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Phylogenetic analysis of *Bacteroidales* 16S rRNA gene sequences from human and animal effluents and assessment of ruminant faecal pollution by real-time PCR

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Abstract:

Aims: The aims of this study were to evaluate the host-specific distribution of *Bacteroidales* 16S rRNA gene sequences from human- and animal-related effluents and faeces, and to define a ruminant-specific marker.

Methods and Results: *Bacteroidales* 16S rRNA gene clone libraries were constructed from samples of effluent (sewage, bovine manure and pig slurry) and faeces (human, bovine, pig and wild bird), using PCR primers targeting order *Bacteroidales*. The phylogenetic analysis revealed six main distinct human-, bovine-, pig- and wild bird-specific clusters. From the bovine-specific cluster II, we designed a ruminant-specific marker, Rum-2-Bac, and this showed 97% sensitivity (n = 30) and 100% specificity (n = 40) when tested by TaqMan® real-time PCR. Average concentrations of this marker in bovine and sheep faeces and in bovine manure were 8·2 ± 0·5, 8·4 ± 1·3 and 7 ± 0·5 $\log_{10}$ copies per gram, respectively. It was also quantified in samples of runoff water impacted by bovine manure, with average concentrations of 5·1 ± 0·3 $\log_{10}$ copies per millilitre water.

Conclusions: Our results confirmed that some members of *Bacteroidales* isolated from effluents and faeces had host-specific distributions. Identification of a bovine-specific cluster made it possible to design a reliable ruminant-specific marker.

Significance and Impact of the Study: The host-specific distribution of *Bacteroidales* sequences from effluents mirrored the host-specific distribution of sequences observed in individual faeces. This efficient new ruminant-specific *Bacteroidales* 16S rRNA marker represents a useful addition to the microbial source tracking toolbox.

Keywords: 16S rRNA gene • *Bacteroidales* • faecal contamination • Microbial Source Tracking • sewage and manure

1. Introduction

Human and animal faecal pollution affects environmental water quality in both inland and coastal areas, with negative implications for recreational uses, public safety and shellfish sanitary status (Feldhusen, 2000; Gourmelon *et al.* 2006; Dorfman and Sinclair Rosselot 2008). Such pollution can also lead to economic losses if there are shellfish market closures or restrictions on bathing (Rabinovici *et al.* 2004).

France is an important animal producer in the European Union. It is the top cattle producer and ranks third for pig production. Indeed, in 2006, more than 19.5 million cattle and 14.8 million pigs were reared in France. Brittany and Normandy account for 10.5% and 11.7% of total national cattle production (Agreste Scees 2007). Furthermore, in Normandy, the presence of sheep on salt meadows could lead to human health risks in coastal waters (Hubbard *et al.* 2004). The coastal areas of these regions are thus particularly sensitive to animal faecal pollution. Bovine faecal contamination can arise due to slurry or manure spreading on arable land, cattle grazing or drinking near rivers, or stock movements.

Due to difficulties in the identification of non-point sources and the combined presence of urban and agricultural activities on some watersheds, Microbial Source Tracking (MST) methods are crucial for effective resource management and remediation. Many MST techniques have been developed to discriminate between human and non-human sources of faecal contamination, and to distinguish contamination from different animal species (Wiggins 1996; Parveen *et al.* 1999; Bernhard and Field 2000a and b; USEPA 2005). These methods aim to detect microbial or chemical targets associated with human and animal faeces, or sewage- or animal-related effluents (Marvin *et al.* 2001; Glassmeyer *et al*. 2005).

Among these methods, the detection of host-specific bacterial genetic markers such as *Bacteroidales* 16S rRNA genes in DNA extracts is a promising method. Detection of these anaerobic bacteria was proposed as an alternative and possibly superior water quality indicator (Fiksdal *et al.* 1985), as this group represents the main component of human and animal flora. Members of *Bacteroidales* are present at high concentrations in faeces (e.g. about 10^9-10^{11} per g of human faeces) but do not maintain culturability for very long once released into fresh or marine waters, due to their low oxygen tolerance (Allsop and Stickler; 1985; Fiskdal *et al.* 1985). Furthermore, some faecal members of *Bacteroidales* are described as having host species-specific or group-specific distributions (Kreader 1995; Bernhard and Field, 2000b). To quantify the relative contributions of specific hosts in an environmental sample, real-time PCR assays have recently been developed targeting human-specific (Seurinck *et al.* 2005; Layton *et al.* 2006; Kildare *et al.* 2007; Okabe *et al.* 2007; Reischer *et al.* 2007), ruminant-specific (Layton *et al.* 2006; Reischer *et al.* 2006) and pig-specific (Okabe *et al.* 2007; Mieszkin *et al.* 2009) *Bacteroidales* markers. As preliminary assays in our laboratory with previously developed ruminant-specific *Bacteroidales* markers showed some lack of specificity on pig or human feacal samples from France with these primer sets, we decided to develop a new ruminant-specific marker. Furthermore, identification of partial *Bacteroidales* 16S rRNA gene sequences from hosts in different locations is still needed to improve host-specific *Bacteroidales* PCR assays. Moreover there are few *Bacteroidales* sequences obtained from effluents or, particularly, from animal waste such as slurries or manure, available in databases. Designing host-specific markers based on sequences obtained from this kind of sample should improve the efficiency of 16S rRNA genetic markers for identifying faecal contamination in the environment.

The aims of this study were (i) to determine host-specific *Bacteroidales* 16S rRNA gene sequence distribution with sequences isolated from wastewater treatment plants (WWTP) water, bovine manure and pig slurry samples, and from human, bovine, pig and wild bird faeces; (ii) to design new primers and a probe to quantify the ruminant-specific *Bacteroidales* 16S rRNA gene by TaqMan® analysis; and (iii) to validate this marker on samples of runoff water impacted by bovine manure.

To our knowledge, this is the first study on the distribution of *Bacteroidales* 16S rRNA gene sequences that focuses on sewage and animal waste samples.

2. Materials and methods

Sample collection

To consider *Bacteroidales* from human and animal sources at the individual and population level, a total of 82 faeces and 52 effluent samples were collected from August 2004 to April 2008 in France.

Faecal samples

Human faeces were obtained from 17 healthy adult and child volunteers from Brittany. Animal faeces were collected immediately after excretion from apparently healthy animals (19 cows, 10 sheep, 10 horses, 16 pigs, and 10 wild birds). Cow faeces were sampled from animals kept on pasture or housed in stalls on 6 farms in Brittany and Normandy. Sheep faeces were collected from one farm in Brittany and from salt meadows on two farms in Normandy. Horse faeces were collected from stud farms in Brittany and Pays de la Loire. Pig faeces were collected from sows, adult males, young pigs and piglets mainly housed in stalls on 7 farms in Brittany. Wild bird faeces were collected mainly from seagulls, gulls, Eurasian widgeon, and brent goose on two beaches in Brittany.

Effluent and water samples

Ten WWTP water samples were collected at the outlets of 10 independent municipal WWTP not connected to slaughterhouses, in Brittany, Normandy and Pays de la Loire. Twenty bovine manure and 10 pig slurry samples were collected from 11 independent farms in Brittany.

For the run-off experiments, bovine manure with a concentration of 30 t per ha was spread on experimental arable land (75 cm by 150 cm). Runoff water samples were collected after 15 and 120 min of rainfall simulations with a rainfall intensity ranging from 50 to 55 mm per hour, corresponding to a storm event. A total of 12 field runoff water samples were collected, either previously spread with bovine manure (6 samples) or left as an untreated control (6 samples). Rain was simulated by watering with deep well water used, which was also sampled for testing.

All samples were placed in sterile containers and transported to the laboratory in insulated coolers. Faeces and bovine manure samples were aliquoted and cells from water and effluent samples were captured on filters upon arrival at the laboratory. All the samples were then stored at -20°C until analysis.

Sample preparation and DNA extraction

For faeces and bovine manure samples, DNA was extracted from 250 mg wet weight of sample using the Fast DNA Spin Kit for Soil (MP Biomedical, Illkirsh, France), according to Dick *et al.* (2005). Pig slurry and runoff water samples (50 ml) were centrifuged at 9000 × g for 15 minutes and DNA extracted from 250 mg of the pellet using the Fast DNA Spin Kit for Soil. For WWTP water samples, 20-100 ml were filtered through 0.22 µm Nuclepore membrane filters (Whatman, Boumath, France), respectively. Filters were then placed in 0.5 ml of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH = 8.0], 0.5% Sarkosyl) and kept frozen at -20°C until extraction (Dick and Field 2004). DNA was then extracted using the DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) as described in Dick and Field (2004).

PCR and 16S rRNA gene library construction

The primers Bac32F and Bac708R (Bernhard and Field, 2000b) were used to selectively amplify Bacteroidales 16S rRNA genes from DNA extracted from n=10 samples each of human, porcine, bovine and wild bird faeces; WWTP water, pig slurry and bovine manure.

Two microlitres of DNA extracts were added to a 40 µl-reaction mixture containing 1X PCR buffer (Roche Diagnostics, Meylan, France), 200 µM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 2 mM $MgCl_2$ and 1U Taq DNA polymerase (Roche Diagnostics). Reactions were carried out for 30 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s in a Peltier Thermal Cycler (PTC 200; MJ Research, Waltham, MA, USA), apart from the DNA from wild bird faeces. To obtain enough PCR products from wild bird faeces, 40 cycles of amplification were needed. Positive (DNA from faeces previously found positive) and negative (water) controls were included in each experiment. PCR products obtained from each library, which were then pooled to obtain seven clone libraries, were gel purified (Nusieve GTG agarose 2%; BMA, Rockland, USA) using the QiaQuick gel purification kit (Qiagen, France). Libraries were cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The vectors were transformed into chemically competent *Escherichia coli* (*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 inoculate 96-well culture plates (Deep-Well; Millipore) containing one ml LB 2X broth with ampicillin (50 µg ml^{-1}). Culture plates were incubated at 37°C for 24 h with shaking (130 rpm). After centrifugation, they were stored at -20°C until the plasmid purification step, performed with the Plasmid Miniprep 96 Kit (Millipore),3 the inserts were then sequenced.

Sequencing and phylogenetic analysis

Sequencing was performed on an ABI PRISM 9700 capillary sequencer using the ABI Prism Big Dye Terminator Cycle sequencing kit with M13-f and M13-r primers, as described by the manufacturer (Perkin-Elmer Applied Biosystems).

Sequences were edited using BioEdit (Hall, 1999). DNA sequences (approximately 690 bp) were processed using MALLARD software (Ashelford *et al. 2006*) to eliminate chimeric sequences. Sequences were aligned using MAFFT (version 5) and the distance matrix calculated using DNADIST (version 3.5c). This matrix was used with DOTUR software (Shloss and Handelsman 2005) to assign sequences to operational taxonomic units (OTUs). OTUs were defined by assigning 16S rRNA gene sequences of >98% similarity to the same species. Representative OTU sequences were retrieved rationally with the pipeline application RapidOTU (Legrand *et al.* 2008) and were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/) using BLAST. Sixty-five uncultured *Bacteroidales* sequences isolated from human and animal effluent sources (bovine manure, pig slurry and sewage) from the GenBank database were added. Multiple alignments were performed with CLUSTAL W (Thompson *et al.* 1997). A phylogenetic tree was constructed using PHYLO-WIN (Galtier *et al.* 1996). The root was determined using the 16S rRNA gene sequence of *Cytophaga fermentans* (M58766) as an outgroup. Distance trees were constructed using Neighbour-Joining algorithms (Saitou and Nei 1987) with the Kimura two-parameter correction (Kimura 1980). The statistical significance of tree branches was evaluated by bootstrap analysis using 500 resamplings.

Oligonucleotide primers and probes

The ruminant-specific *Bacteroidales* primers and probe (Rum-2-Bac) was designed from multiple alignments of partial *Bacteroidales* 16S rRNA gene sequences obtained in this study (Table 1). The primers and probe All-*Bacteroidales* (AllBac) from Layton *et al.* (2006) was used to amplify total *Bacteroidales* 16S rRNA genes. Oligonucleotide specificity for ruminant-associated *Bacteroidales* 16S rRNA genes was verified using the BLAST (NCBI) and Probe Match (Ribosomal Database Project: RDP II) programs.

Table 1: Oligonucleotide sequences and amplification conditions for conventional and real-time PCR assays

Primers and Probes	Primer and probe sequence (5'→ 3') *	Size of amplicon (bp)	Annealing Temp.(°C)	Final concentration (nmol)	Target	Reference
Bac32F Bac708R	AACGCTAGCTACAGGCTT CAATCGGAGTTCTTCGTG	690	60	500 500	All *Bacteroidales*	Bernhard and Field 2000a
AllBac						
AllBac296F AllBac467R AllBac375Bhqr	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG (FAM)CCATTGACCAATATTCCTCA CTGCTGCT(BHQ-1)	106	60	200 200 100	All *Bacteroidales*	Layton *et al.* 2006
Rum-2-Bac						
BacB2-590F Bac708Rm BacB2-626P	ACAGCCCGCGATTGATACTGGTAA CAATCGGAGTTCTTCGTGAT (FAM)ATGAGGTGGATGGAATTCG TGGTGT(BHQ-1)	99	60	200 200 200	Ruminant-specific *Bacteroidales*	This study

*FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1

Real-time PCR assays

All real-time PCR were performed using the TaqMan® Brilliant II QPCR Master Mix kit (Stratagene). Amplifications were performed using a Stratagene MX 3000 P 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, 60°C for 1 min. TaqMan reactions were carried out in a final volume of 25 µl with appropriate final concentrations of the primers and probe (Table 1).

The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC; Applied Biosystems, France). Samples were diluted if inhibitors were present. Negative controls (no template DNA) were performed in triplicate for each run.

The sensitivity and the specificity of the ruminant-specific *Bacteroidales* marker (Rum-2-Bac) was tested on target DNA (*n=30*) represented by bovine and sheep faeces and by bovine manure samples and non target DNA (*n=40*) represented by human, pig, horse and wild bird faeces and by pig slurry samples.

DNA standard curves and quantification

Linear plasmid DNA used to generate standard curves was extracted with the QIAquick Miniprep Extraction Kit (Qiagen), following the manufacturer's instructions. The linear forms of bovine plasmid 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 (Ct) against 16S rRNA gene copy numbers.

For Rum-2-Bac and AllBac, bovine plasmid DNA containing partial 16S rRNA, gene sequence inserts were used as standards at 10-fold dilutions ranging from 1.6×10^7 to 1.6×10^0 copies per real-time PCR, with a quantification limit of 1.6 target copies per reaction.

Consequently, the lower quantification limit of both the ruminant-specific *Bacteroidales* and All-*Bacteroidales* markers was 4.5 $\log_{10}$ copies per g in faeces. For the runoff experiments, the lower quantification limit of the markers was 2.9 $\log_{10}$ copies per millilitre.

Data treatment

The results of general and ruminant-specific *Bacteroidales* marker quantifications were expressed in 16S rRNA gene copies per g of faeces or bovine manure and per ml of runoff water.

To evaluate the performance of the ruminant-specific marker on faecal samples, sensitivity (r) and specificity (s) were defined as r = a/(a+c) and s = d/(b+d), where a is the number of faecal samples that were positive for the marker of their own species (true positive); b is the number of faecal samples that were positive for a marker of another species (false positive); c, is the number of faecal samples that were negative for a marker of their own species (false negative); and d is the number of faecal samples that were negative for a marker of another species (true negative) (Fisher and Van Bell 1993).

Nucleotide sequence accession numbers

Sequence data used in this study has been submitted to the GenBank database under numbers EU797125 to EU797175 and EU913511 to EU913643.

3. Results

Sequence and phylogenetic analysis

From a total of 672 clones sequenced from the seven clone libraries, 594 partial *Bacteroidales* 16S rRNA gene sequences were obtained. One hundred and seventeen sequences were identified as potentially chimeric and were removed from the phylogenetic analysis. The final analysis contained 477 unambiguous sequences corresponding to 185 OTUs
(Table 2).

Analysis of Bacteroidales 16S rRNA genes from human and animal effluent and faeces samples

Table 2 presents the number of sequences and OTUs, obtained from each type of faeces or effluent sample, shown the similarities of the OTUs to sequences from the NCBI database. Pig faeces and slurry are different from the other samples, as their *Bacteriodales* were predominantly *Prevotella*-like rather than *Bacteroides*-like.

Finally, most of the OTUs isolated from a host were similar to sequences isolated from the same origin found in the Genbank database. For example, of the 27 OTUs isolated from pig faeces, 60% were closest to sequences from Genbank database isolated from pig sources whereas 26% were closest to sequences from human sources.

Table 2: Distribution and characterization of *Bacteroidales* operational taxonomic units (OTUs) from French samples of faeces and effluents

Types of samples	Main host-sources of the samples	Seq ⁎ (n)	OTUs (n)	% of OUT with <98% similarity to GenBank sequences	Bacteroides- or Prevotella-like† (%)	Host-sources of sequences from GenBank‡ closest to OTUs with 100% similarity	References
Faeces	**Human**	73	16	94	*Bacteroides* (94)	82% Human; 12.5% Turkey	Eckburg *et al.* 2005; Manichanh *et al.* 2008; Scupham 2007
	Bovine	75	37	58	*Bacteroides* (89)	92% Ruminant; 8% Environmental	Dick *et al.* 2005; Layton *et al.* 2006; Bernhard and Field 2000a
	Pig	63	27	33	*Prevotella* (85)	60% Pig; 26% Human	Leser *et al.* 2002; Dick *et al.* 2005; Eckburg *et al.* 2005
	Wild bird	61	15	45	*Bacteroides* (87)	40% Chicken; 33% Human; 13% Horse; 13% Environmental	Layton *et al.* 2006; Scupham 2007; Eckburg *et al.* 2005; Simpson *et al.* 2004; Lamendella *et al.* 2007
Effluents	**WWTP waters**	68	30	70	*Bacteroides* (67)	57% Human; 27% Ruminant; 10% Dog	Ley *et al.* 2006; Li *et al.* 2008 Ley *et al.* 2008; Layton *et al.* 2006
	Bovine manure	79	36	57	*Bacteroides* (92)	78% Ruminant; 22% Environmental	Layton *et al.* 2006; Ueki *et al.* 2008
	Pig slurry	58	24	60	*Prevotella* (55)	55% Pig; 13% Environmental	Leser *et al.* 2002; Dick *et al.* 2005; Eckburg *et al.* 2005; Okabe *et al.* 2007
Total		477	185				

WWTP: Wastewater Treatment Plant.

⁎Seq: unambiguous sequences.

†From RDPII (>98% similarity) ‡From NCBI with Blast program.

Distribution of Bacteroidales 16S rRNA OTUs in host-specific clusters and design of ruminant-specific Bacteroidales marker

Phylogenetic analysis of the *Bacteroidales* OTUs identified in this study and the *Bacteroidales* sequences isolated from human and animal effluents from the Genbank database showed six main distinct host-specific clusters with sequences from both effluent and faeces samples (Fig. 1). Several clusters containing sequences from multiple faecal sources were also identified in the phylogenetic tree.

For reasons of clarity of presentation, only 22 and 17 OTUs from bovine and pig faeces, respectively, were included in the phylogenetic tree.

Two human-specific clusters were distinguished, HUMAN I and II, which contained 12 and 5 OTUs, respectively. The HUMAN I cluster contained the 'Fhc 10' OTU, which was 99% identical to *Bacteroides thetaiotaomicron* (AE015928), and the 'Fhc17' and 'STEPc48' OTUs, which were 99% identical to *Bacteroides ovatus* (AY652736, EU722734 and DQ100446). The HUMAN II cluster contained the OTUs 'Fhc31' and 'STEPc77', which were 100% identical to cultured *Bacteroides vulgatus* (EU728705, and EU136687 and CP000139, respectively). The 'Fhc11' and 'STEPc81' OTUs were 100% and 99% identical to cultured bacteria *Bacteroides dorei* (EU722737), respectively, and 99% identical to the clones 'HF145' and 'HF8' (Bernhard and Field 2000a; AF233408 and AF233411), respectively. This cluster also contained four sequences isolated from human sewage samples (EU573838, EU573839, EU573847 and EU573852).

One pig-specific cluster was identified: PIG. This cluster contained 10 OTUs, of which OTU 'Fpc8' was 99% identical to the clone 'PigC1' (AY695691) described by Dick *et al.* (2005). The OTUs 'Fpc35' and 'Fpc3' were closer to clones 'P93' (AB237869) and 'P80' (AB237867) obtained by Okabe *et al.* (2007) (96% and 94% similarity, respectively). This cluster also contained three sequences isolated from aerated pig manure slurry samples (AB331475, AB331465 and AB331464).

Only one cluster was identified for wild bird *Bacteroidales* OTUs, WILD BIRD, which contained seven OTUs.

Two bovine-specific clusters were isolated, BOVINE I and II, which contained 10 and 24 OTUs, respectively. The BOVINE I cluster contained one sequence isolated from a continuously stirred tank reactor of bovine waste samples (EU551115). The BOVINE II cluster contained 1 OTU, 'Fbc86', which was 97% identical to the clone 'CF123' (Bernhard and Field 2000a; AF233400). This cluster was used to design a ruminant-associated real-time PCR assay that we named Rum-2-Bac.To validate this PCR assay, oligonucleotide specificity for ruminant-associated *Bacteroidales* 16S rRNA genes were tested *in silico* using the BLAST (NCBI) and Probe Match (RDP II) programs. The primer BacB2-590F and the probe BacB2-626P were found highly specific to ruminant source.

Figure 1: Phylogenetic relationships of 185 operational taxonomic units from partial *Bacteroidales* 16S rRNA gene sequences isolated from effluent samples (WWTP water, STEPc; bovine manure, Fubc; and pig slurry, Lpc) and faeces (human, Fhc; bovine, Fbc; wild bird, Foc; and pig, Fpc). The scale bar represents 2.8% sequence divergence. Bootstrap values were calculated with 500 resamplings, and values below 75% were omitted. The 16S rRNA gene sequence from *Cytophaga fermentans* (M58766) was used as an outgroup for rooting the tree. Uncultured *Bacteroidales* isolated from faecal and effluent origin and sequences of cultivated and described *Bacteroides* and *Prevotella* species obtained from GenBank were also included.

Determination of faecal concentration of general and ruminant-specific *Bacteroidales* markers in effluent and faeces

Table 3 presents the percentage of positive results and average concentrations of ruminant-specific and general *Bacteroidales* markers in human and animal faeces and bovine manure. The Rum-2-Bac marker showed 97% sensitivity (*n=30*) and 100% specificity (*n=40*). Average concentrations of the Rum-2-Bac marker obtained on bovine and sheep faeces were similar (8.2 ± 0.5 and 8.4 ± 1.3 $\log_{10}$ copies per g, respectively) whereas in bovine manure, average concentration was lower with 7 ± 0.5 $\log_{10}$ copies per g. No amplification was observed with DNA from human, horse, pig and wild bird faeces samples.

The average concentrations of the All-*Bacteroidales* marker in faeces samples ranged between 5.3 ± 1.1 $\log_{10}$ copies per g in wild bird faeces to 10.2 ± 0.5 $\log_{10}$ copies per g in human faeces. Furthermore, for one faeces sample from a seagull, no amplification was obtained. For bovine manure samples, average concentrations of the All-*Bacteroidales* marker were 9.6 ± 0.3 $\log_{10}$ copies per g (Table 3).

Table 3: Quantification of All-*Bacteroidales* (AllBac) and ruminant-specific markers in faecal and effluent samples; percentage of positive samples and concentrations

	AllBac		**Rum-2-Bac**	
Samples	**Positive samples (%)**	**Concentration***	**Positive samples (%)**	**Concentration**
Bovine faeces (*n=10*)	100	9.8 ± 0.2	100	8.2 ± 0.5
Sheep faeces (*n=10*)	100	9.8 ± 0.9	100	8.4 ± 1.3
Horse faeces (*n=10*)	100	8.8 ± 0.3	0	<4.5
Pig faeces (*n=10*)	100	10.1 ± 0.8	0	<4.5
Human faeces (*n=10*)	100	10.2 ± 0.5	0	<4.5
Wild bird faeces (*n=10*)	90	5.3 ± 1.1	0	<4.5
Bovine manure (*n=10*)	100	9.6 ± 0.3	90	7 ± 0.5

* Concentrations are expressed in $\log_{10}$ copies per gram in faeces and bovine manure samples

Application of general and ruminant-specific *Bacteroidales* markers in runoff waters impacted by bovine manure

Run-off experiments were performed in order to test the capacity of the ruminant-specific marker to be detected after storm events in run-off waters from arable land where bovine manure had previously been spread. The ruminant-specific and general *Bacteroidales* markers were quantified in runoff waters impacted by bovine manure, giving with mean estimated concentrations of 5.1 ± 0.3 and 7.1 ± 0.5 $\log_{10}$ copies per ml of water, respectively. In the control runoff water, all the results with the Rum-2-Bac and AllBac markers were below the quantification limit (2.9 $\log_{10}$ copies per mlilitre).

4. Discussion

Coastal waters and rivers can be contaminated in multiple ways, especially by human sources, farm animals and wildlife (Griffin *et al.* 2001; Shanks *et al.* 2006; Savichtcheva *et al.* 2007). While wild bird droppings from roosting seagulls and faeces from pastured cattle have been described as potential sources of faecal pollution, municipal wastewater effluents and bovine or pig slurry manure spreading on arable land are also known to be also important sources of faecal contamination (Griffin *et al.* 2001; Savichtcheva *et al.* 2007; Lu *et al.* 2008). *Bacteroidales* have previously been noted to have several desirable characteristics as faecal

source identifiers, including the possibility of quantitative assessment, broad geographic stability and broad distribution in target host animals (USEPA, 2005).

Our work has provided interesting data on *Bacteroidales* 16S rRNA gene diversity among different hosts, by identifying 185 OTUs and six host-specific clusters (two for human, two for bovine, one for pig and one for wild bird). Interestingly, all human-, bovine- and pig-specific *Bacteroidales* 16S rRNA gene sequence clusters identified contained sequences from both effluents and faeces. These results suggest that i) some host-specific *Bacteroidales* 16S rRNA gene sequences identified in faeces could still be present in effluents (Peu *et al.* 2006) and ii) they could persist in the environment, even after a passage though the sewage network, or stockage/treatment of these wastes (Kreader, 1998; Peu *et al.* 2006; Okabe and Shimazu 2007).

Most *Bacteroidales* OTUs identified were not closely related to cultured bacteria species, indicating a high level of (as yet uncultured) diversity, as observed by Dick *et al.* (2005), Layton *et al.* (2006) and Okabe *et al.* (2007). In contrast, some OTUs from humans were found to be >99% identical to sequences of cultured *Bacteroides,* as in Dick *et al.* (2005). However, as *Bacteroides*-related sequences from bird, cat and dog faeces were also closely related to these sequences, cultured *Bacteroidales* do not seem to be efficient MST targets.

Compared to high number of 16S rRNA gene sequences isolated from faeces samples in the database, there are few *Bacteroidales* sequences isolated from effluent samples and still fewer derived from animal wastes (Whitehead *et al.* 2005; Shipin *et al.* 2007; Ueki *et al.* 2008; Hanajima *et al.* 2009). Phylogenetic analysis with effluent sequences from this study and database supported the host specificity of the clusters. Furthermore, some of the sequences found to be host-specific in this study were also found specific to the same host in geographic areas other than France, such as the United States or Japan (Bernhard and Field 2000b; Okabe *et al.* 2007). For example, the HUMAN II cluster contained four sequences, present in the Genbank database, that had been isolated from sewage in Ireland, and the clones 'HF8' and 'HF145', isolated in the United States, that were used to design the human-specific HF 183 marker (Bernhard and Field 2000b; Seurinck *et al.* 2005). The PIG cluster contained sequences that were used to design the pig-specific markers in the studies of Okabe *et al.* (2007) and Mieszkin *et al.* (2009).

These results suggest the existence of host-specific *Bacteroidales* sequences with a broad geographic distribution. One explanation for the presence of host-specific *Bacteroidales* could be the differences in digestive tract physiology and diet of humans and animal species, rather than the physiology of these hosts (Dick *et al.* 2005).

This study also provided data on the presence of *Bacteroidales* in different faecal samples. Indeed, all samples tested were found to contain *Bacteroidales*, and quantitative results were obtained on these samples (except for one seagull faeces sample) by performing All-*Bacteroidales* real-time PCR assays. All-*Bacteroidales* marker concentrations were similar in bovine faeces and manure and in horse, sheep, pig and human faeces whereas lower concentrations were obtained in wild bird faeces. A lower level of *Bacteroidales* in wild bird samples than in other origin faeces has already been observed in previous studies. For example, no positive results on wild bird faeces by conventional PCR assay were observed by Fogarty and Voytek (2005). Furthermore, Lu *et al.* (2003; 2008) by phylogenetic analysis of faecal flora, demonstrated that *Bacteroidales* are poorly represented in avian faeces (about 1%), while *Lactobacillaceae* (70%) and *Clostridiaceae* (11%) are dominant.

The ruminant-specific marker (Rum-2-Bac), which we developed from the bovine-specific cluster Bovine II, was quantified in bovine and ovine faecal samples suggesting that this marker could be used to detect bovine and sheep faecal contamination that arises in the coastal environment due to grazing alongside rivers and on salt meadows. This marker represents 1-5% of the total *Bacteroidales* populations in bovine and sheep feces samples. These results are similar of those that we obtained for the human-specific *Bacteroidales* marker developed by Seurinck *et al.* (2005) and for the two pig-specific *Bacteroidales* markers developed by Mieszkin *et al.* (2009). Furthermore, the high Rum-2-Bac marker concentrations obtained in all bovine manure samples showed that this marker is reliable for investigating faecal pollution from bovine manure spread on arable land. Indeed, this marker

was detected and quantified in water samples impacted by bovine manure (concentration 30 t ha^{-1}) during runoff simulations with a rain intensity corresponding to a storm event.
The Rum-2-Bac marker was not amplified in horse faeces suggesting that this marker is able to discriminate between horse and cattle faecal pollution.
The design of a new ruminant-specific *Bacteroidales* 16S rRNA gene marker to discriminate ruminant faecal contaminations from other faecal sources represents an efficient tool for a microbial source tracking toolbox to improve and monitor water quality in watersheds and coastal areas impacted by faecal pollution.

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