Evaluation of selective media for the isolation of *Brachyspira aalborgi* from human faeces

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The purposes of this study were to identify a solid medium that supports improved growth of the anaerobic intestinal spirochaete *Brachyspira aalborgi*, to modify this for use as a selective isolation medium and then to test the medium for its effectiveness in isolating *B. aalborgi* from patients' faeces. Of the media evaluated, brain heart infusion agar (BHIA) with 10 % bovine blood (BB) was the most effective base–supplement combination for growth, with colonies attaining 1.2 mm in diameter by 21 days. Incubation in an anaerobic jar (94 % H₂, 6 % CO₂) permitted growth of larger colonies than incubation in an anaerobic chamber (80 % N₂, 10 % H₂, 10 % CO₂). Growth was improved only slightly at 38.5 °C compared with 37 °C. Selection of *B. aalborgi* from artificially seeded faeces was achieved equally well on eight different solid media containing spectinomycin (400 µg ml⁻¹) alone or in combinations with polymyxin B (5 µg ml⁻¹), colistin (25 µg ml⁻¹) and rifampicin (12.5 µg ml⁻¹). By using BHIA 10 % BB with spectinomycin plus polymyxin B, *B. aalborgi* was isolated from one of five human faecal samples that were positive for *B. aalborgi* by PCR amplification. This is the first report of the isolation of *B. aalborgi* from human faeces.

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INTRODUCTION

The anaerobic intestinal spirochaetes *Brachyspira aalborgi* and *Brachyspira pilosicoli* both colonize the large intestine of humans (Hovind-Hougen *et al.*, 1982; Trivett-Moore *et al.*, 1998). Both have also been associated with a condition called 'intestinal spirochaetosis', in which spirochaetes may be found attached by one cell end to the colorectal epithelium, forming a 'false brush border' (Harland & Lee, 1967; Mikosza & Hampson, 2001). Extensive colonization has been reported to result in a variety of intestinal disturbances, including chronic diarrhoea, abdominal pain and rectal bleeding (Gad *et al.*, 1977; Crucioli & Busuttil, 1981; Douglas & Crucioli, 1981; Padmanabhan *et al.*, 1996; Peghini *et al.*, 2000).

Of the two spirochaete species, *B. aalborgi* is the more slowly growing and fastidious, requiring at least 2–3 weeks for isolation (Hovind-Hougen *et al.*, 1982). Consequently, *B. aalborgi* has been relatively poorly studied and detection has relied on the use of PCR amplification of *B. aalborgi* DNA from colorectal biopsies (Mikosza *et al.*, 1999, 2001a; Kraaz *et al.*, 2000) or faeces (Mikosza *et al.*, 2001b). Isolation of *B. aalborgi* has been reported only three times, in each case following culture of fresh colonic biopsy specimens taken

from Scandinavian patients (Hovind-Hougen *et al.*, 1982; Kraaz *et al.*, 2000; Jensen *et al.*, 2001). The isolation medium in these studies was tryptose soy agar supplemented with 10 % bovine blood (BB), containing 400 µg spectinomycin and 5 µg polymyxin ml⁻¹. To date, *B. aalborgi* has not been isolated from human faeces, although the organism was recently isolated from the faeces of captive non-human primates (Munshi *et al.*, 2003). The lack of isolates has hampered comprehensive characterization and pathogenicity studies with this organism.

The purposes of the current study were to identify a solid medium that enhanced the growth of *B. aalborgi*, to modify this as an improved isolation medium and then to use it to isolate *B. aalborgi* from the faeces of human patients.

METHODS

Strategy for media development. The strategy followed was to test two laboratory-adapted reference strains of *B. aalborgi* for their growth on a range of agar bases, initially supplemented with 10 % BB as for the original isolation medium (Hovind-Hougen *et al.*, 1982). The base that supported the best growth then was tested with different primary supplements (blood) and the optimal combination was then tested with different secondary supplements and under different environmental conditions. To make the medium selective for *B. aalborgi*, a range of potentially inhibitory substances was evaluated, using human faeces

Abbreviations: BB, bovine blood; BHIA, brain heart infusion agar.

seeded with the *B. aalborgi* strains. Finally, the optimized isolation medium was tested on the faeces of human patients.

Reference strains and preparation of inocula. *B. aalborgi* strains 513A^T (=ATCC 43994^T) (Hovind-Hougen *et al.*, 1982) and W1 (Kraaz *et al.*, 2000) were obtained from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. The reference strains were routinely cultured on Columbia base agar containing 6 % equine blood (BA), incubated for 10 days at 37 °C in an anaerobic jar with an atmosphere of 94 % H₂ and 6 % CO₂ generated with a Gaspak Plus envelope (BBL). For media assessment, spirochaete growth was resuspended in PBS or Brucella broth using a sterile cotton-tipped swab and the suspension was returned to anaerobic conditions until inoculation. The organisms were counted in a Helber counting chamber, the concentration was adjusted to 1 × 10⁸ organisms ml⁻¹ and their viability was assessed by inoculation on BA using the spread plate method. Volumes of 50 µl were added to plates and the suspensions were streaked for single colonies in a standard manner.

Growth media. The following agar bases, all acquired from Oxoid, were used in the study: brain heart infusion agar (BHIA), Columbia base agar, Brucella base agar plus vitamin K (1 mg ml⁻¹) and haemin (5 mg ml⁻¹), Wilkins–Chalgren agar and anaerobe basal agar. In addition, trypticase soy agar (TSA) from Becton Dickinson was used by itself and as a base for a modification of Kunkle’s broth medium (Kunkle *et al.*, 1986). The latter contained 2 % fetal calf serum (FCS), 1.5 % alcoholic cholesterol solution, 1 % (w/v) yeast extract, 0.5 % (w/v) glucose, 0.2 % (w/v) NaHCO₃ and 0.05 % (w/v) cysteine hydrochloride monohydrate. Primary supplements that were tested with each of the agar bases were 5 and 10 % ovine blood (OB), 10 % BB and 10 % defibrinated BB. Secondary supplements were 2 % FCS, 1.5 % ethanolic cholesterol solution and 5 % pig faeces extract. The latter was prepared by emulsifying one part faeces obtained from normal adult pigs that had not received antimicrobials in four parts sterile PBS, stirring the emulsion for 24 h at 4 °C and then centrifuging for 1 h at 9950 g at 4 °C. The supernatant was stored at –20 °C until required and filter-sterilized (450 nm) before being added to agar.

Additional parameters assessed included 30 min pre-reduction of the plates prior to inoculation and incubation either in an anaerobic jar using the Gaspak Plus system or in an anaerobic chamber (80 % N₂, 10 % H₂, 10 % CO₂) (Don Whitley Scientific). Growth in anaerobic jars was compared at 37, 38.5 and 42 °C. The influence of pH was assessed by adding 4 ml 10 mM KCl to 2 ml set TSA 10 % BB (Carson *et al.*, 2000) and the pH was measured after 30 min incubation at room temperature. BA was used as a control medium in all tests.

Isolation media. The susceptibility of *B. aalborgi* strains to a range of antimicrobials for potential use as selective agents was determined with Microring MWAC and MWAN discs (Medical Wire & Equipment). The selective compounds and their disc strengths are listed in Table 1. In addition, spectinomycin (S), vancomycin (V), colistin (C), rifampicin (R) and polymyxin B (P) (Sigma) were incorporated directly into the media. Susceptibility to bile (dried ox bile, pure for microbiology; Merck) was assessed by making bile discs containing 5, 10 or 15 mg bile (Weinberg *et al.*, 1983) and placing these on BA swabbed with the suspensions.

The selective capacity of the media was examined by adding 1 ml bacterial suspension (10⁸ cells) to 1 g faeces from a healthy donor who had been confirmed free of *B. aalborgi* and *B. pilosicoli* by faecal PCR. The suspension was mixed thoroughly and 10 µl was added to a plate with a standard loop and streaked as above. Selection of *B. aalborgi* from seeded faeces was tested on TSA 10 % BB with all combinations of spectinomycin (400 µg ml⁻¹), vancomycin (25 µg ml⁻¹) and colistin (25 µg ml⁻¹). Additionally, spectinomycin (400 µg ml⁻¹) plus all combinations of polymyxin B (5 µg ml⁻¹), rifampicin (12.5 µg ml⁻¹)

Table 1. Compounds tested for use in development of selective media for *B. aalborgi*

Compound	Concentration
Added to agar	
Spectinomycin	400 µg ml ⁻¹
Vancomycin	25 µg ml ⁻¹
Colistin	25 µg ml ⁻¹
Polymyxin B	5 µg ml ⁻¹
Rifampicin	12.5 µg ml ⁻¹
Discs*	
Ox bile ^a	5, 10 and 15 mg
Novobiocin ^b	5 µg
Erythromycin ^c	60 µg
Kanamycin ^c	1000 µg
Colisitin ^c	10 µg
Metronidazole ^b	5 µg
Penicillin G ^c	2 IU
Rifampicin ^c	15 µg
Sodium polyethol sulfonate ^b	1 mg
Vancomycin ^c	5 µg

*Compounds were tested as discs prepared in-house (a) or were contained in Microring MWAC (b) or Microring MWAN (c) discs in the amounts shown.

and colistin (25 µg ml⁻¹) was tested. The lower limit of selection of *B. aalborgi* from seeded faeces on spectinomycin plus polymyxin B (SP) was determined by performing 10-fold dilutions on the bacterial suspension. Faeces were seeded, mixed and streaked as above.

Both growth and isolation media were examined for *B. aalborgi* after 10, 14, 18 and 21 days incubation. Growth was assessed by recording colony size and scoring the extent of growth along streaks on the test plates. A grading method was developed where the best medium was scored ‘1’ at a given time-point, the next best medium ‘2’ and so on, and a mean was determined for each medium after 21 days. All procedures were repeated at least once.

Isolation. The efficacy of the best medium was tested by attempting isolation of *B. aalborgi* from fresh faecal samples from 60 apparently healthy individuals taking part in a study examining carriage of intestinal spirochaetes, using faecal PCR as described by Mikosza *et al.* (2001b). Five of these individuals had positive faecal PCRs for *B. aalborgi* (data not shown). Isolation of *B. aalborgi* was attempted on BHIA 10 % BB SP, incubated in an anaerobic jar at 37 °C for 21 days before being exposed to oxygen. Spirochaete growth was subcultured to BA and colonial and phase-contrast morphologies were recorded. For electron microscopy, the cultured spirochaetes were scraped from the plates and resuspended in distilled water. Drops of the suspensions were placed on Formvar-coated reinforced grids and stained with phosphotungstic acid (3 %, pH 7.2). Grids were examined in a Philips CM100 Biotwin transmission electron microscope.

Spirochaete identity was confirmed by adding cells directly to PCRs designed to amplify portions of the 16S rRNA genes of *B. aalborgi* or *B. pilosicoli* (Mikosza *et al.*, 1999, 2001a). Cycling for *B. aalborgi* was carried out at an annealing temperature of 48 °C. The PCR product was sequenced in both directions using a commercially available cycle sequencing kit (ABI PRISM BigDye dye terminator cycle sequencing ready reaction kit, version 1.0; Applied Biosystems), according to the manufacturer’s instructions. The sequence was analysed on the ABI

PRISM 310 Genetic Analyzer (Applied Biosystems). The sequence data obtained were aligned and compared with 16S rDNA sequences of the *B. aalborgi* and *B. pilosicoli* type strains by BLAST search with the National Centre for Biotechnology Information server at the National Library of Medicine. Multiple sequence alignments were performed by the CLUSTAL method using BioManager with the Angis server.

RESULTS

Growth media

The most effective agar base for *B. aalborgi* growth was BHIA with 10 % BB. After 21 days, colonies on this medium were 1.2 mm in diameter, light grey and weakly β -haemolytic. A diameter of 1 mm was attained after 14 days incubation. The next best growth was on TSA with 10 % BB, on which colonies only reached 0.8 mm diameter and had a transparent appearance. All the other bases supported growth, but colonies were small and slow to appear.

As a supplement, 10 % BB was superior to 10 % OB, which, in turn, allowed larger colonies and faster growth than 5 % OB, where a maximum size of only 0.5 mm was achieved after 21 days. Additional supplements of 2 % FCS, 1.5 % cholesterol and 5 % pig faecal extract offered no growth advantage. Defibrination of BB or pre-reduction of the media did not improve growth of *B. aalborgi*. Growth of *B. aalborgi* did not occur at 42 °C. Colonies on BHIA incubated at 38.5 °C were at best only 0.2 mm larger at some time-points than colonies on BHIA incubated at 37 °C. The best pH for growth of *B. aalborgi* on TSA 10 % BB was pH 7. Growth to pH 8 was satisfactory, but agar below pH 6.5 would not withstand prolonged incubation. The pH of BHIA 10 % BB was 7.6. For all media, incubation in an anaerobic jar resulted in growth of larger colonies, by 0.2–0.5 mm, compared with incubation in an anaerobic chamber.

Selective media

Both reference strains of *B. aalborgi* were susceptible to bile, metronidazole, penicillin G, kanamycin and vancomycin and resistant to spectinomycin, polymyxin B, colistin, erythromycin and rifampicin. Equivocal results were obtained for sodium polyethol sulfonate and novobiocin for strain W1, while strain 513A^T was susceptible to these compounds. On media with antimicrobials incorporated, clear growth of *B. aalborgi* occurred on all plates except those containing vancomycin, although colonies were frequently 0.2 mm smaller than on the medium without antimicrobials. Selection of *B. aalborgi* from seeded faeces was achieved on media containing S, SP, SC, SR, SRC, SPR, SPC and SRPC, and no single medium produced consistently superior growth. The lower limit of detection of *B. aalborgi* from seeded faeces on SP was around 200 c.f.u. per 10 μ l seeded faeces or 2×10^4 c.f.u. g⁻¹.

Spirochaetes were isolated from one of the five faecal samples that were previously shown by PCR to contain *B. aalborgi*. After 21 days incubation, two types of weakly β -haemolytic colonies were seen on BHIA 10 % BB: these were grey,

spreading colonies of 1.2 mm diameter with a raised, pinpoint centre inside an irregular flat edge (type A) or smooth, pinpoint colonies (< 0.1 mm diameter) (type B). On subculture, a mixture of the two colony types persisted; however, the type A colonies predominated.

PCR and sequencing

PCR of both colony types from the primary isolation plate confirmed their identity as *B. aalborgi*. A band of 472 bp corresponding to the expected product size in the *B. aalborgi* 16S rRNA PCR was obtained, while no band was obtained in a 16S rRNA PCR for *B. pilosicoli*. Sequencing of the PCR product from each colony type showed 99 % similarity with the sequences for *B. aalborgi* 513^T, W1 and nine of the uncultured *Brachyspira* isolates sequenced by Pettersson *et al.* (2000). For each colony type, polymorphisms occurred at the base equivalent to position 416 in *Escherichia coli* (Brosius *et al.*, 1978), where a guanidine residue was replaced by thymidine, and at the base equivalent to position 436, where thymidine was replaced by cytosine.

Phase-contrast and electron-microscope morphology

Under the phase-contrast microscope, the spirochaetes appeared as short, helical cells with a pronounced flexuous motility. Cells from the two colony types had a similar appearance. Electron microscopy revealed two cell types. One was a plump cell type, varying from 2.0 to 6.0 μ m long and about 250 nm wide. Cells were typically 'S'- or comma-shaped with a 2 μ m wavelength. The other cell type was similar in length, shape and amplitude, but was only approximately 120–130 nm in width (Fig. 1). A mixture of cell types was observed in the large, grey, spreading colonies, whilst only the thin cell type was observed in the pinpoint colonies. Both cell types had four periplasmic flagella inserted in a row at each end of the cell.

DISCUSSION

The overall objectives of this study were attained, in that a selective medium was developed and used to isolate *B. aalborgi* from a human faecal sample. This is the first time that this has been achieved, with all isolates previously obtained having been grown directly from biopsy samples. Intestinal biopsies are useful for investigating individual cases of intestinal spirochaetosis, since they can be used to link the presence of spirochaetes to specific pathological changes. Nevertheless, taking biopsies is an invasive process that is only undertaken routinely in developed countries. Collection and examination of faeces for studies on the presence and disease associations of *B. aalborgi* is easier, less expensive and more acceptable to patients. Although the organism can be detected in faeces using PCR (Mikosza *et al.*, 2001b), the ability to culture *B. aalborgi* will permit future characterization of the isolates and facilitate molecular epi-

Fig. 1. Electron micrograph showing the two spirochaete cell types isolated from the faeces of a human volunteer. The larger spirochaete cell is 5 µm long.

demology (strain typing) and pathogenicity studies. Hence, the development of an improved selective medium for the isolation of *B. aalborgi* from human faeces is a useful advance.

The best base medium for growth of *B. aalborgi* was BHIA, supplemented with 10 % BB, whilst selection of *B. aalborgi* from seeded faeces was achieved with media containing S, SC, SP, SR, SRC, SPR, SPC or SRPC. The medium used in previous isolations of *B. aalborgi* was TSA 10 % BB SP (Hovind-Hougen *et al.*, 1982; Kraaz *et al.*, 2000; Jensen *et al.*, 2001). As the performance of these combinations appeared equal in this study, SP was chosen because of the history of successful isolation in previous studies. In this investigation, BHIA 10 % BB supported better growth than TSA 10 % BB, and it is possible that the additional nutrients provided by the BHIA allowed the *B. aalborgi* strain that was isolated in this study to out-compete the other gut microbiota on the selective plate.

TSA with 5 % OB, S (400 µg ml⁻¹), V (25 µg ml⁻¹) and C (25 µg ml⁻¹) has been used to isolate *B. pilosicoli* from human faeces (Lee & Hampson, 1992). Results from the current study indicate that this medium does not support the growth of *B. aalborgi*, presumably as a result of the strains being susceptible to vancomycin. Poor growth of *B. aalborgi* W1 on a very similar medium has been reported previously (Kraaz *et al.*, 2000). These results indicate that studies in which TSA 5 % OB SVC is used to isolate human intestinal spirochaetes are likely to fail to isolate *B. aalborgi*.

Pre-reduction of media did not influence growth of *B. aalborgi*; however, tests were carried out on laboratory-adapted strains. It is possible that pre-reduction of media might have allowed the isolation of other strains in the

samples tested. Likewise, growth of these strains was barely improved at 38.5 °C, despite a previous report of optimal growth at this higher temperature (Hovind-Hougen *et al.*, 1982). This may again be due to the laboratory adaptation of strains, or the difference observed by Hovind-Hougen *et al.* (1982) was possibly more marked because of the use of TSA as the base medium. The superior growth achieved in the anaerobic jars compared with an anaerobic chamber was unexpected. Humidity levels tended to be higher in jars, and the gas composition differed between the two atmospheres. In particular, anaerobic jars may contain traces of oxygen, and it is known that the growth of *Brachyspira hyodysenteriae* is enhanced by the presence of 1 % oxygen, associated with the production of NADH oxidase (Stanton & Lebo, 1988; Stanton, 1989). *B. aalborgi* also has a gene encoding NADH oxidase (Mikosza *et al.*, 1999). It is possible that some or all of these factors may have impacted upon growth of *B. aalborgi*. It is also likely that these organisms are quite sensitive to changes in the environment, as it was noted that growth of *B. aalborgi* was inhibited in the presence of large numbers of contaminating organisms.

Antimicrobial susceptibilities of *B. aalborgi* were not determined, as growth of the organism was poor and inconsistent on the NCCLS-recommended media for testing anaerobes (Summanen *et al.*, 1993). Results of the disc sensitivity tests did not elicit any more compounds that were considered useful for selective media, as *B. aalborgi* was susceptible to most compounds tested. Resistance to colistin and rifampicin was already suspected and was thus utilized in this study, and erythromycin is not suitable for use under anaerobic conditions. Demonstration of metronidazole susceptibility at 5 µg per disc corresponded to reports of the successful use of this agent in a clinical setting, and this result confirms its role as the drug of choice in intestinal spirochaetosis (Peghini *et al.*, 2000; Heine *et al.*, 2001).

In common with previous descriptions (Hovind-Hougen *et al.*, 1982; Kraaz *et al.*, 2000), two colony types grew from the positive faecal sample. The colony types were similar to those described previously, and the size and morphology of one of the isolates was consistent with previous descriptions of *B. aalborgi* (Hovind-Hougen *et al.*, 1982; Kraaz *et al.*, 2000; Jensen *et al.*, 2001). Sequencing of PCR products from the two colony types gave identical 16S rDNA sequences, suggesting that the two types were identical in this region. An unusual feature, however, was the identification of some cells that were much thinner by electron microscopy than the 'typical' cells. The smaller colony types appeared to be made up of these thinner types, although they were also present in the larger colonies. The differences in cell size might have been an artifact, the result of the presence of two distinct types of organism, or might reflect a genuine difference in the phenotype of a given strain. These possibilities require further investigation.

Isolation of *B. aalborgi* was achieved from only one of five PCR-positive faecal samples. The lower limit of selection from seeded faeces was quite poor, at 2×10^4 c.f.u. (g

faeces)⁻¹, but was comparable to that described for faecal PCR (Mikosza *et al.*, 2001b). While the remainder of these samples may have contained non-viable organisms that therefore were only detected by PCR, these results suggest that further modifications may be required to optimize media and conditions for isolation of all *B. aalborgi*. The individual from whom the *B. aalborgi* isolate was obtained was self-diagnosed as having 'chronic diarrhoea', but was unfortunately lost to follow-up because he moved overseas shortly after the faecal sample was submitted. A faecal sample from his female partner was PCR- and culture-negative for *B. aalborgi*. The availability of an improved isolation medium should now facilitate further epidemiological studies on the possible role of this spirochaete in human colorectal disease.

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NOTE ADDED IN PROOF

After this paper was accepted for publication, another paper describing the isolation of *Brachyspira aalborgi* from human faeces was published (Calderaro *et al.*, 2003).

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