Fecal pollution source tracking in waters intended for human supply based on archaeal and bacterial genetic markers

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ABSTRACT

The determination of fecal pollution sources in aquatic ecosystems is essential to estimate associated health risks. In this study, we evaluate eight microbial source tracking (MST) markers including host-specific *Bacteroidales* and *Methanobrevibacter* spp. for discrimination between human, bovine, equine, and swine fecal contamination in waters intended for human supply. Overall, the novel host-specific archaeal and bacterial primers proposed in this study demonstrated high sensitivity and specificity. Markers for the Archaea domain were more prevalent in the fecal and water samples studied. We conclude that the investigations regarding the sources of fecal pollution in public water supplies can contribute to improve the quality of human health. To our knowledge, this is the first analysis using both archaeal and bacterial fecal MST markers on tropical water bodies of Rio de Janeiro city, Brazil.

Key words | *Bacteroidales*, fecal pollution, *Methanobrevibacter* spp., microbial source tracking, water quality

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INTRODUCTION

Concerns about water quality have increased in recent years, partly due to frequent contamination of coastal and inland water resources by sewage carrying waterborne pathogens. Waterborne diseases are mainly caused by enteric pathogenic micro-organisms, which are transmitted primarily by the fecal–oral route (USDA 2012). Consequently, this situation has been aggravated in recent years due to frequent contamination of drinking, recreational, and irrigation waters by emerging pathogens such as *Giardia lamblia*, *Cryptosporidium parvum*, Enterohemorrhagic *Escherichia coli* O157:H7, *Vibrio cholera*, among others (WHO 2006).

Fecal pollution can reach water bodies through discharge of fecal waste or raw sewage, wastewater from livestock, hospitals, slaughterhouses, and industrial activities, among other sources (USEPA 2005). The possibility of animal waste reaching underground sources of drinking water represents a significant public health threat. In addition, aquifers worldwide are experiencing increasing pollution threats from urbanization, industrial development, agricultural activities, and mining enterprises. Groundwater is a vital natural resource for the economic and secure provision of potable water supply in both urban and rural settings (Foster *et al.* 2002).

Owing to the economic development of metropolitan regions, the exploitation of environmental resources impacts on water availability in rivers, reservoirs, and other water sources, both in terms of quantity and quality. Consequently, there is increasing pollution of waterbodies located in the vicinity of urban regions where popular demand for proper pollution control is ever increasing (Gonçalves 2009). Thus, the monitoring of raw water is of fundamental importance in the water treatment station operational

doi: 10.2166/wh.2015.292

routine, since water quality is dynamic in time and space (Carmo *et al.* 2008; Di Bernardo & Paz 2008). This dynamism has a direct influence in water treatment processes, as contaminated waters require complex procedures that generally lead to increases in water prices.

Worldwide, water quality is evaluated using culture-based enumeration of fecal indicator bacteria (e.g., *E. coli*, *enterococci*) and, more recently, by quantitative real-time polymerase chain reaction (PCR) (USEPA 2012). However, neither of these approaches provide information about the source of fecal pollution since these organisms are normal inhabitants of the gastrointestinal tracts of several mammals, birds, and insects (Whitman *et al.* 2005; Doud & Zurek 2012). This apparent lack of reliability of traditional indicators has driven the development and implementation of complementary indicators to detect fecal pollution in aquatic environments as microbial source tracking (MST) procedures, resulting in a high diversity of microbial biomarkers (Malakoff 2002; Wu *et al.* 2008; Ahmed *et al.* 2013; Harwood *et al.* 2014).

The determination of fecal pollution sources in aquatic ecosystems is essential to estimate associated health risks and to provide measures to remediate polluted waters (Blanch *et al.* 2008). However, field studies for the determination of the origin of fecal pollution using various available microbiological biomarkers have shown that the current methods present limitations, indicating the need for other markers (Blanch *et al.* 2008; McQuaig *et al.* 2009; Ahmed *et al.* 2012).

Anaerobic micro-organisms constitute the major part of human and other animal microflora. Considering their complex nutritional requirements and inability to grow below 30 °C, which limits their survival in extra-enteric environments, anaerobes such as *Bifidobacterium* spp., *Clostridium perfringens*, *Methanobrevibacter* spp., and members of the order *Bacteroidales* have been currently used as alternative microbial indicators of recent fecal contamination (Stewart *et al.* 2003; Bonjoch *et al.* 2004; Ufnar *et al.* 2006). In addition, their presence can be correlated to host-specific sources of fecal pollution (Bernhard & Field 2000; Bower *et al.* 2005; Savichtcheva & Okabe 2006).

The *Bacteroidales* have been proposed as a host-specific fecal biomarker as they are abundant in the feces of many warm-blooded animals, including humans, and do not survive long in the environment (Bernhard & Field 2000; Meays *et al.* 2004; Dick *et al.* 2005). Other good candidates are archaeal representatives of the genus *Methanobrevibacter* (order *Methanobacteriales*) that includes 16 known species. These microbes inhabit the intestinal tract of animals, decaying plants, and anaerobic sludge from wastewater treatment plants. As only a few species occur in more than one host, they could be seen as specific microbial indicators of fecal pollution in environmental samples (Lai *et al.* 2004; Lee *et al.* 2013). Within this context, a variety of other biomarkers have also been proposed to discriminate fecal pollution events in environmental waters (Scott *et al.* 2002; Martellini *et al.* 2005; Lamendella *et al.* 2007; Fremaux *et al.* 2009).

The main objective of this study was to evaluate the presence of fecal host-specific markers in water bodies intended for human consumption of Região dos Lagos, Rio de Janeiro, Brazil. The São João river is the main water body of the São João basin and together with Bacaxá and Capivari rivers flows to Juturnaíba dam that provides water supply throughout the Região dos Lagos (Figure 1). As in many developing countries, large parts of the Brazilian population have no regular access to improved sanitation. This situation is all the more blatant in rural areas. Thus, there is a lack of information about wastewater disposal sources and no quality control data. The suspected sources of fecal pollution in these waterbodies are animal husbandry waste from small farms in their vicinity with direct access to the reservoir, native and feral wildlife and, to a lesser extent, human recreational activities.

In this work, microbial indicators from biologically significant fecal pollution sources such as human, equine, bovine, and swine were implemented. The host-specificity and sensitivity of *Bacteroidales* and *Methanobrevibacter* markers were assessed in fecal samples from target and non-target host groups. Subsequently, environmental water samples from São João basin were evaluated.

METHODS

Fecal sampling

To determine the host-specificity and sensitivity of the markers, 49 fecal samples were collected from six host groups. Human feces ($n = 12$) were obtained from volunteers of the laboratory

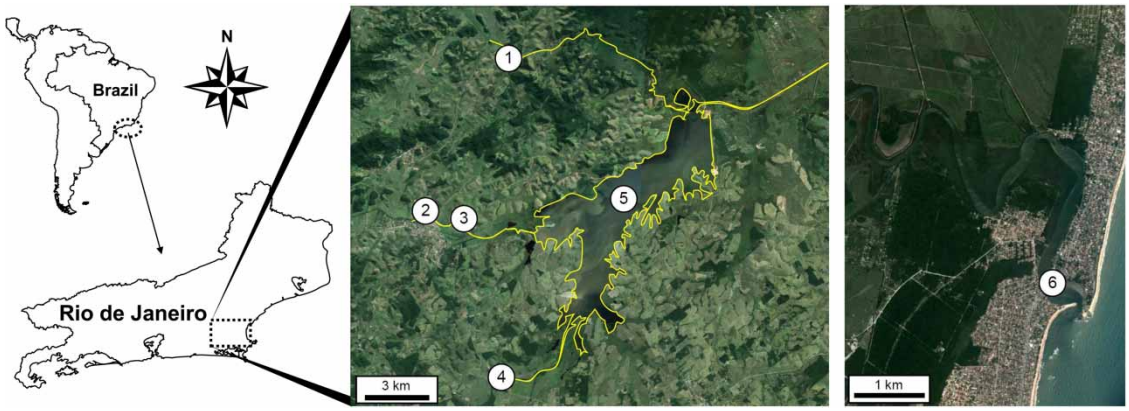

Figure 1 | Geographical location of the São João river basin in the northern state of Rio de Janeiro: (1) São João river, (2) Capivari river, (3) Capivari railway, (4) Bacaxá river, (5) Juturnaíba dam, (6) São João river mouth.

staff. Fecal samples of horse ($n = 12$), pig ($n = 8$), sheep ($n = 3$), chicken ($n = 3$), and cow ($n = 11$) came from two different farms in Mato Grosso do Sul, Brazil. Fresh animal stool samples (2–20 g) were taken in sterile Falcon tubes, maintained on ice and transported to the laboratory. Between 0.3 and 0.5 g of fecal material were resuspended in 1 mL of sodium phosphate buffer (PBS) (0.12 mol L^{-1}, pH 8.0). The diluted fecal mixture was mixed on a rotating platform to produce a homogeneous suspension and stored at −20 °C.

Environmental water sampling

Environmental water samples ($n = 12$) were collected in two different seasons (November 2013 and May 2014) from Juturnaíba dam (22 °38′S/42 °18′W), São João river (22 °35′S/41 °59′), São João river mouth (22 °35′/41 °59′W), Capivari river (22 °38′S/42 °24′), Capivari railway (22 °38′S/42 °22′W) and Bacaxá river (22 °42′S/42 °21′) (Figure 1). Six of the 12 samples were collected in dry weather and the remaining six were collected following wet weather events. The water samples (5.0 L) were taken at a depth of approximately 15–20 cm below the surface in a sterile polyethylene bottle. All samples were stored on ice and conducted to the laboratory within 4 h. The enumeration of *E. coli* in 100 mL was carried out using the defined substrate method (Colilert, IDEXX), according to the protocol described in *Standard Methods for the Examination of Water and Wastewater* (APHA 2012). Then, 4 L of each water sample were filtered through a 0.22 μm Stericup® system (Millipore). In case of filter clogging, additional filters were added. The filters were placed in 2 mL microcentrifuge tubes with 1 mL of PBS and kept at −20 °C overnight.

DNA extraction

The procedure used for DNA extraction was a modified version of previously described protocols (Ogram *et al.* 1987; Smalla *et al.* 1993). Briefly, tubes containing water sample filters and the homogeneous suspension of fecal samples were submitted to three cycles of freezing and thawing (−70 °C/2 min, 65 °C/2 min). Then, an equal volume of glass beads (0.1 mm diameter) was added and the suspension was shaken three times for 80 s at maximum speed in a Bead-Beater. The liquid phase was extracted with phenol–chloroform [1:1 (v/v)] and chloroform–isoamyl alcohol [24:1 (v/v)]. The DNA was precipitated from the aqueous phase with three volumes of ethanol and after being dried the pellet was resuspended in 100 μL of deionized water. For further purification of the DNA, we used the Dneasy Tissue Kit (Qiagen GmgH, Hilden, Germany) according to the manufacturer's instructions to remove possible PCR inhibitors (Clementino *et al.* 2007). Purified DNA from fecal and water samples was quantified in order to standardize the DNA concentration for further PCR reactions. These measures were obtained with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The possible presence of DNA amplification inhibitors in fecal and water samples was assessed by PCR reactions targeting *Bacteroidales* 16S rRNA gene with universal primers Bac32F and Bac708R (Bernhard & Field

2000) and *Methanobrevibacter* spp. *mcrA* gene with universal primers mcrAf and mcrAr (Luton *et al.* 2002). *Bacteroides fragilis* INCQS 00068/ATCC 25285 and *Methanobrevibacter smithii* INCQS A45D/DSM 11975 were used as positive control for *Bacteroidales* and *Methanobrevibacter* spp., respectively. *E. coli* INCQS 00043/ATCC 23229, *Klebsiella pneumoniae* INCQS 00629/ATCC BAA-1706, *Pantoea agglomerans* INCQS 00721/ATCC 33243 were used as negative control for both reactions. DNA templates that yielded negative results were diluted 1:10 with sterile distilled H_2O and re-tested. Approximately 25% of all fecal and water samples tested showed initial inhibition before dilution. All samples producing fragments of the expected sizes were further analyzed with the MST markers.

Primer development

PCR reactions for the detection of human- and equine-associated *Bacteroidales* were performed according to previously described protocols (Bernhard & Field 2000; Dick *et al.* 2005). However, there were no amplified fragments of the expected size even after several optimization procedures. To circumvent this issue, we designed new equine and human *Bacteroidales* specific oligonucleotides based on 16S rRNA sequences (AY212554 and AF233411, respectively) available in public databases. We also designed a new primer set for the detection of *M. gottschalkii* based on the *mcrA* gene (EU919431). The primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Invitrogen (Carlsbad, CA, USA). Primer specificity analysis was done *in silico* using BLASTn against the NCBI database and *In Silico*-PCR amplification against archaeal and bacterial genomic DNA (http://insilico.ehu.es/PCR).

Specificity, sensitivity, and limit of detection

MST primer sensitivity (r) and specificity (s) were tested using DNA from human, pig, horse, cow, sheep, and chicken feces. The r and s values were calculated according to the following formulas: $r = [TP/(TP + FN)]$ and $s = [TN/(TN + FP)]$, where TP is the number of samples that were positive for the PCR marker of their own species (true positive); FN is the number of samples that were negative for a PCR marker of their own species (false negative); TN is the number of samples that were negative for a PCR marker of another species (true negative); and FP is the number of samples that were positive for a PCR marker of another species (false positive).

To determine the limit of detection (LOD) of the equine- and human-associated *Bacteroidales* and equine-associated *Methanobrevibacter* markers, serial dilutions (10^{-1}–10^{-11}) from 20 ng of fecal DNA samples were made.

PCR conditions

PCR analysis was carried out in 50 μL amplification reaction mixtures containing 1 × PCR buffer (Invitrogen), 5% dimethyl sulfoxide (w/v), 200 μmol L^{-1} dNTPs (Invitrogen), 2 U of Platinum *Taq* DNA Polymerase (Invitrogen), 1 pmol of each primer, optimum $MgCl_2$ concentration (Table 1) and about 20 ng of DNA template. The cycling conditions consisted of an initial 95 °C step for 5 min and 40 cycles of amplification at 95 °C for 1 min, an annealing temperature specific for each primer set (Table 1) for 1 min, 72 °C for 1 min and a final elongation at 72 °C for 6 min. PCR products were loaded onto a 1% (v/v) agarose gel, and were separated by electrophoresis at 70 V for 2 h in 1 × TAE (40 mmol L^{-1} Tris base, 20 mmol L^{-1} sodium acetate, 1 mmol L^{-1} EDTA, pH 8.0) buffer with a 100 bp DNA ladder (Invitrogen) as molecular weight standard. The gels were stained with ethidium bromide and gel images were digitalized with the Video Documentation System and analyzed with ImageMaster software (Amersham Pharmacia Biotech).

The reproducibility for amplification of host-specific *Bacteroidales* and archaeal markers was evaluated in triplicate PCR reactions with at least two fecal DNA samples from target hosts. In addition, for archaeal markers, DNA from the reference strains *Methanobrevibacter smithii* INCQS A45D/DSM 11975, *M. ruminantium* INCQS A36D/DSM 1093, and *M. gottschalkii* INCQS A49D/DSM 11977 were used as positive controls for human, bovine, and equine MST markers, respectively.

Host-specific *Bacteroidales* PCR fragments were purified using the QIAquick® PCR Purification kit (Qiagen GmgH, Hilden, Germany) and sequenced with the Big Dye Terminator Kit and analyzed on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1 | Oligonucleotides, target genes, and PCR conditions

Primer	Sequence (5′-3′)	Target	MgCl2 (mM)	Annealing temp (°C)	Product size (bp)	Reference
HuM113F	ACTCTTGGCCAGCCTTCTGA	16S rRNA/Human-associated *Bacteroidales*	1.5	57	290	This study
HuM403R	ACCCATAGGGCAGTCATCCT					
Mnif-342F	AACAGAAAACCCAGTGAAGAG	*nifH*/*Methanobrevibacter smithii*	2.0	58	222	Ufnar *et al.* (2006)
Mnif-363R	ACGTAAAGGCACTGAAAAACC					
CF128F	CCAACYTTCCCGWTACTC	16S rRNA/Bovine-associated *Bacteroidales*	1.5	56	464	Bernhard & Field (2000)
CF592R	AYMTCCCGTCTACGCTCC					Liu *et al.* (2012)
Mrnif-F	AATATTGCAGCAGCTTACAGTGAA	*nifH*/*Methanobrevibacter ruminantium*	2.0	56	336	Ufnar *et al.* (2007a)
Mrnif-R	TGAAAATCCTCCGCAGACC					
PF163F	GCGGATTAATACCGTATGA	*16S rRNA*/Swine-associated *Bacteroidales*	1.5	55	385	Dick *et al.* (2005)
PF548R	CCCAATAAATCCGGATAACG					This study
P23-2 F	TCTGCGACACCGGTAGCCATTGA	*mcrA*/P23-2 Clone	2.0	60	258	Ufnar *et al.* (2007b)
P23-2 R	ATACACTGGCGACATTCTTGAGGATTAC					
HoR201F	TGGGGATGCGTCTGATTAGC	*16S rRNA*/Equine-associated *Bacteroidales*	1.5	53	242	This study
HoR442R	CCCACACGTGGGTCACTTTA					
GoT285F	GCACAAACTGGTTTAAGCGGA	*mcrA*/*Methanobrevibacter gottschalkii*	1.5	54	120	This study
GoT404R	GGAGAATACGTTAGCAGCACCA					

Electropherograms were converted to fasta format through Sequencher 3.0 software (Gene Codes Corporation, Ann Harbor, MI, USA). Nucleotide similarity searches were carried out online with BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) against GenBank (NCBI). The sequences reported in this study were submitted to GenBank/NCBI database under accession numbers KM924823–KM924826.

RESULTS

Enumeration of *E. coli*

Ten water samples showed *E. coli* levels within acceptable limits (0–920 MPN/100 mL) according to Brazilian standards (CONAMA 2005). The two other samples, Capivari railway and São João river mouth, had *E. coli* counts above the recommended limit (1,119.9 and 2,682 MPN/100 mL, respectively).

Specificity, sensitivity, and LOD

Host-specificity and -sensitivity of human-, bovine-, equine-, and swine-associated markers were evaluated by screening 49 fecal samples from six host groups. The archaeal human marker was detected in all (12/12) human fecal DNA samples tested but not in non-target host groups (0/37). However, the *Bacteroidales* human marker was positive in eight of 12 human samples and one of 37 animal fecal DNA samples (Table 2). The bovine archaeal marker was positive for 10 of

Table 2 | Specificity assays for host-specific markers in fecal samples

		No. of positive PCR results							
		Human markers		Bovine markers		Swine markers		Equine markers	
Target	No. of samples tested	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
Human	12	12/12 (100%)	8/12 (67%)	ND	ND	ND	ND	ND	ND
Swine	8	ND	ND	ND	ND	8/8 (100%)	7/8 (91%)	ND	ND
Cow	11	ND	ND	(10/11) 91%	11/11 (100%)	ND	ND	ND	ND
Horse	12	ND	ND	ND	ND	ND	ND	12/12 (100%)	10/12 (83%)
Chicken	3	ND	ND	ND	ND	ND	ND	ND	ND
Sheep	3	ND	1/3 (33%)	ND	ND	ND	ND	ND	ND

ND, not detected.

11 bovine DNA samples while the *Bacteroidales* bovine marker was detected in all cow fecal samples analyzed (11/11). The swine archaeal marker was detected in all pig fecal samples (8/8) while the archaeal equine marker was amplified in all equine samples (12/12). On the other hand, the swine and equine *Bacteroidales* markers were detected in seven of eight and 10 of 12 of each host's samples, respectively (Table 2).

The overall sensitivity of the human-, swine-, and equine-associated archaeal markers to differentiate between its own host group and other animal fecal samples was 1 (maximum value of 1) as was the bovine-associated bacterial marker. Human-, equine-, and swine-associated *Bacteroidales* indicator sensitivity values were 0.67, 0.83, and 0.88, respectively, whereas for the bovine-associated archaeal marker it was 0.91. The specificity value for all archaeal markers and for swine- and equine-associated *Bacteroidales* markers was 1 (maximum value of 1). The specificity value for both human and bovine *Bacteroidales* markers was 0.97. All sensitivity and specificity results are given in Table 3.

The equine archaeal marker was able to amplify a visible fragment up to a dilution of 10^{-5} of horse fecal DNA. The LOD of the remaining biomarkers was established at a 10^{-2} DNA dilution. To determine the reproducibility of the assays, several replicates ($n = 5$) of serially diluted genomic DNA were tested.

Biomarker detection in water samples

M. smithii and *M. ruminantium* were detected in all (12/12) water samples while human- and ruminant-associated *Bacteroidales* markers were detected in nine and eight of 12 samples, respectively. The P23-2 clone (*mcrA* gene) was present in eight of 12 water samples and swine-associated *Bacteroidales* was present in all samples (12/12). The *M. gottschalkii* marker was found in 12 of 12 water samples, while the equine-associated *Bacteroidales* was detected in seven of 12 samples analyzed (Figure 2).

Table 3 | Sensitivity and specifity of the MST markers

	Human markers		Bovine markers		Swine markers		Equine markers	
Value	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
Sensitivity (r)	1	0.67	0.91	1	1	0.88	1	0.83
Specificity (s)	1	0.97	1	0.97	1	1	1	1

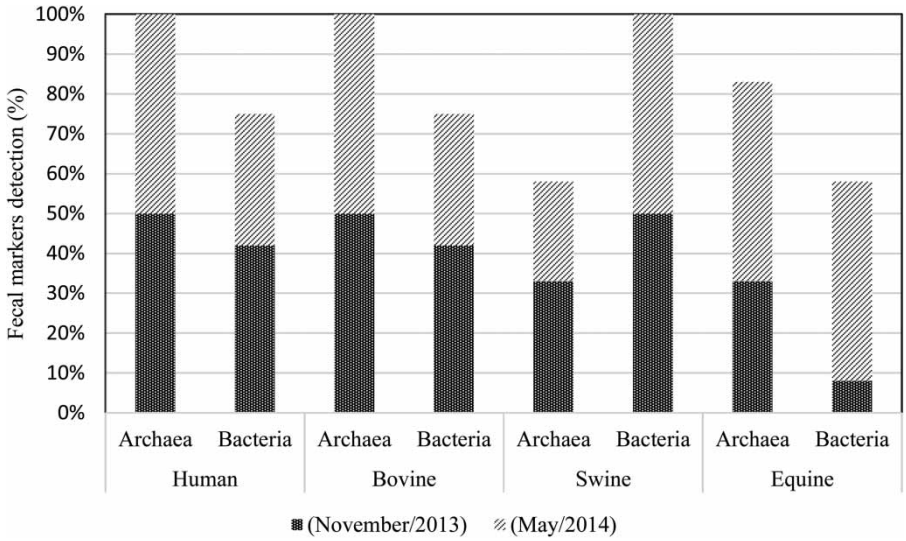

Figure 2 | Detection of biomarkers for different fecal sources.

16S rRNA sequences were analyzed by BLAST to search for sequence similarity with other sequences already available in the GenBank database. The 16S rRNA gene sequences of *Bacteroidales* markers showed 98% identity with those of bovine, human, and equine and 100% with porcine *Bacteroidales* sequences deposited in GenBank.

DISCUSSION

The aim of this study was to evaluate the presence of fecal host-specific markers in water bodies intended for human consumption. In order to increase the likelihood of identifying fecal contamination sources, novel specific molecular markers, including human-, swine-, and equine-associated *Bacteroidales* and *M. gottschalkii* were proposed (Table 1). In addition, previously described bovine-associated *Bacteroidales* and *M. smithii*, *M. ruminantium*, and swine-associated P23 clone markers were used (Bernhard & Field 2000; Ufnar *et al.* 2006, 2007a, b; Liu *et al.* 2012). To the best of our knowledge, this is the first analysis using both archaeal and bacterial fecal biomarkers on tropical water bodies of Rio de Janeiro city, Brazil.

The correlation between some MST markers and traditional fecal indicators is not well documented. A study in northwest France showed a significant correlation between *E. coli* concentrations and the presence of human markers whereas no correlation was observed for other animal markers. Some inconsistencies between *E. coli* concentrations and some host-specific *Bacteroidales* in environmental samples have been found previously (Shanks *et al.* 2006; Gourmelon *et al.* 2007). In the present study, all 10 samples presenting *E. coli* levels that were within acceptable limits according to Brazilian standards (<1,000 MPN/100 mL), showed fecal contamination with at least five MST biomarkers. However, a sample with low and two others with high *E. coli* counts showed the presence of all eight host-specific markers.

Many hypotheses could explain a lack of correlation in some situations: differential fecal inputs, persistence and survival of *E. coli*, methanogens, and *Bacteroidales* in the environment and differences in detection methods, including molecular techniques for host-specific markers and culture for *E. coli* and enterococci (Brion *et al.* 2002; Cole *et al.* 2003; Shanks *et al.* 2006). To identify the origin of fecal contamination, which is currently determined by *E. coli* or enterococcus counts, more information is needed about the persistence of the biomarkers in the environment and their correlation to these fecal indicator bacteria (Long & Sobsey 2004; Shanks *et al.* 2006).

M. smithii and *M. ruminantium nifH* genes were detected in 100% (12/12) of the water samples while human and bovine *Bacteroidales* 16S rDNA sequences were detected in 75% (9/12) of the samples tested. In

addition, the human-associated archaeal marker showed higher specificity and sensitivity than the bacterial one. On the other hand, the bovine-associated archaeal marker showed higher specificity and lower sensitivity compared with the bacterial marker (Table 3).

A study conducted in Australia evaluated the host specificity and sensitivity of the *nifH* gene marker in fecal and wastewater samples from 11 animal species, including humans. The host specificity reported in this particular study was 96% while sensitivity of the marker for human-derived sewage was 81%. The prevalence of the marker in environmental water samples was relatively low compared with the others tested (*esp*, HF183, HPyVs, and adenoviruses). The authors concluded that this marker alone might not be sensitive enough to detect fecal pollution in environmental waters; however, its relatively high host specificity argues for its use in conjunction with other human markers (Ahmed *et al.* 2012). Nevertheless, we observed 100% of sensitivity and specificity for the human host *nifH* marker in our study, supporting its use in future investigations.

Our results also demonstrated that swine fecal contamination was observed in 100% (12/12) of the water samples by the amplification of the *Bacteroidales* marker compared to 66.6% (8/12) detected by the *mcrA* gene from methanogenic organisms. Nevertheless, the *mcrA* gene marker was more sensitive and specific than the swine-associated *Bacteroidales* marker. It is noteworthy that the equine-associated *mcrA* primers described in this study showed high specificity and sensitivity, being detected in 100% (12/12) of the water samples tested in contrast to 58.3% (7/12) using equine-associated *Bacteroidales* markers.

The environmental pollution caused by swine waste is a serious problem due to the high number of contaminants, causing a powerful degradation of air, soil, and mostly of water resources (surface and groundwater) (Seganfredo 2000). Pig waste has a high concentration of biodegradable matter, pathogenic micro-organisms (*Salmonella* spp., *Campylobacter* spp., *Giardia lamblia*, *Taenia solium*), nitrogen, and minerals such as copper, zinc, and arsenic (Schmidt *et al.* 2007; Scanlon *et al.* 2013). In agricultural areas, and even urban areas, we can also observe equine husbandry and horse-breeding, producing about 7–8 kg/animal/day of waste (20% urine and 80% solid material), that is richer in nitrogen than pig or cow feces (Matos 2005). Infections of humans with *Bacillus anthracis* and *Salmonella enterica* that cause anthrax and salmonellosis, respectively, and worms such as *Trichinella spirallis* have been associated with contaminated horse feces (Molinaro 2009).

Despite the importance of the São João river basin, little is known about its water body quality, where monitoring only started in May 2011. Moreover, there are no records of Juturnaíba dam water quality control. Although the dam is considered a wildlife sanctuary, the results of our study demonstrated the presence of fecal pollution from human, bovine, swine, and equine sources.

The novel archaeal and bacterial primers proposed in this study demonstrated high sensitivity and specificity. Interestingly, markers of the Archaea domain were more prevalent, in both the feces and water bodies studied. Nevertheless, a study in northwest France showed that host-specific *Bacteroidales* markers were found to be more sensitive than other biomarkers tested in environmental water samples, especially where low numbers of *E. coli* were found (Gourmelon *et al.* 2007).

Specificity and sensitivity of host-associated markers may vary according to the studied region and should be locally validated before their inclusion in regular water. Variations may occur due to diet or feeding differences and decay rates of the markers at different environmental conditions (Walters & Field 2006; Field & Samadpour 2007; Eichmiller *et al.* 2014). The bovine-associated marker *Bacteroidales* CF128, for instance, has been detected in $\leq$10% of pig feces samples in Oregon and Ireland but was observed in $\geq$90% of pig feces samples in France, Portugal, and the UK (Bernhard & Field 2000; Gawler *et al.* 2007).

The MST approach should be carried out using several water samples, with regular sampling intervals combined with conventional fecal indicator monitoring and also include samples taken in both dry and rainfall weather events. Our data suggest that the use of more than one MST marker to identify the source of fecal contamination is valuable because each of these methods has its strengths and weaknesses that can limit the usefulness of MST. The most commonly used marker, for example, *Bacteroides* HF183, is not entirely specific for human waste. However, it has the advantages of being widely distributed in the human population and being present at relatively high

concentration in wastewater (Carson *et al.* 2005; Gawler *et al.* 2007; Gourmelon *et al.* 2007; Ahmed *et al.* 2009).

Finally, we conclude that although MST tools are widely accepted as alternative methods to evaluate sources of pollution, threshold values have not yet been entirely determined to assess the microbial quality of a water body. Meanwhile, the investigations regarding the sources of fecal pollution in public water supplies can contribute to the implementation of better monitoring programs and remediation strategies in order to improve the quality of human health and ecosystems.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Genome Sequencing Core-PDTIS/FIOCRUZ. We also would like to thank Dr Ivano de Filippis for his critical reading of the manuscript. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), and INCQS/FIOCRUZ.

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First received 22 December 2014; accepted in revised form 10 April 2015. Available online 1 June 2015