intervals.

Table 8.4 Incidence of norovirus-associated IID in the community from alternative methods for estimating the proportion of IID cases with norovirus infection and disease attributable to norovirus and incidence based on electron microscopy and all RT-PCR positive IID cases. Incidence is per 100 person years; 95% credibility intervals are given in brackets.

	Alternative methods					Electron Microscopy	All RT-PCR positive
	Adjustment factor *A*	Ct value cut-off	Ct value cut-off plus probable cases	Subtract control prevalence	Mixture modelling		
Community							
Crude	4.1 (3.4 - 4.8)	3.9 (3.2 - 4.7)	4.4 (3.8 - 5.0)	4.8 (3.9 - 5.8)	3.1 (2.3 - 3.9)	1.4 (1.0 - 1.9)	8.0 (7.1 - 9.1)
Age-adjusted	4.5 (3.8 - 5.2)	4.0 (3.3 - 4.8)	4.6 (4.0 - 5.3)	4.3 (3.3 - 5.4)	-	1.6 (1.1 - 2.1)	8.5 (7.4 - 9.6)
< 5 years	21.4 (15.9 - 27.7)	20.3 (13.8 - 28.0)	21.1 (16.7 - 26.3)	33.4 (25.2 - 42.5)	-	9.1 (5.1 - 14.4)	44.3 (35.2 - 54.4)
≥5 years	3.3 (2.6 - 3.9)	3.1 (2.4 - 3.9)	3.4 (2.9 - 4.1)	2.3 (1.4 - 3.2)	-	1.0 (0.7 - 1.5)	5.9 (5.1 - 6.9)

Abbreviations: Ct, cycle threshold; GP, general practice; RT-PCR, reverse transcription-polymerase chain reaction

8.4. Discussion

In this analysis, viral load measurements from the Study of Infectious Intestinal Disease specimen archive retesting were used to update estimates of the burden of community disease and general practice consultations caused by norovirus in England. This is the first time that this quantitative approach has been used. Results from a recent volunteer study[80], and the analysis of viral load data in Chapter 4, show that low norovirus viral loads detectable by RT-PCR are associated with asymptomatic infection. Consideration of viral load therefore provides the greatest diagnostic accuracy for identifying cases of norovirus-associated IID. Estimates of norovirus disease burden based on viral load are more accurate than those based on electron microscopy, because electron microscopy has poor diagnostic sensitivity; they are more accurate than those based on RT-PCR, because it is possible to exclude IID cases who are RT-PCR positive, but have low viral loads, and are therefore unlikely to have disease caused by norovirus. Using this new method, it was estimated that five percent of the general population in England experienced an episode of norovirus-associated IID between 1993 and 1996, equating to two million episodes per year. Incidence was highest amongst children aged less than five years, with one fifth suffering norovirus-associated IID and 100 000 of these children visiting their general practitioner each year.

The method used to calculate norovirus-associated IID incidence allowed statistical uncertainty in the viral load measurements to be incorporated into the incidence confidence limits. This was only possible with the use of Monte Carlo simulation methods to combine the multiple components of the formulae and their associated statistical uncertainty; this would have been extremely difficult using standard frequentist approaches, such as the Delta Method, because of the large number of variables in the calculation. Uncertainty in diagnostic test results, in terms of sensitivity and specificity, is not normally considered in estimation of pathogen-specific disease incidence, probably because the measurement of diagnostic validity is contingent upon the existence of an appropriate and accurate gold standard. However, with the use of viral load measurements from both IID cases and healthy controls, it was possible to calculate Adjustment Factor *A*, which is an adapted version of the predictive value of each Ct value for identifying norovirus-associated IID. Adjustment factor *A* makes use of the information contained in the whole of the viral load distributions, rather than using a single cut-off, which may be more heavily reliant on the quality of the ROC analysis reference groups.

There was limited resolution for estimating age-stratified incidence in the community because of the small sample size. Genogroup I and genogroup II norovirus infections were combined in this analysis, rather than estimating Adjustment Factor *A* separately for each genogroup, also because of limited sample size. Similarly, in Alternative Method 2 the Ct value cut-off for genogroup II specimens was applied to all norovirus infections, because of the problems with the genogroup I cut-off, described in Chapters 3 and 4. There is evidence that the real-time RT-PCR assay has lower efficiency for genogroup I norovirus strains; for a particular viral load, a genogroup I norovirus may have a higher Ct value than a genogroup II virus. Genogroup I noroviruses constituted less than 10% of the norovirus isolates in IID cases, meaning that treating the Ct values in the two genogroups as equivalent will probably lead to slight underestimation of norovirus incidence; more genogroup I positive IID cases are excluded at a particular viral load because they have a higher Ct value than genogroup II viruses at that viral load. However, the degree of underestimation introduced by grouping all norovirus infections together is likely to be small, because of the low prevalence of genogroup I (Table 4.2).

The concentration of norovirus excretion decreases substantially after symptom resolution[80], but in the method using Adjustment Factor *A* there was no direct adjustment for the possibility that some IID cases with high Ct values may have had disease caused by norovirus, but had low viral loads at the time of specimen collection because their symptoms had already resolved. However, Adjustment Factor *A* represents the probability at each Ct value that a norovirus-positive IID case has disease caused by norovirus; even at very high Ct values, Adjustment Factor *A* was greater than zero and therefore did allow some IID cases with high norovirus Ct values to be incorporated into the incidence estimate (see Figure 8.2). The method therefore indirectly allows for the possibility that some IID cases who truly had norovirus-associated IID had low viral loads at the time of testing. Direct consideration of delay in specimen collection requires classification of norovirus disease status at the individual level, as was done when applying the Ct value cut-off (Alternative Method 2). The cut-off based incidence of norovirus-associated IID was recalculated, including probable cases (defined as having a high Ct value, a late specimen and no other detected pathogens); the resulting incidence was slightly higher than when using only the cases below the Ct value cut-off, but still very similar to the results obtained using Adjustment Factor *A*. However, caution should be exercised in using such an approach, because the number of probable cases will be highly dependent on assay sensitivity and on the number of cycles for which the real-time RT-PCR assay is run; not all IID cases with

norovirus detected and a late specimen may have actually had disease caused by norovirus.

The method described here is dependent on the recruitment and testing of a large control group, which is not always possible in studies of disease aetiology, and on the use of real-time RT-PCR for norovirus detection. Therefore a number of alternative methods were used to adjust the prevalence of norovirus in IID cases, to explore whether these produce suitably similar results to the method using Adjustment Factor *A*, which is believed to be the most robust. As expected, using the Ct value cut-off produced slightly lower incidence estimates, because no IID cases with Ct values above the cut-off were included, and with narrower confidence intervals, because the uncertainty in the cut-off was not represented in the calculations. Mixture modelling gave similar results to the Ct value cut-off, although there was a tendency towards underestimation in comparison to Adjustment Factor *A*; mixture modelling also requires larger sample sizes than the other methods, preventing estimation of detailed age-stratified incidence with the data used in this analysis. Estimates produced by subtracting the prevalence of norovirus in controls from that in IID cases were very different to those produced using the other methods; incidence in young children was substantially overestimated and incidence in older children and adults was underestimated compared to the incidence based on Adjustment Factor *A*. Furthermore, estimates produced by subtracting the control norovirus prevalence from the case norovirus prevalence will be highly dependent on the case definition used, the source of the controls and the study setting.

The new estimates of norovirus-associated IID incidence presented here are approximately three times higher in the community and two and half times higher at the general practice level, than the estimates based electron microscopy testing (Table 8.4). Accordingly, the ratio of community cases to cases presenting to general practitioners increased from 6:1 using electron microscopy diagnosis, to 8:1 using viral load measurements. The incidence estimates are approximately half those that would be obtained by assuming that any IID cases with a positive RT-PCR result for norovirus had disease caused by norovirus. The incidence of general practice consultations for norovirus-associated IID in children aged less than five years was very similar to the incidence of consultations for rotavirus-associated IID in this age group in the Study of Infectious Intestinal Disease (shown in Table 8.2); the analysis of routine surveillance data in Chapter 7 also indicated that norovirus causes an equivalent frequency of general practice consultations in young children as rotavirus. However, the ratio of

community cases to general practice cases was much higher for norovirus, indicating that norovirus causes more illness amongst young children in the community, but that symptoms are generally milder and therefore less likely to lead to a healthcare consultation. This is consistent with knowledge of the development of immunity to these viruses; immunity protective against symptomatic illness develops after the first two or three rotavirus infections[128], whereas immunity to a particular norovirus strain tends to last no more than six months, and with multiple strains co-circulating[209], repeated symptomatic infections are common throughout life.

The community incidence of norovirus-associated IID estimated here is comparable to that from a study in the Netherlands, which only used RT-PCR testing, not viral load measurements, to identify cases of norovirus-associated IID. However the study in the Netherlands had a narrower case definition for IID (three or more loose stools, or two or more episodes of vomiting in 24 hours), which may not have been sensitive enough to ascertain all episodes of norovirus-associated IID at the community level[5]. Similarly, the incidence of general practice consultations for norovirus in this analysis was only slightly lower than that from a recent study in Germany, which used RT-PCR diagnosis for norovirus, but again had a narrower case definition for IID (two or more loose stools, or two or more vomiting episodes in 24 hours)[22]. The incidence of norovirus-associated IID may also have been higher than normal during the Study of Infectious Intestinal Disease because a new variant of norovirus emerged during 1995 and 1996[201, 221, 537]; emergence of norovirus variants is associated with increased disease incidence[222, 224, 229, 575, 590].

The incidence of norovirus-associated IID in the community showed a slight peak in the winter and autumn months, whilst general practice consultations were reasonably constant throughout the year. Outbreaks of norovirus-associated IID in community settings in the UK show very little seasonality, in strong contrast to outbreaks in healthcare settings, which show marked winter-time seasonality[588]. A number of factors may contribute to these differing patterns of seasonality between community disease and outbreaks in different settings. Firstly, community norovirus outbreaks notified to national surveillance in the UK are more commonly reported from catering settings, with transmission occurring through food contamination during preparation; whilst the prevalence of norovirus infection amongst food handlers is likely determined by the incidence of community disease, the driving factor in these outbreaks is breakdown in food hygiene practices, which is not a seasonal phenomenon. Secondly, it has been suggested that the marked winter-time increase in hospital admissions for respiratory

infections may drive the strong seasonality of norovirus outbreaks in this setting, and there are also distinct norovirus strains circulating in hospital populations compared to the community, which may have different transmission characteristics[588]. Therefore the incidence of community disease or GP consultations would not necessarily show the marked seasonality seen in healthcare-associated outbreaks. However, more detailed characterisation of the molecular epidemiology of norovirus infections in the community is needed, for comparison to the extensive data that already exist for hospital-acquired infections[199, 592], to better understand the factors driving the different seasonality of healthcare outbreaks and community disease. Finally, it is also possible that there was more out-of-season norovirus transmission during the Study of Infectious Intestinal Disease because of the emergence of a new norovirus variant, as described above[590]. The seasonality of norovirus-associated IID in the community matches the seasonality of asymptomatic infections, described in Chapter 5, although the asymptomatic infection winter time peak was more distinct.

With the widespread use of RT-PCR for norovirus diagnosis in community-based studies, it is recommended that a real-time platform is used to allow consideration of viral load when calculating norovirus incidence, as has been done in this analysis. This approach is preferable to including all IID cases who are RT-PCR positive, regardless of their viral load, because many may be shedding norovirus at low concentrations, with disease caused by another pathogen. Using the method described here, additional real-time testing in a subset of norovirus-infected IID cases would be sufficient, rather than testing all IID cases, if resources are limited, providing the subset is of a reasonable size and is representative.

Accurate estimates of norovirus-associated IID incidence at the community level are important for understanding the introduction of norovirus into healthcare settings, where outbreaks cause substantial economic burden and service disruption[14], and for informing potential vaccination programmes[20, 162] or other public health interventions against norovirus. Whilst the estimates presented here were based on data collected between 1993 and 1996, they provide the best available information on the burden of norovirus-associated IID in England; the only other published incidence estimates are those from the original electron microscopy testing in Study of Infectious Intestinal Disease, which most probably greatly underestimate norovirus incidence. Furthermore, these results are based on current diagnostic methods; as new studies are carried out, they will provide a baseline from which to assess changes in norovirus incidence over time that are not confounded by concurrent changes in the sensitivity of diagnostic

methods, which is a problem for routinely collected surveillance data, such as the HPA norovirus laboratory reports described in Chapter 7.

Chapter 9: Discussion of key findings and recommendations

In this thesis, viral load measurements have been used to diagnose sporadic, community-acquired norovirus-associated IID. This is the first time that viral load has been systematically considered in the diagnosis of IID caused by any enteric viruses, despite quantitative real-time RT-PCR being widely available[110, 261, 269-277, 281, 593-601]. The availability of viral load measurements from healthy controls was essential for interpretation of the viral loads detected in IID cases; the Study of Infectious Intestinal Disease specimen archive provided a unique dataset with which to address this issue. The new quantitative diagnostic method facilitated the production of improved estimates of the incidence of IID caused by norovirus in the community in England, by overcoming both the poor diagnostic sensitivity of electron microscopy and the poor diagnostic specificity of RT-PCR. In addition, a new and comprehensive description of the characteristics of symptomatic and asymptomatic norovirus infection, and of risk factors for their acquisition, was produced. Key findings from the work presented were:

i. The optimal cut-off for attributing disease to norovirus with the real-time RT-PCR assay used in this study was at Ct value 31, which excluded IID cases in the top third of the Ct value distribution (the range of Ct values was 15 to 39);

ii. Using real-time RT-PCR testing and the Ct value cut-off, norovirus disease aetiology was rejected in 48% of norovirus-infected IID cases in the community and 57% of norovirus-infected IID cases presenting to general practitioners; the equivalent excluded fractions using the viral load-based adjustment factor were 50% in the community cohort and 55% amongst general practice patients;

iii. Using viral load to diagnose norovirus-associated IID, the number of IID cases with disease attributable to norovirus increased approximately 2.5 times in the community and two times amongst individuals presenting to general practitioners, compared to electron microscopy diagnosis;

iv. Approximately 5% of the general population in England experienced an episode of norovirus-associated IID each year between 1993 and 1996 (age-adjusted incidence 4.5 episodes per 100 person-years [95% credibility

interval (CI): 3.8 – 5.2]); incidence was highest amongst children aged less than five years (21 episodes per 100 person-years [95% CI: 16 – 28]) but still substantial amongst older children and adults aged five years and older (3 episodes per 100 person-years [95% CI: 3 – 4]);

v. Among children aged less than two years with norovirus-associated IID, one in four was taken for a general practice consultation; during the study, there were 100 000 consultations amongst children aged less than five years for norovirus-associated IID (95% CI: 85 000 – 123 000), a similar number to that due to rotavirus;

vi. Infectious contacts accounted for a large proportion of norovirus transmission leading to norovirus-associated IID; within households, contact with infectious children presented the greatest risk of norovirus transmission, both to adults and other children; the risk of transmission also increased with the number of symptomatic individuals in the household;

vii. No risk factors were identified for asymptomatic norovirus infection, although behaviours such as water sports participation, fruit and vegetable consumption and animal contact were associated with decreased risk of both symptomatic and asymptomatic norovirus infection.

9.1. Diagnosing norovirus-associated IID and asymptomatic norovirus infection

Success in detecting norovirus in clinical and environmental samples has been greatly enhanced by the development of RT-PCR assays. However, when diagnosing norovirus as the cause of sporadic, community-acquired IID, simple detection by RT-PCR is not sufficient for a confident diagnosis because of the high population prevalence of asymptomatic norovirus infection detected by RT-PCR[5, 21-24, 43, 490]. This is in contrast to pathogens such as *Campylobacter* spp. and *Salmonella* spp., which are found at very low prevalence in the general population[5, 21, 22, 43, 51, 334]; detection of these bacteria by culture in an individual with IID provides convincing evidence that the bacteria are the cause of illness. The quantitative approach developed in this thesis provides a major advance in the accuracy of norovirus-associated IID diagnosis by RT-PCR; using the Ct value cut-off at the individual level, or the adjustment factor for calculating population incidence, greatly increased the diagnostic specificity of RT-

PCR, to match the high diagnostic sensitivity of the assay. These two parallel approaches were developed to: (i) allow examination of norovirus case characteristics and risk factors for norovirus-associated IID, which requires classification of norovirus aetiology in individual IID cases (with the Ct value cut-off); and (ii) to make full use of the information contained in the whole viral load distributions when calculating incidence at the population level (using the adjustment factor.)

The predictive value of viral load for diagnosing norovirus-associated IID will decrease as the proportion of specimens collected late in the clinical course of IID increases, because the viral load shed by norovirus cases after symptom resolution quickly returns to levels found in asymptomatically infected individuals[80]. However, this is a problem in the interpretation of diagnostic tests for any pathogen and is not unique to the quantitative method developed here for norovirus. Whilst tests such as ELISA, light/electron microscopy and bacterial culture are not normally considered or discussed quantitatively, they are all implicitly quantitative because they have a lower limit of detection. A specimen may be ELISA negative for rotavirus, despite there being rotavirus present, because there is too little virus for it to be detectable by ELISA, even though there may be little actual difference in viral load compared to an ELISA-positive specimen with a rotavirus concentration just above the detection limit of the assay[497]. It is not routine practice to adjust for specimen collection delay when using ELISA, microscopy or bacterial culture to estimate the burden of sporadic IID caused by other pathogens. However, in the calculation of norovirus incidence using the adjustment factor in this thesis, the problem of low viral loads caused by late specimen collection was indirectly accounted for because the method is probability-based, and therefore does not exclude all IID cases with low norovirus viral loads.

Whilst the diagnostic sensitivity and specificity are of concern when diagnosing norovirus-associated IID by RT-PCR, the analytical sensitivity (i.e. the detection limit) and specificity (i.e. only producing positive results when norovirus is present in a specimen) of RT-PCR must be considered in the diagnosis of asymptomatic norovirus infections. It is likely that some healthy controls in the Study of Infectious Intestinal Disease were shedding norovirus at levels not detectable by the RT-PCR assays used, which have a detection limit of approximately 10^4 norovirus particles per gram of stool[110, 280]; this is evidenced by the apparently right-truncated normal distribution of Ct values in controls shown in Chapter 4. Therefore it is likely that the true prevalence of asymptomatic norovirus infection is higher than that reported in Chapter 5. Given the extremely low infectious dose of norovirus[98], which is well below the detection limit of

the RT-PCR assays, this underestimation may be particularly important for future studies examining the population-level risk of norovirus transmission due to asymptomatic carriage. Evaluations of RT-PCR using panels of stool specimens containing other enteric viruses have demonstrated that current RT-PCR assays have 100% analytical specificity [110, 248, 249], meaning that very few, if any, of the asymptomatic infections reported here are likely to be false positives.

Real-time RT-PCR is now used for routine norovirus diagnosis in clinical virology laboratories across the UK[488, 489]. Whilst it is likely that clinical virologists already informally consider the Ct values associated with a positive norovirus RT-PCR result when reporting a diagnosis to a clinician, the cut-off based approach developed in this thesis provides a more structured approach to assigning disease aetiology to norovirus in individual cases of sporadic IID. However, it is important that the cut-off described here is validated before it is applied in other laboratories, because of potential performance differences in the assays used[495], because the cut-off will be applied to a different group of IID cases (population and primary health care patients in this study versus predominantly hospital patients in clinical virology laboratories) and because the specimens used in this study had been stored for more than ten years prior to application of the real-time RT-PCR. Even after this validation, the application of such a cut-off in a clinical setting must be considered in the context of the patient's symptoms and the detection or absence of other pathogens in the specimen. In a clinical setting, the aim of diagnostic testing is predominantly to guide patient treatment; given that there are currently no specific anti-viral treatments for viral gastroenteritis, the emphasis of clinical diagnosis for IID must be to exclude bacterial causes and the use of antibiotics, leaving oral or intravenous rehydration therapy as the main treatment option[67]. In contrast, during research studies investigating the burden of disease caused by norovirus in a particular study population, the aim is to provide an appropriate estimate of the number of IID cases attributable to norovirus. In these population-based studies, a small amount of aetiological misclassification has no individual patient-level consequences, and will have little effect on the overall estimate, provided positive and negative misclassification is balanced, i.e. a similar number of individuals are wrongly classified as norovirus cases as the number of norovirus cases who are wrongly classed as not having disease caused by norovirus.

In addition to the diagnosis of predominantly paediatric, sporadic, community-acquired norovirus-associated IID, clinical virology laboratories use real-time RT-PCR to investigate the role of norovirus in IID outbreaks, predominantly those occurring in

healthcare settings. Whilst consideration of the Ct value for a norovirus-positive specimen may still be informative when testing specimens as part of an outbreak investigation, the availability of specimens from multiple exposed individuals makes the exact viral load detected in a single individual less important. If multiple exposed individuals are positive for norovirus by RT-PCR, this provides robust microbiological evidence that norovirus is the cause of the outbreak, which is then considered along with epidemiological and clinical information to determine the appropriate course of intervention to prevent further transmission[487, 511]. Use of a Ct value cut-off is therefore less important in the context of norovirus outbreak investigations. However, the concept that not all RT-PCR positive individuals may have disease caused by norovirus is informative in situations where only one or a very small proportion of outbreak specimens is norovirus-positive. The prevalence of asymptomatic norovirus infection provides an indication of the probability that one specimen collected during an outbreak caused by another pathogen may be norovirus-positive by chance, although the prevalence of asymptomatic infection in hospitals and healthcare settings may differ from that reported in the general population in this thesis and may require separate measurement.

A major limitation of the analyses of norovirus viral load presented in this thesis is the lack of norovirus genotype information for the specimens in the Study of Infectious Intestinal Disease specimen archive. Whilst the efficiency of the real-time RT-PCR assay is similar for all the common genotypes in genogroup II, the efficiency is lower for a number of the genogroup I genotypes and for the rarer genogroup II genotypes GII.7 and GII.8 (see Chapters 3 and 4, Appendix A1.3). Whilst analysing all norovirus genotypes together is likely to have had only a minimal, although currently unquantifiable, effect on the incidence estimates and risk factor analyses, it may not be appropriate to treat all genotypes equally in a clinical setting, where errors in classifying norovirus aetiology may have consequences for patient care. However, even if sufficient specimens were collected for the identification of genotype-specific Ct value cut-offs in this or future studies, application of the genotype-specific cut-offs would require specimen genotyping to be part of routine clinical diagnostic practice, which may not be economically or logistically feasible. Further work on a genogroup I-specific cut-off would, however, provide some improvement in diagnostic accuracy compared to universal application of the genogroup II cut-off.

9.2. Incidence of norovirus-associated IID

The updated estimates of norovirus-associated IID incidence based on viral load measurements, presented in Chapter 8, confirm that norovirus is the most common cause of IID in the community in England. In comparison to the other common pathogens causing IID in the community cohort in the Study of Infectious Intestinal Disease, norovirus caused approximately five times more episodes per 100 person-years than *Campylobacter* spp., eight times more episodes per 100 person-years than enteroaggregative *E. coli* and six times more episodes per 100 person-years than rotavirus, across all ages (comparing crude incidence)[8]. Norovirus was also amongst the most common causes of general practice consultations for IID in the Study of Infectious Intestinal Disease[8], although the majority of consultations were by paediatric norovirus cases, with the number of consultations in children aged less than five years very similar to the number due to rotavirus-associated IID during the study.

The viral-load based norovirus incidence estimates were between two and three fold higher than those based on electron microscopy, with a slightly greater relative increase in incidence in the community cohort compared to the general practice study. This is probably because of the small, but significant, difference in the norovirus Ct value distribution in children aged less than five years in the community cohort and the general practice case-control study (Table 4.4), combined with the predominance of young children amongst norovirus-infected IID cases in the general practice study component. Half of the norovirus-infected IID cases in the community cohort and slightly more than half of those norovirus-infected general practice patients with IID were classified as not having disease caused by norovirus, using the viral load cut-off or the population-level adjustment factor (Appendix A6.3). Consideration of viral load has therefore prevented substantial overestimation of norovirus incidence based on RT-PCR testing.

Modelling of routinely collected surveillance data on general practice consultations for IID and unlinked norovirus laboratory diagnosis reports in England and Wales produced similar estimates of the incidence of norovirus general practice consultations as the prospective case ascertainment used in the Study of Infectious Intestinal Disease. Due to the poor fit of the models for children and adults aged five years and older, the method is probably unsuitable for reliably estimating norovirus consultations in these age groups. However, the model for children aged less than five years provided a good fit to the general practice consultation data that were used as the outcome variable and reproduced the expected seasonal pattern of norovirus activity.

The indirect modelling method is therefore suitable for producing regular updates to the estimates of paediatric general practice consultations caused by norovirus-associated IID. Given that the large majority of the general practice consultations for norovirus in the Study of Infectious Intestinal Disease were in children aged less than five years, they would be the priority group for any public health intervention aiming to reduce consultations for sporadic norovirus-associated IID; it is therefore most important to continue monitoring norovirus consultations in young children using routine surveillance data and the method presented in Chapter 7.

The incidence of norovirus-associated IID in the community and of general practice consultations for norovirus was low in older adults (aged 45 or 65 years and older for the two study components, respectively), in spite of a significant recognised burden of norovirus disease in the elderly[14, 38, 347]. However, the high incidence of norovirus-associated IID amongst the elderly is reported mostly as norovirus outbreaks in hospitals and other healthcare or institutional settings[14, 38, 40, 347, 602], which were not included in the Study of Infectious Intestinal Disease community cohort[83, 480], so were not captured in the incidence estimates. It is also unlikely that many of these elderly individuals who become ill during outbreaks in community settings are seen by general practitioners; nursing home staff are likely to be experienced in the recognition and management of norovirus-associated IID, and given that there is no specific antiviral treatment, would be unlikely to consult a general practitioner for the majority of cases. In light of the concentration of the norovirus disease burden in elderly individuals in easily-identified and well-defined populations in health and community care settings, it is less important to have ongoing monitoring of general practice consultations for norovirus-associated IID through modelling of routine surveillance data. Enhanced surveillance in these vulnerable elderly populations is likely to provide sufficient information on the major burden of norovirus disease in this age group; one example of such monitoring is the hospital norovirus outbreak surveillance system recently introduced by the HPA in England and Wales, which collates reports of norovirus outbreaks in elderly hospitalised populations[348].

9.3. Norovirus transmission

The analysis of risk factors for sporadic, community-acquired norovirus-associated IID indicated that the predominant source of norovirus infection is other symptomatic, norovirus-infected individuals. The risk of acquiring norovirus-associated IID was highest when the symptomatic contact was a young child, although transmission was associated with infectious contacts of all ages. Furthermore, adults living in a

household with young children or a baby were at an increased risk of norovirus-associated IID, regardless of whether these children were symptomatic during the 10-day exposure period used in the epidemiological questionnaire. This most likely reflects the high incidence of symptomatic norovirus infection in young children, demonstrated in Chapter 8.

However, only slightly more than half of the cases of norovirus-associated IID in the Study of Infectious Intestinal Disease could be attributed to reporting contact with an infectious individual and there was no significant risk associated with other transmission routes commonly reported for norovirus, such as drinking water and food (except oyster consumption, which was reported by only a very small number of norovirus cases) [38, 349, 351, 360, 397, 400, 410-418, 444, 445, 451-457]. It is likely that some norovirus cases failed to report contact with symptomatic individuals because of poor recall or not knowing about the symptoms experienced by individuals with whom they had contact. In addition, it is possible that environmental contamination may contribute to the transmission of norovirus leading to sporadic norovirus-associated IID. Environmental contamination of common surfaces such as door-handles, telephones, computer keyboards and a range of other materials have been demonstrated in both environmental sampling, within and independently from outbreak investigations, and in experimental virus transfer studies[18, 328, 329, 398-403, 403-408]. It is therefore possible that some norovirus cases in this study did not actually come into direct contact with a person with symptomatic norovirus infection, but were in contact with surfaces contaminated by such individuals.

Similarly, it is possible that transmission may have occurred through contact with asymptomatically infected individuals or surfaces that they have contaminated, with the norovirus cases being unaware of the infection status of these contacts. In addition to the description of viral loads shed by healthy controls in the Study of Infectious Intestinal Disease specimen archive, presented in Chapter 4, several other studies have demonstrated that asymptomatically infected individuals shed norovirus at concentrations far greater than the estimated infectious dose for norovirus[24, 80, 98, 114]. However, there have been no studies examining the direct person-to-person transmission risk from asymptomatically infected individuals in the household or other community settings, although some outbreak investigations have attributed foodborne norovirus transmission to contamination by asymptomatically infected foodhandlers[360, 397, 412, 414, 416]. Quantifying the transmission risk from asymptomatically infected individuals is important for informing public health action against norovirus. Exclusion of

norovirus cases from work in the food industry and healthcare sector currently only covers the period of symptoms and the subsequent 48 hours[603], but given the extended period of low-level norovirus shedding demonstrated in a number of studies, revision of these guidelines would be necessary if this level of shedding poses a significant transmission risk. However, the major public health intervention against norovirus transmission, in any setting, is good hand and environmental hygiene[389, 409, 604, 605] and these actions can be promoted without the need for detailed knowledge about the exact risks of norovirus transmission from symptomatically and asymptomatically infected individuals. Furthermore, the highest burden of asymptomatic infection is in young children, so the risk of transmission in the general population from asymptomatic infections may be lower than indicated from the age-adjusted community prevalence of 12% presented in Chapter 5, because of the high degree of assortative social mixing amongst young children and adults[606].

No risk factors for asymptomatic norovirus infection were identified. It is likely that a small proportion of the asymptomatic infections were actually pre- or post-symptomatic norovirus shedding. Just under one tenth of the individuals with asymptomatic norovirus infection had experienced gastrointestinal symptoms prior to the ten day exclusion period for control recruitment in the Study of Infectious Intestinal Disease, but within three weeks of recruitment, although the excess prevalence of gastrointestinal symptoms compared to norovirus negative healthy individuals was less than 5%. Unfortunately study participants were only asked to report potential infectious contacts that occurred within the previous ten days in the epidemiological questionnaire. Where asymptomatic norovirus infection was due to contact with symptomatic individuals, this information may not have been captured in the questionnaire if norovirus shedding had occurred for more than 10 days before recruitment into the study. Elucidation of the transmission routes for asymptomatic norovirus infection in the community would require studies with frequent and regular testing of healthy individuals to identify incident asymptomatic norovirus infections and then capture recent exposure history. However, it is unlikely that the economic and human cost of such studies could be justified. Norovirus testing of asymptomatic individuals exposed during norovirus outbreaks and collection of exposure information from these individuals would probably provide more direct and easily available evidence on the routes of transmission leading to asymptomatic norovirus infection. Current evidence from volunteer inoculation studies indicates that pre-existing immunity, and possibly the inoculum dose, probably determine whether symptomatic or asymptomatic infection develops after norovirus

exposure, meaning that there may not be different transmission routes, only a different outcome of exposure depending on these host and viral characteristics[98, 138].

A major limitation of the risk factor analysis was that where study participants reported symptomatic contacts, the aetiology of IID in these contacts was not established. Therefore, whilst the strong association of norovirus-associated IID with symptomatic contacts suggests that a large majority of these contacts had a norovirus infection, this is an assumption and has not been verified with microbiological testing.

9.4. Further limitations

The seasonality and molecular epidemiology of norovirus-associated IID across different age groups in the community in the UK are poorly characterised; this information is important for understanding the epidemiological relationship between sporadic community norovirus cases and norovirus outbreaks, particularly those outbreaks occurring in healthcare settings, which have a distinct but unexplained seasonality and norovirus genotype distribution[97, 197-204, 588]. Whilst GII.4 noroviruses predominate in causing highly seasonal norovirus outbreaks amongst adults in healthcare settings, a broader range of genotypes has been detected both in outbreaks in community settings and amongst sporadic paediatric norovirus cases presenting to healthcare services, which also have a more constant, year-round incidence. However, it remains unclear whether the predominance of GII.4 in causing hospital outbreaks, which occur mostly amongst adults, is because GII.4 is at high prevalence in adult norovirus cases in the community with winter seasonality, or whether higher hospital admission rates in the winter lead to more GII.4 introduction events, or alternatively whether the GII.4 viruses are at lower prevalence in the community but have greater transmissibility in semi-closed settings than other genotypes and are therefore more likely to cause an outbreak after they are introduced.

The season-stratified incidence estimates for norovirus-associated IID in the community showed a slight winter-time peak. However, the seasonal peak was not as marked as that observed in hospital norovirus outbreak incidence[588] and unfortunately it was not possible to examine the seasonality of norovirus-associated IID separately amongst children and adults in the community, because of sample size limitations. In addition, no genotype information was available for any of the symptomatic or asymptomatic norovirus infections in the Study of Infectious Intestinal Disease specimen archive; only the genogroup of the infections was determined in the RT-PCR assay. It was therefore not possible to specifically compare the GII.4 incidence

between children and adults, or to examine the seasonality of GII.4 infection in the community.

The estimates of norovirus-associated IID incidence produced in this thesis are based on data collected in the mid 1990s. The epidemiology of norovirus is particularly complex[97, 581], with substantial variation in norovirus activity across successive years identified in surveillance of outbreaks and laboratory diagnoses in England and Wales. Reporting artefacts and changes in diagnostic methods may account for some, but certainly not all, of the inter-seasonal variation in norovirus activity. Furthermore, a new variant of norovirus GII.4 emerged during 1995 and 1996, when the Study of Infectious Intestinal Disease was carried out[201, 221, 537]. Emergence of new GII.4 variants has been linked to substantial increases in both the number of infections and outbreaks reported during the normal season and in out-of-season norovirus activity as well[222, 224, 229, 575, 590]. It is therefore impossible to judge how representative these new estimates of norovirus-associated IID incidence are of normal norovirus activity in England, especially without genotype characterisation to confirm that the emergent GII.4 variant from 1995/1996 was causing illness in the study population. However these new estimates are based on current diagnostic methods, and the new quantitative approach to norovirus-associated IID diagnosis can now be used in new studies of sporadic, community-acquired IID. The results from these new studies will then be directly comparable to the incidence estimates presented in this thesis, to allow characterisation of recent changes in norovirus community incidence, without problems of confounding due to changes in diagnostic methods over the same time period.

This is the first description of the distribution of viral loads present in naturally occurring symptomatic and asymptomatic norovirus infections in the community. Whilst the increase in norovirus disease incidence associated with new GII.4 variants has been reported in many countries, the effect of these novel variants on the prevalence of asymptomatic infection is unknown; both an increase or decrease in asymptomatic prevalence are biologically plausible. Decreased asymptomatic prevalence could be expected because the novel GII.4 variants are antibody-escape mutants[179, 223].; if the occurrence of symptoms after norovirus infection is predominantly determined by pre-existing, short-term immunity, a higher proportion of infections may be symptomatic immediately after the emergence of a GII.4 antibody-escape mutant. Conversely, if other mechanisms are involved in the occurrence of asymptomatic infection, such as the infecting dose, an increase in asymptomatic prevalence may be expected simply because of the greater circulation of norovirus in the population caused by the increase

in disease incidence. The prevalence of asymptomatic infections in the community will directly affect the degree of overestimation expected by using RT-PCR to diagnose norovirus associated-IID; with a higher prevalence of asymptomatic norovirus infection in the population, a greater proportion of norovirus-infected IID cases may have a coincidental norovirus infection with disease actually caused by another pathogen.

9.5. Recommendations

The analysis of viral loads in symptomatic and asymptomatic norovirus infection presented in this thesis has shown that the community incidence of sporadic norovirus-associated IID would have been greatly over-estimated if all RT-PCR positive IID cases in the Study of Infectious Intestinal Disease specimen archive were considered to have disease caused by norovirus. Up to 50% of these IID cases were shedding norovirus at the same levels seen in healthy controls, indicating that they probably did not have disease caused by norovirus, despite being infected at the time of their illness. In similar future studies of the population incidence of IID, it is therefore recommended that real-time RT-PCR is used for norovirus diagnosis and that a group of healthy controls is recruited concurrently to allow application of the adjustment factor method described in Chapter 8. If recruitment of controls is not possible, application of an appropriately validated Ct value cut-off for the assay in use would provide suitably similar estimates to those using the adjustment factor method. Whilst the cut-off based estimates would not acknowledge the uncertainty in the viral load measurements, it is not common practice to explicitly incorporate diagnostic validity when estimating pathogen-specific disease incidence with other diagnostic tests. If it is not possible to test all IID cases with a real-time assay, testing a representative and sizeable proportion would be sufficient to use any of the methods presented in Chapter 8.

Whilst the focus of the methods developed for norovirus diagnosis in this thesis was to identify norovirus cases for the calculation of norovirus-associated IID incidence and examination of risk factors for norovirus disease, the work has implications for the clinical diagnosis of norovirus-associated IID used to guide patient care. Other information on patient history, symptoms and co-infections should still be considered in the clinical setting, but development of a cut-off, as described here, for use in clinical virology laboratories would better inform the interpretation of norovirus RT-PCR testing.

The importance of direct person-to-person transmission in the acquisition of sporadic norovirus-associated IID indicates that further reinforcement of existing public health messages regarding hand and domestic environmental hygiene could facilitate a

reduction in norovirus transmission. Although, given the low infectious dose and the current lack of any sanitizers that completely inactivate norovirus, reductions in disease incidence may be limited.

During the Study of Infectious Intestinal Disease, norovirus caused a similar number of paediatric general practice consultations as rotavirus. Universal introduction of the licensed rotavirus vaccines is under ongoing consideration by the UK Department of Health; the results from this study indicate that norovirus may also be a priority for public health action. However, it is unlikely that a norovirus vaccine could be recommended for use in young children because of the high valency required to cover the large number of immunologically distinct genotypes detected in paediatric norovirus cases. Further public education about the causes of acute paediatric gastroenteritis and appropriate care action may be a more suitable intervention. Conversely, vaccination against GII.4 noroviruses may help to reduce the burden of norovirus outbreaks in healthcare settings, although protection will only be short-lived and vaccine response may be worse in the predominantly elderly populations that are affected by nosocomial norovirus outbreaks. Further characterisation of the molecular epidemiology of norovirus in the community would be essential before a GII.4 vaccine could be recommended and also after introduction, to ensure that other strains are not likely to replace GII.4 in healthcare settings and maintain the current level of outbreak incidence.

The quantitative methods developed here for norovirus diagnosis are very likely to be applicable to other enteric viruses and to viruses causing other acute infectious disease syndromes, such as respiratory viruses. Indeed, a quantitative approach to diagnosis would be useful for any pathogen that is at high prevalence in healthy individuals in the general population, providing there is evidence that pathogen load is well correlated with the occurrence of symptoms.

9.6. Perspective

The body of work presented in this thesis has provided a novel approach to the diagnosis of norovirus-associated IID, updated estimates of sporadic norovirus incidence in England and new evidence to support existing insights into the transmission patterns of norovirus in the community. However, the data were collected in a single high income country and there is now an emerging interest in norovirus as a cause of acute paediatric IID in low and middle income countries[49]. Whilst the epidemiology of norovirus is likely to be different in low income countries, the

quantitative method for norovirus-associated IID diagnosis will certainly be of great importance in assessing the disease burden caused by norovirus in these settings. Indeed, interim results from ongoing studies in low income countries indicate that the prevalence of asymptomatic infection is very high, in some places almost equivalent to the prevalence of norovirus amongst IID cases[607]. The quantitative diagnostic methods developed in this thesis will therefore be essential for determining the relative importance of norovirus as a cause of IID in low income countries, which is currently poorly understood.

The potential certainly exists for a high norovirus disease burden in low income countries, given the evidence of food- and water-borne transmission from outbreak investigations in high income countries, in addition to direct person-to-person transmission. However, it remains to be seen whether the hypothesised higher norovirus exposure levels in low income countries actually lead to a higher incidence of disease. Volunteer studies have indicated that repeated homotypic norovirus strain exposure can lead to longer-lasting immunity that is protective against disease beyond the six to twelve months of protection provided by a single infection episode[56, 77]; if there truly is higher exposure to norovirus in low income country settings this may serve to boost immunity and prevent more illness. However nutritional status is also likely to play an important role in moderating any immune protection resulting from frequent norovirus exposure, especially amongst young children. Antigenic drift resulting in immune response evasion has been well characterised in GII.4 noroviruses from high income countries[179]; norovirus therefore has the potential to respond to prevailing population immunity, adding further complexity to the potential picture of norovirus epidemiology in low income countries.

The studies of paediatric IID burden currently being conducted in low income countries will hopefully provide the answer to the most important question: how important is norovirus as a cause of severe paediatric IID and associated mortality? Whilst there is an abundance of work using indirect methods to estimate mortality associated with rotavirus-associated IID globally[608], it is not appropriate to simply extrapolate the spectrum of pathogens detected in children presenting to primary and emergency care services directly to the causes of diarrhoeal mortality in this population: outcomes after consultation or hospital admission are likely to differ substantially between pathogens. Therefore, whilst a recent systematic review has suggested that norovirus may cause 200000 deaths in children aged less than five years globally[49], more robust data are required to verify the accuracy of this figure. However, it will be interesting to see where

norovirus falls on the public health agenda in low income countries. There can be little hope of a vaccine that will be effective in preventing norovirus disease amongst children in the general population because of the high antigenic diversity of noroviruses and the demonstrated potential for rapid evolution to escape host immune responses[179, 223]. Therefore limited public health funding and resources may be better utilised in improving nutrition and sanitation, which will act to simultaneously reduce the burden and mitigate the effects of a host of infectious diseases in low income countries.

However, norovirus remains firmly on the public agenda in high income countries, with extensive media coverage of both nosocomial outbreaks and disease in the general population each winter in the UK. Public health priorities are very different in high income countries; with very few deaths from norovirus-associated IID, the focus is on the disruption to care and particularly the economic costs of hospital norovirus outbreaks and the costs of paediatric primary care consultations. Indeed, the incidence estimates presented here suggest that norovirus could become the leading cause of paediatric primary care consultations for IID if rotavirus vaccination is introduced into routine childhood schedules in the UK, given the substantial reduction in severe rotavirus disease seen in the United States and Australia since the start of rotavirus vaccination[609-612]. However, the greatest potential for effective action against norovirus probably lies in the healthcare sector, where a GII.4 specific vaccine may reduce disease burden amongst patients and staff, provided strain replacement does not occur in this setting. The first tentative steps towards commercial production of a norovirus vaccine are now being taken[613], making this approach more realistic. However, the community of norovirus researchers still has a lot to learn about the pathogenesis and transmission dynamics of norovirus and it may be unwise to put all our hopes in a GII.4 vaccine: there is a need for controlled trials of interventions addressing the effectiveness of other measures such as case isolation, ward closure and environmental cleaning for reduction of norovirus outbreaks in healthcare settings, to improve the evidence base underpinning current guidelines. Norovirus appears to be a clever pathogen: highly infectious, resistant to environmental degradation and cleaning, antigenically diverse and constantly evolving. The battle to control norovirus infection is likely to be long, hard and intriguing.

References

1. Djuretic T, Wall PG, Ryan MJ et al. General outbreaks of infectious intestinal disease in England and Wales 1992 to 1994. *Commun.Dis.Rep.CDR Rev.* 1996;**6**(4):R57-R63.

2. Evans HS, Madden P, Douglas C et al. General outbreaks of infectious intestinal disease in England and Wales: 1995 and 1996. *Commun.Dis.Public Health.* 1998;**1**(3):165-71.

3. Evans HS, Maguire H. Outbreaks of infectious intestinal disease in schools and nurseries in England and Wales 1992 to 1994. *Commun.Dis.Rep.CDR Rev.* 1996;**6**(7):R103-R108.

4. Kroneman A, Verhoef L, Harris J et al. Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the foodborne viruses in Europe Network from 1 July 2001 to 30 June 2006. *J.Clin.Microbiol.* 2008;**46**(9):2959-65.

5. de Wit MA, Koopmans MP, Kortbeek LM et al. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *Am.J.Epidemiol.* 2001;**154**(7):666-74.

6. Hellard ME, Sinclair MI, Forbes AB et al. A randomized, blinded, controlled trial investigating the gastrointestinal health effects of drinking water quality. *Environ.Health Perspect.* 2001;**109**(8):773-8.

7. Marshall JA, Hellard ME, Sinclair MI et al. Incidence and characteristics of endemic Norwalk-like virus-associated gastroenteritis. *J.Med.Virol.* 2003;**69**(4):568-78.

8. Wheeler JG, Sethi D, Cowden JM et al. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The Infectious Intestinal Disease Study Executive. *BMJ.* 1999;**318**(7190):1046-50.

9. Mead PS, Slutsker L, Dietz V et al. Food-related illness and death in the United States. *Emerg.Infect.Dis.* 1999;**5**(5):607-25.

10. Kapikian AZ, Wyatt RG, Dolin R et al. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J.Virol.* 1972;**10**(5):1075-81.

11. Castriciano S, Luinstra K, Petrich A et al. Comparison of the RIDASCREEN norovirus enzyme immunoassay to IDEIA NLV GI/GII by testing stools also assayed by RT-PCR and electron microscopy. *J.Virol.Methods.* 2007;**141**(2):216-9.

12. Gallimore CI, Green J, Richards AF et al. Methods for the detection and characterisation of noroviruses associated with outbreaks of gastroenteritis: outbreaks occurring in the north-west of England during two norovirus seasons. *J.Med.Virol.* 2004;**73**(2):280-8.

13. Froggatt PC, Barry V, I, Ashley CR et al. Surveillance of norovirus infection in a study of sporadic childhood gastroenteritis in South West England and South Wales, during one winter season (1999-2000). *J.Med.Virol.* 2004;**72**(2):307-11.

14. Lopman BA, Reacher MH, Vipond IB et al. Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002-2003. *Emerg.Infect.Dis.* 2004;**10**(10):1827-34.

15. Gordon SM, Oshiro LS, Jarvis WR et al. Foodborne Snow Mountain agent gastroenteritis with secondary person-to-person spread in a retirement community. *Am.J.Epidemiol.* 1990;**131**(4):702-10.

16. Greig JD, Lee MB. Enteric outbreaks in long-term care facilities and recommendations for prevention: a review. *Epidemiol.Infect.* 2009;**137**(2):145-55.

17. Okada M, Tanaka T, Oseto M et al. Genetic analysis of noroviruses associated with fatalities in healthcare facilities. *Arch.Virol.* 2006;**151**(8):1635-41.

18. Wu HM, Fornek M, Schwab KJ et al. A norovirus outbreak at a long-term-care facility: the role of environmental surface contamination. *Infect.Control Hosp.Epidemiol.* 2005;**26**(10):802-10.

19. Jansen A, Beyer A, Brandt C et al. [The norovirus-epidemic in Berlin - clinic, epidemiology, and prevention]. *Z.Gastroenterol.* 2004;**42**(4):311-6.

20. Ball JM, Graham DY, Opekun AR et al. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. *Gastroenterology.* 1999;**117**(1):40-8.

21. Amar CF, East CL, Gray J et al. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993-1996). *Eur.J.Clin.Microbiol.Infect.Dis.* 2007;**26**(5):311-23.

22. Karsten C, Baumgarte S, Friedrich AW et al. Incidence and risk factors for community-acquired acute gastroenteritis in north-west Germany in 2004. *Eur.J.Clin.Microbiol.Infect.Dis.* 2009.

23. Okabayashi T, Yokota S, Ohkoshi Y et al. Occurrence of norovirus infections unrelated to norovirus outbreaks in an asymptomatic food handler population. *J.Clin.Microbiol.* 2008;**46**(6):1985-8.

24. Barreira DM, Ferreira MS, Fumian TM et al. Viral load and genotypes of noroviruses in symptomatic and asymptomatic children in Southeastern Brazil. *J.Clin.Virol.* 2009.

25. Lopez AD, Mathers CD. Measuring the global burden of disease and epidemiological transitions: 2002-2030. *Ann.Trop.Med.Parasitol.* 2006;**100**(5-6):481-99.

26. Boschi-Pinto C, Velebit L, Shibuya K. Estimating child mortality due to diarrhoea in developing countries. *Bull.World Health Organ.* 2008;**86**(9):710-7.

27. Guerrant RL, Kosek M, Lima AA et al. Updating the DALYs for diarrhoeal disease. *Trends Parasitol.* 2002;**18**(5):191-3.

28. Akgun S, Rao C, Yardim N et al. Estimating mortality and causes of death in Turkey: methods, results and policy implications. *Eur.J.Public Health.* 2007;**17**(6):593-9.

29. Lopez AD, Mathers CD, Ezzati M et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet.* 2006;**367**(9524):1747-57.

30. Gauci C, Gilles H, O'Brien S et al. The magnitude and distribution of infectious intestinal disease in Malta: a population-based study. *Epidemiol.Infect.* 2007;**135**(8):1282-9.

31. Herikstad H, Yang S, Van Gilder TJ et al. A population-based estimate of the burden of diarrhoeal illness in the United States: FoodNet, 1996-7. *Epidemiol.Infect.* 2002;**129**(1):9-17.

32. Majowicz SE, Dore K, Flint JA et al. Magnitude and distribution of acute, self-reported gastrointestinal illness in a Canadian community. *Epidemiol.Infect.* 2004;**132**(4):607-17.

33. Scallan E, Fitzgerald M, Collins C et al. Acute gastroenteritis in northern Ireland and the Republic of Ireland: a telephone survey. *Commun.Dis.Public Health.* 2004;**7**(1):61-7.

34. Roberts JA, Cumberland P, Sockett PN et al. The study of infectious intestinal disease in England: socio-economic impact. *Epidemiol.Infect.* 2003;**130**(1):1-11.

35. van den Brandhof WE, De Wit GA, de Wit MA et al. Costs of gastroenteritis in The Netherlands. *Epidemiol.Infect.* 2004;**132**(2):211-21.

36. Giaquinto C, Van DP, Huet F et al. Costs of community-acquired pediatric rotavirus gastroenteritis in 7 European countries: the REVEAL Study. *J.Infect.Dis.* 2007;**195 Suppl 1**S36-S44.

37. Bailey MS, Gallimore CI, Lines LD et al. Viral gastroenteritis outbreaks in deployed British troops during 2002-7. *J.R.Army Med.Corps.* 2008;**154**(3):156-9.

38. Dedman D, Laurichesse H, Caul EO et al. Surveillance of small round structured virus (SRSV) infection in England and Wales, 1990-5. *Epidemiol.Infect.* 1998;**121**(1):139-49.

39. Little CL, Barrett NJ, Grant K et al. Microbiological safety of sandwiches from hospitals and other health care establishments in the United Kingdom with a focus on Listeria monocytogenes and other Listeria species. *J.Food Prot.* 2008;**71**(2):309-18.

40. Meakins SM, Adak GK, Lopman BA et al. General outbreaks of infectious intestinal disease (IID) in hospitals, England and Wales, 1992-2000. *J.Hosp.Infect.* 2003;**53**(1):1-5.

41. Wilcox MH, Cunniffe JG, Trundle C et al. Financial burden of hospital-acquired Clostridium difficile infection. *J.Hosp.Infect.* 1996;**34**(1):23-30.

42. Zerey M, Paton BL, Lincourt AE et al. The burden of Clostridium difficile in surgical patients in the United States. *Surg.Infect.(Larchmt.).* 2007;**8**(6):557-66.

43. de Wit MA, Koopmans MP, Kortbeek LM et al. Etiology of gastroenteritis in sentinel general practices in the netherlands. *Clin.Infect.Dis.* 2001;**33**(3):280-8.

44. Junquera CG, de Baranda CS, Mialdea OG et al. Prevalence and Clinical Characteristics of Norovirus Gastroenteritis Among Hospitalized Children in Spain. *Pediatr.Infect.Dis.J.* 2009;**28**(7):604-7.

45. Jansen A, Stark K, Kunkel J et al. Aetiology of community-acquired, acute gastroenteritis in hospitalised adults: a prospective cohort study. *BMC.Infect.Dis.* 2008;**8**(1):143.

46. Fleury MD, Stratton J, Tinga C et al. A descriptive analysis of hospitalization due to acute gastrointestinal illness in Canada, 1995-2004. *Can.J.Public Health.* 2008;**99**(6):489-93.

47. Djuretic T, Ramsay M, Gay N et al. An estimate of the proportion of diarrhoeal disease episodes seen by general practitioners attributable to rotavirus in children under 5 y of age in England and Wales. *Acta Paediatr.Suppl.* 1999;**88**(426):38-41.

48. Harris JP, Jit M, Cooper D et al. Evaluating rotavirus vaccination in England and Wales. Part I. Estimating the burden of disease. *Vaccine.* 2007;**25**(20):3962-70.

49. Patel MM, Widdowson MA, Glass RI et al. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg.Infect.Dis.* 2008;**14**(8):1224-31.

50. Tate JE, Curns AT, Cortese MM et al. Burden of acute gastroenteritis hospitalizations and emergency department visits in US children that is potentially preventable by rotavirus vaccination: a probe study using the now-withdrawn rotashield vaccine. *Pediatrics.* 2009;**123**(3):744-9.

51. Tompkins DS, Hudson MJ, Smith HR et al. A study of infectious intestinal disease in England: microbiological findings in cases and controls. *Commun.Dis.Public Health.* 1999;**2**(2):108-13.

52. Van DP, Giaquinto C, Huet F et al. Multicenter prospective study of the burden of rotavirus acute gastroenteritis in Europe, 2004-2005: the REVEAL study. *J.Infect.Dis.* 2007;**195 Suppl 1**S4-S16.

53. Glass RI, Bresee J, Jiang B et al. Gastroenteritis viruses: an overview. *Novartis.Found.Symp.* 2001;**2**385-19.

54. Cukor G, Nowak NA, Blacklow NR. Immunoglobulin M responses to the Norwalk virus of gastroenteritis. *Infect.Immun.* 1982;**37**(2):463-8.

55. Glass RI, Noel J, Mitchell D et al. The changing epidemiology of astrovirus-associated gastroenteritis: a review. *Arch.Virol.Suppl.* 1996;**12**287-300.

56. Parrino TA, Schreiber DS, Trier JS et al. Clinical immunity in acute gastroenteritis caused by Norwalk agent. *N.Engl.J.Med.* 1977;**297**(2):86-9.

57. Velazquez FR, Matson DO, Calva JJ et al. Rotavirus infections in infants as protection against subsequent infections. *N.Engl.J.Med.* 1996;**335**(14):1022-8.

58. Agus SG, Dolin R, Wyatt RG et al. Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. *Ann.Intern.Med.* 1973;**79**(1):18-25.

59. Schreiber DS, Blacklow NR, Trier JS. The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis. *N.Engl.J.Med.* 1973;**288**(25):1318-23.

60. Schreiber DS, Blacklow NR, Trier JS. The small intestinal lesion induced by Hawaii agent acute infectious nonbacterial gastroenteritis. *J.Infect.Dis.* 1974;**129**(6):705-8.

61. Dolin R, Levy AG, Wyatt RG et al. Viral gastroenteritis induced by the Hawaii agent. Jejunal histopathology and serologic response. *Am.J.Med.* 1975;**59**(6):761-8.

62. Kaplan JE, Feldman R, Campbell DS et al. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. *Am.J.Public Health.* 1982;**72**(12):1329-32.

63. Cheetham S, Souza M, Meulia T et al. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. *J.Virol.* 2006;**80**(21):10372-81.

64. Souza M, Azevedo MS, Jung K et al. Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus. *J.Virol.* 2008;**82**(4):1777-86.

65. Troeger H, Loddenkemper C, Schneider T et al. Structural And Functional Changes Of The Duodenum In Human Norovirus Infection. *Gut.* 2008;**e-pub ahead of print**.

66. Meeroff JC, Schreiber DS, Trier JS et al. Abnormal gastric motor function in viral gastroenteritis. *Annals of Internal Medicine.* 1980;. **92**(3).

67. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. *N.Engl.J.Med.* 2009;**361**(18):1776-85.

68. Clarke IN, Lambden PR. Organization and expression of calicivirus genes. *J.Infect.Dis.* 2000;**181 Suppl 2**S309-S316.

69. Zahorsky J. Hyperemesis hiemis or the winter vomiting disease. *Arch.Pediat.* 1929;**46**391-5.

70. Adler JL, Zickl R. Winter vomiting disease. *J.Infect.Dis.* 1969;**119**(6):668-73.

71. Dolin R, Blacklow NR, DuPont H et al. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. *J.Infect.Dis.* 1971;**123**(3):307-12.

72. Blacklow NR, Cukor G, Bedigian MK. Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. *Journal of Clinical Microbiology.* 1979;. **10**(6).

73. Erdman DD, Gary GW, Anderson LJ. Serum immunoglobulin A response to Norwalk virus infection. *J.Clin.Microbiol.* 1989;**27**(6):1417-8.

74. Gary GW, Anderson LJ, Keswick BH et al. Norwalk virus antigen and antibody response in an adult volunteer study. *J.Clin.Microbiol.* 1987;**25**(10):2001-3.

75. Graham DY, Jiang X, Tanaka T et al. Norwalk virus infection of volunteers: new insights based on improved assays. *J.Infect.Dis.* 1994;**170**(1):34-43.

76. Gray JJ, Cunliffe C, Ball J et al. Detection of immunoglobulin M (IgM), IgA, and IgG Norwalk virus-specific antibodies by indirect enzyme-linked immunosorbent assay with baculovirus-expressed Norwalk virus capsid antigen in adult volunteers challenged with Norwalk virus. *J.Clin.Microbiol.* 1994;**32**(12):3059-63.

77. Johnson PC, Mathewson JJ, DuPont HL et al. Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults. *J.Infect.Dis.* 1990;**161**(1):18-21.

78. Okhuysen PC, Jiang X, Ye L et al. Viral shedding and fecal IgA response after Norwalk virus infection. *J.Infect.Dis.* 1995;**171**(3):566-9.

79. Wyatt RG, Dolin R, Blacklow NR et al. Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers. *J.Infect.Dis.* 1974;**129**(6):709-14.

80. Atmar RL, Opekun AA, Gilger MA et al. Norwalk virus shedding after experimental human infection. *Emerg.Infect.Dis.* 2008;**14**(10):1553-7.

81. Dolin R, Blacklow NR, DuPont H et al. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. 1971. *J.Infect.Dis.* 2004;**189**(11):2142-7, discussion.

82. Lindesmith L, Moe C, LePendu J et al. Cellular and humoral immunity following Snow Mountain virus challenge. *J.Virol.* 2005;**79**(5):2900-9.

83. Food Standards Agency: A report of the study of infectious intestinal disease in England. London: HMSO; 2000.

84. Rockx B, de WM, Vennema H et al. Natural history of human calicivirus infection: a prospective cohort study. *Clin.Infect.Dis.* 2002;**35**(3):246-53.

85. Gotz H, Ekdahl K, Lindback J et al. Clinical spectrum and transmission characteristics of infection with Norwalk-like virus: findings from a large community outbreak in Sweden. *Clin.Infect.Dis.* 2001;**33**(5):622-8.

86. Nakagomi T, Correia JB, Nakagomi O et al. Norovirus infection among children with acute gastroenteritis in Recife, Brazil: disease severity is comparable to rotavirus gastroenteritis. *Arch.Virol.* 2008;**153**(5):957-60.

87. Narkeviciute I, Tamusauskaite I. Peculiarities of norovirus and rotavirus infections in hospitalised young children. *J.Pediatr.Gastroenterol.Nutr.* 2008;**46**(3):289-92.

88. Sakai Y, Nakata S, Honma S et al. Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan. *Pediatr.Infect.Dis.J.* 2001;**20**(9):849-53.

89. Chen SY, Tsai CN, Lai MW et al. Norovirus Infection as a Cause of Diarrhea-Associated Benign Infantile Seizures. *Clin.Infect.Dis.* 2009.

90. Ito S, Takeshita S, Nezu A et al. Norovirus-associated encephalopathy. *Pediatric Infectious Disease Journal.* 2006;. **25**(7).

91. Takanashi S, Hashira S, Matsunaga T et al. Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis. *J.Clin.Virol.* 2009.

92. Lopman BA, Reacher MH, Vipond IB et al. Clinical manifestation of norovirus gastroenteritis in health care settings. *Clin.Infect.Dis.* 2004;**39**(3):318-24.

93. Tsang OT, Wong AT, Chow CB et al. Clinical characteristics of nosocomial norovirus outbreaks in Hong Kong. *J.Hosp.Infect.* 2008.

94. Iversen AM, Gill M, Bartlett CL et al. Two outbreaks of foodborne gastroenteritis caused by a small round structured virus: evidence of prolonged infectivity in a food handler. *Lancet.* 1987;**2**(8558):556-8.

95. Truman BI, Madore HP, Menegus MA. Snow mountain agent gastroenteritis from clams. *American Journal of Epidemiology.* 1987;. **126**(3).

96. Kappus KD, Marks JS, Holman RC et al. An outbreak of Norwalk gastroenteritis associated with swimming in a pool and secondary person-to-person transmission. *Am.J.Epidemiol.* 1982;**116**(5):834-9.

97. Lopman B. Noroviruses: simple detection for complex epidemiology. *Clin.Infect.Dis.* 2006;**42**(7):970-1.

98. Teunis PF, Moe CL, Liu P et al. Norwalk virus: How infectious is it? *J.Med.Virol.* 2008;**80**(8):1468-76.

99. Thornhill TS, Kalica AR, Wyatt RG et al. Pattern of shedding of the Norwalk particle in stools during experimentally induced gastroenteritis in volunteers as determined by immune electron microscopy. *J.Infect.Dis.* 1975;**132**(1):28-34.

100. Chan MC, Sung JJ, Lam RK et al. Fecal viral load and norovirus-associated gastroenteritis. *Emerg.Infect.Dis.* 2006;**12**(8):1278-80.

101. Kageyama T, Shinohara M, Uchida K et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. *J.Clin.Microbiol.* 2004;**42**(7):2988-95.

102. Lee N, Chan MC, Wong B et al. Fecal viral concentration and diarrhea in norovirus gastroenteritis. *Emerg.Infect.Dis.* 2007;**13**(9):1399-401.

103. Haruki K, Seto Y, Murakami T et al. Pattern of shedding of small, round-structured virus particles in stools of patients of outbreaks of food-poisoning from raw oysters. *Microbiol.Immunol.* 1991;**35**(1):83-6.

104. Goller JL, Dimitriadis A, Tan A et al. Long-term features of norovirus gastroenteritis in the elderly. *J.Hosp.Infect.* 2004;**58**(4):286-91.

105. Kirkwood CD, Streitberg R. Calicivirus shedding in children after recovery from diarrhoeal disease. *J.Clin.Virol.* 2008;**43**(3):346-8.

106. Tu ET, Bull RA, Kim MJ et al. Norovirus excretion in an age-care setting. *J.Clin.Microbiol.* 2008.

107. Aoki Y, Suto A, Mizuta K et al. Duration of norovirus excretion and the longitudinal course of viral load in norovirus-infected elderly patients. *J.Hosp.Infect.* 2010;**75**(1):42-6.

108. Murata T, Katsushima N, Mizuta K et al. Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis. *Pediatr.Infect.Dis.J.* 2007;**26**(1):46-9.

109. Harada S, Okada M, Yahiro S et al. Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto Prefecture, Japan. *J.Med.Virol.* 2009;**81**(6):1117-27.

110. Kageyama T, Kojima S, Shinohara M et al. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J.Clin.Microbiol.* 2003;**41**(4):1548-57.

111. Duizer E, van Duynhoven Y, Vennema H et al. Failure to detect norovirus in a large group of asymptomatic individuals by Marshall et al. (Public Health Vol 118 (3) 230-233). *Public Health.* 2004;**118**(6):455-6.

112. Marshall JA, Hellard ME, Sinclair MI et al. Failure to detect norovirus in a large group of asymptomatic individuals. *Public Health.* 2004;**118**(3):230-3.

113. Marshall JA, Kelly H, Wright PJ. Asymptomatic carriage of norovirus. A reply to the comments by Duizer et al. *Public Health.* 2004;**118**(6):456-7.

114. Ozawa K, Oka T, Takeda N et al. Norovirus infections in symptomatic and asymptomatic food handlers in Japan. *J.Clin.Microbiol.* 2007;**45**(12):3996-4005.

115. Baron RC, Greenberg HB, Cukor G et al. Serological responses among teenagers after natural exposure to Norwalk virus. *J.Infect.Dis.* 1984;**150**(4):531-4.

116. Ettayebi K, Hardy ME. Recombinant norovirus-specific scFv inhibit virus-like particle binding to cellular ligands. *Virol.J.* 2008;**521**.

117. Harrington PR, Lindesmith L, Yount B et al. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. *J.Virol.* 2002;**76**(23):12335-43.

118. Madore HP, Treanor JJ, Buja R et al. Antigenic relatedness among the Norwalk-like agents by serum antibody rises. *J.Med.Virol.* 1990;**32**(2):96-101.

119. Jiang X, Zhong WM, Farkas T et al. Baculovirus expression and antigenic characterization of the capsid proteins of three Norwalk-like viruses. *Arch.Virol.* 2002;**147**(1):119-30.

120. Kitamoto N, Tanaka T, Natori K et al. Cross-reactivity among several recombinant calicivirus virus-like particles (VLPs) with monoclonal antibodies obtained from mice immunized orally with one type of VLP. *J.Clin.Microbiol.* 2002;**40**(7):2459-65.

121. Parker TD, Kitamoto N, Tanaka T et al. Identification of Genogroup I and Genogroup II broadly reactive epitopes on the norovirus capsid. *J.Virol.* 2005;**79**(12):7402-9.

122. Hansman GS, Natori K, Shirato-Horikoshi H et al. Genetic and antigenic diversity among noroviruses. *J.Gen.Virol.* 2006;**87**(Pt 4):909-19.

123. Hale AD, Lewis DC, Jiang X et al. Homotypic and heterotypic IgG and IgM antibody responses in adults infected with small round structured viruses. *J.Med.Virol.* 1998;**54**(4):305-12.

124. Iritani N, Seto T, Hattori H et al. Humoral immune responses against norovirus infections of children. *J.Med.Virol.* 2007;**79**(8):1187-93.

125. Hale AD, Tanaka TN, Kitamoto N et al. Identification of an epitope common to genogroup 1 "norwalk-like viruses". *J.Clin.Microbiol.* 2000;**38**(4):1656-60.

126. LoBue AD, Lindesmith L, Yount B et al. Multivalent norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains. *Vaccine.* 2006;**24**(24):5220-34.

127. Lindesmith LC, Donaldson E, Leon J et al. Heterotypic Humoral and Cellular Immune Responses Following Norwalk Virus Infection. *J.Virol.* 2009.

128. Velazquez FR. Protective effects of natural rotavirus infection. *Pediatr.Infect.Dis.J.* 2009;**28**(3 Suppl):S54-S56.

129. Franco MA, Angel J, Greenberg HB. Immunity and correlates of protection for rotavirus vaccines. *Vaccine.* 2006;**24**(15):2718-31.

130. Karst SM, Wobus CE, Lay M et al. STAT1-dependent innate immunity to a Norwalk-like virus. *Science.* 2003;**299**(5612):1575-8.

131. Hutson AM, Atmar RL, Marcus DM et al. Norwalk virus-like particle hemagglutination by binding to h histo-blood group antigens. *J.Virol.* 2003;**77**(1):405-15.

132. Tamura M, Natori K, Kobayashi M et al. Interaction of recombinant norwalk virus particles with the 105-kilodalton cellular binding protein, a candidate receptor molecule for virus attachment. *J.Virol.* 2000;**74**(24):11589-97.

133. Cao S, Lou Z, Tan M et al. Structural basis for the recognition of blood group trisaccharides by norovirus. *J.Virol.* 2007;**81**(11):5949-57.

134. Marionneau S, Ruvoen N, Moullac-Vaidye B et al. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology.* 2002;**122**(7):1967-77.

135. Hutson AM, Atmar RL, Estes MK. Norovirus disease: changing epidemiology and host susceptibility factors. *Trends Microbiol.* 2004;**12**(6):279-87.

136. Hutson AM, Atmar RL, Graham DY et al. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J.Infect.Dis.* 2002;**185**(9):1335-7.

137. Hutson AM, Airaud F, LePendu J et al. Norwalk virus infection associates with secretor status genotyped from sera. *J.Med.Virol.* 2005;**77**(1):116-20.

138. Lindesmith L, Moe C, Marionneau S et al. Human susceptibility and resistance to Norwalk virus infection. *Nat.Med.* 2003;**9**(5):548-53.

139. Ewald D, Franks C, Thompson S et al. Possible community immunity to small round structured virus gastroenteritis in a rural aboriginal community. *Commun.Dis.Intell.* 2000;**24**(3):48-50.

140. Fretz R, Svoboda P, Schorr D et al. Risk factors for infections with Norovirus gastrointestinal illness in Switzerland. *Eur.J.Clin.Microbiol.Infect.Dis.* 2005;**24**(4):256-61.

141. Halperin T, Vennema H, Koopmans M et al. No Association between Histo-Blood Group Antigens and Susceptibility to Clinical Infections with Genogroup II Norovirus. *J.Infect.Dis.* 2008;**197**(1):63-5.

142. Hennessy EP, Green AD, Connor MP et al. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J.Infect.Dis.* 2003;**188**(1):176-7.

143. Kindberg E, Akerlind B, Johnsen C et al. Host genetic resistance to symptomatic norovirus (GGII.4) infections in Denmark. *J.Clin.Microbiol.* 2007;**45**(8):2720-2.

144. Meyer E, Ebner W, Scholz R et al. Nosocomial outbreak of norovirus gastroenteritis and investigation of ABO histo-blood group type in infected staff and patients. *J.Hosp.Infect.* 2004;**56**(1):64-6.

145. Miyoshi M, Yoshizumi S, Sato C et al. [Relationship between ABO histo-blood group type and an outbreak of norovirus gastroenteritis among primary and junior high school students: results of questionnaire-based study]. *Kansenshogaku Zasshi.* 2005;**79**(9):664-71.

146. Thorven M, Grahn A, Hedlund KO et al. A homozygous nonsense mutation (428G-->A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GGII) infections. *J.Virol.* 2005;**79**(24):15351-5.

147. Nordgren J, Kindberg E, Lindgren PE et al. Norovirus Gastroenteritis Outbreak with a Secretor-independent Susceptibility Pattern, Sweden. *Emerg.Infect.Dis.* 2010;**16**(1):81-7.

148. Larsson MM, Rydell GE, Grahn A et al. Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype. *J.Infect.Dis.* 2006;**194**(10):1422-7.

149. Harrington PR, Vinje J, Moe CL et al. Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. *J.Virol.* 2004;**78**(6):3035-45.

150. Huang P, Farkas T, Marionneau S et al. Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *J.Infect.Dis.* 2003;**188**(1):19-31.

151. Marionneau S, Airaud F, Bovin NV et al. Influence of the combined ABO, FUT2, and FUT3 polymorphism on susceptibility to Norwalk virus attachment. *J.Infect.Dis.* 2005;**192**(6):1071-7.

152. Rydell GE, Nilsson J, Rodriguez-Diaz J et al. Human noroviruses recognize sialyl Lewis x neoglycoprotein. *Glycobiology.* 2009;**19**(3):309-20.

153. Shirato H, Ogawa S, Ito H et al. Noroviruses distinguish between type 1 and type 2 histo-blood group antigens for binding. *J.Virol.* 2008.

154. Cheetham S, Souza M, McGregor R et al. Binding patterns of human norovirus-like particles to buccal and intestinal tissues of gnotobiotic pigs in relation to A/H histo-blood group antigen expression. *J.Virol.* 2007;**81**(7):3535-44.

155. Huang P, Farkas T, Zhong W et al. Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. *J.Virol.* 2005;**79**(11):6714-22.

156. Ball JM, Estes MK, Hardy ME et al. Recombinant Norwalk virus-like particles as an oral vaccine. *Arch.Virol.Suppl.* 1996;**12**243-9.

157. Ball JM, Hardy ME, Atmar RL et al. Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. *J.Virol.* 1998;**72**(2):1345-53.

158. Guerrero RA, Ball JM, Krater SS et al. Recombinant Norwalk virus-like particles administered intranasally to mice induce systemic and mucosal (fecal and vaginal) immune responses. *J.Virol.* 2001;**75**(20):9713-22.

159. Guo L, Wang J, Zhou H et al. Intranasal administration of a recombinant adenovirus expressing the norovirus capsid protein stimulates specific humoral, mucosal, and cellular immune responses in mice. *Vaccine.* 2008;**26**(4):460-8.

160. Harrington PR, Yount B, Johnston RE et al. Systemic, mucosal, and heterotypic immune induction in mice inoculated with Venezuelan equine encephalitis replicons expressing Norwalk virus-like particles. *J.Virol.* 2002;**76**(2):730-42.

161. Tacket CO, Mason HS, Losonsky G et al. Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. *J.Infect.Dis.* 2000;**182**(1):302-5.

162. Tacket CO, Sztein MB, Losonsky GA et al. Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. *Clin.Immunol.* 2003;**108**(3):241-7.

163. Scipioni A, Mauroy A, Vinje J et al. Animal noroviruses. *Vet.J.* 2008;**178**(1):32-45.

164. Wang QH, Han MG, Cheetham S et al. Porcine noroviruses related to human noroviruses. *Emerg.Infect.Dis.* 2005;**11**(12):1874-81.

165. Jiang X, Wang M, Wang K et al. Sequence and genomic organization of Norwalk virus. *Virology.* 1993;**195**(1):51-61.

166. Lambden PR, Caul EO, Ashley CR et al. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. *Science.* 1993;**259**(5094):516-9.

167. Pletneva MA, Sosnovtsev SV, Green KY. The genome of hawaii virus and its relationship with other members of the caliciviridae. *Virus Genes.* 2001;**23**(1):5-16.

168. Glass PJ, White LJ, Ball JM et al. Norwalk virus open reading frame 3 encodes a minor structural protein. *J.Virol.* 2000;**74**(14):6581-91.

169. Glass PJ, Zeng CQ, Estes MK. Two nonoverlapping domains on the Norwalk virus open reading frame 3 (ORF3) protein are involved in the formation of the phosphorylated 35K protein and in ORF3-capsid protein interactions. *J.Virol.* 2003;**77**(6):3569-77.

170. Kojima S, Kageyama T, Fukushi S et al. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J.Virol.Methods.* 2002;**100**(1-2):107-14.

171. Bhella D, Gatherer D, Chaudhry Y et al. Structural insights into calicivirus attachment and uncoating. *J.Virol.* 2008;**82**(16):8051-8.

172. Chen R, Neill JD, Noel JS et al. Inter- and intragenus structural variations in caliciviruses and their functional implications. *J.Virol.* 2004;**78**(12):6469-79.

173. Lochridge VP, Jutila KL, Graff JW et al. Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions. *J.Gen.Virol.* 2005;**86**(Pt 10):2799-806.

174. Prasad BV, Hardy ME, Jiang X et al. Structure of Norwalk virus. *Arch.Virol.Suppl.* 1996;**12**237-42.

175. Prasad BV, Hardy ME, Dokland T et al. X-ray crystallographic structure of the Norwalk virus capsid. *Science.* 1999;**286**(5438):287-90.

176. White LJ, Ball JM, Hardy ME et al. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. *J.Virol.* 1996;**70**(10):6589-97.

177. Allen DJ, Gray JJ, Gallimore CI et al. Analysis of Amino Acid Variation in the P2 Domain of the GII-4 Norovirus VP1 Protein Reveals Putative Variant-Specific Epitopes. *PLoS.ONE.* 2008;**3**(1):e1485.

178. Hardy ME, Tanaka TN, Kitamoto N et al. Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies. *Virology.* 1996;**217**(1):252-61.

179. Lindesmith LC, Donaldson EF, LoBue AD et al. Mechanisms of GII.4 Norovirus Persistence in Human Populations. *PLoS.Med.* 2008;**5**(2):e31.

180. Tan M, Huang P, Meller J et al. Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. *J.Virol.* 2003;**77**(23):12562-71.

181. Zheng DP, Ando T, Fankhauser RL et al. Norovirus classification and proposed strain nomenclature. *Virology.* 2006;**346**(2):312-23.

182. Green J, Vinje J, Gallimore CI et al. Capsid protein diversity among Norwalk-like viruses. *Virus Genes.* 2000;**20**(3):227-36.

183. Norcott JP, Green J, Lewis D et al. Genomic diversity of small round structured viruses in the United Kingdom. *J.Med.Virol.* 1994;**44**(3):280-6.

184. Ando T, Mulders MN, Lewis DC et al. Comparison of the polymerase region of small round structured virus strains previously classified in three antigenic types by solid-phase immune electron microscopy. *Arch.Virol.* 1994;**135**(1-2):217-26.

185. Noel JS, Ando T, Leite JP et al. Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995. *J.Med.Virol.* 1997;**53**(4):372-83.

186. Wang J, Jiang X, Madore HP et al. Sequence diversity of small, round-structured viruses in the Norwalk virus group. *J.Virol.* 1994;**68**(9):5982-90.

187. Ando T, Noel JS, Fankhauser RL. Genetic classification of "Norwalk-like viruses. *J.Infect.Dis.* 2000;**181 Suppl 2**S336-S348.

188. Katayama K, Shirato-Horikoshi H, Kojima S et al. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology.* 2002;**299**(2):225-39.

189. Seto Y, Iritani N, Kubo H et al. Genotyping of Norovirus strains detected in outbreaks between April 2002 and March 2003 in Osaka City, Japan. *Microbiol.Immunol.* 2005;**49**(3):275-83.

190. Vinje J, Green J, Lewis DC et al. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses". *Arch.Virol.* 2000;**145**(2):223-41.

191. Farkas T, Thornton SA, Wilton N et al. Homologous versus heterologous immune responses to Norwalk-like viruses among crew members after acute gastroenteritis outbreaks on 2 US Navy vessels. *J.Infect.Dis.* 2003;**187**(2):187-93.

192. Hale AD, Crawford SE, Ciarlet M et al. Expression and self-assembly of Grimsby virus: antigenic distinction from Norwalk and Mexico viruses. *Clin.Diagn.Lab Immunol.* 1999;**6**(1):142-5.

193. Kasuga K, Tokieda M, Ohtawara M et al. Small round structured virus associated with an outbreak of acute gastroenteritis in Chiba, Japan. *Jpn.J.Med.Sci.Biol.* 1990;**43**(4):111-21.

194. Okada S, Sekine S, Ando T et al. Antigenic characterization of small, round-structured viruses by immune electron microscopy. *J.Clin.Microbiol.* 1990;**28**(6):1244-8.

195. Treanor JJ, Jiang X, Madore HP et al. Subclass-specific serum antibody responses to recombinant Norwalk virus capsid antigen (rNV) in adults infected with Norwalk, Snow Mountain, or Hawaii virus. *J.Clin.Microbiol.* 1993;**31**(6):1630-4.

196. Vinje J, Hamidjaja RA, Sobsey MD. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. *J.Virol.Methods.* 2004;**116**(2):109-17.

197. Chhabra P, Dhongade RK, Kalrao VR et al. Epidemiological, clinical, and molecular features of norovirus infections in western India. *J.Med.Virol.* 2009;**81**(5):922-32.

198. Fankhauser RL, Monroe SS, Noel JS et al. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J.Infect.Dis.* 2002;**186**(1):1-7.

199. Gallimore CI, Green J, Lewis D et al. Diversity of noroviruses cocirculating in the north of England from 1998 to 2001. *J.Clin.Microbiol.* 2004;**42**(4):1396-401.

200. Johansen K, Mannerqvist K, Allard A et al. Norovirus strains belonging to the GII.4 genotype dominate as a cause of nosocomial outbreaks of viral gastroenteritis in Sweden 1997--2005. Arrival of new variants is associated with large nation-wide epidemics. *J.Clin.Virol.* 2008;**42**(2):129-34.

201. Noel JS, Fankhauser RL, Ando T et al. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. *J.Infect.Dis.* 1999;**179**(6):1334-44.

202. Waters A, Coughlan S, Dunford L et al. Molecular epidemiology of norovirus strains circulating in Ireland from 2003 to 2004. *Epidemiol.Infect.* 2006;**134**(5):917-25.

203. Waters A, Dunford L, Tuite G et al. Significant prevalence and genetic diversity of norovirus infection in Irish children. *Pediatr.Res.* 2008.

204. Widdowson MA, Cramer EH, Hadley L et al. Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus--United States, 2002. *J.Infect.Dis.* 2004;**190**(1):27-36.

205. Blanton LH, Adams SM, Beard RS et al. Molecular and epidemiologic trends of caliciviruses associated with outbreaks of acute gastroenteritis in the United States, 2000-2004. *J.Infect.Dis.* 2006;**193**(3):413-21.

206. Bon F, mbert-Balay K, Giraudon H et al. Molecular epidemiology of caliciviruses detected in sporadic and outbreak cases of gastroenteritis in France from December 1998 to February 2004. *J.Clin.Microbiol.* 2005;**43**(9):4659-64.

207. Verhoef LP, Kroneman A, van DY et al. Selection tool for foodborne norovirus outbreaks. *Emerg.Infect.Dis.* 2009;**15**(1):31-8.

208. Okada M, Ogawa T, Kaiho I et al. Genetic analysis of noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. *J.Clin.Microbiol.* 2005;**43**(9):4391-401.

209. Iturriza-Gomara M, Elliot AJ, Dockery C et al. Structured surveillance of infectious intestinal disease in pre-school children in the community: 'The Nappy Study'. *Epidemiol.Infect.* 2008;1-10.

210. Lee BE, Preiksaitis JK, Chui N et al. Genetic relatedness of noroviruses identified in sporadic gastroenteritis in children and gastroenteritis outbreaks in northern Alberta. *J.Med.Virol.* 2008;**80**(2):330-7.

211. Bull RA, Hansman GS, Clancy LE et al. Norovirus recombination in ORF1/ORF2 overlap. *Emerg.Infect.Dis.* 2005;**11**(7):1079-85.

212. Bull RA, Tanaka MM, White PA. Norovirus recombination. *J.Gen.Virol.* 2007;**88**(Pt 12):3347-59.

213. Etherington GJ, Dicks J, Roberts IN. High throughput sequence analysis reveals hitherto unreported recombination in the genus Norovirus. *Virology.* 2006;**345**(1):88-95.

214. Jiang X, Espul C, Zhong WM et al. Characterization of a novel human calicivirus that may be a naturally occurring recombinant. *Arch.Virol.* 1999;**144**(12):2377-87.

215. Rohayem J, Munch J, Rethwilm A. Evidence of recombination in the norovirus capsid gene. *J.Virol.* 2005;**79**(8):4977-90.

216. Siebenga JJ, Vennema H, Renckens B et al. Epochal evolution of GGII.4 norovirus capsid proteins from 1995 to 2006. *J.Virol.* 2007;**81**(18):9932-41.

217. Ambert-Balay K, Bon F, Le Guyader F et al. Characterization of new recombinant noroviruses. *J.Clin.Microbiol.* 2005;**43**(10):5179-86.

218. Chhabra P, Chitambar SD. Norovirus genotype IIb associated acute gastroenteritis in India. *J.Clin.Virol.* 2008;**42**(4):429-32.

219. Phan TG, Kuroiwa T, Kaneshi K et al. Changing distribution of norovirus genotypes and genetic analysis of recombinant GIIb among infants and children with diarrhea in Japan. *J.Med.Virol.* 2006;**78**(7):971-8.

220. Reuter G, Vennema H, Koopmans M et al. Epidemic spread of recombinant noroviruses with four capsid types in Hungary. *J.Clin.Virol.* 2006;**35**(1):84-8.

221. Bull RA, Tu ET, McIver CJ et al. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. *J.Clin.Microbiol.* 2006;**44**(2):327-33.

222. Lopman B, Vennema H, Kohli E et al. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet.* 2004;**363**(9410):682-8.

223. Cannon JL, Lindesmith LC, Donaldson EF et al. Herd immunity to GII.4 noroviruses is supported by outbreak patient sera. *J.Virol.* 2009;**83**(11):5363-74.

224. Adamson WE, Gunson RN, Maclean A et al. Emergence of a new norovirus variant in Scotland in 2006. *J.Clin.Microbiol.* 2007;**45**(12):4058-60.

225. Buesa J, Montava R, bu-Mallouh R et al. Sequential evolution of genotype GII.4 norovirus variants causing gastroenteritis outbreaks from 2001 to 2006 in Eastern Spain. *J.Med.Virol.* 2008;**80**(7):1288-95.

226. Ho EC, Cheng PK, Wong DA et al. Correlation of norovirus variants with epidemics of acute viral gastroenteritis in Hong Kong. *J.Med.Virol.* 2006;**78**(11):1473-9.

227. Ho EC, Cheng PK, Lau AW et al. Atypical norovirus epidemic in Hong Kong during summer of 2006 was caused by a new genogroup II/4 variant. *J.Clin.Microbiol.* 2007.

228. Jin M, Xie HP, Duan ZJ et al. Emergence of the GII4/2006b variant and recombinant noroviruses in China. *J.Med.Virol.* 2008;**80**(11):1997-2004.

229. Lyon MJ, Wei G, Smith GA. Epidemic viral gastroenteritis in Queensland coincides with the emergence of a new norovirus variant. *Commun.Dis.Intell.* 2005;**29**(4):370-3.

230. Okada M, Ogawa T, Yoshizumi H et al. Genetic variation of the norovirus GII-4 genotype associated with a large number of outbreaks in Chiba prefecture, Japan. *Arch.Virol.* 2007.

231. Ramirez S, Giammanco GM, De GS et al. Emerging GII.4 norovirus variants affect children with diarrhea in Palermo, Italy in 2006. *J.Med.Virol.* 2009;**81**(1):139-45.

232. Reuter G, Krisztalovics K, Vennema H et al. Evidence of the etiological predominance of norovirus in gastroenteritis outbreaks--emerging new-variant and recombinant noroviruses in Hungary. *J.Med.Virol.* 2005;**76**(4):598-607.

233. White PA, Hansman GS, Li A et al. Norwalk-like virus 95/96-US strain is a major cause of gastroenteritis outbreaks in Australia. *J.Med.Virol.* 2002;**68**(1):113-8.

234. Vipond IB, Caul EO, Hirst D et al. National epidemic of Lordsdale Norovirus in the UK. *J.Clin.Virol.* 2004;**30**(3):243-7.

235. Allen DJ, Noad R, Samuel D et al. Characterisation of a GII-4 norovirus variant-specific surface-exposed site involved in antibody binding. *Virol.J.* 2009;**6**150.

236. Bok K, Abente EJ, Realpe-Quintero M et al. Evolutionary dynamics of GII.4 noroviruses over a 34-year period. *J.Virol.* 2009;**83**(22):11890-901.

237. Carlsson B, Lindberg AM, Rodriguez-Diaz J et al. Quasispecies dynamics and molecular evolution of human norovirus capsid P region during chronic infection. *J.Gen.Virol.* 2009;**90**(Pt 2):432-41.

238. Tan M, Xia M, Chen Y et al. Conservation of carbohydrate binding interfaces: evidence of human HBGA selection in norovirus evolution. *PLoS.ONE.* 2009;**4**(4):e5058.

239. Curry A, Bryden A, Morgan-Capner P et al. A rationalised virological electron microscope specimen testing policy. PHLS North West Viral Gastroenteritis and Electron Microscopy Subcommittee. *J.Clin.Pathol.* 1999;**52**(6):471-4.

240. Almeida JD. Practical aspects of diagnostic electron microscopy. *Yale J.Biol.Med.* 1980;**53**(1):5-18.

241. Biel SS, Gelderblom HR. Diagnostic electron microscopy is still a timely and rewarding method. *J.Clin.Virol.* 1999;**13**(1-2):105-19.

242. CROMPTON K. The role of technician in electron microsopy. *Can.J.Med.Technol.* 1960;**22**68-70.

243. Curry A. Electron microscopy and the investigation of new infectious diseases. *Int.J.Infect.Dis.* 2003;**7**(4):251-7.

244. Saah AJ, Hoover DR. "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Ann.Intern.Med.* 1997;**126**(1):91-4.

245. Vipond IB, Pelosi E, Williams J et al. A diagnostic EIA for detection of the prevalent SRSV strain in United Kingdom outbreaks of gastroenteritis. *J.Med.Virol.* 2000;**61**(1):132-7.

246. Miller I, Gunson R, Carman WF. Norwalk like virus by light cycler PCR. *J.Clin.Virol.* 2002;**25**(2):231-2.

247. Dingle KE, Lambden PR, Caul EO et al. Human enteric Caliciviridae: the complete genome sequence and expression of virus-like particles from a

genetic group II small round structured virus. *J.Gen.Virol.* 1995;**76 (Pt 9)**2349-55.

248. Ando T, Monroe SS, Gentsch JR et al. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization. *J.Clin.Microbiol.* 1995;**33**(1):64-71.

249. Green J, Gallimore CI, Norcott JP et al. Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. *J.Med.Virol.* 1995;**47**(4):392-8.

250. Le GF, Estes MK, Hardy ME et al. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. *Arch.Virol.* 1996;**141**(11):2225-35.

251. Greene SR, Moe CL, Jaykus LA et al. Evaluation of the NucliSens Basic Kit assay for detection of Norwalk virus RNA in stool specimens. *J.Virol.Methods.* 2003;**108**(1):123-31.

252. Moore C, Clark EM, Gallimore CI et al. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. *J.Clin.Virol.* 2004;**29**(4):290-6.

253. Patterson SS, Smith MW, Casper ET et al. A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II. *J.Appl.Microbiol.* 2006;**101**(4):956-63.

254. Fukuda S, Takao S, Kuwayama M et al. Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay. *J.Clin.Microbiol.* 2006;**44**(4):1376-81.

255. Yoda T, Suzuki Y, Yamazaki K et al. Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. *J.Med.Virol.* 2007;**79**(3):326-34.

256. Hale AD, Green J, Brown DW. Comparison of four RNA extraction methods for the detection of small round structured viruses in faecal specimens. *J.Virol.Methods.* 1996;**57**(2):195-201.

257. Kok T, Wati S, Bayly B et al. Comparison of six nucleic acid extraction methods for detection of viral DNA or RNA sequences in four different non-serum specimen types. *J.Clin.Virol.* 2000;**16**(1):59-63.

258. Boom R, Sol CJ, Salimans MM et al. Rapid and simple method for purification of nucleic acids. *J.Clin.Microbiol.* 1990;**28**(3):495-503.

259. Witlox KJ, Nguyen TN, Bruggink LD et al. A comparative evaluation of the sensitivity of two automated and two manual nucleic acid extraction methods for the detection of norovirus by RT-PCR. *J.Virol.Methods.* 2008.

260. Kleiboeker SB. Applications of competitor RNA in diagnostic reverse transcription-PCR. *J.Clin.Microbiol.* 2003;**41**(5):2055-61.

261. Rolfe KJ, Parmar S, Mururi D et al. An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. *J.Clin.Virol.* 2007;**39**(4):318-21.

262. Mullis KB. The unusual origin of the polymerase chain reaction. *Sci.Am.* 1990;**262**(4):56-5.

263. Mullis KB. Target amplification for DNA analysis by the polymerase chain reaction. *Ann.Biol.Clin.(Paris).* 1990;**48**(8):579-82.

264. Moe CL, Gentsch J, Ando T et al. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. *J.Clin.Microbiol.* 1994;**32**(3):642-8.

265. Vinje J, Koopmans MP. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. *J.Infect.Dis.* 1996;**174**(3):610-5.

266. Willcocks MM, Silcock JG, Carter MJ. Detection of Norwalk virus in the UK by the polymerase chain reaction. *FEMS Microbiol.Lett.* 1993;**112**(1):7-12.

267. Yamazaki K, Oseto M, Seto Y et al. Reverse transcription-polymerase chain reaction detection and sequence analysis of small round-structured viruses in Japan. *Arch.Virol.Suppl.* 1996;**12**271-6.

268. Iturriza-Gomara M, Green J, Brown DW et al. Comparison of specific and random priming in the reverse transcriptase polymerase chain reaction for genotyping group A rotaviruses. *J.Virol.Methods.* 1999;**78**(1-2):93-103.

269. Richards GP, Watson MA, Kingsley DH. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. *J.Virol.Methods.* 2004;**116**(1):63-70.

270. Pang X, Lee B, Chui L et al. Evaluation and validation of real-time reverse transcription-pcr assay using the LightCycler system for detection and quantitation of norovirus. *J.Clin.Microbiol.* 2004;**42**(10):4679-85.

271. Nordgren J, Bucardo F, Dienus O et al. Novel light-upon-extension real-time PCR assays for detection and quantification of genogroup I and II noroviruses in clinical specimens. *J.Clin.Microbiol.* 2008;**46**(1):164-70.

272. Ishida S, Yoshizumi S, Ikeda T et al. Sensitive and rapid detection of norovirus using duplex TaqMan reverse transcription-polymerase chain reaction. *J.Med.Virol.* 2008;**80**(5):913-20.

273. Trujillo AA, McCaustland KA, Zheng DP et al. Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. *J.Clin.Microbiol.* 2006;**44**(4):1405-12.

274. Menton JF, Kearney K, Morgan JG. Development of a real-time RT-PCR and Reverse Line probe Hybridisation assay for the routine detection and genotyping of Noroviruses in Ireland. *Virol.J.* 2007;486.

275. Dreier J, Stormer M, Made D et al. Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. *J.Clin.Microbiol.* 2006;**44**(8):2714-20.

276. Hohne M, Schreier E. Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. *J.Med.Virol.* 2004;**72**(2):312-9.

277. Gunson RN, Miller J, Carman WF. Comparison of real-time PCR and EIA for the detection of outbreaks of acute gastroenteritis caused by norovirus. *Commun.Dis.Public Health.* 2003;**6**(4):297-9.

278. Orlando C, Pinzani P, Pazzagli M. Developments in quantitative PCR. *Clin.Chem.Lab Med.* 1998;**36**(5):255-69.

279. Houde A, Leblanc D, Poitras E et al. Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. *J.Virol.Methods.* 2006;**135**(2):163-72.

280. Medici MC, Martinelli M, Ruggeri FM et al. Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples. *J.Clin.Microbiol.* 2005;**43**(8):3772-8.

281. Logan C, O'leary JJ, O'sullivan N. Real-time reverse transcription PCR detection of norovirus, sapovirus and astrovirus as causative agents of acute viral gastroenteritis. *J.Virol.Methods.* 2007.

282. Iturriza-Gomara M, Xerry J, Gallimore CI et al. Evaluation of the Loopamp((R)) (loop-mediated isothermal amplification) kit for detecting Norovirus RNA in faecal samples. *J.Clin.Virol.* 2008.

283. Lamhoujeb S, Charest H, Fliss I et al. Real-time molecular beacon NASBA for rapid and sensitive detection of norovirus GII in clinical samples. *Can.J.Microbiol.* 2009;**55**(12):1375-80.

284. Dolin R, Roessner KD, Treanor JJ et al. Radioimmunoassay for detection of the Snow Mountain Agent of viral gastroenteritis. *J.Med.Virol.* 1986;**19**(1):11-8.

285. Greenberg HB, Valdesuso J, Kapikian AZ et al. Prevalence of antibody to the Norwalk virus in various countries. *Infect.Immun.* 1979;**26**(1):270-3.

286. Hayashi Y, Ando T, Utagawa E et al. Western blot (immunoblot) assay of small, round-structured virus associated with an acute gastroenteritis outbreak in Tokyo. *J.Clin.Microbiol.* 1989;**27**(8):1728-33.

287. Herrmann JE, Nowak NA, Blacklow NR. Detection of Norwalk virus in stools by enzyme immunoassay. *J.Med.Virol.* 1985;**17**(2):127-33.

288. Madore HP, Treanor JJ, Pray KA et al. Enzyme-linked immunosorbent assays for Snow Mountain and Norwalk agents of viral gastroenteritis. *J.Clin.Microbiol.* 1986;**24**(3):456-9.

289. Treanor JJ, Madore HP, Dolin R. Development of an enzyme immunoassay for the Hawaii agent of viral gastroenteritis. *J.Virol.Methods.* 1988;**22**(2-3):207-14.

290. Lew JF, Kapikian AZ, Valdesuso J et al. Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. *J.Infect.Dis.* 1994;**170**(3):535-42.

291. Lew JF, Kapikian AZ, Jiang X et al. Molecular characterization and expression of the capsid protein of a Norwalk-like virus recovered from a Desert Shield troop with gastroenteritis. *Virology.* 1994;**200**(1):319-25.

292. Jiang X, Wang M, Graham DY et al. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J.Virol.* 1992;**66**(11):6527-32.

293. Jiang X, Matson DO, Ruiz-Palacios GM et al. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. *J.Clin.Microbiol.* 1995;**33**(6):1452-5.

294. Leite JP, Ando T, Noel JS et al. Characterization of Toronto virus capsid protein expressed in baculovirus. *Arch.Virol.* 1996;**141**(5):865-75.

295. Green KY, Kapikian AZ, Valdesuso J et al. Expression and self-assembly of recombinant capsid protein from the antigenically distinct Hawaii human calicivirus. *J.Clin.Microbiol.* 1997;**35**(7):1909-14.

296. Jiang X, Wilton N, Zhong WM et al. Diagnosis of human caliciviruses by use of enzyme immunoassays. *J.Infect.Dis.* 2000;**181 Suppl 2**S349-S359.

297. Brinker JP, Blacklow NR, Estes MK et al. Detection of Norwalk virus and other genogroup 1 human caliciviruses by a monoclonal antibody, recombinant-antigen-based immunoglobulin M capture enzyme immunoassay. *J.Clin.Microbiol.* 1998;**36**(4):1064-9.

298. Hale AD, Lewis D, Green J et al. Evaluation of an antigen capture ELISA based on recombinant mexico virus capsid protein. *Clin.Diagn.Virol.* 1996;**5**(1):27-35.

299. Jiang X, Cubitt D, Hu J et al. Development of an ELISA to detect MX virus, a human calicivirus in the snow Mountain agent genogroup. *J.Gen.Virol.* 1995;**76 (Pt 11)**2739-47.

300. Parker S, Cubitt D, Jiang JX et al. Efficacy of a recombinant Norwalk virus protein enzyme immunoassay for the diagnosis of infections with Norwalk virus and other human "candidate" caliciviruses. *J.Med.Virol.* 1993;**41**(3):179-84.

301. Okame M, Shiota T, Hansman G et al. Anti-norovirus polyclonal antibody and its potential for development of an antigen-ELISA. *J.Med.Virol.* 2007;**79**(8):1180-6.

302. Cubitt WD, Green KY, Payment P. Prevalence of antibodies to the Hawaii strain of human calicivirus as measured by a recombinant protein based immunoassay. *J.Med.Virol.* 1998;**54**(2):135-9.

303. Dimitrov DH, Dashti SA, Ball JM et al. Prevalence of antibodies to human caliciviruses (HuCVs) in Kuwait established by ELISA using baculovirus-expressed capsid antigens representing two genogroups of HuCVs. *J.Med.Virol.* 1997;**51**(2):115-8.

304. Gray JJ, Jiang X, Morgan-Capner P et al. Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. *J.Clin.Microbiol.* 1993;**31**(4):1022-5.

305. Honma S, Nakata S, Numata K et al. Epidemiological study of prevalence of genogroup II human calicivirus (Mexico virus) infections in Japan and Southeast Asia as determined by enzyme-linked immunosorbent assays. *J.Clin.Microbiol.* 1998;**36**(9):2481-4.

306. Jing Y, Qian Y, Huo Y et al. Seroprevalence against Norwalk-like human caliciviruses in beijing, China. *J.Med.Virol.* 2000;**60**(1):97-101.

307. Lew JF, Valdesuso J, Vesikari T et al. Detection of Norwalk virus or Norwalk-like virus infections in Finnish infants and young children. *J.Infect.Dis.* 1994;**169**(6):1364-7.

308. Nakata S, Honma S, Numata K et al. Prevalence of human calicivirus infections in Kenya as determined by enzyme immunoassays for three genogroups of the virus. *J.Clin.Microbiol.* 1998;**36**(11):3160-3.

309. Numata K, Nakata S, Jiang X et al. Epidemiological study of Norwalk virus infections in Japan and Southeast Asia by enzyme-linked immunosorbent assays with Norwalk virus capsid protein produced by the baculovirus expression system. *J.Clin.Microbiol.* 1994;**32**(1):121-6.

310. O'Ryan ML, Vial PA, Mamani N et al. Seroprevalence of Norwalk virus and Mexico virus in Chilean individuals: assessment of independent risk factors for antibody acquisition. *Clin.Infect.Dis.* 1998;**27**(4):789-95.

311. Parker SP, Cubitt WD, Jiang XJ et al. Seroprevalence studies using a recombinant Norwalk virus protein enzyme immunoassay. *J.Med.Virol.* 1994;**42**(2):146-50.

312. Parker SP, Cubitt WD, Jiang X. Enzyme immunoassay using baculovirus-expressed human calicivirus (Mexico) for the measurement of IgG responses and determining its seroprevalence in London, UK. *J.Med.Virol.* 1995;**46**(3):194-200.

313. Pujol FH, Vasquez G, Rojas AM et al. Norwalk virus infection in Venezuela. *Ann.Trop.Med.Parasitol.* 1998;**92**(2):205-11.

314. Smit TK, Bos P, Peenze I et al. Seroepidemiological study of genogroup I and II calicivirus infections in South and southern Africa. *J.Med.Virol.* 1999;**59**(2):227-31.

315. Okitsu-Negishi S, Okame M, Shimizu Y et al. Detection of norovirus antigens from recombinant virus-like particles and stool samples by a commercial norovirus enzyme-linked immunosorbent assay kit. *J.Clin.Microbiol.* 2006;**44**(10):3784-6.

316. Dimitriadis A, Bruggink LD, Marshall JA. Evaluation of the Dako IDEIA norovirus EIA assay for detection of norovirus using faecal specimens from Australian gastroenteritis outbreaks. *Pathology.* 2006;**38**(2):157-65.

317. Dimitriadis A, Marshall JA. Evaluation of a commercial enzyme immunoassay for detection of norovirus in outbreak specimens. *Eur.J.Clin.Microbiol.Infect.Dis.* 2005;**24**(9):615-8.

318. de BE, Duizer E, Vennema H et al. Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR. *J.Virol.Methods.* 2006;**137**(2):259-64.

319. Khamrin P, Nguyen TA, Phan TG et al. Evaluation of immunochromatography and commercial enzyme-linked immunosorbent assay for rapid detection of norovirus antigen in stool samples. *J.Virol.Methods.* 2008;**147**(2):360-3.

320. Khamrin P, Takanashi S, Chan-It W et al. Immunochromatography test for rapid detection of norovirus in fecal specimens. *J.Virol.Methods.* 2009.

321. Mutoh K, Hakamata A, Yagi H et al. Evaluation of new commercial immunochromatography kit for norovirus in feces. *Pediatr.Int.* 2009;**51**(1):164-6.

322. Takanashi S, Okame M, Shiota T et al. Development of a rapid immunochromatographic test for noroviruses genogroups I and II. *J.Virol.Methods.* 2007.

323. Okame M, Yan H, Akihara S et al. Evaluation of a newly developed immunochromatographic method for detection of norovirus. *Kansenshogaku Zasshi.* 2003;**77**(8):637-9.

324. Albert MJ, Bishop RF. Cultivation of human rotaviruses in cell culture. *J.Med.Virol.* 1984;**13**(4):377-83.

325. Cromeans TL, Lu X, Erdman DD et al. Development of plaque assays for adenoviruses 40 and 41. *J.Virol.Methods.* 2008;**151**(1):140-5.

326. Duizer E, Schwab KJ, Neill FH et al. Laboratory efforts to cultivate noroviruses. *J.Gen.Virol.* 2004;**85**(Pt 1):79-87.

327. Straub TM, Zu Bentrup KH, Orosz-Coghlan P et al. In vitro cell culture infectivity assay for human noroviruses. *Emerging Infectious Diseases.* 2007;. **13**(3).

328. Clay S, Maherchandani S, Malik YS et al. Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses. *Am.J.Infect.Control.* 2006;**34**(1):41-3.

329. D'Souza DH, Sair A, Williams K et al. Persistence of caliciviruses on environmental surfaces and their transfer to food. *Int.J.Food Microbiol.* 2006;**108**(1):84-91.

330. Lages SL, Ramakrishnan MA, Goyal SM. In-vivo efficacy of hand sanitisers against feline calicivirus: a surrogate for norovirus. *J.Hosp.Infect.* 2008;**68**(2):159-63.

331. Lamhoujeb S, Fliss I, Ngazoa SE et al. Evaluation of the Persistence of Infectious Human Noroviruses on Food Surfaces using Molecular Beacon-Based Nucleic Acid Sequence-Based Amplification. *Appl.Environ.Microbiol.* 2008.

332. Mattison K, Karthikeyan K, Abebe M et al. Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus. *J.Food Prot.* 2007;**70**(2):500-3.

333. Patel MM, Hall AJ, Vinje J et al. Noroviruses: a comprehensive review. *J.Clin.Virol.* 2009;**44**(1):1-8.

334. Vernacchio L, Vezina RM, Mitchell AA et al. Diarrhea in American infants and young children in the community setting: incidence, clinical presentation and microbiology. *Pediatr.Infect.Dis.J.* 2006;**25**(1):2-7.

335. Kobayashi S, Fujiwara N, Takeda N et al. Seroepidemiological study of norovirus infection in Aichi Prefecture, Japan. *Microbiol.Immunol.* 2009;**53**(6):356-9.

336. Peasey AE, Ruiz-Palacios GM, Quigley M et al. Seroepidemiology and risk factors for sporadic norovirus/Mexico strain. *J.Infect.Dis.* 2004;**189**(11):2027-36.

337. Nicollier-Jamot B, Pico V, Pothier P et al. Molecular cloning, expression, self-assembly, antigenicity, and seroepidemiology of a genogroup II norovirus isolated in France. *J.Clin.Microbiol.* 2003;**41**(8):3901-4.

338. Harris JP, Edmunds WJ, Pebody R et al. Deaths from norovirus among the elderly, England and Wales. *Emerg.Infect.Dis.* 2008;**14**(10):1546-52.

339. Haustein T, Harris JP, Pebody R et al. Hospital Admissions Due to Norovirus in Adult and Elderly Patients in England. *Clin.Infect.Dis.* 2009.

340. Huhulescu S, Kiss R, Brettlecker M et al. Etiology of Acute Gastroenteritis in Three Sentinel General Practices, Austria 2007. *Infection.* 2008.

341. Levidiotou S, Gartzonika C, Papaventsis D et al. Viral agents of acute gastroenteritis in hospitalized children in Greece. *Clin.Microbiol.Infect.* 2009;**15**(6):596-8.

342. Nakanishi K, Tsugawa T, Honma S et al. Detection of enteric viruses in rectal swabs from children with acute gastroenteritis attending the pediatric outpatient clinics in Sapporo, Japan. *J.Clin.Virol.* 2009;**46**(1):94-7.

343. de Wit MA, Koopmans MP, Kortbeek LM et al. Gastroenteritis in sentinel general practices,The Netherlands. *Emerg.Infect.Dis.* 2001;**7**(1):82-91.

344. Payment P, Franco E, Fout GS. Incidence of Norwalk virus infections during a prospective epidemiological study of drinking water-related gastrointestinal illness. *Can.J.Microbiol.* 1994;**40**(10):805-9.

345. Gomara MI, Simpson R, Perault AM et al. Structured surveillance of infantile gastroenteritis in East Anglia, UK: incidence of infection with common viral gastroenteric pathogens. *Epidemiol.Infect.* 2008;**136**(1):23-33.

346. Adak GK, Caul EO, Cowden JM. Infection with small round structured viruses: England and Wales 1981-1990. *CDR (Lond Engl.Rev.).* 1991;**1**(13):R141-R144.

347. Djuretic T, Ryan MJ, Fleming DM et al. Infectious intestinal disease in elderly people. *Commun.Dis.Rep.CDR Rev.* 1996;**6**(8):R107-R112.

348. Health Protection Agency. Web-based real-time surveillance for norovirus hospital outbreaks. Health Protection Report 3[6]. 13-2-2009.
Ref Type: Magazine Article

349. de Wit MA, Widdowson MA, Vennema H et al. Large outbreak of norovirus: the baker who should have known better. *J.Infect.* 2007;**55**(2):188-93.

350. Evans MR, Meldrum R, Lane W et al. An outbreak of viral gastroenteritis following environmental contamination at a concert hall. *Epidemiol.Infect.* 2002;**129**(2):355-60.

351. Patterson W, Haswell P, Fryers PT et al. Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited. *Commun.Dis.Rep.CDR Rev.* 1997;**7**(7):R101-R103.

352. Becker KM, Moe CL, Southwick KL et al. Transmission of Norwalk virus during football game. *N.Engl.J.Med.* 2000;**343**(17):1223-7.

353. Cannon RO, Poliner JR, Hirschhorn RB et al. A multistate outbreak of Norwalk virus gastroenteritis associated with consumption of commercial ice. *J.Infect.Dis.* 1991;**164**(5):860-3.

354. Doyle A, Barataud D, Gallay A et al. Norovirus foodborne outbreaks associated with the consumption of oysters from the Etang de Thau, France, December 2002. *Euro.Surveill.* 2004;**9**(3):24-6.

355. Godoy P, Torres J, Guix S et al. [Norwalk virus-like food poisoning after eating oysters]. *Med.Clin.(Barc.).* 2000;**114**(20):765-8.

356. Godoy P, Prat A, Alseda M et al. [Outbreak of foodborne disease by Norwalk-like viruses genogroup II]. *Med.Clin.(Barc.).* 2002;**119**(1):13-5.

357. Godoy P, Izcara J, Bartolome R et al. [Outbreak of food-borne Norovirus associated with the consumption of sandwiches]. *Med.Clin.(Barc.).* 2005;**124**(5):161-4.

358. Godoy P, Nuin C, Alseda M et al. [Waterborne outbreak of gastroenteritis caused by Norovirus transmitted through drinking water]. *Rev.Clin.Esp.* 2006;**206**(9):435-7.

359. Gotz H, de JB, Lindback J et al. Epidemiological investigation of a food-borne gastroenteritis outbreak caused by Norwalk-like virus in 30 day-care centres. *Scand.J.Infect.Dis.* 2002;**34**(2):115-21.

360. Kuo HW, Schmid D, Jelovcan S et al. A foodborne outbreak due to norovirus in Austria, 2007. *J.Food Prot.* 2009;**72**(1):193-6.

361. Mertens PLJM, Thijs C, Vinje J et al. A gastroenteritis epidemic caused by a Norwalk-like virus after two weddings in a restaurant; a plea for integral microbiological investigation. *Nederlands Tijdschrift voor Geneeskunde.* 2002;. **146**(50):14.

362. Noda M, Fukuda S, Nishio O. Statistical analysis of attack rate in norovirus foodborne outbreaks. *Int.J.Food Microbiol.* 2008;**122**(1-2):216-20.

363. Nygard K, Vold L, Halvorsen E et al. Waterborne outbreak of gastroenteritis in a religious summer camp in Norway, 2002. *Epidemiology & Infection.* 2004;. **132**(2).

364. Simmons G, Garbutt C, Hewitt J et al. A New Zealand outbreak of norovirus gastroenteritis linked to the consumption of imported raw Korean oysters. *N.Z.Med.J.* 2007;**120**(1264):U2773.

365. Heun EM, Vogt RL, Hudson PJ et al. Risk factors for secondary transmission in households after a common-source outbreak of Norwalk gastroenteritis. *Am.J.Epidemiol.* 1987;**126**(6):1181-6.

366. Billgren M, Christenson B, Hedlund KO et al. Epidemiology of Norwalk-like human caliciviruses in hospital outbreaks of acute gastroenteritis in the Stockholm area in 1996. *J.Infect.* 2002;**44**(1):26-32.

367. Green J, Wright PA, Gallimore CI et al. The role of environmental contamination with small round structured viruses in a hospital outbreak investigated by reverse-transcriptase polymerase chain reaction assay. *J.Hosp.Infect.* 1998;**39**(1):39-45.

368. Itoda I, Hidai H, Kikuchi K et al. [Hospital outbreak of viral gastroenteritis attributed to Norwalk-like viruses in Japan]. *Kansenshogaku Zasshi.* 2002;**76**(1):32-40.

369. Lynn S, Toop J, Hanger C et al. Norovirus outbreaks in a hospital setting: the role of infection control. *N.Z.Med.J.* 2004;**117**(1189):U771.

370. Almagro ND, Conti CF, Espinola GE et al. [Outbreak of gastroenteritis caused by Norwalk virus at a senior citizens assisted living facility in Granada, Spain]. *Rev.Esp.Salud Publica.* 2003;**77**(2):287-95.

371. Augustin AK, Simor AE, Shorrock C et al. Outbreaks of gastroenteritis due to Norwalk-like virus in two long-term care facilities for the elderly. *Can.J.Infect.Control.* 1995;**10**(4):111-3.

372. Gellert GA, Waterman SH, Ewert D et al. An outbreak of acute gastroenteritis caused by a small round structured virus in a geriatric convalescent facility. *Infect.Control Hosp.Epidemiol.* 1990;**11**(9):459-64.

373. Godoy P, Artigues A, Bartolome R et al. [Norovirus gastroenteritis outbreak by person-to-person transmission in a nursing home]. *Med.Clin.(Barc.).* 2006;**127**(14):538-41.

374. Gonzalez MF, Moreno CA, de Mateo OS et al. [Nosocomial epidemic outbreak of acute gastroenteritis by Norwalk-like virus]. *Med.Clin.(Barc.).* 2002;**118**(16):611-5.

375. Odelin MF, Ruel N, Berthelot P et al. [Investigation of an outbreak of norovirus gastroenteritis in a geriatric hospital]. *Ann.Biol.Clin.(Paris).* 2006;**64**(2):141-7.

376. Schmid D, Lederer I, Pichler AM et al. An outbreak of Norovirus infection affecting an Austrian nursing home and a hospital. *Wien.Klin.Wochenschr.* 2005;**117**(23-24):802-8.

377. Kuo HW, Schmid D, Schwarz K et al. A non-foodborne norovirus outbreak among school children during a skiing holiday, Austria, 2007. *Wien.Klin.Wochenschr.* 2009;**121**(3-4):120-4.

378. Michel A, Fitzgerald R, Whyte D et al. Norovirus outbreak associated with a hotel in the west of Ireland, 2006. *Euro.Surveill.* 2007;**12**(7):E11-E12.

379. Oppermann H, Mueller B, Takkinen J et al. An outbreak of viral gastroenteritis in a mother-and-child health clinic. *Int.J.Hyg.Environ.Health.* 2001;**203**(4):369-73.

380. de Wit MA, Koopmans MP, Van Duynhoven YT. Risk factors for norovirus, Sapporo-like virus, and group A rotavirus gastroenteritis. *Emerg.Infect.Dis.* 2003;**9**(12):1563-70.

381. Tam CC, Rodrigues LC, O'Brien SJ. The study of infectious intestinal disease in England: what risk factors for presentation to general practice tell us about potential for selection bias in case-control studies of reported cases of diarrhoea. *Int.J.Epidemiol.* 2003;**32**(1):99-105.

382. Dastjerdi AM, Green J, Gallimore CI et al. The bovine Newbury agent-2 is genetically more closely related to human SRSVs than to animal caliciviruses. *Virology.* 1999;**254**(1):1-5.

383. Mattison K, Shukla A, Cook A et al. Human noroviruses in swine and cattle. *Emerg.Infect.Dis.* 2007;**13**(8):1184-8.

384. Sugieda M, Nakajima S. Viruses detected in the caecum contents of healthy pigs representing a new genetic cluster in genogroup II of the genus "Norwalk-like viruses". *Virus Res.* 2002;**87**(2):165-72.

385. Bartlett AV, III, Reves RR, Pickering LK. Rotavirus in infant-toddler day care centers: epidemiology relevant to disease control strategies. *J.Pediatr.* 1988;**113**(3):435-41.

386. Bartlett AV, Moore M, Gary GW et al. Diarrheal illness among infants and toddlers in day care centers. II. Comparison with day care homes and households. *J.Pediatr.* 1985;**107**(4):503-9.

387. Reves RR, Morrow AL, Bartlett AV, III et al. Child day care increases the risk of clinic visits for acute diarrhea and diarrhea due to rotavirus. *Am.J.Epidemiol.* 1993;**137**(1):97-107.

388. Butz AM, Fosarelli P, Dick J et al. Prevalence of rotavirus on high-risk fomites in day-care facilities. *Pediatrics.* 1993;**92**(2):202-5.

389. Barker J, Stevens D, Bloomfield SF. Spread and prevention of some common viral infections in community facilities and domestic homes. *Journal of Applied Microbiology.* 2001;**91**(1):7-21.

390. Fretz R, Svoboda P, Luthi TM et al. Outbreaks of gastroenteritis due to infections with Norovirus in Switzerland, 2001-2003. *Epidemiol.Infect.* 2005;**133**(3):429-37.

391. Kelly S, Foley B, Dunford L et al. Establishment of a national database to link epidemiological and molecular data from norovirus outbreaks in Ireland. *Epidemiol.Infect.* 2008;**136**(11):1472-9.

392. Lopman BA, Reacher MH, van DY et al. Viral gastroenteritis outbreaks in Europe, 1995-2000. *Emerg.Infect.Dis.* 2003;**9**(1):90-6.

393. Chadwick PR, McCann R. Transmission of a small round structured virus by vomiting during a hospital outbreak of gastroenteritis. *J.Hosp.Infect.* 1994;**26**(4):251-9.

394. Marks PJ, Vipond IB, Carlisle D et al. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. *Epidemiol.Infect.* 2000;**124**(3):481-7.

395. Marks PJ, Vipond IB, Regan FM et al. A school outbreak of Norwalk-like virus: evidence for airborne transmission. *Epidemiol.Infect.* 2003;**131**(1):727-36.

396. Brown CM, Cann JW, Simons G et al. Outbreak of Norwalk virus in a Caribbean island resort: application of molecular diagnostics to ascertain the vehicle of infection. *Epidemiol.Infect.* 2001;**126**(3):425-32.

397. Daniels NA, Bergmire-Sweat DA, Schwab KJ et al. A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: first molecular traceback to deli sandwiches contaminated during preparation. *J.Infect.Dis.* 2000;**181**(4):1467-70.

398. Boxman IL, Dijkman R, te Loeke NA et al. Environmental swabs as a tool in norovirus outbreak investigation, including outbreaks on cruise ships. *J.Food Prot.* 2009;**72**(1):111-9.

399. Gallimore CI, Taylor C, Gennery AR et al. Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit. *J.Clin.Microbiol.* 2006;**44**(2):395-9.

400. Boxman I, Dijkman R, Verhoef L et al. Norovirus on swabs taken from hands illustrate route of transmission: a case study. *J.Food Prot.* 2009;**72**(8):1753-5.

401. Carling PC, Bruno-Murtha LA, Griffiths JK. Cruise ship environmental hygiene and the risk of norovirus infection outbreaks: an objective assessment of 56 vessels over 3 years. *Clin.Infect.Dis.* 2009;**49**(9):1312-7.

402. Norwalk-like virus outbreaks at two summer camps--Wisconsin, June 2001. *MMWR Morb.Mortal.Wkly.Rep.* 2001;**50**(30):642-3.

403. Norovirus outbreak in an elementary school--District of Columbia, February 2007. *MMWR Morb.Mortal.Wkly.Rep.* 2008;**56**(51-52):1340-3.

404. Cheesbrough JS, Barkess-Jones L, Brown DW. Possible prolonged environmental survival of small round structured viruses. *J.Hosp.Infect.* 1997;**35**(4):325-6.

405. Cheesbrough JS, Green J, Gallimore CI et al. Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiol.Infect.* 2000;**125**(1):93-8.

406. Chimonas MA, Vaughan GH, Andre Z et al. Passenger behaviors associated with norovirus infection on board a cruise ship--Alaska, May to June 2004. *J.Travel.Med.* 2008;**15**(3):177-83.

407. Kuusi M, Nuorti JP, Maunula L et al. A prolonged outbreak of Norwalk-like calicivirus (NLV) gastroenteritis in a rehabilitation centre due to environmental contamination. *Epidemiol.Infect.* 2002;**129**(1):133-8.

408. Bright KR, Boone SA, Gerba CP. Occurrence of Bacteria and Viruses on Elementary Classroom Surfaces and the Potential Role of Classroom Hygiene in the Spread of Infectious Diseases. *J.Sch Nurs.* 2009.

409. Heijne JC, Teunis P, Morroy G et al. Enhanced hygiene measures and norovirus transmission during an outbreak. *Emerg.Infect.Dis.* 2009;**15**(1):24-30.

410. Baert L, Uyttendaele M, Stals A et al. Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context. *Epidemiol.Infect.* 2009;**137**(3):316-25.

411. Kassa H. An outbreak of Norwalk-like viral gastroenteritis in a frequently penalized food service operation: a case for mandatory training of food handlers in safety and hygiene. *J.Environ.Health.* 2001;**64**(5):9-12, 33.

412. Lo SV, Connolly AM, Palmer SR et al. The role of the pre-symptomatic food handler in a common source outbreak of food-borne SRSV gastroenteritis in a group of hospitals. *Epidemiol.Infect.* 1994;**113**(3):513-21.

413. Parashar UD, Dow L, Fankhauser RL et al. An outbreak of viral gastroenteritis associated with consumption of sandwiches: implications for the control of transmission by food handlers. *Epidemiol.Infect.* 1998;**121**(3):615-21.

414. Patterson T, Hutchings P, Palmer S. Outbreak of SRSV gastroenteritis at an international conference traced to food handled by a post-symptomatic caterer. *Epidemiol.Infect.* 1993;**111**(1):157-62.

415. Reid JA, Caul EO, White DG et al. Role of infected food handler in hotel outbreak of Norwalk-like viral gastroenteritis: implications for control. *Lancet.* 1988;**2**(8606):321-3.

416. Vivancos R, Shroufi A, Sillis M et al. Food-related norovirus outbreak among people attending two barbeques: epidemiological, virological, and environmental investigation. *Int.J.Infect.Dis.* 2009;**13**(5):629-35.

417. Zomer TP, de JB, Kuhlmann-Berenzon S et al. A foodborne norovirus outbreak at a manufacturing company. *Epidemiol.Infect.* 2009;1-6.

418. McLean M, Giles S, Roseveare C et al. Norwalk-like virus gastroenteritis linked to a food handler. *New Zealand Public Health Report.* 2001;. 8(9).

419. Greig JD, Todd EC, Bartleson CA et al. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 1. Description of the problem, methods, and agents involved. *J.Food Prot.* 2007;**70**(7):1752-61.

420. Moe CL. Preventing Norovirus Transmission: How Should We Handle Food Handlers? *Clin.Infect.Dis.* 2008.

421. Ang LH. An outbreak of viral gastroenteritis associated with eating raw oysters. *Commun.Dis.Public Health.* 1998;**1**(1):38-40.

422. Berg DE, Kohn MA, Farley TA et al. Multi-state outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. *J.Infect.Dis.* 2000;**181 Suppl 2**S381-S386.

423. David ST, McIntyre L, Macdougall L et al. An outbreak of norovirus caused by consumption of oysters from geographically dispersed harvest sites, british columbia, Canada, 2004. *Foodborne.Pathog.Dis.* 2007;**4**(3):349-58.

424. Le Guyader F, Neill FH, Estes MK et al. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl.Environ.Microbiol.* 1996;**62**(11):4268-72.

425. Le Guyader FS, Bon F, DeMedici D et al. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. *J.Clin.Microbiol.* 2006;**44**(11):3878-82.

426. Rizzo C, Di B, I, Santantonio M et al. Epidemiological and virological investigation of a Norovirus outbreak in a resort in Puglia, Italy. *BMC.Infect.Dis.* 2007;**7**135.

427. Webby RJ, Carville KS, Kirk MD et al. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. *Clin.Infect.Dis.* 2007;**44**(8):1026-31.

428. Prato R, Lopalco PL, Chironna M et al. Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy. *BMC.Infect.Dis.* 2004;**437**.

429. Shieh Y, Monroe SS, Fankhauser RL et al. Detection of norwalk-like virus in shellfish implicated in illness. *J.Infect.Dis.* 2000;**181 Suppl 2**S360-S366.

430. Stafford R, Strain D, Heymer M et al. An outbreak of Norwalk virus gastroenteritis following consumption of oysters. *Commun.Dis.Intell.* 1997;**21**(21):317-20.

431. Beuret C, Baumgartner A, Schluep J. Virus-contaminated oysters: a three-month monitoring of oysters imported to Switzerland. *Appl.Environ.Microbiol.* 2003;**69**(4):2292-7.

432. Boxman IL, Tilburg JJ, te Loeke NA et al. Detection of noroviruses in shellfish in the Netherlands. *Int.J.Food Microbiol.* 2006;**108**(3):391-6.

433. Cheng PK, Wong DK, Chung TW et al. Norovirus contamination found in oysters worldwide. *J.Med.Virol.* 2005;**76**(4):593-7.

434. Costantini V, Loisy F, Joens L et al. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Appl.Environ.Microbiol.* 2006;**72**(3):1800-9.

435. Croci L, Losio MN, Suffredini E et al. Assessment of human enteric viruses in shellfish from the northern Adriatic sea. *Int.J.Food Microbiol.* 2007;**114**(2):252-7.

436. Hansman GS, Oka T, Li TC et al. Detection of human enteric viruses in Japanese clams. *J.Food Prot.* 2008;**71**(8):1689-95.

437. Terio V, Martella V, Moschidou P et al. Norovirus in retail shellfish. *Food Microbiol.* 2010;**27(1)**:29-32.

438. Ueki Y, Akiyama K, Watanabe T et al. Genetic analysis of noroviruses taken from gastroenteritis patients, river water and oysters. *Water Sci.Technol.* 2004;**50(1)**:51-6.

439. Ueki Y, Sano D, Watanabe T et al. Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. *Water Res.* 2005;**39**(18):4271-80.

440. Myrmel M, Berg EM, Rimstad E et al. Detection of enteric viruses in shellfish from the Norwegian coast. *Appl.Environ.Microbiol.* 2004;**70**(5):2678-84.

441. McDonnell S, Kirkland KB, Hlady WG et al. Failure of cooking to prevent shellfish-associated viral gastroenteritis. *Arch.Intern.Med.* 1997;**157**(1):111-6.

442. Chalmers JW, McMillan JH. An outbreak of viral gastroenteritis associated with adequately prepared oysters. *Epidemiol.Infect.* 1995;**115**(1):163-7.

443. Kingsley DH, Meade GK, Richards GP. Detection of both hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. *Appl.Environ.Microbiol.* 2002;**68**(8):3914-8.

444. Hjertqvist M, Johansson A, Svensson N et al. Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June-August 2006. *Euro.Surveill.* 2006;**11**(9):E060907.

445. Korsager B, Hede S, Boggild H et al. Two outbreaks of norovirus infections associated with the consumption of imported frozen raspberries, Denmark, May-June 2005. *Euro.Surveill.* 2005;**10**(6):E050623.

446. Anderson AD, Heryford AG, Sarisky JP et al. A waterborne outbreak of Norwalk-like virus among snowmobilers-Wyoming, 2001. *J.Infect.Dis.* 2003;**187**(2):303-6.

447. Beller M, Ellis A, Lee SH et al. Outbreak of viral gastroenteritis due to a contaminated well. International consequences. *JAMA.* 1997;**278**(7):563-8.

448. Carrique-Mas J, Andersson Y, Petersen B et al. A norwalk-like virus waterborne community outbreak in a Swedish village during peak holiday season. *Epidemiol.Infect.* 2003;**131**(1):737-44.

449. Khan AS, Moe CL, Glass RI et al. Norwalk virus-associated gastroenteritis traced to ice consumption aboard a cruise ship in Hawaii: comparison and application of molecular method-based assays. *J.Clin.Microbiol.* 1994;**32**(2):318-22.

450. Maunula L, Miettinen IT, von Bonsdorff CH. Norovirus outbreaks from drinking water. *Emerg.Infect.Dis.* 2005;**11**(11):1716-21.

451. Boccia D, Tozzi AE, Cotter B et al. Waterborne outbreak of Norwalk-like virus gastroenteritis at a tourist resort, Italy. *Emerg.Infect.Dis.* 2002;**8**(6):563-8.

452. Gallay A, de VH, Cournot M et al. A large multi-pathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. *Clin.Microbiol.Infect.* 2006;**12**(6):561-70.

453. Goodman RA, Buehler JW, Greenberg HB et al. Norwalk gastroenteritis associated with a water system in a rural Georgia community. *Arch.Environ.Health.* 1982;**37**(6):358-60.

454. Kaplan JE, Goodman RA, Schonberger LB et al. Gastroenteritis due to Norwalk virus: an outbreak associated with a municipal water system. *J.Infect.Dis.* 1982;**146**(2):190-7.

455. Kukkula M, Maunula L, Silvennoinen E et al. Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses. *J.Infect.Dis.* 1999;**180**(6):1771-6.

456. Scarcella C, Carasi S, Cadoria F et al. An outbreak of viral gastroenteritis linked to municipal water supply, Lombardy, Italy, June 2009. *Euro.Surveill.* 2009;**14**(29).

457. Taylor J, Foster K, Berrington A. Clostridium difficile: a questionnaire survey of laboratory practice in England, Wales, and Northern Ireland. *Commun.Dis.Public Health.* 2004;**7**(4):322-7.

458. Gray JJ, Green J, Cunliffe C et al. Mixed genogroup SRSV infections among a party of canoeists exposed to contaminated recreational water. *J.Med.Virol.* 1997;**52**(4):425-9.

459. Maunula L, Kalso S, von Bonsdorff CH et al. Wading pool water contaminated with both noroviruses and astroviruses as the source of a gastroenteritis outbreak. *Epidemiol.Infect.* 2004;**132**(4):737-43.

460. Podewils LJ, Zanardi BL, Hagenbuch M et al. Outbreak of norovirus illness associated with a swimming pool. *Epidemiol.Infect.* 2006;1-7.

461. Sartorius B, Andersson Y, Velicko I et al. Outbreak of norovirus in Vastra Gotaland associated with recreational activities at two lakes during August 2004. *Scand.J.Infect.Dis.* 2007;**39**(4):323-31.

462. Hoebe CJ, Vennema H, de Roda Husman AM et al. Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. *J.Infect.Dis.* 2004;**189**(4):699-705.

463. Cannon JL, Papafragkou E, Park GW et al. Surrogates for the study of norovirus stability and inactivation in the environment: aA comparison of murine norovirus and feline calicivirus. *J.Food Prot.* 2006;**69**(11):2761-5.

464. Hewitt J, Rivera-Aban M, Greening GE. Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *J.Appl.Microbiol.* 2009.

465. Topping JR, Schnerr H, Haines J et al. Temperature inactivation of Feline calicivirus vaccine strain FCV F-9 in comparison with human noroviruses using an RNA exposure assay and reverse transcribed quantitative real-time polymerase chain reaction-A novel method for predicting virus infectivity. *J.Virol.Methods.* 2009;**156**(1-2):89-95.

466. Barker J, Vipond IB, Bloomfield SF. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. *J.Hosp.Infect.* 2004;**58**(1):42-9.

467. Baert L, Uyttendaele M, Vermeersch M et al. Survival and transfer of murine norovirus 1, a surrogate for human noroviruses, during the production process of deep-frozen onions and spinach. *J.Food Prot.* 2008;**71**(8):1590-7.

468. Mormann S, Dabisch M, Becker B. Effects of technological processes on the tenacity and inactivation of norovirus GGII in experimentally contaminated foods. *Appl.Environ.Microbiol.* 2009.

469. Buckow R, Isbarn S, Knorr D et al. Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. *Appl.Environ.Microbiol.* 2007.

470. Zoni R, Zanelli R, Riboldi E et al. Investigation on virucidal activity of chlorine dioxide. experimental data on feline calicivirus, HAV and Coxsackie B5. *J.Prev.Med.Hyg.* 2007;**48**(3):91-5.

471. Belliot G, Lavaux A, Souihel D et al. Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants. *Appl.Environ.Microbiol.* 2008;**74**(10):3315-8.

472. Gehrke C, Steinmann J, Goroncy-Bermes P. Inactivation of feline calicivirus, a surrogate of norovirus (formerly Norwalk-like viruses), by different types of alcohol in vitro and in vivo. *J.Hosp.Infect.* 2004;**56**(1):49-55.

473. Kampf G. Efficacy of ethanol-based hand disinfectants against the feline calicivirus (FCV) under practical conditions. *Krankenhaus-Hygiene + Infektionsverhutung.* 2004;. **26**(1).

474. Kampf G, Grotheer D, Steinmann J. Efficacy of three ethanol-based hand rubs against feline calicivirus, a surrogate virus for norovirus. *J.Hosp.Infect.* 2005;**60**(2):144-9.

475. Macinga DR, Sattar SA, Jaykus LA et al. Improved inactivation of nonenveloped enteric viruses and their surrogates by a novel alcohol-based hand sanitizer. *Appl.Environ.Microbiol.* 2008;**74**(16):5047-52.

476. Whitehead K, McCue KA. Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. *Am.J.Infect.Control.* 2009.

477. Liu P, Yuen Y, Hsiao HM et al. Effectiveness of Liquid Soap and Hand Sanitizer against Norwalk Virus on Contaminated Hands. *Appl.Environ.Microbiol.* 2009.

478. Duizer E, Bijkerk P, Rockx B et al. Inactivation of caliciviruses. *Appl.Environ.Microbiol.* 2004;**70**(8):4538-43.

479. Advisory Committee on the Microbiological Safety of Food: The Microbiological Safety of Food. London: HMSO; 1990.

480. Sethi D, Wheeler JG, Cowden JM et al. A study of infectious intestinal disease in England: plan and methods of data collection. *Commun.Dis.Public Health.* 1999;**2**(2):101-7.

481. Rodrigues LC, Cowden JM, Wheeler JG et al. The study of infectious intestinal disease in England: risk factors for cases of infectious intestinal disease with Campylobacter jejuni infection. *Epidemiol.Infect.* 2001;**127**(2):185-93.

482. Sethi D, Cumberland P, Hudson MJ et al. A study of infectious intestinal disease in England: risk factors associated with group A rotavirus in children. *Epidemiol.Infect.* 2001;**126**(1):63-70.

483. Royal College of General Pracitioners Research and Surveillance Centre. 2009.
Ref Type: Internet Communication

484. Fleming DM. Weekly Returns Service of the Royal College of General Practitioners. *Commun.Dis.Public Health.* 1999;**2**(2):96-100.

485. Royal College of General Practitioners Birmingham Research Unit: Royal College of General Practitioners Weekly Returns Service annual report 2007. 2006.

486. Health Protection Agency. Gastroenteritis: Sporadic cases. 2007. National Standard Method VSOP 2 Issue 5.
Ref Type: Case

487. Health Protection Agency. Gastroenteritis: Outbreaks. 2007. National Standard Method VSOP 3 Issue 6.
Ref Type: Case

488. Gunson RN, Collins TC, Carman WF. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. *J.Clin.Virol.* 2006;**35**(4):355-67.

489. Gunson RN, Bennett S, Maclean A et al. Using multiplex real time PCR in order to streamline a routine diagnostic service. *J.Clin.Virol.* 2008;**43**(4):372-5.

490. Gallimore CI, Cubitt D, du PN et al. Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis. *J.Clin.Microbiol.* 2004;**42**(5):2271-4.

491. Carman WF, Niesters HG. The end of cell culture in diagnostics: Is molecular diagnosis the Harry Potter or the Lord Voldemort of clinical virology as a specialty? *S.Afr.Med.J.* 2007;**97**(11):1169-76.

492. Guiver M. Review of the introduction of respiratory virus PCR in HPA Regional Laboratories. Health Protection Conference . 2008.
Ref Type: Abstract

493. Gunson RN, Miller J, Leonard A et al. Importance of PCR in the diagnosis and understanding of rotavirus illness in the community. *Commun.Dis.Public Health.* 2003;**6**(1):63-5.

494. Stata Corporation. Stata Statistical Software, release 10. College Station, TX: Stata Corporation LP; 2007.

495. Fryer JF, Baylis SA, Gottlieb AL et al. Development of working reference materials for clinical virology. *J.Clin.Virol.* 2008;**43**(4):367-71.

496. Health Protection Agency. Investigation of faecal specimens for bacterial pathogens. 2007. National Standard Methods BSOP 30 Issue 6.
Ref Type: Case

497. Phillips G, Lopman B, Tam CC et al. Diagnosing rotavirus A associated IID: Using ELISA to identify a cut-off for real time RT-PCR. *J.Clin.Virol.* 2009;**44**(3):242-5.

498. Rubenstein AS, Miller MF. Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus. *J.Clin.Microbiol.* 1982;**15**(5):938-44.

499. Bewick V, Cheek L, Ball J. Statistics review 13: receiver operating characteristic curves. *Crit Care.* 2004;**8**(6):508-12.

500. Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. *Biom.J.* 2005;**47**(4):458-72.

501. Youden W. Index for rating diagnostic tests. *Cancer.* 1950;**3**(1):32-5.

502. World Health Organization: Generic protocol for (i) hospital-based surveillance to estimate the burden of rotavirus among children and (ii) a community-based survey on utilization of health care services for gastroenteritis in children. Geneva, Switzerland: World Health Organization; 2002.

503. Galil A, Antverg R, Katzir G et al. Involvement of infants, children, and adults in a rotavirus gastroenteritis outbreak in a kibbutz in southern Israel. *J.Med.Virol.* 1986;**18**(4):317-26.

504. Kirkwood CD, Clark R, Bogdanovic-Sakran N et al. A 5-year study of the prevalence and genetic diversity of human caliciviruses associated with sporadic cases of acute gastroenteritis in young children admitted to hospital in Melbourne, Australia (1998-2002). *J.Med.Virol.* 2005;**77**(1):96-101.

505. Medici MC, Martinelli M, Abelli LA et al. Molecular epidemiology of norovirus infections in sporadic cases of viral gastroenteritis among children in Northern Italy. *J.Med.Virol.* 2006;**78**(11):1486-92.

506. Allen U, Hebert D, Petric M et al. Utility of semiquantitative polymerase chain reaction for Epstein-Barr virus to measure virus load in pediatric organ transplant recipients with and without posttransplant lymphoproliferative disease. *Clin.Infect.Dis.* 2001;**33**(2):145-50.

507. Ghisetti V, Barbui A, Franchello A et al. Quantitation of cytomegalovirus DNA by the polymerase chain reaction as a predictor of disease in solid organ transplantation. *J.Med.Virol.* 2004;**73**(2):223-9.

508. Wittek M, Sturmer M, Doerr HW et al. Molecular assays for monitoring HIV infection and antiretroviral therapy. *Expert.Rev.Mol.Diagn.* 2007;**7**(3):237-46.

509. Lee SC, Antony A, Lee N et al. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. *J.Clin.Microbiol.* 2000;**38**(11):4171-9.

510. Adaui V, Verdonck K, Best I et al. SYBR Green-based quantitation of human T-lymphotropic virus type 1 proviral load in Peruvian patients with neurological disease and asymptomatic carriers: influence of clinical status, sex, and familial relatedness. *J.Neurovirol.* 2006;**12**(6):456-65.

511. Turcios RM, Widdowson MA, Sulka AC et al. Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. *Clin.Infect.Dis.* 2006;**42**(7):964-9.

512. Mattner F, Sohr D, Heim A et al. Risk groups for clinical complications of norovirus infections: an outbreak investigation. *Clin.Microbiol.Infect.* 2006;**12**(1):69-74.

513. Tsugawa T, Numata-Kinoshita K, Honma S et al. Virological, serological, and clinical features of an outbreak of acute gastroenteritis due to recombinant genogroup II norovirus in an infant home. *J.Clin.Microbiol.* 2006;**44**(1):177-82.

514. Barnes GL, Uren E, Stevens KB et al. Etiology of acute gastroenteritis in hospitalized children in Melbourne, Australia, from April 1980 to March 1993. *J.Clin.Microbiol.* 1998;**36**(1):133-8.

515. Bon F, Fascia P, Dauvergne M et al. Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. *J.Clin.Microbiol.* 1999;**37**(9):3055-8.

516. Chen SY, Tsai CN, Chao HC et al. Acute gastroenteritis caused by multiple enteric pathogens in children. *Epidemiol.Infect.* 2008;1-4.

517. Colomba C, De GS, Giammanco GM et al. Viral gastroenteritis in children hospitalised in Sicily, Italy. *Eur.J.Clin.Microbiol.Infect.Dis.* 2006;**25**(9):570-5.

518. Giordano MO, Ferreyra LJ, Isa MB et al. The epidemiology of acute viral gastroenteritis in hospitalized children in Cordoba City, Argentina: an insight of disease burden. *Rev.Inst.Med.Trop.Sao Paulo.* 2001;**43**(4):193-7.

519. Guerrero ML, Noel JS, Mitchell DK et al. A prospective study of astrovirus diarrhea of infancy in Mexico City. *Pediatr.Infect.Dis.J.* 1998;**17**(8):723-7.

520. Jafari F, Garcia-Gil LJ, Salmanzadeh-Ahrabi S et al. Diagnosis and prevalence of enteropathogenic bacteria in children less than 5 years of age with acute diarrhea in Tehran children's hospitals. *J.Infect.* 2008.

521. Kaur R, Rawat D, Kakkar M et al. Intestinal parasites in children with diarrhea in Delhi, India. *Southeast Asian J.Trop.Med.Public Health.* 2002;**33**(4):725-9.

522. Khan MM, Iqbal J, Ghafoor A et al. Aetiologic agents of diarrhoeal diseases in hospitalised children in Rawalpindi, Pakistan. *J.Diarrhoeal Dis.Res.* 1988;**6**(3-4):228-31.

523. Kim KH, Yang JM, Joo SI et al. Importance of rotavirus and adenovirus types 40 and 41 in acute gastroenteritis in Korean children. *J.Clin.Microbiol.* 1990;**28**(10):2279-84.

524. Medici MC, Martinelli M, Arcangeletti MC et al. Epidemiological aspects of human rotavirus infection in children hospitalized with acute gastroenteritis in an area of northern Italy. *Acta Biomed.* 2004;**75**(2):100-6.

525. Monica B, Ramani S, Banerjee I et al. Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India. *Journal of Medical Virology.* 2007;. **79**(5).

526. Nguyen TA, Yagyu F, Okame M et al. Diversity of viruses associated with acute gastroenteritis in children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. *Journal of Medical Virology.* 2007;. **79**(5).

527. Oh DY, Gaedicke G, Schreier E. Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity. *J.Med.Virol.* 2003;**71**(1):82-93.

528. Orlandi PP, Silva T, Magalhaes GF et al. Enteropathogens associated with diarrheal disease in infants of poor urban areas of Porto Velho, Rondonia: a preliminary study. *Mem.Inst.Oswaldo Cruz.* 2001;**96**(5):621-5.

529. Phan TG, Nguyen TA, Yan H et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. *Clin.Lab.* 2005;**51**(7-8):429-35.

530. Roman E, Wilhelmi I, Colomina J et al. Acute viral gastroenteritis: proportion and clinical relevance of multiple infections in Spanish children. *J.Med.Microbiol.* 2003;**52**(Pt 5):435-40.

531. Rosenfeldt V, Vesikari T, Pang XL et al. Viral etiology and incidence of acute gastroenteritis in young children attending day-care centers. *Pediatr.Infect.Dis.J.* 2005;**24**(11):962-5.

532. Sdiri-Loulizi K, Gharbi-Khelifi H, de Rougemont A et al. Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. *J.Clin.Microbiol.* 2008;**46**(4):1349-55.

533. Grimprel E, Rodrido C, Desselberger U. Rotavirus disease: Impact of coinfections. *Pediatr.Infect.Dis.J.* 2008;**27**(1):S3-S10.

534. Hung TY, Liu MC, Hsu CF et al. Rotavirus infection increases the risk of bacteremia in children with nontyphoid Salmonella gastroenteritis. *Eur.J.Clin.Microbiol.Infect.Dis.* 2009;**28**(4):425-8.

535. Chunge RN, Wamola IA, Kinoti SN et al. Mixed infections in childhood diarrhoea: results of a community study in Kiambu District, Kenya. *East Afr.Med.J.* 1989;**66**(11):715-23.

536. Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. *Scand.J.Infect.Dis.* 1990;**22**(3):259-67.

537. Koopmans M, Vinj inverted question mJ, de WM et al. Molecular epidemiology of human enteric caliciviruses in The Netherlands. *J.Infect.Dis.* 2000;**181 Suppl 2**S262-S269.

538. Dennehy PH. Transmission of rotavirus and other enteric pathogens in the home. *Pediatr.Infect.Dis.J.* 2000;**19**(10 Suppl):S103-S105.

539. Goldmann DA. Transmission of viral respiratory infections in the home. *Pediatr.Infect.Dis.J.* 2000;**19**(10 Suppl):S97-102.

540. Clark B, McKendrick M. A review of viral gastroenteritis. *Curr.Opin.Infect.Dis.* 2004;**17**(5):461-9.

541. Denno DM, Keene WE, Hutter CM et al. Tri-county comprehensive assessment of risk factors for sporadic reportable bacterial enteric infection in children. *J.Infect.Dis.* 2009;**199**(4):467-76.

542. Gallay A, Bousquet V, Siret V et al. Risk factors for acquiring sporadic Campylobacter infection in France: results from a national case-control study. *J.Infect.Dis.* 2008;**197**(10):1477-84.

543. Caceres VM, Kim DK, Bresee JS et al. A viral gastroenteritis outbreak associated with person-to-person spread among hospital staff. *Infect.Control Hosp.Epidemiol.* 1998;**19**(3):162-7.

544. McDonnell RJ, Wall PG, Adak GK et al. Outbreaks of infectious intestinal disease associated with person to person spread in hotels and restaurants. *Commun.Dis.Rep.CDR Rev.* 1995;**5**(10):R150-R152.

545. Norovirus outbreak associated with ill food-service workers--Michigan, January-February 2006. *MMWR Morb.Mortal.Wkly.Rep.* 2007;**56**(46):1212-6.

546. Widdowson MA, Glass R, Monroe S et al. Probable transmission of norovirus on an airplane. *JAMA.* 2005;**293**(15):1859-60.

547. Caul EO. Small round structured viruses: airborne transmission and hospital control. *Lancet.* 1994;**343**(8908):1240-2.

548. Chadwick PR, Walker M, Rees AE. Airborne transmission of a small round structured virus. *Lancet.* 1994;**343**(8890):171.

549. Chapin AR, Carpenter CM, Dudley WC et al. Prevalence of norovirus among visitors from the United States to Mexico and Guatemala who experience traveler's diarrhea. *J.Clin.Microbiol.* 2005;**43**(3):1112-7.

550. Fretz R, Schmid H, Kayser U et al. Rapid propagation of norovirus gastrointestinal illness through multiple nursing homes following a pilgrimage. *Eur.J.Clin.Microbiol.Infect.Dis.* 2003;**22**(10):625-7.

551. Verhoef L, Duizer E, Vennema H et al. Import of norovirus infections in the Netherlands and Ireland following pilgrimages to Lourdes, 2008--preliminary report. *Euro.Surveill.* 2008;**13**(44):ii.

552. Isakbaeva ET, Widdowson MA, Beard RS et al. Norovirus transmission on cruise ship. *Emerg.Infect.Dis.* 2005;**11**(1):154-8.

553. McEvoy M, Blake W, Brown D et al. An outbreak of viral gastroenteritis on a cruise ship. *Commun.Dis.Rep.CDR Rev.* 1996;**6**(13):R188-R192.

554. Verhoef L, Boxman IL, Duizer E et al. Multiple exposures during a norovirus outbreak on a river-cruise sailing through Europe, 2006. *Euro.Surveill.* 2008;**13**(24).

555. Royston P. Multiple imputation of missing values. *Stata Journal.* 2004;**4**(3):227-41.

556. Royston P. Multiple imputation of missing values: Update of ice. *Stata Journal.* 2005;**5**(4):527-36.

557. Royston P. Multiple imputation of missing values: update. *Stata Journal.* 2005;**5**(2):188-201.

558. Royston P. Multiple imputation of missing values: further update of ice, with an emphasis on interval censoring. *Stata Journal.* 2007;**7**(4):445-64.

559. Royston P, Roys. Multiple imputation of missing values: Further update of ice, with an emphasis on categorical variables. *Stata Journal.* 2009;**9**(3):446-77.

560. Royston P, Carlin JB, White IR. Multiple imputation of missing values: New features for mim. *Stata Journal.* 2009;**9**(2):252-64.

561. Victora CG, Huttly SR, Fuchs SC et al. The role of conceptual frameworks in epidemiological analysis: a hierarchical approach. *Int.J.Epidemiol.* 1997;**26**(1):224-7.

562. Isakbaeva ET, Bulens SN, Beard RS et al. Norovirus and child care: challenges in outbreak control. *Pediatr.Infect.Dis.J.* 2005;**24**(6):561-3.

563. Multistate outbreak of viral gastroenteritis related to consumption of oysters--Louisiana, Maryland, Mississippi and North Carolina, 1993. *MMWR Morb.Mortal.Wkly.Rep.* 1993;**42**(49):945-8.

564. Thomas R, Elias P. Development of the Standard Occupational Classification. *Population Trends.* 1989;**55**16-21.

565. Brady T. Adjusted population attributable fractions from logistic regression. *Stata Technical Bulletin.* 2009;**428**-12.

566. Greenland S, Drescher K. Maximum likelihood estimation of the attributable fraction from logistic models. *Biometrics.* 1993;**49**(3):865-72.

567. Sobel J, Gomes TA, Ramos RT et al. Pathogen-specific risk factors and protective factors for acute diarrheal illness in children aged 12-59 months in Sao Paulo, Brazil. *Clin.Infect.Dis.* 2004;**38**(11):1545-51.

568. Makita K, Hayakawa Y, Okame M et al. First detection of IgA against norovirus in breast milk. *Clin.Lab.* 2007;**53**(3-4):125-8.

569. Koo HL, Ajami NJ, Jiang ZD et al. Noroviruses as a Cause of Diarrhea in Travelers to Guatemala, India, and Mexico. *J.Clin.Microbiol.* 2010.

570. Curtis V, Cairncross S. Effect of washing hands with soap on diarrhoea risk in the community: a systematic review. *Lancet Infect.Dis.* 2003;**3**(5):275-81.

571. Ejemot RI, Ehiri JE, Meremikwu MM et al. Hand washing for preventing diarrhoea. *Cochrane.Database.Syst.Rev.* 2008;(1):CD004265.

572. Surgeoner BV, Chapman BJ, Powell DA. University students' hand hygiene practice during a gastrointestinal outbreak in residence: what they say they do and what they actually do. *J.Environ.Health.* 2009;**72**(2):24-8.

573. Multisite outbreak of norovirus associated with a franchise restaurant--Kent County, Michigan, May 2005. *MMWR Morb.Mortal.Wkly.Rep.* 2006;**55**(14):395-7.

574. Yoder J, Roberts V, Craun GF et al. Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking--United States, 2005-2006. *MMWR Surveill Summ.* 2008;**57**(9):39-62.

575. Tu ET, Bull RA, Greening GE et al. Epidemics of gastroenteritis during 2006 were associated with the spread of norovirus GII.4 variants 2006a and 2006b. *Clin.Infect.Dis.* 2008;**46**(3):413-20.

576. Bland JM, Altman DG. Matching. *BMJ.* 1994;**309**(6962):1128.

577. Ambler G, Omar RZ, Royston P. A comparison of imputation techniques for handling missing predictor values in a risk model with a binary outcome. *Stat.Methods Med.Res.* 2007;**16**(3):277-98.

578. Schafer JL. Multiple imputation: a primer. *Stat.Methods Med.Res.* 1999;**8**(1):3-15.

579. House T, Keeling MJ. Household structure and infectious disease transmission. *Epidemiol.Infect.* 2009;**137**(5):654-61.

580. Viboud C, Boelle PY, Cauchemez S et al. Risk factors of influenza transmission in households. *Br.J.Gen.Pract.* 2004;**54**(506):684-9.

581. Lopman B, Armstrong B, Atchison C et al. Host, weather and virological factors drive norovirus epidemiology: time-series analysis of laboratory surveillance data in England and Wales. *PLoS.ONE.* 2009;**4**(8):e6671.

582. Palmer S, Houston H, Lervy B et al. Problems in the diagnosis of foodborne infection in general practice. *Epidemiol.Infect.* 1996;**117**(3):479-84.

583. Cooper DL, Smith GE, Edmunds WJ et al. The contribution of respiratory pathogens to the seasonality of NHS Direct calls. *J.Infect.* 2007;**55**(3):240-8.

584. Mangtani P, Hajat S, Kovats S et al. The association of respiratory syncytial virus infection and influenza with emergency admissions for respiratory disease in London: an analysis of routine surveillance data. *Clin.Infect.Dis.* 2006;**42**(5):640-6.

585. Ryan MJ, Ramsay M, Brown D et al. Hospital admissions attributable to rotavirus infection in England and Wales. *J.Infect.Dis.* 1996;**174 Suppl 1**S12-S18.

586. Gillespie IA, O'Brien SJ, Adak GK et al. Point source outbreaks of Campylobacter jejuni infection--are they more common than we think and what might cause them? *Epidemiol.Infect.* 2003;**130**(3):367-75.

587. Gillespie IA, O'Brien SJ, Adak GK et al. Foodborne general outbreaks of Salmonella Enteritidis phage type 4 infection, England and Wales, 1992-2002: where are the risks? *Epidemiol.Infect.* 2005;**133**(5):795-801.

588. Lopman BA, Adak GK, Reacher MH et al. Two epidemiologic patterns of norovirus outbreaks: surveillance in England and wales, 1992-2000. *Emerg.Infect.Dis.* 2003;**9**(1):71-7.

589. Tam CC, Higgins CD, Neal KR et al. Chicken consumption and use of acid-suppressing medications as risk factors for Campylobacter enteritis, England. *Emerg.Infect.Dis.* 2009;**15**(9):1402-8.

590. Lopman BA, Reacher M, Gallimore C et al. A summertime peak of "winter vomiting disease": surveillance of noroviruses in England and Wales, 1995 to 2002. *BMC.Public Health.* 2003;**313**.

591. Lunn DJ, Thomas A, Best N et al. WinBUGS - a Bayesian modelling framework: concepts, structure and extensibility. *Statistics and Computing.* 2000;**10**325-37.

592. Gallimore CI, Iturriza-Gomara M, Xerry J et al. Inter-seasonal diversity of norovirus genotypes: emergence and selection of virus variants. *Arch.Virol.* 2007;**152**(7):1295-303.

593. Freeman MM, Kerin T, Hull J et al. Enhancement of detection and quantification of rotavirus in stool using a modified real-time RT-PCR assay. *J.Med.Virol.* 2008;**80**(8):1489-96.

594. Gutierrez-Aguirre I, Steyer A, Boben J et al. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. *J.Clin.Microbiol.* 2008;**46**(8):2547-54.

595. Jothikumar N, Cromeans TL, Hill VR et al. Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. *Appl.Environ.Microbiol.* 2005;**71**(6):3131-6.

596. Logan C, O'leary JJ, O'sullivan N. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. *J.Clin.Microbiol.* 2006;**44**(9):3189-95.

597. Oka T, Katayama K, Hansman GS et al. Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. *J.Med.Virol.* 2006;**78**(10):1347-53.

598. Pang XL, Preiksaitis JK, Lee B. Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. *J.Clin.Virol.* 2005;**33**(2):168-71.

599. Schwarz BA, Bange R, Vahlenkamp TW et al. Detection and quantitation of group A rotaviruses by competitive and real-time reverse transcription-polymerase chain reaction. *J.Virol.Methods.* 2002;**105**(2):277-85.

600. Zhang Z, Mitchell DK, Afflerbach C et al. Quantitation of human astrovirus by real-time reverse-transcription-polymerase chain reaction to examine correlation with clinical illness. *J.Virol.Methods.* 2006;**134**(1-2):190-6.

601. Chan MC, Sung JJ, Lam RK et al. Sapovirus detection by quantitative real-time RT-PCR in clinical stool specimens. *J.Virol.Methods.* 2006;**134**(1-2):146-53.

602. Ryan MJ, Wall PG, Adak GK et al. Outbreaks of infectious intestinal disease in residential institutions in England and Wales 1992-1994. *J.Infect.* 1997;**34**(1):49-54.

603. Food Standards Agency: Food Handlers: Fitness to work - regulatory guidance and best practice advice for food business operators. 2008.

604. Chadwick PR, Beards G, Brown D et al. Management of hospital outbreaks of gastro-enteritis due to small roundstructured viruses. *J.Hosp.Infect.* 2000;**45**(1):1-10.

605. Friesema IH, Vennema H, Heijne JC et al. Norovirus outbreaks in nursing homes: the evaluation of infection control measures. *Epidemiol.Infect.* 2009;**137**(12):1722-33.

606. Mossong J, Hens N, Jit M et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. *PLoS.Med.* 2008;**5**(3):e74.

607. Kotloff, K.: Data on severe diarrheal disease burden and etiology from the Global Enteric Multi-Center Study (GEMS). Malaga, Spain: 5th International Conference on Vaccines for Enteric Diseases; 9-9-2009.

608. Parashar UD, Burton A, Lanata C et al. Global mortality associated with rotavirus disease among children in 2004. *J.Infect.Dis.* 2009;**200 Suppl 1**S9-S15.

609. Delayed onset and diminished magnitude of rotavirus activity--United States, November 2007-May 2008. *MMWR Morb.Mortal.Wkly.Rep.* 2008;**57**(25):697-700.

610. Reduction in rotavirus after vaccine introduction--United States, 2000-2009. *MMWR Morb.Mortal.Wkly.Rep.* 2009;**58**(41):1146-9.

611. Belshaw DA, Muscatello DJ, Ferson MJ et al. Rotavirus vaccination one year on.. *Commun.Dis.Intell.* 2009;**33**(3):337-40.

612. Cortese MM, Tate JE, Simonsen L et al. Reduction in Gastroenteritis in United States Children and Correlation With Early Rotavirus Vaccine Uptake From National Medical Claims Databases. *Pediatr.Infect.Dis.J.* 2010.

613. Richardson, C., Steadman, B., Foubert, T., Klas, S., and Tino, W.: Preclinical development of multivalent Norovirus VLP vaccines. Malaga, Spain: 5th International Conference on Vaccines for Enteric Diseases; 9-9-2009.

Appendix 1: Additional information for Chapter 3

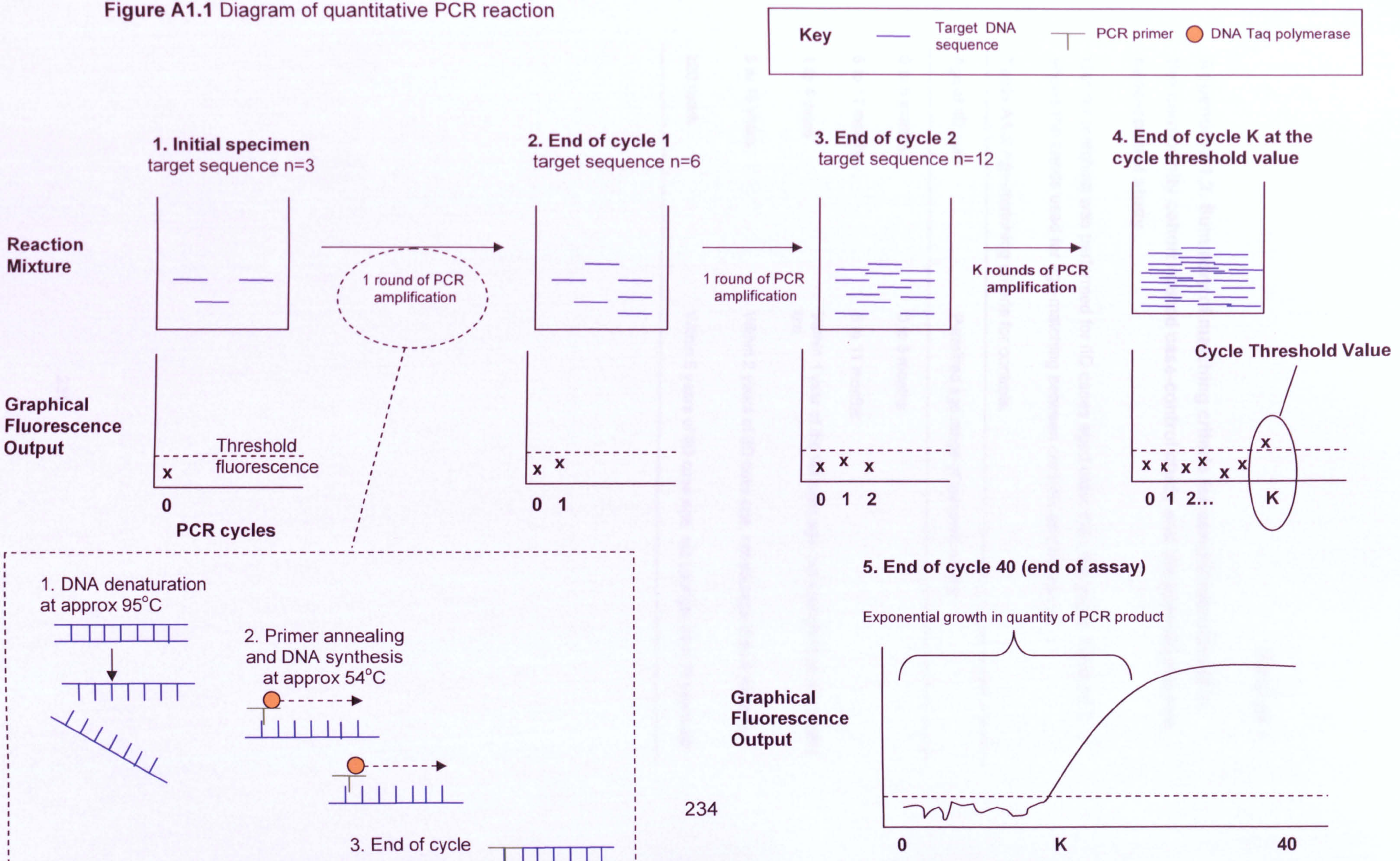
Appendix 1.1. Description of the real-time PCR reaction

In a real-time RT-PCR assay for norovirus, the norovirus RNA is reverse transcribed to generate copy DNA (cDNA). The cDNA is then replicated in the real-time PCR assay. Primer DNA molecules bind to the target sequence on the cDNA molecules, initiating replication of the cDNA. Before the replication reaction, the primer consists of a short DNA sequence, complimentary to a sequence in the norovirus cDNA, and a fluorescent compound bound to a quencher compound, which suppresses fluorescent activity. As the primers are incorporated into the newly synthesised DNA molecule, the fluorescent compound and the quencher dissociate and the fluorescent compound emits coloured light when white light is directed onto the reaction mixture at the end of the PCR cycle using an optical fibre. The primers are designed to be specific to norovirus, so that DNA replication and fluorescence only occur in the presence of norovirus cDNA.

The intensity of the optical signal in the PCR reaction mixture is measured at the end of each PCR cycle using a computer and camera connected to the optical fibre in the reaction mixture. In each round of the PCR reaction, the number of copies of the target will double if the efficiency of the reaction is 100%, i.e. the primers bind to all copies of the target sequence in the reaction mixture. The PCR reaction will have 100% efficiency in the initial stages of the reaction if the primer is perfectly matched to the target sequence. As the number of target sequence copies increases with each successive round of PCR replication, the intensity of the fluorescent signal increases. The cycle threshold (Ct) value is the number of PCR cycles after which the amount of fluorescence emitted by the reaction mixture rises above background noise (see Figure A1.1). The Ct value is therefore inversely proportional to the amount of the target sequence present in the original specimen, because the lower the initial concentration of the target sequence, the more cycles will be required to generate sufficient PCR product for the fluorescent activity to rise above this threshold.

The Ct value therefore acts as a good proxy for the relative amount of norovirus in the original specimen, although it may vary with the conditions in the PCR machine, which determine the level of background light activity ('noise'), and the efficiency of the PCR reaction, which determines the replication rate per PCR cycle.

Figure A1.1 Diagram of quantitative PCR reaction



Appendix A1.2. Summary of matching criteria for control recruitment in the community cohort nested case-control study and the general practice case-control study

Gender matching was performed for IID cases aged older than five years. Table A1.2 shows the bands used for age-matching between controls and IID cases.

Table A1.2 Age-matching criteria for controls.

Age of IID case	Permitted age range of matched control
0 to 5 months	0 to 5 months
6 to 11 months	6 to 11 months
1 to 4 years	Within 1 year of the IID case age, not younger than 11 months old
5 to 19 years	Within 2 years of IID case age, not younger than 4 years old
≥20 years	Within 5 years of IID case age, not younger than 18 years old

Appendix A1.3. Detection limit of the norovirus real-time RT-PCR assay for common norovirus genotypes

Genotype	Genome copies required for detection before cycle 40[i]
Genogroup I	
GI-1	10^2
GI-2	10^3
GI-3	10^3
GI-4	10^2
GI-5	10^1
GI-6	10^2
GI-7	10^3
Genogroup II	
GII-1	10^1
GII-2	10^1
GII-3	10^1
GII-4	10^1
GII-5	10^1
GII-6	10^1
GII-7	10^6
GII-8	10^4

[i] In the assay used to test the Study of Infectious Intestinal Disease specimen archive.

Appendix A1.4. Age distribution of recruited and archived IID cases and controls providing stool specimens for diagnostic testing in the Study of Infectious Intestinal Disease

Figure A1.4a IID cases in the general practice case-control study.

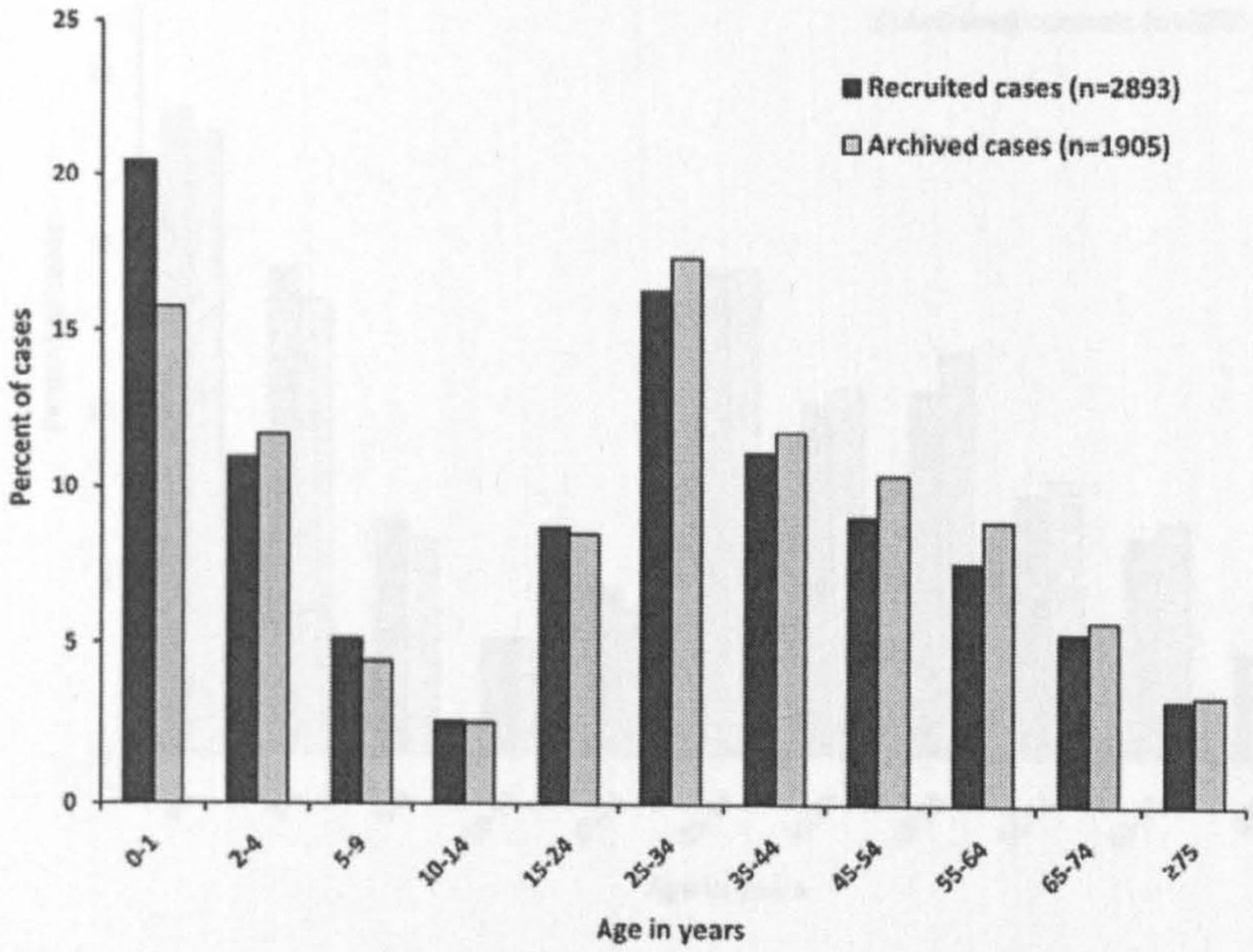


Figure A1.4b IID cases in the community cohort.

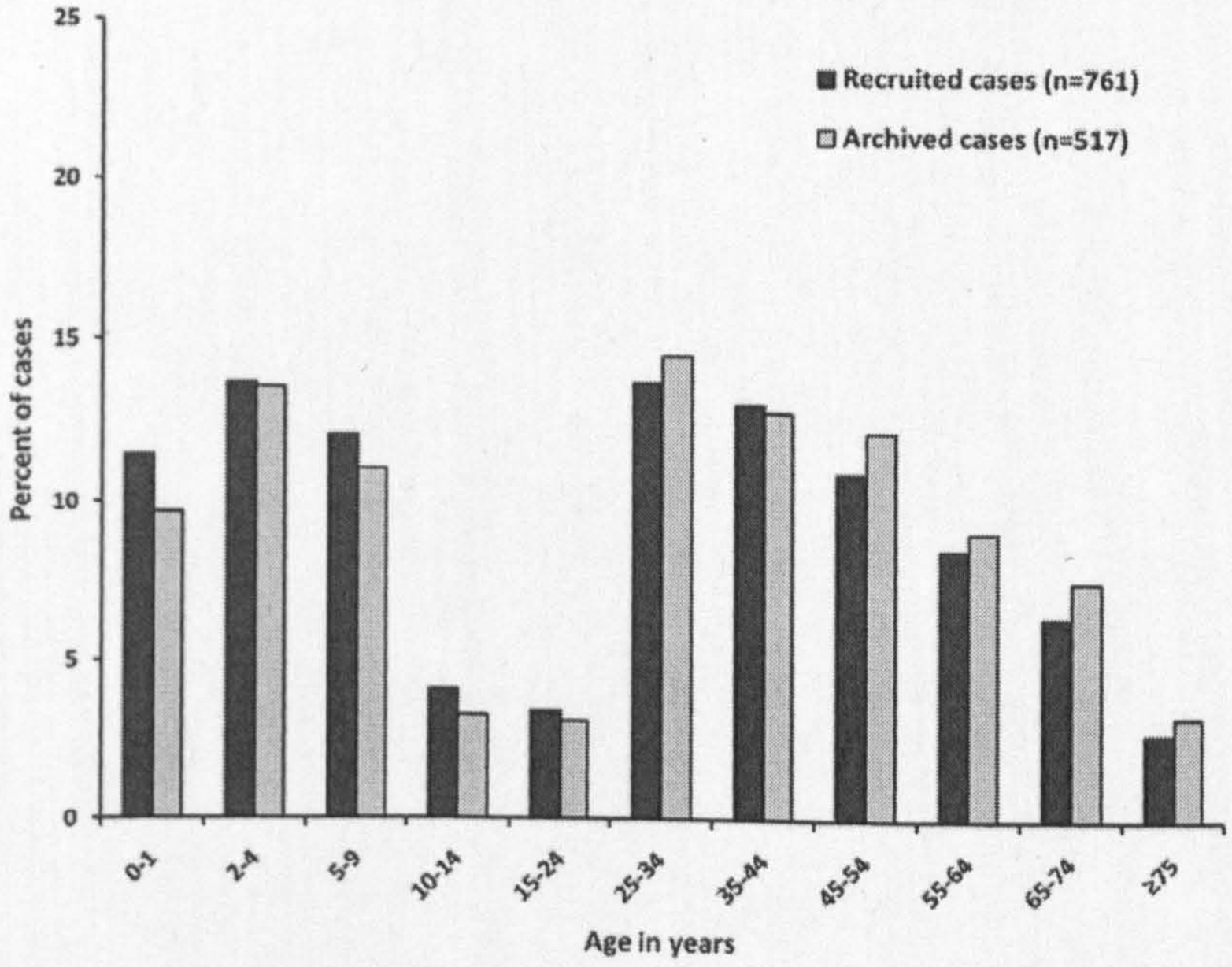
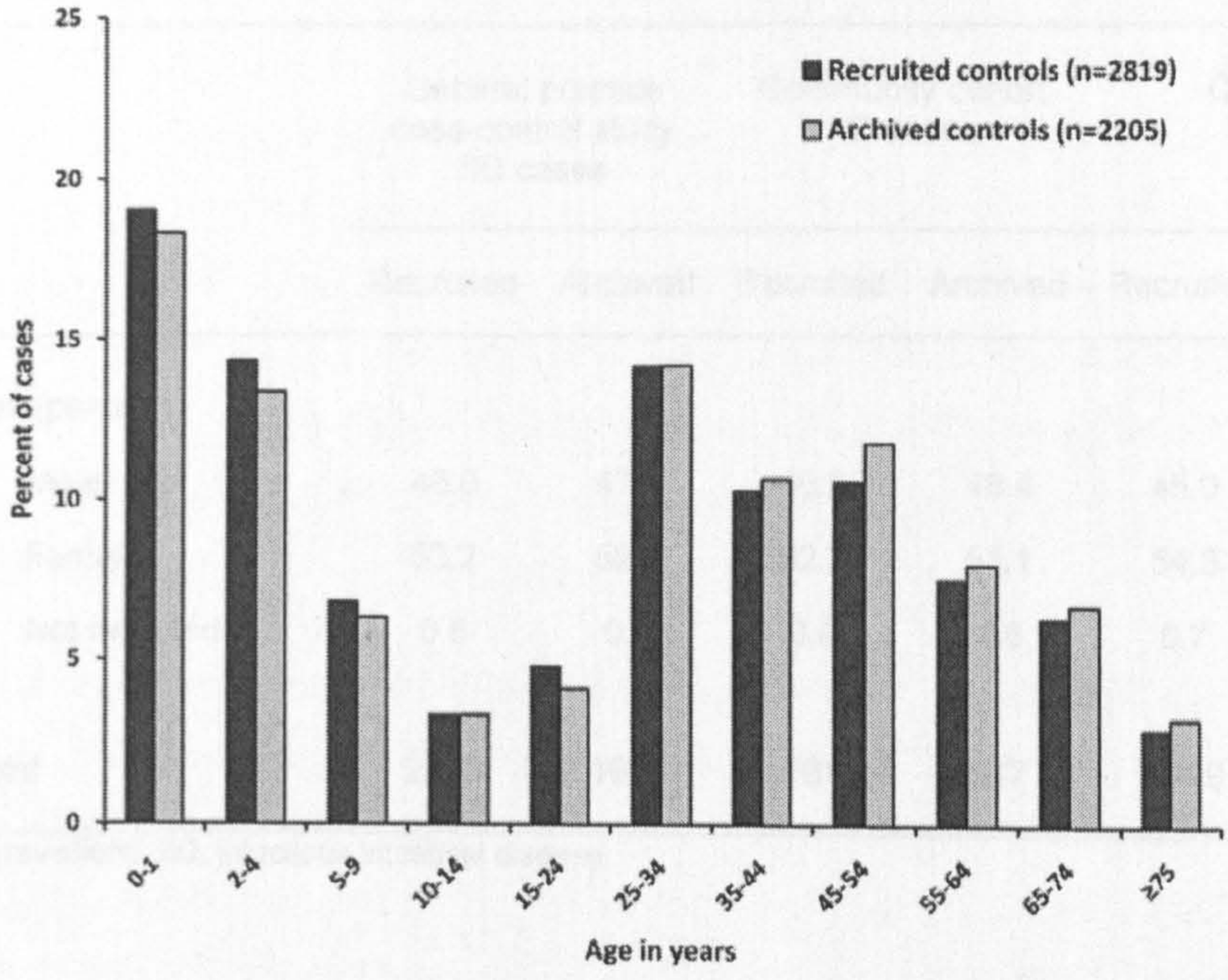


Figure A1.4c Controls.



Appendix A1.5: Sex of recruited and archived IID cases and controls providing stool specimens for diagnostic testing in the Study of Infectious Intestinal Disease

	General practice case-control study IID cases		Community cohort IID cases		Controls	
	Recruited	Archived	Recruited	Archived	Recruited	Archived
Sex (percent)						
Male	46.0	47.1	46.9	48.4	45.0	46.1
Female	53.2	52.3	52.2	51.1	54.3	53.2
Not reported	0.8	0.6	0.9	0.6	0.7	0.7
Total	2893	1905	761	517	2819	2205

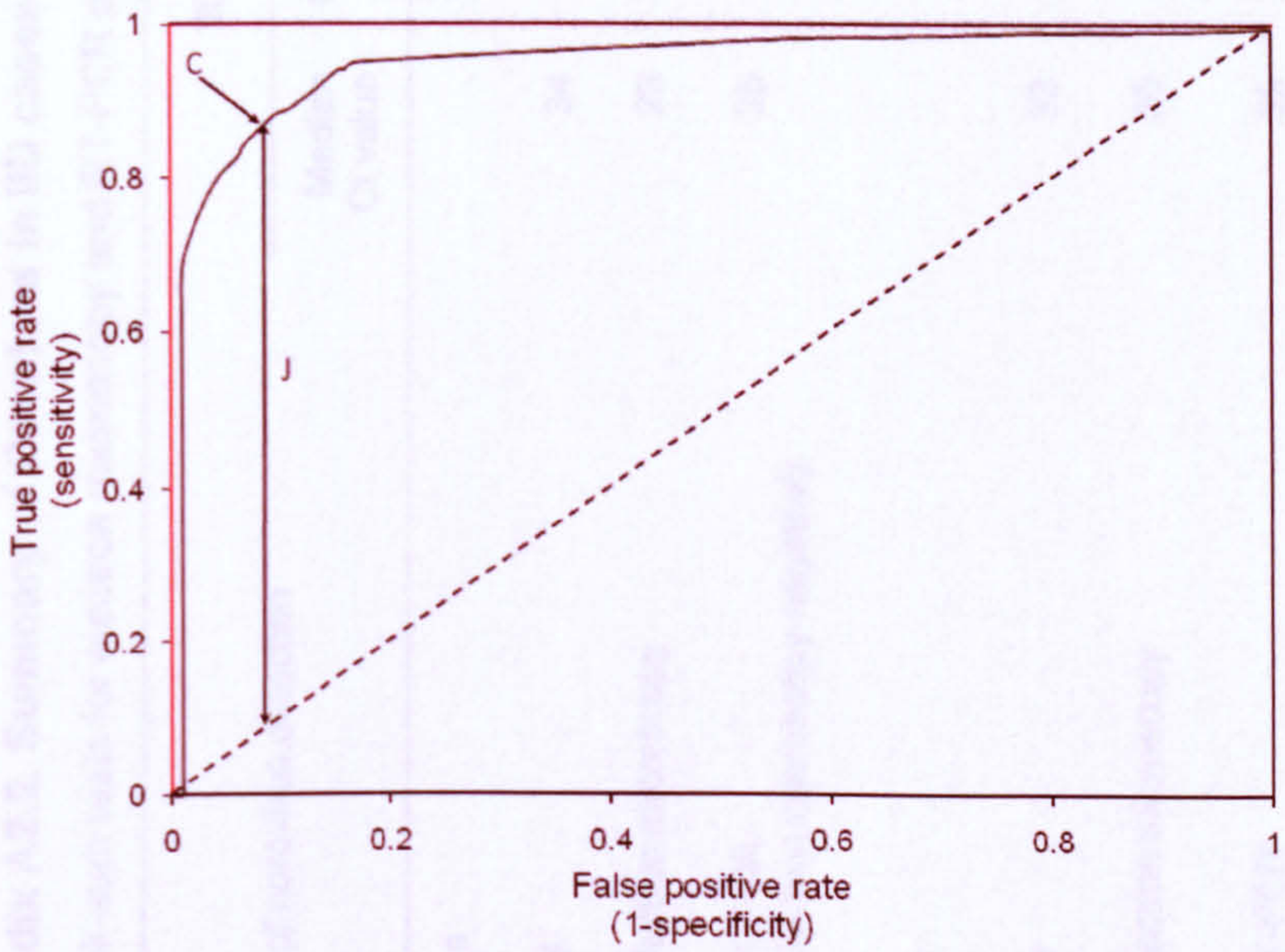
Abbreviations: IID, infectious intestinal disease

Appendix 2: Additional information and results for Chapter 4

Appendix A2.1. Production and interpretation of an empirical ROC curve.

An empirical ROC curve is produced by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) at each possible cut-off in the range of quantitative measurements from the test, in this case at each Ct value. The sensitivity and specificity are calculated by comparing the classification of individuals using a cut-off at a particular Ct value with the classification of individuals in the reference groups. The point on the ROC curve closest to the top left hand corner of the plot represents the optimal cut-off in the quantitative test data and has the maximum Youden index. The area under the ROC curve (AUC) represents the discriminatory power of the quantitative test compared to the reference groups.

Figure A2.1 Features of an empirical ROC plot. Black dashed line shows a ROC curve for a test with no discriminatory power (AUC=0.5); red line shows a ROC curve for a theoretical perfect test, which matches the classification of the reference test (AUC=1); the black solid line shows a ROC curve for a test with good discriminatory power. J is the maximum Youden index value; C indicates the point on the ROC curve representing the optimal cut-off in the quantitative test value, which is at the maximum value of the Youden index.



Appendix A2.2. Summary of Ct values in IID cases and controls by age group

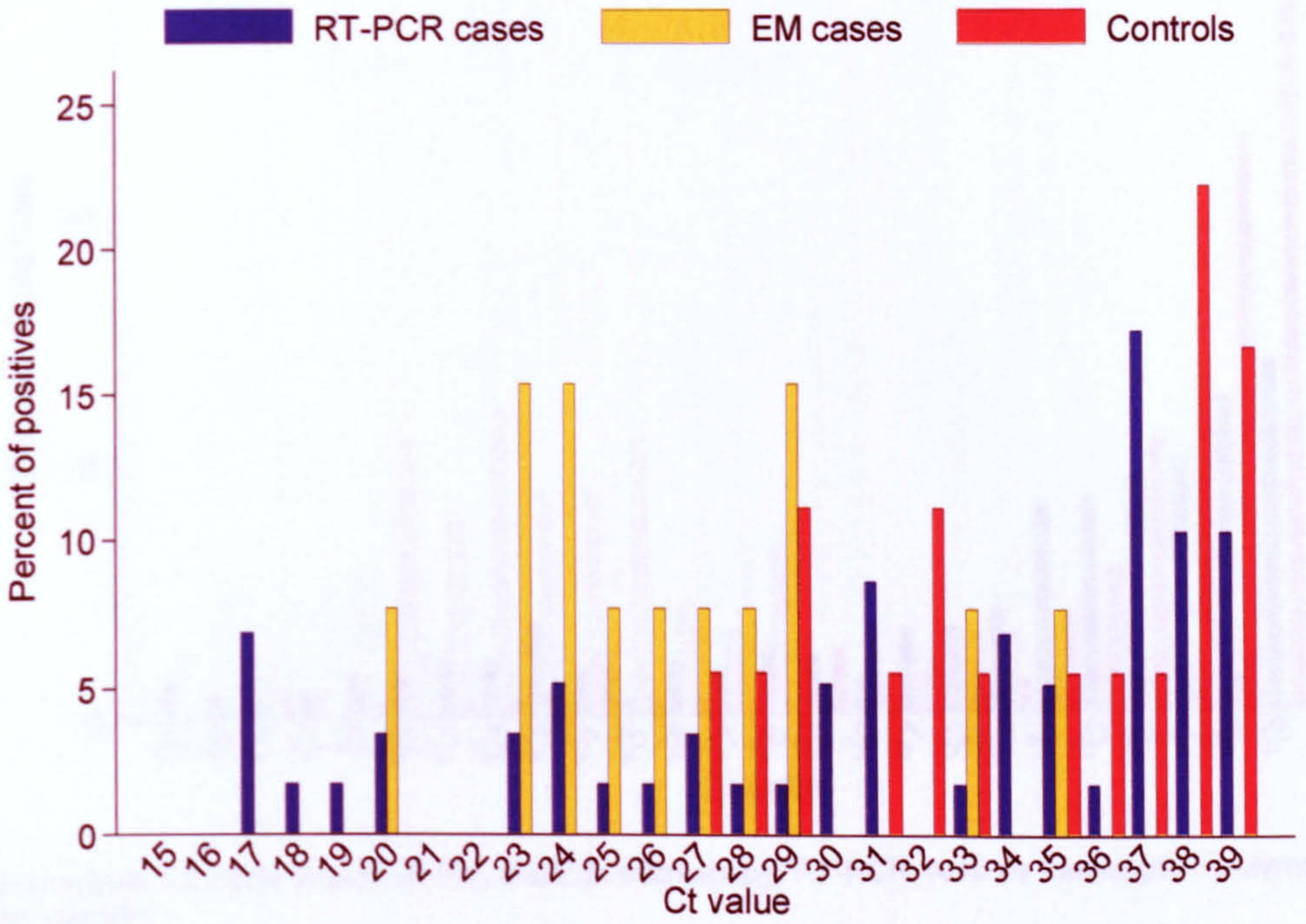
The rank-sum tests for electron microscopy and RT-PCR positive IID cases compare them to all controls.

Method of norovirus detection	IID Cases			Controls			Rank-sum test *P* value comparing cases to controls
	Median Ct value	Ct value IQR	Sample size	Median Ct value	Ct value IQR	Sample size	
< 5 years							
All	34	25 - 37	286	37	32 - 38	120	<0.001
Electron microscopy	23	21 - 25	53				<0.001
RT-PCR (Electron microscopy negative)	35	31 – 37	233				0.001
≥5 years							
All	33	25 - 37	430	38	36 - 39	79	<0.001
Electron microscopy	26	23 - 29	66				<0.001
RT-PCR (Electron microscopy negative)	35	26 – 38	364				<0.001

Abbreviations: Ct, cycle threshold; IQR, interquartile range.
Age was not recorded for two IID cases.

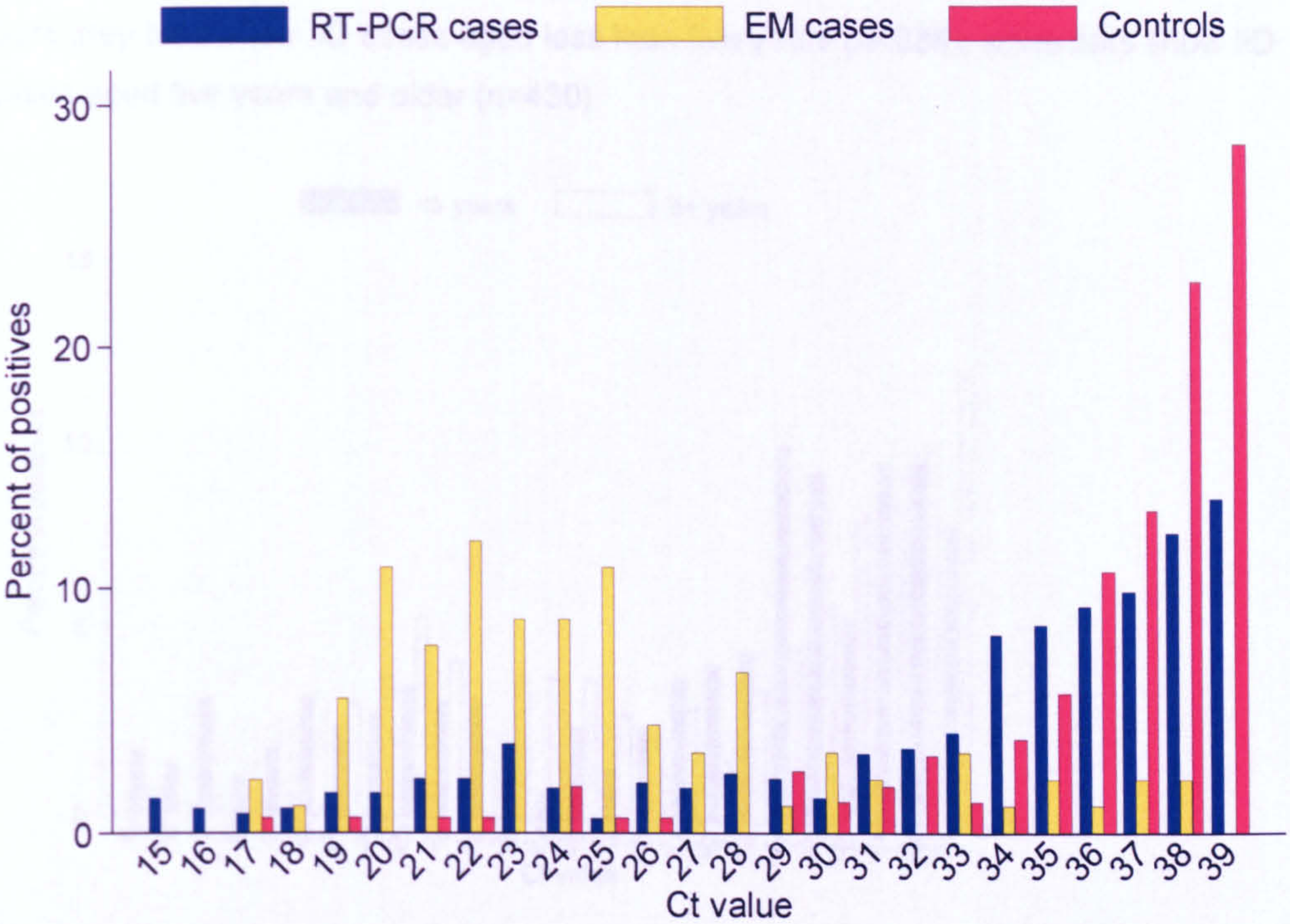
Appendix A2.3. Distribution of norovirus Ct values

Figure A2.3a Norovirus genogroup I. 'EM cases' are IID cases positive by electron microscopy, 'RT-PCR cases' are IID cases negative by electron microscopy and subsequently positive by RT-PCR. Sample sizes: EM cases = 13, RT-PCR cases = 58, controls = 18.



Abbreviations: Ct, cycle threshold; EM, electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction.

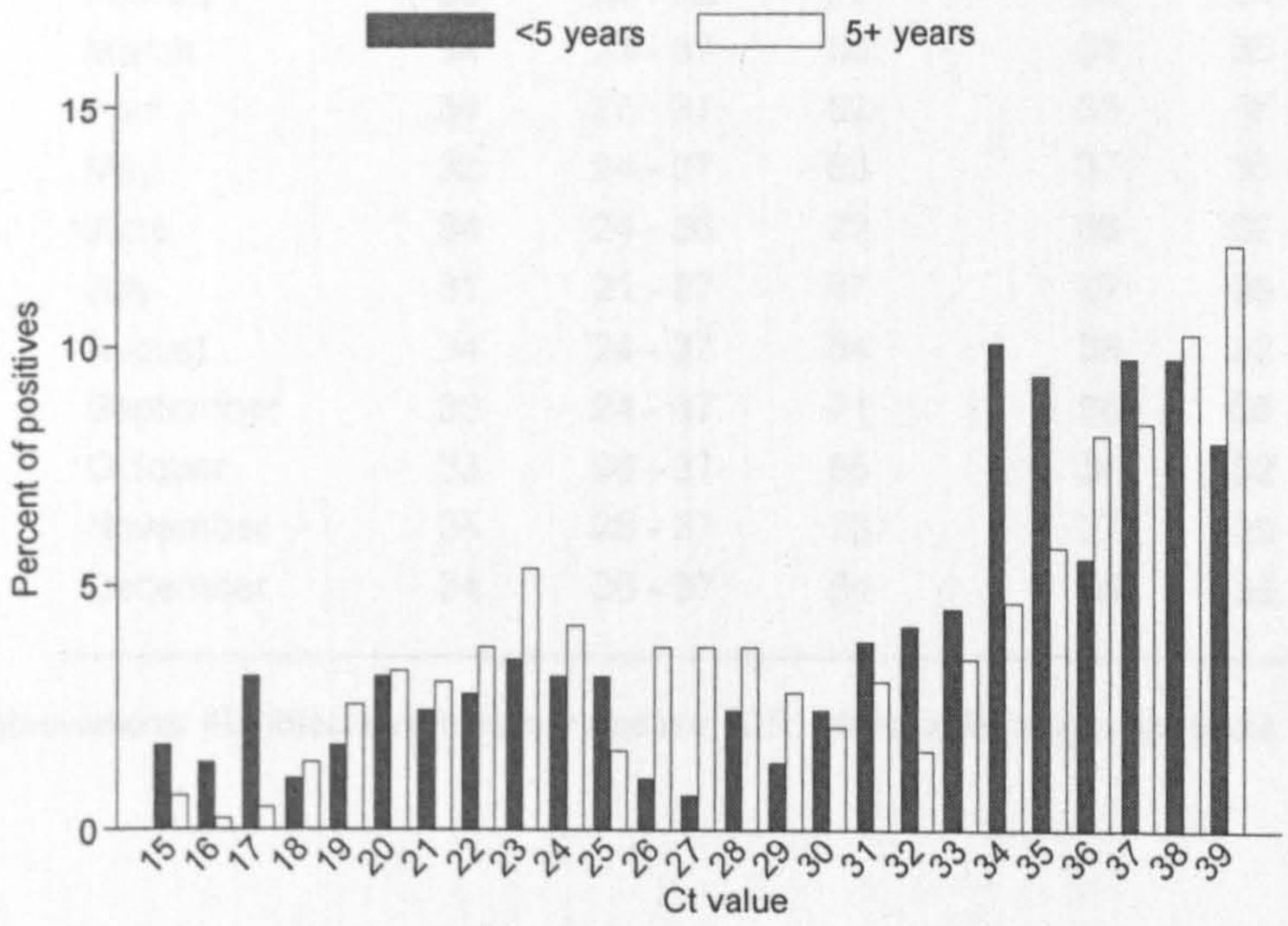
Figure A2.3b Norovirus genogroup II. 'EM cases' are IID cases positive by electron microscopy, 'RT-PCR cases' are IID cases negative by electron microscopy and subsequently positive by RT-PCR. Sample sizes: EM cases = 92, RT-PCR cases = 497, controls = 159.



Abbreviations: Ct, cycle threshold; EM, electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction.

Appendix 2.4. Distribution of Ct values in IID cases aged less than five years and IID cases aged five years and older

Dark grey bars show IID cases aged less than five years (n=288); white bars show IID cases aged five years and older (n=430).



Abbreviations: Ct, cycle threshold.

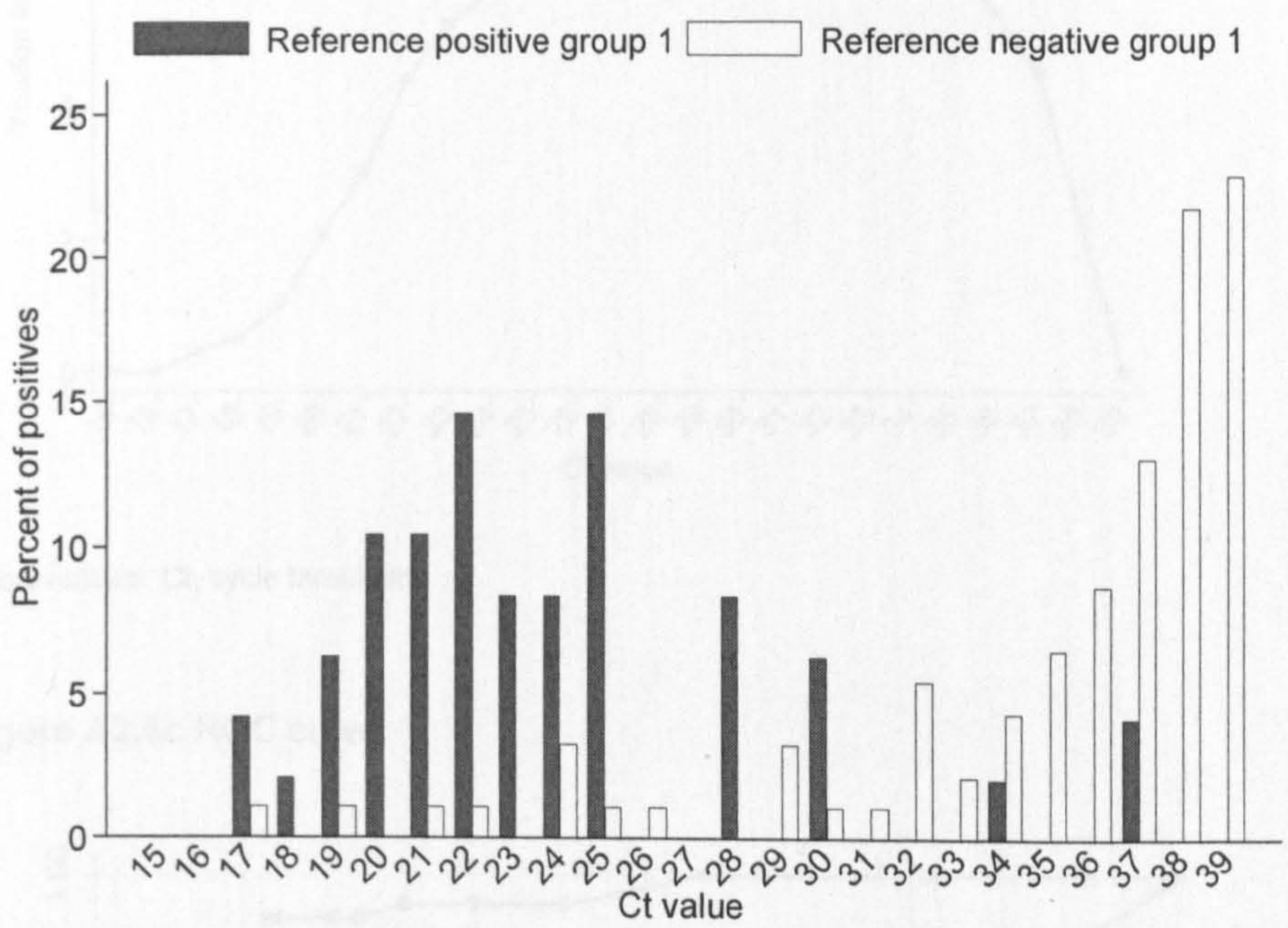
Appendix A2.5. Monthly median Ct values in IID cases and controls.

	IID cases			Controls		
	Median	IQR	Total	Median	IQR	Total
January	34	27 - 37	63	38	35 - 39	17
February	36	26 - 38	32	36	30 - 38	12
March	34	23 - 37	50	38	35 - 39	14
April	34	27 - 37	52	38	36 - 39	15
May	32	24 - 37	63	37	35 - 38	12
June	34	24 - 36	72	36	32 - 37	23
July	31	21 - 37	47	37	35 - 38	21
August	34	24 - 37	54	38	32 - 39	13
September	30	24 - 37	71	38	35 - 39	9
October	33	28 - 37	85	36	32 - 38	21
November	34	28 - 37	73	37	29 - 39	25
December	34	26 - 37	54	38	36 - 39	17

Abbreviations: IID, infectious intestinal disease; IQR, interquartile range. Appendix

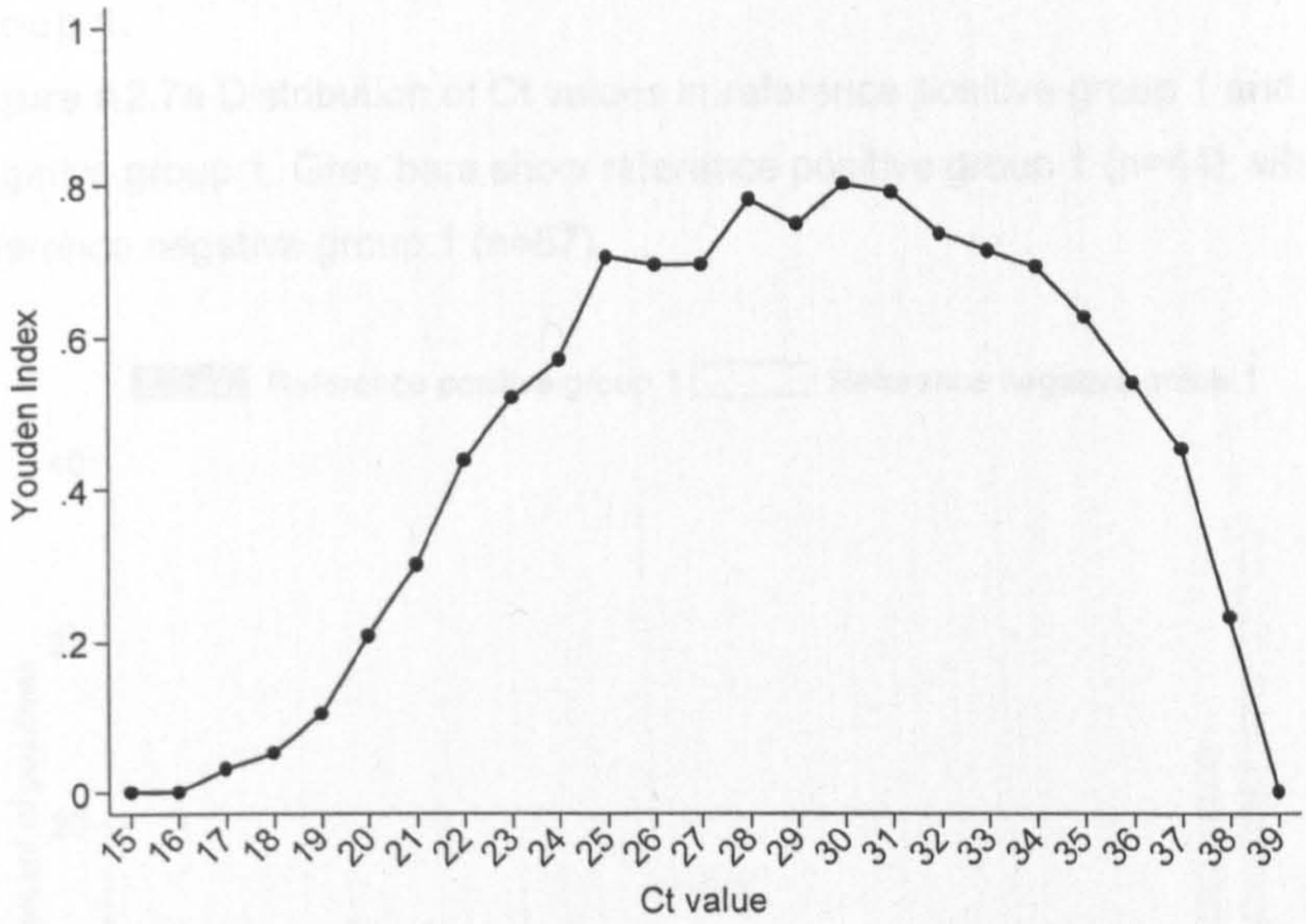
Appendix A2.6. ROC analysis for genogroup II, children aged less than five years, reference positive group 1 and reference negative group 1.

Figure A2.6a Distribution of Ct values in reference positive group 1 and reference negative group 1. Grey bars show reference positive group 1 (n=48); white bars show reference negative group 1 (n=92).



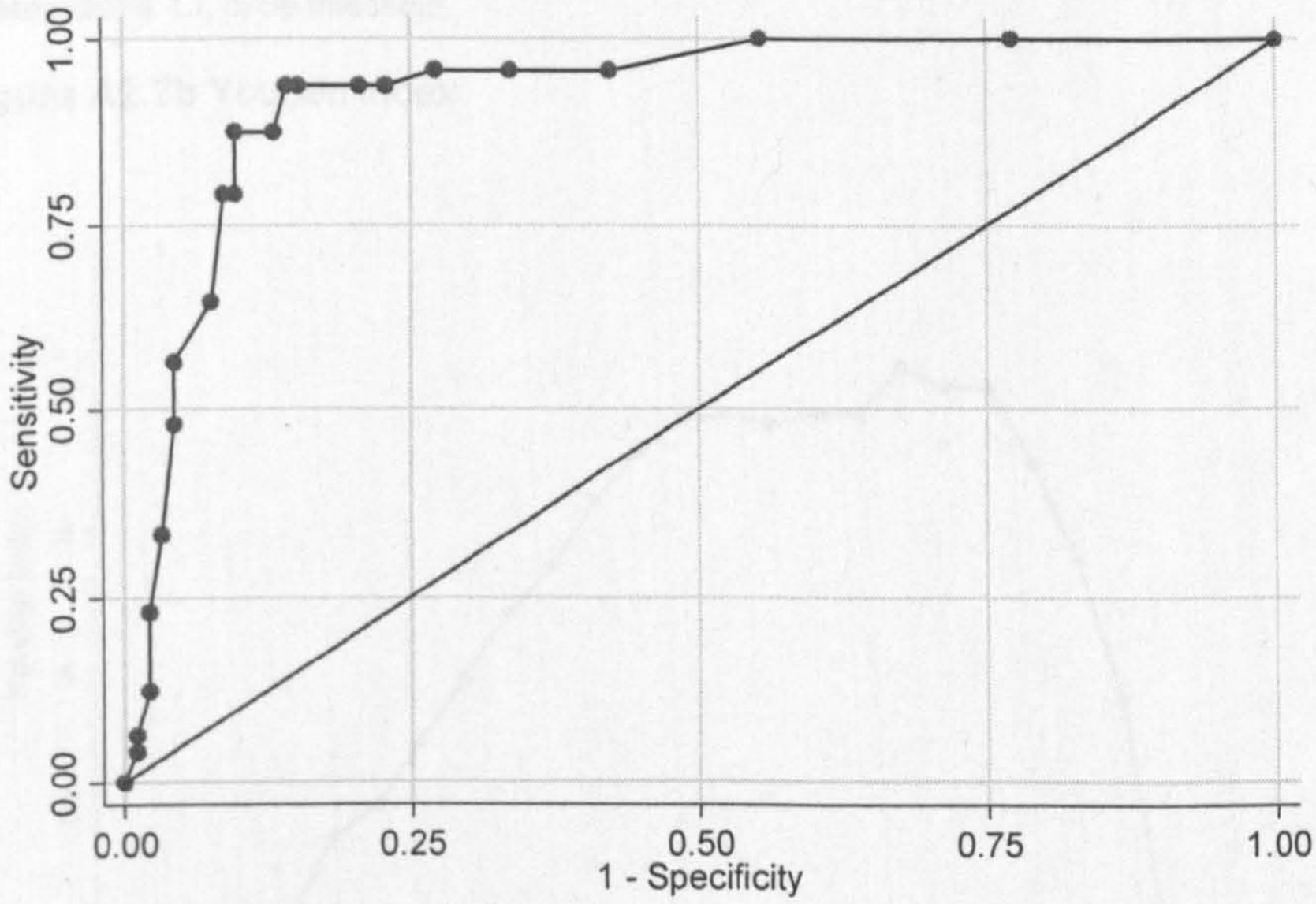
Abbreviations: Ct, cycle threshold.

Figure A2.6b Youden index.



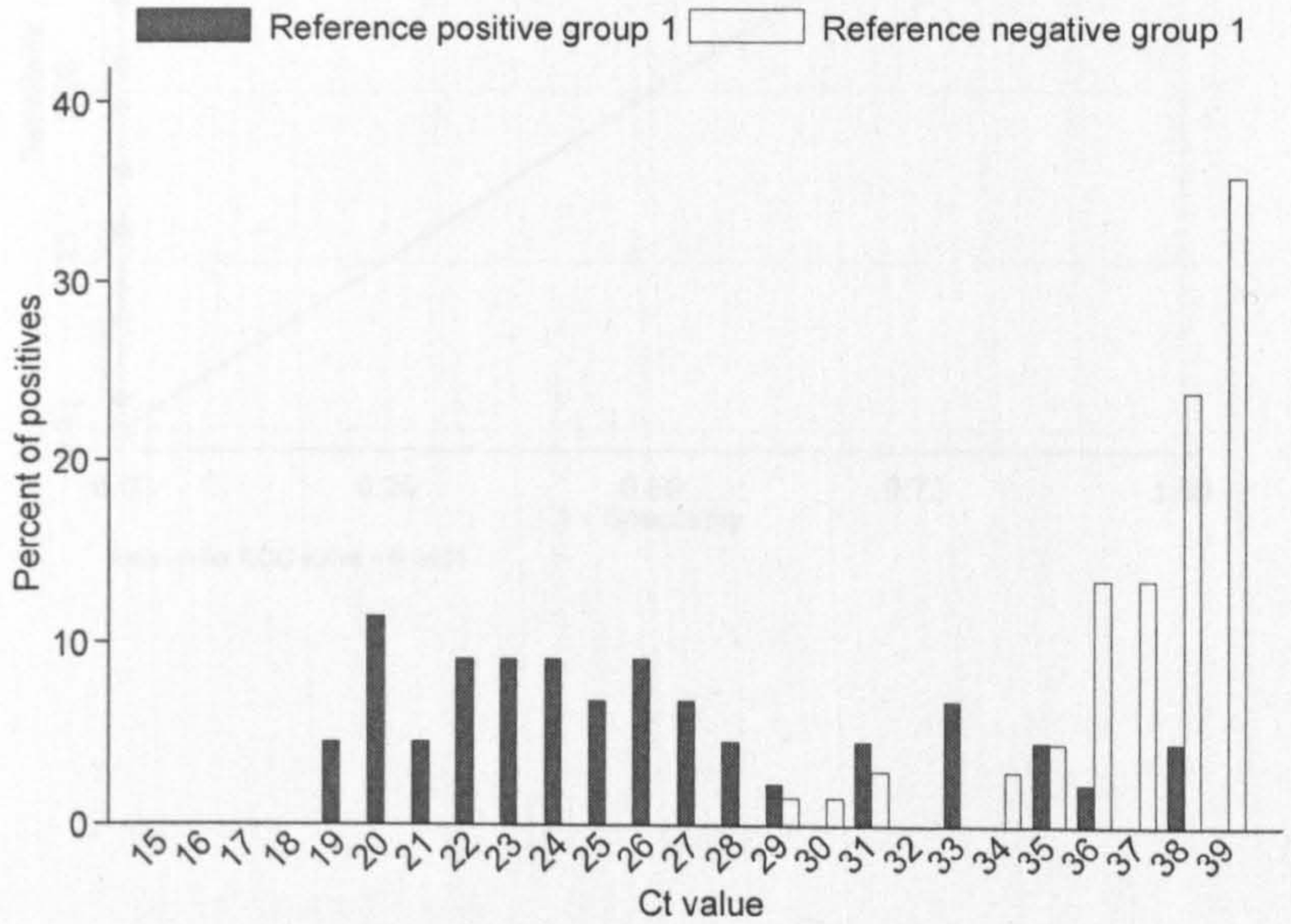
Abbreviations: Ct, cycle threshold.

Figure A2.6c ROC curve.



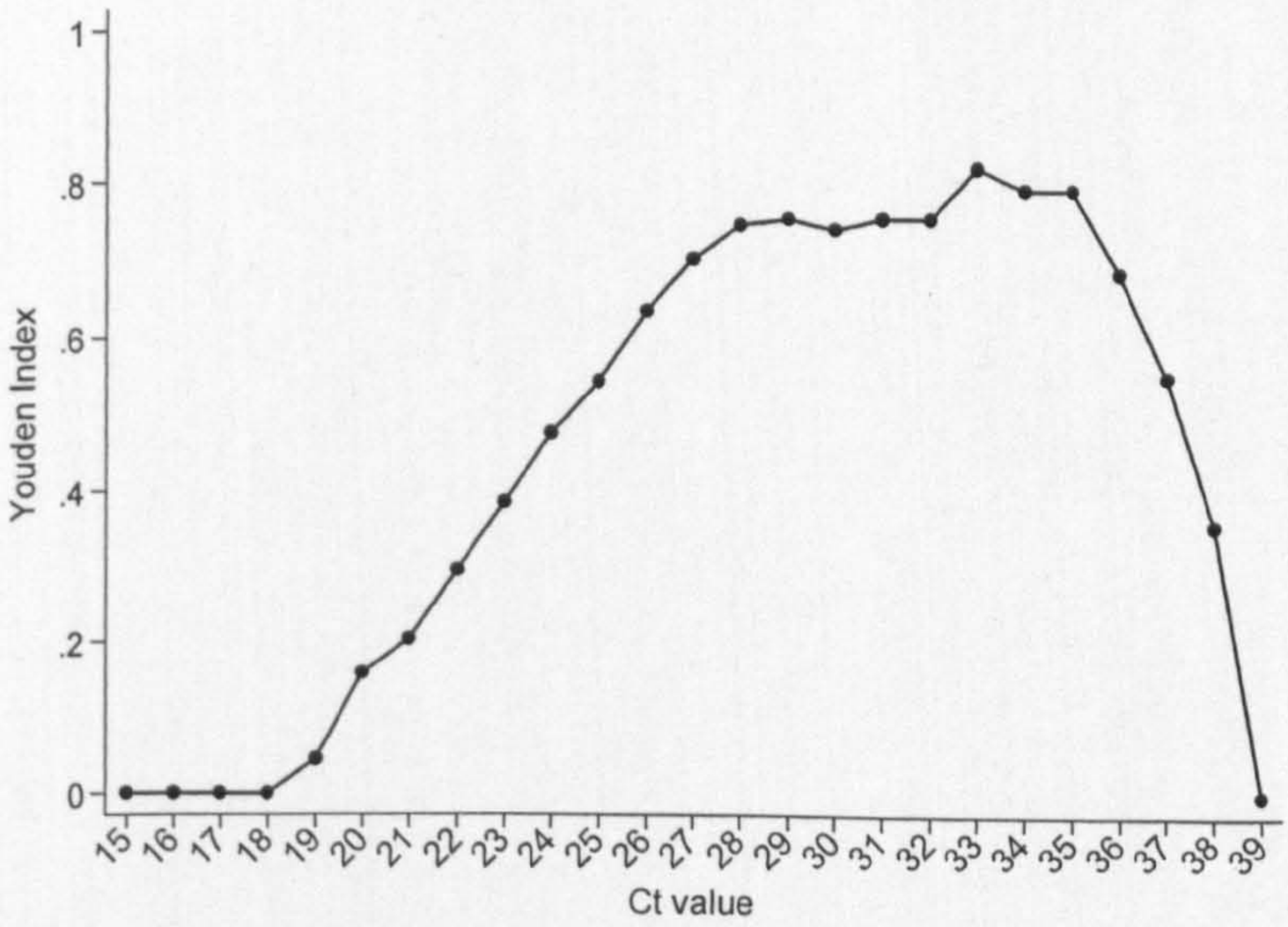
Appendix 2.7. ROC analysis for genogroup II, children and adults aged five years and older, reference positive group 1 and reference negative group 1.

Figure A2.7a Distribution of Ct values in reference positive group 1 and reference negative group 1. Grey bars show reference positive group 1 (n=44); white bars show reference negative group 1 (n=67).



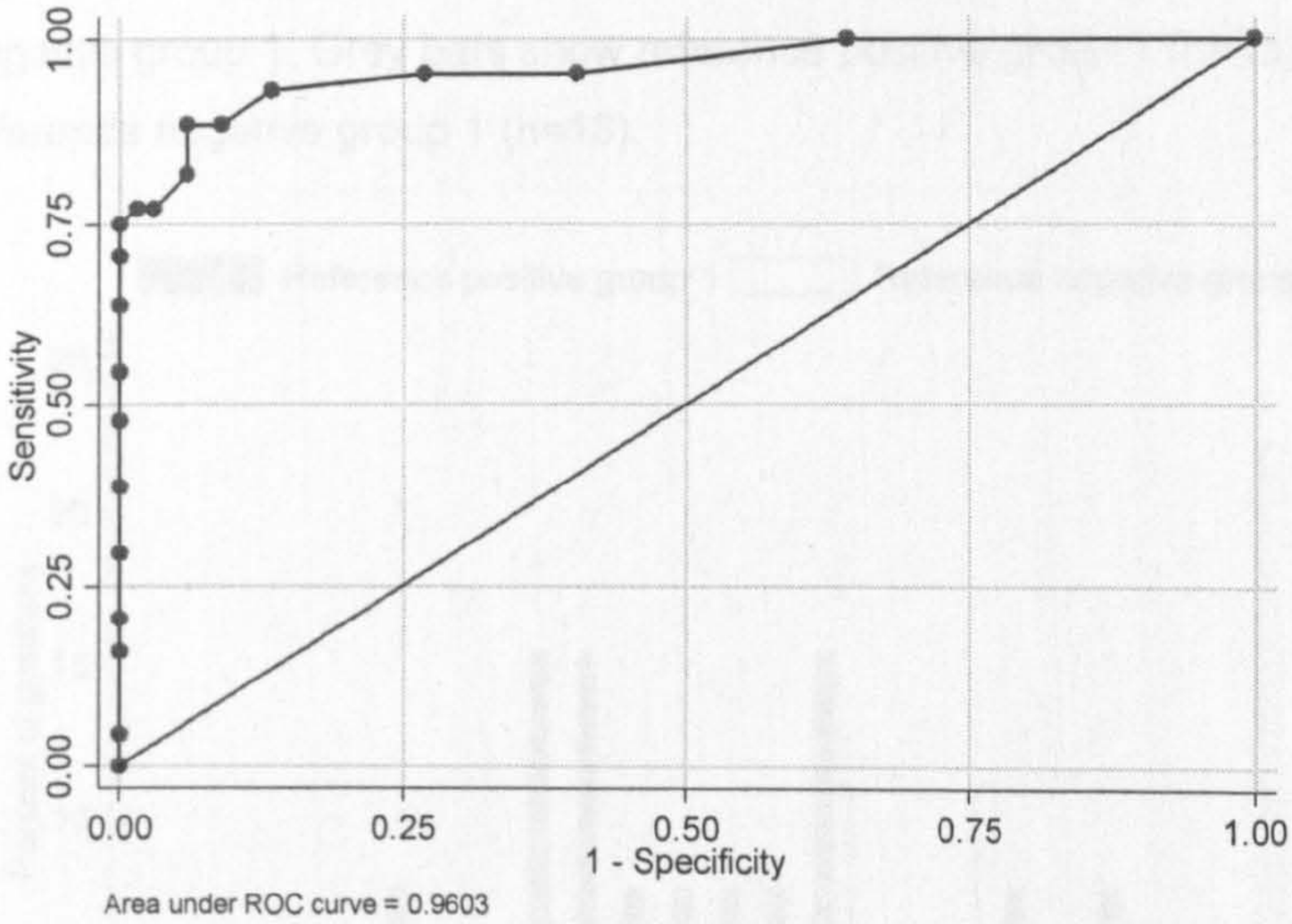
Abbreviations: Ct, cycle threshold.

Figure A2.7b Youden index.



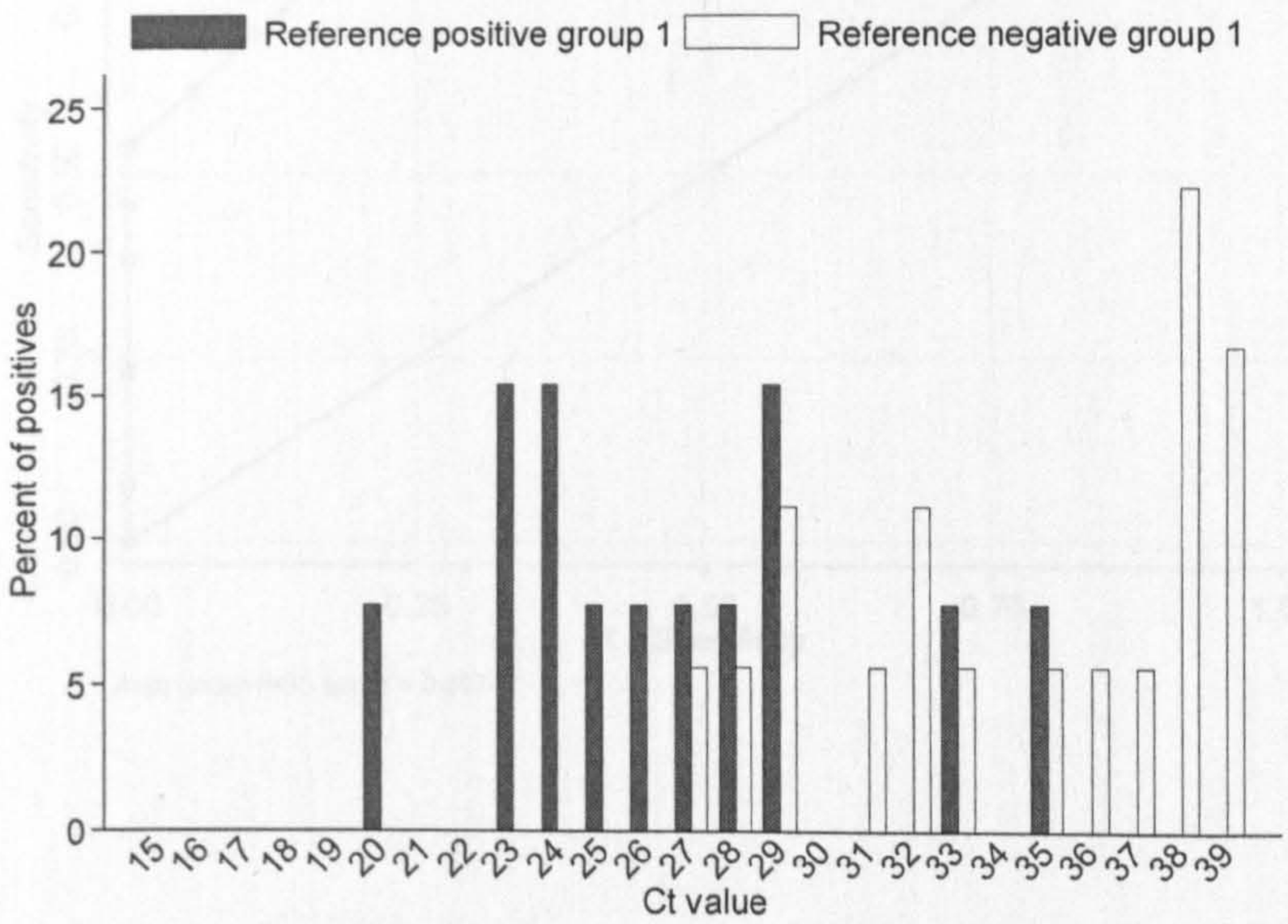
Abbreviations: Ct, cycle threshold.

Figure A2.7c ROC curve.



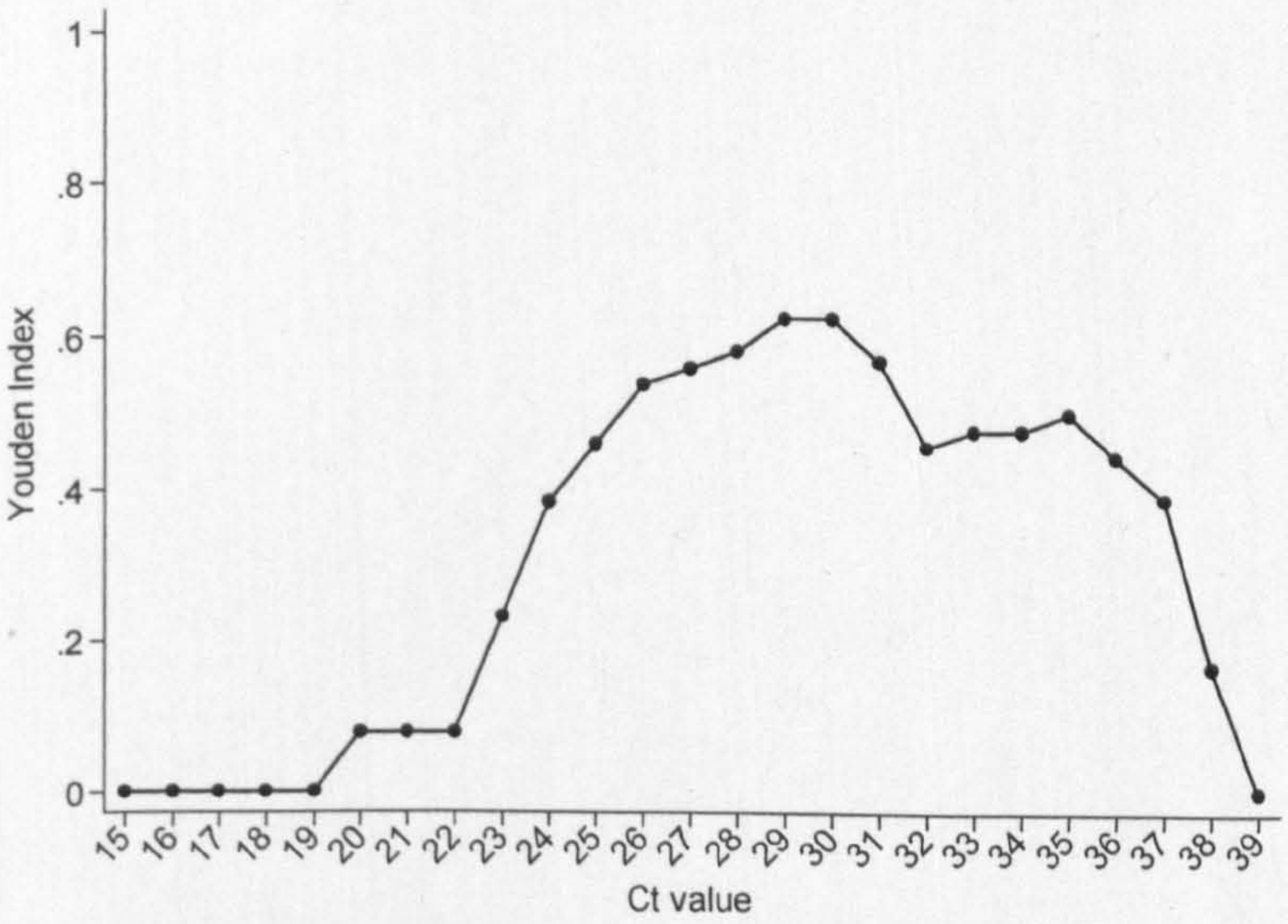
Appendix 2.8. ROC analysis for genogroup I, all ages, reference positive group 1 and reference negative group 1

Figure A2.8a Distribution of Ct values in reference positive group 1 and reference negative group 1. Grey bars show reference positive group 1 (n=13); white bars show reference negative group 1 (n=18).



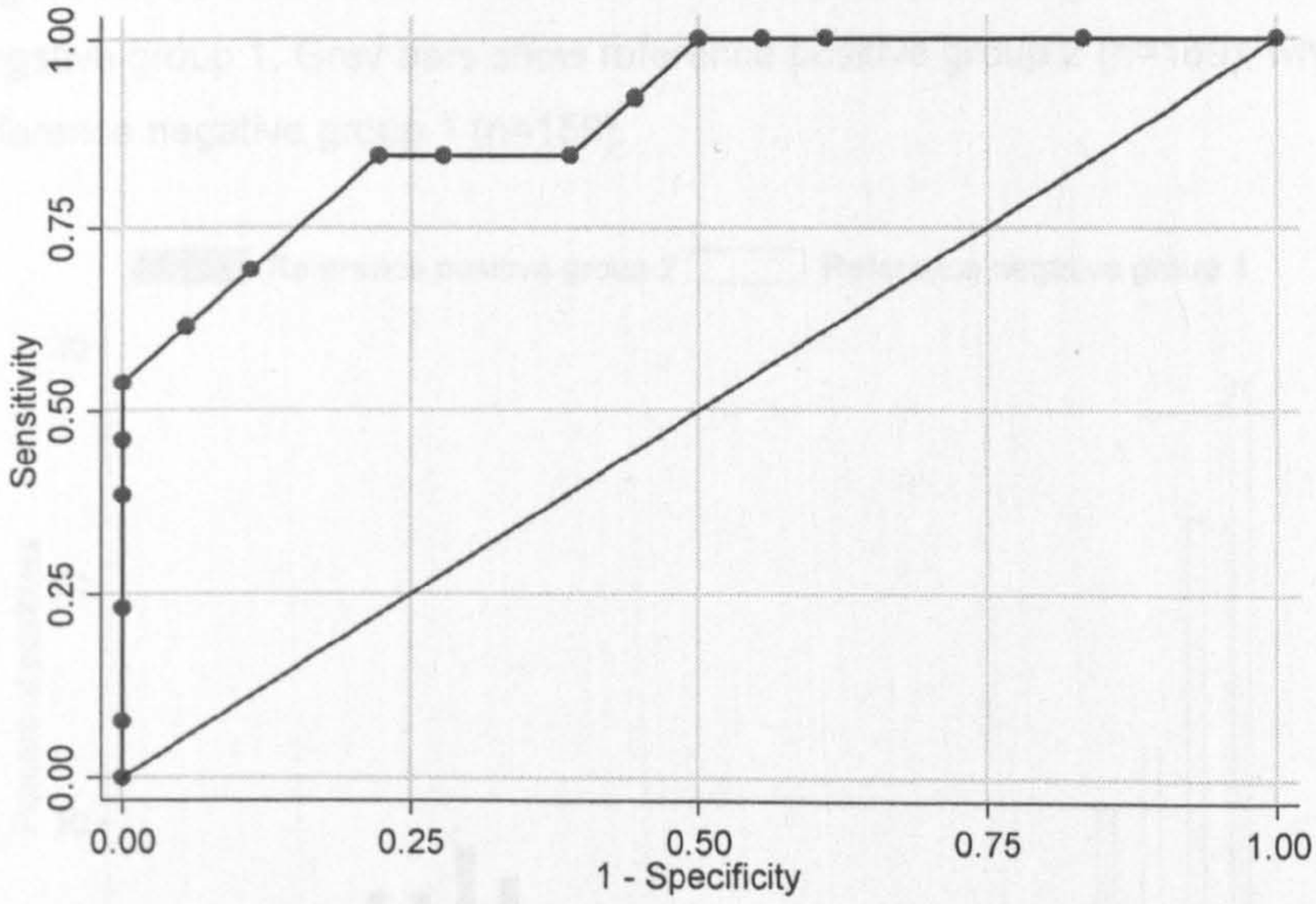
Abbreviations: Ct, cycle threshold.

Figure A2.8b Youden index.



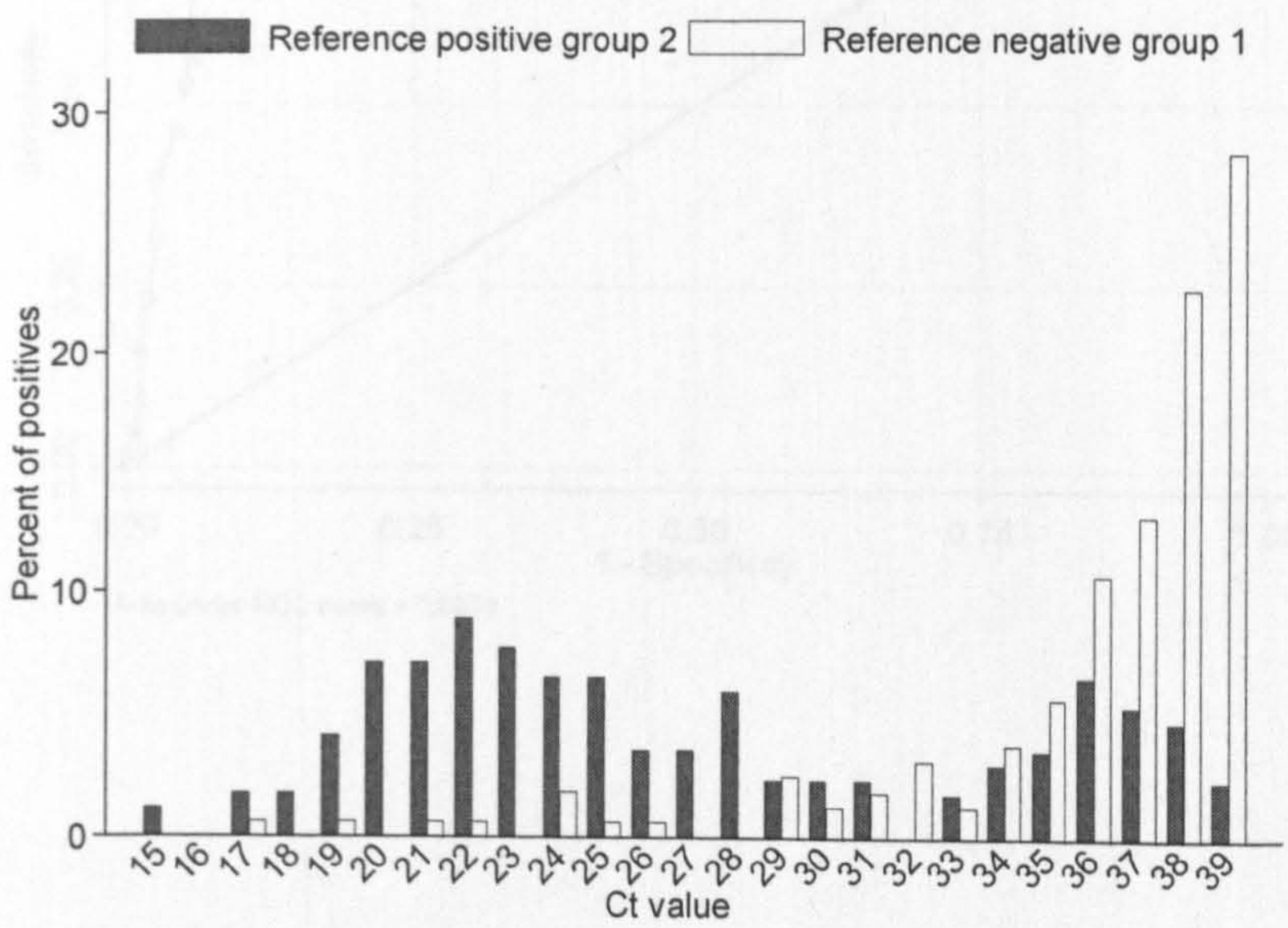
Abbreviations: Ct, cycle threshold.

Figure A2.8c ROC curve.



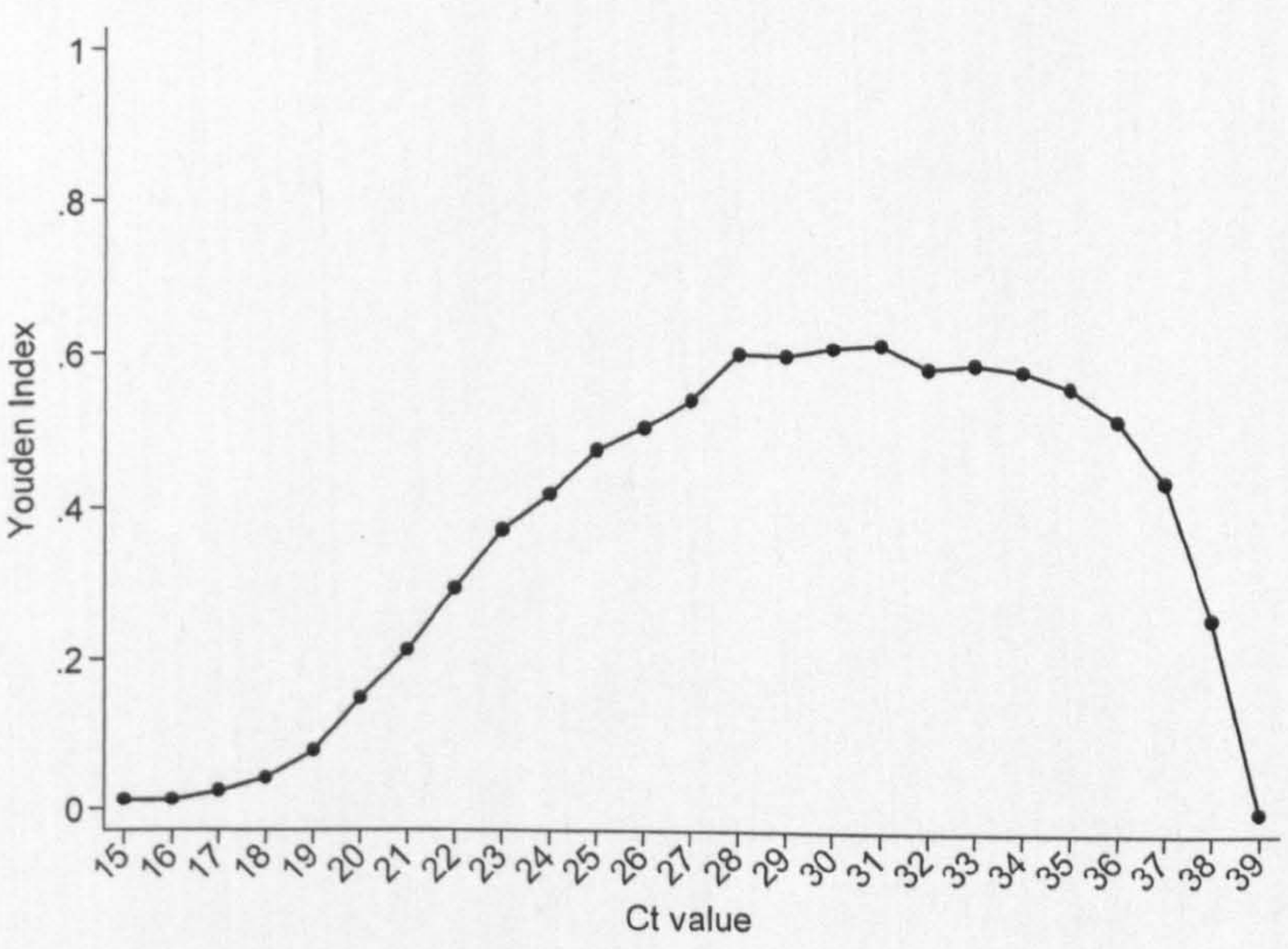
Appendix 2.9. ROC analysis for genogroup II, all ages, reference positive group 2 and reference negative group 1.

Figure A2.9a Distribution of Ct values in reference positive group 2 and reference negative group 1. Grey bars show reference positive group 2 (n=169); white bars show reference negative group 1 (n=159).



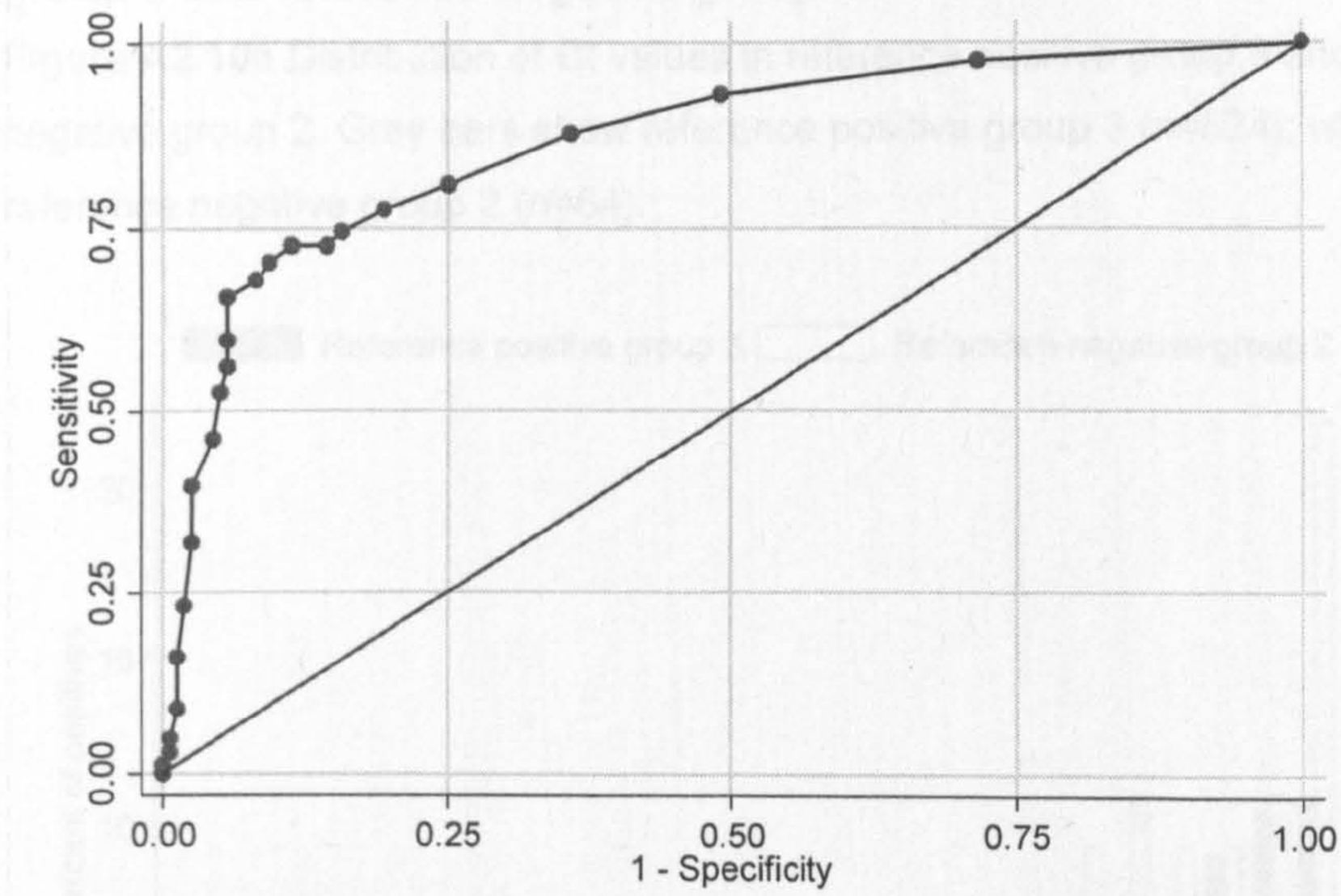
Abbreviations: Ct, cycle threshold.

Figure A2.9b Youden index.



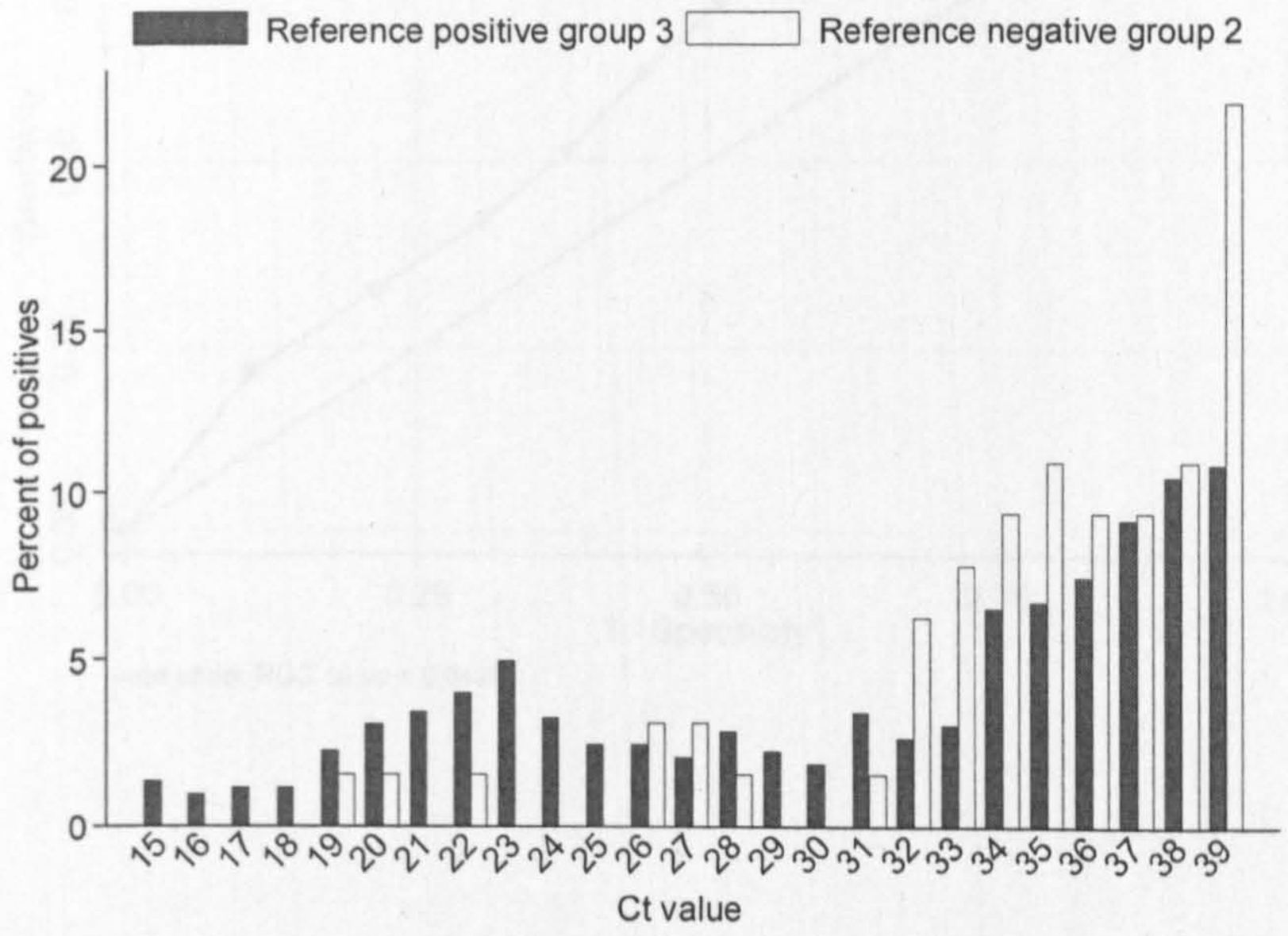
Abbreviations: Ct, cycle threshold.

Figure A2.9c ROC curve.



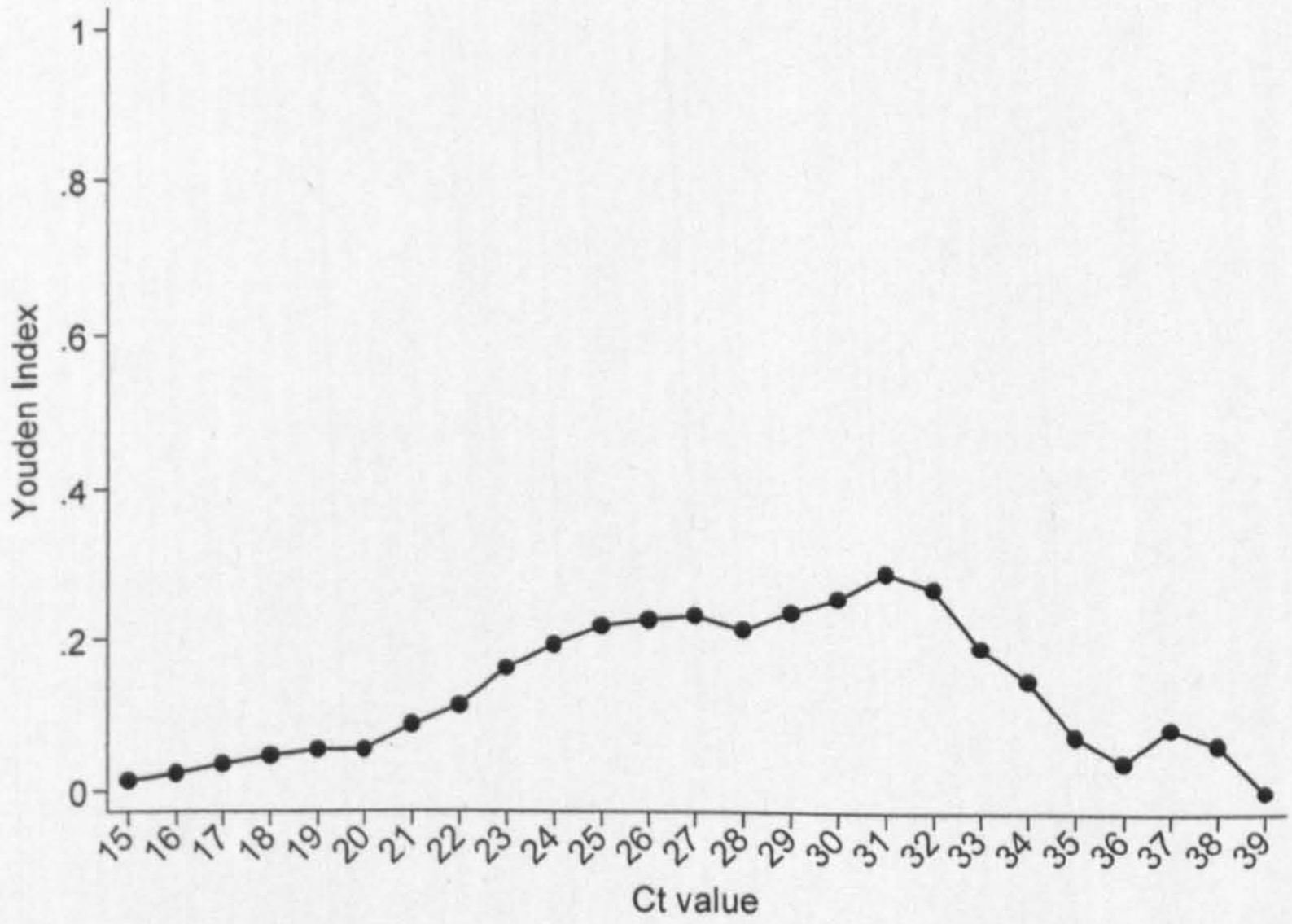
Appendix 2.10. ROC analysis for genogroup II, all ages, reference positive group 3 and reference negative group 2.

Figure A2.10a Distribution of Ct values in reference positive group 3 and reference negative group 2. Grey bars show reference positive group 3 (n=524); white bars show reference negative group 2 (n=64).



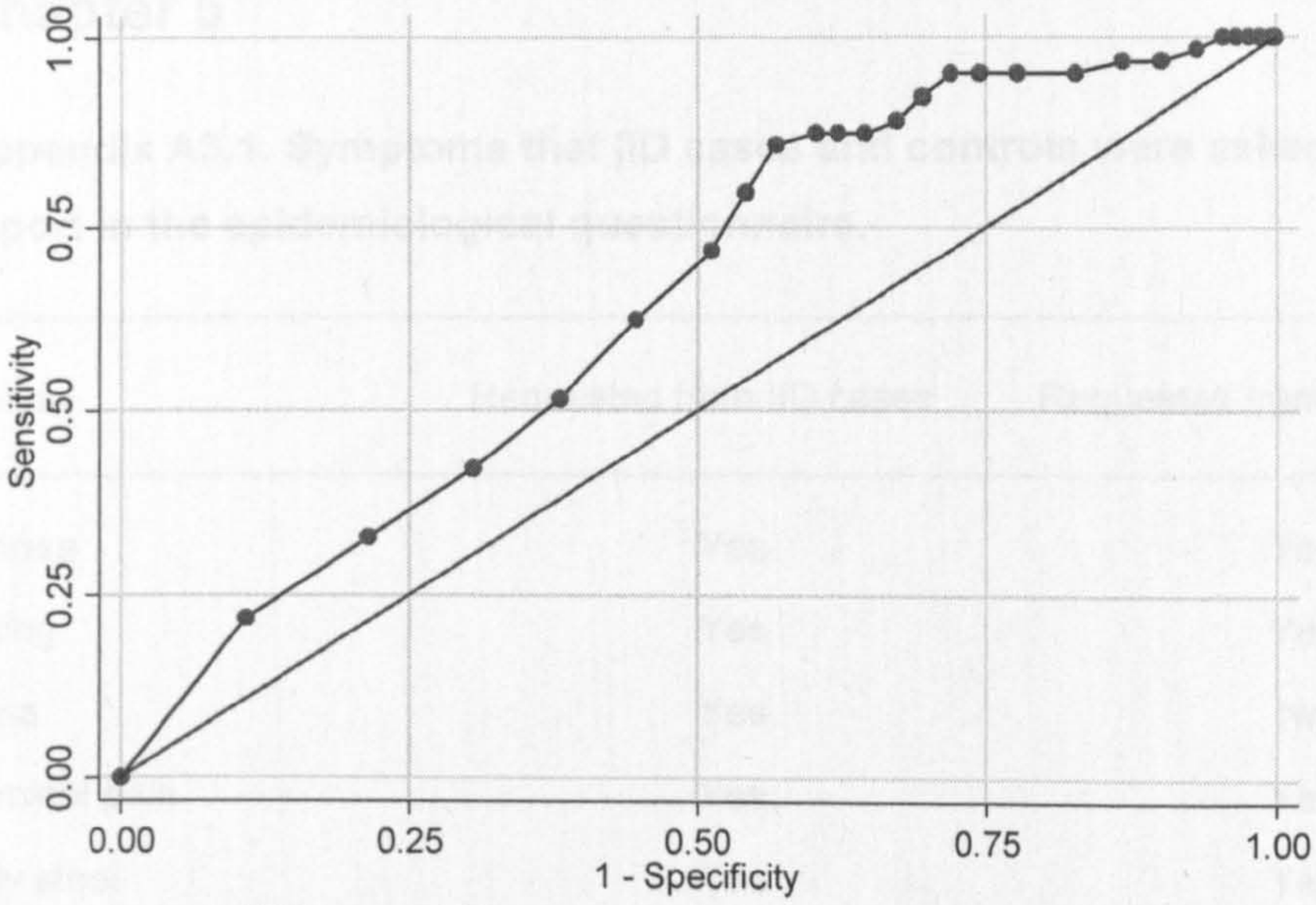
Abbreviations: Ct, cycle threshold.

Figure A2.10b Youden index.



Abbreviations: Ct, cycle threshold.

Figure A2.10c ROC curve.



Appendix 3: Additional information and results for Chapter 5

Appendix A3.1. Symptoms that IID cases and controls were asked to report in the epidemiological questionnaire.

Symptom	Requested from IID cases	Requested from Controls
Diarrhoea	Yes	Yes
Vomiting	Yes	Yes
Nausea	Yes	No
Abdominal pain	Yes	Yes
Bloody stool	Yes	Yes
Loss of appetite	Yes	Yes
High temperature	Yes	No
Cough, runny/blocked nose, sore throat	Yes	Yes
Headache	Yes	Yes
Aching muscles	Yes	Yes
Joint pains /stiffness	Yes	Yes
Back or neck pain /stiffness	Yes	Yes
Joint swelling	Yes	Yes
Painful red eyes	Yes	Yes
Dizziness /faintness	Yes	Yes

Appendix A3.2. Seasonality of asymptomatic norovirus infection.

Figure A3.2a Seasonality of asymptomatic norovirus infection in children aged less than five years.

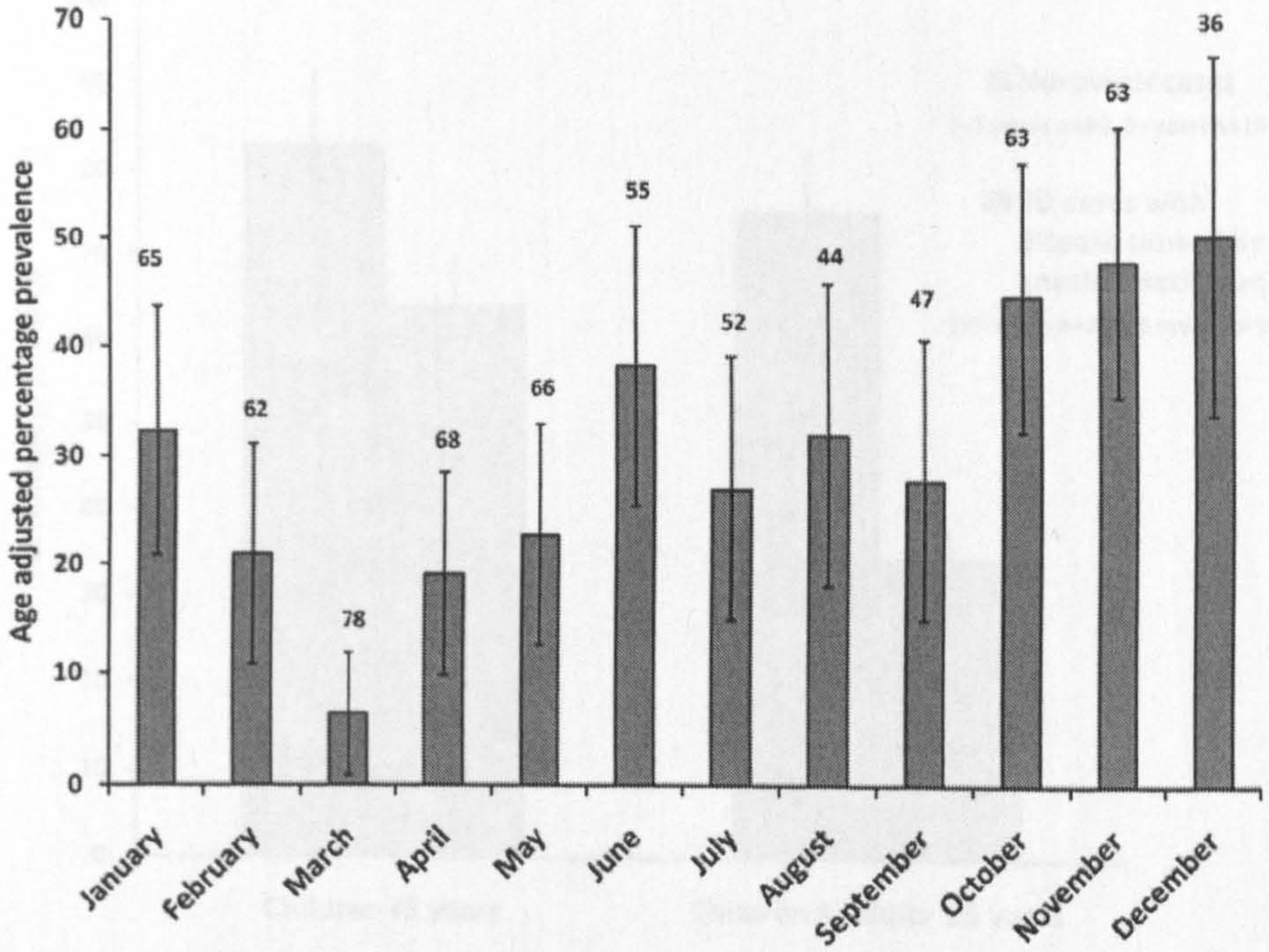
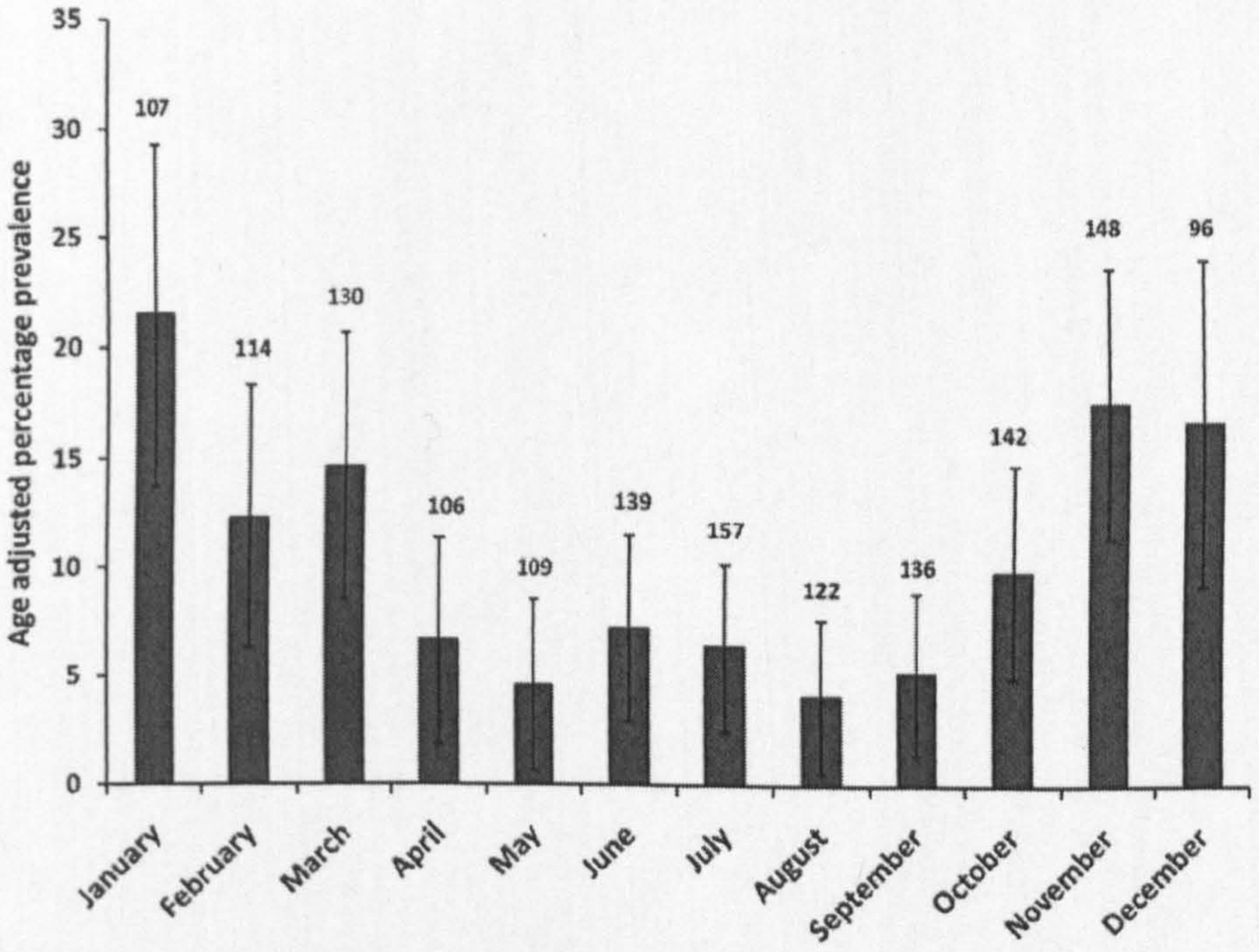
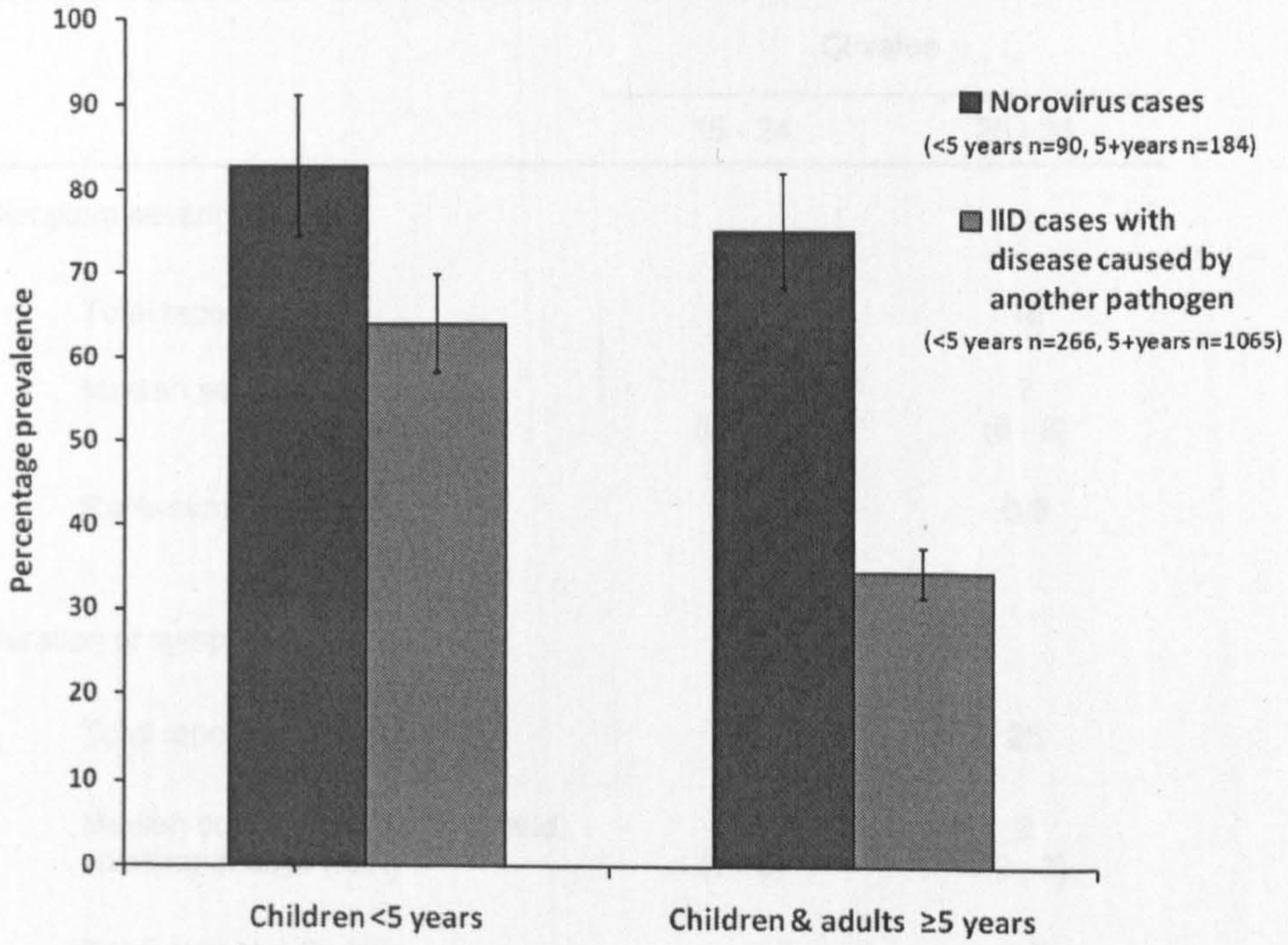


Figure A3.2b Seasonality of asymptomatic norovirus infection in children and adults aged five years and older.



Appendix A3.3. Prevalence of vomiting in norovirus cases and IID cases with disease caused by another pathogen.



Appendix A3.4. Variation in symptom severity and duration with norovirus viral load in norovirus cases.

	Ct value	
	15 - 24	25 - 33
Symptom severity		
Total reporting	23	16
Median severity score (IQR)	7 (6 - 8)	7 (6 - 8)
Rank-sum test *P* value		0.9
Duration of symptoms		
Total reporting	36	25
Median duration of diarrhoea and vomiting in days (IQR)	2 (1 - 3)	2 (2 - 3)
Rank-sum test *P* value		0.3

Abbreviations: Ct, cycle threshold; IQR, interquartile range.

Appendix A3.5. Comparison of symptoms in norovirus cases who were positive by electron microscopy and norovirus cases diagnosed only by the Ct value cut-off.

Figure A3.5 Prevalence of gastrointestinal symptoms.

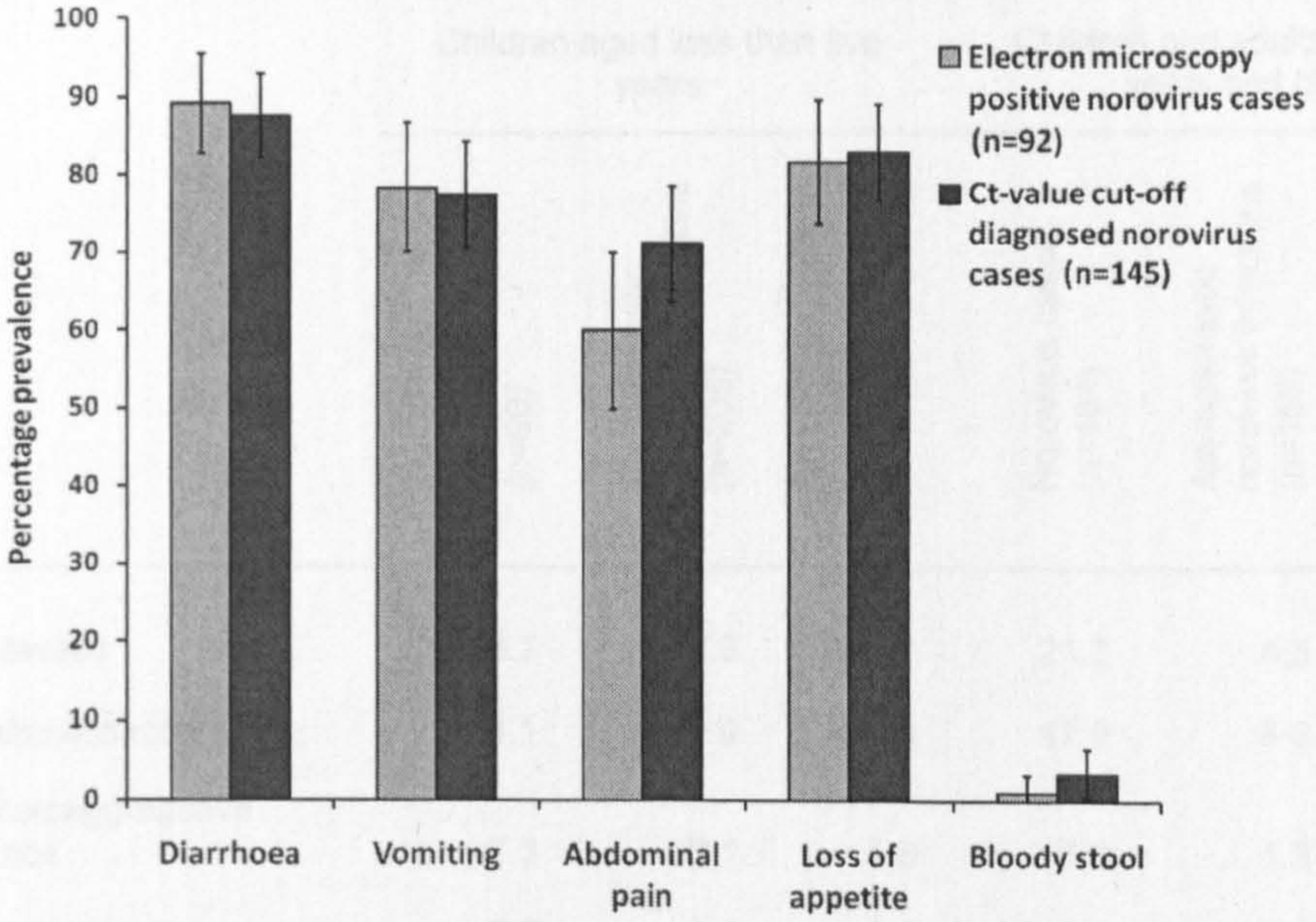


Table A3.5 Severity and duration of symptoms in norovirus cases. Rank-sum test is comparing the difference in score/duration in the two groups of norovirus cases.

	Electron microscopy positive	Ct value cut-off diagnosed
Symptom severity		
Total reporting	47	67
Median severity score (IQR	7 (6 - 9)	7 (6 - 8)
Rank-sum test *P* value		0.8
Duration of symptoms		
Total reporting	69	109
Median duration of diarrhoea and vomiting in days (IQR)	3 (2 - 4)	3 (2 - 5)
Rank-sum test *P* value		0.6

Abbreviations: Ct, cycle threshold; IQR, interquartile range.

Appendix A3.6. Percent prevalence of additional pathogens in norovirus cases, asymptomatic norovirus infections and norovirus negative controls.

	Children aged less than five years			Children and adults aged five years and older		
	Norovirus cases (n=90)	Asymptomatic norovirus infections (n=205)	Norovirus negative controls (n=494)	Norovirus cases (n=184)	Asymptomatic norovirus infections (n=156)	Norovirus negative controls (n=1350)
Rotavirus	36.7	27.3	21.7	21.2	4.5	10.5
Campylobacter spp.	21.1	2.0	4.5	17.9	8.3	5.3
Enteroaggregative *E. coli*	7.3	2.1	1.6	7.6	1.3	2.2
Salmonella spp.	2.2	1.0	0.8	3.8	0.0	0.7
Aeromonas spp.	7.8	4.4	7.5	2.7	0.6	3.9
Cl. perfringens	3.3	0.5	1.2	1.6	0.0	0.7
Sapovirus	6.7	3.4	1.4	0.5	5.1	1.3
Adenovirus	2.2	1.0	0.4	0.5	0.0	0.0
Cryptosporidium spp.	1.1	0.0	1.0	0.0	0.0	0.5
Astrovirus	3.3	1.5	0.2	0.5	0.0	0.0
Yersinia spp.	2.2	2.0	3.6	3.3	0.0	2.6
Giardia spp.	1.1	2.0	2.0	0.5	0.0	1.3
Cl. difficile	4.4	5.4	5.3	0.5	0.0	0.2

Appendix 4: Additional information and results for Chapter 6

Appendix A4.1. Questions from the baseline and epidemiological questionnaires used in the risk factor analysis.

The exposure period for proximal risk factor reporting by IID cases was the 10 days before their illness started and 10 days before questionnaire completion for controls. Q is questionnaire item; R is response options.

Variable	Questionnaire Item
Social class	Answered by respondent or the main wage earner in the house What is your occupation? (if unemployed, what was your most recent occupation; if retired what was your main occupation?) R: Job title and job description including business/industry of employment This information was used by the study team to assign IID cases and controls to a Standard Occupational Class[564].
Household size (number of people)	Number of permanent household members listed when reporting details of household infectious contacts (see below), plus the respondent.
Household age structure (number of children <5 years)	Number of permanent household members listed when reporting details of infectious contacts (see below) who were aged less than five years.

Variable	Questionnaire Item
Household crowding (number of people per room)	Number of permanent household members listed when reporting details of infectious contacts (see below) divided by the total number of rooms reported in the question: Q: How many rooms does your household have? (exclude WC/toilet, hall and landing.) R: Number
Baby in nappies in the household	Q: Do you still have a baby in nappies? R: Yes/No
Pet ownership	Q: Do you have any pets? R: Yes/No
Sharing a bathroom or toilet with another household	Q: Does any other household share the bathroom and/or WC with you? R: Yes/No

Variable	Questionnaire Item
Nursery/day care attendance (Child questionnaire only)	Q: Does your child attend any of the following?
	R: Day nursery/crèche
	Toddlers' play group
	Child minder
	Nursery school or class
	Local Authority school
	Private school
	Other (specified in free text)
Breast feeding (Child questionnaire only)	Q: Is your child less than 1 year old?
	R: Yes/No
	Q: How is your baby currently being fed milk?
	R: Breast feed only
	Bottle feed only
	Mixed breast/bottle feed
	Other

Variable	Questionnaire Item
Hand hygiene	Q: Please indicate whether you think the following statement is true or false (tick one box): It doesn't matter whether you wash your hands or not before handling food. R: Agree/Disagree/Don't know
Water sports	Q: During the *10 days before your illness started* (for cases)/ *last 10 days* (for controls) did you go swimming or join in water sports (e.g. sailing, water skiing) in the UK or abroad? R: Yes/No
Foreign travel	Q: During the *10 days before your illness started* (for cases)/ *last 10 days* (for controls) did you spend one or more nights away from home? R: Yes/No Q: If yes, were you staying in the UK or abroad? R: In the UK/Abroad

Variable	Questionnaire Item
Animal contact	Q: Were you in contact with any animals [other than pets] (e.g. zoo, farm, other people's pets) in the *10 days before your illness started* (for cases)/*last 10 days* (for controls)? R: Yes/No
Food (raw fruit/vegetables/shellfish/meals prepared outside home)	Q: Did you eat any of the following foods in the *10 days before your illness started* (for cases)/*last 10 days* (for controls)? Raw salad/vegetable/coleslaw prepared at home Raw salad/vegetable/coleslaw from a shop Raw salad/vegetable/coleslaw eaten at a restaurant Apple/pear/peach/nectarine/grapes Oysters Cockles/mussels/clams/whelks/winkles Any meals prepared outside the home R: Yes/not sure (respondents left blank if foods were not eaten)

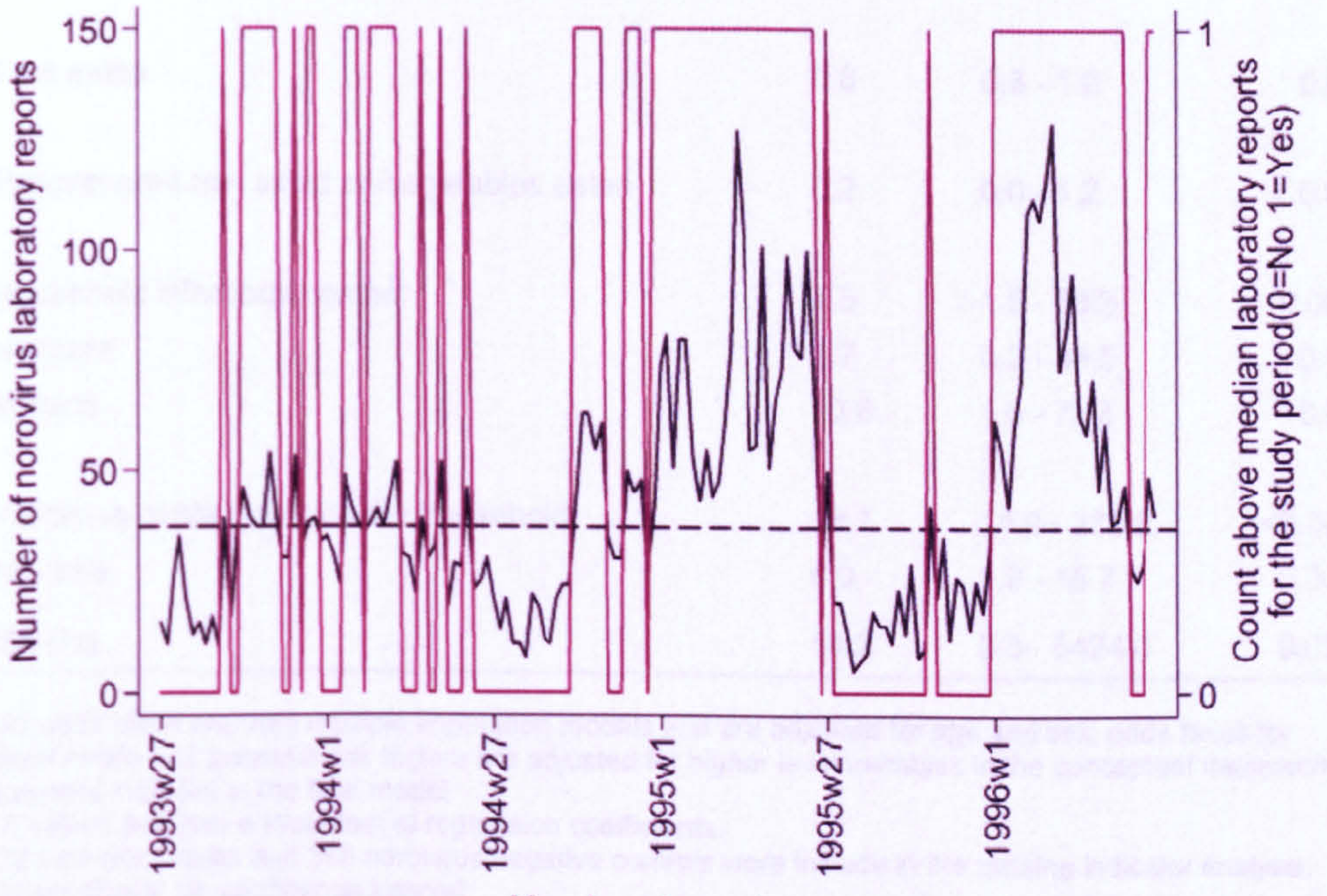
Variable	Questionnaire Item
Household infectious contact	IID cases and controls were asked to provide details of any individuals who lived in or stayed a night in their household during the *10 days before their illness* (for cases)*/ last 10 days* (for controls): Age Sex Whether they were a permanent household member or a visitor Whether they had diarrhoea or vomiting symptoms during this 10 day period (yes/no/not sure)
Infectious contact outside the household	Q: Did you have contact in the *10 days before your illness started* (for cases)*/last 10 days* (for controls) with any other people outside the household how you know were suffering with diarrhoea or vomiting? R: Yes/No/Not sure

Appendix A4.2. Definition of the Norovirus Season.

Reports of norovirus diagnoses in England and Wales from the Health Protection Agency National Surveillance of Laboratory-Confirmed Infections were used to identify the times of peak norovirus activity between 1993 and 1996, during the Study of Infectious Intestinal Disease. Further details about the norovirus laboratory report data are provided in Chapter 3.

The weekly counts of norovirus laboratory reports between 1993 and 1996 are shown in Figure A4.2. The start of the norovirus season was defined as the first of five consecutive weeks when the number of norovirus laboratory reports was above the median for the whole study period (the dashed black line in Figure A4.2). The end of the norovirus season was defined as the first subsequent week of five consecutive weeks when the number of laboratory reports was below the median for the whole study period.

Figure A4.2 Weekly norovirus laboratory reports in England and Wales during the Study of Infectious Intestinal Disease (1993 to 1996).



[a] Red line indicates when the number of laboratory reports is above the median for the study period.
[b] The black dashed line shows the median number of weekly laboratory reports for the study period.
[c] The black solid line shows the weekly count of norovirus laboratory reports.

Appendix A4.3. Risk Factors for Norovirus IID in Children Aged Less Than Five Years in England, 1993 to 1996, From the Missing Indicator Analysis.

	Odds Ratio[a]	95% CI	*P* value[b]
Social class			
Non-manual	1.0		
Manual/Unskilled	2.9	1.6 - 5.1	<0.001
Military	3.6	0.3 - 38.1	0.29
Housewife/student/carer	4.1	1.1 - 15.2	0.03
Missing	3.8	1.2 - 12.1	0.02
Foreign travel	10.2	0.9 - 109.4	0.06
Missing	2.0	0.1 - 26.1	0.61
Animal Contact	0.5	0.3 - 1.0	0.03
Not sure	0.8	0.1 - 8.5	0.83
Missing	0.2	0.0 - 1.4	0.10
Fruit eaten	0.6	0.3 - 1.0	0.06
Pre-prepared raw salad or /vegetables eaten	0.2	0.0 - 1.2	0.07
Household infectious contact	4.5	1.5 - 13.3	0.006
Not sure	2.7	0.2 - 34.5	0.45
Missing	10.8	1.6 - 72.5	0.02
Infectious contact outside the household	58.7	14.9 - 230.9	<0.001
Not sure	6.0	1.9 - 18.7	0.002
Missing	54.2	0.5 - 5424.0	0.089

[a] All odds ratios are from multiple imputation models and are adjusted for age and sex; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model.
[b] *P* values are from a Wald test of regression coefficients.
[c] 72 norovirus cases and 348 norovirus negative controls were include in the missing indicator analysis.
Abbreviations: CI, confidence interval.

Appendix A4.4. Risk Factors for Norovirus IID in Older Children and Adults (Aged Five Years and Older) in England, 1993 to 1996, From the Missing Indicator Analysis.

	Odds Ratio[a]	95% CI	*P* value[b]
Household structure			
Single person household	0.9	0.4 - 2.0	0.77
Adults and children aged ≥5 years only	1.0		
≥1 children aged <5 years	2.4	1.5 - 3.8	<0.001
Missing	1.8	1.0 - 3.4	0.05
Baby wearing nappies in the household	2.8	1.8 - 4.5	<0.001
Missing	0.9	0.3 - 3.0	0.84
Water sports in last 10 days	0.5	0.3 - 0.8	0.01
Missing	1.4	0.6 - 3.1	0.44
Foreign travel	3.3	1.4 - 7.5	0.005
Missing	0.6	0.1 - 2.3	0.44
Animal Contact	0.4	0.3 - 0.7	<0.001
Not sure	3.6	1.1 - 12.6	0.03
Missing	1.6	0.6 - 3.7	0.32
Oysters eaten	16.6	1.3 - 206.4	0.03
Whelks/winkles eaten	19.5	1.5 - 251.2	0.03
Fruit eaten	0.6	0.4 - 1.0	0.03
Household infectious contact	4.9	2.7 - 9.0	<0.001
Not sure	2.0	0.6 - 6.7	0.25
Missing	2.9	1.1 - 7.5	0.03
Infectious contact outside the household	4.8	2.6 - 8.8	<0.001
Not sure	2.1	0.6 - 6.8	0.23
Missing	2.9	1.1 - 7.5	0.02

[a] All odds ratios are from multiple imputation models and are adjusted for age, sex and social class; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model, except the presence of a baby in the household.
[b] *P* values are from a Wald test of regression coefficients.
[c] 152 norovirus cases and 1097 norovirus negative controls were include in the missing indicator analysis.
Abbreviations: CI, confidence interval

Appendix A 4.5. Association of Asymptomatic Norovirus Infection With Variables Associated With Norovirus-associated IID

Table A4.5a Children Aged Less Than Five Years (Multiple Imputation Model).

	Exposure prevalence				
	Asymptomatic norovirus infections	Norovirus negative controls	Odds Ratio[a]	95% CI	*P* value[b]
Total	193	461			
Social class					
Non-manual	59.1	56.8	1.0		
Manual/Unskilled	33.2	35.6	0.9	0.6 - 1.2	0.4
Military	1.5	0.9	1.7	0.4 - 7.6	0.5
Housewife/student/carer	1.5	2.6	0.7	0.2 - 2.4	0.6
Missing	4.7	4.1			
Foreign travel	0.5	0.9	0.6	0.1 - 5.6	0.6
Missing	2.1	1.3			
Animal Contact	43.5	44.5	0.9	0.7 - 1.4	0.8
Not sure	1.0	2.0	0.5	0.1 - 2.6	0.4
Missing	2.6	3.0			
Fruit eaten	71.0	75.9	0.8	0.5 - 1.2	0.3
Pre-prepared raw salad or /vegetables eaten	6.7	7.2	1.0	0.5 - 2.0	1.0
Household infectious contact	11.9	9.3	1.2	0.6 - 2.4	0.6
Not sure	2.1	1.3	2.6	0.5 - 13.8	0.3
Missing	2.6	3.3			
Infectious contact outside the household	11.4	6.7	1.9	0.9 - 4.0	0.1
Not sure	13.5	13.9	0.8	0.4 - 1.4	0.4
Missing	0.5	0.7			

[a] All odds ratios are from multiple imputation models and are adjusted for age and sex; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model.
[b] *P* values are from a Wald test of regression coefficients.
Abbreviations: CI, confidence interval.

Table A4.5b Older Children and adults (Multiple Imputation Model).

	Exposure prevalence				
	Asymptomatic norovirus infections	Norovirus negative controls	Odds Ratio[a]	95% CI	*P* value[b]
Total	156	1260			
Household structure					
Single person household	6.9	6.9	1.3	0.7 - 2.6	0.4
Adults and children aged ≥ years only	64.2	71.0	1.0		
≥1 children aged <5 years	18.5	14.1	1.4	0.8 - 2.2	0.2
Missing	8.6	8.0			
Baby wearing nappies in the household	12.6	9.0	1.3	0.7 - 2.3	0.4
Missing	2.7	2.9			
Foreign travel	2.0	2.5	1.0	0.3 - 3.5	1.0
Missing	2.0	2.4			
Animal Contact	38.4	36.3	1.1	0.7- 1.5	0.8
Not sure	-	1.0			
Missing	4.0	3.2			
Fruit eaten	78.2	82.5	0.8	0.5 - 1.2	0.2
Household infectious contact	6.6	6.7	0.9	0.4 - 2.0	0.8
Not sure	2.7	2.5	1.1	0.3 - 3.9	0.8
Missing	12.6	9.8			
Infectious contact outside the household	8.3	9.2	1.0	0.5, 1.9	1.0
Not sure	15.9	14.5	1.0	0.6, 1.7	0.9
Missing	1.3	1.7			

[a] All odds ratios are from multiple imputation models and are adjusted for age, sex and social class; odds ratios for intermediate and proximal risk factors are adjusted for higher level variables in the conceptual framework that were included in the final model, except the presence of a baby in the household.
[b] *P* values are from a Wald test of regression coefficients.
Abbreviations: CI, confidence interval.

Appendix 5: Additional information and results for Chapter 7

Appendix A5.1. Development of the confounder model for children aged less than five years.

The black line shows the observed weekly counts of general practice consultations for IID in children aged less than five years; the red line shows the fitted values from the model.

Figure A5.1a Model including eight Fourier terms

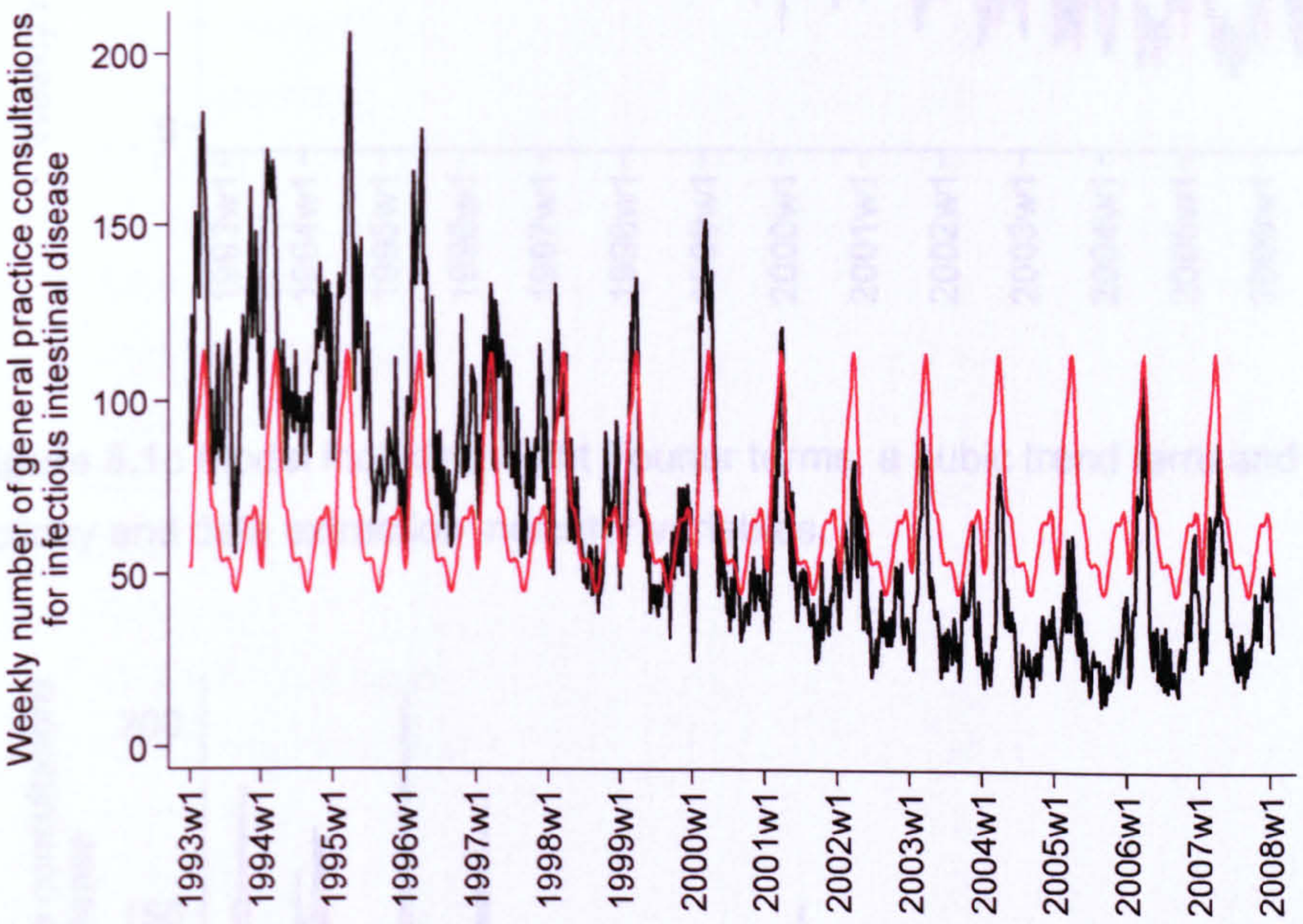


Figure 5.1b Model including eight Fourier terms and a cubic trend term.

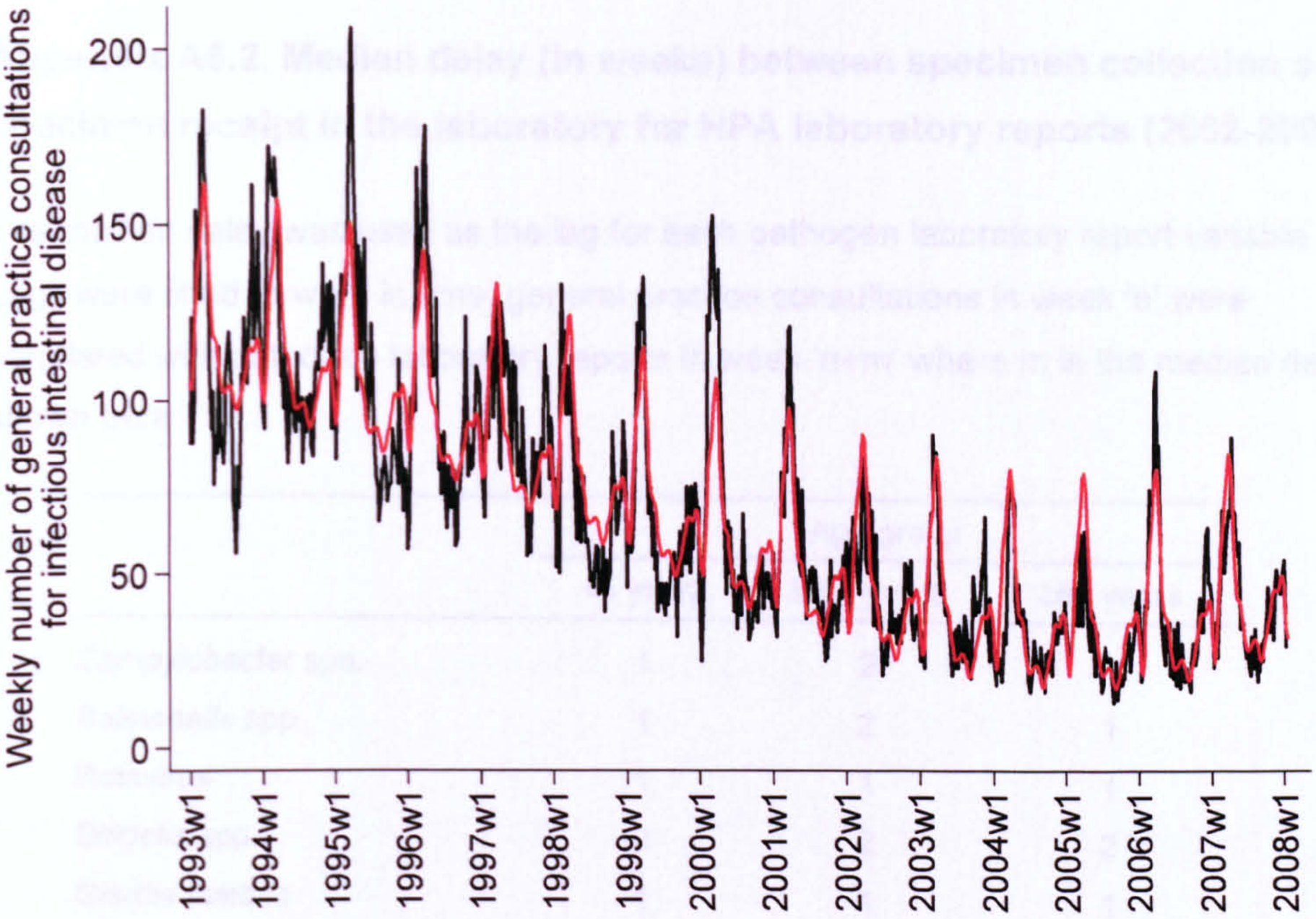
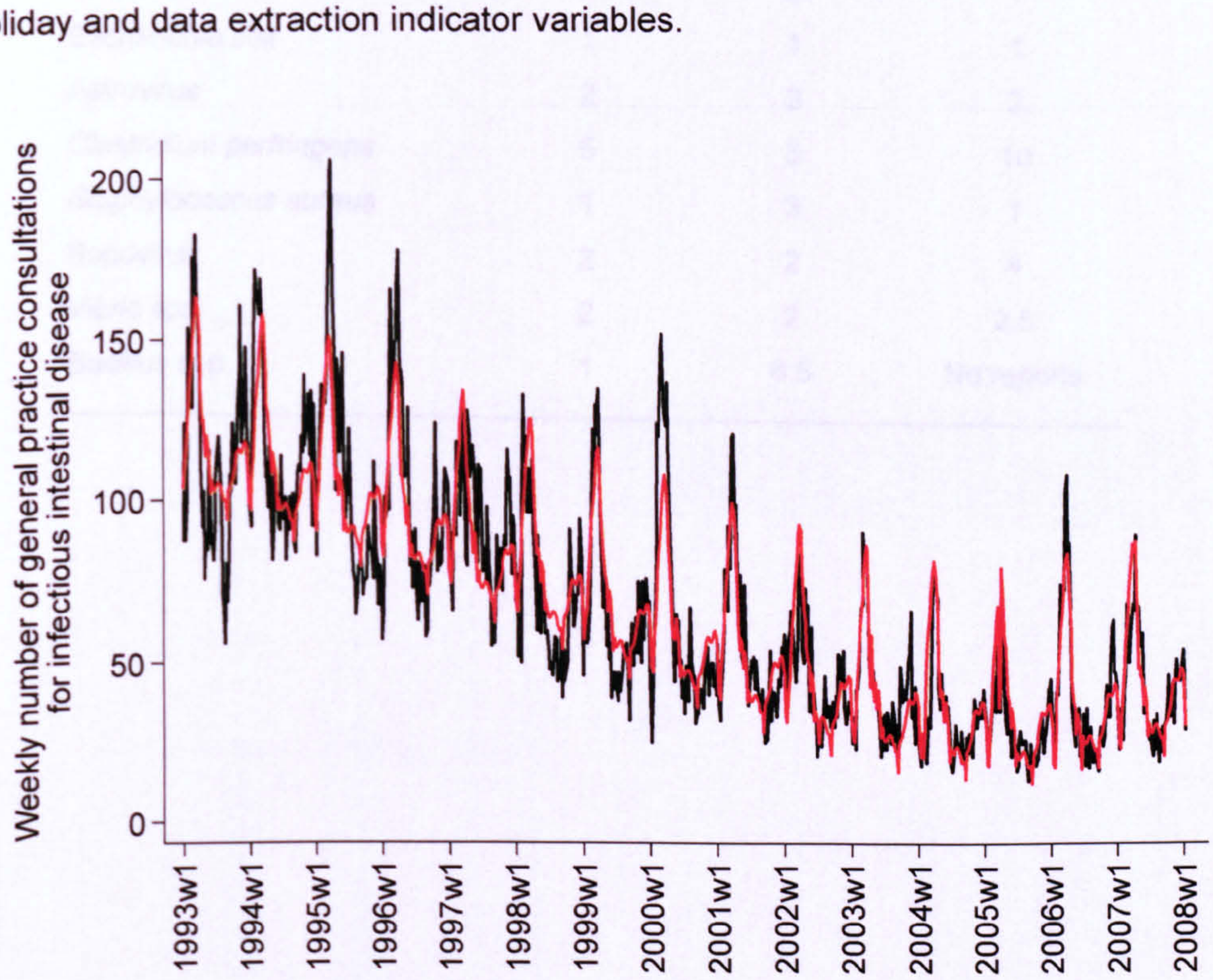


Figure 5.1c Model including eight Fourier terms, a cubic trend term and the bank holiday and data extraction indicator variables.



Appendix A5.2. Median delay (in weeks) between specimen collection and specimen receipt in the laboratory for HPA laboratory reports (2002-2007).

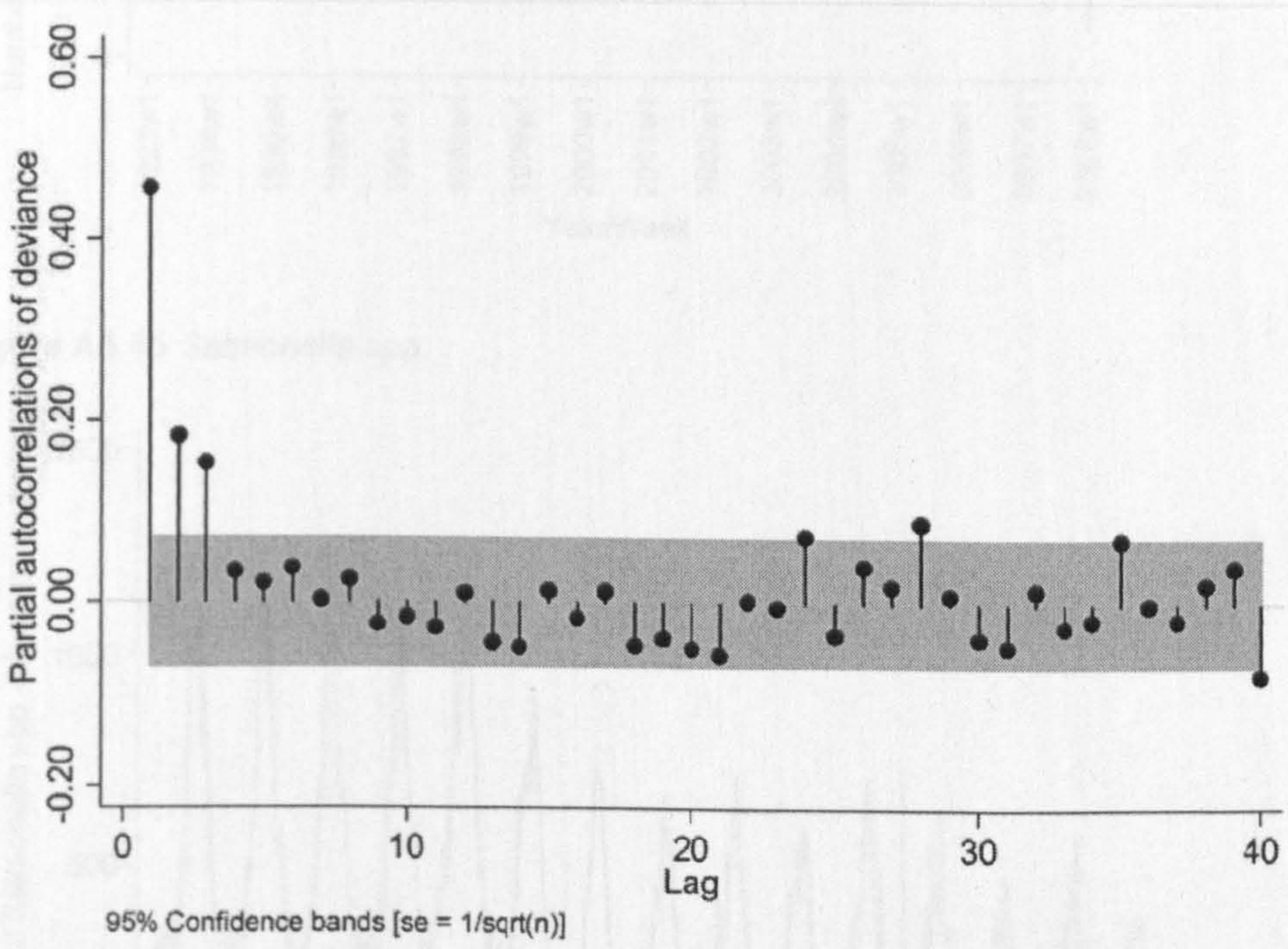
The median delay was used as the lag for each pathogen laboratory report variable. Lags were fitted forward in time; general practice consultations in week 'n' were correlated with pathogen laboratory reports in week 'n+m' where m is the median delay shown here.

	Age group		
	<5 years	5-64 years	≥65 years
Campylobacter spp.	1	2	1
Salmonella spp.	1	2	1
Rotavirus	1	1	1
Shigella spp.	2	2	2
Giardia lamblia	1	1	1
Cryptosporidium spp.	1	1	1
Adenovirus	1	1	1
Norovirus	2	2	1
Escherichia coli	1	1	1
Astrovirus	2	3	2
Clostridium perfringens	5	3	10
Staphylococcus aureus	1	3	1
Sapovirus	2	2	4
Vibrio spp.	2	2	2.5
Bacillus spp.	1	6.5	No reports

Appendix A5.3. Explanation of selection of autoregressive terms using the partial autocorrelation function

The partial autocorrelation (PAC) of the deviance residuals from a regression model of time series data shows the degree of correlation between the deviance residual in week 'n' with week 'n-x', adjusted for correlation in weeks with lower values of x. The PAC in week 'n-3' is adjusted for correlation between week 'n' and both weeks 'n-1' and 'n-2'. A significant PAC must lie outside the 95% confidence interval of zero, shown in grey on Figure A5.3. In Figure A5.3, there are three significant PAC terms at weeks 'n-1', 'n-2' and 'n-3', therefore the deviance residuals for each of the preceding three weeks would be fitted against the general practice consultations in week 'n' to adjust for autocorrelation.

Figure A5.3 Example of a PAC plot used to select the number of autoregressive terms to adjust for autocorrelation.



Appendix A5.4. Weekly counts of HPA pathogen laboratory reports, 1993 to 2007.

Only those pathogens with a median weekly count greater than one are shown.

Figure A5.4a *Campylobacter* spp.

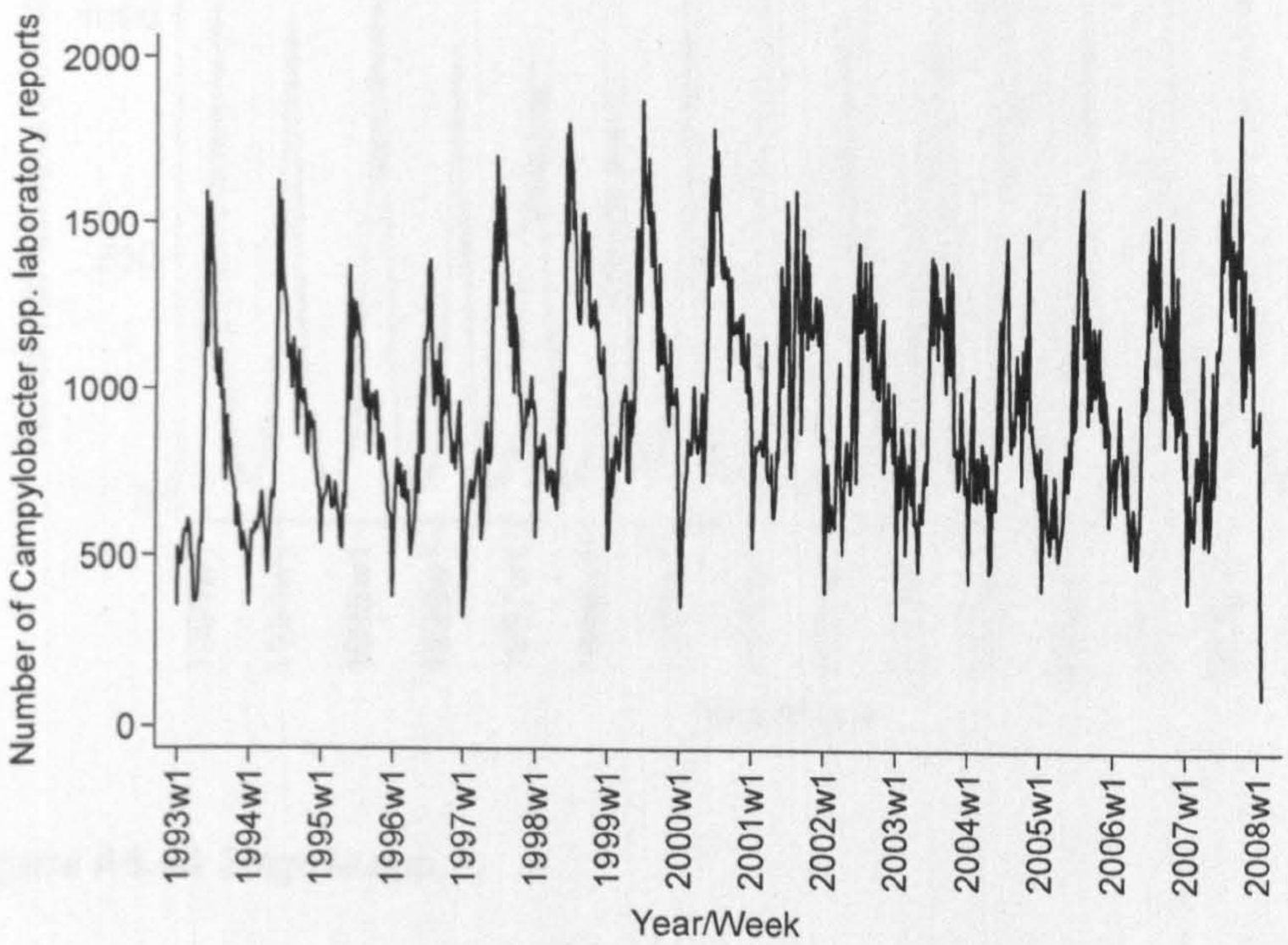


Figure A5.4b *Salmonella* spp.

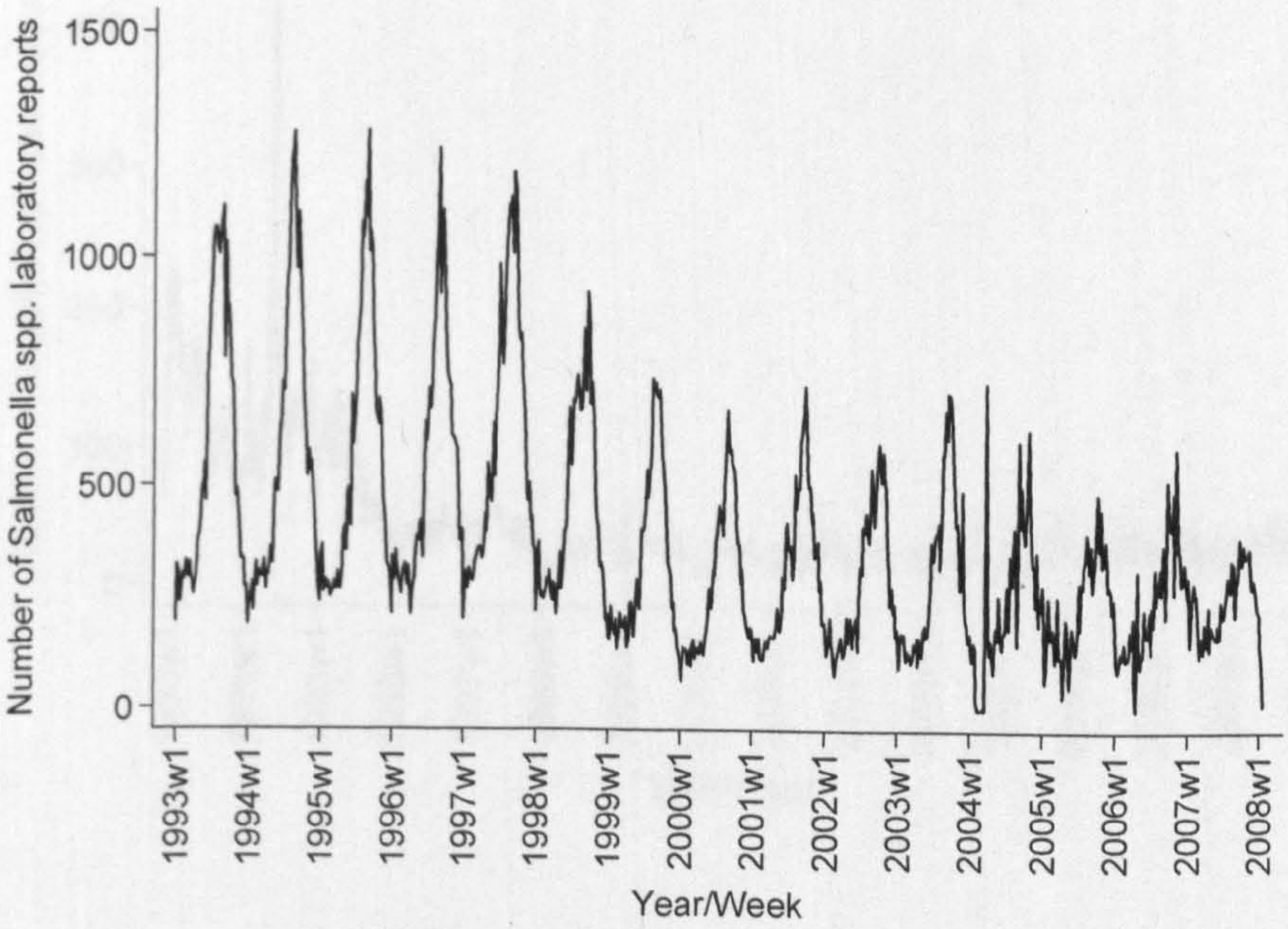


Figure A5.4c Rotavirus.

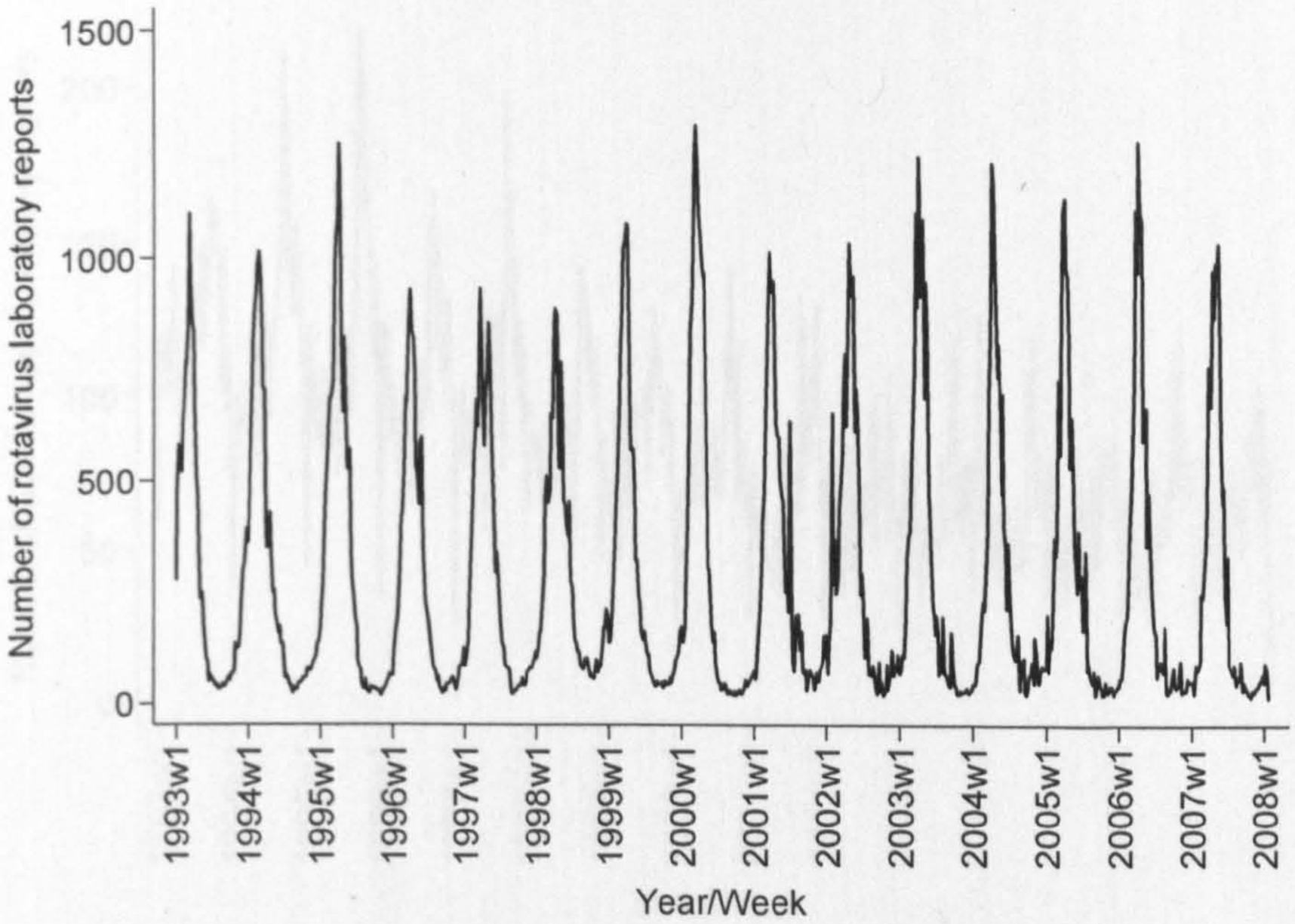


Figure A5.4d *Shigella* spp.

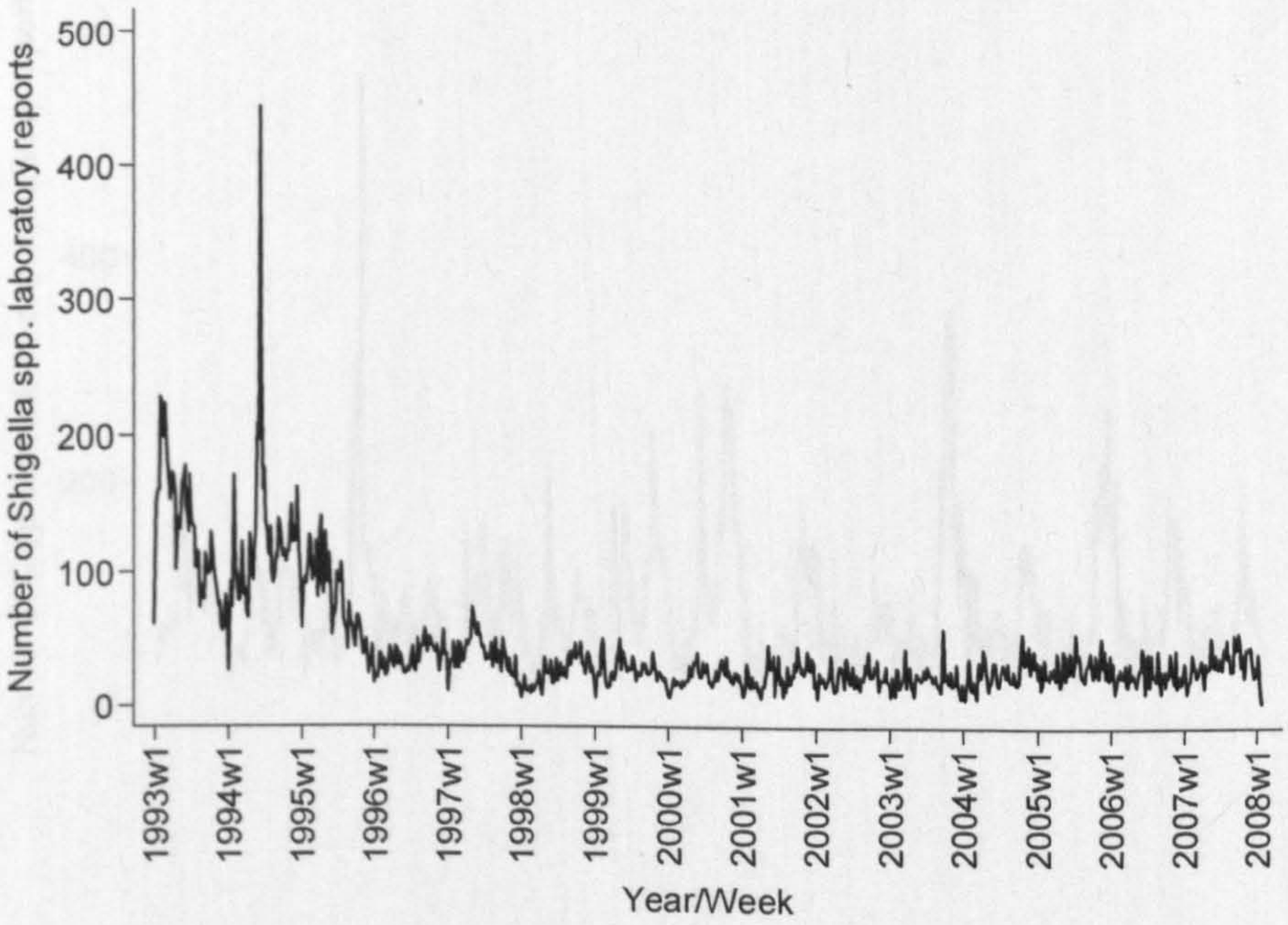


Figure A5.4e *Giardia* spp.

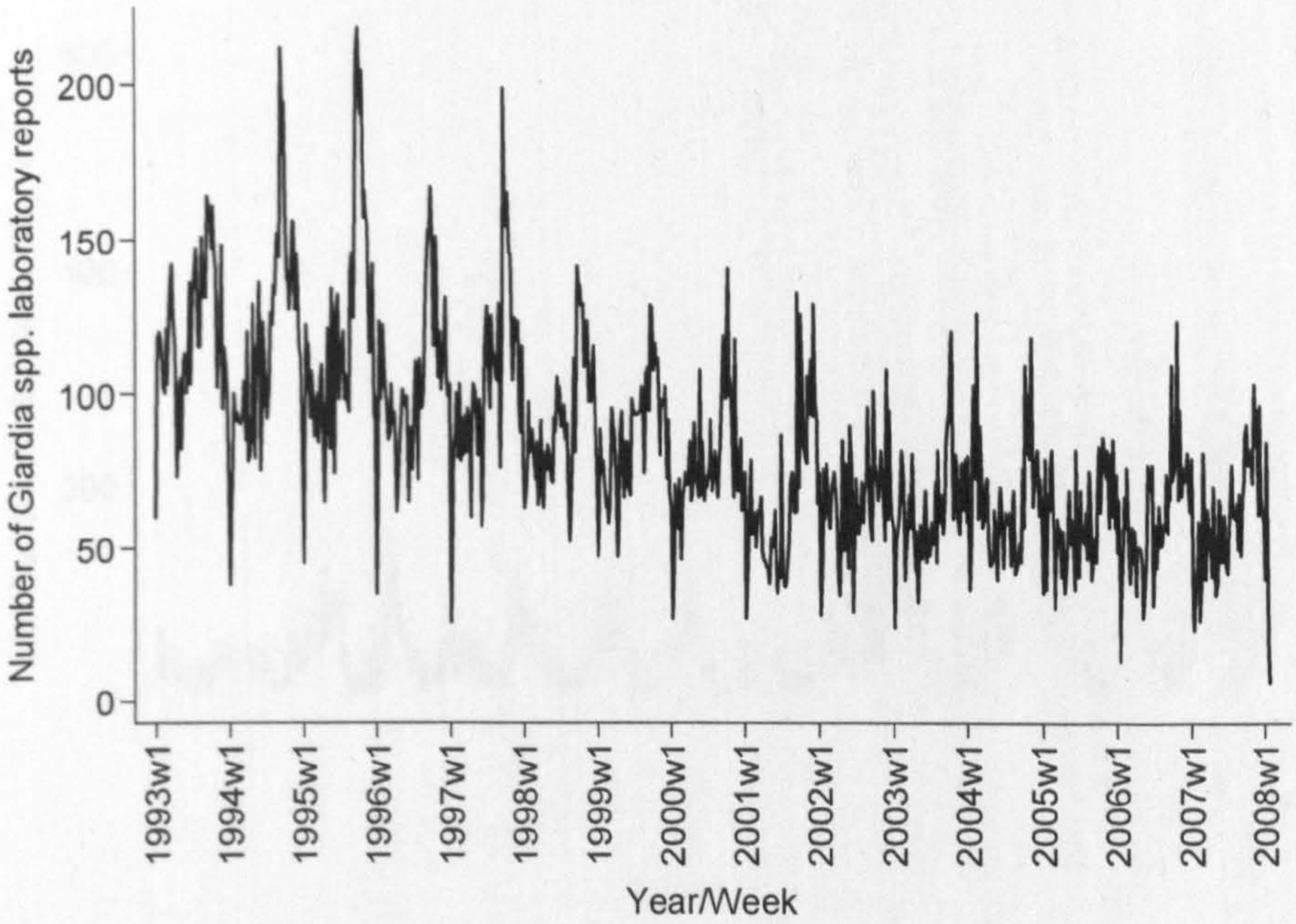


Figure A5.4f *Cryptosporidium* spp.

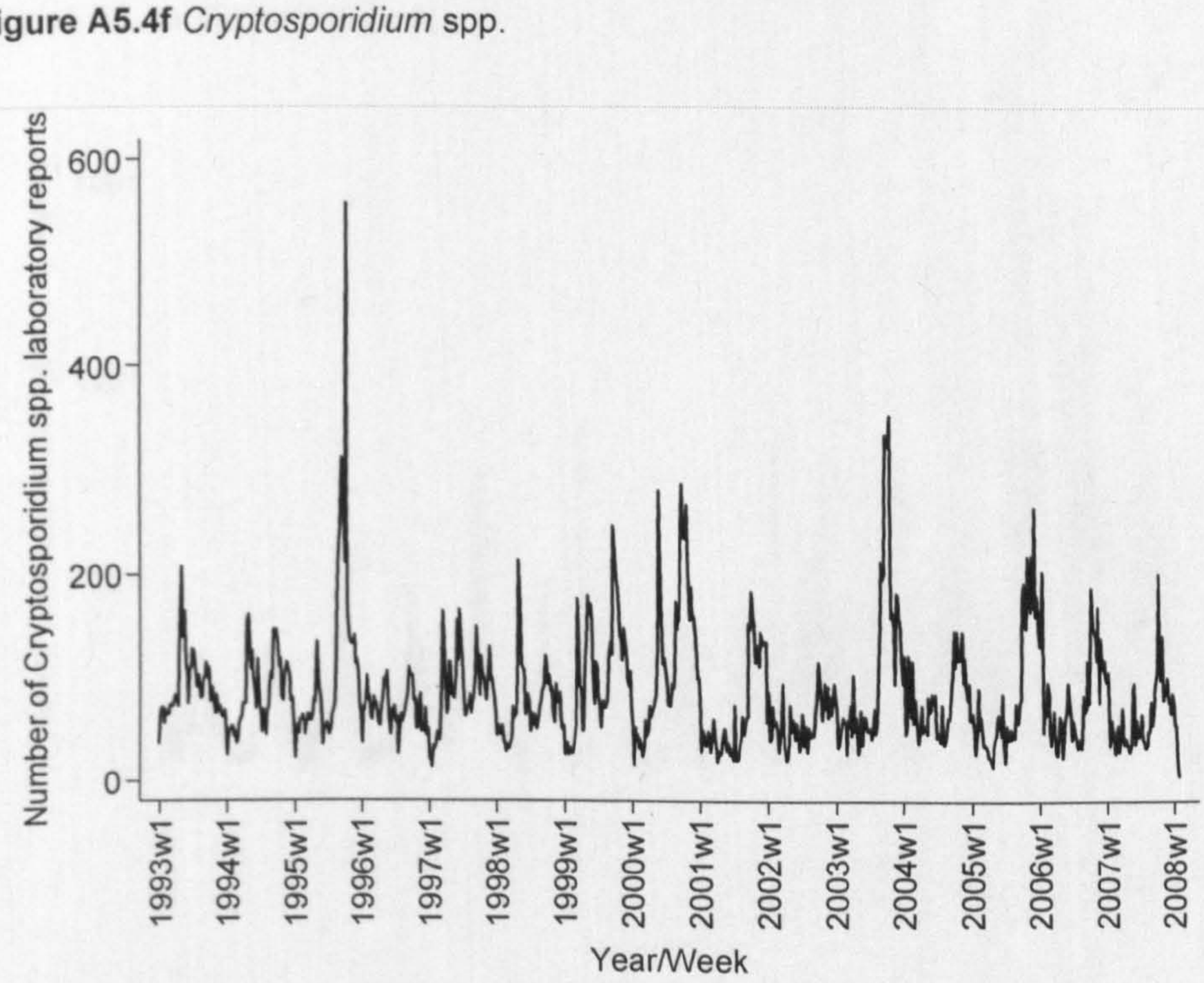


Figure A5.4g Norovirus.

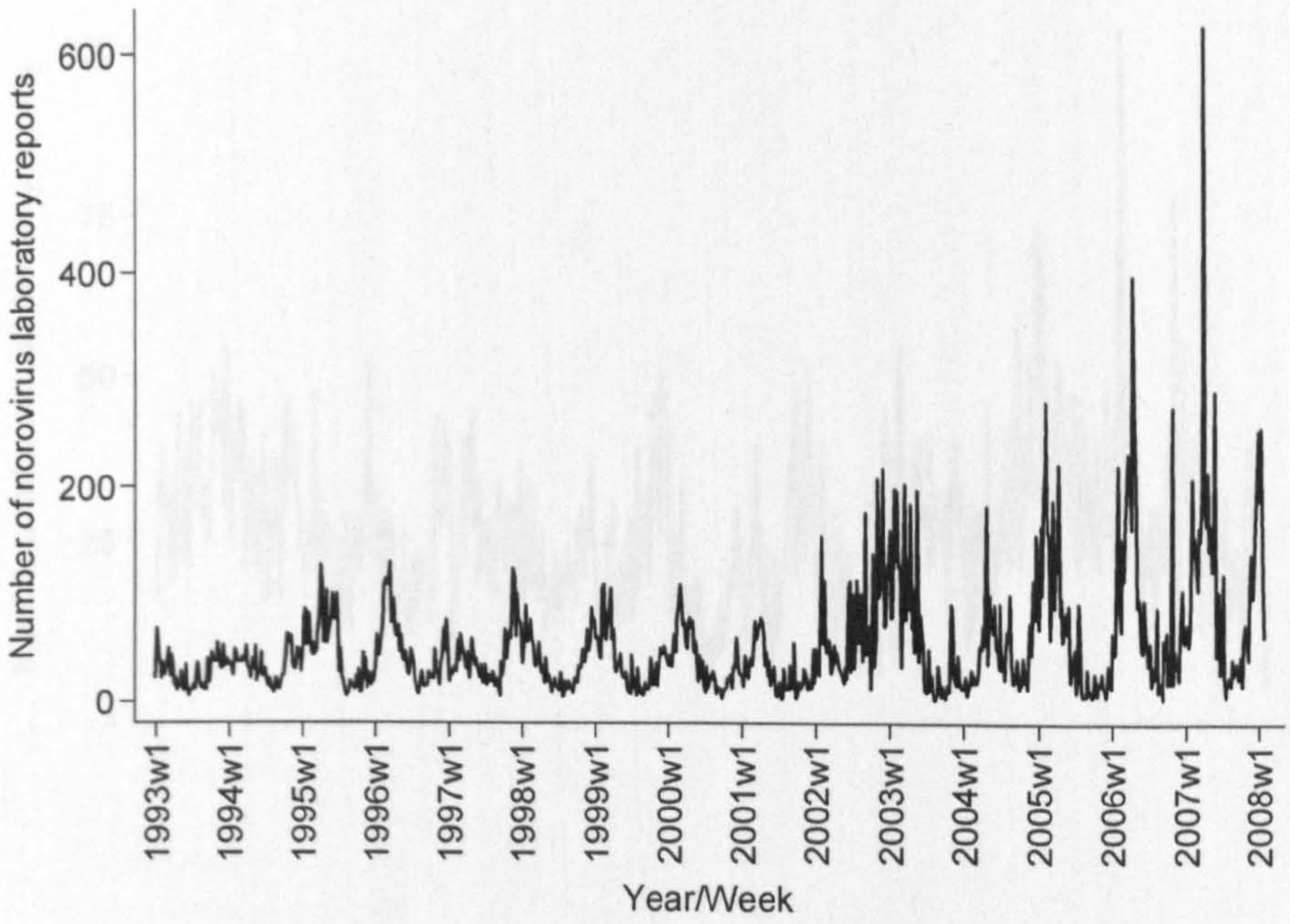


Figure A5.4h *Escherichia coli*.

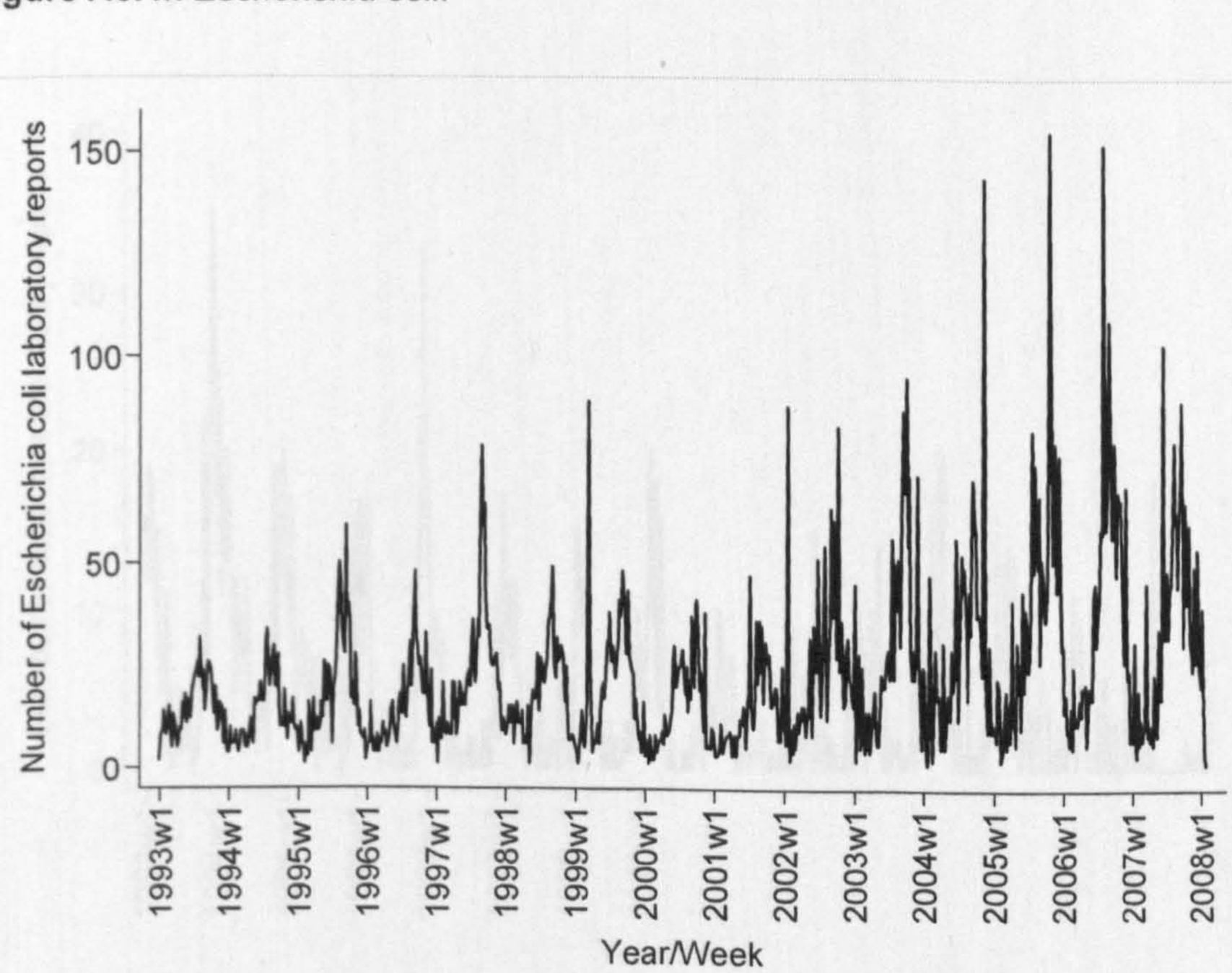


Figure A5.4i Adenovirus.

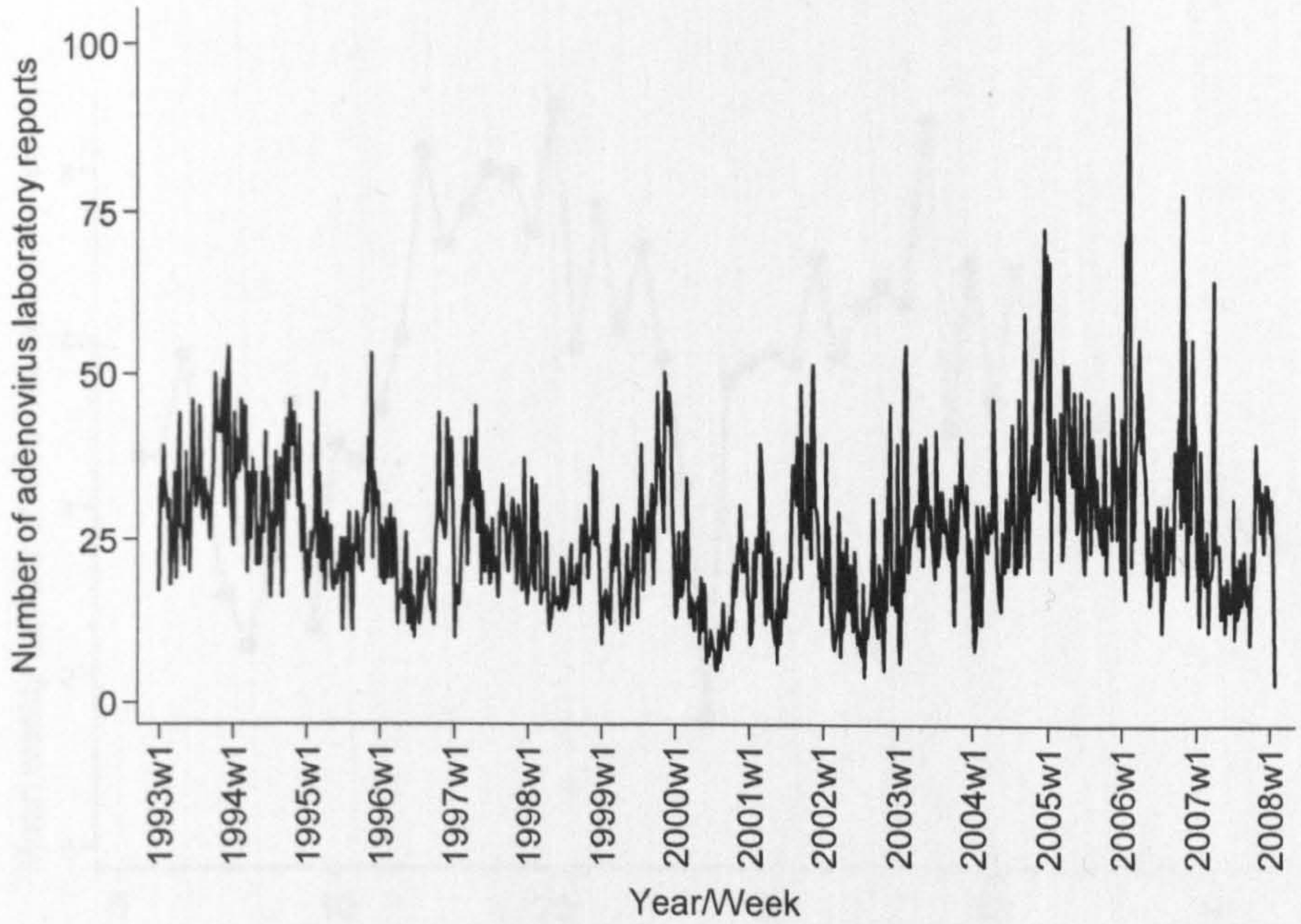
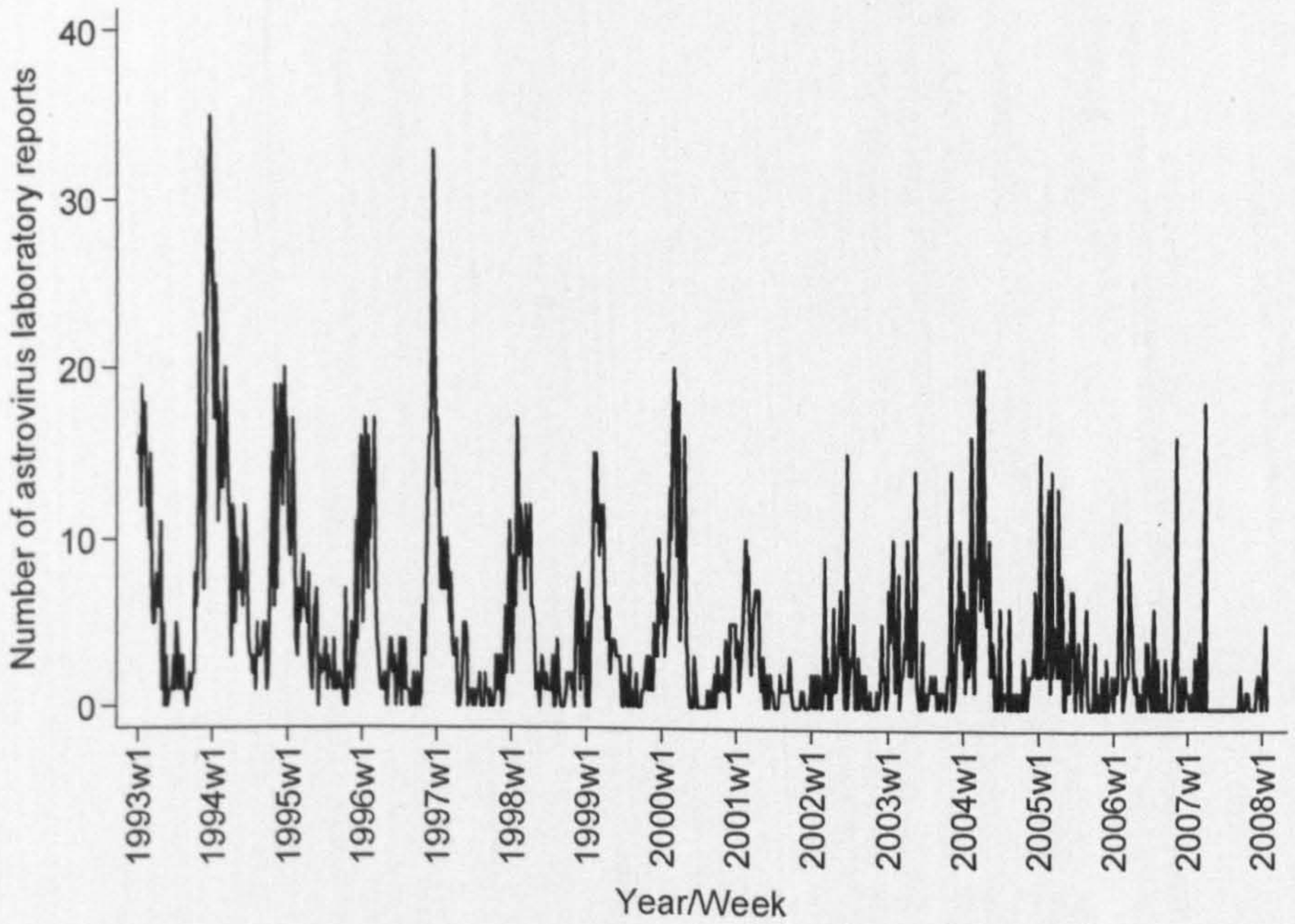
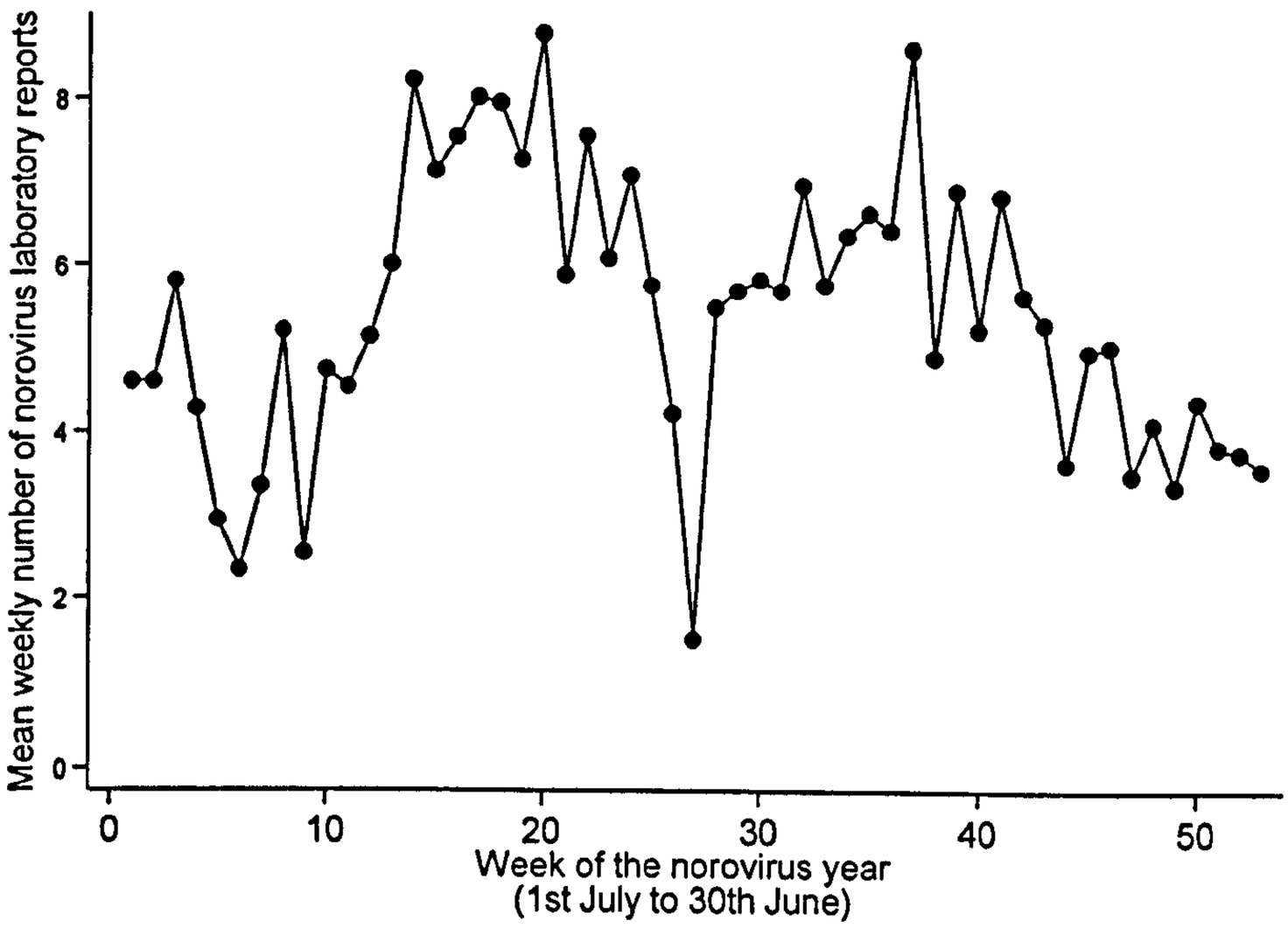


Figure A5.4j Astrovirus.



Appendix 5.5. Seasonality of norovirus laboratory reports in children aged less than five years.



Appendix A5.6. Summary statistics for RCGP general practice IID consultations and HPA pathogen laboratory reports.

Table A5.6a Children aged less than five years.

	Median	IQR	Min	Max	% zero weeks
RCGP IID episodes	59	37 - 92	13	206	0
Campylobacter spp.	76	60 - 96	5	317	0
Salmonella spp.	55	34 - 83	0	195	0.9
Rotavirus	120	45 - 424	12	1200	0
Shigella spp.	3	1 - 6	0	64	9.8
Giardia lamblia	11	6 - 17	0	50	0.1
Cryptosporidium spp.	25	16 - 37	2	260	0
Norovirus	4	2 - 8	0	37	7.9
Adenovirus	23	16 - 30	3	98	0
Escherichia coli	6	3 - 10	0	54	6.4
Astrovirus	2	0 - 5	0	33	30.0
Staphylococcus aureus	0	0	0	23	94.0
Sapovirus	1	0 - 2	0	9	48.5
Vibrio spp.	0	0	0	3	94.5
Clostridium perfringens	0	0	0	3	96.9
Bacillus spp.	0	0	0	1	99.6

Abbreviations: IID, infectious intestinal disease; IQR, interquartile range; RCGP, Royal College of General Practitioners; spp., species.

Table A5.6b Children and adults aged 5-64 years.

	Median	IQR	Min	Max	% zero weeks
RCGP IID episodes	117	90 - 168	25	305	0
Campylobacter spp.	685	539 - 904	63	1407	0
Salmonella spp.	207	131 - 354	0	919	0.5
Rotavirus	6	2 - 13	0	34	5.4
Shigella spp.	24	16 - 36	3	367	0
Giardia lamblia	60	48 - 74	4	167	0
Cryptosporidium spp.	41	28 - 63	3	255	0
Norovirus	7	4 - 13	0	136	2.2
Adenovirus	1	0 - 3	0	18	25.8
Escherichia coli	9	4 - 17	0	113	3.7
Astrovirus	0	0 - 1	0	7	71.6
Staphylococcus aureus	0	0 - 1	0	273	65.9
Sapovirus	0	0	0	8	92.0
Vibrio spp.	1	0 - 2	0	8	36.8
Clostridium perfringens	0	0	0	11	76.1
Bacillus spp.	0	0	0	4	91.2

Abbreviations: IID, infectious intestinal disease; IQR, interquartile range; RCGP, Royal College of General Practitioners; spp., species.

Table A5.6c Adults aged 65 years and older.

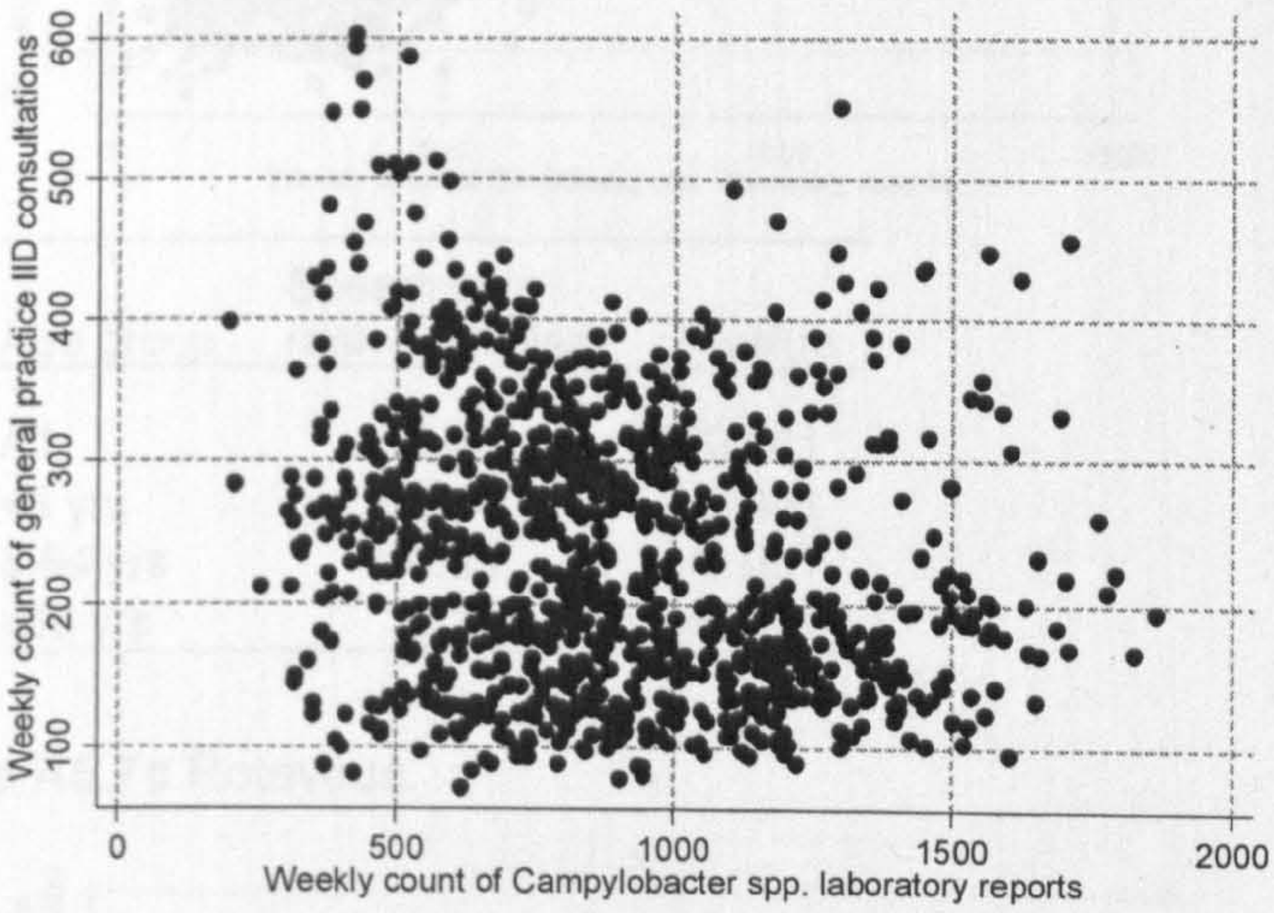
	Median	IQR	Min	Max	% zero weeks
RCGP IID episodes	23	17 - 31	5	55	0
Campylobacter spp.	106	80 - 105	28	306	0
Salmonella spp.	29	20 - 44	0	119	0.8
Rotavirus	3	1 - 8	0	44	14.7
Shigella spp.	1	0 - 2	0	24	29.1
Giardia lamblia	3	2 - 5	0	27	4.7
Cryptosporidium spp.	2	1 - 3	0	35	22.2
Norovirus	18	8 - 40	0	431	2.3
Adenovirus	0	0	0	8	81.1
Escherichia coli	2	1 - 3	0	29	24.8
Astrovirus	0	0	0	7	87.1
Staphylococcus aureus	0	0 - 1	0	128	59.5
Sapovirus	0	0	0	5	96.0
Vibrio spp.	0	0	0	2	88.6
Clostridium perfringens	0	0	0	63	78.3
Bacillus spp.	0	0	0	1	99.7

Abbreviations: IID, infectious intestinal disease; IQR, interquartile range; RCGP, Royal College of General Practitioners; spp., species.

Appendix A5.7. Correlation of HPA pathogen laboratory reports with RCGP general practice consultations for IID, 1993-2007.

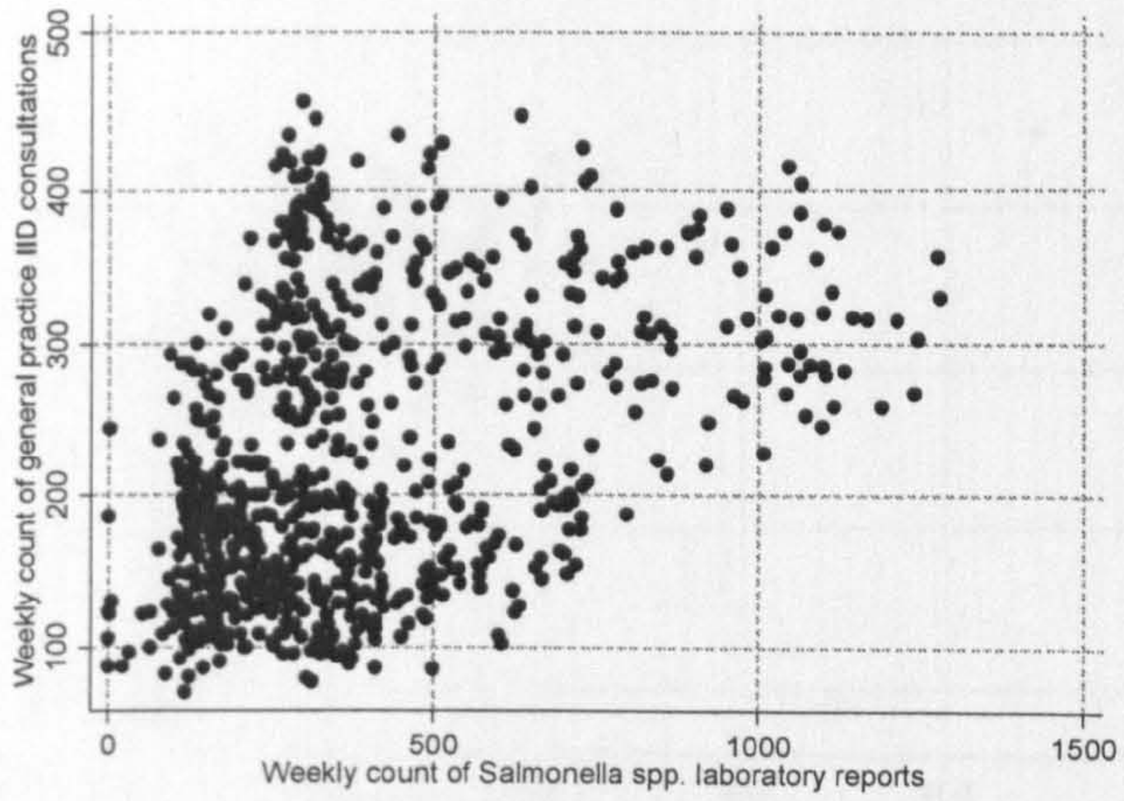
Graphs show data from all age groups, tables provide age group specific Spearman's rank correlations statistics.

Figure A5.7a *Campylobacter* spp.



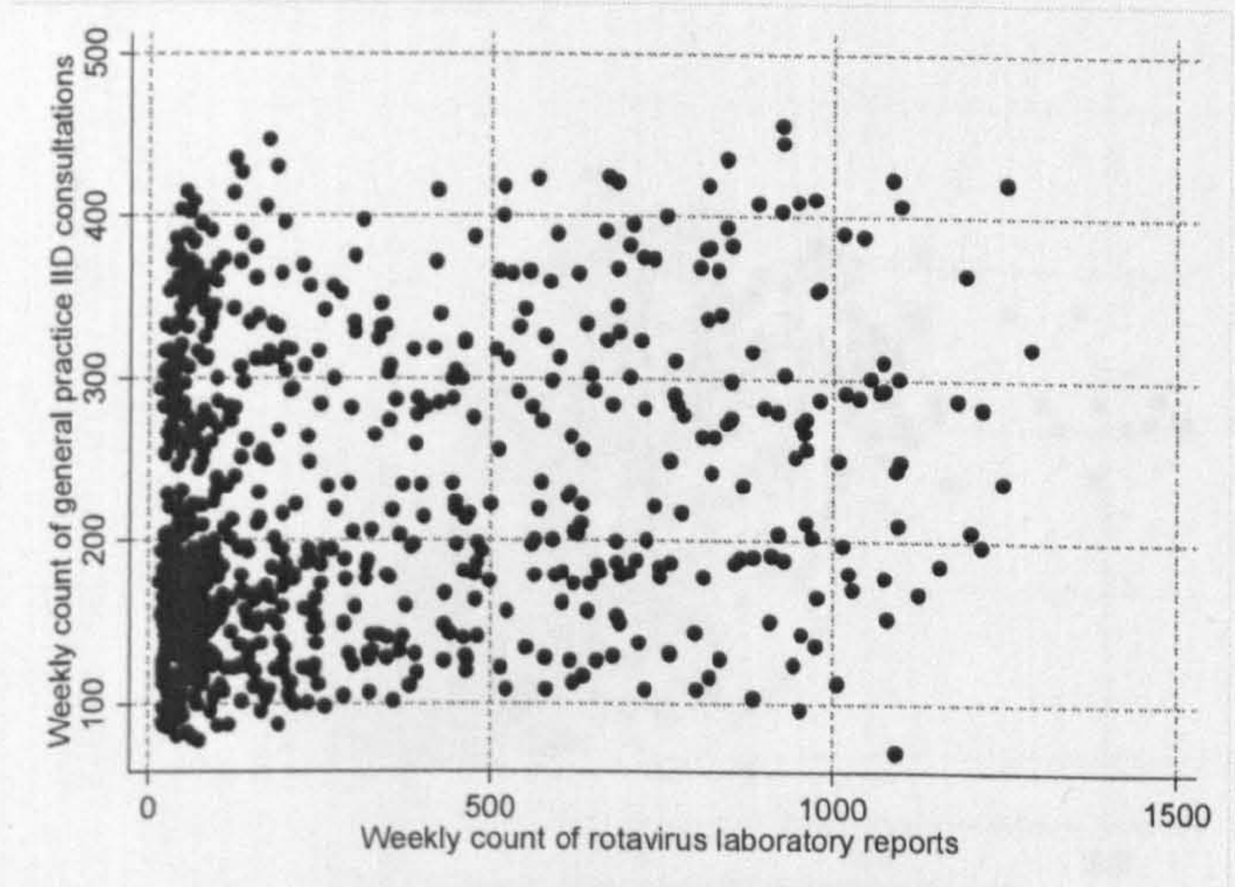
Age group	Spearman's rank coefficient	P value
All	-0.13	<0.001
<5 yrs	0.23	<0.001
5-64 yrs	-0.30	<0.001
≥65 yrs	-0.44	<0.001

Figure A5.7b *Salmonella* spp.



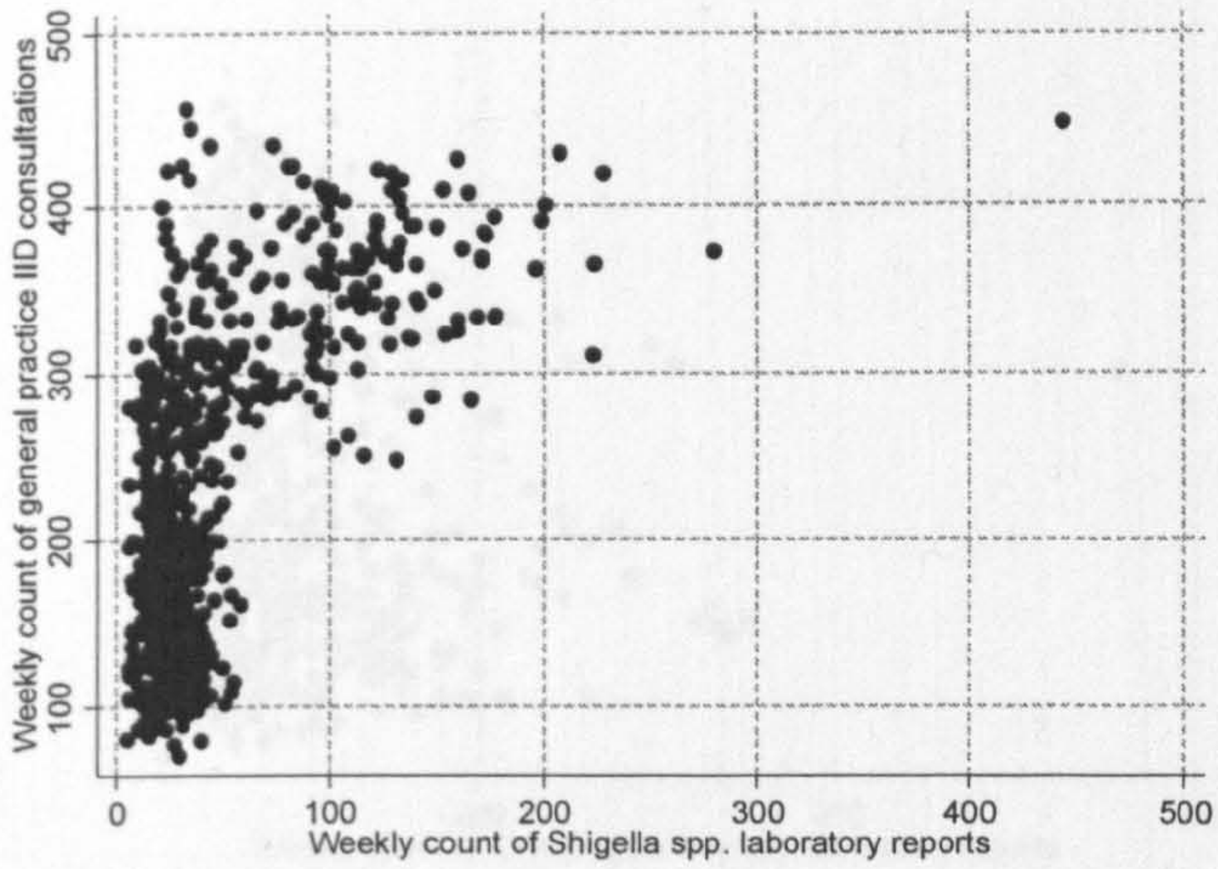
Age group	Spearman's rank coefficient	P value
All	0.40	<0.001
<5 yrs	0.44	<0.001
5-64 yrs	0.39	<0.001
≥65 yrs	0.31	<0.001

Figure A5.7c Rotavirus.



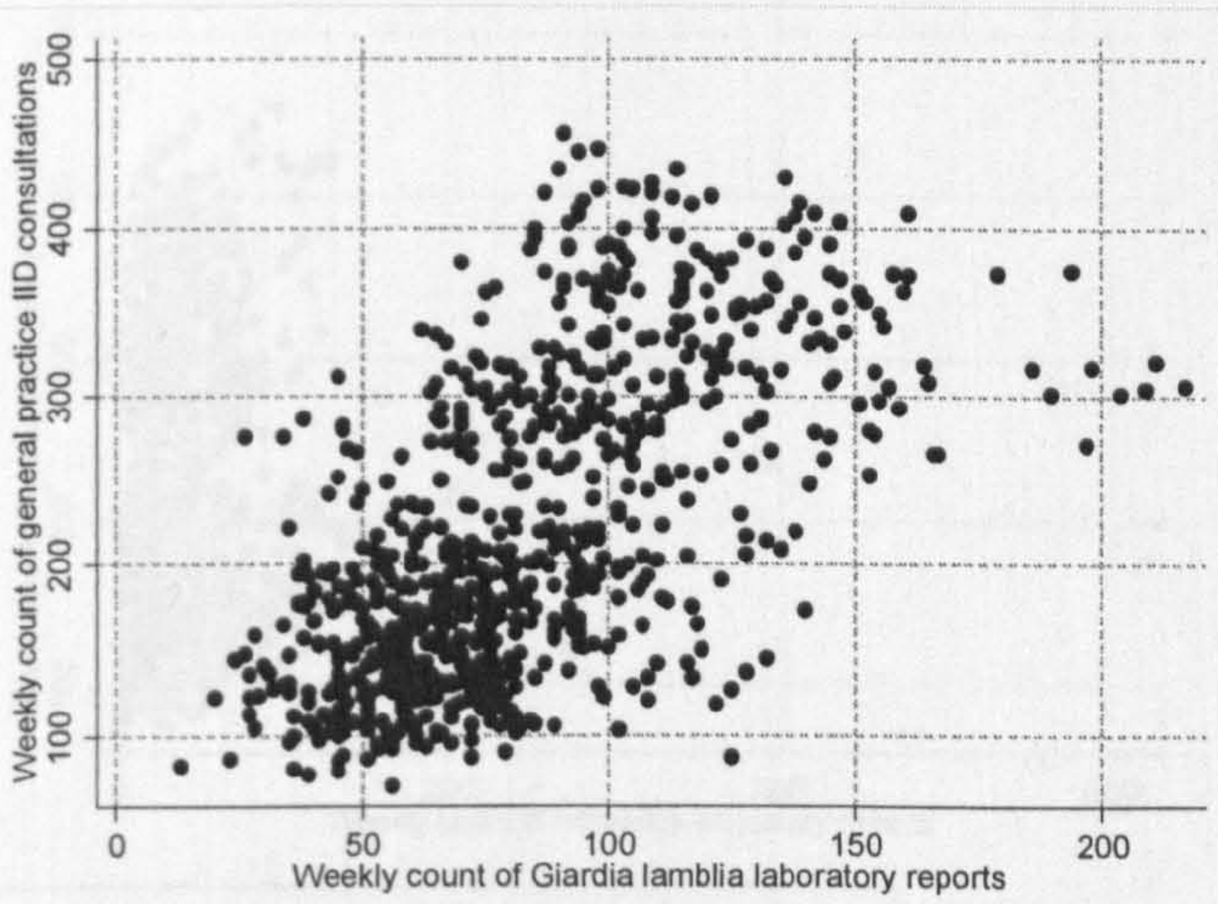
Age group	Spearman's rank coefficient	P value
All	0.20	<0.0001
<5 yrs	0.39	<0.0001
5-64 yrs	0.00	0.01
≥65 yrs	-0.003	0.93

Figure A5.7d *Shigella* spp.



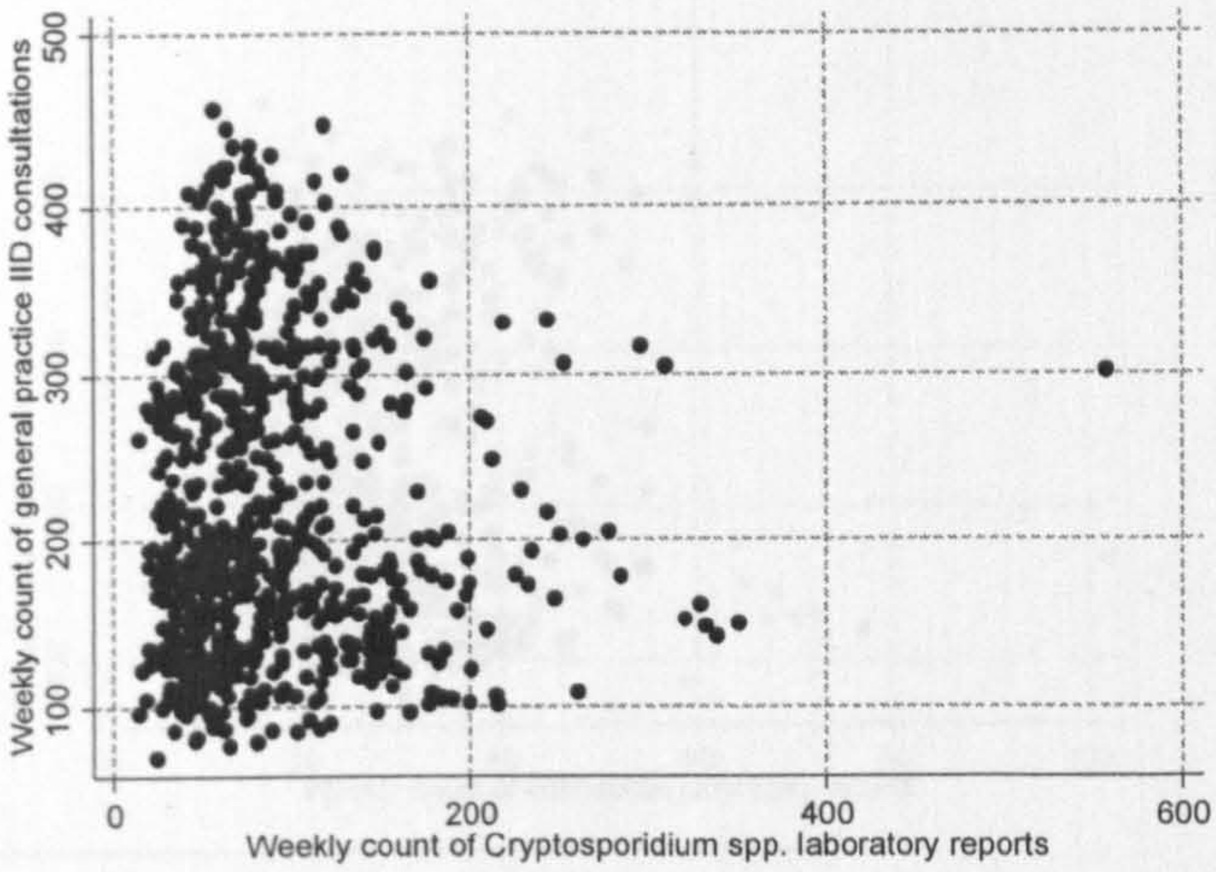
Age group	Spearman's rank coefficient	P value
All	0.52	<0.001
<5 yrs	0.61	<0.001
5-64 yrs	0.32	<0.001
≥65 yrs	0.17	<0.001

Figure A5.7e *Giardia* spp.



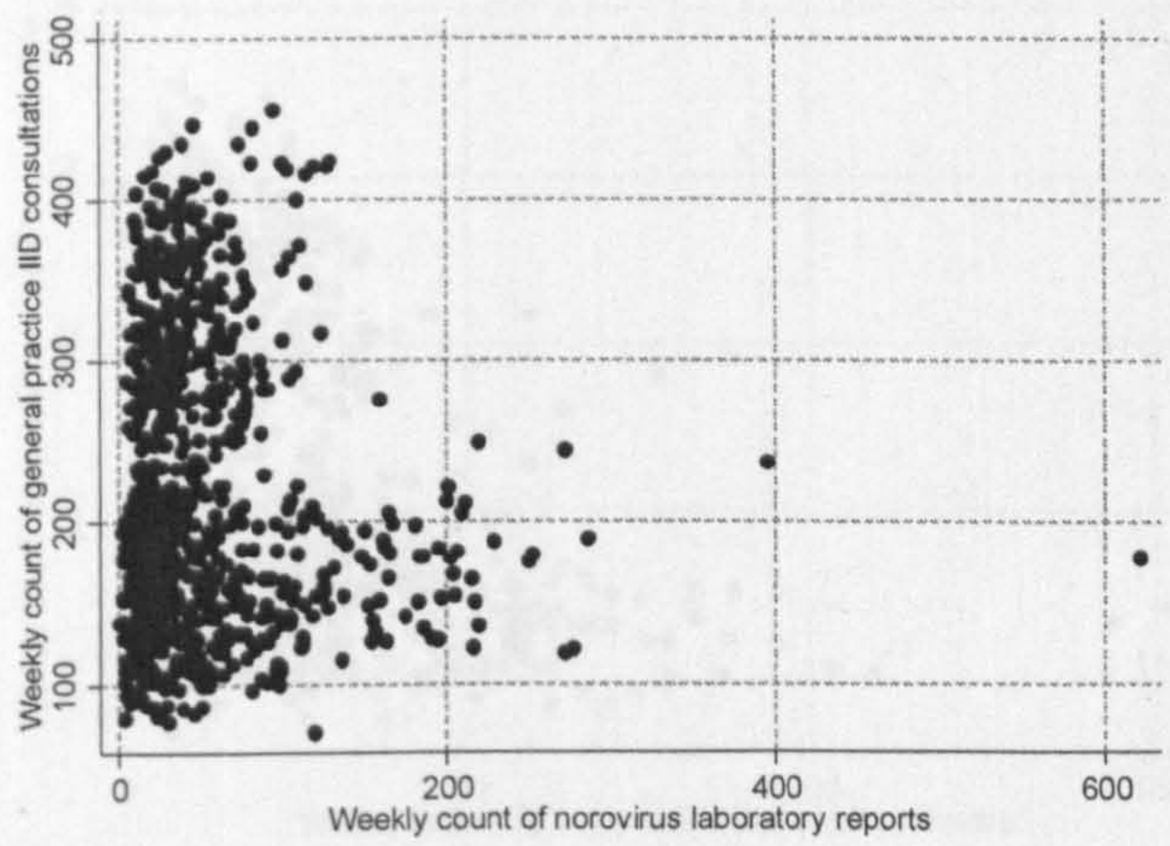
Age group	Spearman's rank coefficient	P value
All	0.64	<0.001
<5 yrs	0.57	<0.001
5-64 yrs	0.20	<0.001
≥65 yrs	0.17	<0.001

Figure A5.7f *Cryptosporidium* spp.



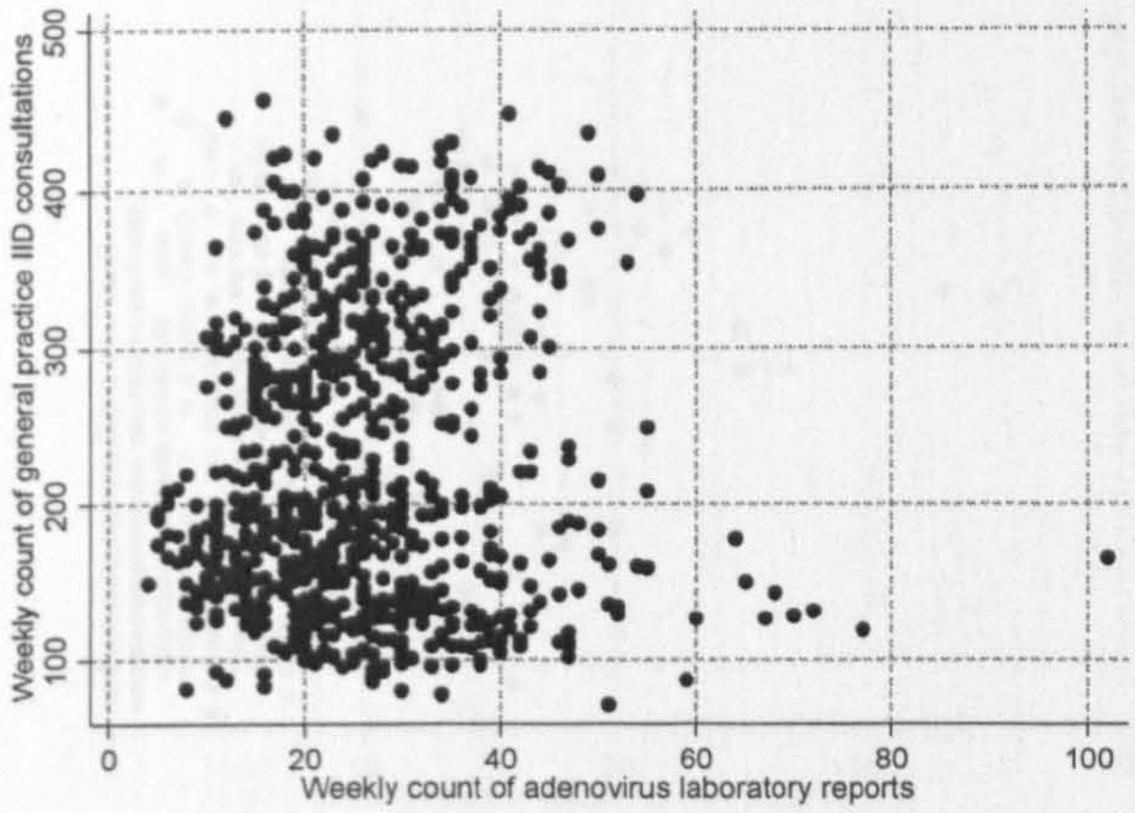
Age group	Spearman's rank coefficient	P value
All	0.09	0.009
<5 yrs	0.17	<0.001
5-64 yrs	-0.16	<0.001
≥65 yrs	0.02	0.54

Figure A5.7g Norovirus.



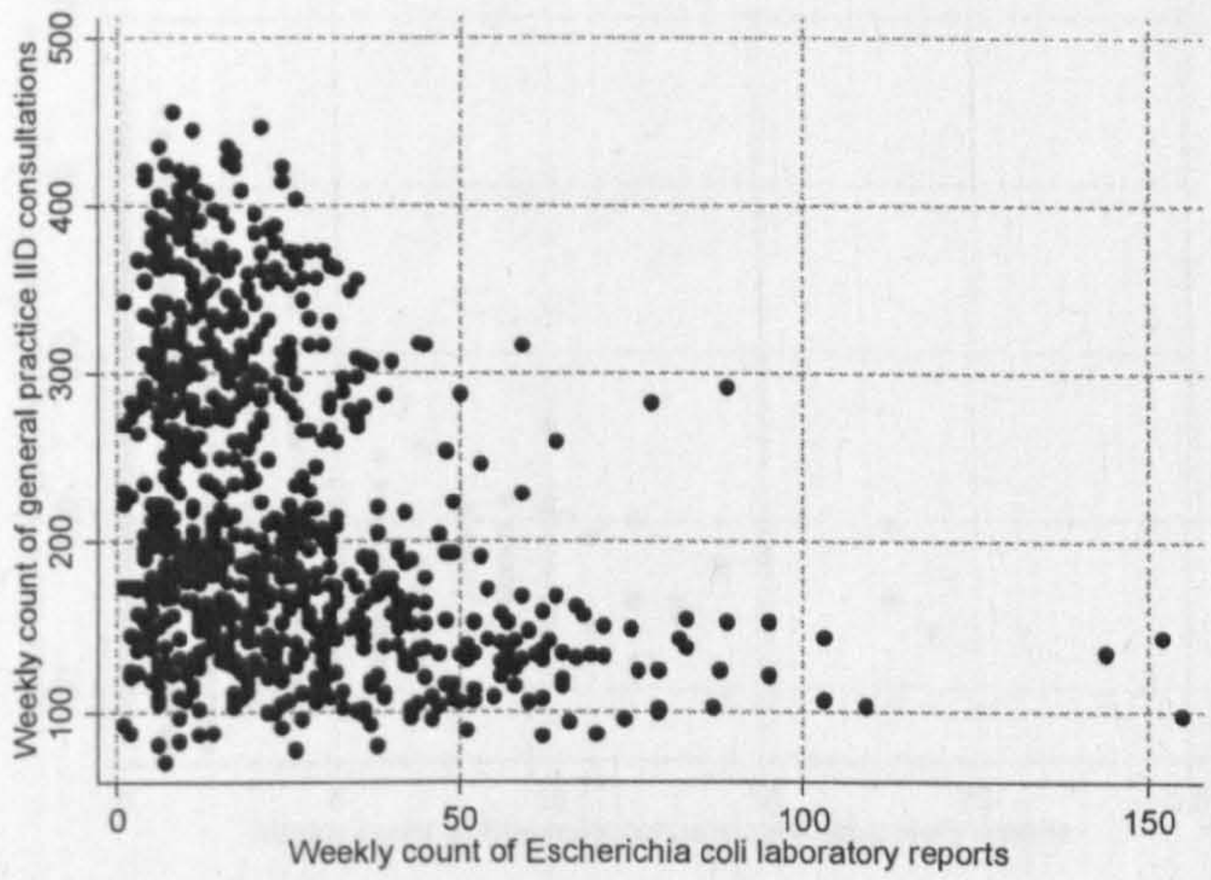
Age group	Spearman's rank coefficient	P value
All	0.03	0.44
<5 yrs	0.48	<0.001
5-64 yrs	-0.28	<0.001
≥65 yrs	-0.05	0.15

Figure A5.7h Adenovirus.



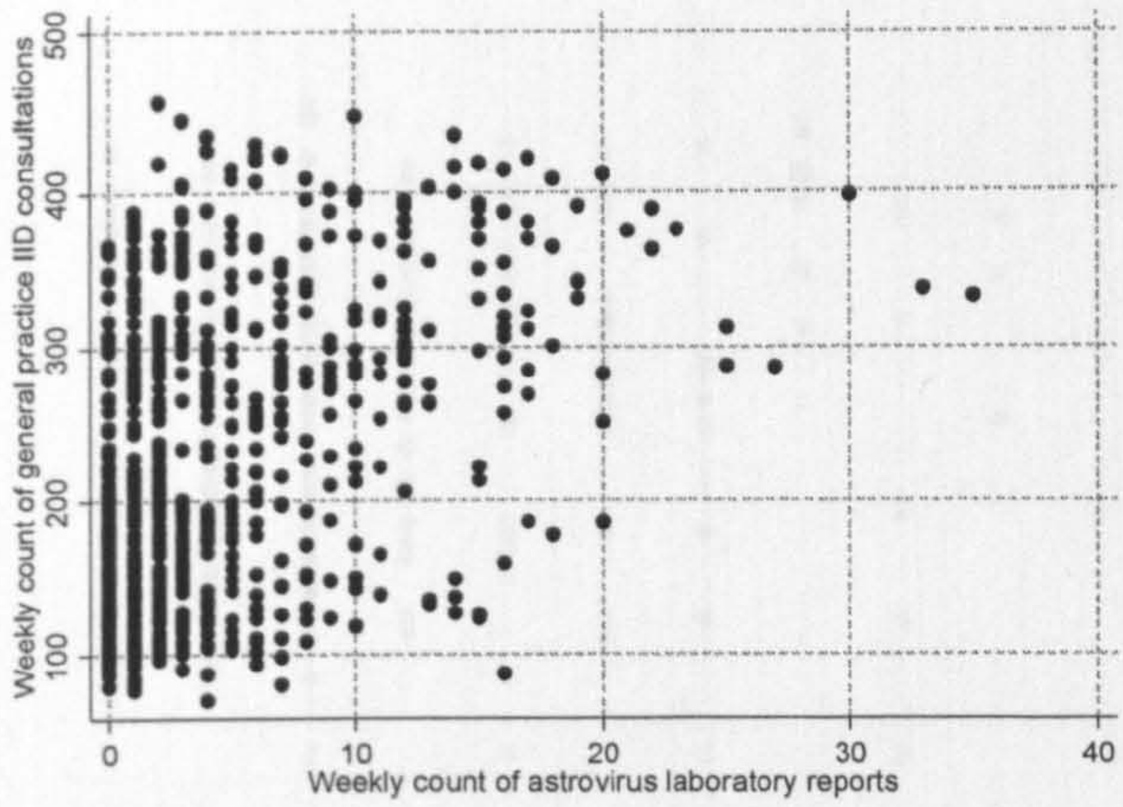
Age group	Spearman's rank coefficient	P value
All	0.06	0.08
<5 yrs	0.10	0.004
5-64 yrs	-0.10	0.006
≥65 yrs	-0.05	0.18

Figure A5.7i *Escherichia coli.*



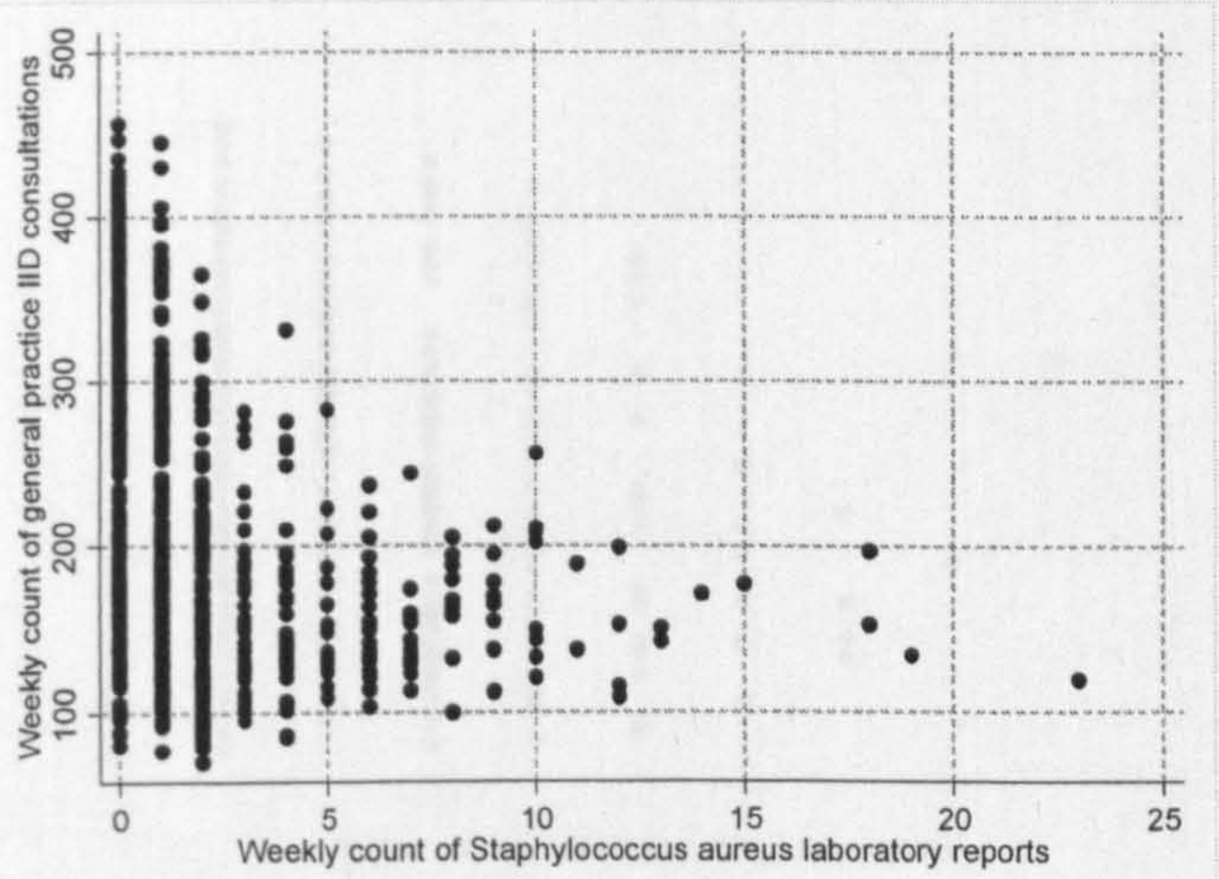
Age group	Spearman's rank coefficient	P value
All	-0.34	<0.001
<5 yrs	-0.11	0.002
5-64 yrs	-0.32	<0.001
≥65 yrs	-0.33	<0.001

Figure A5.7j Astrovirus.



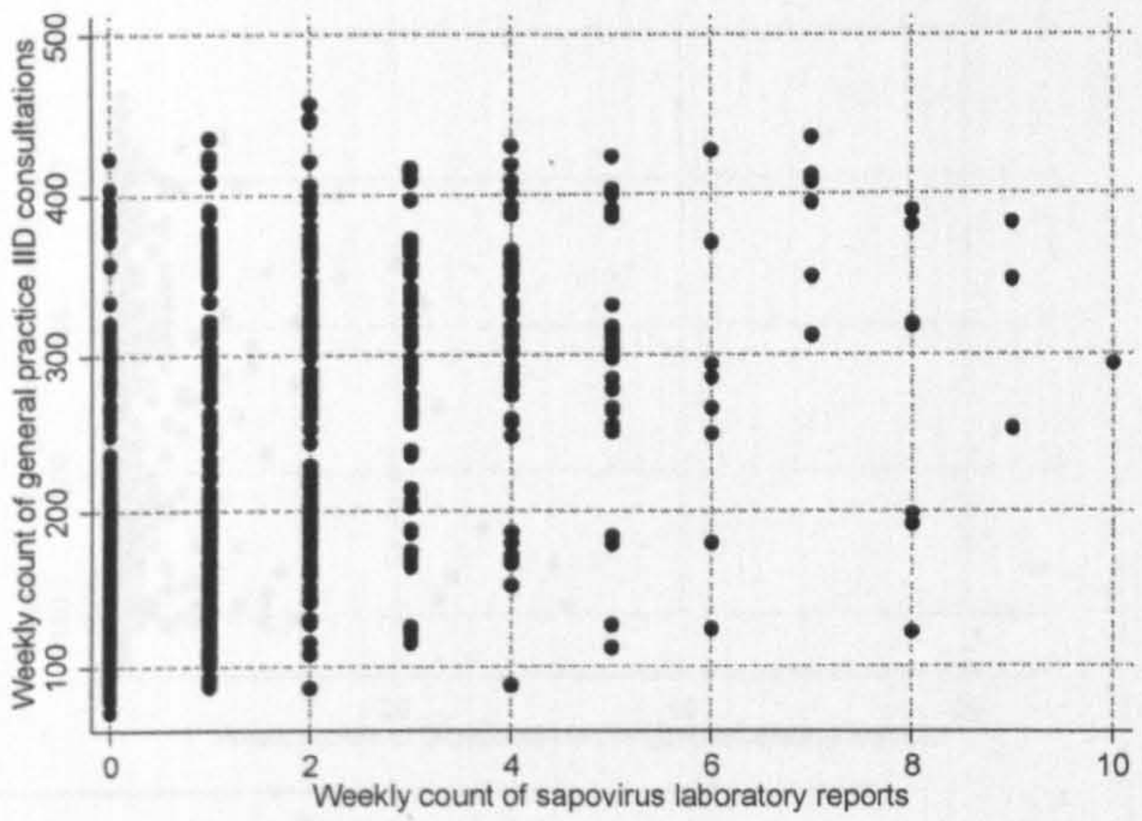
Age group	Spearman's rank coefficient	P value
All	0.40	<0.001
<5 yrs	0.49	<0.001
5-64 yrs	0.03	0.41
≥65 yrs	0.03	0.34

Figure A5.7l *Staphylococcus aureus*.



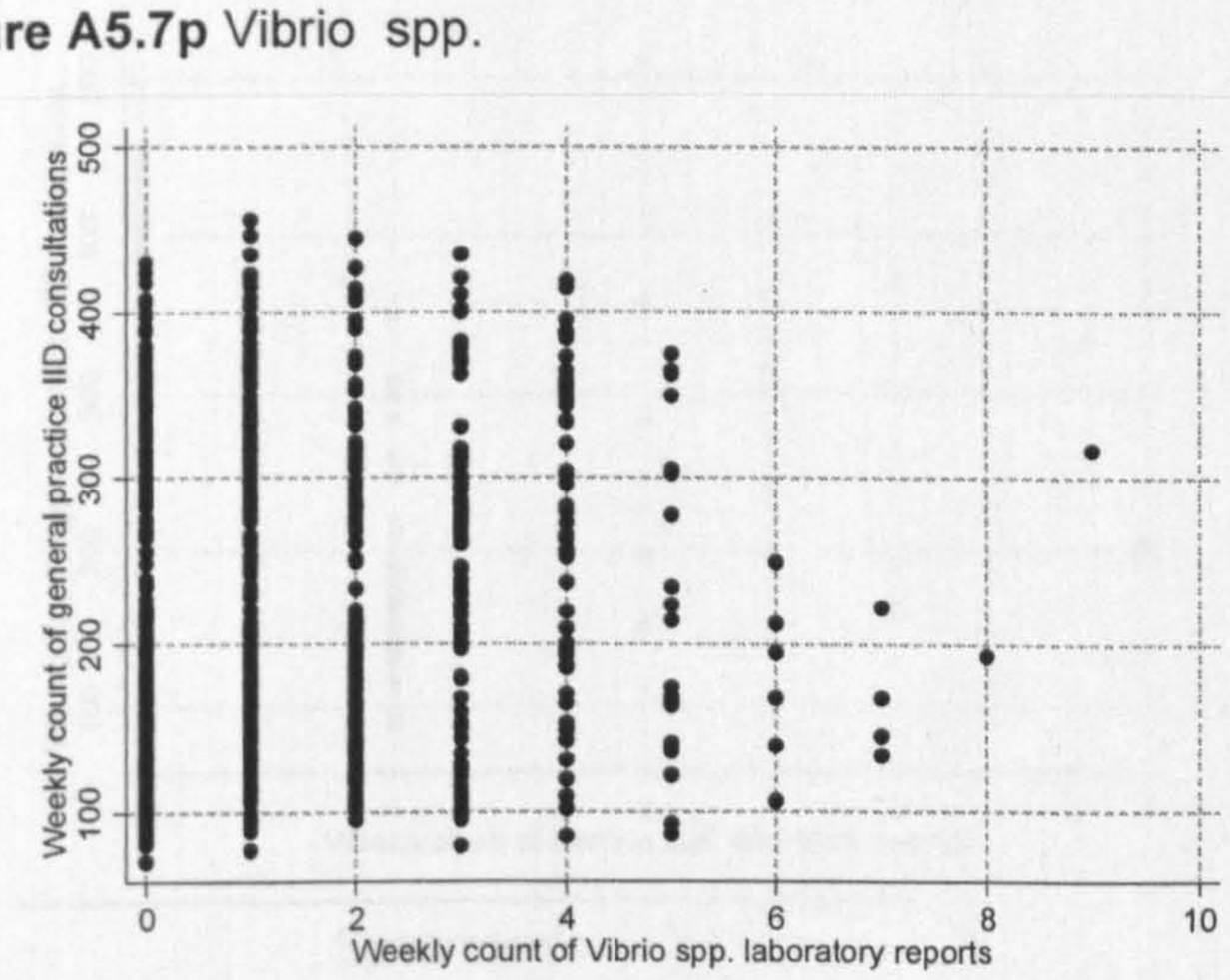
Age group	Spearman's rank coefficient	P value
All	-0.49	<0.001
<5 yrs	-0.12	<0.001
5-64 yrs	-0.27	<0.001
≥65 yrs	-0.28	<0.001

Figure A5.7m Sapovirus.



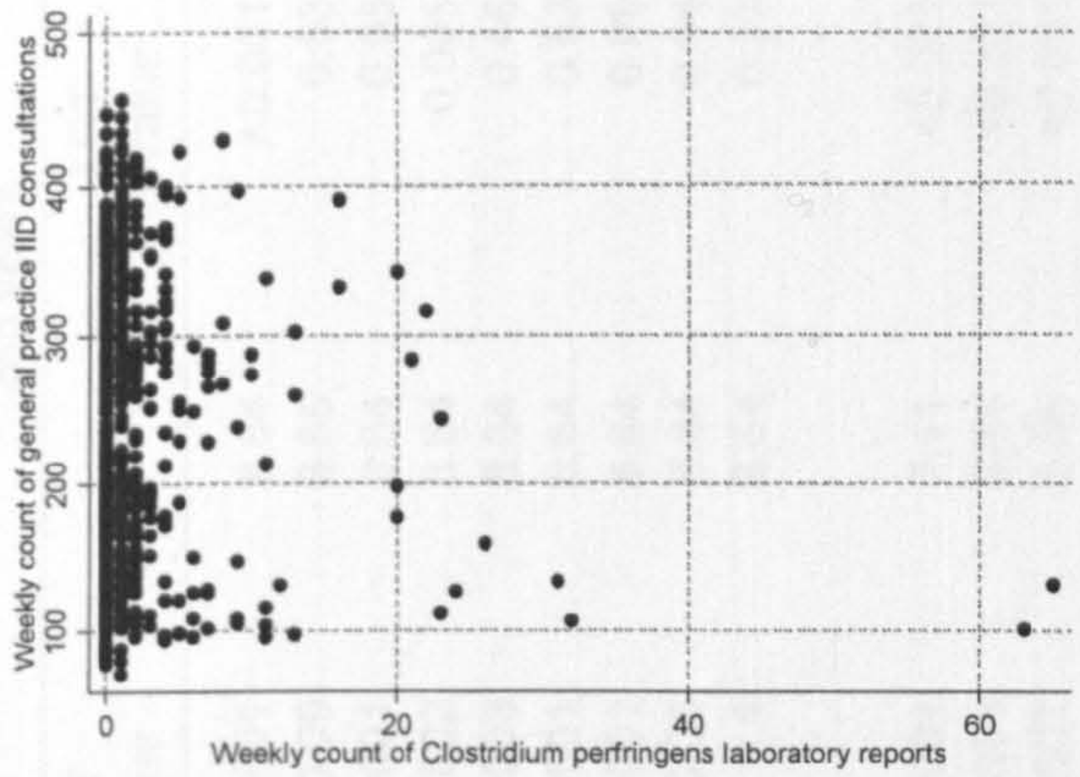
Age group	Spearman's rank coefficient	P value
All	0.47	<0.001
<5 yrs	0.45	<0.001
5-64 yrs	0.02	0.52
≥65 yrs	0.08	0.03

Figure A5.7p Vibrio spp.



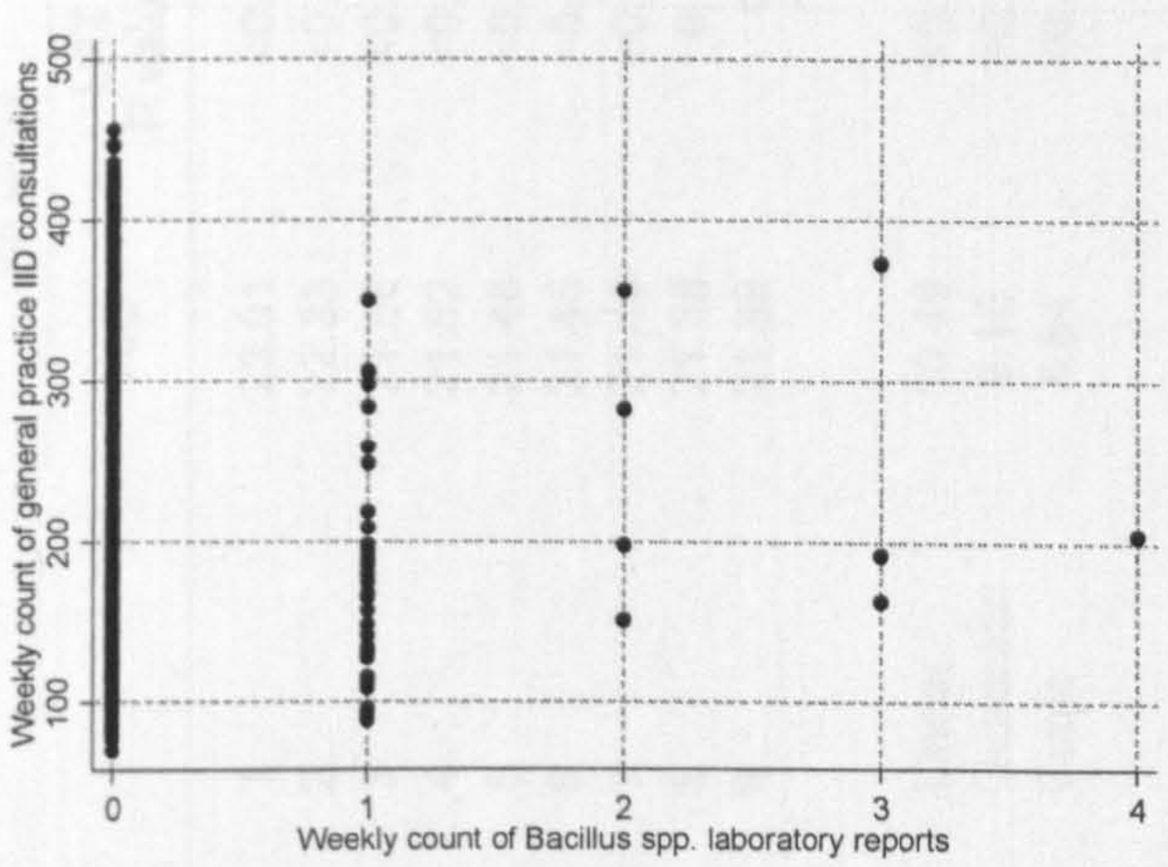
Age group	Spearman's rank coefficient	P value
All	0.09	0.01
<5 yrs	-0.03	0.39
5-64 yrs	0.05	0.13
≥65 yrs	0.02	0.60

Figure A5.7k *Clostridium perfringens*.



Age group	Spearman's rank coefficient	P value
All	0.15	<0.001
<5 yrs	0.11	0.002
5-64 yrs	0.08	0.03
≥65 yrs	0.01	0.75

Figure A5.7q *Bacillus* spp.



Age group	Spearman's rank coefficient	P value
All	-0.07	0.06
<5 yrs	-0.07	0.06
5-64 yrs	0.004	0.90
≥65 yrs	-0.008	0.82

Appendix 5.8. Results of fitting the confounder models.

		<5 years		5-64 years		≥65 years	
		AIC	LRT P value	AIC	LRT P value	AIC	LRT P value
Fourier terms	1	23.01	<0.001	26.25	<0.001	8.64	<0.001
	2	22.33	<0.001	26.25	0.09	8.65	0.63
	3	21.58	<0.001	26.01	<0.001	8.64	0.05
	4	21.52	<0.001	26.00	0.002	8.64	0.005
	5	21.48	<0.001	25.99	0.003	8.64	0.46
	6	21.45	<0.001	25.93	<0.001	8.64	0.53
	7	21.40	<0.001	25.85	<0.001	8.64	0.05
	8	21.38	0.002	25.85	0.08	8.64	0.04
	9	21.39	0.35	25.86	0.4	8.64	0.23
Trend terms							
Fourier model	Linear	10.49	<0.001	12.44	<0.001	7.11	<0.001
	Quadratic	9.15	<0.001	10.65	<0.001	6.66	<0.001
	Cubic	8.84	<0.001	9.97	<0.001	6.56	<0.001
4 week model	Linear	7.84	0.37	8.77	1.0	6.40	0.87
	Quadratic	7.84	0.02	8.76	0.01	6.40	0.07
	Cubic	7.84	0.26	8.76	0.26	6.40	0.40
13 week model	Linear	10.38	0.87	9.33	0.87	6.36	0.04
	Quadratic	10.38	0.96	9.33	0.17	6.36	0.93
	Cubic	10.39	0.54	9.33	0.17	6.37	0.51
No seasonal adjustment	Linear	14.70	<0.001	12.76	<0.001	6.58	0.20
	Quadratic	13.54	<0.001	11.05	<0.001	6.30	<0.0001
	Cubic	13.26	<0.001	10.34	<0.001	6.27	0.0002

Abbreviations: AIC, Akaike's information criterion; LRT, likelihood ratio test.

Appendix A5.9. Pathogen regression coefficients and Wald test P values used to select pathogens for inclusion in the final models.

Table A5.9a Direct models using Fourier terms for seasonal adjustment (Direct Model 1).

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	-0.03	0.2	-0.02	0.007	0.0	1.0
Salmonella spp.	0.03	0.3	0.05	<0.001	0.02	0.4
Rotavirus	0.05	<0.001	0.60	0.001	0.17	<0.001
Shigella spp.	0.44	0.002	0.15	0.006	0.12	0.5
Giardia lamblia	-0.05	0.7	0.10	0.1	-0.13	0.4
Cryptosporidium spp.	0.006	0.9	0.03	0.3	-0.02	0.9
Adenovirus	-0.03	0.6	-0.34	0.5	-0.31	0.5
Norovirus	0.74	<0.001	0.47	<0.001	0.05	<0.001
Escherichia coli	-0.05	0.6	-0.10	0.2	-0.17	0.1
Astrovirus	-0.18	0.3	2.3	0.1	0.95	0.1
Clostridium perfringens	4.9	0.3	0.08	0.9	-0.05	0.7
Staphylococcus aureus	-0.59	0.3	0.07	0.6	-0.06	0.3
Sapovirus	-0.58	0.3	0.31	0.9	0.11	0.9
Vibrio spp.	0.54	0.8	-1.0	0.3	0.18	0.9
Bacillus spp.	10.9	0.07	-6.7	0.3	-	-

Table A5.9b Direct models using a 13-week indicator variable for seasonal adjustment (Direct Model 2).

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	-0.02	0.2	0.01	0.01	0.01	0.2
Salmonella spp.	-0.04	0.3	0.02	0.07	0.005	0.8
Rotavirus	0.06	<0.001	0.36	0.009	0.09	0.09
Shigella spp.	0.50	0.02	0.19	0.007	0.35	0.1
Giardia lamblia	0.15	0.3	0.07	0.2	-0.06	0.7
Cryptosporidium spp.	-0.06	0.2	-0.04	0.2	-0.13	0.3
Adenovirus	-0.03	0.7	-0.33	0.5	-0.18	0.7
Norovirus	0.35	0.08	0.18	0.09	0.02	0.02
Escherichia coli	-0.12	0.2	-0.12	0.2	-0.12	0.3
Astrovirus	-0.15	0.5	0.21	0.9	0.20	0.8
Clostridium perfringens	-17.7	0.007	-0.85	0.4	0.22	0.3
Staphylococcus aureus	0.22	0.7	-0.09	0.5	-0.07	0.3
Sapovirus	1.1	0.08	1.6	0.5	0.29	0.9
Vibrio spp.	2.7	0.2	-0.27	0.8	-1.8	0.1
Bacillus spp.	9.5	0.1	7.7	0.3		

Table A5.9c Direct models using a 4-week indicator variable for seasonal adjustment.

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	0.004	0.9	0.02	0.08	0.02	0.2
Salmonella spp.	-0.02	0.7	-0.02	0.2	0.05	0.3
Rotavirus	0.06	<0.001	0.61	0.007	0.08	0.3
Shigella spp.	0.02	1.0	0.13	0.2	0.36	0.3
Giardia lamblia	0.26	0.1	0.13	0.10	-0.07	0.7
Cryptosporidium spp.	-0.03	0.6	0.05	0.3	-0.19	0.3
Adenovirus	-0.10	0.2	-0.20	0.7	-0.79	0.2
Norovirus	0.17	0.4	-0.05	0.7	0.03	0.1
Escherichia coli	-0.07	0.6	-0.12	0.2	-0.13	0.4
Astrovirus	0.08	0.8	1.5	0.5	1.8	0.03
Clostridium perfringens	1.6	0.8	1.5	0.3	-0.55	0.3
Staphylococcus aureus	0.17	0.8	-0.30	0.07	-0.08	0.2
Sapovirus	0.40	0.6	1.8	0.5	-6.1	0.05
Vibrio spp.	-0.32	0.9	-1.5	0.2	-0.09	1.0
Bacillus spp.	12.1	0.06	2.5	0.8	-	-

Table A5.9d Indirect model with seasonal and autocorrelation adjustment (Indirect Model 1).

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	-0.03	0.3	-0.02	0.003	-0.005	0.6
Salmonella spp.	0.04	0.1	0.06	<0.001	0.04	0.03
Rotavirus	0.06	<0.001	0.72	<0.001	0.19	<0.001
Shigella spp.	0.51	0.001	0.16	0.004	0.17	0.4
Giardia lamblia	-0.01	0.9	0.10	0.1	-0.04	0.8
Cryptosporidium spp.	-0.03	0.5	0.01	0.7	-0.09	0.5
Adenovirus	-0.02	0.8	0.07	0.1	0.25	0.6
Norovirus						
Escherichia coli	-0.02	0.8	-0.16	0.05	-0.28	0.01
Astrovirus	-0.03	0.9	2.4	0.1	1.3	0.03
Clostridium perfringens	2.5	0.6	0.35	0.7	-0.01	0.9
Staphylococcus aureus	-0.48	0.4	0.11	0.4	-0.03	0.6
Sapovirus	-0.09	0.9	-0.06	1.0	1.6	0.2
Vibrio spp.	0.37	0.9	-0.80	0.4	-0.08	0.9
Bacillus spp.	9.8	0.1	-4.6	0.5	-	-

Table A5.9e Indirect model with adjustment only for long-term trends (Indirect Model 2).

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	-0.10	<0.001	-0.006	0.2	-0.01	0.07
Salmonella spp.	0.0	0.8	0.05	<0.001	0.03	0.03
Rotavirus	0.05	<0.001	0.32	0.002	0.16	<0.001
Shigella spp.	0.51	0.001	0.13	0.02	0.16	0.4
Giardia lamblia	0.27	0.07	0.12	0.05	-0.02	0.9
Cryptosporidium spp.	0.06	0.07	-0.03	0.2	-0.06	0.6
Adenovirus	0.08	0.2	0.12	0.8	0.28	0.6
Norovirus						
Escherichia coli	-0.17	0.06	-0.20	0.01	-0.30	0.004
Astrovirus	0.53	0.008	3.1	0.05	1.4	0.02
Clostridium perfringens	-0.67	0.9	0.01	1.0	-0.03	0.8
Staphylococcus aureus	0.51	0.4	0.17	0.2	-0.02	0.8
Sapovirus	0.89	0.1	-1.5	0.5	2.1	0.1
Vibrio spp.	-0.72	0.8	-1.9	0.05	-0.01	1.0
Bacillus spp.	5.2	0.4	-8.9	0.2	-	-

Table A5.9f Indirect model with adjustment for long-term trend and no pathogen laboratory report lags (Indirect Model 3)

	<5 years		5-64 years		≥65 years	
	Coefficient	Wald test P value	Coefficient	Wald test P value	Coefficient	Wald test P value
Campylobacter spp.	-0.11	<0.001	0.0	0.8	-0.01	0.09
Salmonella spp.	-0.03	0.2	0.04	<0.001	0.006	0.7
Rotavirus	0.05	<0.001	0.28	0.003	0.10	0.004
Shigella spp.	0.43	0.001	0.12	0.01	0.12	0.4
Giardia lamblia	0.23	0.04	0.07	0.2	0.07	0.5
Cryptosporidium spp.	0.04	0.2	-0.03	0.2	0.02	0.9
Adenovirus	0.10	0.04	-0.01	1.0	0.26	0.5
Norovirus						
Escherichia coli	-0.12	0.1	-0.15	0.01	-0.23	0.004
Astrovirus	0.38	0.02	1.2	0.2	0.34	0.3
Clostridium perfringens	0.78	0.7	-0.21	0.7	-0.03	0.5
Staphylococcus aureus	0.18	0.7	0.03	0.6	0.05	0.3
Sapovirus	0.39	0.4	0.26	0.9	0.93	0.2
Vibrio spp.	-1.9	0.2	-0.70	0.2	1.2	0.04
Bacillus spp.	2.3	0.7	-2.0	0.4	-	-

Appendix A5.10. Results of fitting interactions between norovirus and rotavirus laboratory reports and time.

Age group	Interaction pathogen	Null model	Pathogen coefficient	Interaction coefficient	LRT P value	Null Model AIC	Interaction model AIC
< 5 years	Norovirus	Direct 1	1.99	-0.16	<0.001	7.67	8.02
	Rotavirus	Indirect 2	0.10	-0.003	<0.001	8.89	8.57
5-64 years	Norovirus	Direct 1	1.53	-0.08	<0.001	8.69	9.10
≥65 years	Norovirus	Direct 1	0.14	-0.007	<0.001	6.39	6.41

Abbreviations: AIC, Akaike's information criterion; LRT, likelihood ratio test.

Appendix A5.11. Partial autocorrelation plots for the final models

Figure A5.11a Children aged less than five years Direct Model 1.

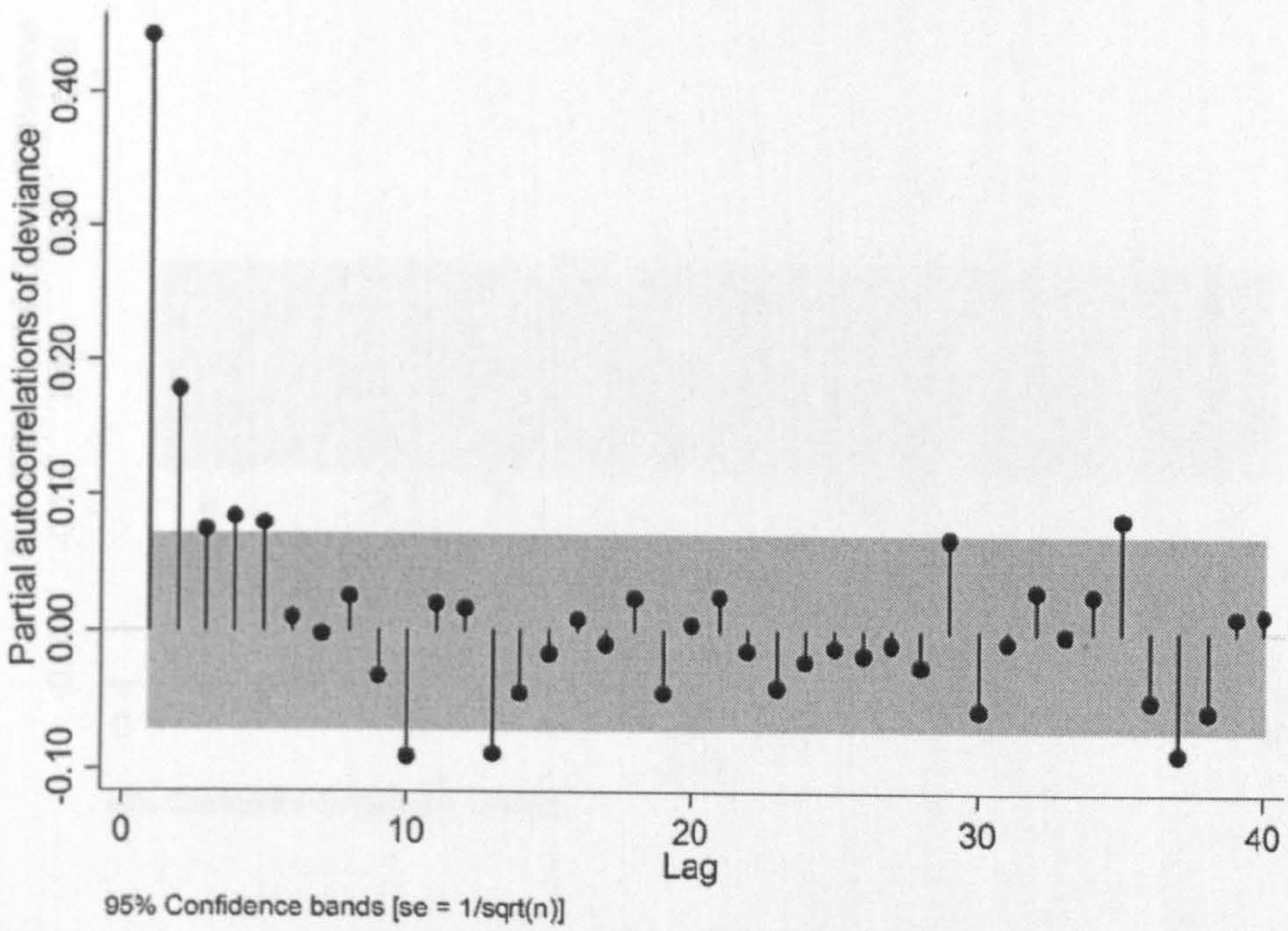


Figure A5.11b Children aged less than five years Direct Model 2.

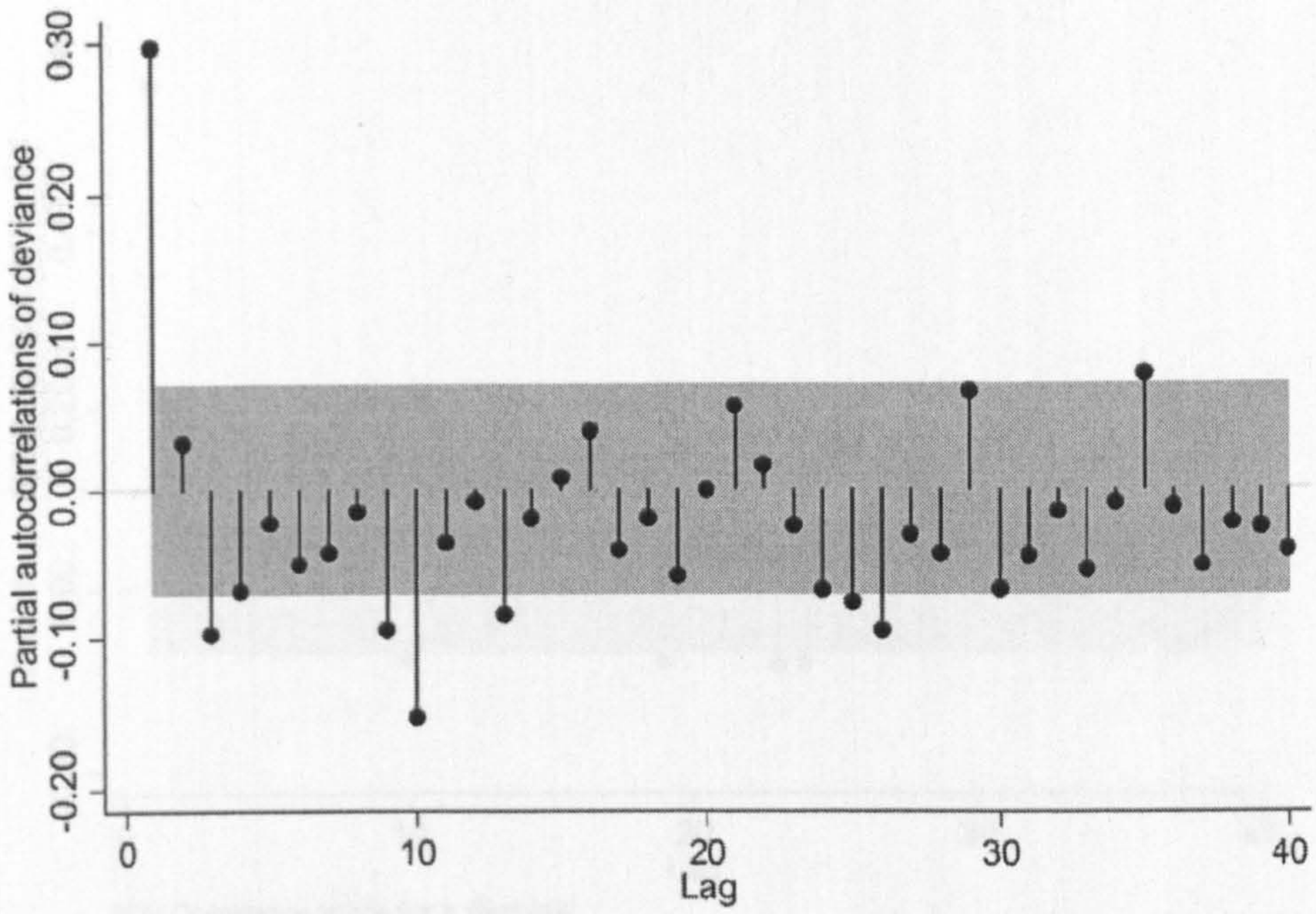


Figure A5.11c Children aged less than five years Indirect Model 1.

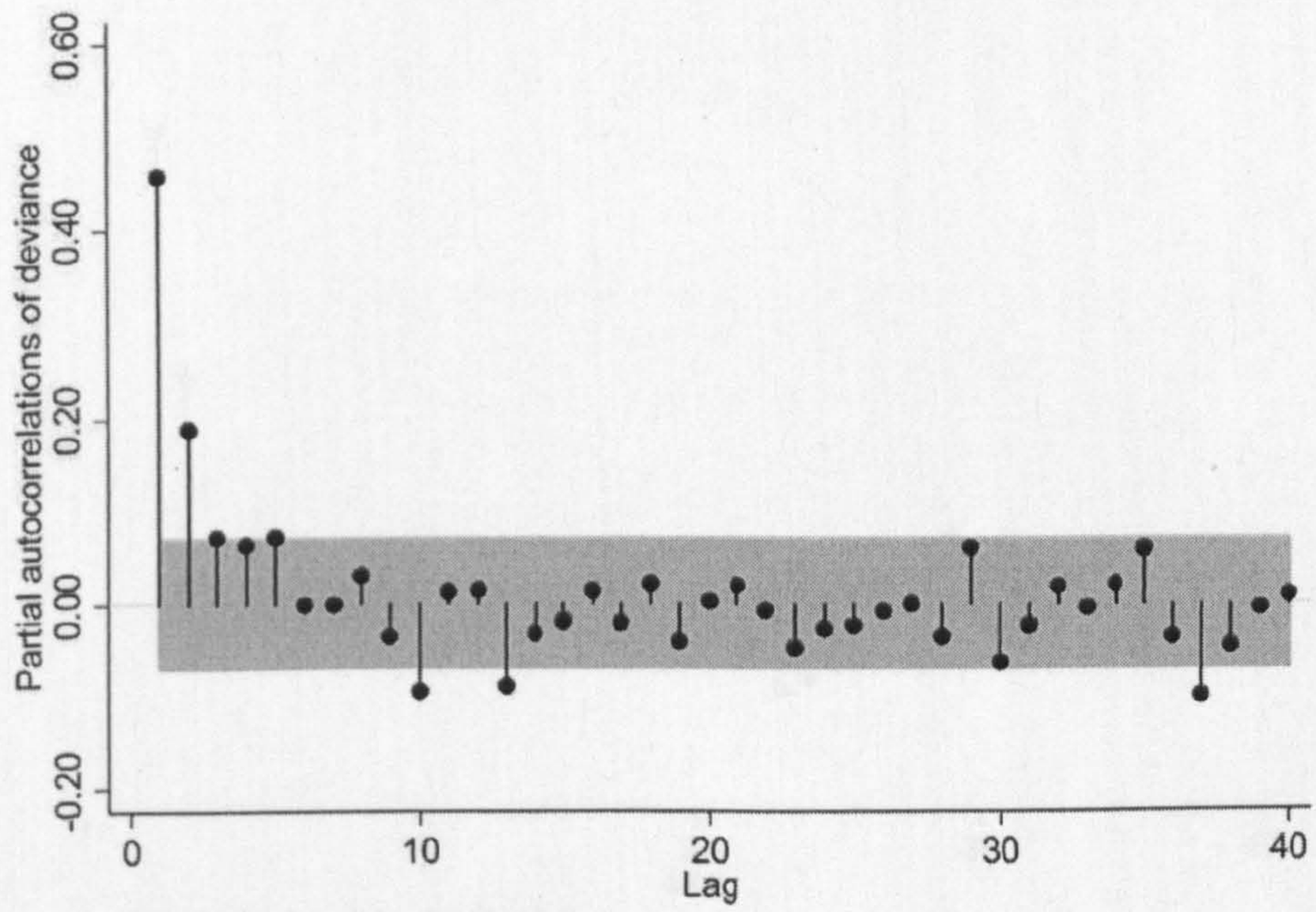


Figure A5.11d Children aged less than five years Indirect Model 2.

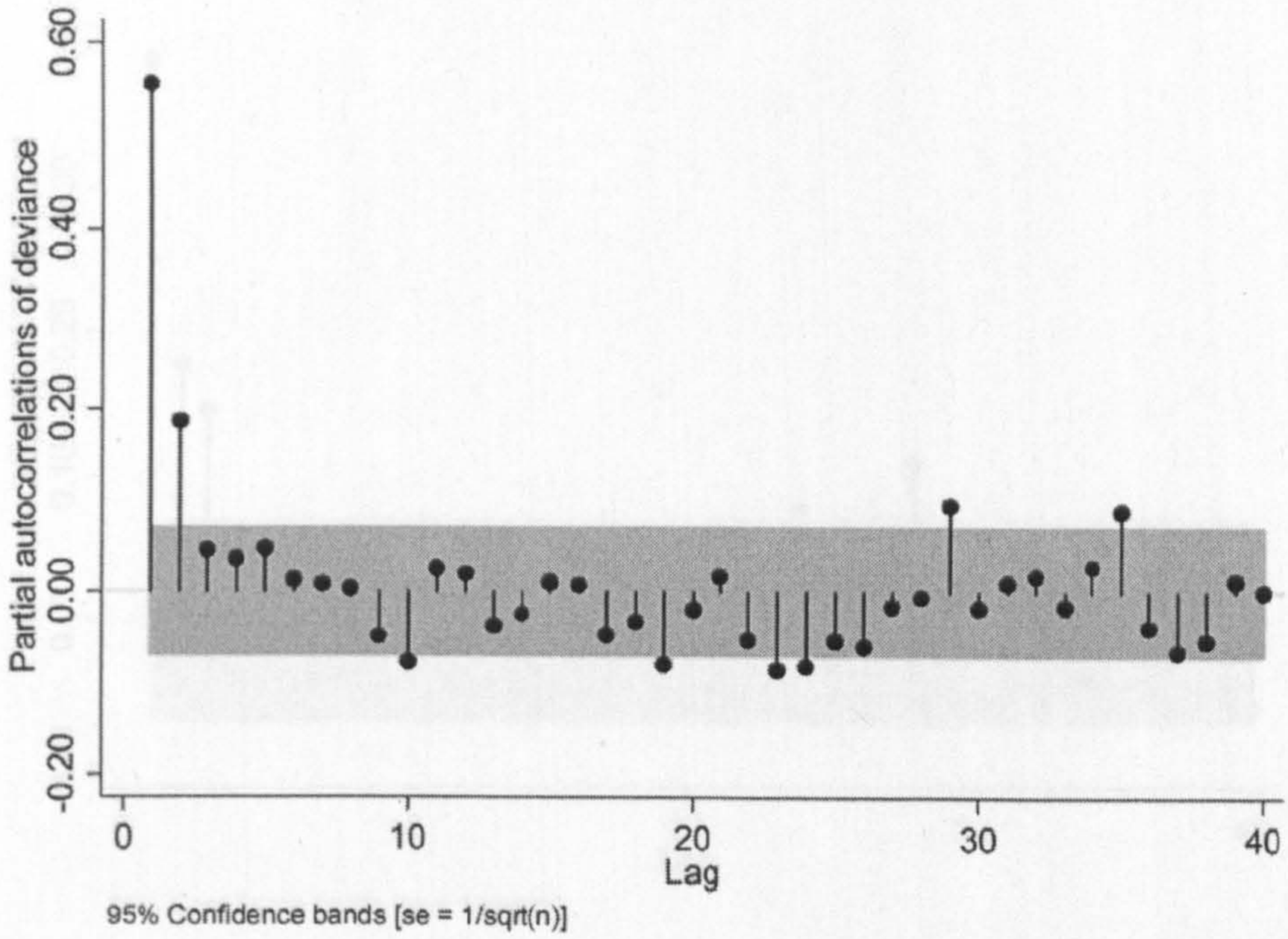


Figure A5.11e Children aged less than five years Indirect Model 3.

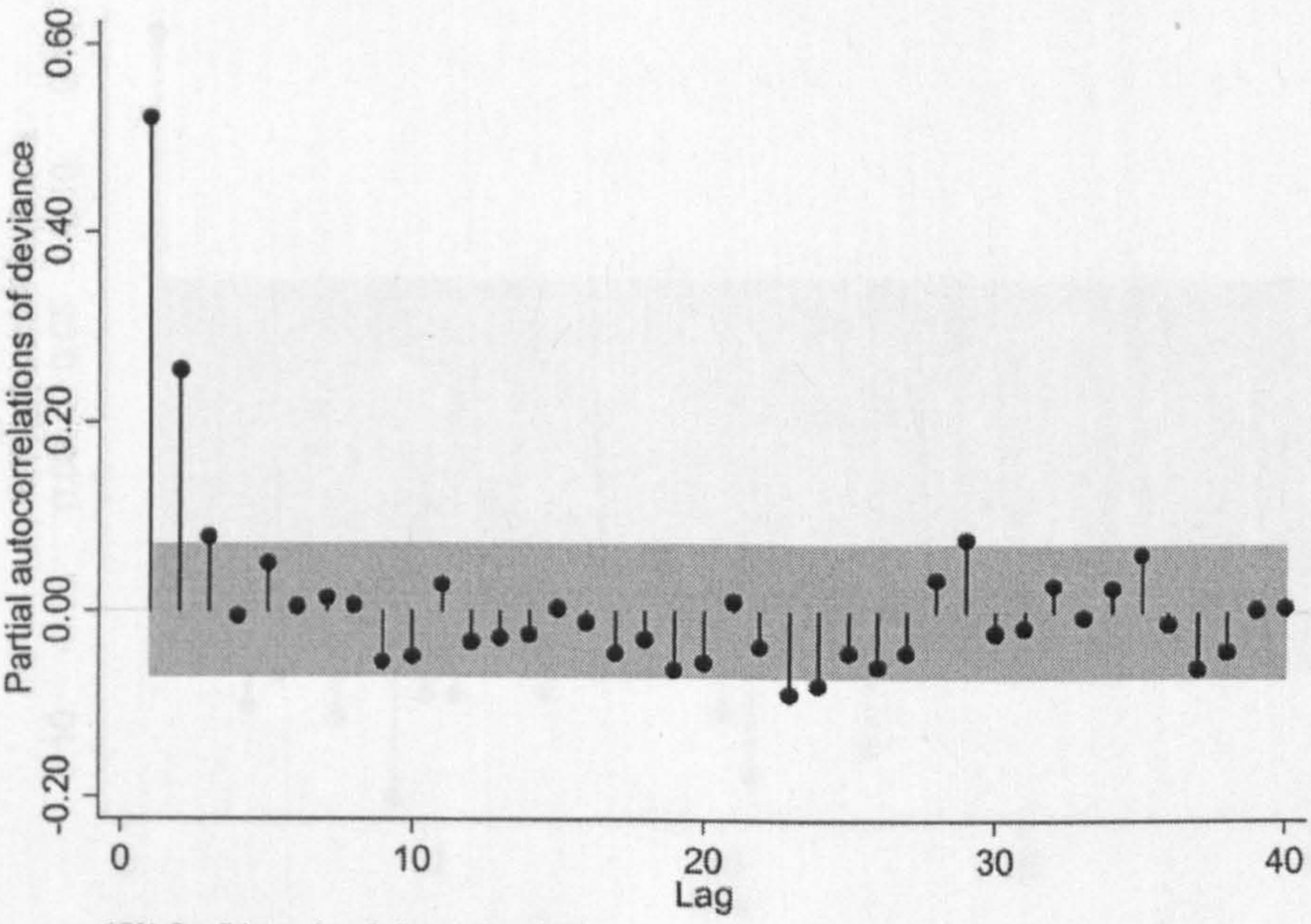


Figure A5.11g Children and adults aged between five and 64 years Direct Model 1.

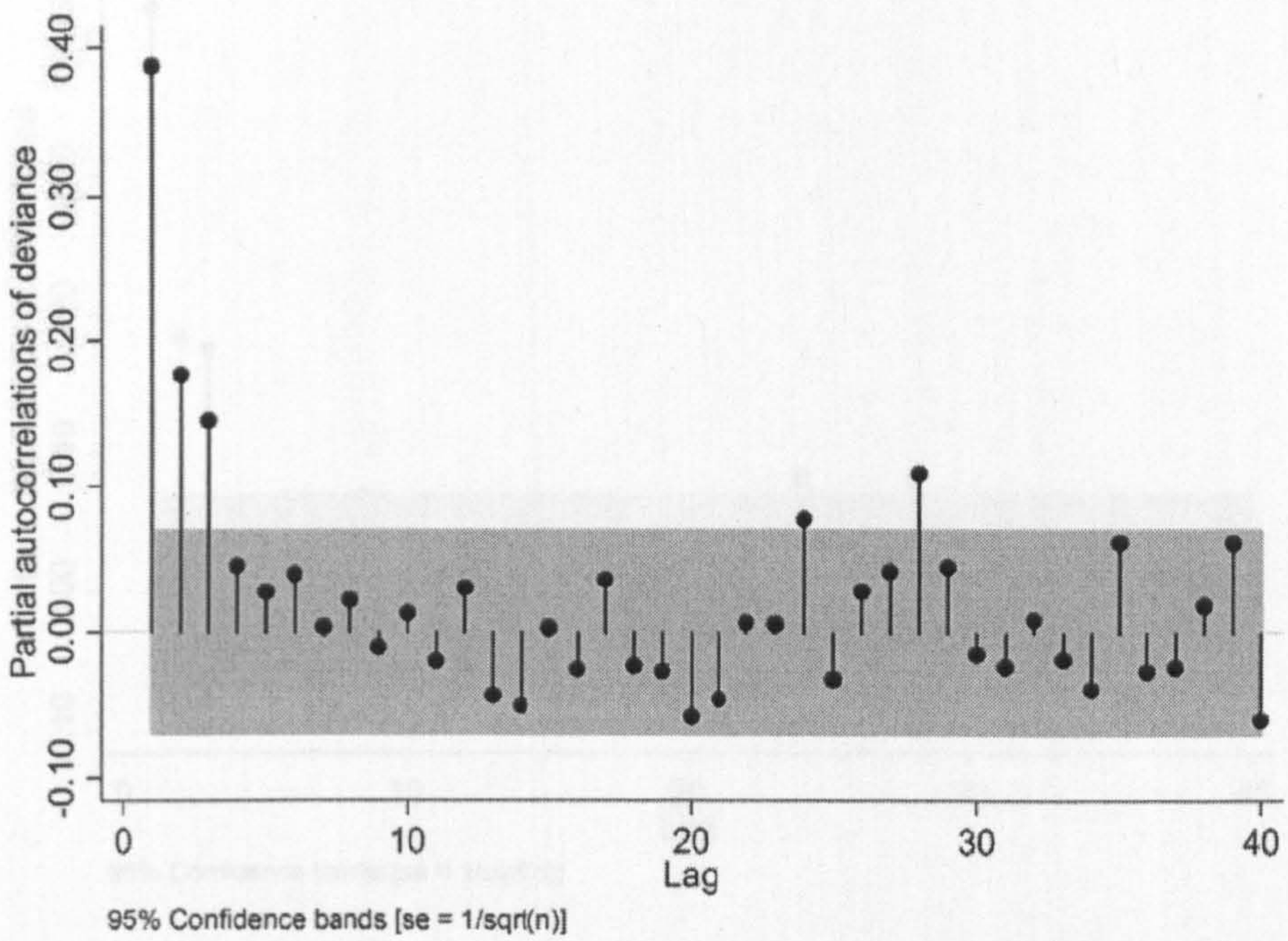


Figure A5.11h Children and adults aged between five and 64 years Direct Model 2.

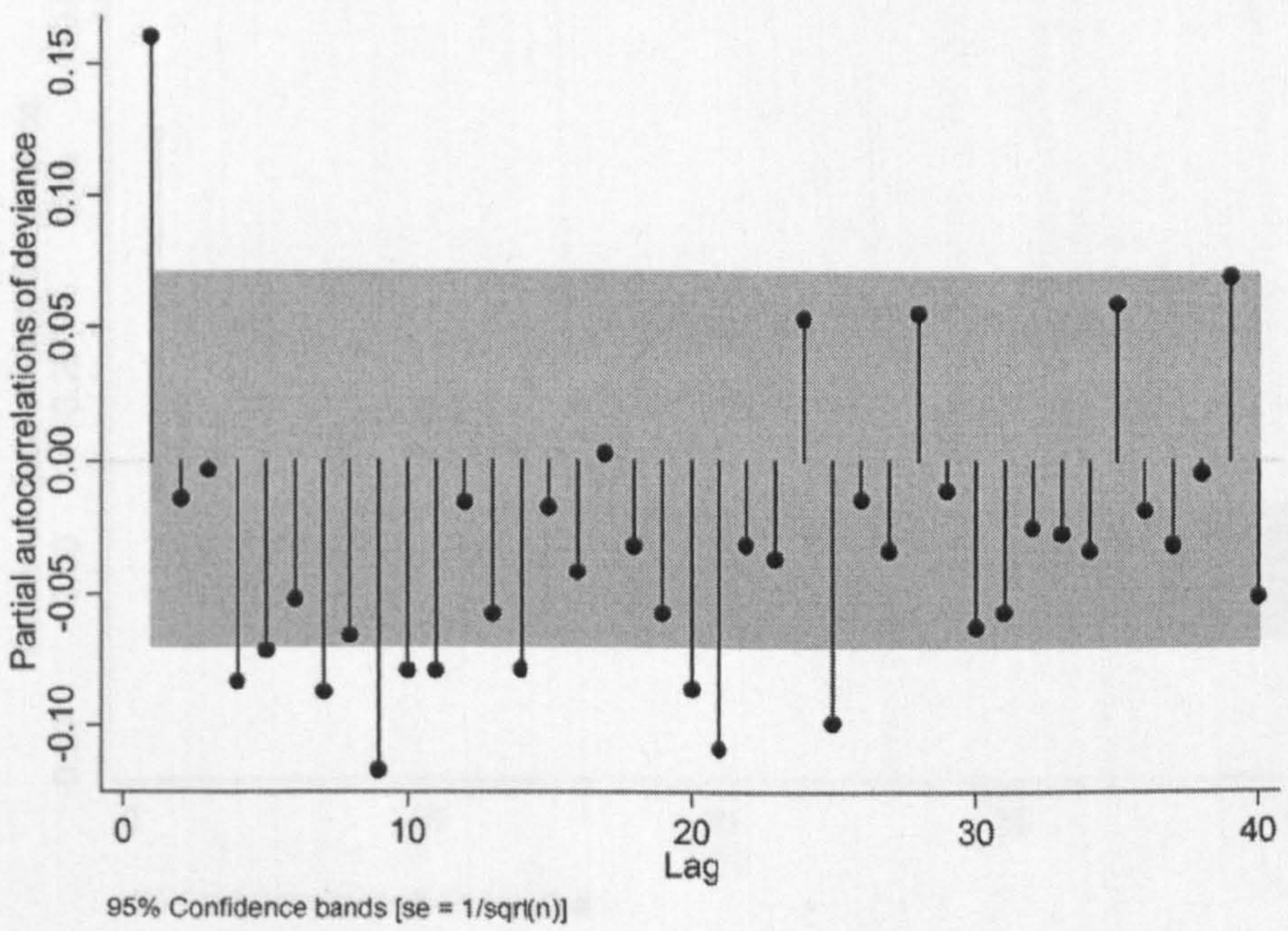


Figure A5.11i Children and adults aged between five and 64 years Indirect Model 1.

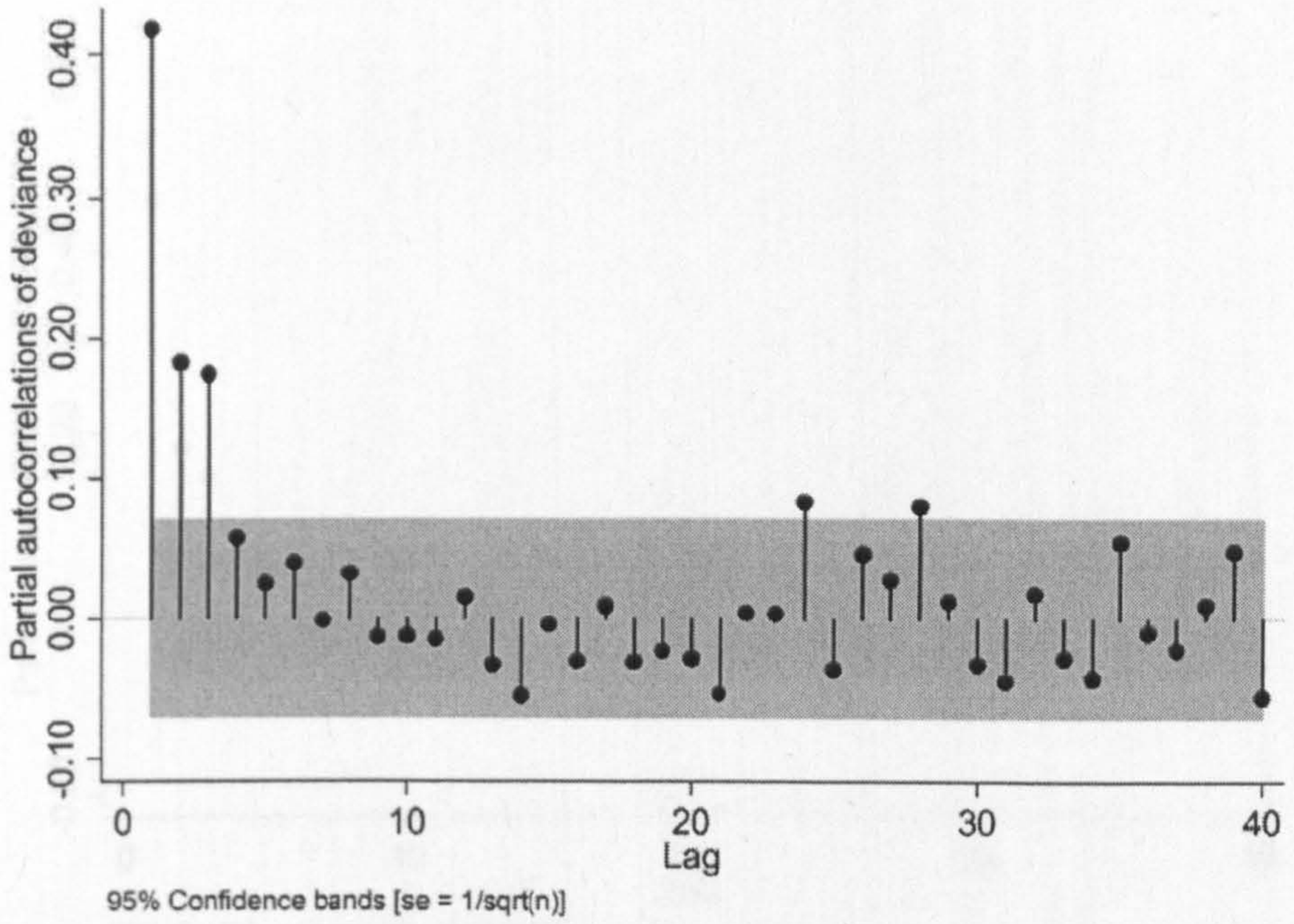


Figure A5.11j Children and adults aged between five and 64 years Indirect Model 2.

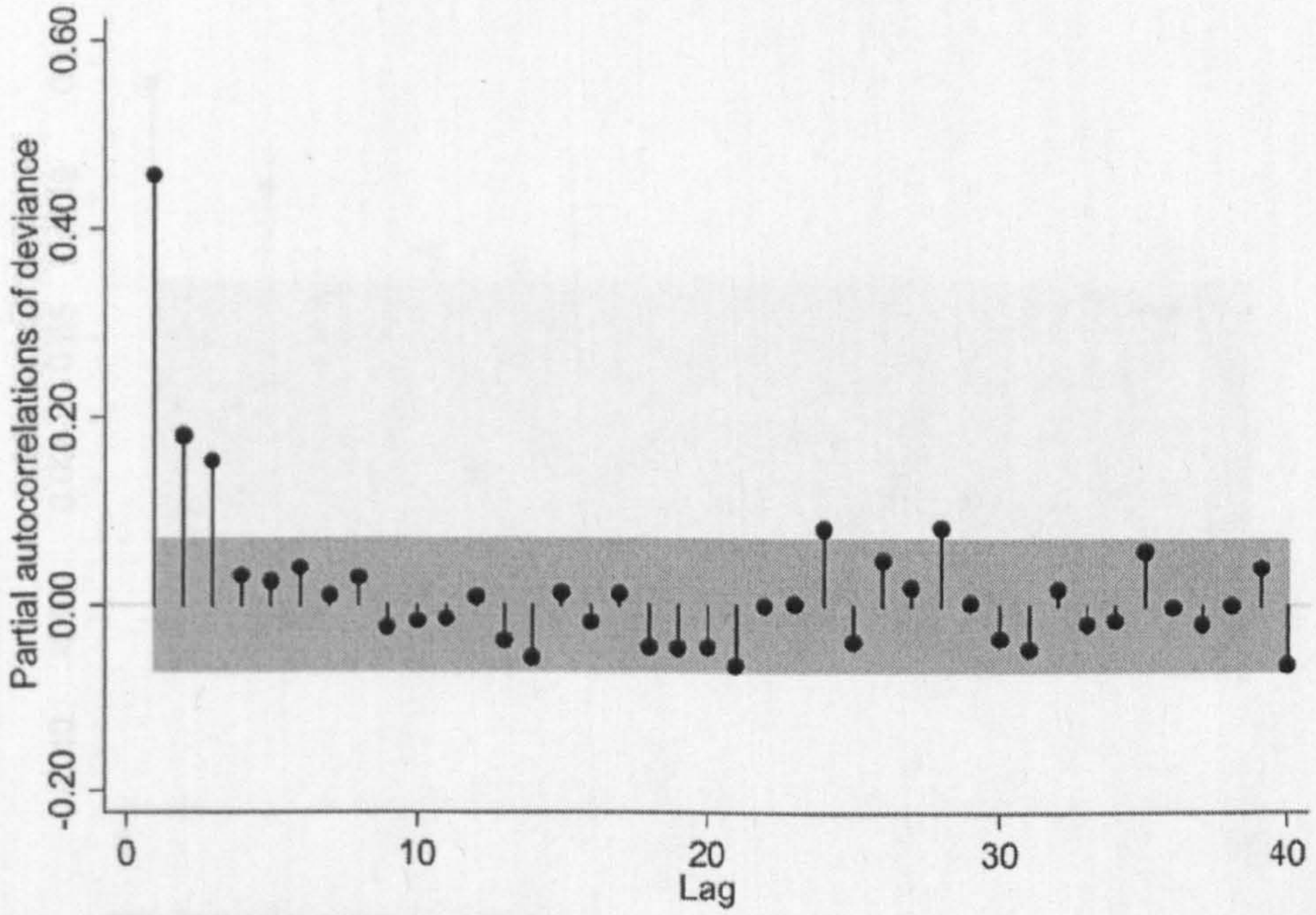


Figure A5.11k Children and adults aged between five and 64 years Indirect Model 3.

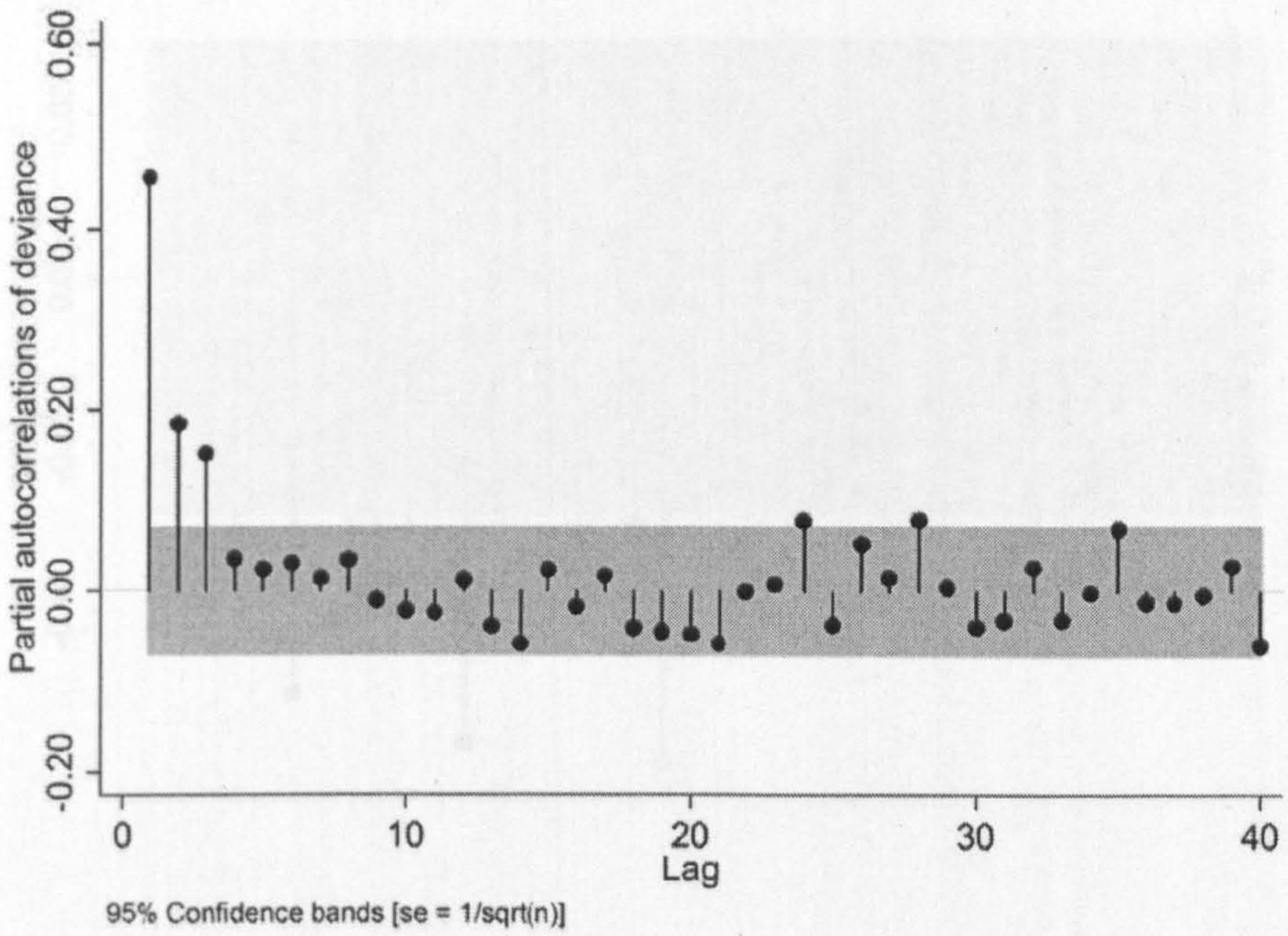


Figure A5.11m Adults aged 65 years or older Direct Model 1.

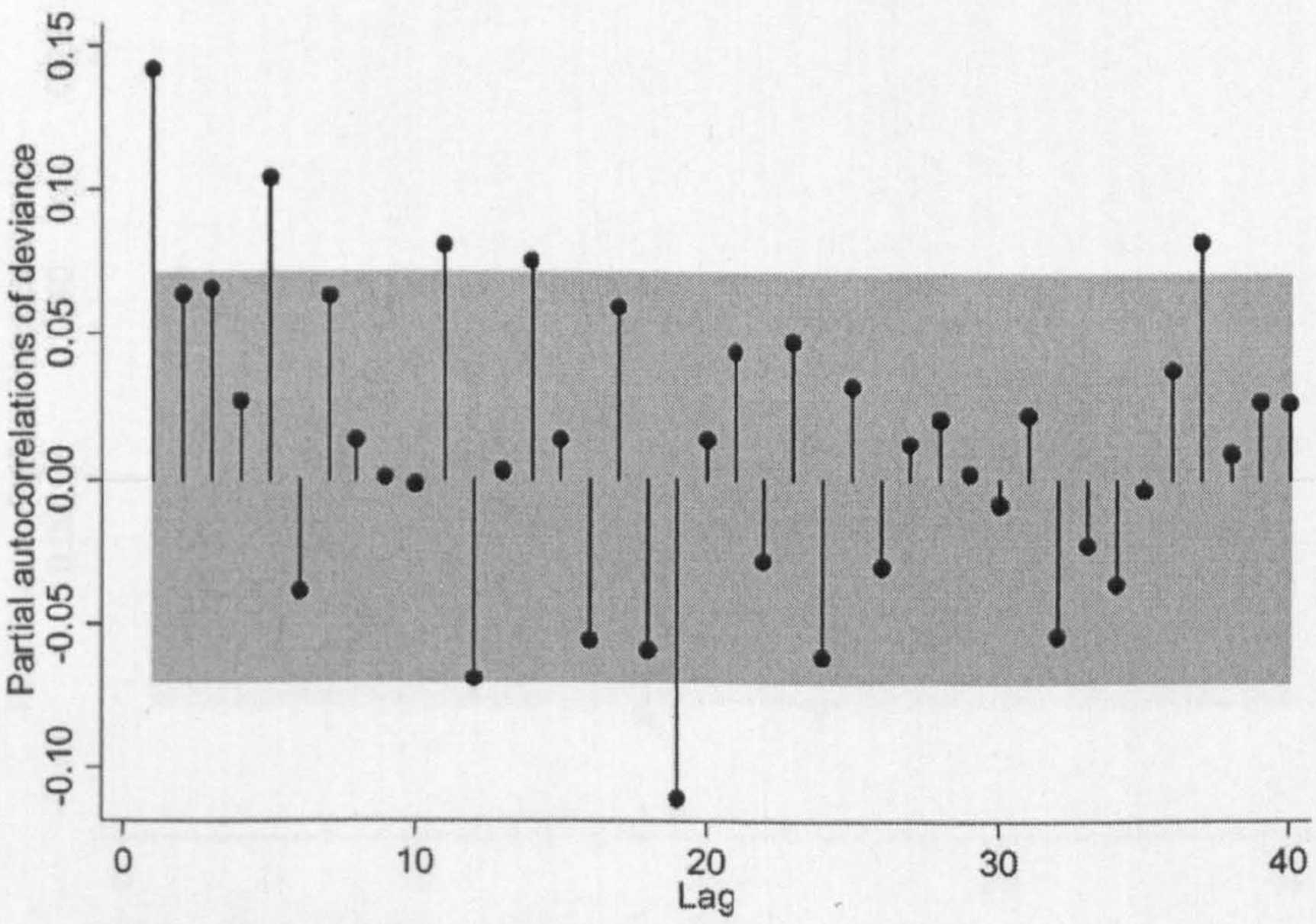


Figure A5.11n Adults aged 65 years or older Direct Model 2.

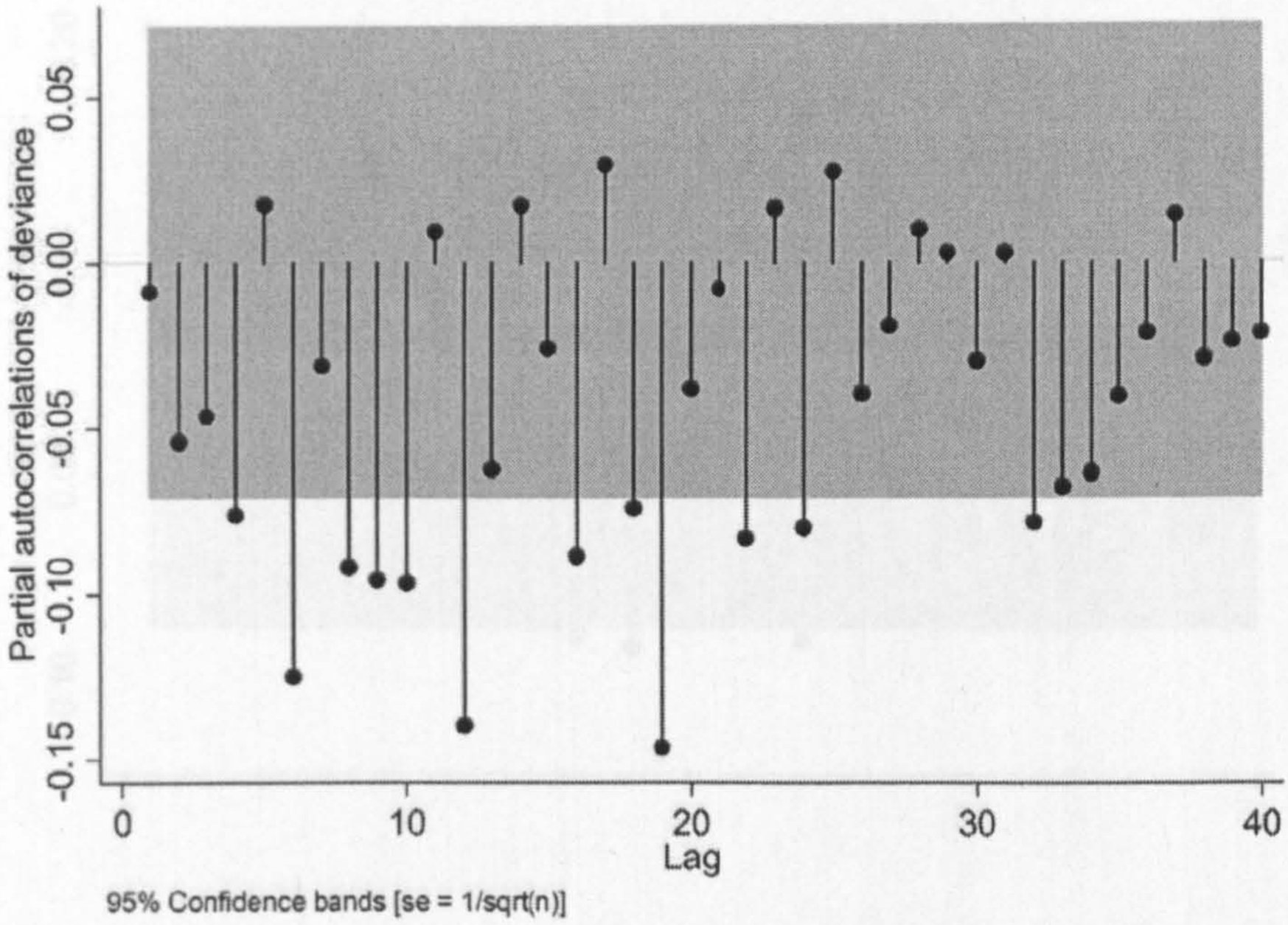


Figure A5.11o Adults aged 65 years or older Indirect Model 1.

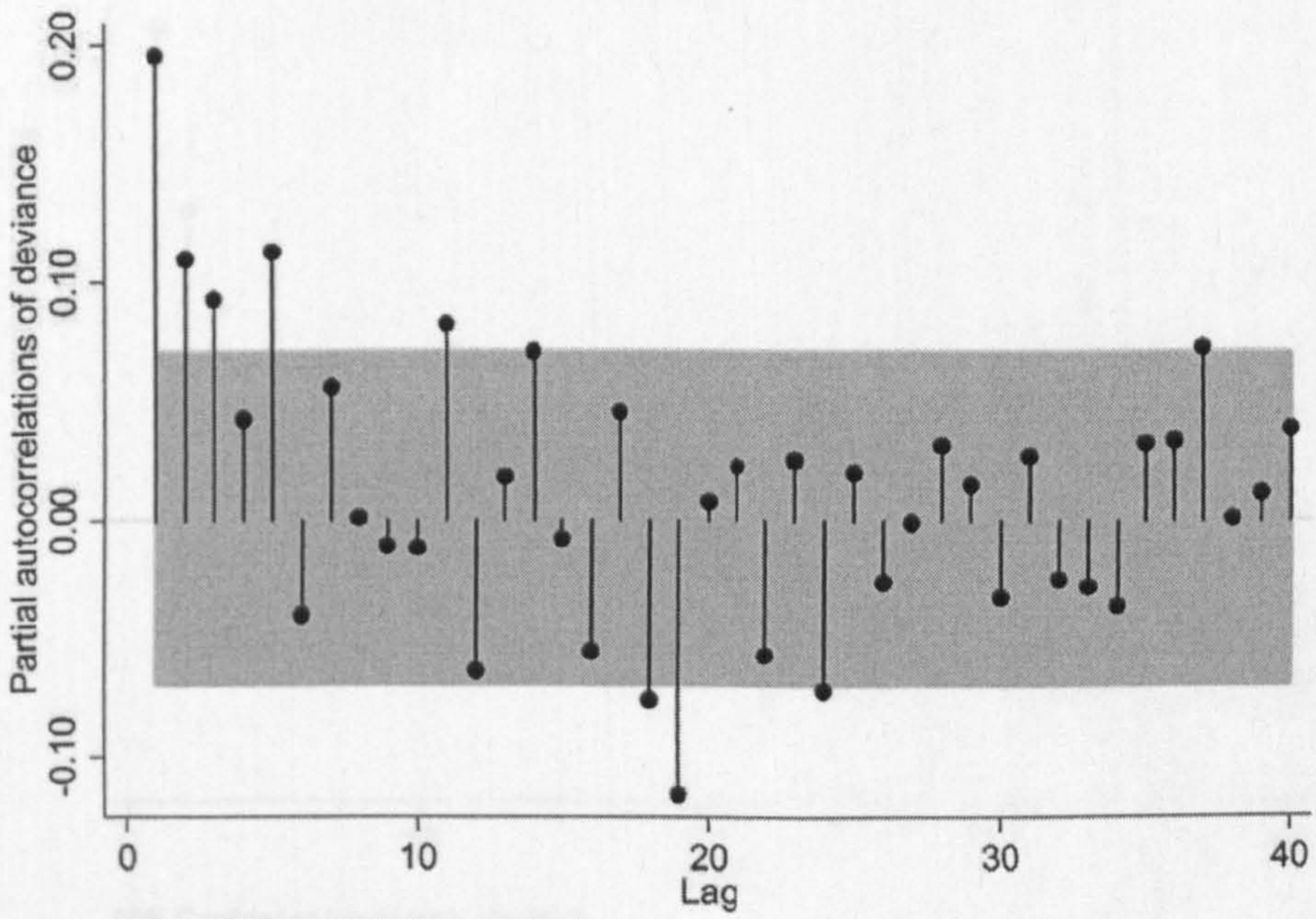


Figure A5.11p Adults aged 65 years or older Indirect Model 2.

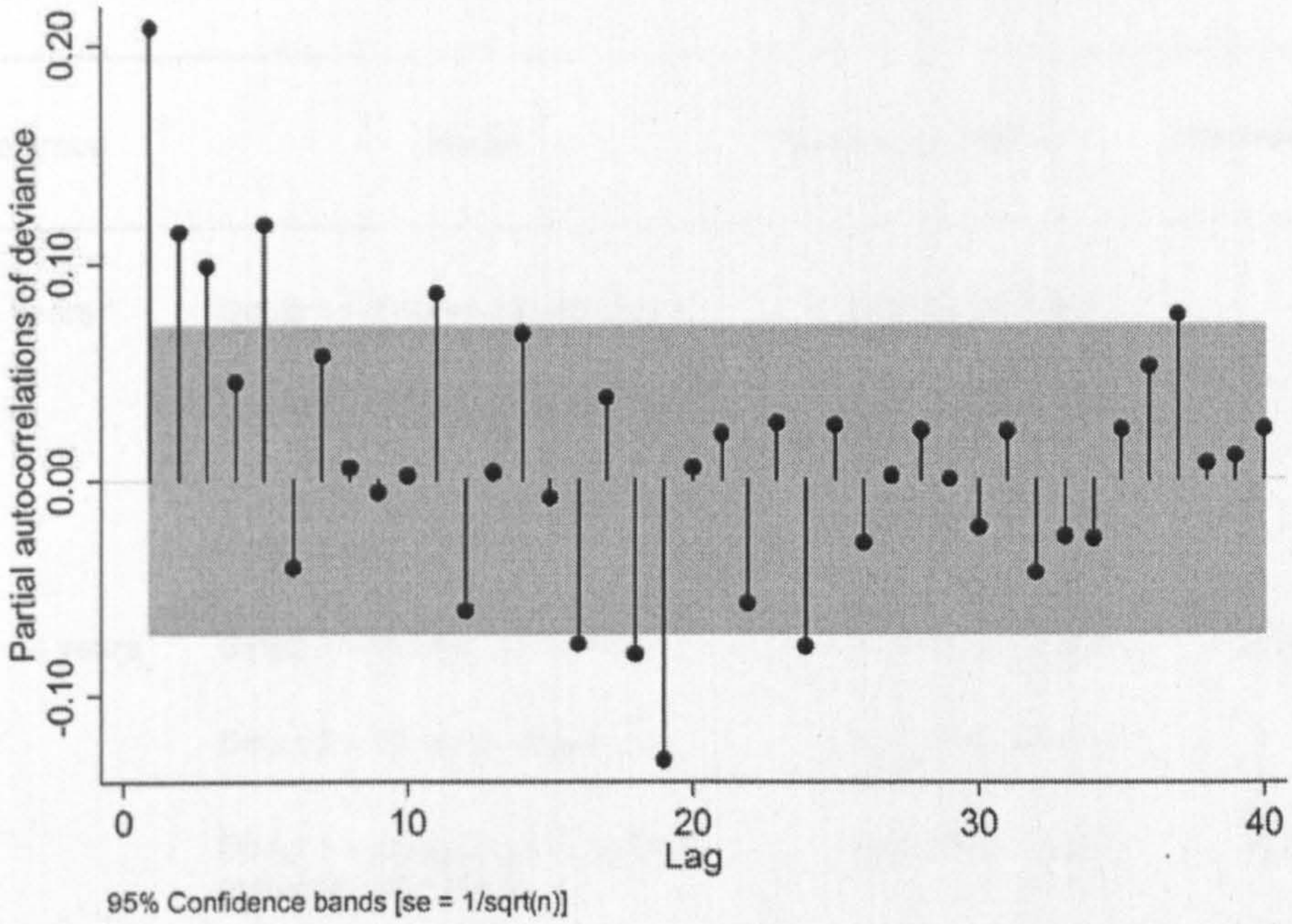
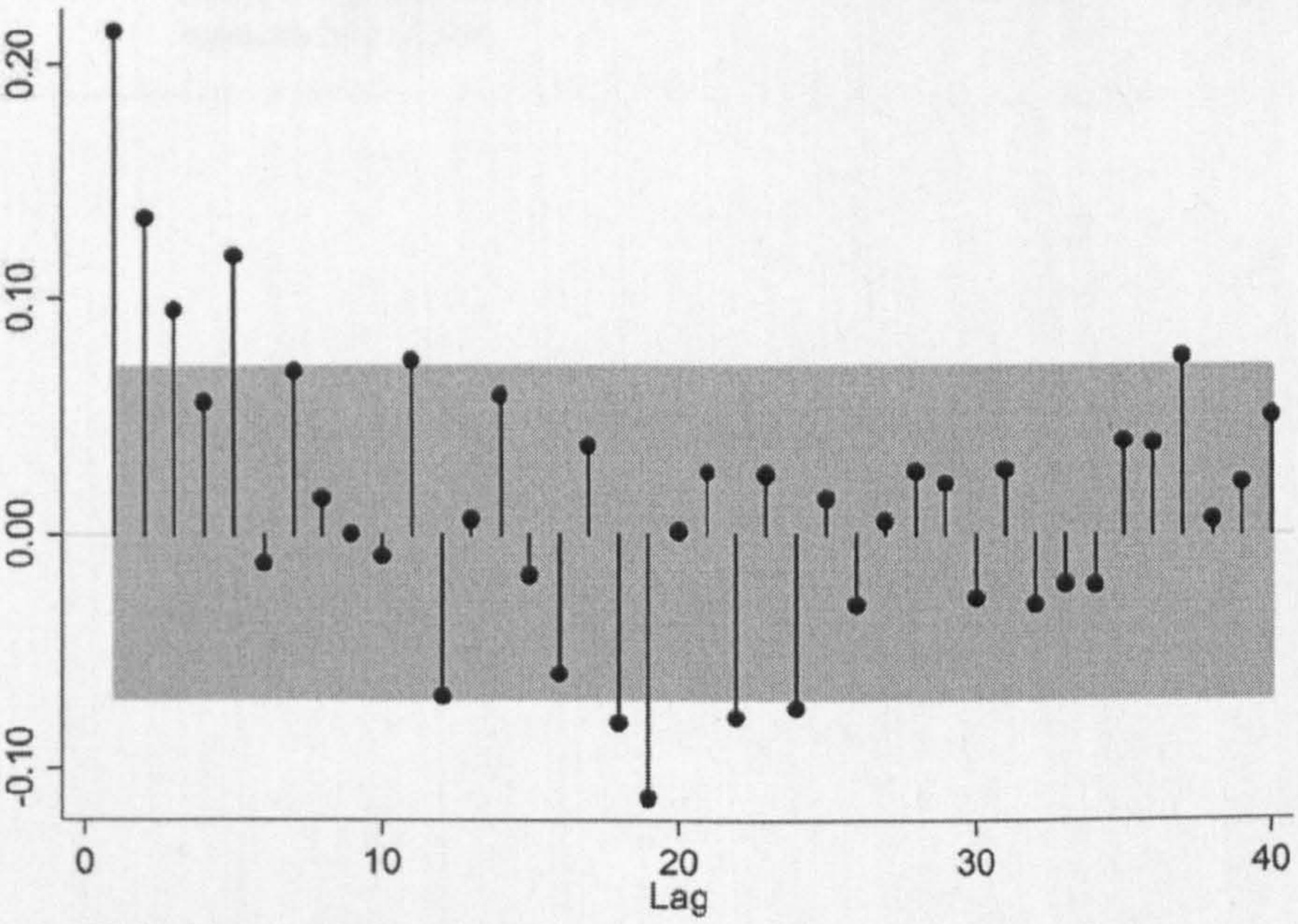


Figure A5.11q Adults aged 65 years or older Indirect Model 3.



Appendix A5.12. Norovirus coefficients in the final direct models

Age group	Model	Norovirus coefficient	Interaction coefficient
< 5 years	Direct 1 – Fourier adjustment	0.59 (0.33, 0.86)	
	Direct 2 – 13 week adjustment	0.36 (0.00 , 0.72)	
	Direct 1 – categorical year term replaces cubic trend	0.54 (0.25, 0.83)	
5 – 64 years	Direct 1 – Fourier adjustment	1.39 (0.73, 2.06)	-0.08 (-0.13, -0.03)
	Direct 2 – 13 week adjustment	0.20 (0.00, 0.41)	
	Direct 1 – categorical year term replaces cubic trend	1.39 (0.68, 2.09)	-0.09 (-0.14, -0.04)
≥65 years	Direct 1 – Fourier adjustment	0.14 (0.07, 0.20)	-0.007 (-0.01, -0.002)
	Direct 2 – 13 week adjustment	0.02 (0.003, 0.041)	
	Direct 1 – categorical year term replaces cubic trend	0.12 (0.05, 0.19)	-0.006 (-0.01, -0.0007)

Appendix A5.13. Changes in Pearson's residuals over the study period

Figure A5.13a Children aged less than five years Direct Model 1.

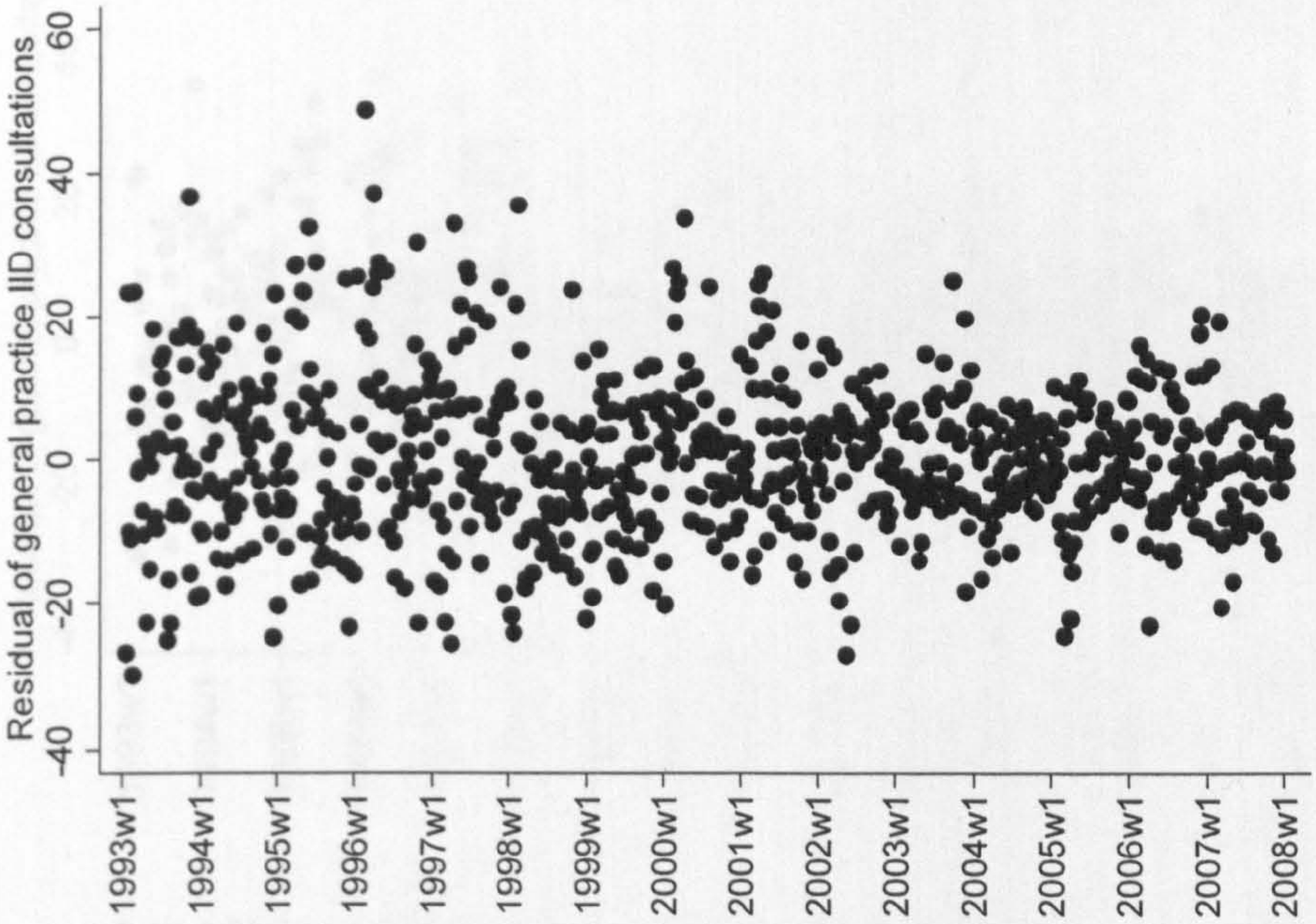


Figure A5.13b Children aged less than five years Direct Model 2.

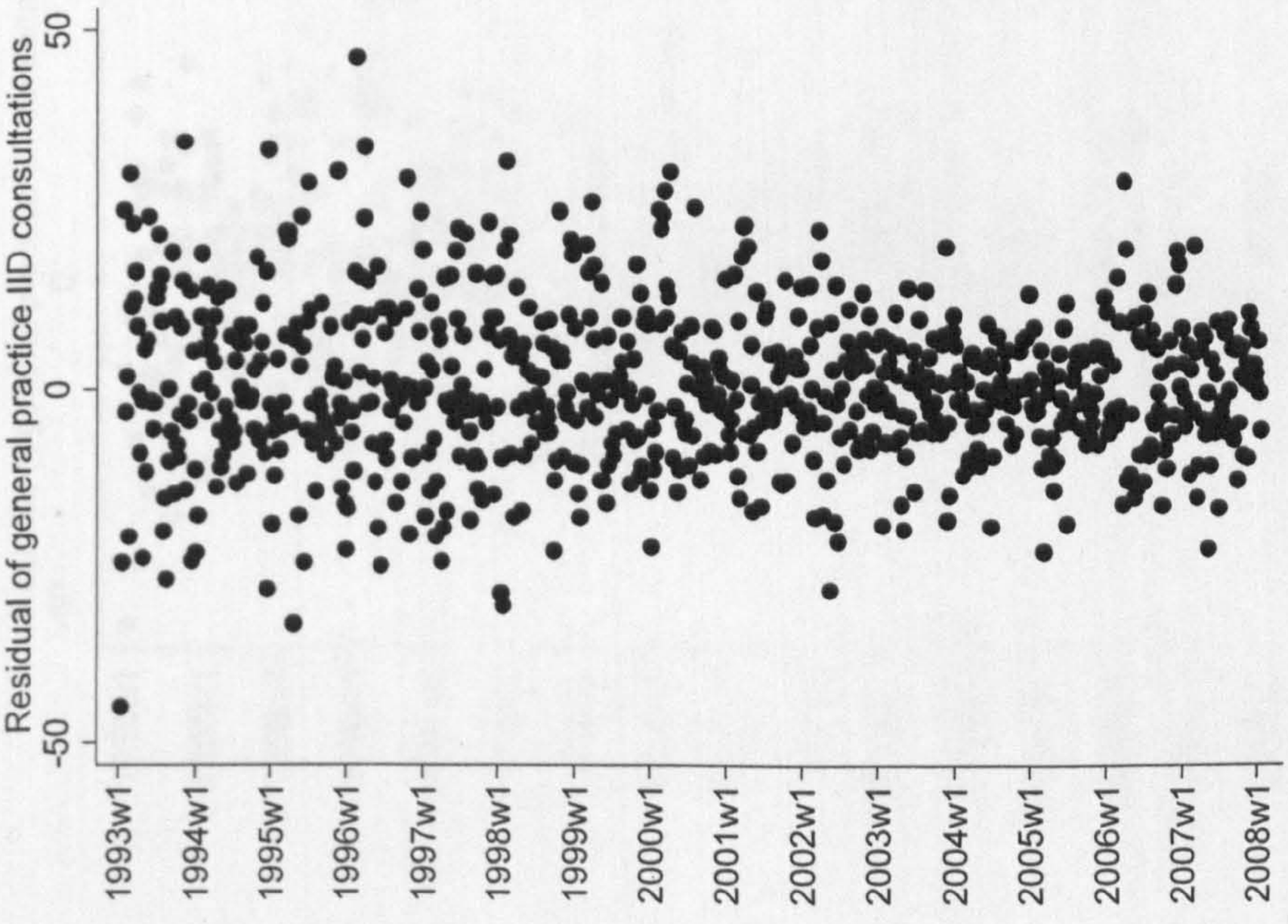


Figure A5.13c Children aged less than five years Indirect Model 1.

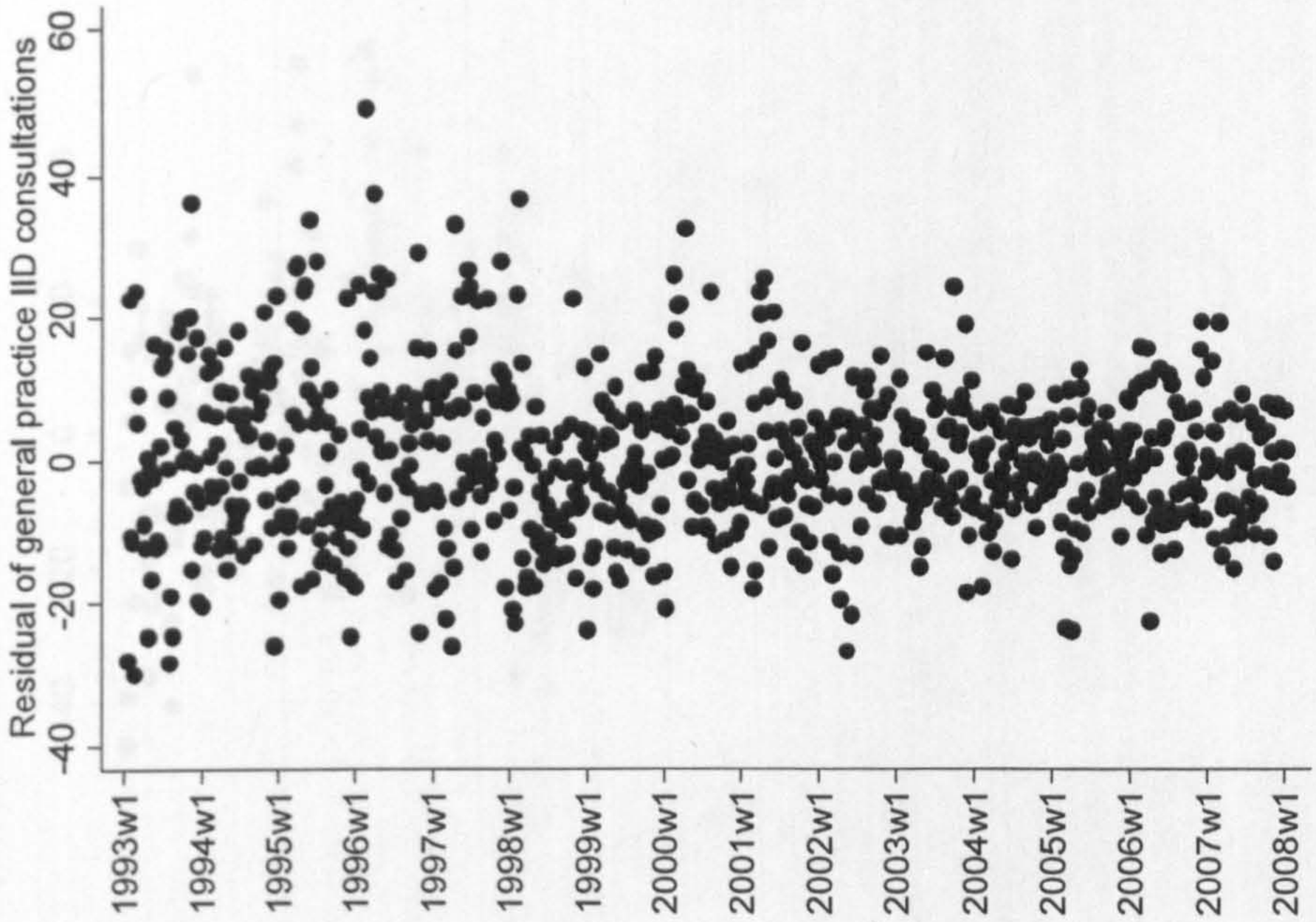


Figure A5.13d Children aged less than five years Indirect Model 2.

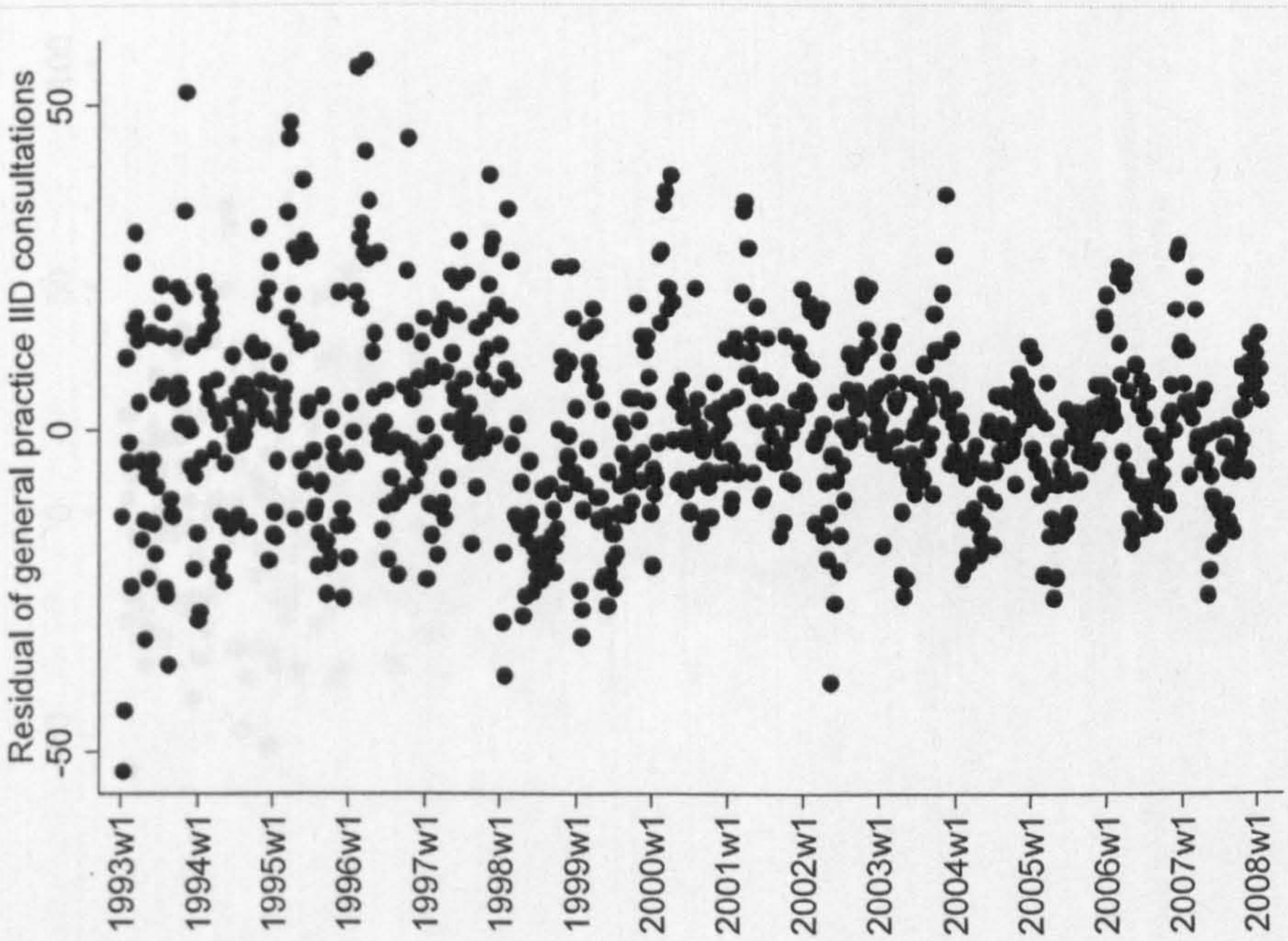


Figure A5.13e Children aged less than five years Indirect Model 3.

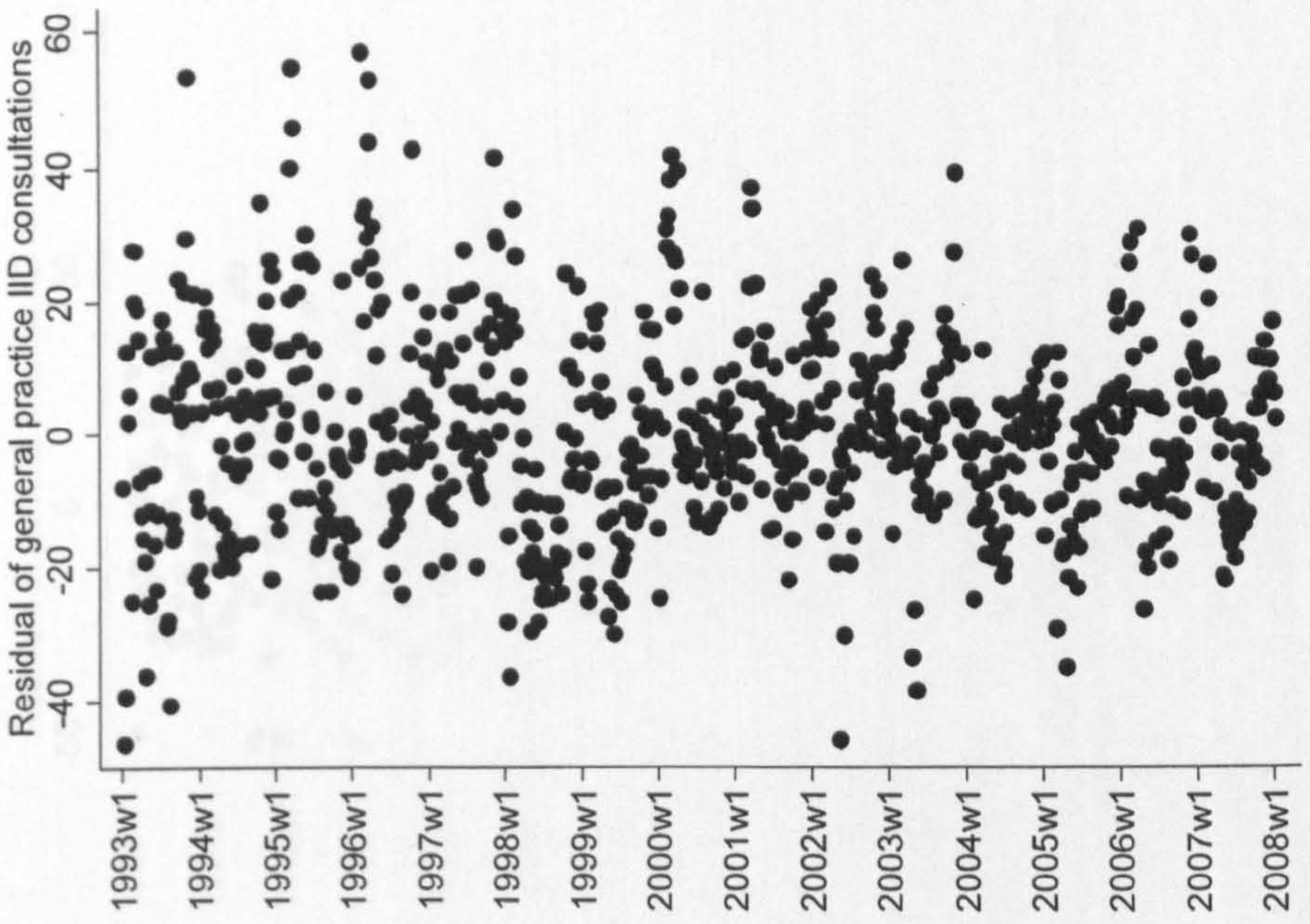


Figure A5.13g Children and adults aged between five and 64 years Direct Model 1.

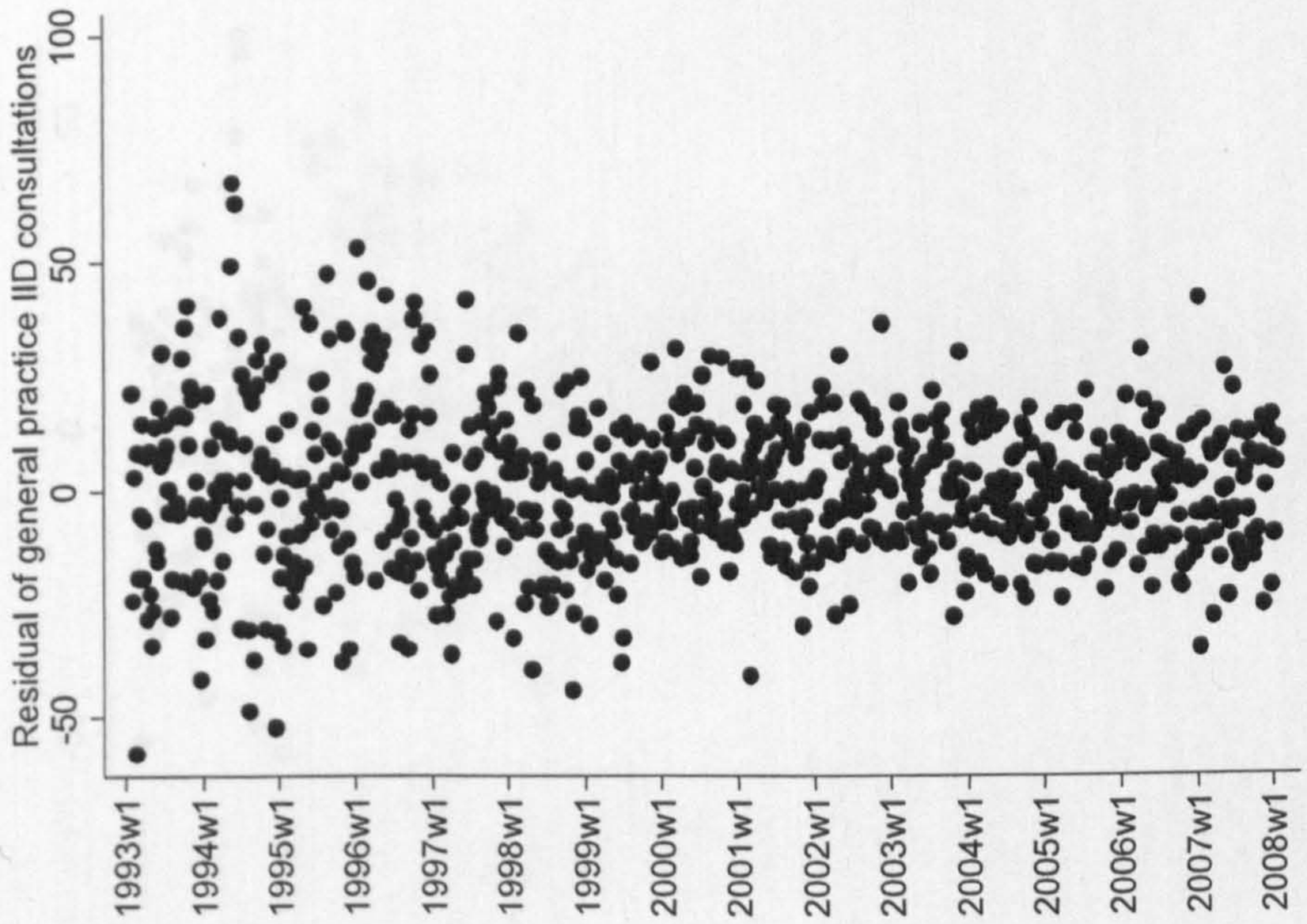


Figure A5.13h Children and adults aged between five and 64 years Direct Model 2.

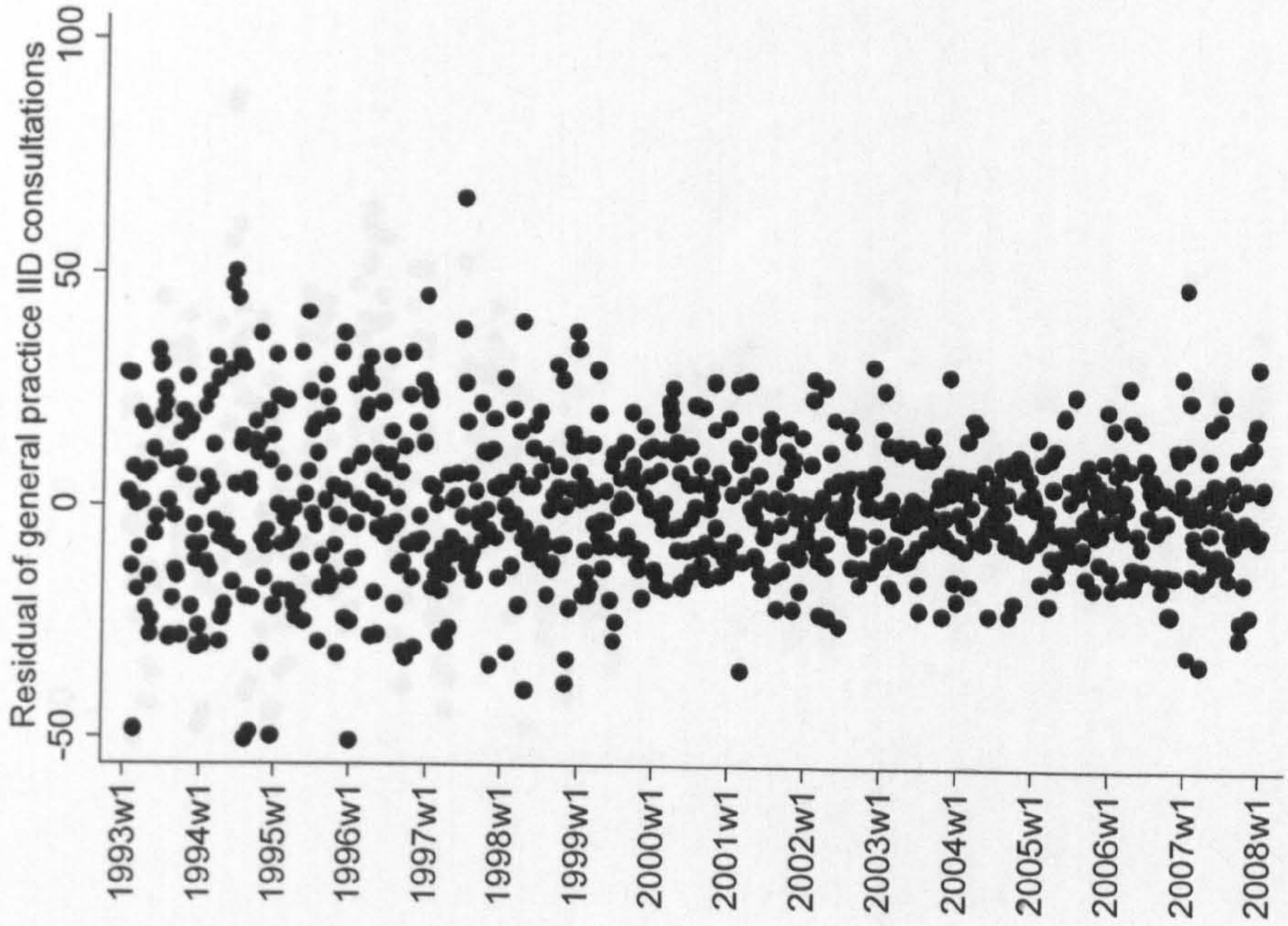


Figure A5.13i Children and adults aged between five and 64 years Indirect Model 1.

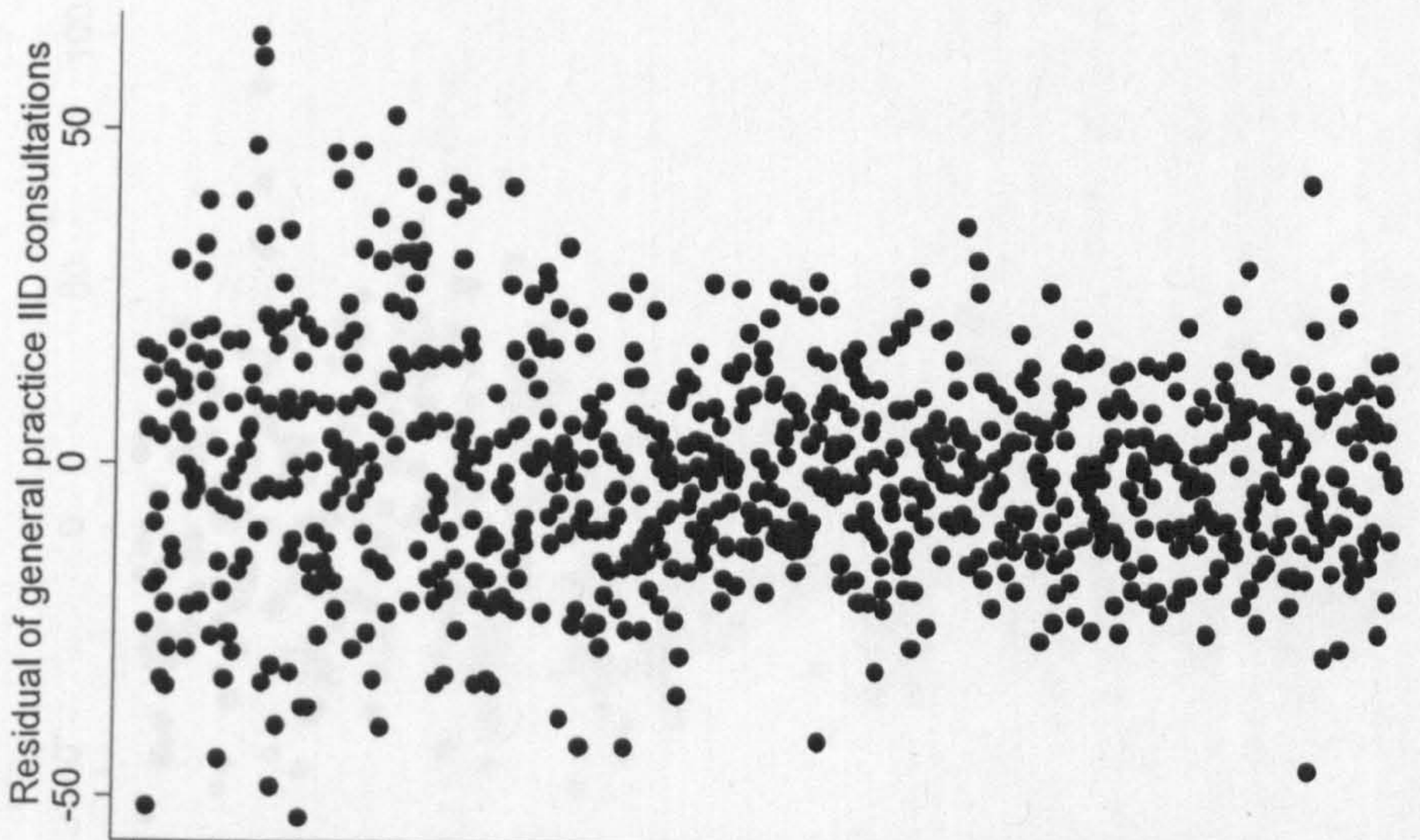


Figure A5.13j Children and adults aged between five and 64 years Indirect Model 2.

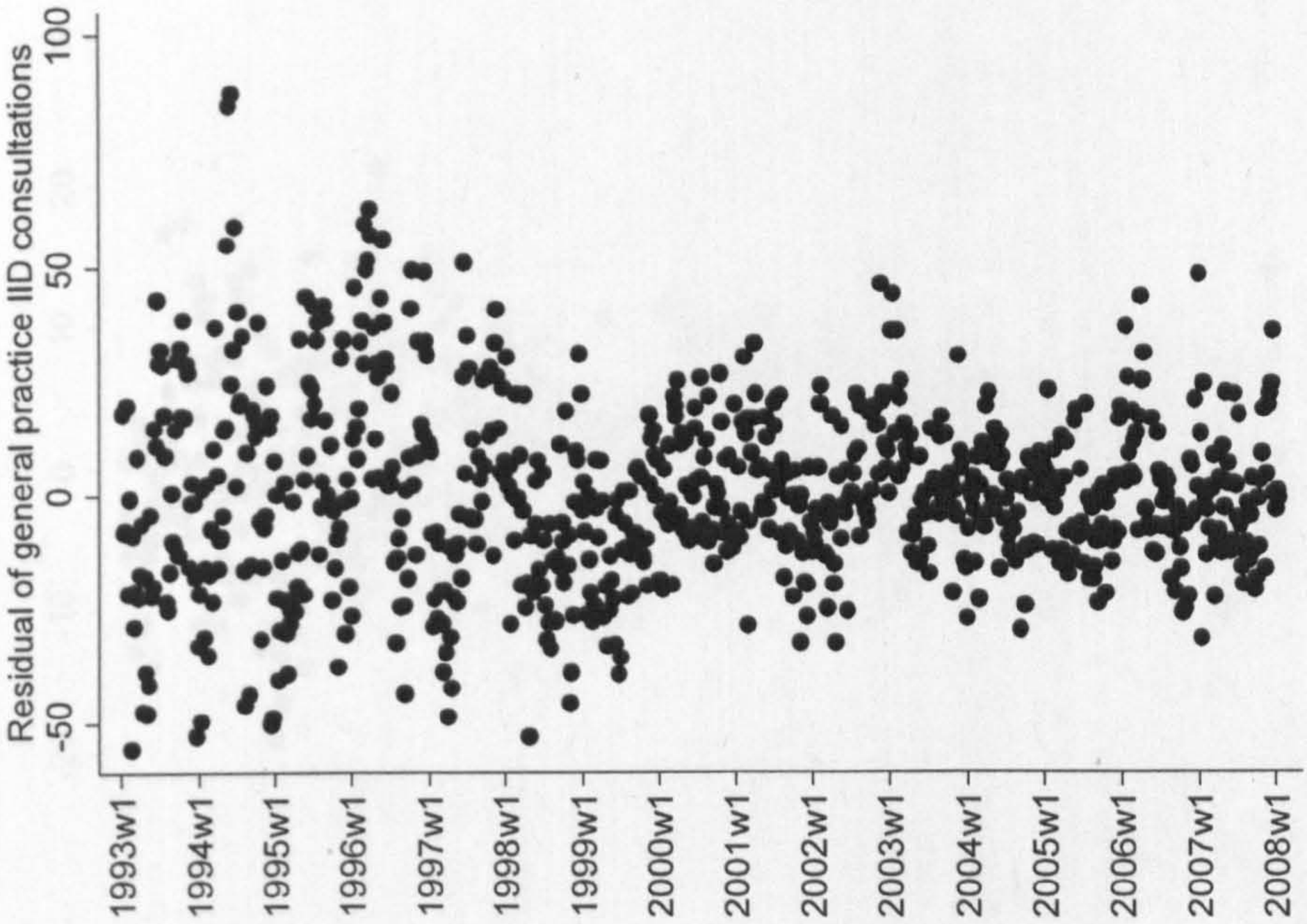


Figure A5.13k Children and adults aged between five and 64 years Indirect Model 3.

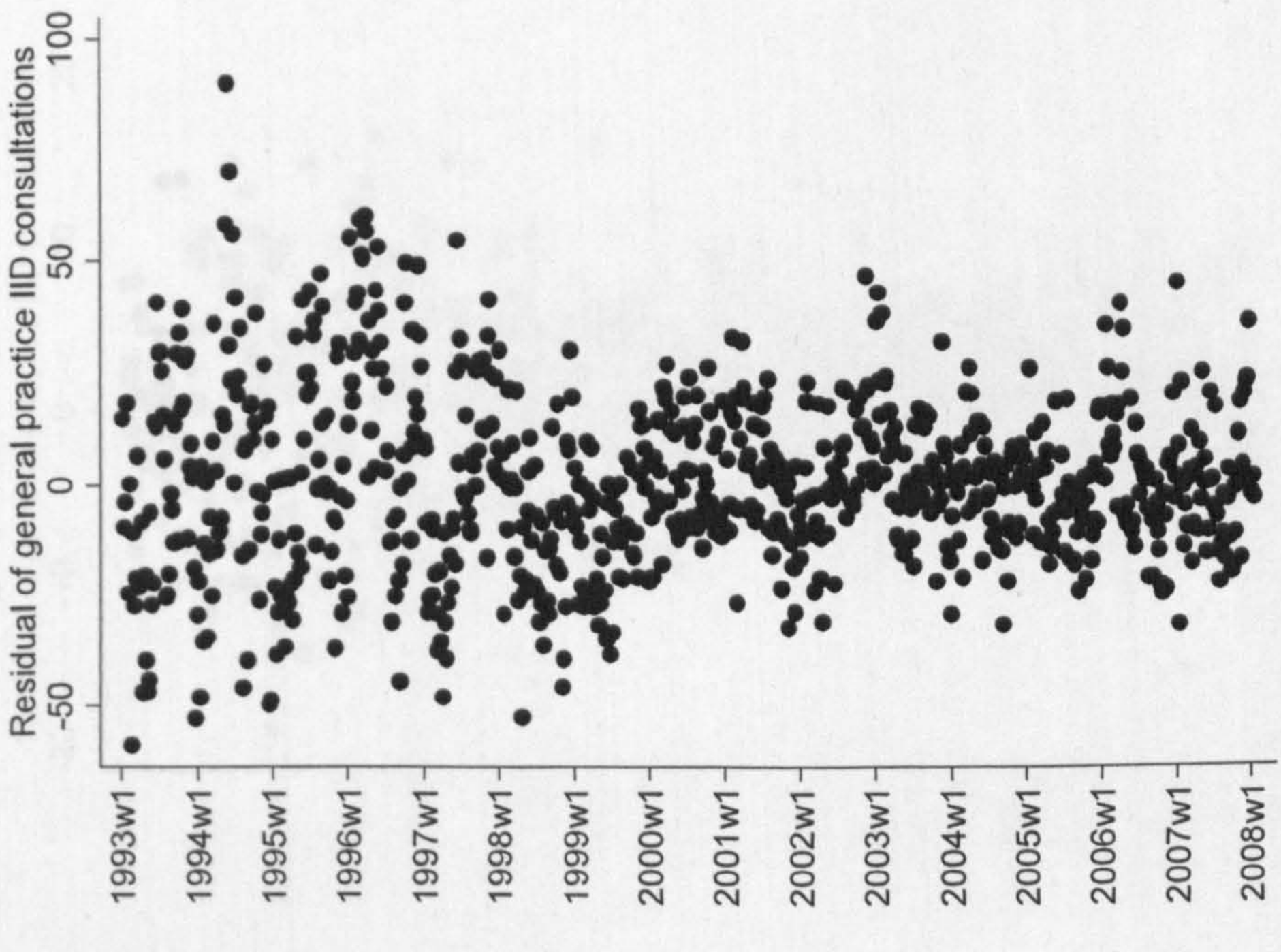


Figure A5.13m Adults aged 65 years or older Direct Model 1.

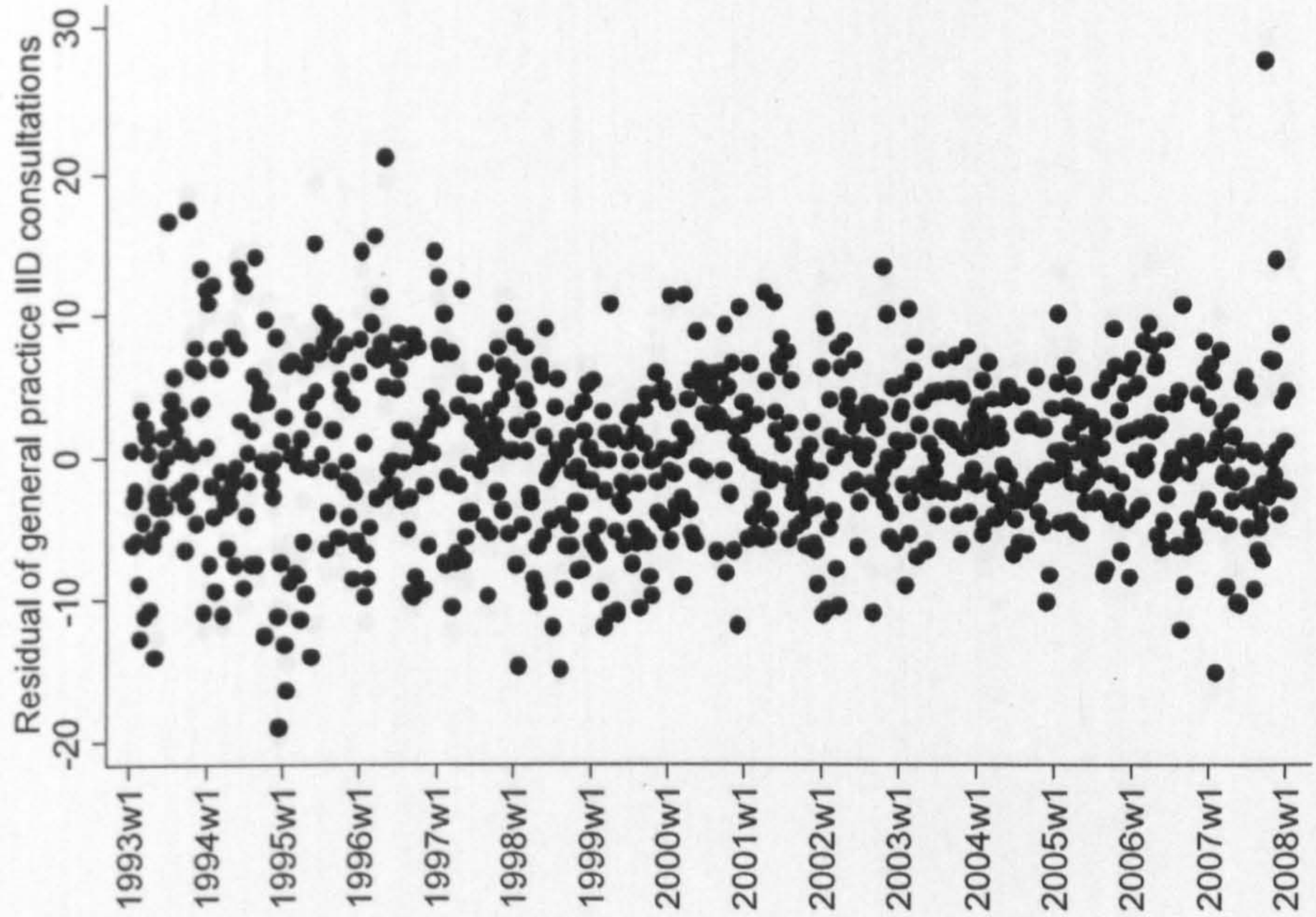


Figure A5.13n Adults aged 65 years or older Direct Model 2.

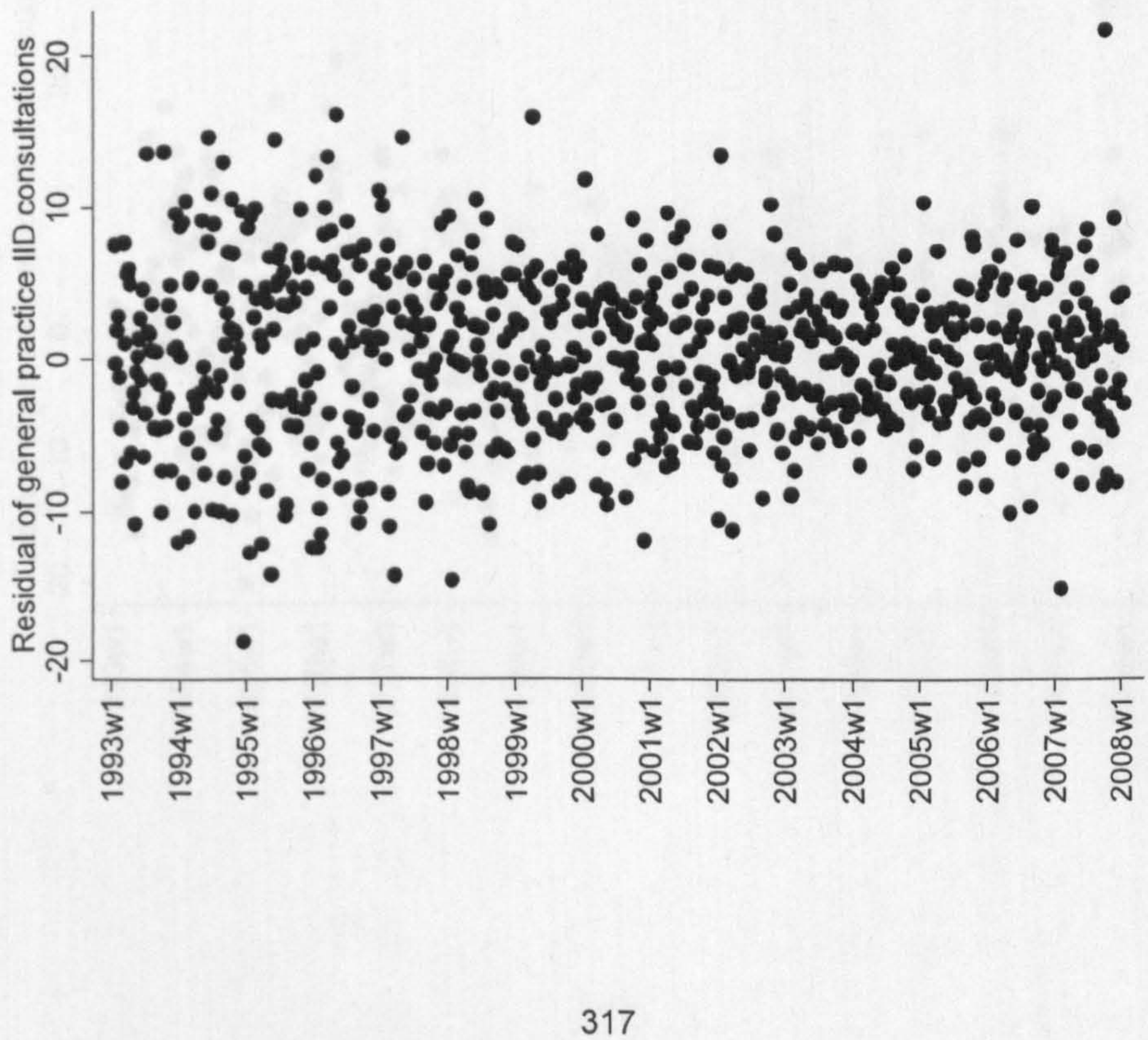


Figure A5.13o Adults aged 65 years or older Indirect Model 1.

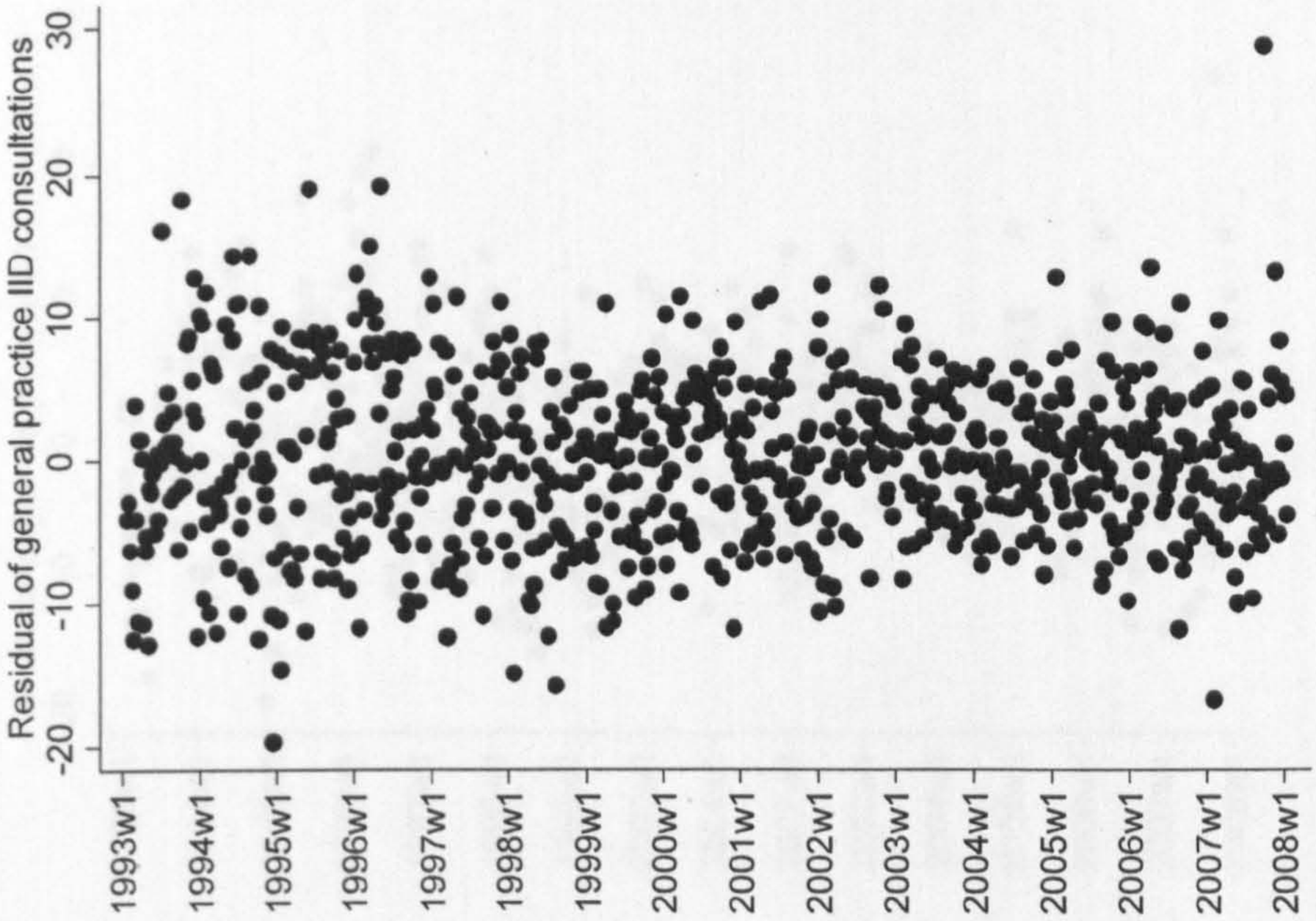


Figure A5.13p Adults aged 65 years or older Indirect Model 2.

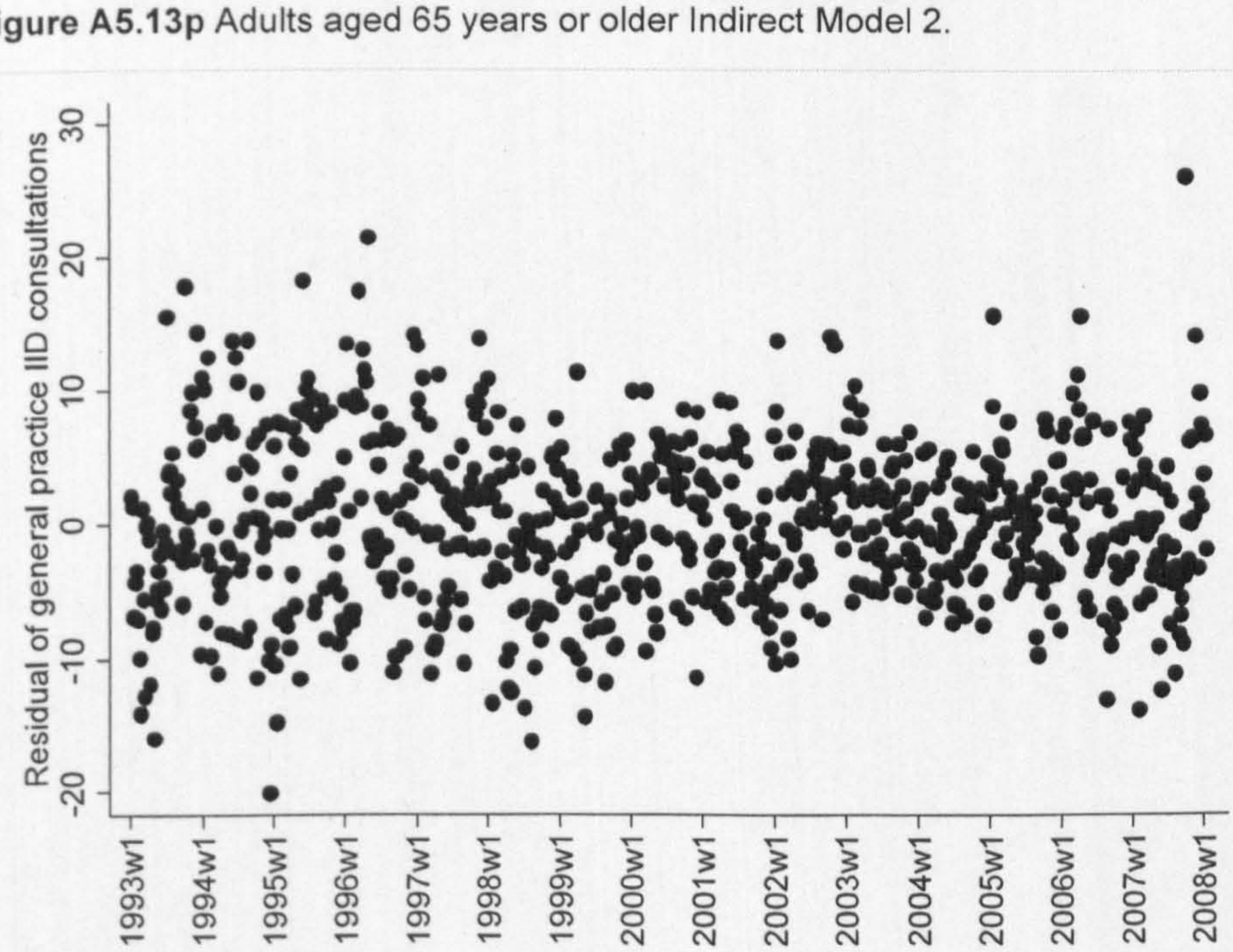
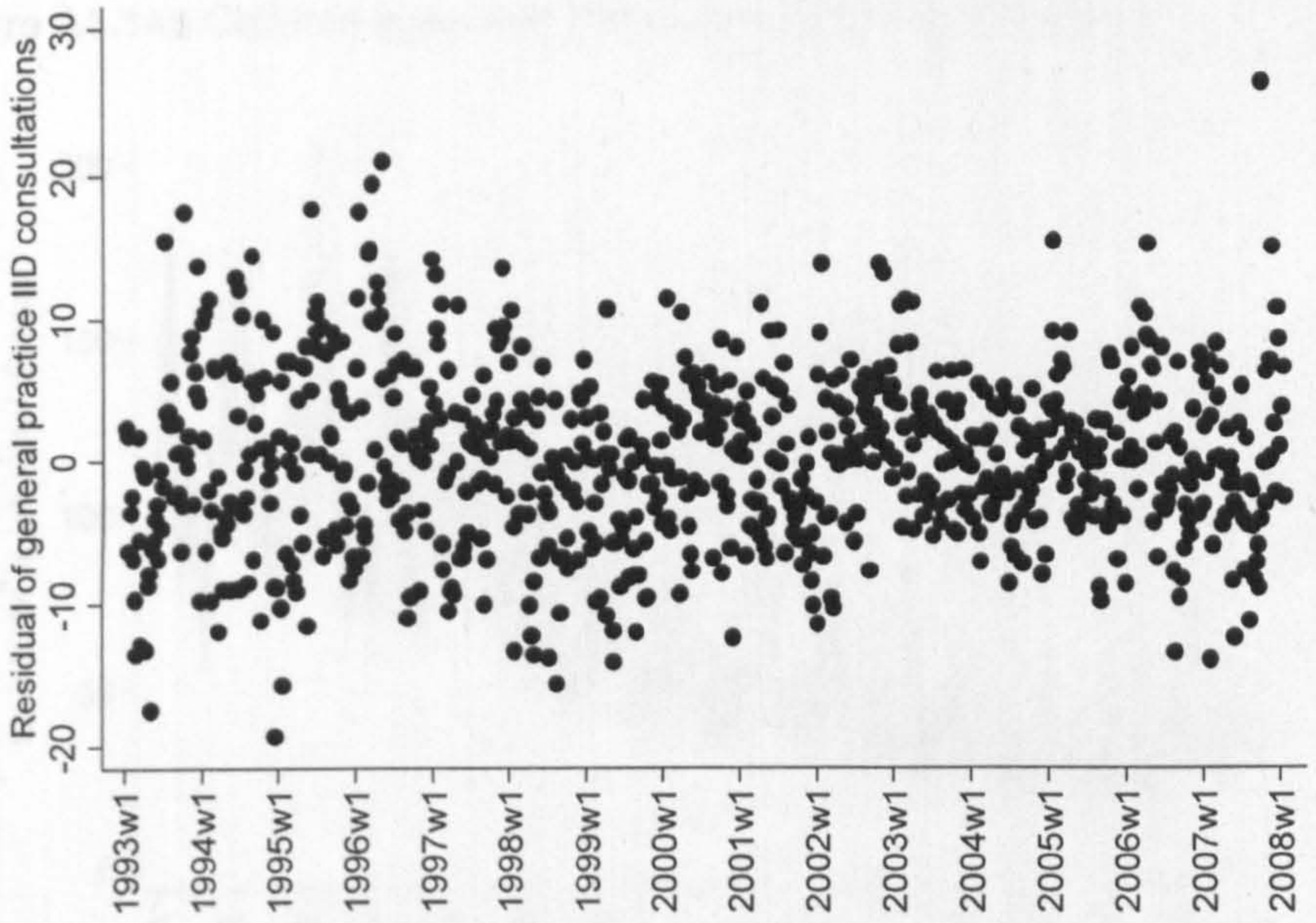


Figure A5.13q Adults aged 65 years or older Indirect Model 3.



Appendix A5.14. Fitted values from final models

The black line shows the observed weekly counts of general practice consultations for IID; the red line shows the fitted values from the model.

Figure A5.14a Children aged less than five years Direct Model 1.

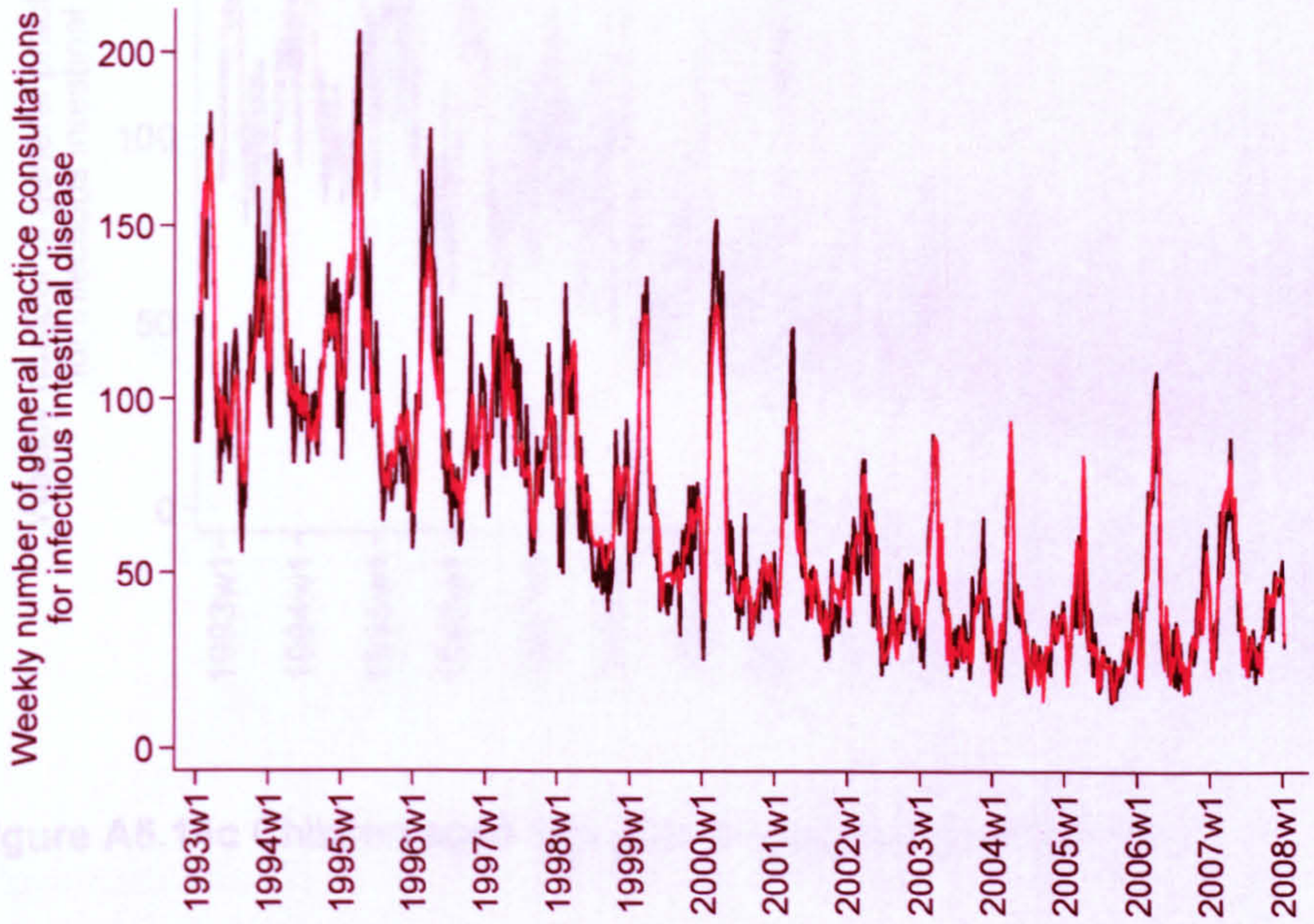


Figure A5.14b Children aged less than five years Direct Model 2.

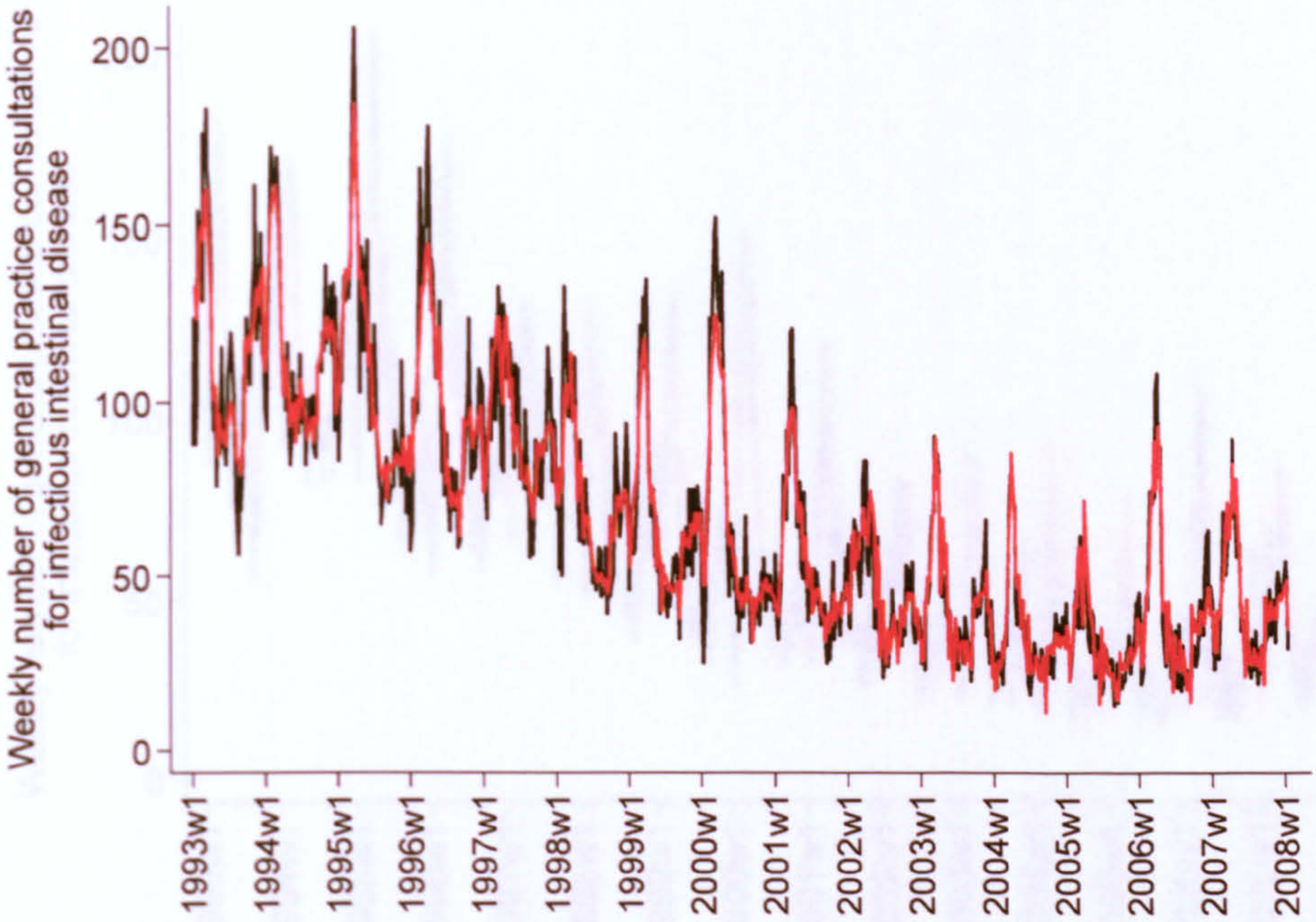


Figure A5.14c Children aged less than five years Indirect Model 1.

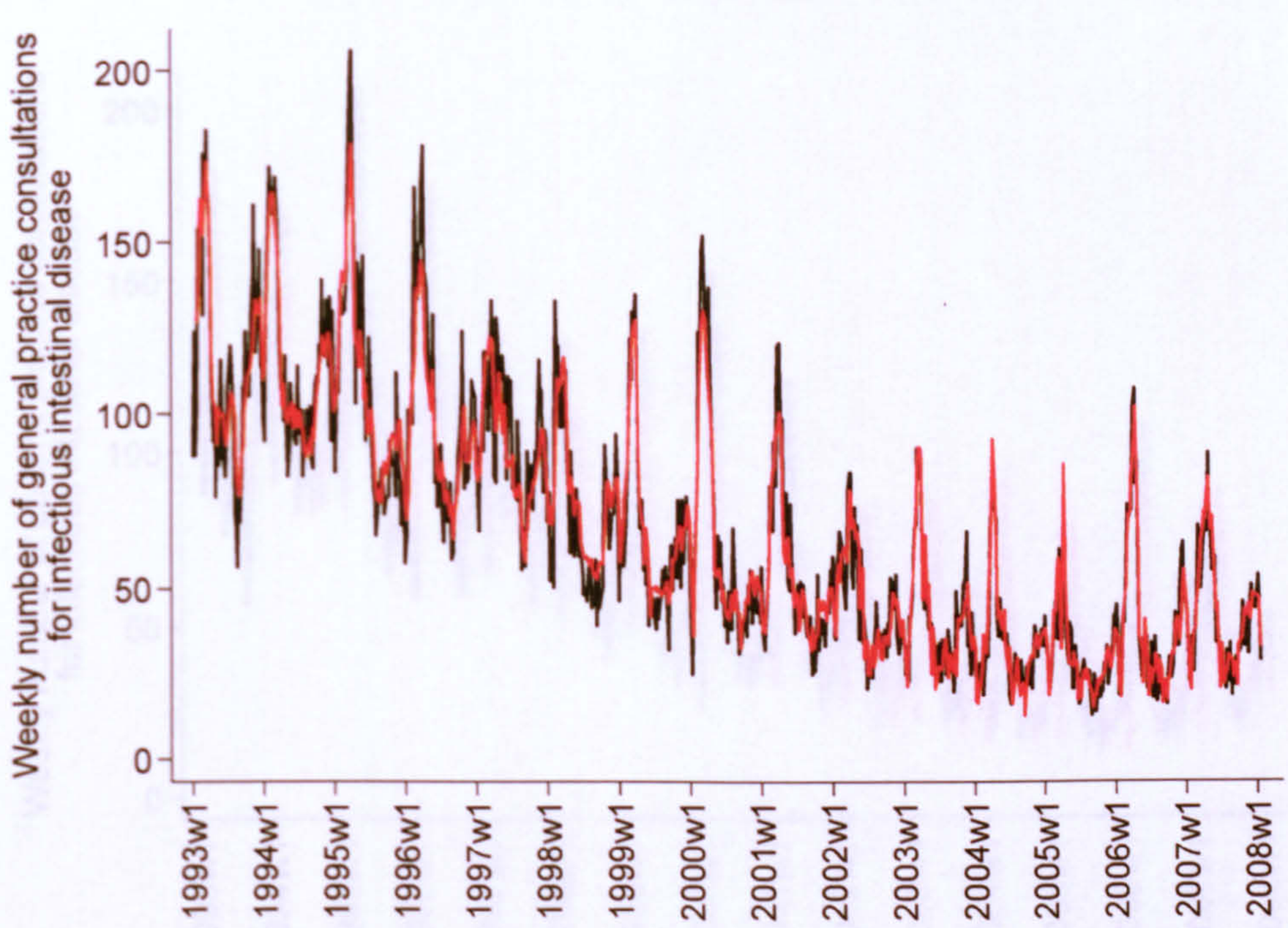


Figure A5.14d Children aged less than five years Indirect Model 2.

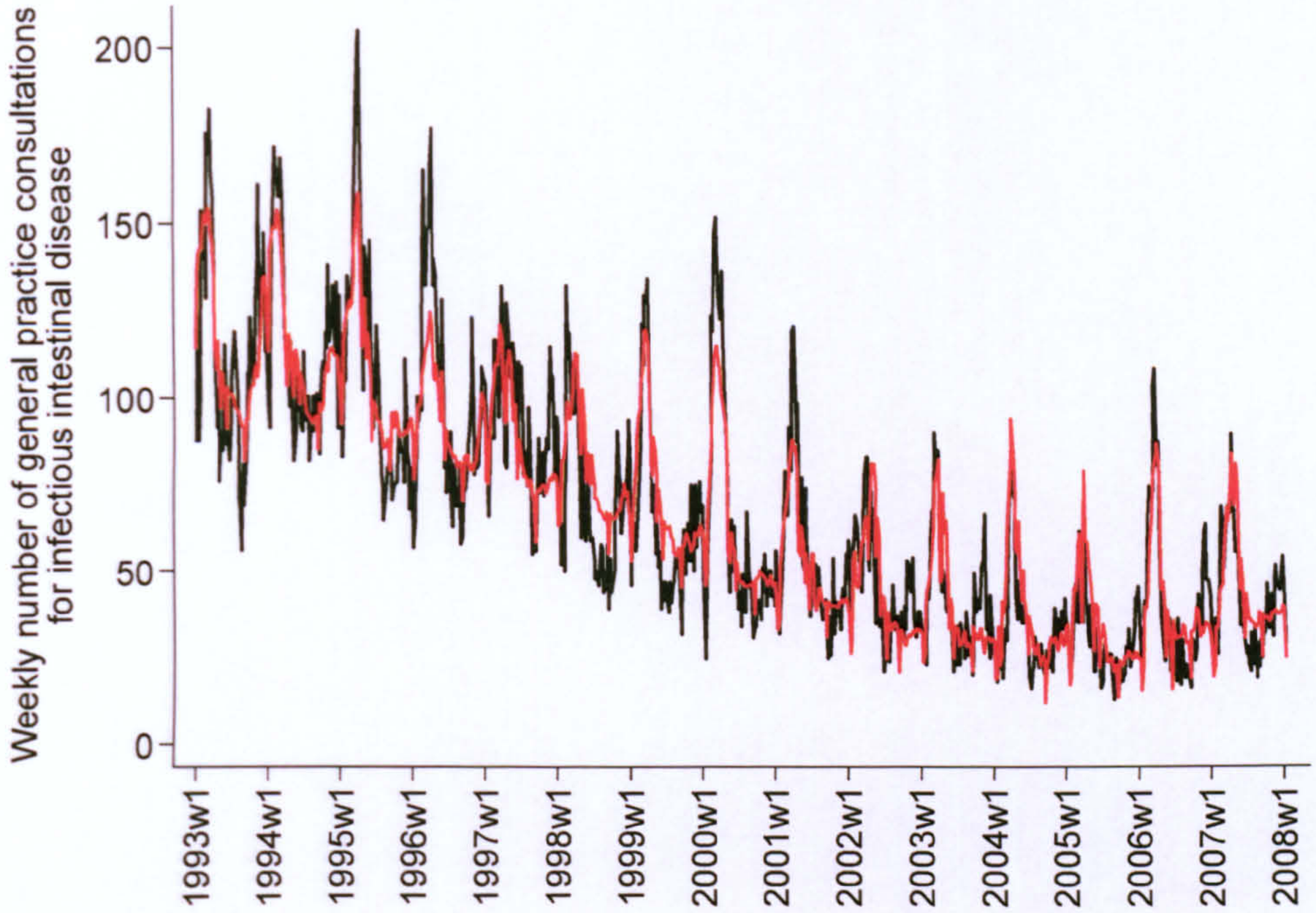


Figure A5.14e Children aged less than five years Indirect Model 3.

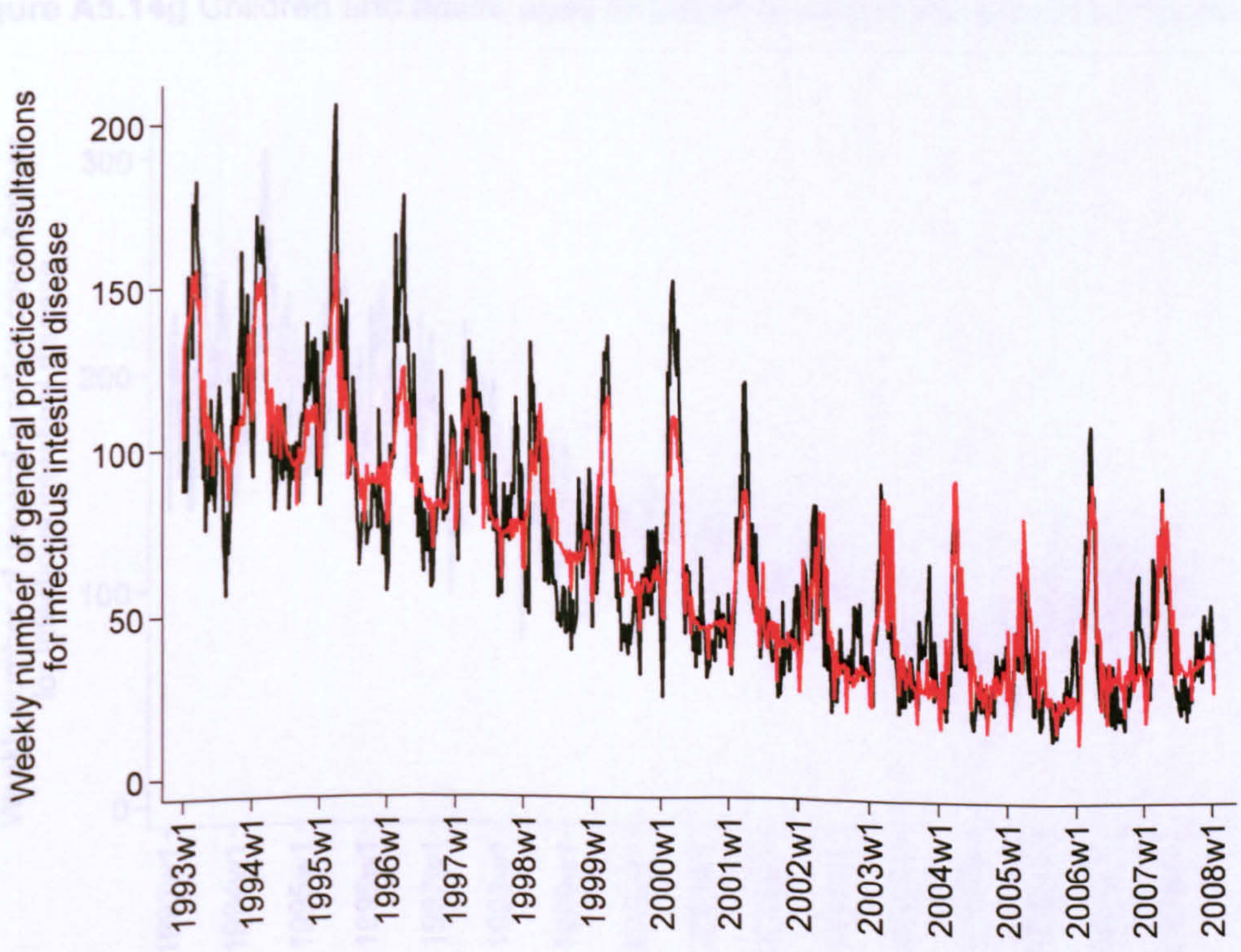


Figure A5.14e Children aged less than five years Indirect Model 4.

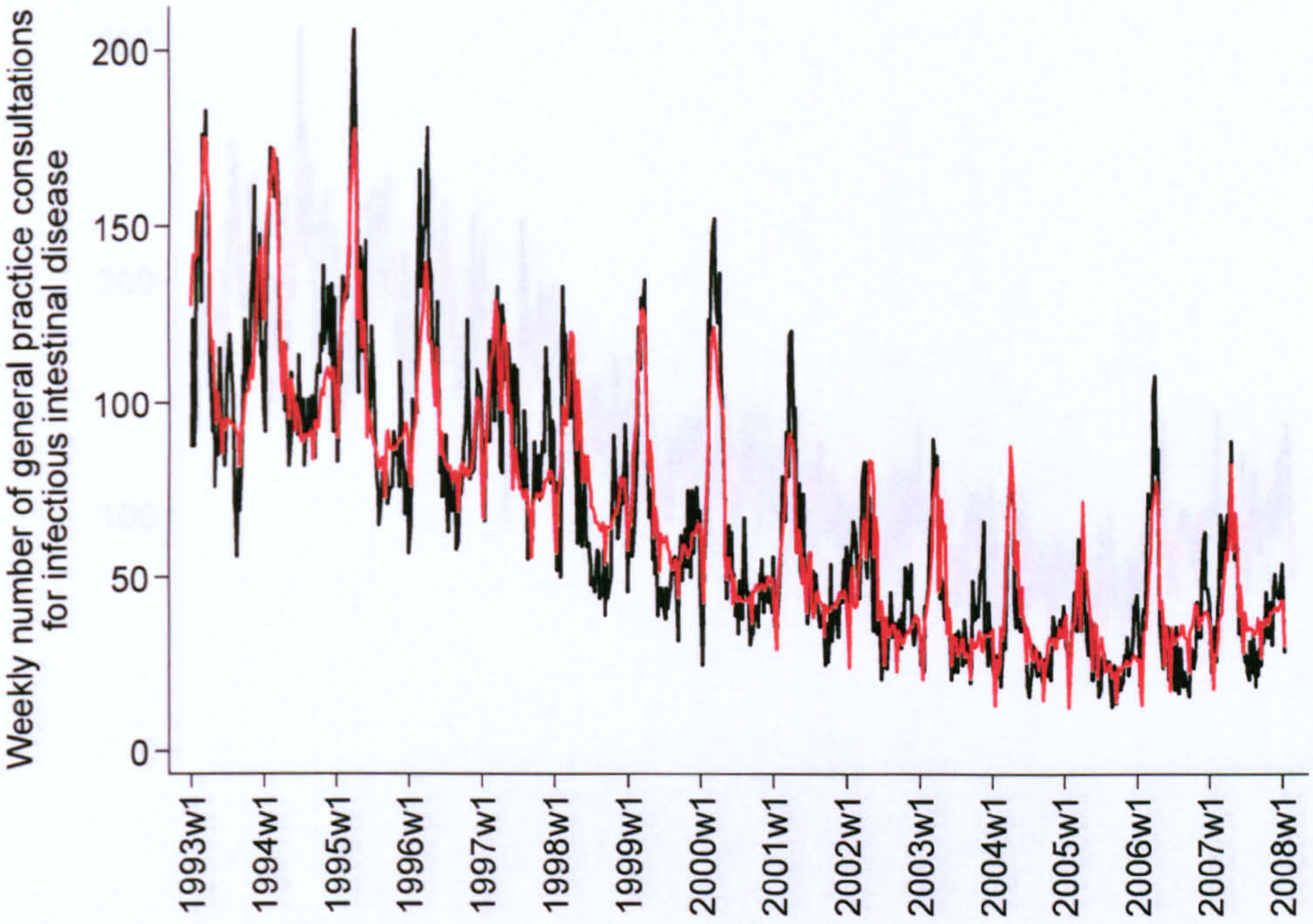


Figure A5.14g Children and adults aged between five and 64 years Direct Model 1.

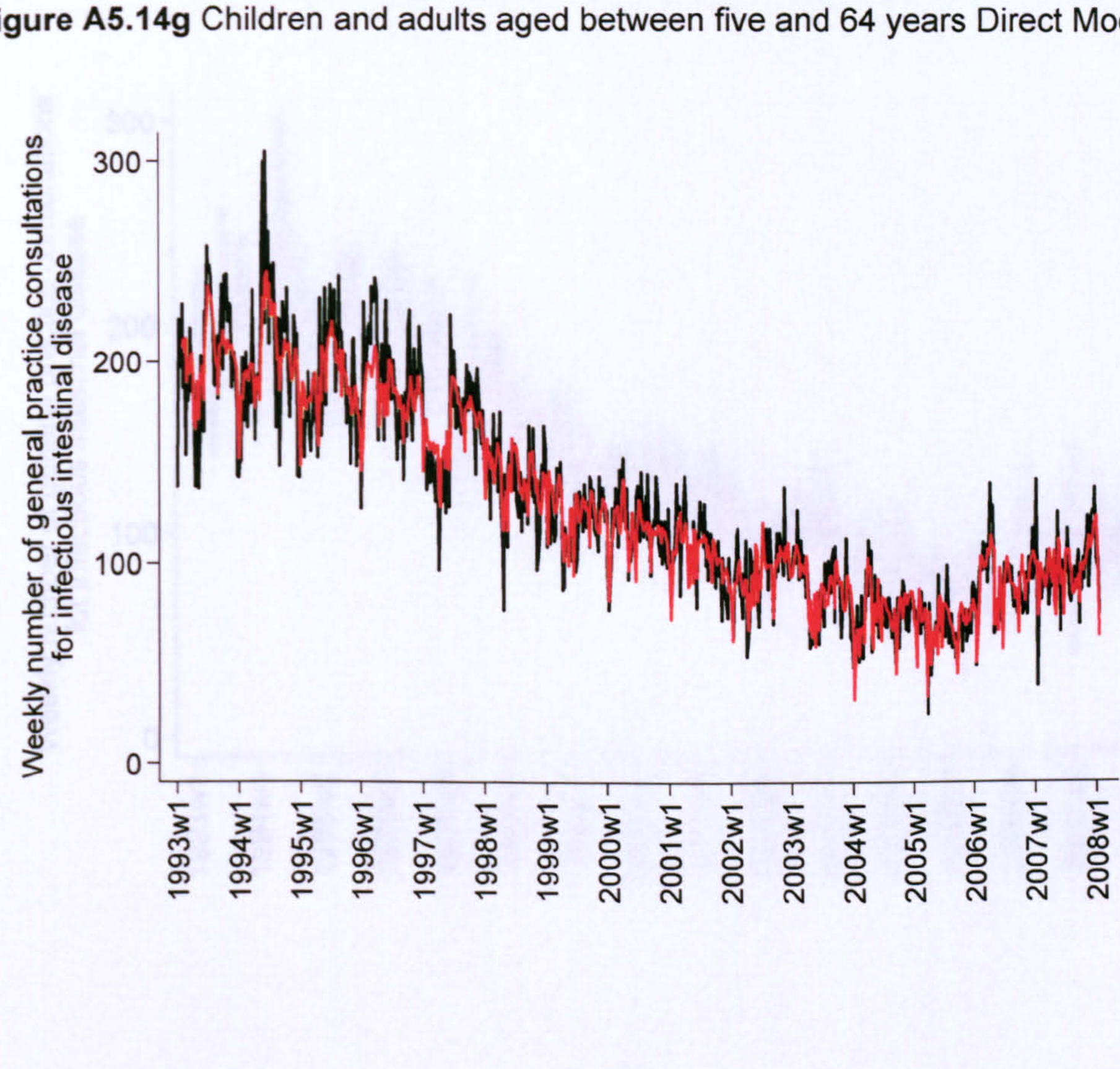


Figure A5.14h Children and adults aged between five and 64 years Direct Model 2.

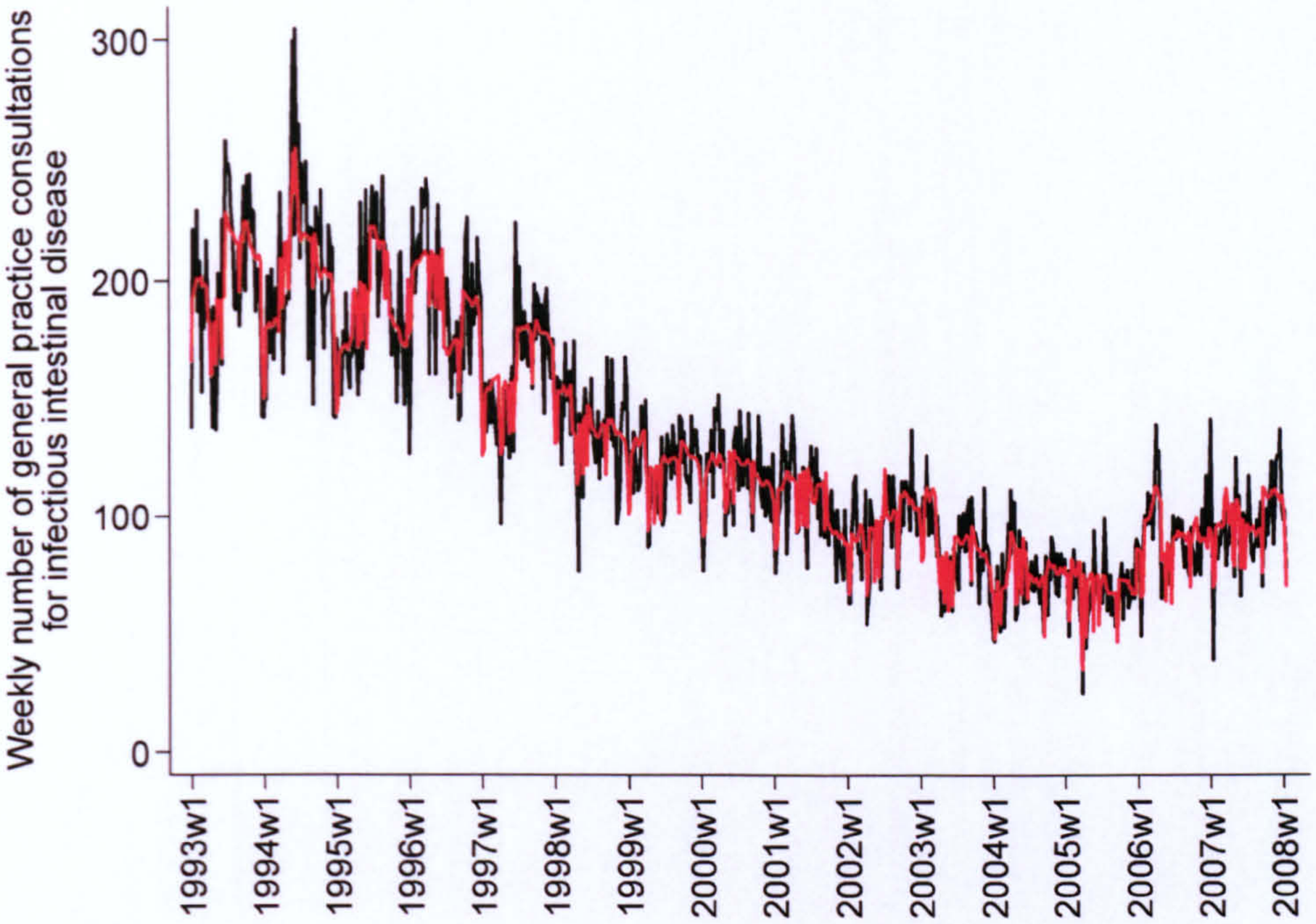


Figure A5.14i: Children and adults aged between five and 64 years Indirect Model 1.

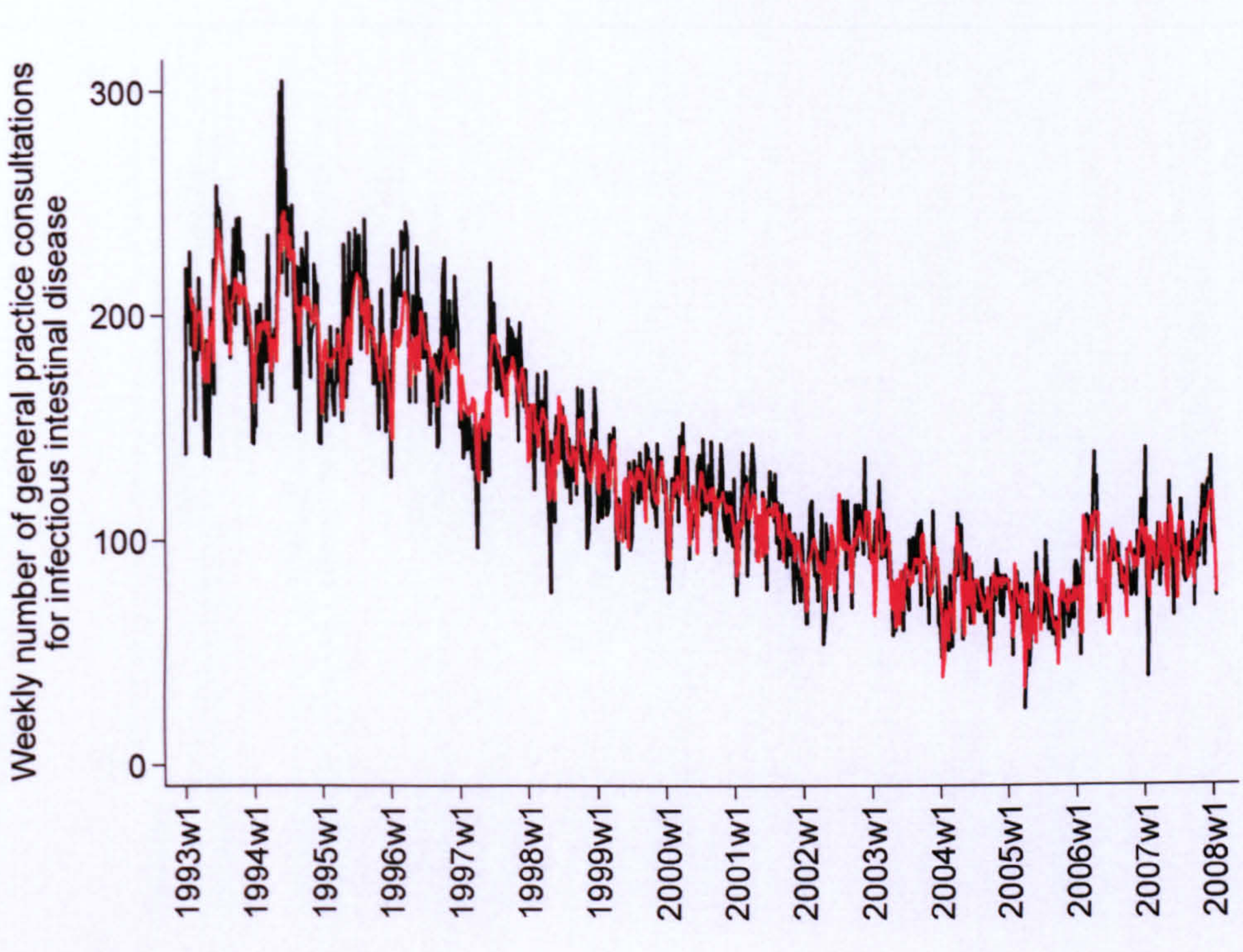


Figure A5.14j Children and adults aged between five and 64 years Indirect Model 2.

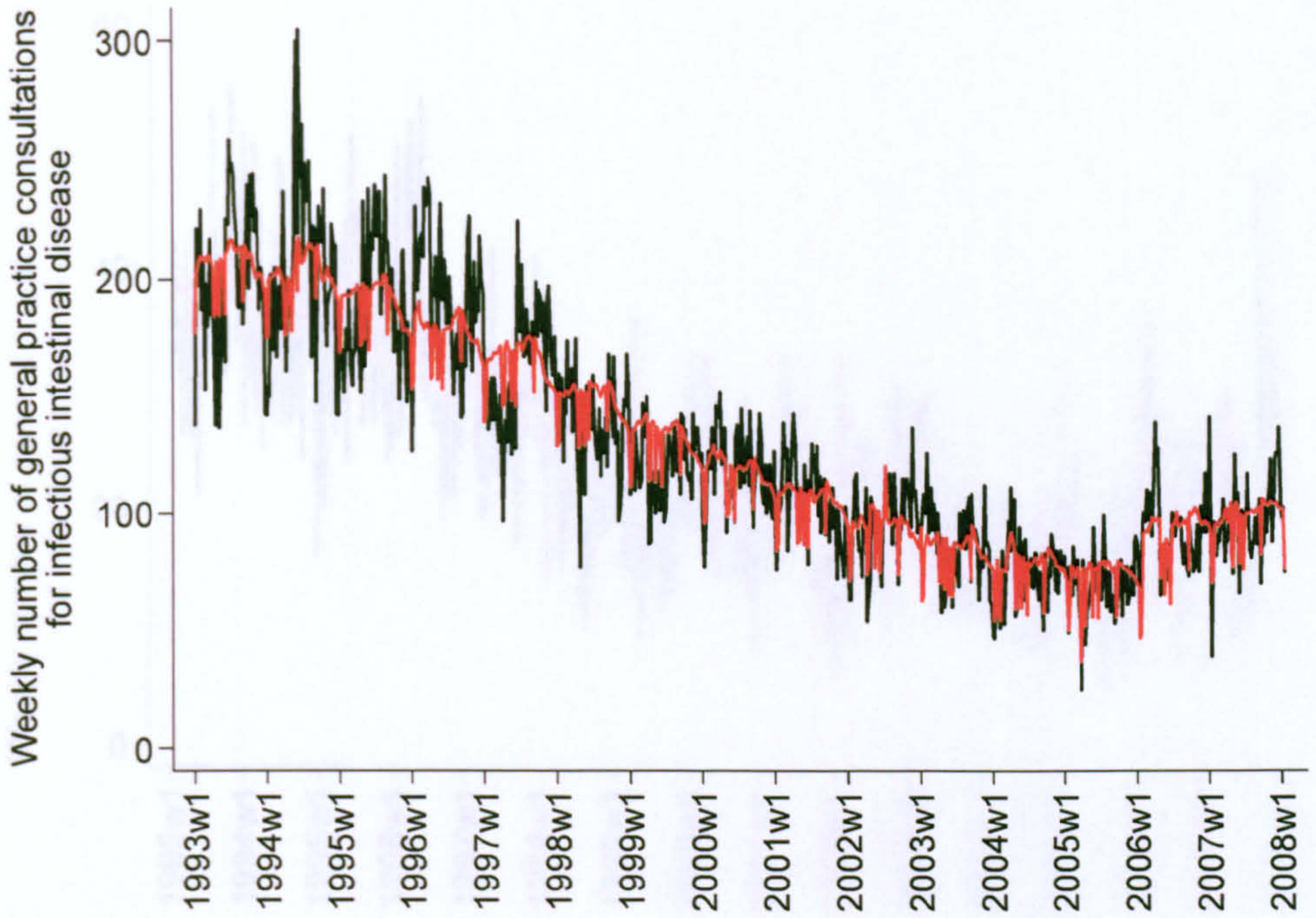


Figure A5.14k Children and adults aged between five and 64 years Indirect Model 3.

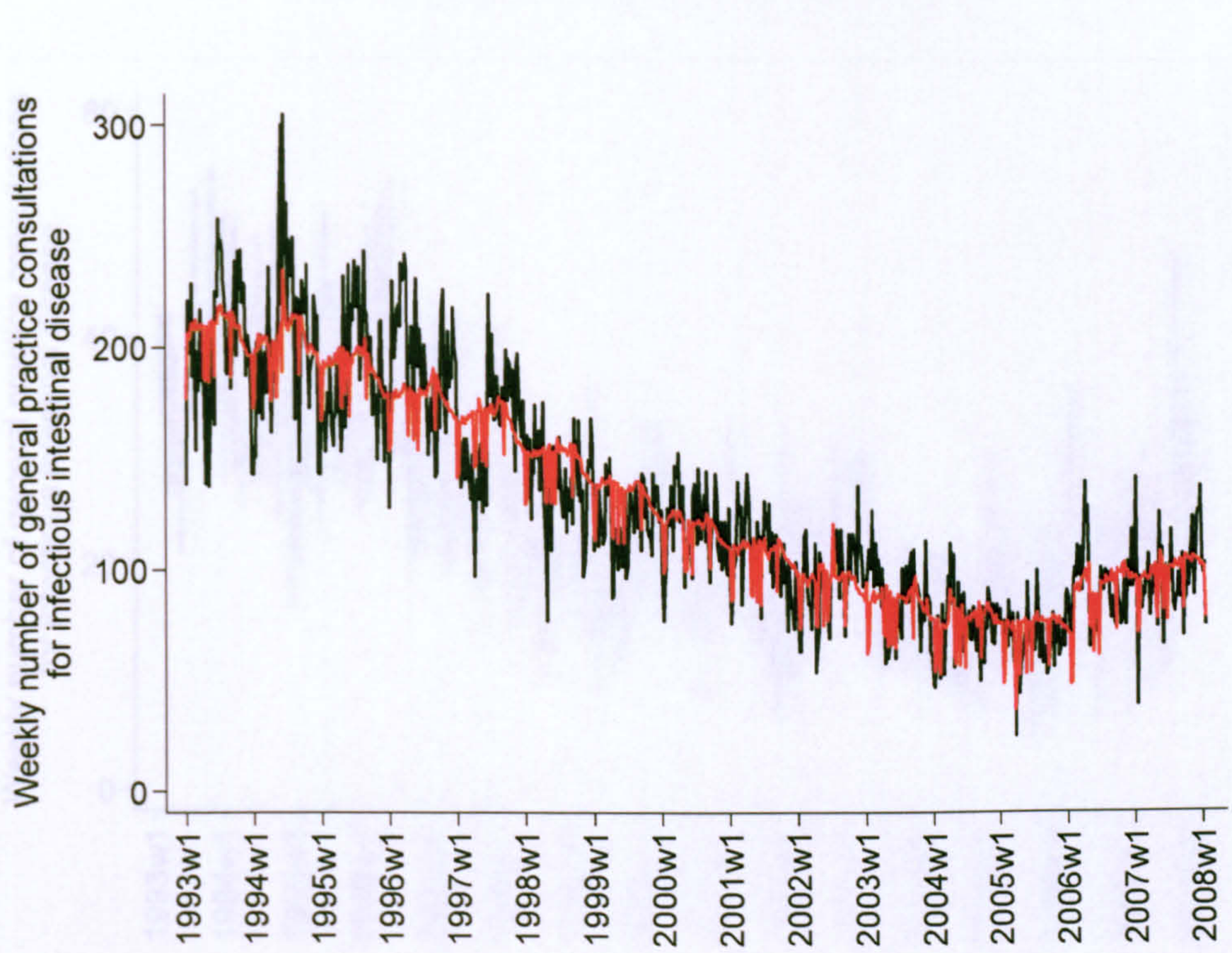


Figure A5.14m Adults aged 65 years or older Direct Model 1.

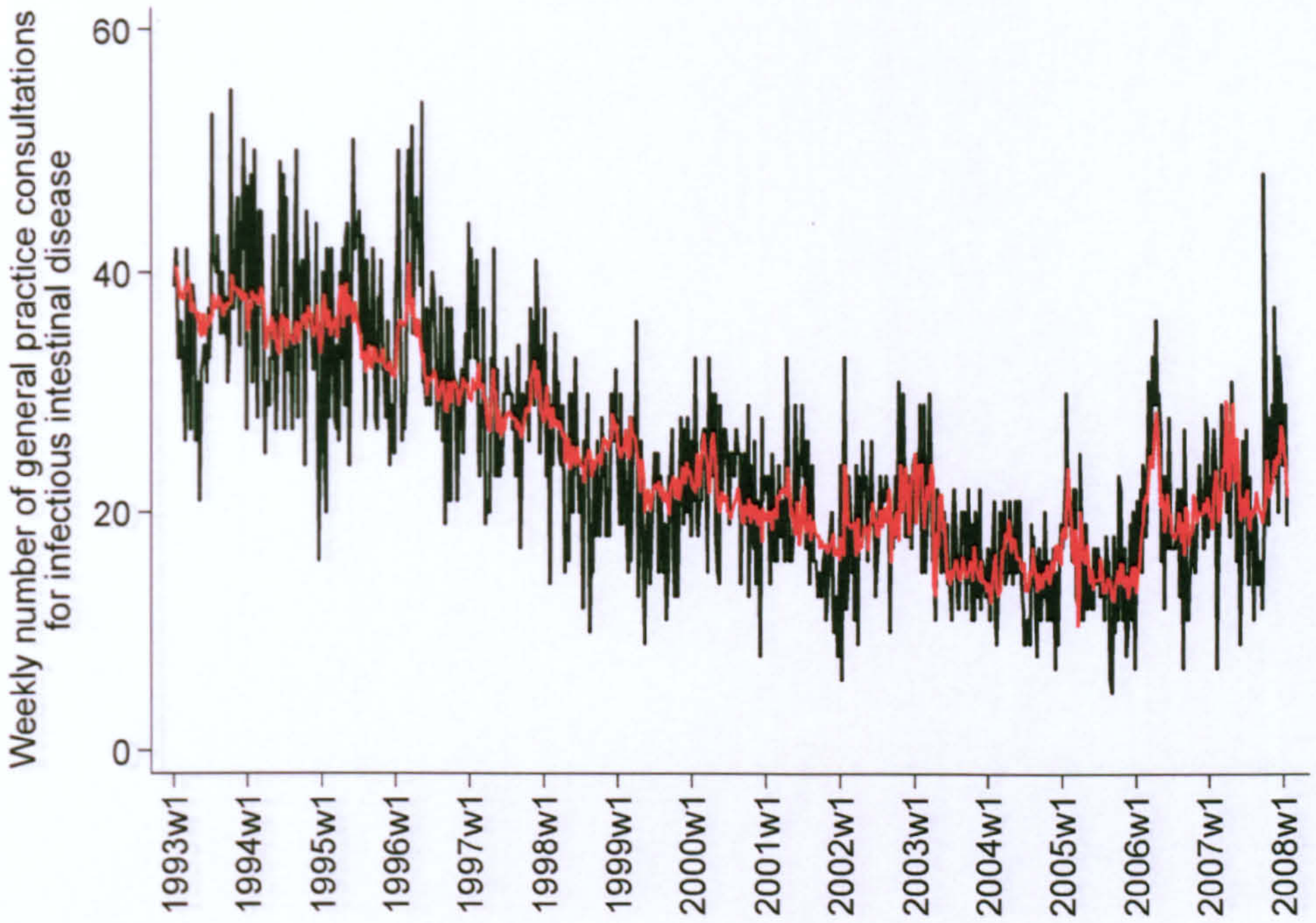


Figure A5.14n Adults aged 65 years or older Direct Model 2.

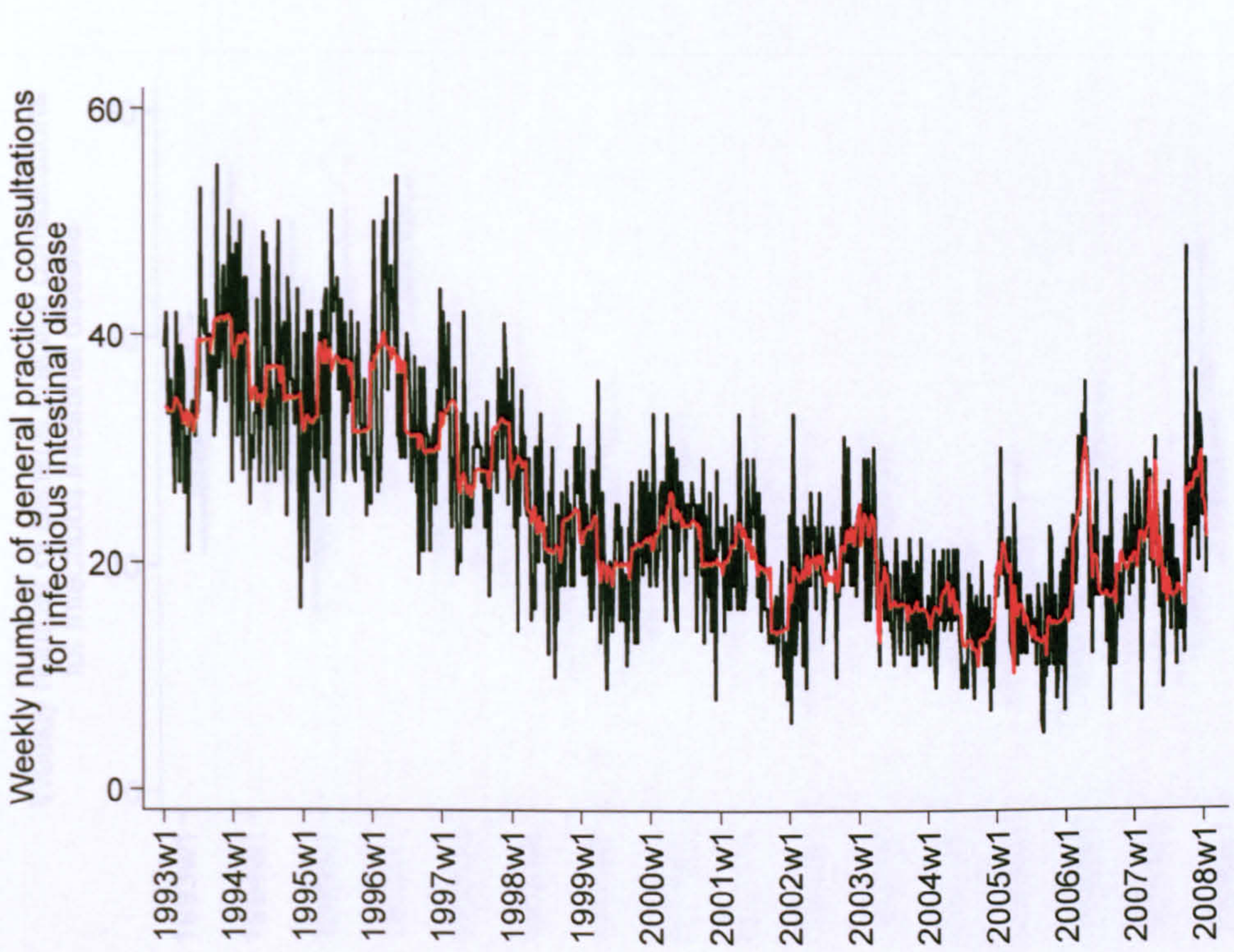


Figure A5.14o Adults aged 65 years or older Indirect Model 1.

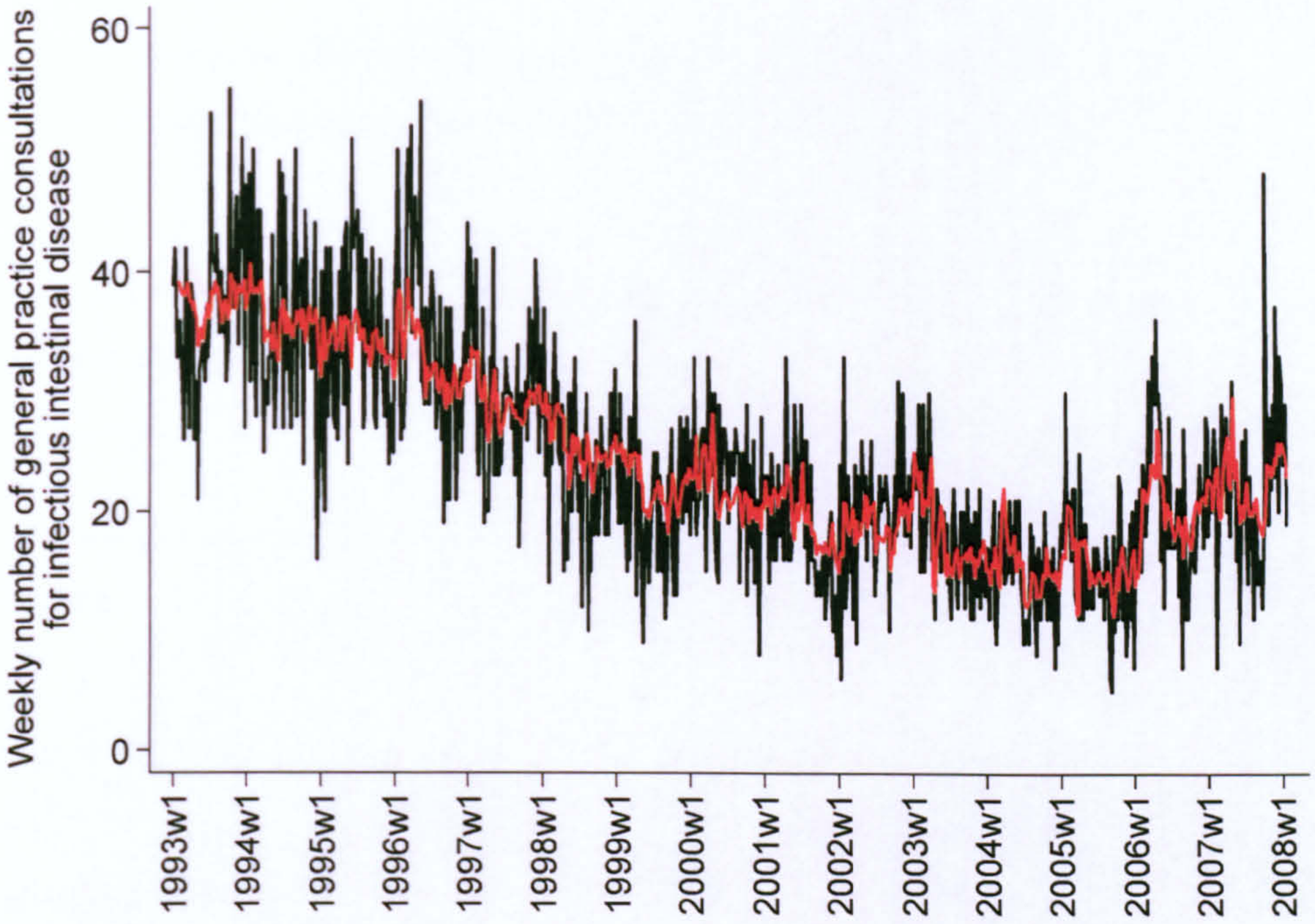


Figure A5.14p Adults aged 65 years or older Indirect Model 2.

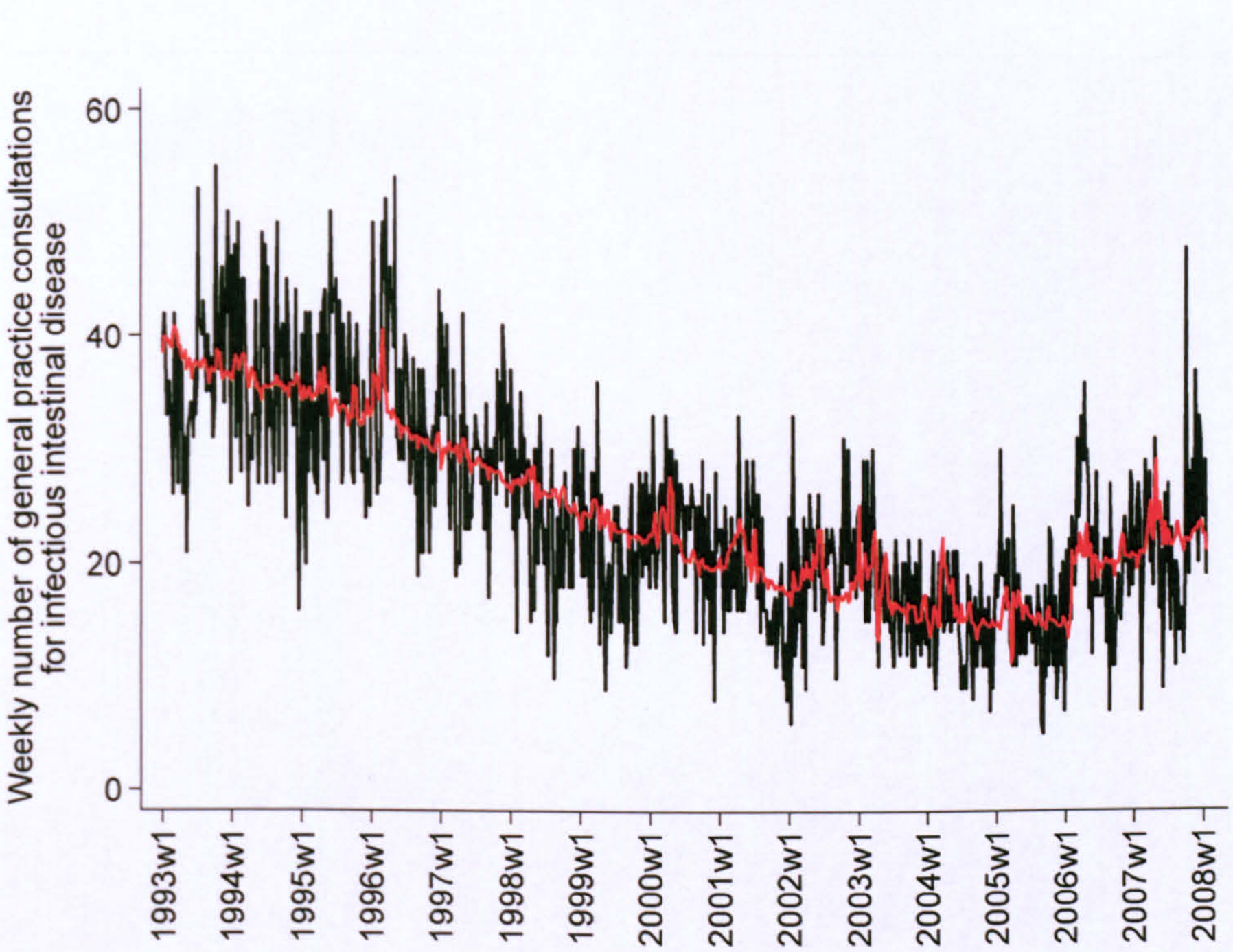
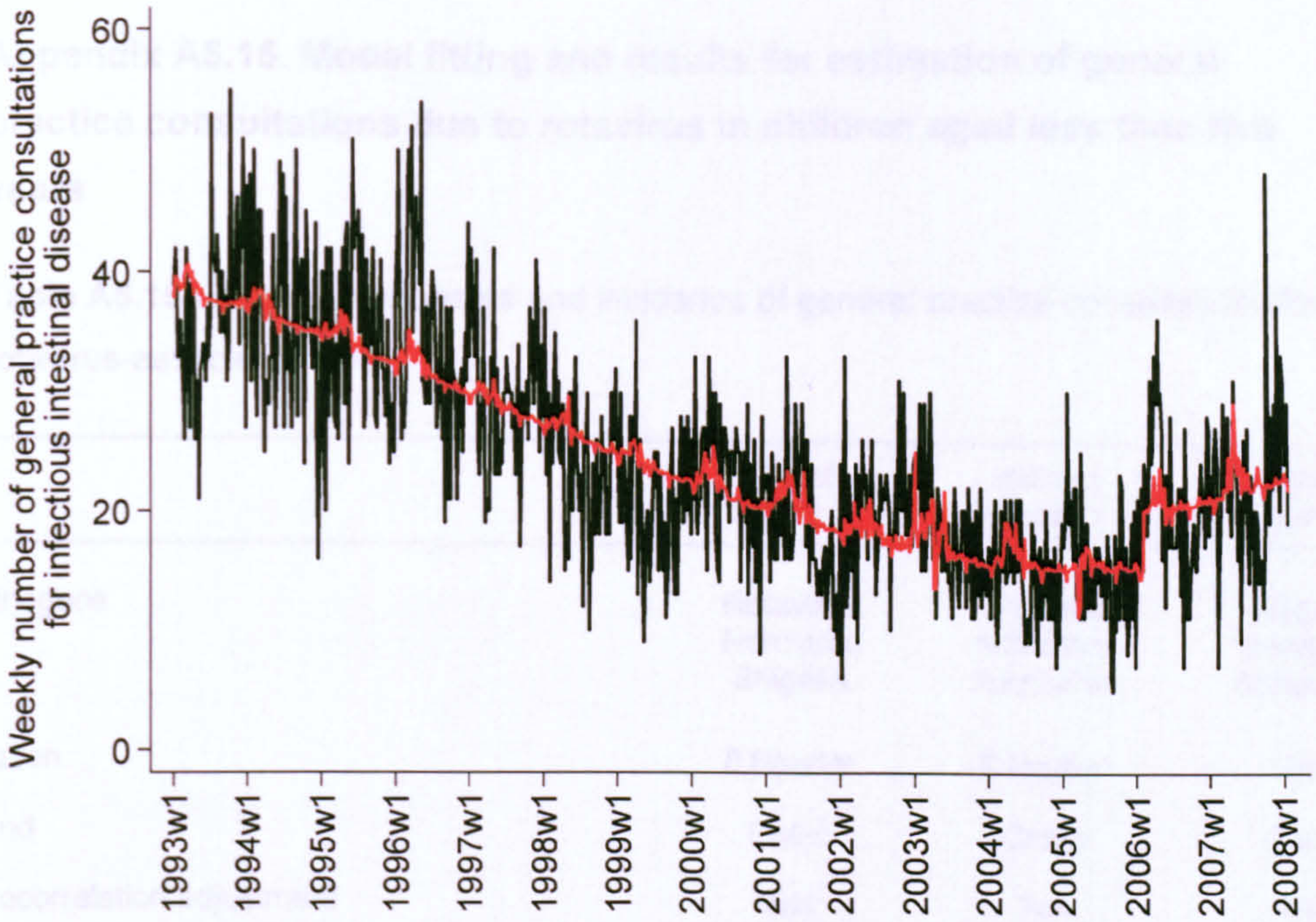


Figure A5.14q Adults aged 65 years or older Indirect Model 3.



Appendix A5.15. Model fitting and results for estimation of general practice consultations due to rotavirus in children aged less than five years

Table A5.15a Model components and incidence of general practice consultations for rotavirus-associated IID.

		Direct Model 1	Indirect Model 1	Indirect Model 2
Pathogens		Rotavirus Norovirus Shigella	Shigella Norovirus Astrovirus	Shigella Norovirus Astrovirus
Season		8 Fourier	8 Fourier	No
Trend		Cubic	Cubic	Cubic
Autocorrelation adjustment		Yes (2 AR terms)	Yes (2 AR terms)	No
Interaction pathogen*time		Rotavirus *time[i]	No	No
AIC		7.62	7.86	11.26
Incidence of general practice consultations per 1000 population				
1993-2007	Direct	23.2 (18.9, 27.5)[ii]	-	-
	Indirect			
	25th percentile	-	10.3	20.5
	Positive only	-	6.1	10.3
	Lowest	-	32.0	44.4
1994-1996	Direct	30.3 (18.3, 42.2)[ii]	-	-
	Indirect			
	25th percentile	-	15.3	28.3
	Positive only	-	9.7	16.1
	Lowest	-	40.0	57.0

[i] Interaction P value <0.001; rotavirus coefficient 0.09 (95% confidence interval: 0.08, 0.10); interaction coefficient -0.003 (95% confidence interval :-0.004, -0.002)
[ii] 95% Confidence intervals
Abbreviations: AIC, Akaike's information criterion.

Table A5.15b Rotavirus coefficients in the direct models.

Age group	Model	Rotavirus coefficient	Interaction coefficient
< 5 years	Direct 1 – Fourier adjustment	0.09 (0.08, 0.10)	-0.003 (-0.004, -0.002)
	Direct 2 – 13 week adjustment	0.12 (0.10, 0.13)	-0.005 (-0.007, -0.004)
	Direct 1 – categorical year term replaces cubic trend	0.10 (0.08, 0.11)	-0.004 (-0.005, -0.003)

Figure 5.15 Mean weekly incidence of general practice consultations for rotavirus-associated IID.

Figure 5.15a Direct model 1.

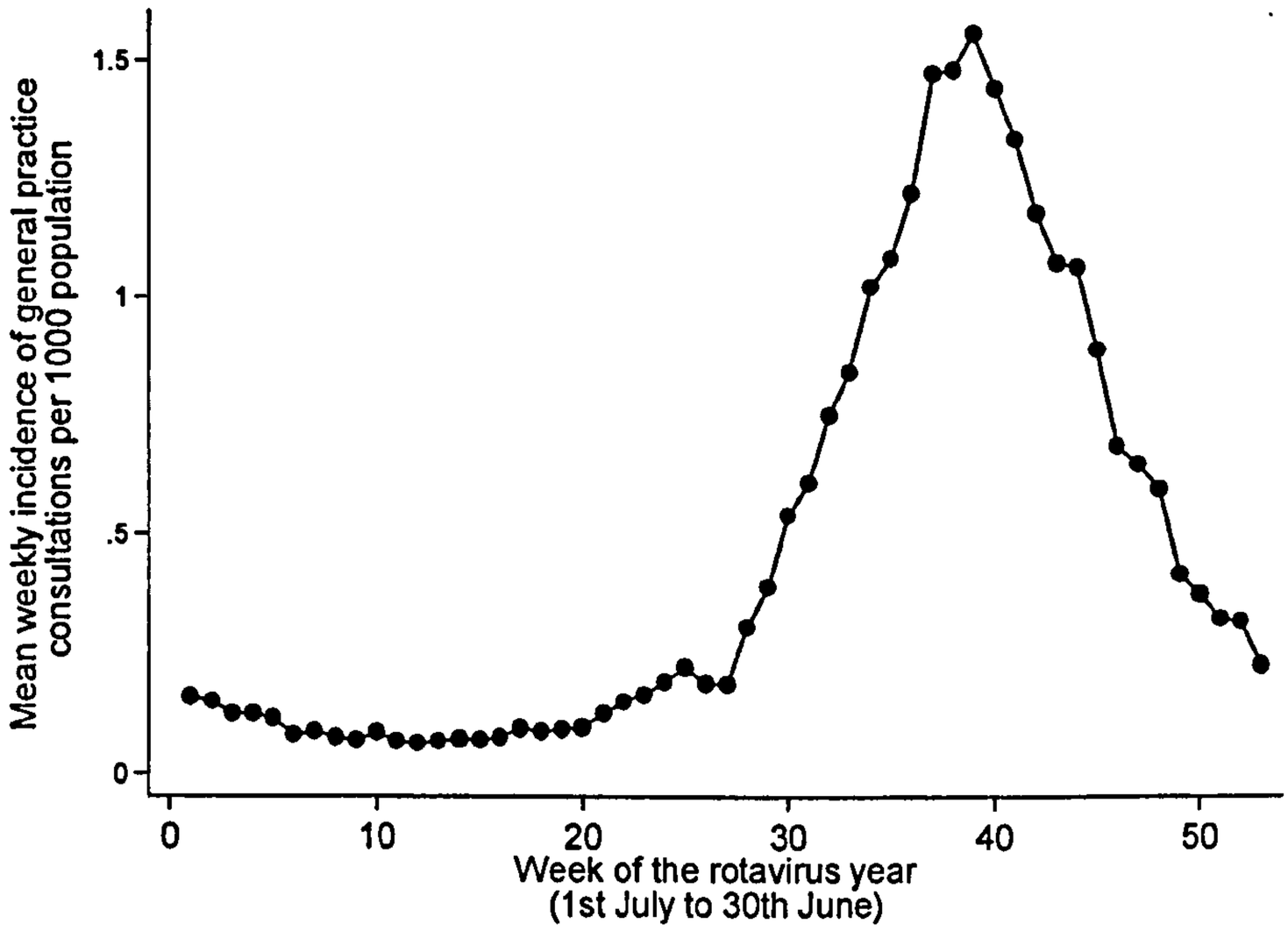


Figure 5.15b Indirect model 1 (25th percentile standardised residuals).

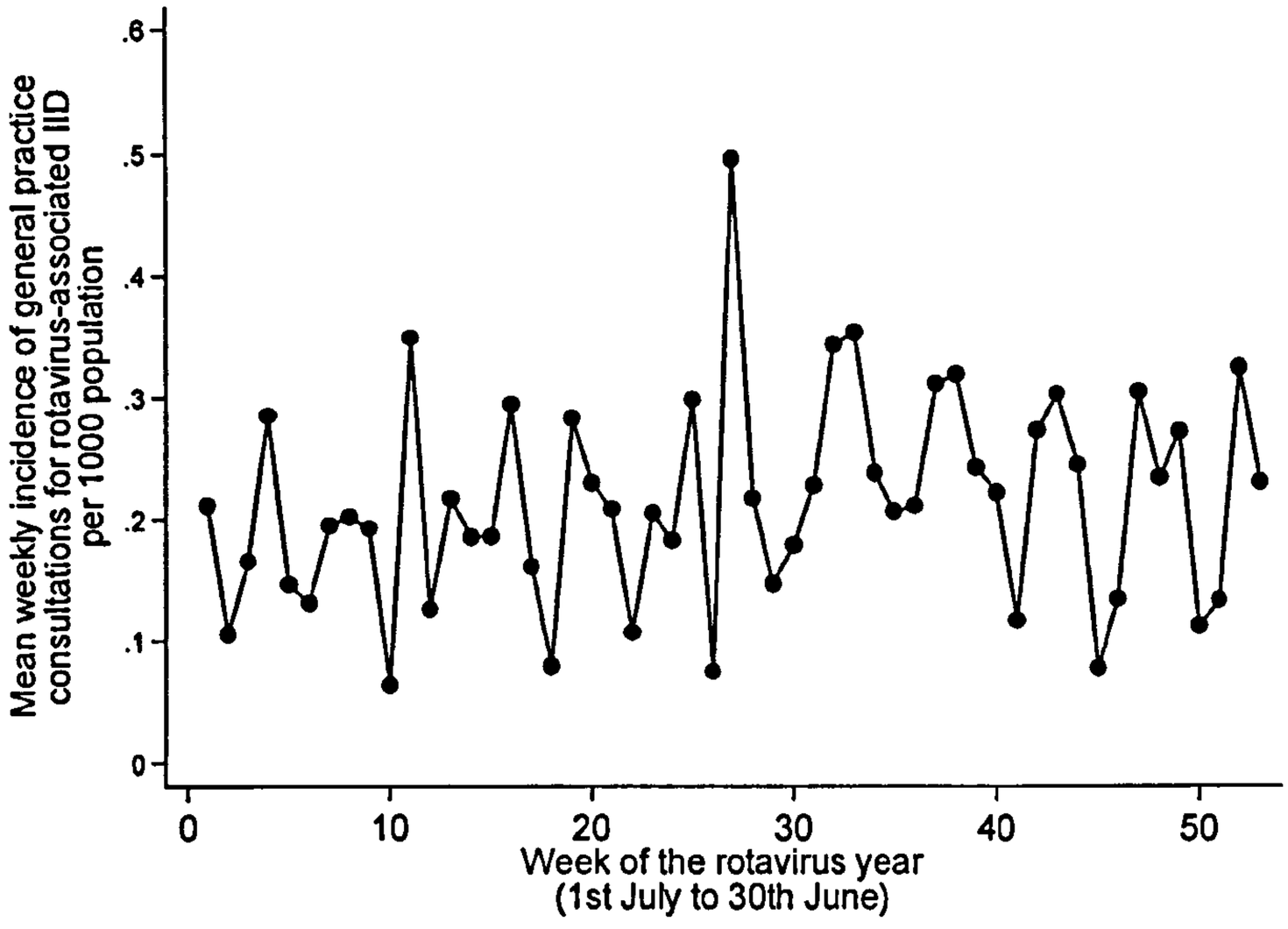
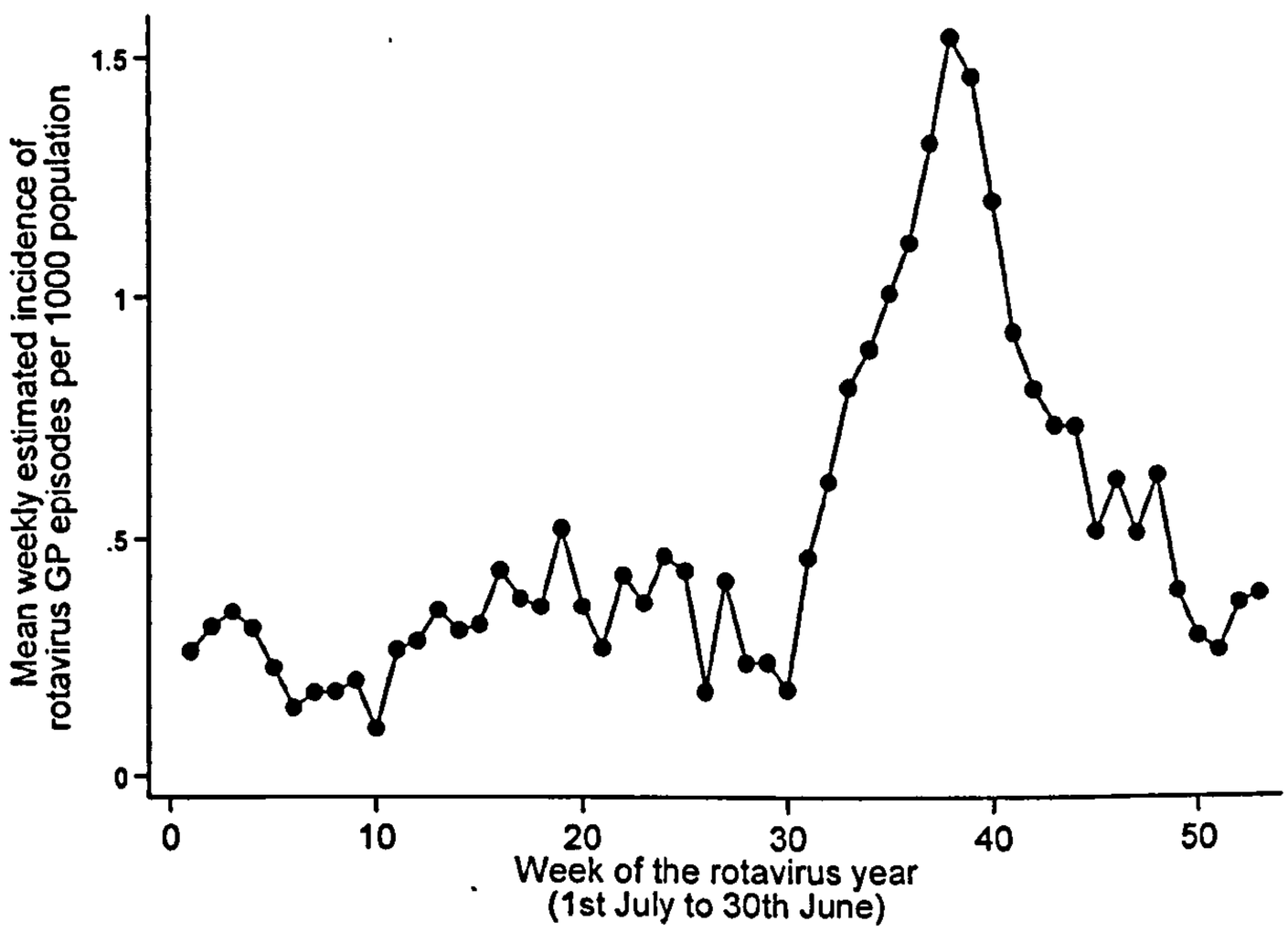


Figure 5.15c Indirect model 2 (25th percentile standardised residuals).



Appendix 6: Additional information and results for Chapter 8

Appendix A6.1. Description of mixture model of norovirus Ct values

A model with two component distributions was fitted to the Ct value distribution from IID cases only. Two model components were used because it was believed *a priori* that there were two groups of IID cases: those with high viral loads and disease caused by norovirus; and those with low norovirus load and disease caused by another pathogen. Modelling was carried out separately for IID cases in the community cohort and the general practice study, to allow a different proportion of cases to have disease caused by norovirus in the two study components.

A maximum likelihood method was used to estimate the mean and standard deviation of the two component distributions imposed on the Ct value data. The model and maximum likelihood method have been described previously*. IID cases who were positive for norovirus by non-quantitative RT-PCR, but negative by real time RT-PCR were represented as censored values at Ct value 39, to allow a normal distribution to be fitted to both component distributions in the model. The model provided the proportion of IID cases in the group with disease caused by norovirus, with a likelihood-based 95% confidence interval. This proportion was substituted for Adjustment Factor *A* in Formula 1.

For the crude incidence estimates, data from IID cases of all ages were analysed together, therefore assuming that the distribution of Ct values is the same across all ages. To produce age-stratified and age-adjusted incidence estimates, the mixture model was fitted separately to the data from children aged less than five years and to older children and adults. Age-adjusted and age-stratified estimates were only produced for the general practice study; it was not possible to fit age-stratified models in the community cohort, because of the smaller sample size. Figures A1.1 to A1.4 show the fit of the modelled distributions to the observed data.

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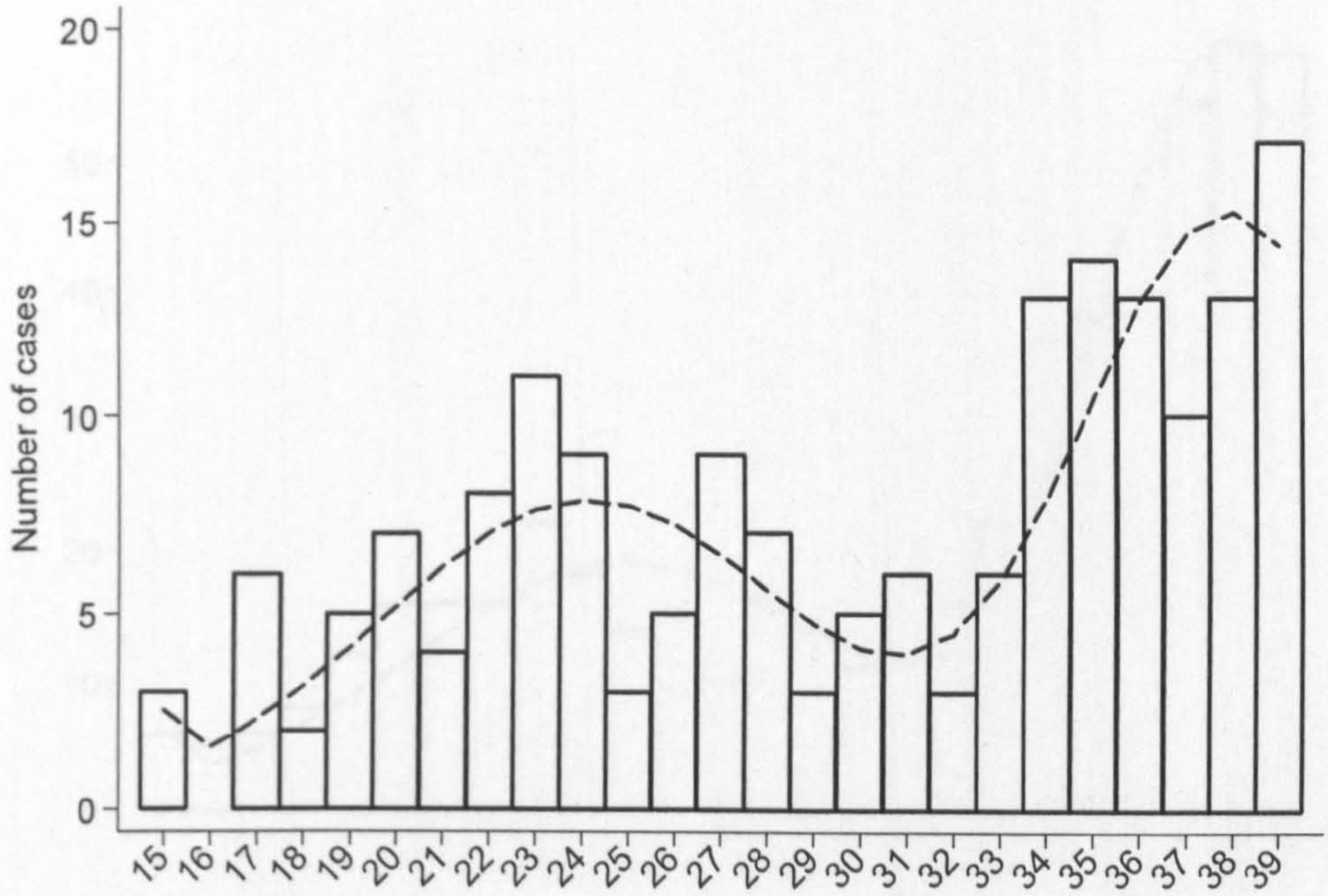
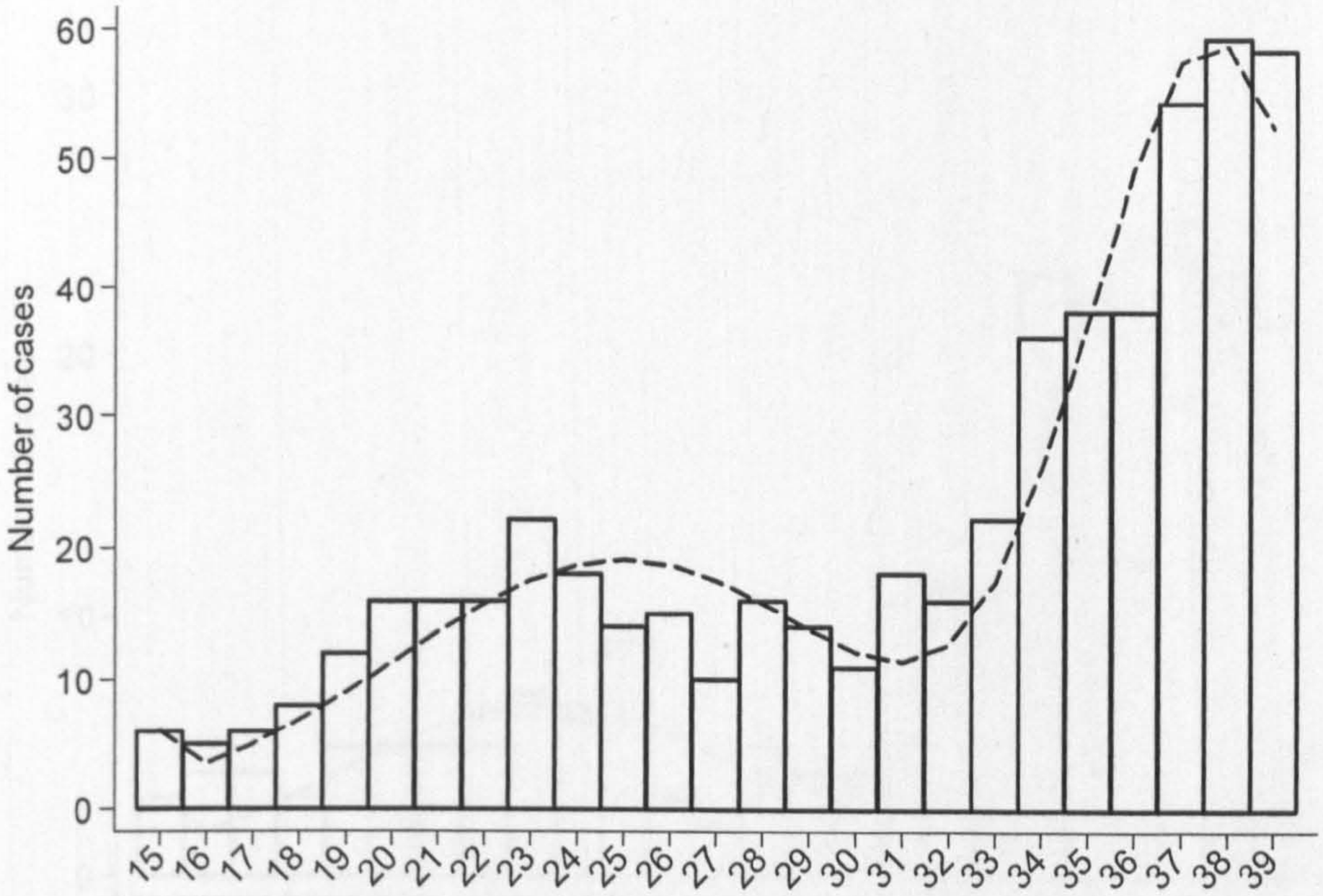
* Gay NJ. Analysis of serological surveys using mixture models: application to a survey of parvovirus B19. *Stat.Med.* 1996;15(14):1567-73.

Figure A6.1a Fit of mixture model to distribution of Ct values from IID cases of all ages in the community cohort. Bars show the number of IID cases at each Ct value in the community cohort. Black line shows the fitted number of IID cases at each Ct value predicted by the model.



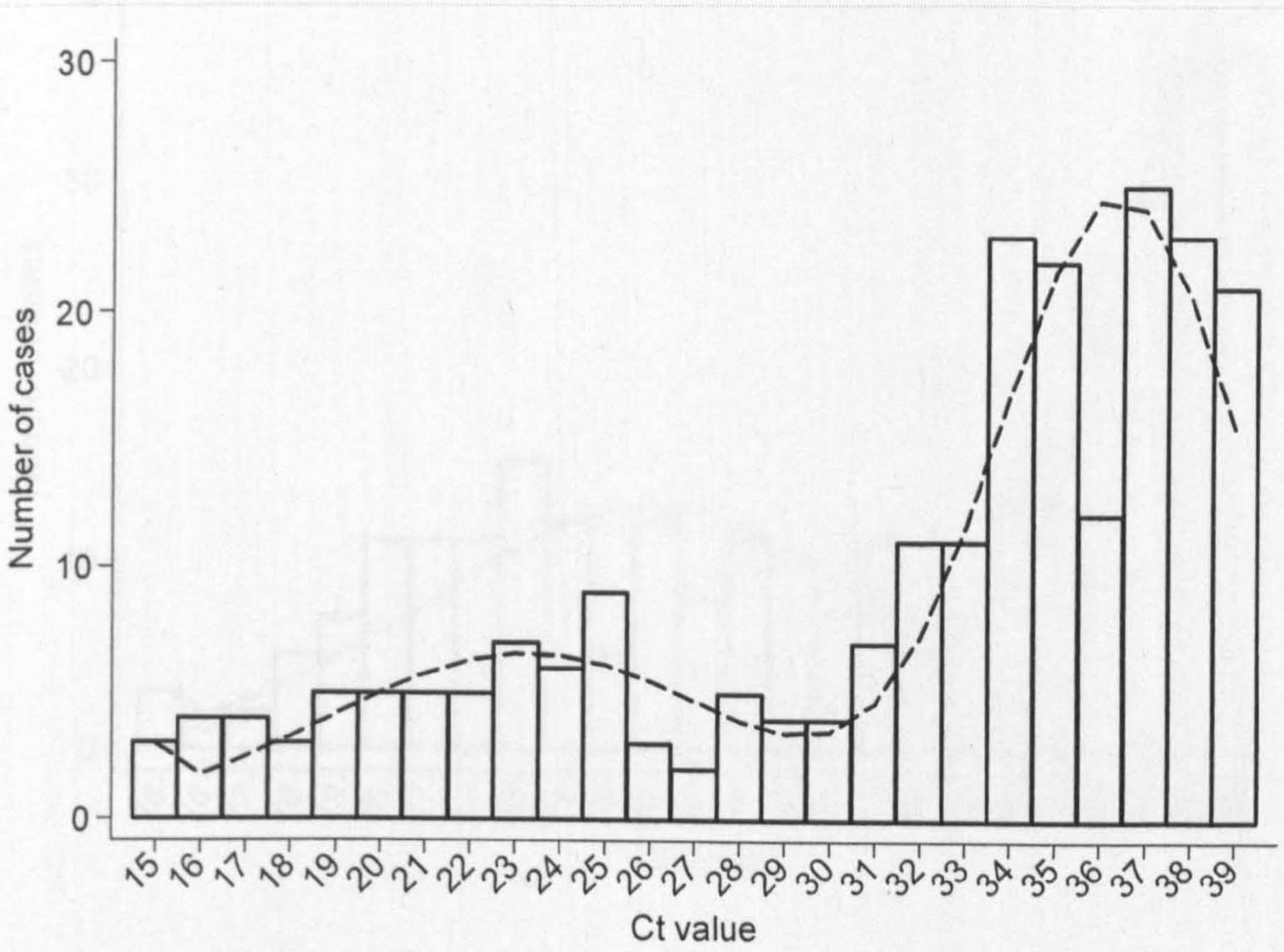
Abbreviations: Ct, cycle threshold.

Figure A6.1b Fit of mixture model to distribution of Ct values from IID cases of all ages in the general practice study. Bars show the number of IID cases at each Ct value in the general practice study. Black line shows the fitted number of IID cases at each Ct value predicted by the model.



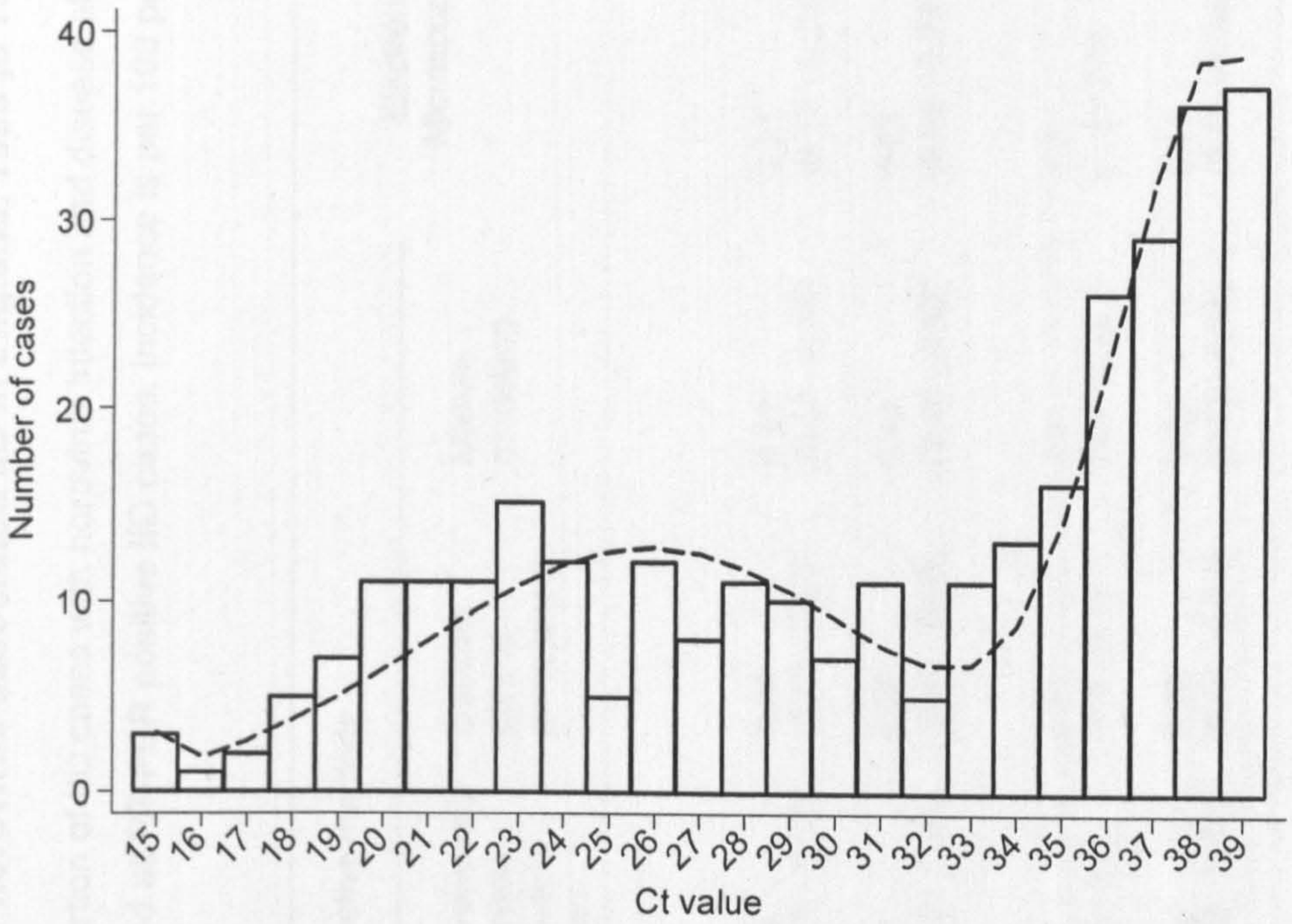
Abbreviations: Ct, cycle threshold.

Figure A6.1c Fit of mixture model to distribution of Ct values from IID cases aged less than five years in the general practice study. Bars show the number of IID cases aged less than five years at each Ct value in the general practice study. Black line shows the fitted number of IID cases at each Ct value predicted by the model.



Abbreviations: Ct, cycle threshold.

Figure A6.1d Fit of mixture model to distribution of Ct values from IID cases aged five years or older in the general practice study. Bars show the number of IID cases aged five years or older at each Ct value in the general practice study. Black line shows the fitted number of IID cases at each Ct value predicted by the model.



Abbreviations: Ct, cycle threshold.

Appendix A6.2. Incidence of GP consultations for norovirus-associated IID in England, 1993 to 1996.

Results from alternative methods for estimating the proportion of IID cases with norovirus infection and disease attributable to norovirus and incidence based on electron microscopy and all RT-PCR positive IID cases. Incidence is per 100 person-years; 95% credibility Intervals are given in brackets.

	Alternative methods					Electron Microscopy	All RT-PCR positive
	Adjustment factor *A*	Ct value cut-off	Ct value cut-off plus probable cases	Subtract control prevalence	Mixture modelling		
GP consultations							
Crude	0.49 (0.43 - 0.55)	0.46 (0.41 - 0.52)	0.58 (0.53 - 0.63)	0.69 (0.61 - 0.77)	0.41 (0.33 - 0.48)	0.21 (0.17 - 0.24)	1.1 (1.0 - 1.2)
Age -adjusted	0.54 (0.48 - 0.60)	0.49 (0.43 - 0.55)	0.61 (0.56 - 0.66)	0.50 (0.39 - 0.60)	0.43 (0.36 - 0.49)	0.21 (0.18 - 0.25)	1.2 (1.1 - 1.2)
< 5 years	3.2 (2.6 - 3.8)	2.6 (2.1 - 3.2)	3.2 (2.8 - 3.7)	5.7 (4.8 - 6.5)	2.5 (1.9 - 3.2)	1.8 (1.4 - 2.3)	8.1 (7.3 - 8.9)
≥5 years	0.35 (0.30 - 0.39)	0.34 (0.29 - 0.38)	0.42 (0.38 - 0.47)	0.13 (0.04 - 0.22)	0.28 (0.23 - 0.34)	0.11 (0.10 - 0.14)	0.68 (0.61 - 0.73)

Abbreviations: Ct, cycle threshold; GP, general practice; RT-PCR, reverse transcription-polymerase chain reaction.

Appendix A6.3. Age-specific adjustment factors calculated in each study component.

	Adjustment factor *A*	
	Community	General Practice
All ages	0.50	0.45
<5 years	0.48	0.40
≥5 years	0.55	0.52

Appendix 7: Published papers and conference presentations

Appendix 7.1. Phillips G, Lopman B, Tam C C, Iturriza-Gomara M, Brown D, & Gray J. Diagnosing rotavirus A associated IID: Using ELISA to identify a cut-off for real time RT-PCR. J. Clin. Virol. 2009; 44(3):242-5.

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Short Communication

Diagnosing rotavirus A associated IID: Using ELISA to identify a cut-off for real time RT-PCR

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ABSTRACT

Background: The use of RT-PCR for diagnosis of group A rotaviruses is increasing, but up to 14% of healthy individuals may be positive by RT-PCR. If RT-PCR is not well correlated with disease, rotavirus A may not always be the cause of illness in RT-PCR positive patients with infectious intestinal disease (IID).
Objectives: To describe the differences in faecal viral load between ELISA positive IID cases, RT-PCR positive cases and healthy controls. To develop a cut-off in faecal viral load for attributing illness to rotavirus A in RT-PCR positive IID cases.
Study design: Faecal viral load was measured, using real time RT-PCR, in 118 community IID cases and 65 healthy controls, previously tested by ELISA. Cycle threshold (Ct) values from the real-time RT-PCR were used as a proxy measure of viral load. A cut-off for attributing illness to rotavirus A was selected, using ROC analysis.
Results: There was little overlap in viral load between ELISA positive IID cases (median Ct 17) and healthy controls (median Ct 37), but ELISA negative, RT-PCR positive IID cases (median Ct 37) had viral loads similar to healthy controls, indicating that RT-PCR is not detecting extra cases of group A rotavirus associated IID, only sub-clinical infections. The optimal cut-off in the real time RT-PCR was at Ct value 24–27.
Conclusion: ELISA is the best method for the laboratory diagnosis of rotavirus A associated IID. If RT-PCR is used, it is advisable to use a real time platform and to use a viral load cut-off equivalent to the detection limit of ELISA.

1. Background

Enzyme linked immunosorbent assay (ELISA) has traditionally been the method of choice for laboratory diagnosis of group A rotavirus associated infectious intestinal disease (IID).[1,2] However, with the availability of reverse transcription-polymerase chain reaction (RT-PCR) assays for rotavirus A[3,4] and the move towards multiplexing in clinical virology,[5,6] the use of RT-PCR is increasing. Whilst RT-PCR does identify more rotavirus A infections than ELISA,[7] up to 14% of healthy individuals may be positive by RT-PCR,[8] indicating that in some RT-PCR positive IID cases, rotavirus A may not actually be the cause of illness.

Differences in faecal viral load between symptomatically and asymptomatically infected individuals have been demonstrated using real time RT-PCR[9] and histopathological studies indicate that damage to intestinal epithelial cells, caused by viral replication, may play a role in pathogenesis.[10] It may therefore be possible to use faecal viral load to indicate where rotavirus A is the cause of illness in RT-PCR positive IID cases.

2. Objectives

Research objectives were to describe the differences in rotavirus A viral load detected in IID cases positive by ELISA, IID cases negative by ELISA but positive by RT-PCR and healthy controls; to develop a cut-off in faecal viral load for attributing illness to rotavirus A in IID cases.

Abbreviations: AUC, area under the ROC curve; Ct, cycle threshold; ELISA, enzyme linked immunosorbent assay; IID, infectious intestinal disease; IQR, interquartile range; RT-PCR, reverse transcription-polymerase chain reaction; ROC, receiver operating characteristic.

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Appendix 7

Table 1
Distribution of rotavirus A real time RT-PCR Ct values in IID cases and healthy controls. IQR is the interquartile range. ELISA indicates that the IID case was ELISA positive, RT-PCR indicates that the IID case was ELISA negative and RT-PCR positive.

Method of rotavirus A detection	IID cases			Controls			Rank sum test p-value comparing cases to controls[a]
	Median Ct value	IQR	Sample size	Median Ct value	IQR	Sample size	
All ages							
All	18	15–30	153	37	33–39	65	<0.0001
ELISA	17	15–20	118				<0.0001
RT-PCR (ELISA negative)	37	32–39	35				0.96
<5 years							
All	17	15–22	92	37	33–40	46	<0.0001
ELISA	16	15–20	79				<0.0001
RT-PCR (ELISA negative)	35	32–38	13				0.37

[a] The rank sum test for ELISA and RT-PCR positive IID cases compare them to all controls.

3. Study design

3.1. Specimens

Faecal specimens were collected from IID cases and healthy controls during the Infectious Intestinal Disease Study for England (1993–1996).[11] IID cases were recruited from a community cohort, or at consultation with their general practitioner. IID cases had acute diarrhoea or vomiting lasting less than 2 weeks, with no known non-infectious cause, preceded by a symptom-free period of 3 weeks.[12] Healthy controls, with no history of IID for the preceding 3 weeks, were recruited concurrently to IID cases, from the community cohort or from the registration list of participating general practices (not after consultation for another condition).[12] IID cases provided a faecal specimen during acute illness; controls provided a specimen at recruitment.

3.2. Testing

In the original study, faecal specimens from IID cases and controls were tested for rotavirus A using ELISA.[11] Specimens with sufficient volume remaining after testing were archived in frozen storage.[13] Subsequently, all archived specimens were retested for rotavirus A using RT-PCR.[8] In this study, a real time RT-PCR assay (method previously described[14]) was used to determine the viral load in specimens that were previously positive for rotavirus A by ELISA or RT-PCR.

The cycle threshold (Ct) values from the real time RT-PCR were used as a proxy measure of viral load. The Ct value is inversely proportional to the amount of virus present in the specimen, so the lower the Ct value the higher the faecal viral load. The real time RT-PCR assay was run for 45 cycles so the maximum possible Ct value for a positive specimen was 44.

3.3. Descriptive analysis

The median Ct value and interquartile range were calculated for IID cases and controls and comparisons were made between groups using the rank-sum test in Stata 10.[15]

3.4. Receiver operating characteristic analysis

Receiver operating characteristic (ROC) analysis was used to select a cut-off in faecal viral load for attributing illness to rotavirus A. ELISA was used as the gold standard reference test to indicate where rotavirus A was the cause of illness in IID cases. The optimal cut-off was identified at the maximum value of the Youden index (sensitivity + specificity − 1).[16–18] The ROC analysis was repeated, using healthy controls in the reference negative group, to increase the sample size for the analysis in children aged less than 5 years. Healthy controls should serve as a suitable reference negative group because they have viral loads representative of rotavirus A infection without disease.

4. Results

4.1. Descriptive analysis

IID cases were aged up to 83 years and controls were aged up to 46 years; 60% of cases and 71% of controls were aged less than

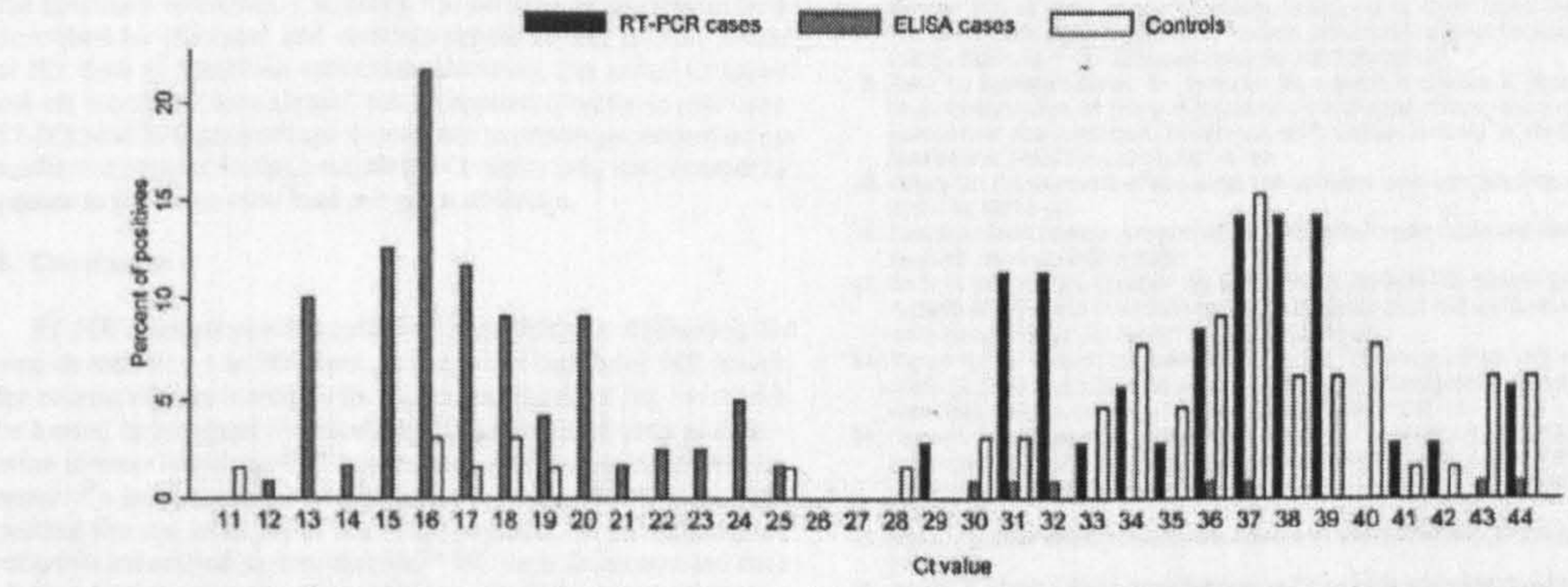

Fig. 1. Percentage distribution of real time RT-PCR Ct values in IID cases and controls. Low Ct values correspond to high viral loads; the viral load decreases with increasing Ct value. 'ELISA cases' are IID cases positive by ELISA, 'RT-PCR cases' are IID cases negative by ELISA and subsequently positive by RT-PCR. Sample sizes: ELISA cases = 118, RT-PCR cases = 35, controls = 65.

Appendix 7

Table 2
ROC analysis results, AUC is the area under the ROC curve. CI is confidence interval.

	Ct cut-off	Youden Index	Sensitivity (95% CI)	Specificity (95% CI)	AUC	Sample size	
						Reference positive	Reference negative
All ages							
ELISA gold standard	25–28	0.96	0.96 (0.92–0.99)	1	0.99	118	35
Reference negative group controls	24–27	0.82	0.94 (0.90–0.98)	0.88 (0.80–0.96)	0.92	118	65
Aged < 5 years							
Reference negative group controls	24	0.84	0.95 (0.90–1.0)	0.89 (0.80–0.98)	0.95	79	46

5 years. The median Ct value in ELISA positive IID cases was substantially lower than in controls (Table 1) and there was very little overlap between the distributions of Ct values in these two groups (Fig. 1). There was no evidence of a difference in Ct value distribution between the ELISA negative, RT-PCR positive IID cases and the controls (Fig. 1, Table 1), in all ages and when the analysis was restricted to children aged less than 5 years.

4.2. ROC analysis

Using ELISA as the gold standard, the optimal Ct value cut-off for attributing illness to rotavirus A in IID cases, for all ages, was in the range 25–28. There was a clear bimodal distribution of Ct values, with few observations in the range 25–28, so it was not possible to distinguish between these cut-off values. Using healthy controls as the reference negative group produced similar results (Table 2). The optimal cut-off for children aged less than 5 years was at Ct value 24, although the Youden index declined only slightly (difference less than 0.1) at Ct values of 25–27 (data not shown).

5. Discussion

We have used faecal viral load to demonstrate that ELISA diagnosis is highly correlated with disease in rotavirus A infection, in accordance with other community and hospital-based studies,[13,18–21] and that RT-PCR is probably only detecting additional infections in IID cases at levels not associated with illness. We have selected a cut-off in the real time RT-PCR assay to improve the specificity of diagnosing rotavirus A associated IID by this method.

A major strength of this study is the availability of specimens from healthy controls, which were essential for interpreting the RT-PCR results in IID cases. Degradation of the rotavirus A genome, during the prolonged storage of these specimens, is likely to be minimal, because the double stranded RNA is relatively stable. It is also unlikely that degradation will have occurred differentially across the specimen collection. Therefore, the patterns in faecal viral load described for IID cases and controls should reflect relative levels at the time of specimen collection. However, the actual Ct value cut-off identified here should not be applied directly to real time RT-PCR results from fresh specimens, nor to results generated using a different assay protocol, because the Ct values may not necessarily equate to the same viral load per gram of faeces.

6. Conclusion

RT-PCR does not provide sufficient specificity for attributing illness to rotavirus A in IID cases. As the use of multiplex PCR assays for enteric viruses increases in routine diagnosis of IID, there will be a need to interpret the results of these sensitive tests to determine disease aetiology.[22,23] We have shown that ELISA positivity remains a good correlate of disease in rotavirus A infection, supporting the use of ELISA in the WHO protocol for surveillance of rotavirus associated gastroenteritis.[24] We have demonstrated that clinical laboratories can use real time RT-PCR testing of ELISA-positive and ELISA-negative specimens to define a suitable cut-off for their real time RT-PCR assays. Accurate diagnosis of disease aetiology is important both for individual patient care, where correct identification of the cause of illness is essential for clinical management, and at the population level, to ensure that estimates of rotavirus A disease burden are accurate, for assessing vaccine impact in immunized populations.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

1. Cheung EY, Hnatko SI, Gunning H, Wilson J. Comparison of rotazyme and direct electron microscopy for detection of rotavirus in human stools. *J Clin Microbiol* 1982;16:562–3.
2. Kjeldsberg E, Mortensson-Egnund K. Comparison of solid-phase immune electron microscopy, direct electron microscopy and enzyme-linked immunosorbent assay for detection of rotavirus in faecal samples. *J Virol Methods* 1982;4:45–53.
3. Wilde J, Yolken R, Willoughby R, Eiden J. Improved detection of rotavirus shedding by polymerase chain reaction. *Lancet* 1991;337:323–6.
4. Xu L, Harbour D, McCrae MA. The application of polymerase chain reaction to the detection of rotaviruses in faeces. *J Virol Methods* 1990;27:29–37.
5. Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. *Clin Lab* 2005;51:429–35.
6. Rohayem J, Berger S, Juretzek T, Herchenroder O, Mogel M, Poppe M, et al. A simple and rapid single-step multiplex RT-PCR to detect norovirus, astrovirus and adenovirus in clinical stool samples. *J Virol Methods* 2004;118:49–59.
7. Gunson RN, Miller J, Leonard A, Carman WF. Importance of PCR in the diagnosis and understanding of rotavirus illness in the community. *Commun Dis Public Health* 2003;6:63–5.
8. Amar CF, East CL, Gray J, Iturriza-Gomara M, Mackie EA, McLaughlin J. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). *Eur J Clin Microbiol Infect Dis* 2007;26:311–23.
9. Kang G, Iturriza-Gomara M, Wheeler JG, Crystal P, Monica B, Ramani S, et al. Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: correlation with clinical severity in children in South India. *J Med Virol* 2004;73:118–22.
10. Ramig RF. Pathogenesis of intestinal and systemic rotavirus infection. *J Virol* 2004;78:10213–20.
11. Food Standards Agency. A report of the study of infectious intestinal disease in England. London: HMSO; 2000.
12. Sethi D, Wheeler JG, Cowden JM, Rodrigues LC, Sockett PN, Roberts JA, et al. A study of infectious intestinal disease in England: plan and methods of data collection. *Commun Dis Public Health* 1999;2:101–7.
13. Tompkins DS, Hudson MJ, Smith HR, Eglin RP, Wheeler JG, Brett MM, et al. A study of infectious intestinal disease in England: microbiological findings in cases and controls. *Commun Dis Public Health* 1999;2:108–13.
14. Gomara MI, Simpson R, Perault AM, Redpath C, Largelly P, Joshi D, et al. Structured surveillance of infantile gastroenteritis in East Anglia, UK: incidence of infection with common viral gastroenteric pathogens. *Epidemiol Infect* 2007;1–11.
15. StataCorp. Stata Statistical Software: Release 10. College Station, (TX): StataCorp LP; 2007.
16. Bewick V, Cheek L, Ball J. Statistics review 13: receiver operating characteristic curves. *Crit Care* 2004;8:508–12.
17. Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. *Biomed J* 2005;47:458–72.

Appendix 7

18. Youden W. Index for rating diagnostic tests. *Cancer* 1950;3:32–5.
19. de Wit MA, Koopmans MP, Kortbeek LM, Wannet WJ, Vinje J, van Leusden F, et al. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *Am J Epidemiol* 2001;154:666–74.
20. de Wit MA, Koopmans MP, Kortbeek LM, van Leeuwen NJ, Vinje J, Van Duynhoven YT. Etiology of gastroenteritis in sentinel general practices in the Netherlands. *Clin Infect Dis* 2001;33:280–8.
21. Nokes DJ, Abwao J, Pamba A, Peenze I, Dewar J, Maghenda JK, et al. Incidence and clinical characteristics of group A rotavirus infections among children admitted to hospital in Kilifi, Kenya. *PLoS Med* 2008;5:e153.
22. Gunson RN, Bennett S, Maclean A, Carman WF. Using multiplex real time PCR in order to streamline a routine diagnostic service. *J Clin Virol* 2008.
23. O'Neill HJ, McCaughey C, Coyle PV, Wyatt DE, Mitchell F. Clinical utility of nested multiplex RT-PCR for group F adenovirus, rotavirus and norwalk-like viruses in acute viral gastroenteritis in children and adults. *J Clin Virol* 2002;25:335–43.
24. World Health Organization. Generic protocol for (i) hospital-based surveillance to estimate the burden of rotavirus among children and (ii) a community-based survey on utilization of health care services for gastroenteritis in children. Geneva, Switzerland: World Health Organization; 2002.

Appendix 7.2. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, & Gray J. Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect Dis*. 2009; 9(63).

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Diagnosing norovirus-associated infectious intestinal disease using viral load

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Abstract

Background: Reverse transcription-polymerase chain reaction (RT-PCR) is the main method for laboratory diagnosis of norovirus-associated infectious intestinal disease (IID). However, up to 16% of healthy individuals in the community, with no recent history of IID, may be RT-PCR positive; so it is unclear whether norovirus is actually the cause of illness in an IID case when they are RT-PCR positive. It is important to identify the pathogen causing illness in sporadic IID cases, for clinical management and for community based incidence studies. The aim of this study was to investigate how faecal viral load can be used to determine when norovirus is the most likely cause of illness in an IID case.

Methods: Real-time RT-PCR was used to determine the viral load in faecal specimens collected from 589 IID cases and 159 healthy controls, who were infected with genogroup II noroviruses. Cycle threshold (Ct) values from the real-time RT-PCR were used as a proxy measure of viral load. Receiver-operating characteristic (ROC) analysis was used to identify a cut-off in viral load for attributing illness to norovirus in IID cases.

Results: One hundred and sixty-nine IID cases and 159 controls met the inclusion criteria for the ROC analysis. The optimal Ct value cut-off for attributing IID to norovirus was 31. The same cut-off was selected when using healthy controls, or IID cases who were positive by culture for bacterial pathogens, as the reference negative group. This alternative reference negative group can be identified amongst specimens routinely received in clinical virology laboratories.

Conclusion: We demonstrated that ROC analysis can be used to select a cut-off for a norovirus real time RT-PCR assay, to aid clinical interpretation and diagnose when norovirus is the cause of IID. Specimens routinely received for diagnosis in clinical virology laboratories can be used to select an appropriate cut-off. Individual laboratories can use this method to define in-house cut-offs for their assays, to provide the best possible diagnostic service to clinicians and public health workers. Other clinical and epidemiological information should also be considered for patients with Ct values close to the cut-off, for the most accurate diagnosis of IID aetiology.

Appendix 7

Background

Infectious intestinal disease (IID) is a syndrome of mixed aetiology; many different pathogens can infect the human gastrointestinal tract and produce diarrhoea, vomiting and other characteristic symptoms. Mixed gastrointestinal infections are frequently detected, especially in infants and young children and when polymerase chain reaction (PCR) assays are used for diagnosis [1,2]. It is important to determine which pathogen is the cause of illness, in order to direct clinical management for individual patients and to advance epidemiological understanding of IID.

Reverse transcription- PCR (RT-PCR) is now the method of choice for detecting norovirus in clinical specimens. RT-PCR detects norovirus at lower concentrations and is less affected by specimen quality and preparation than electron microscopy [3-5]; large numbers of specimens can be tested simultaneously, compared to the single throughput for electron microscopy. RT-PCR also detects a much wider range of norovirus genetic variants than enzyme-linked immunosorbent assays (ELISA) and may be more easily adaptable for detection of new strains [6].

However, many healthy individuals, with no recent history of IID, are RT-PCR positive [7-9], meaning that virus detection by RT-PCR is not well correlated with disease in norovirus infection. If RT-PCR positivity does not necessarily equate to norovirus-associated IID, it cannot be used alone to attribute illness to norovirus in IID cases; it is possible that the norovirus infection is 'asymptomatic' in the IID case, with another pathogen, detected or undetected, actually causing the symptoms. The poor diagnostic specificity of PCR and the associated difficulties for clinical interpretation of test results have been highlighted for other viral pathogens [10,11].

Previous studies have demonstrated differences in faecal norovirus load between symptomatically and asymptomatically infected individuals [7,12]. Histopathological investigations of experimentally inoculated volunteers and naturally infected individuals also indicate that the mechanism of pathogenesis in norovirus infection may rely on damage to the intestinal epithelium, caused by viral replication [13-15], so that symptoms may be a result of high viral loads. The aim of this study was to use faecal viral load measurements to determine when illness is attributable to norovirus in IID cases.

Methods

Specimens

Faecal specimens were collected from IID cases and healthy controls during the Infectious Intestinal Disease Study for England (1993–1996) [16]. IID cases were recruited from a prospectively followed cohort in the community, or on consultation with their general practitioner for IID. IID cases had acute diarrhoea or vomiting, lasting less than two weeks, with no known non-infectious cause, preceded by a symptom free period of at least three weeks [17]. Healthy controls, with no history of IID for the preceding three weeks, were recruited from within the community cohort or from the registration lists of participating general practices (but not after consultation for another condition) [17]. Controls were recruited concurrently to IID cases. IID cases were asked to provide a faecal specimen during acute illness and controls provided a specimen at recruitment.

Testing

In the original study, norovirus was detected using electron microscopy. Faecal specimens were also tested for a range of other bacterial, viral and protozoal pathogens, using bacterial culture, microscopy or ELISA. Specimens with sufficient volume remaining after testing were archived in frozen storage [18]. Subsequently the archived specimens were all re-tested for norovirus using RT-PCR [7,19]. PCR testing was also used to detect seven other common bacterial, viral and protozoal pathogens.

For the present study, norovirus RNA was re-extracted from the stored faecal specimens that were previously positive for norovirus by EM or RT-PCR, and real-time RT-PCR (method previously described [7]) was used to determine the viral load. The real time RT-PCR assay has separate primer pairs for norovirus genogroup I and genogroup II, so it was possible to use the assay to identify the genogroup of norovirus present. Only viral load measurements from norovirus genogroup II positive specimens were used for this analysis; differences in the performance of the two genogroup specific assays mean that it is not appropriate to directly compare the results between the two genogroups (J. Gray, personal communication). Specimen collection and testing for norovirus is summarised in Additional File 1.

Data

The cycle threshold (Ct) value from the real time RT-PCR was used as a proxy measure of faecal viral load. The Ct value is inversely proportional to the amount of virus present in the specimen, so the lower the Ct value the higher the faecal viral load. The Ct value represents the number of rounds of PCR replication required to raise the number of copies of the target sequence in the reaction mixture above a pre-determined threshold [20]. The real time RT-PCR assay was run for 40 cycles, so the maximum possible Ct value for positive specimens in this study was 39.

Descriptive analysis

The median Ct value and inter-quartile range were calculated for IID cases and controls; comparisons were made between groups using the rank-sum test in Stata 10 [21].

Appendix 7

Receiver-operating characteristic analysis

Receiver-operating characteristic (ROC) analysis was used to define a cut-off in the Ct values, to attribute disease to norovirus in IID cases. There is no gold standard test for diagnosing norovirus-associated IID. We therefore used microbiological and clinical characteristics to select reference groups for the ROC analysis.

Reference positive groups

We defined three reference positive groups, selected to have Ct values that are representative of where norovirus is causing illness (Table 1). Reference positive group 1 included only IID cases who were diagnosed as norovirus positive by electron microscopy; the high viral loads required for detection by electron microscopy correspond to viral shedding during acute infection in experimentally inoculated volunteers [22,23], so these IID cases are highly likely to have IID caused by norovirus.

In reference positive group 2, we additionally included IID cases who were electron microscopy negative and subsequently RT-PCR positive, providing that they had no other pathogens identified in their stool and that they had collected a specimen early in their illness (less than three days since symptom onset). These two restrictions were used to ensure that norovirus was the most likely cause of their illness and to ensure that their faecal viral load is representative of acute symptomatic norovirus infection [12,22,23]. We defined this second reference group to determine whether using only electron microscopy positive cases in reference group 1 biased the cut-off to lower Ct values (higher viral loads).

Reference positive group 3 included IID cases who were RT-PCR positive for norovirus (including those previously positive by EM) and who were negative for other bacterial, protozoal and viral pathogens that are routinely detected in clinical diagnostic algorithms for sporadic IID in National Health Service laboratories in the UK [24,25]. This restriction was used to make norovirus the most likely cause of illness in these IID cases, so that their Ct values should be representative of where norovirus is causing illness. We defined this third reference positive group to explore whether it is suitable for selecting a Ct value cut-off, because electron microscopy diagnosis is no longer used in clinical laboratories in the UK, so cannot be used to select a reference positive group in future studies.

Reference negative groups

We defined two reference negative groups, selected to have Ct values representative of where norovirus is not causing illness (Table 1). Reference negative group 1 included norovirus-infected healthy controls. Reference negative group 2 included norovirus infected IID cases

Table 1: Inclusion criteria for the ROC analysis reference groups

Reference group	Inclusion Criteria
Reference positive 1	1. IID 2. Norovirus detected by electron microscopy 3. Norovirus infection confirmed by RT-PCR
Reference positive 2	1. IID 2. Norovirus detected by electron microscopy 3. Norovirus infection confirmed by RT-PCR Or 1. IID 2. Electron microscopy negative 3. Norovirus detected by RT-PCR 4. No other pathogen detected 5. Specimen collected within 3 days of symptom onset
Reference positive 3	1. IID 2. Norovirus detected by electron microscopy and/or RT-PCR 3. Negative for *Campylobacter* spp., *Salmonella* spp. and *Shigella* spp. by bacterial culture and *Cryptosporidium* spp. by light microscopy (and rotavirus A by ELISA in children aged less than five years only)
Reference negative 1	1. No history of IID in previous 3 weeks 2. Norovirus detected by RT-PCR
Reference negative 2	1. IID 2. Norovirus detected by RT-PCR 3. Infection with *Salmonella* spp., *Campylobacter* spp. or *Shigella* spp. detected by bacterial culture or *Cryptosporidium* spp. detected by light microscopy (or rotavirus A by ELISA in children aged less than five years only)

with a bacterial infection diagnosed by culture or rotavirus A infection diagnosed by ELISA (for children aged less than five years only). Bacterial culture without enrichment may indicate the presence of high concentrations of viable bacterial cells, meaning that the bacteria detected are likely to be causing illness, rather than the norovirus infection. Similarly, ELISA for rotavirus A has a high detection limit that correlates well with disease [26,27], so rotavirus A is probably the cause of illness in ELISA positive individuals, rather than the norovirus infection. We defined this second reference negative group to explore whether it is suitable for selecting a cut-off, because specimens from healthy controls are not routinely received in clinical laboratories, so cannot be used as the reference negative group if other laboratories want to use this method to develop a cut-off for their real time assays.

In each ROC analysis, the sensitivity and specificity were calculated for each potential cut-off in the range of Ct values and an empirical ROC plot created using Stata 10 [21]. The Youden index (sensitivity + specificity-1) was calculated and the maximum value used to identify the optimal cut-off [28-30]. The analysis was done for all ages together and then separately, in children aged less than five years and individuals aged five years or older.

Ethics

Ethical approval was granted from both local and national research ethics committees (Royal College of General Practitioners, London School of Hygiene and Tropical Medicine, Public Health Laboratory Service) for the IID study, including creation of the faecal specimen archive [17]. Written, informed consent was obtained from all cases and controls. The faecal specimen archive was anonymised and no further ethical approval was sought for the retesting in this study.

Results

Descriptive analysis

Ct values were generated for 589 IID cases and 159 healthy controls, who were infected with genogroup II noroviruses; 92 of the IID cases were positive by electron microscopy and 497 were negative by electron microscopy but subsequently positive by RT-PCR. IID cases were aged up to 94 years and controls up to 84 years; 40% of IID cases and 60% of controls were aged less than five years.

The median Ct value was lower in IID cases (median 34) than in controls (median 38) (Table 2). The difference compared to controls was greatest for IID cases positive by electron microscopy (median 24); there was very little overlap in the distribution of Ct values in electron microscopy positive IID cases and controls (Figure 1). The distribution of Ct values for the IID cases who were negative

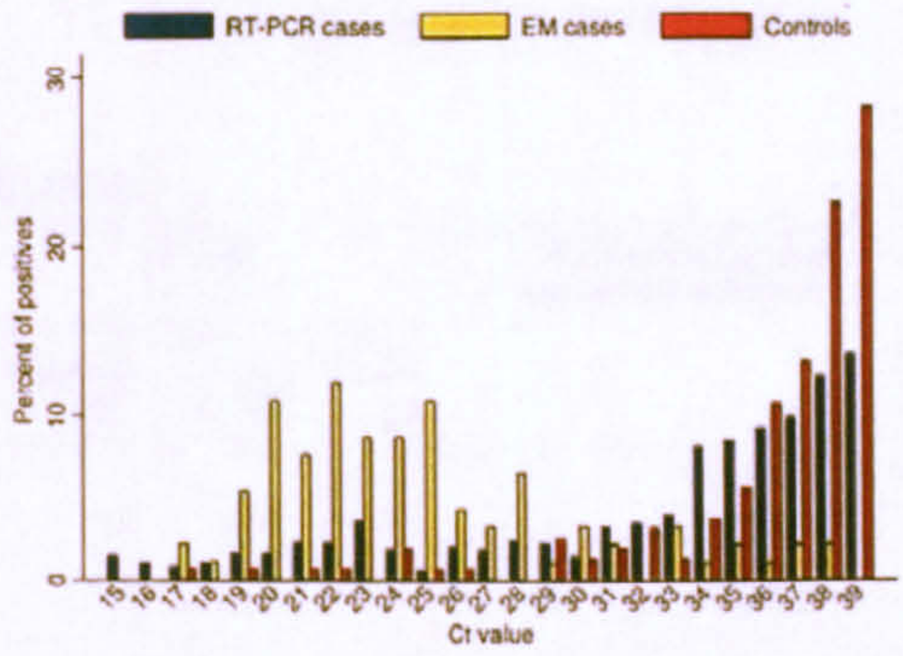

Figure 1
Percentage distribution of real time RT-PCR Ct values in IID cases and controls. Low Ct values correspond to high viral loads; the viral load decreases with increasing Ct value. 'EM cases' are IID cases positive by electron microscopy, 'RT-PCR cases' are IID cases negative by electron microscopy and subsequently positive by RT-PCR. Sample sizes: EM cases = 92, RT-PCR cases = 497, controls = 159.

by electron microscopy and subsequently RT-PCR positive overlaps substantially with the controls, although a small proportion have the higher viral loads seen in the electron microscopy positive IID cases (Figure 1, Table 2).

ROC analysis

The numbers of specimens meeting the inclusion criteria for each of the reference groups are shown in Table 3.

The optimal cut-off for attributing illness to genogroup II noroviruses in IID cases was at Ct value 31, corresponding to the maximum Youden index for the ROC analysis with reference positive group 1 and reference negative group 1 (Figure 2).

Using this cut-off, IID cases with Ct values of 31 or below are classified as 'positive' for norovirus-associated IID: they have disease caused by norovirus. IID cases with Ct values above 31 are classified as 'negative' for norovirus-associated IID: they have disease but their norovirus infection was not the cause of their symptoms.

The optimal cut-off for children aged less than five years was at Ct value 30, whereas for older children and adults it was at Ct value 33 (Table 3). There was some evidence of a difference in Ct value distribution between electron microscopy positive IID cases in these two age groups (rank sum test p = 0.036), with the median in children aged less than five years at Ct value 23 and at Ct value 25

Appendix 7

Table 2: Ct values in genogroup II norovirus positive IID cases and healthy controls.

Method of norovirus detection	IID Cases			Controls			Rank-sum test p-value comparing cases to controls
	Median Ct value	Ct value IQR	Sample size	Median Ct value	Ct value IQR	Sample size	
All ages							
All	34	25–37	589	38	35–39	159	<0.0001
Electron microscopy	24	21–27.5	92				<0.0001
RT-PCR (Electron microscopy negative)	35	29–38	497				<0.0001
< 5 years							
All	34	26–37	253	37	34–38	92	<0.0001
Electron microscopy	23	21–25	48				<0.0001
RT-PCR (Electron microscopy negative)	35	32–37	205				0.0001
5 years +							
All	34	25–38	334	38	36–39	67	<0.0001
Electron microscopy	25	22–28.5	44				<0.0001
RT-PCR (Electron microscopy negative)	35	27–38	290				<0.0001

The rank-sum tests for electron microscopy and RT-PCR positive IID cases compare them to all controls. Age was not recorded for two IID cases. IQR is the interquartile range.

for older children and adults (Table 2). This indicates that the different cut-offs may reflect a true difference in viral load between these age groups.

The optimal cut-off (all ages) was also at Ct value 31 when RT-PCR positive cases with no other pathogen detected and early specimen collection were included in the reference positive group (reference positive group 2) (Table 3). This was also true for the age-group specific ROC analyses (data not shown). The optimal cut-off was also at Ct value 31 when norovirus positive IID cases who were negative for other commonly tested enteric pathogens were used as

Table 3: ROC analysis results.

Reference groups used	Optimal Ct cut-off	Youden Index	Sensitivity (95% CI)	Specificity (95% CI)	AUC	Sample size	
						Reference positive	Reference negative
Ref positive 1 Ref negative 1							
All	31	0.77	0.88 (0.65–1.00)	0.89 (0.84–0.94)	0.93	92	159
aged <5 years	30	0.80	0.94 (0.84–1.00)	0.86 (0.79–0.93)	0.93	48	92
aged >5 years	33	0.83	0.89 (0.79–0.98)	0.94 (0.88–1.00)	0.96	44	67
Ref positive 2 Ref negative 1	31	0.61	0.72 (0.66–0.79)	0.89 (0.84–0.94)	0.87	169	159
Ref positive 3 Ref negative 2	31	0.29	0.43 (0.39–0.47)	0.86 (0.77–0.94)	0.64	524	64

The reference groups are described in Table 1. AUC is the area under the curve.

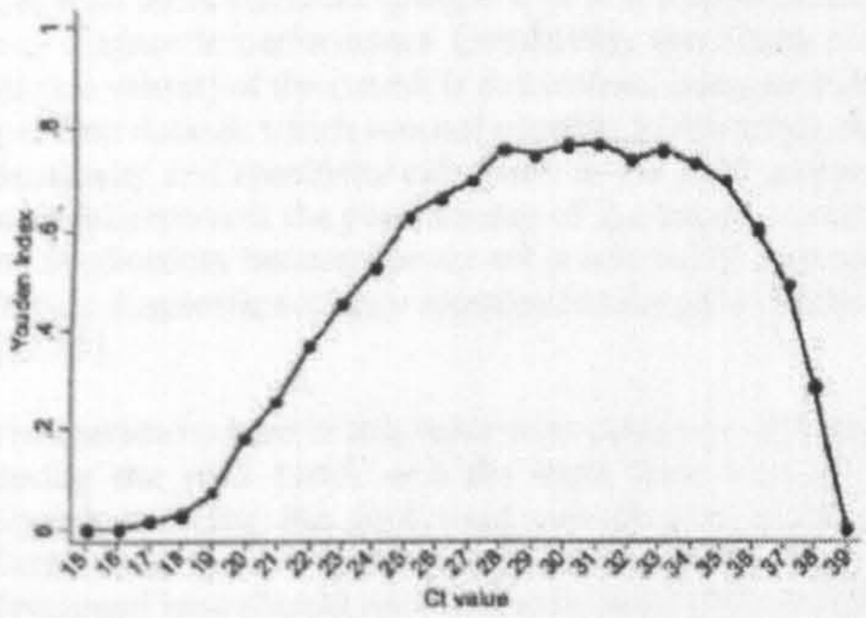

Figure 2
Youden Index from ROC analysis for reference positive group 1 and reference negative group 1. Reference positive group 1 were electron microscopy positive IID cases and reference negative group 1 were RT-PCR positive healthy controls.

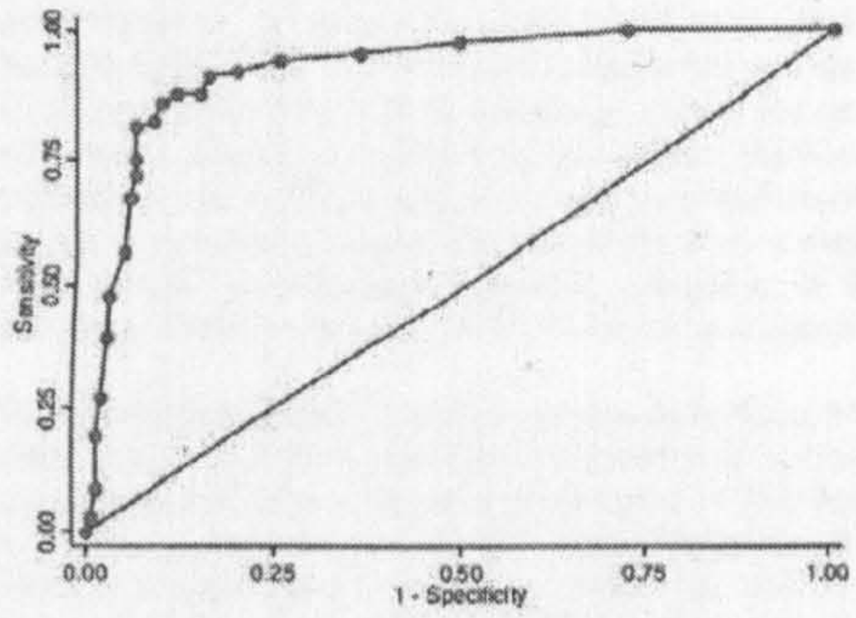

Figure 3
ROC plot for reference positive group 1 and reference negative group 1. Reference positive group 1 were electron microscopy positive IID cases and reference negative group 1 were RT-PCR positive healthy controls. The diagonal line represents a ROC plot for a test with no discriminatory power.

the reference positive group (reference positive group 3), and the bacterial culture positive IID cases were used as the reference negative group (reference negative group 2).

The Ct values discriminated well between reference positive group 1 and reference negative group 1, because the area under the ROC curve was close to the maximum value of one (Figure 3, Table 3). The discriminatory power of the Ct values was poorer for the ROC analysis when RT-PCR positive cases with no other pathogen detected and early specimen collection were included in the reference positive group (reference positive group 2). The discriminatory power was very low for distinguishing between reference positive group 3 and reference negative group 2 because the area under the curve was close to 0.5, which is indicative of a test with no discriminatory power.

Discussion

In this study we have demonstrated a difference in viral load between symptomatic and asymptomatic norovirus infection. A substantial proportion of IID cases who were positive only by RT-PCR had viral loads equivalent to those in healthy controls. This supports the hypothesis that norovirus is not always the cause of illness where it is detected by RT-PCR. We have shown that it is possible to use the viral load in clinical specimens to indicate where norovirus is the most likely cause of illness, by selecting a cut-off for the norovirus real time RT-PCR assay. We have also shown that the method of cut-off selection can be adapted for use with specimens that are routinely received and tested in clinical laboratories, to help other laboratories develop in-house cut-offs for their assays. This is essential because there is substantial variability between UK virology reference laboratories in the Ct values produced from standard reference specimens [31]; the same cut-off may not be appropriate for all laboratories because of these differences in assay performance.

A major strength of this study is the availability of specimens from healthy controls. There are few community studies of IID with large control groups available, but they are essential for interpreting the RT-PCR data in IID cases. Importantly, it has been possible to validate the use of bacterial culture positive IID cases as a reference negative group, by comparison to the ROC analysis using healthy controls; this removes the need to collect further control specimens in future studies. We have also shown that RT-PCR positive IID cases, who are negative for other common bacterial, protozoal and viral pathogens, are a suitable reference positive group, so that the method can be used by laboratories without EM testing facilities. These reference groups can now be used by other laboratories for development of cut-offs for their assays.

The area under the ROC curve for the alternative reference groups is very low, possibly because the viral loads in many of the IID cases in the reference positive group were not representative of symptomatic norovirus infection; this is reflected in the low sensitivity for the cut-off at Ct value 31 when using these groups in the ROC analysis. However, selection of an appropriate cut-off is the main aim of this method and we have shown that this is possi-

ble with these reference groups. It is also important that the diagnostic performance (sensitivity, specificity, predictive values) of the cut-off is determined using an independent dataset, which was not possible in this study; the sensitivity and specificity calculated in the ROC analysis may misrepresent the performance of the cut-off in clinical application, because the cut-off is selected by optimising the diagnostic accuracy compared to the gold standard [32,33].

The specimens used in this study were originally collected during the mid 1990s and the viral RNA may have degraded during the prolonged storage and repeated freeze-thaw cycles for re-testing. Therefore the cut-off developed here should not be directly applicable to real-time RT-PCR results from fresh specimens without validation. Similarly, the cut-off should not be applied to assays with different protocols, because the Ct values may not equate to the same viral load per gram of faeces. It is unlikely, however, that there will have been differential degradation of RNA between specimens during storage, so it is still valid to compare the viral load between specimens in this collection, and to assume that the relative differences observed between IID cases and controls are a true reflection of symptomatic and asymptomatic infection. It is also important to note that any cut-off in viral load can only be applied to specimens collected from IID patients during acute symptoms, when the viral load is representative of disease aetiology. After symptoms resolve in norovirus-associated IID, the viral load quickly drops to levels seen in asymptomatic infection [12] and the predictive value of the cut-off will be greatly reduced.

The cut-off developed here is not applicable to two of the rarer genotypes in genogroup II (GII-7 and GII-8), because the real time RT-PCR assay has poorer efficiency (a higher detection limit) for these genotypes (J. Gray, personal communication), so the Ct values do not represent the same faecal viral loads as for the other genotypes. At a population level, the degree of misclassification would be small because of the low prevalence of GII-7 and GII-8 [34-36]. However, correct identification of illness caused by these genotypes may be important for clinical management, but would require development of genotype-specific cut-offs. Similarly, we have excluded genogroup I noroviruses from this analysis because the efficiency of the assay is highly variable for genotypes within in this genogroup. Development of a cut-off for GII-7 and GII-8 or genogroup I noroviruses would require collection of sufficient specimens for genotype-specific ROC analyses; clinical application would require genotyping to be part of routine diagnosis, which may not be economically or logistically feasible. Further work is also needed to characterise the kinetics of the real time RT-PCR assay, to determine whether a Ct value of 31 translates to the same faecal viral load for all genogroup II genotypes with the same assay efficiency. Selection of a single cut-off may also not be appropriate if the Youden index is similar for a range of Ct values between 28 and 33, as was the case in this analysis. With a larger sample size, in future studies, there may be better power to discriminate between potential cut-offs in this range. Nevertheless, the cut-off provides a major improvement in diagnostic specificity compared to the current qualitative use of RT-PCR in norovirus diagnosis.

The causal relationship between disease symptoms and viral load has not been established. However, if the relationship between the occurrence of disease and viral load is consistent, regardless of whether high viral loads are a cause or a consequence of disease, viral load will be a good marker of norovirus-associated IID and the approach developed here is valid. Viral load is routinely used to predict outcome and guide clinical management for a number of viruses that cause chronic infections, such as Epstein-Barr virus [37] and cytomegalovirus [38] in transplant patients, HIV [39], hepatitis C [40] and HTLV [41]. However this is the first time, to our knowledge, that viral load has been used as a tool for diagnosing enteric viruses as the cause of acute IID.

Conclusion

As PCR diagnosis is applied to an increasing number of viral pathogens, the debate is growing about the clinical interpretation of positive results and the utility of PCR in diagnostic services [10,42,43]. PCR has many advantages over traditional diagnostic methods, including higher throughput, shorter turnaround time, adaptability to new strains and production of data for molecular epidemiological surveillance. It is therefore important to ensure that clinically informative results are produced from PCR assays, to provide a high standard of patient care alongside these other benefits. The method developed here shows that the real-time RT-PCR output for norovirus can be used to attribute disease to norovirus in IID cases, where simple detection may not be sufficient to give a confident diagnosis of norovirus-associated IID. This semi-quantitative approach to diagnosis can improve both the accuracy of community-based estimates of norovirus associated IID incidence and the interpretability of diagnostic results provided to clinicians from clinical virology laboratories. However it is important that clinical and epidemiological information is considered in the diagnosis of disease aetiology for individual patients with Ct values close to the cut-off.

Independent validation of this method is required prior to application in other studies and laboratories; we have provided a method for validation without the need for collection of specimens from healthy controls or further use of EM. The method may also be useful for other viral patho-

gens, for which the same problems with the interpretability of PCR have been described. Future work will focus on applying this approach for estimation of norovirus associated IID incidence and describing the implications for diagnosis of norovirus outbreaks.

Abbreviations

AUC: area under the ROC curve; Ct: cycle threshold; ELISA: enzyme linked immunosorbent assay; EM: electron microscopy; IID: infectious intestinal disease; IQR: interquartile range; PCR: polymerase chain reaction; ROC: receiver operating characteristic; RT-PCRL: reverse transcription-polymerase chain reaction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GP performed analyses and drafted the manuscript. CT and BL advised on analysis. JG and MIG led the laboratory work and advised on analysis. JG and DB led the study design. All authors contributed to the drafting and revisions of the manuscript.

Additional material

Additional File 1

Testing Summary. Summary of specimen processing and testing.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2334-9-63-S1.pdf]

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References

1. Gomara MI, Simpson R, Perault AM, Redpath C, Lorgelly P, Joshi D, *et al.*: **Structured surveillance of infantile gastroenteritis in East Anglia, UK: incidence of infection with common viral gastroenteric pathogens.** *Epidemiol Infect* 2008, 136:23-33.
2. Roman E, Wilhelmi I, Colomina J, Villar J, Cilleruelo ML, Nebreda V, *et al.*: **Acute viral gastroenteritis: proportion and clinical relevance of multiple infections in Spanish children.** *J Med Microbiol* 2003, 52:435-440.
3. Houde A, Lebianc D, Pokrus E, Ward P, Brassard J, Simard C, *et al.*: **Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material.** *J Virol Methods* 2006, 135:163-172.
4. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, *et al.*: **Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR.** *J Clin Microbiol* 2003, 41:1548-1557.
5. Medici MC, Martinelli M, Ruggeri FM, Abelli LA, Bosco S, Arcangeletti MC, *et al.*: **Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples.** *J Clin Microbiol* 2005, 43:3772-3778.
6. Jiang X, Wilton N, Zhong WM, Farkas T, Huang PW, Barrett E, *et al.*: **Diagnosis of human caliciviruses by use of enzyme immunoassays.** *J Infect Dis* 2000, 181(Suppl 2):S349-S359.
7. Amar CF, East CL, Gray J, Iturriza-Gomara M, Maclure EA, McLauchlin J: **Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996).** *Eur J Clin Microbiol Infect Dis* 2007, 26:311-323.
8. de Wit MA, Koopmans MP, Kortbeek LM, Wannet WJ, Vinje J, van Leusden F, *et al.*: **Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology.** *Am J Epidemiol* 2001, 154:666-674.
9. Gallimore CI, Cubitt D, du PN, Gray JJ: **Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis.** *J Clin Microbiol* 2004, 42:2271-2274.
10. Carman WF, Niesters HG: **The end of cell culture in diagnostics: is molecular diagnosis the Harry Potter or the Lord Voldemort of clinical virology as a specialty?** *S Afr Med J* 2007, 97:1169-1176.
11. Gunson RN, Miller J, Leonard A, Carman WF: **Importance of PCR in the diagnosis and understanding of rotavirus illness in the community.** *Commun Dis Public Health* 2003, 6:63-65.
12. Atmar RL, Opekun AA, Gilger MA, Estes MK, Crawford SE, Neill FH, *et al.*: **Norwalk virus shedding after experimental human infection.** *Emerg Infect Dis* 2008, 14:1553-1557.
13. Agus SG, Dolin R, Wyatt RG, Tousimis AJ, Northrup RS: **Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man.** *Ann Intern Med* 1973, 79:18-25.
14. Schreiber DS, Blacklow NR, Trier JS: **The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis.** *N Engl J Med* 1973, 288:1318-1323.
15. Troeger H, Loddenkemper C, Schneider T, Schreier E, Epple HJ, Zeitz M, *et al.*: **Structural And Functional Changes Of The Duodenum In Human Norovirus Infection.** *Gut* 2008 in press.
16. Food Standards Agency: **A report of the study of infectious intestinal disease in England.** London, HMSO; 2000.
17. Sethi D, Wheeler JG, Cowden JM, Rodrigues LC, Sockett PN, Roberts JA, *et al.*: **A study of infectious intestinal disease in England: plan and methods of data collection.** *Commun Dis Public Health* 1999, 2:101-107.
18. Tompkins DS, Hudson MJ, Smith HR, Eglin RP, Wheeler JG, Brett MM, *et al.*: **A study of infectious intestinal disease in England: microbiological findings in cases and controls.** *Commun Dis Public Health* 1999, 2:108-113.
19. Amar CF, East CL, Grant KA, Gray J, Iturriza-Gomara M, Maclure EA, *et al.*: **Detection of viral, bacterial, and parasitological RNA or DNA of nine intestinal pathogens in faecal samples archived as part of the english Infectious Intestinal disease study: assessment of the stability of target nucleic acid.** *Diagn Mol Pathol* 2005, 14:90-96.
20. Grove DS: **Quantitative real-time polymerase chain reaction for the core facility using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 sequence detector.** *Journal of Biomolecular Techniques* 2007, 10:11-16.
21. StataCorp: **Stata Statistical Software: Release 10.** College Station, TX: StataCorp LP; 2007.
22. Haruki K, Seto Y, Murakami T, Kimura T: **Pattern of shedding of small, round-structured virus particles in stools of patients of outbreaks of food-poisoning from raw oysters.** *Microbiol Immunol* 1991, 35:83-86.
23. Thornhill TS, Kalica AR, Wyatt RG, Kapikian AZ, Chanock RM: **Pattern of shedding of the Norwalk particle in stools during experimentally induced gastroenteritis in volunteers as determined by immune electron microscopy.** *J Infect Dis* 1975, 132:28-34.
24. Health Protection Agency 2007: **Gastroenteritis: Sporadic cases.** *National Standard Method VSOP 2 Issue 5* .
25. Health Protection Agency 2007: **Investigation of faecal specimens for bacterial pathogens.** *National Standard Methods BSOP 30 Issue 6* .
26. Rubenstein AS, Miller MF: **Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus.** *J Clin Microbiol* 1982, 15:938-944.

27. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, Gray J: **Diagnosing rotavirus A associated IID: Using ELISA to identify a cut-off for real time RT-PCR.** *J Clin Virol* 2009, **44**:242-245.
28. Bewick V, Cheek L, Ball J: **Statistics review 13: receiver operating characteristic curves.** *Crit Care* 2004, **8**:508-512.
29. Fluss R, Faraggi D, Reiser B: **Estimation of the Youden Index and its associated cutoff point.** *Biom J* 2005, **47**:458-472.
30. Youden W: **Index for rating diagnostic tests.** *Cancer* 1950, **3**:32-35.
31. Fryer JF, Baylis SA, Gottlieb AL, Ferguson M, Vincini GA, Bevan VM, *et al.*: **Development of working reference materials for clinical virology.** *J Clin Virol* 2008, **43**:367-371.
32. Leeflang MM, Moons KG, Reitsma JB, Zwinderman AH: **Bias in sensitivity and specificity caused by data-driven selection of optimal cutoff values: mechanisms, magnitude, and solutions.** *Clin Chem* 2008, **54**:729-737.
33. Ewald B: **Post hoc choice of cut points introduced bias to diagnostic research.** *J Clin Epidemiol* 2006, **59**:798-801.
34. Bon F, mbert-Balay K, Giraudon H, Kaplon J, Le GS, Pommepuy M, *et al.*: **Molecular epidemiology of caliciviruses detected in sporadic and outbreak cases of gastroenteritis in France from December 1998 to February 2004.** *J Clin Microbiol* 2005, **43**:4659-4664.
35. Kirkwood CD, Clark R, Bogdanovic-Sakran N, Bishop RF: **A 5-year study of the prevalence and genetic diversity of human caliciviruses associated with sporadic cases of acute gastroenteritis in young children admitted to hospital in Melbourne, Australia (1998–2002).** *J Med Virol* 2005, **77**:96-101.
36. Medici MC, Martinelli M, Abelli LA, Ruggeri FM, Di BI, Arcangeletti MC, *et al.*: **Molecular epidemiology of norovirus infections in sporadic cases of viral gastroenteritis among children in Northern Italy.** *J Med Virol* 2006, **78**:1486-1492.
37. Allen U, Hebert D, Petric M, Tellier R, Tran D, Superina R, *et al.*: **Utility of semiquantitative polymerase chain reaction for Epstein-Barr virus to measure virus load in pediatric organ transplant recipients with and without posttransplant lymphoproliferative disease.** *Clin Infect Dis* 2001, **33**:145-150.
38. Ghisetti V, Barbui A, Franchello A, Varetto S, Pittaluga F, Bobbio M, *et al.*: **Quantitation of cytomegalovirus DNA by the polymerase chain reaction as a predictor of disease in solid organ transplantation.** *J Med Virol* 2004, **73**:223-229.
39. Wittek M, Sturmer M, Doerr HW, Berger A: **Molecular assays for monitoring HIV infection and antiretroviral therapy.** *Expert Rev Mol Diagn* 2007, **7**:237-246.
40. Lee SC, Antony A, Lee N, Leibow J, Yang JQ, Soviero S, *et al.*: **Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics.** *J Clin Microbiol* 2000, **38**:4171-4179.
41. Adaui V, Verdonck K, Best I, Gonzalez E, Tipismana M, Arevalo J, *et al.*: **SYBR Green-based quantitation of human T-lymphotropic virus type I proviral load in Peruvian patients with neurological disease and asymptomatic carriers: influence of clinical status, sex, and familial relatedness.** *J Neurovirol* 2006, **12**:456-465.
42. Gunson RN, Bennett S, Maclean A, Carman WF: **Using multiplex real time PCR in order to streamline a routine diagnostic service.** *J Clin Virol* 2008, **43**:372-375.
43. O'Neill HJ, McCaughey C, Coyle PV, Wyatt DE, Mitchell F: **Clinical utility of nested multiplex RT-PCR for group F adenovirus, rotavirus and norwalk-like viruses in acute viral gastroenteritis in children and adults.** *J Clin Virol* 2002, **25**:335-343.

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Original Contribution

Community Incidence of Norovirus-associated Infectious Intestinal Disease in England: Improved Estimates Using Viral Load for Norovirus Diagnosis

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Existing estimates of the incidence of infectious intestinal disease (IID) caused by norovirus are based on electron microscopy or reverse transcription-polymerase chain reaction (RT-PCR). Neither method accurately represents norovirus disease burden: Electron microscopy has poor diagnostic sensitivity, and RT-PCR has poor diagnostic specificity. In this study, viral load measurements were used to identify cases of norovirus-associated IID and to produce new incidence estimates for England. IID cases were ascertained in the Study of Infectious Intestinal Disease in England (1993–1996), and stool specimens were tested by semiquantitative real-time RT-PCR for norovirus. The age-adjusted community incidence of norovirus-associated IID was 4.5/100 person-years (95% credibility interval: 3.8, 5.2), equating to 2 million episodes/year. Among children aged less than 5 years, the community incidence was 21.4/100 person-years (95% credibility interval: 15.9, 27.7), and the incidence of consultations to general practitioners for norovirus-associated IID was 3.2/100 person-years (95% credibility interval: 2.6, 3.8), with 100,000 children visiting their general practitioner for norovirus-associated IID each year. Norovirus is the most common cause of IID in the community in England and is responsible for a similar number of pediatric primary care consultations as rotavirus.

England; gastroenteritis; incidence; Monte Carlo method; *Norovirus*; primary health care; reverse transcriptase polymerase chain reaction

Abbreviations: IID, infectious intestinal disease; ROC, receiver operating characteristic; RT-PCR, reverse transcription-polymerase chain reaction.

Norovirus is the most common cause of infectious intestinal disease (IID) in the community in high-income countries (1–4), and a substantial prevalence of norovirus infection has been reported among IID cases seeking medical care (5). Existing estimates of norovirus-associated IID incidence in the community and among individuals presenting to their general practitioner in England are based on electron microscopy, which has poor diagnostic sensitivity for identifying norovirus-associated IID (6–8); it is very likely that these estimates underrepresent the burden of norovirus disease.

Reverse transcription-polymerase chain reaction (RT-PCR) is now the preferred diagnostic method for norovirus. However, semiquantitative real-time RT-PCR testing has demonstrated a wide range of viral loads in norovirus-infected IID cases (8); many IID cases shed norovirus at the same concentration as healthy individuals, with no recent history of IID (8, 9). It is therefore unlikely that all IID cases with norovirus infection detected by RT-PCR have disease caused by norovirus; another pathogen is probably causing illness in IID cases shedding norovirus at very low concentrations. Only individuals with IID caused by norovirus should be included in estimates of norovirus disease burden.

We demonstrated in previous work that viral load measurements can be used to identify IID cases with disease caused by norovirus and to exclude IID cases with "asymptomatic" norovirus infection concurrent with disease caused

by another pathogen (8). In this study, we used viral load measurements from IID cases in the Study of Infectious Intestinal Disease in England to improve estimates of the incidence of norovirus-associated IID in the community and leading to general practice consultations. Accurate estimates of norovirus-associated IID incidence at the community level are essential for understanding the introduction of norovirus into health-care settings, where outbreaks cause substantial economic burden and service disruption (10), and for informing potential vaccination programs (11, 12).

MATERIALS AND METHODS

Recruitment and all-cause IID incidence

Data are taken from the Study of Infectious Intestinal Disease in England ("the IID Study"), conducted between 1993 and 1996 (13). The incidence of IID in the community, caused by any pathogen, was estimated in a prospective cohort, which was demographically representative of the population of England. Cohort members were actively followed up, with weekly null reporting, to ensure that all IID episodes were recorded (14).

The incidence of general practitioner consultations for IID, caused by any pathogen, was estimated by recruiting individuals with IID presenting to one of the 70 participating general practices (14). Incidence numerators were adjusted for underascertainment of IID cases, and denominators were adjusted for registered patients no longer using the practices (4, 14).

IID cases were individuals with diarrhea (any loose stools) or significant vomiting (≥2 vomiting episodes/24 hours), lasting less than 2 weeks, without a known noninfectious cause, preceded by a symptom-free period of at least 3 weeks (14). Healthy controls, with no recent history of IID, were recruited concurrently to cases in both study components, from the community cohort or from the general practice patient registration lists (13). Informed consent was obtained from all participants at the time of recruitment.

Specimens and testing

IID cases provided a fecal specimen during acute illness, and controls provided a specimen at recruitment. Norovirus was detected by electron microscopy, and specimens were archived in frozen storage (15). All specimens, including those previously positive by electron microscopy, were later retested for norovirus using a more sensitive RT-PCR assay. All norovirus RT-PCR-positive specimens were retested by using a semiquantitative RT-PCR assay (run for 40 cycles) (16). Recruitment and stool testing in IID cases are summarized in Table 1.

The cycle threshold value from the real-time RT-PCR assay provides a proxy measure of fecal viral load; it is inversely proportional to the amount of virus present in the specimen. The distribution of norovirus cycle threshold values in IID cases and controls used in this study has been described previously (8).

Table 1. Summary of Case Recruitment and Stool Specimen Testing in the Community Cohort and General Practice Component of the Study of Infectious Intestinal Disease, England, 1993–1996

	Community Cohort	General Practice Study
Base population, person-years of follow-up	4,026	409,876[a]
Ascertained cases, no.	781	13,619[b]
Stool specimens, no.	761	2,893[c]
Electron microscopy positive for norovirus, no.	50	169
Stool specimen archived, no.	517	1,905
RT-PCR positive for norovirus, no.[d]	211	623
Cycle threshold value determined with real-time RT-PCR, no.	174	544

Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction.

[a] Adjusted for registered patients no longer actively using participating general practices.

[b] Adjusted for underascertainment.

[c] Stool specimens were collected from patients in only 34 of the 70 general practices recruiting cases.

[d] Includes those previously positive by electron microscopy.

Calculating norovirus incidence

The incidence of norovirus-associated IID (INV) was calculated as follows:

$$\mathrm{INV} = I \times \mathrm{p(NV)} \times A, \qquad (1)$$

where I is the incidence of all-cause IID/100 person-years, p(NV) is the proportion of IID cases positive for norovirus by RT-PCR, and A is a factor used to adjust for those IID cases with norovirus infection at low viral loads who therefore do not have disease caused by norovirus.

In a previous analysis of norovirus cycle threshold values from the IID Study, we used receiver operating characteristic (ROC) analysis to select a cutoff for attributing disease to norovirus in IID cases (8). However, standard ROC analysis does not provide confidence limits around the selected cutoff. In this analysis, the cycle threshold value distributions from the reference groups in the ROC analysis were used to calculate adjustment factor A, incorporating uncertainty in these distributions due to sampling error into the incidence estimate. The reference-positive group included IID cases with norovirus detected by electron microscopy, because they have viral loads representative of where norovirus infection is causing disease (17, 18). The reference-negative group included healthy controls, because they have viral loads representative of where norovirus infection is not causing any illness.

Adjustment factor A was calculated as follows:

$$A = \sum_{i=15}^{i=39} \mathrm{Ct}_i \times \frac{\mathrm{RP}_i}{\mathrm{RP}_i + \mathrm{RN}_i}, \qquad (2)$$

where RP_i is the moving average of the proportion of the

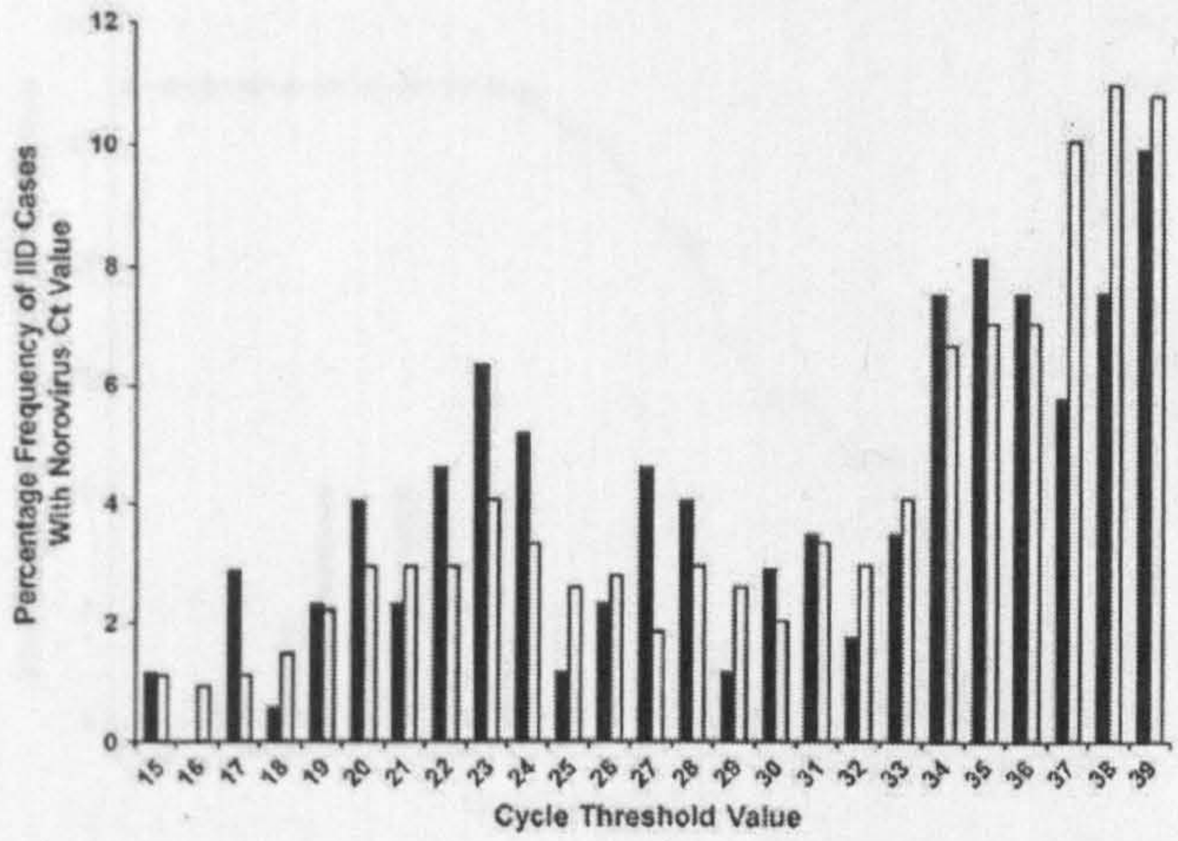

Figure 1. Distribution of norovirus cycle threshold values in IID cases from the Study of Infectious Intestinal Disease, England, 1993–1996. Black bars are IID cases from the community cohort ($n = 174$); white bars are IID cases from the general practitioner study ($n = 544$). Ct, cycle threshold; IID, infectious intestinal disease.

reference-positive group at cycle threshold (Ct) value i (over $i - 2$ to $i + 2$); RN_i is the moving average of the proportion of the reference-negative group at cycle threshold value i (over $i - 2$ to $i + 2$); and Ct_i is the proportion of IID cases positive by real-time RT-PCR with cycle threshold value i. Adjustment factor A varies between 0 and 1. Adjustment factor A is a weighted average of the relative frequency of the reference-positive and reference-negative groups at each cycle threshold value, weighted by the proportion of all norovirus-infected IID cases at each cycle threshold value (Figure 1).

Figure 2 shows the distribution of cycle threshold values in the reference groups and the value of the subcomponent $(RP_i/(RP_i + RN_i))$, which represents the relative frequency of the reference groups. At low-cycle threshold values, where viral loads are high and there are few individuals from the reference-negative group, the subcomponent $(RP_i/(RP_i + RN_i))$ is close to 1, indicating that the majority of IID cases with norovirus infection at these concentrations have disease caused by norovirus. In contrast, at the high-cycle threshold values (low viral loads) found in the majority of the disease-free reference-negative group, the subcomponent $(RP_i/(RP_i + RN_i))$ is close to 0, indicating that very few IID cases with norovirus infection at these concentrations have disease caused by norovirus.

Adjustment factor A was calculated separately for children aged less than 5 years and for older children and adults (aged 5 years or older) in the age-stratified and age-adjusted incidence.

Incidence estimation by Monte Carlo simulation in WinBUGS

The incidence of norovirus-associated IID was calculated by using Monte Carlo simulation in WinBUGS, version 1.4, software (19). Confidence limits for norovirus-associated IID incidence are provided as Bayesian credibility intervals from the posterior sampling distribution. The all-cause IID incidence/100 person-years (I) from the IID Study was modeled by using a log-normal distribution. Proportions were modeled by using binomial distributions with noninformative uniform priors. Multinomial distributions were used to model the cycle threshold value distributions, with noninformative Dirichlet prior distributions. The simulation was run for 300,000 iterations, from 3 different sets of initial values, to check convergence.

Separate simulations were run to estimate the incidence of norovirus in the community and the incidence of general practice consultations and to calculate age- and season-stratified incidence. The numbers of IID cases with norovirus cycle threshold values limited the number of age groups in which the community incidence could be presented. Age-adjusted incidence was calculated as a weighted average of the incidence in children aged less than 5 years and in older children and adults (aged 5 years or older); weights were taken from the mid-1994 population estimate for England, obtained from the Office of National Statistics, United Kingdom. The annual numbers of cases of norovirus-associated IID were calculated from the incidence estimates and the age-stratified mid-1994 population estimate for England.

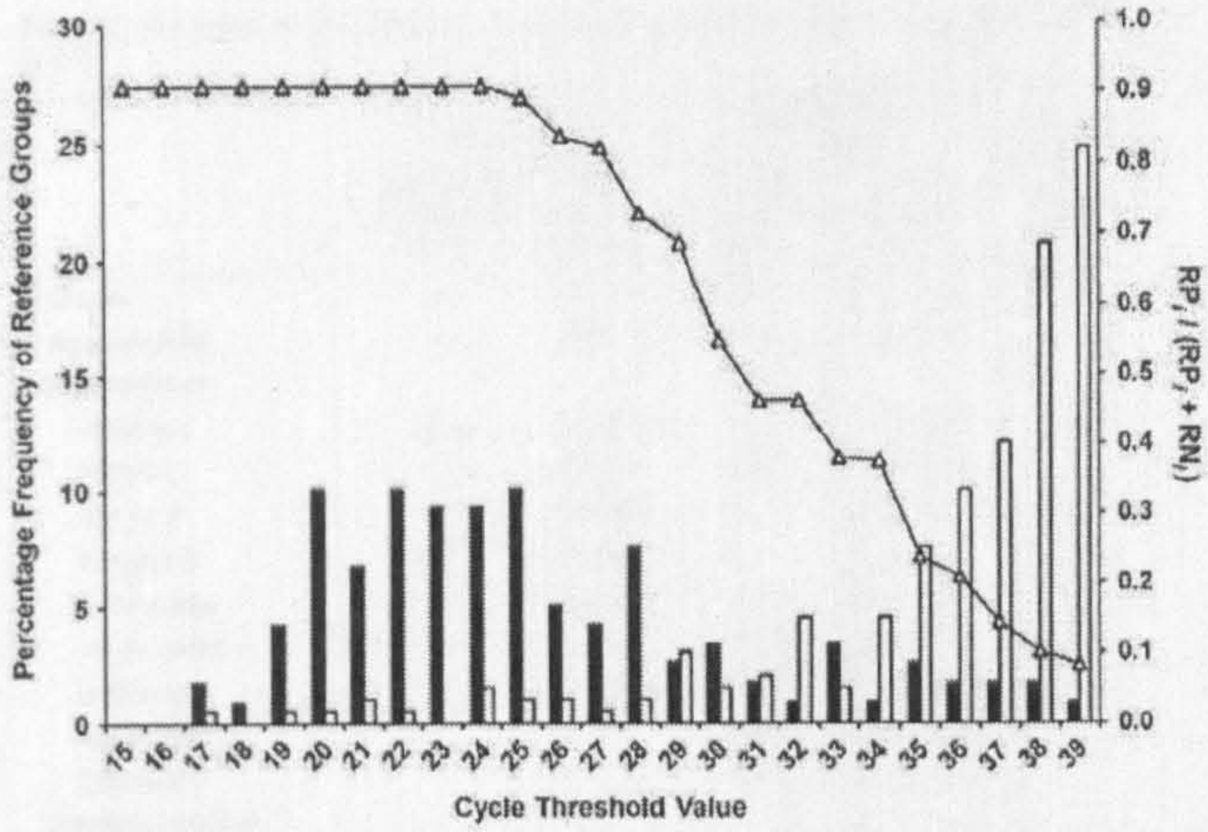

Figure 2. Distribution of norovirus cycle threshold values in reference-positive and reference-negative groups, selected from participants in the Study of Infectious Intestinal Disease, England, 1993–1996, and adjustment factor subcomponent $RP_i/(RP_i + RN_i)$. Black bars are the reference-positive group ($n = 119$); white bars are the reference-negative group ($n = 199$); triangle symbols show the adjustment factor subcomponent $RP_i/(RP_i + RN_i)$. RP_i, moving average of the proportion of the reference-positive group at cycle threshold value i; RN_i, moving average of the proportion of the reference-negative group at cycle threshold value i.

Alternative methods for estimating the proportion of IID cases with disease attributable to norovirus

We used 3 further methods to estimate the proportion of IID cases with disease attributable to norovirus, which either do not require a control group or have been used in previous studies.

Alternative method 1. In previous studies using only RT-PCR, not semiquantitative real-time RT-PCR, the proportion of norovirus-infected IID cases with disease attributable to norovirus has been estimated as the difference in norovirus prevalence between the control group and IID cases (5). We calculated norovirus-associated IID incidence as follows:

$$\mathrm{INV} = I \times \left(\mathrm{p(NV)}_{\mathrm{case}} - \mathrm{p(NV)}_{\mathrm{control}}\right), \qquad (3)$$

where $\mathrm{p(NV)}_{\mathrm{case}}$ represents the norovirus prevalence among IID cases, and $\mathrm{p(NV)}_{\mathrm{control}}$ represents the norovirus prevalence among controls.

Alternative method 2. We have previously defined a cutoff in norovirus genogroup II cycle threshold values for attributing disease to norovirus (8). We applied this cutoff (at cycle threshold value 30 for children aged <5 years and at cycle threshold value 33 for older children and adults) to IID cases with a cycle threshold value for either norovirus genogroup I or genogroup II. The proportion of IID cases with a norovirus cycle threshold value at or below the cycle threshold value cutoff was substituted for adjustment factor A in equation 1. To explore the effect of late specimen collection on norovirus incidence, we defined probable cases of norovirus-associated IID as those IID cases with a cycle threshold value above the cutoff, a specimen collected 5 or more days after symptom onset, and no other pathogen detected. These probable cases were added to the IID cases with a norovirus cycle threshold value at or below the cutoff.

Alternative method 3. We used mixture modeling to estimate the proportion of IID cases with a norovirus cycle threshold value that have disease attributable to norovirus, using only data from IID cases. This proportion was substituted for adjustment factor A in equation 1 and uncertainty represented by using a beta distribution, based on the confidence interval provided from the mixture model. Details of the mixture model are provided in the Web Appendix (http://aje.oxfordjournals.org/).

We also estimated the incidence of norovirus-associated IID based on electron microscopy testing using equation 3:

$$\mathrm{INV} = I \times P, \qquad (4)$$

where P is the proportion of cases positive by electron microscopy. The incidence of norovirus-associated IID based on classifying any norovirus RT-PCR-positive IID case as a case of norovirus-associated IID and the incidence of rotavirus-associated IID based on enzyme-linked immunosorbent assay diagnosis (20) (in children aged <5 years only) were calculated in the same way.

RESULTS

The crude community incidence of norovirus-associated IID was 4.1/100 person-years (Table 2); after age

Table 2. Incidence of Norovirus-associated Infectious Intestinal Disease in England, 1993–1996[a]

	Community		General Practice Consultation		Ratio of Community to General Practice Cases
	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	
Crude	4.1	3.4, 4.8	0.49	0.43, 0.55	8.4
Age adjusted	4.5	3.8, 5.2	0.54	0.48, 0.60	8.3
Age stratified					
<5 years	21.4	15.9, 27.7	3.2	2.6, 3.8	6.7
≥5 years	3.3	2 6, 3.9	0.35	0.30, 0.39	9.7
0–1 year	27.2	17.9, 36.6	6 4	5.2, 7.7	4.3
2–4 years	16.7	11.4, 23.3	1.5	1.2, 2.0	11.1
5–14 years	6.5	4.5, 8.9	0.44	0.31, 0.59	14.8
15–44 years	4.1	3.1, 5.3	0.38	0.32, 0.45	10.8
≥45 years	1.7	1.1, 2.3	0.29	0.24, 0.35	5.9
45–64 years			0.26	0.20, 0.32	
≥65 years			0.37	0.27, 0.47	
Season stratified					
January–March	4.7	3.4, 6.3	0 48	0.37, 0.57	
April–June	3.8	2.7, 5.1	0.52	0.43, 0.62	
July–September	3.3	2.4, 4.5	0.43	0.35, 0.51	
October–December	4.8	3.6, 6.3	0.56	0.46, 0.66	
Rotavirus-associated IID					
0–1 year	13.7	5.6, 25.1	6.4	5.2, 7.7	2.1
2–4 years	6.2	2.5, 11.5	1.5	1.2, 2.0	4.1
>5 years	8.5	4.6, 13.6	3.2	2.6, 3.8	2.7

Abbreviation: IID, infectious intestinal disease.

[a] The incidence of rotavirus-associated infectious intestinal disease is shown also for children aged less than 5 years.

adjustment, the community incidence was 4.5 episodes/100 person-years (Table 2). Incidence was highest in children aged less than 5 years, with 20% experiencing norovirus-associated IID every year. Community norovirus–associated IID incidence peaked between October and March (Table 2).

There were 0.5 general practice consultations for norovirus-associated IID/100 person-years (Table 2). The incidence of general practice consultations was highest among children aged less than 2 years, at 6.4/100 person-years. Approximately 1 of 7 children aged less than 5 years with norovirus-associated IID consulted a general practitioner, compared with 1 of 3 of those with rotavirus-associated IID in this study population (Table 2). The seasonality of general practice consultations for norovirus-associated IID was less pronounced than in the community (Table 2).

Incidence based on the cycle threshold value cutoff was slightly lower than using adjustment factor *A*, and the credibility intervals were narrower, as shown in Table 3 and the Web table (http://aje.oxfordjournals.org/). Subtracting the control norovirus prevalence from that in IID cases produced higher incidence estimates in young children, but lower estimates in older children and adults. Mixture modeling produced the lowest estimates.

DISCUSSION

This is the first study to use viral load measurements to estimate the incidence of norovirus-associated IID. A recent volunteer study showed that low norovirus viral loads, detectable by RT-PCR, are associated with asymptomatic infection (9). Consideration of viral load therefore provides the greatest diagnostic accuracy for identifying cases of norovirus-associated IID. Using such an approach, we have demonstrated that norovirus is the most common cause of IID, across all age groups, in the community in England (4), and that there is a substantial incidence of general practice consultations for norovirus-associated IID among young children (Table 4), similar to that caused by rotavirus.

Estimates of norovirus disease burden based on viral load are very likely to be more accurate than those based on electron microscopy, because electron microscopy has poor diagnostic sensitivity, or those based on RT-PCR, because it is possible to exclude IID cases who are RT-PCR positive

Table 3. Sensitivity of Estimates of Community Norovirus-associated IID Incidence in England, 1993–1996, to the Method of Calculating the Proportion of IID Cases Attributable to Norovirus[a]

Community	Alternative Methods													
	Adjustment Factor A		Cycle Threshold Value Cutoff		Cycle Threshold Value Cutoff Plus Probable Cases		Subtract Control Prevalence		Mixture Modeling		Electron Microscopy		All RT-PCR Positive	
	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval	Incidence/ 100 Person-Years	95% Credibility Interval
Crude	4.1	3.4, 4.8	3.9	3.2, 4.7	4.4	3.8, 5.0	4.8	3.9, 5.8	3.1	2.3, 3.9	1.4	1.0, 1.9	8.0	7.1, 9.1
Age adjusted	4.5	3.8, 5.2	4.0	3.3, 4.8	4.6	4.0, 5.3	4.3	3.3, 5.4			1.6	1.1, 2.1	8.5	7.4, 9.6
<5 years	21.4	15.9, 27.7	20.3	13.8, 28.0	21.1	16.7, 26.3	33.4	25.2, 42.5			9.1	5.1, 14.4	44.3	35.2, 54.4
≥5 years	3.3	2.6, 3.9	3.1	2.4, 3.9	3.4	2.9, 4.1	2.3	1.4, 3.2			1.0	0.7, 1.5	5.9	5.1, 6.9

Abbreviations: IID, infectious intestinal disease; RT-PCR, reverse transcription-polymerase chain reaction.

[a] Results are from alternative methods for estimating the proportion of IID cases with norovirus infection and disease attributable to norovirus and incidence based on electron microscopy and all RT-PCR-positive IID cases.

but have low viral loads and are therefore unlikely to have disease caused by norovirus. We developed a method for calculating norovirus-associated IID incidence that allowed statistical uncertainty in the viral load measurements to be incorporated into the confidence limits. This was only possible with the use of Monte Carlo simulation methods to combine the multiple components of the calculation and their associated statistical uncertainty; this would have been extremely difficult using standard frequentist approaches, such as the Delta Method, because of the large number of variables in the calculation. Although the estimates presented here are based on data collected between 1993 and 1996, they provide the best available information on the burden of norovirus disease in England. Furthermore, these results are based on current diagnostic methods; as new studies are carried out, they will provide a baseline from which to assess changes in norovirus incidence over time that are not confounded by concurrent changes in the sensitivity of diagnostic methods.

There was limited resolution for estimating age-stratified incidence in the community because of the small sample size. We combined genogroup I and genogroup II norovirus infections in this analysis, rather than estimating adjustment factor *A* separately for each genogroup, also because of limited sample size. Similarly, in alternative method 2, we used a cycle threshold value cutoff developed for genogroup II specimens only, because no published cutoff exists for genogroup I. There is evidence that the real-time RT-PCR assay has lower efficiency for genogroup I norovirus strains (Jim Gray, Health Protection Agency Centre for Infections, personal communication, 2009), so that a given cycle threshold value may represent a higher viral load in the original stool specimen for some genogroup I strains, compared with genogroup II strains. Genogroup I noroviruses constituted less than 10% of the norovirus isolates in the study, so we believe that grouping the genogroups would result in conservative incidence estimates, rather than overestimation.

The concentration of norovirus excretion decreases substantially after symptom resolution (9). Although we made no direct adjustment for the possibility that some IID cases with high cycle threshold values may have had disease caused by norovirus, but had low viral loads at the time of specimen collection because their symptoms had already resolved, the method used to calculate adjustment factor *A* does allow some IID cases with high norovirus cycle threshold values to be incorporated into the incidence estimate (Figure 2). It therefore indirectly allows for the possibility that some IID cases who truly had norovirus-associated IID had low viral loads at the time of testing. It is not possible to directly allow for late specimen collection using adjustment factor *A*, because it is calculated at the population level. Direct consideration of delay in specimen collection requires classification of norovirus disease status at the individual level, as was done when applying the cycle threshold value cutoff (alternative method 2). We recalculated the cutoff-based incidence of norovirus-associated IID, including probable cases (defined as having a high cycle threshold value, a late specimen, and no other detected pathogens) and found that the incidence was

Table 4. Estimated Annual Numbers of Norovirus-associated Infectious Intestinal Disease Cases in the Community and Consulting a General Practitioner in England, 1993–1996

Age Group	Community		General Practice Consultation	
	Thousands of Cases	95% Credibility Interval	Thousands of Cases	95% Credibility Interval
Age adjusted	2,175.8	1,836.8, 2,543.0	261.5	233.4, 290.6
0–1 year			81.0	65.4, 97.8
2–4 years			30.4	22.7, 38.8
<5 years	691.4	513.4, 897.1	103.7	85.2, 123.2
5–14 years	403.1	279.0, 550.3	27.1	18.9, 36.6
15–44 years	854.9	635.6, 1,104.9	78.6	65.1, 93.0
≥45 years	306.4	211.4, 426.8	54.9	45.0, 65.8
45–64 years			26.2	21.9, 35.4
≥65 years			28.1	21.0, 36.2

slightly higher than using only the cases below the cycle threshold value cutoff, but still very similar to those obtained using adjustment factor *A*. However, we would urge caution in using such an approach, because the number of probable cases will be highly dependent on assay sensitivity and on the number of cycles for which the real-time RT-PCR assay is run; not all IID cases with norovirus detected and a late specimen may have actually had disease caused by norovirus.

The method we used is dependent on the recruitment and testing of a large control group, which are not always possible. We used a number of alternative methods to adjust the prevalence of norovirus in IID cases, to explore whether these produce suitably similar results to our method, which we believe to be most robust. As expected, using the cycle threshold value cutoff produced slightly lower incidence estimates with narrower credibility intervals, because the uncertainty in the cutoff was not represented in the calculations. We found that mixture modeling gave similar results to the cycle threshold value cutoff, although there was a tendency toward underestimation; mixture modeling also requires larger sample sizes than the other methods, preventing estimation of detailed age-stratified incidence estimates. Estimates produced by subtracting the prevalence of norovirus in controls from that in IID cases were very different from those produced using the other methods; incidence in young children was substantially overestimated, and incidence in older children and adults was underestimated. Furthermore, estimates produced with this method will be highly dependent on the case definition used, the source of the controls, and the study setting.

The new estimates of norovirus-associated IID incidence presented here are approximately 3 times higher in the community and 2.5 times higher at the general practitioner level than previous estimates for England based on electron microscopy (4). Accordingly, the ratio of community cases to cases presenting to general practitioners increased from 6 to 1, using electron microscopy diagnosis, to 8 to 1, using viral load measurements (4). The incidence estimates are approximately half those obtained by assuming that any IID cases with a positive RT-PCR result for norovirus has disease caused by norovirus, indicating that without consideration of viral load there is the potential for substantial overestimation of the burden of norovirus disease.

The community incidence estimates are comparable to those from a study in the Netherlands, which used RT-PCR testing to identify cases of norovirus-associated IID but had a narrower case definition for IID (3 or more loose stools, or 2 or more episodes of vomiting in 24 hours), which may not have been sensitive enough to ascertain all episodes of norovirus-associated IID at the community level (1). Similarly, the incidence of general practitioner consultations for norovirus was only slightly lower than that from a recent study in Germany, which used RT-PCR diagnosis for norovirus, but again this study had a narrower case definition for IID (2 or more loose stools, or 2 or more vomiting episodes in 24 hours) (21). The incidence of norovirus-associated IID may also have been higher than normal during our study because a new variant of norovirus emerged during 1995 and 1996 (22–24); emergence of norovirus variants has been associated with increased disease incidence (25–28).

The incidence of norovirus-associated IID in the community showed a slight peak in the winter and autumn months, while general practice consultations were reasonably constant throughout the year. Outbreaks of norovirus-associated IID in community settings in the United Kingdom show very little seasonality, in strong contrast to outbreaks in health-care settings, which show marked winter-time seasonality (29). A number of factors may contribute to these differing patterns of seasonality between community disease and outbreaks in different settings. First, community norovirus outbreaks are more commonly reported from catering settings, with transmission occurring through food contamination; while the prevalence of norovirus infection among food handlers is likely determined by the incidence of community disease, the driving factor in these outbreaks is breakdown in food hygiene practices, which is not a seasonal phenomenon. Second, it has been suggested that the

marked winter-time increase in hospital admissions for respiratory infections may drive the strong seasonality of norovirus outbreaks in this setting, and that there are distinct norovirus strains circulating in hospital populations and in the community that may have different transmission characteristics (29); therefore, the incidence of community disease or general practitioner consultations would not necessarily show the marked seasonality seen in health care-associated outbreaks. However, detailed characterization of the molecular epidemiology of norovirus infections in the community is needed, for comparison with the extensive data that already exist for hospital-acquired infections (30, 31), to understand better the factors driving the different seasonality of health-care outbreaks and community disease. Finally, it is also possible that there was more out-of-season norovirus transmission during this study because of the emergence of a new norovirus variant, as described above (32).

We have demonstrated, for the first time, how viral load measurements can be used to make improved estimates of norovirus disease burden. This approach is preferable to including all IID cases who are RT-PCR positive, regardless of their viral load, because many may be shedding norovirus at low concentrations, with disease caused by another pathogen. With the widespread use of RT-PCR for norovirus diagnosis in community-based studies, we recommend using a real-time platform to allow consideration of viral load when calculating norovirus incidence; we have shown that additional real-time testing in a subset of norovirus-infected IID cases would be sufficient to use this approach, providing the subset is of a reasonable size and is representative. Further work is needed to validate the use of a cycle threshold value cutoff for use in studies without a control group. Asymptomatic norovirus infection is very common (1, 16, 21, 33–35). Therefore, this quantitative approach provides the most rigorous estimate of norovirus disease burden.

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REFERENCES

1. de Wit MA, Koopmans MP, Kortbeek LM, et al. Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *Am J Epidemiol.* 2001; 154(7).666–674.
2. Hellard ME, Sinclair MI, Forbes AB, et al. A randomized, blinded, controlled trial investigating the gastrointestinal health effects of drinking water quality. *Environ Health Perspect.* 2001;109(8):773–778.
3. Marshall JA, Hellard ME, Sinclair MI, et al. Incidence and characteristics of endemic Norwalk-like virus-associated gastroenteritis. *J Med Virol.* 2003;69(4):568–578.
4. Wheeler JG, Sethi D, Cowden JM, et al. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The Infectious Intestinal Disease Study Executive. *BMJ.* 1999;318(7190):1046–1050.
5. Patel MM, Widdowson MA, Glass RI, et al. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis.* 2008;14(8):1224–1231.
6. Froggatt PC, Vipond IB, Ashley CR, et al. Surveillance of norovirus infection in a study of sporadic childhood gastroenteritis in South West England and South Wales, during one winter season (1999–2000). *J Med Virol.* 2004;72(2):307–311.
7. Lau CS, Wong DA, Tong LK, et al. High rate and changing molecular epidemiology pattern of norovirus infections in sporadic cases and outbreaks of gastroenteritis in Hong Kong. *J Med Virol.* 2004;73(1):113–117.
8. Phillips G, Lopman B, Tam CC, et al. Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect Dis* [serial online]. 9. , 2009. 163Available at: http://www.biomedcentral.com/1471-2334/9/63.
9. Atmar RL, Opekun AR, Gilger MA, et al. Norwalk virus shedding after experimental human infection. *Emerg Infect Dis.* 2008;14(10):1553–1557.
10. Lopman BA, Reacher MH, Vipond IB, et al. Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002–2003. *Emerg Infect Dis.* 2004;10(10):1827–1834.
11. Ball JM, Graham DY, Opekun AR, et al. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. *Gastroenterology.* 1999;117(1):40–48.
12. Tacket CO, Sztein MB, Losonsky GA, et al. Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. *Clin Immunol.* 2003;108(3):241–247.
13. Food Standards Agency. *A Report of the Study of Infectious Intestinal Disease in England.* London, United Kingdom: HMSO; 2000.
14. Sethi D, Wheeler JG, Cowden JM, et al. A study of infectious intestinal disease in England: plan and methods of data collection. *Commun Dis Public Health.* 1999;2(2):101–107.
15. Tompkins DS, Hudson MJ, Smith HR, et al. A study of infectious intestinal disease in England: microbiological findings in cases and controls. *Commun Dis Public Health.* 1999; 2(2):108–113.
16. Amar CF, East CL, Gray J, et al. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal

Disease Study (1993–1996). *Eur J Clin Microbiol Infect Dis.* 2007:26(5):311–323.
17. Haruki K, Seto Y, Murakami T, et al. Pattern of shedding of small, round-structured virus particles in stools of patients of outbreaks of food-poisoning from raw oysters. *Microbiol Immunol.* 1991;35(1):83–86.
18. Thornhill TS, Kalica AR, Wyatt RG, et al. Pattern of shedding of the Norwalk particle in stools during experimentally induced gastroenteritis in volunteers as determined by immune electron microscopy. *J Infect Dis.* 1975; 132(1):28–34.
19. Lunn DJ, Thomas A, Best N, et al. WinBUGS—a Bayesian modelling framework: concepts, structure and extensibility. *Stat Comput.* 2000;10:325–337.
20. Phillips G, Lopman B, Tam CC, et al. Diagnosing rotavirus A associated IID: using ELISA to identify a cut-off for real time RT-PCR. *J Clin Virol.* 2009;44(3):242–245.
21. Karsten C, Baumgarte S, Friedrich AW, et al. Incidence and risk factors for community-acquired acute gastroenteritis in north-west Germany in 2004. *Eur J Clin Microbiol Infect Dis.* 2009;28(8):935–943.
22. Bull RA, Tu ET, McIver CJ, et al. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. *J Clin Microbiol.* 2006;44(2): 327–333.
23. Koopmans M, Vinjé J, de Wit M, et al. Molecular epidemiology of human enteric caliciviruses in the Netherlands. *J Infect Dis.* 2000;181(suppl 2):S262–S269.
24. Noel JS, Fankhauser RL, Ando T, et al. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. *J Infect Dis.* 1999;179(6):1334–1344.
25. Adamson WE, Gunson RN, Maclean A, et al. Emergence of a new norovirus variant in Scotland in 2006. *J Clin Microbiol.* 2007;45(12):4058–4060.
26. Lopman B, Vennema H, Kohli E, et al. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet.* 2004;363(9410):682–688.
27. Lyon MJ, Wei G, Smith GA. Epidemic viral gastroenteritis in Queensland coincides with the emergence of a new norovirus variant. *Commun Dis Intell.* 2005;29(4):370–373.
28. Tu ET, Bull RA, Greening GE, et al. Epidemics of gastroenteritis during 2006 were associated with the spread of norovirus GII.4 variants 2006a and 2006b. *Clin Infect Dis.* 2008; 46(3):413–420.
29. Lopman BA, Adak GK, Reacher MH, et al. Two epidemiologic patterns of norovirus outbreaks: surveillance in England and Wales, 1992–2000. *Emerg Infect Dis.* 2003;9(1):71–77.
30. Gallimore CI, Green J, Lewis D, et al. Diversity of noroviruses cocirculating in the north of England from 1998 to 2001. *J Clin Microbiol.* 2004;42(4):1396–1401.
31. Gallimore CI, Iturriza-Gomara M, Xerry J, et al. Inter-seasonal diversity of norovirus genotypes: emergence and selection of virus variants. *Arch Virol.* 2007;152(7):1295–1303.
32. Lopman BA, Reacher M, Gallimore C, et al. A summertime peak of "winter vomiting disease": surveillance of noroviruses in England and Wales, 1995 to 2002. *BMC Public Health* [serial online]. 2003;3:13. Available at: http://www.biomedcentral.com/1471-2458/3/13.
33. García C, DuPont HL, Long KZ, et al. Asymptomatic norovirus infection in Mexican children. *J Clin Microbiol.* 2006; 44(8):2997–3000.
34. Oh DY, Gaedicke G, Schreier E. Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity. *J Med Virol.* 2003;71(1):82–93.
35. O'Ryan ML, Lucero Y, Prado V, et al. Symptomatic and asymptomatic rotavirus and norovirus infections during infancy in a Chilean birth cohort. *Pediatr Infect Dis J.* 2009; 28(10):879–884.

Appendix 7.4. Phillips G, Tam CC, Rodrigues LC, Lopman B. Prevalence and characteristics of asymptomatic norovirus infections in the community in England. *Epidemiol. Infect.* 2010 Mar 3:1-5. [Epub ahead of print].

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Prevalence and characteristics of asymptomatic norovirus infection in the community in England

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SUMMARY

Norovirus is a major cause of infectious intestinal disease, and a substantial prevalence of asymptomatic infection has been reported. We describe the prevalence, seasonality and characteristics of asymptomatic norovirus infection in England. Healthy individuals were recruited at random from the general population during the Study of Infectious Intestinal Disease (1993–1996). Norovirus was identified using real-time RT-PCR. The age-adjusted prevalence of asymptomatic norovirus infection was 12%; prevalence was highest in children aged <5 years and showed wintertime seasonality. More work is needed to understand whether asymptomatic infections are important for norovirus transmission leading to sporadic illness and outbreaks.

Key words: Asymptomatic viral infections, England, infectious disease epidemiology, norovirus, prevalence.

Norovirus is the most common cause of infectious intestinal disease (IID) in the community in high-income countries [1]. Norovirus infection has also been identified in a substantial proportion of individuals with no IID symptoms in several community-based studies, with crude prevalences of up to 16% reported in high-income countries [1–3]. Volunteer studies have demonstrated the occurrence of norovirus infection with no concurrent IID after experimental inoculation [4]. While these volunteer individuals experienced no IID symptoms, some reported other non-specific symptoms such as headache, fever, muscle ache, abdominal pain and nausea.

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The objectives of this study was to describe the age and seasonal distribution of norovirus infection without IID (hereafter referred to as 'asymptomatic norovirus infection') in the community in England and to describe the characteristics of these infections.

We used data from participants in the Study of Infectious Intestinal Disease in England, conducted between 1993 and 1996 [5]. These individuals were recruited as controls for a case-control study, either from a prospectively followed community cohort, or from the registration lists of general practitioners participating in the study [5]. Informed consent was obtained at the time of recruitment [5]. The inclusion criteria specified that participants should have no recent history of diarrhoea (any loose stools) or significant vomiting (⩾2 vomiting episodes per 24 h) prior to recruitment [5].

At recruitment, participants submitted stool specimens for microbiological testing, in order to detect

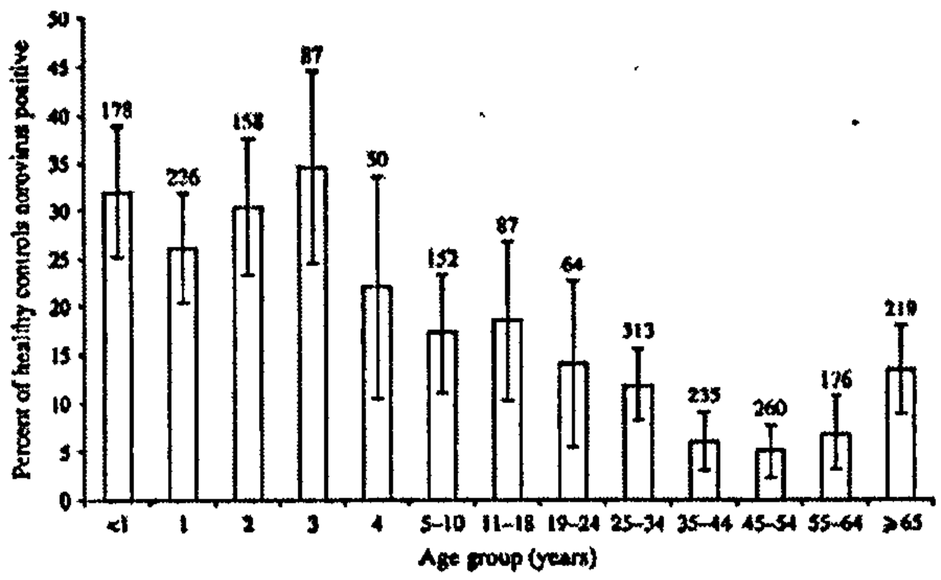

Fig. 1. Age-specific prevalence of asymptomatic norovirus infection in the Study of Infectious Intestinal Disease, England (1993–1996). Numbers above the histograms show the number of participants tested in each age group. Black bars (I) show the 95% confidence intervals.

a range of 18 bacterial, viral and protozoal gastrointestinal pathogens. Norovirus was detected by electron microscopy in the original study [6]. Stool specimens were archived and subsequently retested, using real-time reverse transcription–polymerase chain reaction (RT-PCR) to detect norovirus [1, 6]. In the current study, participants were classified as having norovirus infection if they tested positive either by electron microscopy or real-time RT-PCR, or both. The real-time RT-PCR assay has separate sets of primers and probes for genogroup I and genogroup II noroviruses, making it possible to distinguish the genogroup of norovirus present in the positive specimens. No further genotyping was performed.

Participants provided details of gastrointestinal and non-specific symptoms in the previous 3 weeks in an epidemiological questionnaire (although details of fever and nausea were not collected). Adults completed the questionnaire themselves; a parent or guardian completed the questionnaire on behalf of children aged <16 years [5]. For this analysis, participants who had been free of diarrhoea and vomiting for at least 10 days prior to recruitment were considered asymptomatic with respect to IID, although they may have experienced other symptoms during that period.

Stool specimens were received from 2205 asymptomatic participants and 2065 returned the questionnaire providing information on recent symptoms. Of the 2205 asymptomatic participants, 361 had an asymptomatic norovirus infection and 1844 tested negative for norovirus; the age- and season-specific prevalence of asymptomatic norovirus infection was based on these 2205 participants. Of the 2065 asymptomatic participants who returned questionnaires, 344 had an asymptomatic norovirus infection and 1721 were norovirus negative; these 2065 participants were used for the analysis of recent symptoms.

The age-adjusted prevalence of asymptomatic norovirus infection in the community in England was calculated by standardizing against the age-stratified mid-1994 population estimate for England, obtained from the Office for National Statistics, UK. Symptoms that were in excess in asymptomatic norovirus infections compared to norovirus-negative participants are presented. The analysis of symptoms is intended to be exploratory, to generate hypotheses for future work; the original study was not designed or powered to examine differences in symptom profiles between asymptomatic norovirus infections and norovirus-negative participants. Accordingly, confidence intervals are provided for symptom prevalences, prevalence differences and prevalence ratios, but no hypothesis tests (or *P* values) are presented.

The age-adjusted, community prevalence of asymptomatic norovirus infection was 12% [95% confidence interval (CI) 11–14], with the highest prevalence in children aged <5 years, although more than 5% of individuals in older age groups were infected (Fig. 1). The prevalence of asymptomatic infection showed a wintertime peak of 20% during November, December and January (Fig. 2); the seasonal pattern was less distinct for children aged <5 years compared to older children and adults (data not shown).

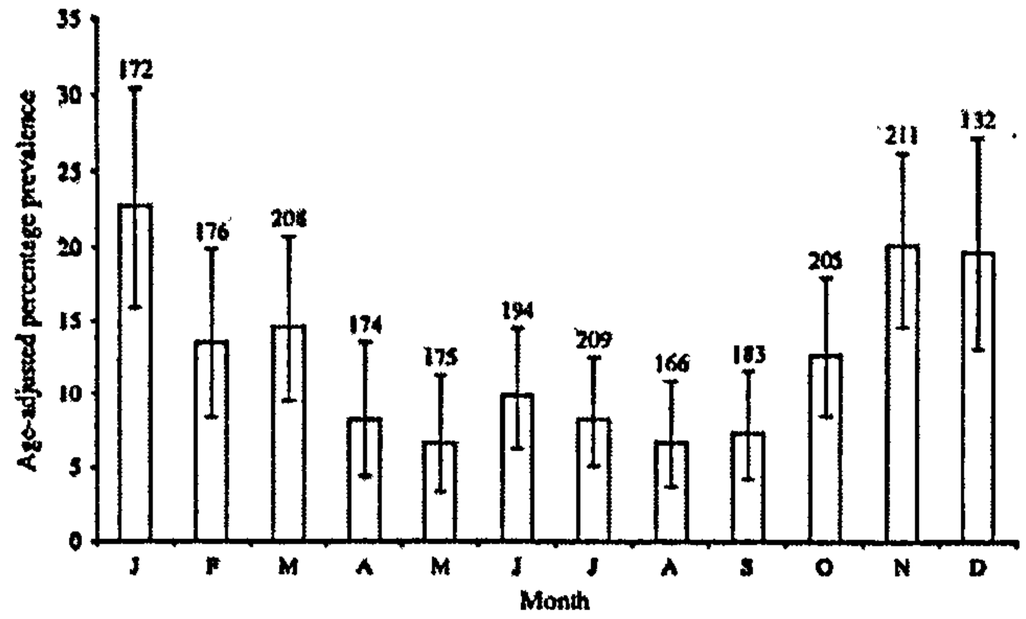

Fig. 2. Age-adjusted monthly prevalence of asymptomatic norovirus infection in the Study of Infectious Intestinal Disease, England (1993–1996). Numbers above the histograms show the number of participants tested in each month. Black bars (I) show the 95% confidence intervals.

Genogroup II noroviruses were most common, representing 78% of the 361 asymptomatic norovirus infections, with 13% of specimens positive for genogroup I and 9% positive for both genogroups. The prevalence of genogroup II, compared to genogroup I and mixed genogroup infections, varied between 63% and 86% over the year, with the highest prevalence during October–December, and in April and May. However, the number of asymptomatic infections occurring per month was <40 throughout most of the year, so some of this variation could be due to sampling error.

During the 3 weeks preceding questionnaire completion, a cough, sore throat and other cold-like symptoms were reported by 61% of participants aged <5 years with asymptomatic norovirus infection (95% CI 54–68), compared to 52% (95% CI 47–56) of norovirus-negative participants in this age group [prevalence difference 9% (95% CI 0·7–17); prevalence ratio adjusted for month of the year 1·2 (95% CI 1·0–1·4)]. There was a smaller excess of cold-like symptoms in older children and adults with asymptomatic norovirus infection; the prevalence in individuals with asymptomatic norovirus infection was 12% (95% CI 7–17) and 9% in norovirus-negative participants (95% CI 7–10) [prevalence difference 3% (95% CI −2 to 8); prevalence ratio adjusted for month of the year 1·3 (95% CI 0·8–2·0)]. No other non-gastrointestinal symptoms were found to be in excess in participants with asymptomatic norovirus infection.

Nine percent of participants with asymptomatic norovirus infection experienced diarrhoea and/or vomiting prior to the 10-day exclusion period, but within 3 weeks of questionnaire completion (95% CI 6–12). The prevalence was higher in participants with asymptomatic norovirus infection compared to norovirus-negative participants, for both children aged <5 years [asymptomatic norovirus infection 10% (95% CI 6–15); norovirus negative 7% (95% CI 5–10); prevalence difference 3% (95% CI −2 to 8)], and older children and adults [asymptomatic norovirus infection 8% (95% CI 4–12); norovirus negative 4% (95% CI 3–5); prevalence difference 4% (95% CI −0·5 to 8)]. Older children and adults with asymptomatic norovirus infection also reported loss of appetite more often than norovirus-negative participants in this age group [asymptomatic norovirus infection 9% (95% CI 4–13); norovirus negative 3% (95% CI 2–4); prevalence difference 6% (95% CI 1–11)].

The prevalence of asymptomatic norovirus infection in our study is higher than that reported in previous studies conducted in other high-income countries, which had comparable samples of asymptomatic individuals [2, 3]. Real-time RT–PCR is known to have slightly higher sensitivity than gel-based RT–PCR [7]. However, this is unlikely to account for the difference of 7% between the prevalence of asymptomatic norovirus infection in the current study and the prevalence in a previous study in The Netherlands [2], which used gel-based RT–PCR.

A previous study conducted in Germany used nested gel-based RT-PCR [3]; the use of nested PCR primers increases the sensitivity of the gel-based assay [8], meaning that the assay used in the study in Germany is likely to have comparable sensitivity to the real-time RT-PCR used in the current study. It is possible that the differences in asymptomatic norovirus prevalence between the studies are due to differences in the genetic strains of norovirus circulating at the time that the studies were performed. Periodic emergence of new norovirus strains has been associated with increases in the incidence of infection and a new strain emerged in 1995–1996, during recruitment of participants into the Study of Infectious Intestinal Disease [9, 10].

Diagnostic evaluation studies using panels of stool specimens containing other enteric viruses have demonstrated that current norovirus RT-PCR assays have 100% analytical specificity, including the assay used in the current study [11–13]. Therefore, very few, if any, of the asymptomatic norovirus infections reported here are likely to be false positives. Some asymptomatic participants in this study may have been shedding norovirus at levels not detectable by the RT-PCR assay used, which has a detection limit of $\sim 10^6$ norovirus particles/g stool [8, 13]; it is therefore possible that the true prevalence of asymptomatic norovirus infection is higher than reported.

Asymptomatic norovirus infection showed wintertime seasonality. Outbreaks of norovirus-associated IID in healthcare settings in England and Wales show strong wintertime seasonality, but, in contrast, there is little seasonality in norovirus outbreaks reported from community settings [14]. The seasonality of norovirus-associated IID incidence at the community level in England has not been described.

Gastrointestinal and cold-like symptoms were more common in asymptomatic norovirus infections than norovirus-negative participants. The original study was not designed or powered to examine differences in symptom prevalence between these groups, and we had no *a priori* hypotheses about the relative frequency of symptoms. Therefore, while the 95% confidence intervals for the majority of symptom-prevalence differences did include zero, potential reasons for the observed excess prevalence in asymptomatic norovirus infections are discussed below.

Even after adjustment for season, cold-like symptoms were at higher prevalence in participants with asymptomatic norovirus infection; this may be due to a co-infection with a respiratory virus, because viruses causing the common cold and influenza are transmitted via similar routes to norovirus, e.g. through direct person-to-person contact or from contaminated environmental surfaces [15, 16]. In previous studies, experimentally inoculated volunteers have reported non-specific symptoms such as headache, fever and muscle ache during norovirus infection [4]; details of fever were not collected from asymptomatic participants in the Study of Infectious Intestinal Disease, so it is also possible that the excess of cold-like symptoms may represent non-specific symptoms associated with norovirus infection. The prevalence of headache and muscle ache in individuals with asymptomatic norovirus infections was slightly lower than that in norovirus-negative participants; while these symptoms have been reported in experimentally inoculated volunteers, symptoms may have been more accurately reported over the shorter clinical observation period in the inoculation studies, compared to the 3-week recall period used for self-reporting of symptoms in the current study.

Participants in the current study were recruited because they had been free of diarrhoea and/or vomiting for at least 10 days; the aetiology of any recent IID symptoms prior to this period was not established. Therefore, we do not know how many of the norovirus infections detected were truly asymptomatic rather than post-symptomatic shedding. Post-symptomatic shedding after experimental inoculation has been demonstrated, lasting up to 8 weeks [4], so it is likely that some of the asymptomatic infections reported here are the result of prolonged post-symptomatic shedding. This is consistent with the small excess of diarrhoea and vomiting symptoms in participants with asymptomatic norovirus infection. It is also possible that some asymptomatic norovirus infections were due to pre-symptomatic shedding, although the short incubation period of 24–48 h for norovirus disease [17] means that only a small number of the infections in the current study are likely to be pre-symptomatic shedding.

Irrespective of the source of asymptomatic norovirus infection, further work is needed to understand whether these infections contribute substantially to norovirus transmission leading to sporadic illness or outbreaks. A few published foodborne norovirus outbreak investigations have attributed illness to food contamination by asymptomatically infected food handlers [18]. However, the importance of asymptomatic infections for norovirus transmission outside of food catering settings has not been investigated.

While norovirus is shed at much lower concentrations by asymptomatically infected individuals compared to those with disease [19], the estimated infectious dose is exceptionally small [20], so norovirus shedding at low concentrations could still potentially lead to transmission. Only studies identifying incident asymptomatic infections, with follow-up of contacts during infection, will reveal the importance of asymptomatic infections for continued norovirus transmission.

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DECLARATION OF INTEREST

None.

REFERENCES

1. Amar CF, *et al.* Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). *European Journal of Clinical Microbiology and Infectious Diseases* 2007; 26: 311–323.
2. de Wit MA, *et al.* Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *American Journal of Epidemiology* 2001; 154: 666–674.
3. Karsten C, *et al.* Incidence and risk factors for community-acquired acute gastroenteritis in north-west Germany in 2004. *European Journal of Clinical Microbiology and Infectious Diseases* 2009; 28: 935–943.
4. Atmar RL, *et al.* Norwalk virus shedding after experimental human infection. *Emerging Infectious Diseases* 2008; 14: 1553–1557.
5. Sethi D, *et al.* A study of infectious intestinal disease in England: plan and methods of data collection. *Communicable Disease and Public Health* 1999; 2: 101–107.
6. Tompkins DS, *et al.* A study of infectious intestinal disease in England: microbiological findings in cases and controls. *Communicable Disease and Public Health* 1999; 2: 108–113.
7. Houde A, *et al.* Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. *Journal of Virological Methods* 2006; 135: 163–172.
8. Medici MC, *et al.* Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples. *Journal of Clinical Microbiology* 2005; 43: 3772–3778.
9. Koopmans M, *et al.* Molecular epidemiology of human enteric caliciviruses in The Netherlands. *Journal of Infectious Diseases* 2000; 181 (Suppl. 2): S262–S269.
10. Noel JS, *et al.* Identification of a distinct common strain of 'Norwalk-like viruses' having a global distribution. *Journal of Infectious Diseases* 1999; 179: 1334–1344.
11. Ando T, *et al.* Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization. *Journal of Clinical Microbiology* 1995; 33: 64–71.
12. Green J, *et al.* Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis. *Journal of Medical Virology* 1995; 47: 392–398.
13. Kageyama T, *et al.* Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology* 2003; 41: 1548–1557.
14. Lopman BA, *et al.* Two epidemiologic patterns of norovirus outbreaks: surveillance in England and wales, 1992–2000. *Emerging Infectious Diseases* 2003; 9: 71–77.
15. Dennehy PH. Transmission of rotavirus and other enteric pathogens in the home. *Pediatric Infectious Disease Journal* 2000; 19: S103–S105.
16. Goldmann DA. Transmission of viral respiratory infections in the home. *Pediatric Infectious Disease Journal* 2000; 19: S97–102.
17. Gotz H, *et al.* Clinical spectrum and transmission characteristics of infection with Norwalk-like virus: findings from a large community outbreak in Sweden. *Clinical Infectious Diseases* 2001; 33: 622–628.
18. Lo SV, *et al.* The role of the pre-symptomatic food handler in a common source outbreak of food-borne SRSV gastroenteritis in a group of hospitals. *Epidemiology and Infection* 1994; 113: 513–521.
19. Phillips G, *et al.* Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infectious Diseases* 2009; 9: 63.
20. Teunis PF, *et al.* Norwalk virus: how infectious is it? *Journal of Medical Virology* 2008; 80: 1468–1476.

Appendix 7.5. Papers under review.

Phillips G, Tam CC, Rodrigues LC, Lopman B. Risk factors for symptomatic and asymptomatic norovirus infections in the community. *BMC Infect. Dis.* under review.

Appendix 7.6. Conference presentations.

Vaccines Enteric Diseases Conference; Malaga, Spain, September 2009 (**oral**). *Improving estimates of norovirus-associated infectious intestinal disease in England.*

European Scientific Conference on Applied Infectious Disease Epidemiology; Berlin, Germany, November 2008 (**oral**). *Determining norovirus aetiology from viral load.*

Health Protection Agency Annual Scientific Conference; Warwick, UK, September 2008 (**poster**). *Estimating the incidence of GP consultations for norovirus using routine surveillance data.*

Eighth International Rotavirus Symposium; Istanbul, Turkey, June 2008 (**poster**). *Association of rotavirus with other gastrointestinal pathogens in symptomatic and asymptomatic individual*