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Occurrence and Magnitude of Norovirus Contamination and Quantitative Microbial Risk Assessment (QMRA) of Norovirus in Accra, Ghana

By

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Master of Public Health

Epidemiology

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Abstract

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By Han-Hsuan Tsai

Noroviruses (NoVs) are the major cause of nonbacterial gastroenteritis worldwide. NoV genogroup I (GI) and genogroup II (GII) are the most predominant strains that cause disease among humans. NoVs are primarily transmitted via the fecal-oral route and through contaminated environments. However, research on NoV exposure pathways, contamination levels, and the corresponding risk of NoV infection is limited, particularly in low income urban settings.

In this study, environmental samples were collected from a variety of domains in Accra, Ghana, in order to investigate NoV GI and GII levels in the environment. Samples were screened for the presence of NoV GI and/or GII, and the samples that contained PCR inhibitors or were NoV positive in the screening step were further quantified. The results of the environmental investigation showed that a high percentage (41% for NoV GI and 52% for NoV GII) of septage samples collected from public latrines were NoV-positive. In addition, 5% to 15% of samples collected from public drains, flooded areas, irrigation systems, and farms were also NoV-positive. The NoV GI and GII concentrations in irrigation and farm water samples were used to conduct Quantitative Microbial Risk Assessment (QMRA) to investigate the potential risk of NoV infection among farmers who unintentionally ingest NoV contaminated water while working. The QMRA results showed that, for farmers who work for seven days in the field, the estimated average probabilities of NoV GI and GII infection are 0.28 and 0.42, respectively,

The PCR inhibition for NoVs and Adenovirus and the sensitivity analysis of the decision making scheme used to determine whether the samples were positive were also analyzed. The results of sensitivity analysis showed that the changes in frequencies of positive, negative, inhibition positive, and inhibition negative samples were significant if the decision scheme became stricter; however, the changes in average concentrations among different schemes were not significant. The results of PCR inhibition showed that there 18%, 19%, and 2% of samples inhibited for NoV GI, NoV GII, and Adenovirus, respectively, and 18 out of 25 (72%) of samples that were inhibited for Adenovirus were also inhibited for NoVs.

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Chapter 1: Literature Review

NOROVIRUS EPIDEMIOLOGY

Noroviruses (NoVs) are now classified into six genetic groups (genogroups), GI to GVI [1, 2]. Genogroups GI, GII, GIV, and GVI are capable of infecting humans[3], with GI and GII the predominant human pathogenic genogroups that cause acute gastroenteritis[4].GIII are primarily bovine and ovine viruses, GV NoV primarily infect mice[5]; in addition, GIV and GVI are capable of infecting dogs[2]. The most common symptoms of NoV infections are diarrhea and vomiting; some patients also experience abdominal pain and cramping, bloating, body aches, and fever[6].

Noroviruses are the most common viral cause of gastroenteritis outbreaks worldwide among all age groups[5, 6]. Atmar and Estes summarized published studies from 1996 to 2004 and found that NoVs were identified as the cause of 71% all gastroenteritis outbreaks in the US, Europe, Japan, and Australia[5]. Among foodborne outbreaks with a known cause in the US, NoV accounted for 46% of outbreaks from 2001 to 2008 and accounted for 42% of outbreaks from 2009 to 2010[7, 8].

NoVs are also frequently reported as the cause of sporadic acute gastroenteritis cases. Atmar and Estes reported that between 1993 and 2003, 5.4% to 21.2% of global sporadic acute gastroenteritis cases were due to NoV infections[5]. Sai et al. also

reported that NoVs were detected in 8.75% to 10.4% of clinical stool samples collected in China during 2010 to 2012[6, 9]. NoVs have been recognized as one of the most common causative agents of childhood diarrhea[9]. Medici et al. reported that the prevalence of NoVs was up to 48% among children with acute gastroenteritis based on previous studies[10].

NoVs infect humans throughout the year, though the infections are more likely to occur during the winter season[5, 11]. The results of a systematic review by Ahmed et al. showed that NoV outbreaks and cases that reported during 1998 to 2009 exhibited a seasonal pattern in most areas with a temperate climate, with around 41% of outbreaks and 53% of cases reported during the winter season[11]. Sai et al. also analyzed the seasonal distribution of NoV infections in Ji'nan, China in 2010, by calculating the detection rates of NoV in clinical stool samples every month. The results of the study showed that NoV infections were the highest between September and December (55% of samples were positive), and the infections were the lowest in June and July (8.6% of samples were positive)[6]. However, the data from many tropical areas were still limited for analyzing the seasonal pattern of NoV outbreaks in these regions[11].

Since the end of last century, NoV GII strains have become the most predominant cause of acute gastroenteritis disease among all genogroups that are

capable of infecting humans [5, 12]. Studies show that NoV GII strains were responsible for 66% to 96% of NoV outbreaks between 2001 to 2009 in Europe, and 81% to 100% of outbreaks between 1994 to 2009 in Asia, Australia, Africa, America [3]. Hall et al. reported that approximately 80% of outbreaks were caused by NoV GII strains among all confirmed foodborne outbreaks of NoV during 2001 to 2008 in the US[8]. Sai et al. also reported that 95% to 100% of NoV-positive stool samples collected from clinics in Ji'nan, China during 2010 to 2012 belonged to genogroup II[6, 9].

GII.4 noroviruses are the predominant cause of outbreaks in developed countries and are disproportionately reported as the pathogens when the route of transmission was person-to-person[13]. GII.4 strains are genetically heterogeneous, and new GII.4 strains emerge every 2 to 3 years[10]. Compared to other NoV strains, GII.4 viruses are more frequently detected among patients in hospitals or long-term care facilities[14, 15]. Bernerd et al. reported that GII.4 strains were detected in more than 82% of nursing home and hospital outbreaks in Germany from 2001 to 2009[14]. In addition, GII.4 viruses are correlated with more severe outcomes, such as hospitalizations or deaths [12, 16]. Desai et al. estimated that the hospitalization rate and mortality rates caused by GII.4 viruses are 9.4-times and 3.1-times higher than those caused by non-GII.4 viruses, respectively[16].

GENOTYPE, STRUCTURE AND DETECTION OF NOROVIRUS

NoVs belong to the Caliciviridae family and are non-enveloped, single stranded, positive sense RNA viruses with a genome of 7.5 to 7.7 kilobasepairs[3, 17]. The genome of NoVs contains three open reading frames (ORFs): ORF1 encodes a large polyprotein that is cleaved after synthesis into several nonstructural proteins, which include nucleotide triphosphatase, genome-linked viral protein, viral protease, and viral polymerase[18]; ORF2 encodes the major capsid protein VP1; and ORF3 encodes the minor capsid protein VP2, which is thought to stabilize VP1[1, 19].

Reverse transcription-polymerase chain reaction (RT-PCR) assays are commonly used for NoV detection. Virus-specific primers are used for amplifying part of NoV genome, such as regions encoding viral polymerase or VP1[5]. Due to high genetic diversity, separate sets of primers are usually used to detect NoV strains that belong to different genogroups [20]. Recently, real-time RT-PCR assays have proven to be able to detect lower NoV concentrations and, thus, have been used to detect NoVs in fecal samples and environmental samples[20, 21]. Nevertheless, it is still difficult to precisely quantify NoV concentrations in samples due to variety of extraction methods and the presence of environmental compounds that may inhibit the PCR performance[21].

In addition to PCR assays, there are several methods for detecting NoVs in clinical samples. One method is using enzyme immunoassays to detect the viral

antigens in stool samples. Previous studies tested the performance of a commercial enzyme immunoassay kit (IDEIA NVL assay) and reported that the sensitivity and the specificity ranged from 39% to 100% [22, 23]. Serologic assays, which can detect the host's immune responses to viral infection, have been developed [24, 25]. Electron microscopy has also been used in many laboratories to visualize viruses directly in stool samples; however, this approach is not sensitive (less than 25%) compared with molecular assays[5].

NoV genotyping is a useful to identify and track the patterns and the source of NoV transmission and evolution[5]. In addition, correctly identifying the genetic differences among NoVs is critical because the infectivity, the host range, the incidence, and the stability in the environment vary from strain to strain [1]. Since human NoVs cannot be cultured, most laboratories use genetic analysis to classify NoVs[5]. However, there is no standardized region for NoV sequencing. Laboratories usually use one of 5 representative regions (region A to E) in the NoV genome for NoV genotyping. Regions A and B are located in ORF1, regions D and E in ORF2, and region C includes portions of both of ORF1 and ORF2 [26]. The ability to accurately type NoV-positive samples varies when sequencing different regions [26]. Mattison et al. evaluated the success rates of typing NoVs GI and GII positive samples from 9 laboratories in Canada and the US, and the results showed that the

success rates of typing region C (78%) were higher than the success rates of typing region D (52%), indicating that typing region C is a better protocol for genotyping NoVs[26]. Nevertheless, the authors also argued that the protocol of typing region D has a better resolution to distinguish GII.4 strains [26].

Since the mid 1990s, new NoV genotypes have been assigned when the complete VP1 amino acid sequences showed more than 20% differences compared to the sequences of other NoV genotypes. However, due to the increased recognition of NoV genotype diversity, a consistent nomenclature system for NoV is needed to classify the NoVs that have been identified and that may be identified in the future [1]. Kroneman et al. proposed to use a new NoV classification and nomenclature scheme that sequences partial ORF1, uses the 2×SD criterion, which is twice the standard deviation values of average distances within and between phylogenetic clusters, to group sequences into a particular genotype, and names NoVs based on both ORF1 and VP1 sequences.

NoVs have high rates of RNA recombination, which may be the reason for high genetic diversity among NoVs[1]. RNA recombination of NoVs usually occurs at the junction region of ORF1 and ORF2[27]. RNA recombination produces novel strains of NoVs, which may have increased virulence and environmental stability, enabling them to replace the older strains circulating in population[6]. Recognizing NoVs

recombination in the junction region in the ORF1 and ORF2 is common, the nomenclature system proposed by Kroneman et al., which suggested to note the partial ORF1 according to their phylogenetic clustering in front of the genotype designated based on VP1, was suggested to differentiate the recombinant strains [1].

TRANSMISSION OF NOROVIRUS

NoVs can be transmitted person-to-person, via fecal-to-oral contact, or by consuming contaminated food[28]. Airborne transmission and contact with contaminated surfaces are also possible routes of transmission[28]. Although NoV disease is usually self-limiting, NoVs can be shed in the feces of individuals with either symptomatic or asymptomatic infection for up to 3 weeks[29]; moreover, immune-impaired individuals may continue shedding NoV particles for years[30]. The transmission of NoV is facilitated by their stability in the environment, resistance to inactivation and an infectious dose as low as 10 viral particles[19, 29, 31, 32]. In addition, people who have been infected by NoVs do not develop long-term immunity and are still susceptible to re-infection due to the variety of viral strains[29].

Person-to-person transmission is the most dominant route of transmission of NoVs, and has been documented extensively in semi-closed settings, such as hospitals, health care centers, and cruise ships[33]. Between 2002 and 2006, 74% of outbreaks reported by the Foodborne Viruses in Europe network involved person-to-person

transmission[33].

Although person-to-person transmission is the major route of transmission, infection due to consuming NoV-contaminated food is also very common [33]. According to US CDC reports, foodborne transmission accounts for 39% of 348 outbreaks from 1996 to 2000[28]. Seafood, especially mollusks, which can accumulate NoV particles by filtering large amounts of water, is the most often implicated food vehicle among NoV foodborne outbreaks[32]. Martinez et al. reported that 26.7% of identified NoV outbreaks were due to contaminated seafood[34].

Food products can be contaminated with NoVs through contact with a NoV-infected person's fecal materials or vomitus during different steps of food production, ot though contact with NoV-contaminated surfaces [33, 35]. The contamination may occur through crop irrigation with NoV- contaminated water or through the application of manure that contains NoVs[33]. Food handlers have played an important role in NoV transmission. They may transmit NoVs during food harvesting, processing, preparation, or service via contaminated hands, and NoV transmission via food handlers usually affects many people[33, 36]. A review showed that food handlers were responsible for 42.5% of foodborne or waterborne outbreaks due to NoVs during 2000 to 2007[37].

Different transmission routes are associated with different genogroups of NoVs. Matthew et al. conducted a systematic review, which collected the reported outbreaks in the US between December 1983 and March 2010, and the results showed that GII strains were significantly less likely to be associated with water-borne transmission compared to GI strains (adjusted odds ratio = 0.19, 95% CI 0.05 - 0.67) [38]. On the other hand, GII strains are more often linked to outbreaks in health care settings (adjusted odds ratio =33.67, 95% CI 1.76 - 644.18) [38].

PREVALENCE OF NOVS IN ENVIRONMENTAL AND FOOD SAMPLES

NoVs have been found in variety of environmental surface water samples in Asia, Europe, Africa, and South America[31, 39, 40]. Kittigul et al. reported that 22% and 4% of the river samples and irrigation water samples in Thailand, respectively, were NoV- positive[41]. Lodder and de Roda Husman detected NoVs in all water samples collected from two rivers in the Netherlands[39]. Aw et al. also found that 43 out of 60 (72%) river water samples from Singapore were NoV-positive, of which 27 samples (45%) were GI positive, and 39 samples (65%) were GII positive[42]. Mans et al. reported that 95 of 151 samples (63%) collected from rivers in South Africa were NoV-positive, of which 67 samples (44%) and 64 samples (42%) were positive for GI and GII NoVs, respectively[31]. Fernandez et al. evaluated contamination levels in five rivers in Argentina between 2005 and 2010, and reported that 53 out of

209 river water samples were NoV-positive[40].

NoVs have frequently been detected in food samples obtained from food processing companies or supermarkets[37, 43]. Nevertheless, the food sample types, the methods to process food samples, and even the assays to detect NoVs vary from study to study; therefore, the differences in NoV prevalence results in food samples among different studies may be due to different methods that were used for sample processing and viral detection. For example, Baert et al. summarized the results of NoV prevalence in the produce samples collected from produce processing companies in Belgium, supermarkets in France, and food companies in Canada. The results showed that NoVs were detected in 33%, 50%, and 28% of leafy green samples collected during 2009 to 2010 in Belgium, France, and Canada, respectively, using real-time RT-PCR; and GI NoVs accounted for at least 67% of these NoV-positive samples[37]. Laura et al. also analyzed 80 leafy green samples and 30 tomato samples collected from open-air markets in Italy and in Turkey using RT-booster PCR. The results showed that 15 tomato samples were NoV-positive but no leafy green sample was NoV-positive. Furthermore, the 15 NoV-positive samples were then tested with RT-hemi-nested PCR to determine the genogroups of the NoV-positive sample, and the results showed that all NoV-positive samples belonged to NoV GII[43]. Since the virus extraction methods and RT-PCR assays varied considerably between these two

studies, it is difficult to compare the results and draw conclusions.

Environmental swabbing has been proven to be a useful tool for detecting NoVs in different environmental settings, such as catering companies, hospitals, or cruise ships[44-46]. Boxman et al. collected swab samples from January 2008 to February 2009 from catering companies in the Netherlands, and found that 1.7% of swab samples were NoV-positive, although these companies had not been associated with any outbreaks[44]. Morter et al. found that 31.4% of environmental swab samples collected from a hospital in the UK from 2009 to 2010 were NoV-positive, even after cleaning the sampling sites[45].

Unfortunately, there is no standard protocol for swab collection or for swab processing to detect NoVs on swab samples; thus, the discrepancies may also generate different results. Ronnqvist et al. used swabs made of polyester, flocked nylon, cotton wool, or microfiber to swab four different surfaces, which were latex, plastic, stainless steel, and cucumber, and then eluted with two different buffers[46]. The results showed that the levels of recovery varied considerably among different combinations of swab materials, surfaces, and elute solutions; the highest recovery (89%) was achieved when using microfiber to swab a plastic surface and elute with glycine buffer, and the recovery was lowest (11%) when using a flocked swab to sample a latex surface and elute with phosphate-buffered saline[46].

QUANTITATIVE MICROBIAL RISK ASSESSMENT

Introduction of Quantitative Microbial Risk Assessment

Quantitative microbial risk assessment (QMRA) has been shown to be an effective method for estimating the potential health risks associated with exposure to pathogens in water[47]. QMRA is capable of quantifying the risk of infection associated with the pathogens of interest by directly using the concentrations of the pathogens. It is also capable of being performed indirectly by using the relationship between the pathogens of interest and a fecal indicator [47].

There are four steps in QMRA process. The first step is identifying the context, including hazardous events that might lead to exposure to pathogens[48]. The second step is calculating the exposure, which includes both quantifying pathogen concentrations in sources and the amount or volume of the source that is consumed[48]. Combining the information on the pathogen concentrations and the volume consumed, the total exposure or dose can be calculated:

$$\text{Dose} = \mu \times V$$

where μ indicates the pathogen concentration and V indicates the volume or amount of the source that is consumed[48].

The third step is modeling the dose-response relationship, which connects the exposure and the probability of infection. This step is usually considered to be the key element of the QMRA process, and several models have been developed to fit the

reported dose-response data and describe the probability of infection[49, 50]. The first model is a quantitative dose-response model, which assumes that each consumed pathogen behaves independently and the distribution of the pathogen in the environment is random and follows a Poisson distribution[48]. The outcomes of infection by each pathogen can be described as a binomial process: infection or no infection. The process requires the translation of dose (continuous values) to actual number of pathogens being consumed (discrete)[48, 51]. The probability of infection given exposure to n pathogens (P(inf | n)) can be described as:

$$P(inf \mid n)=1-(1-r)^n$$

where r is the probability of infection caused by an individual pathogen, and the probability of infection given the mean pathogen concentration (P(inf |μ)) is:

$$P(inf \mid \mu) = \sum_{n=0}^{\infty} [P(n \mid \mu) \times P(inf \mid n)]$$

where P(n | μ) is the probability of exposure to n pathogens given the mean concentration μ[48].

Beta Poisson models are one of the most common models for QMRA[49]. The Beta Poisson model is derived from an exponential model, which assumes that pathogens are distributed randomly and thus follow Poisson distribution. The Beta Poisson model, which contains two parameters α and β, overcomes the limitation that a simple exponential model does not consider the variation of pathogen

infectivity and host susceptibility[48, 49]. The model is described as:

$$P_{inf} \approx 1 - \left(1 + \frac{\mu}{\beta}\right)^{\alpha}$$

The Beta Poisson model holds only when $\beta \geqq 1$and $\beta >> \alpha$ [48, 49].

The fourth step is characterizing the risk, which integrates the previous three steps to estimate the risks in a given population[49]. The risks can be estimated in different ways, such as the probability of one or more infections every year, or the Disability Adjusted Life Years (DALYs)[48].

The simplest way of conducting QMRA is to calculate a point estimate, which is useful for identifying whether further analyses with more complicated models are necessary[48]. Although point estimation is straightforward, the actual infection risk of exposure to certain pathogens is better described by a statistical distribution due to high uncertainty and variability, which can be addressed by a Monte Carlo simulation that incorporates the parameters of uncertainty and variability in the model[52, 53].

Using QMRA to estimate NoV related health adverse outcomes

NoVs have been estimated to be the most dominant health risk in environmental water or recreational water in several states using QMRA[54-56]. McBride et al. collected agricultural stormwater in the US and measured the concentrations of NoV GII using qPCR [55]. The QMRA results showed that up to 22 out of 100 children who are exposed to undiluted agricultural stormwater will be infected with Norovirus

GII, given a water intake of between 3.75 mL and 300 mL, and median and maximum concentrations of agricultural stormwater of 100 genomic copies /mL and 2×10^4 genomic copies /mL, respectively [55]. Genthe et al. estimated that the daily probability of NoV infection ranged from 6% to 14% if consuming 1.2 liters of untreated river water per day from the sampling sites in South Africa, given median and maximum NoV concentrations of 24 NoV particles/100 mL and 830 NoV particles/100 mL, respectively [54]. Viau et al. also used QMRA to estimate a swimmers' risk of gastroenteritis due to exposure to NoVs based on the assumption that the swimmer is exposed to NoVs when swimming in the coastal area adjacent to a stream discharge, where the water is diluted to 100 times by the surrounding pathogen-free water. The results showed that the swimmers' risk of illness could be up to 0.5, given the mean amount of ingestion of 2.92 mL and the maximum GI concentration of 1440.6 genomic copies /100 mL [56].

QMRA has also been used to estimate farmers' occupational risk of NoV infection and a population's risk of NoV infection when using greywater or wastewater to irrigate crops[57-59]. Mara et al. used QMRA-Monte Carlo simulation to estimate the NoV infection risk of consuming wastewater-irrigated raw lettuce. The results showed that the *E. coli* concentration per 100 mL wastewater needed to be reduced at least 6 log units(from 10^7-10^8 to 10-100) for the NoV infection risk to fall

close to the tolerable level of 1.4×10^{-3} per person per year (pppy)[59]. Barker et al. estimated that in Melbourne, Australia, the daily probabilities of illness due to NoV exposure ranged from 6×10^{-11} to 2×10^{-4} if consuming unwashed lettuce that had been irrigated with untreated water[60]. In addition, the 95th percentile of annual probabilities of NoV infection ranged from 2×10^{-2} to 1 if consuming greywater-irrigated lettuce without washing. These results exceeded the USEPA's acceptable threshold of 10^{-4} annual probability of infection[60]. Mara et al. reported that if a farmer used wastewater to irrigate crops and involuntarily ingested 1 mg to10 mg of soil per day for 300 days a year, the estimated occupational median NoV infection risks for the farmers were 0.038, 0.32, and 0.98 pppy when the NoVs concentrations were 1-10, 10-100, and 100-1000 per 100 g soil, respectively[57].

Using QMRA to estimate the health outcomes in urban settings

Poor wastewater and sanitation infrastructure in low -income urban settings poses many health issues related to waterborne pathogens infection[61]. The risk of infection may increase due to high population density, limited access to basic services, and poor hygiene practices[61]. To understand the magnitude of the problem, Katukiza et al. investigated the level of diarrhea-causing pathogens in surface water, grey water, water sources, and soil in a typical urban slum in Sub-Saharan Africa, and used QMRA to estimate slum dwellers' risk of infection from pathogenic

microorganisms via different exposure pathways[61]. The results showed that the bacteria concentrations were up to 8×10^{6} CFU/100 mL, 2×10^{5} CFU/100 mL, 5×10^{7} CFU/100 mL for *E. coli* in greywater, *Salmonella* spp. in surface water, and total coliforms in greywater, respectively; and the viral concentrations in surface water were up to 26.5 Genomic copies /mL and 5.12 Genomic copies /mL for adenovirus and rotavirus, respectively[61]. The disease burden was estimated to be 680 DALYs per 1,000 people per year, a value that exceeds the WHO reference level of tolerable risk to human pathogens[61].

Likewise, Machdar et al. investigated *E. coli* concentrations of drinking water in a crowded, low-income urban area in Accra, Ghana, and used ratios of *E. coli* to pathogens to estimate the concentrations of *E. coli* O157:H7, *Campylobacter*, rotavirus, cryptosporidium, and ascaris in drinking water; the estimated pathogens concentrations were then used to estimate the disease burden using QMRA[62]. The results showed that the disease burden expressed in DALYs of *E. coli* O157:H7, *Campylobacter*, rotavirus, cryptosporidium, and ascaris were 3.95×10^{-1}, 8.13×10^{-2}, 2.60×10^{-2} , 2.50×10^{-5}, and 1.40×10^{-3} DALYs per person per year, respectively[62].

EPIDEMIOLOGICAL AND ENVIRONMENTAL NOV RESEARCH IN GHANA

Due to lack of access to improved water sources and sanitation, diarrheal disease is one of the leading causes of morbidity and mortality among children in Ghana[63].

WHO and UNICEF reported that about 36% of people who live in rural areas of Ghana lack access to improved drinking water sources, and 89% do not have adequate sanitation; furthermore, it was estimated that 13% of deaths in those under 5 years old were due to diarrheal disease[63].

Although epidemiological research on NoV in Ghana has not been well-established and related studies are limited, NoVs have been reported as one of the major pathogens that cause diarrhea in children, and GII NoVs have also been identified as the most dominant strains among Ghanaian children[64, 65]. Silva et al. tested 367 clinical stool samples from children between 2005 and 2006 and found that 27 samples (7.4%) were NoV-positive[65]. Among the NoV-positive samples, 22 samples belonged to GII, and GII.4 strains were the most commonly detected genotype[65]. Armah et al. also reported that NoVs were detected in 15.9% of stool samples collected from Ghanaian children with acute diarrhea, and 76.9% of NoV-positive samples were GII-positive [64].

Ghanaians' risk of viral infection increases due to increased chances of exposure to contaminated water, which results from limited wastewater treatment facilities in Ghana, leaky septic systems, and open defecation[66]. The environmental research conducted by Silverman et al. showed that 80% of river water samples collected in Accra, Ghana were NoV GII-positive[67]. Gibson et al. also detected NoVs in

groundwater, surface water, and treated drinking water samples collected in Ghana in 2009[63].

FUTURE RESEARCH

The population of many low-income urban settings like Accra is growing rapidly, but the wastewater treatment and sanitation infrastructure is often not expanding at the same rate. Additionally, open defecation may facilitate the spread of NoVs. However, the research regarding the risk of NoV exposure in such settings is still limited.

To conduct QMRA for estimating NoV-related adverse health outcomes, it is critical to thoroughly understand the NoV contamination levels in various environments. Although some studies used concentration ratios of *E. coli* to viruses to estimate the disease burden of a population, the burden may be underestimated or overestimated. In addition, the *E. coli* to viruses ratio may change over time [61]. Therefore, environmental data of NoVs are required to estimate the health risk of NoV infection. Moreover, a comprehensive understanding of potential exposure pathways is needed to estimate citizens' risk of exposure and risk of infection while carrying out a variety of daily activities.

In addition to assessing NoV contamination in the environment, it is also important to investigate the behaviors of members in the community in different scenarios associated with NoV exposure to estimate the risk of infection for those

living in low-income urban settings. Although several QMRA studies have been conducted to address the risk of NoV infection or illness, the exposure scenarios used in these studies may not reflect typical exposure scenarios in low-income urban settings like Accra[57]. In addition, people of different age groups may behave differently. For example, children may need more attention regarding NoV exposure because they not only have weaker immune systems, but also have higher water ingestion rates and inhalation rates than adults[55]. Therefore, behavioral investigation is especially important for vulnerable groups like children and elderly.

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Chapter 2: Viral Testing and Quantitative Microbial Risk Assessment

ABSTRACT

Noroviruses (NoVs) are the major cause of nonbacterial gastroenteritis worldwide. NoV genogroup I (GI) and genogroup II (GII) are the most predominant strains that cause disease among humans. NoVs are primarily transmitted via the fecal-oral route, and are commonly found in the environment. However, research related to NoV exposure pathways, contamination levels, and the corresponding risk of NoV infection is limited, particularly in low income urban settings.

In this study, environmental samples were collected from a variety of domains in Accra, Ghana, in order to investigate NoV GI and GII levels in the environment. Samples were screened for the presence of NoV GI or/and GII with an internal control to detect inhibition. NoV contamination was quantified in sample that screened positive or after dilution in samples with evidence of inhibition. The results of environmental investigation showed that no samples collected from vendors, markets, schools, or nurseries were NoV-positive; however, a high percentage (41% for NoV GI and 52% for NoV GII) of septage samples collected from public latrines were NoV-positive. In addition, 5% to 15% of samples collected from public drains, flooded areas, irrigation, and farms were also NoV-positive.

The data on NoV GI and GII concentrations in irrigation and farm water samples were used to conduct Quantitative Microbial Risk Assessment (QMRA) to investigate the potential risk of NoV infection among farmers who

unintentionally ingest NoV contaminated water while irrigating. The QMRA results showed that the estimated average probabilities of NoV GI and GII infection for farmers who work for seven days in the field are 0.28 and 0.42, respectively.

INTRODUCTION

Noroviruses (NoVs), which are classified into six genogroups (GI to GVI), are the major cause of nonbacterial gastroenteritis disease worldwide [1, 2]. NoVs GI and GII are the most predominant strains that cause acute gastroenteritis disease in humans [3]. Mead et al. estimated that the incidence of NoV is 23 million infections per year, and a review reported that NoVs are responsible for 47% to 96% of gastroenteritis outbreaks in Europe, the U.S., Japan and Australia[1, 4]. NoVs are capable of infecting persons of all age groups; they are able to infect not only school-aged children and adults, but some studies have shown that NoVs are one of the major causes of hospitalization among infants and young children[1]. The symptoms of NoV infection include vomiting, watery diarrhea, and nausea[5]; some patients also develop symptoms of abdominal pain and fever[6].

NoVs are primarily transmitted via the fecal-oral route[1]. Outbreaks are often associated with exposure to contaminated food, water, or surfaces contaminated with fecal matter[1, 7]; in addition, person-to-person transmission is an important route of NoV transmission[7]. Transmission is facilitated by the stability of NoV in various environments, its resistance to inactivation, the low infectious dose, and rapid genetic mutation[8]. Lack of long-term immunity and prolonged shedding of viral particles by asymptomatic or immune-compromised hosts also facilitate the transmission of NoVs[8]. Outbreaks are commonly associated with NoV-infected food-handlers[9].

NoVs are ubiquitous in the environment; they have been detected in surface water[10-12], wastewater[13-15], ground water[16], recreational

water[5, 17], and even drinking water[18]. NoVs have also been commonly found in food products[9], such as ready-to-eat foods[19], seafood[20], and produce[21]. Mathijs et al. argued in a review that food products may be contaminated through irrigation with NoV-contaminated water, contact with contaminated manure, or processing by food-handlers who are shedding NoV particles[9]. In addition to water and food, environmental swabbing has detected NoVs in a variety of settings, including health care facilities[22-24], catering companies[25, 26], and cruise ships[26]

The genetic diversity of NoVs in the environment has been reported in both temperate areas and tropical regions[10]. A study conducted in Singapore showed that NoV GI strains were detected in 27 (63%) out of 43 NoV-positive water samples, and GII strains were detected in 39 (91%) out of 43 NoV-positive water samples[10]. Fernandez et al. also sequenced 33 NoV-positive river water samples collected in Argentina and found that all samples belonged to NoV GII[11]. Similarly, a study in Korea showed that 64% of NoV-positive groundwater samples belonged to NoV GII, whereas only 36% belonged to NoV GI[16].

Quantitative Microbial Risk Assessment (QMRA) is a tool that combines the characteristics of pathogens and hosts, quantitative exposure data to the pathogens, and mathematical models to estimate the risk of adverse health effects. Usually, there are four steps to implementing QMRA, including: 1) defining the pathogens and the conditions that need to be investigated; 2) obtaining the quantitative exposure data to the pathogens in the conditions defined in the previous step; 3) linking exposure data to the probability of adverse health outcomes via dose-response models; and 4) integrating the

information of exposure and dose-response assessment to estimate the risk of outcomes of interest[27].

QMRA has been used to estimate the risk of NoV infection in different scenarios, including swimming in contaminated water, inadvertently ingesting untreated river water, and children exposed to undiluted agricultural water[28-30]. In addition, the general population's risk of NoV infection from consuming greywater-irrigated crops and farmers' risk of infection from exposure to NoVs while irrigating crops with wasterwater were also estimated using QMRA[31-34]. Although the use of QMRA to estimate the risk of NoV infection is not comprehensive, all the studies to date have reported a high risk of NoV infection.

Accra, the capital of Ghana, consists of 201 km^2 of land and the total population is approximately 1.7 million[35]. It is the most populated and fast-growing city in Africa; however, the sanitation conditions in Accra are substandard [35]. It is estimated that the city generates up to 1,800 tons of waste daily, but the municipal waste system is only capable of collecting 1,200 tons per day[35]. Furthermore, the city's water sources are contaminated due to the limited functioning of the city's wastewater treatment infrastructure, leaky septic systems, and open defecation[36].

Accra residents may have a high risk of exposure to NoV-contaminated environments and, thus, are likely to be infected by NoVs. The environmental studies related to NoV and the risk of NoV infection in Accra are limited. In addition, because NoV exposure pathways are complicated, it is important to recognize the prevalence and concentration of NoVs in different domains to identify the potential pathways of exposure and measure the risk of NoV

infection in Accra.

The objectives of this study are to investigate the NoV prevalence and concentrations in variety of environments in Accra and to use QMRA to estimate the occupational risk of NoV infection among farmers who unintentionally ingest NoV contaminated irrigation water.

METHODS

Environmental samples were collected from multiple domains from four low-income neighborhoods in Accra, Ghana to investigate the predominant routes of NoV transmission. These domains included markets and street vendors, households, public latrines, schools, nurseries, public drains, oceans and beaches. In addition, flooding samples were collected from flooded areas where human activities were occurring to identify the NoV levels.

Irrigation samples were also collected to detect the NoV contamination levels because wastewater is usually the primary source of irrigation in Accra due to limited wastewater treatment infrastructure[36]. Through this pathway, the farmers' occupational risk of NoV infection may increase while using untreated water to irrigate crops and incidentally make contact with NoVs. In addition, consumers' risks of infection may also increase if they eat contaminated raw produce without washing it.

Sample collection and pretreatment

In total 1,506 samples, which included 40 septage samples, 376 particulate samples, 40 large volume environmental water samples, 244 drinking water samples, 197 small volume environmental water samples, 336 food samples, and 273 swab samples, were collected for NoV detection. The

sample collection and pretreatment procedures for each sample type are described below.

Septage samples

Septage samples were collected from the holding tank of public latrines. The samples were used for RNA extraction directly without any concentration or pretreatment steps.

Particulate samples

Particulate samples (soil, sand, or sediment) were collected from the grounds surrounding households, public latrines, beaches, schools, nurseries, farms and other public settings. A sterile spatula was used to collect soil, sand or sediment to a depth of 5 cm, and the sample was transferred to a sterile 250 mL Whirl-Pak bag. The sample was transported to the lab within 6 hours and stored at 4°C until further pretreatment procedures.

Viruses were eluted from the particulate samples by suspending 10g sample in 20 mL of sterile phosphate buffer saline (PBS), adjusting the pH to 9.0, and vigorously shaking the suspension for 30 minutes at room temperature. The particulates were allowed to settle for 15 minutes and the clarified supernatant was collected for testing. Viruses were concentrated by adding 12% polyethylene glycol (PEG) to 1 ml of the supernatant. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 3 minutes and resuspended in lysis buffer for RNA extraction.

Food samples

Food samples, which included produce and prepared food, were collected from markets, street vendors, households, a school, and farms. The sampling

worker was asked to open a 1-liter or 3-liter Whirl-Pak bag and let the vendor or caretaker of the household or the school put the produce or unpackaged food into the bag. If the food was packaged, the whole package was put directly into the Whirl-Pak bag. The food sample was then transport to the lab within 6 hours. The weight and the volume were recorded, and the sample was stored at 4°C refrigerator until further pretreatment procedures.

Each produce sample was incubated in 500 mL of sterile PBS with 0.04% Tween-80 (PBST) for 10 minutes. The sample was then vigorously shaken for 30 seconds, the surface was gently massaged for 60 seconds, and then shaken again for 30 seconds. After removing the produce, 160 mL of the PBST solution was further concentrated by PEG precipitation by the 12% PEG 8000, 1% bovine serum albumin (BSA) and 0.9M NaCl. The pH of the solution was adjusted to 7.0-7.5 and incubated overnight at 4°C. After incubation, the sample was centrifuged at 4,800 rpm for 60 minutes at 4°C. The resulting pellet was resuspended in 20ml PBST. Viruses were concentrated for testing by adding 12% polyethylene glycol (PEG) to 1 ml of the primary concentrate. After incubating overnight at 4C, the viruses were pelleted by centrifugation at 13,000 rpm for 2 min and resuspended in lysis buffer for RNA extraction.

Viruses were eluted from the prepared food samples by suspending 10g sample in 20 mL of sterile phosphate buffer saline (PBS), adjusting the pH to 9.0, and vigorously shaking the suspension for 30 minutes at room temperature. The particulates were allowed to settle for 15 minutes and the clarified supernatant was collected for testing. Viruses were concentrated by adding 12% PEG to 1 mL of the supernatant. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 3 minutes

and resuspended in lysis buffer for RNA extraction.

Swab samples

EnviroMax Plus Sterile Environmental Sampling Swabs (Puritan Medical) pre-wetted with 50 μL of sterile-filtered 0.5 mm dihydrogen sulfate (H_2SO_4) buffer were used to collect swab samples. A framing square was used to sample XX cm^2 by gently swabbing the surface, flipping the swab over halfway through. The sample was transported to the lab within 6 hours and stored at 4°C refrigerator until further pretreatment procedures.

Each swab was consecutively eluted with two 4mL volumes of PBST. Each elute volume was vortexed for 30 seconds, incubated for 5 minutes at RT, vortexed again, before removing the solution. The elutes were combined for a total volume of ~6 mL. Viruses were concentrated for testing by adding 12% PEG to 1 mL of the primary elute. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 3 minutes and resuspended in lysis buffer for RNA extraction.

Small-volume drinking water samples

If the participant stored sachet water to drink, the sampling worker was asked to open a 1-liter Whirl-Pak bag and ask the participant to put the sachet into the Whirl-Pak bag. If the participant stored water in a container, the participant was asked to fill the 500 mL Whirl-Pak bag from the stored water reservoir in the same way that they normally access the water for drinking. The water samples were placed in a cooler and transported to the lab within 6 hours. The samples were stored at 4ºC until further pretreatment procedures.

For each small-volume water sample, the viruses in 160 mL of water

were concentrated by PEG precipitation (final concentration:12% PEG 8000, 1% bovine serum albumin (BSA) and 0.9M NaCl). The pH of the solution was adjusted to 7.0-7.5 and the sample was incubated overnight at 4°C. After incubation, the sample was centrifuged at 4,800 rpm for 60 minutes at 4°C. The resulting pellet was resuspended in 20ml PBST. Viruses were concentrated for testing by adding 12%PEG to 1 mL of the primary concentrate. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 2 min and resuspended in lysis buffer for RNA extraction.

Small-volume environmental water samples

A sterile sample collection device was used to collect environmental water samples without collecting sediment or trash in the water. The water sample was transferred to a 500 mL Whirl-Pak bag. The water sample was placed in a cooler and transported to the lab within 6 hours. Samples were stored at 4°C refrigerator for further pretreatment procedures.

Viruses were concentrated for testing by adding 12%PEG to 1 mL of the raw water sample. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 2 min and resuspended in lysis buffer for RNA extraction.

Large-volume water samples

Sterile 20 liter containers were used for collecting large-volume water samples. For collecting each large-volume piped water sample, the tap was flushed for about 30 seconds prior to sample collection. The water sample was transported to the lab within 6 hours and was stored at 4ºC until further

pretreatment procedures.

For collecting each ocean water sample, the sampling worker was asked to collect ocean water where the water surface was above the knee. The bucket was submerged and filled to the top without catching too much sand. The bucket was placed on the beach with a clean lid on it and settled for 5 minutes. The settled ocean water was then carefully decanted into a clean 20 liter container. The water sample was stored in a cooler, transported to the lab within 6 hours and stored at 4ºC.

Two large-volume piped water samples and 38 ocean water samples were processed by an ultrafiltration approach that is described in detail elsewhere [37]. The viruses in the elute were concentrated for testing by adding 12% polyethylene glycol (PEG) to 1 ml of the primary concentrate. After incubating overnight at 4°C, the viruses were pelleted by centrifugation at 13,000 rpm for 2 min and resuspended in lysis buffer for RNA extraction.

RNA extraction and detection

In total, 1,241 samples were analyzed for NoV GI detection, and 1,210 samples were analyzed for NoV GII detection. QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) was used for RNA extraction, which was performed according to the manufacturer's instructions. The final elution volume of all samples was 50 μL. The extracted RNA samples were stored at −20°C until they were processed with RT-PCR.

QuantiFast Pathogen RT-PCR +IC Kit (Qiagen, Valencia, CA) was used to screen for the presence of NoVs in each sample. This kit contains a universal internal control which was used to monitor for reaction inhibitors in the samples. The RT-PCR mix in each tube consisted of 1x QuantiFast

Pathogen Master Mix, 10 μM forward primer, 10 μM reverse primer, 5 μM probe, 1x Internal Control Assay, 1x internal control RNA, 1x QuantiFast Pathogen RT Mix, and 5.0 μL of extracted RNA sample. The genogroup-specific COG1 and COG2 primer and probe sets described by Kageyama et al. were used to detect NoVs GI and GII, respectively[38]. The RT-qPCR program was performed under the following conditions: 50°C for 20 minutes and 95°C for 5 minutes followed by 45 cycles of 95°C for 15 seconds, and then 60°C for 30 seconds. Two negative controls and two positive controls were run with each batch.

Samples that were positive in the screening assay described above were further quantified using OneStep PCR Kit (Qiagen, Valencia, CA). For quantifying NoV GI and GII, the RT-PCR mix in each tube consisted 1x Qiagen OneStep RT-PCR Buffer, 10 mM deoxynucleoside triphosphate (dNTP) mix, 10 μM forward primer, 10 μM reverse primer, 10 μM probe, 1x Qiagen OneStep RT-PCR Enzyme Mix, and 5.0 μL of extracted RNA sample. The COG1 and COG2 primer and probe sets described by Kageyama et al. were used to detect NoVs GI and GII, respectively[38]. RT-qPCR program was performed under the following conditions: 50°C for 32 minutes and 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, and then 56°C for one minute. Viral copy numbers were estimated from standard curves generated using in vitro-transcribed RNA samples of known concentrations[39]. Two negative controls were run with each batch.

Data analysis

Decision Scheme for screening RT-PCR

The process of determining if the sample is NoV GI positive and/or GII positive is presented as Figure 1. The sample was determined to be presumptive positive if the results of the QuantiFast assay showed a clear amplification curve in either duplicate. The sample was determined to be presumptive inhibition positive if the internal control did not amplify or the mean Ct value of internal control was at least 5 Ct greater than the negative controls. The sample was determined to be negative if there was no clear amplification curve in either duplicate.

Presumptive positive and presumptive inhibition positive samples were quantified with the OneStep RT-PCR kit. Each sample was run in duplicate. Presumptive inhibition positive samples were diluted 1:5 prior to OneStep analysis. Samples were determined to be positive or inhibition positive sample if at least one of the duplicated OneStep run was positive; otherwise the sample would be determined to be negative or inhibition negative.

Analyzing the concentrations of the positive samples

The samples processed with OneStep were quantified based on the following rules (figure 1). If both duplicates had a Ct value <41 and the difference of the two Ct values was smaller than 4, the quantification was then based on averaging the results of the two duplicates. If one of the duplicates had a Ct value ≧41 and the other did not have clear amplification, the quantity of NoVs in the sample was calculated by averaging the Ct value and 0.5. If one of the duplicates had a Ct value <41 and the other duplicate did not have

clear amplification, or if both duplicates had a Ct value <41 but the difference of the two Ct values was larger than 4, the sample was determined to be not quantifiable.

QMRA method

The NoV GI and GII concentration data in irrigation water and water collected from farms were used to estimate the risks of NoV GI and GII infection for farmers who unintentionally ingest NoVs in contaminated irrigation water. The effects of immunity and secondary transmission were not considered in this analysis [30]. Monte Carlo simulation (n = 10,000) was used to estimate the uncertainties associated with: 1) NoV GI and GII concentrations and 2) volume of water unintentionally ingested.

The amount of wastewater accidentally ingested by farmers in Accra was estimated to be uniformly distributed from 1.0 to 5.0 mL per day (82). This scenario was based on the assumptions that the farmers' farming activities in Accra are labor intensive, and that they generally do not wear any protective boots, mouth covers, or gloves, and that they are in direct contact with contaminated irrigation water (82).

The distributions of 10-base logarithm of NoV GI and GII concentrations were assumed to be normally distributed. Data on NoV GI and GII in irrigation or farm water samples were used to estimate (log) concentrations. This was done by fitting the probability of density function of four distributions: 1) data of sample concentrations of positive and quantifiable samples on OneStep, 2) the frequency of samples that were negative on QuantiFast, 3) the frequency of samples that were positive using QuantiFast but not on OneStep, and 4) the frequency of the number of samples that were

positive but not quantifiable using OneStep. The four distributions above were combined with the same maximum likelihood parameters (means and standard deviations of the log-transformed concentrations) to fit a lognormal distribution for the concentrations of NoV GI and NoV GII. The maximum likelihood parameters of the four distributions above were obtained by calculating the minimum deviance using R. The R codes of conducting QMRA are attached in the appendix.

Because the limits of detection of small environmental water samples for the OneStep and QuantiFast assays were not tested, theoretical limits of detection were used for constructing the distributions of concentrations. The limits of detection of NoV GI and GII on QuantiFast were assumed to be 10 and 100 genomic copies per 100 mL water, respectively; and the limits of detection of NoV GI and GII on OneStep were assumed to be 100 and 1000 genomic copies per 100 mL.

The probability of infection with NoV given a certain (discrete) dose is given by the Beta Binomial relation:

$$P_{inf} = 1 - (\frac{\Gamma(\alpha+\beta)\Gamma(\beta+n)}{\Gamma(\beta)\Gamma(\alpha+\beta+n)}$$

where both α and β are the parameters defining infectivity. In a challenge study with Norwalk virus (GI,1) in human volunteers these parameters were estimated at α =0.04 and β=0.055(48); n is the number of NoV genomes ingested, which is assumed to be a Poisson sample, with parameter (mean dose) calculated by multiplying the concentration and the amount of water ingested during one exposure event.

A farmer's probability of NoV GI or GII infection after working for 7 days was determined as:

$$P = 1 - \prod_{i=1}^{7}(1 - P_i)$$

where P_i is the probability of infection per exposure event.

RESULTS

Number and concentrations of positive samples from different domains

Following the decision scheme describing in figure 1, 42 (3.3%) samples were determined to be NoV GI positive, and 58 (4.6%) samples were NoV GII positive.

The samples collected from several domains, which included markets, street vendors, schools, and nurseries, were all NoV-negative, regardless of sample types. All food samples collected from markets or street vendors were NoV-negative (table 1). Similarly, the all particulate samples, drinking water samples, and swab samples collected from schools and nurseries were also NoV-negative, and the only food sample obtained from the school was also NoV-negative (table 4 and table 7).

From households, particulate samples, drinking water samples, one food sample, and swab samples were collected (table 2). However, none of the samples were NoV GI positive. Nevertheless, one particulate sample, which the soil was collected within 3 meters of drains and trash, and one swab sample, which was swabbed from tomato paste can in a household, were NoV GII positive. The concentrations of the particulate sample, and the swab sample were 7.5×10^5 per gram of particulate and 4.2×10^5 per swab, respectively (table 10).

High percentage of samples collected from public latrines was NoV-positive, compared with the samples collected from other domains (table 3). Sixteen (41%) and 18 (42%) of septage sample collected from public

latrines were NoV GI and NoV GII positive, respectively (table 3); ten of GI positive septage samples and 15 of GII positive septage samples were also quantifiable, which the mean GI and GII concentrations were 3.8×10^6 per gram (SD = 6.7×10^6) and 1.5×10^6 per gram (SD = 2.2×10^6), respectively (table 10). One swab sample collected from anal cleansing container in a public latrine was NoV GI positive and also quantifiable, which the concentration was 8.6×10^5 per swab.

Sea water samples and particulate samples were collected from ocean and beach areas (table 5). Two water samples collected from oceans were NoV GII positive (table 5), and the 2 samples were also quantifiable, which the concentrations were 5.0 per 100 mL water and 8.1 per 100 mL water (table 10). However, none of the samples collected from oceans or beaches were NoV GI positive.

Both NoV GI and GII were detected in particulate samples and water samples collected from flood, public drains, or other public domains (table 6). Eleven (9%) and 17 (15%) particulate samples were NoV GI and GII positive, respectively. One particulate samples taken from public domains (concentration = 5.0×10^5 per gram sample) and 8 samples collected from public drains (mean concentration = 6.1×10^5 per gram particulate sample, SD = 9.7×10^5) were also NoV GI quantifiable (table 9); one sample taken from public domains (concentration = 7.4×10^5 per gram sample), 13 samples collected from public drains (mean concentration = 4.2×10^5 per gram sample, SD = 4.8×10^5), and 2 flood particulate samples (concentration = 4.8×10^5 and 9.48×10^5 per gram sample) were NoV GII quantifiable (table 10). Four environmental water samples collected from public drains were NoV GI

positive, and all 4 samples were also quantifiable, with a mean concentration of 5.4×10^4 per 100 mL water sample (SD = 4.4×10^4) (table 6 and table 9). One environmental water sample collected from a public drain and 3 flood water samples were NoV GII positive and also quantifiable (table 6 and table 9); the concentration of the water sample collected from the public drain was 4.2×10^4 per 100 mL water sample, and the mean concentration of the flood samples was 1.2×10^5 per 100 mL water sample (SD = 1.1×10^5).

Although only two particulate samples were NoV GII positive and none of the food samples were NoV-positive, several irrigation water samples and water samples collected from farms were NoV-positive (table 8). Ten (11%) and 13 (15%) of the irrigation water samples or environmental water samples collected from farms were NoV GI and GII positive, respectively (table 8). Among the 10 NoV GI positive samples, one irrigation water sample and 6 water samples collected from farms were quantifiable, and the NoV GI concentration of the irrigation water sample was 1.6×10^5 per 100 mL water sample, and the mean GI concentration of the 6 samples collected from farms was 1.2×10^5 per 100 mL water sample (SD = 1.1×10^5) (table 9). Thirteen NoV GII positive environmental water samples collected from farms were also GII quantifiable, with a mean concentration of 2.9×10^5 per 100 mL water sample (SD = 8.3×10^5) (table 10). Two particulate samples collected from farms were NoV GII positive, with GII concentrations of 2.3×10^5 and 2.5×10^5 per gram sample (table 8 and table 10).

QMRA analysis of occupational risk of NoV infection among farmers

Distribution of NoV GI and GII log concentrations

The results of fitting the NoV GI and GII data for irrigation water

samples and water samples collected from farms are presented in Figure 2. The maximum likelihood estimates of the mean of the log NoV GI concentration was -4.81 with an SD of 5.79, and the maximum likelihood estimate of the mean of the log NoV GII concentration was -2.02 with SD of 4.25.

The estimated risks of NoV infection among farmers

Farmers' estimated probability of NoV GI infection following a single exposure event ranges from 0 to 0.53, and the mean probability of infection is 0.05. Farmers' mean probability of NoV GII infection after a single exposure event is 0.09, which is higher than that of NoV GI; and the minimum and maximum probability of GII infection is 0 and 1, respectively. Although the probability of NoV infection is zero for most of the exposure events, the model shows that the probability would increase to at least 0.42 if the farmer accidentally ingests contaminated irrigation water.

Figures 3 and 4 show the distribution of the estimated 7-day probability of NoV GI and GII infection for farmers who unintentionally ingest contaminated water while irrigating crops assuming daily irrigation. These estimates were obtained by using Monte Carlo simulation. The estimated mean, minimum, and maximum probability of NoV GI infection is 0.28, 0, and 0.96, respectively. The mean probability of NoV GII infection is 0.42, which is higher than that of NoV GI; and the minimum and maximum probability of GII infection is 0 and 1, respectively.

DISCUSSION

The environmental NoV investigation in this study showed that no samples collected from markets, street vendors, schools, and nurseries were NoV-positive (Tables 1, 4, and 7), and only one particulate sample collected from households and two ocean water samples were NoV GII positive (Tables 2 and 5). Nevertheless, a high percentage (41% for NoV GI and 52% for NoV GII, table 3) of septage samples and one swab sample collected in public latrines were NoV-positive, and 11% and 15% of environmental water samples collected from irrigation and farms were NoV GI and GII positive, respectively (Table 8). In addition, 9% and 5% of particulate samples and environmental water samples collected from public drains and flooding areas were NoV GI positive, respectively; and 15% and 7% of particulate samples and environmental water samples collected from these areas were NoV GII positive, respectively.

Although one food sample was NoV GI positive by the QuantiFast assay (data not shown), the food samples collected in this study were all determined to be NoV GI negative using the decision scheme, and the results were quite different from the results of previous studies [21, 40]. Thirty-nine food samples were inhibited for NoV GI, but none of these samples were tested positive in OneStep. The possible explanations for no positive samples include there were no NoVs in these food samples, NoV concentrations of the food samples were too low to be detected in OneStep, or many NoVs were lost during sample processing. However, due to the lack of recovery data of the procedures of food samples processing, it is difficult to estimate the impact of recovery of food sample processing procedures on NoV detection. In addition,

the step of RNA purification may reduce the ability of accurately detecting NoV-positive samples if the NoV concentrations in food samples were low. Stals et al. tested the successful detection rates of NoV inoculated salad to test the influence of low viral inoculums level on recovery, and found that the successful detection rates were only 0/6 to 3/6 for GI inoculated at 4.02×10^3 genomic copies per 10 g of penne salad and 0/6 to 1/6 for GII inoculation at 4.91×10^4 genomic copies per 10 g of penne salad [41]. Nevertheless, the health threat of NoV infection via ingesting contaminated food cannot be ruled out because the infectious dose of NoV is very low [42].

This study showed that a high percentage of septage samples collected in public latrines were NoV-positive and the finding indicated that public latrines may be an important exposure pathway for Ghanaians. Baker and Bloomfield found that *Salmonella* spp. were capable of spreading from toilets to surfaces and then to hands[43]. Since the NoVs are stable in the environment, the NoV transmission may increase through this exposure pathway.

Five percent and 7% of water samples collected from public domains, which included flooding areas and public drains, were NoV GI and GII positive, respectively. The fractions of the NoV-positive water samples were lower than the findings of other studies. Ngaosuwankul et al. reported that 14% of floodwater samples collected in Thailand were NoV-positive. Each water sample of the study was concentrated from 400 mL, compared to the sample volume of the present study (1 mL), and this increased sensitivity likely explains the difference in detection [44]. However, de Man et al. collected 1 to 5 mL of water samples and directly extracted RNA, methods similar to those used in this study. The study showed that 12% and 24% of

water samples collected in flooding areas or from overflowing storm sewers in the Netherlands were NoV GI and GII positive, respectively [15]. Nevertheless, the authors reported that the flooding water samples were quite muddy and thus might explain the higher fractions of the NoV-positive samples than those of the current study[15], which the fractions of NoV-positive particulate samples (9% were NoV GI positive and 15% were GII positive) collected in flooding areas, public drains, and public domains were higher than the water samples.

For farmers who work for seven days in the field, the estimated average probabilities of NoV GI and GII infection are 0.28 and 0.42, respectively. As shown in Figures 3 and 4, the first peak of NoV GI infection is when a farmer is exposed to around 1-5 viruses for one day during the seven days; the second peak is when he is exposed to 1-5 viruses for two days; and the third peak is when he is exposed to 1-5 viruses for three days. The results imply that the farmer would be sick even if he is exposed to only a few viral particles for fewer than three days during seven days of work. In addition, although a farmers' seven-day probability of NoV infection is 0 for most of the simulation results, once he/she is exposed to NoV particles for one day, his/hers seven-day probability of NoV infection would drastically increase to at least 0.42. The results indicate that although the prevalence of NoVs in irrigation water or water collected from farms may be low, the corresponding risk of NoV infection among farmers may still be considerable.

The QMRA estimated results have several limitations because many assumptions have been made to construct the QMRA to estimate the risk of NoV infection among farmers in Accra. First, because the number of genomic

copies was used instead of actual viral particles ingested when calculating the probability of infection, the true probability of NoV infection may be overestimated since the genome detected may not be infectious. However, the dose-response relationship that was referred to in this study used RT-PCR to quantify the concentrations of virus suspension in the human challenge study, and therefore, the risk estimates may still be unbiased given the assumption that the ratio of total to infectious numbers of NoVs is constant and the use of RT-PCR to detect environmental NoV[45].

The second limitation is that the theoretical limits of detection of NoV GI and GII in the QuantiFast and OneStep assays -- which were used to incorporate the NoV quantitative data and presence/absence data in irrigation water samples and water samples collected from farms into the same distribution -- may be lower than the actual values. If the true limits of detection were five-times the theoretical values, the seven-day mean probability of NoV GI infection would increase from 0.28 to 0.35, and the seven-day mean probability of NoV GII infection would increase from 0.42 to 0.43. These results indicate that the real risk of NoV infection may be even higher than what were estimated in this study.

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FIGURES

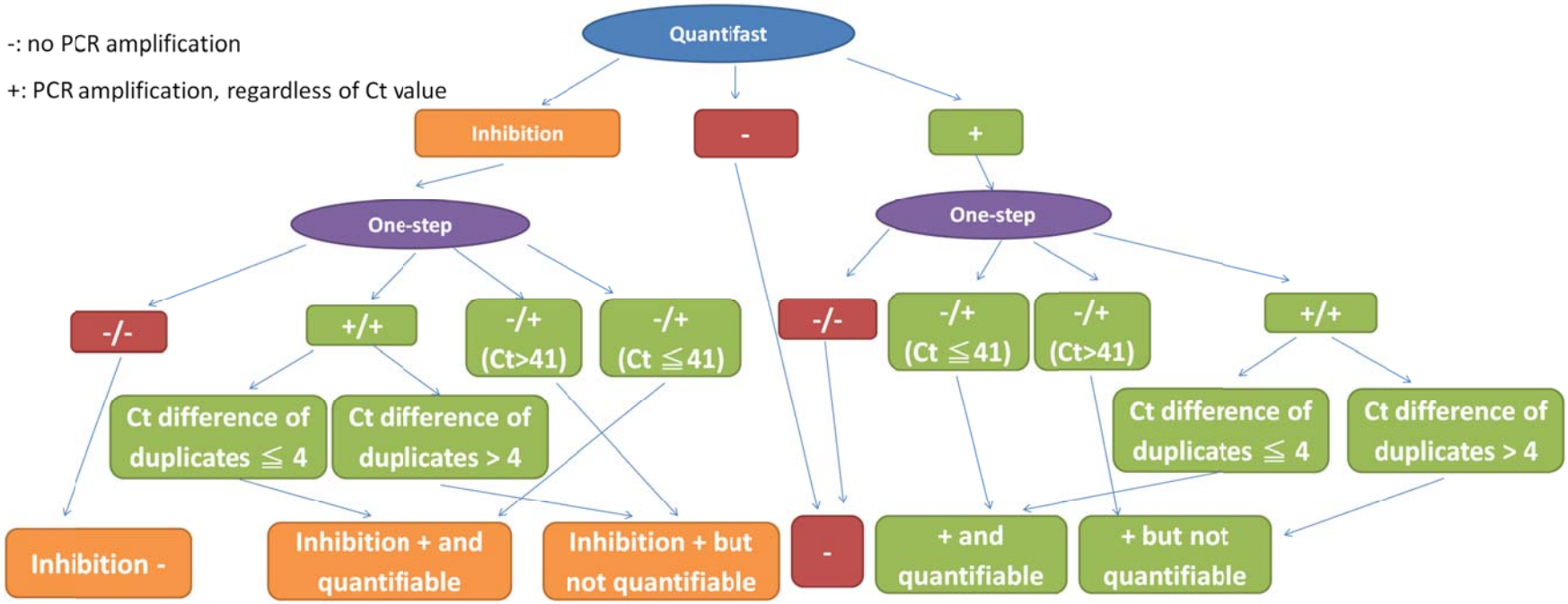

Figure 1. The decision scheme of determining whether the sample was positive and/or quantifiable

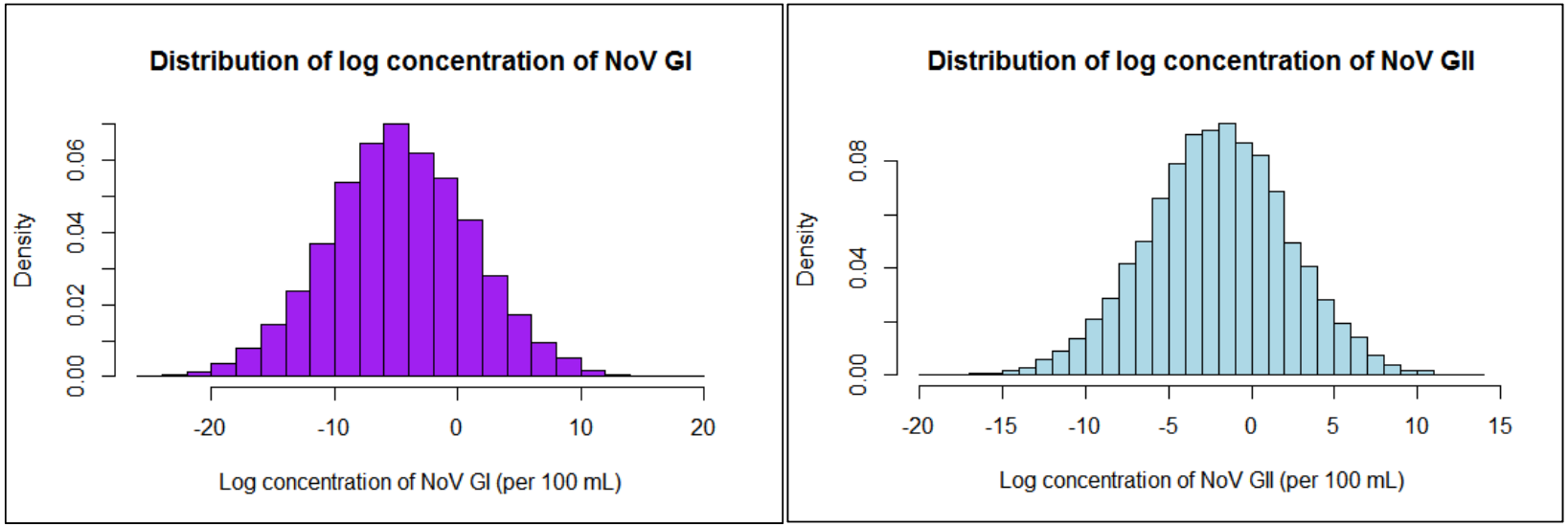

Figure 2. The distribution of log NoV GI concentration (left) and log NoV GII concentration (right)

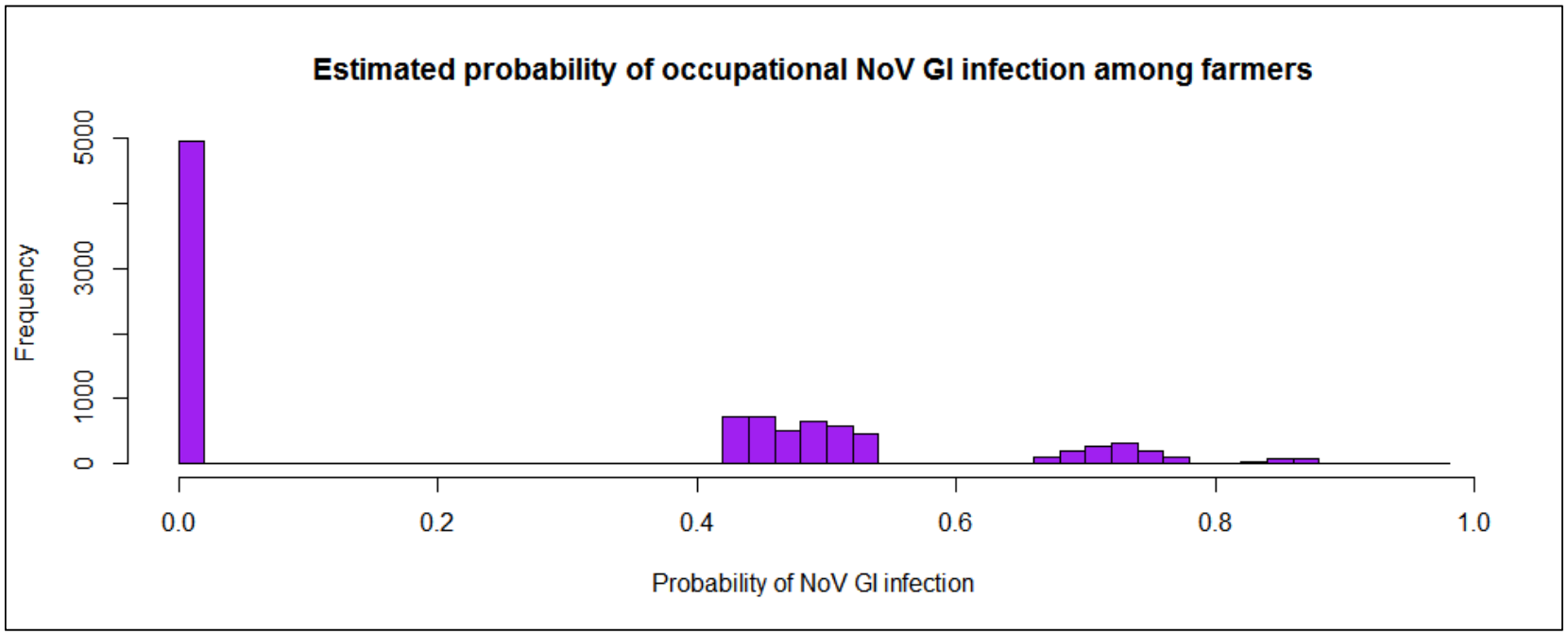

Figure 3. Estimated seven-day probability of NoV GI infection among farmers who unintentionally ingest contaminated water

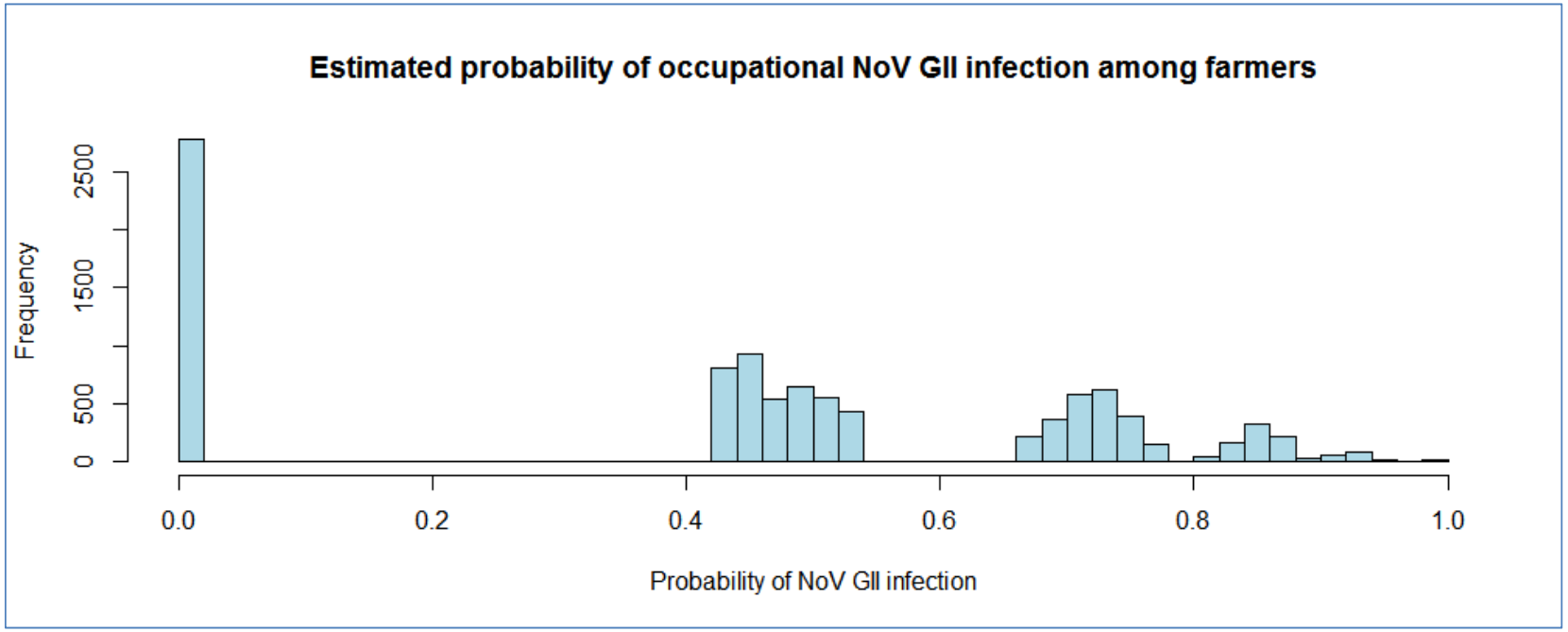

Figure 4. Estimated seven-day probability of NoV GII infection among farmers who unintentionally ingest contaminated water

TABLES

Table 1. NoV detection results for food samples collected from markets and street vendors

Final decision	Number of NoV GI samples (%) (n=189)	Number of NoV GII samples (%) (n=189)
Positive	0 (0)	0 (0)
Negative	179 (95)	182 (96)
Inhibition Positive	0 (0)	0 (0)
Inhibition Negative	10 (5)	7 (4)

Table 2. NoV detection results for samples collected from households

Final decision	Number of NoV GI samples (%)				Number of NoV GII samples (%)			
	Particulate (n=50)	Drinking water (n=114)	Food (n=4)	Swabs (n=111)	Particulate (n=50)	Drinking water (n=122)	Food (n=4)	Swabs (n=105)
Positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Negative	29 (58)	112 (98)	4 (100)	111 (100)	29 (58)	117 (96)	4 (100)	102 (97)
Inhibition Positive	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
Inhibition Negative	21 (42)	2 (2)	0 (0)	0 (0)	20 (40)	5 (4)	0 (0)	2 (0)

Table 3. NoV detection results for samples collected from public latrines

Final decision	Number of NoV GI samples (%)			Number of NoV GII samples (%)		
	Septage (n=40)	Particulate (n=11)	Swabs (n=64)	Septage (n=35)	Particulate (n=11)	Swabs (n=62)
Positive	15 (38)	0 (0)	1 (2)	16 (46)	0 (0)	0 (0)
Negative	20 (50)	9 (82)	50 (78)	17 (49)	5 (45)	53(85)
Inhibition Positive	1(3)	0 (0)	0 (0)	2 (6)	0 (0)	0 (0)
Inhibition Negative	4 (10)	2 (18)	13 (20)	0 (0)	6 (55)	9 (15)

Table 4. NoV detection results for samples collected from schools

Final decision	Number of NoV GI samples (%)				Number of NoV GII samples (%)			
	Particulate (n=8)	Drinking Water (n=35)	Food (n=1)	Swabs (n=38)	Particulate (n=8)	Drinking Water (n=35)	Food (n=1)	Swabs (n=38)
Positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Negative	7 (88)	34 (97)	1 (100)	37 (97)	5 (63)	34 (97)	1 (100)	37 (97)
Inhibition Positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Inhibition Negative	1 (13)	1 (3)	0 (0)	1 (3)	3 (38)	1 (3)	0 (0)	1 (3)

Table 5. NoV detection results for samples collected from oceans and beaches

Final decision	Number of NoV GI samples (%)		Number of NoV GII samples (%)	
	Particulate (n=32)	Ocean Water (n=29)	Particulate (n=32)	Ocean Water (n=31)
Positive	0 (0)	0 (0)	0 (0)	0 (0)
Negative	29 (91)	20 (69)	29 (91)	23 (74)
Inhibition Positive	0 (0)	0 (0)	0 (0)	2 (6)
Inhibition Negative	3 (9)	9 (31)	3 (9)	6 (19)

Table 6. NoV detection results for samples collected from public domains, flood, or public drains

Final decision	Number of NoV GI samples (%)		Number of NoV GII samples (%)	
	Particulate (n=118)	Environmental water (n=87)	Particulate (n=117)	Environmental water (n=58)
Positive	10 (8)	4 (5)	9 (8)	0 (0)
Negative	65 (55)	77 (89)	62 (53)	50 (86)
Inhibition Positive	1 (1)	0 (0)	8 (7)	4 (7)
Inhibition Negative	42 36)	6 (7)	38 (32)	4 (7)

Table 7. NoV detection results for samples collected from nurseries

Final decision	Number of NoV GI samples (%)			Number of NoV GII samples (%)		
	Particulate (n=6)	Drinking water (n=30)	Swabs (n=29)	Particulate (n=6)	Drinking water (n=30)	Swabs (n=29)
Positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Negative	3 (50)	28 (93)	28 (97)	4 (67)	29 (97)	29 (100)
Inhibition Positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Inhibition Negative	3 (50)	2 (7)	1 (3)	2 (33)	1 (3)	0 (0)

Table 8. NoV detection results for samples collected from farms and irrigation water

Final decision	Number of NoV GI samples (%)			Number of NoV GII samples (%)		
	Particulate (n=81)	Environmental Water (n=82)	Food (n=77)	Particulate (n=83)	Environmental Water (n=82)	Food (n=77)
Positive	0 (0)	9 (10)	0 (0)	0 (0)	9 (10)	0 (0)
Negative	17 (21)	67 (77)	48 (62)	21 (25)	66 (76)	48 (62)
Inhibition Positive	0 (0)	1 (1)	0 (0)	2 (2)	4 (5)	0 (0)
Inhibition Negative	64 (79)	10 (11)	29 (38)	60 (72)	8 (9)	29 (38)

Table 9. Summary of NoV GI concentrations

Sample type	Septage (per gram)	Particulate (per gram)		Environmental water (per 100 mL)			Swab (per swab)
Environment of sampling	Public latrine (n=10)	Public drain (n=8)	Public domain (n=1)	Irrigation (n=1)	Public drain (n=4)	Farm (n=6)	Public latrine (n=1)
Concentration range	7.8×10^4 to 2.2×10^7	5.4×10^4 to 3.0×10^6	5.0×10^5	1.6×10^5	6.5×10^3 to 1.1×10^5	1.2×10^4 to 3.1×10^5	8.6×10^5
Mean (SD)	3.8×10^6 (6.7×10^6)	6.1×10^5 (9.7×10^5)	-	-	5.3×10^4 (4.4×10^4)	1.2×10^5 (1.1×10^5)	-

Table 10. Summary of NoV GII concentrations

Sample type	Septage (per gram)	Particulate (per gram)					Environmental water (per 100 mL)				Swab (per swab)
Environment of sampling	Public latrine (n=15)	Household (n=1)	Public drain (n=13)	Public domain (n=1)	Flood (n=2)	Farm (n=2)	Ocean/ beach (n=2)	Public drain (n=1)	Flood (n=3)	Farm (n=13)	Household (n=1)
Concentration range	5.8×10^4 to 8.2×10^6	7.5×10^4	4.1×10^4 to 1.7×10^6	7.4×10^5	4.8×10^5 to 9.4×10^5	2.3×10^5 to 2.5×10^5	5.0 to 8.1	4.2×10^4	1.6×10^4 to 2.3×10^5	2.4×10^3 to 3.1×10^6	4.2×10^4
Mean (SD)	1.4×10^6 (2.1×10^6)	-	4.2×10^5 (4.8×10^5)	-	7.1×10^5 (3.3×10^5)	2.4×10^5 (19,274)	6.5 (2.2)	-	1.2×10^5 (1.1×10^5)	2.9×10^5 (8.3×10^5)	-

APPENDIX

R codes for QMRA

```
rawdata <- read.csv("Irrigation_concentration.csv",header=TRUE,sep=",");

# a < detection limit Quantifast
# b > detection limit Quantifast and < detection limit Onestep
# c inhibition negative
# d difference between Ct values > 4

# For the genogroup I    assay
dtq.g1 <-    10; # detection limit (genome copies/l) Quantifast
dto.g1 <- 100; # detection limit (genome copies/l) Onestep
# For the genogroup II    assay
dtq.g2 <-    100; # detection limit (genome copies/l) Quantifast
dto.g2 <- 1000; # detection limit (genome copies/l) Onestep

# Set up data array for genogroup I data (log10 concentrations)
conc.g1 <- (rawdata[,2]!="a" & rawdata[,2]!="b" &
                    rawdata[,2]!="c" & rawdata[,2]!="d"); # measurable concentrations
data.g1 <- array(NA,dim=c(nrow(rawdata),3));
conc.g1
data.g1[conc.g1,1] <- log10(as.numeric(as.character(rawdata[conc.g1,2])));
data.g1[rawdata[,2]=="a",2] <-    -Inf;
data.g1[rawdata[,2]=="a",3] <- log10(dtq.g1); # below QF DL
data.g1[rawdata[,2]=="b",2] <- log10(dtq.g1);
data.g1[rawdata[,2]=="b",3] <- log10(dto.g1); # between QF and OS DL
data.g1[rawdata[,2]=="d",2] <- log10(dto.g1);
data.g1[rawdata[,2]=="d",3] <- Inf;                    # above OS DL

# Set up data array for genogroup II data (log10 concentrations)
conc.g2 <- (rawdata[,3]!="a" & rawdata[,3]!="b" &
                    rawdata[,3]!="c" & rawdata[,3]!="d"); # measurable concentrations
data.g2 <- array(NA,dim=c(nrow(rawdata),3));
data.g2[conc.g2,1] <- log10(as.numeric(as.character(rawdata[conc.g2,3])));
data.g2[rawdata[,3]=="a",2] <-    -Inf;
data.g2[rawdata[,3]=="a",3] <- log10(dtq.g2); # below QF DL
```

```
data.g2[rawdata[,3]=="b",2] <- log10(dtq.g2);
data.g2[rawdata[,3]=="b",3] <- log10(dto.g2); # between QF and OS DL
data.g2[rawdata[,3]=="d",2] <- log10(dto.g2);
data.g2[rawdata[,3]=="d",3] <- Inf;                    # above OS DL

# Define likelihood function
lik <- function(par,data){
   pdens <- dnorm(data[!is.na(data[,1]),1],mean=par[1],sd=par[2]);
   cdist <- pnorm(data[!is.na(data[,3]),3],mean=par[1],sd=par[2]) -
         pnorm(data[!is.na(data[,2]),2],mean=par[1],sd=par[2]);
   return(c(pdens,cdist)); # list of likelihoods for each observation
}



# Define deviance function: sum of -2*log(likelihood) of all observations
dev <- function(par,data) -2*sum(log(lik(c(par[1],exp(par[2])),data)));

# start search for maximum likelihood (= minimum deviance) parameters
# from here
par.init <- c(6,log(2));

# Calculate MaxLik (MinDev) parameters
res.g1 <- optim(par.init,dev,data=data.g1,method="Nelder-Mead")
res.g2 <- optim(par.init,dev,data=data.g2,method="Nelder-Mead")

# Retrieve MaxLik (MinDev) parameters from above results
par.g1 <- c(res.g1$par[1],exp(res.g1$par[2]));
par.g2 <- c(res.g2$par[1],exp(res.g2$par[2]));

# Simulate concentrations from the fitted distributions
nsim <- 10000; # number of Monte Carlo samples

log_concentration.GI <- rnorm(n=nsim,mean=par.g1[1],sd=par.g1[2]);

mean(log_concentration.GI)
log_concentration.GII <- rnorm(n=nsim,mean=par.g2[1],sd=par.g2[2]);

hist(log_concentration.GI, breaks=25, probability = TRUE,col="purple", main =
```

```
"Distribution of log concentration of NoV GI", xlab = "Log concentration of NoV GI
(per 100 mL)")
hist(log_concentration.GII, breaks=25, probability = TRUE,col="light blue", main =
"Distribution of log concentration of NoV GII", xlab = "Log concentration of NoV GII
(per 100 mL)")

Concentration.GI <- 10^log_concentration.GI;
Concentration.GII <- 10^log_concentration.GII;

hist(Concentration.GI[Concentration.GI<0.1])

hist(mcconc.g1[mcconc.g1<10])
#dose-response relationship
discrdr <- function(n,alpha,beta)
   1-(gamma(alpha+beta)*gamma(beta+n))/(gamma(beta)*gamma(alpha+beta+n));
# alpha = 0.040; beta = 0.055: Teunis et al 2008.

#if uniformly consumed
intake <- runif(nsim, 0.1, 0.5)

# intake in ml, must convert to l if conc is in 1/0.1l

dose.g1 <- rep(NA,nsim);
dose.g2 <- rep(NA,nsim);
pinf.g1 <- rep(NA,nsim);
pinf.g2 <- rep(NA,nsim);
for(k in 1:nsim){
   dose.g1[k] <- rpois(n=1,lambda=Concentration.GI[k]*intake[k]);
   dose.g2[k] <- rpois(n=1,lambda=Concentration.GII[k]*intake[k]);
   pinf.g1[k] <- discrdr(dose.g1[k],0.040,0.055);
   pinf.g2[k] <- discrdr(dose.g2[k],0.040,0.055);
}
help(rpois)
hist(dose.g1[dose.g1<10])
pinf.g1
ndays <- 7;
risk.g1 <- rep(NA,nsim);
risk.g2 <- rep(NA,nsim);
```

```
for(k in 1:nsim){
   risk.g1[k] <- 1-prod(1-pinf.g1[sample((1:nsim),size=ndays)],na.rm=TRUE);
   risk.g2[k] <- 1-prod(1-pinf.g2[sample((1:nsim),size=ndays)],na.rm=TRUE);
}

hist(risk.g1, breaks=50, col="purple", main="Estimated probability of occupational
NoV GI infection among farmers", xlab="Probability of NoV GI infection")
mean(risk.g1)
min(risk.g1)
max(risk.g1)
summary(risk.g1)

hist(risk.g2, breaks=50, col="light blue", main="Estimated probability of occupational
NoV GII infection among farmers", xlab="Probability of NoV GII infection")
mean(risk.g2)
min(risk.g2)
max(risk.g2)
summary(risk.g2)

par.g1
par.g2

pinf.g1
risk.g1
summary(pinf.g1)
sort(pinf.g1)
sort(pinf.g2)
summary(pinf.g2)
```

Chapter 3: Sensitivity Analysis of the real-time PCR Decision Scheme and Inhibition Analysis

SENSITIVITY ANALYSIS OF THE DECISION SCHEME

In this study, two RT-PCR assays -- QuantiFast Pathogen RT-PCR +IC Kit (Qiagen, Valencia, CA) and OneStep PCR Kit (Qiagen, Valencia, CA) -- were used to determine if a sample was NoV-positive, NoV-negative, inhibition positive, or inhibition negative. Because QuantiFast was considered to be more sensitive but was less reliable for quantification than OneStep, it was used to screen for the presence of NoVs in each sample. In contrast, OneStep was considered to be more reliable for quantification than QuantiFast, so the sample was tested on OneStep for quantification if at least one of the sample duplications was presumptive positive on QuantiFast, or if the sample was presumptive inhibition positive, meaning that the average Ct value of the internal amplification controls of the sample was larger than five compared with negative controls. To minimize the possibility of obtaining false negative results, the most liberal decision scheme was established, which indicates that a presumptive positive or presumptive inhibition positive sample would be determined to be positive or inhibition positive if at least one of the duplicated OneStep runs was positive (figure 5). Therefore, it is critical to conduct a sensitivity analysis to investigate whether the frequencies of positive, negative, inhibition positive, or inhibition negative samples changed considerably when the decision scheme became more strict.

Methods of analyzing modified decision schemes

The original decision scheme that was used to determine the positive or negative

samples was biased towards assigning a positive result over a negative result (a liberal decision scheme). Therefore, two stricter decision schemes were designed to assess the impact of the decision tree on study outcomes. For one of the modified schemes (modified scheme A), the sample would be determined to be positive only if both of the OneStep duplicates are positive. For the other modified scheme (modified scheme B), the sample would be determined to be positive only if both of the duplicates are positive and the Ct values smaller than 41. The results of the frequencies of positive, negative, inhibition positive, and inhibition negative samples of the two modified schemes were compared with those of the original scheme using Fisher's exact test with a significance level of 0.05.

The concentrations of positive and quantifiable samples were also re-analyzed based on the two modified decision schemes. For modified scheme A, the sample would be determined to be quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is no greater than 4. For modified scheme B, the sample would be determined to be positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is no greater than 4.The results of quantification based on the two modified decision schemes were separately compared with the results of the original scheme using a two-sample pooled t-test with a significance level of 0.05.

Results of modifying the decision scheme

Comparison of NoVs positive results among three decision schemes

Table 11 and table 12 summarize the results of NoV GI and GII assays from different decision schemes, respectively. If the original decision scheme was changed to the modified scheme A, 8 out of 39 NoV GI- positive samples and 6 out of 35 NoV

GII-positive samples would become negative, and one out of three NoV GI- inhibition positive sample and 6 out of 23 NoV GII-inhibition positive samples would be determined to be inhibition negative. If we further ruled out those samples with one or both the Ct values larger than 41, 15 NoV GI-positive samples and 10 NoV GII-positive samples would become negative, and 1 NoV GI-inhibition positive samples and 11 NoV GII-inhibition positive samples would be determined to be inhibition negative, compared with the original scheme. Fisher's exact test showed that the results of both the modified scheme were significantly different from those of the original scheme (all p values <0.0001).

Comparison of NoV quantification results among three decision schemes

In total, 31 samples were determined to be NoV GI positive and quantifiable using the original decision scheme, while 30 samples and 23 samples would still be determined to be positive and quantifiable if using the modified scheme A and the modified scheme B, respectively, instead of the original scheme (table 13 and table 14). If the modified scheme A was used instead of the original scheme, the number of positive and quantifiable samples would decrease from 10 to 7 (table 13). If using the modified scheme B instead of the original scheme, three septage samples (table 13), one particulate sample collected from public drains (table 13), two environmental water samples collected from public drains (table 14), and two environmental water samples collected from farms (table 14) would become NoV GI negative. Nevertheless, the results of pooled t-tests show that the differences of NoV GI mean concentration between the modified schemes and the original scheme were all not significant.

In total, 54 samples were determined to be NoV GII positive and quantifiable using the original decision scheme, while 45 samples and 36 samples would still be

determined to be positive and quantifiable if using the modified scheme A and the modified scheme B, respectively, instead of using the original scheme (table 15 to table 18). If the modified scheme A was used instead of the original scheme, one septage sample (table 15), one swab sample collected from households (table 15), one particulate sample collected from households (table 16), one public drain particulate sample, one particulate sample collected from farms (table 17), one flooding water sample, and three farm samples (table 18) would become NoV GII negative. If the modified scheme B was used instead of the original scheme, one septage sample (table 15), one household swabbing sample (table 15), one household particulate sample (table 16), two particulate samples collected from public drains (table 17), and one particulate sample collected from farm (table 17) would be determined to be NoV GII negative; in addition, one ocean water sample, two flooding water samples, one water samples collected from public drains, and six farm water samples would be determined to be NoV GII negative if the decision scheme changed from the original to the modified scheme B (table 18). Although the number of NoV GII positive and quantifiable samples varied from scheme to scheme, all differences of mean concentrations of NoV GII positive and quantifiable water samples using different schemes were insignificant.

INHIBITION ANALYSIS

Environmental compounds, such as humic acid and fulvic acid, can be co-concentrated with viruses when processing environmental samples for virus detection [1, 2]. These environmental compounds may inhibit downstream molecular assays such as PCR and RT-PCR, causing false-negative results or underestimating viral concentrations. Although DNA or RNA purification methods are used to remove environmental inhibitors, inhibition of PCR signals may still be observed due to the

incomplete removal of the inhibitors. Laverick et al. measured the NoV GII inhibition levels in inlet sewage water, sewage effluent water, and seawater by spiking known concentrations of NoV GII quantitative standards DNA into the NoV GII negative water samples. The results showed that only 47% to 58% of spiked NoV GII were recovered using PCR, and the seawater also showed NoV inhibition, from which the NoV GII concentrations were inhibited from 57% to 74% [3].

Inhibitors may interfere with the polymerase activities not only at the step of DNA amplification but also at the step of RNA reverse transcription for RT-PCR assays [4, 5]. Studies show that the step of reverse transcription could be prone to inhibition when detecting viruses in a variety of samples, such as shellfish, water, and food samples[3, 6-8].

To investigate the hypothesis that samples are more likely to be inhibited for NoVs (RNA viruses) than to be inhibited for Adenovirus (a DNA virus), the frequency of inhibition was analyzed and compared for NoV GI, NoV GII, and/or Adenovirus by the QuantiFast Pathogen + IC assay kit. The QuantiFast Pathogen + IC assay kit that was used in this study contained an RNA template of the Internal Control that was capable of identifying possible PCR inhibition, and an Internal Control Assay that could amplify the Internal Control RNA. Therefore, by comparing the Ct values of internal amplification control of the sample with those of negative controls, the potential inhibition could be identified when low Ct values of internal amplification control of negative controls were observed. A sample would be determined to be inhibited if the Ct value of internal amplification control of the sample was larger than 5 compared with negative controls.

Results of inhibition analysis

There were 229 samples (18%), 229 samples (19%), and 27 samples (2%)

inhibited for NoV GI, NoV GII, and Adenovirus, respectively (table 16). The percentages of samples inhibited for NoV GI and GII were similar, whereas the percentage of samples inhibited for Adenovirus was much lower compared with the percentage of samples inhibited for NoVs. The results are consistent with the hypothesis that samples were more likely to be inhibited for NoVs than be inhibited for Adenovirus. Since RNA of NoVs needs to be reverse transcribed to cDNA before PCR amplification, the inhibitors in the sample may not only influence the step of amplification step, but also influence the step of reverse transcription. In contrast, Adenovirus is a DNA virus, and hence only the step of DNA amplification is subject to environmental inhibitors.

Table 17 summarizes the results of NoV GI, GII, and Adenovirus inhibition results for each sample by sample type. In total 143 of 224 (64%) samples inhibited for NoV GI were also inhibited for GII, and 143 of 227 (63%) samples inhibited for NoV GII were inhibited for GI. The potential mechanisms for genogroup-specific inhibition are unknown. The inhibition analysis also shows that 18 of 25 (72%) samples that were inhibited for Adenovirus were also inhibited for NoV GI or GII. The results indicate that if the sample is inhibited for Adenovirus, it may have a high chance to be inhibited for NoVs also because the step of reverse-transcription, which was also subjective to PCR inhibitors, was needed for detecting NoVs with RT-PCR.

Particulate samples were the most susceptible to environmental inhibitors. Table 17 shows that 60% of particulate samples were inhibited for NoV GI, GII, or Adenovirus. Although ultrafiltration, which was used to concentrate the large volume water samples in this study, may have some effect on removing environmental inhibitors [1], large volume environmental water samples were also very susceptible to inhibitors, and the results show that 46% of large water samples were inhibited for

at least one of the three viruses. On the contrary, only 21% of small volume environmental samples were inhibited for NoVs or Adenovirus. These results are similar to the study results of Hata et al., which also show that samples concentrated from larger volumes tended to cause inhibition more frequently than samples concentrated from small volumes[9]. The possible explanation of the difference is that environmental inhibitors are also co-concentrated when concentrating water samples [1], and hence larger amount of environmental inhibitors tends to be concentrated from larger samples compared with smaller samples [9].

RECOMMENDATIONS FOR FUTURE RESEARCH

While the modified schemes may be more sensitive than the original scheme because they required both duplicated wells to be positive or have Ct values less than 41, the probability of a false negative may also be increased. Since the NoV-positive rates of this study were low (only 42 out of 1,241 samples were NoV GI positive/inhibition positive and 58 out of 1,210 samples were NoV GII positive/inhibition positive), it is reasonable to use a liberal decision scheme to include as many positive samples as possible.

The results of sensitivity analysis showed that the NoV-positive rates changed significantly if the decision scheme changed from the original to either modified scheme A or modified scheme B; nevertheless, all changes of mean concentrations of the positive and quantifiable samples were insignificant. These results imply that when the positive rates are low but the concentration ranges of the quantifiable samples are wide, the frequency of positive or negative samples may change significantly; however, a change of decision scheme may not have significant an impact on the change of mean concentrations of the samples because the standard deviations of the concentrations are wide and the sample sizes are small.

The construction of a decision scheme is based on the purpose of the research. If the purpose of the study is to investigate the presence/absence of NoVs in environmental samples, using QuantiFast alone to determine whether a sample is NoV-positive may reduce the chances of a false negative, because QuantiFast was considered to be more sensitive than OneStep. However, if the purpose of the study includes investigating the NoVs contamination levels in the environment, using QuantiFast alone may not be sufficient. An RT-PCR assay with high reliability on quantification, such as OneStep, may need to be combined with the screening assay.

The results of inhibition analyses in this study showed that PCR and RT-PCR inhibition is an important issue for detecting Adenovirus and NoVs in environmental samples. Therefore, it will be critical to evaluate the level of PCR and/or RT-PCR inhibition when implementing environmental viral investigations in the future. For assays with internal amplification control, such as QuantiFast, the presence of PCR and/or RT-PCR inhibition may be able to be identified by comparing the amplification signals of internal controls in negative controls with those in samples. However, if the research goal is to measure the concentration, or in the case of using the assays without internal amplification control, such as OneStep, one approach to evaluating the impact of PCR inhibitors on viral concentrations in samples is to spike a known amount of viruses in pre-determined negative samples and to measure the percentage of recovery[3].

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FIGURE

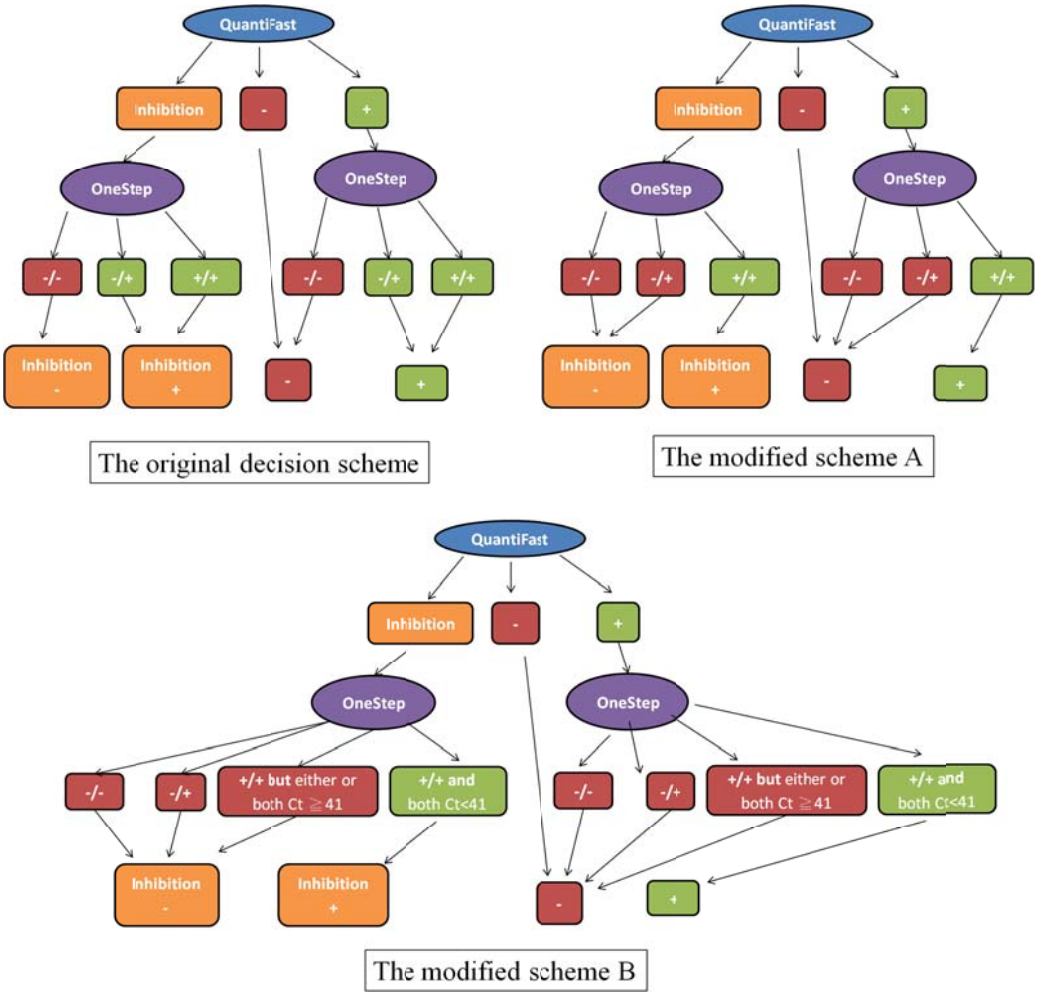

Figure 5. The original decision scheme, the modified scheme A, and the modified scheme B

TABLES

Table 11. Frequency of NoV GI positive results using the three decision schemes

	Original Scheme		Modified scheme A		Modified scheme B	
	Number of samples	(%)	Number of samples	(%)	Number of samples	(%)
Positive	39	(3.1)	31	(2.5)	24	(1.9)
Negative	975	(78.6)	983	(79.2)	990	(79.8)
Inhibition Positive	3	(0.2)	2	(0.16)	2	(0.2)
Inhibition Negative	224	(18.1)	225	(18.13)	225	(18.1)
Fisher's Exact Test*	-		p<0.0001		p<0.0001	

*The original scheme as the reference group

Table 12. Frequency of NoV GII positive results using the three decision schemes

	Original Scheme		Modified scheme A		Modified scheme B	
	Number of samples	(%)	Number of samples	(%)	Number of samples	(%)
Positive	35	(2.9)	29	(2.4)	25	(2.1)
Negative	947	(78.3)	953	(78.8)	957	(79.1)
Inhibition Positive	23	(1.9)	17	(1.4)	12	(0.9)
Inhibition Negative	205	(16.9)	211	(17.4)	216	(17.9)
Fisher's Exact Test*	-		p<0.0001		p<0.0001	

*The original scheme as the reference group

Table 13. Comparison of NoV GI concentrations in septage and particulate samples using the three decision schemes

Sample type	Septage (per gram)			Particulate (per gram)					
Environment of sampling	Public latrine			Public drain			Public domain		
	Original Scheme (n=10)	Modified Scheme A[a] (n=9)	Modified Scheme B[b] (n=7)	Original Scheme (n=8)	Modified Scheme A[a] (n=8)	Modified Scheme B[b] (n=7)	Original Scheme (n=1)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=1)
Concentration range	7.8×10^4-2.2×10^7	2.6×10^5-2.2×10^7	3.4×10^5-2.2×10^7	5.4×10^4-3.0×10^6	5.4×10^4-3.0×10^6	1.1×10^5-3.0×10^6	5.0×10^5	5.0×10^5	5.0×10^5
Mean (SD)	$3.8\times10^6(6.7\times10^6)$	$4.2\times10^6(6.9\times10^6)$	$5.3\times10^6(7.6\times10^6)$	$6.1\times10^5(9.7\times10^5)$	$6.1\times10^5(9.7\times10^5)$	$6.9\times10^5(1.0\times10^6)$	-	-	-
Pooled t-test*	-	p=0.90	p=0.67	-	-	p=0.88	-	-	-

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 14. Comparison of NoV GI concentrations in environmental water and swab samples using the three decision schemes

Sample type	Environmental water (per 100 mL)									Swab (per swab)		
Environment of sampling	Irrigation			Public drain			Farm			Public latrine		
	Original Scheme (n=1)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=1)	Original Scheme (n=4)	Modified Scheme A[a] (n=4)	Modified Scheme B[b] (n=2)	Original Scheme (n=6)	Modified Scheme A[a] (n=6)	Modified Scheme B[b] (n=4)	Original Scheme (n=1)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=1)
Concentration range	1.6×10^5	1.6×10^5	1.6×10^5	6.5×10^3-1.1×10^5	6.5×10^3-1.1×10^5	3.3×10^4-6.8×10^4	1.2×10^4-3.1×10^6	1.2×10^4-3.1×10^6	6.7×10^4-3.1×10^6	8.6×10^5	8.6×10^5	8.6×10^5
Mean (SD)	-	-	-	$5.3\times10^4(4.4\times10^4)$	$5.3\times10^4(4.4\times10^4)$	$5.1\times10^4(2.4\times10^4)$	$1.2\times10^5(1.1\times10^5)$	$1.2\times10^5(1.1\times10^5)$	$1.8\times10^5(1.0\times10^5)$	-	-	-
Pooled t-test*	-	-	-	-	-	p=0.93	-	-	p=0.50	-	-	-

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 15. Comparison of NoV GII concentrations of septage and swab samples using the three decision schemes

Sample type	Septage (per gram)			Swab (per swab)		
Environment of sampling	Public latrine			Household		
	Original Scheme (n=15)	Modified Scheme A[a] (n=14)	Modified Scheme B[b] (n=12)	Original Scheme (n=1)	Modified Scheme A[a] (n=0)	Modified Scheme B[b] (n=0)
Concentration range	5.8×10^4-8.2×10^6	5.8×10^4-8.2×10^6	1.5×10^5-8.2×10^6	4.2×10^4	-	-
Mean (SD)	1.4×10^6 (2.1×10^6)	1.5×10^6 (2.3×10^6)	1.7×10^6 (2.3×10^6)	-	-	-
Pooled t-test*	-	p=0.90	p=0.78	-	-	-

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 16. Comparison of NoV GII concentrations of particulate samples collected from households and public domain using the three decision schemes

Sample type	Particulate (per gram)					
Environment of sampling	Household			Public domain		
	Original Scheme (n=1)	Modified Scheme A[a] (n=0)	Modified Scheme B[b] (n=0)	Original Scheme (n=1)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=1)
Concentration range	7.5×10^4	-	-	7.4×10^5	7.4×10^5	7.4×10^5
Pooled t-test*	-	-	-	-	-	-

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 17. Comparison of NoV GII concentrations of particulate samples collected from public drain, flooding areas, and farms using the three decision schemes

Sample type	Particulate (per gram)								
Environment of sampling	Public drain			Flood			Farm		
	Original Scheme (n=13)	Modified Scheme A[a] (n=12)	Modified Scheme B[b] (n=11)	Original Scheme (n=2)	Modified Scheme A[a] (n=2)	Modified Scheme B[b] (n=2)	Original Scheme (n=2)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=1)
Concentration range	4.1×10^4-1.7×10^6	4.1×10^4-1.7×10^6	4.1×10^4- 1.7×10^6	4.8×10^5-9.4×10^5	4.8×10^5-9.4×10^5	4.8×10^5-9.4×10^5	2.3×10^5-2.5×10^5	2.5×10^5	2.5×10^5
Mean (SD)	$4.2\times10^5(4.8\times10^5)$	$4.3\times10^5(5.0\times10^5)$	$4.5\times10^5(5.2\times10^5)$	$7.1\times10^5(3.3\times10^5)$	$7.1\times10^5(3.3\times10^5)$	$7.1\times10^5(3.3\times10^5)$	$2.4\times10^5(1.9\times10^4)$	-	-
Pooled t-test*	-	p=0.93	p=0.89	-	-	-	-	p=0.67	p=0.67

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 18. Comparison of NoV GII concentrations of environmental water samples using the three decision schemes

Sample type	Environmental water (per 100 mL)											
Environment of sampling	Ocean and Beach			Flood			Public Drain			Farm		
	Original Scheme (n=2)	Modified Scheme A[a] (n=2)	Modified Scheme B[b] (n=1)	Original Scheme (n=3)	Modified Scheme A[a] (n=2)	Modified Scheme B[b] (n=1)	Original Scheme (n=1)	Modified Scheme A[a] (n=1)	Modified Scheme B[b] (n=0)	Original Scheme (n=13)	Modified Scheme A[a] (n=10)	Modified Scheme B[b] (n=7)
Concentration range	5.0-8.1	5.0-8.1	8.1	1.6×10^4-2.3×10^5	1.2×10^5-2.3×10^5	2.3×10^5	4.2×10^4	4.2×10^4	-	2.4×10^3-3.1×10^6	7.7×10^3-3.1×10^6	1.5×10^4-3.1×10^6
Mean (SD)	6.5 (2.2)	6.5 (2.2)	-	1.2×10^5 (1.1×10^5)	1.7×10^5 (7.6×10^4)	-	-	-	-	$2.9\times10^5(8.3\times10^5)$	3.8×10^5 (9.4×10^5)	$5.2\times10^5(1.1\times10^5)$
Pooled t-test*	-	-	p=0.67	-	p=0.59	p=0.48	-	-	-	-	p=0.82	p=0.61

*:Original scheme as the reference group; [a]: the sample would be determined as quantifiable only if both of the OneStep duplicates are positive and the Ct difference between the two duplicates is ≦4; [b]: the sample would be determined as positive and quantifiable only if the Ct values of both the duplicates are smaller than 41, and the difference of the two Ct values is ≦4.

Table 19. Frequency of target detection and inhibition with QuantiFast Pathogen + IC Assay

Quantifast Decision	Number of samples (%)		
	NoV GI (n=1243)	NoV GII (n=1213)	Adenovirus (n=1414)
Positive	72 (6)	42 (3)	367 (26)
Negative	942 (76)	942 (78)	1020 (72)
Inhibition	229 (18)	229 (19)	27 (2)

Table 20. Co-occurrence of inhibition across assays and sample types

Quantifast Decision			Number of samples (%)							Total number (n=1138)
NoVs GI	NoVs GII	Adenovirus	Septage (n=41)	Particulate (n=298)	Large environmental water (n=28)	Drinking water (n=179)	Small environmental water (n=144)	Food (n=264)	Swabs (n=229)	
Not inhibited	Not inhibited	Inhibited	1	1	1	0	0	3	1	7
Not inhibited	Inhibited	Not inhibited	0	48	3	2	17	8	3	81
Inhibited	Not inhibited	Not inhibited	3	37	5	2	10	12	5	74
Inhibited	Not inhibited	Inhibited	6	0	0	0	0	0	0	6
Inhibited	Inhibited	Not inhibited	2	85	4	3	3	23	9	129
Inhibited	Inhibited	Inhibited	0	9	0	0	0	3	0	12
Total number of inhibited samples (%)			**12 (29)**	**180 (60)**	**13 (46)**	**7 (4)**	**30 (21)**	**49 (19)**	**18(8)**	**309 (26)**

Chapter 4: Public Health Implications and Future Research

PUBLIC HEALTH IMPLICATIONS

Understanding the exposure pathways of noroviruses (NoVs) is the first and the most critical step in forming policies to reduce the risk of NoV infection and illness. It is estimated that NoVs are the cause of 21 million illnesses and 71,000 hospitalizations in the U.S. annually[1]. Globally, NoVs are responsible for an estimated 218,000 deaths among children aged < 5 years and 1.1 million hospitalizations each year [2]. Although most NoV cases are mild and self-limiting, NoVs can be shed in human feces and thus may be transmitted from toilets to the environment with either symptomatic or asymptomatic infection for up to three weeks [3]. Because there is no effective vaccine or antiviral treatment against NoV infection, interventions that target reducing NoV exposure and contamination are important for preventing NoV transmission.

Due to inadequate water and sanitation infrastructure, high population density, leaky septic systems, and the behavior of open defecation [4], it was suspected that NoVs may be present in drains and many public domains in Accra. The results of NoV environmental investigation in this study showed that 9% and 5% of particulate samples and water samples that were collected in drains and flooding areas were NoV GI positive, respectively; and 15% and 7% of the particulate samples and water

samples were NoV GII positive, respectively. The results indicate that NoVs may be transported through drainage systems or the next flooding event and cause waterborne or foodborne outbreaks by polluting receiving waters or shellfish that may be consumed by humans[5].

Eleven percent and 15% of the irrigation water samples and water samples collected from farms were NoV GI and GII positive, respectively; and the NoV concentrations were up to 3.1×10^5 genomic copies per 100 mL and 3.1×10^6 genomic copies per 100 mL for NoV GI and GII, respectively. In addition, the results of QMRA show that farmers' occupational risk of NoV infection would be at least 0.42 if they unintentionally ingest NoV contaminated irrigation water for only one day during seven days of work. These findings highlight not only the potential risk of NoV infection among farmers, but also the risk among populations who consume crops that are irrigated by the contaminated water.

FUTURE RESEARCH

The goals of this study were to investigate NoV contamination levels in Accra, Ghana, and to estimate the corresponding occupational risk of infection for farmers who unintentionally ingest contaminated irrigation water. However, Accra is only one of the low-income urban settings where the population is growing rapidly but the water and sanitation infrastructure remains inadequate. Although NoV contamination

levels in multiple environments in Accra have been investigated in this study, future research should place more emphasis on environmental NoV investigation in other low-income urban settings to comprehensively understand the NoV contamination levels and the potential exposure pathways.

Although most of our samples collected in markets and street vendors were NoV-negative, it does not mean that the risk of NoV infection via ingesting food in these areas is zero. A certain amount of NoVs may be lost during the food sample processing procedures. However, the recovery data of food samples are absent in this study, so the potential impact of NoVs lost during sample processing is unknown. In addition, the ability to accurately detect NoV-positive food samples may be reduced by environmental inhibitors in the sample because 46 food samples (17%) were inhibited for NoV GI or/and GII. Therefore, the recovery and limits of detection data of the food samples should be tested to understand the possibility of false negatives. Moreover, future research should be focused on developing food sample processing methods that have high recovery and that decrease the influence of environmental inhibitors on RT-PCR in order to increase the sensitivity of NoV detection in food samples.

It is critical to test the limits of detection on both QuantiFast and OneStep for all sample types and the limits of quantification on OneStep. If the data on limits of

detection and limits of quantification are available, the information about negative samples may be more valuable because the researchers and the readers would be able to know that the viral levels of the negative samples are actually below a certain level. In addition, the data on limits of detection and quantification can be further used to construct mathematical models that use probability density function to describe potential distributions of viral concentrations in the environment.

Quantitative Microbial Risk Assessment (QMRA) is critical to understanding the health burden of gastroenteritis. However, QMRA research regarding the NoV infection or illness in low-income urban settings is still limited. Future research should focus on risk assessment of NoV infection or illness related to a variety of human activities based on the results of quantitative NoV environmental investigation and behavioral analyses among people who live in low-income urban settings.

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