Estimation of Pig Fecal Contamination in a River Catchment by Real-Time PCR Using Two Pig-Specific Bacteroidales 16S rRNA Genetic Markers

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The microbiological quality of coastal or river water can be affected by fecal contamination from human or animal sources. To discriminate pig fecal pollution from other pollution, a library-independent microbial source tracking method targeting Bacteroidales host-specific 16S rRNA gene markers by real-time PCR was designed. Two pig-specific Bacteroidales markers (Pig-1-Bac and Pig-2-Bac) were designed using 16S rRNA gene Bacteroidales clone libraries from pig feces and slurry. For these two pig markers, 98 to 100% sensitivity and 100% specificity were obtained when tested by TaqMan real-time PCR. A decrease in the concentrations of Pig-1-Bac and Pig-2-Bac markers was observed throughout the slurry treatment chain. The two newly designed pig-specific Bacteroidales markers, plus the human-specific (HF183) and ruminant-specific (BacR) Bacteroidales markers, were then applied to river water samples (n = 24) representing 14 different sites from the French Daoulas River catchment (Brittany, France). Pig-1-Bac and Pig-2-Bac were quantified in 25% and 62.5%, respectively, of samples collected around pig farms, with concentrations ranging from 3.6 to 4.1 log10 copies per 100 ml of water. They were detected in water samples collected downstream from pig farms but never detected near cattle farms. HF183 was quantified in 90% of water samples collected downstream near Daoulas town, with concentrations ranging between 3.6 and 4.4 log10 copies per 100 ml of water, and BacR in all water samples collected around cattle farms, with concentrations ranging between 4.6 and 6.0 log10 copies per 100 ml of water. The results of this study highlight that pig fecal contamination was not as frequent as human or bovine fecal contamination and that fecal pollution generally came from multiple origins. The two pig-specific Bacteroidales markers can be applied to environmental water samples to detect pig fecal pollution.

Human and animal fecal pollution of coastal environments affects shellfish and recreational water quality and safety, in addition to causing economic losses from the closure of shellfish harvesting areas and from bathing restrictions (13, 19, 33). Human feces are known to contain human-specific enteric pathogens (3, 18, 28), but animals can also be reservoirs for numerous enteric human pathogens, such as Escherichia coli O157:H17, Salmonella spp., Mycobacterium spp., or Listeria spp., that may persist in the soil or surface waters (6, 8, 22, 24). Among animals, pigs are known to carry human pathogens that are excreted with fecal wastes. There are approximately 125 million pigs in the European Union (EU) and 114 million in North America (12, 36, 48), generating an estimated 100 and 91 million tons of pig slurry per year, respectively (4). France, the third largest pig producer in the EU, with about 23,000 farms, generates 8 to 10 million tons of pig slurry per year. Brittany accounts for 56.1% of the total national pig production on only 6% (27,200 km2) of the French territory, though it has 40% (2,700 km) of the coastline. This production could affect shellfish and recreational water quality and safety, in addition to being currently evaluated by the detection and enumeration of culturable facultative-anaerobic bacteria, such as E. coli, enterococci, or fecal coliforms (11), in shellfish and bathing waters (European Directives 2006/113/CE and 2006/7/CE). Pigs are among the potential sources of E. coli inputs to the environment; a pig produces approximately 1 × 107 E. coli bacteria per gram of feces, which corresponds to an E. coli flow rate per day that is 28 times higher than that for one human (16, 34, 55).

E. coli is not a good indicator of fecal sources of pollution in water because of its presence in both human and animal feces; therefore, alternative fecal indicators must be used. Microbial source tracking methods (44) are being developed to discriminate between human and nonhuman sources of fecal contamination and to distinguish contamination from different animal species (17, 46, 54). Many of these methods are library dependent, requiring a large number of isolates to be cultured and tested, which is time consuming and labor intensive. For these reasons, library-independent methods are preferred for the detection of host-specific markers.

The detection of host-specific Bacteroidales markers is a promising library-independent method and has been used for identifying contamination from human and bovine origins (25, 29, 39, 40, 45). In this study, we selected Bacteroidales 16S rRNA gene markers and real-time PCR to focus on fecal contamination from pigs. To date, only one pig-specific Bacteroidales 16S rRNA gene marker has been developed and used on water samples for the identification of pig fecal contamina-
tion by real-time PCR assay (SYBR green) (37). When this pig-specific Bacteroidales marker was tested on a small number of fecal samples (n = 16), it showed some cross-reaction with human and cow feces.

The present study investigated pig fecal contamination in a French catchment, the Daoulas estuary (Brittany), which has commercial and recreational shellfish harvesting areas and which is potentially subject to fecal contamination. The aims of the present study were (i) to design new primers for the detection and quantification of pig-specific Bacteroidales 16S rRNA genes by TaqMan analysis; (ii) to validate the sensitivity and specificity of the new primers and TaqMan assay using a target (feces, slurry, compost, and lagoon water samples) and nontarget (human and other animal sources) DNA, respectively; and (iii) to evaluate the TaqMan assay for proper detection and quantitative estimation of pig-associated fecal pollution. The study represents the first application of pig-specific Bacteroidales markers using a TaqMan assay in Europe and included a monitoring study of marker levels throughout the various stages of slurry treatment.

MATERIALS AND METHODS

Sample collection. (i) Fecal samples. Individual human and animal fecal samples were collected from April 2004 to March 2008. Human fecal samples were obtained from 24 healthy adult and child volunteers from Brittany (France). Animal fecal samples were collected immediately after excretion from apparently healthy animals (25 pigs, 10 cows, 10 sheep, and 10 horses). Pig fecal samples were collected from sows and male adults, young pigs, and piglets mainly housed in stalls on 15 farms in Brittany. Cow fecal samples were collected from animals kept on pasture or housed in stalls and included samples from adults and heifers on six independent beef and dairy farms in Brittany and Normandy. Samples from sheep were collected from one farm in Brittany and from salt meadows on two farms in Normandy. Samples from horses were collected from stud farms in Brittany and Pays de la Loire.

(ii) Pig effluent samples. Twenty-three slurry and 14 lagoon surface water samples were collected from 14 and 9 independent farms, respectively, in Brittany during April and December 2007 and March 2008. Seven compost samples obtained by slurry centrifugation were also collected on one of these farms.

(iii) Case study of the pig-specific markers through a slurry treatment process on a pig farm. A pig farm that performs slurry treatment was also selected to evaluate the new pig markers during a slurry treatment process. This case illustrates the most-frequently used slurry treatment process in Brittany. Indeed, 71% of farms in Brittany use this biologically activated sludge method to treat slurry effluents, while 17% use composting (32). In this case, the main farm collects and processes its own slurry along with slurries from four other surrounding farms, corresponding to the wastes of approximately 800 sows in total. Mixed slurries are centrifuged as part of compost production (60 m3 per day), collects and processes its own slurry along with slurries from four other sources (37). The resulting slurry is biologically treated through an activated sludge process. After decantation, surface water is stored in a lagoon basin (13,000 m3). A main farm (the main farm) were sampled twice, while sites 5 and 6 were sampled three times. All samples were placed in sterile containers and transported in insulated coolers. Cells were captured on filters upon arrival to the laboratory and stored at −20°C.

Sample preparation and DNA extraction. For water samples, amounts of approximately 200 ml were filtered through 0.22-μm Nuclepore membrane filters (Whatman, Schleicher and Schuell, Germany). Filters were then placed in 0.5 ml of GTC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) (8) and frozen at −20°C until extraction. DNA was extracted by using a DNeasy blood mini kit (Qiagen, Courtaboeuf, France), with two modifications: the proteinase K step was omitted, and 700 ml of Qiangen buffer AL was added to the filters after the GTC buffer (9).

For fecal and compost samples, DNA was extracted from amounts of 250 mg (wet weight) by using a FastDNA spin kit for soil (MP Biomedical, Illkirch, France) according to the supplier's instructions, with an additional wash using SEWS-M reagent as suggested by Dick and Field (9). Pig slurry samples (50 ml) were centrifuged at 9,000 × g for 15 min. DNA was then extracted from 250 ml of the pellet by using a FastDNA spin kit for soil.

PCR and 16S rRNA gene library construction. The primers Bac32F and Bac708R (Table 1) were used to selectively amplify Bacteroidales 16S rRNA genes from DNA extracts of 10 pig feces and 10 pig slurry samples. Reactions were performed in a Peltier thermal cycler (PTC 200; MJ Research, Waltham, MA) for 30 cycles of 94°C for 5 min, 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s. Final extension was continued for 7°C for 7 min. PCR products from each individual sample were pooled to obtain a library of two different clone libraries (from pig feces and pig slurry). Pooled PCR products were gel purified (Nuiseiv GTG agarose 2%; BMA, Rockland, ME) using a QuiaQuick gel purification kit (Qiagen, France). They were cloned into the pCR2.1 vector by using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The vectors were transformed into chemically competent E. coli cells (One Shot TOP10F; Invitrogen). Ninety-six transformants from each host-specific library were randomly picked on Luria-Bertani (LB) agar plates and used to transform two different colony libraries (from pig feces and pig slurry). Real-time PCR assays. All real-time quantitative PCRs were performed using SYBR green (Perkin-Elmer Applied Biosystems).

Sequences were edited using BioEdit (21). DNA sequences (approximately 690 bp) were processed using MALLARD software (2) to eliminate chimeric sequences. Sequences were aligned using MAFFT (version 5), and the distance matrix calculated using the software DNADIST (version 3.5c). The distance matrix was used with DOTUR software (43) to assign sequences to operational taxonomic units (OTUs). OTUs were defined by assigning 16S rRNA gene sequences >98% similarity to the same species. Representative OTU sequences were retrieved rationed with the pipeline RapidOTU (30; http://genome.jouy.inra.fr/rapidotu/) and compared with the GenBank database (http://www.ncbi.nlm.nih.gov/) by using the basic local alignment search tool (BLAST). Multiple alignments were performed using CLUSTALW (51). A phylogenetic tree was constructed by using the PHYL-O-WIN program (15). The root was determined using the 16S rRNA gene sequence of Cytophaga fermen- tans (GenBank accession number M85760) as an out-group. Distance trees were constructed by using neighbor-joining algorithms (41) with the Kimura two-parameter correction (26). The statistical significance of tree branches was evaluated by bootstrap analysis using 500 resamplings (branches with values of <70 are not shown).

Oligonucleotide primers and probes. The primer and probe sets for all-Bac- teria markers (Bac2; modified from reference 30) and all-Bacteroidales markers (AllBac; from Layton et al. (29)) were used to amplify total bacterial and total Bacteroidales 16S rRNA genes from a mixture of human and rumen-specific Bac- teroidales 16S rRNA gene markers (HF183 and BacR) was performed with the primers and probe described by Seurinck et al. (45) and Reisicher et al. (39), respectively (Table 1). Two pig-specific Bacteroidales primer and probe sets (Pig-1-Bac and Pig-2-Bac) were designed from multiple alignments of partial Bacteroidales 16S rRNA genes obtained in this study (Table 1). Oligonucleotide specificity for pig-associated Bacteroidales 16S rRNA genes was verified by using the BLAST (NCBI) and the Probe Match (Ribosomal Database Project II) programs. The pig-specific Bacteroidales marker (Pig-Bac2) described by Okabe et al. (37) was also tested on target and nontarget DNA preparations and compared with the two pig-specific Bacteroidales markers designed in this study.

Real-time PCR assays. All real-time quantitative PCRs were performed using a TaqMan Brilliant QPCR core reagent kit (Strategene), except for those with
the human-specific and the pig-specific markers defined by Okabe et al. (36), which used Brilliant SYBR green QPCR master mix (Stratagene). Amplifications were performed using a Stratagene MX3000P with software version 4. Each reaction was run in duplicate with the following cycle conditions: 1 cycle at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. 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 25 μl with appropriate final concentrations of primers and probe (Table 1). The presence/absence of PCR inhibitors was verified by using an internal positive control (Applied Biosystems, France). Samples were diluted if inhibitors were present. Negative controls (no template DNA) were performed in triplicate for each run.

Host-specific Bacteroidales, all-Bacteroidales, and all-Bacteria markers were tested on all feces, pig waste effluent, and river water samples.

DNA standard curves and quantification. Linear plasmid DNA used to generate standard curves was extracted with a QIAquick miniprep extraction kit (Qiagen), following the manufacturer’s instructions. The linear forms of pig, bovine, and human plasmids were obtained with NotI enzyme (Roche Diagnostics) in a final volume of 50 μl for 3 h at 37°C. For the quantification of Bacteroidales markers, standard curves were generated from serial dilutions of a known concentration of plasmid DNA. Standard curves were generated by plotting threshold cycles against 16S rRNA copy numbers.

A PCR standard for the all-Bacteria marker was prepared by using a 10-fold dilution of bacterial genomic DNA extracted from a pure culture of E. coli with a Wizard genomic DNA purification kit (Promega) according to the manufacturer’s instructions. The copy number of 16S rRNA genome was considered to be 7 copies for the E. coli strain (27).

Enumeration of E. coli bacteria. E. coli bacteria were enumerated in subsets of the pig fecal samples and effluent samples and in all water samples from the Daoulas catchment by the microplate method (standard NF EN ISO 9308-3 [1]), with a detection limit of <10 most probable number (MPN) per g for feces and compost and <15 MPN per 100 ml of water.

Data treatment and statistical analyses. The results of the general and host-specific marker quantifications were expressed in 16S rRNA gene copies per g or ml of feces, compost, slurry, or water.

All statistical analyses were performed using STATISTICA version 6.1 (StatSoft, France). To evaluate the performance of the microbial source tracking methods on fecal samples, sensitivity (r) and specificity (s) were defined as $r = a/(a + c)$ and $s = d/(b + d)$, where $a$ is a fecal sample positive for the marker of its own species (true positive); $b$ is a fecal sample positive for a marker of another species (false positive); $c$ is a fecal sample negative for a marker of its own species (false negative); and $d$ is a fecal sample negative for a marker of another species (true negative) (14). Fisher’s exact test was used to verify if the observed differences in the frequency of detection of the various markers in their target or nontarget fecal samples were significantly different. Analysis of variance (ANOVA) followed by comparisons of means using Fisher’s least significant difference test was used to test for significant differences between the concentrations of the different markers at the same stage of slurry treatment and between the different stages of the slurry treatment ($P < 0.05$). Linear regression analysis (analysis of the completed model) was used to verify if differences in concentration between Bacteroidales markers and E. coli bacteria were significant.

Nucleotide sequence accession numbers. Sequence data used in this study have been submitted to the GenBank database under accession numbers EU797125 to EU797175.
RESULTS

Phylogenetic analysis of Bacteroidales 16S rRNA genes from pig feces and pig slurry samples. Of the 96 clones obtained from Bacteroidales 16S rRNA gene libraries derived from pig feces and pig slurry samples, 94 and 86 clones, respectively, yielded unambiguous sequence data. Twenty-seven and 24 different OTUs were obtained for pig feces and slurry, respectively, with 16 OTUs showing clone sequences common to both feces and slurry. Forty-five and 60% of the sequences from pig feces and pig slurry libraries had more than 98% similarity to bacterial 16S rRNA gene sequences published in GenBank (NCBI).

The 16S rRNA pig Bacteroidales sequences were predominantly Prevotella-like, 85% from feces and 55% from slurry. From pig feces, 60% of the sequences were closely related to isolates from pig feces and 26% were related to sequences derived from human tissues and stools. From pig slurry samples, 55% of the sequences showed >98% similarity with bacterial 16S rRNA gene sequences isolated from pig fecal samples, 21% with sequences associated with human tissues or stools, and 13% corresponded to environmental clones.

To design Bacteroidales pig-specific primers, clusters of pig-specific sequences were investigated using (i) the partial Bacteroidales 16S rRNA gene sequences obtained from the slurry and fecal samples and (ii) partial Bacteroidales 16S rRNA gene sequences of human and bovine fecal origin in the GenBank database. Phylogenetic analysis of the 51 OTUs showed two distinct clusters of pig-specific sequences (Fig. 2). Sequences from clusters I and II were selected to design Bacteroidales pig-specific primers to detect the pig-specific Bacteroidales markers. Cluster I contained only one OTU (Fpc59), which represented eight Bacteroidales 16S rRNA gene sequences, with five sequences isolated from pig feces and three from pig slurry. The “Fpc59” OTU was closely related (99% similarity) to PigA4 uncultured Bacteroidales sequences from a pig fecal sample (10) and, to a lesser extent, to a sequence from Pre-

TABLE 1. Oligonucleotide sequences for conventional and real-time PCR assays with the annealing temperature, the final concentration, and the expected size for each amplified product

| Primer or probe | Primer and probe sequence (5’ → 3’)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac32F</td>
<td>AACGCTAGCTACAGGCTT</td>
</tr>
<tr>
<td>Bac708R</td>
<td>CAATCGGAGTTCCTTCG</td>
</tr>
<tr>
<td>BacT1369F</td>
<td>CGTGAATACGGTTCCGG</td>
</tr>
<tr>
<td>BACT1492R</td>
<td>GAGCTGACCTTGGTTCAGCTT</td>
</tr>
<tr>
<td>TM1389F</td>
<td>GAGAGGAAGTTCCCCCAC</td>
</tr>
<tr>
<td>AllBac</td>
<td>CGCTACGTCGTTCCGAG</td>
</tr>
<tr>
<td>AllBac296F</td>
<td>CCATTGACACATATGTCGG</td>
</tr>
<tr>
<td>AllBac467R</td>
<td>TACAGGAGTTACATGTCGG</td>
</tr>
<tr>
<td>AllBac375Bhqr</td>
<td>ATGCTCTCCTACAGATTCGG</td>
</tr>
<tr>
<td>HFl183</td>
<td>ATCAGGTTTACACAGATTCGG</td>
</tr>
<tr>
<td>HFl183f</td>
<td>TACCCCGCTACTAATGTAAGGTCCCCCAC</td>
</tr>
<tr>
<td>HFl183r</td>
<td>TACCCCGCTACTAATCTAATG</td>
</tr>
<tr>
<td>BacR</td>
<td>GCGTATCCAACCTTCCCG</td>
</tr>
<tr>
<td>BacR_f</td>
<td>CATCCTCCATCCGTACCG</td>
</tr>
<tr>
<td>BacR_r</td>
<td>(FAM)CTTCCGAAAGGAGATT(NFQ-MGB)</td>
</tr>
<tr>
<td>BacR_p</td>
<td>CTGATGAGTTCCAGCTTGGTTCAGCTT</td>
</tr>
<tr>
<td>Pig-Bac2</td>
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</tr>
<tr>
<td>qBac41F</td>
<td>CTCCGCTTTAATCCGGCTTCTCTGGT</td>
</tr>
<tr>
<td>qPS183R</td>
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</tr>
<tr>
<td>Pig-1-Bac</td>
<td>AAGCGTATCAGGCTTACACAGATTCGG</td>
</tr>
<tr>
<td>Pig-1-Bac32Fm</td>
<td>CGGCCCTATTCGATTGCTATGGG(FAM)</td>
</tr>
<tr>
<td>Pig-1-Bac108R</td>
<td>ATCAGAAGCTTGCTTTGATAGATGGCTG(BHO-1)</td>
</tr>
<tr>
<td>Pig-1-Bac44P</td>
<td>AACGCTAGCTACAGGCTT</td>
</tr>
<tr>
<td>Pig-2-Bac</td>
<td>GATGCCTACAGGCTTACACAGATTCGG</td>
</tr>
<tr>
<td>Pig-2-Bac41F</td>
<td>ATCAGAAGCTTGCTTTGATAGATGGCTG(BHO-1)</td>
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<tr>
<td>Pig-2-Bac163Rm</td>
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</tr>
<tr>
<td>Pig-2-Bac113MGB</td>
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<td>Pig-2-Bac44P</td>
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<tr>
<td>Pig-2-BacMGB</td>
<td>ACCTCAGGGGTCTATGGCTATGGCTT</td>
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</table>

<table>
<thead>
<tr>
<th>Size of amplicon (bp)</th>
<th>Annealing temp (°C)</th>
<th>Final concn (nmol)</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>690</td>
<td>60</td>
<td>500</td>
<td>All Bacteroidales</td>
<td>5</td>
</tr>
<tr>
<td>142</td>
<td>60</td>
<td>200</td>
<td>All Bacteria</td>
<td>50</td>
</tr>
<tr>
<td>106</td>
<td>60</td>
<td>200</td>
<td>All Bacteroidales</td>
<td>29</td>
</tr>
<tr>
<td>83</td>
<td>60</td>
<td>200</td>
<td>Human Bacteroidales</td>
<td>44</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>100</td>
<td>Ruminant Bacteroidales</td>
<td>39</td>
</tr>
<tr>
<td>145</td>
<td>60</td>
<td>300</td>
<td>Pig-specific Bacteroidales</td>
<td>37</td>
</tr>
<tr>
<td>129</td>
<td>60</td>
<td>200</td>
<td>Pig-specific Bacteroidales This study</td>
<td></td>
</tr>
<tr>
<td>116</td>
<td>60</td>
<td>300</td>
<td>Pig-specific Bacteroidales This study</td>
<td></td>
</tr>
</tbody>
</table>

a Modified from reference 50.

b FAM, 6-carboxyfluorescein; NFQ-MGB, nonfluorescent quencher group-minor groove binder; BHO-1, black hole quencher 1.
FIG. 2. Phylogenetic relationships of 51 OTUs obtained from partial 16S rRNA gene sequences from pig fecal (Fpc) and pig slurry (Lpc) samples using the Bacteroidales-specific primers Bac32F and Bac708R (5). An OTU is defined by assigning 16S rRNA gene sequences of ≥98% similarity to the same species. The numbers above the branch points are the percentages of bootstrap replicates that support the branching order. Scale bar represents 2.2% sequence divergence. The numbers in parentheses indicate the frequency of identical clones (for example, 1/8 OTU represents 1 of 8 sequences). Accession numbers of known Bacteroides and Prevotella sequences obtained from GenBank are also included.
**TABLE 2. All-Bacteria and Bacteroidales markers tested with different fecal and effluent samples from human, pig, and other animal sources**

<table>
<thead>
<tr>
<th>Source of sample (no. of samples)</th>
<th>All Bacteria; Bac2</th>
<th>All Bacteroidales; AllBac</th>
<th>Pig-specific Bacteroidales</th>
<th>Human-specific Bacteroidales; HF183</th>
<th>Ruminant-specific Bacteroidales; BacR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig Feces (25)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Pig Slurry (23)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lagoon water (14)</td>
<td>100</td>
<td>100</td>
<td>93</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pig Compost (7)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human feces (24)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Bovine feces (10)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
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<tr>
<td>Ovine feces (10)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Equine feces (10)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* A result was considered positive when marker concentration was greater than 4.5 log10 copies per g in feces and compost and 3.5 log10 copies per 100 ml of water.

**votella brevis** (GenBank sequence accession no. AJ011682; 90% similarity). This cluster was used to design the first pig-associated real-time PCR assay, Pig-1-Bac. Cluster II contained 12 OTUs: 8 from pig feces OTUs and 4 from pig slurry. In cluster II, the OTUs “Fpc8,” “Lpc61,” and “Fpc37” were closely related to the pig marker PigC1 described by Dick et al. (37) demonstrated 100% sensitivity (n = 10; pig feces and effluent samples). However, positive results were also observed for nontarget DNA (n = 30; human, bovine, sheep, and horse feces samples), with an overall score of 54% specificity.

For the two pig-specific Bacteroidales markers designed in this study and for the all-Bacteroidales, HF183, and BacR markers, plasmid DNAs containing partial 16S rRNA gene sequence inserts were run as standards, using 10-fold dilutions ranging from 1.6 × 10^7 to 1.6 × 10^10 copies per PCR mixture, with a quantification limit of 1.6 target copies per reaction.

Genomic DNA dilutions from a pure culture of *E. coli*, ranging from 7 × 10^6 to 7 × 10^10 16S rRNA gene copies per PCR, were run as standards for the all-Bacteria assay, with a quantification limit of 70 target copies per reaction mixture.

Consequently, the lower limits for quantification of the all-Bacteroidales and host-specific Bacteroidales markers were 4.5 log10 copies per g in feces and composts and 3.5 log10 copies per 100 ml of water.

**Testing general and host-specific Bacteroidales markers in feces and effluent samples.** The sensitivities and specificities of the general and host-specific Bacteroidales primers and probes developed in this present study were also evaluated (29, 39, 45) were tested on target and nontarget fecal DNA samples. The two pig-specific Bacteroidales primer and probe sets were both applied to 69 samples of pig origin (Table 2). For Pig-1-Bac and Pig-2-Bac, average concentrations were estimated to be 8.6 ± 0.8 (mean ± standard deviation) and 8.5 ± 0.6 log10 copies per g feces (wet weight), 4.8 ± 0.9 and 4.9 ± 0.7 log10 copies per ml of slurry, 2.4 ± 0.4 and 2.6 ± 0.4 log10 copies per ml of lagoon water, and 5.3 ± 0.5 and 5.3 ± 0.6 log10 copies per g of compost samples. No amplification was observed with nontarget DNA. The two pig-specific Bacteroidales markers showed 98 to 100% sensitivity and 100% specificity. The pig-specific Bacteroidales primers (Pig-Bac2) described by Okabe et al. (37) demonstrated 100% sensitivity (n = 10; pig feces and effluent samples). However, positive results were also observed for nontarget DNA (n = 30; human, bovine, sheep, and horse feces samples), with an overall score of 54% specificity.

All fecal and pig waste samples were positive for both the all-Bacteria and the all-Bacteroidales markers (Table 2). For samples of pig origin, the all-Bacteroidales marker concentrations were 10.1 ± 0.7 log10 copies per g of pig feces, 6.9 ± 1.1 log10 copies per ml of slurry, 4.7 ± 0.6 log10 copies per ml of lagoon water, and 9.5 ± 0.4 log10 copies per g of compost samples. The human-specific Bacteroidales marker HF183 was present in 13 of 24 human stool samples, with average concentrations of 7.8 ± 2.1 log10 copies per g of wet feces, implying 54% sensitivity. Negative results with HF183 were obtained for all nontarget DNA, showing 100% specificity. The ruminant-specific Bacteroidales marker BacR gave positive results on all bovine and sheep feces, with average concentrations estimated at 10 ± 0.3 log10 copies per g of wet feces, corresponding to 100% specificity. Amplifications were observed with pig effluent samples and human feces samples, showing 89% specificity. The observed differences in the frequency of detection of host-specific Bacteroidales markers in target and nontarget samples were significant (P < 0.05).

**Case study of the pig-specific markers throughout a slurry treatment process on a pig farm.** All markers and, especially, the pig-specific markers and *E. coli* bacteria enumerated by a culture technique were detected throughout the pig waste treatment chain, and their concentrations were seen to decrease throughout the slurry treatment process (Fig. 3). Indeed, Pig-1-Bac concentrations were 8.5 ± 0.7 log10 copies per g in feces, 4.8 ± 0.7 log10 copies per ml in slurry, 2.1 ± 0.3 log10 copies per ml in lagoon water, and 5.3 ± 0.5 log10 copies per g in compost samples. Pig-2-Bac concentrations were 8.6 ± 0.5 log10 copies per g in feces, 4.9 ± 0.7 log10 copies per ml in slurry, 2.4 ± 0.2 log10 copies per ml in lagoon water, and 5.1 ± 0.5 log10 copies per g in compost samples. ANOVA confirmed that the concentrations of the two markers were not significantly different in any of the stages of slurry treatment (P > 0.05). Concentrations of the all-Bacteroidales marker were 10.5 ± 0.1 log10 copies per g in feces, 7.8 ± 0.4 log10 copies per
ml in slurry, $2.1 \pm 0.3 \log_{10}$ copies per ml in lagoon water, and $5.3 \pm 0.3 \log_{10}$ copies per g in compost samples (Fig. 3). Concentrations of E. coli by culture were $6.9 \pm 0.7 \log_{10}$ MPN per g in feces, $6.6 \pm 0.8 \log_{10}$ MPN per ml in slurry, $2.4 \pm 0.1 \log_{10}$ MPN per ml in lagoon water, and $4.3 \pm 0.4 \log_{10}$ MPN per g in compost samples (Fig. 3). ANOVA indicated that concentrations of Pig-1-Bac and Pig-2-Bac markers were not significantly different from E. coli concentrations either in slurry or in lagoon water samples, whereas concentrations of the all-Bacteroidales marker were significantly different from E. coli concentrations at all treatment stages. A high level of correlation was found between the concentrations of the two pig-specific Bacteroidales markers and E. coli concentrations obtained by culture in pig wastes (for Pig-1-Bac, $R^2 = 0.77$, and for Pig-2-Bac, $R^2 = 0.88$) (Fig. 4).

**Marker concentrations in environmental river water samples.** Escherichia coli was found in all samples at concentrations that varied with sampling point and date from 2.5 to 4.3 $\log_{10}$ MPN per 100 ml (Table 3). The all-Bacteroidales marker was quantified in all samples, at concentrations that ranged between 4.6 and 7.8 $\log_{10}$ copies per 100 ml of water. At least one host-specific marker was quantified in samples from all sites, with most cases having multiple markers. The Pig-1-Bac marker was quantified in 25% of water samples collected around pig farms corresponding to site 10, with 4 $\log_{10}$ copies per 100 ml, and to site 11, with 3.9 $\log_{10}$ copies per 100 ml, and it was detected in 37.5% of these samples. It was detected in 30% of water samples collected downstream, corresponding to site 6, and never detected in samples collected near cattle farms. The Pig-2-Bac marker was quantified in 62.5% of water samples collected around pig farms (site 2, 4.1 $\log_{10}$ copies per 100 ml; site 10, 4 $\log_{10}$ copies per 100 ml; site 11, 3.6 and 3.8 $\log_{10}$ copies per 100 ml; and site 14, 3.6 $\log_{10}$ copies per 100 ml) and detected in 87.5% of these samples. It was detected in 20% of water samples collected downstream, corresponding to sites 6 and 7, and never detected in samples collected near cattle farms.

The HF183 marker was quantified in 90% of water samples collected downstream near Daoulas town, corresponding to sites 3, 4, 5, 6, and 7. The concentrations of the marker in these sites ranged between 3.5 and 4.4 $\log_{10}$ copies per 100 ml. It was quantified in 50% of water samples collected around pig farms, corresponding to sites 11, 12, and 13. The concentrations in samples from these sites ranged between 3.9 and 5.1 $\log_{10}$ copies per 100 ml. It was also quantified in 17% of water samples collected around cattle farms, corresponding to site 9, with 4.3 $\log_{10}$ copies per 100 ml. The BacR marker was quantified in all water samples collected around cattle farms, corresponding to sites 1, 7, and 8. The concentrations of the marker in these sites ranged between 4.6 and 6.0 $\log_{10}$ copies per 100 ml. It was quantified in 62.5% of water samples collected around pig farms, corresponding to sites 2, 10, 12, and 13, and concentrations ranged between 4.5 and 6 $\log_{10}$ copies per 100 ml. It was also quantified in 60% of water samples collected downstream, corresponding to sites 5, 6, and 7, and concentrations ranged between 5.7 and 6 $\log_{10}$ copies per 100 ml.

No significant correlation was observed between E. coli concentration and the concentrations of the two pig-specific Bacteroidales markers ($R^2 = 0.12$ and $R^2 = 0.11$), the human- and ruminant-specific Bacteroidales markers ($R^2 = 0.11$ and $R^2 = 0.10$), or the all-Bacteroidales marker ($R^2 = 0.15$) in the river water samples.

**DISCUSSION**

In this study, Bacteroidales 16S rRNA gene sequences were obtained from pig feces and slurries, and host-specific TaqMan
real-time PCR primers and probes were designed to identify pig fecal contamination in natural water samples. Most of the *Bacteroidales* sequences identified in the present study were related to uncultured *Prevotella* bacteria, thus indicating a high level of (as yet uncultured) diversity similar to the levels observed by Dick et al. (10) and Okabe et al. (37). Sequence analyses showed that clusters with only pig-specific sequences (from both feces and slurry samples) were represented in the genus *Prevotella*. In the phylogenetic tree, among a total of 51 sequences identified in the present study were closely related to the clone sequence "PigC1" obtained from human samples that have been published in GenBank. The similarity between *Bacteroidales* marker relative to the pig-specific PCR primer and probe sets for real-time PCR assays. The Pig-1-Bac and Pig-2-Bac markers were designed from clusters I and II, respectively. These pig-specific markers succeeded in identifying pig fecal pollution in target samples, and their concentrations were correlated with culturable *E. coli* concentrations throughout a pig waste treatment chain (from feces to compost or lagoon waters). However, concentrations of these pig-specific markers were low in comparison to all-*Bacteroidales* marker concentrations. One explanation for these lower concentrations could be that the total *Bacteroidales* 16S rRNA gene sequences from pig samples are not all pig specific. Indeed, 30% of the *Bacteroidales* 16S rRNA gene sequences from pig samples obtained in this study showed 98% similarity with *Bacteroidales* 16S rRNA gene sequences isolated from human samples that have been published in GenBank. The similarity between *Bacteroidales* sequences from pig and human *Bacteroidales* sequences was also found previously (29, 49) and was explained by their common omnivorous diet and similar digestive tract (10). The proportion of pig-specific markers relative to the all-*Bacteroidales* marker and proportion of the all-*Bacteroidales* marker relative to the all-*Bacteria* marker decreased along the waste treatment chain. Explanations could include (i) a loss of *Bacteroidales* during slurry storage, slurry treatment under aerobic conditions, or in activated sludge or (ii) dilution of *Bacteroidales* in the bacterial community during pig slurry storage. Indeed, Peu et al. (38) observed changes in the dominant microbial population between feces and slurry and between a slurry storage tank and a pond by obtaining PCR single-strand conformation polymorphism profiles. Furthermore, aerobic bacteria, such as *Bacillus thuringiensis*, *Sphingobacterium mizutae*, or *Paenibacillus* spp., have been shown to appear during slurry storage (31).

Previously, pig-specific *Bacteroidales* primers were described and found to be specific to pig feces from geographically dis-
Detection of pig-specific *Bacteroidales* markers in river water samples was performed in two previous studies. The pig-specific *Bacteroidales* marker described by Dick et al. (10) was previously tested on another French catchment (the Aber Benoît estuary that is also important for pig production, with approximately 225,500 pigs). It was rarely detected in river water samples from this area (20). In contrast, the pig-specific *Bacteroidales* marker designed by Okabe et al. (37) was detected in all four Japanese rivers sampled and at higher levels than the all-*Bacteroidales* marker (42). Savichtcheva et al. (42) recommended further validation of this pig-specific marker. Of the two pig-specific markers described in the present study, the Pig-2-Bac marker was detected more often than the Pig-1-Bac marker in environmental waters, although both were detected in similar concentrations in pig feces and effluents from different farms and geographical areas. Thus, a study of the persistence of both of these markers in the environment could be useful to evaluate the difference in detection in river samples.

In conclusion, this study has provided efficient TaqMan real-time PCR assays targeting pig-specific *Bacteroidales* 16S rRNA genes to discriminate pig fecal contamination in natural waters. Moreover, the detection of the pig-specific *Bacteroidales* markers over a 48-month period demonstrates their temporal stability. Among the two pig *Bacteroidales* markers designed, the Pig-2-Bac marker appears to be the most suitable, as it was detected more frequently in rivers. This study confirms that fecal pollution in river waters often comes from multiple sources; it was mainly of human and bovine origin on the sampling dates investigated in the Daoulas catchment. However, additional sampling should be carried out during high-rainfall events within the pig slurry spreading period to determine whether the pig markers and thus pollution from pigs could be more prevalent then. These pig-specific *Bacteroidales* markers could represent an efficient tool in a microbial source tracking toolbox, to discriminate between fecal pollution from pigs and other fecal sources. This tool will assist in the management of microbial water quality of bathing and shellfish farming areas.

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