The Epidemiology of Noroviruses in Ghana: A Case Study of Norovirus Detection

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Abstract

Diarrheal diseases cause significant morbidity and mortality worldwide, particularly in children under age five. Most of the 2.5 million annual diarrhea-related deaths occur in developing countries, where access to treatment is limited by geographic, cultural and knowledge-based factors. Noroviruses, the leading cause of diarrhea in adults, are the second leading viral etiology of diarrhea in children behind rotaviruses, causing a roughly estimated 218,000 child deaths a year. The recent introduction of rotavirus vaccines throughout the world may open a path for noroviruses to take over as the leading viral etiology of diarrhea in children. However, while norovirus vaccines are still currently in development, the burden of norovirus disease in many developing nations has yet to be established. Through the analysis of 152 stool specimens collected from Ghanaian children presenting with diarrhea from 3 sites, this study demonstrates that noroviruses are a significant contributor to the childhood diarrheal disease burden in Ghana, responsible for at least 16.4% of all cases. The results help establish the baseline norovirus prevalence in this developing nation prior to the introduction of rotavirus vaccines and explore the potential for future interventions against this major viral etiology of childhood diarrhea. Moreover, a survey of the disparities in norovirus studies around the world leads to a call for standardized detection protocols.

Introduction

Noroviruses are the second most common cause of viral diarrhea in children under age five around the world, behind rotaviruses. Noroviruses cause an estimated 1.1 million hospitalizations and up to 218,000 deaths in this age group per year.[1,2] Given the effectiveness of current rotavirus vaccines in reducing the most globally common strains of rotaviruses, it is possible that noroviruses will soon take over as the leading cause of viral diarrhea in children. Indeed, noroviruses have recently exceeded rotaviruses as the leading cause of viral gastroenteritis in children under age five in the US.[3]

Diarrheal diseases disproportionately contribute to morbidity and mortality in low-income, developing countries relative to developed nations. For example, of the approximately 527,000 deaths a year of children under age five attributed to rotavirus infection, an estimated 252,000 occur in sub-Saharan Africa and 196,000 in Southeast Asia.[4] In these low-income settings, children suffering from diarrhea often have limited access to medical care and oral rehydration therapy, a formulated solution of water and essential electrolytes that prevents potentially fatal dehydration. Focusing on noroviruses in a specific country provides a closer examination of viral diarrhea in the underdeveloped world. The following case study in Ghana, a developing nation in sub-Saharan Africa, focuses on measuring the disease burden imposed by noroviruses in the country. Given the recent advances in development of a norovirus vaccine, this step is critically important to establishing the potential for interventions against noroviruses.[5,6]

Noroviruses belong to one of five genogroups that are further subclassified by genotype.[7] In general, the nucleotide sequences of norovirus strains belonging to different genogroups can vary by up to 50%.[8] Most noroviruses that infect humans globally belong to either genogroup I or II.[9] Overall, genogroup II noroviruses are believed to be responsible for 80-90% of all cases of norovirus gastroenteritis in the world.[10] The mutation rate of the norovirus genome is estimated to be about 1.21 x 10-2 to 1.41 x 10-2 nucleotide substitutions/site/year, higher than most other rapidly evolving RNA viruses.[11] The rapid rate of evolution complicates the constant monitoring of norovirus epidemiology as well as the development of interventions such as vaccines.

Noroviruses infect humans primarily during cool, dry seasons.[11] Often, a single strain is responsible for the majority of norovirus gastroenteritis cases each season. For example, during the 1995-1996 season, GII.4 noroviruses were responsible for about 90% of norovirus gastroenteritis in the United States.[12] GII.4 noroviruses continue to be the leading cause of norovirus outbreaks in the world, responsible for up to 60% of all cases of norovirus diarrhea during most seasons.[13] However, recombination and point mutations drive the rapid evolution of noroviruses, allowing new strains to emerge every season.[13] Moreover, the mutation rate of the norovirus genome is higher than most other rapidly evolving RNA viruses.[14] Together, these factors complicate the constant monitoring of norovirus epidemiology as well as the development of interventions such as vaccines.

As rotaviruses, not noroviruses, have been the predominant cause of acute diarrhea in children throughout the world, extensive surveillance efforts have monitored the local rotavirus strain distribution in Ghana. In contrast, knowledge of the regional epidemiology of noroviruses is currently limited to two studies that examined samples collected in 2000 and 2005-2006, respectively. Both exam-

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ined only samples from Northern Ghana and included relatively small sample sizes.[15] Given the recent introduction of a rotavirus vaccine and the predicted decline in rotavirus gastroenteritis, noroviruses may soon become the most epidemiologically prevalent and clinically relevant cause of viral diarrhea in children in this country. This study aims to provide an updated assessment of epidemiology of noroviruses in Ghana by using samples collected from sites throughout the nation.

Table 1. Oligonucleotide primers used for norovirus detection and genogrouping.

Genogroup	Primer (Polarity)	Sequence*
GI	GIFFN (+)	5'-GGAGATCGCAATCTCCTGCCC-3'
	GISKR (-)	5'-CCAACCCARCCATTRTACA-3'
GII	GIIFBN (+)	5'-TGGGAGGGCGATCGCAATCT-3'
	GIISKR (-)	5'-CCRCCNGCATRHCCRTTRTACAT-3'

**According to the IUPAC nucleotide ambiguity codes: R = A or G; H = A or C or T; N = A or C or G or T*

Materials & Methods

Site Descriptions: Samples were collected between February 2011 and February 2012 from three distinct locations: Agogo Presbyterian Hospital, a rural medical center with a catchment area containing 55,587 children; Navrongo War Memorial Hospital, a peri-urban facility that serves a district populated by 69,906 children; and the Korle Bu Teaching Hospital in Accra, the capital city of Ghana with a population containing 531,719 children.[16] The cities of Navrongo (Kassena Nankana District), Agogo (Asante Akim North District) and Accra (Accra Metropolitan District), listed in increasing population size, are located in Northern, Central and Southern Ghana, respectively, and provide coverage for both urban and rural populations.

Study Population: Fecal specimens were obtained with parental consent from children under five years of age hospitalized with acute diarrhea. In accordance with the WHO's definition of diarrhea, children were eligible if they had at least three episodes of non-bloody watery stools within 24 hours for fewer than seven days. In total, 152 samples were collected for this study: 53 samples from Agogo, 50 from Navrongo and 49 from Korle Bu. Demographic and clinical data was obtained from hospitals and missing values were excluded from analysis.

Sample Collection and Analysis: Stool specimens were collected in sterile containers, stored at 4°C and transported to the Noguchi Memorial Institute for Medical Research (NMIMR) near Accra, where they were stored at -20°C until analysis. Samples were tested on an enzyme-linked immunosorbent assay (ELISA) using the commercially available IDEIA-Rotavirus kit (DAKO; Glostrup, Denmark) following the manufacturer's instructions. This study was approved by the Institutional Review Boards of Princeton University (#5794) and NMIMR at the University of Ghana (#002/12-13).

Norovirus RNA Extraction and RT-PCR: RNA was extracted using the guanidinium thiocyanate (GITC) phenol/chloroform method described by Boom et al., eluted with 100 µL DEPC-treated water and purified with the RNAid kit (QBIOgene; Carlsbad, CA) according to the manufacturer's instructions.17 RT-PCR analysis was performed with the protocol described by Kojima et al. with some modifications.[18,19] Briefly, amplification of the cDNA used 1.0 µL of 20 pmol solutions of either the GIFFN or GIIFBN forward primer 2.0 µL of 20 pmol solutions of either the GISKR or GIISKR reverse primer (Table 1). These primers target the 5' end of ORF2, amplifying genogroup-specific sequences of the N-terminal and shell (N/S) region of the VP1 gene.[20] Thus, these primers can distinguish between genogroup I and genogroup II viruses.

Data Analysis: Statistical associations between norovirus status and demographic or clinical data were calculated using multinomial logistic regressions performed on the Stata software package (College Station, TX).

Results

Prevalence of Norovirus Infection: To determine the burden of norovirus disease in Ghanaian children, diarrheic stool samples were analyzed by RT-PCR for the presence of norovirus RNA (Table 2). Of the 152 fecal specimens collected from children under age five hospitalized with acute gastroenteritis between February 2011 and February 2012, norovirus RNA was detected in 25 (16.4%) samples. The prevalence of norovirus-positive samples differed by study site: 11 (22.4%) in Korle Bu, 11 (20.8%) in Agogo and three (6.0%) in Navrongo, though the lower prevalence in the northernmost site was not statistically significant ($p = 0.07$). A greater proportion of samples obtained from girls tested positive (11/62; 17.7%) than did specimens from boys (9/77; 11.7%). The majority of children who were hospitalized with acute diarrhea and had positive test results for norovirus were less than two years old (71.4%), with the 18-24 month group experiencing the highest rates of infection. The mean age of norovirus-positive children was 20.3 months. Positive norovirus status was significantly associated with the presence of vomiting ($p = 0.041$).

Prevalence of Rotavirus and Co-Infections: To further explore other causes of viral diarrhea within the study population, the samples were tested for the presence of rotavirus antigen with a commercial ELISA kit that uses a polyclonal antibody (Table 3). Of the 152 fecal specimens, rotavirus antigens were detected in 73 (48.0%) samples. The prevalence of rotavirus-positive samples also differed by study site: 30 (61.2%) in Korle Bu, 24 (45.3%) in Agogo and 19 (38.0%) in Navrongo. A larger proportion of specimens from boys tested positive for rotavirus (39/77; 50.6%) than did samples from girls (23/62; 37.1%). The vast majority of hospitalized children with rotavirus-positive samples were under two years old (90.5%), with the 6-11 month group experiencing the highest rates of infection. The mean age of rotavirus-positive children was 14.1 months. Rotavirus-positive children (40.0%) were more likely to experience vomiting than rotavirus-negative children (28.9%). Of the 152 samples, ten tested positive for both rotavirus and norovirus infection, equally distributed between boys and girls (five each). Most of these co-infections (seven) were detected in samples obtained from Korle Bu, though three were detected in Agogo, while none were found in Navrongo. The highest rates of co-infection were in the 18-23 month age group.

Seasonality of Norovirus Infection: As rotavirus infection has been determined to be seasonal in Ghana as well as in other tropical parts of the world, dates of sample collection of all norovirus-positive, norovirus-negative and rotavirus-positive samples were plotted to determine if noroviruses follow any seasonal patterns of infection in causing acute diarrhea in children (Fig. 1).[21] Only samples collected from October through May tested positive for norovirus on the RT-PCR, which encompasses the entire dry season in Ghana. Only three samples were detected in May, which falls during the transition between the dry and wet seasons. The peak of detected norovirus infections was in December 2011, followed by January 2012, with six and five positive samples respectively. These months fall in the middle of the dry season throughout Ghana. The peaks of norovirus-positive samples coincided with the seasonal peaks in overall diarrhea, including norovirus-negative samples, as well as with the peaks in rotavirus-positive diarrhea; this observation will be explained further in the discussion.

Distribution of Norovirus Genogroups: To provide a preliminary understanding of norovirus diversity throughout all parts of Ghana, the fecal specimens were analyzed with an RT-PCR assay using genogroup-specific primers directed at the N/S domain of ORF2 (Table 1).[18] Of the 25 samples that tested positive for norovirus, 23 (92%) were successfully amplified with GII-specific primers and two with GI-specific primers (8%) (Fig. 6). GII noroviruses were detected in samples provided from children of all ages from all three study sites during all months between October and May. GI noroviruses were detected only at Korle Bu Teaching Hospital in children aged 3 weeks and 42 months in the months of March and May, respectively. Thus, in this study of Ghanaian children, detected cases of norovirus diarrhea were more commonly caused by GII viruses, which caused infections in broader ranges of location, age and date, relative to the incidence of GI noroviruses.

Table 2. Norovirus detection in samples from Ghanaian children hospitalized with diarrhea.

Variable	Norovirus - (%)	Norovirus + (%)	P Value*
All Samples	127 (83.6%)	25 (16.4%)	
Study Site			Ref
Korle Bu	38 (77.6%)	11 22.4%)	0.777
Agogo	42 (79.2 %)	11 (20.8%)	0.072
Navrongo	47 (94.0%)	3 (6.0%)	
Age			
<6 months	16 (88.9%)	2 (11.1%)	
6-11 months	30 (90.9%)	3 (9.1%)	
12-17 months	27 (90.0%)	3 (10.1%)	
18-23 months	18 (72.0%)	7 (28.0%)	
24-35 months	13 (81.3%)	3 (18.8%)	
36-59 months	14 (87.5%)	3 (12.5%)	
Gender			0.208
Male	68 (88.3%)	9 (11.1%)	
Female	51 (82.3%)	11 (17.1%)	
Vomiting			0.041
Present	17 (73.9%)	6 (26.1%)	
Not Present	61 (89.7%)	7 (10.3%)	

**Multinomial logistic regression against the reference (Ref) variable for norovirus-positive samples*

Discussion: A Call for Standardized Norovirus Detection Protocols

The results from the present study suggest that noroviruses contribute significantly to the disease burden of childhood diarrhea in Ghana. Compared to other published studies, the norovirus prevalence of 16.4% was higher than that reported in a pooled analysis of studies conducted in seven developing countries (12.1%), spanning from Malawi to Thailand to Peru.[2] However, the figure in the present study was lower than that found recently for children in other countries such as the US (21%) and in Nigeria (37.5%).[3,19]

A number of reasons can potentially explain these disparities in norovirus prevalence across different studies. One possible factor is the type of assay used to detect and group noroviruses. The present study used an RT-PCR method described by Kojima et al. in 2002 that uses primers that target the 5' end of ORF2. These primers amplify genogroup-specific sequences of the N-terminal and shell (N/S) region of the VP1 gene.18 Incidentally, the VP1 gene is the most hypervariable part of the genome (i.e. there are many variations), as it encodes the domain of the virus involved in receptor binding.[22] There is a considerable chance that some of the stool samples in this study contained noroviruses that these primers, developed in 2002, did not fully anneal to in the initial steps of the PCR protocol. Given the constant genetic drift and shift of noroviruses, many viruses in these samples may have escaped detection with these primers.

Indeed, there are other sets of primers used in RT-PCR assays to detect and group noroviruses. The primers in this study amplify a segment known as "Region C" within the norovirus genome. Correspondingly, Regions A and B are within ORF1, while Region D is downstream of Region C within ORF2.20 Studies have shown that different assays have varying sensitivities to different norovirus genotypes, with overall sensitivities ranging from 52-73% depending on the strain.[23] The most broadly reactive norovirus primers target Region B within ORF1, which codes for the viral RNA polymerase.[24] This region contains the most conserved sequence within the entire norovirus genome.[25] However, because the region is so well conserved, primers targeting this area are used only for detection rather than distinguishing between different virus genogroups. Thus, assays targeting Region B using real-time quantitative RT-PCR, which enables the detection of extremely low titers of norovirus, are now a gold standard in labs around the world for the diagnosis of norovirus infection.[26]

Overall, of the protocols capable of genogrouping viruses, the Region C primers used in the present study yield the highest sensitivity assays.[23] Even still, these primers likely provide a low estimate of the norovirus prevalence in Ghanaian children. A study examining norovirus infection in Nigerian children under age five compared the same RT-PCR assay to a commercially available norovirus ELISA kit.[19] In this case, the Region C protocol yielded norovirus-specific gene amplicons for only 53.3% of all ELISA-positive samples, failing to amplify the remaining samples. A possible explanation of the dismal norovirus sensitivity is the diversity of norovirus strains circulating within the region that are non-reactive to the specific primers and antibodies used in the RT-PCR and ELISA assays respectively. In nearby Ghana, the molecular epidemiology of noroviruses is presumably just as diverse. Thus, genogrouping and detection tests face many challenges given the rapid mutation and evolution of noroviruses, especially in this developing region.

After extrapolating the data using the extremely low sensitivity value in the Nigerian study and a higher value from a multi-center pooled analysis (78%), a reasonable estimated range for the norovirus prevalence in the present study in Ghanaian children is 21.1-30.8% (ie the prevalence in the present study divided by the sensitivity values from the other studies). This figure, taking into account the possibility that the Kojima et al. RT-PCR assay missed a number of norovirus-positive samples, better aligns with most other published studies. Extrapolating from the sensitivity values presented in the 2010 study in Nigeria, which likely had a similar norovirus strain epidemiology based on the geographical proximity to Ghana, the actual prevalence of viruses in children from Navrongo is closer to 11.3%. This figure is more comparable to that reported in the 2006 norovirus study in Navrongo, which used multiple sets of primers and both single and sensitive seminested RT-PCR protocols.[16]

Another factor contributing to the disparities among results was the variance in inclusion criteria among the various study populations. In the present study, the duration of diarrhea was restricted to less than seven days and children had to be less than five years of age, which is the age range most affected by diarrhea. Samples were selected randomly from those collected between February 2011 and February 2012 without regard to rotavirus status, age, sex or clinical symptoms. In the 2006 norovirus study conducted in Navrongo, the study population was restricted to rotavirus-negative samples collected from children under two years of age. As the peak age group of norovirus infection in the present study was 18-23 months, limiting this population to younger children would have increased the prevalence.[16]

Moreover, while the 2006 report neglected to list limits in the duration of illness, many other studies in Africa enroll children up to 14 days after onset of symptoms.[27] Rotavirus and norovirus shedding generally peaks within the first week of illness but can last for nearly two months, reflecting how the duration of illness can affect the outcome of each study.28 Furthermore, while the 2006 norovirus study failed to report the dates of sample collection, the study in Nigeria only examined samples collected between November 2007 and May 2008. As these dates coincide with the dry season in West Africa, it is unsurprising that the norovirus prevalence in that study was so high (37.5%). Clearly, the inclusion criteria of a study can influence the calculated prevalence of the viruses.

Nonetheless, the distribution of norovirus genogroups in the present study was consistent with other reports. In this report, 92% of all detected noroviruses belonged to genogroup GII, while the remaining 8% belonged to GI. Similar ratios were detected in other studies examining the norovirus molecular epidemiology throughout the world, including Ghana.[3,15,16,29] Indeed, GII.[4] noroviruses generally comprise the most predominant genotype, depending on the season.[10] Thus, the molecular epidemiology surveyed in this study is consistent with previously published literature.[3,15,16,29]

These disparities in practices illustrate that for noroviruses, a stan-

Table 3. Rotaviruses and co-infection in samples from Ghanaian children hospitalized with diarrhea.

Variable	Norovirus + (%)	Co-Infection (%)	P Value*
All Samples	25 (16.4%)	10 (6.6%)	
Study Site			Ref
Korle Bu	11 (22.4%)	7 (13.3%)	0.722
Agogo	11 (20.8%)	3 (5.7%)	0.138
Navrongo	3 (6.0%)	0 (0.0%)	
Age			
<6 months	2 (11.1%)	1 (5.6 %)	
6-11 months	3 (9.1%)	2 (6.1%)	
12-17 months	3 (10.1%)	2 (6.7%)	
18-23 months	7 (28.0%)	4 (16.0%)	
24-35 months	3 (18.8%)	0 (0.0%)	
36-59 months	3 (12.5%)	0 (0.0%)	
Gender			0.093
Male	9 (11.1%)	5 (6.5%)	
Female	11 (17.1%)	5 (8.1%)	

**Multinomial logistic regression against reference (Ref) variable*

dardized set of inclusion criteria and assays used for analysis would greatly benefit global molecular surveillance. For comparison with other etiologies of childhood diarrhea, children in norovirus studies should be five years of age or under and follow the WHO definition of diarrhea, i.e., pass loose or liquid stools at least three times a day for at most seven days.[30] Samples that test positive for rotavirus infection should not be excluded from study, as co-infections can comprise a non-negligible portion of detected norovirus cases. The assays used to detect and classify noroviruses should also be standardized. Currently, various studies use different primer sets that target disparate regions within the norovirus genome. These regions experience different degrees of variability, leading to inconsistent results. Until more sensitive and accurate assays are developed, a standardized primer set should be used. Ideally, studies would all start with a round of RT-qPCR using primers targeting the conserved Region B, allowing the detection of noroviruses even in low titers. Subsequent failure of amplification in Region C or D would then prompt sequencing analyses, enabling the discovery of entirely novel norovirus strains. However, given the limited funding and tools available in the surveillance lab in Ghana, these sensitivity-boosting steps were not possible. Nonetheless, it is crucial that norovirus prevalence is reported in accordance with standard criteria so that the burden of disease can be reliably monitored and compared in different regions of the world. These data would indicate the areas in most need of specific interventions such as a norovirus vaccine.

Most of the demographic, temporal, and clinical characteristics of the norovirus and rotavirus infections in Ghanaian children were consistent with those in other studies. As reported in other studies, children infected with rotaviruses and noroviruses were more likely to experience vomiting than those whose disease was caused by other agents.[29,31] As both of these viral etiologies of childhood diarrhea are spread through the fecal-oral route, the implications for transmissibility are considerable. Noroviruses in particular are infamous for the ability to spread through infectious vomitus, especially within enclosed settings such as hospitals.[31]

Moreover, both rotavirus and norovirus infection followed a distinct pattern of seasonality, peaking during the dry season months of October-May while all but disappearing during the wet season. These patterns are consistent with previous studies in Ghana as well as other countries with tropical wet and dry climate such as Kenya and Nicaragua.[15,21,29,32] This seasonality is often attributed to the airborne transmission of virus particles through dust and fecal matter, which occurs more easily during the colder and windier dry season.[19] Nevertheless, the patterns of seasonality are less pronounced areas with tropical climates as in regions with temperate climates, such as Europe and the US.[33]

The age distribution of noroviruses was also similar to that of other studies, proportionally affecting infants younger than age 2 more than older children.[3,29] Interestingly, prior to the introduction of rotavirus vaccines, norovirus infection in developed countries was more prevalent in children older than two years of age.[15] Meanwhile, children have been experiencing norovirus infections at younger ages in the US and elsewhere following the introduction of rotavirus vaccines.[3] This trend suggests that following the 2012 introduction of Rotarix in Ghana, noroviruses could begin to impose a larger disease burden upon younger age groups throughout the nation. Due to their greater body surface-volume ratios, higher metabolic rates, and smaller fluid reserves, younger infants are especially prone to dehydration.[34] Moreover, previously unexposed infants are less immunologically primed against the agents that cause acute gastroenteritis. Consequently, the morbidity and mortality of norovirus diarrhea would be disproportionately higher in younger age groups.

Future studies will need to continue to monitor noroviruses in Ghana as well as other developing nations to measure the changes in the viral etiologies of childhood diarrhea over multiple years. Longitudinal monitoring is especially crucial following the recent introduction of a rotavirus vaccine into the national immunization program. Ultimately, continued surveillance of the burden of norovirus disease in Ghana can help determine the necessity of further preventative interventions targeting noroviruses.

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