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Detection and characterization of human rotavirus in tap water by multiplex RT-PCR

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More than a billion diarrheal cases occur each year among children below five years resulting in high morbidity and mortality. Rotaviruses, the single leading cause of diarrhea in children below five years, cause more than 130 million episodes of severe diarrhea throughout the world. While treated water has been identified as a route of transmission, rotavirus in treated water has not been investigated in Ghana. Since the presence of rotaviruses in drinking water is unacceptable, a virological survey to detect and characterize rotavirus was conducted on tap water in the western part of Accra. Treated water samples were collected from five zones within the distribution network of Weija Water Works. Two litres of each sample were concentrated 4,000 fold and the viral particles extracted. Viral RNA was extracted from all concentrates using phenol/chloroform, purified with the RNaid[®] kit and reverse transcribed. The cDNA was amplified by semi-nested PCR using P and G genotype-specific primers and analyzed an agarose gel. Rotavirus was detected in 48.1% of samples. Nine rotavirus P-types and 12 G-types were detected. The detection pattern showed increased viral pollution with distance from the treatment plant and in areas with high human activity.

Keywords: Characterization, detection; Ghana; rotavirus diarrhea; RT-PCR; tap water.

INTRODUCTION

Diarrhea remains one of the principal causes of pediatric morbidity and mortality 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). A contributing factor to the high incidence of diarrheal cases in developing countries is consumption of contaminated food and water resulting from inadequate sanitation and access to clean water. A broad range of enteric viruses including rotavirus, norovirus, astrovirus, adenovirus types 40 and 41 have been implicated in the aetiology of diarrhea (Akan et al., 2009; Sack et al., 1997; Tayeb et al., 2008; Walter et al., 2001).

Several studies have documented the presence of enteric viruses in both raw water (surface and ground waters) and treated drinking water (Federal-Provincial-Territorial Committee on Drinking Water, 2010; Lee and Kim, 2002). Common water treatment processes including chlorination have not been shown to completely eliminate these viruses in water (De Leon and Jaykus, 1997; Tyrrell et al., 1995). The presence of enteric viruses in treated drinking water and their sources has become a public health concern (Craig, 2000; Espinosa et al., 2009). This is because enteric viruses are more resistant compared to coliform bacterial indicators in the water environment and can cause serious disease in susceptible individuals (Craig, 2000). A low viral dose of between 1 and 50 tissue culture infectious units is enough to cause illness (Moe, 1991). For these reasons, new and advanced molecular diagnostic assays which are simple and can rapidly detect and simultaneously

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quantify viruses in the water environment are urgently needed.

Although contaminated food and drinking water have been associated with diarrheal outbreaks, the role of rotavirus contaminated water in the transmission and spread of rotavirus disease is unknown and has never been studied in developing countries like Ghana. Rotaviruses have been estimated to cause 25 - 35% of all cases of severe diarrheal disease (Glass et al., 2005) resulting in a significant economic impact on society in terms of direct medical costs, loss of work hours or school hours, quality of life and mortality. More than 130 million episodes of severe diarrhea cases in children under five years throughout the world are caused by rotavirus infection (Glass et al., 1999). Annually, rotavirus gastroenteritis is responsible for over 1,250,000 episodes of diarrhea and about 527,000 deaths, mainly in developing countries (Racaniello 2010). Gastroenteritis outbreaks resulting from rotavirus contamination of treated municipal drinking water have been documented (Ali et al., 2004; Cho et al., 2000). Therefore, more rotavirus transmission research in Africa, institution of preventive measures against rotavirus spread and an urgent need to complete clinical trials and implement the use of rotavirus vaccines including *RotaTeq* and *Rotarix* in Africa will be required (CDC 2010; De Vos et al., 2004; Vesikari et al., 2006).

First discovered in 1973 from duodenal biopsies of children with diarrhea (Bishop et al., 1973) rotaviruses are the most important cause of pediatric diarrhea. The genus rotavirus has been classified into groups, subgroups and serotypes based on viral capsid proteins. Seven (7) Groups (A, B, C, D, E, F and G) have been identified. The outer capsid of rotavirus group A consists of VP7 and VP4 proteins that carry independent neutralization and protective antigens (Blutt et al., 2004; Estes 2001; Kapikian et al., 2001; Santos et al., 2005). Based on the type of antibodies induced by either protein in humans and animals, group A rotaviruses have been divided into 2 genotypes; VP7 and VP4 genotypes. So far 15 VP7 (G) types have been identified (Santos et al., 2005) of which 4 (G1 – G4) are epidemiologically important. Similarly, 24 VP4 (P) types have been reported, of which two (P [8] and P [4]) are common globally (Santos et al., 2005). Clinical symptoms of rotavirus infection include diarrhea, fever and vomiting (Chiu et al., 2000; Sungkapalee et al., 2006).

In Ghana, there is little information on viral contaminants of drinking water. This is probably due to the absence of infrastructure and technology for assessing viral contamination of water. In fact, the Ghana Standards Board does not include criteria for virological quality of water in its certification due to cost implications, lack of capacity and the lengthy nature of virological analysis (Ghana Standards Board, 1997). This in turn makes it difficult to determine the risks associated with

the presence of viruses in drinking water and the development of diarrheal disease.

In the Greater Accra Region of Ghana, there are two Head Works that treat water from two dams for consumption by the metropolis. These are the Weija and Kpong Head Works. Water from the Weija dam which is relatively more polluted is treated by the Weija Head Works and distributed to over 1,452,863 consumers living in West Accra via a network of pipes (Tawiah and Wadieh, 2005). These distribution pipes, which are mostly old and made of plastic, are often subjected to various degrees of physical damage, providing routes for fecal and other contamination to enter the drinking water system. The aim of this investigation was, therefore, to test treated water along the distribution lines encompassing West Accra for rotavirus contamination by a multiplex semi-nested PCR using type-specific primers. The information generated should provide justification for research in the supply of clean, safe virus-free drinking water in developing countries and to identify interventions that prevent or destroy viral contaminants of water and advocate for the introduction of rotavirus vaccines in Africa.

MATERIALS AND METHODS

Collection of samples

A map of the distribution network from Weija Water Works was obtained and divided into 5 zones (Figure 1). Zoning was done according to distance from the treatment plant. Two and half litres of tap water samples were obtained in sterilized rubber containers from 27 sites. Five samples were obtained in each of 5 zones within the distribution network and two samples were obtained from the Weija dam and immediately after treatment at the Adam Clark Plant, Weija Water Works (Site A) and stored at 20°C until concentration procedure.

Concentration of water samples and elusion of viruses from filter

Two litres of each sample was concentrated 4,000 fold using the stirred ultrafiltration cells (Amicon®Ltd, Great, Britain) at a maximum pressure of 5.3kg/cm². Regenerated cellulose ultrafiltration membranes (Amicon® Bioseparations, Bedford, USA) of 10⁵ nominal molecular weight limit (NMWL) were used for filtration. Viruses that had adsorbed to the filter were eluted with 0.5 ml of 3% beef extract (DIFCO Laboratories, Detroit, USA) in 0.05M glycine (Introgen Life Technologies, Auckland, New Zealand) buffer, pH 9.5. The concentrates were stored at 4°C.

Extraction and purification of viral RNA

Viral RNA was extracted from all concentrates by the phenol/chloroform method, purified with the RNaïd® kit (Bio 101, Carlsbad, USA), and stored at 4°C (Boom et al., 1990). Briefly, sodium acetate containing 1% sodium dodecyl sulphate; pH 5.0 was used to stabilize the RNA and to disrupt proteins. Phenol/chloroform and guanidinium isothiocyanate were used to

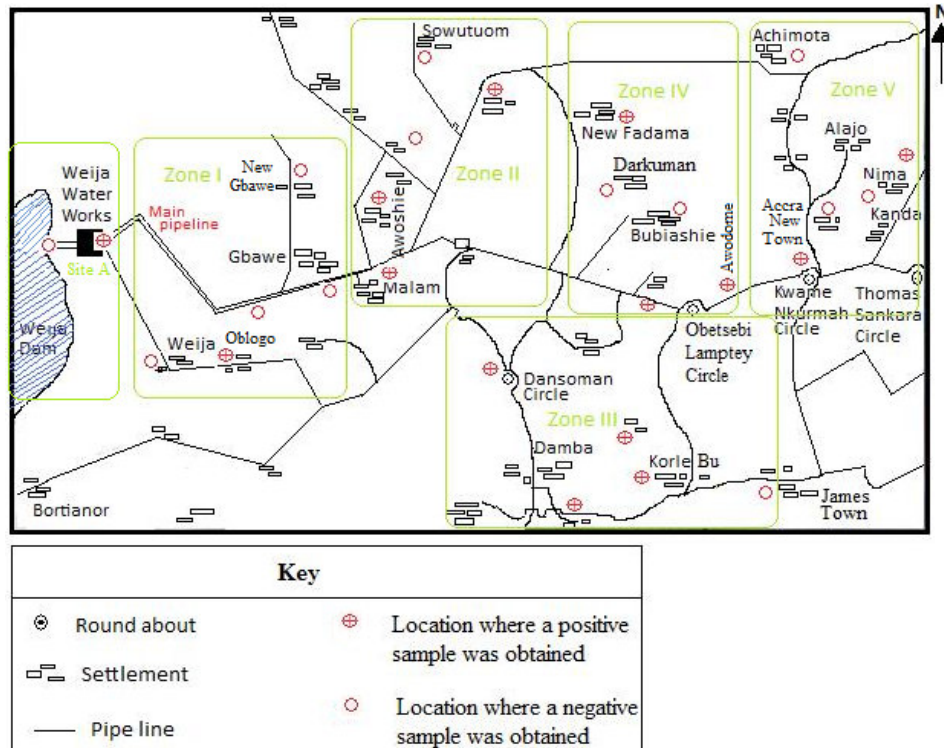


Figure 1. Schematic representation of Weija Water Works, the water distribution network and the five defined zones where drinking water was collected.

Table 1. Oligonucleotide primers used for VP7 or G and VP4 or P genotyping of rotavirus strains (Gouvea et al., 1990; Gentsch et al., 1992)

Primer	Sequence	Position (nt)	Strain (genotype)
sBeg 9	GGCTTTAAAAGAGAGAATTC	1-21	Group A
End 9	GGTCACATCATAAATTCTAATCTAAG	1062-1036	Group A
aBT-1	CAAGTACTCAAATCAATGATGG	314-335	Wa (G1)
aCT-2	CAATGATATTAACACATTTTCTGTG	411-435	DS-1(G2)
aET-3	CGTTTGAAGAAGTTGCAACAG	689-709	P (G3)
aDT-4	CGTTTCTGGTGAGGAGTTG	480-498	ST-3 (G4)
aAT-8	GTCACACCATTTGTAAATTCG	178-198	69M (G8)
aFT-9	CTAGATGTAACTACAACACTAC	757-776	W161(G9)
RVG-9	GGTCACATCATAAATTCT	1062-1044	Group A
Con 2	ATTTCCGACCATTTATAACC	868-887	Group A
Con 3	TGGCTTCGCCATTTATAGACA	11-32	Group A
1T-1	ACTTGGATAACGTGC	339-356	KU(P8)
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5(P4)
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076(P6)
4T-1	TGAGACATGCAATTGGAC	385-402	K8(P9)
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M(P10)

further disrupt and degrade lipids, proteins and other compounds present in the water samples and to inhibit RNase. The RNaïd[®] matrix was used to adsorb the extracted RNA on silica particles, remove any unwanted proteins or salts with RNaïd[®] wash buffer and re-suspended the purified RNA in DEPC treated water.

Detection and characterization of rotavirus

A cDNA of rotavirus VP7 and VP4 genes was produced in a reverse transcription reaction with VP7 and VP4 specific primers

(Table 1). For the VP7 reaction, sBeg 9 and End 9 (GIBCO BRL, Gaithersburg, USA) were added to purified dsRNA in a reaction mixture containing dNTPs (Promega, Madison, USA), avian myeloblastosis (AMV) reverse transcriptase and buffer (Promega, Madison, USA) (Gouvea et al., 1990). Similarly, Con 2 and Con 3 (GIBCO BRL, Gaithersburg, USA) primer pair was added to dsRNA in a reaction mixture as above for the VP4 reverse transcription reaction (Gentsch et al., 1992). The mixtures were then incubated for 20 min at 42°C. Each cDNA obtained was amplified by PCR in a reaction mix containing dNTPs, 25 mM MgCl₂ and Taq polymerase

Table 2. Total number of samples collected and number of rotavirus positive samples per zone. The average distance from the water treatment plant has also been indicated.

Zone/ Site	№ of positive samples (per zone)	Total № of samples (per zone)	Average distance from water treatment plant (km)
A	1	2	0.00
I	1	5	4.99
II	2	5	9.59
III	4	5	13.36
IV	3	5	13.59
V	2	5	17.66
Total	13	27	

(Promega, Madison, USA). Negative controls containing double distilled water in place of sample were incorporated simultaneously in all steps in each experiment. Rotavirus VP4 and VP7 standard positive samples were run along with the water samples to examine the quality of extraction, PCR and electrophoresis procedures. The PCR was run for 30 cycles preset at 94°C for 1 min (denaturation), 42°C for 2 min (annealing) and 72°C for 3 min (amplification) with a final elongation step at 72°C for 7 min.

First round VP7 cDNA was added to another PCR reaction mixture containing 6 serotype specific primers (aBT-1, aCT-2, aET-3, aDT-4, aAT-8 and aFT-9) and an RVG9 primer (Table 1) and subjected to a second round (nested) PCR cycle as above (Gouvea et al., 1990). Similarly, the nested PCR amplification of partial length VP4 cDNA was performed using a consensus primer (Con 3) and a cocktail of serotype specific primers (1T-1, 2T-1, 3T-1, 4T-1 and 5T-1) and amplified in a reaction mixture as above (Gentsch et al., 1992). The nested PCR amplified products were electrophoresed at 100V for 1hr on a 2.0% agarose gel containing ethidium bromide and visualized on a UV Transilluminator. The amplicon sizes were estimated using a 100bp molecular weight marker (Bioron, GmbH, Germany).

RESULTS

Rotavirus VP7 or VP4 amplicons were detected in 48.1% (13/27) of samples examined (Table 2). Of the 2 samples collected in site A, 1 sample (1002) tested positive for rotavirus while 1/5, 2/5, 4/5, 3/5 and 2/5 samples each from zones I, II, III, IV and V, respectively, tested positive for rotavirus (Table 2). Within the 5 zones in the distribution network, 13 samples were collected from public taps and 12 from private supply lines of which 6/13 of samples collected from public taps and 7/12 samples collected from private lines were positive for rotavirus (Table 3).

Figures 2 and 3 indicate genotype amplicons generated during second round PCR amplification of selected samples for VP4 and VP7, respectively. Two samples, 1203 and 1403 (Figure 2) produced substantial amplicons that were noted as bands with high intensity on the agarose gels. Of the 9 water samples where a P types could be identified, P[6] (3/21) and P[8] (6/21) genotypes were detected. The remaining 12 water samples contained group A rotavirus G types including G1 (1/21), G2 (8/21), G3 (1/21) and G9 (2/21). The

complete genotype conformation (G and P type) was only found in 4 samples.

The highest number of positive water samples occurred in zones III and IV with zone IV having the highest rotavirus strain diversity (Table 3). Of the 9 P types detected, 6 were P[8] and occurred frequently throughout all the zones studied. The remaining 3 rotavirus strains were P[6] and occurred in 2 samples in zone III. Mixed infections of P[8]P[6] were also detected in zone I. Rotavirus G2 strains (8/12) were frequently detected in zones II, III, IV and V. Mixed infections of G2G9 (1/12) and G2G3G9 (1/12) were detected in zone IV. G and P combinations detected included P[6]G2 (2/30) and P[8]G2 (2/30).

DISCUSSIONS

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

Reverse transcription multiplex semi nested PCR showed that 13/27 tap water samples collected from public and private taps were positive for rotavirus. While rotavirus was not detected in the untreated water (1001) by nested PCR amplification, it was detected at the treatment plant (1002) immediately after treatment. This suggests that the water is polluted from source (Weija dam) and that the treatment process, particularly disinfection, was inefficient. Possible sources of contamination of the dam may include surface runoff, flooding and poor sewage disposal among others. In fact, a refuse dump owned by the Accra Metropolitan Assembly (AMA) located at Oblogo (zone I, ≈ 5.0km away from the dam) might be a big source of contamination by surface runoff or flooding, which are frequent in the area. In addition, viruses could have escaped disinfection due to operational factors such as

Table 3. Characterization of rotavirus VP4 and VP7 by RT-PCR in tap water obtained from the western part of Accra. Public tap samples were obtained from taps for general use and private line samples were obtained from inside homes.

Sample №	Source	Zone	Rotavirus RT-PCR	Genotyping result	
				VP7	VP4
1001	Untreated water (dam)	Site A	Neg	Neg	Neg
1002	Treated water	Site A	Pos	G1	Neg
1101	Public tap	I	Neg	Neg	Neg
1102	Private line	I	Pos	Neg	P[8]P[6]
1103	Private line	I	Neg	Neg	Neg
1104	Public tap	I	Neg	Neg	Neg
1105	Public tap	I	Neg	Neg	Neg
1201	Private line	II	Neg	Neg	Neg
1202	Private line	II	Neg	Neg	Neg
1203	Private line	II	Pos	G2	Neg
1204	Public tap	II	Neg	Neg	Neg
1205	Public tap	II	Pos	G2	P[8]
1301	Private line	III	Neg	Neg	Neg
1302	Private line	III	Pos	G2	Neg
1303	Public tap	III	Pos	G2	P[6]
1304	Public tap	III	Pos	G2	P[6]
1305	Private line	III	Pos	Neg	P[8]
1401	Private line	IV	Pos	Neg	P[8]
1402	Public tap	IV	Pos	G2G3G9	Neg
1403	Private line	IV	Pos	G2G9	Neg
1404	Public tap	IV	Neg	Neg	Neg
1405	Private line	IV	Neg	Neg	Neg
1501	Public tap	V	Pos	G2	P[8]
1502	Public tap	V	Neg	Neg	Neg
1503	Public tap	V	Pos	Neg	P[8]
1504	Public tap	V	Neg	Neg	Neg
1505	Private line	V	Neg	Neg	Neg

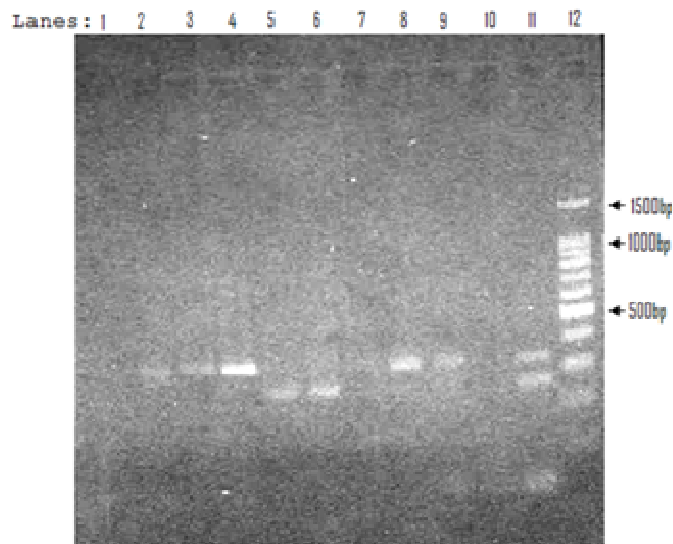


Figure 2. Electrophoresis of rotavirus VP4 genotyping PCR amplification products on 2.0% agarose gel. Lane 1 shows the negative control. Lanes 2 to 11 represent samples 1503 (P[8]), 1401 (P[8]), 1305 (P[8]), 1304 (P[6]), 1303 (P[6]), 1302 (Neg), 1301 (P[8]), 1205 (P[8]), 1103 (Neg) and 1102 (P[8]P[6]), respectively. Lane 12 shows the 100bp molecular marker.

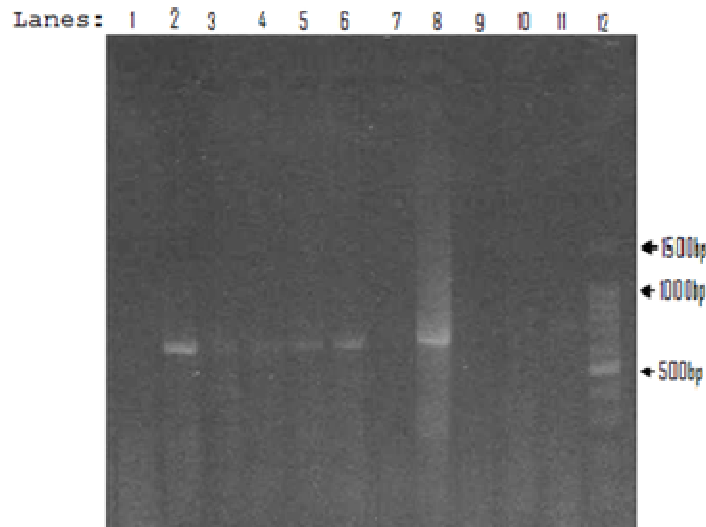


Figure 3. Electrophoresis of rotavirus VP7 genotyping PCR amplification products obtained on 2.0% agarose gel. Lane 1 shows the negative control. Lanes 2 to 11 represent samples 1304 (G2), 1303 (G2), 1302 (G2), 1501 (G2), 1205 (G2), 1204 (Neg), 1203 (G2), 1103 (Neg), 1102 (Neg) and 1002 (G1), respectively. Lane 12 shows the 100bp molecular marker.

inadequate floc formation, floc break down, filter overloading, etc. (Ali et al., 2004).

The failure to detect rotavirus in sample 1001 may be due to numerous factors including the small sample volume utilized for analysis, only one sample was taken from each location, time of sampling, location on the dam the sample was taken and material or compounds in the original water sample that inhibited the PCR reaction (Richards 1999). Most of the amplicon bands obtained after electrophoresis were faint, indicating a relatively low concentration of rotaviruses or the presence of inhibitors affecting the PCR result. This may be partially due to small volumes (2 L) of samples analyzed since the concentration of viruses in environmental samples is low (Ali et al., 2004). The higher percentage of positive samples collected from private lines may be an indication that the reservoirs and water storage facilities may be a source of contaminations or that members of the households are asymptomatic carriers. The circulating rotavirus genotypes isolated (Table 3) from treated water are in concordance with earlier findings by Asmah et al (2001) and Armah et al (2003) from clinical samples in rural Ghana, however G2 was most frequently isolated in treated drinking water.

The gradual increase in rotavirus positive samples with increasing distance from the water treatment plant (Table 1) may be due to the increasing level of contamination due to poorly maintained water pipes or may indicate pockets of infected individuals or asymptomatic carriers. High rotavirus contamination in zone III suggests that the water distribution system in this area is not adequate and

that water pipes may be broken, exposing communities along the pipe to extensive viral and fecal contamination.

Enteric viruses are known to be stable over a wide range of pH (3-10) (Fong and Lipp, 2005) and are considered to be resistant to many disinfection processes (Ali et al., 2004; Cronin et al., 2001). Residual chlorine level recorded by the Mile 4 laboratory (GWCL) located approximately 18km away from the Weija Water Works range between 0.1 and 0.15mg/L (data not shown). Recent data on human astrovirus survival in chlorinated drinking water showed that inactivation required between 0.5 and 1.0mg/L_{vv} (Abad 1997; WHO 2002). These results suggest that the level of free chlorine in the water from the treatment plant is insufficient to inactivate the enteric viruses present.

Insufficient disinfection may also be due to a combined effect of physical factors such as pH and temperature, where an increase in pH from 6 to 9 reduces the effectiveness of free chlorine by a factor of 3 (LeChevallier et al., 1981). In fact, the average pH of water measured 18km away from Weija Water Works is 8.00 (data not shown), suggesting that lower levels of HOCl will be released and that disinfection is suboptimal. This phenomenon could also account for the persistence of rotaviruses in municipal water (Sack et al., 1997).

Since rotaviruses seem to persist in drinking water, it is important to protect both open and stored water and to use more stringent water purification methods to prevent waterborne disease outbreaks. Furthermore, based on the finding in this paper, the introduction of vaccines to prevent rotavirus infection is vital. The rotavirus vaccine

program is currently facing challenges with the US Food and Drug Administration (FDA) advising that the administration of the Rotarix vaccine (GlaxoSmithKline, United States) be suspended. This action comes after an independent research group found that the vaccine contains DNA of porcine circovirus type 1 (Racaniello, 2010). Also, RotaTeq (Merck and Co, Inc., United States) has faced challenges with pronounced site effects including intussusceptions. However, the European Medicines Agency (EMA) and the World Health Organization (WHO) have not withdrawn recommendations for the use of rotavirus vaccines. Therefore, future interventions to combat rotavirus disease may include both the introduction of a safe effective rotavirus vaccine and the supply of safe clean virus-free water.

Conclusion

Water treated and distributed by the Weija Water Works was found to contain several strains of rotavirus. The level of viral contamination increased with distance from the plant. The prevalence of rotaviruses was also found to increase in areas of high human activity. Disinfection methods currently in use do not seem to be adequate in inactivating viruses in the water. Increase in chlorine dosage which will in turn raise residual chlorine levels in the water should be considered by water companies. Legislative measures for regular viral monitoring as part of microbial risk assessment in drinking water should also be considered by the national quality monitoring bodies. Future studies may have to include conducting epidemiological studies to relate rotavirus occurrence to a defined health end point and to ascertain the public health significance of rotavirus in drinking water. Cell culture and quantitative RT-PCR, larger sample volumes and multiple samples from water sources and sequential analysis of viral genomes would be required to obtain conclusive evidence on the public health significance of the results presented in this paper.

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