Surveillance of human and swine adenovirus, human norovirus and swine circovirus in water samples in Santa Catarina, Brazil

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ABSTRACT

Animal and human wastewater can potentially contaminate water sources and the treatment of drinking water may not effectively remove all contaminants, especially viruses. The purpose of the present study was to evaluate the viral contamination of water used for human and animal consumption in the city of Concórdia, located in southern Brazil. Porcine circovirus type 2 (PCV2), porcine adenovirus (PAdV), human adenovirus (HAdV) and human norovirus (NoV) were searched for using quantitative polymerase chain reaction (qPCR). HAdV-positive samples were tested for viral infectivity by plaque assay. The qPCR results showed that PAdV, PCV2 and HAdV genetic material were present in all sampling sites. NoV was absent in all samples. The presence of genetic material from PAdV and PCV2 was detected in 30% and 45% of the 36 analyzed samples, respectively, with an average of 10^2 gc mL^{-1} for PAdV and 10^4 gc mL^{-1} for PCV2. HAdV was present in 100% of the samples, with an average of 10^4 gc mL^{-1}. However, in plaque assay, only 36% of the samples were positive. As viable particles of HAdV were found in drinking water, these results confirm that swine manure and human sewage impact surface water and groundwater, endangering water quality and indicating a potential risk to public health.

Key words | adenovirus, circovirus, drinking water, norovirus, plaque assay, qPCR

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INTRODUCTION

Water quality is one of the main concerns of this century, in particular because of the contamination caused by inadequate discharge of human and animal waste in the environment. The water used for human consumption is usually submitted to a treatment system; however, if not properly performed, microorganisms such as bacteria and virus can remain viable. Many human diseases can be related to contaminated food and water (Figueras & Borrego 2010). Water can also be contaminated by swine manure, increasingly so as swine production has increased in the last decade (ABIPECS, http://www.abipecs.org.br), consequently increasing the manure volume which, as with human waste, can compromise water quality if not correctly treated.

Among the microorganisms of fecal origin that can be present in the aquatic environment, human adenovirus (HAdV) is the most prevalent and resistant to sewage treatment, increasing the risk of human contamination after ingestion of contaminated food and water (Enriquez *et al.* 1995). Another virus transmitted by contaminated food and water is norovirus (NoV), which is the main cause of non-bacterial gastroenteritis worldwide, accounting for approximately 23 million cases per year in the USA (Gentry *et al.* 2009). Three genogroups of NoV (GI, GII and GIV) can infect humans.

Among the swine viruses, two have received special attention: adenovirus (PAdV) and circovirus type 2 (PCV2). PAdV is an icosahedral, non-enveloped, double-stranded

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DNA virus that is prevalent within swine populations. It is found in feces, residual water and sludge (De Motes *et al.* 2004; Hundesa *et al.* 2006), making PAdV a good fecal contamination marker for the environment. PCV2 is a small, non-enveloped virus, containing a circular, single-stranded genome DNA which can potentially contaminate swine through the ingestion of contaminated water. It causes significant economic losses because it is associated with post-weaning multisystemic wasting syndrome (PMWS; Chae 2005).

The swine population in Brazil is estimated to be approximately 35 million, making the country the fourth largest producer (3 million tons/year), fourth largest exporter (600,000 tons/year) and the sixth largest consumer (11–13 kg/inhabitant/year) in the world. Swine production in Brazil is concentrated in the southern part of the country (Kunz *et al.* 2009); the city of Concórdia, located in Santa Catarina state, south of Brazil is the main swine producer in this region. Swine facility water usage and manure production has consequently increased significantly.

Considering these factors, the present study aimed to evaluate the contamination of different water sources used for human and animal consumption in the city of Concórdia by PCV2, PAdV, HAdV and NoV GI and GII using quantitative polymerase chain reaction (qPCR), and to study the HAdV viability using a plaque assay.

METHODS

Water samples

Two liters of water from six different sites in Concórdia (Santa Catarina, Brazil) were collected monthly from August 2010 to January 2011. The collection points were chosen to represent most of Concórdia city and neighborhood as well as different water sources for human and animal consumption. The following sampling sites were selected: (A) water after conventional treatment followed by chlorination used for swine consumption; (B) water after conventional treatment followed by chlorination from a tap used for human consumption; (C) surface water without treatment used for swine consumption; (D) water from Pinhal River, which crosses many swine production farms; (E) groundwater collected from a tap located at a school; (F) water from Jacutinga River, which is uptaken and treated for population supply, including the site B. Samples were transported on ice and stored at 4 °C until concentration on the following day.

Concentration and nucleic acid extraction

All the samples had the pH adjusted to 5.0–6.0 and were subsequently filtered and concentrated as described by Katayama *et al.* (2002), where 25 mL $MgCl_2$ (2.0 M) was added to 2 L of water sample and filtered through a negative membrane (Nihon Millipore). The membrane was washed with H_2SO_4 (0.5 mM, pH 3.0) and eluted with 11 mL NaOH (1.0 mM, pH 10.0). Additionally, a centrifugation step (1.500 × g for 5 min at 4 °C) using the Centriprep® YM 50 (Millipore) filtration system was performed, and samples were concentrated in a final volume of 4 mL and stored at −80 °C until nucleic acid extraction. Nucleic acid extraction was performed using a QIAmp MinElute Virus Spin Kit (Qiagen) following the manufacturer's instructions, where 200 μL of concentrated sample was treated with protease, followed by serial washes and centrifugations and finally nucleic acid elution in 60 μL of elution buffer and storage at –80 °C until further analysis.

RT-PCR

A reverse transcriptase reaction (RT) was performed to generate cDNA for NoV detection using random primers in the presence of reverse transcriptase enzyme. The reaction was performed in a final volume of 25 μL, containing 5.0 μL ribonucleic acid (RNA). After RT, the samples were subjected to qPCR to quantify specific NoV genomes.

Quantitative PCR

Table 1 lists the sequences of primers and probes used for HAdV, NoV GI and GII, PAdV and PCV2 genome quantification as well as annealing temperatures using the TaqMan assay. All amplifications were performed in a StepOne Plus real-time PCR system (Applied Biosystems). Standard curves were generated by using serial dilutions (range 10^0–10^6) of known amounts of linearized plasmids containing a specific

Table 1 | Primers and probes sequences from qPCR

Virus	Primers and probes	Sequence	Annealing temperature (°C)	References
PAdV	Forward primer	5′-AACGGCCGCTACTGCAAG-3′	60	Hundesa *et al.* (2009)
	Reverse primer	5′-AGCAGCAGGCTCTTGAGG-3′		
	Probe	5′-6FAM-CACATCCAGGTGCCGC-BHQ1-3′		
PCV2	Forward primer	5′-TGGCCCGCAGTATTCTGATT-3′	60	Opriessnig *et al.* (2003)
	Reverse primer	5′-CAGCTGGGACAGCAGTTGAG-3′		
	Probe	5′-6FAM-CCAGCAATCAGACCCCGTTGGAATG-TAMRA-3′		
HAdV	Forward primer	5′-C(AT)TACATGCACATC(GT)C(CG)GG-3′	60	Hernroth *et al.* (2002)
	Reverse primer	5′-C(AG)CGGGC(GA)AA(CT)TGCACCAG-3′		
	Probe	5′-6FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-TAMRA-3′		
NoV GI	Forward primer	5′CG(CT) TGGATGCG(ACGT)TT(CT)CATGA-3′	56	Kageyama *et al.* (2003)
	Reverse primer	5′-CTTAGACGCCATCATCATT(CT)AC-3′		
	Probe A	5′-6FAM-AGAT(CT)GCGATC(CT)CCTGTCCA-TAMRA-3′		
	Probe B	5′-6FAM-AGATCGCGGTCTCCTGTCCA-TAMRA-3′		
NoV GII	Forward primer	5′-CA(AG)GA(AG)(GTC)C(ACGT)ATGTT(CT)AG(AG) TGGATGAG-3′	56	Kageyama *et al.* (2003)
	Reverse primer	5′-TCGACGCCATCTTCATTCACA-3′		
	Probe	5′-6FAM-TGGGAGGGCGATCGCAATCT-TAMRA-3′		

region of each virus. The genome copies (gc) were defined as the mean of the data obtained. Each sample was carried out in duplicate and 10-fold diluted. Ultrapure water was used as the non-template control for each assay.

Plaque assay

The plaque assay was performed for HAdV2 as described by Cromeans *et al.* (2008). Briefly, A549 cell monolayers were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Gibco), 1.0 mM sodium pyruvate, 10 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin. Cells were seeded in six-well tissue culture plates at a density of 5×10^5 cells/well and incubated at 37 °C in 5% CO_2 over 24 h. Concentrated water samples, treated with 10 U mL^{-1} penicillin, 10 μg mL^{-1} streptomycin and 0.025 μg mL^{-1} amphotericin B, were diluted in high-glucose DMEM in a non-cytotoxic dilution, and 0.25 mL was inoculated in triplicate onto monolayers that had been prewashed with Phosphate Buffered Saline (PBS). After 1 h of incubation at 37 °C with rotation every 15 min, the inocula were removed and cell monolayers were overlaid with 0.6% Bacto-agar in high glucose DMEM 2X containing 4% FBS, 0.1 mM sodium pyruvate, 10 U mL^{-1} penicillin, 10 μg mL^{-1} streptomycin, 26 mM $MgCl_2$ and incubated at 37 °C for 7 days. After this period, the agar overlay was removed, cells were stained with 20% Gram's crystal violet and plaques were counted macroscopically once the stain was removed.

RESULTS AND DISCUSSION

From August 2010 to January 2011, 36 water samples were harvested from rivers, groundwater and treated and untreated water sources. After concentration and nucleic acid extraction, qPCR was performed to quantify viruses. The average PAdV, PCV2 and HAdV genome copies per L (gc L^{-1}) of original water samples found at each sampling sites are listed in Table 2.

PAdV was positive in 30% of samples, with an average of 2.06×10^5 gc L^{-1}, ranging from 1.08×10^4 to 1.49×10^6 gc L^{-1}. PCV2 was positive in 41% of samples, with an average of 7.91×10^7 gc L^{-1}, ranging from 1.14×10^6 to 9.86×10^8 gc L^{-1}. The treated water used for human consumption (site B) exhibited the highest number of PCV2 positive samples (4/6), with two positive samples presenting a high number of PAdV gc L^{-1} with an average of 2.49×10^5 gc L^{-1}. The groundwater samples (site E) had one positive sample for PAdV (8.73×10^5 gc L^{-1}) and three for PCV2 (average of 2.14×10^7 gc L^{-1}).

Table 2 | Quantification by qPCR of PAdV, PCV2 and HAdV in water samples collected at six sampling sites in Concórdia, SC, from August 2010 to January 2011. The numbers in parentheses represent the positive samples number of all six samples

Sampling sites	PadV (gc L^{-1})		PCV2 (gc L^{-1})		HadV (gc L^{-1})	
	Average	Interval	Average	Interval	Average	Interval
Site A	4.40×10^{5} (2/6)	1.15×10^{6}–1.49×10^{6}	1.08×10^{8} (2/6)	1.14×10^{6}–6.47×10^{8}	1.55×10^{7} (6/6)	7.58×10^{6}–2.43×10^{7}
Site B	2.49×10^{5} (2/6)	1.56×10^{4}–1.48×10^{6}	4.74×10^{7} (4/6)	2.30×10^{6}–2.20×10^{8}	3.67×10^{7} (6/6)	6.95×10^{6}–1.53×10^{8}
Site C	1.43×10^{5} (2/6)	1.48×10^{4}–8.41×10^{5}	1.66×10^{8} (2/6)	1.28×10^{7}–9.86×10^{8}	1.36×10^{7} (6/6)	9.36×10^{6}–1.70×10^{7}
Site D	1.35×10^{5} (2/6)	1.08×10^{4}–7.97×10^{5}	7.75×10^{7} (2/6)	2.47×10^{6}–4.63×10^{8}	1.48×10^{7} (6/6)	1.30×10^{6}–3.18×10^{7}
Site E	1.46×10^{5} (1/6)	0–8.73×10^{5}	2.14×10^{7} (3/6)	2.24×10^{6}–1.21×10^{8}	1.30×10^{7} (6/6)	6.89×10^{6}–2.73×10^{7}
Site F	1.25×10^{5} (2/6)	2.07×10^{4}–7.32×10^{5}	5.38×10^{8} (2/6)	4.10×10^{6}–1.66×10^{8}	1.60×10^{7} (6/6)	6.11×10^{6}–3.55×10^{7}
TOTAL	11/36		15/36		36/36	

The high presence of PAdV in the water samples corroborates other studies that found 70% of samples tested positive (Hundesa *et al.* 2006) but in a greater amounts than that found for Hundesa *et al.* (2009) (average of 9.0×10^{1} gc L^{-1}). Because of the high prevalence and resistance of PAdV to environmental conditions, this virus has been considered an indicator of manure contamination. However, PCV2 was also found in the water samples in large amounts and was even more prevalent than PAdV. Consequently, PCV2 can potentially be proposed as another viral marker of swine manure contamination in environmental samples.

One hundred percent of the samples were positive for HAdV, with an average of 1.83×10^{7} gc L^{-1} ranging from 1.30×10^{6} to 1.53×10^{8} gc L^{-1}. The treated water used for human consumption (site B) showed the highest average and the greatest amount of HAdV genomic copies/L at 1.53×10^{8}. For all samples analyzed for HAdV, the quantification was never less than 10^{6} gc L^{-1}. NoV, both GI and GII, was absent from the 36 samples.

The absence of NoV GI and GII was expected in treated water samples (sites A and B), as these viruses have an RNA genome and are more susceptible to traditional methods of water treatment by chlorination. However, its absence in untreated sampling sites was not expected because the presence of NoV GI and GII is frequently reported in groundwater (Hill *et al.* 2010) in the affluent and effluent treatment plant samples of human sewage (La Rosa *et al.* 2010) and in river water (Lodder & De Roda Husman 2005). The amount of collected water (2 L) was probably not enough to detect NoV.

In contrast, the high percentage of HAdV-positive samples is greater compared to other studies which found HAdV in 61% of river water samples in Japan, with an average of 10^{4} gc L^{-1} (Haramoto *et al.* 2010), 10^{2} gc L^{-1} HAdV in Spanish rivers (Hundesa *et al.* 2009) and 14% positive groundwater samples in Africa (Verheyen *et al.* 2009). Sewage treatment plant effluent is commonly discarded in the environment and can reach river water, which will later become a source of the human pollution and may represent a health risk (Albinana-Gimenez *et al.* 2009). This discharge into the environment could be one of the reasons for the high presence of HAdV in this study. The lower presence of HAdV in river water compared to conventionally treated finished water samples may be due to the presence

of higher impurities in source water and the possibility of carryover chemical residues in this matrix; these can act as inhibitors in the qPCR assay and result in a false negative.

Figure 1 shows the gc L^{-1} detected in each sampling site during the months of collection for PAdV, PCV2 and HAdV. In October 2010, PCV2 was detected in all sampling sites at

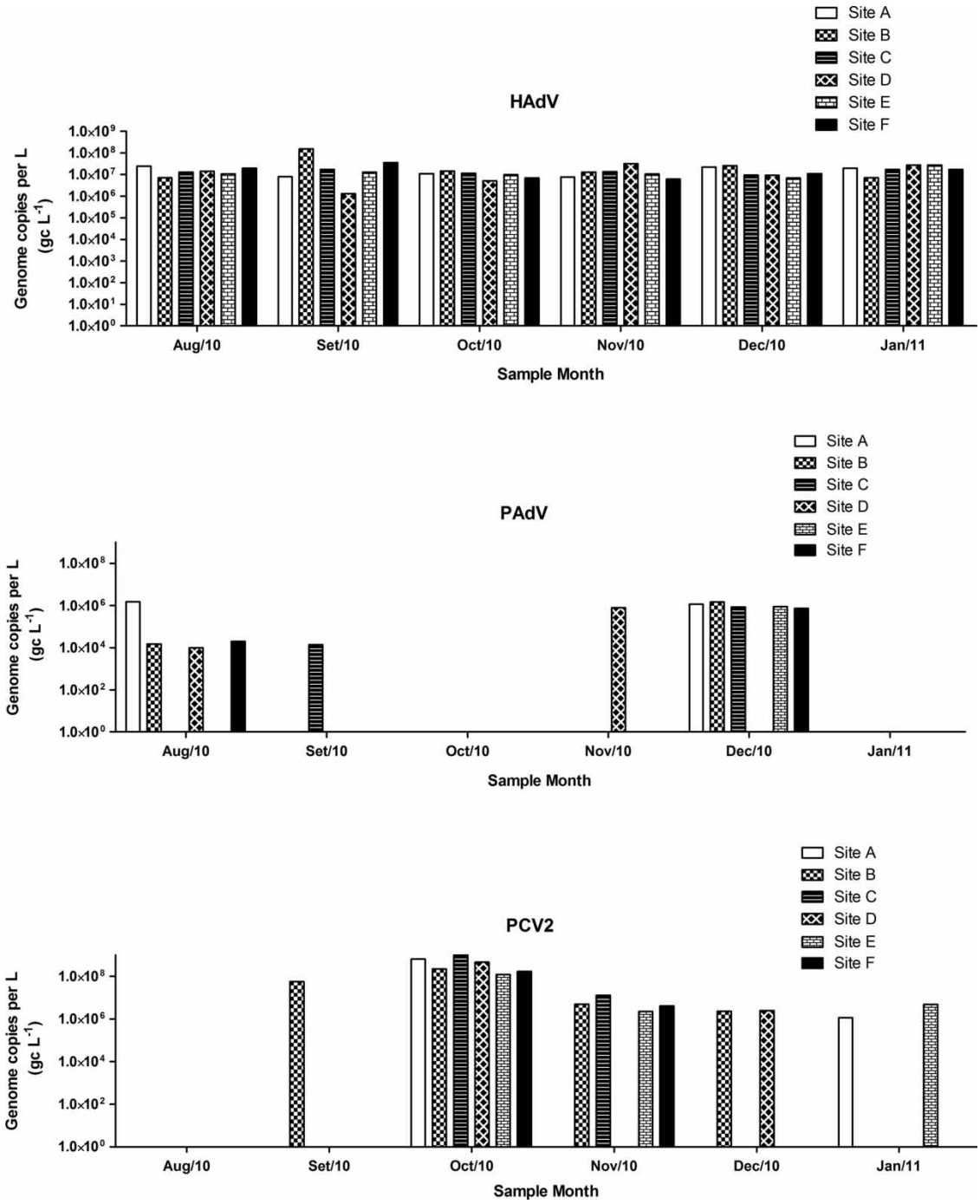

Figure 1 | Genome copies per L (gc L^{-1}) of HAdV, PAdV and PCV2 detected by qPCR of water samples collected at six sampling sites from August 2010 to January 2011. Site A: treated water used for swine consumption; Site B: treated water used for human consumption; Site C: surface water without treatment used for swine consumption; Site D: water from Pinhal River; Site E: groundwater for human consumption; Site F: water from Jacutinga River.

the highest amount. PAdV was detected at five sites and in high quantities in December 2010, which is the only month in which PAdV was detected in groundwater (site E). In August 2010, PCV2 was not detected at any sampling site. PAdV was absent in October 2010 and January 2011 in all samples. Only one sample (site B in December 2010) was positive for both swine viruses. HAdV did not greatly vary during the study period.

There were heavy rains on the 2 days immediately prior to the collection performed in December 2010 (18 and 52 mm, respectively) (Embrapa, http://www.cnpsa.embrapa.br), which may explain the detection of PAdV at five sites, including groundwater (site E). The relationship between the presence of the virus in environmental samples and the precipitation level has been reported by Maalouf *et al.* (2010). However, this explanation cannot be applied to the presence of PCV2. In addition, it is possible that there is no relationship between the detection of PCV2 and PAdV, as both viruses were detected together only in one sample.

The plaque assay for assessing HAdV viability revealed that 13 of 36 samples (36%) were positive for viable HAdV. The number of plaque-forming units per liter (PFU L^{-1}), the average at all sampling sites and each month, are listed in Table 3. The most viable viral particles were detected in September 2010. The treated water used for swine consumption (site A), from Pinhal River (site D) and Jacutinga River (sites F), had the highest frequency of viable viral particles. No viable viral particles were found in groundwater (site E).

The divergence between qPCR and plaque assay results for HAdV can be explained by the fact that, for the qPCR, the primers are used to amplify a conserved region of all human adenoviruses and the plaque assay technique only determines the presence of viable viruses that are able to replicate and produce plaques. Likewise, the study of enteric adenoviruses in water has been hindered because they are generally more difficultcult to culture than other HAdV species and may infect cells without producing visible cytopathic effects or plaques (Rigotto *et al.* 2011). It is therefore not possible to conclude that there were more positive samples by qPCR, which could be contaminated with enteric or fastidious adenovirus.

Figure 2 shows the average gc L^{-1} and the PFU L^{-1} of HAdV in all sampling sites for each month of collection. The highest number of viable viral particles was observed in the first 3 months, whereas in January 2011 no positive result was observed. In September 2010, a slight increase in gc L^{-1} and PFU L^{-1} was observed.

The presence of a higher number of viable HAdV in the first 3 months of collection may be related to the lower temperature at this time of year (winter), resulting in the virus existing at its lowest temperature and thereby preserving its integrity and potential infectivity. In warmer periods there was a visible decay in viral viability in water samples, and no PFU were detected in January 2011 (summer). The relation of the survival of HAdV to temperature has previously been observed by Rigotto *et al.* (2011) and may be related to increased viral inactivation by ultraviolet radiation from the sun (Thurston-Enriquez *et al.* 2003).

It is known that enteric viruses lose viability in groundwater due to several factors including temperature, dissolved oxygen, pH, turbidity, chloride concentration, conductivity

Table 3 | Number of plaque-forming unit HAdV per liter (PFU L^{-1}) detected by plaque assay in water samples collected at six sampling sites in Concordia, SC, from August 2010 to January 2011

Sampling sites	Aug/10	Sept/10	Oct/10	Nov/10	Dec/10	Jan/11
Site A	0	133	53	0	26	0
Site B	0	213	0	26	0	0
Site C	346	0	53	0	0	0
Site D	53	160	0	0	133	0
Site E	0	0	0	0	0	0
Site F	0	26	186	26	0	0

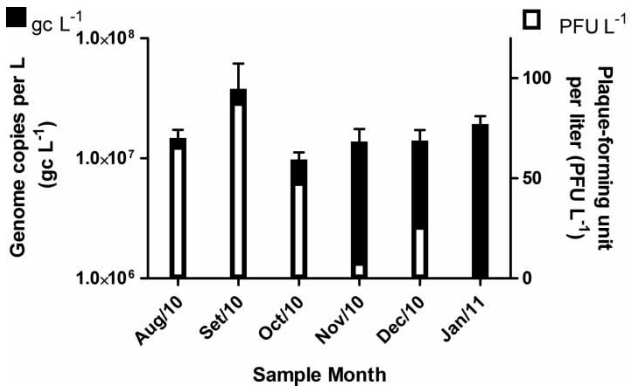

Figure 2 | Average genome copies per L (gc L^{-1}) (black bars) and PFU L^{-1} (white bars) of HAdV in all sampling sites on each collection month, from August 2010 to January 2011.

and the presence of other microorganisms (Gordon & Toze 2003). There may be a loss of HAdV viability in groundwater from sampling site E, as no HAdV PFU was detected in any sample. More information about the physical-chemical and microbiological parameters of the water could help explain these results.

The detection of HAdV in treated drinking water samples is alarming and has been reported previously by Van Heerden *et al.* (2003, 2005). Despite chlorination in water treatment plants capable of inactivating HAdV (Page *et al.* 2009; Kahler *et al.* 2010), the virus was still found to be present and viable in the treated water. It is assumed that either the chlorine concentration or time for water disinfection was not enough or there was contamination in the treatment system itself or in the water distribution system.

As a result of this study, it is necessary to increase the proper disposal of swine manure so that it does not have direct contact with rivers, preventing contamination of surface water and groundwater and eliminating potential pathways for diseases. Furthermore, it is essential to implement effective techniques for eliminating viruses in sewage and drinking water and to include virological parameters when testing water quality.

CONCLUSIONS

The contamination of rivers and water for human consumption by viable HAdV is concerning, and urgent implementation of more efficient methods for the removal of viruses in sewage treatment and the water supply is necessary. The same principle can be applied to swine manure treatment systems, since they are not enough to eliminate all virus particles. The presence of PAdV and PCV2 at all collection points indicates the possible contact of swine manure with water sources.

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