Rotavirus analyses by SYBR Green real-time PCR and microbiological contamination in bivalves cultivated in coastal water of Amazonian Brazil

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ABSTRACT

The contamination of mussels and oysters by viruses and bacteria is often associated with water contamination and gastroenteritis in humans. The present study evaluated viral and bacterial contamination in 380 samples, from nine mollusk-producing regions in coastal water north of the Brazilian Amazon. Rotavirus contamination was studied for groups A to H, using a two-step SYBR Green RT-qPCR (quantitative reverse transcription polymerase chain reaction), and bacterial families Enterobacteriaceae, Vibrionaceae, and Aeromonadaceae by classical and molecular methods. From the 19 pools analyzed, 26.3% (5/19) were positive for group A Rotavirus, I2 genotype for VP6 region, without amplifications for groups B–H. Bacteriological analysis identified *Escherichia coli* isolates in 89.5% (17/19) with identification of atypical enteropathogenic *E. coli* aEPEC in 10.5% (2/19), *Salmonella* (Groups C1 and G) (10.5%, 2/19), *Vibrio alginolyticus* (57.9%, 11/19) *V. parahaemolyticus* (63.2%, 12/19), *V. fluvialis* (42.1%, 8/19), *V. vulnificus* (10.5%, 2/19), *V. cholerae* non-O1, non O139 (10.5%, 2/19) and *Aeromonas salmonicida* (52.6%, 10/19). All the samples investigated presented some level of contamination by enterobacteria, rotavirus, or both, and these results may reflect the level of contamination in the Northern Amazon Region, due to the natural maintenance of some of these agents or by the proximity with human populations and their sewer.

Key words | bacteriology, bivalve mollusks, qPCR, rotavirus

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INTRODUCTION

The marine environment is known as an important source of natural resources, which benefits mainly mollusk cultivation whose demands are fully satisfied. Increase in mollusk cultivation has favored their consumption, especially in coastal regions (Pereira *et al.* 2007).

These animals feed on particles and plankton during the process of water filtration which averages 4.8 L/h (Carver & Mallet 1990). However, because bivalve mollusks have this filtering behavior, they not only adsorb the nutrients they need but also retain and concentrate toxins, chemical pollutants, including heavy metals, found in water, as well as microorganisms present in the water following failures in the treatment of sewage (Pereira *et al.* 2007).

Many disease outbreaks associated with consumption of contaminated bivalve mollusk have been reported throughout the world, especially those related to consumption of raw or lightly cooked mollusks, such as oysters or mussels contaminated with norovirus, hepatitis A, poliovirus, and adenovirus (Fong & Lipp 2005; Westrell *et al.* 2010). Other

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viral agents such as hepatitis E virus and rotavirus (RV) have also been associated with outbreaks (Renou *et al.* 2008). These viruses are excreted in large numbers during infections in human beings, with around 100 billion viral particles per gram of feces, and the fact that they are stable in the environment, combined with a low infectious dose, facilitates the environmental contamination and dissemination of these pathogens in filter feeding animals (Fong & Lipp 2005).

Among these agents, the rotaviruses have great epidemiological importance. Rotaviruses belong to the Reoviridae family and have a genome formed by double-stranded RNA divided into 11 segments which codify 6 non-structural proteins (NSP–NSP6) and 6 structural proteins (VP1-VP4, VP6, and VP7). Currently, there are eight groups classified as A–H; groups A, B, C and H stand out because they infect human beings and animals (Matthijnssens *et al.* 2012).

Bacterial contaminants are also of great interest for case reports of diarrhea associated with consumption of mollusks. According to Feldhusen (2000), there are three groups of pathogenic bacteria, associated with diseases caused by ingestion of bivalve mollusks and other marine products, divided according to their source of contamination: bacteria naturally present in the aquatic ecosystem (*Aeromonas hydrophila, Clostridium botulinum, Vibrio parahaemolyticus, V. cholerae, V. vulnificus* and *Listeria monocytogenes*); bacteria found in that environment as a result of contamination by animal feces (*Salmonella* spp., *Shigella* spp. and *Escherichia coli*); and bacteria that contaminate products during manipulation and processing (*Staphylococcus aureus*).

Besides the indicators of fecal contamination, which are widely used to evaluate the microbiological quality of bivalve mollusks, different species of *Vibrio* occur naturally in marine, coastal, and estuarine environments, although some species, such as *V. parahaemolyticus, V. vulnificus*, and *V. cholerae* are potentially pathogenic for humans, and may be present in both raw and partially cooked fish and mollusks (Thompson *et al.* 2004). The occurrence of these bacteria is not related to counts of *E. coli* or thermotolerant coliforms but they are responsible for most gastroenteritis related to ingestion of seafood (Pereira *et al.* 2007).

Considering the increasing importance of shellfish consumption and production and the scarcity of studies in Amazonia, our objective was to evaluate the presence of these microorganisms in mussels and oysters from nine mollusk-producing regions located in the Brazilian Amazon.

MATERIALS AND METHODS

Sample collection and analyses

The samples in the study were collected from nine localities where bivalve mollusks are cultivated (Augusto Corrêa, Bragança, Curuçá, Maracanã, Marapanim, Primavera, Salinópolis, São Caetano de Odivelas and Tracuateua) (Figure 1). From each locality, an average of 19 pooled samples were collected, with 20 oysters each. The mollusks were opened under aseptic conditions, removing the soft part using scalpels and sterile tweezers and placed in sterile Petri dishes.

Processing

The samples were washed individually with sterile distilled water and alcohol at 70%. The valves were opened with scalpels under sterile conditions in a laminar flow cabinet. After removing the soft parts from a pool of mollusks, the tissue was triturated using a pestle and mortar. The necessary amount of material for bacteriological analyses (25 to 75 g) was obtained.

Viral analysis

Detection of rotavirus

The viral genome isolation was obtained from the digestive tissue using the silica method (Boom *et al.* 1990). To identify the virus, real-time PCR (polymerase chain reaction) was employed using the relative fluorescent quantification method SYBR Green with specific indicators (primers), for groups A–H that flank the same target region used in the PCR for regions VP6 and VP7 (Table 1).

The amplification was obtained by the relative quantification method SYBR Green, based on the binding of the

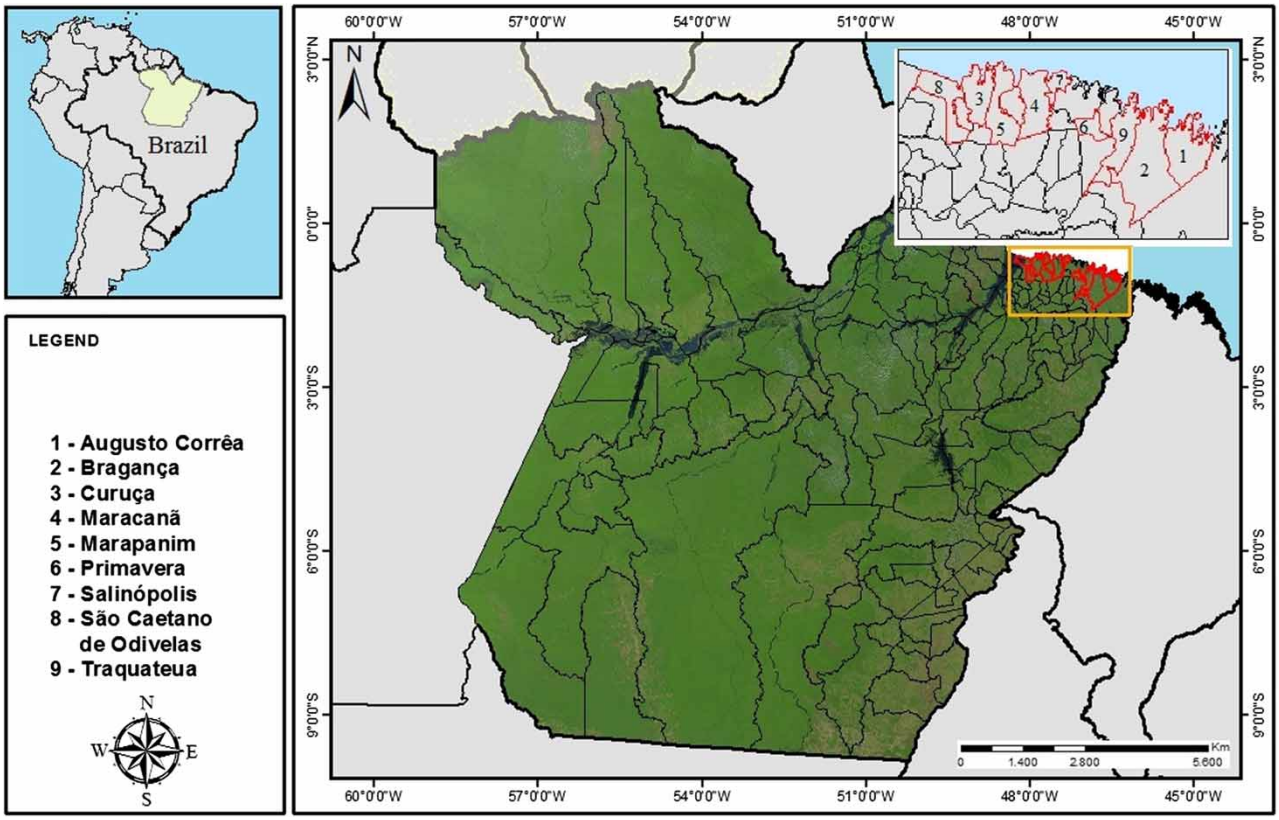

Figure 1 | Location of the oyster-producing regions.

Table 1 | Sequence of primers for VP6 and VP7 genes

Primer	Sequence 5′–3′	Target gene	Amplicon bp
RAVP6HUCOW-F	ATC GGC AAG TAC GGA TTC AC	VP6	122
RAVP6HUCOW-R	CGC TGG TGT CAT ATT TGG TG		122
RBVP7HUM-F	GGC AAT AAA ATG GCT TCA TTG C	VP7	814
RBVP7HUM-R	CTA GCC GAA GCT GTA AAA ACC C		814
RCVP6HUCOWPO-F	CTG GCG CTC CAA ATG TTA AT	VP6	502
RCVP6HUCOWPO-R	ACC ATT CTC TTC ACG GAT GC		502
RDVP6CHIC2-F	TGG ACT TTT GAT TTG CCA CA	VP6	560
RDVP6CHIC2-R	TGT GTG GCA GCT TGA TTT CT		560
RFVP6CHIC2-F	TGG AGT TGC ACC ACT TTA CG	VP6	583
RFVP6CHIC2-R	CGT GAA GCG AGT CAG TGG TA		583
RGVP6CHIC-F	CTC CAA CCT AGC TTT CAG CA	VP6	846
RGVP6CHIC-R	TGG AAT GTT CCG GAT CCA CC		846
RHVP6POR1-F	GAA TGT ATA ATC TGC GGG ATC CA	VP6	1,027
RHVP6POR1-R	TTA AAC ATG CAA TTT TCC TTG ACC		1,027

fluorescent dye SYBR Green into the PCR product (PE Applied Biosystems, Warrington, UK). The reaction was developed in 25 μL volumes, composed of SYBR Green Universal PCR Master Mix containing: Buffer at 1x, magnesium chloride 5.5 mM, dNTP 300 mM, AmpErase UNG (1 U/μL) 0.5 U, AmpliTaq Gold DNA Polymerase (5 U/μL) 1.25 U, specific primers for each target gene, and cDNA at 30 ng/μL. Sample incubation occurred in a Real Time 7500 PCR System (Applied Biosystem, USA) programmed for 95 °C for 10 minutes and 40 cycles of 92 °C for 15 seconds and 60 °C for 1 minute. The amplification was analyzed according to its fluorescence threshold and the resulting melt curve was compared with a positive control. Samples were considered positive when they presented the cycle threshold (Ct) below 35, followed by the melt curve and comparison with what was observed in the SA11 positive control. All reactions were accompanied by negative controls without RNA.

Sequencing

The analyzed samples were sequenced for mutations screening, phylogenetic analysis, and construction of a sequence data base. Thereby, the direct sequencing was performed in the ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using kit ABI PRISM™ Big Dye Terminator Cycle Sequencing V 3.1 (Applied Biosystems, USA) and the same PCR primers.

Sequence analysis

The resulting sequencing data were analyzed by the BioEdit v 7.2.5 software to evaluate mutations. The tree of phylogenetic relationships was constructed using the MEGA V.6.0 program, with neighbor-joining model and bootstrap of 10,000.

Microbiological analyses

For enterobacteria identification, the biochemical characterization and isolation method was performed. About 25 g of macerate was added to 225 mL of buffered peptone water (BPW pH 7.0), homogenized, and incubated at 35 °C for 18 hours. For *Salmonella* detection 0.1 mL of the culture in BPW was inoculated in Rappaport-Vassiliadis (RV) broth and incubated at 42.2 °C for 18 hours. Another 0.1 mL aliquot of BPW was inoculated in EC broth and incubated at 35 °C for 18 hours for *E. coli* isolation. Next, the cultures from RV and EC broths were plated on selective medium and indicators: SS agar and MacConkey agar, respectively, using methods described by the American Public Health Association (APHA). Suspect *Salmonella* and *E. coli* were subcultured to triple sugar iron (TSI) agar test and identified biochemically. To detect vibrios, 75 g of the samples were diluted in APW 1% NaCl, APW 3% NaCl, and BPW, and further isolated in SS, MC, and TCBS media using method described in the US Food and Drug Administration *Bacterial Analytical Manual* (Kaysner & DePaola 2001). About 5 to 10 suspect colonies were plated using TSI and Kligler agar medium, followed by biochemical and serological identification.

Molecular detection of diarrheagenic *E. coli*

The *E. coli* samples that had been previously identified biochemically were cultivated in nutrient agar (Difco) at 35–37 °C for 18–24 hours. The reference strains used as positive control were *E. coli*: EPEC E2348/69 (genes *eae* and *bfpA*), EAEC O42 (gene *aggR*), ETEC H10407 (genes *elt* and *est*), EIEC EDL1284 (gene *ipaH*) and EHEC EDL931 (gene *stx*) and a negative control *E. coli* K12 DH5α. The diarrheagenic *E. coli* were classified according to the presence of these genes.

Extraction of bacterial DNA and selection of indicators

DNA extraction, followed the protocol of Baloda *et al.* (1995). Multiplex PCR follows the protocol developed by Aranda *et al.* (2007) with modifications (Table 2).

RESULTS

Rotavirus identification

From the 19 pools analyzed using the qPCR technique, 26% (5/19) were positive for group A rotavirus, with Ct between 28 and 32 (Figure 2) with melt curve corresponding to

Table 2 | Oligonucleotides used for multiplex PCR and respective amplicons

Primer	Sequence 5′– 3′	Gene	Amplicon bp	Reference
eae-1	CTGAACGGCGATTACGCGAA	*eae*	917	Aranda *et al.* (2004)
eae-2	CGAGACGATACGATCCAG			
BFP-1	AATGGTGCTTGCGCTTGCTGC	*bfpA*	326	Aranda *et al.* (2004)
BFP-2	GCCGCTTTATCCAACCTGGTA			
aggRks-1	GTATACACAAAAGAAGGAAGC	*aggR*	254	Toma *et al.* (2003)
aggRksa-2	ACAGAATCGTCAGCATCAGC			
LT-f	GGCGACAGATTATACCGTGC	*elt*	450	Aranda *et al.* (2004)
LT-r	CGGTCTCTATATTCCCTGTT			
ST-f	ATTTTTMTTTCTGATTTRTCTT	*est*	190	Aranda *et al.* (2004)
ST-r	CACCCGGTACARGCAGGATT			
IpaH-1	GTTCCTTGACCGCCTTTCCGATACCGTC	*ipaH*	600	Aranda *et al.* (2004)
IpaH-2	GCCGGTCAGCCACCCTCTGAGAGTAC			
VTcom-u	GAGCGAAATAATTTATATGTG	*stx*1/ *stx*2	518	Toma *et al.* (2003)
VTcom-d	TGATGATGGCAATTCAGTAT			

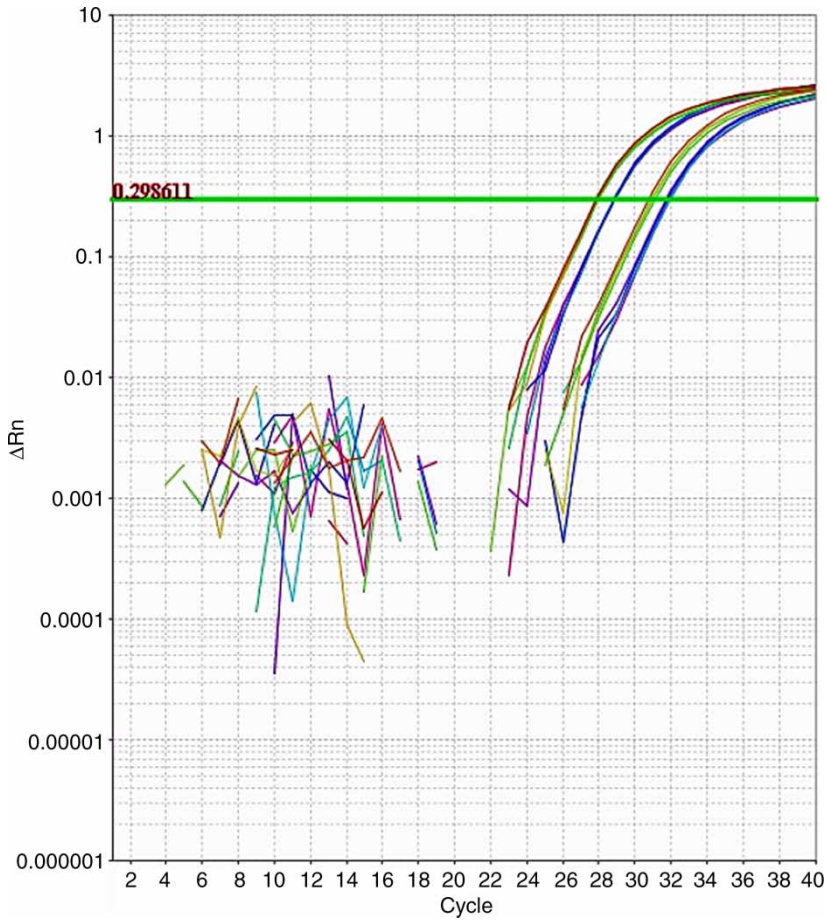

Figure 2 | Ct interval observed from five positive samples for group A rotavirus, with Ct between 28 and 32. Only assays with Ct below 35 were considered positive.

observed positive control SA11 (Figure 3), without observing amplifications for the other investigated groups (B–H) (Table 3). Next, for validation, the samples were re-amplified by RT-PCR (Figure 4) and sequenced. After sequencing the VP6 region, they were compared and aligned with a database obtained from Rotavirus Classification Working Group (RCWG) (http://rotac.regatools.be/). After analysis in a BioEdit v 7.2.5 program and MEGA V.6.0, we obtained a phylogenetic tree of the five samples isolated from the pools (OST 4, MEX 5, MEX 8 MEX 13 and OST 14) (Figure 5) where it was possible to observe that all samples grouped in one branch together with the I2 genotype samples for RVA VP6.

Isolation of bacterial pathogens

After bacteriological analyses for Enterobacteriaceae, *E. coli* isolates were observed in 90% (17/19) of the pools being analysed (12 pools of mussels and 5 pools of oysters), with the identification of a typical enteropathogenic *E. coli* aEPEC in two pools (MEX2 and MEX13) using multiplex PCR. Two of these pools (MEX3 and MEX11) were also positive for *Salmonella* isolates (Groups C1 and G). Additionally, *V. alginolyticus* (11/19), *V. parahaemolyticus* (12/19), *V. fluvialis* (8/19), *V. vulnificus* (2/19), two samples of *V. cholerae* non-O1, non-O139 and *Aeromonas salmonicida* (10/19) were also isolated (Table 3).

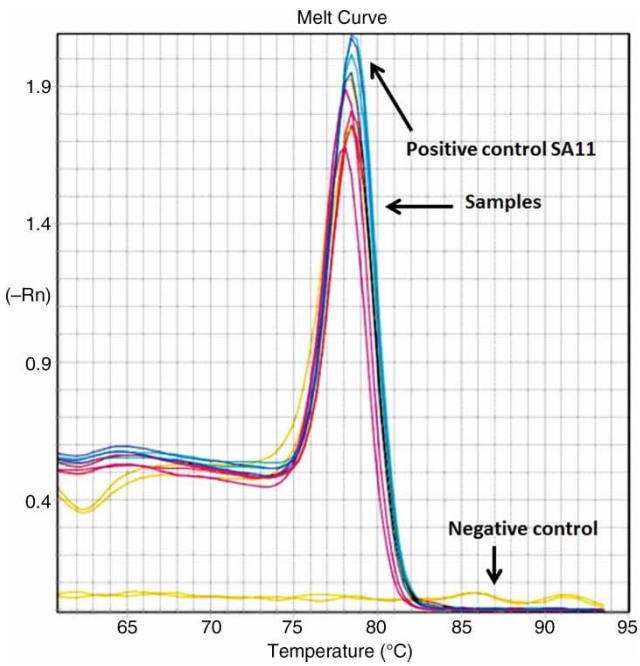

Figure 3 | Melt curve: The qPCR assays demonstrated to be uniform with only one peak for a sample, generating products similar to the SA11 positive control, no amplification was observed in the negative controls.

DISCUSSION

Rotaviruses identified

This study encompassed detection of rotavirus (groups A–H) in bivalve mollusks taken from nine producing regions in Northern Amazon region of Brazil. The results showed the presence of group A rotavirus in 26% (5/19) of the samples, while the other groups (B–H) were not detected, similar to Santiago *et al.* (2014), who showed the existence of rotavirus in samples collected from street markets in research carried out in the City of Mexico. The Santiago *et al.* (2014) study analyzed 30 oyster samples, 10 of which were positive for Group A rotavirus (33%), using RT-PCR technique, higher than the values observed by Kou *et al.* (2008) with rotavirus and norovirus detected in 21% (32/150) of the oyster samples, of which 3.3% (5/150) were positive for rotavirus, and 14% (21/150) for norovirus GII and 4% (6/150) for norovirus GI.

All groups of rotavirus were analyzed, but only rotavirus A was identified, possibly by its high rate of excretion (about 100 billion viral particles per gram of feces) and because it is the most frequent group in cases of diarrhea in humans (Matthijnssens *et al.* 2012). The results presented may reflect the contamination caused by human occupation near the points of collection and cultivation of mollusks, similar to the case described by Krog *et al.* (2014) showing that analysis of bivalves gives overview of the contamination in the environment investigated.

According to Lees (2000), a range of viruses has been identified in samples of bivalve mollusks, among them rotavirus. In this study, a new qPCR methodology was used to identify the presence of rotavirus (groups A–H) where RVA VP6 was efficient in identifying the presence of contamination in 26% (5/19) of the samples analyzed. After sequencing for validation of the methods, the samples were characterized as I2 according to analysis in RCWG

Table 3 | Summarized data for all samples

	Rotavirus group searches							Bacteriology analyses			
Sample[a]	A	B	C	D	F	G	H	Enterobacteriacea	Vibrionacea	Aeromonadaceae	ORIGIN[b]
MEX 1	–	–	–	–	–	–	–		*V. parahaemolyticus*		7
MEX 2	–	–	–	–	–	–	–	*E. coli* (aEPEC)	*V. parahaemolyticus*	*A. salmonicida*	2
MEX 3	–	–	–	–	–	–	–	*E. coli*, *Salmonella* Group G	*V. parahaemolyticus*	*A. salmonicida*	2
OST 4	+	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus*	*A. salmonicida*	1
MEX 5	+	–	–	–	–	–	–		*V. alginolyticus*	*A. salmonicida*	8
MEX 6	–	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. alginolyticus*		9
MEX 7	–	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. alginolyticus* *V. fluvialis*	*A. salmonicida*	8
MEX 8	+	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. vulnificus* *V. alginolyticus* *V. fluvialis*		5
OST 9	–	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. alginolyticus* *V. fluvialis*	*A. salmonicida*	4
MEX 10	–	–	–	–	–	–	–	*E. coli*	*V. alginolyticus* *V. fluvialis*		8
MEX 11	–	–	–	–	–	–	–	*E. coli; Salmonella* Group C1	*V. parahaemolyticus* *V. alginolyticus* *V. fluvialis*		7
OST 12	–	–	–	–	–	–	–	*E. coli*	*V. alginolyticus* *V. fluvialis*	*A. salmonicida*	3
MEX 13	+	–	–	–	–	–	–	*E. coli* (aEPEC)	*V. parahaemolyticus* *V. alginolyticus* *V. cholerae*	*A. salmonicida*	2
OST 14	+	–	–	–	–	–	–	*E. coli*	*V. fluvialis*		4
MEX 15	–	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. alginolyticus* *V. fluvialis* *V. cholerae*	*A. salmonicida*	8
MEX 16	–	–	–	–	–	–	–	*E. coli*	*V. parahaemolyticus* *V. alginolyticus*	*A. salmonicida*	8
MEX 17	–	–	–	–	–	–	–	*E. coli*			7
MEX 18	–	–	–	–	–	–	–	*E. coli*	*Vibrio vulnificus*		6
OST 19	–	–	–	–	–	–	–	*E. coli*			7

[a]MEX (mussel), OST (oyster); [b]Producing regions: 1, Augusto Corrêa; 2, Bragança; 3, Curuçá; 4, Maracanã; 5, Marapanim; 6, Primavera; 7, Salinópolis; 8, São Caetano de Odivelas; 9, Tracuateua.

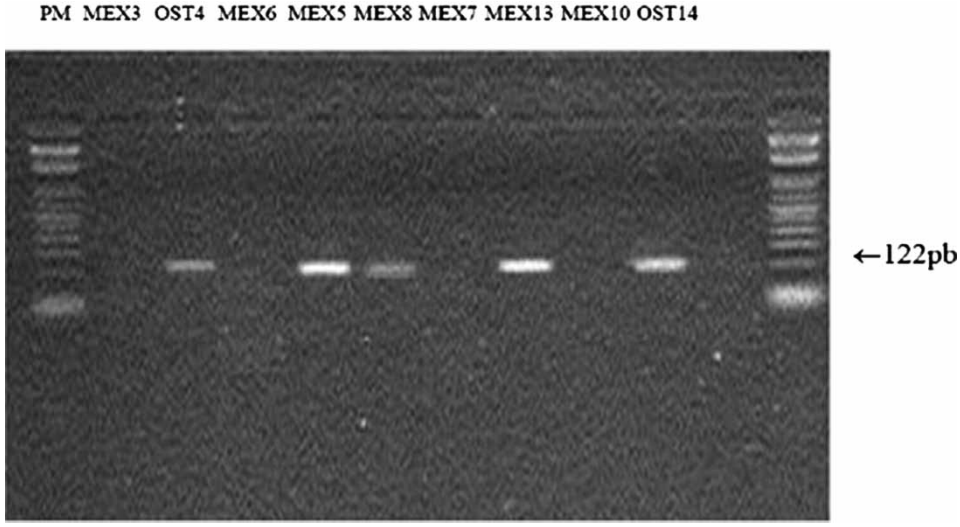

Figure 4 | Agarose gel 2% amplification for VP6 gene, RVA with 122pb for samples MEX 4, MEX 5, 8 MEX, MEX 13 and MEX 14.

(http://rotac.regatools.be/). Kou *et al.* (2008) found similar frequencies (21.33%) with the identification of human genotypes G1, G3, and G9. Kittigul *et al.* (2015) identified in bivalve mollusks genotypes G1, G3, G9, and G12, however, with lower frequency (8.0%). Genotype I2 can be observed in association with G6-P[13]-I2, G2/8–P[4]–I2, G8-P[4]-I2, G3-P[14]-I2, and G3-P[9]-I2 (Grazia *et al.* 2010; Bonica *et al.* 2015). Doan *et al.* (2015) showed a possible zoonotic potential. In our analyses we observed that after phylogenetic analysis the samples grouped with isolated strains of sheep, bovines, and humans showing that it is possible that contamination by RVA may have more than one source as described by Ghosh *et al.* (2015) who identified G6-P[13]-I2 from swine that shared origins with human strains.

Pathogenic bacteria identified

The analyzed samples showed the presence of other bacteria (*V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus*, *E. coli,* and *Salmonella* spp.). It is because the Amazon region has a propitious environment for the maintenance of these agents, such as water temperature, pH, salinity, and nutrient concentrations favorable to their development (DePaola *et al.* 2010).

In the present study, *E. coli* was observed in a high percentage of samples (90%, 17/19) were similar to results described by Papadopoulou *et al.* (2007), who observed 100% contamination by *E. coli* in samples of mussels.

Brands *et al.* (2005) showed that the most probable number (MPN) of fecal coliforms permitted is not an indicator of the absence of *Salmonella*. In addition, detection of *Salmonella* spp. in 25 g of bivalve mollusks make its commercialization for human consumption economically unfeasible. Also, to be taken into consideration is the epidemiological distribution of certain waterborne food diseases, such as *V. cholerae*, considered endemic in aquatic ecosystems of the Brazilian Amazon (Thompson *et al.* 2011) and that was responsible for the important cholera epidemic in 1991.

CONCLUSIONS

All samples showed some degree of contamination bacterial, viral, or both. Although the presence of bacterial agents such as *V. alginolyticus, V. parahaemolyticus, V. fluvialis, V. vulnificus, E. coli,* and *Salmonella* spp. is due to the Amazonian ecosystem being favorable for the maintenance of these agents, the observed presence of *E. coli* aEPEC, *Salmonella* (Groups C1 and G), and rotavirus group A (genotype I2), found in human diarrheal cases, suggest the contamination of the environment by human populations in mollusk-production areas.

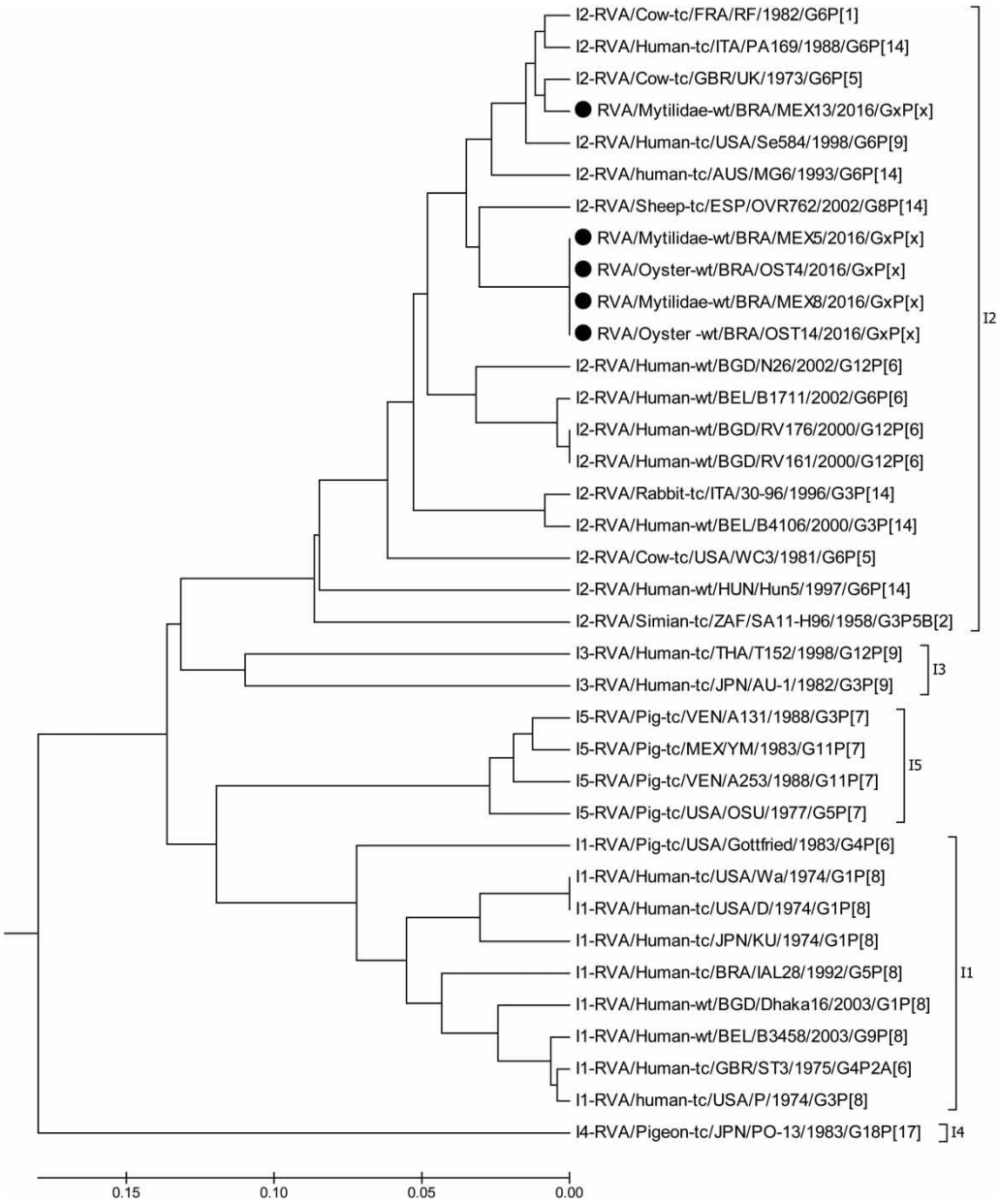

Figure 5 | Phylogenetic analysis of the VP6 gene. Phylogenetic tree constructed from rotavirus VP6 gene sequences amplified from five pooled fecal samples. The scale bar is proportional to the phylogenetic distance.

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CONFLICT OF INTEREST

No conflict of interest declared.

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