

# Search for genetic markers to trace swine-manure contamination of the environment

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## Introduction

Brittany is the main pig production area in France. About 14 million fatteners are produced a year in this province. Such a high concentration of confined-pig feeding operations on only 7% of the French territory has led to an over-application of manures to local soils. To address nitrogen and phosphorus pollution, biological treatments and mechanical separation of slurries have been developed. However, even when treated, manure may contain pathogenic microorganisms which can enter water systems through surface runoff and drainage. However, it is not easy to relate such diffuse pollution with the actual source. It is thus important to develop analytic tools to specifically distinguish sources of contamination found in the surface waters. Faecal contamination is usually highlighted by counting viable faecal indicator bacteria (e.g. *E. coli*, faecal coliforms or enterococci) but which do not give information on the human or animal origin of the contamination.

Specific bacterial marker for pigs has been described by Okabe *et al.* (11) and Gourmelon *et al.* (3) but they were obtained from just two farms and 5 stored manures respectively. These studies focused on the presence of specific markers only in faecal samples and in few manures and did not take into account the potential evolution of markers during storage or biological treatment of manure.

Because of the lack of data concerning the persistence of intestinal bacterial population in manure, the aim of this study was to compare, using a molecular method, the behaviour of four bacterial groups in 17 raw manures and throughout 10 biological treatments in order to propose a potential swine manure marker. The manure microbial communities were analyzed by PCR amplification of microbial 16S ribosomal RNA genes (16S DNA V3 region) and Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) of the resulting PCR products. Total bacteria and four microbial groups were analysed. Three groups, *Bacillus-Streptococcus-Lactobacillus* (BSL), *Eubacterium-Clostridiaceae* and *Bacteroides-Prevotella* belong to the dominant bacterial population of pig faeces (7). The genus *Bifidobacterium*, less dominant, has also been selected as this genus presents a significant host specificity (2).

## Material and methods

### Sampling

Sampling was carried out between March and July 2006 at 10 piggeries located in Brittany (France). Samples were taken from 27 anaerobic storage tanks: 17 from raw manure storage tanks (primary tank) and 10 from treated manure storage tanks after aerobic digestion (secondary tank). The storage time of the pig slurries varied from two weeks to two months in the primary tanks, three to four weeks in the aeration tank and three to nine months in the secondary tanks. All manures stored in tanks were homogenised by mixing for at least 30 minutes before sampling. Cowpats were sampled at 8 farms and human faeces were collected from 6 healthy adults.

## DNA extraction

Manure samples were centrifuged for 10 min at 17,500 g. Approximately 250 mg (wet weight) from each pellet were transferred to a microtube and immediately stored at -20°C. DNA extractions were performed on one pellet using the QIAamp DNA stool kit (QIAGEN) following the manufacturer's protocol.

## Total bacteria and group PCRs

For *Eubacterium-Clostridiaceae*, *Bacteroides-Prevotella* and the BSL group, the primers and the PCR program used in this study have been described by Peu *et al.* (12). For the *Bifidobacterium* group, the primers were g-Bifid-F and g-Bifid-R which were described by Matsuki *et al.* (9). The reaction mix was: deoxyNucleotide TriPhosphate (dNTP) 0.2 mM, primers 2 ng  $\mu\text{L}^{-1}$ , 1x AccuPrime *Taq* DNA polymerase buffer, AccuPrime *Taq* DNA polymerase (Invitrogen, the Netherlands) 2.5 U and 1  $\mu\text{L}$  of manure DNA diluted in water. The final volume was 20  $\mu\text{L}$ . The amplification program was as follows: after a denaturation step at 94°C, 2min, reactions were carried out for 30 cycles of 94°C for 30 sec, 53°C for 90sec and 68°C for 90sec. No final elongation was performed, as recommended by supplier (Invitrogen). The reaction was stopped by cooling the mixture to 10°C.

The protocol of the SSCP PCR used to amplify the variable V3 region of 16S DNA in order to separate the DNA fragment according to their sequences is described by Peu *et al.* (12). The total ITS PCR was performed with the primers pair designed by Cardinale *et al.* (1): ITSF and ITSReub using the amplification program described by the author (1). PCR products were sequenced by Ouest Genopol (Roscoff, France).

## Specific PCRs

For *Bifidobacterium*, the primers targeted the ITS region. The number of ITS total copies was enhanced by a first round PCR with ITSF and ITSReub primers, then a nested PCR was realised with specific primers GE35 (5' to 3': ATGGTATCGCGGGGTCGTC). GE36 (5' to 3': GAACACCCGGGAAGGAA). The reaction mix was the same as that for *Bifidobacterium* group PCR. The amplification program was also identical except for hybridization step which was done at 59°C. Specific PCR of *Lactobacillus sobrius* was performed with a protocol adapted from Konstantinov *et al.* (6).

## Cloning and sequencing

Product obtained from total bacteria and group PCRs were cloned in plasmids and transformed into *E. coli* competent cells using the StrataClone PCR cloning kit (Stratagene) and following the instructions of manufacturer except for the ligation time that has been increased from 5 to 15 min. Then 24 to 48 clones from each transformation were randomly picked and their insert was screened by nested PCR SSCP. In a first step, insert were amplified from colonies. The reaction mix was: dNTP 0.2mM, primers 4 ng. $\mu\text{L}^{-1}$ , 1x Red *Taq* Buffer, Red *Taq* polymerase 2.5 U and deionised water to complete the volume at 25  $\mu\text{L}$ . The amplification conditions were: 10 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final elongation step run for 10 min at 72°C. One  $\mu\text{L}$  of these PCR products was used to perform a SSCP PCR as described above. Inserts giving a peak that co-migrated with distinguishable peaks of the manure SSCP profile were sequenced to finalize the peak identification.

After total ITS PCR of *Bifidobacterium*, the amplified products were cloned and re-amplified by the protocol described above, except that no SSCP PCR was done. PCR products obtained from colonies were sequenced by Ouest Genopole using a T7 primer. DNA sequences were identified by comparison with their closest relatives available in databases using BlastN

from the National Centre for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) and the Ribosomal Database Project II (<http://rdp.cme.msu.edu/>).

## Results

The analysis of the four bacterial groups was carried out on 17 raw manures and 10 treated manures. All profiles were aligned and compared together. However, to simplify the visualization of the data, only 5 profiles per bacterial groups are presented in figures 1 to 4. The *Eubacterium-Clostridiaceae* profiles presented the lowest resolution with a high background level under the peak, indicating the complexity of this group of bacteria (Figures 1A and 1B). The raw manure profiles presented several co-migrated peaks and the number of distinct peaks was close before and after treatment (between 9 and 11). However, none of the 11 peaks present in raw manures was still detected in treated manures.

Figure 1: Comparison of *Eubacterium-Clostridiaceae* group profiles of raw manures (A) and treated manures (B). White markers correspond to peaks not identified, grey markers to peaks identified by one sequence and black markers to peaks identified by at least two sequences

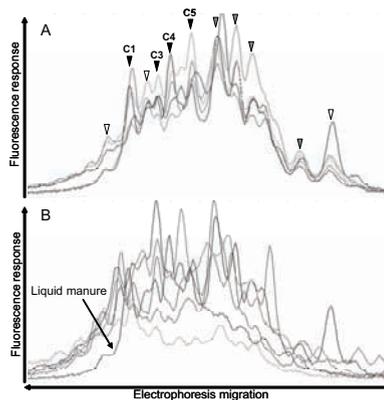
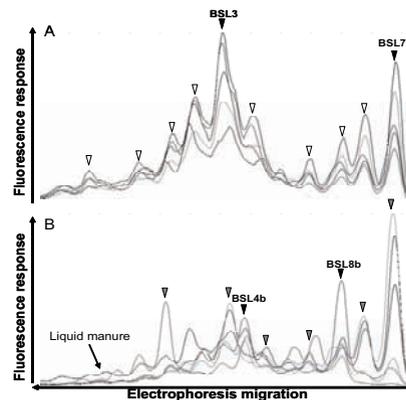


Figure 2 Comparison of BSL group profiles of raw manures (A) and treated manures (B). White markers correspond to peaks not identified, grey markers to peaks identified by one sequence and black markers to peaks identified by at least two sequences



The BSL group profiles presented also a background which was however weaker than *Eubacterium-Clostridiaceae* profiles (Figures 2A and 2B).

The profiles of raw and treated manures comprised 10 and 12 peaks, respectively. After aerobic treatment, 8 peaks co-migrated with peaks from raw manure profiles.

The profiles *Bacteroides-Prevotella* and *Bifidobacterium* groups differed from the profiles of the BSL and *Eubacterium-Clostridiaceae* groups by the absence of background and the weak number of peaks systematically preceded by artefacts (figures 3A and 3B, 4A and 4B). The three peaks of the *Bacteroides-Prevotella* group present in raw manures were not detected in treated manures, which presented two other distinguishable peaks (Figure 3B). The first peak was common to all samples whereas the position of the second peak differed from one treated manure to another. Furthermore, none of these peaks co-migrated with those of the raw manure (Figure 3B).

The profile of *Bifidobacterium* was characterized by two prominent peaks which were detected both in all raw and treated manures (figure 4A and 4B).

Most of the closest matching peaks belonging to the *Eubacterium-Clostridiaceae* and to *Bacteroides-Prevotella* profiles were not identified and had an environmental origin (Table 1). Nevertheless, the sequences of two peaks belonging to *Bifidobacterium* and BSL corresponded to identified species isolated from animal faeces: *Bifidobacterium thermacidophilum* ssp. *porcinum* and *Lactobacillus sobrius*.

Figure 3: Comparison of *Bacteroides-Prevotella* group profiles of liquid manures (A) and treated manures (B). White markers correspond to peaks not identified and black markers to peaks identified by at least two sequences

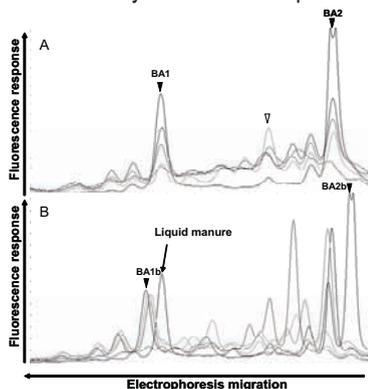


Fig. 4: Comparison of *Bifidobacterium* group profiles of liquid manures (A) and treated manures (B). Black markers correspond to peaks identified by at least two sequences

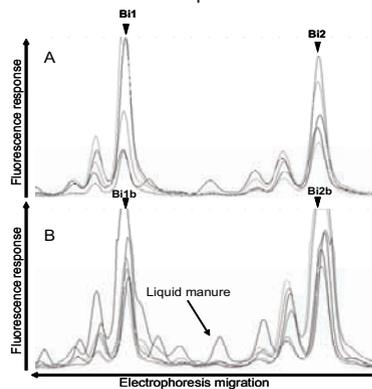
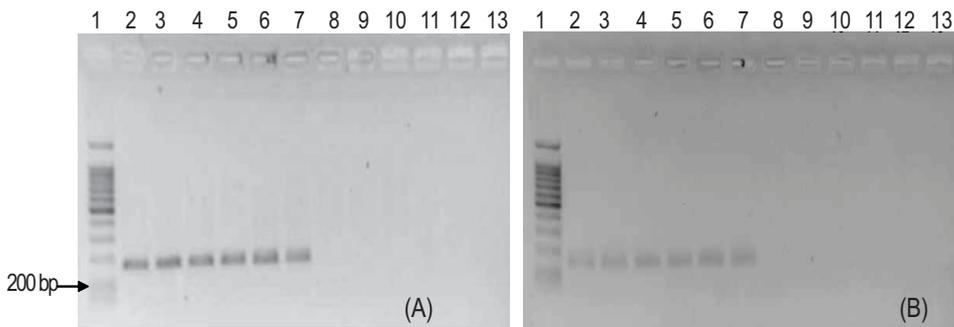


Table 1: List of the identified peaks

peak designation	sequence length (pb)	Name (accession no.)	Closest relative		
			Affiliation group	% similarity	Source
C1	525	Clone B-87 (AY676487)	<i>Clostridiaceae</i>	97	Bovine teat canal
C3	530	Clone M75 (DQ640962)	<i>Clostridiaceae</i>	88	Effluent treatment plant
C4	524	Clone A35 D28 L B B12 (EF559222)	<i>Clostridiaceae</i>	99	Mesophilic anaerobic solid waste digester
C5	525	Clone P316 (AF261803)	<i>Clostridiaceae</i>	98	Manure storage pits
BA1	707	Clone BRC82 (EF436368)	<i>Bacteroidetes</i>	92	Rumen of water buffalo
BA2	844	Clone SRRT42 (AB240481)	<i>Bacteroidetes</i>	92	Rhizosphere biofilm of phragmites
BA1 b	662	Clone Z144 (EU029356)	<i>Bacteroidetes</i>	94	Raw milk
BA2 b	405	Clone oca46 (AY491639)	<i>Bacteroidetes</i>	94	Waste water
BSL3	674	clone WTB_Y48 (EU009859)	<i>Mollicutes</i>	91	Turkey intestinal tract
BSL 7	674	<i>Lactobacillus sobrius</i> (AY700063)	<i>Lactobacillus</i>	100	Piglet intestinal tract
BSL4 b	645	Clone R8C-A3 (AY678482)	<i>Mollicutes</i>	88	Estuarine sediment
Bi1	513	<i>Bifidobacterium thermacidophilum</i> ssp. <i>Porcinum</i> (AY148470)	<i>Bifidobacterium</i>	99	Piglet intestine tra
Bi2	522	<i>Bifidobacterium pseudolongum</i> subsp. <i>Pseudolongum</i> (AY174109)	<i>Bifidobacterium</i>	100	Porcine cecum
Bi1 b	513	<i>Bifidobacterium thermacidophilum</i> ssp. <i>Porcinum</i> (AY148470)	<i>Bifidobacterium</i>	100	Piglet intestinal tract
Bi2 b	514	<i>Bifidobacterium pseudolongum</i> subsp. <i>Seudolongum</i> (AY174109)	<i>Bifidobacterium</i>	98	Porcine cecum

The presence of these two species was looked for in manures and faecal matters using primers designed by Konstantinov *et al.* (6) for *L. sobrius* and primers constructed in this study for *B. thermacidophilum* ssp. *porcinum*, targeting the ITS region. The matrices tested corresponded to the 27 manures, 8 cowpats and 6 human faeces. Representative results obtained for 3 samples of each matrix are shown on Figure 6.

Fig. 6: gel Electrophoresis (1.5 % agarose) of ITS specific PCR for *B. thermacidophilum* ssp. *porcinum* (A) and specific PCR for *L. sobrius* (B). Lane 1: Ladder 100 bp, lane 2 to 4: raw manures, lane 5 to 7: treated manures, lane 8 to 10: cowpats, lane 11 to 13: human faeces



The presence of both species was only observed for DNA extracted from pig manure.

## Discussion

Each bacterial group studied was observed to have a consistent profile during storage in raw manures regardless the geographical localisation of the piggeries. This result is in agreement with the data reported by Leung *et al.* (8) and Peu *et al.* (12) who have respectively observed similar DGGE and SSCP profiles from pig manures analysed at three month intervals. However, to be considered as representative, a microbial indicator of swine contamination must be both abundant and found in all types of stored manures, whether treated or not, before spreading on agricultural soil.

The biological treatment caused significant changes in two of the four bacterial populations. The profiles obtained by CE-SSCP highlighted that the composition of the *Eubacterium-Clostridium* and *Bacteroides-Prevotella* populations significantly evolved throughout the treatment process whereas the profiles of BSL and *Bifidobacterium* were largely unchanged between raw and treated manures. According to the SSCP profiles, the dominant *Eubacterium-Clostridium* and *Bacteroides-Prevotella* species detected in raw manures were affected by the treatment whereas BSL appeared to be more relevant to indicate manure contamination as 8 of the 10 peaks present in the raw manures remained after the biological treatment. One of the peak presented 100% of homology with *Lactobacillus sobrius* which has been previously described in the faeces of piglets (5) and pigs (4). The two peaks of *Bifidobacterium* found in the 17 raw manures are also found in all the treated manures. One peak was closely related to *B. thermacidophilum* subsp. *porcinum* which has been recently described in piglet and pig and faeces (10, 13).

These two species belonging to *Lactobacillus* and *Bifidobacterium* which persisted throughout the treatment are indeed present in manure prior to land spreading. Given their systematic presence in manure and the absence of detection in human and bovine faeces, using specific primers, these two species may represent markers of swine manure in the environment.

To confirm the specificity of these two proposed bacterial markers, further PCR analyses are necessary on samples taken from more faecal matters from different sources.

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