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Research Paper

Detection of human adenovirus, rotavirus and enterovirus in water samples collected on dairy farms from Tenente Portela, Northwest of Rio Grande do Sul, Brazil

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Abstract

Viral gastroenteritis and other waterborne diseases are a major concern for health in Brazil. A number of studies were conducted about the presence of viruses on water samples from Brazilian areas. However, the knowledge about the occurrence of viral contamination of drinking water sources in rural settings of the country is insufficient. On the present work, 15 samples from 5 dairy farms located at the municipality of Tenente Portela were collected and analysed for the presence of human adenoviruses (HAdV), as well as human enteroviruses (EV) and rotaviruses (RV). HAdV was present on 66.66% of the water samples, and have been found in all samples from artesian wells and springs, which are used as sources of drinking water for the individuals inhabiting those farms. EV and RV found only in one sample each. The detection rates of HAdV on the water from these dairy farms are alarming and point towards a situation of elevated environmental contamination by fecal microorganisms of human origin and poor basic sanitation conditions.

Key words: human adenovirus; water quality; dairy farms.

Introduction

Access to safe water in rural areas in Brazil is scarce; it is easily observed in the farms used for dairy milking in southern Brazil, where the production is usually conducted on small properties. Low income, poor access to technical information and improper disposal of animal waste, as well as the lack of sanitation facilities for the farmers and families, lead to a common frame of degradation of environmental quality in these locations (Amaral *et al.*, 2003; de Medeiros and de Souza, 2009).

Studies involving the analysis of microbial contamination and chemical pollution of water in dairy farms have been conducted in different parts of the world and some studies were made on South America (Amaral *et al.*, 2003; Bettera *et al.*, 2011; Derbyshire and Brown, 1978; Schwarte *et al.*, 2011; Weatherley *et al.*, 2011). In most of these studies, it is noticeable the contamination of surface and groundwater by bacteria and protozoa, but there are few studies that address the detection of enteric viruses (Ahmed *et al.*, 2010; Schwarte *et al.*, 2011; Verheyen *et al.*, 2009). Enteric viruses have a number of characteristics that make them excellent markers for fecal contamination of water: i) they are extremely resistant in the environment due to its non-enveloped structure, ii) they are eliminated in large quantities in the feces of humans and animals sick or subclinical infections in iii) in most cases these viruses are

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host-specific and thus allow screening of the species which is the source of fecal contamination (Fong and Lipp, 2005; Silva *et al.*, 2011; Wolf *et al.*, 2010; Wu *et al.*, 2011). Among the enteric viruses three of the most studied as environmental contaminants are the adenoviruses (AdV, *Adenoviridae* family, *Mastadenovirus* genus, double-stranded DNA), enteroviruses (EV, *Picornavirales* order, *Picornaviridae* family, *Enterovirus* genus, single-stranded RNA, positive sense) and rotaviruses (RV, *Reoviridae* family, *Sedoreovirinae* subfamily, genus *Rotavirus*) (Comerlato *et al.*, 2011; Fong and Lipp, 2005; Matthijnssens *et al.*, 2008; Sibley *et al.*, 2011). These agents are transmitted by the fecal-oral route, being associated with a number of diseases, especially gastroenteritis, either in human beings or animals (Ahmad *et al.*, 2009; Hamza *et al.*, 2011). In recent years, the detection of these viruses in surface waters, sewage and coastal waters using the previous concentration of viral particles by different methods and molecular methods for the identification of viral genomes has allowed the conclusion that there is a wide contamination of water by viruses in various ecosystems (Wu *et al.*, 2011). In rural areas, these viruses have been found contaminating ground and surface waters and their presence may represent a risk not only the health of humans and domestic animals, but can also have adverse effects on the health of wildlife (Ahmed *et al.*, 2010; Jiménez-Clavero *et al.*, 2005; Ley *et al.*, 2002).

In this study, water samples were collected from different points on farms devoted to milk production in the municipality of Tenente Portela, in southern Brazil, which is inserted in a wide geographic region devoted mainly to agriculture and livestock, especially dairy. These properties have the typical characteristics of small farms attached to the chain of milk production in southern Brazil, described before. These water samples were tested by the polymerase chain reaction for the presence of human adenovirus (HadV) as an effort to determine whether the human beings are a source o fecal pollution to the water on these farms. The samples were tested also for EV and RV genomes. For HAdV and EV the primers used were capable of detecting viruses from human beings, whereas the primers for RV are pan-reactive to the group A of RV from different species. This is the first study on the contamination of water by enteric viruses at the Northwest of the state of Rio Grande do Sul.

Materials and Methods

Sampling sites and samples

Tenente Portela is a municipality in the northwest region of Rio Grande do Sul (27°22'16" S and 53°45'30" W), the southernmost state of Brazil. The estimated population of 13,719 inhabitants is decreasing through the years and the primary sector is responsible for a third of the income. From the total area of 390 km^2, 19,968 ha are divided by 1,352 farms, from these 1,105 are used for dairy production. The collections were made on different water sources from 5 (five) farms on August 2009, under dry weather conditions. Water samples (500 mL each) were collected aseptically from each farm. The 15 (fifteen) samples obtained were transported to the laboratory under refrigeration, and were kept at 4 °C until sample concentration.

Sample concentration

Water samples were concentrated using an adsorption-elution method previously described (Katayama *et al.*, 2002) with minor modifications (Vecchia *et al.*, 2012). Briefly, 0.6 g of $MgCl_2.6H_2O$ were mixed with 500 mL of water sample and pH was adjusted to 5.0 using a solution of 10% HCl. After, the resulting mixture was vacuum filtered through negatively sterile membrane (type HA, 0.45 μm pore size; 47 mm diameter). The membrane was rinsed through the washing with 87.5 mL of a 0.5 mM H_2SO_4 (pH 3.0) followed by elution of viral particles adsorbed to the membrane with 2.5 mL of 1 mM NaOH (pH 10.5). The pH of the filtrate was neutralized with 12.5 μL of 50 mM H_2SO_4 and 12.5 μL in 100X Tris-EDTA (TE) buffer. The eluate was aliquoted and stored at -80 °C until further processing.

Viral nucleic acid extraction

The commercial kit RTP DNA / RNA Virus Mini Kit (Invitek, Germany) was used for extraction of viral nucleic acids, according to the manufacturer's instructions, using an initial volume of 400 μL of each concentrated water sample. The viral DNA or RNA obtained was stored at -80 °C for later processing.

Polymerase chain reaction (PCR)

In order to achieve amplification EV and RV genomes, a previous step of cDNA synthesis was carried out before amplification. It was performed using the High Capacity cDNA Reverse Transcription commercial kit (Applied Biosciences, USA), using a set of random primers and RNAse Inhibitor (Applied Biosciences, USA), following manufacturer's instructions.

The sequences of the primers and their location in the viruses' genomes are described on Table 1. PCR conditions were optimized and reactions were standardized as following: (a) AdV and RV: 50 μL reaction mixtures consisting 25 μL of GoTaq® Green Master Mix (Promega, USA), 18 μL of nuclease-free water, 1 μL of each primer (20 pM) and 5 μL of nucleic acid; (b) EV: 25 μL final volume containing 12,5 μL of 2x PCR Master Mix (LGCbio, Brazil), 7,5 μL of nuclease-free water, 1 μL of each primer (20 pM) and 3 μL of cDNA product; DNase/RNase free water was used as a negative control during all PCR assays. The positive controls used were Poliovirus-1 (Sabin strain), kindly provided by Dr. Carlos Nozawa; HAdV types 2 and 5,

Table 1 - Primers and conditions used for PCR amplification of AdV, EV and RV genomes used on the present study.

Viruses	Primer			Annealing temperature	Amplicon length
	Name	Sequence	Position		
HAdV (Hexon)	VTB2-HAdVCf	5'-GAGACGTACTTCAGCCTGAAT-3'	106-126[a]	55 °C	101 bp
	VTB2-HAdVCr	5'-GATGAACCGCAGCGTCAA-3'	190-207[a]		
EV (5'UTR)	ENT-F1	5'-CCTCCGGCCCCTGAATG-3'	443-459[b]	56 °C	116 bp
	ENT-R2	5'-ACACGGACACCCAAAGTAG-3'	541-559[c]		
RV (VP6)	ROTAFEEVALE-FW	5'-GATGTCCTGTACTCCTTGT-3'	7-25[d]	54 °C[f]	160 bp
	ROTAFEEVALE-REV	5'-GGTAGATTACCAATTCCTCC-3'	148-167[d]		

[a]Primers sequences reported by Wolf *et al.* (2010).
[b]Primers sequences reported by Tsai *et al.* (1993).
[c]Vecchia *et al.* (2012), Genome position of primers based on GenBank accession number FJ859064.
[d]Vecchia *et al.* (2012), Genome position of primers based on GenBank accession number HM34874.
[e]Initial annealing temperature, which was decreased by 0.5 °C at each of the 39 subsequent cycles (Touchdown-PCR).

kindly provided by Dr. Célia Barardi; Human-RV (isolate C-5, VP6 I-2 Genotype) was isolated from a clinical sample collected from a children with diarrhea (Vecchia *et al.*, 2012).

Amplification of the target genomic fragments was performed using a thermal cycler (MultiGene®, Labnet International, USA). The PCR conditions were optimized for each virus group and were as follows: (a) AdV: 98 °C for 7 min, 40 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; (b) EV: 98 °C for 5 min, 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min; (c) RV: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 54 °C for 1 min (which was decreased by 0.5 °C at each of the 39 subsequent cycles), 72 °C for 1 min. After, all reactions were left at 72 ° C for 7 min for final elongation and submitted to an infinite cycle at 4 °C.

To determine the analytical sensitivity of the assays, 10-fold serial dilutions of each EV, HAdV and RV positive controls grown on cell culture were experimentally inoculated onto sterile 500 mL water samples and after processed on the same manner described above for the samples. All the PCRs have analytical sensitivity enough to detect 1-10 tissue culture infective doses ($TCID_{50}$) diluted on water. These tests and results are described elsewhere (Vecchia *et al.*, 2012).

PCR products were stained with nontoxic fluorescent dye SYBR® SAFE DNA Gel Stain (Invitrogen, USA), analyzed by electrophoresis on 2% (w/v) agarose gel and visualized under ultraviolet (UV) light.

Results

From the 15 samples, 10 showed HAdV genomes (66.66%), and only one sample showed contamination by EV and another by RV (Table 2). HAdV genomes were detected in at least one collection point from the 5 farms. Samples from only one farm resulted positive for EV and RV, and the stream contaminated by RV was also contaminated by HAdV (farm #344). Among the eight surface water samples collected from streams and ponds, only 3 presented viral genomes, while for the six groundwater samples, all were positive HAdV. A sample of tap water was analyzed and was contaminated by HAdV.

Discussion

In areas and facilities where dairy cows are milked staged between the various phases of the milking process, wastes are removed using large volumes of water. Without appropriate treatment, the sludge generated may allow the transportation of fecal microorganisms into ponds, creeks and groundwater (Pullar *et al.*, 2011; Weatherley *et al.*, 2011; Wilcock *et al.*, 2011). In southern Brazil, dairy cows

Table 2 - Detection of HAdV, EV and RV genomes, and coliforms quantification, in water samples collected from springs, creeks, ponds and artesian wells on dairy farms at the municipality of Tenente Portela, Rio Grande do Sul, Brazil.

Farm	Sample	HAdV	EV	RV
#326	Artesian well #1	•	°	°
	Artesian well #2	•	°	°
	Spring	•	°	°
#329	Artesian well	•	°	°
	Creek	°	°	°
	Pond	°	°	°
#330	Spring	•	°	°
	Tap (milking parlor)	•	°	°
	Creek	°	°	°
	Pond	°	°	°
#343	Spring	•	°	°
	Creek	•	°	°
	Pond	•	°	°
#344	Creek	•	°	•
	Pond	°	•	°

• = positive; ° = negative.

are generally raised on a semi-intensive system, and the excreta deposited on pastures may be also a source of fecal pollution since contaminants may be transported into water bodies by superficial runoff (Ahmad *et al.*, 2009; Ahmed *et al.*, 2010). Another major problem of the farms located in this region is the poor access to treated water and absence of basic sanitation in most cases.

HAdV genomes were detected in all samples taken from wells and springs on the present study, thus indicating a high rate of contamination of the subsoil and consequently aquifers. This may be an effect of the poor construction of latrines and wells on these farms, which can permit the infiltration of the subsoil by microorganisms, and viruses may thus accumulate on the groundwater resources (Jung *et al.*, 2011; Pujari *et al.*, 2012; Steyer *et al.*, 2011; Wilcock *et al.*, 2011). The concern is that water from artesian wells and springs is often thought to be free of contaminants and the farmers and families living on these locations have been using this as the solely source of drinking water.

The rates of detection of human HAdV on the present work are higher than those found on urban areas on the north of Brazil (Miagostovich *et al.*, 2008), and very similar to the rates for the southeast (Piranha *et al.*, 2006; Santos *et al.*, 2004) and south of Brazil (Moresco *et al.*, 2012; Rigotto *et al.*, 2010). The detection rate is also very close to the found on another study conducted on pig farms, aiming the detection of porcine adenovirus (PoAdV) (Viancelli *et al.*, 2012). Indeed, HAdV and other adenoviruses are often found as highly prevalent on environmental waters, but one may expect lower levels of detection when analyzing water from areas of low population density. Thus, it is concluded that the impact of poor sanitation conditions within these farms overpasses the small number of individuals on each local. Nevertheless, when comparing to other studies on rural areas, the rates of adenoviral contamination of water on the present study are very high. In a study conducted in Benin, only 12.9% of the sampling sites were positive for AdV genomes (Verheyen *et al.*, 2009). On the other hand, the results for rotaviruses are very similar, in both studies the rates were very low for the molecular detection of RV (Verheyen *et al.*, 2009). Other authors also found lower rates for the detection of HAdV on wastewater collected from rural areas in Australia (Ahmed *et al.*, 2010). Lower rates of detection for AdV were reported on a previous investigation conducted on dairy farms from another watershed in Rio Grande do Sul. The detection levels also differed for the RV and EV (De Oliveira *et al.*, 2012). This low rate of detection was also found on water from dairy farms at the Paranhana watershed (De Oliveira *et al.*, 2012). Although BEV was proposed as reliable marker of fecal contamination of water by cattle manure (Comerlato *et al.*, 2011; Jiménez-Clavero *et al.*, 2005; Ley *et al.*, 2002), those samples were also submitted for molecular detection using the same protocols. However, all showed negative (data not shown). A single sample was positive for EV on the farm #344. It is remarkable that these differences may occur in the same state, but one has to consider the possibility of interference from a range of factors, such as the diversity of the landscapes, the climatic factors at the time of collection, management of the animals and wastes or even the particular epidemiology of these viruses in animal and human population living at the sites of study. These findings points that there it would be difficult to find an universal viral markers of fecal contaminations, at least on rural areas.

The detection rates of HAdV in these water samples in a rural setting in southern Brazil are alarming and point towards a situation of elevated environmental contamination by fecal microorganisms of human origin. Given the resistance of waterborne pathogens and its transportation on the environment, this can be a health risk to individuals inhabiting these farms and even to rural and urban areas present in the same watershed. Unfortunately, rural communities are often neglected by the authorities when dealing with investments in basic sanitation.

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