

Detection of the host-specific *Bacterioides* and *Bifidobacteriumsaeculare* markers in surface water in Hanam, Vietnam with real-time PCR

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Abstract

The microbiological quality of coastal or river waters can be affected by faecal pollution from human or animal sources. Contamination of fresh produce with pathogenic bacteria can be attributed directly or indirectly through the raw wastewater (domestic, industrial effluents, human and animal excrement). This includes water sources that are routinely used for crop irrigation and produce washing. An efficient MST (Microbial Source Tracking) toolbox consisting of several host-specific markers would therefore be valuable for identifying the origin of the faecal pollution in the environment and thus for effective resource management and remediation. As a result, the need for expanding from traditional techniques to molecular methods has been highly recommended. The aim of the study was to investigate the presence of several host-specific markers in surface water in Vietnam using real-time PCR assay. The study was conducted through collecting 145 river water samples at the two communes (Thuong and Quan Nha Villages) in Ha Nam Province in both wet and dry seasons. The AllBac marker was found 21 at the rate of 79.2% (57/72) in Quan Nha and 93.2% (68/73) in Thuong Village, following by the 22 HF-183 with the rate of 25% (18/72) and 44% (32/73), respectively. The Pig-2-Bac marker was found 23 at the lower rate with 19.2% (14/72) in Quan Nha Village and 5.55% (4/72) in Thuong Village. No poultry marker (*Bifidobacteriumsaeculare*) was detected in water samples at both sampling sites. All these markers were found to be higher in Thuong than in Quan Nha Village and in wet than in dry season. The derived results were significant in the evaluation of the quality of surface water and play a vital role for tracking the source of bacterial contamination of the areas where the vegetables come from.

Keywords: Real-time PCR; AllBac Marker; *Bifidobacteriumsaeculare*; Waterway Contamination; Ha Nam

Introduction

Contamination of waterways with faecal material leads to the dissemination of pathogens, antibiotic resistant bacteria, and excess nutrients that have serious impacts on human and environmental health [1-4]. Major sources of contaminants include human derived sources (sewage leaks or combined sewer overflows), and agricultural sources (animal feeding operations and manure management of agricultural fields) [5]. In addition, domestic animals and wildlife may also contribute to aquatic faecal loads in some watersheds. Thus reduction of aquatic faecal loads and increased protection of human and environmental health depend on distinguishing which of the many possible contaminating sources impact a particular body of water and products grown in contaminated water [6].

Water pollution has been significantly important to know in Vietnam. Untreated water can contaminate crops through irrigation activities. These may bring unhealthy and unsafe food to consumers. Increased public awareness of the health-related and economic impact of water contamination and illness has resulted in greater efforts to apply more sensitive methods of pathogen detection and identification. Advances in molecular biology technology, particularly the real-time PCR, have allowed more reliable bacteria identification and surveillance in not only clinical but also environmental samples. Real-time PCR has become a valuable tool in investigating Microbial Source Tracking (MST) related the faecal bacteria population, have little potential for growth in the environment, and have a high degree of host specificity that likely reflects differences in host animal digestive systems.

There is a need to increase the awareness of the impact of bacteria which belong to the genus *Bacteroides* on the water supply in Vietnam. The impact of *Bacteroides* presenting in human, animal faeces is difficult to assess, primarily due to the lack of a uniform standard and laboratory equipment for monitoring incidence and diagnosis of waterborne illness. Bacterial markers were selected among *Bacteroides* and *Bifidobacterium*, two bacterial groups that have been reported to contain host-specific bacterial species [7]. These markers have been widely used to monitor faecal pollution in water and to distinguish human and animal sources [8]. In order to improve water quality, management and remediation plans need methods of identifying faecal pollution sources. Thus, the aim of this study was to use microbial source tracking method (real-time PCR) for detecting and differentiating human and animal pollution in surface water in Vietnam.

Materials and Methods

Site selection and sampling design

Yen Bac and Tien Ngoai are communes of the Duy Tien District in Hanam province which is located at about 60km southern Hanoi. A total of 145 water samples were collected from rivers in Quan Nha Village, Yen Bac Commune and Thuong Village, Tien Ngoai Commune along the Nhue and Nhue-Day River basin (Figure 1). The sites were selected on the basis of husbandry, aquaculture and agriculture activities which may contribute to the fresh surface water pollution. The population in Thuong Village in 2015 was 478, living in 158 households. This is the village which has high intensity of a small- and medium- scale pig and poultry farms with the average number of 16.1 pig per household and 37.8 chicken per household, relatively. In 2015, the population in Quan Nha Village was 1062, living in 367 households. Most farmers are doing rice, pig and poultry farming. Farms are small-scale and raise 1-20 pigs and 1-10 chickens [9]. The pig and chicken pens are typically located near the house and husbandry wastes are discharged to a common canal, which is connected to the Nhue river. In both villages, the drinking water source is mainly originated from rainy water. Water from dug well or drilled well is used for bathing, cleaning and other household activities. The flushing toilet is the most common type of latrine in these villages. Double-vault latrine is also another type of toilet used at 94 households in Quan Nha and at only 8 households in Thuong Village. Water samples analyzed in this study were collected from each village in two seasons with 72 in Quan Nha (including 33 in wet and 39 in dry season) and 73 in Thuong (including 42 in wet and 31 in dry season), respectively. Seasons were categorized as wet (February to July) and dry (August to January). All samples were tested for *Bacteroidales* markers as described below.

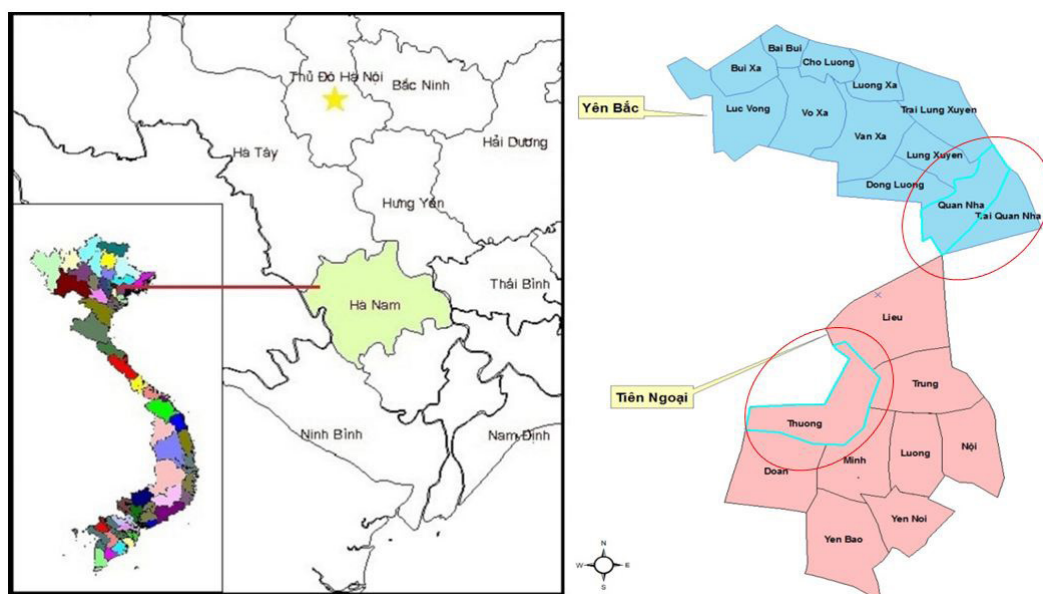


Figure 1: Surface water sampling sites in Ha Nam province

(Source: Luu Quynh Huong, 2014 [10]. PhD thesis and Lien *et al.* 2015 [11])

Sample collection and processing

Surface water samples that are used to irrigate rice and vegetable field fill up fishponds and vegetable washing were collected at field sites during daylight hours. One liter of sample was gathered just below the water surface after sterile, wide-mouth and screw-capped 1-L collection bottles were pre-rinsed twice times in the river. After collection, all samples were stored in a cooling box and transported to the laboratory on the day of sampling for further analysis.

Sample preparation and DNA extraction

Approximately 200ml of water samples were filtered through 0.22 μ m nuclepore track-etch membrane (Whatman, Schleicher and

Schuell, Germany). Filters were then placed in 0.5ml of GITC buffer (5M guanidineisothiocyanate; 100mM EDTA, pH 8.0; 0.5% Sarkosyl) (Cox et al. 2005) and kept at -20 °C until extraction. Genomic DNA was extracted using the Fast DNA kit for soil (MP Biomedicals, Illkirsh, France) according to the manufacturer's instructions.

Realtime PCR assay

All real time quantitative PCRs were performed using a TagMan@ Universal PCR Master Mix (Applied Biosystems), except the human-specific marker defined by Okabe et al. 2007, which used Applied Biosystems™SYBR™green mastermix (Thermo Fisher) [12].

Amplification was performed using an Applied Biosystems 7500 Fast Instrument with software 7500 v2.3. Each reaction was run with the following cycle condition: 1 cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. For poultry marker, the thermal-cycler conditions were as follows: an initial set up of 2 min at 50 °C, followed by 10 min at 95 °C, 40 cycles of 15s of denaturation at 95 °C, and 1 min of annealing/extension at 60 °C. For SYBR green amplifications, a dissociation step was added to improve amplification specificity.

TaqMan and SYBR green reactions were carried out in a final volume of 25µl with appropriate final concentrations of primer and probe (Table 1).

Primer or probe	Primer and probe sequence (5' - 3')	Final concen (nmol)	Reference	Target
AllBac				
AllBac296F	GAGAGGAAGGTCCCCAC	200	Layton et al. 2006 [9]	All Bacteroidales
AllBac467R	CGCTACTTGGCTGGTCA	200		
AllBac375Bhqr	(FAM)CCATTGACCAATATTCCTCCTGCTGCT(BHQ_1)	100		
HF183				
HF183F	ATCATGAGTTCACATGTCGG	200	Seurinck et al. 2005 [18]	Human Bacteroidales
HF183R	TACCCCGCTACTATCTAATG	200		
Pig-2-Bac				
Pig-2-Bac163Rm	ACCTCATA CGGTATTAATCCGC	300	Mieszkin et al. 2009 [13]	Pig-specific Bacteroidales
Pig-2-Bac41F	GCATGAATTTAGCTTGCTAAATTTGAT	300		
Pig-2-Bac113MGB	(VIC)TCCACGGGATAGCC(NFQ-MGB)	100		
<i>Bifidobacterium saeculare</i>				
Bif-F	TTCGGGTTGTAACCGCTTTT	900	Gómez-Doñate et al. 2012 [5]	Poultry Bacteroidales
Bif-R	TACGTATTACCGGGCTGCT	900		
PLprobe	(FAM)GAGAGTGAGTGTACCCGTT(NFQ)	250		

Table 1: Oligonucleotide sequences for real-time PCR assays

Statistical analysis

Data were analyzed using the SPSS software (Statistical Package for Social Sciences) version 11.0. Data was reported as percentage. The association of bacterial marker positive samples with sampling locations and seasons were tested by chi-square (χ^2). The P values of ≤ 0.05 were chosen statistical significance level.

Results

Quantitative PCR detections of the human-associated marker (AllBac) was consistent at the two sampling sites (Quan Nha and Thuong Villages) (Table 2). The all-*Bacteroides* marker (AllBac) was identified in 57 out of 72 sample collected (79.2%) in Quan Nha and 68 out of 73 samples (93.2%) in Thuong Village. At least one host-specific marker was identified in samples from two sites with no case having multiple markers. The HF-183 marker was identified in 18 of water samples collected in the Quan Nha village (25.0%) and in 32 of water samples collected in Thuong Village (44%), respectively. The percentage of water samples which had the Pig-2-Bac marker in Thuong Village 19.2% (n=14) and the figure for Quan Nha Village was only 5.55% (n=4). No poultry marker (*Bifidobacterium saeculare*) was detected in water samples at both sampling sites. It was found that AllBac and Pig-2-Bac markers were significantly higher in Thuong than in Quan Nha (97.6% vs 78.8%, p=0.018 and 28.6% vs 6.1%, p=0.0025). In Thuong village, the Pig-2-Bac marker was significantly higher in wet than dry season (28.6% vs 6.5%, p=0.005) (Table 4). No statistically significant

difference was found with all markers between the two seasons in Quan Nha Village (Table 3).

Sampling time	Sampling location	Host-specific Bacteroides markers			
		AllBac (%)	HF 183 (%)	Pig-2-Bac (%)	Poultry (%)
Wet		p = 0.018	p = 0.25	p = 0.0025	-
	Quan Nha (n = 33)	26 (78.8)	12 (36.4)	2 (6.1)	0 (0)
	Thuong (n = 42)	41 (97.6)	22 (52.4)	12 (28.6)	0 (0)
	Total (N = 75)	67 (89.3)	34 (45.3)	14 (18.7)	0 (0)
Dry		p = 0.52	p = 0.15	p = 1	-
	Quan Nha (n = 39)	31 (79.5)	6 (15.4)	2 (5.1)	0 (0)
	Thuong (n = 31)	27 (87.1)	10 (32.3)	2 (6.5)	0 (0)
	Total (N = 70)	58 (82.8)	16 (22.9)	4 (5.7)	0 (0)

Table 2: Proportion of water samples positive with host-specific *Bacteroidales* markers

Sampling time	Host-specific Bacteroides markers			
	AllBac (%)	HF 183 (%)	Pig-2-Bac (%)	Poultry (%)
	p = 1	p = 0.056	p = 1	-
Wet (n = 33)	26 (78.8)	12 (36.4)	2 (6.1)	0 (0)
Dry (n = 39)	31 (79.5)	6 (15.4)	2 (5.1)	0 (0)

Table 3: Proportion of water samples positive with host-specific *Bacteroidales* markers in Quan Nha village between two seasons

Sampling time	Host-specific Bacteroides markers			
	AllBac (%)	HF 183 (%)	Pig-2-Bac (%)	Poultry (%)
	p = 0.15	p = 0.14	p = 0.005	-
Wet (n = 42)	41 (97.6)	22 (52.4)	12 (28.6)	0 (0)
Dry (n = 31)	27 (87.1)	10 (32.3)	2 (6.5)	0 (0)

Table 4: Proportion of water samples positive with host-specific *Bacteroidales* markers in Thuong village between two seasons

Discussion

The results of this study and previously published research indicate the widespread faecal contamination of river water in Ha Nam over wide range of conditions. AllBac marker was found with the considerable high percentage of the water samples (79.2% - 93.2%) examined at the two sampling sites [13,14]. This is an agreement with findings in a study done in France where 25%-90% of coastal or river water samples were affected by faecal contamination from human and animal sources [15]. These results suggest that real-time PCR assays provide preliminary faecal source identification in watersheds.

Host-specific qPCR assays has been considered as a rapid tool in assessing faecal contamination in river water and other environments [16]. Advantages include the speed of assays, specificity, reasonable cost, and no need for a cultivation step [17]. In this study, we first applied host-specific qPCR assays for rapid determination of human and animal-originated faecal contamination in surface water. One human and two animal-specific qPCR assays targeting human, pig and poultry were used to identify the extent of household and farm animal faecal contamination in water. These hosts were chosen because they are regarded as major sources of faecal contamination in river water and water used for agriculture.

The findings in our study show that Thuong Village was having the higher proportion of samples positive with host-specific markers. This can be explained that the densely populated pig farming activities were presented in this area may cause the more serious surface water pollution due to the poor animal manure management. On the other hand, sewage and household wastewater was discharged to septic tanks located on each household, or to a common pond or canal. Thus, more widespread faecal pollution of the surface water to the household wells originating from human and animal excreta could be possible, as AllBac, HF-183 and Pig-2-Bac markers were found in 20% - 93% of river water samples investigated.

The origin of pollution in the river flowing through the two villages was clearly identified by the HF183 and Pig-2-Bac and by the absence of poultry-specific *Bacteroidales*. The presence of the human and pig markers indicate that river water in Ha Nam can be frequently impacted by human and animal faecal contamination. Alarmingly, the river water is commonly used for numerous recreational activities including swimming, aquaculture and agriculture activities. In fact, the faecal contamination of vegetables can be caused by fertilization of plants with human and animal waste or indirectly via contaminated water used in crop irrigation. Therefore, the two previously mentioned indicators can be used to detect human and animal faecal contamination either in surface

water or in leafy vegetable to help minimize public health risk.

Part of the variability observed in the presence of HF-183, Pig-2-Bac may be explained by the half-life occurrence of these two indicators between the two seasons. Since we found the higher number of water samples positive with these markers in the wet than in dry season. This finding was corresponding to the study done by with the view that human-derived faecal bacteria is more likely to be common in the wet season along with increased concentration of wastewater compounds [18,19]. In addition, previous observations indicate the presence of extreme low flows during the dry season that significantly decrease the transport of bacterial indicators [20].

In conclusion, the increased public awareness of water pollution and health-related risks is extremely needed. The discharge of waste or polluted water from households or animal farms to common canals, ponds, rivers could contaminate surface and groundwater sources with pathogens through surface run-off, leaching and direct faecal deposition into the water bodies [21]. Also the use of polluted water or waste for vegetable irrigation may pose a risk to human health. Therefore improved animal manure management is essential for reducing the risk of surface and groundwater contamination and sustainable water supply [22].

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