

Molecular isolation of human norovirus and astrovirus in tap water by RT- PCR

Julius Tieroyaare Dongdem^{1*}, Susan Damanka² and Richard Asmah³

¹Department of Medical Biochemistry, School of Medicine and Health Sciences.
University for Development Studies, Tamale. Ghana

²Department of Electron Microscopy and Histopathology.
Noguchi Memorial Institute for Medical Research, Legon, Ghana

³School of Allied Health Science, Korle Bu Teaching Hospital. Accra, Ghana

Viral gastroenteritis is responsible for pediatric morbidity and mortality, time loss and also an economic burden in developing countries. Norovirus is known to cause 90% of all epidemic non-bacterial outbreaks worldwide. Astroviruses are second only to rotavirus as cause of viral gastroenteritis worldwide. Nevertheless, in most diarrheal cases, neither norovirus nor astrovirus is routinely screened for in stool or environmental samples, thus, data on health impact of waterborne disease is lacking in developing countries like Ghana. Since the presence of these viruses in drinking water are potential health threats, the aim of this study was to collect water samples within the distribution network of the Weija Water Works and test for norovirus and astrovirus contamination using molecular methods. Two litres of each sample was concentrated 4,000 fold. Viral ssRNA was extracted from all concentrates by the phenol /chloroform method and purified with the RNaid® kit. Ten samples which had previously tested positive for rotaviruses were selected for norovirus and astrovirus detection. Each sample was reverse transcribed, amplified by PCR and analyzed by gel electrophoresis. Norovirus GII type was not detected. Four and three out of the ten samples tested positive for GI norovirus and astrovirus respectively. By virtue of molecular weight, we isolated different strains of norovirus and one strain type of astrovirus. The level of viral contamination increased particularly at the region furthest from the treatment plant.

Keywords: Detection, norovirus, astrovirus, Diarrhea, Ghana, Tap water, RT-PCR

INTRODUCTION

Acute viral gastroenteritis is a very common illness that is not only responsible for particularly pediatric morbidity and mortality, but also a great deal of time loss from school and work. Though less harmful to adults, gastroenteritis also represents an economic burden in developing countries. Over a billion diarrheal cases occur each year among children below five years resulting in approximately 2.5 million deaths (O'Ryan et al., 2005). Viral gastroenteritis however, affects all age groups worldwide and occurs in both endemic and epidermic forms (Blacklow et al., 1981).

Clinical manifestation of acute viral gastroenteritis is variable. Generally, it is self limiting, has an explosive onset and is manifested by varying combinations of diarrhea, nausea, vomiting, low grade fever, abdominal cramps, headache, anorexia, myalgia and malaise (Khan et al., 1992). Other symptoms include loss of taste, general lethargy, weakness, muscle aches, dehydration and Loss of weight.

Both human norovirus and astrovirus have also been implicated in the aetiology of viral gastroenteritis aside rotavirus and adenovirus. Norovirus is an RNA virus and belongs to the family *Caliciviridae* which causes approximately 90% of epidemic non-bacterial outbreaks of gastroenteritis worldwide (Lindesmith et al., 2003). Norovirus is responsible for 50% of all foodborne

outbreaks of gastroenteritis in the U.S (Widdowson et al., 2005). Noroviruses are now considered to be the most common cause of outbreaks of acute gastroenteritis worldwide (Lindesmith, 2003). Most outbreaks are triggered by contamination of food by an infected food handler. Norovirus outbreaks can affect persons of all ages and occur in a wide variety of settings e.g., nursing homes, hospitals, restaurants, communities, schools, day care centers, military barracks, cruise ships, etc. (Widdowson et al., 2005). Although waterborne outbreaks are far less common than foodborne outbreaks, gastroenteritis outbreaks attributable to norovirus infections have been reliably linked to sources of contaminated water including municipal water, well water, stream water, commercial ice, lake water and swimming pool water which has not been investigated in most sub Saharan African countries like Ghana. One such study looked for patterns that might explain norovirus outbreaks in Toronto. It was found that winter flare ups of the highly contagious condition were more likely to happen in the week after water temperatures in Lake Ontario dipped below 4°C or flow from the Don river into Lake Ontario was high (Branswell, 2008). The findings suggested that under certain environmental conditions, noroviruses from human sewage may proliferate in bodies of water that are used both as municipal water sources and sewage treatment outlets, eventually finding their way back into the human gastrointestinal tracts through drinking water (Branswell, 2008). Other waterborne outbreaks were investigated in Finland for viruses. The diagnostic procedure included analysis of patients' stool samples by electron microscopy and RT-PCR for noroviruses and astroviruses. Epidemiological studies discovered that, of the 41 waterborne outbreaks reported during the observation period (1998 - 2003), samples from 28 outbreaks which were available for analysis showed that noroviruses caused 18 outbreaks (CDC, 2001). During fall 2008, 3 norovirus outbreaks occurring on college campuses in California, Michigan and Wisconsin were reported by Roberts et al., 2009. Public health investigations led by the respective state and local health departments to characterize the extent of the outbreaks resulted in approximately 1,000 cases of reported illness including at least 10 hospitalizations and prompted closure of one of the 3 campuses (Roberts et al., 2009). The number of deaths from norovirus in the U.S is estimated to be about 300 yearly, most of which occur in young children, elderly and persons with weakened immune systems. Severe illness is rare however, symptoms may become life-threatening in these groups if dehydration is ignored or untreated (Goodgame, 2006).

In most cases, contamination occurs almost exclusively by sewage that contains enteric pathogens. Enteric viruses that affect humans are mostly species-specific. Their abundance may therefore be explained by the high concentrations in stool of patients which can easily make way into drinking water sources and escape disinfection.

According to Branswell (2008), the waterborne outbreaks of norovirus that occur are typically due to drinking water sources in which chlorination or other disinfection systems have broken down, or in recreational situations in which either disinfection breaks down or chlorination breaks down in the case of a swimming pool.

The genus astrovirus on the other hand, has been classified within the newly established *Astroviridae* family. Even though several studies have revealed the public health significance of human astrovirus (HAstV), very little is understood about the epidemiology of this virus. Astroviruses were first observed by electron microscopy in stool specimens from infants with gastroenteritis (Appleton and Higgins, 1975). Like noroviruses, astroviruses are now recognized as important aetiological agents of viral gastroenteritis in all age groups. In fact, the development of RT-PCR has revealed that astroviruses are second only to rotavirus as the most common cause of viral gastroenteritis in children worldwide (Cruz et al., 1992; Glass et al., 1996; Herrmann et al., 1991; Lew et al., 1991). Also, the impending licensing of a rotavirus vaccine (Tucker et al., 1998) may lead to astrovirus becoming the primary cause of viral gastroenteritis among young children in the near future. However, in most diarrheal cases, astrovirus is neither routinely screened for in stool samples nor in environmental samples. Also, data on the health impact of waterborne astrovirus is lacking especially in developing countries like Ghana.

Large outbreaks of astrovirus diarrhea are described with increasing incidence (Oishi et al., 1994). In July 2001, an outbreak of gastroenteritis occurred in Helsinki among children and adults after bathing in an outdoor wading pool. Epidemiological survey revealed that at least 242 persons were affected. Microbiological testing of both patient stool samples and of the pool water revealed the presence of two different gastroenteritis viruses: a norovirus and an astrovirus (Maunula et al., 2004). Mitchell et al.(1995) evaluated astrovirus as a cause of diarrheal outbreaks among infants and toddlers in day care centers. Astrovirus was detected in 6 (7%) of the 81 outbreaks investigated and concluded that astrovirus is an important cause of outbreaks of diarrhea among children attending day care centers, more frequently infecting younger children and often produced asymptomatic infections.

Current analytic methods do not authorize direct monitoring of norovirus and astrovirus in treated drinking water nor are they enforced by quality monitoring bodies. Indicator organisms e.g. coliform bacteria, have been used as proxy indicators of fecal contamination. However, the size, physiology and susceptibility of bacterial indicators to physical treatment and disinfection differ from those of enteric viruses. In other words, inherent limitations of this approach exist. Enteric viruses are known to be highly resistant to common disinfection methods. More so, many studies have revealed the

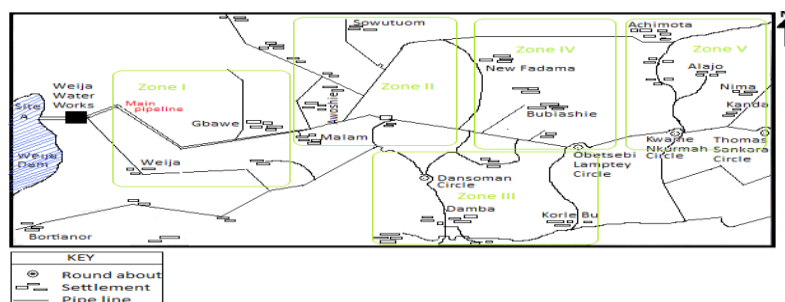


Figure 1: Weija Water Works, distribution network and marked zones

presence of enteric viruses in treated water that complied with safety parameters for bacterial indicators. Therefore, until reliable methods for assessing the occurrence and susceptibility to treatment of noroviruses and astroviruses among others are available, prevention methods should focus on reducing human waste contamination of drinking water supplies. If drinking or recreational water is suspected as being an outbreak source, high-level chlorination (i.e., 10ppm or 10mg/L for > 30 minutes) might be required for adequate disinfection. However, even this method might still be insufficient in some cases (Keswick et al., 1985).

The ubiquity of waterborne norovirus and astrovirus outbreaks therefore advocates for measures to monitor water for viruses. Since the presence of these viruses in drinking water are potential health threats, the aim of this preliminary study was to collect untreated and treated water samples from the Weija Water Works and along its distribution network and test specifically for norovirus and astrovirus contamination by a multiplex semi-nested PCR using specific primers.

MATERIALS AND METHODS

Collection of samples

A map of the distribution network of the Weija Water Works was obtained and divided into 5 zones according to distance from the treatment plant (Figure 1). Two and a half litres of tap water samples were obtained in sterilized rubber containers from 27 sites within the distribution system. This included 5 samples from each zone, one sample from the Weija dam and one treated water sample immediately after treatment at the Adam Clark Plant, Weija Water Works and stored at 20°C until ready for concentration.

Concentration of water samples and elution of viruses from filter

Two litres of each sample was concentrated (Nadan et

al., 2003) by the stirred ultrafiltration cell (Amicon®Ltd, Great, Britain) method using regenerated cellulose membrane filter (Amicon® Bioseparations, Bedford, USA) of 10⁵NMWL. The concentration was carried out in a 350ml amicon ultrafiltration cell. A cell pressure of 5.3kg/cm² was maintained and the concentration was carried out till each water sample was concentrated to a final volume of 0.5ml (usually in 5 hours). The viruses adsorbed to the filter were eluted with 0.5ml of 3% beef extract (DIFCO Laboratories, Detroit, USA) containing glycine (Introgen Life Technologies, Auckland, New Zealand) buffer pH 9.5. The concentrates were stored at 20°C until required for RNA extraction and purification.

Extraction and purification of viral RNA

Viral single strand RNA (ssRNA) was extracted from all concentrates by the phenol /chloroform method (Boom et al., 1990). The ssRNA extract was purified with the RNaid® kit (Bio 101, Carlsbad, USA), and stored at 4°C. Briefly, sodium acetate containing 1% sodium dodecyl sulphate (SDS pH = 5.0) was used to solubilize phospholipids and membrane protein. Guanidinium isothiocyanate was used to further dissolve proteins leaving the nucleic acids (ssRNA). RNaid® matrix was used to adsorb the extracted RNA on pelletes, washed with RNaid® wash buffer and re-suspended in DEPC treated water.

Detection of norovirus and astrovirus

Reverse transcription of ssRNA extract

Ten samples which had previously tested positive for rotaviruses (Dongdem et al., 2010) were selected for norovirus and astrovirus detection. Purified ssRNA from each selected sample was reverse transcribed by adding 0.5µl of random primers (Amersham pharmacia biotech, Maryland, USA) to 5µl of each ssRNA template in a reaction mix containing dNTPs, avian myeloblastosis (AMV) reverse transcriptase and buffer. The mixtures

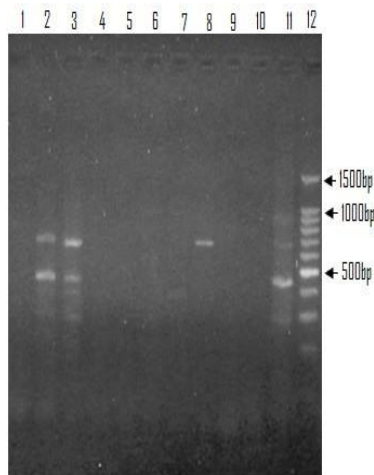
Table 1: Oligonucleotide primers used for GI, GII genogrouping of norovirus and detection of astrovirus (Kageyama et al., 2003; Kojima et al., 2002; Nadan et al., 2003; Yao et al., 2003)

Primer	Sequence	Region (nt)
GIFFN	GGAGATCGCAATCTTCCTGCC (Forward) ³	ORF1/ORF2
GISKR	CCAACCCARCCATTTTACA (Reverse) ²	" "
GIIFBN	TGGGAGGGCGATCGCAATTCT(Forward) ³	" "
GIISKR	CCRCCNGCATRHCCRTTTRACAT(Reverse) ²	" "
Mon2	CCGGCTGGGAGGAGTCGG	6513 – 6781 (3' end of the ORF2)
prBeg	ACCGTCTAACCCTCCTCTC	296 – 324 (3' end of ORF2)
Random primers	5'pd (N) ₆	

H: A, T or C

R: A or G

N: A, T, C or G

**Figure 2:** Electrophoresis of norovirus (GI) PCR amplification products on 2.0% agarose gel. Lane 12 shows the 100bp molecular marker, Lanes 2 to 11 represent samples 1504, 1501, 1404, 1403, 1305, 1304, 1205, 1203, 1002 and 1001 respectively. Lane 1 shows the negative control. 500bp, 1000bp and 1500bp markers are indicated with arrows.

were incubated at 42°C in a water bath for 4 hrs.

GI, GII genogrouping of norovirus by PCR

Each cDNA obtained was amplified in a reaction mixture containing *Taq* polymerase, dNTPs, 25 mM MgCl₂ and the primer pair GIFFN/GISKR for genogrouping GI and GIIFBN/GIISKR primer pair (Table 1) for grouping GII noroviruses (Gallimore et al., 2005). The mixtures were subjected to 40 cycles of PCR (30 sec for denaturation at 95°C, 30 sec at 48°C for annealing and 2 min at 72°C for extension with a final extension cycle at 72°C for 5 min).

Visualization of amplified products

The nested PCR amplified products were electrophoresed at 100V for 1 hour on a 2.0% agarose gel containing ethidium bromide. The gel was visualized under UV light using a UV Trans illuminator and the amplicon sizes read using a molecular weight marker.

Astrovirus PCR amplification

Each cDNA obtained was added to Mon 2/prBeg primer pair (Table 1) in a reaction mixture as done for norovirus above and subjected to 40 cycles of PCR (1 min for denaturation at 94°C, 1 min at 50°C for annealing, 5 min for extension at 72°C with a final extension at 72°C for 15 min) (Walter et al., 2001). Negative controls containing double distilled water in place of sample were incorporated simultaneously in all steps for each experiment from reverse transcription to gel electrophoresis. The PCR amplified products were electrophoresed and visualized as above.

RESULTS

Norovirus genogroup II (GII) type was not detected in any of the samples. Four (40%) of selected samples however tested positive for genogroup I (GI) type norovirus (Table 2). In all, thirteen norovirus GI ssRNA were isolated with approximate molecular weights as follows. Whereas one out of thirteen (1/13) isolates had a molecular weight approximately equal to (≈) 900bp, four out of the thirteen (4/13) isolates had molecular weights ≈ 680bp. Of the 13 isolates also, 3 (3/13) had molecular weights ≈ 450bp, two (2/13) had molecular weights ≈ 350bp with 3 (3/13) having molecular weights ≈ 290bp. This suggests the occurrence of 5 different GI strain types (Figure 2). Only ssRNA norovirus GI with molecular weight ≈ 680bp was

Table 2.0: Detection of norovirus and astrovirus by RT-PCR

Sample №	Zone	Average distance from Head works (km)	RT-PCR Results		
			Norovirus GII	Norovirus GI	Astrovirus
1001	A	0.00	Neg	Pos	Pos
1002	A	4.99	Neg	Neg	Neg
1202	2	9.59	Neg	Neg	Neg
1205	2	9.59	Neg	Pos	Neg
1304	3	13.36	Neg	Neg	Neg
1305	3	13.36	Neg	Neg	Neg
1403	4	13.59	Neg	Neg	Neg
1404	4	13.59	Neg	Neg	Neg
1501	5	17.66	Neg	Pos	Pos
1504	5	17.66	Neg	Pos	Pos

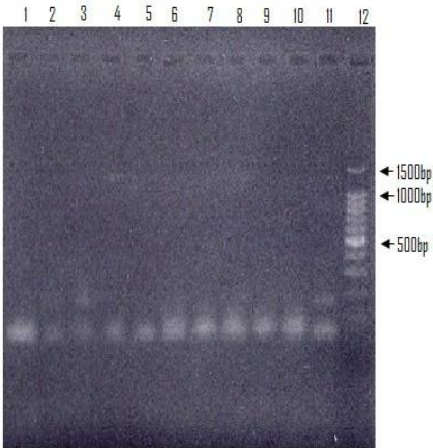


Figure 3: Electrophoresis of astrovirus PCR amplification products on 2.0% agarose gel. Lane 12 shows the 100bp molecular marker, Lanes 2 to 11 represent samples 1504, 1501, 1404, 1403, 1305, 1304, 1205, 1203, 1002 and 1001 respectively. Lane 1 shows the negative control. 500bp, 1000bp and 1500bp markers are indicated with arrows

treatment plant (Table 2). Bands observed for all positive samples had molecular weights \approx 170bp (Figure 3).

DISCUSSIONS

Nested PCR, an *in vitro* method for directed enzymatic amplification of specific targeted DNA sequences was employed. Nested PCR ensures very sensitive, specific and rapid detection of viruses with relative ease. Nested PCR also provides the ability to analyze a large number of samples including potential to eliminate false-positive results encountered with microscopy (Ali et al., 2004).

Rotavirus is known to be more resistant to environmental factors as compared to other enteric viruses including norovirus and astrovirus. Thus rotavirus is sometimes used as a surrogate for determining the occurrence of enteric viruses. As such 10 samples; 2 from each zone /site (Figure 1) which had previously tested positive for rotaviruses (Dongdem et al., 2010) were selected at random from each zone for norovirus and astrovirus detection. Noroviruses have been frequently associated with outbreaks of gastroenteritis linked to faecally contaminated oysters, shrimps and shellfish (Gallimore et al., 2005). In this study however, drinking water was examined for the presence of norovirus genotypes by partial characterization using genogroup specific RT-PCR. Norovirus GII was not detected in any of the 10 selected samples. As such no mixed infections of norovirus GI and GII was recorded. This observation is not out of place since observations made by Gallimore et al. (2005) showed similar findings. According to Kojima et al. (2002), the GII strains are more predominant in foods (e.g. shellfish, shrimps, etc.) and faecally contaminated specimens. However, since shellfish and shrimps contract viruses from the water environment, it is not clear why noroviruses are often present in fish and human specimens as GII strains but GI in the water environment. It is known that both

isolated from sample 1205. Four different GI types with molecular weights \approx 680bp, \approx 450bp, \approx 350bp and \approx 290bp were all however detected in the untreated sample (1001).
Four (40%) of selected samples contained GI strains with \approx 680bp, 3 (30%) contained norovirus GI type with \approx 290bp. Only the untreated water sample (1001) tested positive for norovirus GI type with molecular weight \approx 900bp. On the other hand, only samples 1501 and 1504 were found to contain norovirus GI type with molecular weight \approx 350bp (Figure 2).
Human astrovirus was detected in 3 (30%) of the 10 selected water samples. This included the raw water sample (1001) and 2 others (sample № 1501 and 1504) both located in zone V which was the furthest from the

norovirus genogroups occur in waterborne epidemics. For instance, in a 5-year study (1998 - 2002) in Finland, GII outbreaks even outnumbered those caused by GI noroviruses (86.9% vs. 13.1%) (Maunula and von Bonsdorff, 2005). In other waterborne outbreaks however, nearly half were caused by GI viruses (Maunula et al., 2005). This pattern of occurrence can be attributed to some differences in stability resulting from genetic differences. It may also be attributed to the ability of these viruses to spread from person to person in different genotypes or genogroups.

By virtue of the difference in molecular weight, 5 different norovirus GI strains with molecular weights approximately equal to (900bp, 680bp, 450bp, 350bp and 290bp) were identified (Figure 2). The strain with molecular weight approximately equal to 680bp was the most frequently detected, occurring in all 4 positive samples. Thus, the strain with molecular weight approximately equal to 680bp was the most resistant to environmental conditions and disinfection methods. The strain with molecular weight approximately equal to 900bp was detected in only the raw water sample (1001) as expected, an indication that norovirus PCR of untreated water sample was not inhibited. The pattern demonstrated by norovirus positive samples indicates a fair increase in number of contaminated samples with distance from the treatment plant through site A, and zones II and V.

Astrovirus typing RT-PCR analysis was also a rapid and effective detection tool. Even though sequence analysis was not done to reveal specific strains, Walter et al. (2001) considers the type specific primer pair; Mon 2 /prBeg employed as a confirmatory enough protocol. As confirmed by sequence study, this primer pair detects all HAstVs except HAstV-4 (Nadan et al., 2003). As such the possibility exists that HAstV-4 if present could not have been detected.

Astrovirus PCR of untreated water sample (1001) was not also inhibited. Astrovirus was detected as expected in the raw water but not in treated water through the distribution system except in samples 1501 and 1504 both in zone V (Figure 1). The fact that HAstV was detected at the ends of the distribution system (Figure 1) but not in any other area indicates that enteric viruses might have been introduced into the distribution system through damaged pipes. The HAstV type detected in all 3 samples (1001, 1501 and 1504) (Table 2) have the same molecular weight of approximately 170bp (Figure 3) showing that this strain is predominant in the municipal water distributed within western Accra.

Rotaviruses, noroviruses and HAstVs are usually considered to be less resistant to disinfection and environmental conditions as compared to many other types of viruses in municipal water. Their presence in municipal drinking water therefore suggests that there could be a broader range of pathogenic viruses which are comparatively more resistant in the water samples that

could not be detected by the methods employed (specific for detecting norovirus and astrovirus).

The average pH of all the water samples determined was 8.00, however, enteric viruses are known to be stable over a wide range of pH, for example between 3 and 10 (Fong and Lipp, 2005). This stability enables virus particles to survive a variety of food production, processing, storage conditions and disinfection. This property protects the viral genome from inactivation outside of infected cells and to enzymes that are present in the human gastrointestinal tract (Sair et al., 2002). Survival is however prolonged at neutral pH. Since the average pH (8.00) is within 3 – 10, pH (singly) cannot be a determining factor in the occurrence of the detected viruses in the water (Fong and Lipp, 2005).

Generally, enteric viruses are considered to be resistant to many disinfection processes (Ali et al., 2004; Cronin et al., 2001; WHO, 2002;). However, Abad et al. (1997) have shown that viruses inactivated by free chlorine were still detectable by molecular techniques. This suggests that molecular techniques might over estimate the true occurrence of active enteric viruses in treated water. On the other hand, evidence show that outbreak of noroviruses occurred with chlorine dosage between 0.07 - 0.3mg/L and required superchlorination of the municipal water system for effective disinfection. Recent data on astrovirus survival in chlorinated drinking water for instance showed that inactivation required between 0.5 – 1.0mg/L chlorine (Abad et al., 1997). The residual chlorine level recorded by the Mile 4 Laboratory (GWCL) located approximately 17.66 km from the treatment plant was between 0.1 – 0.15mg/L. This suggests that the level of free chlorine is insufficient to inactivate viruses, an indication that the detected viruses may be active.

Insufficiency in disinfection may also have been due to combined effect of pH and temperature. Physical characteristics of water especially temperature, pH, turbidity and exposure to sunlight affect disinfection. These environmental variables among others have been shown to have statistically significant effect on the stability of enteric viruses in surface fresh waters and can have a major impact on disinfection and pathogenic removal. For example, many disinfectants are pH dependent and may be inefficient when water is alkaline. An increase in pH from 6 to 9 reduces the effectiveness of free chlorine by a factor of 3 (LeChevallier et al., 1981). Therefore, pH has a great influence on the disinfection activity (Biojen, 1995). Thus, at an average pH of 8.00, minimal levels of HOCl is released and consequently lower disinfection ability. This phenomenon which explains the effect of pH on disinfection could also account for the persistence of viruses in the municipal water samples receiving what was believed to be adequate disinfection (Payment et al., 1985; Rose et al., 1986). The fact that these viruses survive disinfection practices raises concerns over whether these organisms

are more resistant to disinfectants or possibly their genomic organizations are altered by the disinfection process. Certainly, the above mentioned evidence suggests that the effectiveness of our disinfection proceedings is being over estimated while the elusiveness and survival of certain viral populations is being under estimated.

National recommendations for volumes of water to be tested vary between tens and hundreds of liters. Such volumes pose a serious practical problem for the testing laboratory. For viral detection by RT-PCR, a smaller volume (1 L) is preferred, as suggested by Gilgen et al. (1997). Independent of the concentration method, the increase in RT-PCR inhibitors usually sets limits on the water concentration. Sensitive methods are needed to detect viruses in environmental samples. Recent reports on the applicability and sensitivity of real-time RT-PCR for noroviruses also offer new possibilities to enhance its sensitivity (Beuret, 2004; Hohne and Schreier, 2004; Kageyama et al., 2003). Another factor is that the test then becomes more rapid, which is essential in monitoring water quality, particularly in epidemic situations. The third advantage is that a quantitative estimate of the contamination level is obtained (Maunula et al., 2005).

CONCLUSION

By virtue of molecular weights, we isolated different strains of norovirus and one strain type of astrovirus from water treated and distributed by the Weija Water Works. The level of viral contamination increased particularly at the region furthest away from the treatment plant. Disinfection methods currently in use do not seem to be adequate in reducing the viral load therefore increase in chlorine dosage should be considered by the water company in order to increase residual levels. Regular viral monitoring as part of microbial risk assessment in drinking water should also be considered by the national quality monitoring bodies. To ascertain the public health significance of these findings it will be necessary to conduct epidemiological studies relating virus occurrence to a defined health end point. Cell culture and quantitative RT-PCR, larger sample volumes and sequential analysis of viral genomes would be required to obtain conclusive evidence on the public health significance of this investigation.

ACKNOWLEDGEMENTS

We thank John Tetteh Amissah, Janathan Adjimani, George Armah, Isaac Odoi, Theresa Manful and Belinda Lartey of Noguchi Memorial Institute for Medical Research and Irenious Soyiri of the School of Public Health, University of Ghana for technical assistance. We

also thank Peace Colerangle, Teiku Cudjo, Newlove Amegashie and John Vitenu all of the Ghana Water Company Ltd for clearance, maps and technical advice.

REFERENCES

- Abad FX, Pinto RM, Villena C, Gajardo R, Bosch A (1997). Astrovirus survival in drinking water. *Appl. Environ. Microbiol.* 63: 3119 – 3122.
- Ali MA, Al-Herrawy AZ, El-Hawaary SE (2004). Detection of enteric viruses, Giardia and Cryptosporidium in two different types of drinking water treatment facilities. *Water Res.* 38: 3931 – 3939.
- Appleton H, Higgins PG (1975). Letter: Viruses and gastroenteritis in infants. *Lancet.* 1975 Jun 7;1(7919).
- Beuret C (2004). Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. *J. Virol Methods.* 115:1 – 8.
- Biojen (1995). Technical information; Chlorine_{rd} release. (<http://www.biojen.com/technical.htm>) (Accessed March 3rd, 2006).
- Blacklow NR, Cukor G (1981). Viral gastroenteritis. *N. Engl. J. Med.* 304: 397 – 406.
- Boom R, Sol CJ, Saliman MM, Jansen CL, Wertheim-van Dillen PM, van der Noord J (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28: 495 – 503.
- Branswell H (2008). Study suggests drinking water may be source of winter norovirus outbreaks. *The Canadian Press. Sunday Issue*, 2nd November, 2008.
- CDC (Centres for Disease Control and Prevention) (2001). "Norwalk-Like Viruses" Public Health Consequences and Outbreak Management ;Recommendations and Reports. *MMWW.* 1 – 18.
- Cronin J, Fox J, Maillard JY (2001). Variability and resistance of enteroviruses to disinfection. *Spotlight N° 80.* Wales Office of Research and Development for Health and Social Care. The National Assembly for Wales, U.K.
- Cruz RJ, Bartlett VA, Herrman EJ, Caceres P, Blacklow RN, Cano F (1992). Astrovirus - associated diarrhoea among Guatemalan ambulatory rural children. *J. Clin. Microbiol.* 30: 1140 – 1144.
- Dongdem JT, Adjimani J, Armah G (2010). Detection and characterization of human rotavirus in tap water by multiplex RT-PCR. *JMMS.* 1:223 – 230.
- Fong TT, Lipp EK (2005). Enteric viruses of humans and animals in aquatic environments: Health risks, detection and potential water quality assessment tools. *MMBR.* 69: 357 – 371.
- Gallimore CI, Cheesbrough JS, Lamden K., Bingham C, Gray JJ (2005). Multiple norovirus genotypes characterized from an oyster-associated outbreak of gastroenteritis. *Inter. J. Food Microbiol.* 103: 323 – 330.
- Gilgen M, Germann D, Luthy J, Hubner P (1997). Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. *Int. J. Food Microbiol.* 37(2-3): 189 – 199.
- Glass RI, Noel J, Mitchell D, Herrmann JE, Blacklow NR, Pickering LK, Dennehy P, Ruiz-Palacios G, de Guerrero ML, Monroe SS (1996). The changing epidemiology of astrovirus-associated gastroenteritis: a review. *Achieves of Virol.-Supplementum*, 12: 287 – 300
- Goodgame R (2006). Norovirus gastroenteritis. *Curr Gastroenterol Rep.* 8: 401 – 408.
- Herrmann JE, Taylor DN, Echeverria P, Blacklow NR (1991). Astroviruses as a cause of viral gastroenteritis in children. *New Engl. J. Med.*, 324: 1757-1760.
- Hohne M, Schreier E (2004). Detection and characterization of norovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. *J. Med. Virol.*, 72: 312 – 319.
- Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K (2003). Broadly reactive and highly sensitive assay for Norwalk-Like Viruses based on Real-Time Quantitative Reverse Transcription-PCR. *J. Clin. Microbiol.*, 41: 1548 – 1557.
- Keswick BH, Satterwhite TK, Johnson PC, DuPont HL, Secor SL, Bitsura JA, Gary GW, Hoff JC (1985). Inactivation of Norwalk virus in drinking water by chlorine. *Appl. Environ. Microbiol.* 50:261-264.
- Khan SA, Ahmed A, Khalid SM (1992). Diarrhea due to rotavirus and

- probability of sewage contaminations. *Journal of Islamic Academy of Sciences*, 5: 142-144.
- Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, Natori K, Takeda N, Katayama K (2002). Genogroup-specific PCR primers for detection of Norwalk-Like Viruses. *J. Virol. Methods*. 100: 107 – 114.
- LeChevallier MW, Evans TM, Seiderler RJ (1981). Effect of turbidity on chlorination efficiency and bacteria persistence in drinking water. *Appl. Environ. Microbiol.* 42: 159 – 167.
- Lew JF, Moe CL, Monroe SS, Allen JR, Harrison BM, Forrester DB, Stine SE, Woods PA, Hierholzer JC, Hermann JE (1991). Astrovirus and adenovirus associated with diarrhea in children in day care settings. *J Infect Dis.*, 164: 673 – 678.
- Lindesmith L, Moe C, Marionneau S, Ruvoen N, Jiang X, Lindblad J, Steward P, LePendur J, Baric R (2003). Human susceptibility and resistance to Norwalk virus infection. *Nature Medicine*, 9: 548 – 553.
- Maunula L, Kalso S, von Bonsdorff CH, Ponka A (2004). Wading pool water contaminated with both noroviruses and astroviruses as the source of a gastroenteritis outbreak. *Epidemiol. and Infection*, 132: 737 – 743.
- Maunula L, Miettinen IT, von Bonsdorff C-H (2005). Norovirus Outbreaks from Drinking Water. *Emerging Infectious Diseases*. *Emerg. Infect. Dis.*, 11: 1716 – 1721.
- Maunula L, von Bonsdorff CH (2005). Norovirus genotypes causing gastroenteritis outbreaks in Finland 1998–2002. *J. Clin. Virol.*, 34:186 – 94.
- Mitchell DK, Monroe SS, Jiang X, Matson DO, Glass RI and Pickering LK (1995). Virologic features of an astrovirus diarrhea outbreak in a day care center revealed by reverse transcriptase- Polymerase Chain Reaction. *J. Infect Dis.*, 6:1437 – 1414.
- Nadan S, Walter JE, Grabow WOK, Mitchell DK, Taylor MB (2003). Molecular characterization of astroviruses by reverse transcriptase PCR and sequence analysis: Comparison of clinical and environmental isolates from South Africa. *Appl. Environ. Microbiol.*, 69: 747 – 753.
- Oishi I, Yamazaki K, Kimoto T, Minekawa Y, Utagawa E, Yamazaki S, Inouye S, Grohmann GS, Monroe SS, Stine SE (1994). A large outbreak of acute gastroenteritis associated with astrovirus among students and teachers in Osaka, Japan. *J. Infect. Dis.*, 170: 439 – 443.
- O'Ryan M, Prado V, Pickering LK (2005). A millennium update on pediatric diarrheal illness in the developing world. *Semin Pediatr Infect Dis.* 16:125 – 136.
- Payment P, Tremblay M, Trudel M (1985). Relative resistance to chlorine of poliovirus and coxsackievirus isolates from environmental sources and drinking water. *Appl. Environ. Microbiol.*, 49: 981 – 983.
- Roberts CM, Archer J, Renner T, Heidel PA, VandeBunte DL, Brennan BM, Croker C, Reporter R, Nakagawa-Ota S, Hall AJ (2009). Norovirus Outbreaks on three College Campuses - California, Michigan and Wisconsin, 2008. *Morbidity and Mortality Weekly Report*. 58:1095 – 1100.
- Rose JB, Gerba CP, Singh SN, Toranzos GH, Keswick B (1986). Isolating viruses from finished water. *AWWA*. 78: 56 – 61.
- Sair AI, D'Souza DH, Jaykus LA (2002). Human enteric viruses as causes of food-borne disease. *Comprehensive Reviews in Food Science and Food Safety*. 1: 73 – 77.
- Tucker AW, Haddix AC, Bresee JS, Holman RC, Parashar UD, Glass RI (1998). Cost-effectiveness analysis of a rotavirus immunization program for the United States. *JAMA*. 279: 1371-1376.
- Walter JE, Mitchell DK, Guerrero ML, Berke T, Matson DO, Monroe SS, Pickering LK, Ruiz-Palacios GR (2001). Molecular epidemiology of human astrovirus diarrhea among children from a Periurban community of Mexico city. *J. Infect. Dis.*, 183: 681 – 686.
- WHO (World Health Organization) (2002). Enteroviruses-non polio. (<http://www.who.int/mediacentre/factsheets/fs174/en/>) (Accessed January 19th, 2005).
- Widdowson MA, Sulka A, Buleus SN, Beard RS, Chaves SS, Hammond R, Salehi ED, Swanson E, Totaro J, Woron R, Mead PS, Bresee JS, Monroe SS, Glass R (2005). Norovirus and foodborne disease, United States, 1991 - 2000. *Emerg Infect Dis.* 11: 95 – 102.
- Yao X, Hu J-F, Daniels M, Shiran H, Zhan X, Yan H, Lu H, Zeng Z, Wang Q, Li T, Hoffman AR (2003). A methylated oligonucleotide inhibits IGF2 expression and enhances survival in a model of hepatocellular carcinoma. *J. Clin. Invest.* 111:265 – 273.