DETECTION OF ROTAVIRUS FROM PIG LIVESTOCK WASTEWATER OF SÃO PAULO STATE, BRAZIL

DETECÇÃO DE ROTAVÍRUS EM EFLUENTES DE CRIAÇÕES DE SUÍNOS NO ESTADO DE SÃO PAULO, BRASIL

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SUMMARY

Rotavirus is one of the most important viral agents of gastroenteritis among child and animals from different species. It's high environmental resistance and the fecal-oral way of transmission makes this virus likely to be transmitted by wastewater. This study seeks to detect the wastewater elimination and circulation of group A rotavirus in low technified pig farms from São Paulo State, Brazil. A total of 25 samples, including piglet feces with diarrhea and untreated wastewater samples, from 7 different farms, were submitted in a parallel screening scheme of rotavirus infection through polyacrilamide gel electrophoresis (PAGE) and ELISA, which the positive samples were further confirmed by RT-PCR (reverse-transcription polimerase chain reaction). The PAGE revealed only one positive sample (1/25 or 4%) from feces, while by ELISA, 6 (6/25 or 24%) samples were positive, which 4 were from feces and 2 from wastewater. The RT-PCR confirmed all positive PAGE and ELISA results. Therefore, the rotavirus was found in 3 of 7 (42.86%) researched farms, which in 2 of these were detected both in animals and wastewater and one were found virus only in fecal samples. In view of these results, there was rotavirus detection from untreated pig farm wastewater, posing as a risk of spreading for humans and animals, implying the need of assuring microbiological and environmental safety measures with this material.

KEY WORDS: Diarrhea. Environment. Rotavirus. Swine. Wastewater.

RESUMO

Os rotavírus são um dos principais agentes virais envolvidos na ocorrência de gastroenterites em crianças e em animais de diferentes espécies. Sua elevada resistência ambiental aliada à via de transmissão fecal-oral, torna-o um agente propício de se propagar pela água, principalmente nos efluentes. O objetivo deste estudo foi o de se detectar a circulação e eliminação de rotavírus em criações de suínos de baixa tecnificação do Estado de São Paulo, Brasil. Um total de 25 amostras, incluindo fezes de leitões com diarréia e efluentes não tratados, de 7 diferentes propriedades, foram testadas em paralelo para detecção do rotavírus através da eletroforese em gel de poliacrilamida (PAGE) e ELISA, sendo as positivas confirmadas por RT-PCR (transcrição reversa - reação em cadeia pela polimerase). A PAGE evidenciou apenas uma amostra positiva (1/25 ou 4%) proveniente de material fecal, enquanto que pela ELISA, 6 (6/25 ou 24%) amostras positivas, das quais 4 de material fecal e 2 de efluentes. A RT-PCR confirmou todos os resultados positivos de PAGE e ELISA. Portanto, os rotavírus foram encontrados em 3 de 7 (42,86%) das criações pesquisadas, das quais em duas destas, o vírus foi detectado tanto no efluente quanto nos animais. Face a estes resultados, houve a detecção de rotavírus nos efluentes não tratados de criações de suínos, constituindo um risco para a disseminação do agente para humanos e animais, implicando na necessidade de assegurarem-se medidas de segurança ambiental e microbiológica deste material.

PALAVRAS-CHAVE: Diarreia. Efluente. Meio-Ambiente. Rotavírus. Suíno.

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INTRODUCTION

The intensification of the pig farming increased not only the environmental concerns, but also health issues due to toxic substances and great variety of microbial disease agents in the wastewater (WHO, 2004). These sewage pathogenic agents are not well studied and many of them can be transmitted from animals to human by various routes, such as by manure management work or eating raw food fertilized with manure slurry (CONSTANTINI et al. 2007, GERBA & SMITH JUNIOR, 2005).

As the low technified pig farms may have poor wastewater treatment practices, once the short production-scale and restricted profitability pose as limiting factors in order to adopt treatment systems like stabilization ponds, biodigesters and composting manure, there is, as consequence, a potential risk of spreading of enteric pathogens arising from the improper disposal of this material.

Rotavirus is one the most important viral agents of gastroenteritis in children and different animals species, by oral-fecal route, in which water plays an important role in the transmission (ESTES & KAPIKIAN, 2007, PAHO, 2001), and both bovine and swine act as a genetic and antigenic reservoir of the human rotavirus diversity (COOK et al. 2007, MARTELLA et al. 2005, PAHO, 2001). In pig, it affects mostly animals aged from 5 to 35 days, causing economic losses by weight reduction, mortality (MORES et al. 1987).

There are several reports about the animal-human rotavirus interaction in the world. Laird et al. (2003) detected P genotypes with porcine and canine origin in Mexico; BanerjeE et al. (2007) found in India, a rotavirus G11P[25] which they considered a possible zoonotic transmission, once the G11 genotype is more commonly found in swines and bovines. These same authors also found a G3P[3] sample, which the G3 genotype had greater nucleotide identity with animal strains, when compared with humans.

In Brazil, Gabbay et al. (2008) detected by PCR a group C rotavirus that had porcine virus characteristics in children feces and Mascarenhas et al. (2007) found human rotavirus with same RNA sequences of VP4 and NSP4 from swine origin

Considering the zoonotic and inter-species aspects of the infection of rotavirus and need of information about the agent environmental contamination by wastewater, the aim of this study was the detection of rotavirus in piglets and low technified pig farms wastewater.

MATERIAL AND METHODS

Between May 2007 and June 2008, 7 farms located in São Paulo Brazil were visited and the animal feces with diarrhea and 300ml of wastewater collected, using for both stool container. A total of 25 samples (18 of feces and 7 of wastewater) were submitted in a parallel screening scheme of rotavirus infection through polyacrilamide gel electrophoresis (PAGE) and ELISA, which the positive samples were further confirmed by RT-PCR (reverse-transcription polimerase chain reaction).

Stool samples were prepared as 20% suspensions in TRIS-HCl 0,1M pH 7,3 buffer and clarified at 12,000x g/ 30 minutes at 4ºC, and the supernatant was stored at -20ºC until analysis.

The wastewater samples were initially clarified at 12,000x g/ 30 minutes at 4ºC, and after concentrated by osmosis, using dialysis bags (LaBELLE & GERBA, 1980) (SIGMA® D6191) and saccharose, in order to reduce the volume to approximately 5 mL.

After, the samples were screened for rotavirus 11-segmented RNA in PAGE (polyacrylamide gel electrophoresis) according to Herring et al. (1982). Total RNA was extracted with phenol/ chlorophorm, precipitated with ethanol and resolved in 3.5% / 7.5% discontinuous polyacrylamide gel under 20 mA for 2 hours and silver stained. The NCDV (Nebraska Calf Diarrhea Virus) rotavirus strain (MEBUS et al. 1969, WHITE et al. 1970) was included as positive and TRIS (base) 0.1M pH 7.3 solution as negative controls, respectively.

As a parallel test, a double-sandwich ELISA for group A rotavirus detection (GREGORI et al. 2000) was also applied to the same fecal and wastewater suspensions, with the NCDV strain as positive and TRIS (base) 0.1M pH 7.3 solution as negative controls, respectively.

For the RT-PCR, the TRIzol® (Invitrogen™) reagent was used to extract the total RNA from fecal suspensions or wastewater, according to the manufacturer's instructions. After, 5.6 µL of this RNA solution was mixed with 1.4 µL of DMSO and denatured at 95ºC for 5 minutes.

Reverse transcription (cDNA synthesis) was carried out at 42°C for 60 min in a reaction mix with 1×First Strand Buffer (Invitrogen™), 1mM of each dNTP, 10 mM DTT, 1µM of each primer (Con2 and Con3, for P detection; Beg9, End9, End9UK, and End9CRW8, for G detection, according to GOUVEA et al. 1994a,b), 7µL of RNA sample (as described) and 200 U of M-MLV Reverse Transcriptase (Invitrogen™) in a 20µL final reaction volume.

As next step, 5 µL of the cDNA were added at the PCR solution composed of 1x PCR Buffer (Invitrogen™), 0,2 mM of each dNTP, 0,5 pmol/ µL of each primer (Con2 and Con3, for P detection; Beg9, End9, End9UK, and End9CRW8, for G detection), 1,5 mM of $MgCl_2$, 1,25 U of Taq DNA Polymerase (Invitrogen™), ultrapure water in a 50 µL. The solution, then, was submitted in thermal cycler, at 30 cycles of 94°C/ 1 minute, 42 °C/ 2 minutes, 72°C/1 minute, and then, at 72°C/1 0 for final extension.

The second round amplification ("nested-PCR"), was consisted in mixing 5µL of amplified DNA from the previous stage with a solution composed of 1x PCR Buffer (Invitrogen™), 0,2 mM of each dNTP, 0,5 pmol/µL of each primer (Con2, pUK, pNCDV, pGOTT, pB223, and pOSU for P detection; sBeg9, FT5, ET10, DT6, BT11, and HT8 for G detection), 1,5 mM of $MgCl_2$, 1,25 U of Taq DNA Polymerase (Invitrogen™), ultrapure water q.s. 50µL, and submitted at 25 cycles of 94°C/1 minute, 55°C/2

minutes, 72°C/1 minutes, followed by a final step of 72°C/10 minutes for final extension.

As following, 10µL from the first and second amplifications were analyzed by agarosis gel eletrophoresis at 1.5% (p/v) in 0.045 M TRIS-borate buffer; EDTA 0.001 M pH 8.0, colorating the gel in water bath with 0.5 µg/mL of ethidium bromide for 10 minutes. Each RT-PCR step (extration, reverse transcription, PCR and eletrophoresis) were conducted in separate rooms, using as positive control the NCDV rotavirus sample and the ultrapure water as negative.

Samples with visible bands of correspondent size from those described by GOUVEA et al. (1994a,b), which are: for G; 876 bp – first amplification, 780 bp - G5, 500 bp - G6, 274 bp - G8, 715 bp - G10, and 337 bp for G11 and to P; 876 bp – first amplification, 622 bp - P1, 555 bp - P5, 423 bp - P6, 502 bp - P7, 314 bp - P11) in any PCR round (first or second), were considered positives, having as reference the adding of 10 µL of 100 bp DNA ladder (Invitrogen™).

RESULTS

The PAGE test detected rotavirus in one sample (1/25 or 4%), whereas in ELISA a total of 6 (6/25 or 24%) samples were positive, which 4 (4/18 or 22.23%) were from feces and 2 (2/7 or 28.57%) of wastewater (Table 1). The RT-PCR confirmed all positive results from the previously cited techniques (Table 1).

Table 1 - Rotavirus fecal and wastewater results

Sample	Site	Type	PAGE	ELISA	RT-PCR			
					1st G	2nd G	1st P	2nd G
1	1	Fecal	-	+	+	-	+	-
2	1	Fecal	-	-	NT	NT	NT	NT
3	1	Fecal	-	-	NT	NT	NT	NT
4	1	Fecal	-	-	NT	NT	NT	NT
5	1	Wastewater	-	-	NT	NT	NT	NT
6	2	Fecal	+	+	-	-	+	P [6]
7	2	Fecal	-	+	+	G [10]	+	-
8	2	Fecal	-	-	NT	NT	NT	NT
9	2	Fecal	-	-	NT	NT	NT	NT
10	2	Fecal	-	-	NT	NT	NT	NT
11	2	Wastewater	-	+	+	G [10]	+	-
12	3	Fecal	-	-	NT	NT	NT	NT
13	3	Wastewater	-	-	NT	NT	NT	NT
14	4	Fecal	-	-	NT	NT	NT	NT
15	4	Fecal	-	-	NT	NT	NT	NT
16	4	Fecal	-	-	NT	NT	NT	NT
17	4	Fecal	-	-	NT	NT	NT	NT
18	4	Wastewater	-	-	NT	NT	NT	NT
19	5	Fecal	-	-	NT	NT	NT	NT
20	5	Fecal	-	-	NT	NT	NT	NT
21	5	Wastewater	-	-	NT	NT	NT	NT
22	6	Fecal	-	+	+	-	-	-
23	6	Wastewater	-	+	+	-	-	-
24	7	Fecal	-	-	NT	NT	NT	NT
25	7	Wastewater	-	-	NT	NT	NT	NT

a- "+" signal is positive result
b- "-" signal is negative result
c- "NT" signal is "non-tested sample"
d- For the second amplification of P and G, the showed genotype is the positive result

DISCUSSION

This study detected rotavirus in fecal and sewage samples using of ELISA and PAGE, with further confirmation by RT-PCR in 3 farms, what means 42.85% (3/7), demonstrating the rotavirus circulation in these piggeries and their viral environmental contamination by fecal sludge (Table 1).

Rotavirus was detected by PAGE in only one fecal sample (1/25 or 4%). However, when ELISA results are taken into consideration, 6 (6/25 or 24%) samples were positive which 4 were from feces and 2 of sewage, all of them confirmed by RT-PCR (Table 1).

The difference between these two diagnostic techniques might be due to the lower ELISA detection limit when compared with PAGE (WINIARCZYK & GRADZKI, 2002), given that the virus on wastewater is diluted and it may be not possible to be detected by PAGE even with the use of concentration method, but because of the reduced number of samples (n), this possibility cannot be confirmed.

An alternative explanation would be the viral RNA degradation under high moisture and presence of unspecific inhibitors condition, as well as the diversity of wastewater in composition and environmental conditions (WILSON, 1997). However, the detection of the virus in 2 wastewater samples by RT-PCR makes this hypothesis unlikely, as this diagnostic technique depends on the viral genetic material integrity (Table 1).

Regarding the rotavirus variability mechanisms (TANIGUCHI & URASAWA, 1995), the use of RT-PCR for direct diagnosis was done with primers described by GOUVEA et al. (1994a; 1994b), although they were intended to group A rotavirus genotyping. Samples were considered positive by RT-PCR if any specific amplification band in the first and/or second amplification rounds targeting VP4 or VP7.

Using our described primers, it was not possible to identify every genotype. The samples 1, 22 and 23 showed to be only G positive (had a 876 bp amplified) while 1, 7 and 11 were only P positive (with a 876 bp amplified). Our found genotypes were G [10] (which is a 715 bp amplified) in samples 7 and 11, and a P [6] in the number 6 (with a 502 bp amplified). The G [10] was found in São Paulo state pigs by GREGORI (2003), and P [6], according to ALFIERI et al. (1999), it's common in Parana state swines, which is a neighbor of São Paulo state.

In one farm (1/7 or 14.28%), the agent was detected exclusively in a fecal sample, arising at least two hypothesis. The first might be the low circulation of rotavirus in this farm, what could explain the negative results in wastewater. Secondly, would be the high dilution or integrity of viral particles in the collect time.

Concerning the positive results both in fecal and manure sludge samples, it should be pointed out that this do not necessarily means that the viral particles are infectious in wastewater, as they are under adverse conditions such as high moisture, which may inactivate the rotavirus (ESTES & KAPIKIAN, 2007). Although molecular techniques are sensitive, they are not able to evaluate the viral viability, requesting further studies that demonstrate this biological characteristic. However, Limsawat & Ohgaki (1997) related that it is very likely that the viral particle is able to infect when detected in wastewater, as the nucleic acid have quick degeneration when in direct contact with sewer. About human rotavirus, there are several reports about the virus detection in wastewater, such as by Mehnert & Stenwien (1993) in São Paulo sewage and Gerba (1996), about a waterborne rotavirus outbreak in Brazil.

Constantini et al. (2007) succeed to inoculate treated wastewater rotavirus in gnotobiotic animals, which were asymptomatic and eliminated the virus by feces. A viral isolation attempt in cell culture would be the test of choice to evaluate viability, however, this technique have low sensibility and high detection threshold (GREEN & LEWIS, 1999, LIMSAWAT & OHGAKI, 1997).

The viability of the swine rotavirus in the water also pose as a zoonotic risk, even though the rotavirus inter-species transmission is rare, but exists (ESTES & KAPIKIAN, 2007, PAHO, 2001, WHO, 2004), what arises environmental health concerns in agriculture and consequently food; drinkwater for humans, animals (mainly bovines and chickens) and wild fauna (WALTER, 2001, WHO, 2004, WHO, 2006).

The rotavirus environmental contamination by wastewater can be a route of spreading the disease to another farms, but also in humans and wild animals. As an example, Gerba et al. (1996) reported 9 rotavirus waterborne outbreaks caused by direct fecal contamination or improper treatment of water. Although this work don't make evaluation of the agent viral viability, it was possible to detect the rotavirus wastewater presence. The study presented herein, open new perspectives to studies towards better understanding of possible swine rotavirus environmental implications and its consequences like, viral particle viability and efficiency of different swine wastewater treatment (RZEZUTKA & COOK, 2004, WALTER, 2001, WHO, 2004).

In conclusion, it was demonstrated the rotavirus were present in 3 farms, which in 2 was possible to observe the virus not only in feces, but also in wastewater contaminating the environment.

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