Molecular Detection of hepatitis A Virus in Water Samples from Buffalo River, Eastern Cape, South Africa

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Abstract: Contaminated surface waters have been implicated in outbreaks of enteric viral infections. Hepatitis A virus (HAV) is the common cause of infectious hepatitis, and hepatitis A outbreaks associated with contaminated water supply have been reported in various countries. The aim of this study was to assess the virological quality of water samples from Buffalo River, a major resource located in Eastern Cape, South Africa. Using standard methods, A total of 18 water samples were collected monthly, for a period of three months (August-October 2010), from a total of six sites on Buffalo River and its dams, including Maden, Rooikrantz and Bridle Drift dams) and examined for the occurrence HAV. The virus was concentrated by the adsorption-elution method using an electronegative filter and amplified via semi-nested reverse transcriptase-polymerase chain reaction (RT-PCR). HAV was detected in 7 (38.9%) of the total of 18 water samples collected. While the virus was never detected at Maden dam and Eluxolzweni, it was detected at the other four sampling sites at varying degrees throughout the study period. The detection rate for HAV was 100% at the King William's Town sampling site and 66.7% at the estuary (Parkside, East London). The virus was detected only once at Rooikrantz dam (in September) and at Bridle Drift dam, Mdanlsane (in October). The presence of HAV in water samples constitutes public health risks. The consistent occurrence of the virus in Buffalo River suggests the need for assessing viral contamination of other water sources.

Keywords: Hepatitis A virus, human settlements, semi-nested PCR, surface waters, water pollution.

1. Introduction

Human enteric viruses (HEntVs) are major causes of waterborne diseases (Bosch *et al.* 2008) and have been detected in water sources, worldwide (Pina *et al.* 2001; Griffin *et al.*, 1999; Pusch *et al.*, 2005; Gersberg *et al.*, 2006; De Paula *et al.*, 2007; Kiulia *et al.*, 2010). HEntVs represent diverse and commonly studied groups of viruses belonging to the families *Reoviridae* (rotaviruses), *Caliciviridae* (noroviruses and saporovirus), *Adenoviridae* (adenoviruses), *Astroviridae* (astroviruses) and *Picornaviridae* (polioviruses, enteroviruses, coxsackieviruses, echoviruses, and hepatitis A virus) (Fong and Lipp, 2005; Colbere-Garapin *et al.*, 2007). Hepatitis A virus (HAV) is a 27- to 32-nm, icosahedral, non-enveloped, single-stranded, positive-sense RNA virus belonging to the family *Picornaviridae* (Venter *et al.* 2007). It is the only member of the *Hepatovirus* genus, and is the most common cause of infectious hepatitis throughout the world with more than 1.5 million clinical cases reported annually (Roque-Afonso *et al.*, 2010).

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HAV is spread person to person by the fecal-oral route and causes acute hepatitis that can lead to jaundice and, in certain cases, liver failure (Tallon *et al.*, 2008). Outbreaks of hepatitis A associated with contaminated water supply have been reported in various countries (Tallon *et al.*, 2008; Pinto *et al.*, 2009).

South Africa is a country of intermediate to high Hepatitis A endemicity (Venter *et al.*, 2007) and the global problem of pollution is heightened there because the country is a semi-arid, water-stressed and depends largely on surface waters, including rivers, streams, ponds and dams. Studies on viruses in water were pioneered in South Africa, in the 1980s, by WOK Grabow and his colleagues in Gauteng Province (Grabow *et al.*, 1983). Further studies followed in the same province (Grabow *et al.*, 1996; Grabow, 2001; Taylor *et al.*, 2001 Grabow, 2007; Venter *et al.*, 2007), and there are also reports of studies on some other provinces including Western Cape, Eastern Cape and Limpopo (van Heerden *et al.* 2005; van Zyl *et al.* 2006; Chigor and Okoh, 2012; Sibanda and Okoh, 2013; Osuolale and Okoh, 2015).Available statistics, based on Census 2011 (Statistics South Africa, 2012) shows that, of the about seven million Eastern Cape population, only 13.6% has access to pipe borne water either in

their dwelling places or within 200 metres. Consequently, many households rely solely on untreated surface waters (River Health Programme, 2004). Such water bodies have been shown to be very vulnerable to contamination (Shuval, 1990; Chigor *et al.*, 2010; Kiulia *et al.*, 2010; Chigor *et al.*, 2012), and have been implicated in outbreaks of enteric infections caused by bacterial, protozoan and viral pathogens (Okoh *et al.*, 2010; WHO. 2009; Bosch, 1998; Pina *et al.* 2001; Fong and Lipp, 2005; Chigor *et al.*, 2010). Contaminated surface waters when used for domestic, agricultural and recreational purposes therefore constitute public health hazards (Pruss *et al.* 2002; Chigor *et al.*, 2012).

It has been shown that faecal indicator bacteria are not reliable indices for enteric viruses in water (Jurzik *et al.*, 2010). Investigation of source waters for viral pathogens is therefore necessary to protect public health (Bosch *et al.*, 2008). Although culture propagation procedure is still the best method to enumerate viruses and demonstrate their infectivity (Fong and Lipp, 2005), such procedure is unsuitable for the detection of hepatitis A virus and other enteric viruses, for which appropriate cell cultures are not available or their growth is limited (Schvoerer *et al.*, 2000). Molecular tests have been successfully applied on environmental samples, allowing a rapid and specific detection of human enteric viruses (Costafreda *et al.*, 2006; De Paula *et al.*, 2007; Bosch *et al.*, 2008).Buffalo River, located in the Eastern Cape Province, is important as the major water source and as a channel for sewage disposal, as well as for recreational purposes in one of the most populous areas on the East coast of southern Africa. The aim of this study was to apply semi-nested RT-PCR in assessing the prevalence of hepatitis A virus and the virological quality of water samples from Buffalo River.

2. Materials and Methods

2.1. Study Area and Sampling

Buffalo River is important as the major water source, for recreational purposes, and as a channel for sewage disposal in one of the most populous areas on the East coast of southern Africa. Located in the Eastern Cape Province of South Africa. It has its source in the Amathola mountain range and empties into the Indian Ocean at East London. Along the Buffalo River, there are four dams supplying water to the urban areas of King William's Town, Zwelitsha, Mdantsane and East London. In the catchments, blockages in the sewerage systems, inadequate treatment capacity and poor management result in the discharge of partially treated and untreated sewage into the river and dams. Also, industrial effluents are either inadequately treated or not treated at all (River Health Programme, 2004).

Collection of Samples: From August to October, 2010 water samples were collected monthly from six different sites on Buffalo River (Figure 1) including S-1 Maden Dam (32°44'370"S; 27°17'905"E), S-2 Rooikrantz Dam (32°45'318"S; 27°19'580"E), S-3 King William's Town (32°53'386"S; 27°23'286"E), S-4 Eluxolzweni (32°56'263"S; 027°27'928"E), S-5 Bridle Drift Dam (32°58'497"S; 027°42'374"E) and S-6 Parkside, East London (33°01'375"S; 027°51'514"E). Water samples were collected in sterile 1.75-litre screw-capped plastic containers and transported on ice to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa where they were kept at -4°C until processing as recommended by American Public Health Association (APHA, 2005).

2.2 Concentration of Viruses in Water

Viruses in water samples were concentrated by first passing one litre through glass fibre prefilters (Millipore, Ireland) following the adsorption-elution method of Haramoto *et al.* (2005), with some modifications. Five millilitres of 250 mM aluminium chloride ($AlCl_3$) were passed through a sterile membrane filter (Millipore; S-Pak Type HA filters, 47 mm diameter, 0.45 µm pore size) to form a cation (Al^{3+})-coated filter, attached to a glass filter holder. The pre-filtered water sample was passed through the filter. A volume of 200 mL of 0.5 mM H_2SO_4 was then passed through the membrane, and viral particles were eluted with 10 mL of 1 mM NaOH. The eluate was recovered in a tube containing 50 µL of 100 mM H_2SO_4 (pH 1.0) and 100 µL of 100x Tris-EDTA (TE) buffer (pH 8.0) for neutralization, followed by centrifugation using a Centriprep YM-50 (Millipore) equipped with an ultrafiltration membrane. The filtrate was added to the Centriprep YM-50 and centrifuged according to the manufacturer's protocol; centrifugation at 2,500 rpm for 10 min, followed by removal of the sample that passed through the ultrafiltration membrane (8 mL) and further centrifugation at 2,500 rpm for 5 min to obtain a final volume of approximately 700 µL. The concentrates were pooled together per sample and stored at -80°C until further analysis (Haramoto *et al.*, 2005)

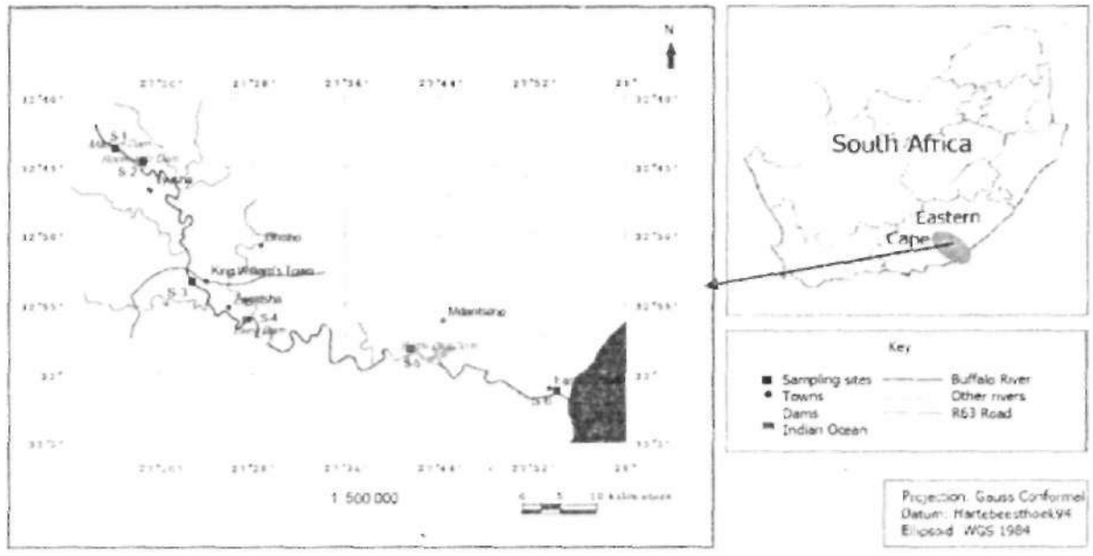

Figure 1: **The study area and sampling sites**(With kind permission from Springer Science+Business Media: Food and Environmental Virology, Quantitative Detection and Characterization of Human Adenoviruses in the Buffalo River in the Eastern Cape Province of South Africa, 4, 2012, 200, VN Chigor and AI Okoh, Fig. 1).

2.3 Extraction of Viral Nucleic Acids

Viral RNA was extracted using 150 µl of the final concentrated sample and commercial RNA purification kits, *Quick-RNA*^{TM} MiniPrep (Zymo Research, USA), following the manufacturer's instructions to obtain a final volume of 60 µl. Viruses were first lysed in a column containing silica gel-based membrane and the chaotropic agent guanidinium thiocyanate that inactivates RNase to ensure isolation of intact viral RNA (Boom *et al.*, 1990). The mixture was then centrifuged to aid the selective adsorption of the viral RNA to the silica gel membrane. A two-step wash to free the bound RNA of contaminants was followed by elution in RNase-free water, containing sodium-azide to prevent microbial growth and subsequent recontamination with RNase. To remove contaminating DNA, the eluate was digested with RNase-free DNase followed by heating at 70°C to in activate the DNase.

2.4 Reverse Transcription for Detection of HAV

To 10 µl of extracted RNA was added, in this order, 1 µL of 100 µM random hexamers, 2 µL of dNTP Mix (10 mM each of the four deoxynucleoside triphosphate stocks), 5 µl of nuclease-free water, 4 µL of 5x RT buffer, 1 µL of Ribolock RNAse Inhibitor, and 2 µL of 200-U/ µLRevertAid^{TM} Premium reverse transcriptase (Fermentas, Canada). The reaction mixture (25 µl) was then incubated for 10min at 25°C followed by 60 min at 42°C for cDNA synthesis. Then the reaction mixture was heated at 70°C for 10 min (to inactivate the reverse transcriptase and terminate the reaction) and cooled to 4°C. The resulting cDNA solution was divided into six aliquots of 5 µL and stored at -20°C for use as templates for PCR amplification (Haramoto *et al.* 2005).
according to the manufacturer's recommendation, and the RNA was stored at -20°C for future analysis.

2.5 Detection of HAV by TaqMan PCR

The cDNA was amplified using MyCycler^{TM} Thermal Cycler PCR system (BioRad, USA).Each aliquot (5 µL) of cDNA was mixed with 1.5 µL of 10x PCR buffer (DreamTaq buffer [Fermentas, Canada]), 1 µL of dNTP Mix (10 mM each dNTP), 0.2 µL Taq polymerase (DreamTaq DNA polymerase [Fermentas, Canada]), 0.5 µl of the sense primer (1mg/ml), 0.5 µl of the antisense primer (1mg/ml) and nuclease-free water (adjust to a final volume of 50 µl). The reaction mixture was incubated at 50°C for 2 min and 94°C for 15 min to activate the Taq polymerase. Thermocycling conditions consisted of 40 cycles of 1min at 94°C (denaturation), 1 min at 60°C (annealing), 1 min at 72°C (extension), and a final extension of 72°C for 10 min (Schvoerer *et al.*, 2000). This is the first PCR round. The primers used (shown in Table 1) target the VP1 capsid protein gene and were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

For the second PCR round, 5 µL of amplified DNA was added to a 15µL mix identical to the first one except for the primers. The PCR conditions on the thermocycler were as follows: 12 min at 94 °C for Taq polymerase activation, denaturation at 94 °C for 1min, annealing for 1min at 55 °C. The final elongation was at 72 °C for 10 min, after 40 cycles of amplification. The amplicons were separated by gel electrophoresis on a 0.8% agarose gel in TBE (Tris-borate-EDTA) buffer (containing GB Green DNA stain) using a horizontal electrophoresis on apparatus and voltage source (BioRad PowerPac Basic) operated at 100Vfor 2h. After electrophoretic separation of DNA, the gel slab was observed under UV light using a UV trans-illuminator (Alliance 4.7), and images were digitized with a one-dimensional gel documentation system.

2.6 Statistical analysis

IBM SPSS Statistics 19 was used for statistical analysis, and significance was set at P values <0.05.

3. Results and Discussion

The result of qualitative detection of HAV is shown in Figure 2. HAV was detected in 7 (38.9%) of the total of 18 water samples collected.

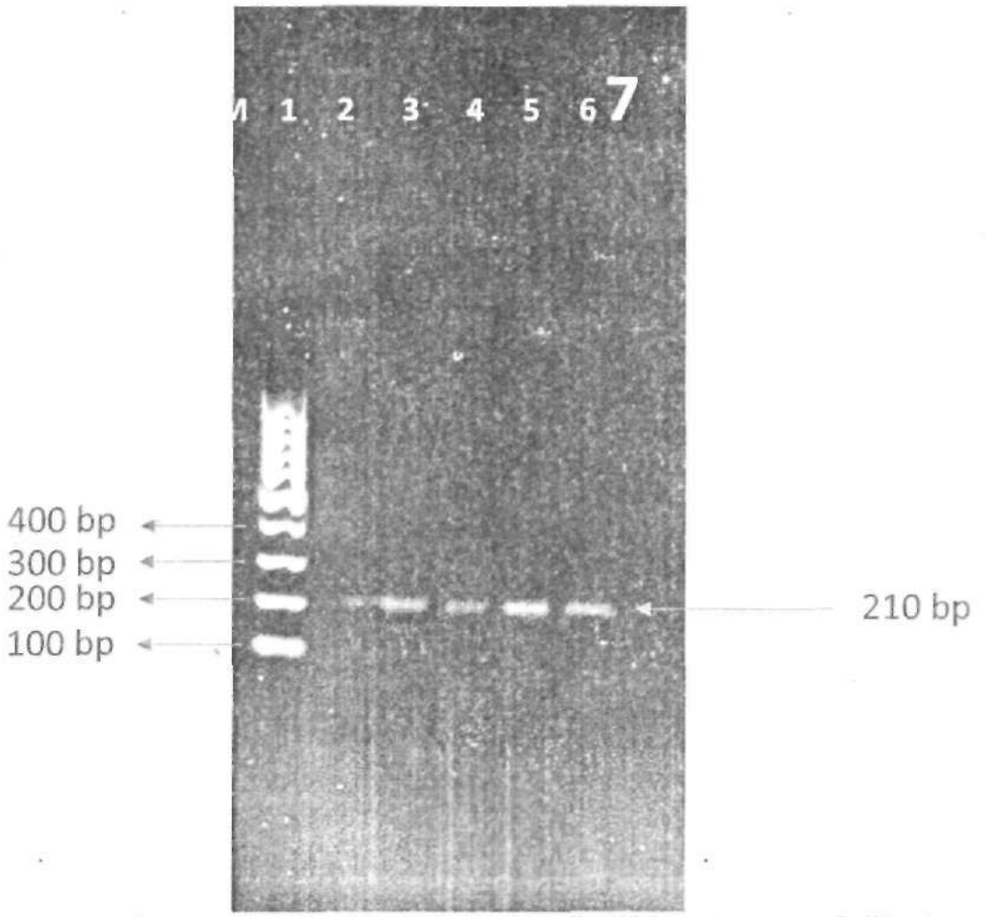

Figure 2: Detection of hepatitis A virus in water samples from King William's Town site on Buffalo River by RT-PCR. Lanes: M. DNA molecular weight marker; 1. MD8; 2. KT8; 3. KT9; 4. KT10; 5. Positive control; and 6. Negative control

While the virus was never detected at Maden dam and Eluxolzweni, it was detected at the other four sampling sites at varying degrees throughout the study period.

Figure 3. reveals that HAV was detected in 100% of the sample collected from the King William's Town sampling site (Lanes 2 to 4 in Figure 1), 66.7% at the estuary (Parkside, East London), and in 33.3% of samples from each of Maden and Bridle Drift dams.

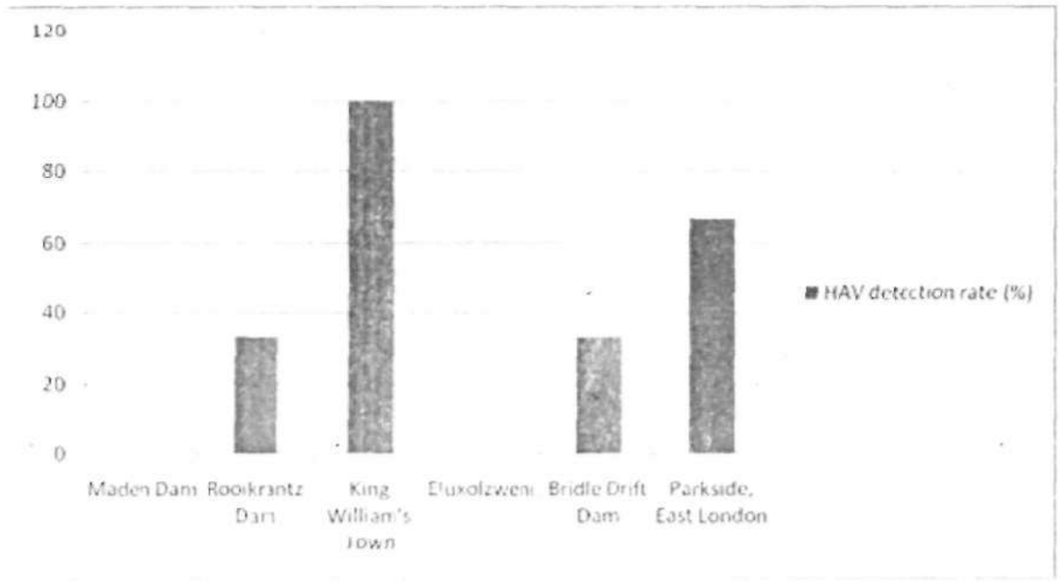

Figure 3: Detection of HAV at six sampling sites on the Buffalo River

The virus was detected only once at Rooikrantz dam (in September) and at Bridle Drift dam, Mdantsane (in October). The detection of hepatitis A virus in Buffalo River is similar to other studies in other provinces in South Africa (Taylor *et al.*, 2001; Venter *et al.*, 2007) and elsewhere (De Paula *et al.*, 2007). The detection of HAV at the study sites correlates with human settlements.Our previous report (Chigor *et al.*, 2013), on bacteriological parameters, showed that significantly higher mean concentrations of faecal indicator bacteria were recorded at the sampling sites located at the lower reaches (eg. King William's Town and Bridle Drift Dam) of the river compared to those at the upper reaches (Maden Dam and Rooikrantz Dam). This was attributable to anthropogenic activities and increased populations in the different catchments. In their report, Williams *et al.* (2012) highlighted the need for land-use types associated with the catchment areas of a watercourse to be considered as a central factor in models that aim to predict pathogen risk in environmental waters. HAV was not detected at Maden dam and Eluxolzweni. The latter is a sparsely populated settlement, and the catchment upstream of this dam is a protected state forest, so pressures from human activity are limited to forest management and recreational activities.

Table 1: Sequence, primers and amplicon size for the detection of Hepatitis A virus

Primer	Sequence (5'–3')	Amplicon Size (bp)	Reference
First PCR			
H2Forward	GTTTTGCTCCTCTTTATCATGCTATG		
H1Reverse	GGAAATGTCTCAGGTACTTTCTTTG	247	Schvoerer *et al.*, 2000
Second PCR			
H2 Forward	GTTTTGCTCCTCTTTATCATGCTATG		
H3 Reverse	TCCTCAATTGTTGTGATAGC	210	

Previous reports show that South Africa is a country of intermediate to high Hepatitis A endemicity (Venter *et al.*, 2007). This presence of HAV for three consecutive months may suggest that the virus is consistently present at the King William's Town site on Buffalo River.This could

be attributable to the virus possibly being continously shed in the faeces of infected individuals.Enteroviruses were also detected at the same site in both August and October (Chigor and Okoh, 2012).It should be noted that, based on bacteriological parameters (Chigor *et al.*, 2013), the King William's Town site, where HAV was constantly detected during the study period, is the most polluted of the six sites.

4. Conclusions

The presence of HAV in water samples constitutes public health risks. Our results show that the virus is consistently present in Buffalo River, and the steady occurrence of the virus in the River suggests the need for assessing for viral contamination of water sources.

Acknowledgements

The authors are grateful to the Water Research Commission (WRC), South Africa for funding this research. VNC also thanks TETFund Nigeria for the offer of doctoral grant.

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