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Classification of *Brachyspira* spp. isolated from Swedish dogs

Claes Fellström[1]*, Bertil Pettersson[2], Ulla Zimmerman[3], Anders Gunnarsson[3] and Ricardo Feinstein[4]

[1]*Department of Medicine and Surgery, Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala,* [2]*Department of Biotechnology, The Royal Institute of Technology, Stockholm,* [3]*Department of Bacteriology and* [4]*Department of Pathology, The National Veterinary Institute, Uppsala, Sweden*

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Abstract

Brachyspira spp. were isolated from 21 of 32 sampled dogs (66%) in a colony of Swedish beagle dogs with a history of diarrhea and from 3 of 17 Swedish pet dogs (17%) with diarrhea. All Swedish isolates were weakly β-hemolytic and gave a negative indole reaction. Eighty-eight percent showed negative α-galactosidase and hippurate reactions, but a positive β-glucosidase reaction. Two isolates were hippurate positive with a negative β-glucosidase reaction. One additional German isolate diverged by showing a positive indole reaction in combination with a positive hippurate reaction. Sequencing of 16S rDNA indicated that the hippurate-positive isolates belonged to the species *Brachyspira pilosicoli*. Four representative isolates were examined using pulsed-field gel electrophoresis (PFGE) and compared with six reference strains and five porcine isolates of *Brachyspira* spp. The canine isolates clustered together in the PFGE analysis. Necropsy examination of a culture-positive *B. pilosicoli* colony-raised beagle dog revealed macro- and microscopical lesions of colitis with numerous spiral-shaped bacteria in the lumens of the crypts, in goblet cells and within the colonic epithelium.

Introduction

Brachyspira spp., formerly *Serpulina* spp., are intestinal spirochetes. In pigs they cause two major diarrheic diseases, i.e. *B. hyodysenteriae* (Ochiai *et al*., 1997) causes swine dysentery (SD) (Harris, 1999) and *B. pilosicoli* causes porcine intestinal spirochetosis (PIS) (Trott *et al*., 1996b). In addition, two presumably non-pathogenic species, namely *B. innocens* (Kinyon and Harris, 1979) and *B. murdochii* (Stanton *et al*., 1997), and one species, *B. intermedia* (Stanton *et al*., 1997) for which the pathogenic potential still remains controversial, have been isolated from pigs. Porcine intestinal spirochetes can also be classified into schemes based on their biochemical properties (Fellström *et al*., 1999), multilocus enzyme electrophoresis (MEE) (Lee *et al*., 1993) or 16S rDNA phylogeny (Pettersson *et al*., 1996).

Besides pigs, *Brachyspira* spp. have been isolated from birds, dogs, humans, non-human primates, guinea-pigs, opossums and wild rodents (Duhamel, 1997; Swayne and McLaren, 1997). The consequences of colonization by intestinal spirochetes in animals other than pigs and in humans are controversial. Some investigators believe that the bacteria may be responsible for various gastrointestinal disturbances, while others have questioned their clinical importance and claimed that shedding of large numbers of spirochetes in connection with diarrhea may be blamed on dislodgment from the crypts by diarrhea induced by other etiological factors (Leach *et al*., 1973). In chicken and pullets, *B. intermedia, B. pilosicoli* and *B.* (*Serpulina*) *alvinipulli* (Stanton *et al*., 1998), for example, have been associated with retarded growth, diarrhea, delayed onset of egg production and cecal lesions (Swayne and McLaren, 1997). In addition, spirochetes isolated from a rhea with necrotizing typhlocolitis could not be distinguished from *B. hyodysenteriae* by hemolysis, indole production, RFLP or 16S rRNA sequencing (Jensen *et al*., 1996; Stanton *et al*., 1996).

* Corresponding author: Department of Large Animal Clinical Sciences, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Box 7018, SE-750 07 Uppsala, Sweden. E-mail: Claes.Fellstrom@kirmed.slu.se

Various phenotypes of *Brachyspira*-like spirochetes have been isolated from captured mice and rats (Joens and Kinyon, 1982; Blaha, 1983), from non-human primates (Duhamel, 1997), cats (Weber and Schramm, 1989), nutria (Molnár, 1986) and opossums (Turek and Meyer, 1979). The isolates from cats and opossums were obtained from normal healthy individuals. In nutria, however, an infectious disease was observed resembling SD in both clinical symptoms and pathological lesions (Molnár, 1986). In the guinea-pig, spirochetosis has been reported in connection with Tyzzer's disease (*Bacillus piliforme* infection) (Vanrobaeys *et al*., 1998). Two species of intestinal spirochetes have been isolated from humans, namely *B. pilosicoli* (Trott *et al*., 1996b) and *B. aalborgi* (Hovind-Hougen *et al*., 1982). Several studies have suggested that *B. pilosicoli* may be responsible for intestinal disturbances in humans. Furthermore, Trott *et al*. (1995, 1996a) and Trott and Hampson (1998) produced diarrhea and colonic lesions both in pigs and chicks, in challenge studies with human *B. pilosicoli* strains.

Intestinal spirochetes have been found in both healthy and diarrheic dogs (Pindak *et al*., 1965; Turek and Meyer, 1978; Meier *et al*., 1982; Weber and Schramm, 1989; Lee and Hampson, 1994, 1996; Duhamel *et al*., 1995; Trott and Hampson, 1998). In a recent study it was suggested that canine intestinal spirochetes consist of *B. pilosicoli* and a group of non-pathogenic spirochetes, provisionally designated '*Brachyspira* (*Serpulina*) *canis*' (Duhamel *et al*., 1998).

A role for spirochetes in dogs as either a primary or secondary pathogen has been proposed (Meier *et al*., 1982; Duhamel *et al*., 1995, 1996), but also questioned (Leach *et al*., 1973; Weber and Schramm, 1989). Duhamel *et al*. (1995) suggested that infection with *B. pilosicoli* in the colon of dogs might be subclinical, but massive infection, which may occur in environments with poor hygiene or in dogs with a comprised intestinal function because of concurrent etiological factors, may cause diarrhea. Koopman *et al*. (1993) found similar restriction enzyme analysis (REA) patterns between spirochetes isolated from dogs and humans. Lee and Hampson (1994) studied human and canine spirochetes by MEE and found a close genetic relationship between isolates from Aboriginal children and an isolate from a dog living with these children, although they also claimed that most isolates obtained from dogs were morphologically and genetically different from *B. pilosicoli*. In addition, Trott *et al*. (1998) were able to isolate strains with the same PFGE pattern from humans and dogs, suggesting that cross-species transmission of *B. pilosicoli* may occur naturally and that the infection can be zoonotic.

The aim of the present study was to describe intestinal spirochetosis (IS) in Swedish dogs, to classify the isolates and compare them to reference and field strains of porcine *Brachyspira* spp.

Materials and methods

Dogs and strains

Due to a previous outbreak of diarrhea, 29 dogs in a colony of purpose-bred laboratory beagles were sampled for *Brachyspira* spp. on five occasions over a period of 18 months. Three dogs were resampled 5 months after the initial sampling occasion. The problems of diarrhea preceded the use of the animals in experiments. The diarrhea was successfully controlled using a rice-based diet. During the diarrhea outbreak, one dog (aged 13 months) from the colony was submitted for diagnostic necropsy at The National Veterinary Institute (Uppsala). Intestinal samples from this animal were examined for aerobic and anaerobic bacteria. However, virus isolation was not attempted. It was not possible to identify any management or experimental factors that might have influenced the occurrence of diarrhea in the colony. Three months later four additional, apparently healthy, dogs from the same colony were also necropsied.

In addition, 17 stool specimens from pet dogs with acute or chronic diarrhea problems were examined for *Brachyspira* spp. The dogs originated from the south, middle and north of Sweden. Breed, age and sex were not available, nor other causes of diarrhea found in the pet dogs. One canine strain, A3077, was obtained from Jutta Verspohl, Institut für Mikrobiologie und Tierseuchen, Tierärtztliche Hochschule, Hannover. All results of biochemical tests, PFGE and 16S rDNA analysis of the canine isolates were compared with the corresponding results of *Brachyspira* reference strains and porcine Swedish field strains (Fellström and Gunnarsson, 1995; Pettersson *et al*., 1996; Fellström *et al*., 1999) (Table 1).

Isolation and culture of Brachyspira *isolates*

Rectal swabs were transported in Amies medium and examined for growth of *Brachyspira* spp. at the National Veterinary Institute. The time from sampling to inoculation did not exceed 48 hours. The swabs were inoculated on selective medium, Serpulina-agar plates [blood agar base no. 2 (Oxide code CM 271) supplemented with 5% citrated sheep blood, colistin sulfate solution (0.025 mg/ml), vancomycin–HCl solution (0.025 mg/ml), 1% spectinomycin solution (0.8 mg/ml) and 1% sodium ribonucleate]. The plates were incubated under anaerobic conditions at 42°C under an atmosphere of 90–95% H_2 and 5–10% CO_2.

After 3–4 and 6–7 days, the plates were examined for characteristic spirochetal growth. The isolates were then subcultivated on fastidious anaerobe agar (FA-agar, LabM code LAB 90 Bakt Dla; National Veterinary Institute, Uppsala, Sweden) for assessment of purity by phase-contrast microscopy.

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Table 1. Strains used for comparisons by pulsed-field gel electrophoresis (excluding avian and human strains) and 16S rDNA sequence analysis

Strain	Species	Phenotype	Animal of origin	Reference
B78^T	*B. hyodysenteriae*	I	Pig	Harris *et al.* (1972)
B204	*B. hyodysenteriae*	I	Pig	Kinyon *et al.* (1977)
AN174:92	*B. hyodysenteriae*	I	Pig	Pettersson *et al.* (1996)
AN983:90	*B. intermedia*	II	Pig	Pettersson *et al.* (1996)
ATCC51140^T	*B. intermedia*	II	Pig	Stanton *et al.* (1997)
C378	*B. murdochii*	IIIa	Pig	Pettersson *et al.* (1996)
ATCC51284^T	*B. murdochii*	IIIa	Pig	Stanton *et al.* (1997)
C301	*B. murdochii*	IIIa	Pig	Pettersson *et al.* (1996)
B256^T	*B. innocens*	IIIbc	Pig	Kinyon and Harris (1979)
P43^T/6/78	*B. pilosicoli*	IV	Pig	Pettersson *et al.* (1996)
AN497:93	*B. pilosicoli*	IV	Pig	Pettersson *et al.* (1996)
AN883:98	*Brachyspira*	IIIa	Dog	This study, Sweden
Cb104:95	*Brachyspira*	IIIa	Dog	This study, Sweden
AN2608:97	*B. pilosicoli*	IV	Dog	This study, Sweden
A3077	*B. pilosicoli*	IV	Dog	This study, Sweden
WES-B	*B. pilosicoli*	IV	Human	GenBank U23034
NCTC 11492^T	*B. aalborgi*	nd	Human	Hovind-Hougen *et al.* (1982)
W1	*B. aalborgi*	nd	Human	Kraaz *et al.* (2000)
ATCC51933^T	*B. alvinipulli*	nd	Avian	Stanton *et al.* (1998)

nd = not determined.

Biochemical classification of Brachyspira *isolates*

All *Brachyspira* isolates were inoculated on trypticase soy agar (TSA agar) with 5% beef blood with a cotton swab soaked in beef broth, in order to determine the intensity of β-hemolysis. The agar plates were read after 3–4 and 6–7 days and β-hemolysis was judged as weak or strong. The isolates were retransferred to FA-agar and tested for indole production, hippurate-cleaving capacity, α-galactosidase and β-glucosidase activity. The indole spot test was performed as described by Sutter and Carter (1972). The method of von Rübsamen and Rübsamen (1986) was used for the hippurate-cleavage test. The API ZYM test (API, 69280 Marcy-l'Etoile, France) or diagnostic tablets (Roscoe, 2630 Taastrup, Denmark) were used for the determination of α-galactosidase, α-glucosidase and β-glucosidase activities. The isolates were arranged in groups I–IV according to the classification system proposed by Fellström and Gunnarsson (1995).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed on one German and three Swedish *Brachyspira* strains (AN883:98, Cb104/95, AN2608:97 and A3077) as previously described for *B. hyodysenteriae* (Fellström *et al.*, 1999) with DNA digested in the plugs with *Mlu*I and *Sal*I. The gels were subjected to PFGE in TBE buffer (0.045 M Trisborat, 1 mM EDTA) at 6 V/cm, using a Pharmacia Gene Navigator (Pharmacia, Uppsala, Sweden) unit at 175 V and a temperature of 12°C with buffer circulation. The program for *Mlu*I and *Sal*I was 10 s, 3 h/15 s, 6 h/20 s, 6 h/40 s, 5 h/60 s, 4 h. The gels were stained with ethidium bromide (0.1% in water), examined over a UV transilluminator, and photographed. The photographs were scanned into the GelCompare program. Based on the combined gels of *Mlu*I- and *Sal*I-digested DNA, the program created a dendrogram from a matrix of band-matching coefficients by the unweighted pair group method of arithmetic averages (UPGMA) clustering fusion strategy. The dendrogram also included PFGE patterns from six reference and five porcine field strains previously analysed (unpublished).

Sequence analysis of the 16S rRNA gene

Partial sequences of the 16S rRNA gene of the spirochetes isolated from one German and three Swedish dogs (strains AN883:98, Cb104/95, AN2608:97 and A3077) were determined by direct solid-phase DNA sequencing using primers and protocols as described previously (Pettersson *et al.*, 1996).

Phylogenetic analysis

The nucleotide information from individual traces was compiled into contigs of the 16S rRNA genes, which were subsequently aligned manually with 16S rRNA gene sequences of reference and outgroup strains. Ambiguously aligned positions were not removed prior to phylogenetic calculations and the final data set comprised 1396 aligned positions. The phylogeny was inferred by using DNADIST contained in the Phylogenetic Inference Package, PHYLIP 3.573c (Felsenstein, 1981). Evolutionary distances were calculated from the data set, correcting for

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multiple nucleotide substitutions at single locations by the one parameter model of Jukes and Cantor (1969) and user trees based on distance matrix were computed by neighbor-joining (Saitou and Nei, 1987) with the program, NEIGHBOR. The results obtained were tested statistically by bootstrap analysis using the program SEQBOOT by resampling the data set 1000 times. Majority-rule consensus trees were computed from the user trees by using the CONSENSE program.

Nucleotide accession numbers

The nucleotide information of the 16S rRNA genes of the *Brachyspira* strains AN883:98, Cb104:95, AN2608:97 and A3077 used in this study have been deposited in GenBank under the accession numbers AF245122, AF245121, AF245123 and AF245120, respectively. The GenBank numbers for the 16S rRNA gene sequences of the reference and outgroup strains used for comparison in this study are as follows: *B. aalborgi* NCTC 11492^T, Z22781; *B. aalborgi* W1, AF200693; *B. alvinipulli* ATCC 51933^T, U23030; *B. hyodysenteriae* ATCC 27164^T, U14930; *B. hyodysenteriae* B204, U14932; *B. hyodysenteriae* AN174:92, U14931; *B. intermedia* ATCC 51140^T, U23033; *B. intermedia* AN983:90; *B. innocens* ATCC 29796^T, U14920; *B. murdochii* ATCC 51284, U22838; *B. murdochii* C301, U14917; *B. murdochii* C378, U14918; *B. pilosicoli* ATCC 51139^T, U14927; *B. pilosicoli* WES-B, U23034.

Results

Isolation and classification of spirochetes

In the beagle colony, 21 of the 32 samples (66%) were positive by culture. Two isolates were classified as group IV spirochetes (*B. pilosicoli*). One of them was isolated from the dog which was necropsied. Of the remaining isolates, 18 shared biochemical reaction patterns with the three isolates from the pet dogs. One isolate diverged, showing a positive α-galactosidase reaction and otherwise a typical group IIIbc pattern. Spirochetes could only be isolated from 3 of the 17 pet dogs with diarrhea problems. The non-*B. pilosicoli* and non-group IIIbc isolates were weakly β-hemolytic, α-galactosidase, indole and hippurate negative, and classified as spirochetes of group IIIa in the biochemical classification (Fellström *et al*., 1999). Two of the three dogs which were sampled twice with a 5-month interval were group III spirochete positive on both sampling occasions. The results of the biochemical tests and the biochemical classification of the isolates are presented in Table 2.

Necropsy

The dog from the colony of beagles with a history of diarrhea showed macro- and microscopic lesions of colitis. Apart from *Brachyspira pilosicoli*, only *Escherichia coli* was cultured, but the latter finding was regarded as insignificant. *Salmonella,* clostridia or other enteric pathogens were not found. The intestines were also examined parasitologically with negative results (the dogs had been treated with anthelmintics as part of the sanitary routines at the colony). In addition, the study of intestinal sections using special stains, such as Giemsa, Grocott and Warthin–Starry, did not reveal any fungi or protozoans, but numerous spiral-shaped organisms were observed in the lumen of the crypts, in goblet cells, and within the colonic epithelium (Fig. 1). End-on attachment was not observed. The histological features observed did not correspond to any viral diseases, such as those caused by parvoviruses, rotaviruses or coronaviruses.

The subsequently euthanized dogs displayed thickened colonic mucosas and markedly enlarged Peyer's patches and lymphoid follicles in the large intestines. Histologically, the main finding was a pronounced hyperplasia of the gut-associated lymphoid tissues (GALT) and diffuse infiltration of the intestinal mucosa with lymphocytes and plasma cells. Spiral-shaped bacteria were not observed. Culture for spirochetes was not performed from the latter dogs.

Pulsed-field gel electrophoresis

In the resulting dendrogram of the PFGE analysis, porcine strains of each biochemical group I–IV clustered together (Fig. 2). All four dog isolates clustered in one cluster with two subgroups corresponding to their bio-

Table 2. The biochemical reactions of weakly β-hemolytic intestinal spirochetes isolated from one German and 21 Swedish dogs (25 isolates)

Biochemical group	No. of isolates	Indole	α-galactosidase	β-glucosidase	Hippurate	Species
IIIa	21	–	–	+	–	*Brachyspira*
IIIbc	1	–	+	+	–	*B. innocens*
IV	2	–	+	–	+	*B. pilosicoli*
IV atypical	1	+	–	–	+	*B. pilosicoli*

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chemical type but separated from porcine isolates of the same biochemical groups.

Phylogenetic analysis of the dog isolates

A phylogenetic tree computed by neighbor-joining is presented in Figure 3. The *B. aalborgi* strains NCTC 11492^T and W1 were selected as outgroups based on previous results of Pettersson *et al.* (1996), which showed that *B. aalborgi* branched early from the distinct lineage formed by the brachyspiras (serpulinas) in the spirochetal cladogram. The avian species *B. alvinipulli* ATCC 51933^T was included for comparison. Members of the *B. hyodysenteriae* group formed a distinct clade, but with weak bootstrap support for certain internal nodes. Only the branches having a statistical value of more than 60% are indicated. Vertical lines and roman numer-

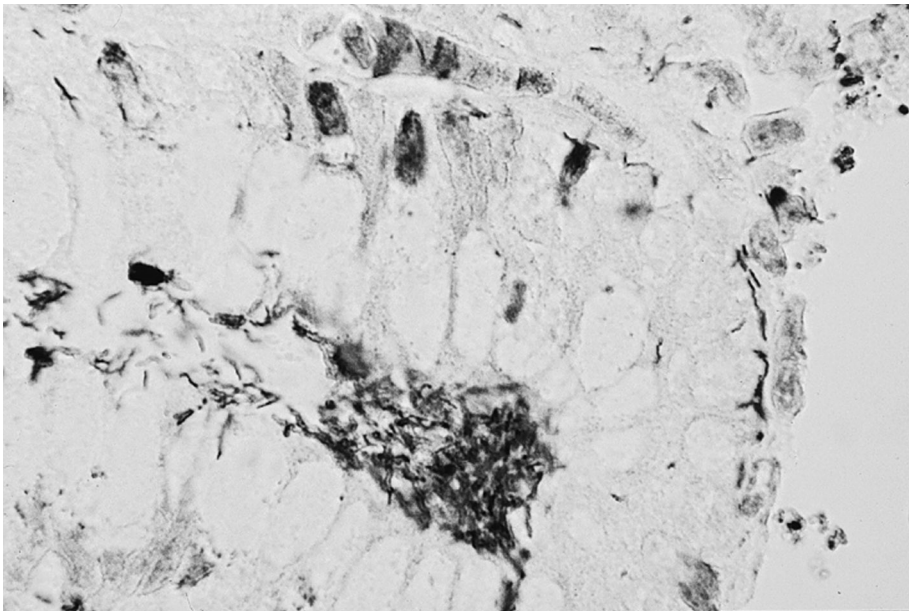

Fig. 1. Numerous spiral-shaped bacteria observed in the lumen of the crypts, in goblet cells and within the colonic epithelium (Warthin–Starry stain; magnification ×400).

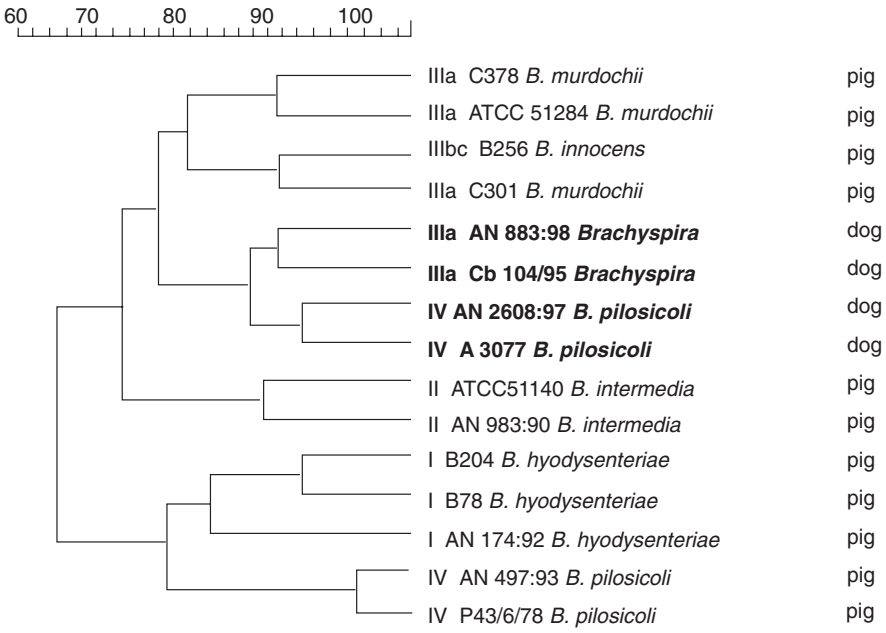

Fig. 2. Dendrogram as deduced from PFGE banding patterns of six reference strains and eight field strains of *Brachyspira* spp. isolated from pigs and dogs. The analyses were performed using the UPGMA clustering fusion strategy (Vauterin and Vauterin, 1992). Roman figures indicate biochemical group.

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als in the tree (Fig. 3) indicate the biochemical profiles. The *Brachyspira* strains AN883:98 and Cb104:95 showing phenotypic properties of IIIa clustered together, but not together with the swine species *B. murdochii* because they were bisected by *B. innocens* ATCC 29796^T. The strains AN883:98 and Cb104:95 did not share the unique nucleotide feature of a C in the helix terminated by the tetra loop in position 187 in the 16S rRNA molecule (*Escherichia coli* numbering), which was found to be a signature for subcluster IIIa in a previous study performed by Pettersson *et al.* (1996). Thus, this position is not a motif, which unifies the intestinal spirochetes of the biochemical profile of IIIa. Strikingly, these strains did not share any of the unique nucleotide sites of the group III line of descent, but the guanosine in position 1260 of *E. coli*. The dog isolates AN2608:97 and A3077, with the ability to hydrolyse hippurate, clustered together with other strains of *B. pilosicoli* characterized by this biochemical attribute. They were closely related to *B. pilosicoli* ATCC 51139^T, being more than 99.5% similar in 16S rDNA sequence. These strains also harbored the higher-order structural attribute that is characteristic for the biochemical group IV, namely the hexamer of uridines in the helix capped by the loop starting at the position 208 (according to *E. coli*) of the resulting 16S rRNA molecule, as modeled by Pettersson *et al.* (1996). Moreover, all 11 of the additional nucleotide positions, which have been described to be unique for this lineage, were also found in the 16S rRNA genes of the strains AN2608:97 and A3077.

Discussion

This study summarizes the present knowledge of canine intestinal spirochetosis in Sweden. It also adds new general information on the genetic background of some typical canine strains of *Brachyspira* spp. Canine intestinal spirochetes in Sweden seem mainly to consist of group IIIa spirochetes (which in pigs are regarded as non-pathogenic) and *B. pilosicoli*. We also found one diverging isolate which was biochemically classified as *B. innocens*. Interestingly, the canine strains clustered together in a separate cluster in the PFGE dendrogram based on porcine intestinal spirochetes (Fig. 2). This finding may indicate an adaption of the genotype to the host and somewhat enlighten the debate regarding the zoonotic properties of *B. pilosicoli*. Another unexpected finding was the strongly positive indole reaction of the German group IV dog isolate, A3077, classified as *B. pilosicoli*. With the exception of *B. intermedia* isolates, this is the first weakly β-hemolytic intestinal spirochete with a positive indole reaction ever recognized by our laboratory.

Phylogenetically, the dog isolates clustered in two different entities. The strains AN2608:97 and A3077 closely resembled members of the *B. pilosicoli* clade, as judged from the presence of unique nucleotide positions and their tight clustering in the evolutionary distance tree (Fig. 3), suggesting their taxonomic affiliation as being members of this species. The canine strains AN883:98 and Cb104:95, which showed phenotypic properties

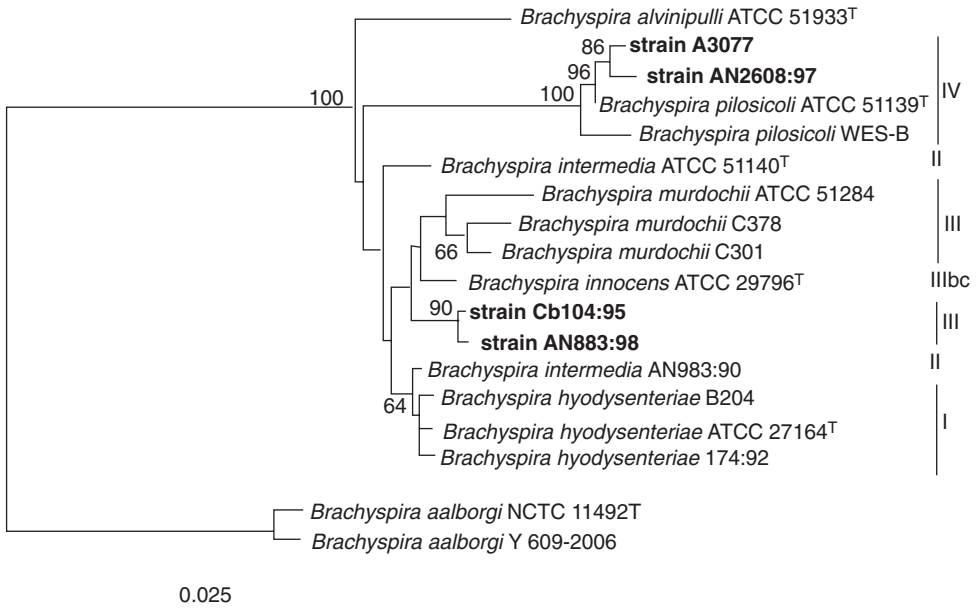

Fig. 3. Phylogenetic tree displaying the evolutionary relationships between *Brachyspira* strains isolated from dogs and reference strains. The dog isolates studied in this work have been marked in bold. The tree was computed with neighbor joining (Saitou and Nei, 1987) from a distance matrix calculated by using the one-parameter model of Jukes and Cantor (1969). Bootstrap percentage values as obtained from resampling the data set 1000 times are shown at the nodes for which the statistical support was greater than 60%. *B. aalborgi* NCTC 11492^T served as the outgroup. The scale bar indicates 0.025 nucleotide substitutions per nucleotide site.

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similar to those of the IIIa subcluster, did not group together with three strains of the species *B. murdochii*. The tree indicates that the phenotypic profile of IIIa is not monophyletic because it is bisected by *B. innocens* ATCC 29796^T (Fig. 3). It remains to be shown whether the phenotype IIIa does not reflect phylogeny or whether it is a problem associated with the use of 16S rDNA sequences for deciphering genealogies of brachyspiras positioned in this locale of the intestinal spirochete tree. It still has to be confirmed whether or not the strains AN883:98 and Cb104:95 are closely related to the canine strains provisionally named '*Brachyspira canis*' (Duhamel *et al*., 1998). PFGE analysis and phylogenetic analysis of 16S rRNA genes of Swedish canine strains indicate that canine non-*B. pilosicoli* strains may belong to a separate species, as has been suggested on the basis of previous multilocus enzyme electrophoresis and ribotyping methods (Duhamel *et al*., 1998).

In this study we were not able to confirm a causal relationship between diarrhea and isolation of spirochetes from dogs. However, in the colony of beagles, a causal relation between infection with *B. pilosicoli* and diarrhea may have been present since this was the only organism found that could explain the lesions of colitis observed in the dog submitted for necropsy due to previous diarrhea problems in the colony. End-on attachment, previously described as a significant finding in IS caused by *B. pilosicoli*, was not observed. However, previous challenge studies with *B. pilosicoli* (Neef *et al*., 1994; Thomson *et al*., 1997) have shown that end-on attachment is not always recognized when pigs are colonized with this agent. Another reason could be that the time between euthanasia and necropsy was about 3–4 hours. During this time a bacterial attachment may have been broken. Although speculative, the changes of GALT hyperplasia and infiltrates of lymphocytic and plasma cells observed in seemingly healthy dogs from the same colony may reflect an immune-mediated reaction to a previous bacterial infection, possibly *B. pilosicoli*.

Our results suggest that IS does not seem to be a common cause of diarrhea among Swedish dogs. However, in closed environments contaminated with feces, e.g. kennels and colonies of research dogs, *B. pilosicoli* should be considered as a possible cause of intestinal disturbances. To prevent IS in these environments it may therefore be important to implement adequate sanitary measures aimed at reducing fecal contamination of the localities.

Acknowledgments

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