Microbiological Assessment and Detection of Adenovirus in Sachet Water Sold In Abeokuta, Nigeria

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Abstract: Microbiological safety of sachet water remains a public health problem in Nigeria. This study was aimed at investigating some packaged sachet water sold in Abeokuta, South-West Nigeria for the microbiological safety including some of the enteric viruses on contaminant candidate list. Sachet water samples from five different producers were obtained over three month's period. Bacterial and fungal analyses were conducted with standard culture method. Targeted protozoans were investigated by microscopic examination of sediments obtained after centrifugation. Nested and semi-nested polymerase chain reaction (PCR) techniques targeting specific genes in adenovirus, norovirus and rotavirus were used for viral analyses. Results were presented in presence-absence score. Contingency table was used to establish relationship between viruses, *Escherichia coli* and protozoans. Out of a total twenty pooled samples analysed**,** adenovirus had a prevalence rate of 10% across the study period, whereas rotavirus and norovirus were absent. *Giardia* cysts and *Cryptosporidium* oocysts were also absent. *Escherichia coli* was present in 40% of the brands. Other bacteria identified were *Salmonella enterica* serovar Typhi, *Shigella dysentariae*, and *Pseudomonas aeruginosa*. *Aspergillus* sp, *Mucor* and *Rhizopus* sp. were present in some samples collected. *A*denovirus was detected by PCR in a pooled sample of sachet water that tested negative for *Escherichia coli*, *Cryptosporidium* oocysts and *Giardia* cysts. There is need for microbiological screening of sachet water periodically in order to enhance public health safety.

Key words: Adenovirus, Fungi, Norovirus, Protozoans, Rotavirus, Sachet water

INTRODUCTION

Poor water quality is associated with an estimated 3.5 billion diarrhoeal episodes and 1.87 million diarrhoeal-associated childhood deaths annually (Arnold and Colford, 2007; Boschi-Pinto *et al.,* 2008). Of these deaths, 90% occur in children from developing countries and this high proportion accounted for nearly 20% of the 10 million total deaths per annum in children under 5 years of age (Boschi-Pinto *et al.,* 2008; UNICEF, 2008). Pathogenic bacteria, viruses and protozoans are well known microbial contaminants of drinking water (Szewzyk *et al.,* 2000), although fungi are considered emerging chronic water quality problem (Hageskal *et al*. 2009; Ashbolt, 2015).

Waterborne viral infection is one of the most important causes of human morbidity (Fongaro *et al.,* 2013). Waterborne viruses have gained attention worldwide as emerging pathogens because of their low infectious dose, survival in water and considerable health impacts (Swenson *et al.,* 2003; Fong and Lipp 2005; Xagoraraki *et al.,* 2007). As part of the Safe Drinking Water Act, United State Environmental Protection Agency (USEPA) listed adenoviruses and noroviruses as two of the four viral groups on the "Contaminant Candidate List'' (CCL) (Miagostovich *et al.,* 2008; Teunis *et al.,* 2008; USEPA, 2009).

In Nigeria, many households in rural and urban areas consume packaged sachet water (Odeyemi, 2015) because it is cheap, affordable, and readily available and also because of its perceived safety (Dada, 2009). There have been several studies on bacteriological quality of different brands of sachet water marketed in Nigeria using bacterial indicators without cognizance of virological quality.

Absence of bacterial indicators of feacal contamination does not necessarily guarantee consumers' safety from enteric pathogens like viruses (USEPA, 1998; Xagoraraki *et al.,* 2007; Hssaine *et al.,* 2011; Armon, 2015) and due to the cost of analysis, frequent virus monitoring programmes are currently impractical (Matthijnssens *et al.,* 2008). Viral contamination is best monitored by direct detection of the pathogens themselves without using indicators as a proxy (Armon, 2015). This study was therefore designed to assess some brands of sachet water sold in Abeokuta, South-West Nigeria for the presence of pathogens including some of the viruses on contaminant candidate list.

MATERIALS AND METHODS

Study Area

Abeokuta is one of the most prominent urban settlements in the SouthWestern Nigeria (Bello and Falano, 2017). It is the capital of Ogun State, lying between latitude 7^0 06' and 7^0 13' North and longitude 3^0 15' and 3^0 25' East (Olowofela *et al*., 2013). Situated within the rainforest belt of the tropics, the city occupies a geographical area of 1256sqkm with a population of about 449,088 inhabitants (National Population Commission, 2006). The city is approximately 100km north of Lagos and 80km Southwest of Ibadan, the Oyo State capital. Inadequate public water supply is a major problem in the city (Odjegba *et al*., 2015).

Sachet water sampling

A total of 60 sachet water samples were collected from five different producers. Water samples for viral analyses were frozen while others were stored at 4°C and analysed within 6 h of collection. Samples were pooled into a total of twenty (20) for analyses. Samples for viral analyses were transported to the virology laboratory of the Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria.

Microbiological analyses

Water samples were inoculated by spread plate method on molten nutrient agar and incubated at 37°C for 24 h for total heterotrophs, MacConkey agar incubated at 37°C for 24 h for coliforms, and eosin methylene blue (EMB) agar incubated at 44°C for 24 h for *Escherichia coli*. Gram's staining and biochemical tests such as oxidase, catalase, sugar fermentation, urease reaction, lysine decarboxylase, indole test, and H_2S production were performed to identify bacterial isolates using Bergey's Manual of Systematic Bacteriology (Garrity *et al.,* 2004).

Fungi were detected by inoculating water samples on Sabouraud dextrose agar (SDA) and incubated at 25 ºC for 7 days (Gottlich *et al.,* 2002). Microscopic identification was based on morphological characteristics of spores, macroconidia, microconidia, rhizoids and stolons according to Barnett and Hunter (2006).

Cryptosporidium parvum and *Gardia lamblia* were detected by repeated centrifuging of ten 50ml of each sample at 2,500 rpm for 5min. and the sediments obtained were subjected to microscopic examination using x 10 and x 40 objective lenses (Kwakye-Nuako *et al*., 2007; Chinyelu *et al.,* 2010).

Viral analysis was carried out by following concentration protocol as described by WHO, (2003) and Hsainne *et al.* (2011). Sachet water (500ml of pooled samples) was collected and 39.5 ml of 22% dextran, 287ml 29% PEG6000, and 35 ml 5N NaCl were added. It was mixed thoroughly and kept in constant agitation for 1 h at 4°C using a horizontal shaker. Afterwards, the mixture was transferred into a sterile one litre separation funnel attached to retort stand and left overnight at 4°C. In the morning, 5.0 to 10 ml of lower layer was collected into sterile 50 mL centrifuge tube and stored at -20^0C until analysed.

Viral nucleic acid was extracted using Qiagen viral mini kit (Qiagen, Germany) according to manufacturer's instructions. For rotavirus and norovirus cDNA synthesis, SCRIPT cDNA synthesis kit (Jena Bioscience, Germany) was used according to manufacturer's instruction. Briefly, for a 20.0µL cDNA mix, 12.0µl of extract was added to 0.5µL SCRIPT reverse-transcriptase, 4.0µL of SCRIPT RT buffer complete, 1.0µL dNTP mix, 1.0µL of DTT, 0.5µL of random hexamers, and 1.0µL RNase inhibitor. The reaction was incubated at 42^0C for 10 minutes followed by 50^0C for 60 minutes in a Veriti Thermal cycler (Applied Biosystems, USA). The cDNA was stored at -80^0C until analysed.

PCR procedure for viral gene amplification

Target genes, and primers for viral gene amplification are presented in Table 1. PCR was done in 25µL volumes containing 5.0µL of Red load Taq (Jenabioscience), 5.0µL of Nucleic acid extract (for the adenovirus screen) or cDNA (for norovirus and rotavirus), 1µL of each primer and 13µL of RNase free water for the first round of PCR. Thermal cycling was 94^0C for 3 min, 45 cycles at 94^0C for 30 s, 56^0C for 30 s and 72^0C for 30 s. This was followed by 72^0C for 7 min and held at 4^0C till terminated. Amplicons from the first round of PCR were used as template for the second set of primers and the second PCR. All PCR assays were executed in a Veriti Thermal cycler (Applied Biosystems, USA) and all PCR products were resolved on 2% agarose gels stained with ethidium bromide and viewed using a transilluminator.

RESULTS

Detection of microorganisms of interest found in sachet water brands

Bacteria, fungi and viruses detected in sachet water samples are presented in Table 2. *Escherichia coli* was not detected in brands 1, 4 and 5 within the period of study while in brands 2 and 3, *Escherichia coli* Also, fungal analysis revealed varying levels of predominance of *Aspergillus sp.* (50%), was detected. Fig. 1 shows *Escherichia coli* (17%), Salmonella *enterica* serovar T*yphi* (14%), *Pseudomonas aeruginosa* (3%), *Enterobacter aerogenes* (6%), *Shigella dysentariae* (11%), *Staphylococcus aureus* (20%), and *Klebsiella aerogenes* (29%). Fig. 2 shows the occurrence of *Aspergillus niger* (50%), *Rhizopus oryzae* (40%) and *Mucor* mucorales (10%) in the sachet water sampled. Also, no ova, cyst, oocyst, trophozoite, or adult form of any protozoans was seen in all samples.

For enteric viruses, only 2 **s**ample tested positive for adenovirus over the study period. Rotavirus and norovirus were not detected in all water samples collected. A cross tabulation of relationship between viruses (norovirus, adenovirus and rotavirus) examined versus the protozoans showed a close relationship of 100%, 93%, 100% respectively. The absence of these protozoans was observed to consistently indicate the absence of rotavirus, and norovirus in sachet water being investigated. *Escherichia coli* on the other hand indicated poor relationship to rotavirus, adenovirus and norovirus (60%, 57% and 60%) respectively (Table 3).

DISCUSSION

Water borne diseases remain a challenge in both developed and developing countries. Infectious diseases predominantly caused by human and animal enteric pathogens and health risks associated with drinking of non-potable water are well documented (WHO/UNICEF, 2006; Reynolds *et al.,* 2008; WHO, 2008). The bacteriological analysis in this study has revealed that some sachet water were heavily contaminated with persistent occurrence of *P. aeruginosa* and *S. dysenteriae*. The presence of *P. aeruginosa* and *Shigella dysentariae* in some vended sachet water has been reported from various parts of the country (Adekunle *et al.,* 2004; Ezeugwunne *et al.,* 2009; Oladipo *et al.,* 2009; Shittu *et al.,* 2013, 2014; Mbah and Muhammed, 2015).

Rhizopus sp. (40%) and *Mucor sp*. (10%). Fungi have been reported from sachet and bottled drinking water (Shittu *et al.,* 2016; Thliza *et al.* 2015; Jonathan *et al.,* 2016). The presence of filamentous fungi in drinking water has become an area worthy of investigation especially with respect to biofilm formation and problems associated with pathogenic fungi and mycotoxins (Siqueira *et al.,* 2011; Paterson and Lima 2015; Oliveira *et al.* 2016; Novak Babič *et al*., 2017; 2018; Paterson, 2019; Mhlongo *et al.,* 2019).

In this study, no oocyst of *Cryptosporodium parvum or* cyst of *Giardia lamblia* was detected in any sample including where adenovirus was found present. The absence of these protozoans could possibly be due to the low sensitivity of the microscopy method used. Molecular detection techniques such as PCR-based methods offer many advantages over microscopic method being rapid, specific, and sensitive (Yu *et al*., 2009; Gotfred-Rasmussen *et al*., 2016). Oocyst *of Cryptosporodium parvum* and cyst of *Giardia lamblia* have been reported in finished water (LeChevallier *et al.,* 1991; Kwakye-Nuako *et al.,* 2007; Chinelu *et al.,* 2010).

The detection of adenovirus in drinking water in this study was consistent with similar studies on tap water and river water (Cho *et al.,* 2000), South-African waters (Genthe *et al.,* 1995), raw and treated water (Van Heerden *et al.,* 2003; 2004); swimming pool (Van Heerden et *al.,* 2005), surface water and drinking water resources in Southern Ghana (Gibson-Schwab *et al*., 2011) and in drinking water sources used in rural areas of Benin, West Africa (Verheyen *et al*. (2009). Human adenoviruses (HAdVs) are the second-leading cause of childhood gastroenteritis worldwide (USEPA, 2005). These are important human pathogens and are responsible for both enteric illnesses and respiratory and eye infections, acute hemorrhagic cystitis, and meningoencephalitis (Mena and Gerba, 2009).

Escherichia coli as a faecal indicator was not detected in the sample that was positive for adenovirus similarly to the study of Pusch *et al*. (2005) in German environmental waters. Adenoviruses have been found to be significantly more stable than faecal indicator bacteria and other enteric viruses during UV treatment (Jiang *et al.,* 2006). Enteric viruses have been isolated from water that indicated microbiological quality. Also, outbreaks of viral gastroenteritis have been reported from ingestion of water that complied with faecal coliform standard (Fong and Lipp, 2005).

From this study, absence of norovirus was in contrast to report of (Jack *et al.,* 2013). Noroviruses (NoVs), previously called Norwalk-like viruses, cause gastroenteritis in all age groups (Maunula *et al.,* 2005) and are the most common cause of acute nonbacterial gastroenteritis worldwide (Karim *et al.,* 2004; Blanton *et al.,* 2006). Noroviruses similar to other enteric viruses and can remain infectious in the environmental waters for long periods, surviving longer than bacteria (Green, 2007; Maunula, 2007; Teunis *et al.,* 2008; Seitz *et al,* 2011).

Also, rotavirus was not detected in this study contrary to Verheyen *et al*. (2009) who found both rotaviruses and adenoviruses in drinking water. Rotavirus (RoV) is the most common cause of diarrhoeal disease primarily in young children less than five worldwide, though infection and disease in older children and adults also occur (Kapikian *et al*., 2001; Bernstein 2009; Matthijnssens *et al,* 2008). By the age of five, nearly every child in the world has been infected with rotavirus at least once (USEPA, 2005).

Though norovirus and rotavirus were not found in this study, both have been reported in clinical samples alongside with adenovirus from Southwest Nigeria (Arowolo *et al*., 2019). This study has provided information on the occurrence of human enteric viruses in water from SW, Nigeria, relevant to research priorities on

human enteric viruses in Africa (Upfold *et al.,* 2021).

CONCLUSION

Public health significant pathogenic bacteria, opportunistic fungi and adenovirus were detected in packaged sachet water sold in Abeokuta. This is indicative of potential microbiological hazards and risks especially, to children and immunocompromised individuals. Packaged sachet water in Nigeria requires increased attention and monitoring by the regulatory agencies.

Table 1. Target genes, PCR assay and primer sequences for viral analyses

Target Genes and PCR assay	Primers	Sequences	References
Adenovirus hexon gene Semi-nested PCR	Forward 1 Reverse 1 Forward 2 Forward 2	JTVXF (5'-GGACGCCTCGGAGTACCTGAG-3'), JTVXR (5'-ACIGTGGGGTTTCTGAACTTGTT-3') JTVXP (5-CTGGTGCAGTTCGCCCGTGCCA-3) JTVXR (5'-ACIGTGGGGTTTCTGAACTTGTT-3')	Xagoraraki *et al.* (2007).
Norovirus ORF gene Nested PCR	Forward 1 Reverse 1 Forward 2 Forward 2	JJGII (5'-CAAGAGTCAATGTTTAGGTGGATGAG-3') GOG2R (5'-TCGACGCCATCTTCATTCACA-3') RingP (5-TGGGAGGGCGATCGCAATCT-3) GOG2R (5-TCGACGCCATCTTCATTCACA-3)	Kageyama *et al.* (2003)
Rotavirus VP7 gene Nested PCR	Forward 1 Reverse 1 Forward 2 Forward 2	ROTAVP7F1 (5'-GGCTTTAAAAGAGAGAATTTC-3') ROTAVP7R1 (5'-GGTCACATCATACAATTCT-3') ROTAVP7F2 (5'-TAGCTCCTTTTAATGTATGG-3') ROTAVP7R2 (5'-AACTTGCCACCATYTYTTCC-3')	Miagostovich *et al.* (2008)

Table 2: Detection of microorganisms of interest found in sachet water brands

Microorganisms	Packaged sachet water brands				
	1	2	3	4	5
Bacteria					
Escherichia coli	_ _ _	+ + +	+ + +	_ _ _	_ _ _
Fungi					
Aspergillus niger	_ _ +	_ _ _	+ + _	_ + _	_ + _
Rhizopus oryzae	_ _ _	_ _ _	+ _ +	_ + +	_ _ _
Mucor mucorales	_ _ _	+ _ _	_ _ _	_ _ _	_ _ _
Enteric viruses					
Adenovirus	+_ _	_ _ _	_ _ _	_ _ _	_ _ _
Norovirus	_ _ _	_ _ _	_ _ _	_ _ _	_ _ _
Rotavirus	_ _ _	_ _ _	_ _ _	_ _ _	_ _ _

Key: + Present; - Absent across the three month study period

Table 3: Cross-tabulation of relationship between Viruses, *Escherichia coli* and Protozoans

		Escherichia coli		*Cryptosporodium parvum*		*Giardia lamblia*	
		Present	Absent	Present	Absent	Present	Absent
Rotavirus	Present	0	0	0	0	0	0
	Absent	6	9	0	15	0	15
Adenovirus	Present	0	1	0	1	0	1
	Absent	6	8	0	14	0	14
Norovirus	Present	0	0	0	0	0	0
	Absent	6	9	0	15	0	15

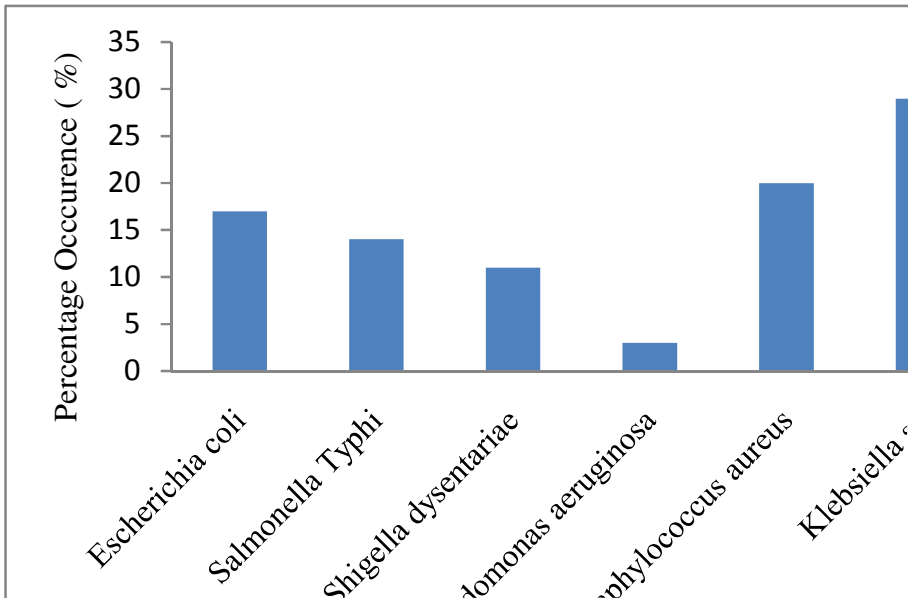

Fig. 1: Percentage of bacteria isolated from sachet water

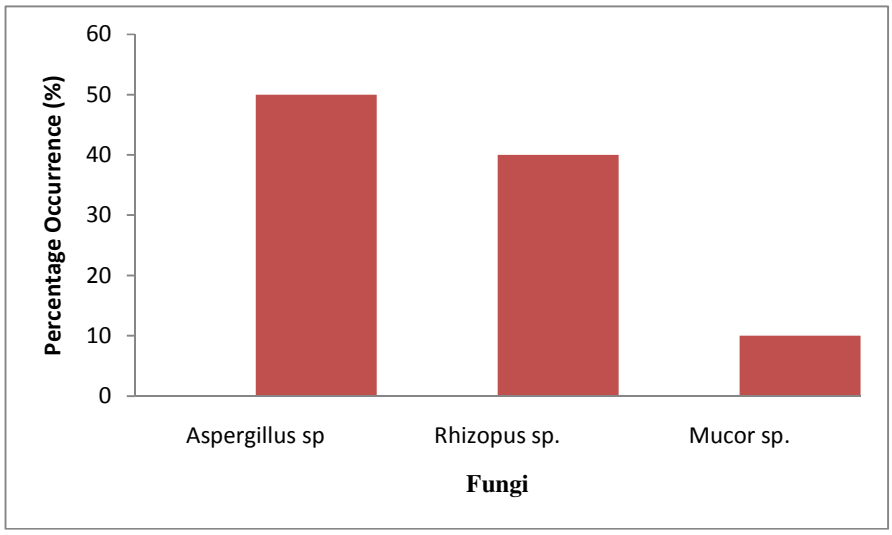

Fig. 2. Percentage of fungi isolated from packaged sachet water brands

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