Detection of human enteric viruses in Umgeni River, Durban, South Africa

Johnson Lin and Atheesha Singh

ABSTRACT

The prevalence of adenovirus (AdV), rotaviruses (RV) and enteroviruses (EV) in Umgeni River waters of Durban, South Africa was assessed qualitatively and quantitatively during April 2011 to January 2012 using polymerase chain reaction (PCR)/reverse transcription-polymerase chain reaction (RT-PCR), nested PCR and quantitative PCR (qPCR), as well as nested integrated cell culture PCR (nested ICC-PCR). The phylogenetic analysis of the adenovirus and enterovirus amplicons was also performed. The nested PCR results effectively detected the presence of AdV and EV in all water samples. The results of qPCR demonstrated that higher populations of EV and of AdV were widely found in the Umgeni River. Rotavirus could only be detected in the upper Umgeni River, mainly during drier seasons. Nested ICC-PCR further confirmed the presence of infectious AdV and EV particles in 100% of water samples using various cell lines. The present study identifies potential viral hazards of Umgeni River water for domestic water supply and recreational activities.

Key words | human enteric viruses, nested ICC-PCR, phylogenetic analysis, quantitative PCR, Umgeni River

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ABBREVIATIONS AND NOTATION

AdV	adenovirus
C_T	threshold cycle
EV	enteroviruses
GC	genome copy
HepV	hepatitis viruses
Nested ICC-PCR	nested integrated cell culture polymerase chain reaction
NoV	noroviruses
PCR	polymerase chain reaction
PyV	polyomavirus
RT-PCR	reverse transcription-polymerase chain reaction
RV	rotaviruses
WHO	World Health Organization

INTRODUCTION

Surface waters and rivers that serve as sources for both domestic and recreational uses carry varying amounts of wastewaters, which may sometimes reach a proportion of 50% and more during periods of low flow (WHO 2004). This coupled with rapid urbanization in developing countries such as South Africa has raised critical issues of water supply and waste disposal. The increasing demands on available water in both developed and developing worlds and the concurrent expansion of industrial activity make the recycling of domestic wastewater inevitable (WHO 1996). The regulatory standards such as faecal coliform count to assess the microbiological quality of water often do not coincide with the presence of pathogens such as parasites and viruses (Poma *et al.* 2012). Increasing attention is being directed to the contamination of waters by viruses on a global scale (Lin & Ganesh 2013).

Over 150 enteric viruses, the most frequently reported enteric viruses in faecal-polluted water are adenoviruses (AdV), enteroviruses (EV), noroviruses (NoV), rotaviruses (RV), hepatitis viruses (HepV) and polyomaviruses (PyV) (Sidhu *et al.* 2012). Enteric viruses are present in the faeces and vomit of infected individuals in high concentrations,

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and are transmitted primarily through the faecal–oral route, either directly through person to person contact or by consumption of contaminated food or water (Wong *et al.* 2012). Protection of public welfare from severe gastro-intestinal infections by enteric viral pathogens depends on the ability to identify and to eliminate the sources of contamination (Poma *et al.* 2012).

There is a strong focus on safe water supplies and water reclamation in South Africa. However, the studies that have assessed for viral agents in South Africa's waters have occurred only in a limited number of locations and provinces (van Heerden *et al.* 2004; van Zyl *et al.* 2004; Sibanda & Okoh 2012; Chigor & Okoh 2012a). There are only limited surveillance data and knowledge of the actual burden of waterborne viruses in the KwaZulu-Natal region. A study by Olaniran *et al.* (2012) demonstrates that viral-like particles can be observed using electron microscope in the wastewater treatment plants and the receiving river watersheds including the Umgeni River near Durban. Previous studies further demonstrate that *Adenoviridae, Herpesviridae, Orthomyxoviridae, Picornaviridae*, *Poxviridae*, and *Reoviridae* were found in the Umgeni River under transmission electron microscopy (Ganesh *et al.* 2013). This study focuses on the detection and quantification of adenovirus, rotaviruses and enteroviruses in the surface waters of the Umgeni River samples collected throughout four seasons. At the same time, phylogenetic analysis of virus-positive samples was conducted to identify some of the dominant strains circulating in the environment.

METHODOLOGY

Sample collection and viral concentration

Focus in this study was on the Umgeni River (five points along the river as shown in Figure 1) due to it being the largest catchment (5,000 km^2) in the KwaZulu-Natal region of South Africa. The Umgeni River is approximately 230 km long, providing water to over 3.5 million people and supporting an area that is responsible for approximately 65% of the total economic production in the province (WRC 2002). The sampling seasons and sites included five vast areas of the river starting from a dam water source, passing through a nature reserve, waste treatment works, informal human settlement and finally ending at the river mouth (where the river and ocean meet). Sampling points were designated as follows: U1 (Umgeni River mouth – estuarine/brackish water), U2 (Reservoir Hills – informal settlement/domestic waste), U3 (New Germany Wastewater Works – treated water after chlorination that enters the adjoining Umgeni River water), U4 (Krantzkloof Nature Reserve – vegetation and conservation area) and U5 (Inanda Dam – restricted water containment). Umgeni River water samples (25 L each) of five different sites were collected every season (April, July, October 2011 and January 2012) and the viral concentration (from 20 L to 5 mL) using a two-step tangential flow filtration as described by Ganesh *et al.* (2013).

Extraction of viral nucleic acids from water samples and cDNA synthesis

All viral concentrates were filtered through a 0.22 μm Sterivex filter (Millipore, USA) and treated with 10% chloroform to remove contaminating microbial cells before use (Rosario *et al.* 2009). Viral DNA and RNA were extracted separately from 1 mL viral concentrates each using the High Pure Viral Nucleic Acid Large Volume Kit (for the isolation of viral DNA for polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR), Roche Diagnostics, Germany) and High Pure Viral RNA Kit (for the isolation of viral RNA for RT-PCR, Roche Diagnostics), respectively, according to the manufacturer's instructions with no modifications. DNA and RNA qualities and quantities were measured using the NanoDrop 2200 Spectrophotometer (Thermo Scientific) and the extracts were stored at −70 °C.

RNA concentrations were standardised to 1 μg/mL with RT-PCR grade water and cDNA was synthesized using the iScript^{TM} cDNA Synthesis kit (BIORAD, South Africa). Each 20 μL reaction contained 4 × iScript Reaction Mix, iScript Reverse Transcriptase (RNAse H$^+$), nuclease-free water and approximately 8–10 μL template RNA (100 fg to 1 μg total RNA). The complete reaction mix was incubated for 5 minutes at 25 °C, 30–40 minutes at 42 °C followed by 5 minutes at 85 °C. The resulting cDNA was stored at −70 °C.

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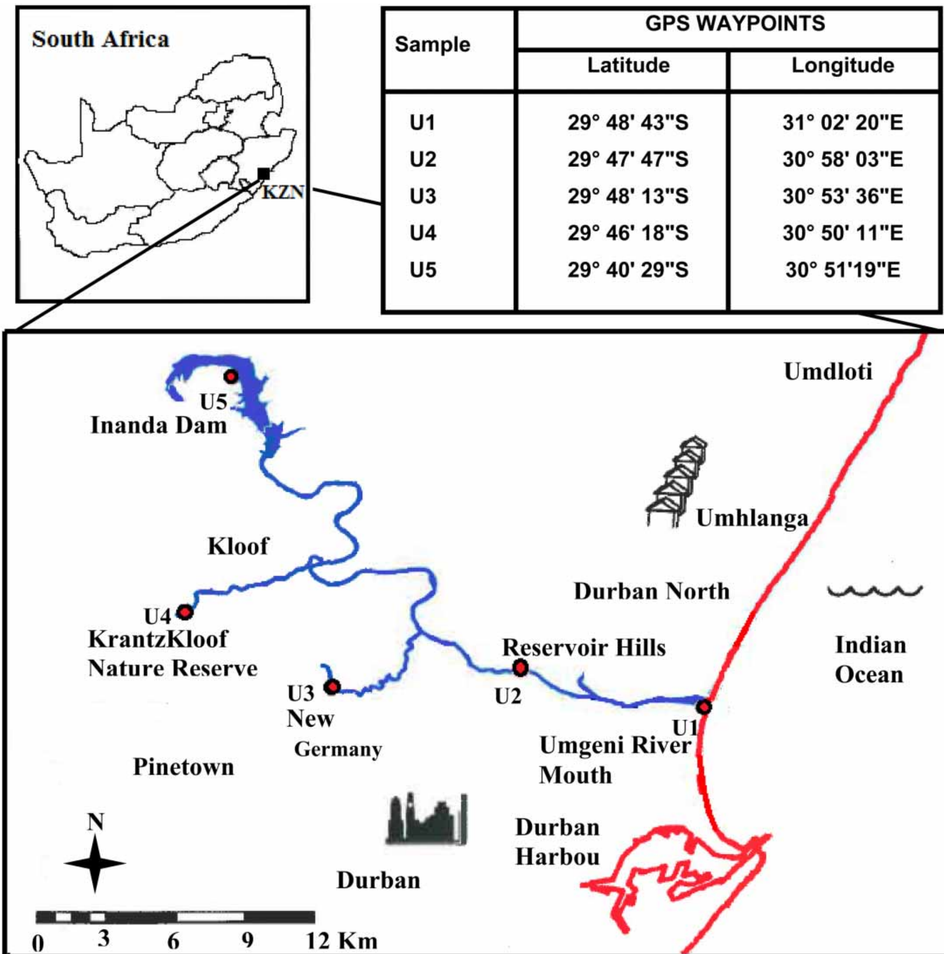

Figure 1 | Map of the study region within Durban and surrounding areas. Shown are the locations of the approximate sampling points of the Umgeni River investigated in this study: U1, Umgeni River mouth – estuarine/brackish water; U2, Reservoir Hills – informal settlement/domestic waste; U3, New Germany Wastewater Works – treated water after chlorination that enters the adjoining Umgeni River water; U4, Krantzkloof Nature Reserve – vegetation and conservation area; U5, Inanda Dam – restricted water containment.

Detection of human viruses from the Umgeni River water by PCR/RT-PCR assay

The primer sequences used to amplify and to detect human enteric viral genomes were selected using previously published data (Table 1) (Allard *et al.* 1992; Symonds *et al.* 2009). PCR for each of the targeted viral groups was performed according to Symonds *et al.* (2009). All PCR mixtures had a total volume of 25 µL and contained 5 µL of target DNA/cDNA, 12.5 µL of 2 × JumpStart™ REDTaq® ReadyMix™ Reaction Mix for High Throughput PCR (Sigma-Aldrich, St Louis, MO, USA), and 10 µM of each primer, unless otherwise stated.

Adenoviruses

Nested PCR was used to amplify the hexon gene conserved in approximately 47 different adenovirus serotypes (Table 1; Allard *et al.* 1992). Five microlitres of the product from the first round of PCR was used as a template for the second PCR reaction. Both rounds of PCR had a final concentration of 0.4 mM $MgCl_2$ in the reaction mixture. Both rounds of the nested PCR were run under the following conditions: 4 minutes at 94 °C, followed by 40 cycles of 92 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minutes and a final incubation step at 72 °C for 5 minutes. In all PCR reactions, a

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Table 1 | Primer and probe sequences for PCR, nested PCR and TaqMan custom gene expression assays designed for qPCR detection of viral groups in the Umgeni River water samples ($n = 20$) used in this study

Virus	Primer/probe	Primer/probe sequence (5′→ 3′)	Amplicon size (bp)	Reference
Adenovirus	AV-A1	5′-GCC GCA GTG GTC TTA CAT GCA CAT C-3′	301	Allard *et al.* (1992)
	AV-A2	5′-CAG CAC GCC GCG GAT GTC AAA GT-3′		
	AV-B1*	5′-GCC ACC GAG ACG TAC TTC AGC CTG-3′	143	
	AV-B2*	5′-TTG TAC GAG TAC GCG GTA TCC TCG CGG TC-3′		
Enterovirus	JP UP	5′-TTA AAA CAG CCT GTG GGT TG-3′	600	Symonds *et al.* (2009)
	JP DOWN	5′-ACC GGA TGG CCA ATC-3′		
	ENT UP*	5′-CCT CCG CCC CTG AAT G-3′	154	
	ENT DOWN*	5′-ATT GTC ACC ATA AGC GAC C-3′		
Enterovirus	ENTV F	5′-CCCTGAATGCGGCTAAT-3′	143	Gregory *et al.* (2006)
	ENTV R	5′-TGTCACCATAAGCAGCCA-3′		
	ENTV probe	5′-FAM–ACGGACACCCAAAGTAGTCGGTTC-MGB-3′		
Adenovirus	ADV F	5′-GCCACGGTGGGGTTTCTAAACTT-3′	129	Heim *et al.* (2003)
	ADV R	5′-GCCCCAGTGGTCTTACATGCACATC-3′		
	ADV probe	5′-FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-MGB-3′		
Rotavirus	ROT F	5′-ACCATCTACACATGACCCTC-3′	87	Pang *et al.* (2004)
	ROT R	5′-GGTCACATAACGCCCC-3′		
	ROT probe	5′-FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-MGB-3′		

*Primers for nested PCR; FAM, 6-carboxyfluorescein (reporter dye); MGB, minor groove binder/nonfluorescent quencher.

positive control (cell-cultured adenovirus type 2 ATCC VR 846) and negative control (distilled water) were included.

Enteroviruses

Nested PCR was used to amplify the 5′-untranslated region conserved in approximately 25 different enterovirus genomes (Table 1; Symonds *et al.* 2009). An additional 1.8 mM $MgCl_2$ and 1.4 mM $MgCl_2$ were added to the first and second round PCR reaction mixtures, respectively. The first round of PCR conditions were 40 cycles of 95 °C for 30 seconds, 57.7 °C for 30 seconds and 72 °C for 45 seconds, followed by 5 minutes at 72 °C. Thereafter 5 μL of amplified PCR product from the first round PCR was used as the template for the second round of PCR, which was amplified by 40 cycles of 95 °C for 30 seconds, 56.5 °C for 30 seconds and 72 °C for 30 seconds, followed by 5 minutes at 72 °C. In all PCR reactions, a positive control (cell-cultured Coxsackie B virus type 1 (Cox B1)) and negative control (distilled water) were included.

Detection and sequencing of PCR/RT-PCR amplicons

The PCR amplicons were electrophoresed on 2% (w/v) agarose gels (Seakem®LE Agarose, BioWhittaker Molecular Applications, Rockland, ME, USA) and visualized by UV transillumination with the Chemi Genius^2 BIO Imaging System and Gene Snap software (Syngene, UK). The band of the PCR product of the correct size was gel extracted, purified using the UltraClean Gelspin kit (Mo Bio Laboratories, Inc.) and re-amplified as before. To confirm the identity of positive nested PCR products, the amplicons were sequenced with their respective primer sets by Inqaba Biotech (South Africa). The obtained nucleotide sequences were analysed by BLAST program at the NCBI (National Centre for Technology Control, NIH, USA) website (http://www.ncbi.nlm.nih.gov/BLAST) under the nucleotide sequences database, to obtain identities of the positive PCR products.

Nucleic acid alignment of the sequences was performed in ClustalX (Larkin *et al.* 2007). A phylogenetic tree was constructed using the neighbour-joining method for each virus

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type. The branching confidence was estimated by bootstrapping with 1,000 re-samplings in MEGA program version 5 (Tamura *et al.* 2011). The accession numbers of the virus prototypes (GenBank accession KM205564–KM205603) used for the alignment and the neighbour-joining tree phylogenetic analysis were retrieved from the BLAST search GenBank database. Nucleotide sequences in the present study are designated according to the sampling location and season for different genotypes or variants.

Integrated cell culture PCR (ICC (RT-)PCR)

Extraction of viral nucleic acids

The ICC (RT-)PCR method includes an infectivity assay and a sensitive (RT-)PCR in order to eliminate false-positive results, to increase amplification signals and to provide a method with high levels of sensitivity and specificity (Reynolds *et al.* 1996). The water sample concentrate (200 μL) from the Umgeni River water sources was inoculated into the various cell lines as described by Ganesh *et al.* (2013). Two known viruses: adenovirus type 2 ATCC VR 846 and Coxsackie B virus type 1 (Cox B1) were used as positive controls. The cell lines used for investigations of human virus in water included A549 (adenocarcinomic human alveolar basal epithelial cell), HEK 293 (human embryonic kidney cells), Hela (Henrietta Lacks – cervical cancer), HepG2 (human hepatocellular carcinoma cells), PLC/PRF/5 (human primary liver carcinoma cells) and Vero (African green monkey kidney cells). The viral RNA and DNA were extracted from 500 μL cell culture supernatants from those cell lines showing cytotoxicity effect using the TRISure™ Reagent (Bioline, Germany) according to the manufacturer's instruction without modification. The RNA and DNA pellets were dissolved in 60 μL TE Buffer (Applied Biosystems), and stored at −70 °C. DNA and RNA of known virus ATCC strains were used as the controls of qPCR experiment.

Viral RNA concentrations were standardized to 1 μg/mL with RT-PCR grade water and cDNA was synthesized using the DyNamo™ cDNA Synthesis kit for RT-PCR (Finnzymes, Thermo Fisher Scientific, Finland). Each 20 μL reaction contained 2 × RT buffer, 300 ng/μL of Random Hexamer Primer set, M-MuLV RNaseH$^+$ reverse transcriptase, nuclease-free water, and approximately 8–10 μL template RNA (100 fg to 1 μg total RNA). The reaction mix was incubated for 10 minutes at 25 °C, 40–60 minutes at 37 °C, followed by 5 minutes at 85 °C. The resulting cDNA was stored at −70 °C.

Detection of human enteric viruses from cell culture by PCR/RT-PCR assay

The presence of potential pathogenic viruses (adenoviruses and enteroviruses) grown on cell culture was confirmed using PCR. PCR/RT-PCR was performed as described in the section 'Detection of human viruses from Umgeni River water by PCR/RT-PCR assay'. Ten to fifteen microlitres of the ICC-(RT-) PCR amplicons were analysed directly by electrophoresis on 2–3% (w/v) agarose gels and visualized by UV transillumination with the Chemi Genius2 BIO Imaging System and Gene Snap software (Syngene, UK).

Quantitative PCR amplification of human viruses from Umgeni River water

Custom TaqMan® Gene Expression Assays, developed by Applied Biosystems (South Africa) using previously published primer and probe sequences (Table 1), were used to quantify the three human viral genomes. The TaqMan® Fast Virus 1-Step master mix (Applied Biosystems, USA) was utilized to perform one-step RT-PCR/qPCR of viral RNA and DNA directly. A typical 20 μL fast real time assay reaction contained 5 μL of 4 × TaqMan® Fast Virus 1-Step master mix, 1 μL of Custom TaqMan® Gene Expression Assay (having a combination of approximately 18 μM primer and 5 μM probe concentration) and RNA or DNA samples up to the maximum allowed by each reaction volume. qPCR was performed in triplicate on an ABI 7500 Fast (Applied Biosystems) using default fast universal thermal cycling conditions of 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C, 30 seconds at 60 °C and 1 minutes at 72 °C. The entire reaction was completed in less than 45 minutes.

The 7500 Fast software for the 7500 Fast Real Time Machine (Applied Biosystems) was used to analyse the data. After determination of RNA concentrations by spectrophotometry, the copy numbers of RNA and DNA molecules

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were calculated using the following formulae (www.qiagen.com):

$$\text{DNA/RNA (copy per } \mu\text{L)} = \frac{X(\text{g per } \mu\text{L})}{\text{DNA/RNA transcript length in nucleotides x 340}} \text{ x } 6.022 \text{ x } 10^{23}$$

where X g/μL (DNA or RNA concentrations) were determined spectrophotometrically.

A sample was considered as positive when the threshold cycle (C_T) value was <35. RNAs obtained from Coxsackie B virus type 1 (Cox B1) and DNAs from adenovirus type 2 ATCC VR 846 and human rotavirus ATCC VR-2018 were used to generate standard curves. A standard curve of genome copies of known virus was generated based on the C_T values of known concentrations. The number of genome copies of each sample was obtained according to the respective C_T value. The obtained genome copy of concentrated viral sample (5 mL) was then converted to the copy number of the original water sample (20 L) by the dilution factor, as presented in Table 2.

RESULTS

Detection of pathogenic human viruses using PCR/RT-PCR

This study used PCR and RT-PCR to determine the presence of adenovirus and enterovirus in Umgeni River water samples. PCR/RT-PCR and nested PCR amplifications of the hexon gene based on 47 different adenovirus serotypes and both of the 5′-untranslated region of the enterovirus genome detecting 25 different enteroviruses were successful,

Table 2 | Number of genome copies of human viruses detected by TaqMan primer/probe sets in the Umgeni River water samples containing mixtures of viral DNA and RNA

		Adenovirus	Enterovirus	Rotavirus			
Sampling points	**Genome copies per ℓ**	**Quantity mean***	**SD**	**Quantity mean***	**SD**	**Quantity mean***	**SD**
Autumn (April 2011)	U1	4.21×10^5	2.05×10^5	2.20×10^9	2.17×10^8	ND	ND
	U2	2.83×10^5	8.55×10^4	2.72×10^{10}	1.15×10^{10}	ND	ND
	U3	1.53×10^5	2.32×10^4	1.58×10^9	9.12×10^8	ND	ND
	U4	5.95×10^4	6.55×10^4	9.26×10^9	6.62×10^9	ND	ND
	U5	ND	ND	3.72×10^{11}	4.62×10^{10}	ND	ND
Winter (July 2011)	U1	1.46×10^4	8.30×10^3	9.61×10^8	1.27×10^9	ND	ND
	U2	1.46×10^5	3.35×10^4	9.80×10^8	3.53×10^8	ND	ND
	U3	4.63×10^4	2.09×10^4	1.10×10^7	1.64×10^7	ND	ND
	U4	ND	ND	2.82×10^9	6.86×10^8	4.15×10^2	2.02×10^2
	U5	3.21×10^4	2.13×10^4	1.92×10^9	1.65×10^9	ND	ND
Spring (October 2011)	U1	ND	ND	2.12×10^8	1.45×10^8	ND	ND
	U2	4.78×10^4	3.34×10^4	1.77×10^{10}	2.76×10^{10}	ND	ND
	U3	5.55×10^5	2.31×10^5	4.40×10^{10}	1.75×10^{10}	ND	ND
	U4	8.95×10^6	2.00×10^6	1.01×10^9	2.75×10^8	58.1	14.7
	U5	4.92×10^6	1.36×10^6	2.54×10^6	1.92×10^6	1.04×10^2	52.2
Summer (January 2012)	U1	6.20×10^4	4.38×10^4	1.48×10^{10}	5.83×10^9	ND	ND
	U2	1.90×10^4	1.87×10^4	6.13×10^8	8.38×10^8	ND	ND
	U3	1.51×10^4	1.10×10^3	1.37×10^9	8.87×10^8	2.62×10^2	24.2
	U4	3.23×10^4	1.88×10^4	1.75×10^9	1.10×10^9	1.01×10^2	19.5
	U5	3.78×10^4	3.59×10^4	8.48×10^8	2.94×10^8	ND	ND

ND, sample with the threshold cycle (C_T) > 35.

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resulting in PCR/RT-PCR amplification products of the expected size (Table 1). The PCR method used for the hexon gene of adenovirus effectively detected the presence of adenoviruses in all water samples (95%) except the winter sample from U5. All the hexon nested PCR products (143 bp) were visible for the positive control and all the water samples tested (100%).

For enterovirus, the PCR method to amplify the 5′-untranslated conserved region in approximately 25 different enterovirus genomes could not detect the presence of enterovirus in most of the water samples. Only 20% of water samples (Site 1: autumn and spring samples; Site 5: autumn and summer samples) produced a desired band (600 bp) after PCR (Table 1), but the nested PCR gene product (154 bp) was successfully amplified using 1st PCR amplicons as the template for all the river water samples tested including those which had tested negative by PCR.

Phylogenetic analysis

Profiles of the virus phylogenetic trees were obtained by comparing the sequences from environmental samples with reference sequences stored in GenBank. It was found that the gene sequences of adenovirus and enterovirus groups had relatively low E-values with sequence identities ranging from 91 to 100% compared to their known counterparts in the GenBank database. The genetic relationships between the viral groups of adenovirus and enterovirus are represented in Figures 2 and 3, respectively.

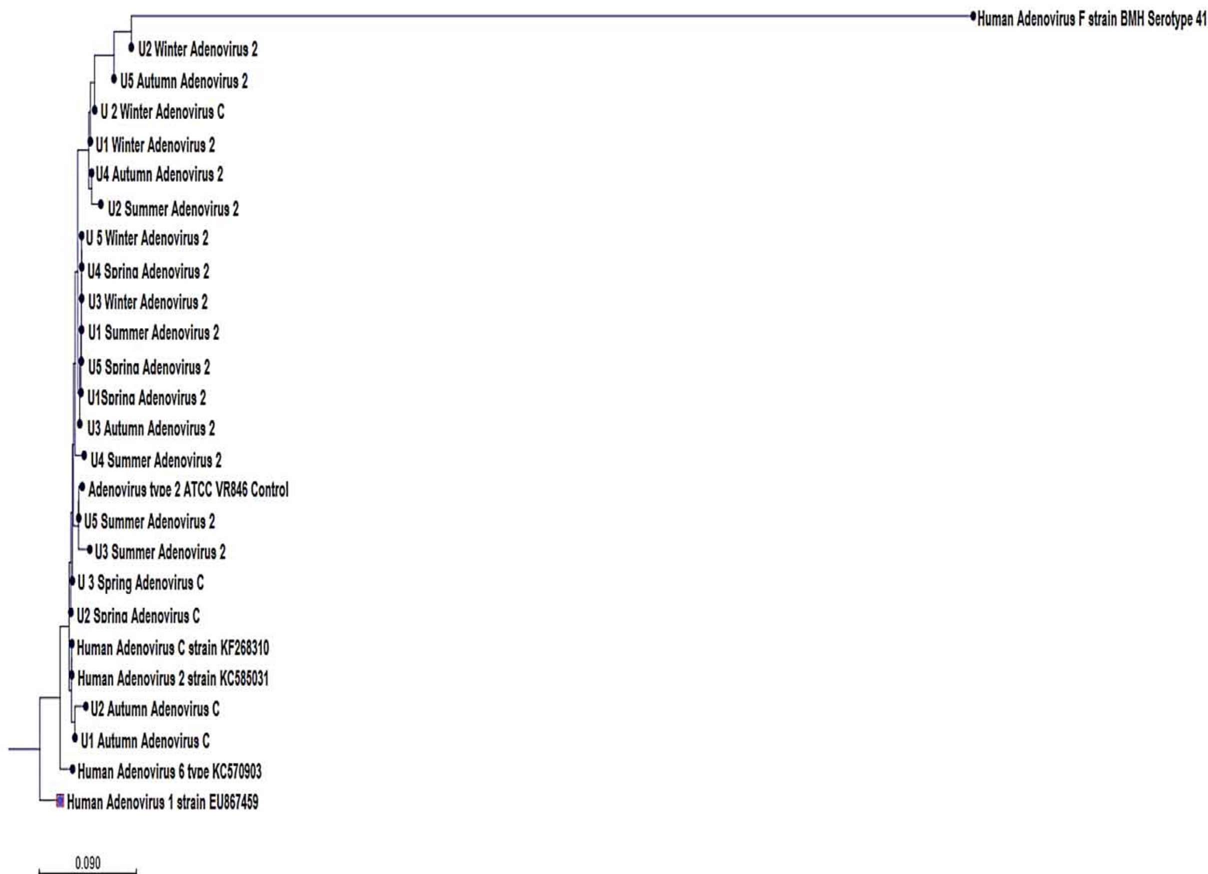

Figure 2 | Neighbour-joining tree representing the phylogenetic relationship between nucleotide sequences of amplicons (143 bp) of the hexon gene from different river water samples (U1–U5, autumn, winter, spring and summer). Each branch represents a sequence or a group of sequences, with identities and sequences being selected from GenBank database using BLAST search of the obtained sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

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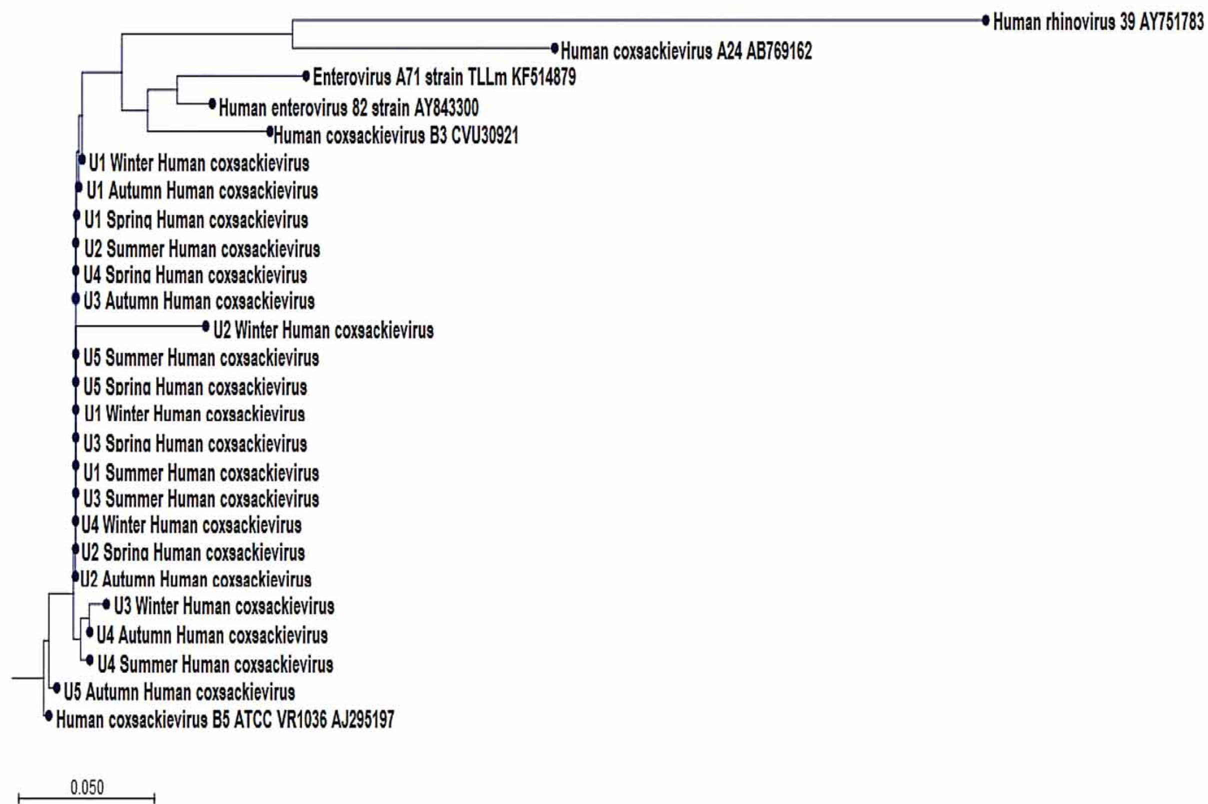

Figure 3 | Neighbour-joining tree representing the phylogenetic relationship between nucleotide sequences of amplicons (154 bp) of the 5′-untranslated region of the enterovirus genome from different river water samples (U1–U5, autumn, winter, spring and summer). Each branch represents a sequence or a group of sequences, with identities and sequences being selected from GenBank database using BLAST search of the obtained sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

Neighbour-joining trees grouped isolates into clades of viruses of the same viral type verifying the identity obtained by BLAST searching. From the neighbour-joining phylogenetic trees in Figures 2 and 3, it can be seen that most of the adenovirus genogroups (Figure 2) were clustered together into one clade with the known adenovirus type 2 ATCC VR 846 (control) while a similar pattern was observed in the human Coxsackievirus groups (Figure 3). Human adenovirus strains during winter (U1, U2, U4) and autumn (U4 and U5) and human Coxsackievirus strains during summer (U4), autumn (U4 and U5) and winter (U3) were separated from the main clades. Most of the identified samples belonged to human adenovirus C strain and human adenovirus 2 strain. All enterovirus samples were identified as human Coxsackievirus B5 strains. These results indicate that the sources of adenoviruses and enteroviruses might be limited.

Quantification of viral nucleic acids using qPCR

To validate the qPCR assays prior to application of environmental samples, the detection limit and amplification efficiency of each reaction were determined. The nucleic acids were standardized to approximately 1 μg/mL where necessary. Standard curves with 10-fold serial dilutions of DNA and RNA controls were prepared and assayed in triplicate. PCR amplification efficiency (E) for each assay was calculated from the slope of the standard curves as 97%, 102% and 102% for adenovirus, enterovirus and rotavirus, respectively.

Table 2 shows qPCR results for adenoviruses, enteroviruses and rotaviruses based on equivalent volumes processed per sample. Each value of qPCR was only considered as positive when the threshold cycle (C_T) of the sample was less than 35. The AdV and EV were detected

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in 85% and 100% of the water samples, respectively. Only five out of the 20 water samples were categorized as EV positive using conventional PCR. Two out of five water samples from the U4 site contained RV and none of the water samples from the U1 and U2 sites contained RV. The population of RV detected in the water samples was significantly lower than that of AdV and EV (Table 2). The results show that the Umgeni River contained higher EV population compared to that of AdV and RV, with the latter being the least.

Adenovirus copy numbers ranged from: not detected (ND) (U5) to 4.21×10^5 GC/L (U1) during autumn, ND (U4) to 1.46×10^5 GC/L (U2) during winter, ND (U1) to 8.95×10^6 GC/L (U4) during spring and 1.51×10^4 (U3) to 6.20×10^4 GC/L (U1) during summer (Table 2). High AdV genome copies were observed during spring season.

Enterovirus copy numbers in this study ranged from: 1.58×10^9 (U3) to 3.72×10^{11} GC/L (U5) during autumn, 1.10×10^7 (U3) to 2.82×10^9 GC/L (U4) during winter, 2.54×10^6 (U5) to 4.40×10^{10} GC/L (U3) during spring and 6.13×10^8 (U2) to 1.48×10^{10} GC/L (U1) during summer (Table 2). High concentrations of EV were detected in all four seasons. Relatively high spatial distribution of EVs occurred throughout all samples and seasons tested (Table 2).

Detection of human enteric viruses by ICC-PCR

The Umgeni River water samples were further analysed by integrated cell culture PCR to provide sensitive detection and confirmation of infectious AdV and EV in various cell culture lines. The percentage of water samples from the Umgeni River which contained positively identified infectious pathogenic viruses is shown in Figure 4. Nested ICC-PCR confirmed the presence of infectious AdV particles in approximately 80, 85 and 100% of water samples using the HEK 293, PLC/PRF/5 and Hep G2 cell lines, respectively. No infectious adenovirus particles were detected at point U4 during the winter and summer seasons on the HEK 293 cell line and at point U5 on HEK 293 during spring and on the PLC/PRF/5 cell during autumn and spring seasons. Nested ICC-RT PCR confirmed the presence of infectious enterovirus particles on all six cell lines tested (Figure 4), with most of the

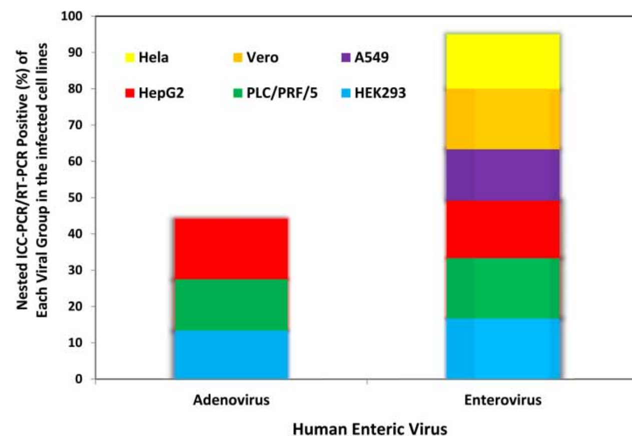

Figure 4 | Proportion of naturally occurring adenovirus and enterovirus genomes detected by nested ICC (RT-)PCR in the various infected cell lines.

infections occurring on the HEK 293, Vero and HeLa cell lines. The results of nested ICC-RT PCR confirmed the presence of infectious viral particles in the water samples of the Umgeni River.

DISCUSSION

Previous studies (Ganesh *et al.* 2013) demonstrate that adenovirus-like, enterovirus-like and rotavirus-like particles were prevalent in the Umgeni River under electron microscope. This study used PCR, nested PCR, qPCR and ICC-nested PCR to determine the presence and the quantity of AdV, EV and RV in Umgeni river water samples.

The PCR/RT-PCR method used for the hexon gene of adenovirus effectively detected the presence of adenoviruses in all water samples except the winter sample from U5. However, the PCR/RT-PCR method to amplify the 5′-untranslated region conserved in approximately 25 different enterovirus genomes failed to detect the presence of EV in most of the water samples (80%; 16 out of 20 samples). Additional nested PCR/RT-PCR step sensed both types of viral particles in 100% of the concentrated river water samples. The use of two phases of amplification enhances the sensitivity of virus detection especially for EV. Other studies demonstrate that nested PCR is more sensitive in detecting viral particles in the environmental samples (Puig *et al.* 1994; Ahmed *et al.* 2010). Nested PCR can increase the yield and specificity of the target DNA

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amplification through the use of two sequential sets of primers (Dongdem *et al.* 2009; Symonds *et al.* 2009). If the first primers bind to and amplify an unwanted DNA sequence, it is very unlikely that the second set of primers will also bind within the unwanted region. Nested PCR assays for AdVs and Evs, as reported by Symonds *et al.* (2009), were shown to have increased sensitivities in 2–3 logarithmic units compared to conventional PCR alone, with detection limits of 100 viral particles per mL for both in wastewater samples. The major disadvantage of the two-step sequential nested PCR is the increased probability of contamination of the negative specimens with products derived from positive amplifications (Picken *et al.* 1996). Nested PCR might also detect non-viable infective agents (Perez de Rozas *et al.* 2008).

The high EV population shown in the results of qPCR study (Table 2) and the detection of EV in the cytotoxic supernatant of almost all tissue cultures tested after infection using the virus concentrates of the Umgeni River in this study are a cause of concern. Enteroviruses have been detected in all types of water including recreational water, tap water and sea water (Lee *et al.* 2005; Ehlers *et al.* 2005a). Ehlers *et al.* (2005b) used a combination of cell culture and nested PCR, and reported the presence of EVs in 42.5% of sewage, 18.7% of treated drinking water, 28.5% of river water, 26.7% of dam/spring water and in 25.3% of borehole water samples. They can survive drinking water treatment and have been detected in drinking water apparently free of coliform bacteria (Vivier *et al.* 2004). Enteroviruses can survive in a wide range of temperatures and salinities (Gregory *et al.* 2006; Chigor & Okoh 2012a). These viruses have been detected in the chlorinated water samples from a water treatment plant (Keswick *et al.* 1984). Among the enteric viruses, human EVs are also one of the most commonly detected viruses in polluted waters (Rusin *et al.* 1999). In addition, enteroviruses are major causes of paralysis, meningitis and myocarditis (Bosch *et al.* 2008). Given the load of EV (high genome copies) found in this river, their resistance to the environmental changes, and the role of EV in waterborne disease, this observation should raise the alarm to authorities as further epidemiological investigation is necessary to confirm the risk of disease to populations exposed to contaminated water.

Adenoviruses (AdVs) are associated with numerous diseases and are responsible for many recreational water disease outbreaks (Enriquez & Gerba 1995). Children and immune-compromised patients are at greater risk of contracting adenovirus infections. Subsequently, adenovirus has been included in EPA's Drinking Water Contaminant Candidate Lists. Adenoviruses have been detected in various waters worldwide. Human AdVs have been shown to frequently occur in raw water sources, treated drinking water supplies, urban rivers and polluted coastal waters (Dongdem *et al.* 2009; Ahmed *et al.* 2010; Fong *et al.* 2010; Jurzik *et al.* 2010; Sidhu *et al.* 2013). Previous detection of adenoviruses from water sources in South Africa has been limited (van Heerden *et al.* 2003, 2004) and our understanding has been burdened by the limited number of scientific outputs, lack of available and precise detection analyses, with regard to virus viability, infectivity and pathogenesis (Grabow *et al.* 2004). AdVs have been detected in about 22% of river water samples and about 6% of treated water samples in selected areas of South Africa excluding KwaZulu-Natal (van Heerden *et al.* 2005).

Adenoviruses have been detected in 92% of water samples at Parkside of the Buffalo River (up to 4.71×10^3 GC/L) (Chigor & Okoh 2012b) and in 39% at Manqulweni of the Tyme River (up to 8.49×10^4 GC/L) (Sibanda & Okoh 2012) in the Eastern Cape province of South Africa, respectively. Sibanda & Okoh (2012) found that adenovirus detection rate increased with distance downstream in the Tyume River. They attributed this to the anthropogenic activities contributing to contamination of natural water sources with enteric pathogens being discharged from partially/untreated wastewater effluents from domestic and municipal sewage into river waters. Our results showed that AdVs were detected in 85% of the water samples of the Umgeni River with the highest concentration of 8.95×10^6 GC/L. Adenoviruses were detected in 16% of the samples, with concentrations ranging from 10^2 to 10^4 GC/L in two southern California urban rivers using qPCR (Choi & Jiang 2005) and in 61% of the samples, with concentrations ranging from 10^3 to 10^5 GC/L in Japan (Haramoto *et al.* 2010). However, the spatial distribution of AdVs seems to be widely spread throughout the Umgeni River. Fong *et al.* (2010) also showed that there are no seasonal variations in the prevalence of these viruses. Due to the

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high detection rates of AdVs and EVs, both viruses have been proposed as water quality indicators (Lin & Ganesh 2013). There are numerous studies reporting the presence of multiple genotypes, or different sequences of enteroviruses (Chen *et al.* 2008; Apostol *et al.* 2012) and of adenoviruses (Fong *et al.* 2010) in environmental water samples. Detailed genotyping analysis has been proposed to identify the sources of contamination (Fong & Lipp 2005; Kroneman *et al.* 2011).

A recent study (Chigor & Okoh 2012a) in the Buffalo River and dams in the Eastern Cape province of South Africa has detected EVs in 9.7% of the water samples, with concentrations ranging from 13 to 86 genome copies/L. In this study, enteroviruses were detected at all sampling points during all seasons according to the results of nested PCR and qPCR, with 90% of the EVs found to be infectious by ICC-PCR with concentrations ranging from 2.54×10^6 to 3.72×10^{11} GC/L which is significantly higher than the other studies in South Africa (Vivier *et al.* 2004; Ehlers *et al.* 2005b; Chigor & Okoh 2012a).

Rotavirus genome copies were significantly lower in the water environment and ranged from 58.1 (U4 in spring) to 415 GC/L (U4 in winter). Only five water samples were categorized as positive samples containing rotaviruses (Table 2). The water samples from U4 contained rotaviruses more frequently than those from other sites and none of the water samples from U1 and U2 were positive for rotavirus. The results of qPCR demonstrate the presence of rotaviruses in this river system.

The World Health Organization (WHO) estimates that globally 527,000 deaths occur each year among children as a result of rotavirus infection, especially in Sub-Saharan Africa (WHO 2004). In South Africa, approximately six children die each day from severe rotavirus gastroenteritis (Parashar *et al.* 2003). Their survival characteristics can be demonstrated by the presence of large amounts of infectious particles in wastewater (Dubois *et al.* 1997), in environmental waters and in drinking water (van Zyl *et al.* 2006). Ansari *et al.* (1991) reported that although waterborne outbreaks of rotavirus gastroenteritis are often recorded, air, body contact and food may be a vehicle for this infection. Although rotavirus is a common cause of diarrhoea clinically in South Africa, few studies have reported on the presence of rotaviruses in raw and treated water in South Africa prior to this study (van Zyl *et al.* 2004, 2006; Sibanda & Okoh 2012). Rotavirus infection has dire consequences with prevention incidence being necessary to limit the spread of disease (Mwenda *et al.* 2010). In a study from Kenya, Kiulia *et al.* (2010) detected Group A rotavirus in 100% of samples collected from a river located in an urban area and in three (25%) of rural river water samples. Sibanda & Okoh (2013) reported that RVs were detected in 13.9% of samples with concentrations ranging from 2.5×10^1 to 2.1×10^3 GC/L in the Tyume River in the Eastern Cape Province of South Africa. Similarly, our study detected 25% of the water samples of the Umgeni River with concentrations ranging from 5.81×10^1 to 4.15×10^2 GC/L. This study demonstrates that the population of RV was higher only during the cooler and drier (July to January) months in the selected river in South Africa (Table 2), as reported by Sibanda & Okoh (2013) and Steele & Glass (2011). However, this study failed to detect rotavirus in water samples collected from urban areas (U1 and U2) and RV was often found in U4 which is a nature reserve and conservation area. Due to the homologous sequences for the rotavirus primers used in this study that can be found in bovine, simian, porcine and human group A rotavirus (Pang *et al.* 2004), it is likely that RVs detected in U4 were from animal sources. Doan *et al.* (2013) confirmed that a bovine RV was transmitted to and caused diarrhoea in a human child in Jerusalem. Thus, the detection of rotavirus is still a concern for public health.

The disadvantage of the PCR-based assays is that they do not allow a conclusion on the infectivity of viral particles in the water samples analysed, since free viral particle nucleic acids would also be detected by the PCR-based assay. The use of ICC-PCR has been described for the detection of infectious EVs (Reynolds *et al.* 1996), hepatitis A virus (Reynolds *et al.* 2001; Jiang *et al.* 2004), enteric adenovirus (Lee & Kim 2002) and astrovirus (Grimm *et al.* 2004). Methods that combine features of cell culture and molecular techniques for a rapid, sensitive detection of infective virus particles in water samples have been developed (Cantera *et al.* 2010) and are found promising to be used in the future. The results of nested ICC-RT PCR further confirmed the presence of infectious viral particles in the water samples of the Umgeni River. Lee *et al.* (2005) demonstrated the simultaneous detection of both EV and AdV in the same cell

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line with this approach. The Umgeni River water samples analysed by integrated cell culture PCR provide confirmation of infectious AdV and EV particles in various cell culture lines. Combining cell culture and PCR, as reported here, will allow researchers to detect more rapidly infectious viruses in clinical and environmental samples. Lee *et al.* (2004) evaluated a cell culture-PCR assay based on a combination of A549 and BGMK cell lines as a tool to monitor infectious adenoviruses and enteroviruses in river water. They found that the number of samples positive for AdV was higher with A549 cells (13 samples) than with BGMK cells (1 sample) and that the number of samples positive for EV were similar with both types of cells. The cell culture-PCR assay established in their study with a combination of A549 and BGMK cells and molecular identification was shown to be a useful tool for monitoring infectious AdV and EV in aquatic environments (Lee *et al.* 2004).

Several studies have assessed the recovery of viruses from different water samples and established that the recovery rate depends not only on concentration and detection methods, but also the source of the environmental water that is tested (Albinana-Gimenez *et al.* 2009; Haramoto *et al.* 2009; Girones *et al.* 2010; Kiulial *et al.* 2010; Victoria *et al.* 2010). The AdV DNA copy numbers as determined by one qPCR assay differed by more than a factor of 1-log from those determined by another qPCR assay (Bofill-Mas *et al.* 2006). The virus particles from the water samples analysed in this study represent only a single time point in the Umgeni River; therefore, it is possible that the types of viruses found could differ if samples were collected at different periods of the year. In all cases, the high prevalence of enteric viruses in surface water highlights the importance of assessing the water sources used for domestic purposes for viral contamination (Kiulia *et al.* 2010).

CONCLUSIONS

Adenoviruses, EVs and RVs were found in all water samples seasonally collected from five points along the Umgeni River. Nested ICC-PCR confirmed viral infectivity in 90% of water samples which contained infectious viral particles. The molecular characterization of these viruses confirmed that the majority of viral isolates were of human origin. Quantification of viral groups using qPCR revealed relatively high genome copies of EV. The results of this study confirmed the presence of infectious viruses in river water potentially used for domestic and recreational activities. The populations of adenoviruses and enteroviruses in the study water samples could serve as indicators for faecal contamination. These results emphasize the need to assess the sources of environmental contamination in interconnected ecosystems to effectively enhance the safety of recreational water. It also helps to demonstrate the need to include virological parameters when determining water quality to reduce the potential exposure of users to contaminated water.

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