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Assessment of the prevalence of enteric viruses in the final effluents of two peri-urban wastewater treatment plants

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ABSTRACT

Objective: To assess the prevalence of enteric viruses in the final effluents of two peri-urban wastewater treatment plants (WWTPs) in Amathole District Municipality in the Eastern Cape Province of South Africa from September 2012 to August 2013.

Methods: Water samples were collected monthly from the final effluents of the selected WWTPs (WWTP-K and WWTP-R) located in Komga and East London, respectively in Amathole District Municipality for a period of 12 months between September 2012 and August 2013. RT-PCR was used for the detection of adenoviruses (AdV), rotaviruses and hepatitis A virus while conventional PCR was used to delineate all detected viruses into their serotypes using specific primer sets.

Results: None of the viruses were detected in samples from WWTP-R. In effluent samples from WWTP-K, rotaviruses were detected in 58% (7/12) of the samples in concentrations ranging from 1.7×10^4 to 2.3×10^6 genome copies/L while AdV and hepatitis A virus were detected in 17% (2/12) of the samples in concentrations ranging from 4.5×10 to 2.8×10^2 and 2.3×10 to 7.1×10 genome copies/L, respectively. Molecular characterization of AdV positive samples showed the presence of species B, species C and species F (AdV41) from the May and June 2013 samples.

Conclusions: Detection of enteric viruses in final effluents reflects the inability of WWTPs to completely remove viruses from final effluents and the likelihood of contaminating receiving watersheds with potentially virulent viral particles, which may pose a serious health risk to people directly utilizing such water either for consumption or full contact purposes.

1. Introduction

As of the year 2004, the World Health Organization (WHO) estimated that some 1.1 billion people did not have access to safe drinking water, and a further 2.6 billion did not have proper sanitation, a position that unfortunately has not been significantly improved[1]. According to the WHO, morbidity and mortality related to diarrheal diseases can only be drastically lowered if the global citizenry could have access to safe water and sanitation[1]. However, this has remained a far-fetched dream especially among developing countries and some developed but water-scarce countries like South Africa that may soon have to resort to water recycling to counter water scarcity problems[2]. What worsens water woes in many countries is the unabated pollution of the already over-exploited water sources by inadequately treated wastewater effluents among other pollutants[3]. With a ballooning global human population, disposal of sewage waste is increasingly becoming a major problem. Disposal of raw or partially treated sewage effluents leads to water-related illnesses such as diarrhea that reportedly kills 3–4 million children each year, and WHO has predicted that water-related diseases could kill 135 million people

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by 2020[1,4]. People that are sick with virally induced illnesses shed large quantities of enteric viruses in stools and the sewage they produce carries those viruses into the environment, putting other people at risk of contracting virally induced acute and sometimes fatal illnesses from ingestion of river and sea water, or poisoned shellfish[1,5].

While faecal indicator bacteria have traditionally been used as the gold standard for assessing the microbiological quality of water worldwide[6,7], their major limitation is their ability to grow naturally in the water environment, mostly in tropical climates[8]. This may produce erroneous and costly water quality assessment results, especially where time and source of pollution are factors of interest. In addition, research has shown a lack of correlation in the presence of viral pathogens and faecal indicator bacteria[9,10] whose sole presence or absence cannot be used to accurately predict the presence of other pathogens such as protozoan parasites and enteric viruses which are highly infectious in low doses[11]. Most sewage treatment plants fail to completely remove enteric viruses from wastewater either due to the resistance of viruses to adverse conditions or simply because the treatment plants are generally not designed to remove viral pathogens as they are to reduce the biochemical oxygen demand levels of the wastewater[12].

In South Africa, wastewater in rural areas and small towns often receives partial or minimal treatment[13,14]. However, even in the sub-urban and urban areas with expectedly a high degree of treatment, pathogens and some chemicals may still be released into the environment[15,16]. The inadequately treated effluents from municipal wastewater treatment plants (WWTPs) often impact so many receiving water bodies[17]. It is a widely accepted notion that the discharge of inadequately treated sewage water has a direct impact on the microbiological quality of surface waters and consequently the potable water derived from it[2,14,17,18]. This makes the inadequately treated wastewater a source of pathogens in the environment, thus a threat to human health[19]. It is therefore needful that both the microbiological qualities of such wastewater should be investigated periodically to make sure that they comply with the standards set by regulatory bodies to prevent or reduce incidences of waterborne disease outbreaks. However, we are of the opinion that not all viruses or groups of viruses are present in sewage effluents at the same time, and that indeed viruses are not omnipresent in sewage effluents except those produced by a diseased host. We here present a study of two WWTPs utilizing the same treatment technology and located in a peri-urban setting to assess the prevalence of human adenovirus (AdV), rotavirus (RV) and hepatitis A virus (HAV) in final effluents so as to serve some peri-urban communities.

2. Materials and methods

2.1. Description of study site

Two WWTPs, namely, WWTP-K and WWTP-R located in Komga and East London, respectively in Amathole District Municipality were selected for this study. WWTP-K has a design capacity of 0.63 ML/day and service area with a population of 38 991 people in approximately 10 310 households comprising 62.2% formal dwellings and 37.8% informal settlements[20]. Only 9.4% of residences have a toilet connected to sewerage and 13.7% have piped water inside their dwellings. WWTP-R has a design capacity of 2.5 ML/day and serves a population of 11 668 people spreading across 3 868 households[21]. Both plants use activated sludge treatment system and discharge their final effluents into the Kei and Buffalo Rivers, respectively.

2.2. Sample collection

Twenty-four water samples were collected monthly from the final effluents of the selected WWTPs for a period of twelve months between September 2012 and August 2013. Sterile bottles (1.7 L) containing 1% sodium thiosulphate that is added for dechlorination were used to collect these samples. Samples were transported to the Applied and Environmental Microbiology Research Group laboratory, University of Fort Hare in Alice, South Africa, in a cooler box containing ice at about 4 ˚C for analysis within 6 h of collection.

2.3. Concentration of viruses

The viral nucleic acids were concentrated by the adsorption-elution method of Haramoto *et al.*[22] as modified using a negatively charged Millipore filter membrane (Merck, Ireland)[12]. Briefly, 5 mL of 250 mmol/L $AlCl_3$ solution was passed through the filter membrane and placed over sterile filters. This was followed by filtration of 500 mL of water sample. Al^+ ions were then washed off using 200 mL of 0.5 mmol/L H_2SO_4. All of the steps through adsorption were aided with the use of the suction pump to speed up the whole process while elution of the adsorbed viral particles was done using 10 mL of 1 mmol/L NaOH solution. The eluate was recovered in a Centriprep YM-50 (Merck, Germany) tube containing 50 μL of 100 mmol/L H_2SO_4 (pH 1.0) and 100 μL of 100× Tris-EDTA buffer (pH 8.0) for neutralisation, followed by centrifugation. This method has been reported to have a recovery efficiency of 56% ± 32% (n = 37)[23-25]. The final concentrated samples were stored at –80 ˚C until further analysis.

2.4. Extraction of viral nucleic acids

Extraction of viral nucleic acids was done in accordance with the method of Boom *et al.*[26] using commercially available extraction kits. For AdVs, DNA was extracted from 200 μL of the concentrated sample using Quick-gDNA™ MiniPrep (Zymo Research, USA) following the manufacturer's instructions. Finally, the purified DNA was eluted in 60 μL of DNA elution buffer. The RNA viruses (RVs and HAV) extraction was carried out using Quick-RNA™ MiniPrep (Zymo Research, USA). The concentrated samples were first lysed in a reaction vessel containing a solid nucleic acid carrier (silica gel-

based membrane) in the presence of the chaotropic agent (guanidine thiocyanate) that inactivated RNase and ensured the isolation of intact viral RNA. The mixture was then centrifuged briefly to aid the selective adsorption of viral RNA to the silica gel membrane. The bound viral RNA was washed free of contaminants in two steps before elution in RNase-free water.

2.5. Reverse transcription of RNA viral genomes

The RNA was reverse-transcribed into cDNA using reverse transcriptase (Fermentas Life Sciences, Europe). For RVs, prior to reverse transcription, RNA sample was subjected to denaturation at 95 ˚C for 5 min and flash chilled in ice for 2 min to ensure complete separation of the double stranded RNA genome following the method of Jothikumar *et al.*[27]. Reverse transcription for all RNA viruses was then done by mixing 10 μL of the template, 1 μL of random hexamer primer, 1 μL deoxynucleotide mix, 2.5 μL diethylpyrocarbonate-treated water, 4 μL of 5× RT buffer, 0.5 μL RiboLock RNase inhibitor and 1 μL RevertAid Premium Reverse Transcriptase (Fermentas Life Sciences), added in the indicated order into a 0.5 mL PCR tube on ice. The mixture was briefly vortexed before incubation at 25 ˚C for 10 min, followed by 30 min at 60 ˚C and terminated by heating at 85 ˚C for 5 min. The produced cDNA was then used for quantification using RT-PCR.

2.6. Formulation of standard curves and RT-PCR sensitivity assays

A method by Haramoto *et al.*[28] was used to plot the standard curve of the quantified viral genomes. Briefly, nucleic acid extracts from ATCC control strains were quantified using a Qubit fluorometer. For human AdV, viral nucleic acid was extracted from ATCC VR-6 (Strain Tonsil 99) reference strain using DNA extraction kits (Quick-gDNA™ MiniPrep; Zymo Research, USA) while transcribed cDNAs from ATCC VR-1357 (Strain PA21) and ATCC VR-2274 (Strain 248) were used to construct the standard curves for HAV and RV, respectively. From these, a range of known concentrations were prepared by ten-fold serial dilutions using nuclease-free water. The serial dilutions that were treated as standard, and together with the extracted cDNA and DNA from the samples were subjected to RT-PCR analysis simultaneously for quantitative enumeration of all viruses. The sensitivity and specificity of the assays were determined according to Simmons and Xagoraraki[29] by using seven-fold serial dilutions of the stock culture extracts and a detection limit of 10 copies of the target DNA per reaction was set for each of the RT-PCR assays.

2.7. Detection of viral genomes by RT-PCR

The concentration of virus genomes in water samples was quantitatively determined using quantitative PCR with TaqMan probes. The virus quantification was done following a one-step reaction in a 96-well plate. The PCR assay used 5 μL of the concentrated cDNA in a total reaction volume of 25 μL, containing 12.5 μL of PCR master mix, 0.5 μL of forward primer, 0.5 μL of reverse primer and 6.5 μL of nuclease-free water. The reaction mixture was loaded into 96-well plates, loaded into the thermocycler and data were collected at the end of 2 h. Primer pairs and probes used for HAV were obtained from Costafreda *et al.*[30] and Pintó *et al.*[31] while those used for RVs and Advs were obtained from Jothikumar *et al.*[27] and Simmons and Xagoraraki[29], respectively.

The thermal cycling protocols used were as follows: HAV: 10 min at 95 ˚C for *Taq* activation, 45 cycles of denaturation at 94 ˚C for 15 s, annealing at 60 ˚C for 1 min and extension at 70 ˚C for 1 min; RV: 15 min at 95 ˚C for *Taq* activation, 45 cycles of denaturation at 95 ˚C for 15 s, annealing at 55 ˚C for 30 s and extension at 72 ˚C for 30 s; Adv: 15 min at 95 ˚C for *Taq* activation, 45 cycles of denaturation at 95 ˚C for 10 s, annealing at 55 ˚C for 30 s and extension at 72 ˚C for 20 s.

At the end of all the PCR cycles, fluorescence activity data were collected and followed by SDS software (Applied Biosystems) analysis to obtain quantitative data on the concentration of viral DNA in each well. Detection limit (< 10 viral genomes per reaction) was determined and positivity of all samples was defined by a threshold cycle value of $\leqslant$ 35.

2.8. Detection of viral species and serotypes

Samples that were positive for any of the viruses were further characterized by conventional PCR into their epidemiological species. For RV, species A, B, and C, primers previously described by Gouvea *et al.*[32] were used following the description of Lai *et al.*[33]. For Adv, the primers described by Xu *et al.*[34] targeting the fibre region of the viral genome were used for the detection of Adv species A to F. The PCR assays for the species detection was carried out as described by Metzgar *et al.*[35] and Tiemessen and Nel[36] but now as singleplex PCR.

2.9. Statistical analysis

Obtained data were analyzed using SPSS (IBM SPSS Statistics 19; IBM, USA) for seasonal variation of these viruses.

3. Results

The target viruses (Adv, RV and HAV) were only detected in WWTP-K and none of them was detected in the WWTP-R. The viral quantities obtained by RT-PCR are shown in Table 1. RVs were the most prevalent and detected in 50% of the analysed samples in concentrations ranging from 1.1×10^4 to 2.3×10^4 genome copies/L. Meanwhile, Advs and HAV were each detected in 16.7% of the samples at concentrations of 45–276 and 23–71 genome copies/L, respectively.

None of the RV-assessed serogroups A, B or C were detected

from the RV positive samples. For the Adv positive samples species F, serotype 41 was detected in all Adv positive samples while only 50% of samples were positive for species C (AdV6 and AdV2). Also species B (AdV7) was detected in 50% of the samples positive for Adv.

Table 1
Mean viral quantities detected in WWTP-K samples by RT-PCR (genome copies/L).

Date	RV	HAV	Adv
Sep 2012	–	–	–
Oct 2012	–	23	–
Nov 2012	10678 (406)*	–	–
Dec 2012	11580 (315)*	–	–
Jan 2013	–	–	–
Feb 2013	12377 (1023)*	–	–
Mar 2013	22533 (225)*	–	–
Apr 2013	–	71	–
May 2013	11214 (112)*	–	45
Jun 2013	20341 (203)*	–	276 (34)*
Jul 2013	–	–	–
Aug 2013	–	–	–

*: Standard deviation (samples analysed in triplicates).

4. Discussion

Enteric viruses are recognized to be important agents of disease following exposure to faecally polluted waters or foods grown in such waters[25] and their detection in final effluents presents a public health hazard because of their highly infectious nature even in low dosages. Therefore, detection of these viruses in wastewater final effluents not only points to the inefficiency of WWTPs in complete pathogen removal but also is an insight into the health status of the host community. While Xagoraraki *et al.*[37] highlighted that WWTPs may not completely remove viruses especially in the secondary treatment stage[38], an accurate study of the inactivation of gastroenteritis-causing viruses following wastewater disinfection is riddled with many tailbacks, main of which is the low and variable levels of enteric virus frequently seen in effluents[38,39]. And, many kinds of viruses have been detected in sewage and wastewater, some of which affect both animals and humans[40,41]. Therefore, viruses may persist at high levels despite the decontamination processes are commonly used for drinking water and sewage treatment which damages the health status of the host community[42,43].

In this study, RVs were both the most prevalent and most abundant of all the viruses, having been detected in high titre in all seasons except spring while both Advs and HAV had a very low incidence. Numerous studies have always concluded that Advs were the most prevalent of enteric viruses in surface waters (including sewage effluents), supposedly because of their double stranded DNA genome which causes them to resist disinfection procedures[25,44,45]. Results of this study, however, showed that it was not only a question of which viruses were able to resist treatment processes but also a question of the prevalence of the viruses in question in the host human population. While both WWTP-K and WWTP-R used activated sludge coupled with chlorine disinfection as a means of pathogen removal by both adsorption and inactivation, no detectable viral genomes were recovered in effluent samples from WWTP-R, further buttressing the point that the prevalence of these viruses in sewage effluents is directly related to the health of the feeder community, and not sewage in general, as may be the case with organisms like *Escherichia coli*. Also, detection of RVs in titres in excess of 1.0×10^4 in all instances in WWTP-K despite the hypothetical removal efficiency of 94% indicates that a sizeable proportion of the host community population could have been infected with this virus. This outcome compares well with reports that RVs are the number one etiological agents of gastroenteritis in children, causing approximately half a million deaths of children and 2.4 million hospitalizations per year[46-48] especially in low-income countries. However, some researchers have found out that a large number of RV infections occur in winter and early spring, but seasonality is less pronounced in tropical and low-income countries[48-50]. Hence, RV genomes in this study were detected in all other seasons except spring, which also points to the fact that ambient temperatures are not necessarily a determinant of the prevalence of RVs in human populations but rather of persistence in the water environment.

The presence of RV in the effluents poses a threat to human health as they can persist for up to 32 days in water[12,25,51]. Importantly, while Advs could have been detected in only 16.7% of the samples, the detection of Adv serotypes Adv2, Adv6, Adv7 and Adv41 was of particular interest because of their significance to public health. Adv41 has replaced Adv40 as the predominant serotype isolated from gastroenteritis patients worldwide[23,52]. This study confirms these reports as Adv40 was not detected. Detection of these Adv serotypes in sewage final effluents is health threatening[53,54]. Moreover, Adv is known to resist sunlight (UV) inactivation[44], which could pose a public health threat to downstream users of Kei River, especially since this river passes through rural communities where the supply of pipe borne water may be erratic, not to mention the use of the river for recreational activities like swimming. Also, Advs have been identified as the second most important viral pathogens of gastroenteritis in infants and children after RVs[2,13]. Of the detected viruses, HAV was detected in the lowest concentrations and we also assumed that the risk of infection posed by HAV during the period of study was negligible. However, HAV is known to cause not only gastroenteritis, but also an acute inflammation of the liver[2,55] and, although it rarely results in fatalities, in developing countries it becomes an economic burden especially in populations who have not been vaccinated.

Assessment of the distribution of these viruses over a twelve-month period revealed that RVs were found in significantly higher concentrations than Advs or HAV ($P < 0.05$) in WWTP-K and the distribution of Advs was skewed towards winter.

Due to the similarities of the treatment technologies employed by both WWTP-K and WWTP-R, it can be concluded with certainty that the occurrence and distribution of enteric viruses and hence other

pathogens in wastewater final effluents are directly related to the occurrence of the same in the host human population. With regard to virus-induced gastroenteritis and other illnesses, we therefore conclude that the host human population for WWTP-R is healthier than that of WWTP-K. We still conclude that the WWTPs show a high degree of inefficiency in viruses removal during sewage treatment, which could possibly be attributed more to design deficiencies than infrastructure inadequacy. The RT-PCR assay used in the current study was not able to evaluate the viability of the detected viruses, which is a serious limitation. We suggest that alternative methods like the integrated cell culture quantitative reverse transcription PCR should be used subsequently, with the goal of assessing both virus removal and the infectivity of the detected viruses.

Conflict of interest statement

We declare that we have no conflict of interest.

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