Understanding the carriage and transmission of non-typhoidal Salmonella infections in Kenya.

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Statement of Own Work

I, Esther Muthumbi, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I have duly acknowledged it.

Signed,

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Abstract of Thesis

Non-typhoidal Salmonellae (NTS) are responsible for enteric disease characterized by self- limiting enterocolitis in most parts of the world. In Africa, however, they cause severe invasive disease and a high case fatality ratio. The serotypes responsible for >80% of invasive NTS disease (iNTS) are *S.* Typhimurium and *S.* Enteritidis. Control efforts against these NTS serotypes are limited by a lack of understanding of their transmission - both mode and rates of transmission. This PhD was designed to improve the understanding of the epidemiology of carriage of NTS and its rate of transmission to enable efficient design of effective control strategies, including vaccination.

Through a cross-sectional study of faecal carriage and seroprevalence in 3 locations in Kenya with varying incidence of iNTS, I observed that Kilifi, had the highest carriage prevalence despite the lowest incidence of disease. However, the majority serotypes were neither *S*. Typhimurium nor *S*. Enteritidis. At all sites, older children and adults had the highest carriage. Analysis of anti-*Salmonella* antibodies showed a decay of maternal antibodies in 4-5 months and a rapid acquisition of NTS infections among infants thereafter. A catalytic model of the seroprevalence data estimated the force of infection (FOI) with Group *O:4,5* serotypes and Group *O:9 serotypes* as ranging from 0.2 to 0.5 episodes/person/year- both were highest in Kilifi. I carried out an additional longitudinal seroprevalence study retrospectively on archived samples in Kilifi. I observed that the FOI of Group *O:9 serotypes* had decreased over time concurrently with a documented decrease in iNTS incidence while the FOI of Group *O:4,5* increased as iNTS incidence decreased in one of the locations. These suggest that interrupting transmission can be a strategy for control of iNTS caused by these serotypes, possibly through vaccines which reduce faecal carriage. Such measures can be focused on older children and adults who are the reservoirs for infection, especially in households with infants who have the highest incidence of infection and are at highest risk of invasive disease. For control of *S*. Typhimurium disease, host risk factors will need to be addressed. The parameters we have

estimated such as FOI could be used to inform dynamic transmission models to predict vaccine effectiveness and cost-effectiveness analyses of different potential vaccination strategies including vaccination of pregnant mothers, infants, or older children.

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AMR	Antimicrobial Resistance
AfOX	Africa – Oxford Initiative
CI	Confidence Interval
CFR	Case Fatality Ratio
CLSI	Clinical & Laboratory Standards Institute
CVD	Centre for Vaccine Development
ELISA	Enzyme linked immunoassay
FOI	Force of Infection
GEMS	Global Enteric Multicenter Study
GMMA	Generalized Modules for Membrane Antigens
GMC	Geometric Mean Concentration
GPDS	Global Pediatric Diarrhea Surveillance
GVGH	GSK Vaccines for Global Health
HAZ	Height for Age Z-score
HIV	Human immunodeficiency virus
IDeAL	Initiative to Develop African Leaders
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
iNTS	Invasive Non-typhoidal *Salmonella*
IVI	International Vaccine Institute

KCH	Kilifi County Hospital
KEMRI	Kenya Medical Research Institute
KHDSS	Kilifi Health and Demographic Surveillance System
KWTRP	KEMRI-Wellcome Trust Research Programme
LMIC	Low and Middle Income Countries
MUAC	Mid Upper Arm Circumference
MLST	Multi-Locus Sequence Typing
NIBSC	National Institute for Biological Standards and Control
NTS	Non-typhoidal *Salmonella*
OMV	Outer Membrane Vesicles
PCR	Polymerase Chain reaction
PCV	Pneumococcal Conjugate Vaccine
ROC	Receiver Operator Curve
SD	Standard Deviation
sSA	sub-Saharan Africa
TCV	Typhoid Conjugate Vaccine
TSAP	Typhoid Surveillance in Africa Project
WASH	Water Sanitation and Hygiene
WAZ	Weight for Age Z-score
WGS	Whole Genome Sequencing
WHO	World Health Organization

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Dedication

This thesis is dedicated to:

my mum - my no. 1 fan.

my children – always reach for the greatest heights.

my husband – now our lives begin.

my brother Mwas, in loving memory.

Preface

My interest in Salmonellosis research began in 2014. I was newly employed at the KEMRI-Wellcome Trust Research Programme (KWTRP) in Kilifi as part of the Invasive Bacterial Diseases group under the leadership of my current PhD supervisor, Prof. Anthony Scott. In that year, he was invited to moderate a session at the "Invasive Salmonella in Africa consensus meeting" meeting (1). The focus of the meeting was to highlight the burden of invasive Salmonellosis in sub-Saharan Africa, where surveillance from sentinel sites had reported an increasing incidence of disease. As an eager student, I offered to analyse the data from our site in Kilifi where bacteraemia surveillance linked with demographic data from the Kilifi Health and Demographic Surveillance System (KHDSS) had been conducted since 1998. The meeting was successful, demonstrated by the Supplement Journal that was published later in the year in the Clinical Infectious Diseases Journal(2), with contributions from 17 countries across sub-Saharan Africa (sSA), including our site in Kilifi which I authored(3).

During preparation for the manuscript, it became apparent that the main knowledge gap in the understanding of the epidemiology of non-typhoidal Salmonella (NTS) was in the epidemiology of transmission. Whilst the epidemiology of disease was poorly described at the time, the epidemiology of infection was almost non-existent. However, it is known that understanding transmission of an infectious disease is key in the design and deployment of any control efforts(4). This was highlighted in an expert opinion piece by Prof. Melita Gordon(5) "The most important remaining gap in our knowledge is probably an understanding of how NTS is transmitted, and the nature of the relationship between diarrhoeal disease, carriage and invasive disease in Africa, so that diagnostic and prevention tools can be appropriately directed"(6).

This PhD has sought to describe the epidemiology of infection and transmission of non-typhoidal Salmonella (NTS) infections in order to understand how we can prevent disease by controlling infection through interventions, particularly vaccination.

I played the main role in conceptualising and coordinating the studies under this PhD. Specifically:

- I conceptualised the carriage study and developed the associated protocol including engaging the different stakeholders in the three sites in Kenya, and in Oxford.
- I coordinated the fieldwork in each of the 3 sites, including training of field teams and day-to-day operations. The studies ran in series and therefore I was able to be present at each of the sites.
- I developed the protocols for the Microbiology laboratory work and trainings for the laboratory teams. Actual benchwork for culture, identification and storage of samples and isolates was performed by the trained laboratory technicians headed by Alfred Mwanzu.
- I received training on the NTS ELISAs as performed at the Jenner Institute and assayed the first 1500 samples. Later, I transferred the assays to KWTRP and trained the study laboratory technicians on the ELISA. The team then conducted the remaining 1500 ELISAs for the seroprevalence chapter and those of the longitudinal study.
- I conceptualised the longitudinal study, developed its protocol and sought for funding (successfully) for the study.
- I conducted the day-to-day data integrity checks and data management.
- I conducted the statistical analyses for all the studies.
- I conducted the mathematical modelling for the studies under guidance of Stefan Flasche.

I was guided and supervised by Anthony Scott.

Narrative literature review

The Pathogen.

Salmonellae are gram-negative bacilli from the Family Enterobacteriaceae. The genus Salmonella consists of 2 Species, *Salmonella enterica* and *Salmonella bongori*; the latter causes disease in cold-blooded animals ,especially lizards(7). *Salmonella enterica* is further divided into 6 subspecies: *S. e. arizonae*, *S. e. diarizonae, S. e. enterica, S. e. salamae, S. e. indica and S. e. houtenae,* which are further divided into >2500 serotypes based on the serological identification of the O and H antigens, the somatic and flagellar antigens , respectively (8). The full names for common serotypes such as Typhimurium would be *Salmonella enterica* subsp. *enterica* serovar Typhimurium, abbreviated as *S.* Typhimurium. This complex nomenclature was originally proposed by Kauffman, and later modified by Le Minor and Popoff(9). The final form is published and maintained by the 'WHO Collaborating Center for Reference and Research on Salmonella', and any new serotypes are listed in the Kauffmann-White scheme, which is updated as new serotypes are discovered(10). There are 2557 serotypes of *S. enterica* listed in the latest update (dated 2007). Given advances in genomics, there have been proposals to improve the *Salmonella* nomenclature by incorporating Sequence Types (ST) based on Multi-Locus Type Sequencing (MLST) in the naming and reporting of Salmonella(11), but these have not taken effect yet.

Salmonellae are further grouped into Typhoidal and non-typhoidal serotypes. Typhoidal serotypes consist of *S.* Typhi and *S.* Paratyphi A, *S.* Paratyphi B or *S.* Paratyphi C which cause enteric fever(12). All other serotypes are designated as non-typhoidal *Salmonella* (NTS). These groups are distinguished by the clinical manifestations of the infections they cause amongst other characteristics briefly summarized below and expanded in the rest of this chapter:

Table 1.1: Differences between Typhoidal and non-typhoidal *Salmonella* serotypes

	Typhoidal *Salmonella*	Non-typhoidal *Salmonella*

Species	The primary species within the typhoidal group is *Salmonella enterica* serovar Typhi.	Various *Salmonella* serotypes, excluding Typhi.
Disease	Typhoid Fever, a systemic illness	Enterocolitis, a localized infection of the gut and invasive non-typhoidal *Salmonella* disease, a systemic illness
Transmission	Fecal-oral route often via contaminated food and water	Fecal-oral route often via consumption of contaminated food such as undercooked eggs, poultry and dairy products; and occasionally via direct contact with animals
Host range	Primarily adapted to humans and does not cause disease in animals	Broader host range and can infect a wide variety of animals including livestock, pets and wildlife.
Vaccination	Several licensed vaccines available including Typhoid Conjugate Vaccines, Ty21a - a live attenuated oral vaccine, Vi Capsular Polysaccaride vaccine,	No licensed vaccine in humans available; several candidate vaccines against invasive disease are in development

Invasive non-typhoidal Salmonella disease.

At the start of this PhD research project, the annual cases of NTS in 2010 were estimated in global models to be 94 million cases of diarrhoea and 3.4 million cases of invasive disease. The models suggested that 3% of the diarrhoeal cases and 57% of the invasive cases occur in sub-Saharan Africa (13, 14). The high burden of invasive NTS disease (iNTS) in Africa was linked to the high prevalence of *Plasmodium falciparum* malaria (henceforth referred to as malaria) and HIV infection, which are known risk factors for iNTS disease and were used for predictions in the models. More recent estimates from the Global Burden of Disease study suggest that in 2017, the number of cases of iNTS disease worldwide was 535,000, 79% of which occurred in sSA(15). The models had a 6-fold difference, likely due to sparse underlying data with wide uncertainties, different assumptions and model choices. However, even with this wide variation in the modelled estimates, it was clear that Africa carries a disproportionate burden of iNTS disease in the world.

The incidence of iNTS in children aged <15years as estimated by the Typhoid Surveillance in Africa study (TSAP) study, a standardized bacteraemia surveillance in 10 African countries conducted between 2010 and 2014, ranged from 0-742 per 100,000 showing marked geographical variation

within the continent(16). Ghana was the country with the highest incidence while Kibera, an urban slum area in Kenya, had an estimated incidence of 31 per 100,000 in children aged <15years (and 49 per 100,000 in children aged <5yars), which was lower than expected. Previous estimates of iNTS incidence in children aged <5years from surveillance sites in Kenya were 3,914 per 100,000 in Siaya (2009-2014), 997 per 100,000 in Kibera (2009-2014) and 36 per 100,000 in Kilifi (1998-2014) (3, 17). The differences were possibly due to differences in methodology, especially of incidence adjustment factors. In several reviews on bacteraemia in Africa(18, 19), NTS was identified as the most commonly isolated bacteria among adults, and second commonest among children, second only to *Streptococcus pneumoniae*, which is currently being controlled by effective vaccines(20-24), and in more recent estimates from the TSAP, NTS isolates were still commonly isolated (Second to *S*. Typhi)(16).These estimates of iNTS incidence are mostly derived from hospital-based surveillance which may be biased by ill-defined catchment populations, by variable health seeking behaviour and by limited access to microbiology facilities, especially in low- and middle-income countries (LMIC). Therefore, these estimates, despite being large, are only a 'Tip of the iceberg', and do not provide an accurate measure of the burden of disease in the population.

Transmission of NTS – Rates and Reservoirs.

Table 1.2 Definition of terms related to transmission of infectious organisms.

Source	*The person, animal, object or substance from which an infectious agent is transferred or disseminated to the host. e.g. Contaminated food for S. Typhi*
Reservoir	*The natural habitat in which the viable infectious agent lives, grows and replicates. e.g. human for S. Typhi*

Portal of entry	*The way an infectious agent enters a susceptible host*
Portal of exit	*The way an infectious agent leaves a susceptible host*
Mode of transmission	*The method that an infectious agent uses to get from source to host e.g. foodborne for S. Typhi*
Route of transmission	*The path taken from portal of exit to portal of entry using the chosen mode e.g. faecal-oral*
Carrier	*An infected person or animal that harbours a specific infectious agent in the absence of clinical disease and can transmit the agent*

Salmonella Typhi are host-adapted, causing infection and disease in humans alone. By contrast, serotypes of non-typhoidal *Salmonella* are known to infect a variety of hosts including domesticated farm and wild animals(25). NTS serotypes causing disease in humans have been isolated in chicken and eggs, pigs, cattle, camels, reptiles, and on the leaves of vegetables commonly eaten raw(26-30). Consequently, transmission of NTS to humans is thought to be linked to consumption of contaminated food and water. The infectious dose for *Salmonella* is 10^5-10^7 bacteria(31, 32), based on challenge studies, and a directly proportional relationship exists between dose of NTS consumed and the severity of disease assessed in food-poisoning outbreaks (33). Data from the WHO Salm-Surv project (2002-05) reports that the most common serotypes associated with NTS infections in humans globally are (in ranked order): *S.* Enteritidis (65%), *S.* Typhimurium (12%) and *S.* Newport (4%) while in non-humans they are *S.* Typhimurium (17%), *S.* Heidelberg (11%) and *S.* Enteritidis (9%) (34). On the serotype level, similar strains tend to affect both humans and animals. However, genomic analyses identified a sequence type (ST313) of *Salmonella* Typhimurium that seemed to occur only in humans, and whose genome had undergone degradation and acquired pseudogenes, features associated with host-restriction(35). This meant that this *S*. Typhimurium ST313 had become host-adapted to humans, just like *S.* Typhi(25). Additionally, in an analysis looking at the

meat pathway in NTS source attribution, with the exception of ST313, sequence types of NTS derived from human samples were comparable with those derived from “The meat pathway” (i.e. farms, animal stool samples, carcasses and slaughter houses) (36). Together, these studies highlighted *S*. Typhimurium ST313 as a host-adapted strain.

However, three studies have reported the presence of *S*. Typhimurium ST313 strains from non-human sources. The first was a study in experimental chickens where *S*. Typhimurium ST313 strains fed to chickens by oral lavage showed evidence of invasion through the ileum(37). The second study identified 2 S. Typhimurium ST313 isolates among faecal and mesenteric lymph nodes samples in pigs at slaughterhouses in Kenya(28). These seemed to contradict the evidence on host-adaptation. However, when a core-gene SNP-phylogeny was constructed to determine the similarities of the porcine isolates and published human *S*. Typhimurium ST313 isolates. It showed that the two isolates were similar to *S*. Typhimurium ST313 isolates from the UK and not the *S*. Typhimurium ST313 from Africa, especially Lineage II isolates which cause invasive disease in sSA (28). Similarly, genomic analyses of *S*. Enteritidis isolates causing invasive disease in Africa have identified distinct clades of sequence type ST11 with signs of host-adaptation (38). Before the widespread use of whole genome sequencing, anthroponotic transmission of NTS had been inferred in a study in Kenya, where NTS isolates from children with iNTS disease were compared with NTS isolates from their household human contacts, household animal contacts and household environment including water. Using pulse gel field electrophoresis, NTS isolates from the children were found to be similar to the isolates from asymptomatic household contacts but different from isolates from animals and the environment(39). Similar results were obtained in a household transmission study from The Gambia, Burkina Faso and in Malawi (40-42). In the third study *S*. Typhimurium ST313 Lineage II isolates were identified in deep organ tissues from urban rats caught in mouse traps in DRC, pointing to rats as a possible reservoir for iNTS disease. However, none of the isolates were recovered from the rats’ faecal samples(43). If humans are a major reservoir of NTS in Africa, then the subset of

individuals with asymptomatic carriage are important in NTS transmission and are a public health concern.

Carriage of NTS

The prevalence of faecal carriage of NTS among asymptomatic food handlers has been reported as 4.7% in Bangkok(44) and 3.1% in Ethiopia(45). In Kenya, in the aforementioned study on iNTS case children and their household contacts, 6.9% of the asymptomatic contacts grew NTS cultured from rectal swabs(39). Population-based studies of carriage of NTS in Africa are few. Im *et al.* reported carriage prevalence of 2.4% and 1% from a study on faecal carriage among children and adults in Guinea Bissau and Senegal, respectively. The prevalence was highest among children aged 5-14 years and older children and adults aged 15-34 years(46). The serotypes could not be determined as the multiplex PCR was targeting 4 of the most common *Salmonella* serotypes in the area (*S.* Typhimurium, *S.* Enteritidis, *S.* Dublin and *S*. Typhi) of which this carriage study had none. In another study of faecal carriage, 38 isolates were recovered from the stool samples of 1,108 healthy children and adults in DRC (prevalence of NTS carriage was 3.4%)(47). Only 9 of those isolates were either *S.* Typhimurium(n=4) or *S.* Enteritidis (n=5). Serogroup B serotypes, other than *S*. Typhimurium were, *S.* Stanleyville (n=5) and *S*. Essen (n=1). This was the predominant serogroup, followed by serogroup O:11 (F) which had 7 isolates, including 4 *S.* Rubislaw. In total, 10 serogroups were represented. In this study, each of the participants gave stool samples on two consecutive days, yielding 24 isolates on day 1 samples and 14 isolates on day 2 samples (1 participant had positive samples on both days), and demonstrating that excretion of NTS from the gut is intermittent, as previously described(12), and that quantifying carriage prevalence from a single sample may lead to underestimation. The epidemiology of carriage from both studies differed from the known epidemiology of invasive disease which is characterized by higher incidence in the younger age-groups (<5years) and a higher preponderance of *S.* Typhimurium and *S.* Enteritidis serotypes. Additional estimates on the prevalence of NTS carriage have been approximated through the control arms of the GEMS study and the Vaccine Impact on Diarrhea in Africa (VIDA) study. In GEMS, the prevalence of *Salmonella*

stool carriage among the asymptomatic controls was 180/13,129 (1.4%) (48). Out of the 180 isolates recovered, serogroup O:4 (B) accounted for 49 (33%), serogroup O:8 (C_2/C_3) accounted for 40 (22%), serogroup O:6,7 (C_1) accounted for 30 (17%), serogroup O:9 (D_1) accounted for 13 (7%), serogroup E_1/E_4 for 11 (6%) and both serogroup O:11 (F) and serogroupO:13 (G) accounted for 9 isolates each (5%). *S.* Typhimurium and *S.* Enteritidis accounted for 28 and 6 isolates, respectively. In VIDA, the prevalence of NTS in controls aged <5y was 45/2138 (2.1%) in The Gambia and 61/2,095 (2.9%) in Kenya (49). Across both sites, *S.* Typhimurium and *S.* Enteritidis accounted for 3 and 12 isolates, respectively. In both studies, *S*. Typhimurium and *S*. Enteritidis accounted for a minority of the isolates.

Colonization of the gallbladder by *S.* Typhi is associated with persistent infections(50). Murine models have demonstrated persistence of NTS infection in the gallbladder, similar to *S*. Typhi in humans (51). The models of *Nramp1* deficient mice infected with *S.* Typhimurium are commonly used to study persistence of *S*. Typhi, even though it is host adapted. The nature of the systemic infection and the histological changes in the gallbladder of the mouse models including biofilm formation mirror typhoid fever in humans(51). In humans, the duration of shedding through the biliary duct can range from weeks to over one year depending on age, concurrent Schistosoma infections, structural abnormalities of the biliary system, use of antibiotics and the serotype of NTS (32, 52-54) The studies showed that younger children shed for longer and excrete larger quantities of NTS. In addition, *S.* Typhimurium serotypes are excreted for a shorter duration compared to other NTS serotypes. Buchwald *et al*. undertook a meta-analysis of 2,800 patients from 32 studies and found that <1% of the subjects have chronic excretion of NTS (defined as excretion for >1 year)(53). Persistence of NTS carriage, ranging from 30 days up to 8 years, was observed in a retrospective study from Israel where >48,000 cases of iNTS occurring over 17 years were analysed to determine duration of infection and frequency of relapses(55). Persistence was defined as isolation of the same serotype at least 30 days after the previous isolation. They identified 1,047 cases of persistent infections (2.2% of the total), with evidence of clonal similarities within the same person, and further

reported within-host genetic changes (i.e., loss and acquisition of mobile genetic elements) in the serotypes during persistence. Symptomatic persistence was associated with younger age, receiving antibiotic treatment, hospitalization, and co-infection with other enteric pathogens. The main symptom, reported from recall, was diarrhoea. A variety of serotypes were associated with persistence to different degrees. The persistent index in the study was calculated as the ratio of persistent cases to sporadic cases. The persistence index of *S.* Typhimurium and *S.* Enteritidis was 0.7 and 0.5, respectively. Recurrence (phylogenetically distinct from index case) and recrudescence (phylogenetically indistinguishable or derived from index case) of iNTS infections has also been described in Malawi. In the study, 19 out of 44 patients with iNTS (43%) had an episode of recurrence, 5 of whom continued to have multiple episodes of recurrence within the first year of follow-up(56). While the episodes of persistence in the Israeli study were in immunocompetent patients, all the recurrent cases in Malawi were in HIV-infected patients. Within host changes in the genetic composition of a *Salmonella* serotype during chronic infection has also been described in Malawi in an immunocompromised patient who carried *S.* Enteritidis for 15 years(57).

Diarrhoeal disease caused by NTS

The global burden of diarrhoea due to *Salmonella* was estimated to be 93.8 million cases worldwide and 155,000 deaths (13) Of these cases, 3% were estimated to be from Africa – a sharp contrast to the disproportionate burden of invasive NTS disease in Africa vis-à-vis the rest of the world. More recently, findings from Global Enteric Multicenter study (GEMS) and the Global Pediatric Diarrhea Surveillance (GPDS), which were large multicentre case-control studies on diarrhoea aetiology, showed that NTS is a rare cause of diarrhoea. In these studies, rotavirus was the leading cause of diarrhoea in children in Africa; while *Shigella* was the leading bacterial cause (48, 58). After the introduction of the rotavirus vaccine, the Vaccine Impact on Diarrhea in Africa (VIDA) study, which was a follow on to GEMS study, reported similar results(49). Across the sites, the attributable fractions for non-typhoidal *Salmonella* infections were low. This is because the infections were

relatively uncommon (1% in GPDS and 2% in GEMS) and occurred almost as frequently in cases as controls, except for serogroup O:11 (F) isolates in GEMS which were isolated only in controls. However, in Kenya, 3.2% and 3.7% of moderate-to-severe diarrhoea episodes in toddlers (12-23months) and older children (24-59months) were attributable to *Salmonella*. In VIDA study, NTS infections were associated with moderate-to-severe diarrhoea in children aged 24-59months in both Kenya and The Gambia, though the attributable fraction was lower than in GEMS. Stool samples from GEMS and VIDA were tested by both conventional culture methods and by PCR through Taqman array cards while for GPDS, only PCR was used. Further analysis of the *Salmonella* isolates from GEMS showed that the serotype distribution differed by age and by site; *S*. Typhimurium isolates were more common in African sites than Asia, while serogroup C isolates were more common in Asia(59). An increase in the proportion of NTS diarrhoea by serogroup C isolates has been observed in European countries and the USA (60) and in Kenya (49). Antimicrobial susceptibility profiles from each site in GEMS were similar among cases and controls except for Kenya and India, where more resistant isolates were found among cases (65%) than controls (40%) in Kenya; and more resistant isolates were found among controls (30%) than cases (0%) in India. Interestingly, all *S* Typhimurium isolates from the Kenyan site belonged to sequence type 313 (ST313) which is associated with invasive disease, signifying that *S.* Typhimurium ST313 can occur in carriage, diarrhoea and invasive NTS disease. iNTS disease, associated with *S.* Typhimurium ST313, seldom presents with diarrhoea. However, co-isolation of NTS, including genetically related *S*. Typhimurium ST313 and *S.* Enteritidis ST11, from paired stool and blood samples of patients admitted with iNTS bacteraemia has been observed(61) showing that the same pathogen can cause both infection states. In the same study, other paired stool and blood samples had non-identical serotypes suggesting that additional factors may be involved in the selection. Once an infective dose of NTS is ingested, the factors that determine whether the bacteria establish an infection in the gut, with or without diarrhoea, or whether the bacteria translocate into the bloodstream causing iNTS disease will depend on both the pathogen and the host susceptibility.

Risk Factors for iNTS

Infection with NTS in the gut can lead to clearance, development of an asymptomatic carrier state, diarrhoeal illness or invasive disease (25). Though the relative proportion of infections that end up in each manifestation is not known, it is thought that only a small subset of NTS infections become invasive and this is based on host susceptibility factors (25, 62) Risk factors for iNTS disease in Africa have been identified as recent malaria infection, concurrent malaria infection, anaemia, HIV infection, malnutrition and sickle cell disease(3, 25, 62-66). Among children in Kilifi, these effects were quantified as: Recent malaria Odds Ratio 1.8 (95% CI 1.0-3.1) (67), malnutrition 2.3 (1.5-3.4) and HIV infection 5.1 (3.3-8.0) (3). Malaria is associated with NTS mostly through dysregulation of iron that occurs when malaria parasites cause haemolysis of red blood cells which in turn increase iron availability enhancing bacterial growth and inhibiting neutrophil and macrophage function(68-71). Malaria also causes increased gut permeability leading to invasion of NTS, similar to observations in malnourished children(72, 73). While immunosuppression due to HIV-infection increases the risk of invasion with NTS(74, 75). In fact, iNTS disease in adults occurs almost exclusively in HIV-infected adults - in a study in Malawi where consecutive cases of iNTS in the adult wards were recruited, 99% (77/78) of the cases of iNTS also had HIV-infection (56). In high income countries, immunodeficiencies caused by defects in the IL-12 Pathway have been associated with increased risk of iNTS disease (76). A composite index of iNTS risk factors (the iNRF index) consisting of malaria prevalence, HIV prevalence, malnutrition and unsafe water, has been proposed for iNTS risk stratification of geographical regions to aid in identification of high-risk areas for implementation of control measures(77). The index correlated with iNTS incidence estimated from bacteraemia surveillance and can in turn be used to estimate the iNTS incidence in areas without systematic surveillance. A similar index using drinking water source, type of toilet facility and population density has been proposed for geographical mapping of typhoid fever (78), and its

components have been incorporated in models forecasting the demand for typhoid conjugate vaccines(79).

The incidence of iNTS declines steeply with age(14). The incidence in children under 5 years of age is consistently higher than that of older children and adults. In Kilifi, Kenya, the highest incidence of iNTS was observed in newborn infants(3), though studies in Malawi have demonstrated relative sparing of iNTS disease in children under 4 months of age, and a peak in late infancy(80). In Malawi and South Africa, a smaller peak in incidence has been observed at around the age of 30 years, attributed to the higher HIV prevalence in this group(81).

The main clinical presentation of iNTS disease is fever, which occurs in 95% of the cases(25, 82). Fever is usually rapid in onset with an unknown focus(25). In areas of high malaria burden, presentation with iNTS is often confused with clinical malaria leading to delays in appropriate management(83, 84). Other symptoms that occur, although less commonly are, features of pneumonia, diarrhoea and vomiting. Clinical signs include hepatomegaly and splenomegaly. Complications of iNTS bacteraemia include osteomyelitis and meningitis(82, 85). The latter is seen most in children and is associated with a higher mortality (3, 86). The case fatality of iNTS is estimated as 15%, while that of iNTS meningitis is 50%(82, 87). Because there are few microbiological facilities that can diagnose iNTS in sSA, management of febrile illnesses, especially in children, is guided by national empirical treatment guidelines which are contextualised from WHO Integrated Management of Childhood Illness (IMCI) recommendations(88). First line antibiotics consists of ampicillin and gentamicin and the commonest second line antibiotic is ceftriaxone, a 3rd generation cephalosporin. As NTS isolates are intrinsically resistant to gentamicin, only ampicillin provides effective treatment in the first line management. In Kilifi County Hospital (KCH), 22% of invasive NTS isolated between 1998-2018 were non-susceptible to ampicillin, while 8% were non-susceptible to ceftriaxone (Unpublished data). Specific management for NTS should depend on the antimicrobial susceptibility patterns reported after culture. However, increasing resistance to

commonly available antibiotics such as amoxicillin, chloramphenicol, co-trimoxazole (the NTS multidrug resistant phenotype) and fluoroquinolones have been reported(89-92). A systematic review and meta-analysis reported ceftriaxone resistance among iNTS isolates in sSA was 1.7%(93), however the prevalence of resistance is increasing. A study in Western Kenya reported increasing resistance to ceftriaxone from 6% in 2009 to 57% among invasive isolates in 2013 (91). The last line of treatment includes carbapenems, which are effective, but are usually unavailable either due to supply or cost, and are on the 'Reserve' list of antibiotics from WHO requiring monitoring and utilization reporting(94). Perhaps because of their low volume of use, resistance to carbapenems is rare among NTS isolates. However, one study has reported imipenem resistance in a hospitalized patient in Tunis and it has also emerged in zoonotic NTS infections(95). As fewer treatment options remain effective, focus should switch to prevention of infection for the management of NTS, however, little is known about the epidemiology of infection of NTS serotypes causing disease in sSA.

Immunobiology of NTS

The cell wall of Salmonella bacteria consists of an outer membrane which is made up of proteins, phospholipids and lipopolysaccharides. Each has a role to play in survival including immunogenicity and virulence. The outer membrane proteins determine the interactions between the bacteria and its environment, it also determine pathogenic processes such as motility, adhesion and antibiotic resistance. The lipopolysaccharide (LPS) chain consists of the Core region, the O-polysaccharide antigen and the Lipid A moiety. The LPS chain is responsible for bacterial immunogenicity and is the target for several vaccines against NTS. The O-antigen is the immunodominant part of the LPS chain. The O-antigen chain differs between serotypes and confers specificity during serotyping. For *S.* Typhimurium it consists of repeating units of mannose-rhamnose-galactose-linked to abequose while, for *S.* Enteritidis, it consists of a similar set of repeating units of mannose-rhamnose-galactose but now linked to tyvelose. Specific antibodies against the O-antigen have been observed to be

protective in mouse models(96-98). In humans, antibodies targeting the O-antigen have been shown to have bactericidal activity which is complement dependent(80, 99). Host immunity, in both cell-mediated immunity and antibody-mediated responses, is important in protection against NTS bacteraemia(100, 101). NTS are intracellular organisms but are also able to exist extracellularly, providing a window for antibody dependent killing mechanisms.

The primary types of antibodies involved in the immune response against NTS are:

Immunoglobulin M (IgM) – this is the first antibody produced in response to infection. It is effective in the early stages of an immune response and is involved in the activation of other immune cells. Its detection in diagnostic tests such as the Typhidot and Typhidot-M screening tests for *S*. Typhi indicates a current infection.

Immunoglobulin G (IgG) - This is the most abundant immunoglobulin in the bloodstream. Concentration of IgG increase as the IgM levels decrease following the initial immune response. Repeated exposure to NTS leads to boosting of IgG concentrations, therefore its presence indicates both present and past infections.

Immunoglobulin A (IgA) – Found on mucosal areas such as the gastrointestinal tract, it prevents the NTS bacteria from attachment in the gut and invasion. Similar to IgG, its presence may indicate both past and present infection, however, whilst concentrations of anti-LPS IgG can persist for 12 months after salmonella diarrhoea, both IgM and IgA concentrations decay to undetectable within 2-4 months(102)

Interpretation of antibody concentrations against NTS, therefore, needs to consider both the rate of acquisition of infection and the decay of the antibodies after the infection. For example, in the previously discussed study, the rate of decay of NTS anti-flagellin IgM and IgA antibodies was faster than anti-LPS antibodies (102). Additionally, the strength and duration of antibody responses would be affected by age of the individual, inherent genetic variations in individuals and presence of chronic illnesses such as HIV-infection that may impact antibody production.

Studies in Malawi, where invasive NTS infections in children are largely confined to those aged 4 - 24 months, have shown that anti-*Salmonella* IgG concentrations increase with age, and are capable of complement-mediated killing of NTS from the age of 16 months onwards (80). The assays measured antibody concentrations against *Salmonella* strain D23580. Other studies of NTS seroprevalence include one in the US, which showed a high prevalence of anti-*Salmonella* antibodies among a healthy population of children and adults,(103) and one, in Uganda, which measured antibody concentrations against O:9 (D_1) and O:4,5 (B) O-antigens, showed a high seroprevalence among healthy adult. In the Ugandan study they also found that most children below 18 months of age lacked IgG (104). Comparison between the Malawian and the US studies is valid since they both used similar methodologies, except for the target isolate; the US study used *S.* Typhimurium ST19, a genotype responsible for enterocolitis, while the Malawian study used an *S.* Typhimurium ST313 strain, a genotype responsible for invasive disease. In both studies there was increase in specific antibodies by age and an increase in killing effect of the respective NTS strains by increased concentration of the antibodies. However, all but one of the sera from the US study were able to kill the NTS strain whilst only sera from children above 16 months in Malawi was able to kill *S.* Typhimurium ST313. The single sample that did not show bactericidal activity in the US study was associated with reduced complement deposition and presence of a specific anti-LPS IgM that inhibited killing of the *S*. Typhimurium strain. Inhibition of bactericidal activity by serum-containing anti-NTS antibodies was been observed in a previous study from Malawi where sera from HIV-positive individuals with a high concentration of antibodies blocked the *in vitro* killing of *S*. Typhimurium(105). This last study is not comparable to the others because it used different methodologies. In addition, the cut-off used for seropositivity in the Ugandan study relied on the assay's lower limit of detection, which could misclassify cross-reactive antibodies. Cross-reaction with LPS antigens from other gram-negative bacteria such as *Escherichia coli*, *Helicobacter pylori* and *Campylobacter jejuni* has been previously demonstrated(102), and it is expected that cross-reactivity

from serotypes within similar *Salmonella* serogroups would also occur in O-antigen based antibody assays.

Another key serological study of NTS was based in Vietnam, where the investigators enrolled 503 pregnant mothers and then enrolled their infants after delivery; they were then followed up for 1 year with measurement of anti-*Salmonella* antibodies against *S*. Typhimurium LPS and *S*. Enteritidis LPS at 4, 9 and 12-months. The study demonstrated transplacental transfer of anti-*Salmonella* LPS antibodies of both *S*. Typhimurium and *S*. Enteritidis from mother to child and the presence of maternal anti-Salmonella LPS antibodies of both *S*. Typhimurium and *S*. Enteritidis in serum of newborn infants at birth which decayed steadily over 4-5 months of life. The lowest concentrations of the IgG were between 20-30 weeks, which provides vital information for scheduling, if an infant iNTS vaccine is to be considered. The transfer ratios, calculated as neonatal IgG divided by maternal IgG, were 1.2-1.4. The maternal antibodies in the infants were protective, as evidenced by lack of seroconversion (measured by anti-NTS IgM) during the first 4 months of life, and later increase in seroincidence associated with the decay of maternal antibodies(106).

Seroepidemiological studies of NTS in Africa are rare; the study from Uganda is the only published population-based study(104). The authors assayed 1,090 samples from children and adults (median age not indicated) for IgG against O-antigens from O:4,5 and O:9 using an in-house ELISA. They found that 83% of the sera were seropositive for O:4,5 IgG and 70% were seropositive for O:9 IgG. The concentration and seroprevalence increased by age, however, only 50% of the children had detectable antibody levels at 18 months. This was in sharp contrast to Malawi where children from 4 months of age had specific IgG antibodies against Salmonella D23580 (an invasive *S*. Typhimurium isolate) from primary infection, though the bactericidal effect of these antibodies was only observed from 16 months onwards(80). If IgG is a good marker for infection with NTS, we could summarize that NTS infections occur more commonly in Malawi than in Uganda since the age-incidence curve is

shifted to the left for Malawi. This is an example of how serological studies can be used to infer the magnitude of transmission in the community.

The applications of serosurveillance in epidemiology are numerous. It complements traditional public health methods for surveillance of infectious diseases providing metrics for cumulative incidence of infections, transmission of infections and population immunity. Serology enables detection of past and present cases of infectious diseases even those that were asymptomatic and could have been missed by case-based surveillance or those occurring among people who did not seek or access health care. For example, *Salmonella* serosurveillance across 8 European countries observed a seroincidence that was 100-2000 times higher than the disease incidence reported in the respective countries' case-based surveillance(107).

Seroprevalence data can also be used to measure the rate at which individuals move from seronegative to seropositive states providing the rate of acquisition of an infection. This measure is estimated using catalytic models, provided there is a valid threshold for 'seropositivity'(108). The rate of acquisition is also known as seroconversion rate, force of infection or transmission intensity. For NTS serology research, a threshold for seropositivity has not yet been established. This would distinguish between those who have been exposed to NTS infections and those who have not. While selecting a threshold, some considerations need to be made, such as:

Specificity and Sensitivity of the assay – the threshold should balance the need for high specificity (minimizing false positives) and sensitivity (identifying true positives). Cross-reactivity may occur with antigens from other enteric bacteria with similar LPS structures, mostly gram-negative Enterobacteriales, and from other *Salmonella* serovars with varying degrees. For example, antibodies against the O:9 antigen maybe present after infection with any of the Group D *Salmonella* serotypes, including *S.* Enteritidis and *S*. Typhi. These cross-reactive antibodies may pose diagnostic challenges if the purpose of the assay was serotype identification, however cross-reactive antibodies may offer some level of protection against related serotypes, albeit with varying effectiveness. This

cross-protection can be explored as an advantage in vaccination strategies. Overall, understanding cross-reactivity in research settings is important for accurate interpretation of serological results.

Clinical Correlation – as seen in vaccine studies, a threshold that correlates with protection from infection or disease can be used to assess the effectiveness of the vaccine (109). In the absence of vaccines, quantification of naturally acquired serum IgG or mucosal IgA concentrations that can neutralize NTS can identify a protective threshold, as seen in Shigella serology studies(110). This method has been applied to identify a threshold from studies in Malawi, though has not been externally validated. Lack of consistency in methodology and lack of established standards/guidelines in NTS serology research has limited comparisons across different populations and laboratories. Serology can also be used to monitor trends of natural infections over time, e.g. for dengue (111), and to monitor infections after implementation of interventions e.g. malaria(112). In the field of vaccinology, age-seroprevalence data, collected before a vaccine is introduced, can identify the age group at risk of exposure, and the age at first infection, which together can identify the optimal age group to vaccinate(113). In countries with a large burden of infectious diseases, especially vaccine preventable diseases, serological assays on multiplex platforms can be used to determine the incidence and prevalence of several diseases simultaneously and focus interventions relative to the burden(114). Serological assays can be applied to sera available in biobanks, or to convenience samples such as leftover samples from blood donors and are therefore applicable in resource limited areas where case-based surveillance would be too costly, though non-random sampling will inevitably introduce biases.

A recent review of the application of serology in public health highlights some challenges limiting the widespread use of serology (115): lack of established reference standards for serological based assays, lack of established thresholds for dichotomizing seronegative and seropositive populations, poor specificity due to cross-reactivity by antigenically similar pathogens, uncertainty about the origin of antibodies such as those from maternal origin, vaccination or primary infection, complexity

of statistical methods for data analysis, lack of established serological correlates of protection and, finally, the fact that not all pathogens produce seroconversion upon infection which causes heterogeneity of immune responses. Many of these examples are directly limiting NTS serology research, (102, 114) and are at the core of my PhD studies.

Control of NTS

NTS disease can be controlled by prevention or treatment. Prevention focuses on averting acquisition of infection or inhibiting progression of infection to disease states. Treatment options, however important, can only reach the few cases that present to medical services and are accurately diagnosed, and the effectiveness of these options is currently limited to a few classes of antibiotics due to increasing resistance to commonly available antimicrobial drugs and the high cost of the newer, more effective drugs. Preventive options are limited by a lack of understanding of the routes and rates of transmission, and the knowledge of the relative contribution of and risk factors for acquisition of infection and the probability of invasion given infection.

In the last 25 years, we have observed a decline in invasive NTS disease in the Kilifi Health and Demographic Surveillance System (KHDSS) – a linked demographic and hospital-based surveillance which is nested within the KEMRI-Wellcome Trust Research Programme (KWTRP). It captures vital events and migration among residents within an area of 900km^2 around the Kilifi County Hospital (KCH) and is linked to morbidity and mortality surveillance at KCH(116). A bacteraemia surveillance was established in 2005 that monitors all febrile admissions at KCH (66). Between 1998-2005, the incidence of iNTS disease among children aged <5 years was 88/10^5 person-years(66). This fell by 16% per year between 1998-2014(3). The incidence of invasive disease caused by *S.* Enteritidis, and *S.* Typhimurium fell by 21% and 14%, respectively, during this period(3). An earlier analysis in Kilifi had linked the fall in incidence of all-bacteraemia, and especially NTS bacteraemia, between 1998-2005 to a simultaneous decrease in malaria admissions during the same period(117). Similar temporal associations between iNTS incidence and malaria have been made in Tanzania(118), The

Gambia(119) and in Western Kenya(17). The relationship between malaria and iNTS has been discussed above and more extensively in a literature review by Takem *et al.* (69). The review supports a causal relationship between malaria and iNTS and suggests that interventions that reduce malaria in the population are likely to reduce the incidence of iNTS, and is echoed by other authors(62). However, the RTS,S/AS01 malaria vaccine, with and without booster, did not have a protective effect on all-bacteraemia or on *Salmonella* bacteraemia (120). The vaccine trial was conducted in several sites in Africa including Kilifi. The authors explained that the observed lack of vaccine efficacy could be due to improved care, both clinical and supportive, offered to the study participants during the conduct of the trial. In both Malawi and South Africa, the observed decline in iNTS disease was temporally associated with improved management of HIV/AIDS(121, 122). A strong inverse correlation in South Africa was observed between the use of antiretroviral therapy and the incidence of iNTS between 2003-2013. During the same period, incidence of viral load testing increased from 75 to 3,620/10^5 indicating improved access to healthcare(122).

It is difficult to disentangle the contributions of declining malaria, declining HIV morbidity and other secular trends in the decreasing incidence of iNTS disease, though there have been some bold attempts. Using Structural Equation Models, researchers from Malawi, modelled the interactions between iNTS disease and malaria, HIV, malnutrition, rainfall, and time. The results showed a direct relationship between iNTS and malaria, iNTS and malnutrition, iNTS and HIV, an indirect relationship between iNTS and rainfall through its effect on malaria and malnutrition, and an additional indirect relationship between iNTS and HIV through its effect on malnutrition. There was a direct negative relationship between time and iNTS, malaria and HIV, interpreted as a marker of improvements in the control programmes for these diseases(123). Tack *et al.*, used principal component analysis to model the contributions of environmental factors (represented by rainfall and temperature) and host risk factors (represented by hospital disease data) to explain the associations between these factors and iNTS disease. They found that there was a direct association between rainfall and iNTS, and an indirect one through host associated factors(124). Finally, Thindwa *et al.*, observed a direct

association between rainfall and iNTS which was short lasting but occurred immediately after the rains(125). The two studies suggested that control of environmental factors would improve control of iNTS. The three studies together increased our understanding on the relationship between iNTS and both host and environmental factors but did not give a measure of their relative contribution or attributable fractions. In addition, changes in the rates of acquisition of infection and pathogen factors such as changing virulence, have not been assessed, and therefore their contribution to iNTS disease incidence remains unknown. While it is possible that controlling one or more of these factors would have an impact on the incidence of iNTS disease, it is not known by how much. Prevention strategies aimed at reducing transmission include improvements of WASH practices, identification and treatment of chronic carriers who might be super-spreaders, control of Salmonellosis in food animals to reduce possible zoonotic spread, increased hospital births vs. home births to reduce possible vertical transmission during delivery through unsanitary practices and treatment of Schistosoma worms that may promote persistence of NTS in the gut. Strategies aimed at reducing invasive disease include management of host factors such as malaria, HIV, and malnutrition.

Vaccination

Vaccination has been proposed as a tool for control of iNTS disease(126). There are no licensed iNTS vaccines in humans currently though several candidate vaccines are in different stages of the vaccine development pipeline (Table 1.3). Only one NTS vaccine candidate has successfully completed clinical trials in the past. This was the WT05 vaccine (Microscience), an oral live attenuated vaccine which elicited specific IgA responses at the highest dose given (10^9 CFU) but was associated with prolonged duration of shedding of the bacteria, which was a major limitation(127).

Two candidates, a Generalised Modules for Membrane Antigen (GMMA) based vaccine from GSK Vaccines for Global Health (GVGH) (128, 129)and a Trivalent Salmonella Conjugate Vaccine (TSCV;CVD1000) from Center for Vaccine Development (CVD) are in Phase 1 clinical trials. The

GMMA vaccine targets both *S.* Typhimurium and *S.* Enteritidis, while the TSCV vaccine targets the same NTS serotypes plus *S.* Typhi. It is unclear what the target age-group and population of the combined NTS-Typhoid vaccine would be as both diseases, though caused by *Salmonella* bacteria, occur at different ages. NTS occurs in infancy while Typhoid fever occurs most commonly in school-age children. In addition, NTS occurs in rural areas while Typhoid is predominantly found in urban areas, especially where access to water and sanitation facilities is poor and hygiene practices are compromised (130, 131). Both have been shown to protect against iNTS disease in pre-clinical studies, though activity against carriage has not been assessed.

Table 0.3: Overview of iNTS vaccines in humans in different phases of vaccine development (adapted from WHO).

Type	Vaccine	Description	Developer/ Manufacturer
Live attenuated vaccine	CVD 1931 and CVD 1944	CVD 1931: S. Typhimurium D65 ΔguaBA ΔclpX CVD 1944:S. Enteritidis R11 ΔguaBA ΔclpX	University of Maryland
Conjugate vaccine	CVD 1000 (Trivalent COPS:FliC and TCV)	Trivalent vaccine with S. Typhimurium O:1,4[5],12:H:i and S. Enteritidis O:1,9,12:H:g,m conjugated to flagellin co-formulated with Vi-TT typhoid conjugate vaccine	University of Maryland's Center for Vaccine Development and Global Health (CVD), and Bharat Biotech International Ltd (India)
Conjugate vaccine	Bivalent NTS conjugate vaccine	Bivalent conjugate vaccine inclusive of S. Typhimurium and S. Enteritidis	International Vaccine Institute (IVI), and SK Bioscience (Republic of Korea)
Conjugate vaccine	Trivalent NTS/Typhoid conjugate vaccine	Trivalent S. Typhi/S. Typhimurium/ S. Enteritidis building on Vi-DT typhoid conjugate vaccine	IVI, and SK Bioscience
OMV-based vaccine	iNTS-GMMA	Bivalent S. Typhimurium Generalized Modules for Membrane Antigens (GMMA) containing O:1,4[5],12 OAg plus S. Enteritidis GMMA containing O:1,9,12 OAg, adsorbed on Alhydrogel	GSK Vaccines Institute for Global Health (GVGH)
MAPS-based vaccine	Bivalent NTS MAPS	Bivalent vaccine against S. Typhimurium and S. Enteritidis Multiple Antigen Presenting System (MAPS)	Boston Children's Hospital
OMV-based vaccine	iNTS-GMMA and TCV	Trivalent vaccine with iNTS-GMMA and Vi-CRM197 typhoid conjugate vaccine	GVGH, and Biological E Ltd (India)
Conjugate vaccine	Bivalent OAg-CRM197	S. Typhimurium O:1,4[5],12 OAg conjugated with CRM197 plus S. Enteritidis O:1,9,12 OAg conjugated with CRM197	GVGH
Protein-based vaccine	OmpD	Outer membrane protein D (OmpD) purified from whole bacteria	University of Birmingham

A vaccine with activity against carriage would have indirect protective effects on the population; by interrupting transmission even unvaccinated populations would benefit from the use of the vaccine, as seen in the control of meningitis through polysaccharide-protein conjugate vaccines against *Streptococcus pneumoniae*, *Hemophilus influenzae b* and *Neisseria meningitidis* serogroup C disease (132).

As the iNTS vaccine development pipeline continues, the knowledge gaps on the epidemiology of transmission and infection identified in this narrative would need to be closed to facilitate effective vaccine implementation strategies and policies. Specifically for Kenya, an iNTS disease endemic country (3, 17) preparing for upcoming iNTS vaccine trials (133), the need for informed vaccine policy is urgent. This doctoral research project seeks to strengthen our knowledge of the transmission of NTS infections in order to inform intervention strategies, especially vaccination.

Aims and Objectives of the Studies

Aims

The overall aim of this research is to Strengthen the understanding of faecal carriage and the rates of transmission of non-typhoidal Salmonella in Kenya to provide the evidence base for informing NTS control options. This aim has been broken down into several objectives, corresponding to the chapters in this thesis as below:

4.1.1 Carriage study:

The aim of this study was to determine the prevalence of carriage of NTS among healthy children and adults and associated risk factors.

Specific objectives:

1. To estimate the age-related prevalence of faecal carriage of NTS across three sites in Kenya.
2. To identify the risk factors associated with faecal carriage of NTS.
3. To describe the phenotypic diversity of the NTS isolated in faecal carriage through serotyping and antibiograms.

4.1.2 Seroprevalence study:

The aim of this study was to estimate the exposure of children and adults to NTS through estimation of antibody responses.

Specific objectives:

1. To estimate the age-related concentrations and seroprevalence of anti-Salmonella antibodies across three sites in Kenya.
2. To describe the presence (or absence) of maternally-acquired anti-Salmonella antibodies in infants.
3. To estimate the rate of decay of maternally-acquired IgG antibodies.

4. To describe the relationship between anti-Salmonella antibody concentrations and faecal carriage of NTS.

4.1.3 Catalytic modelling study:

The aim of this study was to develop a catalytic model of NTS transmission dynamics to estimate rate of acquisition of NTS using serological data.

Specific objectives:

1. To develop and determine the best fitting catalytic model from different assumptions of NTS transmission.
2. To estimate the age-related force of infection (FOI) of NTS across three sites in Kenya from the final model.

4.1.4 Longitudinal trends in transmission of NTS study:

The aim of this study was to determine the changes in force of infection of NTS over time in Kilifi and whether these were associated with the observed decline in incidence of iNTS over time, in the background of changing malaria prevalence.

Specific objectives.

To describe the age-related concentration of antibodies and seroprevalence over time across two locations in Kilifi with differing prevalence of malaria.

1. To estimate the age-related force of infection of NTS across two locations in Kilifi
2. To describe the relationship between changes in force of infection of NTS and changes in incidence of iNTS over time.

2.2 Thesis Structure and bridging information.

This thesis in Chapter format is organized into 6 chapters as follows:

Chapter 1: Introduction:

This consists of a narrative literature review of iNTS burden and NTS transmission according to current knowledge. It highlights the knowledge gaps that the doctoral project is designed to address.

Chapter 2: Aims and Objectives:

This is the current chapter which provides an outline of the thesis.

Chapter 3: Carriage prevalence:

This chapter introduces the field study across 3 sites in Kenya whose results are explored in both the carriage prevalence chapter and the seroprevalence chapter. This was a huge collaborative effort between 3 centres which operate under the umbrella of KEMRI (Kenya Medical Research Institution) but operate independently. To get the fieldwork started, I spearheaded the negotiation of MOUs between the two institutions: KEMRI-Centre for Microbiology Research in Nairobi, KEMRI- Centre for Geographical Health Research in Kisumu and our own, KEMRI-Wellcome Trust Research Programme. This enabled transfer of samples, laboratory consumables, training and staff funds across the institutions. In each field site, we conducted public engagement activities about the study and enteric diseases in general - gatherings that were attended by hundreds of community members. We adapted the study tools (ICF & questionnaire) into the local language for ease of administration and understanding. These experiences enriched my learning and were an informative introduction to project management of field epidemiology in remote places. In addition, during the conduct of the fieldwork in Siaya, violence broke after the Kenya National Elections results were declared and we paused fieldwork for almost 6 weeks until normalcy resumed. During this break, participant follow-up was not possible. Consequently, we were unable to measure the duration of carriage among our participants with positive carriage, which was an additional objective that was subsequently abandoned.

Funding for this work was made possible through a doctoral fellowship from the Initiative to Develop African Leaders (IDeAL) programme housed at KWTRP, and additional funds for fieldwork provided through my supervisor Anthony Scott's senior fellowship funding from the Wellcome Trust.

The results of this chapter have been presented as a poster at the 11th International conference on Typhoid and Other Invasive Salmonellosis and the manuscript is now published at the PLOS Neglected Tropical Diseases journal.

Muthumbi EM, Mwanzu A, Mbae C, Bigogo G, Karani A, Mwarumba S, Verani JR, Kariuki S, Scott JAG. **The epidemiology of fecal carriage of nontyphoidal Salmonella among healthy children and adults in three sites in Kenya**. PLoS Negl Trop Dis. 2023 Oct 26;17(10):e0011716. doi: 10.1371/journal.pntd.0011716. PMID: 37883602; PMCID: PMC10629669.

Additional output from this work includes a collaboration with the viral epidemiology group at KWTRP who utilized the stored stool samples from my study as controls to describe the aetiology of diarrhoea in Kilifi and inform rotavirus interventions. Published work highlighted below:

Agoti, C.N., Curran, M.D., Murunga, N., Ngari, M., **Muthumbi, E.,** Lambisia, A.W., Frost, S.D.W., Blacklaws, B., Nokes, D.J., Drumright, L.N **Differences in epidemiology of enteropathogens in children pre- and post-rotavirus vaccine introduction in Kilifi, coastal Kenya.** *Gut Pathog* **14**, 32 (2022). https://doi.org/10.1186/s13099-022-00506-z

Chapter 4: Seroprevalence:

This chapter consists of the laboratory and statistical analysis of the serology data collected in the 3 site field study. It is divided into two sections for ease of readability. The first section consists of the age-seroprevalence analysis and results while the second section consists of the catalytic modelling. The two sections have a common introduction piece and discussion, but separate methods and results sections.

Having only previously interacted with bench bacteriology in the laboratory, this work introduced me to immunological assays, mostly ELISAs. We established a collaboration with Calman MacLennan and Sean Elias from the Jenner Institute, and I travelled to Oxford to learn the NTS ELISA that was

operational in their laboratory. I spent one week learning ELISAs from basic principles to more advanced analysis, afterwards I spent 2 weeks in the laboratory performing assays for the first batch of samples from the seroprevalence study. I returned to Kilifi and setup the assays to enable further ELISAs to be performed locally.

Funding for this training was possible through a travel grant from AfOx initiative (£5,000). During the visit, we cemented the collaboration and were together granted additional funding for my next chapter.

Additional output from this work:

Elias SC, **Muthumbi E**, Mwanzu A, Wanjiku P, Mutiso A, Simon R, MacLennan CA. **Complementary measurement of nontyphoidal *Salmonella*-specific IgG and IgA antibodies in oral fluid and serum.** Heliyon. 2022 Dec 15;9(1):e12071. doi: 10.1016/j.heliyon.2022.e12071. PMID: 36704288; PMCID: PMC9871079.

Kapulu, M.C., Muthumbi, E., Otieno E., Rossi O., Ferruzzi P., *et al* Age-dependent acquisition of IgG antibodies to *Shigella* serotypes – a retrospective analysis of sero-prevalence in Kenyan children, Manuscript in preparation: Submitted for peer review in Frontiers of Immunology **Chapter 5: Longitudinal study**:

This chapter utilizes results from the seroprevalence chapter analysis to develop catalytic models of NTS transmission dynamics which are then applied to previously published estimates of iNTS incidence in Kilifi.

The start of the laboratory analysis of these samples was delayed due to COVID-19 related delays. Shipment of the laboratory consumables was delayed and access to the laboratories at KWTRP for non-COVID work was halted for several months.

Funding for this work was possible through a BactiVac Catalyst Award (£50,000). The results of this chapter were presented at the 3rd Annual BactiVac Network meeting in 2022. **Chapter 6: Discussion**:

I conclude with an overall discussion of the study's outputs, strengths, and limitations. In it I restate the principal findings, I discuss how the different chapters link together and close with my thoughts on how my PhD has advanced knowledge in the subject area and thoughts of future work.

The epidemiology of faecal carriage of nontyphoidal *Salmonella* among healthy children and adults in three sites in Kenya.

3.1 Introduction

Transmission of Non-typhoidal Salmonellae (NTS) has been associated with contamination of food, and with direct contact with chickens or reptiles(30, 134, 135). These findings come from high-income countries where NTS disease is characterized by a self-limiting enterocolitis, with an incidence of 634 per 100,000 cases per year in Europe and North America and a case fatality ratio (CFR) of 0.2%(13). By contrast, NTS disease in Africa is more frequently invasive, causes little or no diarrhoea, and has a higher CFR(14). In 2017, the incidence of invasive NTS (iNTS) disease was 34.5 cases per 100,000 person-years and CFR of 16% across sub-Saharan Africa(15).

The serotypes most commonly causing iNTS disease in Africa are *Salmonella* Typhimurium and *Salmonella* Enteritidis. These serotypes have been isolated from several sources including humans, animals, water and other environmental sources supporting a generalist lifestyle (25). However, genetic analysis in Kenya has shown that *Salmonella* Typhimurium isolates obtained from index cases of NTS bacteraemia in children and their human contacts are identical but are distinct from those found in their animal contacts(39). Similar observations have been made in The Gambia (41) and Burkina Faso(136). Whole genome sequencing of invasive *S.* Typhimurium and *S.* Enteritidis isolates from sites in Africa led to the discovery of a novel sequence type of *Salmonella* Typhimurium, ST313, and a novel clade of *Salmonella* Enteritidis, ' the African clade', which are leading causes of iNTS in Africa and whose genomes have undergone changes in a pattern that is associated with host restriction, similar to *Salmonella* Typhi(35, 38). If humans are a major reservoir of NTS in Africa, then the subgroup of the population that are carriers may be the main source of infection.

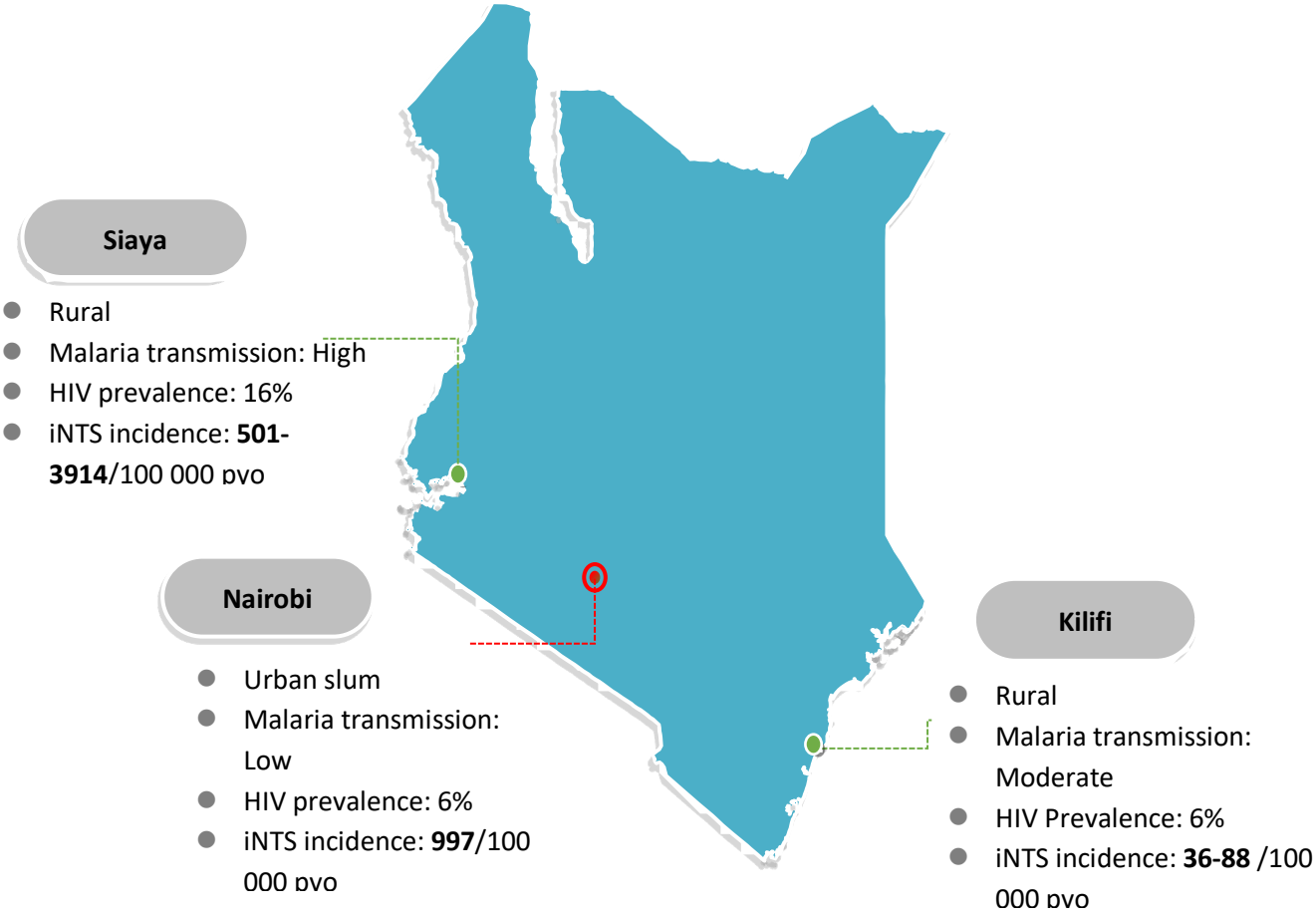
Faecal shedding, especially in settings with suboptimal hygiene and sanitation, could lead to high

transmission rates driving high risk of invasive disease. However, the epidemiology of carriage of NTS and its association with iNTS in Africa, are not well understood. We aimed to explore the epidemiology of carriage in Kenya by studying healthy individuals from three sites with different incidence of iNTS disease.

Method

The study sites were: Junju location in Kilifi County, Mukuru in Nairobi County, and Asembo location in Siaya County. Junju location is a rural setting with a population of approximately 34,000 people over 115 km^2. Mukuru is a densely populated urban slum area, with approx. 250,000 people in an area of 3 km^2, Asembo is a sparsely populated rural location, with a population of approximately 25,000 people over 100 km^2 in a defined surveillance catchment area(137) (138).The distribution of risk factors for iNTS disease differs by site. Malaria prevalence was estimated as 8%, 0.2% and 26% in Kilifi, Nairobi and Siaya(139), respectively, while HIV prevalence was estimated as 3%, 5% and 14%, respectively(140). The incidence of iNTS disease in children under 5 years is estimated as 36-88 per 100,000 person-years in Kilifi and 501- 3,914 per 100,000 person-years in Siaya(3, 17, 66). The incidence of iNTS in Mukuru slums has not been estimated previously but can be inferred from that of neighbouring Kibera slums as 255 – 998 per 100,000 person-years(17).

Figure 3.1: Map of Kenya showing the three study sites: Kilifi, Nairobi, Siaya



In Kilifi and Siaya we used existing population registers as our sampling frame; these were available from the Kilifi Health and Demographic Surveillance System (KHDSS) (116) and from the KEMRI/CDC demographic surveillance system(141), respectively. In Nairobi, we established a household-based sampling frame by interpreting satellite images of the terrain. In Kilifi and Siaya we undertook age-stratified, simple random sampling of the entire population register of the Junju and Asembo locations. In Nairobi, we used random household-block sampling followed by systematic age-based sampling to populate the sample in each age stratum.

At the time of study initialization, there were no published estimates of faecal carriage of NTS in Africa with systematic sampling and laboratory methods. Informal estimates from a Junju based

cohort under surveillance for malaria events indicated a prevalence of 1.2% using standard culture methods (Francis Ndungu, personal communication). In Nairobi, the prevalence of carriage was estimated at 2-4% based on control samples from a Salmonella surveillance project on-going in the Mukuru slum area (Sam Kariuki, personal communication). Using the lower estimate of prevalence, and assuming a confidence level of 95% (alpha=0.05), we calculated that a sample size of 1500 would enable estimation of the prevalence of carriage with a precision of +/-0.5%. Moreover, a sample size of 489 per site would enable estimation of a 3% difference in prevalence between the sites with 80% power and a 95% confidence level. We set out to sample 500 participants per site as this would cover for a 3% loss of stool samples from any cause. At each site we sampled 100 participants in each of five age-strata: 1-11 months, 12-59 months, 5-14 years, 15-54 years, 55 years and above. Random lists were created in each site in excess of the sample size targeted and any selected individuals who were absent or unwilling to participate (or any households in Nairobi that did not contain an age-appropriate participant) were replaced by selecting additional participants from the bottom of the random list.

To create a sampling frame in Mukuru, Nairobi we used Google Earth™, a GIS software (QGIS) and a high-resolution satellite image of the area. On Google Earth™, we selected the study area and used the resulting shape file with coordinates of the study area to acquire a geo-referenced 0.5m resolution satellite image (accuracy 4.5m) from Pleiades satellites through Harris MapMart, a satellite image supplier (Lone Tree, Colorado, USA). The satellite pass had occurred on September 18th, 2016, 8 months prior to the study; we did not expect major changes in spatial distribution of homes within the short period of time. We generated random points in the mapped study area and excluded points on schools, churches and community halls when the points were identified in maps from Open Street Maps (http://openstreetmaps.org). At high resolution, the area had already been mapped into residential blocks by an ongoing Salmonella project(142). In blocks selected by random points we selected homes using the 'spinning pencil' technique and used a systematic approach to

select the age-group of the participant starting with the youngest age group and working up.

Following informed consent, study participants were given the choice to be interviewed at their home or in a local health clinic. A questionnaire was administered, and the participant was asked to provide a stool sample. The questionnaire inquired about hygiene and sanitation practices, food and water consumption, ownership and contact with animals, clinical status in the 2 weeks prior to recruitment including pregnancy and household crowding. These risk factor variables selected for inclusion in the questionnaire were based on factors likely to be associated with food or waterborne transmission of infections. The questionnaire used in the collection of the data can be found in the Appendix. The variables collected were self-reported by the participants or guardians in case of minors. For those interviewed at the clinic, a blood sample was taken for HRP2-based *P. falciparum* malaria rapid test and haemoglobin measurement (Mission strips), as recent malaria, malaria and malnutrition are known risk factors for invasive NTS disease (25). Anthropometric measures (height/length, weight and Mid Upper Arm Circumference [MUAC] for children under 5 years) were also taken.

Those unable to produce a stool sample immediately were given a sterile pot for stool sampling at home the following morning and the sample was collected by study fieldworkers later that day. An aliquot of the stool sample was inserted in Cary-Blair transport media for culture and preserved at 2-4^{o}C in a cooler. Within 8 hours of collection, the stool samples were transported to the Microbiology laboratory for processing.

Sampling was done in October-November 2016 in Kilifi, May-September 2017 in Nairobi and June-September 2017 in Siaya. No samples were collected between 1st and 23rd August 2017 due to the Kenyan National elections.

Samples from Kilifi, Nairobi and Siaya were processed in laboratories at KEMRI-Wellcome Trust Programme in Kilifi, KEMRI-Centre for Microbiological Research in Nairobi and KEMRI Centre for Global Health Research- laboratories in Kisumu, respectively. The data collection tools, and laboratory methods were standardized across all sites.

In the laboratory, culture samples were enriched in Selenite F broth (Oxoid microbiological products) and sub-cultured on to selective media (Xylose Lysine Deoxycholate agar (XLD), Salmonella/Shigella agar (SS) agar and Brilliant Green media) to select for *Salmonella enterica* species. Species were confirmed using biochemical testing (API 20E). *Salmonella* Typhimurium and *Salmonella* Enteritidis were determined using the Kauffman-White scheme and commercial antisera. Anti-Vi antisera was used to determine identity for *S.* Typhi. Antimicrobial susceptibility testing was performed and interpreted, using Clinical Laboratory Standards Institute (CLSI) guidelines, for the following antimicrobials: ampicillin 10µg, amoxicillin/clavulanate 20/10 µg, cotrimoxazole 1.25/23.75µg , cefotaxime 10µg, chloramphenicol 10µg, ciprofloxacin 5µg, ceftazidime 30µg, ceftriaxone 30µg, imipenem 10µg, cefoxitin 30µg and amikacin 30µg(143).

The prevalence of carriage in stool in each age stratum was estimated as the proportion of sampled individuals who had a positive stool culture and exact 95% confidence intervals were calculated. To correct for the bias in the sampling scheme total population prevalence for each site was age-standardized using the age-structure of the local population as population weights.

We defined underweight (weight-for-age Z score [WAZ] <-2); wasting (weight-for-height Z score [WHZ] <-2); and stunting (height-for-age Z score [HAZ] <-2) for children under 5years using WHO Child Growth Standards. Anaemia was defined as haemoglobin <10g/dl, and recent malaria infection was defined as malaria HRP-2 rapid test positive. Risk factor analyses were conducted using Poisson regression with robust standard errors to estimate prevalence ratios. The outcome variable was

presence of NTS in the stool samples. Variables with an association of $p<0.05$ were selected for inclusion in a multivariable regression model, including age, sex and site as *a priori* explanatory variables. Statistical analyses were performed using STATA v.13.

Informed consent was obtained from all adult participants and from the guardians of children aged <18 years old. Assent was obtained from all participants aged 13-17 years old. The study was approved by the KEMRI Scientific and Ethics Research Unit (SERU No. 3221).

Results

In Kilifi, 775 residents were selected for inclusion in the study, 506 (65.3%) were recruited and 494 (63.7%) were sampled. In Nairobi 750 random points were generated by the software, 510 (68%) participants were recruited and 504 (67.2%) were sampled. In Siaya, 607 residents were selected for the study, 506 (83.4%) were recruited and 499 (82.2%) were sampled. In total, we sampled 1,497 participants; the median age was 7y (IQR 1y,30y) and 703 (47%) were male (Table 3.1).

Table 0.1: Participant characteristics at enrolment, by site.

		Kilifi		Nairobi		Siaya	
		n/N	%	n/N	%	n/N	%
Participant characteristics							
Sex	Male	220/494	44.5	266/504	52.8	217/499	43.5
Pregnant, in females >18y		2/115	1.7	3/73	4.1	2/126	1.6
Recent malaria	Positive	23/307	7.5	11/443	2.5	139/489	28.4
Wasting, in < 5y	WHZ <-2	20/191	10.5	16/235	6.8	6/200	3
Stunting, in < 5y	HAZ <-2	50/210	23.8	46/235	19.6	42/200	21
Underweight, in < 5y	WAZ <-2	30/191	15.7	20/235	8.5	16/200	8
Acute Malnutrition, in < 5y	MUAC <12.5cm	34/209	16.3	54/233	23.2	8/200	4
Fever in last 2 weeks	Yes	61/458	13.3	47/486	9.7	152/499	30.5
Diarrhoea in last 2 weeks	Yes	25/458	5.5	61/486	12.6	35/499	7
Admitted in last 2 weeks	Yes	7/458	1.5	11/486	2.3	7/499	1.4
Household characteristics							
Household member fever in last 2 weeks	Yes	44/458	9.6	26/486	5.4	156/499	31.3
Household member diarrhoeal in last 2 weeks	Yes	17/458	3.7	40/486	8.2	39/499	7.8
WASH Practices							
Source of drinking water	Piped water	80/458	17.5	4/486	0.8	18/499	3.6
	Public tap	236/458	51.5	482/486	99.2	214/499	42.8
	River/Lakes	1/458	0.2	0/486	0	77/499	15.4
	Well/Dams/Borehole	141/458	30.8	0/486	0	190/499	38.1
Uses soap for hand washing	Yes	351/458	76.6	471/486	96.9	463/499	92.8
Uses shared basin for washing hands	Yes	192/458	41.9	131/486	26.9	58/499	11.6
Toilet Type	Modern with/without flush	50/458	10.9	63/486	12.9	2/499	0.4
	Pit latrine	394/458	86.1	392/486	80.7	479/499	95.9
	None/Open defecation	10/458	2.2	1/486	0.2	10/499	2
	Other	4/458	0.1	30/486	6.2	8/499	1.6

		Kilifi		Nairobi		Siaya	
		n/N	%	n/N	%	n/N	%
Animal Ownership	Cattle	61/458	13.3	2/486	0.4	305/499	61.1
	Sheep	4/458	0.9	1/486	0.2	142/499	28.5
	Goats	129/458	28.2	8/486	1.7	194/499	38.9
	Pigs	1/458	0.2	1/486	0.2	3/499	0.6
	Chickens	256/458	55.9	57/486	11.7	452/499	90.6
Animal Contact	Cattle	51/458	11.1	228/486	46.9	388/499	77.8
	Sheep	5/458	1.1	294/486	60.5	275/499	55.1
	Goats	71/458	15.5	355/486	73.1	328/499	65.7
	Pigs	5/458	1.1	245/486	50.4	9/499	1.8
	Chicken	178/458	38.9	388/486	79.8	464/499	92.3

Table 0.2: Age and sex-related prevalence of stool carriage of NTS in Kenya

	Kilifi			Nairobi			Siaya		
	n/N*	**%**	**95% CI**	**n/N***	**%**	**95% CI**	**n/N***	**%**	**95% CI**
Female									
0-11 m	3 / 55	5.5	1.1, 15.1	0 / 44	0		1 / 48	2.1	0,11.1
12-59 m	4 / 51	7.8	2.2, 18.8	0 / 50	0		0 / 62	0	
5-14 y	7 / 45	15.6	6.5, 29.5	0 / 72	0		0 / 51	0	
15-54 y	10 / 53	18.9	9.4, 31.9	1 / 57	1.8	0, 9.4	1 / 51	1.9	0,10.4
55+ y	6 / 70	8.6	3.2, 17.7	0 / 15	0		1 / 70	1.4	0, 7.7
Total	30 / 274	10.9	7.5, 15.3	1 / 238	0.4	0, 2.3	3 / 282	1.1	0.2, 3.1
Male									
0-11 m	5 / 71	7.0	2.3, 15.7	0 / 64	0		1 / 49	2	0, 10.9
12-59 m	2 / 43	4.7	0.6, 15.8	0 / 57	0		1 / 41	2.4	0, 12.9
5-14 y	3 / 40	7.5	1.6, 20.4	0 / 79	0		0 / 57	0	
15-54 y	5 / 33	15.2	5.1, 31.9	0 / 49	0		0 / 33	0	
55+ y	1 / 33	3.0	0, 15.8	0 / 17	0		1 / 37	2.7	0, 14.2
Total	16 / 220	7.3	4.2, 11.5	0 / 266	0		3 / 217	1.4	0.3, 4.0
All									
0-11 m	8 / 126	6.4	2.8, 12.1	0 / 108	0		2 / 97	2.1	0.3, 7.3
12-59 m	6 / 94	6.4	2.4, 13.4	0 / 107	0		1 / 103	0.9	0, 5.3
5-14 y	10 / 85	11.8	5.8, 20.6	0 / 151	0		0 / 108	0	
15-54 y	15 / 86	17.4	10.1, 27.1	1 / 106	0.9	0,5.1	1 / 84	1.2	0, 6.5

55+ y	7 / 103	6.8	2.8, 13.5	0 / 32	0		2 / 107	1.9	0.2, 6.6
Total	46 / 494	9.3	6.9, 12.2	1 / 504	0.2	0,1.1	6 / 499	1.2	0.4, 2.6
Age-standardized rates (local population)		13.1	8.8, 17.4		0.9	0, 2.0		0.4	0, 1.3

*n/N number of positive cultures divided by the number of stool samples culture

Out of the 1,497 stool samples collected, 53 NTS isolates were recovered (crude prevalence 3.5%, 95% CI 2.6, 4.6%). Crude carriage prevalence was 9.3% (46/494) in Kilifi, 0.2% (1/504) in Nairobi and 1.2% (6/499) in Siaya (Table 2). In Kilifi, the prevalence varied significantly by age (p=0.035), starting from low in infancy, peaking at 17.4% at 15-54 years and declining in older age groups. The prevalence did not vary significantly by sex (p=0.123). When standardized with the age-specific population weights at each site, the total population prevalence was 13.1%, 0.4%, 0.9% in Kilifi, Nairobi and Siaya, respectively.

Serotype distribution and antimicrobial susceptibility patterns

Out of the 53 NTS isolates, 5 were *S.* Enteritidis, one was *S.* Typhimurium and the remaining 47 could not be typed fully by the anti-sera available (Table 3). Of the *S.* Enteritidis serotypes, 3 were in infants, 1 in a child aged 12-59 months and 1 in an adult aged 15-54 years, while the single isolate of *S.* Typhimurium was in an adult aged >55 years. *S.* Typhi was not isolated in any of the sites. The distribution of *S*. Enteritidis and *S*. Typhimurium, did not vary by site, however the prevalence of the serotypes other than Group B or D was higher in Kilifi (43/494, 8.7%), than in Nairobi (1/504, 0.2%) and Siaya (3/499, 0.6%), p<0.001. All the 53 NTS isolates were susceptible to the antibiotics tested in the panel.

Table 0.3: Serotype distribution of NTS isolates by site

	Kilifi	**Nairobi**	**Siaya**	**Total**
S. Enteritidis	3	0	2	5
S. Typhimurium	0	0	1	1
Other Group D	3	0	0	3
Other Group B	11	0	1	12
Serotypes other than Group B or D	29	1	2	32
Total	**46**	**1**	**6**	**53**

Risk factors associated with faecal carriage of NTS.

The distribution of risk factors among the participants varied by site (Table 3.1). The prevalence of anaemia (haemoglobin <10g/dl) was 20% (58/290) in Kilifi, 7% (31/433) in Nairobi and 9% (20/277) in Siaya. Prevalence of asymptomatic *P. falciparum* infection was 8% (23/307) in Kilifi, 3% (11/443) in Nairobi and 28% (139/489) in Siaya. Among children less than 5 years, those with acute malnutrition (MUAC <12.5cm) were 16% (34/209) in Kilifi, 23% (54/233) in Nairobi and 4% (8/200) in Siaya. The type of animal kept also differed by site: there were more animals kept in Siaya than in any other site, but chicken farming was practiced across all sites. As expected, more animals were kept among participants in the rural areas than in the urban slum area.

In the univariate analysis of risk factors for prevalent NTS carriage among participants, anaemia (haemoglobin <10g/dl), recent malaria and malnutrition were not associated with NTS carriage (Table 3.4). Risk of carriage was higher among participants who use a shared basin for washing hands than among those who wash separately (Prevalence Ratio 2.7, 95% CI 1.6, 4.7), but was not associated with any of the other WASH related exposure categories assessed. Ownership of cattle and sheep was associated with reduced risk of carriage of NTS. In addition, contact with cattle, goats, sheep, or chickens was also associated with a reduced risk of carriage of NTS. We did not include data from Nairobi in this analysis because there was only one positive culture among 504 samples tested.

In the multivariable model, including age and site as covariates, none of the risk factors that were significant on univariate analysis were associated with faecal carriage of NTS.

Table 0.4: Risk factors associated with faecal carriage of NTS in Kilifi and Siaya.

	Prevalence in exposed		Prevalence in unexposed		Prevalence ratio		Adjusted prevalence ratio	
Variable	**n/N**	**%**	**n/N**	**%**	**ratio**	**95% CI**	**ratio**	**95% CI**
Host Factors								
Sex, female	33/556	5.9	19/437	4.4	1.4	(0.8,2.4)		
Recent malaria	4/162	2.5	31/634	4.9	0.5	(0.2,1.4)		
Underweight (WAZ <-2)[†]	2/46	4.4	14/345	4.1	1.1	(0.3,4.6)		
Wasting (WHZ <-2)[†]	3/26	11.5	13/365	3.6	3.2	(0.9,10.7)		
Stunting (HAZ <-2)[†]	4/92	4.4	13/318	4.1	1.1	(0.4,3.2)		
Acute malnutrition (MUAC<12.5)[†]	4/42	9.5	13/367	3.5	2.7	(0.9,7.9)		
Anaemia (Hb<10)	3/78	3.9	27/450	6.0	0.6	(0.2,2.1)		
Clinical illness in last 2 weeks								
Admitted to hospital	0/14	0	47/943	5.0	-			
Diarrhoea	1/60	1.7	46/897	5.1	0.3	(0.04,2.3)		
Fever	8/213	3.8	39/744	5.2	0.7	(0.3,1.5)		
Household illness in last 2 weeks								
Household member had diarrhoea	3/56	5.4	44/901	4.9	1.1	(0.4,3.4)		
Household member had fever	10/200	5.0	37/757	4.9	1.0	(0.5,2.0)		
WASH Practices								
Source of drinking water								
Piped water	8/98	8.2			Ref			
Public tap/vendors	24/450	5.3			0.7	(0.3,1.4)		
River/Stream/Lake	1/78	1.3			1.6	(0.0,1.2)		
Well/Dam/Borehole	14/331	4.2			0.5	(0.2,1.2)		
Uses soap to wash hands	38/814	4.7	9/143	6.3	0.7	(0.4,1.5)		
Uses shared wash basin	23/250	9.2	24/707	3.4	2.7	(1.6,4.7)	1.4	(0.8,2.5)
Type of toilet								
Modern with/without flush	5/52	9.6			Ref			
Pit latrine	39/873	4.5			0.5	(0.2,1.1)		

	Prevalence in exposed		Prevalence in unexposed		Prevalence ratio		Adjusted prevalence ratio	
Variable	**n/N**	**%**	**n/N**	**%**	**ratio**	**95% CI**	**ratio**	**95% CI**
None/Open defecation	1/20	5.0			0.5	(0.1,4.2)		
Other	2/12	16.7			1.7	(0.4,7.9)		
Animal Ownership								
Cattle	9/366	2.5	38/591	6.4	0.4	(0.2,0.8)	1.5	(0.6,4.0)
Sheep	1/146	0.7	46/811	5.7	0.1	(0.0,0.9)	0.5	(0.1,3.6)
Goats	10/323	3.1	37/634	5.8	0.5	(0.3,1.1)		
Pigs	0/4	0	47/953	4.9	-			
Chicken	30/708	4.2	17/249	6.8	0.6	(0.3,1.1)		
Animal Contacts								
Cattle	7/439	1.6	40/518	7.7	0.2	(0.1,0.4)	0.7	(0.2,2.6)
Sheep	2/280	0.7	45/677	6.7	0.1	(0.03,0.4)	0.6	(0.1,2.7)
Goats	7/399	1.8	40/558	7.2	0.2	(0.1,0.5)	0.8	(0.3,1.9)
Pigs	0/14	0	47/943	4.9	-			
Chicken	17/642	2.7	30/315	9.5	0.3	(0.2,0.5)	0.7	(0.3,1.3)

[†]Prevalence of malnutrition estimated only among children <5 years old

Average household size among carriers was 5.3 (SD 2.5) while that of non-carriers was 5.6 (SD 2.6)

We were interested to explore how NTS risk factors varied by serovar, given the varying ecology of the serotypes. However, this was not possible because there were only 5 isolates of *S*. Enteritidis and 1 *S*. Typhimurium. Instead, we did an exploratory analysis of the variation in risk factors by NTS serogroup, comparing each of serogroup B and D in Kilifi with non-carriers of the serogroup.

In the univariate analysis, the prevalence of serogroup B *Salmonella* in Kilifi was higher among participants whose household member had reported having a febrile illness in the previous 2 weeks

(Prevalence Ratio 5.4 [1.6, 17.7]) and whose household used a shared basin for washing hands (Prevalence Ratio 3.7 [1.0, 13.8]). None of the factors remained significant on multivariable analyses. In addition, the prevalence of serogroup D *Salmonella* in Kilifi was not associated with any of the variables tested.

Discussion

In this study the prevalence of NTS carriage varied very markedly by site from 0.4% and 0.9% in Nairobi and Siaya to 13.1% in Kilifi. Surprisingly, only one resident among 504 in the urban slum site in Nairobi was a carrier of NTS. Overall, serotypes commonly associated with invasive disease accounted for only 6 of 53 carriage isolates and the excess prevalence in Kilifi was driven by serotypes other than *S*. Typhimurium/*S.* Enteritidis. Carriage prevalence in Kilifi peaked in young/middle aged adults but was not associated with sex. We observed no *in vitro* resistance to commonly used antimicrobials tested against our NTS isolates.

The observed carriage prevalence in Kilifi (13.1% all ages and 6.4% in <5y) is higher than that reported in all previous population-based studies on faecal carriage of NTS among children and adults in Africa. For example, carriage prevalence across all ages was 1.0% in Senegal, 2.4% in Guinea-Bissau and 3.4% in Democratic Republic of Congo (DRC) (46, 47) while among children aged <5 years in the Global Enteric Multicentre Study (GEMS), the observed prevalence was 1.3% in The Gambia, 0% in Mali and 0.1% in Mozambique. However, the observed prevalence (1.5%) in Siaya among children aged <5 years in our study is comparable to that observed from GEMS study at the same site (3.2%) (59).

The majority of the invasive serotypes isolated across Africa are *S.* Typhimurium or *S.* Enteritidis(18) though these serotypes represent only 11% (6/53) of the carried isolates in our study. In Senegal, Guinea-Bissau, DRC, The Gambia, Mozambique and Siaya (GEMS), these serotypes represented 0/14 (0%), 0/26 (0%), 9/38 (24%), 0/20 (0%), 0/1 (0%) and 25/61 (41%) of all carriage serotypes isolated,

respectively. The invasiveness of a given serotype can be expressed as the proportion of the serotype among invasive isolates divided by the proportion among carriage isolates. In previous studies in Kilifi, these two serotypes were responsible for 89% of iNTS disease (3), yet account for only 6.5% of carriage in this study (relative invasiveness index 13.7); and in Siaya they were responsible for 98% of iNTS disease (131) and 50% carriage in this study (relative invasiveness index 1.8). This crude ratio is >1 in these examples, suggesting relatively higher invasiveness of the serotypes, but also displaying geographic variability. The outcome of an infection with *Salmonella* serotype depends on host susceptibility (25, 144), infective dose (31) and pathogen virulence(145-147). Consequently, these crude calculations are liable to confounding. Furthermore, within-serotype differences in invasiveness have been demonstrated among different sequence types of *S.* Typhimurium(148), suggesting that the broad serotype classification may not capture all the attributes of invasiveness; classifications based on genotype may be more useful(149).

The antibiograms of the 53 isolates showed a full susceptibility profile which is in marked contrast to the multi-drug resistant profiles seen in other NTS isolates, especially among *S*. Typhimurium serotypes. Elsewhere, shared resistance profiles between asymptomatic and symptomatic isolates have been observed(59, 150-153), suggesting that invasive disease may arise out of the pool of carried strains. However, the transmission of isolates causing asymptomatic carriage and of isolates causing iNTS may also differ and the relationship between antimicrobial resistant strains in carriage and the occurrence of drug resistant invasive disease is not clearly understood.

Within country differences in the incidence of iNTS disease have been documented, and have mostly been attributed to differences in urbanization, agricultural practices, and presence of risk factors for invasive disease such, as malaria and HIV(17, 130). In this study, prevalence of recent malaria was higher in Siaya (28%) than in Kilifi (7%) or Nairobi (2%). There were geographical differences in the occurrence of other host risk factors such as undernutrition in children but none of these were

associated with carriage. Factors affecting incidence of iNTS may differ from those associated with NTS carriage. Given the cross-sectional nature of the study, we cannot estimate how many of the cases of NTS carriage were asymptomatic or preceded or followed an invasive episode, though 18% of the participants had a febrile illness and 8% had a diarrhoea episode in the 2 weeks prior to enrolment in the study. None of these risk factors when analysed was associated with carriage.

Age-specific carriage prevalence noted in this study are similar to observations from Guinea-Bissau(46); notably high carriage prevalence among adults and older children and low carriage prevalence among children aged <5 years. This could be explained by age-related differences in (i) acquisition of infection and (ii) persistence of infection. As NTS is a faecal-oral pathogen, neonates are exposed to it during vaginal delivery(154) but, subsequently, due to limited mobility and breastfeeding, their interactions with environmental sources of NTS in infancy are limited. On the other hand, older children and adults interact with a wider range of sources of NTS through the food supply, which may lead to colonization, albeit with diverse serotypes. Previous studies report a median duration of carriage of 7 weeks in children aged <5 years and 3-4 weeks in older children and adults (53). Given that carriage prevalence rises with age in children, this implies that acquisition rates rise even more steeply with age.

This study was limited by the low sensitivity of stool culture for recovery of NTS. Also, given the cross-sectional nature of the sampling and the intermittent shedding of NTS among carriers, the estimates of prevalence in this study are likely to be biased downwards. Use of multiple samples per time point (47) and use of direct-stool PCR, with enrichment(155), could have improved the yield of *Salmonella*. Lin et al., demonstrated a sensitivity of stool culture for *Salmonella* as 57%, while that of direct stool PCR with enrichment step as 85%, among diarrhoeal stool specimens which would have a larger bacterial load than carriage specimens. We also lack full serotype and genotype data of the isolates, and therefore could not comment on the invasiveness of all the isolates identified nor

characterize the sequence types of interest such as *S*. Typhimurium ST313 and the different clades of *S*. Enteritidis. Unfortunately, we were unable to incorporate any of the results from Nairobi in the risk factors analysis because there was only one positive culture. In fact, the entire risk factor analysis was limited by low statistical power (to detect an association if one existed), given the low prevalence of carriage. The sample size was designed to detect the prevalence of carriage with precision and the analysis of risk factors was only a subsidiary objective.

Despite these limitations, this study provides comparative estimates of the prevalence of NTS carriage across different geographical locations in Kenya. Geographical variation in NTS prevalence was strongly inversely associated with incidence of invasive NTS disease. Kilifi, the area with the lowest incidence of iNTS disease (3, 66) had the highest prevalence of carriage (13%), while Siaya and Nairobi, which are areas of high disease incidence(17), had low carriage prevalence (0.9%, 0.2%). The observation suggests the hypothesis that diverse carriage strains, possibly with lower invasive potential, could have a protective advantage if they induce a degree of cross-reactive immunity among the carriers. Further characterization of the isolates recovered, including sequencing, could enable identification of common antibody targets that might support this hypothesis

Force of infection and age-specific concentrations of antibodies against *Salmonella* Enteritidis and *Salmonella* Typhimurium in three sites in Kenya with different invasive NTS disease incidence

4.1 Introduction

The sequelae of Non-typhoidal *Salmonella* (NTS) infections can be clearance, asymptomatic colonisation of the gut, enterocolitis or invasive disease(12, 25). Hospital-based surveillance captures symptomatic cases, such as those with invasive disease and some diarrhoeal cases, while community-based, cross-sectional studies among healthy participants, as described in the previous chapter, give a snapshot of asymptomatic carriage among those actively shedding the bacteria. Antibodies are good biomarkers of acquisition of infection because infection almost always induces a humoral immune response and some isotypes, such as IgG, are known to increase cumulatively with repeated infection. Quantification of these antibodies may therefore provide a reflection of the cumulative incidence of infection in the community i.e. give an estimate of both past and present exposures to NTS infection (107, 114). Analysis of serological profiles can determine the age distribution at infection with NTS and estimate the rate of acquisition of these infections by age,(156) parameters that are critical in the formulation and deployment of interventions, particularly vaccination. For vaccines that might be designed to interrupt human-to-human transmission, understanding the relationship between the antibodies and the carrier state is also critical(132, 157).

There are currently no licensed vaccines against NTS. However, there are ~10 vaccine candidates against NTS in different stages of the vaccine development pipeline(158, 159). Most focus on the serotypes responsible for the majority of iNTS cases, i.e. *S.* Typhimurium and *S.* Enteritidis, and many of the vaccines are being developed as multivalent formulations; for example, some have been formulated to include typhoidal serotypes. The primary target for these vaccines is infants, who bear the highest incidence of iNTS disease(3). Furthermore, invasive disease in this age group is

associated with severe manifestations such as meningitis and has a high case fatality ratio(62). Previous studies have shown evidence of maternally derived anti-NTS antibodies in infants(106).

Maternal antibodies are known to reduce the efficacy of vaccinations (160). The ideal timing for vaccination in this age-group would therefore be after maternal antibodies have decayed but before exposure to primary infection. Assessment of the decay of maternally derived IgG antibodies in infants against the rate of acquisition of IgA antibodies in infants can identify this window for vaccination.

By identifying the threshold associated with infection, antibody concentrations can be dichotomized as seronegative or seropositive, and the rate of conversion from seronegative to seropositive states (seroconversion rate) can be estimated using catalytic models. Where the infection provides lifelong immunity, this seroconversion rate is the force of infection (FOI) i.e. the rate at which susceptible people in the population acquire an infection(161) (108). Catalytic models have been implemented for several infectious diseases since their introduction in 1934(162) and are widely applied in vaccinology. They can be used to: 1) explore the force of infection by age and identify at-risk age groups, which helps to target control strategies at the right population; 2) Identify the age at first infection, which can determine the optimum age window for vaccination and; 3) estimate the reproductive number and the associated herd immunity threshold, which can inform vaccine deployment strategies. There is no previously published catalytic model of NTS transmission dynamics. With several NTS vaccines in clinical trials, a catalytic model for NTS will be both useful and timely.

There are no standardized assays for the quantification of anti-*Salmonella* antibodies(163) and no commonly agreed threshold for determination of seropositivity status. Published serological studies have used in-house ELISAs and comparisons between studies have not been valid due to differing methodologies(80, 103, 164). Assay specificity is also challenging. ELISAs using the

Lipopolysaccharide (LPS) chain of the outer membrane (including those against O-antigen) as antigen have been shown to capture antibodies raised against other gram negative bacteria which have LPS elements with a similar antigenic structure, such as *Escherichia coli* & *Helicobacter pylori* (102). Depending on the relative prevalence of such gram-negative bacteria, this lack of specificity may lead to over estimation of the seroprevalence.

In the absence of a commonly agreed or defined threshold there are several analytic options: 1) to analyse and characterise the data in their continuous form or; 2) to use modelling approaches that reconstitute unobserved populations (latent classes comprising uninfected and infected populations) given a set of assumptions(165, 166). Mixture modelling is one of the latter approaches. The technique assumes that a given set of data contains a number of unobserved groups within it and that these sub-groups have distinct distributions and characteristics which can be inferred from the frequency distribution of antibody concentrations in the whole population. The technique has been used previously in infectious disease epidemiology to dichotomize populations into seronegative and seropositive groups with respect to malaria, dengue and SARS-COV2 antibodies (167-169).

In the first part of this chapter, I aim to quantify the anti-salmonella IgG and IgA antibody concentrations and dynamics and estimate their seroprevalence in three sites in Kenya. Specifically:

1. To estimate the age-specific geometric mean concentration of anti-typhimurium and anti-enteritidis IgG and IgA antibodies by site.
2. To estimate a threshold for defining seropositivity through mixture modelling.
3. To estimate the age-specific seroprevalence of anti-typhimurium and anti-enteritidis IgG and IgA by site using mixture modelling.
4. To estimate the rate of acquisition of anti-typhimurium and anti-enteritidis IgG and IgA concentrations by age
5. To estimate the rate of decay of maternally derived IgG by age

For exploration, I will assess the association between anti-typhimurium and anti-enteritidis IgG and IgA antibodies with faecal NTS carriage from the paired stool samples analysed in the previous chapter.

In the second part of the chapter, I aim to develop a catalytic model of NTS transmission to estimate the serotype-specific Force of Infection (FOI). Specifically:

6. To determine the best fitting catalytic model for NTS using different assumptions of NTS transmission dynamics
7. To estimate the FOI using the best fitting model

The methods and results of the two parts of the chapter will be presented separately but the discussion of the results will be presented together.

Part One:

4.2 Methods

4.2.1 Field methods

The field study has been described in detail in the previous chapter. Briefly, 1,497 healthy children and adult participants from Kilifi, Nairobi and Siaya counties in Kenya were recruited into a study of faecal carriage of NTS. This involved collection of stool for isolation of NTS, anthropometry measures, spot testing for malaria parasites and haemoglobin concentrations and a questionnaire on WASH practices. During the consenting process, the participants were invited into the serology arm of the study, and a separate consent was sought for collection of a venous blood sample (2.5mls) for testing of anti-Salmonella antibodies. Those who consented were invited to the study clinics for sample collection by trained phlebotomists. The samples were transported to the laboratory in cool-boxes at 4^{o}, where the serum was separated, aliquoted and stored at $-80^{o}C$. All sera and stool samples were processed within 8hours of collection.

4.2.2 Laboratory methods

We used an indirect ELISA for detection of anti-O:4,5 antibodies representing *S.* Typhimurium and anti-O:9 antibodies representing *S.* Enteritidis. IgG and IgA antibodies were assayed separately. The ELISA was developed by collaborators from the Jenner Institute, University of Oxford, who provided training on and transfer of the ELISA protocol to the KWTRP laboratory in Kilifi. I learned the IgG antibody ELISAs at the Jenner Institute and thereafter performed the IgA antibody ELISAs in Kilifi having transferred and optimized the assay. Purified O-antigens for plate coating were supplied by Prof. Rafael Simon, University of Maryland (170). This ELISA has been implemented in a previous study by the collaborators(104).

Nunc Maxisorb 96-well plates were coated with 5ug/ml O-antigen in coating buffer (5.3g Na_2CO_3 + 4.2g $NaHCO_3$ + 1L dH_20, pH 9.6) and incubated overnight at +4^oC. The plates were washed 5 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) then blocked with Casein (Thermo Scientific, 37528) for 1 hour, and washed again 5 times with PBS-Tween and x1 with PBS. Test sera were initially diluted at 1:50 and plated in triplicates. Where higher dilutions were required, 1:500 or 1:5000 dilutions of the sera were used.

Previously calibrated reference sera were plated in duplicate following a series of eleven 2-fold dilutions starting at 1:10 (or 1:20 for the O:4,5 IgG assay). Controls were included on each plate as follows: a negative control, consisting of casein dilution buffer added to 2 'blank' wells; a positive control consisting of pooled serum from NTS-positive Malawian adults diluted in casein in 2 wells; an internal control, consisting of the reference sera diluted at 1:160 for IgA assays and 1:320 for IgG assays plated in 4 wells.

Figure 0.1 Plate arrangement for samples and controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	Internal Control
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	Internal Control
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Positive Control	Positive Control
G	1	2	3	4	5	6	7	8	9	10	11	Blank
H	1	2	3	4	5	6	7	8	9	10	11	Blank

The positions G 1-11 and H 1-11 represent the positions of the reference serum used to determine the standard curve of each plate.

The plates containing the test and reference sera were incubated for 2 hours at room temperature and subsequently washed with PBS-Tween x 5 and PBS x1. A corresponding secondary antibody, either the Anti-Human IgA (α-chain specific) peroxidase antibody produced in goat (Sigma, A0295) or the Anti-Human IgG (γ-chain specific) peroxidase antibody produced in goat (Sigma, A6029), was added to the plates which were then incubated at room temperature for 1 hour followed by a washing step with PBS-Tween x 5. The plates were developed using TMB Substrate (AbCam, ab171522) for the colour reaction, which was controlled by addition of the Stop solution (450nm TMB Stop Solution, AbCam ab171529). The development time was calibrated to generate an OD of 1 in the internal control. Once the Stop solution was added, the plates were read within 30 mins using ELISA reader operating Gen5 Software (version 2.0). Bound antibodies were quantified by measuring the absorbance at 450 nm and interpolating the values relative to the reference sera based on a 4-parameter logistic regression curve as ELISA Antibody Units (AU). The 4 parameters are minimum value on curve, maximum value on curve, mid-point of the curve and the slope at the mid-point. One AU is equal to the reciprocal of the dilution of the standard serum giving an OD =1. Repeat testing of the samples was done if the coefficient of variation between the triplicates was >20%. Full plate

repeats were done if the fit of the standard curve was unsatisfactory (R^2<0.994), if the background reaction was high (negative control >0.15 OD) and if the Internal and Positive controls were not within the defined range. Additional dilutions of the samples were recommended if the OD values were higher than the top end of the reference curve, i.e. where the OD of the sample exceeds the OD of the 1st dilution point of the standard curve. Those below the bottom end of the reference curve were considered to be negative.

4.2.3 Statistical analysis

The antibody units were log-transformed (base 10) before further manipulation. Zero values were arbitrarily allocated a value that was half of the lower limit of quantification (LLOQ) for the specific assay, which aided log-transformation. Geometric mean concentrations (GMC) were estimated by age-strata (0-11 months, 12-59 months, 5-14 years, 15-54 years and 55+ years), by site (Kilifi, Nairobi, Siaya) and by faecal NTS carriage status. Comparisons between groups were performed using ANOVA with Tukey's pairwise comparison tests.

We used the Reverse Cumulative Distribution (RCD) curves below to visualize the population distribution of antibody concentrations and how they compared between different age-groups. Each graph shows the antibody concentration on a log scale on the x-axis and on the y axis it shows the cumulative proportion of participants who have that antibody level or higher. Each curve on the graph represents an age-group. The curve begins at 100% and then drops towards the right. A curve located to the right of another curve indicates a higher mean antibody concentration in that age-group.

To assess the rate of change of antibody levels with age, I used a piecewise regression model which assumes that the rate of change is linear between any defined age-band but can differ from one age-band to the next. For example, assuming that the data has one inflection point at age p, two linear regressions will be fitted. One for the line where $x<p$ and the second for the line where $x>=p$. In

Stata this was implemented using the *nl* command. It estimates the inflection/breakpoint age p, the gradient in the age band where x<p, and the gradient in the age band where x>=p.

To assess the mean rate of decay, d, of maternal antibodies, I used linear regression on log antibody concentrations using the formula(171) :

$$(x_a) = (x_o).e^{-da}$$

where (x_a) refers to the mean antibody concentration at age a while (x_o) refers to the mean antibody concentration at birth.

I explored several options to define a threshold for dichotomizing seronegative and seropositive individuals, based on their exposure to NTS, and evaluated each option based on sensitivity, specificity and biological plausibility of the outcome given the known epidemiology of NTS infections. In practice, a Receiver operating characteristic (ROC) curve can be used to determine the optimal balance between sensitivity and specificity, by assessing the performance of the candidate threshold against a 'gold standard', which would ideally have 100% sensitivity and 100% specificity. The gold standard for diagnosis of Salmonellosis is a positive culture, either stool for diarrheal illnesses or blood culture for iNTS bacteraemia. This gold standard has 100% specificity but low sensitivity(49). Therefore, the breadth of antibody responses from culture confirmed individuals would be used to construct the positive reference curve. In our study, we did not have a set of gold standard sera and therefore could not implement the conventional ROC curve analysis. A second approach applied when defining thresholds, is to use the value of the mean plus 2 standard deviations (occasionally + 3 standard deviations) of antibody concentrations derived from a known negative population. The selection of the negative population would be problematic in NTS research as there are no NTS-naïve populations, even among children. Over 90M cases of *Salmonella* infections are reported globally each year (13) . Additionally, in endemic areas, infections are

reported among neonates within the first 7 days of life(154). In malariometric studies, for example, samples from European malaria-naïve populations (except those with history of travel to malaria endemic areas) are commonly used to define negative controls (172).

To define a threshold in this assay, I implemented a data driven approach. I used a 2 –component Gaussian mixture model to separate the data into two latent classes, which I assumed to be a seronegative class and a seropositive class. I used the *fmm* package in Stata to construct the mixture model(173). The mixture modelling approach assumes that the population of observations consists of underlying subclasses that are unobserved and uses the data to reconstruct the subclasses assuming that their distributions are normal and different from each other. By specifying the model as a 2-component mixture model, it reconstructs 2 subclasses. With the resulting distributions as 'gold standard', I plotted a ROC curve and estimated the Youden index, a cut-off on a ROC curve that maximizes sensitivity and specificity of a test (174). This is the point that I used as a threshold for seropositivity, as it gives the maximum potential effectiveness of the test. In addition, for sensitivity analyses, I defined an additional threshold using the mean + 2SD of the first class of the mixture model. The assumption being that it represents the underlying true negative population. I then assessed differences in seroprevalence by age, by site and by faecal carriage status.

To assess the relationship between antibody responses and serogroup specific carriage, I compared the GMCs of O:4,5 IgG and IgA antibodies between those with stool carriage of Group B and those without Group B carriage; and the GMCs of O:9 IgG and IgA antibodies between those with stool carriage of Group D *Salmonella* and those without serogroup specific carriage. These analyses were stratified by age for children under 5 years and those aged 5 years and above.

All statistical analyses were conducted in Stata V15

4.2.4 Ethics statement

The study was approved by the Scientific and Ethics Research Unit at KEMRI under the Protocol #3221

4.3 Results

There were 1521 participants in the parent carriage survey, 1254 of whom (82.4%) also agreed to participate in the seroprevalence study. Participants from Kilifi and guardians of infants were less likely to consent to participate in the sero-survey compared to participants from other sites and older ages (Table 4.1).

Table 0.1 Participant characteristics of those who consented for the sero-survey.

		Total N	Consented n	Proportion. Consented %
Total		1,521	1,254	82
Site	Kilifi	507	319	63
	Nairobi	472	451	96
	Siaya	542	484	89
Age group				
	0-11m	341	219	64
	12-59m	310	256	83
	5-14y	346	313	90
	15-54y	277	250	90
	>55y	246	215	87
Sex	Male	709	571	81
	Female	811	682	84

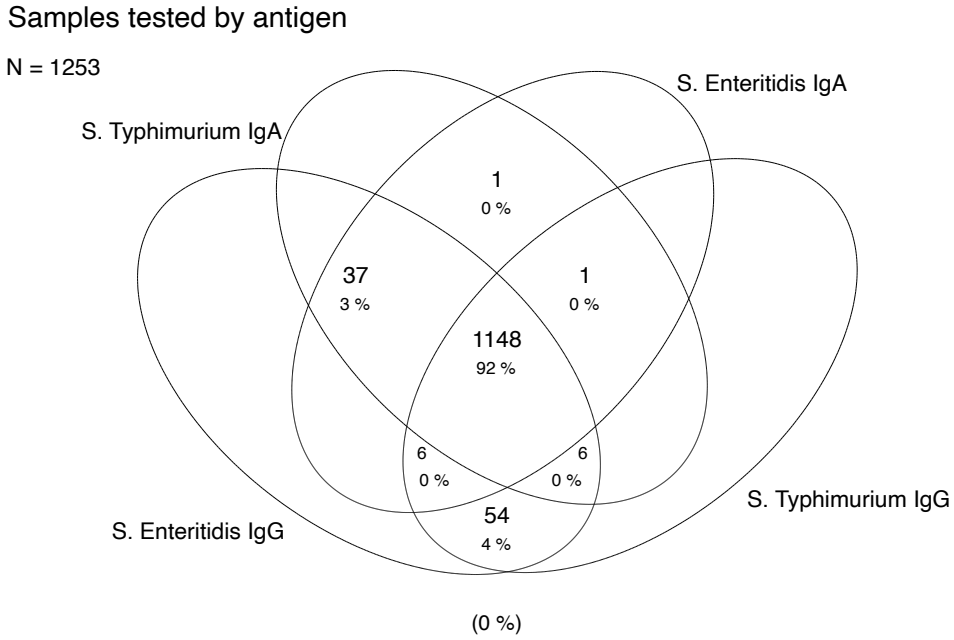
Out of the 1,254 serum samples collected, we assayed 1,252 for O:9 IgG, 1,216 for O:4,5 IgG and 1,194 for both O:9 and O:4,5 IgA (Table 4.2). Among vials that did not have enough sample volume to complete all the assays, we prioritized the IgG assays. We did not repeat the testing of samples that failed QC twice. One sample had incomplete metadata and was excluded. 1,148 samples had results for all 4 antigen-antibody combinations (Figure 4.2).

Table 0.2 Flow of samples

	O:9 IgG	O:4,5 IgG	O:9 IgA	O:4,5IgA
Vials retrieved	1,254	1,254	1,254	1,254
Insufficient Volume	1	2	53	55
Failed CV criterion (>20)	1	12	7	5
Plate Failed*	-	22	-	-
	1,252	1,216	1,194	1,194

*(R^2<0.994 or Controls out of range)

Figure 0.2 Venn diagram showing number of samples tested for each isotype-serotype combination.



Of the 1,148 participants with complete data, the median age was 9.7 years (IQR, 2.8, 34 years) and 518 (45%) were male (Table 4.3). By site, 302 (26%) were from Kilifi, 397 (35%) from Nairobi and 449 (39%) from Siaya. The prevalence of anaemia among those tested was 11% (93/851) and 14% (152/1,093) had evidence of recent malaria infection. By site, Kilifi had the highest prevalence of anaemia (20%) and Siaya had the highest proportion of participants with recent malaria infection (28%). Among 409 children aged <5 years, 28 (7%) had severe acute malnutrition; 22 of them were from Nairobi.

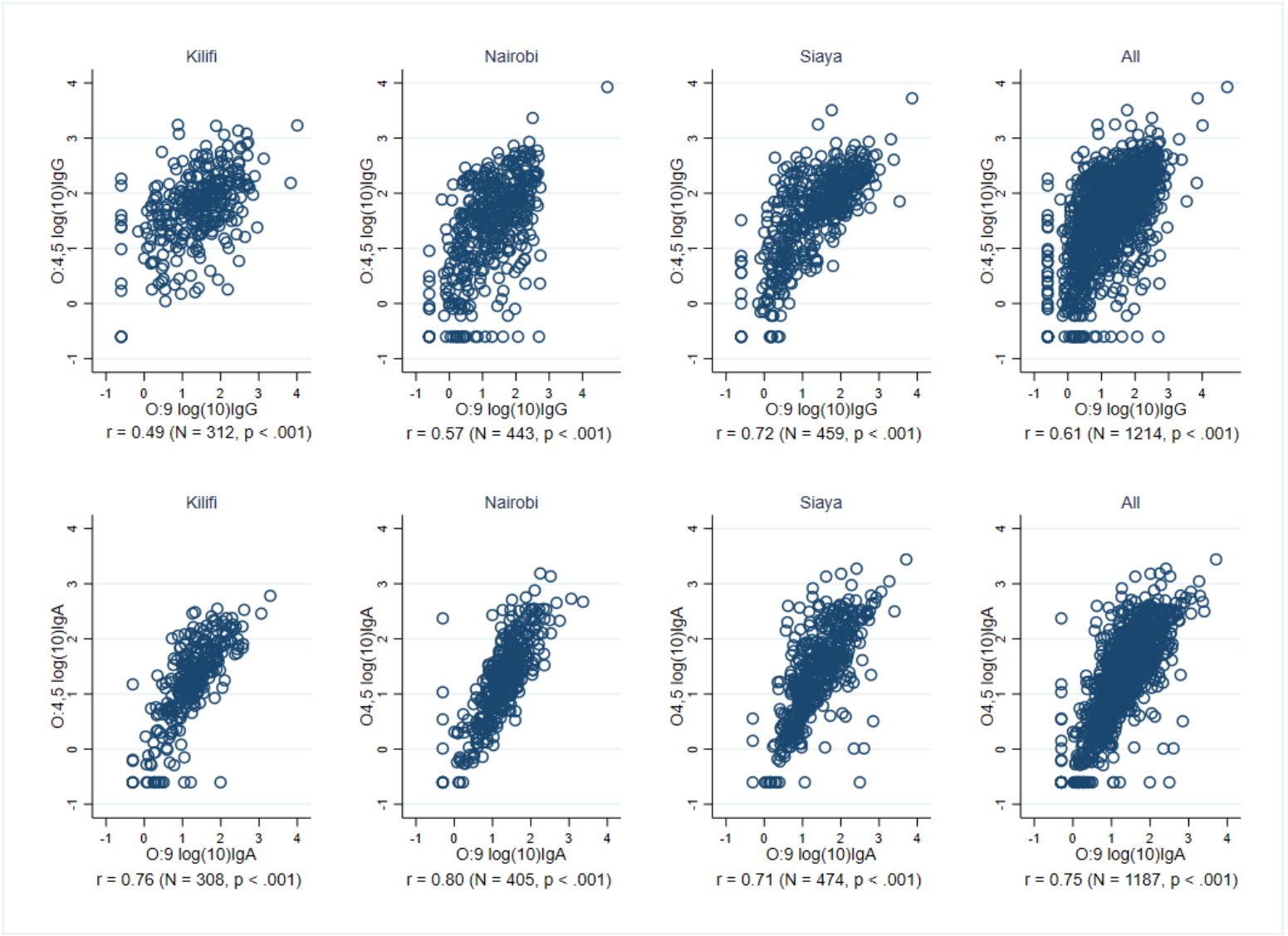
Table 0.3 Participant characteristics among the subset of participants with complete results on all four isotype-serotype combinations tested (n=1,148).

	Kilifi		Nairobi		Siaya		Total	
	n	%	n	%	n	%	n	%
N	302		397		449		1,148	
Age								
0-11m	50	17	54	14	73	16	177	15
12-59m	58	19	78	20	96	21	232	20
5-14y	59	20	137	35	102	23	298	26
15-54y	62	21	97	24	75	17	234	20
>55y	73	24	31	8	103	23	207	18
Sex, male	123	41	198	50	197	44	518	45
MUAC <11.5cm in under 5y†	1	0.9	22	17	5	3	28	7
Number tested for Hb concentration*	291	96	347	87	213	47	851	74
Anaemia (Hb<10g/dl)*	57	20	17	5	19	9	93	11
Number tested for *P. falciparum* malaria*	295	98	357	90	441	98	1,093	95
P. falciparum positive by HRP2*	21	7	9	3	122	28	152	14

†denominator = 409
*Denominators are those tested, which may be smaller than the total sample under study

Exposure to both antigens was present across the sites. By site, there was a strong positive correlation between concentrations of O:4,5 IgA and O:9 IgA antibodies across all sites, and between O:4,5 IgG and O:9 IgG concentrations in Siaya (Figure 4.3).

Figure 0.3: Scatterplots of O:4,5 against O:9 IgG and IgA antibodies by site.



We used the Reverse Cumulative Distribution (RCD) curves below to visualize the population distribution of antibody concentrations and how they compared between different age-groups. RCDs of IgG and IgA concentrations by site and age-group are shown in Figure 4.4. The shape and distribution of the curves at all ages were nearly identical. The curves shifted to the right with increasing age. The shift between the curves was most prominent between the first age-group and the second, and minimal between the last two age-groups.

Figure 0.4 A: Reverse cumulative distribution curves of the concentrations of O:9 IgG and IgA by age and by setting.

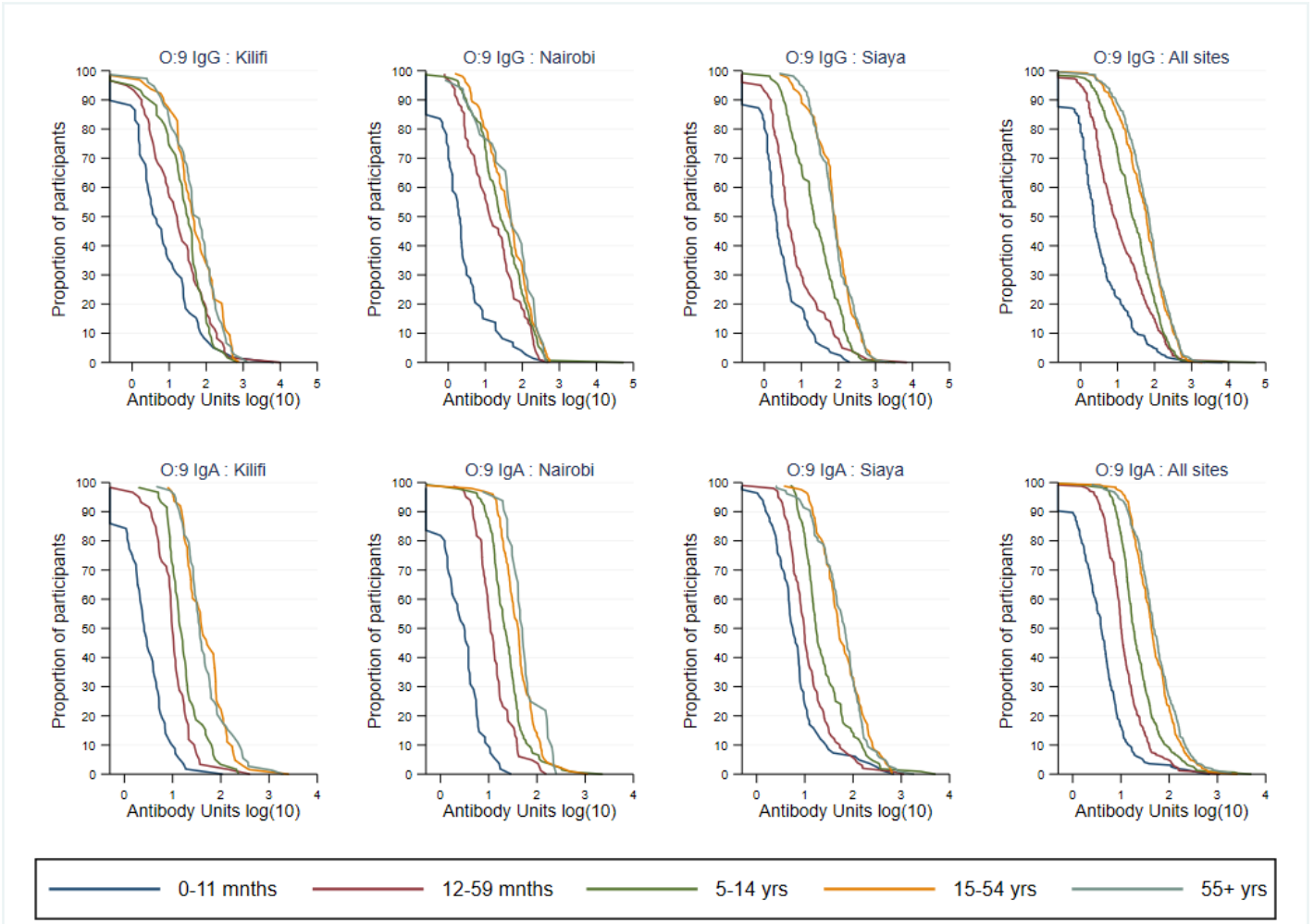
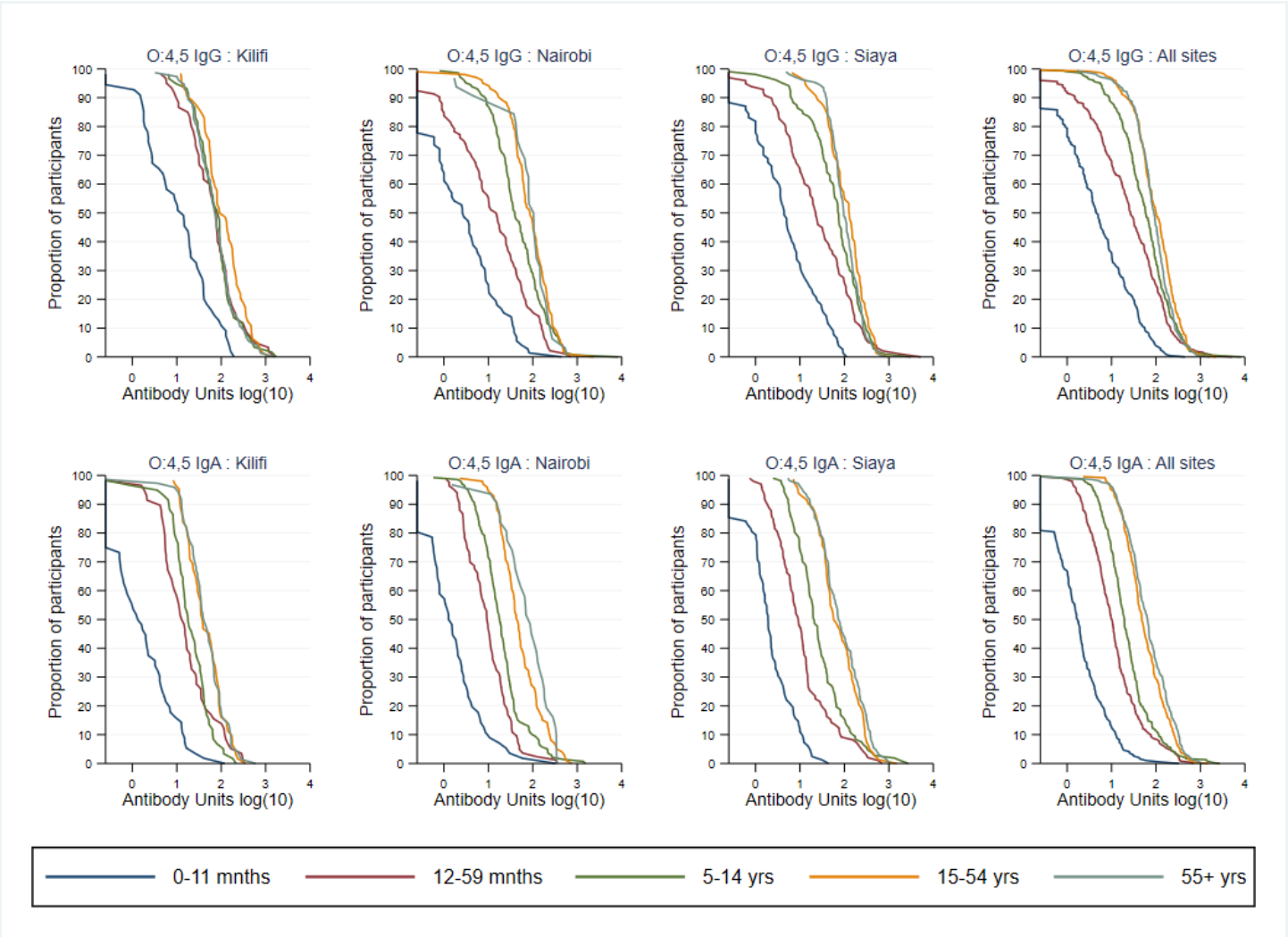

O:9 IgG : Kilifi
O:9 IgG : Nairobi
O:9 IgG : Siaya
O:9 IgG : All sites
O:9 IgA : Kilifi
O:9 IgA : Nairobi
O:9 IgA : Siaya
O:9 IgA : All sites
Proportion of participants
Antibody Units log(10)
0-11 mnths
12-59 mnths
5-14 yrs
15-54 yrs
55+ yrs

Figure 4.4 B: Reverse cumulative distribution curves of the concentrations of O:4,5 IgG and IgA by age and by setting.



The GMCs for IgG against O:9 and O:4,5 antigens increased with age across all sites (Table 4.4). The O:9 IgG GMCs reached the highest concentrations in late adulthood while the O:4,5 IgG GMCs reached a plateau at 15-54years followed by a decline in older persons, most notably in Kilifi but this was not statistically significant. By site, GMCs for O:9 IgG were highest among infants in Kilifi than in Nairobi and Siaya. However, the subsequent rise in concentrations with age was greater in Siaya than Kilifi, and by late adulthood the GMCs in Siaya were highest among all sites. The O:4,5 IgG GMCs differed significantly by site among children under 15 years. The highest GMCs were observed in Kilifi and the lowest in Nairobi.

Table 0.4: Geometric mean concentrations (95% CI) of antibody concentrations by age in the 3 sites

		Kilifi		Nairobi		Siaya		All sites	
		GMC	95% CI	GMC	95% CI	GMC	95% CI	GMC	95% CI
O:9 IgG	0-11 m	6	4-11	2	2-4	3	2-4	3	3-4
	12-59 m	17	11-28	16	11-23	7	5-10	12	9-15
	5-14 y	25	16-38	28	21-37	23	18-32	26	21-31
	15-54 y	49	32-72	41	31-54	76	57-101	52	44-63
	55+ y	51	35-72	44	24-80	76	60-97	61	50-74
O:4,5 IgG	0-11 m	10	6-16	3	2-4	5	3-7	5	4-6
	12-59 m	66	46-93	12	8-18	23	16-35	23	18-30
	5-14 y	76	56-101	44	35-56	60	45-80	54	46-64
	15-54 y	108	81-143	80	63-103	108	85-138	95	82-111
	55+ y	73	56-63	75	46-123	99	83-118	85	74-99
O:9 IgA	0-11 m	3	2-4	3	2-4	7	5-9	4	3-5
	12-59 m	10	8-13	13	11-16	12	10-15	12	10-13
	5-14 y	17	14-22	23	20-28	26	20-33	23	20-26
	15-54 y	48	36-62	40	33-48	60	47-77	48	42-55
	55+ y	46	36-60	56	41-76	58	46-74	53	46-62
O:4,5 IgA	0-11 m	2	1-3	2	1-3	2	2-3	2	1-2
	12-59 m	15	11-22	10	7-12	11	8-15	11	10-14
	5-14 y	19	15-26	20	16-24	27	21-35	22	19-25
	15-54 y	43	34-54	52	41-64	73	57-94	55	48-64
	55+ y	42	32-55	73	46-116	84	67-105	65	55-76

The O:9 and O:4,5 IgA GMCs increased significantly across all age-groups in each site, reaching a maximum in early adulthood which was sustained through adult life. There were only a few significant site differences in the GMCs by age. Siaya had the highest O:9 IgA GMCs in infancy and for O:4,5 IgA the rise in GMCs was highest in Siaya among participants above 15y.

Consistent with the GMC analysis, a scatterplot (with LOWESS curve) of the concentration by age showed that the increase in concentration by age was not similar across age; instead, there appeared to be an increase during childhood, which peaked at a certain age (p) and then remained flat throughout adulthood (Figure 4.5A&B). To mimic the figure, we fit a piecewise linear regression model with one inflection point at age (p) which fits two linear regression lines, one for the section where age<p and another where age>p. The gradients of the slopes represent the rate of increase of antibody concentration by age.

Figure 0.5 A: Scatterplots of antibody concentration by age in years with LOWESS curves fitted: O:9 IgG and IgA

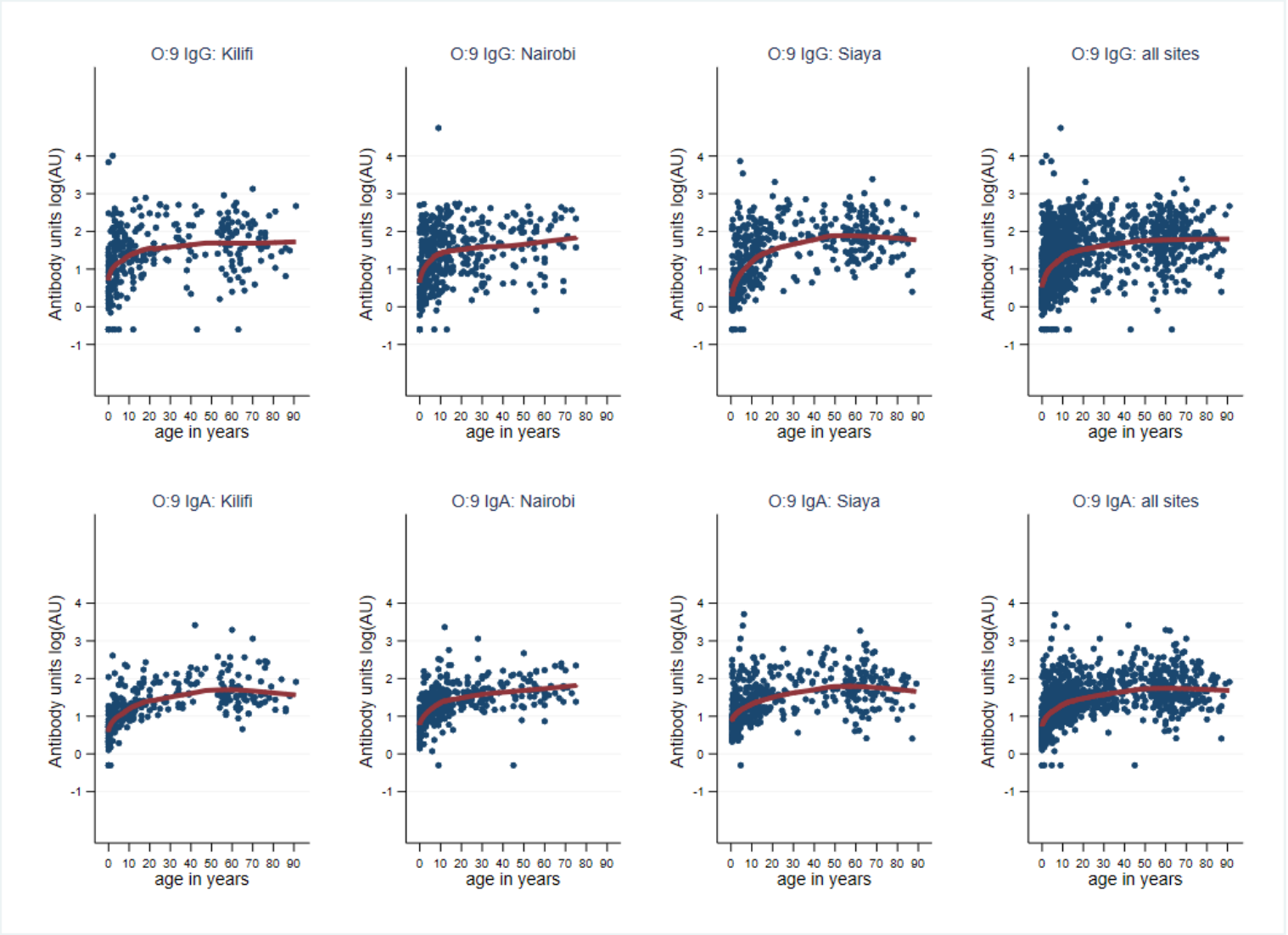
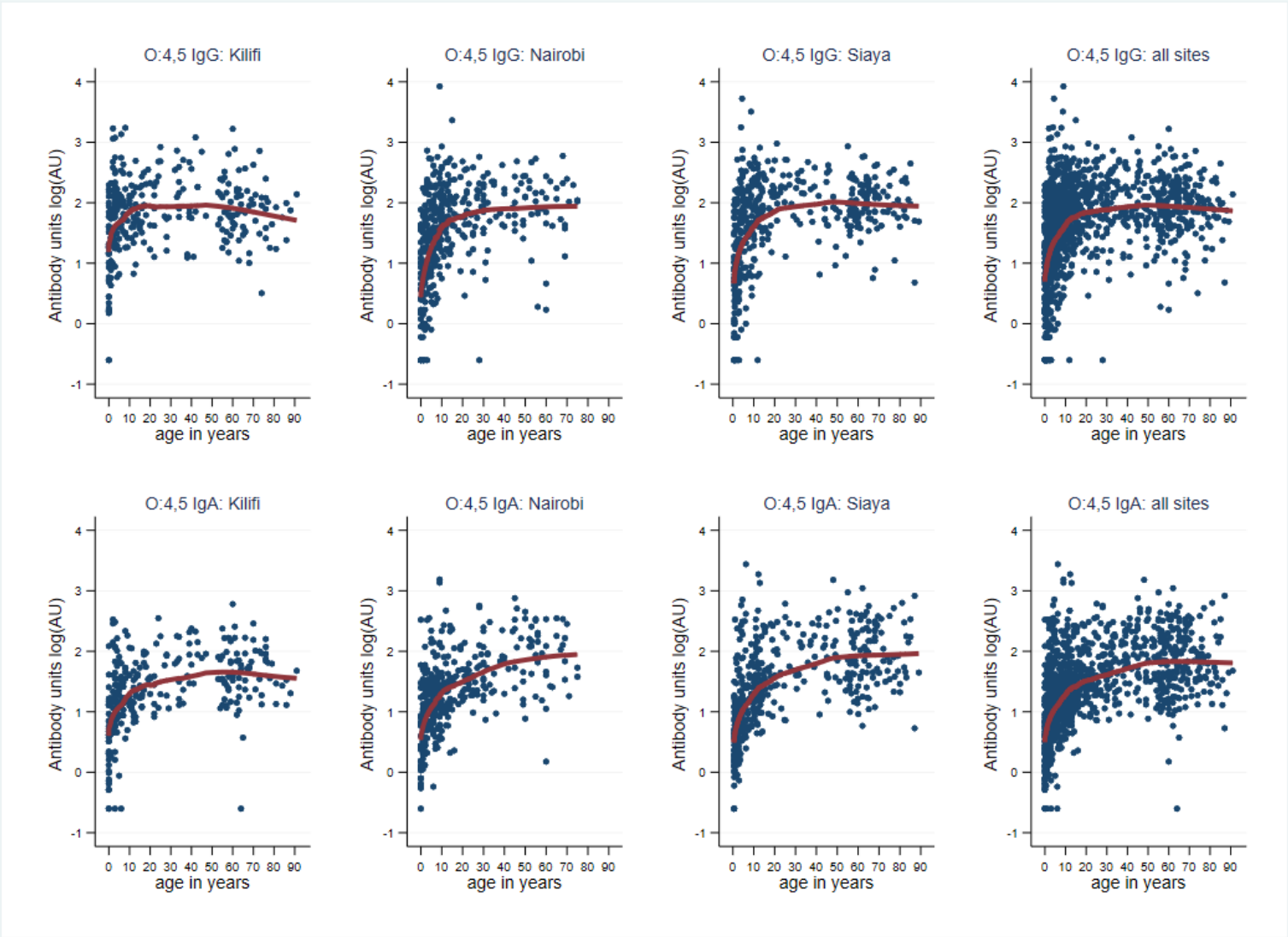


Figure 4.5 B: Scatterplots of antibody concentration by age in years with LOWESS curves fitted: O:4,5 IgG and IgA



Mean IgG against O:9 increases at a rate of 0.7 log AU per year to reach its maximum concentration by 1.4 years in Nairobi whereas, in Kilifi and Siaya, we observe a slower rise in the same antibodies to reach maximum concentrations in late childhood (Table 4.5). A similar pattern is observed for O:9 IgA. The O:4,5 IgG rises at a rate of 0.46 log AU per year to reach its peak by 2 years in Kilifi, which is faster than in Siaya (rate 0.29, breakpoint age 4.6 years) and in Nairobi (rate 0.15, breakpoint age 8.5years). However, the O:4,5 IgA antibodies rise fastest in Kilifi, and slowest in Siaya.

Table 0.5: Piecewise regression analysis of the rate of increase in antibody levels by age in years at each site.

	location	**Breakpoint age, p; 95% CI**		**Gradient age<p; 95% CI**		**Gradient age>p**	**Intercept at age=0; 95% CI**	
O:9 IgG	Kilifi	14.2	6.9-21.5	0.1	0-0.1	0	0.9	0.8-1.1
	Nairobi	1.4	0.9-1.9	0.7	0.4-1	0	0.4	0.2-0.5
	Siaya	17.8	14.4-21.1	0.1	0.1-0.1	0	0.5	0.4-0.6
O:9 IgA	Kilifi	14	10.5-17.5	0.1	0.1-0.1	0	0.6	0.5-0.7
	Nairobi	2.4	1.8-2.9	0.4	0.3-0.5	0	0.4	0.3-0.6
	Siaya	20.4	15-25.8	0.05	0-0.1	0	0.9	0.8-1
O:4,5 IgG	Kilifi	2	0.6-3.4	0.5	0.1-0.8	0	1	0.9-1.2
	Nairobi	8.5	6.8-10.1	0.2	0.1-0.2	0	0.6	0.4-0.7
	Siaya	4.6	3.7-5.5	0.3	0.2-0.4	0	0.5	0.3-0.7
O:4,5 IgA	Kilifi	2	0.8-3.2	0.6	0.2-0.9	0	0.2	0.1-0.4
	Nairobi	2.6	1.9-3.3	0.4	0.3-0.5	0	0.2	0.1-0.4
	Siaya	4.4	3.6-5.2	0.3	0.2-0.4	0	0.2	0-0.3

For a visual representation of the rapid change in antibody concentrations with age in early life, I created a scatterplot with LOWESS curve restricted to participants under the age of 10 (Figure 4.5 C&D).

Figure 4.5 C: Scatterplots of antibody concentration by age in years (<10y) with LOWESS curves fitted: O:9 IgG and IgA

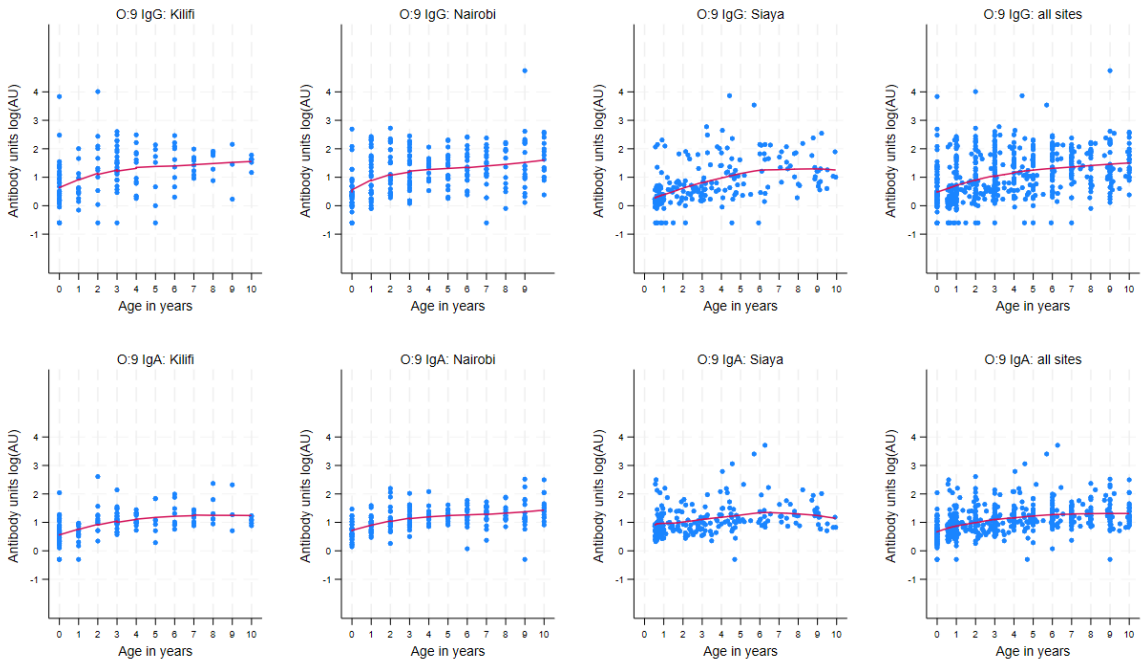
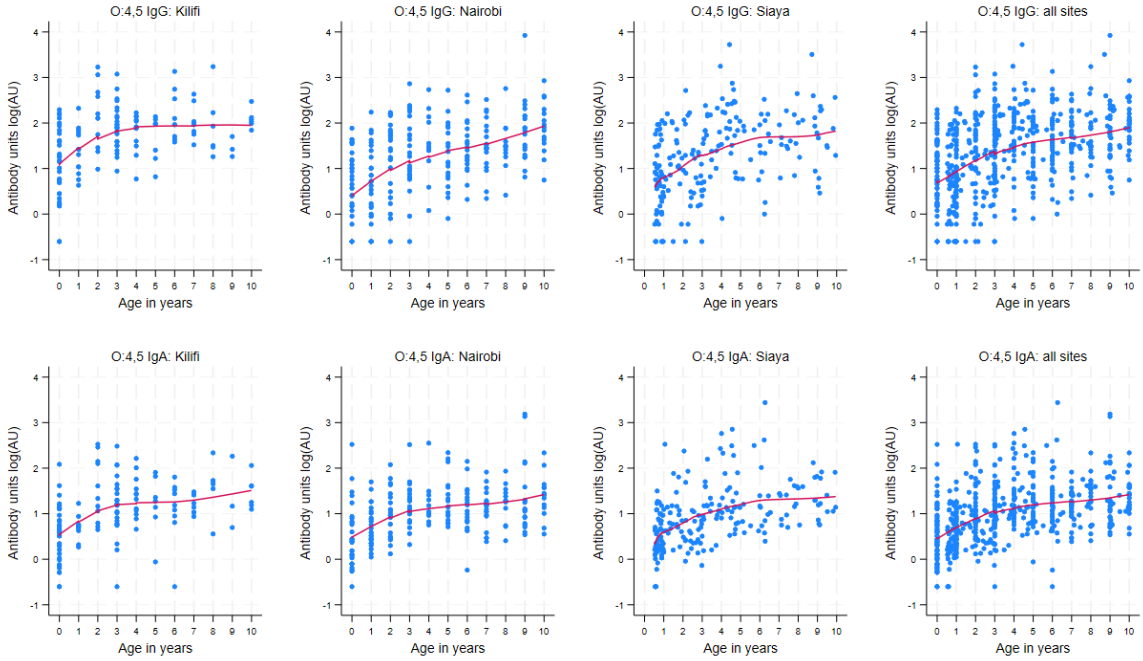
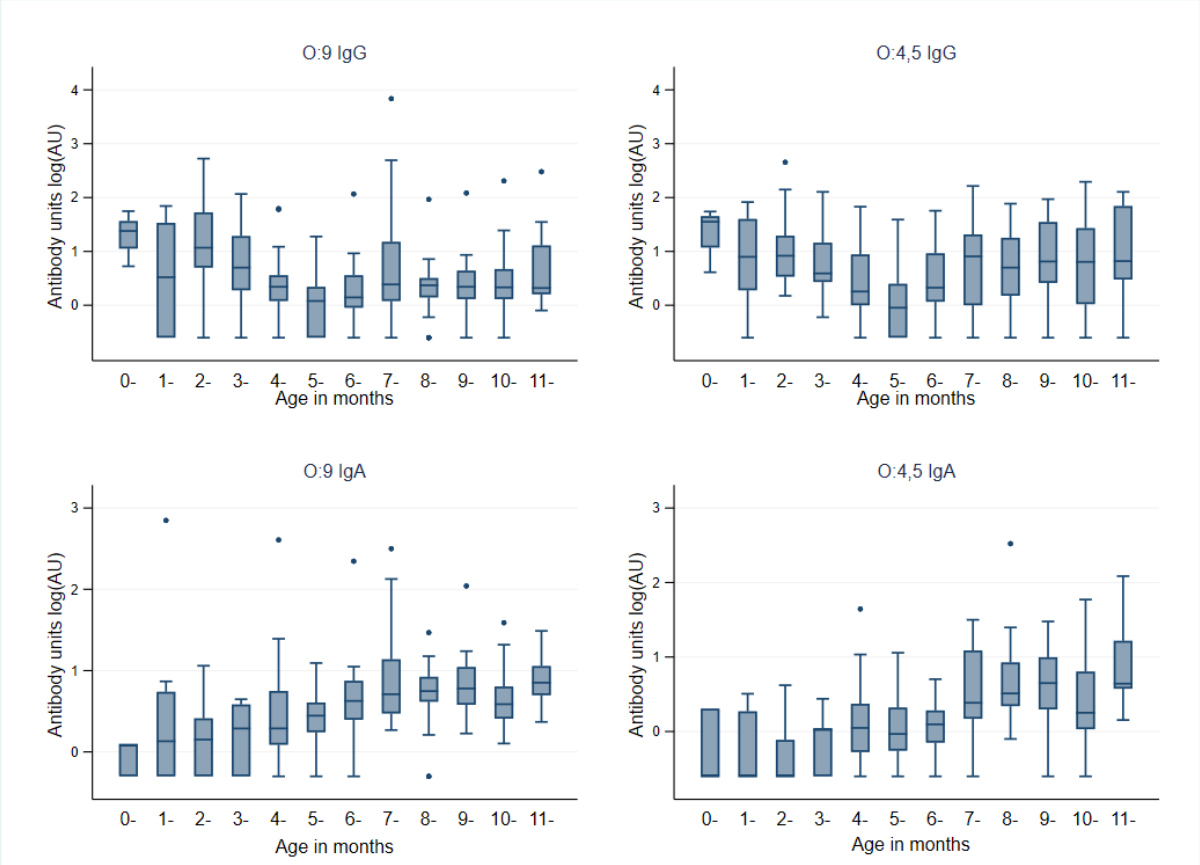


Figure 4.5 D: Scatterplots of antibody concentration by age in years (<10y) with LOWESS curves fitted: O:4,5 IgG and IgA



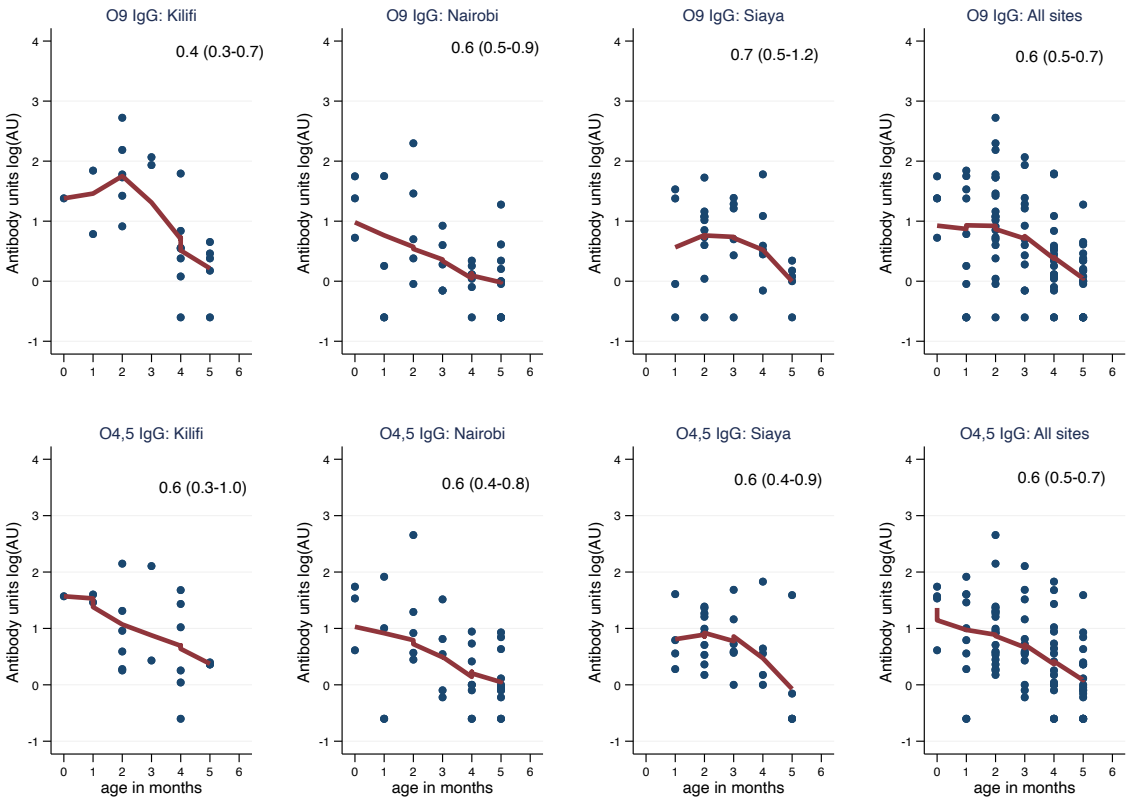
Among infants, we investigated the presence and decay rate of maternally derived O:9 IgG and O:4,5 IgG antibodies. The mean O:9 and O:4,5 IgG concentrations decreased to very low levels in the first 4-5 months of life, before rising again. The pattern is suggestive of the decay of passively acquired maternal antibodies over 4-5 months followed by adaptive antibody responses after natural exposure (Figure 4.6). This is strengthened by a lack of IgA among the infants in the first 4-5 months of life, followed by a gradual increase in the IgA concentration.

Figure 0.6: Decay of maternal antibodies (IgG) and early acquisition of antibodies (IgA) among infants.



The mean rate of decay was estimated using linear regression on log antibody concentration, which assumes an exponential decay of the maternal antibodies (Figure 4.7), applied to IgG antibody data for the first 6 months of life only. The O:9 IgG antibodies in Kilifi decayed at a rate of 60% per month (rate 0.4 [95% CI 0.3-0.7) while in Nairobi the decay rate was 40% (0.6 [0.5-0.9]) and 30% (0.7 [0.5-1.2]) in Siaya. The decay of O:4,5 antibodies was similar across the sites.

Figure 0.7: Scatter plot with LOWESS showing crude rate of decay in maternal antibodies by age at each site.



The numbers represent the crude rate of decay (and 95% CI) estimated for each site and serotype by linear regression.

After adjusting for location, we found that both O:9 IgG and O:4,5 IgG antibodies decreased by 40% per month in the first 6 months of life O:9 IgG rate 0.59 (95% CI 0.47,0.74), O:4,5 IgG 0.60 (0.48,0.75)) and the site differences were not significant. Additionally, since the concentration of IgA antibodies appears to increase from month 4 of life (Fig 4.6), we estimated the rate of decay of maternal antibodies in the first 4 months, and found that both O:9 IgG and O:4,5 IgG antibodies decreased by approximately 10% and 20% per month, respectively, in the first 4 months of life (O:9 IgG crude rate 0.90 (95% CI 0.47,1.72) adjusted rate 0.93 (0.53,1.66); O:4,5 IgG (crude 0.76 (0.45,1.28) adjusted rate 0.76 (0.44,1.30)), but the rates of decrease were not significant.

4.1.5 Anti-*Salmonella* antibody concentrations by NTS-carriage status

Out of 1253 matched stool samples, 34 (3%) were positive for NTS carriage. There were 6 serogroup D *Salmonella* isolates (4 were *S.* Enteritidis) and 10 serogroup B *Salmonella* isolates (1 was *S.* Typhimurium). Eighteen of the isolates could not be fully typed by the anti-sera we had available; however, they were neither serogroup B nor serogroup D serotypes. For the remainder of these analyses, we excluded samples from Nairobi since only one sample was positive for salmonella carriage (out of 451 tested), and this was of an unknown serotype and serogroup.

The GMCs of IgA and IgG antibodies were higher for carriers than non-carriers of homologous serogroup across the ages though the differences were not significant, except for participants over 5years of age with serogroup B *Salmonella* carriage who had 2 times higher O:4,5 IgG GMCs than non-carriers (p=0.04) (Table 4.6). There was no group B carriage among participants under 5 years of age. Among participants who had NTS carriage from serotypes other than group B, the GMCs of O:4,5 IgG in children under 5 years was 14. (95% CI 2-88), while for participants aged >5 years the O:4,5 IgG GMC was 97 (95% CI 52-181). The latter was lower than the GMC for serogroup B carriers of the same age, but this was not statistically significant (p=0.22).

Similarly, among participants who had NTS carriage from serotypes other than group D, the GMCs of O:4,5 IgG in children under 5 years was 11. (95% CI 1-126), while for participants aged >5 years the O:4,5 IgG GMC was 55 (95% CI 25-123). Neither was significantly different from the GMCs of serogroup D carriers of the same age.

Table 0.6 A: Geometric mean concentrations of IgG and IgA antibodies between those with stool carriage of Group B *Salmonella* and those without serogroup specific carriage.

Antibody		Group B Carriers n	Group B Carriers GMC (95% CI)	Does not have Group B carriage n	Does not have Group B carriage GMC (95% CI)	GMC Ratio (95% CI)	p-value*
IgG							
Group B	< 5 y	0	-	290	16.2 (12.8, 20.2)	-	-
	5+ y	10	177.0 (74.1,424.6)	472	82.9 (74.5, 92.2)	2.1 (1.0, 4.5)	0.043
IgA							
Group B	< 5 y	0	-	294	5.1 (4.1, 6.2)		-
	5+ y	10	58.9 (16.3, 212.5)	481	44.2 (39.5, 49.7)	1.3 (0.6, 2.9)	0.487

*p-value from Student's t-test performed to assess the hypothesis that the observed mean differences were not by chance.

Table 0.7B: Geometric mean concentrations of IgG and IgA antibodies between those with stool carriage of Group D *Salmonella* and those without serogroup specific carriage.

Antibody		Group D Carriers n	Group D Carriers GMC (95% CI)	Does not have Group D carriage n	Does not have Group D carriage GMC (95% CI)	GMC Ratio (95% CI)	p-value*
IgG							
Group D	< 5 y	3	27.1 (0, 43.2)	302	6.0 (4.9, 7.5)	4.5 (0.5, 38.8)	0.174
	5+ y	3	55.6 (2.4, 1277)	493	45.7 (39.8, 52.3)	1.2 (0.2, 7.0)	0.825
IgA							
Group D	< 5 y	2	27.9	295	7.4 (6.4, 8.6)	3.7 (0.6, 22.3)	0.145
	5+ y	3	38.4 (7.8, 190.4)	487	39.9 (35.8, 44.5)	0.9 (0.2, 3.8)	0.951

4.1.6 Seroprevalence

For each antigen-antibody combination, finite mixture models were used to explore the underlying distributions and determine seropositivity. The model fits are presented in Figure 4.8 and Table 4.7. There was considerable overlapping of the 2 underlying distributions from the fitted models, especially in the O:9 IgA model fit; while the iterations of the O:4,5 IgA model did not converge at all and there are therefore no results for O:4,5 IgA. Non-convergence suggests that the assumed model may not represent the data well. To improve the model, we modified the base models for the IgA mixture models to include carriage status as a covariate. For O:9 IgA this did not improve the model output and was not better than the base model when assessed by AIC, while for O:4,5 IgA, the modified model still did not converge.

Figure 0.8: Histograms showing the observed vs predicted densities from a 2-component mixture model

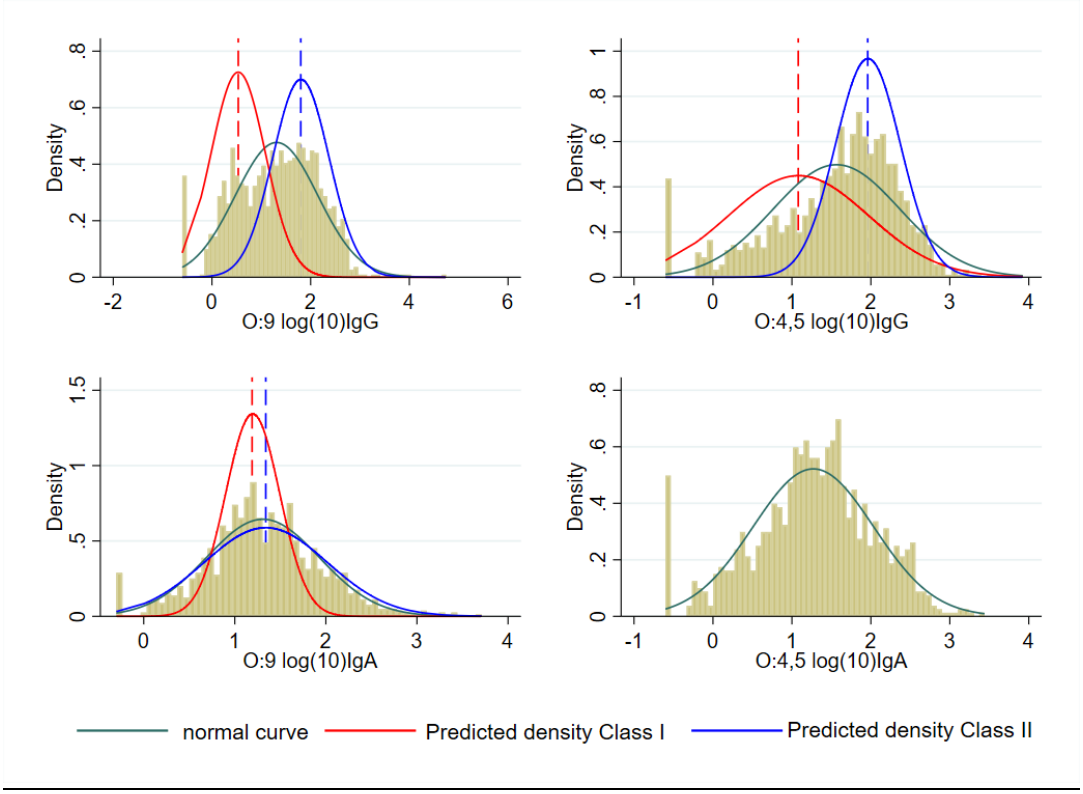


Table 0.8: Predicted class means and associated class proportions from mixture modelling.

	O:9 IgG	O:4,5 IgG	O:9 IgA

	Class 1	Class 2	Class 1	Class 2	Class 1	Class 2
Predicted Mean (Log AU)	0.53	1.8	1.08	1.96	1.19	1.34
(SD)	(0.5)	(0.6)	(0.9)	(0.4)	(0.3)	(0.7)
Predicted Proportion	0.38	0.61	0.45	0.55	0.22	0.78
(95% CI)	(0.27-0.51)	(0.48-0.72)	(0.36-0.54)	(0.45-0.64)	(0.08-0.44)	(0.56-0.92)

Using the IgG distributions from the mixture modelling as a gold standard, we calculated the sensitivity and specificity of different possible cut-offs for defining seropositivity and plotted a ROC curve (Figure 4.9), we then selected a cut-off and compared the resultant seroprevalence with the seroprevalence estimated directly from the model. At 0.5AU, the assay's LLOQ had a 99% sensitivity for both O:9 and O:4,5 IgG but lacked specificity. Using the mean of class 1 of the mixture model + 2 Standard deviations, the specificity was 97% and 98% for O:9 and O:4,5 IgG, respectively, but lacked sensitivity. We used the Youden index, to select a threshold that maximized both the specificity and sensitivity. This was estimated as 1.15 logAU for O:9 IgG (87% sensitivity and 87% specificity) and 1.45 logAU for O:4,5 IgG (89% sensitivity and 66% specificity). We defined seropositivity as any concentration above these thresholds. Table 4.8 shows the sensitivity and specificity of the selected thresholds and how they compare to other possible cut-offs. Since the mixture model could not discriminate the sub-populations from the IgA concentrations, we could not derive a threshold for IgA seropositivity.

Figure 0.9: Receiver Operating Characteristic (ROC) Curve of O:9 and O:4,5 IgG concentrations.

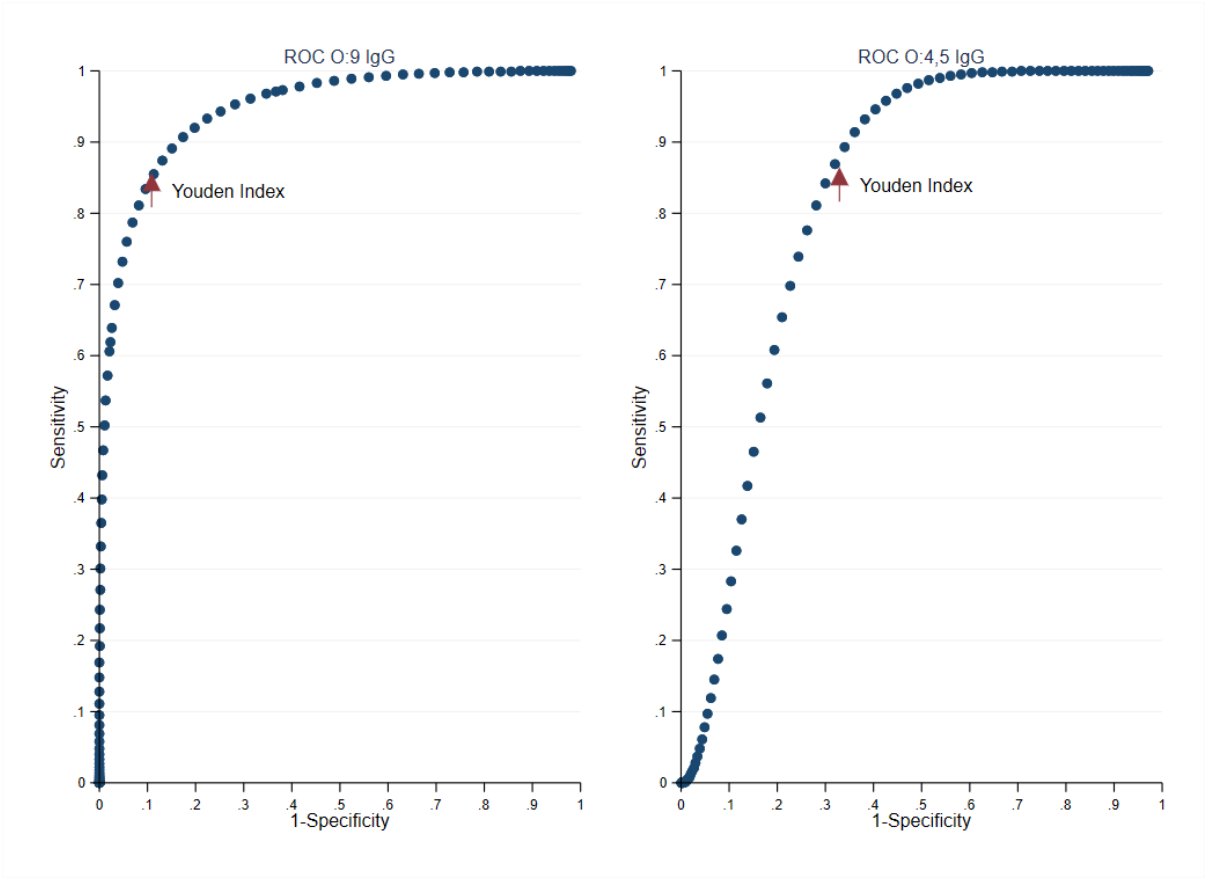

ROC O:9 IgG
Youden Index
Sensitivity
1-Specificity
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1
ROC O:4,5 IgG
Youden Index
Sensitivity
1-Specificity

Table 0.9: Sensitivity and Specificity of different cut-offs.

	O:9 IgG			O:4,5 IgG		
	Mean of Class 1 + 2SD	Youden Index	LLOQ	Mean of Class 1 + 2SD	Youden Index	LLOQ
Thresholds						
Cut off LogAU	1.63	1.15	-0.3	2.88	1.45	-0.3
Cut off AU	42.7	14.1	0.5	758.6	28.2	0.5
Test statistics						
Sensitivity	62%	87%	99%	1%	89%	99%
Specificity	97%	87%	48%	98%	66%	7%
Seroprevalence						
0-11 m	21/218 (10%)	43/218 (20%)	191/218 (87%)	0/204 (0%)	44/204 (22%)	176/204 (86%)
12-59 m	65/256 (25%)	105/256 (41%)	250/256 (98%)	5/250 (2%)	123/250 (49%)	240/250 (96%)
5-14 y	125/312 (40%)	202/312 (65%)	307/312 (98%)	6/307 (2%)	218/307 (71%)	306/307 (100%)
15-54 y	146/250 (58%)	204/250 (82%)	249/250 (100%)	4/241 (2%)	212/241 (88%)	240/241 (100%)
55+ y	133/215 (62%)	184/215 (86%)	214/215 (100%)	3/213 (1%)	190/213 (89%)	213/213 (100%)

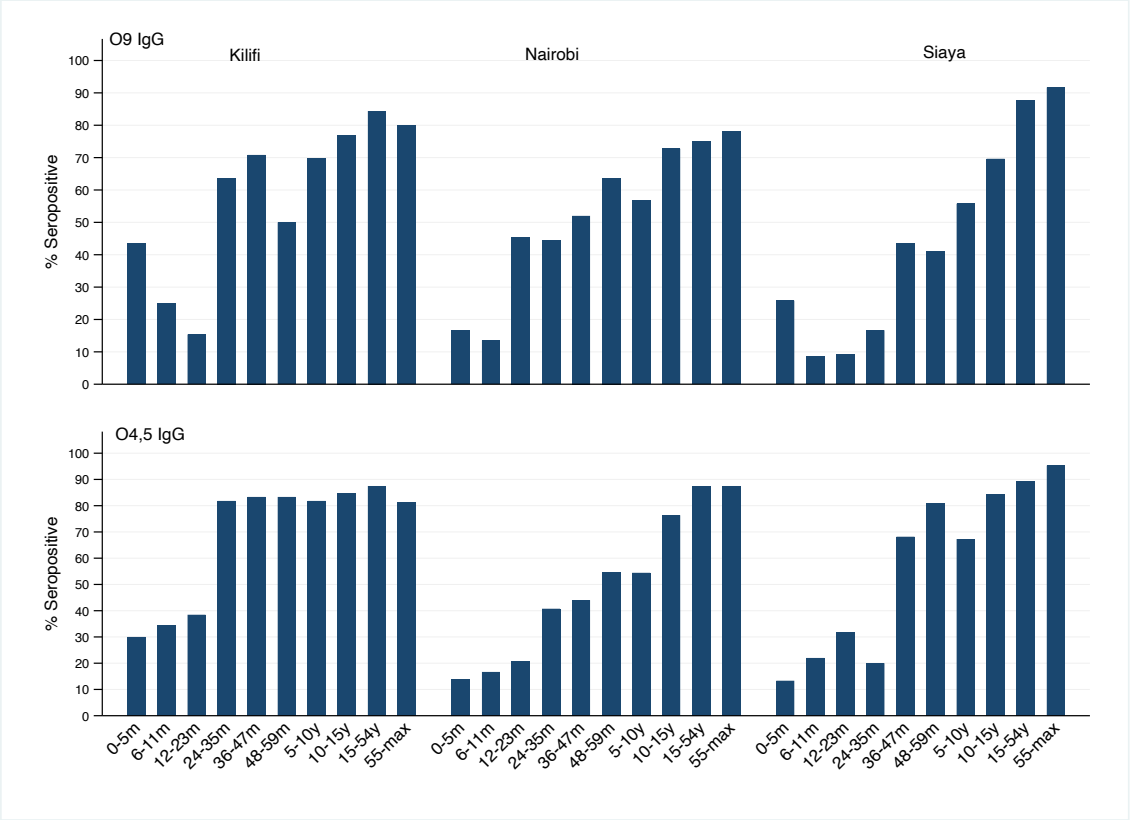
We used the cut-off based on the Youden index in subsequent analysis of the seroprevalence. Out of 1251 tested for O:9 IgG, 738 (59%) were seropositive, while out of 1215 tested for O:4,5 IgG, 787 (65%) were seropositive. Compared to the proportions predicted by the mixture modelling (Table 4.7), the seroprevalence from the selected threshold was higher for O:4,5 IgG but for O:9 IgG the proportions were similar. The seroprevalence differed by age and site (Table 4.9).

Table 0.10: Seroprevalence of O:9 IgG and O:4,5 IgG antibodies by age and site, using thresholds derived from Youden's index.

	Kilifi			Nairobi			Siaya		
	Total	**+ve**	***(%)***	**Total**	**+ve**	***(%)***	**Total**	**+ve**	***(%)***
O:9 IgG									
0-11 m	59	19	*32*	73	11	*15*	86	13	*15*
12-59 m	60	32	*53*	96	47	*49*	100	26	*26*
5-14 y	59	43	*73*	145	91	*63*	108	68	*63*
15-54 y	64	54	*84*	104	78	*75*	82	72	*88*
55+ y	75	60	*80*	32	25	*78*	108	99	*92*
O:4,5 IgG									
0-11 m	55	18	*33*	72	11	*15*	77	15	*19*
12-59 m	60	44	*73*	92	34	*37*	98	45	*46*
5-14 y	59	49	*83*	145	91	*63*	103	78	*76*
15-54 y	64	56	*88*	102	89	*87*	75	67	*89*
55+ y	75	61	*81*	32	28	*88*	106	101	*95*

In infancy, the seroprevalence was higher in Kilifi than in Nairobi and Siaya for both O:9 IgG and O:4,5 IgG (Figure 4.10). The seroprevalence increased by age across all sites. The highest seroprevalence was among adults in Siaya for both O:9 and O:4,5 IgG.

Figure 0.10: Seroprevalence of O:9 IgG and O:4,5 IgG antibodies by age



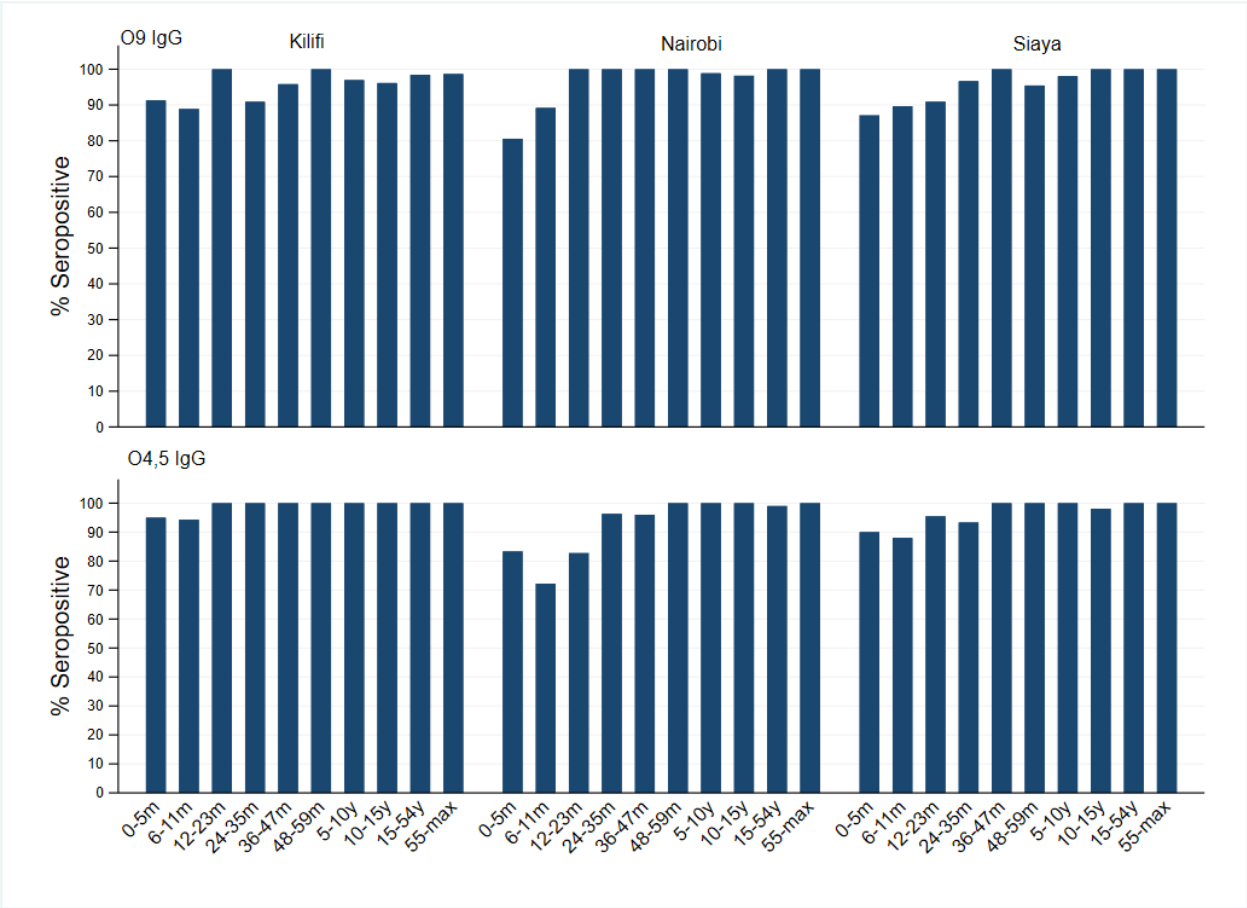
Out of 1148 participants with both O:9 and O:4,5 IgG tested, 583 (51%) were positive for both antigens while 278 (24%) were negative for both antigens. We tested whether there was an association between the seroprevalence of O:9 and O:4,5 IgG, which would imply that infection of the two have common risk factors. We found that the seroprevalence differed significantly in Siaya and Kilifi but not in Nairobi (Table 4.10)

Table 0.11: Association between seroprevalence of O:9 IgG and seroprevalence of O:4,5 IgG

	Kilifi	Nairobi	Siaya	All
N (number of paired samples)	302	397	449	1148
% of paired samples				
Seropositive for both antibodies	170 (56%)	175 (44%)	238 (53%)	583 (51%)
Seronegative for both antibodies	48 (16%)	104 (26%)	126 (28%)	278 (24%)
O:4,5 seropositive, O:9 seronegative	52 (17%)	63 (16%)	63 (14%)	178 (16%)
O:9 seropositive, O:4,5 seronegative	32 (11%)	55 (14%)	22 (5%)	109 (9%)
McNemar Chi	4.76	0.54	19.78	16.6
p-value	0.03	0.461	<0.001	<0.001

For comparison, we estimated the seroprevalence using the assay's LLOD as a threshold(104). The resulting seroprevalence in this case, reflects the proportion in the community with any detectable antibodies to NTS. Seroprevalence estimates, using the assay's LLOD as a threshold, were very high from a young age and reached >90% by the age of 5y for all antibodies across all sites. Overall, 97% (1211/1251) and 97% (1175 out of 1215 tested) were positive for O:9 and O:4,5 IgG, respectively (Figure 4.11). Only 12 samples were negative for IgG antibodies for both serogroups.

Figure 0.11: Seroprevalence of O:9 and O:4,5 IgG by age and site, Lower limit of quantification cut-off.



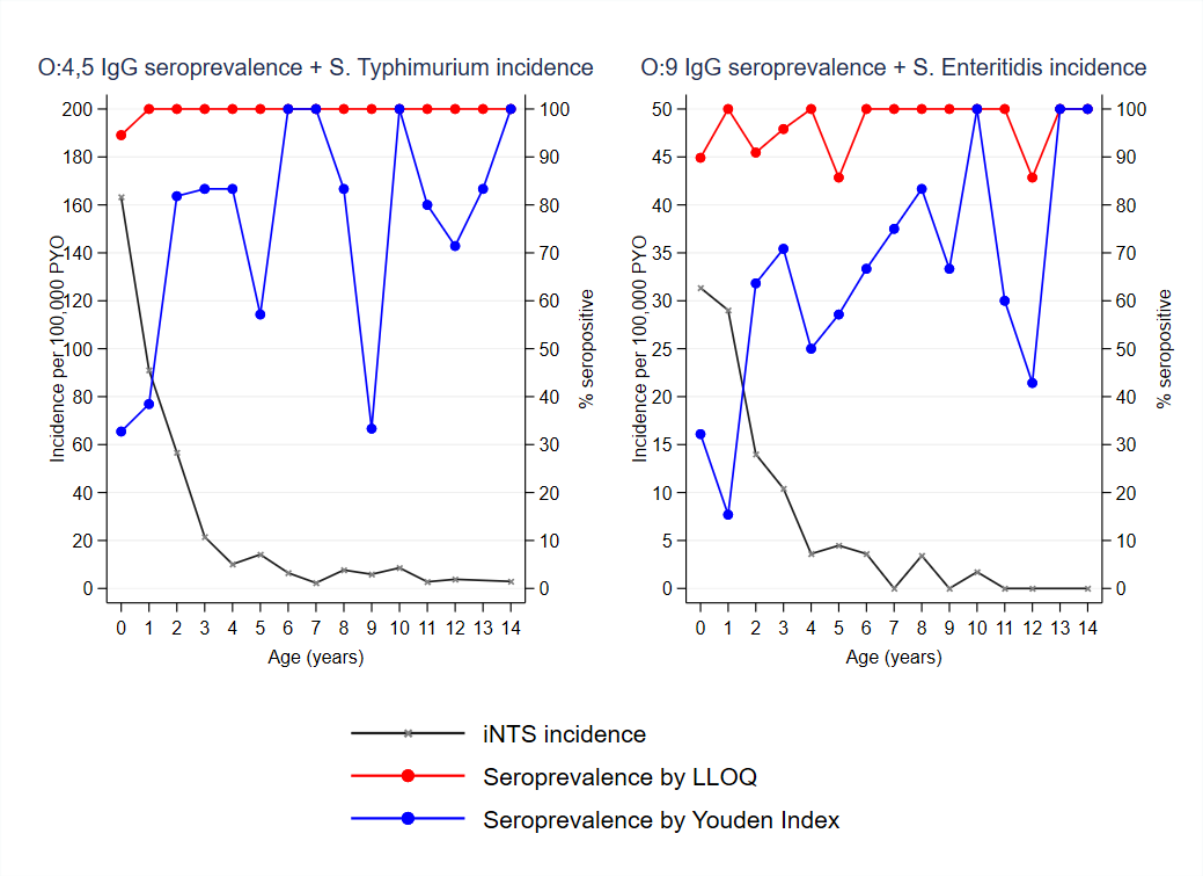
4.1.7 Relationship between seroprevalence and incidence

Finally, to assess the relationship between seroprevalence of NTS and incidence of iNTS by age and serotype, we plotted the age-seroprevalence curve derived from our thresholds with previously described age-incidence data from Kilifi(3). We found that the curves were inverse of each other – the incidence of iNTS was high in infancy and declined with age, while the seroprevalence was lowest in infancy and increased with age. The sharp decrease in incidence from 0-3y is mirrored by the sharp increase in seroprevalence in the same period, the curves intersected at approximately 1.5years (Figure 4.12 A). We also assessed the relationship between seroprevalence of NTS and faecal carriage of NTS, both all NTS serotypes and serogroup specific. Since the number of NTS isolated by faecal carriage was small, we grouped the serotype data by age-categories using similar categories that were used for sampling. We find that both the seroprevalence and culture-

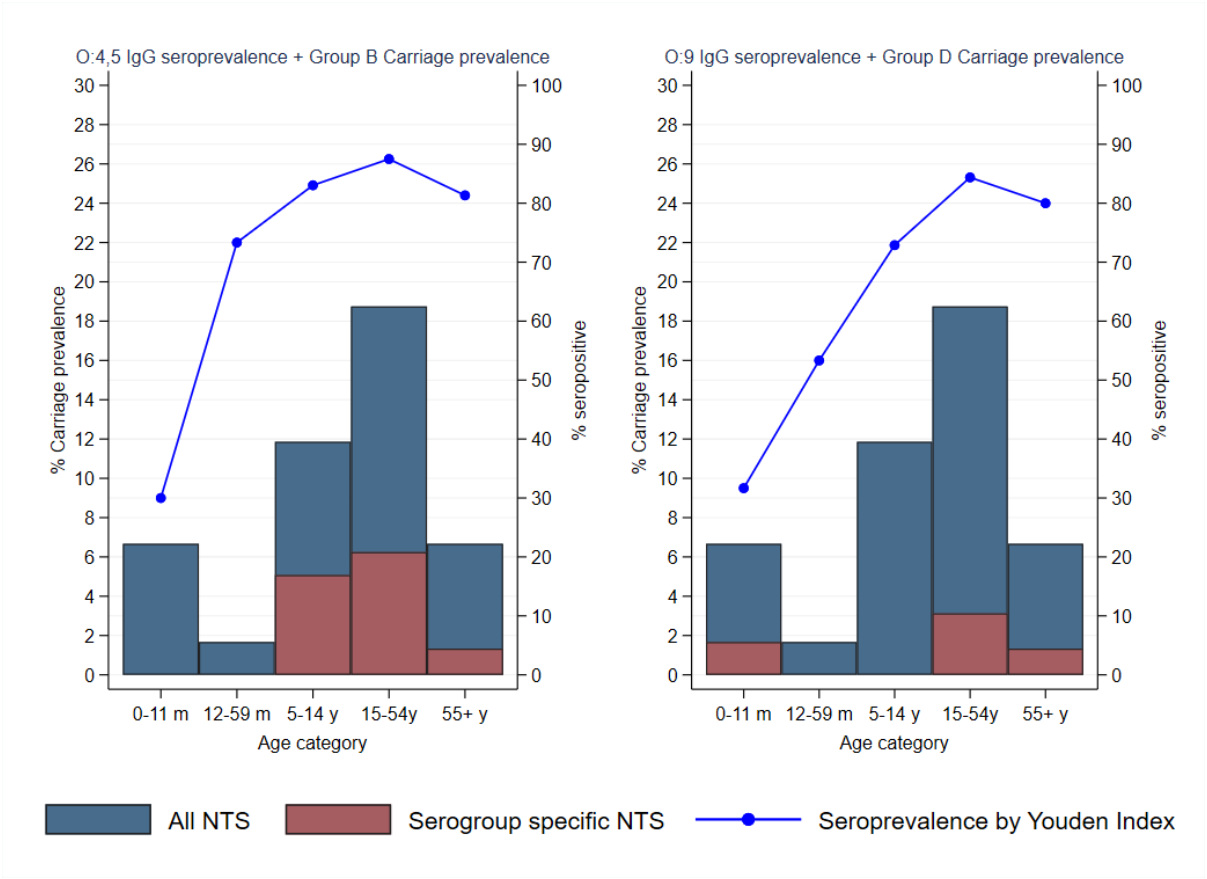
prevalence increase with age (Figure 4.12B). The serogroup specific carriage isolates were too few to make inferences.

Figure 0.12: Relationship between seroprevalence by age and A). Serotype-specific iNTS incidence by age in Kilifi and B). serogroup specific carriage prevalence in Kilifi.

A.



B.


O:4,5 IgG seroprevalence + Group B Carriage prevalence
% Carriage prevalence
% seropositive
0-11 m
12-59 m
5-14 y
15-54y
55+ y
Age category
O:9 IgG seroprevalence + Group D Carriage prevalence
% Carriage prevalence
% seropositive
0-11 m
12-59 m
5-14 y
15-54y
55+ y
Age category
All NTS
Serogroup specific NTS
Seroprevalence by Youden Index

Part Two: A catalytic model of NTS transmission.

Methods

Catalytic models in their simplest form, measure the rate of transition from one state to another. The naming of this class of models is derived from chemistry, where unchanged molecules are subjected to a force ("catalysis") and they become changed molecules. In the case of disease transmission, a catalytic model can measure the rate at which an infection is acquired per unit time among the susceptible population(108). In their classic form catalytic models assume that a population is entirely susceptible at birth, that the force of infection acting on this population exist at an endemic steady state such that a constant rate has been experienced since the birth of the oldest person in the dataset through to the last recorded data point, that the evidence that an infection has taken place is measurable, reliable, and remains so during life, and finally, that the migration or mortality due to infection is negligible. Modifications have been made to the classical catalytic model to accommodate non-immunizing infections such as NTS i.e. infections that do not produce lifelong immunity, by allowing reversion to susceptible states(162). In the classical form, the force of infection, λ, acting on the susceptible proportion (1-y), where y is the proportion seropositive, can be measured by the differential equation describing the rate of change of seropositivity by time:

$$\frac{dy}{da} = \lambda(1 - \lambda)$$

$$y = 1 - e^{-\lambda a}$$

These equations can be modified to reflect biologically plausible assumptions; for example, we can assume that not all infants are susceptible at birth since some could be protected by maternal antibodies:

$y = e^{-\lambda(a-0.5)}$, assuming the maternal antibodies are 100% protective for the first 6 months of life.

or that some people who are seropositive may lose their immunity at a certain rate and become susceptible again:

$y = \frac{\lambda}{\lambda+\rho}\{1 - e^{-(\lambda+\rho)a}\}$, where ρ represents the rate of reversion to the seronegative/susceptible state.

Such adaptations may help the model fit the observed real-life data better, and thereafter generate better estimates of the FOI and subsequent predictions.

I began by considering several models for each serotype:

1. Simple catalytic model (SCM). This is the model in its simplest form assuming that the starting population is fully susceptible, and that the force of infection is constant across age/time.
2. Reverse catalytic model (RCM). This model extends the simple catalytic model to include waning i.e. the average annual rate at which individuals revert to seronegative status from seropositive. It considers that once infected, the population could lose their immunity at a constant rate and become susceptible again.
3. A reverse catalytic model with an age-dependent FOI, estimated by the model. This model assumes that the force of infection is not constant, but changes with age (a), allowing estimation of the FOI before age (a) and the FOI for after age (a). The cut-off age is determined by the model. We explored this option since the results of the piecewise regression modelling from the previous chapter suggested that the FOI could differ by age.

Since we had evidence of the presence and decay rate of maternal antibodies, we excluded infants who were <6 months old. In addition, we did not consider population changes by birth and death processes in our model.

We used the IgG age-seroprevalence data estimated from the previous section to fit the model to the data by maximum likelihood methods. A separate model was developed for each serotype.

Models were fitted independently for each site, except for the reverse catalytic models with common waning, where the models were fitted simultaneously for all sites. This analysis was performed using the *revcat* package (175-177).

All analyses were performed in Stata 15.

Results

The seroprevalence data are shown in Table 4.11. The proportion seropositive for each antigen increases by age until late childhood and then it remains high, though site differences are apparent.

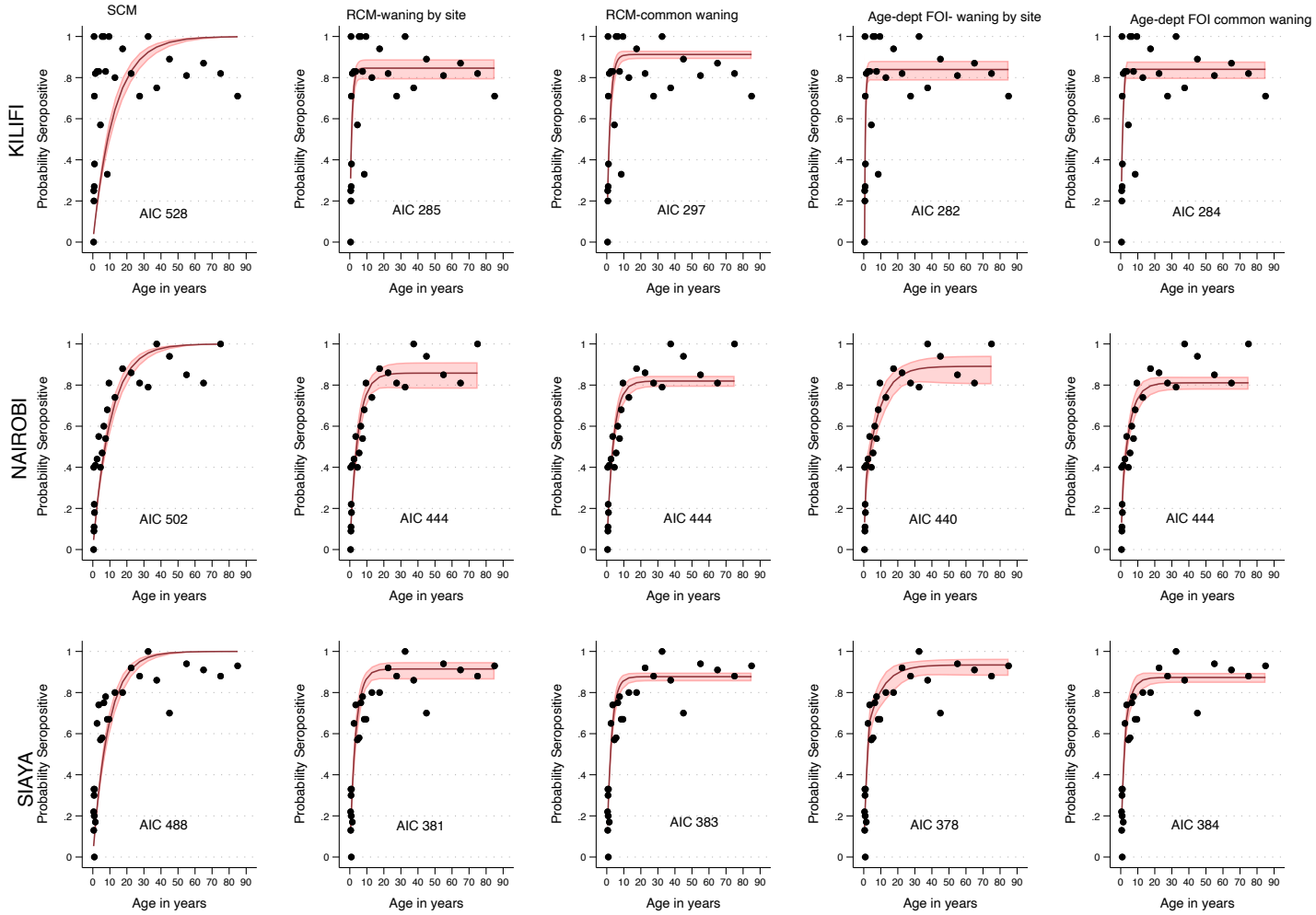
Table 0.12: Proportion of participants seropositive for O:4,5 IgG and O:9 IgG antibodies by age.

	O:4,5 IgG			O:9 IgG		
Age Category	Kilifi	Nairobi	Siaya	Kilifi	Nairobi	Siaya
6m	0.00	0.00	0.25	0.00	0.00	0.11
7m	0.25	0.40	0.15	0.43	0.40	0.13
8m	0.25	0.09	0.33	0.00	0.09	0.00
9m	1.00	0.13	0.38	0.00	0.11	0.00
10m	0.30	0.22	0.22	0.27	0.11	0.20
11m	0.71	-	0.00	0.43	-	0.00
1y	0.38	0.21	0.33	0.15	0.45	0.10
2y	0.82	0.41	0.17	0.64	0.44	0.17
3y	0.83	0.44	0.65	0.71	0.52	0.35
4y	0.83	0.55	0.80	0.50	0.64	0.44
5y	0.57	0.40	0.57	0.57	0.50	0.29
6y	1.00	0.47	0.58	0.67	0.58	0.67
7y	1.00	0.60	0.75	0.75	0.60	0.75
8y	0.83	0.58	0.78	0.83	0.58	0.56
9y	0.33	0.68	0.67	0.67	0.58	0.42
10y	1.00	0.81	0.73	1.00	0.75	0.67
11-<15y	0.80	0.74	0.88	0.70	0.72	0.70
15-<20y	0.94	0.88	0.89	0.75	0.75	0.85
20-<25y	0.82	0.86	1.00	0.91	0.81	1.00
25-<30y	0.71	0.81	0.88	1.00	0.69	0.88
30-<35y	1.00	0.83	1.00	1.00	0.58	0.80
35-<40y	0.75	1.00	1.00	0.75	1.00	0.86
40-<50y	0.89	0.94	0.78	0.78	0.76	0.90
50-<60y	0.81	0.89	0.97	0.85	0.80	0.89
60-<70y	0.87	0.81	0.93	0.70	0.75	0.94
70-<80y	0.82	1.00	0.94	0.94	1.00	1.00
80+y	0.71	-	0.93	0.71	-	0.79

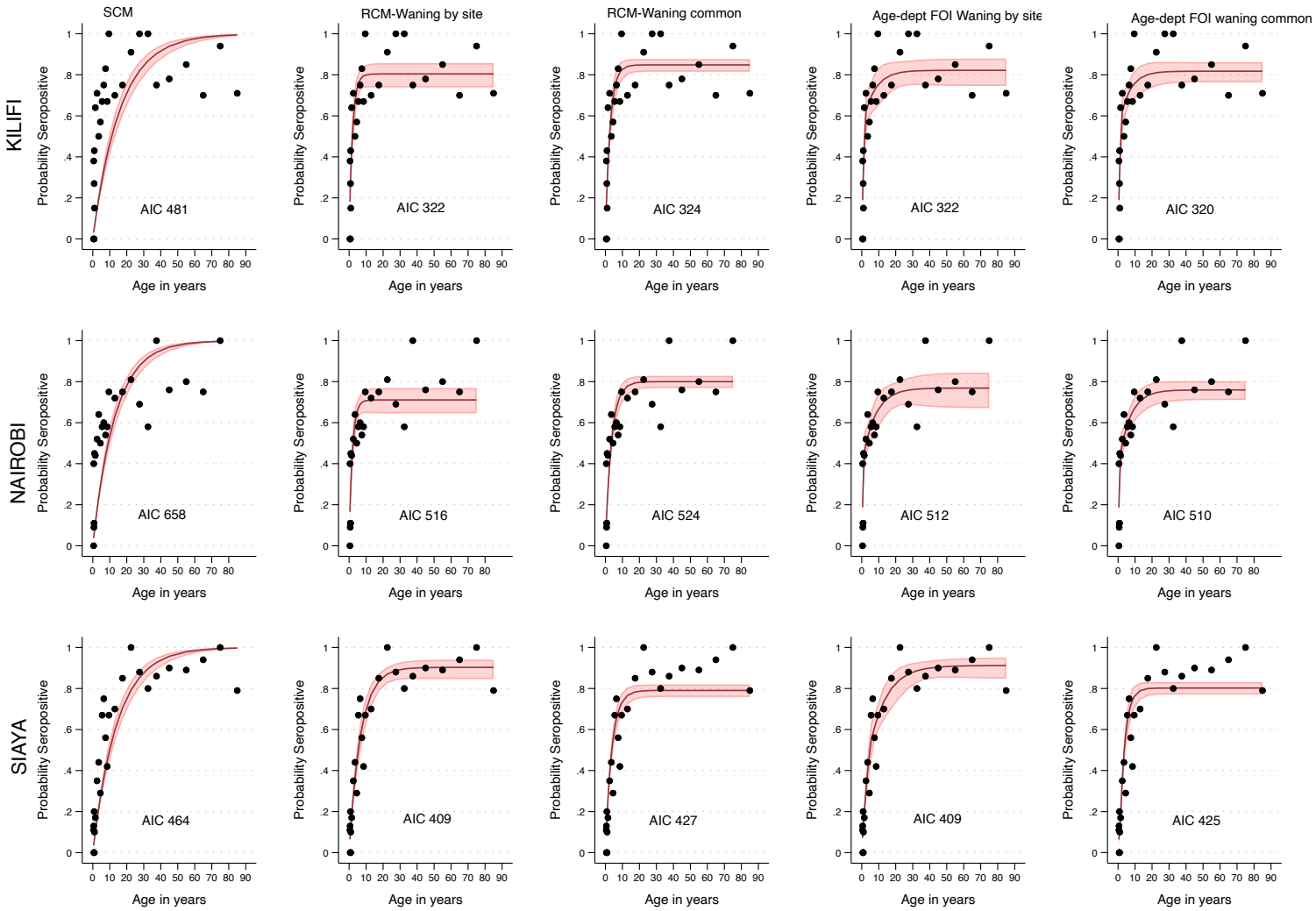
From the catalytic modelling, we find, both visually and according to AIC, that the reversible models incorporating waning had a superior model fit than the simple catalytic models for both O:4,5 IgG and O:9 IgG (Figure 4.13). For O:4,5 infections, allowing the rate of waning to differ by site provided a slightly better fit for Kilifi and Siaya, but was similar for Nairobi. The site-specific waning rates in Nairobi (0.03 per person/year [95% CI .02-.06]) and Siaya (0.03 per person/year [95% CI .01-.05]) were similar but they differed from the rate in Kilifi (0.14 per person/year [95% CI .08-.25]). The common waning rate was estimated at (0.05 per person/year [95% CI .03-.07]). Consequently, application of the common rate of waning had little effect on the estimate of FOI in Siaya and Nairobi but, for Kilifi, the estimated FOIs from the 2 model options were significantly different (Table 3.12). When we considered age dependency of the FOI within the models with waning, the models with age dependent FOIs and site-specific waning provided the best fit overall. In Nairobi, the FOI changed from 0.1 exposures/person/year to 0.3 exposures/person/year at 1.5 years of age while for Siaya the rate changed at the age of 3.5 years. Meaning that, in these two locations, older children experienced a higher rate of infection than the younger ones. These cut-off ages were lower than the ages derived from the earlier piecewise regression model of continuous antibody data, and the direction of change was the inverse. The model from Kilifi showed a higher rate of infection in the first 6 months of life but could not resolve the second rate of infection. Given the additional complexity of the age-dependent models, we selected the reverse catalytic model with common waning as the most parsimonious mode

Figure 0.13: Model fit compared to observed data A. O:4,5 B. O:9

A.



B.


KILIFI
NAIROBI
SIAYA
SCM
RCM-Waning by site
RCM-Waning common
Age-dept FOI Waning by site
Age-dept FOI waning common
Probability Seropositive
Age in years
AIC 481
AIC 322
AIC 324
AIC 322
AIC 320
AIC 658
AIC 516
AIC 524
AIC 512
AIC 510
AIC 464
AIC 409
AIC 427
AIC 409
AIC 425

SCM – Simple catalytic model; RCM- Reverse catalytic model; FOI – Force of infection

Similarly for O:9 models, the reverse catalytic models provided a better fit than the simple catalytic models though the best fit, according to the AIC, was the age-dependent FOI model with common waning, at least for Kilifi and Nairobi; for Siaya, the reverse catalytic model with site specific waning had the best fit. For parsimony also, we selected the reverse catalytic models with common waning as the simplest models with the best fit (Fig 4.13B).

The resultant O:4,5 IgG FOI from these models were 0.50 /person/year [95% CI 0.38, 0.64] for Kilifi, 0.22 (0.18, 0.26) for Nairobi and 0.34 (0.28, 0.41) for Siaya, meaning that in Kilifi each person experiences an infection pressure equivalent to one exposure every two years. This was 2.3 times higher than Nairobi and ~1.5 times higher than in Siaya (Table 4.11).

For O:9 IgG, the FOI in Kilifi was 0.31 /person/year (95% CI 0.25, 0.40), in Nairobi 0.22 (0.19, 0.27) and in Siaya it was 0.21 (0.18, 0.25) meaning that in Kilifi, each person experiences an infection pressure equivalent to 1 exposure in 3.2 years, while in Nairobi and Siaya, the experience is one exposure every 4.5 years approximately.

Table 0.13: Estimates of Force of Infection (FOI) by serotype and site for the different models explored. Rates are in /person/year, 95%CI in brackets.

		O:4,5			O:9		
Model		Kilifi	Nairobi	Siaya	Kilifi	Nairobi	Siaya
Simple catalytic model	FOI	0.08 (0.07,0.09)	0.10 (0.08,0.11)	0.11 (0.09,0.13)	0.06 (0.05, 0.07)	0.08 (0.07, 0.09)	0.07 (0.06, 0.08)
Reverse catalytic model with site-specific waning	FOI	0.77(0.53, 1.11)	0.19 (0.14, 0.25)	0.28 (0.21, 0.36)	0.41 (0.28, 0.61)	0.38 (0.25, 0.57)	0.13 (0.11, 0.17)
	Waning	0.14 (0.08, 0.25)	0.03 (0.02, 0.06)	0.03 (0.01, 0.05)	0.10 (0.05, 0.18)	0.15 (0.08, 0.28)	0.01 (0.01, 0.03)
Reverse catalytic model with common waning	FOI	0.50 (0.38, 0.64)	0.22 (0.18, 0.26)	0.34 (0.28, 0.41)	0.31 (0.25, 0.40)	0.22 (0.19, 0.27)	0.21 (0.18, 0.25)
	Waning	0.05 (0.03, 0.07)	0.05 (0.03, 0.07)	0.05 (0.03, 0.07)	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)
Age-dependent FOI, with site specific waning	FOI-1	1.98 (0.94, 4.17)	0.11 (0.06, 0.18)	0.11 (0.04, 0.25)	0.09 (0.02, 0.54)	0.08 (0.03, 0.26)	0.08 (0.04, 0.20)
	FOI-2	0.01 (o, X)	0.28 (0.20, 0.40)	0.32 (0.25, 0.41)	0.42 (0.31, 0.58)	0.42 (0.31, 0.57)	0.15 (0.11, 0.19)
	Waning	0.18 (0.11, 0.28)	0.02 (0.01, 0.04)	0.01 (0.01, 0.04)	0.05 (0.01, 0.17)	0.05 (0.02, 0.15)	0.01 (0, 0.03)
	Cut-off	0.5y	1.5y	3.5y	2.5y	1.5y	5.5y
Age-dependent FOI, with common waning	FOI-1	0	0.17 (0.12, 0.25)	0.25 (0.11, 0.54)	0.11 (0.04, 0.30)	0.10 (0.05, 0.17)	0.28 (0.20, 0.39)
	FOI-2	0.71 (0.60,0.83)	0.28 (0.20, 0.41)	0.36 (0.28, 0.46)	0.43 (0.31, 0.59)	0.42 (0.31, 0.57)	0.13 (0.08, 0.23)
	Waning	0.05 (0.03,0.07)	0.05 (0.03, 0.07)	0.05 (0.03, 0.07)	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)
	Cut-off	3y	1.5y	3.5y	2.5y	1.5y	1.5y

4.6 Discussion

To the best of our knowledge, this is the first description of the prevalence of anti-*Salmonella* antibodies from community-based studies among children and adults in sub-Saharan Africa. We have observed that both serogroup O:9 (D_1) and serogroup O:4,5 (B) antibodies are present at birth and decay within the first 6 months of life. They are then re-acquired in infancy and increase rapidly in concentration and prevalence by age reflecting the high incidence of infection in this population. This is also the first study to estimate the Force of Infection (FOI) for NTS infections using catalytic models. The model estimated the FOI for serogroup B in Kenya has a range from 0.22 to 0.5 exposures/person/year, the highest transmission intensity being observed in Kilifi, while for serogroup D infections, the FOI varied from 0.21 to 0.31 exposures/person/year, the highest being observed in Kilifi. A rate of 0.2/person/year, the lowest in the region, means that 2 in 10 susceptible persons per year will acquire an NTS infection - a high incidence of infection.

The high force of infection highlights the under-ascertainment of infection rates derived from disease episodes characterised by culture-based isolation of NTS from blood samples through hospital surveillance and emphasizes the importance of serology-based estimates. A study investigating the amount of under-reporting of Salmonellosis in European countries estimated that the incidence rates of NTS infection, estimated from serological surveys, were 100-2000 fold higher than the incidence of culture confirmed NTS infections reported from hospital based surveillance systems, where Salmonellosis is a notifiable illness(107). This is evidence that a majority of the NTS infections in the European setting do not cause disease, which may or may not be the case in our African setting which is characterized by a lower iNTS case ascertainment, more invasive pathogen strains and a higher prevalence of invasion risk factors. These unmeasured infections likely consist of largely subclinical cases, which often contribute to transmission of the infection(55), and later disease in the susceptible.

We have observed the presence of maternally-derived anti-*Salmonella* antibodies and estimated the rate of decay. Both O:4,5 and O:9 antibodies decayed at a rate of 40% per month to very low concentrations in 4-5 months, which is similar to observations from a study in Vietnam(106) where the lowest concentration of maternally-derived anti-NTS antibodies was estimated at 20-30 weeks in infants. We did not assess the *in vitro* bactericidal effect of the maternal antibodies however, we observed that serum anti-NTS IgA antibodies, markers of recent infection, remained at low concentrations for the first half of infancy and increased rapidly thereafter, reflecting infection with O:4,5 and O:9 organisms. The lack of infection in the first 6 months of life, coupled with the presence of maternally derived IgG antibodies, point to the protective effect of systemic maternal antibodies against acquisition of infection. Similar observations have been made in Malawi, where the low incidence of invasive NTS disease among newborn infants has been attributed to the presence of maternally-derived IgG antibodies; this was confirmed in the study from Vietnam where efficient transplacental transfer of antibodies from mother to child was observed and, later, these antibodies exhibited the ability to kill NTS organisms through serum bactericidal assays(80, 101, 106). In Kilifi, however, the incidence of iNTS disease has been shown to be highest among newborn infants(3), yet we have demonstrated the presence of relatively high concentrations of maternal antibodies among infants at birth from the same population. This raises the question: what concentration of antibodies is required for protection against infection or against invasive disease? Additionally, what is the role of host factors, such as malaria, in affecting susceptibility of infants to invasive disease in the presence of (bactericidal) antibodies?

There is no established correlate of protection against NTS infection or invasive disease and we are therefore unable to estimate the proportion immune (proportion above the protective threshold). The studies from Malawi used age as a proxy to determine a threshold associated with protection (80). In these studies, antibody concentrations in children aged ≥16 months were found to confer effective killing against a *S*. Typhimurium variant known to cause invasive disease, which was in-line

with the age-incidence pattern of iNTS in the area where higher incidence of iNTS occurred in children below 16 months old. From our study, the curves showing the association between serotype-specific age-seroprevalence and age-incidence intersect at approximately 1.5y, supporting the observations from Malawi, and in addition, providing support to the selected threshold.

The relationship between antibody concentrations and faecal carriage of NTS has not been explored previously. Whether antibodies protect against acquisition of carriage is not clear. We found higher serum antibody concentrations of both IgA and IgG among carriers than non-carriers. We also found that the increase in seroprevalence by age mirrored the increase in carriage prevalence by age. These are possibly because antibody was stimulated by the recent infection leading to carriage or because carriage itself is a marker of risk for repeated infections. The cases of culture-confirmed carriage of NTS were few and this reduced our statistical power to observe a significant difference, though the effect sizes were large. Additionally, the circulating serotypes inducing these responses were mostly non-pathogenic (evidence from previous chapter) suggesting a role in stimulating cross-reactive antibodies. Repeated infections with less virulent but cross-reacting strains in childhood may elicit systemic immunity (IgG) that provides protection against invasive disease later in life. A similar phenomenon, termed premunition, has been described with other infectious diseases such as malaria, where the presence of asymptomatic parasitaemia is associated with a reduced incidence of disease(178-180); and in studies of urinary tract infections (UTI) where the presence of asymptomatic bacteriuria is associated with long-term protection against UTI(181, 182). For NTS, this observation may not be conclusive as we do not know the direction of the association between antibody concentrations and faecal carriage of NTS, if causal. Additionally, measurement of faecal IgA antibodies may provide a better measure of mucosal immunity to either infection or invasion rather than systemic IgG and IgA from serum samples(183). These data gaps can be addressed through a birth cohort starting with NTS naïve individuals and measuring both the rate of acquisition

of carriage & disease, the rate of seroconversion and the relationship between acquisition of infection and immunity.

We observed an age-related increase in both O:9 and O:4,5 IgG and IgA antibody concentrations and seroprevalence, beginning in infancy in all sites and peaking in late childhood. The rate of increase differed by site highlighting differences in transmission intensities in these endemic regions. For example, the peak GMC for O:4,5 IgG antibodies was reached by 2 years of age in Kilifi and the seroprevalence was ~70% at 5 years of age. By contrast, in Siaya and Nairobi antibody concentrations rose more slowly and peaked at 5 years and 8 years of age, respectively, achieving ~60% seroprevalence at 5 years of age. Coupled with a high FOI for O:4,5 in Kilifi, this shows that the exposure to O:4,5 antigens is more intense in Kilifi than Nairobi and Siaya. Interestingly, Kilifi had the highest prevalence of NTS carriage as observed in the previous chapter, including of Group B serotypes, which could be responsible for the increase in O:4,5 specific antibody concentrations or even non-serotype specific antibodies. At the same time, Kilifi had the lowest incidence of iNTS disease, compared to Nairobi and Siaya, which provides some ecological evidence of serum IgG protection against iNTS disease.

For O:9 antibodies, the mean concentration and FOI was highest in Kilifi too, but the rate of increase in these antibodies was highest in Nairobi. This observation in Nairobi is not supported by carriage data, since the single NTS isolated in Nairobi was not group D; neither is it supported by iNTS disease data since higher iNTS disease incidence by age have been observed in Siaya than Nairobi. It is possible that the assay was detecting antibodies that are cross-reactive with any of the other 165 serotypes within Group D, including with *S*. Typhi, which also belongs to the O:9 serogroup(184), and Typhoid fever is known to cause outbreaks in this urban slum area(185).

The specificity of the ELISAs used may limit the interpretation of our study. The ELISA was designed to detect the O-antigen, a component of LPS found in multiple gram-negative organisms including

NTS. Previous studies from Denmark have demonstrated cross-reactions between *E. coli*, Yersinia and Campylobacter, among other gram-negatives in NTS LPS ELISAs(102, 163). Additionally, for NTS, multiple serotypes exist within one O-antigen serogroup, and their responses might not be differentiated easily using O-antigen based serology. Our assay-based threshold reflected this uncertainty, as it defined seropositivity as any value above the LLOQ. Using this threshold we observed 100% seroconversion during infancy for both O:4,5 and O:9 IgG across all sites. It is likely that the universal presence of antibodies, using this threshold, simply represents a high background incidence of infection with other gram-negative organisms in the environment including non-pathogenic Salmonellae. Seropositivity by this cut-off has previously been interpreted as the proportion of the population that has any detectable antibodies to NTS (104). This was in a study on seroprevalence of anti-NTS antibodies in Kampala, Uganda, where the same assay and LLOQ cut-offs were used to estimate the proportion seropositive. In both our study and the Uganda study, not every sample tested was positive for the two antigens indicating some O-antigen specificity, even against a background of cross-reactivity.

To what extent is waning of anti-NTS antibodies implied by our results? The geometric mean concentrations increased with age and reached a plateau in adulthood but there was no significant evidence of reduced GMCs thereafter suggesting there was no waning. However, seroprevalence increased with age but it reached an asymptote at <100% suggesting that, in the presence of ongoing infection, there is likely to be antibody waning. Not surprisingly, therefore, when we modelled seroprevalence, all the models that incorporated waning had a better fit to the observed data than those that did not - suggesting that waning is important in explaining NTS transmission dynamics. Our interpretation of waning based on seroprevalence is limited by the selected threshold. A lower threshold would mean more seroconversions as observed with the LLOQ threshold demonstration – age seroprevalence curves derived using LLOQ reached 100% seroprevalence by age of 5 years and this was maintained throughout life.

Previous studies have shown that reinfection with NTS is possible, both with the same serotypes and with other different serotypes(12, 55, 56), which means that either natural infection gives partial immune protection or there is waning of immunity. Studies on decay of NTS antibodies are few. In a cohort of Danish patients, antibody concentrations measured at 1, 3, 6 and 12-months after NTS gastroenteritis showed a steady decay in antibody concentrations, though 40% and 16% of the participants, respectively, had detectable IgG to *S.* Enteritidis and *S.* Typhimurium at 12 months(102). Whether this translated to waning of immunity is not clear, as the threshold for immune protection by antibody is not defined.

Our models showed that the rate of waning differed significantly by site- It was highest in Kilifi and lowest in Siaya. Waning of specific antibodies have previously been shown to be associated with age, sex and presence of co-morbidities such as HIV-infection which consume immune cells(186-188). Therefore, geographical differences in waning would be possible if the sites differ in the above factors. In our study, the age and sex distribution of the participants were balanced by site; except for Nairobi where the median age was younger than the other two sites; While the prevalence of HIV-infection and malaria in Kenya is highest in Siaya, which would contradict our observation, as Siaya would be expected to have the highest rate of waning.

We explored two thresholds for defining seropositivity, an assay-based threshold and a threshold derived from mixture modelling, which attempts to dichotomize the population into distinct groups that were unobserved using the breadth and shape of the frequency distribution of antibody concentrations to draw these populations apart. The utility of the method is contingent on the validity of the underlying assumptions. We assumed that the data contained a mixture of 2 populations (seropositive and seronegative) which is the simplest classification possible. Further sub-classifications into recently infected individuals and those with waning antibody could be specified if biologically plausible. However, as this is one of the first studies to look at population distributions of anti-NTS antibodies, and because our understanding of the responses and waning rates is

rudimentary, we selected a two-population model as the most parsimonious option. The mixture modelling method also assumes that antibody concentrations in each population sub-group are normally distributed; the fact that the frequency distribution of antibody concentrations for the whole population is log-normal lends some credence to this assumption. Using this approach, we successfully separated the O:4,5 and O:9 IgG distributions into 2 components each. However, for IgA, the models could not separate the underlying components, assumed to be present. For O:9 IgA, the resulting distributions had considerable overlap with hardly any discrimination between the classes. For O:4,5 IgA, the model did not converge on a result meaning it was unable to discriminate underlying populations in the data, even when adjusted for carriage.

For the IgG concentrations, we used the distribution of the two classes in the mixture models to derive a threshold for seropositivity. This was complicated by the fact that the infected and uninfected groups each had a broad range of concentration values which overlapped markedly across the two groups. Effectively this meant that any threshold would compromise either specificity or sensitivity. Previous studies employing mixture modelling techniques have used the mean of the first class (negative/uninfected group) plus 2 standard deviations as a threshold (Reviewed by Sepulveda N., *et al* for malaria antibodies) (172). In our study this approach produced a threshold with very high specificity but low sensitivity. Optimising specificity may not be biologically justified if the assays lacked serovar specificity, as was likely for the *S.* Typhimurium and *S.* Enteritidis ELISAs. Therefore, we selected a threshold that maximized both sensitivity and specificity using standard analysis of an ROC curve(174). Clearly, the seroprevalence results are highly sensitive to the selection of the threshold, however, the differences observed by age and by site, and the derivation of relative levels of the force of infection are less sensitive to the selection of the threshold. In defence of the external validity of the point chosen in this study, the resulting age-seroprevalence curves showed an increasing seroprevalence by age, which are biologically plausible, as they match the age-incidence of iNTS disease.

Serology based studies of NTS would benefit from standardization of assays and the establishment of an immunological threshold for both exposure and protection. This can be defined through longitudinal studies assessing responses to naturally acquired NTS infections or in challenge studies where the immunological response is assessed before and after infection, using standardized assays. Standardization of NTS assays would enable valid comparisons of study results across different laboratories and populations. Efforts to define the correlates of protection for Shigella (189) and to standardize serological assays for *S.* Typhi (190) through NIBSC-UK are underway and we hope that NTS assays and standards will soon follow. This would enable assessment and monitoring of immunological based interventions, such as vaccines.

4.7 Conclusion

I have used serology to describe the epidemiology of NTS infection and rates of transmission.Using a population-based cross-sectional study approach, I have demonstrated two relevant facts. (1) The transmission intensity of NTS infections by both O:4,5 and O:9 serogroups in Kenyan children is high and it differs by site; therefore, control strategies need to focus on multiple NTS serovars and customize strategy by geographical setting. (2) Protective maternal antibodies are present at birth but decay within first 6 months of life after which primary infections occur rapidly; therefore, infection control strategies need to be implemented in early infancy, and where vaccination is considered, the age window for vaccination may be reduced by the presence of maternal antibodies. In addition, I have highlighted areas that would benefit from further scientific research, including assay standardization and the thresholds for correlates of protection against infection and against invasive disease.

Overall, the study describes the serological profile of anti-NTS antibodies in endemic populations, which have implications for both control strategies and future research.

Temporal changes in the rate of acquisition of NTS infections in Kilifi: 2002 – 2017

5.1 Introduction

Non-typhoidal Salmonellae are a common cause of bacteraemia in Africa, especially in high malaria transmission zones and high HIV prevalence areas. In children, the risk of iNTS disease is higher in the presence of recent *Plasmodium falciparum* malaria, concurrent malaria infection, malnutrition, and sickle cell disease(3, 25, 62, 66) . Among adults, immunosuppression from HIV infection is a risk factor (3, 85). Several sites in Africa have observed a reduction in the incidence of iNTS disease at the same time as they have observed (a) a decrease in malaria prevalence (117, 119) or (b) an increase in the uptake of ARVs (122). This temporal association between risk-factor prevalence and iNTS incidence is assumed to be due to these host-determined changes in the risk of invasion by prevalent NTS infections. However, an alternative hypothesis – that reduction in iNTS incidence is a function of improved living standards leading to reduced transmission of NTS – is also plausible. The distinction is important for vaccine deployment: any future vaccine may reduce invasion or transmission to varying degrees – and its use would depend critically on an understanding of the epidemiology of transmission and the risk factors for invasion.

In the Kilifi Health and Demographic Surveillance System (KHDSS), the proportion of slide-positive malaria admissions at the Kilifi County Hospital has decreased from 0.56 to 0.07 between 1998 and 2009 (191) and a simultaneous decrease in the incidence of severe malaria in children from 7.9 to 1.6 per 1000 between 2003 and 2015 (192). In addition, during the same period, the median age at presentation among admissions with malaria has increased, implying a reduction in parasite transmission in the community(192). The reduction in malaria parasite prevalence has been associated with a reduction in admissions to hospital with all-cause bacteraemia, but especially with non-Typhoidal Salmonella bacteraemia(117) ; Cases of iNTS disease at the KCH have decreased by 16% per year since 1998 (Incidence Rate Ratio 0.84)(3), The mechanisms underlying this temporal relationship are not well understood. It is not known whether the drop in iNTS disease was due to a reduction in transmission of NTS infection in the community which just happens to be in temporal

association with declining malaria transmission, or a reduction in the invasiveness of iNTS due to reduced host-susceptibility as malaria infection declined.

As observed in the previous chapter, one of the most practical approaches to estimate transmission intensity is to measure age-specific seroprevalence. Antibody levels are a sensitive marker of cumulative pathogen exposure and can be used to estimate long-term trends in transmission. By estimating the force of infection over time, we can infer changes in the rate of acquisition of infection. Seroconversion rates from catalytic models provide a measure of force of infection and the rate at which susceptible individuals acquire infection, therefore a declining seroconversion rate can be interpreted to showing a decrease in rate of acquisition of infection. Using archived serum samples collected between 2002 and 2017, we setup a retrospective ecological study to investigate whether the observed changes in iNTS disease incidence in Kilifi were attributable to changes in the incidence of NTS infection or to changes in the probability of invasion caused by changes in the prevalence of malaria. If there was no change in the incidence of NTS infection, that would imply that the change incidence of iNTS disease must have another cause and this is most likely to be a change in the prevalence of risk factors for invasion. If, by contrast we find that the incidence of NTS infection declined over time, then at least some of the change in iNTS disease incidence must be attributable to this change, reducing the role of host risk factor changes on declining iNTS incidence.

5.2 Method

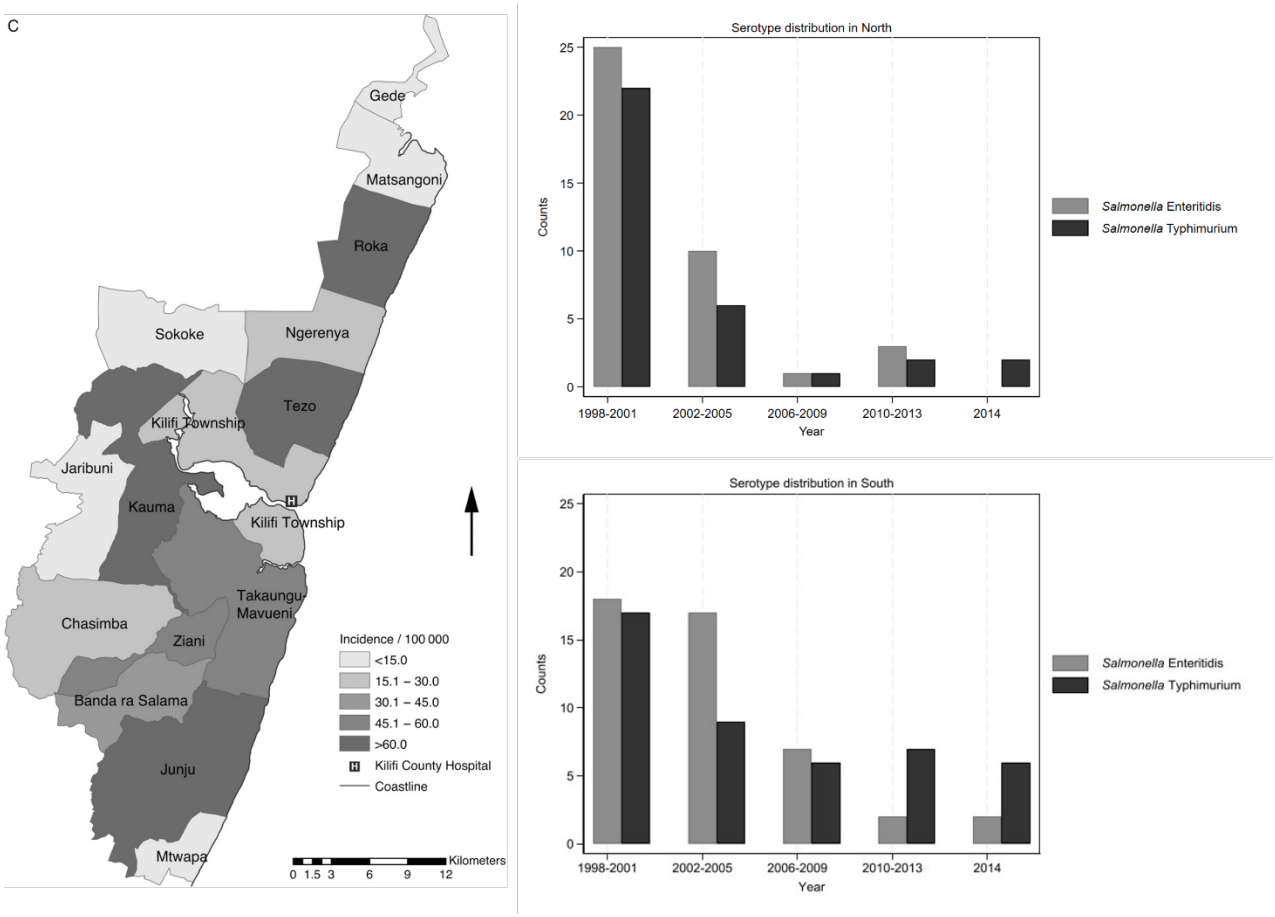
We took advantage of the rich bio bank of cross-sectional serological specimens collected in studies of malaria epidemiology among residents <15y from 2 locations in Kilifi: Ngerenya and Junju locations. Kilifi Creek creates a natural geographic barrier between the northern and southern parts of the KHDSS (Figure 5.1). Ngerenya sub-location lies to the north of the Kilifi creek while Junju is in the south. The epidemiology of both malaria and iNTS have been found to vary between the areas North and South of Kilifi Creek and this analysis takes advantage of this heterogeneity. Since 1998, annual cross-sectional blood sampling has been undertaken in Ngerenya location which is located in

the North. Parasite prevalence (*P. falciparum*) in this location fell from 30% to 0% between 1998-2005 (193), indicating a reduction in malaria transmission intensity. The decline in malaria prevalence in Ngerenya has been attributed to widespread uptake and use of insecticide treated bed nets, which were distributed in this area during the early 1990's(194). Continued surveillance showed that the decline in parasite prevalence continued until 2008 and then increased slightly between 2009-2014 (191). In 2004, a similar surveillance was established in Junju location in the South. A decline in malaria parasite prevalence was also observed in this location; parasite prevalence by microscopy in the Junju cohort decreased from 16% to 4% between 2007 and 2017 (195).

After an initial sample of all children aged <15 years, the studies in both locations were established as rolling cohorts; new members were recruited as infants, and existing cohort members exited the surveillance cohort at 15 years. Cross-sectional surveys were conducted among the cohorts just before the long rains (mostly in May) each year, collecting blood for serology and parasite microscopy.

In Kilifi HDSS, the incidence of iNTS in the KHDSS also varies by location (4.8-69.8/ 100000PYO), after adjusting for access to hospital care. The highest incidence occurred in locations in the South (3). Both *S.* Enteritidis and *S.* Typhimurium are dominant serotypes both North and South of Kilifi Creek.

Figure 0.1: A. incidence of iNTS in KHDSS by location B & C. Serotype distribution of the iNTS cases in North and South of KHDSS (1998-2014)



4.1.8 Laboratory methods

We measured IgG antibodies against the O:9 antigen component representing *S*. Enteritidis and O:4,5-antigen component representing *S.* Typhimurium, by the same ELISA method detailed in the previous chapter. For each vial, we started with O:4,5 assays and then moved to O:9 assays if sufficient volume was available. The optical density readouts were converted into antibody concentrations (AU units) via with reference to relative to a 4-parameter logistic regression curve from previously calibrated reference sera. One AU is equal to the reciprocal of the dilution of the standard serum giving an OD =1.

Serum samples were available from Ngerenya for the period between 2002-2017 and from Junju between 2008-2017. In Ngerenya, the annual cross-sectional bleed did not take place in 2006, 2009 and 2013. For our assays, we included all available serum from all available cross-sectional bleeds.

4.1.9 Data analysis

The antibody units were transformed to log base 10. Zero values were arbitrarily allocated a value that was half of the lower limit of detection (LLOD) for the specific assay, which aided log-transformation. We divided the study time into four, 4-year periods (2002-05, 2006-09, 2010-13, 2014-17). Serotype specific Geometric Mean Concentrations (GMC) were estimated by age-strata (0-5 years, and 5-15 years), by site (Ngerenya and Junju). Comparisons between groups were performed using ANOVA and the trends in annual GMCs for each serotype by calendar time assessed using linear regression of Log_{10} antibody concentrations as AU.

We used the threshold derived from the mixture modelling exercise in the previous chapter to assign seropositive and seronegative status (14.1 AU for O:9 IgG, 28.2 AU for O:4,5 IgG.) The linearity of the trends in proportion seropositive over time were assessed using the Cochrane Armitage test. To estimate the force of infection per year, between sites and serotypes, seroprevalence data stratified by age were analysed using a reverse catalytic model fitted to the observed dataset using the *revcat* command in Stata(175). Based on results from the previous chapter, we assumed a common rate of waning applied to both locations and all time-periods and excluded data from infants under 6 months of age from the analysis as any antibodies detected are likely to represent maternally acquired antibodies rather than those in response to a primary infection. For each time period, we restricted the model to participants under 5years old, which was to keep with the assumption of Muench catalytic models that the force of infection is stable for the duration of the age of the oldest person in the dataset (108). All analyses were carried out in Stata v15.

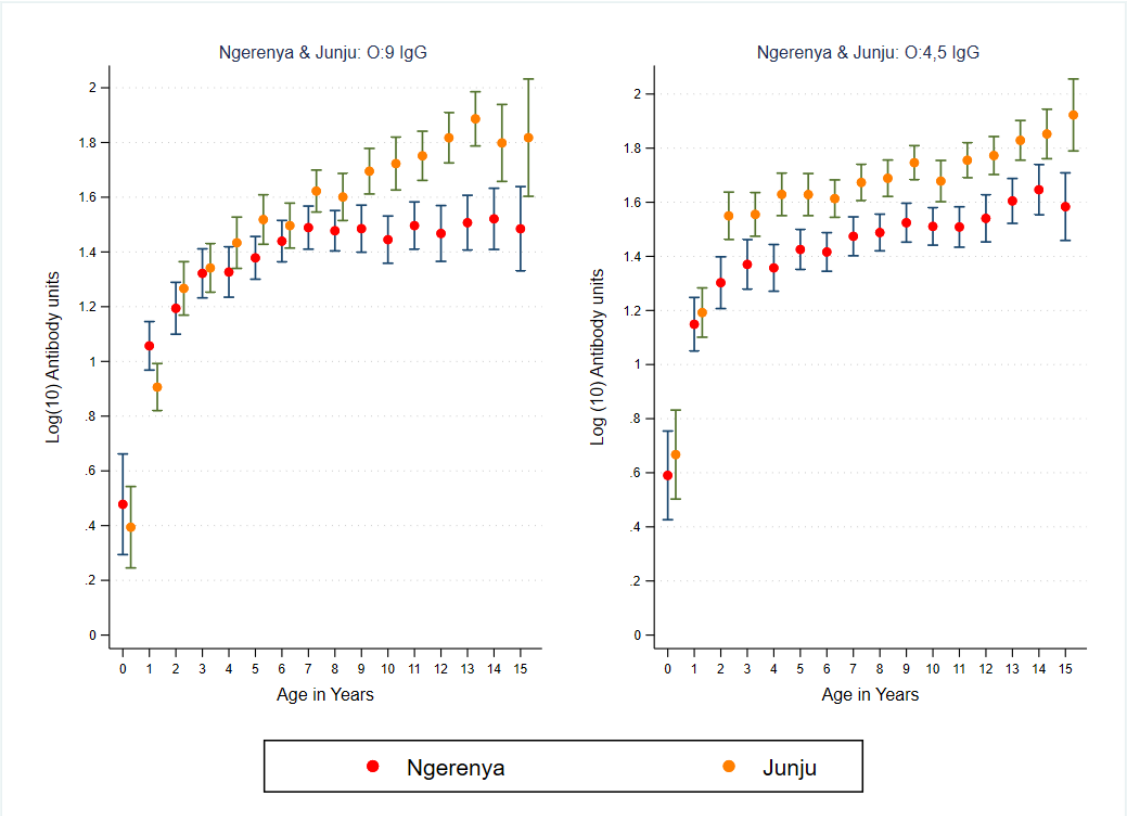
5.3 Results

From the Ngerenya study, we assayed 2704 and 2804 samples for O:9 IgG and O:4,5 IgG, respectively, collected between 2002 and 2017, Of which, 825 and 844, respectively, were from under 5year-olds. From the Junju study, we assayed 2993 and 3109 samples for O:9 IgG and O:4,5 IgG, respectively, collected between 2008 and 201, of which 999 and 974 were from under 5-year-olds. Some vials did not have sufficient sample volume to undertake both assays and, in these, the O:4,5 IgG assay was prioritised.

4.1.10 Antibody concentrations by age

Overall, the anti-O:9 IgG concentrations ranged from 0.02-5489 AU while the anti-O:4,5 IgG concentrations ranged from 0.03-2991 AU (Fig. 5.2).

Figure 0.2: Graph of antibody concentrations in log AU by age in years.



The IgG GMC increased with age. By site, the increase in GMC by age was larger in Junju than in Ngerenya (Table 5.1). Among under 5's, the anti-O:9 IgG means were similar at the two sites (14.4 vs 14.7 AU, p=0.853) while the anti-O:4,5 IgG mean was higher in Junju than in Ngerenya (25.4 Vs 17.3 AU, p<0.001). In the older children, both the O:9 IgG and O:4,5 IgG GMC were significantly higher in Junju than in Ngerenya.

Table 0.1: Geometric mean concentrations of anti-O:9 and anti-O:4,5 IgG by age and site.

	Geometric mean concentrations (95% CI)		
	Ngerenya	Junju	p-value
O:9 IgG			
0-5y	14.7 (13.2, 16.3)	14.5 (12.9, 16.1)	0.854
5-15y	29.1 (27.4, 30.9)	46.3 (43.3, 49.4)	<0.001
Linear change/age year	1.08 (1.07,1.10)	1.18 (1.16,1.19)	
O:4,5 IgG			
0-5y	17.3 (15.6, 19.3)	25.4 (23.0, 28.2)	<0.001
5-15y	31.6 (29.9, 33.3)	50.9 (48.3, 53.6)	<0.001
Linear change/age year	1.08 (1.07,1.10)	1.11 (1.09, 1.12)	

4.1.11 Antibody concentrations by calendar time

We assessed the changes in mean antibody concentrations across time by linear regression. Since there were changes in the age distribution by year - the median age in Ngerenya increased over the years, ranging from 4 to 12 years, while in Junju, the median age of the cohort by year maintained at 5 to 8 years - we adjusted for age in the analysis and present both the crude and age-adjusted estimates in Table 5.2. Across time, the GMC of anti-O:9 IgG decreased significantly in Ngerenya by 6% per calendar year among children aged < 5 years and by 3% per calendar year among older children. In Junju, the changes in anti-O:9 IgG GMC were not significant, after adjusting for age. For anti-O:4,5 IgG, the GMC in Junju increased by 6-7% per year across all age-groups, but the crude increase in GMC in Ngerenya did not retain significance when adjusted for age.

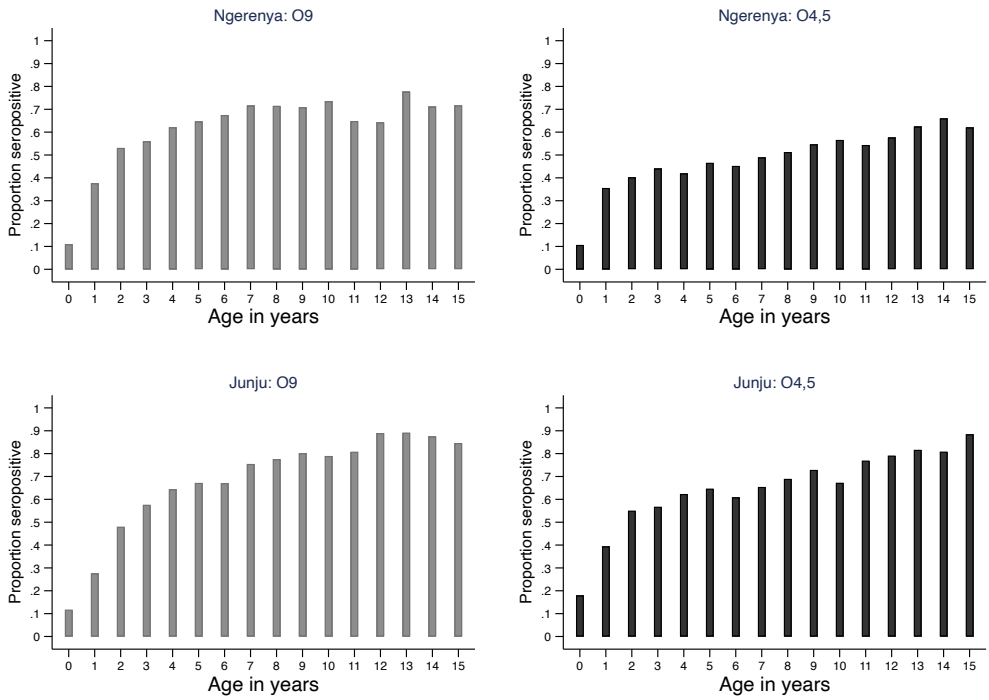
Table 0.2: The geometric mean concentrations (and standard deviations) in Antibody units of anti-O:9 and anti-O:4,5 IgG across time.

	O:9 IgG Geometric Mean Concentrations (SD)				O:4,5 IgG Geometric Mean Concentrations (SD)			
	0-4y		5-14y		0-4y		5-14y	
Year	Ngerenya	Junju	Ngerenya	Junju	Ngerenya	Junju	Ngerenya	Junju
2002	17.0 (3.4)	-	27.4 (3.1)	-	17.6 (4.0)	-	26.9 (2.6)	-
2003	15.9 (6.1)	-	36.8 (3.5)	-	16.5 (6.3)	-	34.3 (4.1)	-
2004	20.4 (5.8)	-	46.8 (3.0)	-	20.2 (5.3)	-	49.8 (3.2)	-
2005	20.1 (4.1)	-	40.2 (3.6)	-	18.1 (4.0)	-	25.2 (3.1)	-
2006	-	-	-	-	-	-	-	-
2007	19.1 (3.6)	-	35.9 (3.2)	-	14.6 (4.3)	-	30.2 (3.2)	-
2008	8.54 (3.5)	15.9 (5.6)	17.7 (4.0)	31.7 (4.2)	13.5 (4.1)	19.6 (4.4)	29.7 (3.3)	34.8 (3.5)
2009	-	19.0 (4.3)	-	40.1 (4.3)	-	21.3 (4.6)	-	41.8 (3.5)
2010	14.1 (3.8)	13.2 (6.1)	33.9 (3.8)	36.4 (4.5)	16.4 (3.6)	24.2 (4.4)	28.0 (2.9)	44.7 (3.4)
2011	8.75 (5.2)	15.1 (6.6)	23.1 (3.8)	49.1 (5.0)	18.7 (4.2)	18.2 (6.4)	27.1 (3.5)	36.7 (3.3)
2012	6.70 (5.7)	10.5 (6.0)	20.2 (3.6)	47.5 (4.2)	9.52 (4.8)	18.1 (5.1)	20.9 (3.5)	47.0 (3.0)
2013	-	18.8 (4.7)	-	65.9 (3.9)	-	33.7 (4.3)	-	73.4 (2.6)
2014	8.99 (2.9)	21.9 (4.3)	27.4 (3.3)	71.1 (3.7)	17.7 (5.5)	54.3 (3.8)	37.5 (2.9)	70.2 (3.1)
2015	-	15.9 (5.5)	22.2 (3.9)	53.4 (3.9)	-	32.9 (5.0)	26.2 (3.1)	64.5 (3.0)
2016	20.5 (4.6)	12.4 (7.0)	44.9 (4.1)	42.0 (5.3)	57.3 (4.0)	47.3 (4.4)	52.5 (3.0)	58.7 (4.0)
2017	10.5 (10.)	8.97 (4.7)	35.0 (4.1)	36.2 (4.9)	33.1 (5.2)	20.5 (6.5)	57.3 (2.8)	58.2 (3.4)
Linear change / year	0.95 (.93,.98)	0.97 (.93,1)	0.98 (.97,1)	1.03 (1-1.05)	1.01 (.99,1.04)	1.06 (1.03,1.1)	1.02 (1,1.03)	1.07 (1.05,1.09)
Adjusted linear change/year	0.94 (.91,.96)	0.97 (.94,1.01)	0.97 (.95,.98)	1 (.97,1.02)	1 (.98,1.03)	1.07 (1.04,1.11)	1 (.98,1.01)	1.06 (1.04,1.08)

4.1.12 Seroprevalence by age and time.

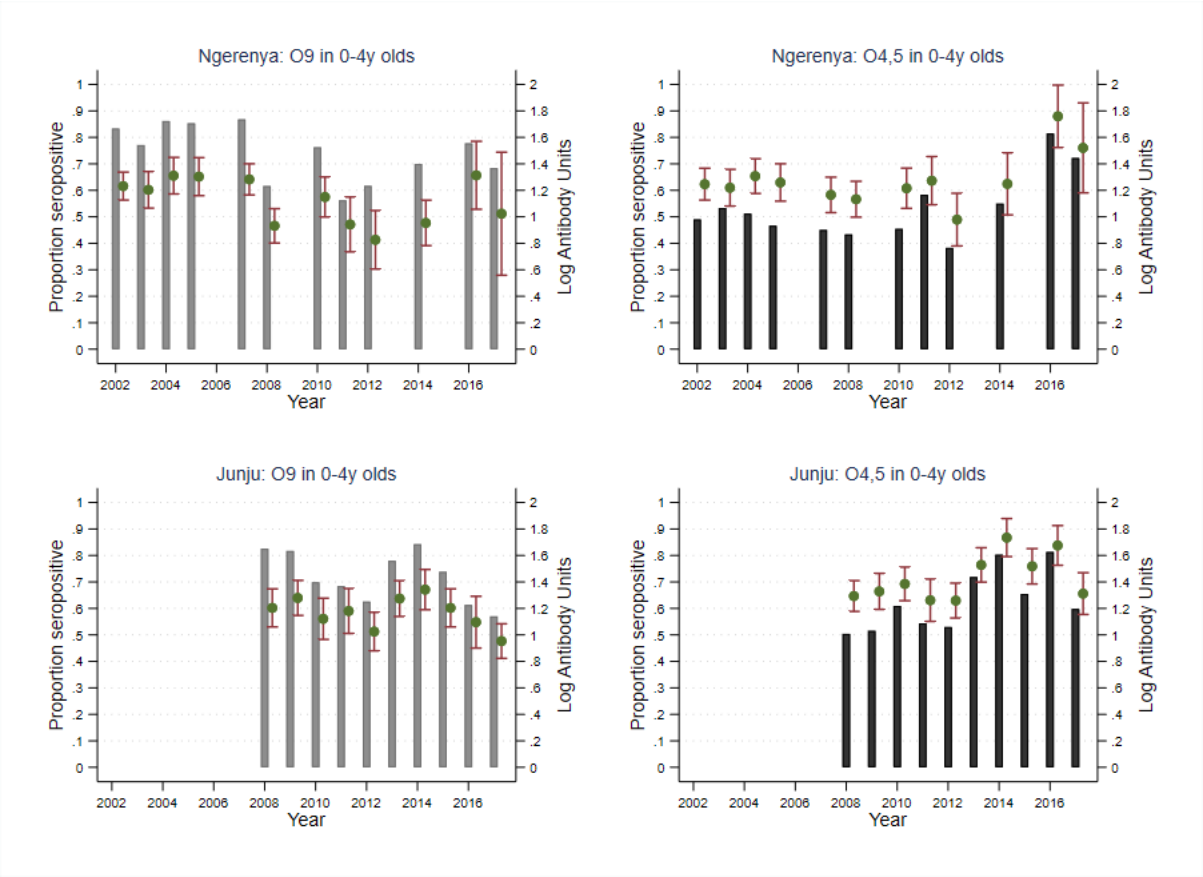
The seroprevalence of both anti-O:9 and O:4,5 IgG antibodies increased by age (Figure 5.3). Overall, the seroprevalence of O:9 IgG in Ngerenya was 11% at 1 year, 62% at 5 years and 71% at 10 years while in Junju it was 12%, 62% and 73% at 1 year, 5 years and 10 years, respectively. For O:4,5 IgG, the seroprevalence in Ngerenya was 11% at 1 year, 42% at 5 years and 55% at 10 years, while in Junju it was higher across the ages, at 18%, 62% and 73% at 1 year, 5 years and 10 years, respectively.

Figure 0.3: Seroprevalence of anti O:9 IgG and O:4,5 IgG by age and site.



Across the study period, there was variation in the seroprevalence by time in both under 5's and older children, though the trends were not linear (Fig. 5.4).

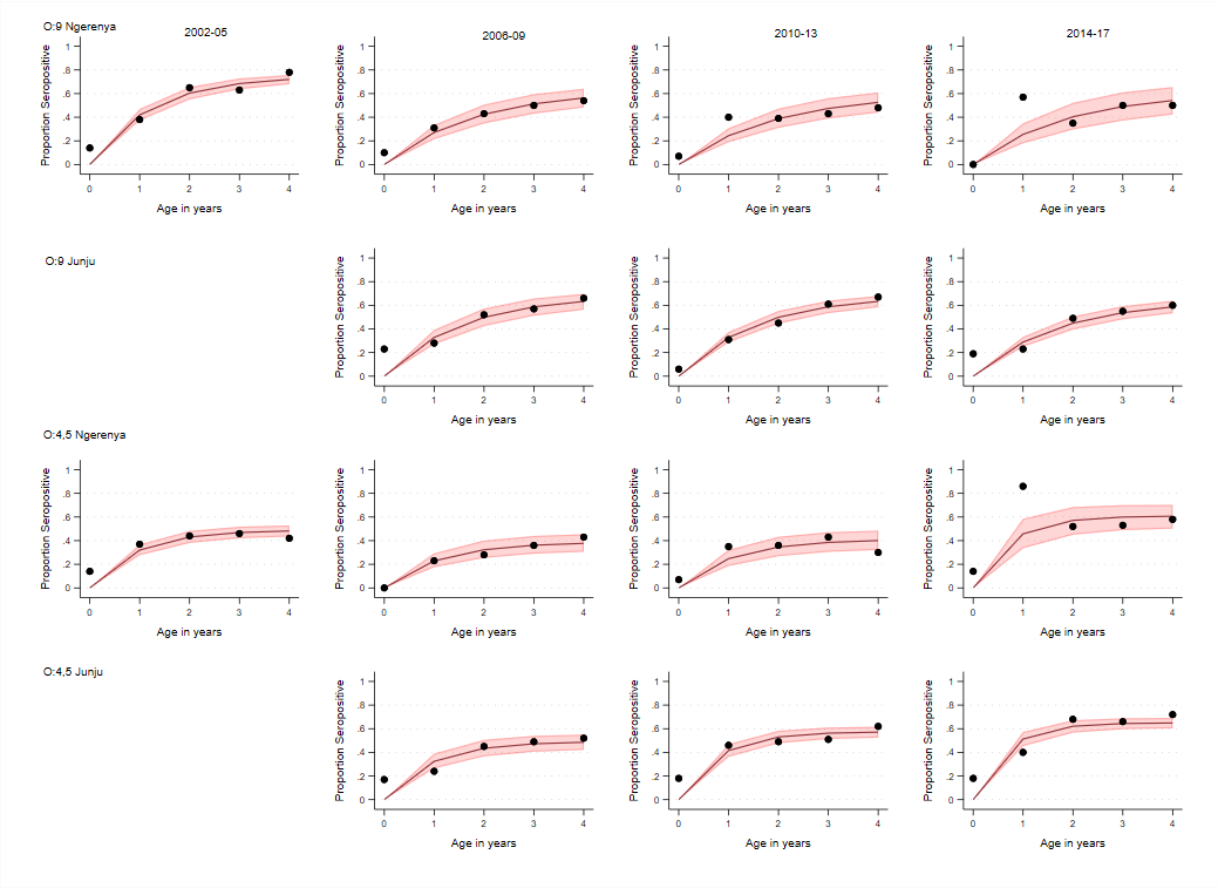
Figure 0.4: Trends in O:9 and O:4,5 IgG GMC and seroprevalence by calendar year.



4.1.13 Modelled estimates.

We modelled the rate of seroconversion using a reverse catalytic model which was fitted for each time-period separately. Incorporation of waning was informed by results of the previous chapter and because the observed seroprevalence in this population was <100% (Fig 5.3). The model fits are presented in Figure 5.5, while the estimated FOI are presented in Table 5.3.

Figure 0.5: Model fitting of the reverse catalytic model.



The FOI differed by site and time-period. Junju had higher FOI than Ngerenya across all time periods for both O:4,5 and O:9 infections. Seroreversion occurred at a rate of 21%/year and 55%/year for O:9 and O:4,5 antibodies, respectively. Over time, the O:9 FOI in Ngerenya decreased from 0.6/person/year in 2002/05 to 0.3/person/year in 2010/13 and later increased to 0.5/person/year in 2014/17. Similarly, for O:4,5, the FOI decreased from 0.5 to 0.4/person/year and later increased to 1.3/person/year over the same time periods. In Junju, the O:9 FOI decreased throughout the study period from 0.5/person/year in 2006-9 to 0.3/person/year in 2014-17, while the FOI for O:4,5 increased from 0.5 to 0.9/person/year over the same period.

Table 0.3: Force of Infection (FOI) and waning from a reverse catalytic model.

Antigen	Period	Force Of Infection (FOI)		Waning
		Ngerenya	Junju	
O:9	2002-05	.62 (.52,.72)	-	.21 (.12, .35)
O:9	2006-09	.35 (.27,.46)	.45 (.35,.56)	
O:9	2010-13	.29 (.22,.37)	.47 (.40,.54)	
O:9	2014-17	.47 (.28,.77)	.34 (.27,.42)	
O:4,5	2002-05	.52 (.43,.62)	-	.55 (.37, .80)
O:4,5	2006-09	.35 (.25,.46)	.53 (.41,.67)	
O:4,5	2010-13	.41 (.30,.54)	.81 (.68,.96)	
O:4,5	2014-17	1.34 (.72,2.5)	.94 (.75,1.1)	

These changes in the transmission intensity can be appreciated through visual assessment of the age-seroprevalence curve across different time periods (Fig. 5.6 & Fig. 5.7). A shift to the right of the age-seroprevalence curves indicates that the population was older at infection, and thus the transmission intensity was lower than the preceding time-period. In Ngerenya, for example, we see a stepwise shift to the right from 2002 to 2013, and then a left shift in the later period for both O:4,5 age-seroprevalence and O:9 age-seroprevalence curves (Fig. 5.6A & 5.7A). This signifies a decrease in the rates of infection from 2002 to 2013 followed by an increase. In Junju, the O:9 age-seroprevalence curves, although shifting to the right over time, were also very close together signifying that the changes observed were minimal (Fig. 5.6C). In the O:9 predicted curve for Junju, the 2006-09 curve appears to be missing only because it is overlaid by the 2013-13 curve. The O:4,5 age-seroprevalence curves in Junju, on the other hand, shifted to the left over time, denoting an increase in the rates of infection over the study period (Fig. 5.7C).

Figure 0.6: O:9 IgG Age-seroprevalence curves by site: observed and predicted.

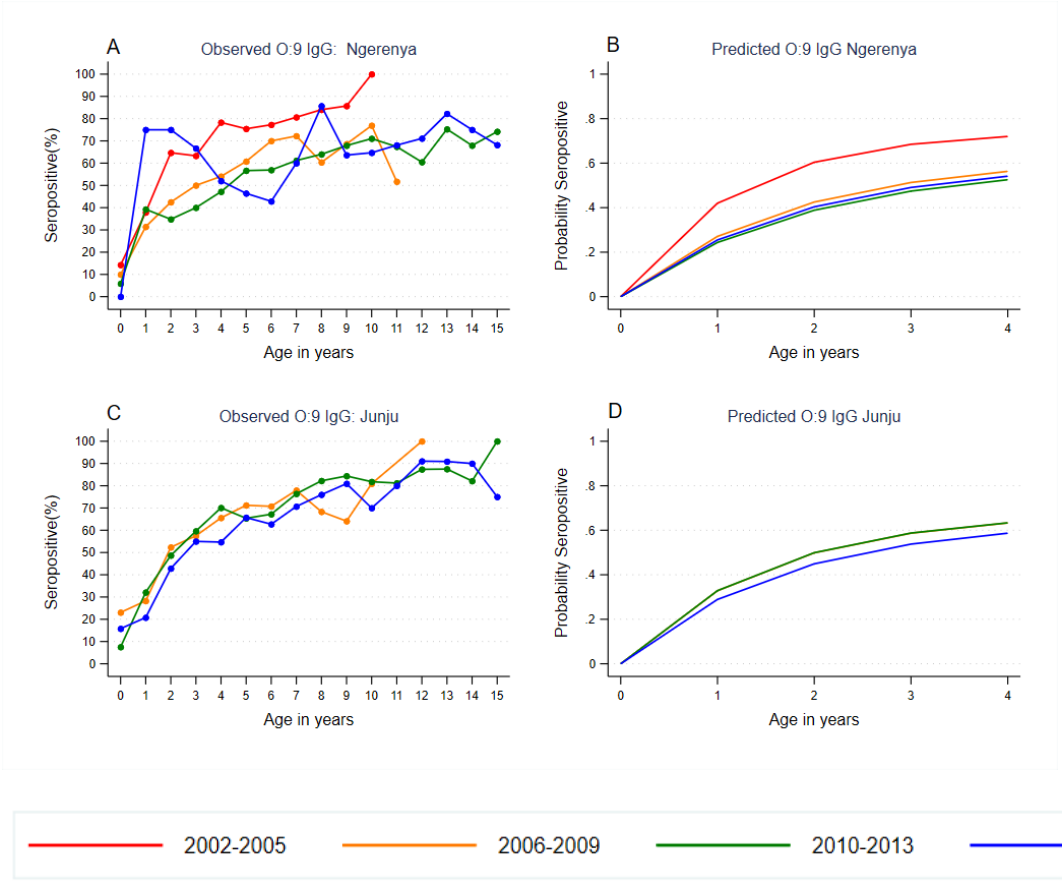
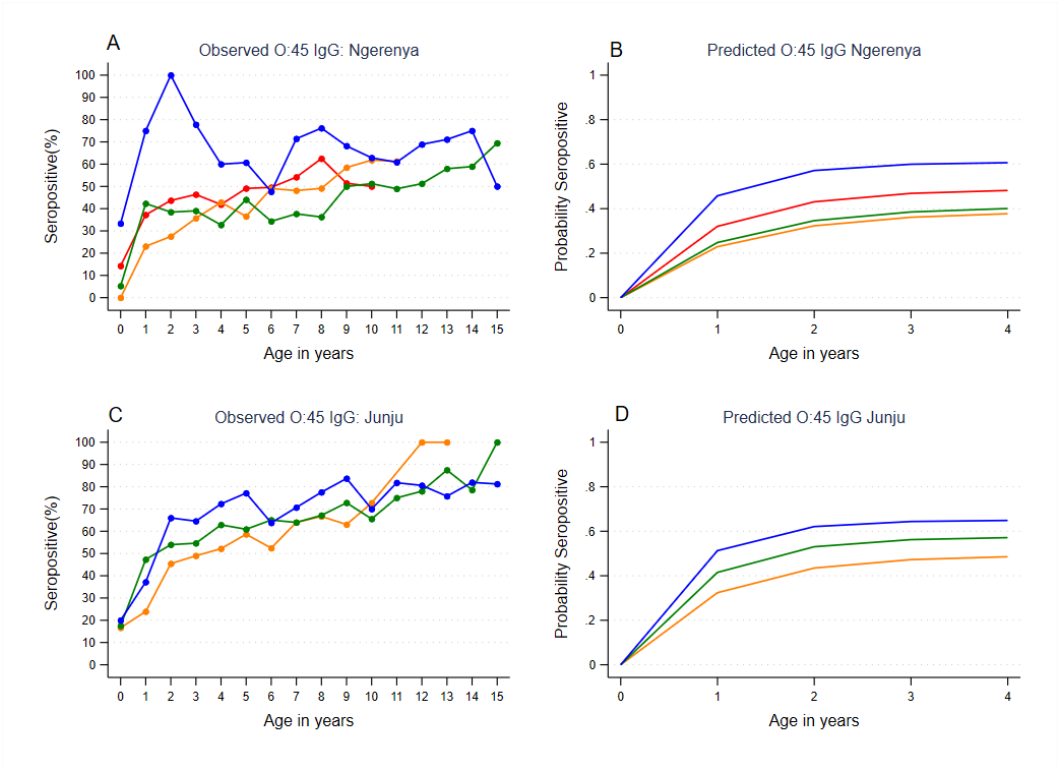
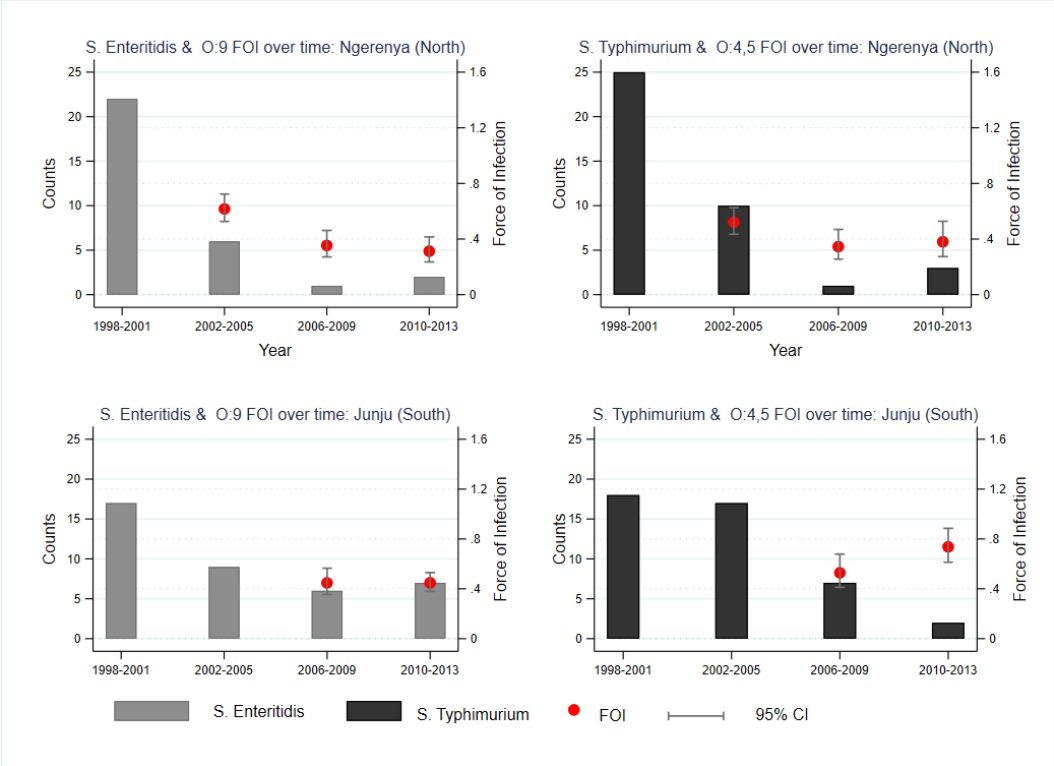


Figure 0.7: O:4,5 IgG Age-seroprevalence curves by site: Observed and predicted.



To visualise the temporal changes in infection and disease together, we graphed the iNTS serotype distribution in the North and South of the KHDSS overlaid with the FOI results, excluding the last study period (2014-2017) where only FOI data was available (Fig. 5.8).

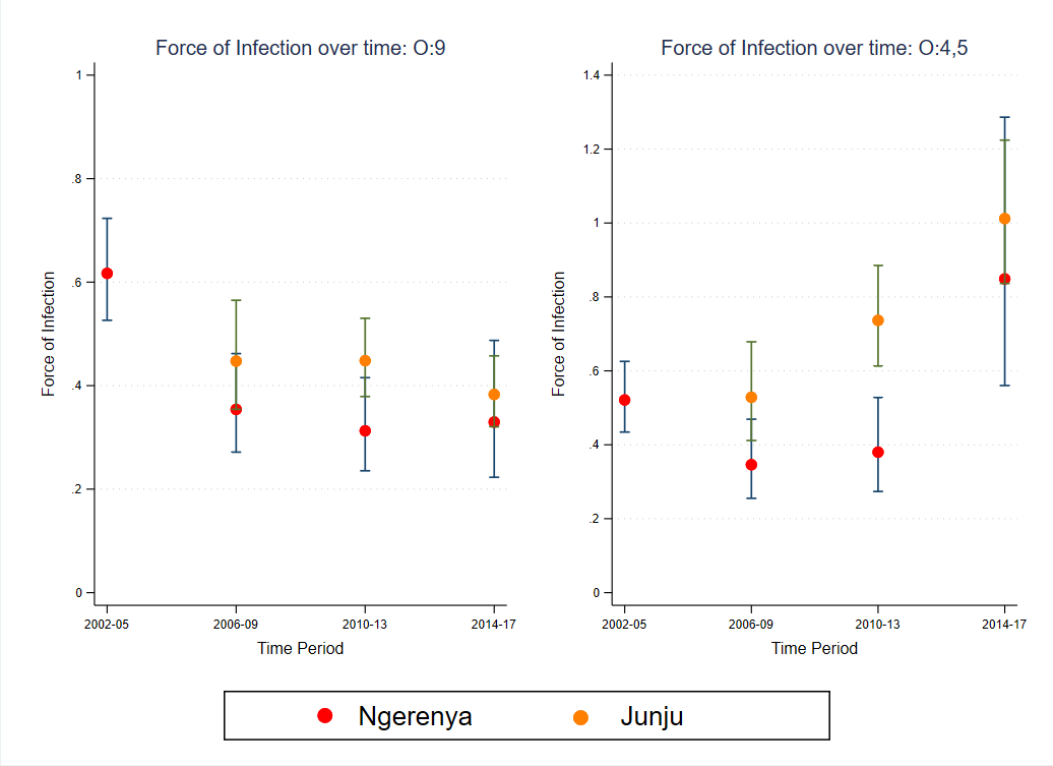
Figure 0.8: Numbers of cases of invasive NTS disease by region (North/South) and estimated force of infection by associated location (Ngerenya/Junju) for O:9 (S. Enteritidis) and O:4,5 (S. Typhimurium) over time.



The trend in incidence of iNTS infection by O:9 serotypes in Ngerenya mirrors the trend in iNTS disease in the North. This is true also for the trend in incidence of infection by O:4,5 serotypes in the North. However, in Junju (South), there are only two overlapping points for the two data series and, whilst there is a consistency of estimates for both infection and disease for Enteritidis/O:4,5, the FOI for O:4,5 in the South increases as disease incidence decreases.

Beyond 2014, there is an increase in the incidence of infection with all serotypes except O:9 in Junju (Fig. 5.9)

Figure 0.9: O:9 and O:4,5 Force of Infection over time (2002-2017)



5.4 Discussion.

This is the first analysis of temporal trends of the rates of infection with non-typhoidal *Salmonella* serotypes in Africa, where these infections cause a significant burden of disease, yet the modes and rates of transmission are largely unknown. For *S*. Enteritidis (O:9 serotypes) temporal trends in both disease incidence and rate of infection are consistent in both Ngerenya and Junju in the time periods where these observations overlap. For *S.* Typhimurium (O:4,5 serotypes) there is also consistency in the trends in disease incidence and rate of infection in the North but the two periods of overlapping observations in the South show a divergence between the two data series. The incidence of invasive disease caused by *S*. Enteritidis in Kilifi HDSS fell by 21% per year between 1998-2014, while that of *S.* Typhimurium fell by only 14% per year during the same period(3) which suggests that, relatively speaking, S. Typhimurium emerged as the more dominant serotype. In both areas, but particularly in Junju the serological data suggest a rising incidence of infection with O:4,5 serogroup *Salmonella*, between 2006 and 2013 and this trend continues in 2014-17. Taken as a whole, and accepting the

ecological nature of the data, the finding suggests that the decline in iNTS disease is due, at least in part, to a decline in the acquisition of infections with iNTS.

What are the factors that contribute to the FOI?

Without a clear understanding of the mode of transmission of NTS infections, it is difficult to estimate which factors affect the FOI. Environmental factors, both rainfall and temperature, have been associated with the risk of iNTS disease directly. Rainfall is associated with increased transmission in the rainy season (124) and *Salmonella* notifications and case load increase both at high temperatures(196, 197) and at lower temperatures (125), though proliferation of *Salmonella* is optimum at warmer temperatures (35-37^{0}C). Since NTS is a faecal-oral pathogen, improved WASH related practices and increased urbanisation are likely to lead to a decline in infection rates(198, 199). Crowding, as seen in urban slum areas, is associated with increased transmission of other invasive *Salmonella* serotypes that are known to be transmitted from human to human via contaminated water such as *S*. Typhi (200), and crowding is likely to have a similar impact on the transmission of NTS strains with human reservoirs, such as the ST313 genotype of *S.* Typhimurium and the 'African clade' of *S*. Enteritidis, which have been isolated in invasive human samples and have genomes that are host-restricted(25, 35, 38). Time as an independent factor has also been associated with iNTS disease, reflecting secular trends in either infection/transmission or invasion though the nature of those secular trends is not clear(123). The FOI has not previously been included in efforts to tease out the relative contribution of the factors associated with iNTS disease, mostly because it had not been possible previously to estimate it; this is the first study to examine the FOI for NTS and how it has changed over time. However, it is difficult to disaggregate the relevant components of FOI which may be changing over time, because it represents a composite of factors including carriage prevalence, mixing probabilities and immunity.

To what extent does the change in transmission intensity explain the changes in iNTS incidence?

Whereas the patterns of change in the rates of infection match those of iNTS disease, the magnitude of change in FOI is not sufficient to explain all of the decline in iNTS disease, suggesting that additional factors were contributing to this decline. These are likely to be changes in risk factors for invasion. In Kilifi, a decrease in malaria had been previously observed and causally linked to the decrease in iNTS disease(117). Malaria infections are thought to increase susceptibility to iNTS(201), by increasing available iron in the body following haemolysis of red blood cells, by causing neutrophil and macrophage dysfunction leading to immune suppression, by increasing gut permeability leading to increased translocation of the NTS, and by causing dysregulation of iron (69, 70). Since NTS is a siderophilic organism, increased iron availability is associated with increased bacterial growth (202). In this study, the concentration of antibodies and the force of infection for both the major serotypes were higher in Junju than in Ngerenya, suggesting NTS transmission intensity was consistently higher in Junju, an area of higher malaria prevalence(193). This creates an association between NTS transmission intensity and malaria prevalence which is independent of host susceptibility. Other factors, such as the decline in the prevalence of HIV, anaemia and malnutrition, and improvements in the management of sickle cell disease could also be driving the declining incidence of iNTS, as they are major risk factors for invasion. Studies in Malawi and South Africa demonstrated a temporal decline in *S*. Typhimurium incidence associated with a decrease in HIV-viral load driven by increased uptake of anti-retroviral drugs (121, 122). Holistically, improvements in social economic status have led to improvements in several disease profiles though the mechanism by which poverty leads to infectious disease are not the same for each infection (203).

In the last study period, 2014-2017, the FOI for O:4,5 increased in both locations and the FOI for O:9 increased in Ngerenya, predicting an increase in iNTS disease. Previous analysis of iNTS incidence data showed an increase in both serotypes in 2014. This highlights the need for continuous surveillance and real-time analysis of surveillance data to enhance preparedness in the

hospitals/health-sector. Although I have focused on environmental and host risk factors, the rising FOI for O:4,5, in particular, could be caused by increased circulation of *S*. Typhimurium ST313 more recently, as this has been reported as an expanding clone in the region(204-206).

It is not possible, within this study, to compare the relative rates of infection of the two principal serotypes of iNTS because we lack a standard method to establish the threshold for seropositivity. If a high antibody threshold is selected for seropositivity this leads to lower seroprevalence estimate and a lower FOI; the FOI is sensitive to the selected threshold. In this study, we adopted the thresholds for seropositivity for each serotype that were determined in the previous chapter through a 2-component mixture model. Mixture models are usually robust in separating latent classes, where a separation exists, except that in our mixture model, there was a large area of overlap between the two populations, especially for O:4,5 serogroup; this means that the assay does not discriminate well between those who have and have not been previously infected. Whilst varying the absolute threshold would give varying point estimates for the FOI, the relative differences in the observed FOI between sites and across time periods are less sensitive to the absolute threshold and are therefore more likely to remain epidemiologically valid.

A significant limitation to the study was the span of available data. We did not have data from Junju for the period before 2008 and were therefore not able to assess the baseline FOI which would have helped determine if the FOI for O:4,5 was increasing even as iNTS disease declined or, as in Ngerenya, the FOI first decreased before later increasing. There were also years when the cross-sectional survey was not conducted and thus we had missing data for those years. We did not perform any data imputation but rather aggregated the time data into 4-year time periods to improve statistical power. Although these features constrained some of the inferences of the study, they are a frequent feature of retrospective data and should be offset against the fact that these two longitudinal data series, in iNTS incidence and serologically derived FOIs, are found rarely in the same geographical setting over the same period.

A significant strength of the study is that it is based on a platform that ensured regular and standardized sample collection (almost) annually for over 15years. Leveraging on this platform, we have demonstrated the annual seroprevalence of anti-NTS antibodies and changes in incidence of infection of NTS, by serotypes, across time revealing that rate of infection is temporally associated with the incidence of disease.

5.5 Conclusion

This study shows that the decline in the incidence of iNTS disease in Kilifi is temporally associated with a decline in the incidence of infection with NTS. It is likely, therefore, that the decline in transmission is responsible to some extent for the decline in invasive disease but the magnitude of the decline in transmission does not explain all of the decline in invasive disease and other factors, such as recent malaria, are known to be risk factors for invasion and have also declined in prevalence during the same period.

5.6 Chapter 5 Supplementary material.

Table 0.4: Median age and Interquartile (IQR) range of participants by calendar year

	Ngerenya		Junju	
Year	Median age (y)	IQR	Median age (y)	IQR
2002	4.2	2.6-6.2	-	-
2003	4.2	2.5-6.2	-	-
2004	4.9	2.9-7.2	-	-
2005	6.1	3.1-8.3	-	-
2006	-	-	-	-
2007	6.4	3.8-8.6	-	-
2008	7.1	4.1-9.3	5.4	3.7-7.4
2009	-	-	6.2	4-8.3
2010	8.2	5.4-11.1	7.0	4-9.1
2011	9.1	5.8-12	7.7	4.3-10
2012	9.8	6.3-12.7	8.0	4-10.7
2013	-	-	8.3	3.9-11.6
2014	10.3	6.6-13	8.8	4.7-12.1
2015	12.8	10.9-14.2	7.8	3.8-12.3
2016	9.9	6.1-12.4	8.0	4.8-11.8
2017	10.5	5.8-12.8	6.1	3.2-10.1

Discussion

The overall focus of this PhD has been on understanding the epidemiology of carriage and transmission of non-typhoidal Salmonella (NTS) infections in Kenya to inform control strategies including vaccination. Specifically, it is to understand the human reservoirs of NTS infection and the rate of infection with age to guide the target population and schedule for the delivery of new vaccines protecting against invasive NTS disease (iNTS).

I have explored this in several chapters through two main studies. The first study was a cross-sectional study of faecal carriage prevalence and seroprevalence of NTS set in 3 locations in Kenya with varying incidence of invasive NTS disease. Here, I designed and led a complex multi-site, age stratified random sample survey which simultaneously examined stool carriage, seroprevalence, clinical status, anthropometry and risk factor data in approximately 1,500 healthy children and adults in Kilifi, Nairobi and Siaya Counties. The second investigation was a retrospective study set in Kilifi where I utilised archived serum samples collected from a rolling cohort of approximately 200 children collected annually between 2002-2017 from two sites with varying incidence of malaria, and on which I performed ELISAs for estimation of anti-Salmonella antibodies.

I found that faecal carriage prevalence in the 3 sites varied by location, between 0.4 and 13%, being highest in Kilifi; carriage prevalence also varied by age, being highest among older children and adults. None of the hypothesised risk factors tested were associated with carriage; these included WASH related factors, which are commonly associated with transmission of faecal-oral pathogens, (207) and host factors, such as malaria and anaemia, which are known risk factors for iNTS disease (25, 62). Of the 53 NTS isolated, only 5 were *S.* Enteritidis and 1 was *S.* Typhimurium, the serotypes that are commonly responsible for iNTS disease.(18) In addition, all 53 serotypes were susceptible to the full range of antibiotics tested which is in marked contrast to the serotypes responsible for invasive disease(82, 208). This suggests that the epidemiology of NTS carriage is likely to be different from that of invasive NTS disease. Furthermore, Kilifi, the location with the lowest incidence of iNTS

had the highest carriage prevalence while Siaya, the location with the highest incidence of iNTS disease had low carriage prevalence. If invasive disease is a direct function of the acquisition or prevalence of carriage, then we would expect to observe a higher incidence of invasive disease in settings with a higher carriage prevalence, whereas, in fact, the opposite was observed. An alternative explanation of these apparently discordant findings is that the non-invasive serotypes observed in areas of high carriage prevalence could induce a form of species-wide immunity that provides protection against invasion with other serotypes leading to a lower incidence of invasive disease.

The serological studies revealed that maternally derived anti-NTS antibodies are present at birth and decay within 4-5 months of birth, after which the concentration of specific antibodies are observed to increase sharply with age until adulthood. The rate of acquisition of *S.* Enteritidis antibodies from primary infection was highest in Nairobi, while that of *S*. Typhimurium antibodies was highest in Kilifi. By the age of 5 years, 80% of children were seropositive to both anti-Enteritidis and anti-Typhimurium antibodies at all sites. The catalytic models confirmed that the incidence of infection differed by site. I concluded that neonates are the likely target age-group for interventions against iNTS in high transmission areas as they have the highest rates of acquisition of infection and have previously been shown to have the highest incidence of invasive disease. Comparison of the age-seroprevalence curve to the age-incidence curve of iNTS disease showed an inverse relationship, confirming that antibodies are associated with natural immunity to iNTS disease. The spatial heterogeneity in occurrence of infections and disease suggest that the different locations should be targeted differently – all settings will benefit from control efforts that reduce transmission, while those areas with less transmission of infection but high disease incidence would in addition benefit from control efforts that reduce host susceptibility to invasion.

From the longitudinal study, I found that there has been a decrease in incidence of infection of both *S.* Typhimurium and *S.* Enteritidis in Ngerenya, where there has also been a decline in the incidence

of iNTS disease by the two serotypes. This shows a direct association between changes in serotype specific transmission of NTS to changes in invasive disease by same serotypes. Meanwhile in Junju, against a background of declining incidence of iNTS disease(3), I observed a decline in incident infections with O:9 but an increase in incident infections with O:4,5, suggesting that additional factors could be associated with the changing incidence of *S*. Typhimurium invasive disease. Previous studies had associated the decline in iNTS in the region to a decrease in malaria transmission, a risk factor for invasion (117). I concluded that although iNTS incidence declined in Kilifi to some extent since 2004 because of the removal of a key invasion risk factor (malaria) its decline is also attributable (at least for one serotype) to a measurable reduction in the FOI in Kilifi - suggesting behaviour changes, possibly driven by access to water and good sewage - may have had a role in disease control already.

Taken as a whole, these three studies illustrate the transmission of NTS and highlight important implications for development and implementation of interventions. Additionally, outputs from this study such as age-seroprevalence, Force of Infection, and carriage by age may now be used as inputs in transmission dynamic models to explore control strategies including vaccination.

Applications of the results in control of iNTS

4.1.14 Vaccination:

There are 2 vaccine candidates in Phase I clinical trials at the moment, several others are in pre-clinical development (Table 1.1) (158). The 2 vaccines, a bivalent Generalized Modules for Membrane Antigens (GMMA) based vaccine targeting *S*. Typhimurium and *S*. Enteritidis (iNTS-GMMA) and a trivalent glycoconjugate *Salmonella* vaccine consisting of Core O-polysaccharides from *S*. Typhimurium and *S*. Enteritidis coupled with the carrier flagellin and a Vi-tetanus toxoid targeting *S*. Typhimurium, *S.* Enteritidis and *S*. Typhi (CVD1000). Both vaccines are immunogenic in murine studies and have demonstrated protection against iNTS through serum bactericidal assays in

challenge studies in mice and rabbits(209, 210). Neither has been assessed on their ability to reduce the transmission of NTS. For a vaccine, this can be brought about by reducing the carriage load, increasing the clearance of NTS in the gut or reducing the rate of acquisition of infections. These vaccines are aiming to reduce the disease burden of iNTS which is highest among infants. For direct protection, vaccines can be administered to infants directly or indirectly through expectant mothers. For indirect protection, vaccines can be administered to older children and adults who we have identified as the main reservoirs for NTS.

The first approach is direct protection by vaccinating infants, which is the one most likely to be implemented. Over 90% of all vaccines are given to infants, despite the challenges of immunising them. Firstly, they have 'immature' immune systems which do not readily mount an immune response when stimulated(211), and they also commonly have maternal antibodies which are known to interfere negatively with expected vaccine responses(212). In addition, diseases such as malaria dampen the immune responses to vaccines(213). These two factors pose a challenge when targeting iNTS vaccines in infants; we have already reported the presence of maternally acquired antibodies against NTS from birth and the new vaccines are likely to be implemented in areas of high prevalence of malaria which overlap with iNTS endemic areas. To stimulate 'immature' immune systems, polysaccharide vaccines conjugated with carrier proteins work through T-cell dependent pathways to enhance immunogenicity and increase protective efficacy(214, 215). The CVD1000 vaccine, is such a glycoconjugate vaccine and may be effective through this mechanism (210). By contrast GMMA based vaccines are able to present multiple polysaccharide molecules and proteins since they are formed from outer membrane vesicles consisting of both the bacterial carbohydrates and proteins in their natural conformation(216). Theoretically therefore, both vaccines would be able to stimulate an immune response in infants.

The correct timing of vaccination would overcome the challenge of inhibitory maternal antibodies – the optimum window for vaccination being the period between depletion of the maternally acquired

antibodies (or if present, when the interference from their concentration is negligible) and primary infection with NTS. Using the serological results presented here, this optimum lies between 4-5 months of age. We have shown that IgG antibodies possibly of maternal origin are present at birth and decline steadily for the first 4-5 months just as IgA antibodies, possibly from the primary adaptive response, appear and increase in concentration rapidly by age. This proposed schedule would work for the GMMA vaccine targeting NTS disease in infants, but would miss neonatal disease. However, scheduling of the CVD1000 vaccine to target both iNTS and Typhoid is not immediately clear as iNTS disease occurs in early infancy while typhoid disease occurs toddlers (217) and in older school going children (5-14 years)(218). The current WHO prequalified Typhoid Conjugate Vaccines are administered in children above 6 months of age, mostly through co-administration with the measles vaccine at 9 months, at which age, according to the TSAP study, 100% of typhoid fever infections would be prevented (16).

Another approach to vaccination would be administration of the NTS vaccines to expectant mothers. This would boost antibody concentration in the mothers and consequently confer protection in neonates, after successful passive transfer of the maternal antibodies. Neonatal iNTS disease in Kilifi carries the highest incidence among infants. Previous studies have additionally demonstrated that most of the cases of neonatal iNTS disease occur in the first 7 days of life (154), which precludes infant vaccination even vaccination of new-borns. The early peak of iNTS disease risk argues strongly in favour of maternal vaccination This approach has been used widely for neonatal diseases including tetanus in low and middle income countries and for pertussis and influenza in high income countries, and is under consideration for use in Respiratory Syncytial Virus (RSV) and Group B Streptococcus vaccines(219-221). These vaccines rely on effective transfer of maternal immunity, either through the placenta or through breast milk, and persistence of immunity in infancy. Efficient transplacental transfer of NTS IgG antibodies acquired by natural infection has previously been demonstrated, and these antibodies have been shown to have bactericidal activity against NTS in

vitro, and after vaccination in murine models (222). Our study also demonstrated that while maternal IgG was present, the infant IgA levels did not rise, pointing to a protective effect of maternal antibodies. Similar observations have been made in a cohort in Vietnam and in Malawi where infections and disease, respectively, increased in incidence after maternal IgG antibodies declined(80, 106). Passive immunity transferred through breast milk provides mucosal immunity, which is also relevant for NTS, a faecal-oral pathogen(223-225). Evidence from Kilifi shows that NTS specific IgA and IgG antibodies in oral fluid samples collected from children and adults increased in concentration with age, reaching a single peak in adulthood for IgG and two peaks for IgA, one in infancy and one in adulthood, although the peak in infancy possibly may simply represent secretory IgA from breastmilk (226). Secretory IgA antibodies are useful to prevent oral infection with NTS or prevent translocation of the pathogens across the gut, as such, oral passive immunization with recombinant human secretory IgA has been proposed for control of NTS(227).

The third approach to vaccination with NTS involves indirect protection of infants by vaccinating populations at highest risk of transmission(157). Reduced carriage in the vaccinated leads to a decrease in disease in the unvaccinated. This concept of herd immunity has been evinced by several vaccines, notably conjugate vaccines(132). For example, by vaccination of adolescents with the meningococcal serogroup C vaccines infants are protected against meningitis, as adolescents are the highest transmitters. PCV vaccines in Kenya and UK provide setting specific examples. In Kenya, introduction of the 10-valent Pneumococcal Conjugate Vaccines (PCV10) among infants and a catch-up campaign among under 5's led to a decline in disease among unvaccinated populations including infants under 2 months of age who were too young to be vaccinated. The incidence of invasive pneumococcal disease in these infants decreased from $173/10^5$ in the pre-vaccine period to 0 in the post-vaccine period(22). In the UK, a setting with a mature PCV programme and high vaccination coverage, recent schedule changes for PCV13 from a 2+1 schedule to a 1+1 were possible due to herd immunity(228). The 1+1 schedule now given to infants at 3 and 12months means that infants

do not rely on direct protection of the vaccine (probability of exposure to infection is low already due to herd immunity), rather the dose in infants serves to prime their immune system for later boosting of antibodies which provides longer term protection into childhood, leading to reduced carriage in older children who then indirectly protect infants by reducing transmission(229); effective in terms of protection and cost. This indirect approach works if the vaccine acts to reduce transmission in the vaccinees and if the vaccine programme achieves high coverage. The target population for vaccination is normally identified by transmission dynamic modelling but in most circumstances, it is those with a combination of high infection prevalence and high probability of mixing with those most susceptible to disease. In the case of NTS, older children (5-14y) and adults (15-54y) have the highest prevalence of carriage and also commonly have the highest rates of contact with infants, especially adult caregivers(230). Currently, we do not know whether the iNTS-GMMA vaccine and the CVD1000 vaccines will have activity against carriage of NTS. In addition, the herd immunity threshold (HIT), proportion of the population required to be immune either by vaccination or natural infection for disease incidence to decrease(231), has not yet been determined. Towards the latter, our study estimated the serotype-specific FOI which is a useful parameter that is used in the more complex transmission dynamic models to estimate the reproduction number, R_0, and subsequently the HIT(232, 233). For coverage, targeting older children might be possible through school vaccination campaigns, similar to human papillomavirus (HPV) vaccine school based vaccination programmes (234) or school based deworming programs (235), that report high coverage in high-income and low-income countries, respectively. In fact, the CVD1000 vaccine might benefit from this approach as this is the target population for typhoid disease control. Targeting adults might however be challenging because currently there is no routine vaccination schedule for adults. The exception here is pregnant women who are offered tetanus toxoid vaccine during routine antenatal care visits. Vaccinating pregnant women with iNTS vaccines during these visits would have a dual effect of conferring passive immunity directly to the infant and

reducing transmission of NTS both horizontally in the household and vertically to the new-born during delivery. However, vaccinating pregnant women alone may not achieve population immunity.

4.1.15 Improving WASH practices

This is likely to lead to control of NTS, given it is a faecal-oral pathogen acquired through ingestion of contaminated food and water. WASH interventions have been shown to be successful in preventing diarrhoeal illnesses(236). This literature review analysed 33 reports from randomized control trials of a variety of WASH related interventions aimed at reducing diarrhoeal illnesses. The studies covered 21 countries, including studies from Kenya. They found that improved access to safe water reduced the incidence of diarrhoeal diseases. WASH interventions are also effective in reducing diarrhoea-associated mortality by 45% (237). Though not tested for NTS, improved WASH practices are likely to have a similar direct effect on NTS by reducing transmission or an indirect effect by reducing other infections, especially helminths, which could potentiate NTS(238, 239). However, low uptake of WASH related interventions could impact effectiveness. For example, it was observed in Malawi that even after sensitization, education and distribution of WaterGuard for free during an ongoing typhoid epidemic, the uptake was low; only 34% of the households with WaterGuard, an in-home chlorination solution for drinking water, reported using it(240). We observed a decrease in iNTS transmission and associated disease over time in Kilifi, which could possibly be linked to programmatic efforts to improve education, urbanisation (excluding slums) and socio-economic economic status which may have translated into improved WASH practices.

A lingering question though, is it actually desirable to eliminate all circulating NTS serotypes? We found that increased circulation of non-invasive serotypes in Kilifi was associated with a higher seroprevalence of antibodies to invasive serotypes and lower incidence of iNTS disease. One possibility here is that infection with less invasive serotypes could be advantageous if they induce a cross-reactive immunity against other more invasive serotypes.

4.1.16 Controlling host risk factors:

We observed that interrupting transmission led to a decline in *S.* Enteritidis disease but not could not explain the changes in *S*. Typhimurium disease which may therefore have varied as a function of fluctuations in host risk factors such as malaria, HIV infection and malnutrition. As well as potentiating the risk of invasion, these comorbidities are known to diminish the body's response to vaccines and are therefore central to the control of iNTS disease(213, 241). Uptake of ARV in HIV-infected patients in South Africa led to a decline in S. Typhimurium disease(122). This strategy could be implemented in areas where both iNTS disease and HIV-infections are endemic, such as Siaya. For *falciparum* malaria, an increase in the uptake of insecticide treated nets led to a decrease in malaria transmission and a concurrent decrease in iNTS disease, assessed ecologically(117). However, in the RTS,S A01 malaria vaccine trial, there was no vaccine effect observed on bacteraemia including iNTS bacteraemia(120). Given that this was a tertiary endpoint, it is possible that the study was not powered to observe a statistically significant effect. Currently, a phase IV trial of the RTS,S A01 malaria vaccine is on-going in western Kenya, including Siaya, against which we can assess and quantify indirect effects of malaria control on iNTS.

Strengths and Limitations of my study approaches

This is the first population based serosurvey for NTS antibodies in Africa and the first implementation of a catalytic model for transmission of NTS. The strength of the study is in the careful design of the fieldwork: firstly, in selecting sites that are distinct based on their epidemiology of iNTS and linking individual level carriage, serology and risk factors data and, secondly, in taking advantage of existing frameworks such as the serum biobank at KWTRP to explore historical perspectives. Kilifi and Siaya, though similar in their rural setting, differ markedly in malaria epidemiology. The site in Nairobi was an urban slum area presenting a third distinct ecology which

was interesting. This has enabled exploration of age-, spatial-, and temporal heterogeneity in epidemiology of NTS transmission.

At the outset of this PhD, there were few prior studies of NTS serology which presented a number of challenges: there was no reference serum, no understanding of the specificity of the NTS assays and no externally validated threshold for seropositivity. Against this background, any threshold is to some extent arbitrary. To try to avoid arbitrary choices, we have used an empiric method to derive a threshold that is valid, but only within the data collected in these field sites. We used the antibody distributions from a 2-component mixture model as a gold standard against which we assessed the sensitivity and specificity of several thresholds before selecting one that maximized specificity and sensitivity, based on the Youden index. The resultant seroprevalence estimates and downstream analyses including the catalytic model, were entirely contingent on the threshold selected. The Youden index (Sensitivity 89% and Specificity 66%) resulted in a O:4,5 IgG seroprevalence of 37% in under 5's while a threshold with 99% specificity and another with 99% sensitivity resulted in seroprevalence estimates that were 36 times lower and 3 times higher, respectively. This shows that the seroprevalence is highly sensitive to the threshold and offers the lower and upper bounds within which to interpret our results. However, although the internal threshold had not been validated externally, it is unlikely to impact on the majority of the analyses presented here which are predominantly focused on the relative comparisons between sites since the same threshold was used at each site. In the cross-sectional serosurvey, I compared seroprevalence results across 3 sites and in the longitudinal serosurvey, compared seroprevalence results across 2 sites. In both examples I focused on the relative differences in the FOI in both the results and discussion sections, rather than the FOI itself. There is an overlap in the results from the 2 studies: The cross-sectional serosurvey in Kilifi was conducted in 2016 in Junju (O:9 FOI 0.3(0.25-0.4) while the Longitudinal study in same location spanned from 2008 to 2017 (2014-17 O:9 FOI 0.3 (0.27-0.42)). The similarity in the estimated FOI provides additional credibility for the methodology. In addition, the age-

seroprevalence curve, showed an increase in seroprevalence by age is an inverse of the known age-incidence curve that shows a decrease in incidence of iNTS by age. This ecological comparison provides further support for the hypothesis that, in this endemic population, increasing antibody concentrations protect against iNTS disease, and it provides a form of external validation for the threshold.

Being the first randomly selected population level serosurvey of NTS, we cannot validate our results through comparisons with other studies. The ELISA implemented has been used in only one other study but the threshold for seropositivity that we selected was different – we used a data driven threshold from a mixture model, whilst the prior Ugandan study used the assay's LLOQ as threshold(104). As discussed in the previous paragraph, this low threshold is likely to increase sensitivity at the expense of specificity, and at this low level of antibody many 'positives' will represent cross-reactive antibodies induced by infection with other gram negative organisms, as seen with other ELISAs targeting LPS(102). Spatial heterogeneity in our results means that it would be imprudent to generalise the results more widely e.g., to the greater African population. However, we have provided a descriptive analysis of the different populations studied including data on key parameters such as prevalence of *falciparum* malaria, anaemia, malnutrition, household crowding, agricultural practices including livestock farming, which could be used to adjust the results to compare with other populations. Trong *et al*. used country groupings by malaria and HIV prevalence to extrapolate iNTS disease incidence with relative accuracy (14). During the course of this PhD, a multi-country serosurvey was commissioned under the Vacc-iNTS consortium (both academia and industry partners) to provide immuno-epidemiological background for the GMMA vaccine deployment and it is likely, therefore, that other investigators will soon publish complementary results from other settings that will help to understand the variations due to methodology and inherent geographical variation (242). My studies provide the evidence for Kenya and could be informative in local policy. Furthermore, a Phase 1 clinical trial of the GMMA vaccine in adults is

scheduled to take place in Kilifi in 2023. The results of this PhD including background immunological responses, baseline faecal carriage prevalence, and the prevalence and decay rate of maternal antibodies will provide a historical perspective on antibody responses after natural infection and baseline data for evaluation of vaccine efficacy.

The suitability of the O-antigen based serology to act as a marker of exposure can be challenged. Cross-reactivity with other gram-negative bacteria reduces its specificity(102); cross-reactivity within Salmonella groups also reduces the specificity. For example, O:9 serogroup is shared across many Salmonella serotypes including *S.* Enteritidis and *S*. Typhi(10). In our seroprevalence study in Nairobi, we found a higher concentration of O:9 antibodies in Nairobi than other sites yet there was no evidence of S. Enteritidis dominance in that area. However, the Mukuru slum area is known to have a high incidence of *S*. Typhi(185), and therefore biologically plausible that these could have contributed to the high concentration of anti-O:9 antibodies observed. *Salmonella* Flagellin antigens would be an alternative as they are both immunogenic and specific(243). They are used in conjunction with the O-antigen in serotyping of Salmonella and have been conjugated with core O-polysaccharides as candidate vaccine against NTS (244) with proven immunogenicity in infants given directly (245) (246)and passively through maternal immunization in murine studies(222).

In the carriage studies, we experienced a low yield of NTS possibly due to the low sensitivity of stool culture for recovery of NTS. In blood cultures for *Salmonella* (evaluated with *S.* Typhi), the sensitivity is approximately 66% against bone-marrow cultures as a gold-standard (247) , and is directly associated with the volume of blood collected (66). In stool studies, the sensitivity of stool culture for NTS is 57% after overnight enrichment with selenite F broth, and is directly associated with the bacterial load(248), while direct stool PCR with enrichment has 85% sensitivity(155). We used conventional microbiology culture methods with enrichment for isolation of NTS which would be expected to underestimate carriage prevalence. We employed similar methods of collection, transport, and culture of the stool samples across all sites, suggesting that the magnitude of this bias

would be similar across sites. The low yield of culture positive 'carriers' limited our ability to examine the relationship between carriage and serology; and excluded the Nairobi site from the risk factor analyses altogether, as only one isolate was recovered out of 500 stool samples cultured. Direct stool PCR with enrichment would increase the yield and statistical power in our analyses, especially if the PCR was serotype specific. Whereas protocols for serotype-specific direct stool PCRs exist(106, 249), the choice of serotypes to multiplex differ by region since the geographical distribution of NTS differs by serotype; and therefore direct adoption of the published protocols for our region would not be useful. Additionally, the best yield would need enriched samples yet our stored stool samples were not enriched, only those that underwent culture directly. This is an important learning point for me for future stool studies.

We also lack full serotype and genotype data of the majority of the isolates recovered. We could only fully serotype *S.* Enteritidis and *S*. Typhimurium isolates using the panel of antisera available. This selection seemed reasonable given that these are the most common serotypes associated with iNTS disease in Kilifi(3). Serogroup data would have enabled comparison of our study results with those of GEMS and VIDA which also took place in Siaya, and reported carriage isolates that were neither *S*. Enteritidis nor *S*. Typhimurium. Lack of genotypic data also meant that we could not characterize the sequence types of interest such as *S*. Typhimurium ST313 and the different clades of *S*. Enteritidis within our carriage isolates. Looking ahead, whole genome sequencing of these carriage isolates in conjunction with invasive isolates archived at KWTRP could provide a platform to compare genetic determinants of invasiveness in our population, which I intend to conduct in the immediate post-doctoral period

Remaining knowledge gaps and possible future directions

The link between faecal carriage and antibodies remains elusive. Does gut colonization with NTS continuously stimulate antibody production such that higher antibody concentrations are expected where carriage prevalence is high OR do antibodies protect from carriage acquisition such that higher antibody concentrations are associated with lower carriage prevalence. My study results have highlighted high concentration of anti-NTS antibodies in carriers than non-carriers, but the interpretation of this finding is not complete without knowledge of the direction of the association. This cannot be determined through a cross-sectional study, such as the one presented here. To answer this question, we would need a longitudinal study of NTS carriage and NTS antibodies using paired samples collected from NTS naïve individuals, possibly neonates, who are then followed up with regular sampling intervals to be able to determine the relative contribution of infection to antibody production and of neutralizing mucosal antibody to the prevention of gut infection.

Does carriage of non-invasive strains protect from invasive disease with other more invasive serotypes through inducing a non-specific protective immunity? This novel hypothesis arising from my studies would also require a longitudinal cohort study that assesses the incidence of invasive disease among children against exposure groups defined by those with antecedent carriage. Such a cohort study would be a massive undertaking, both time and cost, for a number of reasons; 1) the endpoint, with iNTS disease, is relatively rare, 2) The intensity of exposure to NTS infection in the two study groups would need to be similar, which is difficult to control in real life situations and 3) The effect may be modified by the size of the exposure which is difficult to measure. The association could also be explored independently through genomics, if whole genome sequencing of the non-invasive strains would identify immunogenic epitopes that are shared with invasive strains. For example, among antigenically related flaviviruses where immunity to Zika virus confers cross-protection against Dengue viruses(250) and vice versa (251).

What proportion of NTS infections translate to invasive disease? This proportion, also referred to as case-carrier ratio, would help in surveillance of NTS without the necessity to observe disease. As aforementioned, disease surveillance studies require substantial resources, yet disease can be approximated through carriage prevalence obtained through carriage surveys that are relatively easier to conduct. The case-carrier ratios would need to be serotype specific as we have observed from my studies that some serotypes e.g., *S*. Typhimurium are more invasive than others – we found few cases of S. Typhimurium in carriage, yet it is commonly found in invasive samples from the same location.

A big gap remains in identifying the mode of transmission of NTS. The study of strains causing invasive NTS disease in Africa has identified novel genotypes of NTS that are not transmitted through the food pathway, especially meat, challenging our prior understanding of NTS transmission epidemiology (25, 35, 36). The working theory is that humans are the reservoirs of the pathogens, but the mode of transmission is unknown. Household transmission studies, backed with genetic analysis of the isolates, have shown similarities in the strains of isolates causing iNTS disease in children and those circulating in other human household members, but not animals in the household(40, 42). However, these have not been able to establish directionality of the infections, transmission modes, identify any other reservoir for iNTS, risk factors and attack rates of iNTS. Longitudinal cohort studies starting with NTS naïve individuals as aforementioned can provide the platform to study and fill these knowledge gaps.

Finally, assay standardization for antibodies against NTS antigens and development of a reference serum would enable inter-site comparisons of results. Although the field is at an early stage, several studies are underway across Africa and if these yield differences in findings we will not be able to discriminate between variations in epidemiology/immunity and variations in methodology as causes for those differences. Identification of correlates of protection against both infection and disease

would also be extremely valuable in vaccine development as it would enable more meaningful interpretation of the biological implications of antibody concentrations.

Overall, the results of this series of studies have improved our understanding of transmission of NTS and highlights important implications for development and implementation of control interventions, particularly vaccines.

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