

Immunomagnetic separation of the intestinal spirochaetes *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* from porcine faeces

Enrique Corona-Barrera,¹ David G. E. Smith,¹ Tom La,² David J. Hampson² and Jill R. Thomson³

Correspondence

Jill R. Thomson

j.thomson@ed.sac.ac.uk

¹Zoonotic and Animal Pathogens, University of Edinburgh, Edinburgh, Scotland, UK

²School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, Australia

³Scottish Agricultural College, Veterinary Science Division, Penicuik, Scotland, UK

Porcine intestinal spirochaetes are fastidious anaerobic organisms and, as a consequence, it has been necessary to develop various protocols to enhance their isolation from or detection in faeces. Immunomagnetic separation (IMS) is a method developed recently to improve separation of target cells from mixed cell suspensions. The purpose of the present study was to compare the relative sensitivity of IMS for isolation of *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* with current routine diagnostic methods (culture on selective media and PCR) for detection of these micro-organisms in pig faeces. Neither direct nor indirect IMS methods enhanced the sensitivity of detection of either organism when performed with the recommended washings during sample processing. Performance of the IMS procedure without washing gave sensitivity at levels similar to direct culture onto selective medium. Further development of IMS techniques is required to improve isolation rates of *Brachyspira* species from faecal samples.

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INTRODUCTION

Intestinal spirochaetes are mainly members of the genus *Brachyspira*, which includes species commensal and pathogenic for pigs and other animals and humans. *Brachyspira hyodysenteriae* is recognized as the aetiological agent of swine dysentery (SD) (Taylor & Alexander, 1971) and *Brachyspira pilosicoli* is the aetiological agent of a disease of pigs known as intestinal spirochaetosis (Taylor *et al.*, 1980) or porcine colonic spirochaetosis (PCS; Duhamel *et al.*, 1998). Infection with *B. pilosicoli* has been reported in a wide range of hosts, including dogs (Duhamel *et al.*, 1995), chickens and other avian species (Dwars *et al.*, 1992; McLaren *et al.*, 1997), guinea pigs (Vanrobaeys *et al.*, 1998), non-human primates (Takeuchi *et al.*, 1974) and humans (Cooper *et al.*, 1986; Surawicz *et al.*, 1987). As interest in the field of intestinal spirochaetes has grown, further species have been identified recently as pathogens in animal species other than pigs. For instance, *Brachyspira alvinipulli* has been described and

shown to be pathogenic for chickens (Stanton *et al.*, 1998) and the provisionally designated '*Serpulina canis*' has been found in dogs (Duhamel *et al.*, 1998).

Porcine intestinal spirochaetes are Gram-negative, anaerobic and fastidious organisms. As a result, various culture media and incubation conditions have been assessed in order to develop the most reliable and efficient method for their isolation from faecal samples. Typically, isolation of these bacteria is achieved by culture on selective media based on trypticase soy agar or Columbia agar base supplemented with blood and multiple antibiotics to reduce the growth of other bacteria. Various antibiotic combinations have been utilized including spectinomycin (TSA-S400 medium; Songer *et al.*, 1976), spiramycin-colistin-vancomycin (TSA-CVS medium; Jenkinson & Wingar, 1981) and spectinomycin-colistin-vancomycin-spiramycin-rifampicin (BJ medium; Kunkle & Kinyon, 1988). In a comparative study of these media, BJ proved to be the most efficient in eliminating normal faecal flora and enhancing growth of *B. hyodysenteriae* and *Brachyspira innocens* from faecal samples (Achacha & Messier, 1992). It has recently been reported that pre-treatment of faecal samples with antibiotics prior to plating out efficiently reduced the normal intestinal flora and further enhanced isolation of intestinal spirochaetes (Calderaro *et al.*, 2001).

As a consequence of these studies, the main routine diag-

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Abbreviations: IMS, immunomagnetic separation; PCS, porcine colonic spirochaetosis; SD, swine dysentery.

nostic procedure for the porcine intestinal spirochaetes *B. hyodysenteriae* and *B. pilosicoli* has become culture on selective media coupled with biochemical tests for speciation of the isolates obtained (Fellström *et al.*, 1997; Hommez *et al.*, 1998). Despite the usefulness of these methods for detecting intestinal spirochaetes in disease outbreaks, they may lack sensitivity for detection of subclinically affected animals that may be persistently infected with pathogenic spirochaetes. For instance, carrier pigs are considered to be important in the transmission of SD, since animals can carry the bacteria without showing clinical signs (Windsor & Simmons, 1981). Little is known about the role of carriers in PCS, but pigs can remain infected with *B. pilosicoli* for up to 10 weeks (Duhamel *et al.*, 1998), and persistently infected animals may therefore be important in transmission. Carrier pigs may excrete bacteria at numbers below current detection limits [approx. 10^2 – 10^3 c.f.u. (g faeces) $^{-1}$], and the sensitivity of detection methods therefore needs to be as high as possible to isolate or detect intestinal spirochaetes to improve disease control.

The requirement for detection of smaller numbers of bacteria in specimens has led to application of novel methods such as immunomagnetic separation (IMS). IMS incorporates specific antibodies onto magnetic beads, which are then applied for separating specific cells from mixed cell populations. Early applications of IMS were for separation of B lymphocytes from whole blood (Rasmussen *et al.*, 1990); however, this technique has been developed further for separation of specific bacteria from faeces or food samples. For instance, IMS was more sensitive than conventional culture for detecting *Escherichia coli* O157 in faecal samples of cattle and sheep (Heuvelink *et al.*, 1998) and in food samples (Wright *et al.*, 1994). Similarly, IMS improved the separation of *Salmonella* Typhimurium (100 c.f.u.) from faecal samples (Widjoatmodjo *et al.*, 1991). IMS has been further adapted to increase the sensitivity of detection of enteropathogens in faecal samples, e.g. by combining IMS with PCR for detection of pathogens. For instance, improved sensitivities have been achieved in the detection of *Bacteroides fragilis* (Zhang & Weintraub, 1998), *Salmonella* from swine, horses and cattle (Widjoatmodjo *et al.*, 1992; Stone *et al.*, 1994), *Helicobacter pylori* (Enroth & Engstrand, 1995) and *Leptospira* species in urine samples (Taylor *et al.*, 1997).

Improving the sensitivity of detection of pathogens, particularly fastidious organisms such as intestinal spirochaetes, may require a combination of procedures. The aims of this study were to assess the potential of IMS for the isolation of *B. pilosicoli* and *B. hyodysenteriae* from pig faeces and to compare its sensitivity with the existing methods of direct culture and PCR.

METHODS

Brachyspira strains and isolates. *B. pilosicoli* field isolates (P32/2/97, P93/2/94, P595/3/00, P657/3/00, P657/8/00, P676/2/00 and P950/3/00), *B. pilosicoli* type strain ATCC 51139^T, *B. hyodysenteriae* isolates (P93/8 and P944/14/00) and *B. innocens* isolates (P692/9/00, P906/3/00 and

P950/3/00) were all obtained from the Scottish Agricultural College – Veterinary Science Division, Edinburgh. Intestinal spirochaetes were grown over the whole surface of blood agar [BA; 5.0% (v/v) sheep blood in Columbia agar base] plates for 3 days at 37 °C in anaerobic boxes under anaerobic conditions (AnaeroGen; Oxoid). Bacterial suspensions of target cells (*B. hyodysenteriae* and *B. pilosicoli*) for IMS assays were prepared by harvesting the growth from two BA plates using a sterile cotton swab (Bibby Sterilin), which was then dispersed into 5 ml PBS. Bacterial suspensions were centrifuged at 8000 g for 10 min, washed twice with PBS and resuspended in 1 ml PBS. Washed bacterial cells were 10-fold serially diluted (10^1 to 10^{10}) and then used immediately to spike pig faeces. Counting of cells in diluted bacterial suspensions (containing the target cells) was done by a modified Miles and Misra technique. Briefly, 10-fold series dilutions of the neat bacterial suspension were made in 1 ml final volumes. The neat bacterial suspension was mixed thoroughly by pipetting ten times and 100 µl was then transferred to 900 µl PBS diluent to make the first 1:10 dilution; this process was continued to a final dilution 10^{10} . A 10 µl aliquot of each dilution was plated out in quadruplicate onto BA plates (for colony counting), allowed to dry and then incubated anaerobically for 48–60 h. Colonies from the dilution that produced between 20 and 30 c.f.u. were counted and the mean taken and the total number of c.f.u. ml $^{-1}$ was calculated using appropriate dilution and volume multipliers.

Faecal samples and spiking of faeces. Faeces were obtained from pigs on a farm in Scotland and confirmed culture-negative for *Brachyspira* species. Pig faeces were stored frozen in aliquots and then resuspended in PBS to 5.0% (w/v) (i.e. 1 g faeces in 20 ml PBS), and 400 µl aliquots of this faecal suspension were spiked with 30 µl of a 10-fold dilution series of test bacterial suspension containing the target cells *B. hyodysenteriae* or *B. pilosicoli*.

mAbs. Two mAbs of IgM subclass produced in mice were used: one was specific for a 29 kDa outer-membrane protein of *B. pilosicoli* (BJL/AC1; Lee & Hampson, 1995) and the other was specific for a 30 kDa outer-membrane protein of *B. hyodysenteriae* (BJL/SH1; Lee & Hampson, 1996). Titration and specificity of the mAbs was done by dot blotting of crude antigen (Ag) preparations immobilized on nitrocellulose membrane (pore diameter 0.45 µm). Briefly, bacteria were grown to equivalent densities, washed as above and diluted 1:20 in PBS. The nitrocellulose membranes were pre-wet in PBS (pH 7.4) for 10 min and then assembled onto a 96-well Bio-Dot micro-filtration apparatus (Bio-Rad) covering all the 96 holes and properly sealed to avoid cross-contamination. A volume of 100 µl PBS was applied to each well to rehydrate the membrane, the liquid was removed by applying a vacuum and 100 µl inocula of the Ag preparations were then applied to each well and left overnight to pass through the membrane by gravity filtration at room temperature. After Ag preparations had completely drained, each well of the membrane was washed with 200 µl PBS-T [PBS plus 0.05% (v/v) Tween 20] by applying a vacuum. An aliquot of 100 µl blocking solution [5.0% (w/v) skimmed milk in 10 ml PBS-T] was applied to each well and incubated for 1 h to allow gravity filtration and each well was then washed with 200 µl PBS-T by applying a vacuum. For titration, mAbs were reacted against crude Ag preparations of *B. hyodysenteriae* or *B. pilosicoli* containing approximately 1×10^6 spirochaete cells and, for specificity, mAbs were reacted against crude Ag preparations containing similar numbers of bacterial cells (as above) of *B. pilosicoli* ATCC 51139^T, *B. pilosicoli* field isolates P32/2/97, P93/2/94, P595/3/00, P657/3/00, P657/8/00, P676/2/00 and P950/3/00, *B. innocens* isolates P692/9/00, P906/3/00 and P950/3/00, *B. hyodysenteriae* isolates P93/8 and P944/14/00 and several species representative of the normal pig intestinal microflora. The latter were obtained from Kevin Hillman (SAC, Aberdeen, UK) and included *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Clostridium brevis*, *E. coli*, total anaerobic bacteria, mixed enterococci, total aerobic bacteria and mixed coliform bacteria. For titration of mAbs, serial dilutions (1:10 to 1:5120 in PBS) were

prepared in 1 ml volumes and 100 µl of each dilution was incubated in duplicate with the Ag preparations (*B. hyodysenteriae* and *B. pilosicoli* dotted on the nitrocellulose membranes assembled in Bio-Dot apparatus as described above) for 60 min at room temperature to allow gravity filtration and then washed three times with 200 µl PBS-T (containing 0.2% Tween 20) by applying a vacuum. A volume of 100 µl secondary antibody (goat anti-mouse conjugated with horseradish peroxidase; Dako) diluted 1:500 in PBS-T was added to each well and incubated for 60 min to allow gravity filtration and then washed three times with 200 µl PBS-T (containing 0.2% Tween 20) by applying a vacuum. Nitrocellulose membranes were then removed from the Bio-Dot apparatus. Reactions were developed by soaking the membranes in 10 ml of a freshly prepared substrate solution (Vector DAB kit) until a reaction appeared (5–10 min). Optimal titres for mAbs against *B. hyodysenteriae* and *B. pilosicoli* were at 1:160 dilution for both as intensity of reaction remained maximum, and this dilution of mAbs was used in IMS procedures. For specificity, 100 µl of dilution 1:160 of the mAbs was incubated with crude Ag preparations of the bacteria mentioned above; washing, blocking, addition of secondary antibody and development of reaction were performed as described above.

IMS of spirochaetes from faecal suspensions. For IMS, polystyrene magnetic beads (Dynabeads M-450 rat anti-mouse IgM; Dynal) were used, as these beads are pre-coated with anti-mouse IgM. Both direct and indirect IMS methods were applied to separate the target bacterial cells (*B. pilosicoli* ATCC 51139^T or *B. hyodysenteriae* field isolate P944/14/00) from the faecal suspensions.

IMS (direct) method. For the direct IMS method, Dynabeads M-450 were coated with the mAbs either to *B. pilosicoli* (BJL/AC1) or to *B. hyodysenteriae* (BJL/SH1) as follows: 2.5 µl mAb was added to 25 µl Dynabeads M-450 (containing approximately 10⁷ beads), vortexed and incubated with continuous, slow, end-over-end rotation for 30 min at room temperature. Dynabeads M-450 were collected using a magnet (Dynal) and washed four times with washing buffer (per l distilled water: 0.16 g NaH₂PO₄, 1.98 g Na₂HPO₄ · 12H₂O, 8.10 g NaCl, 1 g BSA, pH 7.4). Antibody binding (coating rate) of the rat anti-mouse IgM (Dynabeads M-450) to the IgM of the specific mAb Bjl/AC1 or Bjl/SH1 was verified by measuring the A₂₈₀ of the supernatant antibody solution before and after coating, with decreased absorbance confirming IgM binding. Dynabeads M-450 coated with the specific mAbs were resuspended in 25 µl PBS (original volume as taken from vial before coating) to use in IMS assays. The coating of Dynabeads M-450 with mAbs was scalable as required. Dynabeads M-450 coated with specific mAbs (25 µl) were applied directly to 400 µl (to obtain the optimal 1:160 dilution of the mAbs) of the spiked pig faeces (containing known numbers of target bacterial cells) and incubated at room temperature with continuous, slow, end-over-end rotation for 60 min to allow binding to the cells. After incubation, IMS product was collected using the magnet, washed three to four times with washing buffer and resuspended in a final volume of 100 µl PBS (IMS product).

IMS (indirect) method. In the indirect method, 2.5 µl specific mAbs (BJL/AC1 or Bjl/SH1) were initially incubated at room temperature with 400 µl spiked faeces (containing known numbers of target bacterial cells) with continuous, slow, end-over-end rotation for 60 min, to allow the specific antibody to bind the target cells. Dynabeads M-450 (25 µl) were then added to that suspension and incubated (as above) for 30 min at room temperature. This second incubation allowed the Dynabeads M-450 rat anti-mouse IgM to bind IgM of the specific mAb already bound to the target cells. After that incubation, the IMS product was collected using the magnet and then washed with washing buffer three to four times and resuspended in 100 µl PBS (IMS product).

IMS assays were repeated at least twice by spiking faeces with a 10-fold dilution series of the bacterial suspensions containing *B. pilosicoli* or *B. hyodysenteriae*.

Detection of *B. pilosicoli* and *B. hyodysenteriae* after IMS. Two methods were used to detect the spirochaetes after IMS, culture and PCR.

Culture. For culture, triplicate 10 µl samples of the IMS product (100 µl) were plated onto conventional BA (described above) and TA (selective medium for *Brachyspira* spp.) plates. TA was prepared by adding a selective supplement comprising 4.0% (w/v) spectinomycin dihydrochloride, 5000 IU vancomycin hydrochloride ml⁻¹ and 131 IU colistin methane sulphonate ml⁻¹. Aliquots (10 µl) of each of the IMS washings were also plated out in order to determine whether the target cells were washed off in each washing. Inocula (10 µl) from IMS (direct method) product without washing (i.e. magnetic beads collected after IMS and resuspended in 100 µl PBS without washing) were also plated out. Control inocula (10 µl), i.e. spiked pig faeces containing the 10-fold diluted series of the bacterial suspension, were also plated onto BA and TA. BA and TA plates inoculated with triplicate samples (of each of the 10-fold-diluted series) were incubated anaerobically (AnaeroGen) at two temperatures, 37 and 42 °C, for 3–4 days.

PCR. For PCR amplification, 50 µl samples of the IMS product and controls were used for extraction of DNA. The samples were boiled for 5 min, beads and debris were removed by centrifugation (8000 g for 5 min) and genomic DNA was then extracted from the supernatant using the QiaAmp system (Qiagen). The presence of DNA in extracts was confirmed by electrophoretic separation of 10 µl of the extracted DNA on a 1.0% agarose gel (Sambrook *et al.*, 1989). PCR was performed using primers (SF1, 5'-CAGCTAAGGTCCCAAAATCTATGT-3'; SR1, 5'-GAACCCGAAAGCCAGTCAC-3') that amplify a highly conserved region between nucleotides 874 and 1429 of the 23S rRNA gene of *Brachyspira* species. This generates a characteristic product of 555 bp from *Brachyspira* species (Teran-Dianderas, 1997). The PCR consisted of MegaMix-Blue (Microzone) containing 200 µM of each dNTP, 2.5 mM MgCl₂, *Taq* polymerase (1 U per 50 µl), 1.5 µl 0.2 mM primer SF1, 1.5 µl 0.2 mM primer SR1 and 2 µl DNA template in a final volume of 30.5 µl.

Cycling was carried out with one round of 4 min denaturation at 94 °C, 1 min annealing at 60 °C and 2 min extension at 72 °C, followed by 39 cycles of 1 min denaturation, 1 min annealing and 2 min extension at the same temperatures. After the thermocycling was complete, samples were preserved at 4 °C and PCR products were visualized after electrophoresis on 1.5% agarose gels and staining with ethidium bromide (Sambrook *et al.*, 1989). The sensitivity of PCR was compared with that of culture both with and without IMS.

RESULTS AND DISCUSSION

Infections caused by the porcine intestinal spirochaetes *B. hyodysenteriae* and *B. pilosicoli* are of major importance (Taylor *et al.*, 1980; Lysons & Lemcke, 1983; Fellström & Gunnarson, 1995), resulting in substantial productivity losses as a consequence of detrimental effects on animal health and welfare. Clinically affected animals are a major source of transmission of intestinal spirochaetes via the faecal–oral route; however, there is evidence that asymptomatic carriers are reservoirs and play an important role in the persistence of SD (Windsor & Simmons, 1981). Since carrier animals may excrete small numbers of pathogens, it is unclear whether current methodologies for isolation of intestinal spirochaetes have sufficient sensitivity and practicality for detecting such animals. In the present study, culture of intestinal spirochaetes from IMS and controls (direct

culture) on selective culture media and aliquots of samples tested by PCR were assessed to evaluate their sensitivity for detection of *B. pilosicoli* and *B. hyodysenteriae* in pig faeces.

Usually, inoculation of IMS products and controls onto BA produced mixed bacterial growth, including organisms representative of the normal pig intestinal microbiota, together with *B. pilosicoli* or *B. hyodysenteriae*. In contrast, the same inocula on TA plates with antimicrobials produced pure cultures (confirmed by microscopy) of either *B. pilosicoli* or *B. hyodysenteriae*. Therefore, in accordance with several other reports (Jenkinson & Wingar, 1981; Kunkle & Kinyon, 1988; Calderaro *et al.*, 2001), we observed that selective media were highly effective for the recovery of intestinal spirochaetes from faeces in pure culture; however, in the case of primary field isolates, the sensitivity may be lower.

Incubation at 37 °C supported growth of *B. pilosicoli* and *B. hyodysenteriae* on both BA and TA plates; however, better quality of growth and higher sensitivity of isolation were obtained at 42 °C. Incubation at the higher temperature appeared to improve selectivity and thus was adopted throughout. A summary of relative sensitivities for each of the methods for detection of *B. pilosicoli* and *B. hyodysenteriae* is presented in Table 1. Representative PCR results are shown in Fig. 1.

The indirect IMS method for detection of *B. pilosicoli* and *B. hyodysenteriae* was 10^1 - to 10^2 -fold more sensitive than the direct IMS method. Furthermore, higher sensitivity [in the range 10^1 c.f.u. (g faeces)⁻¹] was obtained from IMS product plated out without washing. Ideally, IMS should be performed with the recommended washing in order to remove organisms other than those targeted. It has been documented that bacteria can be adsorbed non-specifically to solid surfaces in the presence of electrolytes (Marshall *et al.*, 1971). These results confirmed this, since many different bacteria were isolated when samples were cultured on the non-selective medium (mixed cultures), despite the use of a blocking agent (BSA) in the washing buffer, as recommended by the manufacturer. Non-specific adsorption of spirochaetes onto beads may have occurred similarly, thus contributing to the apparently high sensitivity of the IMS

in the absence of washing. On the other hand, non-specific adsorption of bacteria may have obstructed antibody-dependent binding and thus contributed to the poor sensitivity for spirochaetes. However, only one characterized mAb was available for each of the spirochaete species targeted in this investigation. It is possible that the affinity of these antibodies for their antigens was low, thereby resulting in loss of cells during washing. Unfortunately, other mAbs are not currently available for testing in IMS.

It is possible that the lower sensitivity obtained might have been due to a direct effect on viability through aeration occurring during manipulation of targeted cells during the IMS procedure; however, this is unlikely, as *Brachyspira* species can survive in the presence of oxygen through the activity of their NADH oxidase (Stanton *et al.*, 1999). Large numbers of spirochaetes were detected in each of three washing eluates [approx. 10^6 , 10^6 and 10^4 c.f.u. (g faeces)⁻¹], indicating that spirochaetes were removed from beads during the normal washing steps. This may have occurred via disruption of spirochaete binding to immunoglobulin-coated beads as a result of the distinctive morphological characteristics of spirochaetes, as cells of both *B. pilosicoli* (5.2–11.0 µm) and *B. hyodysenteriae* (5.9–12.9 µm) are considerably longer than the diameter (4.5 µm) of the Dynabead M-450 beads used in this study. Nevertheless, IMS has been applied successfully for the isolation of the spirochaete *Leptospira borgpetersenii* from urine samples, in this case with a sensitivity of detection of 10^1 bacterial cells, demonstrating the potentially high sensitivity of IMS for capturing spirochaetes (Taylor *et al.*, 1997). This sensitivity was obtained by a combination of IMS-PCR followed by detection of the PCR product with a labelled probe.

Repeated IMS assays revealed that the sensitivity of IMS in recovering target cells of *B. pilosicoli* or *B. hyodysenteriae* was no higher than controls cultured without IMS. This was unexpected, since limits of detection of other faecal organisms have typically been improved by application of IMS. For instance, *E. coli* O157 has been detected at a level of 12 c.f.u. (g faeces)⁻¹ from asymptomatic patients (Chapman & Siddons,

Table 1. Comparative sensitivity for detection of porcine intestinal spirochaetes in pig faecal samples

Values represent c.f.u. (g faeces)⁻¹ of intestinal spirochaetes detected by each method. ND, Not done.

Assay	<i>B. pilosicoli</i>		<i>B. hyodysenteriae</i>	
	Culture	PCR	Culture	PCR
Direct IMS	4.8×10^5	4.8×10^5	2.5×10^4	2.5×10^3
Direct IMS (without washing)	9.9×10^1	9.9×10^3	ND	ND
Indirect IMS	1.5×10^3	1.5×10^3	9.9×10^3	ND
Control (no IMS)	1.5×10^2	ND	9.9	ND

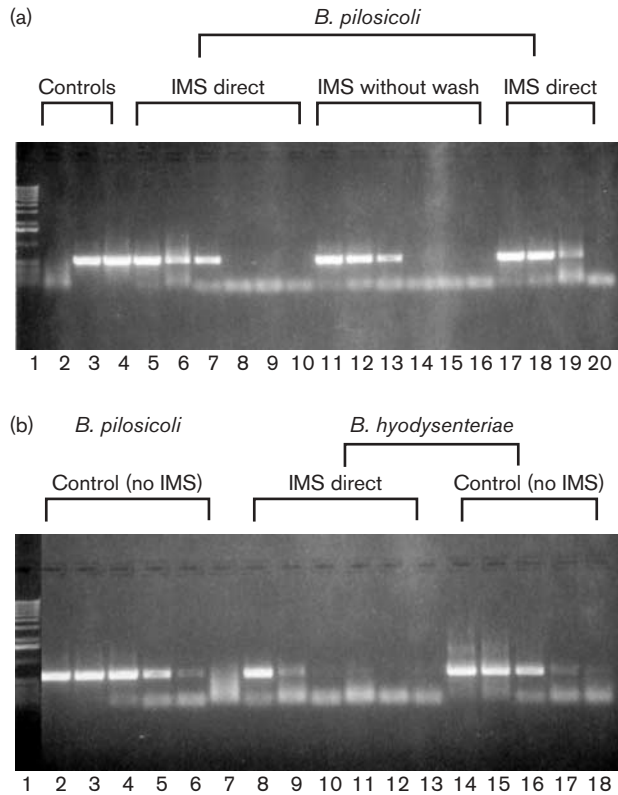


Fig. 1. Agarose gels showing sensitivity of PCR on samples of 10-fold dilutions of bacterial suspensions containing target cells. (a) Lanes: 1, molecular marker 1 KB (0.5–10 kb); 2, negative control; 3, positive control for *B. pilosicoli*; 4, positive control for *B. hyodysenteriae*; 5–10, IMS (direct method) for *B. pilosicoli*, respectively 10^1 to 10^6 dilutions; 11–16, IMS (without washing) for *B. pilosicoli*, 10^1 to 10^6 ; 17–20, IMS (indirect method) for *B. pilosicoli*, 10^1 to 10^4 . (b) Lanes: 1, marker 1 KB; 2–7, controls (no IMS) for *B. pilosicoli*, 10^1 to 10^6 ; 8–13, IMS (direct method) for *B. hyodysenteriae*, 10^1 to 10^6 ; 14–18, controls (no IMS) for *B. hyodysenteriae*, 10^1 to 10^5 .

1996), whilst 100 c.f.u. of *Salmonella* Typhimurium has been recovered from mixed suspensions (Widjoatmodjo *et al.*, 1991).

Previously, isolation of $\sim 10^1$ and $\sim 10^0$ cells of *B. pilosicoli* and *B. hyodysenteriae*, respectively, has been reported (Fellström *et al.*, 1997, 2001) after 7 days of incubation. Our results for sensitivity of detection (incubation period of only 4 days) were approximately 10-fold lower than those of Fellström *et al.* (1997, 2001). In our study, higher sensitivity might have been achieved with a longer incubation period. Taken together, these data indicate that IMS with the reagents currently available does not improve isolation and detection of intestinal spirochaetes and that culture on selective medium may provide adequate sensitivity of detection.

Several reports have identified improved sensitivity for detection of organisms through IMS followed by PCR. In the present study, the sensitivity of IMS-PCR for detection of *B. pilosicoli* was no better than culture either after IMS or

directly from spiked faeces. The sensitivity of PCR for detection of *B. hyodysenteriae* was higher than that for detection of *B. pilosicoli* and similar to the sensitivity of culture. The sensitivities of PCRs targeting the 23S rRNA genes or other genes of *Brachyspira* species for various species of intestinal spirochaetes have been reported (Park *et al.*, 1995; Leser *et al.*, 1997; Atyeo *et al.*, 1998; Barcellos *et al.*, 2000; Mikosza *et al.*, 2001). These PCRs were applied directly to spirochaete cells or to spirochaetal growth on primary isolation plates, and their sensitivities were generally consistent with those of the PCR used in the present study. Therefore, the PCR methodology used in the current study was not responsible for limiting sensitivity of the IMS-PCR assay. Recently, a duplex PCR for the NADH oxidase gene (*nox*) of *B. hyodysenteriae* and the 16S rRNA gene of *B. pilosicoli* was developed and applied to chromosomal DNA extracted directly from pig faeces, and gave a detection limit of 10^3 – 10^4 c.f.u. (g faeces) $^{-1}$ for both spirochaetes (La *et al.*, 2003). In that study, the faecal PCR was more sensitive than culture and subsequent PCR from the growth on the initial isolation plate.

In the present study, the highest sensitivities for recovering *B. pilosicoli* and *B. hyodysenteriae* from pig faeces were obtained by direct plating onto selective media and incubation at 42 °C. In this study, the limits of detection of cells of *B. pilosicoli* and *B. hyodysenteriae* in faecal samples were respectively $\sim 10^2$ and $\sim 10^1$ g $^{-1}$ and are similar to the best detection limits reported by others (Fellström *et al.*, 1997). Those numbers of bacteria are two to three times lower than those excreted by clinically affected pigs, so this procedure may be sufficiently sensitive to detect a proportion of asymptomatic carrier animals. However, high sensitivity of detection of intestinal spirochaetes has been obtained under experimental conditions, or in samples known to be positive, and no reports of the limits of detection of intestinal spirochaetes from field samples are found in the literature. Under the conditions of this study, IMS (performed with washing) followed by culture or PCR offered no improvement in selectivity or sensitivity of detection of intestinal spirochaetes in pig faeces. It is possible that further refinement of IMS will improve its performance for detection of intestinal spirochaetes, but this will require further optimization.

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