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Measuring transfer of human norovirus during sandwich production: Simulating the role of food, food handlers and the environment

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ABSTRACT

Foodborne outbreaks associated with transmission of norovirus are increasingly becoming a public health concern. Foods can be contaminated with faecal material at the point of production or during food preparation, in both the home and in commercial premises. Transmission of norovirus occurs through the faecal-oral route, either via person-to-person contact or through faecal-contamination of food, water, or environmental surfaces. Understanding the role and pathways of norovirus transmission – either via food handlers' hands, contaminated foods or the environment – remains a key public health priority to reduce the burden of norovirus-associated gastroenteritis. However the proportion of norovirus that is typically transferred remains unknown. Understanding this is necessary to estimate the risk of infection and the burden of gastroenteritis caused by norovirus.

In this paper we present a novel method of capture, concentration and molecular detection of norovirus from a wider range of complex food matrices than those demonstrated in existing published methods. We demonstrate that this method can be used as a tool to detect and quantify norovirus from naturally contaminated food, and for monitoring norovirus transfer between food handlers' gloved hands, food or the environment. We measure the effect of introducing contamination at different food production process stages, to the final food product, to determine whether this could cause infection and disease. Between 5.9 and 6.3 Log_{10} cDNA copies/μl of norovirus GII were inoculated onto food handlers' gloved hands, food or the environment and 1.1–7.4% of norovirus contamination was recovered from all samples tested. When interpreted quantitatively, this percentage equates to levels predicted to be sufficient to cause infection and disease through consumption of the final food product, demonstrating a public health risk. Overall detection and quantification of norovirus from foods, food handlers' gloved hands and the environment, when suspected to be implicated in foodborne transmissions, is paramount for appropriate outbreak investigation.

1. Introduction

Foodborne disease is a major cause of morbidity and mortality worldwide, with World Health Organisation (WHO) estimates indicating that each year approximately 600 million people become ill due to consumption of contaminated food, which results in 420,000 deaths (Havelaar et al., 2015). Gastroenteritis accounts for most of this foodborne disease, which can be caused by many different microorganisms, but viruses are amongst the most significant aetiological agents, in particular the noroviruses (Iturriza-Gómara and O'Brien, 2016).

Viruses of the *Norovirus* genus (family *Caliciviridae*) are positive sense, single-stranded RNA viruses with a genome of approximately 7500 nucleotides that is organised as three open reading frames (ORFs). Noroviruses have been recognised as the most common cause of viral gastroenteritis (EFSA, 2013; Kirk et al., 2015) and frequently implicated in outbreaks of disease associated with transmission via food handlers,

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food and water, which poses a significant public health risk (Hall et al., 2013; Pires et al., 2015). Norovirus gastroenteritis is estimated to have a societal cost of $60.3 billion annually (Bartsch et al., 2016) and is accountable for approximately one fifth of all cases of acute gastroenteritis (Ahmed et al., 2014).

Infected individuals may present with symptoms of acute gastroenteritis (Adler and Zickl, 1969) however, asymptomatic infection is also well documented (Ozawa et al., 2007; Sabrià et al., 2016; Teunis et al., 2015). Symptomatic cases typically present with vomiting, often with diarrhoea, which can last 2–4 days in healthy adults, although duration of symptoms can be longer in immunocompromised and hospitalised patients (Lopman et al., 2004). Virus shedding is reported to occur 3–14 h prior to the onset of symptoms (Atmar et al., 2008) and can continue for many weeks in the absence of symptoms (Atmar et al., 2008; Kirby et al., 2014; Sabrià et al., 2016; Teunis et al., 2015), on average from 8 to 60 days (Teunis et al., 2015). The amount of norovirus shed in faeces can range from 10^5 to 10^9 norovirus copies/g faeces, with peak shedding occurring 2–5 days post infection (Atmar et al., 2008). With exposure to as few as 10 to 100 norovirus particles being reported as sufficient to establish infection (Acheson and Fiore, 2004; Teunis et al., 2008), such high levels of virus shedding mean that infected individuals pose a high risk of contaminating their surroundings, allowing sufficient quantities of virus to be transmitted via the environment and cause symptomatic infection. Despite reports of reduced viral shedding in the asymptomatic compared with the symptomatic individuals in norovirus challenge studies (Atmar et al., 2008; Kirby et al., 2014; Reeck et al., 2010), outbreak analyses have found viral loads to be similar between these two groups (Ozawa et al., 2007; Sabrià et al., 2016; Teunis et al., 2015). Whilst those with and without symptoms can transmit the virus, of concern are the food handlers who may be unaware of the role of asymptomatically infected individuals in norovirus transmission (FSA, 2017). Further, many authors have identified asymptomatic cases in food handlers who are working in catering premises (Daniels et al., 2000; Okabayashi et al., 2008; Sabrià et al., 2016).

Transmission of norovirus occurs through the faecal-oral route, either via person-to-person contact or through faecal-contamination of food, water, or environmental surfaces. Foods can be contaminated with faecal material at the point of production or during food preparation, and in both home and commercial premises faecal contamination of food handlers' gloved hands or the environment have been shown to pose a risk of virus transmission (Hardstaff et al., 2018). Norovirus is highly stable in the environment and can be detected on a range of contact surfaces over long periods of time, thereby increasing the likelihood of transfer from one fomite to another, which is of concern in food preparation environments, or from the environment to an individual (Escudero et al., 2012).

Ready-to-eat (RTE) foods pose a significant risk to consumer health if they are contaminated, as these items usually require some aspects of food handler processing and are consumed without cooking. Transfer efficiency studies have demonstrated that norovirus can be transferred to RTE foods and the preparation environment, however, there is considerable variation reported in norovirus transfer efficiency estimates both within and between studies (D'Souza et al., 2006; Escudero et al., 2012; Rönnqvist et al., 2014; Sharps et al., 2012; Stals et al., 2013; Tuladhar et al., 2013; Verhaelen et al., 2013). Few norovirus transfer studies identify the various ways that norovirus may transfer during food preparation by simulating different routes of norovirus introduction and the way in which food is handled. A study by Verhaelen et al. (2013), found that when lettuce and soft berries were manipulated by a gloved hand following a specified protocol, transfer of norovirus and norovirus surrogates was greater from gloves to lettuce (4%–25%) than from gloves to soft berries (<1%), and greater from food to gloves (15–18%) than gloves to food (<1–4%). These data are useful in modelling how much norovirus can be transferred when RTE food items are manipulated by a gloved hand, but do not represent the individual variation in manipulation behaviour in real food handling settings.

Rönnqvist et al. (2014) investigated the proportion of norovirus transferred between food, gloves, utensils and the preparation environment in the simulated preparation of a cucumber sandwich. However, sandwich preparation was modelled using cucumber and plastic as a substitute for bread. Similarly, a greater transfer efficiency was observed from gloves to the food item (1.5% ± 1.9%) than the reverse (0.5% ± 0.4%) as determined by a swab-based recovery method.

In this paper, we present a sample processing and extraction methodology coupled with real-time RT-qPCR which demonstrates a more flexible system for detection of norovirus, and which could be implemented for the detection of norovirus from a wider variety of food samples, expanding on those currently evaluated in the literature. The method was then applied in a food handler contamination simulation as proof of principle study showing the transfer of human norovirus, across food handlers' gloved hands, food ingredients and the environment could be quantified during the preparation of a RTE food product, a cheese and lettuce sandwich.

2. Materials and methods

2.1. Inoculum preparation

The two inocula used in spiking experiments were prepared from two norovirus RNA-positive faecal specimens, representing genotype GI.3 (referred to as "genogroup I inoculum" subsequently in the report) and genotype GII.4 (referred to as "genogroup II inoculum" subsequently in the report). Specimens were selected based on detection of norovirus RNA at a cycle threshold (Ct) of less than 40 cycles. For each specimen, a 10% suspension (w/v) was prepared in a final volume of 40 ml balanced salt solution (Life Technologies, Paisley, UK). The suspension was mixed by vortex and clarified by centrifugation. Specimens were stored at 4 °C.

Specimens were obtained from archives of anonymised residual diagnostic materials held at the Enteric Virus Unit, Public Health England (PHE) Colindale, London. Use of the specimens was reviewed and approved by PHE (and predecessor organisation, Health Protection Agency or HPA), and by the London School of Hygiene and Tropical Medicine ethics committees (ethics approval number 17181).

2.2. Magnetic bead preparation

Using a method based on that described by Tian et al. (2010), magnetic beads were coated at a final concentration of 7.5 mg/ml of partially purified porcine gastric mucin (PGM, Sigma Aldrich) containing 0.5–1.5% sialic acid and 0.2% *N*-acetylneuramic acid (Sigma Aldrich), and covalently coupled with 10 mg/ml of ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) supercarrier immune modulator (Thermo Fisher Scientific). Magnetic beads (MB) were incubated with PGM at 4 °C for a total time of 45 min before separation, and the PGM-coated magnetic beads (PGM-MB) were stored at 4 °C.

2.3. Food spiking

A representative selection of RTE foods were purchased from national supermarket chains or take-away establishments. Foods were selected based on the 13 classifications based on hazard analysis critical control points (HACCP) at production as outlined in the Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market (HPA, 2009). A 25 g portion of food was weighed into a Separator 400 Blender Bag (Grade Ltd., Leicester, UK) (Public Health England, 2016) and the surface of these foods were contaminated with 200 μl of either genogroup I or genogroup II inoculum or a negative control.

2.4. Simulation experiments

A food preparation environment was simulated under containment

level 2 laboratory conditions to allow the transfer of norovirus positive faecal material to be simulated between food handler's gloved hands, the food and the environment. In these experiments, the preparation of a sandwich (consisting of one slice of cheese, two slices of bread and a lettuce leaf) was carried out using three food handlers who undertook three separate tasks in the process (Fig. 1).

The sandwich preparation was performed on stainless steel trays which were sterilised between experiments by treatment with 1000 ppm hypochlorite solution followed by autoclaving. Food handlers were randomly selected from a pool of 20 volunteers amongst microbiology laboratory staff. Only right-handed volunteers were selected for consistency in food handling tasks. Food handlers were protected with a set of SHIELDskin™ Category III PPE nitrile glove (PPE Directive 89/686/EEC) (Bennekom, Netherlands) with a set of vinyl food safe gloves (PAL, Leicestershire, UK) worn over these. All simulations were conducted in a microbiological safety cabinet.

The genogroup I inoculum, genogroup II inoculum or negative control was introduced at one of three points:

(a) the left hand of the volunteer food handler 1 to simulate transmission from an infected individual;
(b) the lettuce leaf to simulate transmission from a contaminated RTE food;
(c) the surface of the sterile metal tray to simulate contamination of a food preparation surface.

Simulations were blinded such that volunteer food handlers were not aware whether the experiment that they were performing was contaminated with one of the two inoculants or a negative control. The use of the negative control was random and allowed experimental controls to be checked to ensure that no environmental residue remained between experiments.

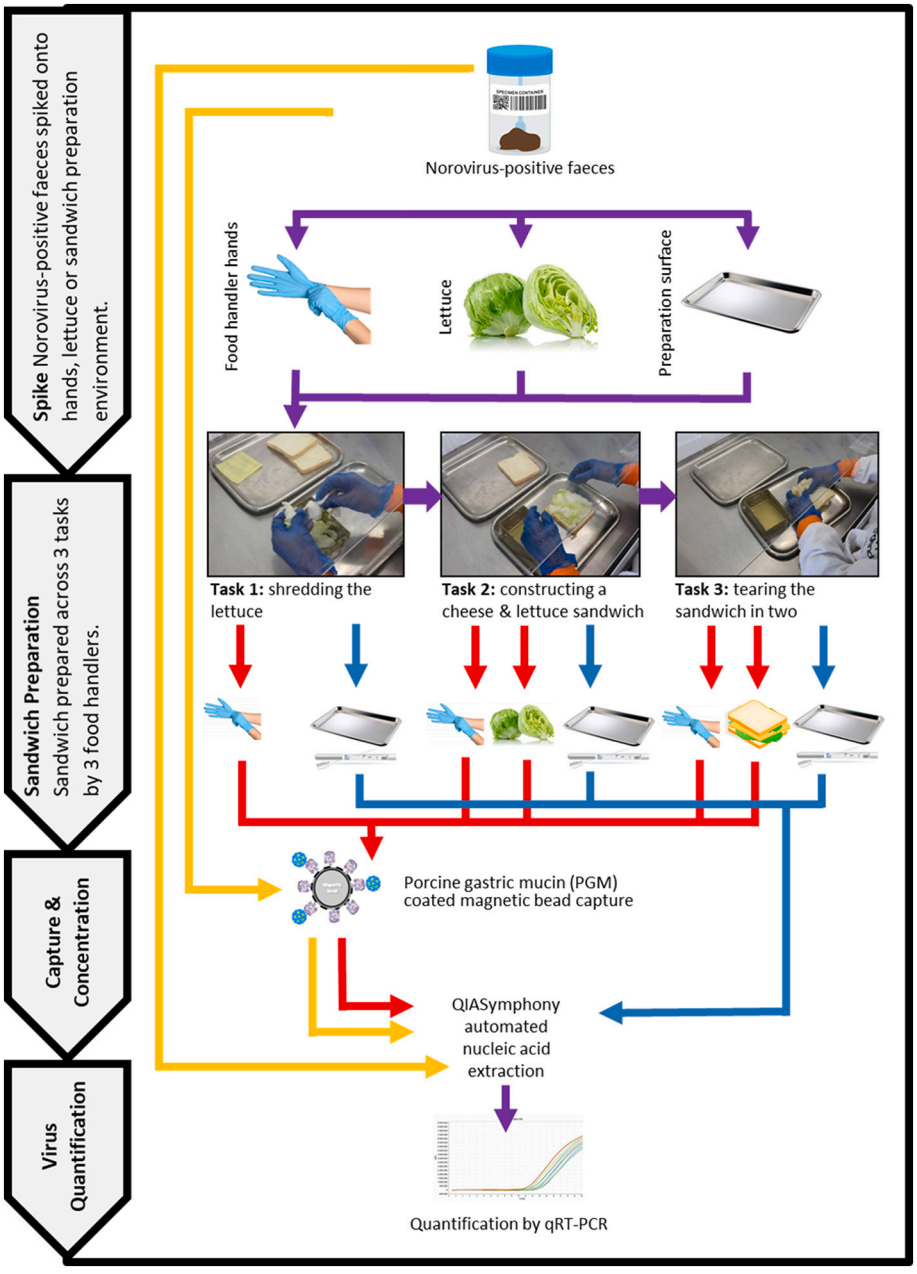

Fig. 1. A diagram of the methods used for preparation and processing of food, environmental and control samples.
Orange pathway used to quantify norovirus present within faecal sample and total amount within spike.
Red pathway used to quantify level of contamination to food handler and food samples.
Blue pathway used to quantify level of contamination to the environment using swabbing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Once construction of the sandwich was completed (Task 3, Fig. 1), each sandwich half was placed into a sterile Separator 400 Blender Bag (Grade Ltd.), one half-sandwich per bag, and prepared by surface washing in 50 ml of PBS (pH 3.5), which was subsequently collected by pipette and transferred to a 50 ml sterile tube.

The outer vinyl gloves were removed using a sterile tongue depressor (S. Murray, Surrey, UK) to prevent the glove from inverting during removal and to limit loss of sample or cross-contamination. They were placed into a sterile Separator 400 Blender Bag, one bag per glove, and prepared by surface washing in 50 ml of PBS (pH 3.5), which was subsequently collected by pipette and transferred to a 50 ml sterile tube.

Environmental surfaces used in the food preparation areas included, two sterile stainless-steel trays approximately 50 × 30 cm and a 10 × 5 cm lettuce bowl. Two sterile viscose swabs pre-moistened in deionised water (TSC Ltd., Lancashire, UK), were used to swab food preparation surfaces in parallel. Each swab was immersed in a 2 ml tube (Sarstedt, Leicester, UK) containing 630 μl of lysis buffer (QIAGEN, Hilden, Germany), mixed, then the viscose swab removed and discarded, and total nucleic acid was extracted from the remaining lysis buffer.

2.5. Virus capture and concentration using PGM conjugated to magnetic beads and nucleic acid extraction and purification

Virus was captured from food and glove surface washes using 100 μl of PGM-MB. The food or glove surface wash samples were incubated on a rotating incubator for 45 min before recovering the PGM-MB by magnetisation for 15 min at 4 °C. Recovered magnetic beads were suspended in 630 μl of lysis buffer (QIAGEN), mixed, then centrifuged to collect magnetic beads and the resulting supernatant pipetted directly into a clean 2 ml tube (Sarstedt). Total nucleic acid was extracted and purified from food, glove and environmental samples suspended in lysis buffer (as described above) using the QIAsymphony™ (QIAGEN) version 4.0.2, using the Complex 200 protocol and the Virus Pathogen Extraction Kit. Mengovirus strain MC_0 (ATCC VR-1597, kindly provided by James Lowther Cefas, Weymouth, UK) was used as a process (spike) control in all experiments.

2.6. Reverse transcription and real-time quantitative PCR

Reverse transcription reactions were conducted in a final volume of 70 μl, in which 40 μl of heat-denatured total nucleic acid was converted to cDNA. The reverse transcription reaction mix consisted of 1 x PCR buffer (Invitrogen), 10 mM $MgCl_2$ (Invitrogen), 2 mM of each deoxyribonucleotide triphosphate (Invitrogen), 458 U of Mu-MLV reverse transcriptase (Invitrogen) and 0.3 μM of random primers. The reaction was incubated at 37 °C for 1 h and was then terminated by incubation at 95 °C for 2 min before being snap cooled on ice for 5 min. Excess cDNA was stored at −20 °C.

Quantitative PCR was undertaken using the oligonucleotide primers COG1 F and COG1 R for detection of norovirus GI and COG2 F and COG2 R reverse for detection of norovirus GII. The RING1(a) TP probe was used for the detection of norovirus GI and RING2 TP probe was used for the detection of norovirus GII as described by Kageyama et al. (2003). A 25 μl total reaction volume was used, which comprised of 1 x Platinum RT-PCR UDG Supermix (Invitrogen), 0.4 mM of each genogroup-specific forward and reverse primer (Invitrogen), 0.1 mM probe (Invitrogen), 1 x ROX dye (Invitrogen) and RNase-free water (Invitrogen). All probes used in norovirus genogroup detection were labelled at the 5′ end with 6-carboxyfluorescein and 3′ end with 5-carboxytetramethylrhodamine. Thermal cycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 56 °C for 1 min. Genotype-matched cDNA controls were included in each assay. Mengovirus RNA was detected as described by Pinto et al. (2009).

The cDNA copies per total nucleic acid extract or inoculum was calculated by multiplying the number of cDNA copies per μl of reaction by 40 and then by 1.5 to represent the dilutions performed on the samples. The conversion of cDNA copies per 0.02 g of faeces into cDNA copies per gram of faeces was calculated by multiplying the cDNA copies per 0.02 g of faeces by 1/dilution factor.

2.7. Plasmid standards

Quantification of norovirus was conducted using genotype matched plasmids kindly provided by James Lowther (Cefas, Weymouth, UK). These plasmids were prepared as a 10-fold dilution series to create a standard curve as described in previous methods (ISO/TS 15216, 2013), and included in duplicate on each assay.

2.8. Statistical analysis

Real time RT-qPCR data was analysed using the Applied Biosystems 7500 Fast Real-Time PCR system version 1.4. Data were exported into Microsoft Excel version 2010 for data curation and calculation of descriptive statistics. All statistical analyses were conducted in GraphPad Prism version 8.1.2.

3. Results

3.1. Limit of detection and quantitation

The limit of detection of the real time RT-qPCR assay was determined using ten replicates of norovirus GI and GII plasmid standards in ten-fold serial dilution series ranging from 10^4 genome copies/μl to 1 genome copy/μl. The limit of detection by real time RT-qPCR for both genotypes was 10 plasmid copies (Supplementary 1). As an additional quality control, the measurement of uncertainty was also established; as a means of validating the quantitative approach and was calculated as ±1.14 Ct for GI and ±1.05 Ct for GII at a confidence level of 95%. This was calculated from the standard deviation of 20 replicates of a positive control multiplied by a coverage factor of 2 (data not shown).

3.2. Experimental design: pH

Different food products and their composition results in variability in their pH and for this reason the pH of food washes was normalised and adjusted to establish optimal conditions for norovirus capture. The capture and concentration of norovirus was determined from two adjusted pH buffers (pH 3.5 and pH 7). The genogroup I inoculum contained 4.3×10^2 (2.6 Log_{10}) cDNA copies/μl of norovirus GI RNA, 1.6×10^2 (2.2 Log_{10}) cDNA copies/μl was recovered from pH 3.5 adjusted buffer and a lower level of 3.1×10^1 (1.5 Log_{10}) cDNA copies/μl from pH 7.0 adjusted buffer. The genogroup II inoculum contained 5.8×10^4 (4.8 Log_{10}) cDNA copies/μl of norovirus GII RNA, and showed the same trend with 6.8×10^3 (3.8 Log_{10}) cDNA copies/μl was recovered from pH 3.5 adjusted buffer and 1.9×10^3 (3.3 Log_{10}) cDNA copies/μl was recovered from pH 7.0 adjusted buffer (Fig. 2). Results indicated a higher level of norovirus recovery from samples normalised to pH 3.5 and this was statistically significant for the results obtained for GII ($p = 0.014$).

3.3. Experimental design: time and temperature

Once the optimal pH was determined to be pH 3.5, we looked to establish the most appropriate incubation time and temperature for the experiment. We quantified the amount of norovirus GI and GII RNA recovered from 50 ml volumes in a buffer adjusted to pH 3.5 incubated over two different time points (60 min or overnight) at two different temperatures (4 °C or ambient temperature) and compared the recovery to an unadjusted buffer (pH 7.0). The greatest amount of norovirus GI RNA was recovered at incubation conditions of 60 min at 4 °C for both norovirus GI 3.9×10^3 (3.6 Log_{10}) cDNA copies/μl and norovirus GII 2.8 $\times 10^6$ (6.5 Log_{10}) cDNA copies/μl resulting in a 46% and 24% increase

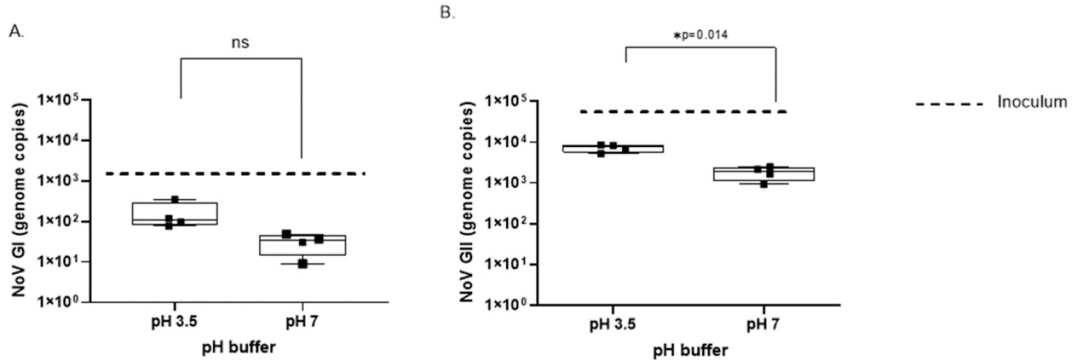

Fig. 2. Validation of pH to use for the norovirus capture and concentration method using porcine gastric mucin (PGM) coated beads. A. 50 ml volume of pH adjusted PBS (pH 3.5 or pH 7) spiked with norovirus GI inoculum resulting in a recovery efficiency of 38% at pH 3.5 and 7% at pH 7 respectively. B. 50 ml volume of pH adjusted PBS (pH 3.5 or pH 7) spiked with norovirus GII inoculum resulting in a recovery efficiency of 12% at pH 3.5 and 3% at pH 7 respectively. Each inoculum comprised 200 μl of a 10% suspension. In all experiments the full capture and concentration experiment was performed twice over on two technical replicates. ns = not significant.

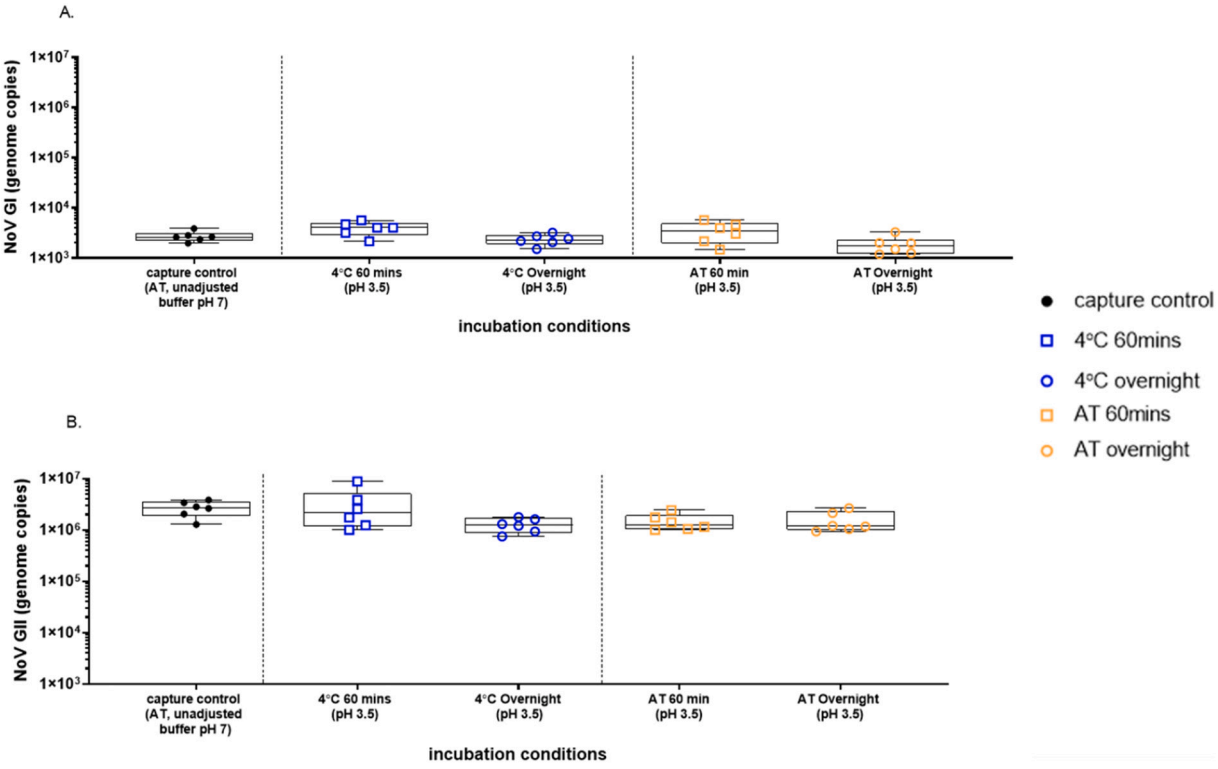

Fig. 3. Validation of the norovirus capture and concentration method using porcine gastric mucin (PGM) coated beads. A. 50 ml volumes of PBS pH 3.5 spiked with norovirus GI, captured and concentrated straight away (capture control) and over different temperatures (4 °C and AT = ambient temperature) and two different time points (60 mins and overnight). B. 50 ml volumes of PBS pH 3.5 spiked with norovirus GII, captured and concentrated straight away (capture control) and over different temperatures (4 °C and AT = ambient temperature) and three different time points (60 mins and overnight). All experiments were performed three times over two technical replicates.

in comparison to the unadjusted buffer (pH 7.0) (Fig. 3). Although not statistically different, there was a clear indication that the 60-minute incubation at 4 °C gave the higher level of recovery and allowed the benefit of a faster turnaround time for testing. Recovery efficiencies were calculated for each condition (Supplementary 2).

3.4. Application to a range of food matrices

Methodologies presented within ISO/TS 15216 (2017) and Tian et al. (2010) are limited by their application to just fresh produce and shellfish. Although food borne outbreaks of norovirus are primarily associated with shellfish and fresh produce, there are examples where more complex food matrices have been contaminated and identified as the source of infection (Morgan et al., 2019; Smith et al., 2017). Using the Ready-to-Eat Guidelines (HPA, 2009), examples of food that represented the 13 food groups determined by their bacterial aerobic colony count (ACC) levels and the HACCP used in their production, were contaminated with known levels of norovirus. The amount of norovirus GI or GII RNA recovered from each food was quantified (Fig. 4). The least amount of RNA loss was identified from food in category 12; the

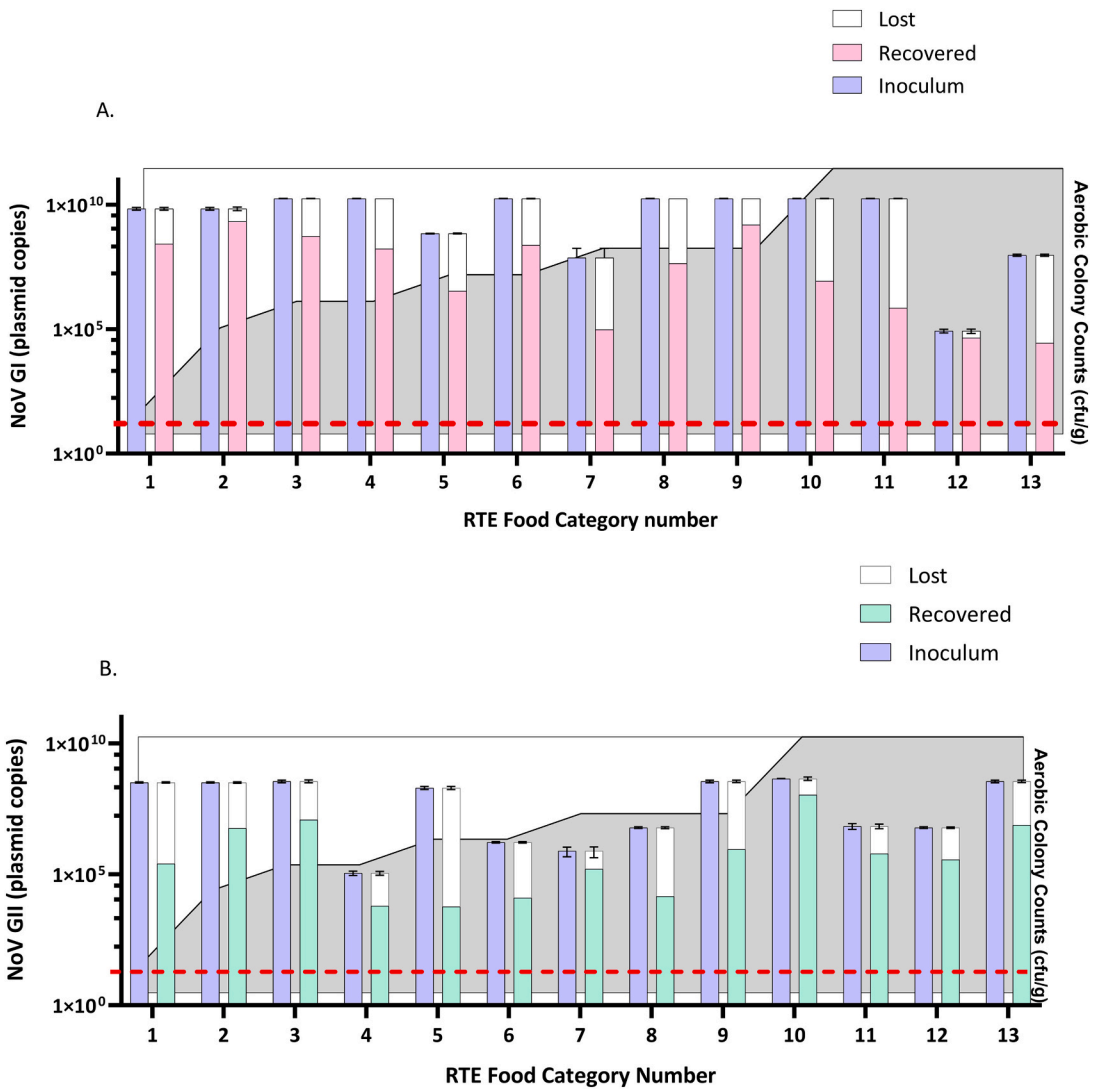

Fig. 4. Recovery of norovirus from an example food from the HPA Ready-to-Eat Food Guidelines (2009). A. recovery of norovirus GI RNA from two replicates of each Ready-to-Eat food B. recovery of norovirus GII RNA from two replicates of each Ready-to-Eat food. Red dotted line = norovirus infectious dose. Grey shading = expected aerobic colony counts (cfu/g) per food category. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

category for pre-packed sandwiches containing salad. For norovirus GI RNA 55%. For norovirus GII RNA 24% of the contaminating input was recovered from food in category 10 (Supplementary 3).

3.5. Detection from cake linked to an outbreak of norovirus infection

Approximately 400 g of cake implicated in a norovirus outbreak was transported to PHE during an outbreak investigation. The type of cake

Table 1
A table showing the genome copies/μl of norovirus recovered from Food handler's gloved hands, the environment or a sandwich and the route of norovirus introduction through Food handler 1, lettuce or the environment.

	Contamination point	Mean recovery after sandwich preparation (cDNA copies/μl ± SD)										
		Input amount	Food handler 1	% recovered[a]	Food handler 2	% recovered	Food handler 3	% recovered	Environment	% recovered	Food (sandwich)	% recovered
GI	Food handler 1	69,376,325 ± 16,232,898	5,728,582 ± 1,642,607	8.3	31,677 ± 14,011	0.05	5176 ± 2357	0.01	141,882 ± 31,292	0.2	371,671 ± 5689	0.5
GII	Food handler 1	790,433 ± 148,929	49,943 ± 4653	6.3	1076 ± 514	0.14	251 ± 27	0.03	5427 ± 869	0.7	1737 ± 133	0.2
	Lettuce	2,291,921 ± 849,419	30,224 ± 3007	1.3	3312 ± 967	0.15	1533 ± 961	0.07	5202 ± 122	0.4	8300 ± 4563	0.3
	Environment	934,171 ± 423,862	Not detected		Not detected		137 ± 15	0.01	9792 ± 1791	1.1	10 ± 5	0.001

[a] Percentage recovery was calculated by either [Food Handler / Input amount] × 100 or [Environment / Input amount] × 100 or [Sandwich / Input amount] × 100.

was a sponge containing a buttercream filing and fondant icing around the exterior, all layers of the cake were tested as a whole slice. Two subsamples of approximately 25 g of the cake were randomly selected and tested. Norovirus cDNA was detected from both 25 g portions of the cake. Quantification of the amount of norovirus detected was 9.13×10^2 and 6.15×10^2 cDNA copies/μl.

3.6. Simulation experiments

The introduction of norovirus contamination to a food preparation environment at three points was simulated experimentally under laboratory conditions. These three points of contamination were; the primary food handler's gloved hands, a food ingredient and the food preparation surface.

Where the first food handler introduced norovirus through contamination from their non-dominant hand into the food preparation process, the highest level of contamination was recovered from all three food handlers' gloved hands after they had completed their task (Table 1, Fig. 5). For norovirus GI the input of the viral RNA was 6.9×10^7 (7.8 Log_{10}) cDNA copies/μl with 6.2×10^6 (6.8 Log_{10}) cDNA copies/μl of norovirus GI RNA being recovered. The distribution of this was 5.8×10^6 (6.8 Log_{10}) cDNA copies/μl recovered from gloved hands, 3.7×10^5 (5.6 Log_{10}) cDNA copies/μl recovered from the sandwich and 1.4×10^5 (5.2 Log_{10}) cDNA copies/μl recovered from the environment. For norovirus GII the input of viral RNA was 7.9×10^5 (5.9 Log_{10}) cDNA copies/μl with 5.8×10^4 (4.8 Log_{10}) cDNA copies/μl of norovirus RNA being recovered. The distribution of this was 5.1×10^4 (4.7 Log_{10}) cDNA copies/μl recovered from gloved hands, 1.7×10^3 (3.2 Log_{10}) cDNA copies/μl recovered from the sandwich and 5.4×10^5 (3.7 Log_{10}) cDNA copies/μl recovered from the environment. Where norovirus was introduced through contamination of a food ingredient, an iceberg lettuce leaf, the food handlers' gloved hands again had the highest recovery of the inoculum (Fig. 6). In this simulation 2.0×10^6 (6.3 Log_{10}) cDNA copies/μl of input RNA was pipetted onto the lettuce leaf, of this 4.9×10^4 (4.7 Log_{10}) cDNA copies/μl was recovered, with 3.5×10^4 (4.5 Log_{10}) cDNA copies/μl recovered from gloved hands during sandwich preparation, 8.3×10^3 (3.9 Log_{10}) cDNA copies/μl was recovered from the sandwich and 5.2×10^3 (3.7 Log_{10}) cDNA copies/μl was recovered from the environment.

In contrast, when the food preparation environment was contaminated, the greatest recovery of virus remained the environment (Fig. 7). Of the input of 9.3×10^5 (6.0 Log_{10}) cDNA copies/μl input RNA, 1.0×10^4 or (4.0 Log_{10}) cDNA copies/μl, was the total RNA recovered, with 1.4×10^2 (2.1 Log_{10}) cDNA copies/μl recovered from the gloved hands, 9.8×10^0 (1.0 Log_{10}) cDNA copies/μl, recovered from the lettuce and 9.8×10^3 (4.0 Log_{10}) cDNA copies/μl, recovered from the environment.

4. Discussion

Understanding the role and pathways of norovirus transmission in the catering environment – either via food handler or contaminated foods – remains a key public health priority to reduce the burden of norovirus-associated gastroenteritis. Here, we present data that contributes to this knowledge gap and through simulation of food preparation, this proof of principle study demonstrates norovirus can be tracked from the point of contamination to the final food product using molecular methods and, furthermore, that the quantity of norovirus in the final food product is likely to be sufficient to cause infection and disease. Bacterial indicators have become a standard measure of hygiene practices within premises, however the NoVAS study identified that the bacterial indicators were not a suitable proxy as an indicator for norovirus being present (NoVAS, 2020). This means that methods are needed to allow direct detection from hands, food and the environment to accurately assess risk. The data presented demonstrates an effective methodology for all three sample types and furthermore is not inhibited by the bacterial composition of the sample matrix or by the ingredients or method of production.

We validated and implemented a rapid, robust, semi-automated method for recovery of viral nucleic acid direct from glove washes, food items and environmental swabs. Typically, suspected foodborne outbreak investigations rely upon detection of infectious agents in clinical samples from affected patients to infer aetiology (CDC, 2006; Daniels et al., 2000; Sala et al., 2005). Development of the ISO/TS-15216 (2017) was a significant advance in detecting viruses associated with food, however, this method is validated for detection of only two viral pathogens – norovirus and hepatitis A virus – in a limited number of food matrices, and is labour-intensive making it less tractable for rapid-response, larger-scale public health outbreak investigations. Here we present a sample processing and extraction methodology coupled

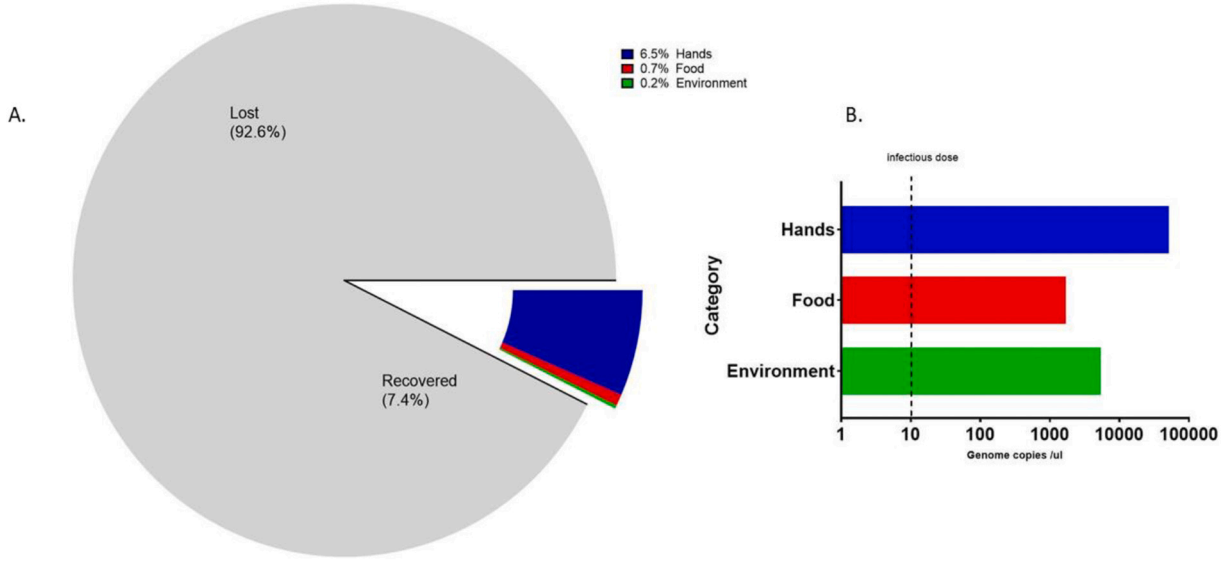

Fig. 5. Recovery and loss of norovirus during food simulation where the food handler's gloved hands were inoculated, and the proportions recovered from gloves, food or the environment. A. Recovery of norovirus GII RNA as a percentage. B. Recovery of norovirus GII RNA as genome copies per microlitre to demonstrate proportion recovered in comparison to the infectious dose.

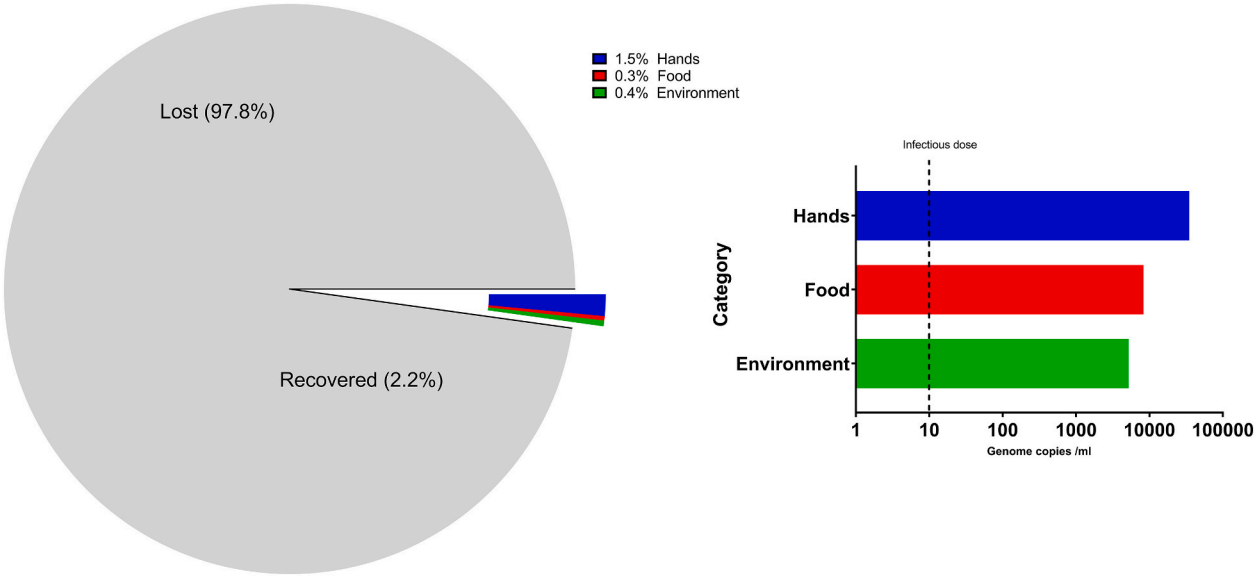

Fig. 6. Recovery and loss of norovirus during food simulation where the lettuce was inoculated, and the proportions recovered from gloves, food or the environment. A. Recovery of norovirus GII RNA as a percentage. B. Recovery of norovirus GII RNA as genome copies per microlitre to demonstrate proportion recovered in comparison to the infectious dose.

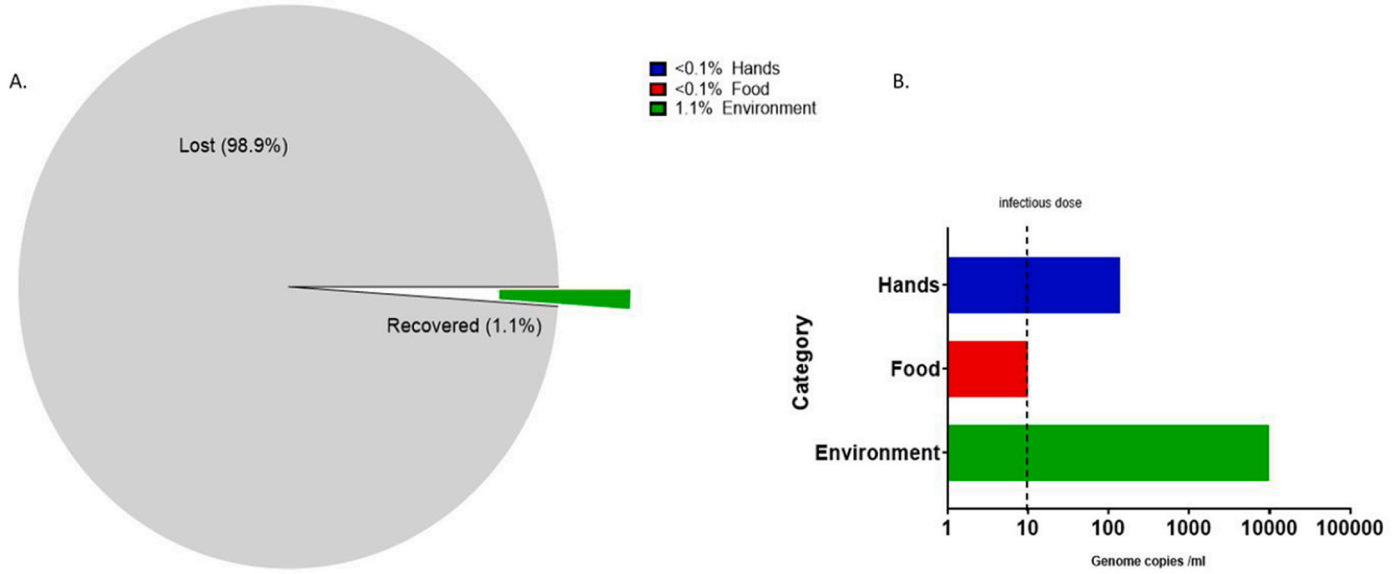

Fig. 7. Recovery and loss of norovirus during food simulation where the food preparation tray was inoculated, and the proportions recovered from gloves, food or the environment. A. Recovery of norovirus GII RNA as a percentage. B. Recovery of norovirus GII RNA as genome copies per microlitre to demonstrate proportion recovered in comparison to the infectious dose.

with real-time RT-qPCR which demonstrates a more flexible system for detection of norovirus, and which could be implemented for other important foodborne pathogens, in a broad range of food matrices.

Simulation studies have been reported in the past, but with some limitations. For example, Rönnqvist et al. (2014) investigated the proportion of norovirus transferred between food, gloves, utensils and the preparation environment in the simulated preparation of a cucumber sandwich. However, sandwich preparation was modelled using cucumber and plastic surfaces as a substitute for bread. Further, in another study by Verhaelen et al. (2013), lettuce and soft berries were minimally manipulated by a glove which is not representative of the complex manipulation of food in food-handling premises. By comparison, our simulation did not use food substitutes, did not use norovirus surrogates, and replicated, as far as possible, the production of a sandwich as would occur in a food-handling premises.

An additional consideration here is human factors, which have become well-recognised for their importance to control foodborne transmission of norovirus and other pathogens at food-handling premises (FSA, 2017). Illness within food handlers is a known issue, alongside asymptomatic carriage of pathogens. However, due to pressures to return to work because of staff shortages in the sector and socio-economic pressures, staff have reported returning to work sooner than the recommended 48 h (FSA, 2011; Harris et al., 2010). Within a recent social sciences study, only five of the 37 respondents could state the correct exclusion of not returning to the workplace until at least 48 h after symptoms have stopped and only one recognised the potential of asymptomatic food handlers as a means of transmitting infection (FSA, 2017).

Inclusion of a quantitative measure of virus contamination is critical for method evaluation and result interpretation, particularly in context of public health response, informing evidence-based guidelines, risk assessment and management. We found that between 1.1 and 7.4% of norovirus contamination was recovered across the range of samples tested. Although low, when interpreted quantitatively, the number of virus genome copies detected in the prepared sandwiches indicated levels predicted to be sufficient to cause infection and disease (Acheson and Fiore, 2004; Teunis et al., 2008).

Recent reports of norovirus outbreaks have identified the need for access to laboratory testing of food items beyond the widely recognised high-risk items of salads, soft fruits and oysters (Morgan et al., 2019; Smith et al., 2017). However, identifying viral contamination of foods remains a challenge, as demonstrated in a recent study in which norovirus was only detected in six out of 352 food samples implicated in 67 norovirus foodborne outbreaks (Somura et al., 2019). Our study shows the ability to detect norovirus from 13 food categories described by the HPA (2009), including cake from an outbreak investigation. Given the epidemiological and laboratory evidence of diverse and complex food matrices associated with outbreaks (Somura et al., 2019; Saito et al., 2015; Stals et al., 2011), there is a clear need to expand testing capability beyond soft fruits, salad vegetables and oysters.

Several limitations were recognised in the presented study. First, although virus contamination of environmental surfaces was addressed, the transfer from contaminated kitchen utensils or high contact kitchen surfaces was not explored. This limitation is important given the observation that norovirus is highly persistent in the environment (Cannon et al., 2006; Escudero et al., 2012) and that norovirus surrogates have been shown to remain infectious on stainless steel surfaces, such as those found in kitchens, for up to 15 days (Fallahi and Mattison, 2011).

Secondly, simulations in this study were conducted with a viral load identified from food handlers from the literature. Sabrià et al. (2016) demonstrated that asymptomatic food handlers shed on average 4.5 ± 1.5 Log_{10} genome copies of norovirus for 19 days after the primary exposure event. However, simulations in this study did not take into account the lower range of the viral load reported. Others have also found asymptomatic individuals shedding lower viral loads (Phillips et al., 2009). Therefore, the risk associated with food handlers shedding at lower viral loads remains unknown and will be crucial for understanding the potential role of asymptomatic food handlers in norovirus transmission. Relatedly, it is recognised that the viral loads used in the simulations were high, and whilst may be representative of heavy contamination on hands, it is recognised that virus loads are likely to be lower in foods. One exception is likely to be oysters which are known to concentrate norovirus from the environment in digestive tissues, as demonstrated in a survey which demonstrated >1.0% of oysters were contaminated with >10,000 copies/g of norovirus (Lowther et al., 2012). Additionally, the focus of the study presented here was proof-of-concept, and following demonstrating the utility of the approach described herein, further simulation experiments with lower viral loads on foods and environmental sources will be important to inform risk assessments for catering environments.

Thirdly, the study design was limited to the use of gloved hands, which may not be truly representative of skin. Indeed, bare hand contact has been identified as a risk factor strongly linked to virus transmission (Todd et al., 2007), and it is possible that the transfer of norovirus from contaminated bare hands may differ due to the complexity of the texture and surface of the skin. Nonetheless, the data here provide useful insight into the role of gloved hands during food preparation, as whilst wearing gloves in commercial food preparation environments is not mandatory in UK food law, gloves are used in commercial food preparation settings depending on requirements set by individual food business operators.

Fourth, although genome detection by RT-qPCR does not provide an indicator of virus infectivity, as challenges remain in the development of in vitro culture systems for norovirus (Estes et al., 2019), one of the advantages of using PGM-MB prior to RT-qPCR is that this method captures virus particles prior to genome amplification, and will therefore remove some non-infectious RNA signal (Manuel et al., 2018). Infectivity is complex and not all virus particles may be able to complete an infectious cycle as defective, marginally damaged capsids or genomes could still be amplified by real time PCR, which only targets amplification of small fragments of the complete genome (Knight et al., 2013). However recent findings combining HBGA-virus capture prior to plaque assays or RT-qPCR found concordance between PGM-MB RT-qPCR and PGM-MB plaque assay results (Li & Chen, 2015; Wang et al., 2014). Whilst PGM-MB may not capture all norovirus genotypes, given the variation in interactions between norovirus and HBGAs (Thornhill et al., 1977; Dolin et al., 1982), those genotypes are regarded as less prevalent (Harris et al., 2019).

Finally, even when detected, the ability to link foodborne viruses with those detected in cases through analysis of virus genome sequences remains a challenge. For example, during an outbreak investigation which found eight GI.6 norovirus positive clinical cases, two out of 30 environmental swabs had norovirus detected, but could not be genotyped (Smith et al., 2017). Norovirus typing remains largely based on partial genome amplicon sequencing, and recovery of sufficient amplicon for sequencing is challenging from food and environmental samples. During the NoVAS study in the UK, the use of metagenomics and whole genome sequencing methods were evaluated, and whilst promising, also demonstrate these methods are susceptible to inherent challenges of low viral loads, inhibitors and loss of genetic material during processing (NoVAS, 2020).

We present an approach for quantification of norovirus from gloved hands, food and the environment. Recently research has shown that in the UK one in every 1200 meals eaten out or take away meals results in a case of norovirus and that norovirus can be detected in food handlers and the environment in food preparation premises (NoVAS, 2020). Our methodology provides a step towards increasing the ability to detect and quantify norovirus from hands, complex food matrices and the food preparation environment as a means of supporting public health response and for developing evidence-based guidelines that are currently lacking. This will be critical for enhancing countermeasures for food-associated gastroenteritis. Further development and extension of these studies are essential to continue the target of reducing the burden of foodborne disease, ensuring the safety of food.

Declaration of competing interest

We have no conflicts of interest to disclose. The research was funded as part of a PhD studentship by Public Health England, to establish a method for detecting viruses from foods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109151.

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