Variation amongst human isolates of *Brachyspira* (*Serpulina*) *pilosicoli* based on biochemical characterization and 16S rRNA gene sequencing

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Brachyspira pilosicoli* (formerly *Serpulina pilosicoli*) causes swine spirochaetosis and can also be isolated from human faeces, although its role in human disease remains unclear. The genetic and biochemical variations amongst 19 isolates of human spirochaetes from five different countries were evaluated and compared to those found amongst swine isolates of *B. pilosicoli*. All isolates were negative for *β*-glucosidase and all but one were positive for hippurate hydrolysis, which are characteristics typical of *B. pilosicoli*. The isolates showed variation in indole production and *α*-galactosidase and *α*-glucosidase activity, other characteristics which can be used to identify *B. pilosicoli*. The DNA sequences of part of the 16S rRNA gene differed from each other and from that of *B. pilosicoli* by 0–3 bp out of 283 bp. It is concluded that there is considerable variation amongst human intestinal spirochaetes. Since few of the isolates reported here match the current criteria for *B. pilosicoli*, it is concluded that this species is more heterogeneous than previously appreciated. However, it cannot be excluded that some isolates may belong to uncharacterized related *Brachyspira/Serpulina* species.

Keywords: human intestinal spirochaete, *Brachyspira pilosicoli*, 16S rRNA analysis

INTRODUCTION

Although both man and animals have been known to harbour intestinal spirochaetes for over a century, their taxonomic divisions and pathological significance have only recently been addressed. At present, there is evidence that there are at least two species of human intestinal spirochaetes: *Brachyspira pilosicoli* (formerly *Serpulina pilosicoli*; Validation List No. 64, 1998) and the rarely reported and human-specific *Brachyspira aalborgi* (Trott *et al.*, 1996c; Hovind-Hougen *et al.*, 1982).

Isolates of *Brachyspira/Serpulina* obtained from swine faeces have received more attention in the last few years, and the number of species in this genus has been extended from two to five (Trott *et al.*, 1996a; Stanton *et al.*, 1997). *Brachyspira hyodysenteriae* (formerly *Serpulina hyodysenteriae*; Validation List No. 64, 1998) is the cause of swine dysentery (Taylor & Alexander, 1971). *Brachyspira innocens* (formerly *Serpulina innocens*; Validation List No. 64, 1998) is closely related, but not pathogenic. The recently described *B. pilosicoli* can cause intestinal spirochaetosis in pigs (Trott *et al.*, 1996b; Muniappa *et al.*, 1997; Thomson *et al.*, 1997). The pathogenic potential of the recently described *Serpulina intermedia* and *Serpulina murdochii* remains largely unknown. A simple biochemical classification has recently been described for swine isolates, dividing *Brachyspira/Serpulina* in six groups that represent the five species mentioned and a sixth, unnamed group (Fellström & Gunnarson, 1995; Stanton *et al.*, 1997).

The phylogeny of human intestinal spirochaetes has been studied using multilocus enzyme electrophoresis (MEE) (Lee & Hampson, 1994), PAGE (Barrett *et al.*,

1996) and PFGE (Atyeo *et al.*, 1996; Rayment *et al.*, 1997). These techniques revealed considerable heterogeneity amongst human isolates, but the isolates fell into a distinct group (Lee & Hampson, 1994; Barrett *et al.*, 1996). This heterogeneous group also contained isolates from swine and other animals, including strain P43/6/78^T, the type strain of *B. pilosicoli* (Stanton *et al.*, 1996; Lee & Hampson, 1994). Although these techniques are useful for epidemiology, they cannot be used to assign species status to the human intestinal spirochaetes. Further evidence that human intestinal spirochaetes are *B. pilosicoli* comes from immunoblotting with a species-specific monoclonal antibody (Rayment *et al.*, 1997), species-specific PCR (Park *et al.*, 1995; Trott *et al.*, 1997) and partial 16S rDNA sequencing of two isolates (Stanton *et al.*, 1996). Biochemical analysis of three human intestinal spirochaetes and seven spirochaetes isolated from human blood identified them as *B. pilosicoli* and it has been suggested that blood isolates may have originated in the intestinal tract (Trott *et al.*, 1996c, 1997).

This study was initiated to further compare human intestinal spirochaetes to swine isolates of *B. pilosicoli* and to assess the genetic and biochemical variability of human isolates of *B. pilosicoli* from five different countries.

METHODS

Isolates and culture. The isolates of spirochaetes from faecal cultures of patients in Australia, Britain, Italy and Oman, and from blood cultures of patients in France have been described previously (Table 1) (Lee & Hampson, 1992; Barrett, 1990; Sanna *et al.*, 1982; Trott *et al.*, 1997). They were stored at $-70\ ^\circ$C in 10% (w/v) glycerol broth and cultured anaerobically at 37 $^\circ$C on Columbia agar base with 5% (v/v) sheep blood. Subculture onto fresh agar was performed after 7 d and the organisms were harvested after a further 2 d incubation.

Phenotypic characterization. Hippurate hydrolysis and indole production were performed as described previously (Fellström & Gunnarson, 1995). β-Haemolysis was assessed on the plates used for culture. α-Galactosidase, α-glucosidase and β-glucosidase were tested and scored using the API-ZYM kit according to manufacturer's instructions (Marcy-l'Etoile). All assays were compared with results obtained using *B. hyodysenteriae* B78^T and *B. innocens* B256^T as reference strains.

Lysis of bacteria and PCR. A loopful of bacteria was suspended in 500 µl 10 mM Tris/HCl buffer pH 8·0 plus 0·1 mM EDTA. The bacteria were collected by centrifugation and the supernatant was discarded. Chloroform (100 µl) was added and the bacteria were resuspended by vigorous vortexing. An equal volume of water was added and the suspension was mixed. After centrifugation to separate the phases, the aqueous phase was recovered and again extracted with chloroform. The resulting aqueous phase was used for PCR.

The primers 285 (sequence 5′ GAGAGTTTGATCCTGG-CTCAG 3′) and 244 (5′ CCCACTGCTGCCTCCCGTAG 3′) (Kirschner *et al.*, 1993; De Smet *et al.*, 1995) amplify around 360 bp of the 5′ end of the 16S rRNA gene. DNA present in 1 µl lysate was amplified in a total volume of 50 µl containing 0·5 µM of each primer, 200 µM of each nucleotide, 5 µl 10× buffer and 2·5 U *Taq* polymerase, (Gibco/BRL) covered by one drop of mineral oil. The DNA was amplified with two cycles of 96 $^\circ$C for 2·5 min, 55 $^\circ$C for 30 s and 72 $^\circ$C for 1 min, followed by 35 cycles of 95 $^\circ$C for 1 min, 55 $^\circ$C for 30 s and 72 $^\circ$C for 1 min, and one cycle of 72 $^\circ$C for 10 min. Blank controls (samples without DNA) were included throughout.

DNA sequencing. The whole PCR sample was run on a 1·5% (w/v) agarose gel to remove primers, the appropriate band was cut out with a scalpel and purified using the QIAEX-II kit (Qiagen) according to the manufacturer's instructions. A quarter of the 20 µl purified PCR product was used for dye terminator fluorescence sequencing by mixing with 1 µl primer (either 285 or 244) and 4 µl ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction mix with AmpliTaq FS (PE Applied Biosystems), and covered with one drop of mineral oil. This was then cycled through 30 cycles of 95 $^\circ$C for 1 min, 55 $^\circ$C for 30 s and 72 $^\circ$C for 1 min. The sequencing product was diluted to 50 µl, precipitated, washed with 70% (v/v) ethanol, dried and dissolved in 12·5 µl Template Suppression Reagent (PE Applied Biosystems). After heating at 95 $^\circ$C for 5 min, the samples were loaded in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) and run according to the manufacturer's instructions.

DNA sequence analysis. All computer analyses were performed using the GCG Wisconsin Sequence Analysis Package, version 8.1.0 (Genetics Computer Group, WI, USA). Homologous sequences were identified by searching the GenBank or EMBL databases using the FASTA program. Retrieved sequences were aligned using PILEUP, which creates a multiple sequence alignment using progressive, pairwise alignments. Default values of the program were used. Pairwise homologies between the aligned sequences were calculated with the DISTANCES program using the uncorrected distances method, with a penalty of 1·0 for each gap and ignoring any ambiguities.

RESULTS

Phenotypic characterization

All 19 isolates were analysed for haemolysis, indole production, hippurate hydrolysis and α-galactosidase, α-glucosidase and β-glucosidase activities; results were compared with an existing scheme for classification of *Brachyspira*/*Serpulina* species (Fellström & Gunnarson, 1995) (Table 1). All of the human intestinal spirochaetes were weakly haemolytic. All but one were positive for hippurate hydrolysis and all were negative for β-glucosidase activity, two characteristics reported to be unique to *B. pilosicoli* (Fellström & Gunnarson, 1995). There was more variation in the results for the other assays, with over half the isolates differing in one to three characteristics from the proposed *B. pilosicoli* scheme. Over half the isolates had weak α-galactosidase activity. None of the isolates fell into any of the other groups proposed for the other species in the genus (Fellström & Gunnarson, 1995). All five countries provided isolates with aberrant biochemical characteristics.

Table 1. Isolates and their characteristics

Biochemical and partial 16S rDNA sequence of the first 19 isolates were determined in this study. −, No activity; ±, weak activity; and +, strong activity. The biochemical data or 16S rDNA sequence of the other isolates were obtained from published work or the GenBank or EMBL DNA databases. References refer to the description of the isolate for those used in this study, or the published biochemical characteristics for other isolates mentioned. Species assignation for published sequences is as described in the database entries or the corresponding paper. Haem, haemolysis; Ind, indole; Hip, hippurate; α-Gal, α-galactosidase; α-Glc, α-glucosidase; β-Glc, β-glucosidase.

Isolate	Clinical symptoms/comments	Country	Haem	Ind	Hip	α-Gal	α-Glc	β-Glc	Sequence	Species	Accession no.	Reference
57		Oman	Weak	−	+	±	−	−	IS-A		Y10314	Barrett *et al.* (1996)
91	Old paralysis	Oman	Weak	+	+	±	−	−	IS-A			Barrett *et al.* (1996)
108	None apparent	Oman	Weak	+	+	±	−	−	IS-A			Barrett *et al.* (1996)
HRM-7	Carcinoma of liver	Italy	Weak	−	+	−	−	−	IS-A			Sanna *et al.* (1982)
81-80	Septicaemia, fever 40·9 °C, stroke, hemiplegia, death	France	Weak	−	+	+	+	−	IS-A			Trott *et al.* (1997)
28-94	Septicaemia	France	Weak	−	+	+	−	−	IS-A			Trott *et al.* (1997)
128-90	Septicaemia, acute peritonitis, necrosis of caecum, recovered	France	Weak	−	+	−	−	−	IS-A			Trott *et al.* (1997)
Gel	Diarrhoea	UK	Weak	−	−	±	−	−	IS-A			Unpublished
GOR	Diarrhoea; HIV+	UK	Weak	−	+	±	−	−	IS-A			Unpublished
Matthew	Diarrhoea	Australia	Weak	−	+	±	−	−	IS-A			Lee & Hampson (1992)
Karlos	Diarrhoea	Australia	Weak	+	+	±	−	−	IS-A			Lee & Hampson (1992)
54	Epigastric pain, vomiting, diarrhoea	Oman	Weak	−	+	±	−	−	IS-B		Y10315	Barrett *et al.* (1996)
85	Acute pain and vomiting	Oman	Weak	−	+	+	−	−	IS-C		Y10316	Barrett *et al.* (1996)
89	Acute pain for one day	Oman	Weak	+	+	−	−	−	IS-D		Y10317	Barrett *et al.* (1996)
HPR-1	Diarrhoea, abdominal pain	Italy	Weak	−	+	−	−	−	IS-D			Sanna *et al.* (1982)
382-91	Septicaemia, alcoholic, myeloma	France	Weak	−	+	±	−	−	IS-D			Trott *et al.* (1997)
FT-7		UK	Weak	−	+	±	−	−	IS-D			Unpublished
Steptoe	Rectal bleeding; HIV+	UK	Weak	−	+	±	−	−	IS-D			Unpublished
Jeremiah	Diarrhoea	Australia	Weak	+	+	−	+	−	IS-D			Lee & Hampson (1992)
N HRM7	Carcinoma of liver									*B. pilosicoli*	Z26968; U23031	
N-Wes-B										*B. pilosicoli*	Z26967; U23034	
P43/6/78ᵀ	Type strain, diarrhoeic pig	Scotland	Weak	−	+	+	−	−	*B. pilosicoli*	U14927; U23032	Fellström & Gunnarsson (1995)
B78ᵀ	Type strain		Strong	+	−	−	+	+	*B. hyodysenteriae*	U14930; M57743	Fellström & Gunnarsson (1995)
B256ᵀ	Type strain		Weak	−	−	+	+	+	*B. innocens*	U14920; M57744	Fellström & Gunnarsson (1995)
PWS/Aᵀ	Type strain, pig isolate		Weak	+	−	−	+	+	*S. intermedia*	U23033	Trott *et al.* (1996c)
56-150ᵀ	Type strain		Weak	−	−	−	−	+	*S. murdochii*		Stanton *et al.* (1997)
155-20									*S. murdochii*	U22838	Stanton *et al.* (1997)
NCTC 11492ᵀ	Type strain								*B. aalborgi*	Z22781	

Table 2. Difference matrix for the 16S rRNA gene of selected *Brachyspira/Serpulina* sequences

The numbers to the right and above the diagonal represent the number of different nucleotides in the 283 bp region analysed in this study. The values to the left and underneath the diagonal represent the percentage difference for published 16S rDNA sequences over a region of 1400 bp.

	IS-A	*B. pilosicoli*	IS-B	IS-C	IS-D	*B. innocens*	*B. hyodysenteriae*	*S. murdochii*	*S. intermedia*	*B. aalborghi*
IS-A	−	0	1	2	1	12	10	15	12	22
B. pilosicoli		−	1	2	1	15	13	18	15	25
IS-B			−	3	1	13	11	16	13	23
IS-C				−	1	12	10	15	12	22
IS-D					−	13	11	16	13	23
B. innocens		3·37%				−	2	7	8	14
B. hyodysenteriae		4·61%				2·78%	−	7	10	14
S. murdochii		3·07%				1·97%	3·80%	−	9	17
S. intermedia		2·34%				2·05%	3·29%	1·02%	−	19
B. aalborghi		5·05%				4·32%	5·64%	4·10%	3·95%	−

Partial sequencing of the 16S rRNA gene

The 5′ end of the 16S rRNA gene from 19 isolates of human intestinal spirochaetes was amplified using primers 285 and 244 (Kirschner *et al.*, 1993; De Smet *et al.*, 1995) to produce a PCR fragment of around 360 bp. This region contains 41% of all substitutions identified in the 16S rRNA genes of around 30 intestinal spirochaete isolates (Fellström *et al.*, 1995; Stanton *et al.*, 1996; Petterson *et al.*, 1996). These PCR products were sequenced on both strands, with the oligonucleotides used for amplification as the sequencing primers. A region of 283 bp was sequenced for all isolates. Sequence differences were confirmed by repeating the PCR and sequencing. This showed that the isolates were highly homologous, with 0–3 bp differences amongst them (Table 2).

Overall, four distinct sequences were obtained, which, for convenience, are identified as IS-A, IS-B, IS-C and
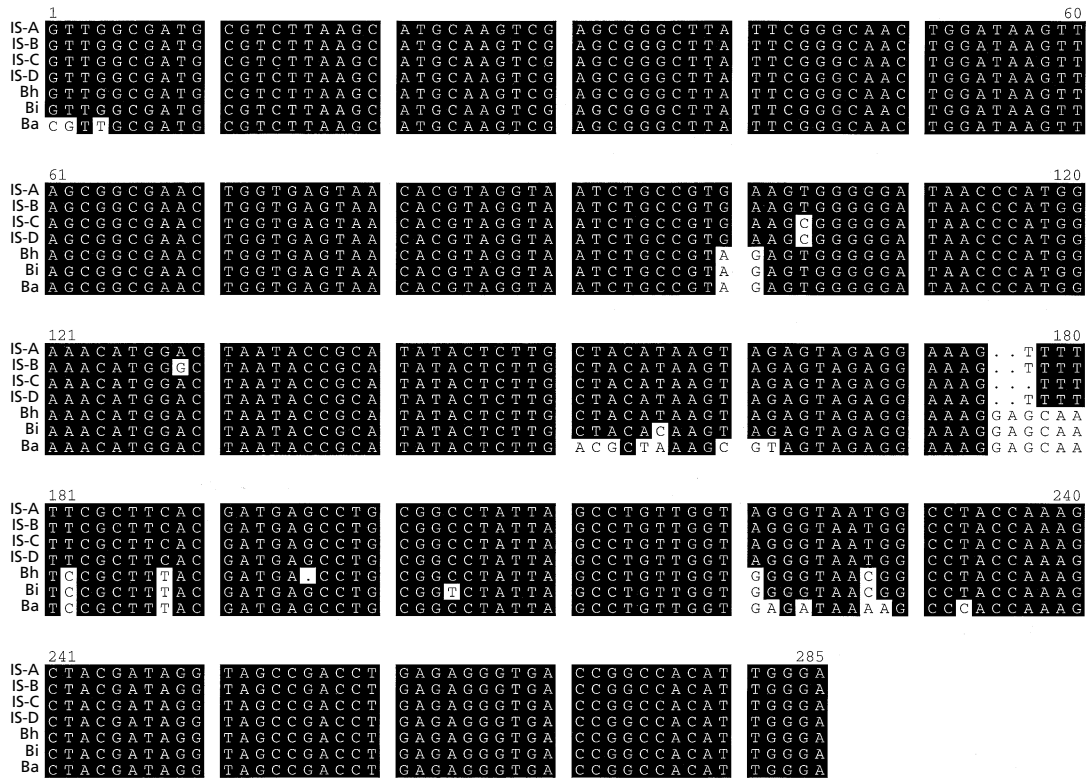
Fig. 1. Alignment of the IS-A, IS-B, IS-C and IS-D sequences with 16S rRNA sequence of *B. hyodysenteriae* (Bh; accession number U14930), *B. innocens* (Bi; accession number U14920) and *B. aalborghi* (Ba; accession number Z2278).

IS-D (IS, intestinal spirochaete) (Fig. 1). Isolates with sequence IS-A accounted for over half of all isolates (Table 1). Only one isolate was found with sequence IS-B and only one had IS-C. All countries provided isolates with sequences IS-A and IS-D. The single isolates with sequences IS-B and IS-C were both from Omani patients. Also, there was no correlation between DNA sequence and biochemical analysis. Samples with an aberrant result for the indole reaction, α-galactosidase or α-glucosidase had either an IS-A or IS-D sequence.

Computer analysis of sequences

The sequences obtained were submitted to the EMBL/GenBank/DDBJ databases (accession nos Y10314, Y10315, Y10316 and Y10317). Sequence database searches using the FASTA program revealed that the IS sequences showed highest homology to those of other intestinal spirochaetes belonging to the *Brachyspira/Serpulina* genus.

A perfect match was found between IS-A and three independent swine *Brachyspira/Serpulina* isolates (accession nos U14927, U14928 and U14929) (Petterson *et al.*, 1996). These three 16S rRNA gene sequences differed outside the 283 bp region analysed here. One of these isolates was the *B. pilosicoli* type strain P43/6/78[T]. The IS-A sequence was also identical to

the partial 16S rRNA gene sequences of the human isolate HRM-7, deposited in the database by J. V. Hookey and others (accession no. Z26968; unpublished data). However, it differed by 1 bp from the sequence submitted by Stanton *et al.* (1996) for the same isolate (accession no. U23031). Sequencing of isolate HRM-7 showed that it was identical to the Hookey sequence and thus belongs to the IS-A type.

Similarly, the IS-D sequence was identical to the partial 16S DNA sequences of the human intestinal isolate N-Wes-B, obtained by J. V. Hookey and others (accession no. Z26967; unpublished data), but differed by 3 bp from the sequence of Stanton *et al.* (1996) for the same isolate (accession no. U23034). There were no perfect matches for the IS-B and IS-C sequences.

All IS sequences differed by at least 10 bp from the other *Brachyspira/Serpulina* sequences, *B. hyodysenteriae*, *B. innocens*, *S. murdochii* and *S. intermedia* and other isolates not yet assigned to any formally recognized species (Fig. 1 and data not shown). All *Brachyspira/Serpulina* sequences differed from that of *B. aalborgi* by 14–25 bp.

DISCUSSION

Recent work has shown that isolates of human intestinal spirochaetes are heterogeneous, but closely related to or indistinguishable from *B. pilosicoli* (Lee &

Hampson, 1994; Trott *et al.*, 1996c; Atyeo *et al.*, 1996; Rayment *et al.*, 1997). Since biochemical analysis and 16S rDNA sequencing were only performed on a few isolates (Stanton *et al.*, 1996; Trott *et al.*, 1997), a larger collection of human intestinal spirochaetes was characterized in this study to confirm their species status and determine intra-species variability.

The biochemical typing presented here shows that all but one of the human isolates were positive for hippurate hydrolysis and negative for β-glucosidase activity, two characteristics unique to *B. pilosicoli*. There was more variation in the other biochemical characteristics, supporting the heterogeneity found using MEE, PAGE and PFGE. The majority of isolates had only weak α-galactosidase activity. It is not clear from published work if a weak or strong reaction is expected as only a positive or negative was indicated (Fellström & Gunnarsson, 1995; Trott *et al.*, 1996c, 1997). Biochemical variation was also found in analysis of the seven human bloodborne spirochaetes, four of which were re-analysed in this study (Trott *et al.*, 1997). This variation is in contrast to results on swine isolates, where 165 *Brachyspira/Serpulina* isolates could be placed into six groups, representing the five species and an unnamed group IIIb, without any apparent variation in biochemical characteristics (Fellström & Gunnarsson, 1995). Of these, 56 swine isolates matched the criteria for *B. pilosicoli*. In this study, only nine of the 19 human isolates fully matched the characteristics in the biochemical characterization criteria proposed for group IV, now known as *B. pilosicoli* (Fellström & Gunnarson, 1995). On the other hand, none of the isolates studied here matched any of the other groups proposed in this classification and were still most closely matched to *B. pilosicoli*. This discrepancy may be because the criteria were established using only swine isolates from one geographic area (Sweden), or because human spirochaete isolates represent a more heterogeneous population of *B. pilosicoli*.

Similarly, DNA sequencing of a variable region of the 16S rRNA gene revealed that human intestinal spirochaetes showed variation, but were still most closely related to each other and to *B. pilosicoli*. Seven swine isolates of *B. pilosicoli* did not show any variation in this region, although they had polymorphisms in other regions of the 16S rDNA sequence (Petterson *et al.*, 1996).

Further analysis of some of these isolates using PCR and Western blotting with a monoclonal antibody, both reported to be specific for *B. pilosicoli*, showed that 16 isolates out of 16 were positive by PCR and nine out of nine were positive by Western blotting (C. Bakatselou & S. P. Barrett, unpublished results; Rayment *et al.*, 1997). It is postulated that the considerable variation in the biochemical data and the variation of up to 3 bp in the 283 bp analysed, is simply intra-species variation. For comparison, there were up to 2 bp differences amongst isolates of *B. hyody-*

senteriae and up to 4 bp differences between different *B. innocens* isolates in the same region (Table 2; Fellström *et al.*, 1995; Petterson *et al.*, 1996). In an unrelated genus, no differences have been found in the same region for the pathogen *Mycobacterium tuberculosis*, but up to 3 bp differences were found for several other mycobacterial species causing opportunist infections (Kirschner *et al.*, 1993). However, it has to be borne in mind that this difference matrix is based on the comparison of only a relatively short sequence (283 bp) with very few informative nucleotides. The data in Table 2 highlight the limitation of this: the region analysed also showed only 2 bp differences between the published sequences of the type strains of *B. hyodysenteriae* and *B. innocens* (Table 2). Analysis of the almost complete published sequence of the 16S rRNA gene shows a different relationship, where different isolates of the same species group together, separated from other species (Petterson *et al.*, 1996). Based on all the current data, the most likely conclusion is that all 19 isolates belong to the same species, *B. pilosicoli*, although it cannot be excluded that some of them are other uncharacterized *Brachyspira/Serpulina* species. In any case, it has been clearly shown that the human isolates in this study form a distinct branch, including *B. pilosicoli*, represented by P43/6/78[T], that differs from other described species of *Brachyspira/Serpulina* or *B. aalborgi*.

Isolates were selected to represent different geographical origins. There was no correlation between geographical origin and the phenotypic variation or the sequence type of the isolates. IS-A and IS-B were found among isolates from all countries. The single isolates with sequence IS-B and IS-C were both from Omani patients. This may simply be because more isolates from Oman than from any other country were included in this study, or it may be that the high prevalence of intestinal spirochaetosis in Oman reflects the presence of multiple genetic types (Barrett, 1990).

It has previously been noted that *B. pilosicoli* contains a unique 'signature' sequence of 5′ AGTTTTTTCG-CTTCA 3′ in their 16S rDNA (173–189 bp; Fig. 1), which is a key factor in identifying *B. pilosicoli* and has been used to design a primer for PCR-based detection of this organism (Park *et al.*, 1995). However, it was found that the single IS-C isolate differed in this region in having only five rather than six 'T' nucleotides. As this difference is in the centre of the primer used which otherwise differs by 7–8 bp from other *Serpulina* species, this may have only minor consequences for the specificity of this assay. In fact, this isolate has produced a positive result in a species-specific PCR (C. Bakatselou & S. P. Barrett, unpublished results).

Based on biochemical and DNA sequencing analyses, it is concluded that all human intestinal spirochaetes belong to the species *B. pilosicoli*. It is of interest to note that none of the other *Brachyspira/Serpulina* species were found. This is in contrast with swine faecal isolates, which represent at least five different

species of *Serpulina*. This may suggest that *B. pilosicoli* has a different host–pathogen interaction allowing it to colonize a broader host range than other *Serpulina* species.

The clinical significance of *B. pilosicoli* in human infection remains unclear and needs further study. *B. pilosicoli* has been demonstrated to cause spirochaetosis in pigs (Trott *et al.*, 1996b; Muniappa *et al.*, 1997; Thomson *et al.*, 1997), and there was a significant correlation between the presence of group IV spirochaetes (now known to be *B. pilosicoli*) and occurrence of diarrhoea in pigs (Fellström & Gunnarson, 1995). Most human intestinal spirochaetes are obtained from humans with diarrhoea (Lee & Hampson, 1994). Also, most, but not all, of the intestinal isolates studied here were obtained from patients with intestinal problems. However, this does not prove an aetiological link. It could be that colonization by other agents causing intestinal problems may 'flush out' spirochaetes that are normally residing in a healthy intestine. It may also be the case that only certain strains of *B. pilosicoli* are pathogenic, depending on their ability to produce specific toxins or other virulence factors. This is, for example, observed with *Escherichia coli*, where different strains can either produce enterotoxins or cytotoxins or penetrate epithelial cells.

In summary, it is concluded, based on phenotypic analysis and DNA sequencing, that there is considerable variation amongst human intestinal spirochaetes, but that they all belong to or are closely related to the species *B. pilosicoli*. The clinical significance of human *B. pilosicoli* infection remains unclear.

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