Epidemiological aspects
and improved differential diagnostics
of porcine *Brachyspira pilosicoli*

Marja Fossi
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Acknowledgements

These studies were mainly conducted at five locations of the National Veterinary and Food Research Institute: Seinäjoki Unit, Kuopio Department, Department of Pathology, Department of Bacteriology and Department of Virology. Some parts of the project were performed at the Pathology Division of the Department of Basic Veterinary Sciences and at the Saari Unit of the Faculty of Veterinary Medicine, University of Helsinki. This work was financially supported by the Ministry of Agriculture and Forestry, the Foundation of Veterinary Science in Finland and the Finnish Association for Food Animal Practitioners.

I am deeply grateful to Professor Sinikka Pelkonen, Head of the Kuopio Department, for her supervision and unceasing support throughout the Brachyspira studies. The General Director, Professor Tuula Honkanen-Buzalski, is warmly thanked for guidance and wise counsel, especially during the final preparation of the manuscript.

My sincere thanks are also extended to all of my co-authors:

– Tarja Pohjanvirta and Sirpa Heinikainen for their essential contributions to the molecular studies and their patience,

– Professor Marjukka Anttila, Head of Department of Pathology, and Teija Kokkonen for skilful assistance with pathology,

– Katja Ahlsten for sharing with me the difficult but interesting days around the infection trial,

– Teresa Skrzypczak for fruitful collaboration in the laboratory and pleasant companionship while attending conferences,

– Kirsti Pelkola for her careful microbiological studies during the infection trial,

– Professor Antti Sukura, Head of the Pathology Division, for helping me with electron microscopy,

– Mari Heinonen and Olli Peltoniemi for their arrangements and actions for the eradication of B. pilosicoli and for control samplings in a sow herd,

– Danish colleagues Tim Kåre Jensen, Mette Boye and Rikke Lindecrona for the molecular studies carried out at the Danish Institute for Food and Veterinary Research.

All of my colleagues and the technicians at the facilities involved deserve my warmest thanks for their positive attitudes and generous assistance with all manner of things.

Veterinary Centre Manager Jill Thomson and Professor Claes Fellström are sincerely thanked for reviewing the thesis and providing valuable comments to improve it. I also thank Carol Ann Pelli for editing the English language of this manuscript and most of the original articles.
Abstract

*Brachyspira pilosicoli* causes porcine intestinal spirochaetosis, which is manifested by a mild, persistent diarrhoea among weaned pigs at the age of 7–14 weeks. The growth of diseased pigs is retarded and their feed conversion is lowered, resulting in diminished production. These studies were designed to investigate the molecular epidemiology of *B. pilosicoli* in Finnish sow herds and to improve laboratory diagnostics for *B. pilosicoli*. Infectivity of the rare hippurate-negative biotype of *B. pilosicoli* was examined by an infection trial and eradication of *B. pilosicoli* from a sow herd was demonstrated.

A high genetic diversity was observed among 131 *B. pilosicoli* strains obtained from 49 sow herds located in the two major pork production areas of Finland. A high discriminatory power of pulsed-field gel electrophoresis (PFGE) was established with either *Sma*I or *Mlu*I used as a restriction enzyme. Common genotypes between the herds were rare, and no clustering of the genotypes according to the two geographical areas was observed. A single genotype could persist in a herd for several years; however, genetic recombination among *B. pilosicoli* strains might occur. The epidemic nature of *B. pilosicoli* infection in Finnish pig farms was shown, and the role of migrating vectors or fomites for horizontal transmission was assessed as minor.

The high discriminatory power of PFGE was further exploited to investigate hippurate-negative phenotypes of *B. pilosicoli*. No relationship between hippurate-negativity and genotypes of *B. pilosicoli* was detected. This finding was substantiated by comparative analyses of 16S rDNA nucleotide sequences of hippurate-negative and -positive isolates; different nucleotide positions of the strains were not predictive of the hippurate hydrolysis reaction. *B. pilosicoli* isolates from the same herd could possess an identical genome despite having a different capacity for hydrolysing hippurate. The congruent phylogeny of hippurate-negative and -positive *B. pilosicoli* was further ascertained by an ultrastructural study; the two biotypes shared all of the features unique to the species *B. pilosicoli*. In conclusion, the expression of hippurate hydrolysis can vary within a single *B. pilosicoli* clone, and thus, the hippurate test cannot be used alone for differentiation of porcine *B. pilosicoli*.

An occasional hippurate-negativity can disturb the phenotype-based differential diagnostics of *B. pilosicoli*. A *B. pilosicoli*-specific D-ribose test was therefore established to strengthen the diagnostics protocol. Sixty unrelated *B. pilosicoli* strains and 35 strains of other porcine *Brachyspira* species were studied for D-ribose utilization by an indirect method based on recording the pH reduction of a broth culture in the presence of D-ribose. All *B. pilosicoli* strains, regardless of the hippurate reaction, could utilise D-ribose, whereas the strains of the other *Brachyspira* species were D-ribose-negative. These results enabled the construction of an amended classification scheme for phenotypic differentiation of porcine *B. pilosicoli*.

Experimental pigs were inoculated either with a hippurate-negative *B. pilosicoli* strain or with a *B. pilosicoli* type strain. Somewhat unexpectedly, the pigs in both trial groups remained healthy. Only the hippurate-negative *B. pilosicoli* strain could be reisolated from two of eight infected pigs. An explanation for the silent infection – or lack of infection –
may lie in the challenge procedure, strain attenuation, environmental conditions, absence of concomitant enteropathogens or the feeding regimen. The pathogenicity of hippurate-negative \( B. \) \textit{pilosicoli} should be further studied in modified conditions for trial pigs.

Eradication of a chronic \( B. \) \textit{pilosicoli} infection without total depopulation was attempted in a 60-sow farrowing herd. The principles for eradication of \( B. \) \textit{hyodysenteriae}, the cause of swine dysentery, were applied. Special attention was paid to sanitary measures, relocation of animals according to age groups to nearby shelters, husbandry principles and adequate medication. The eradication was successful; the diarrhoea of the young growers disappeared, and \( B. \) \textit{pilosicoli} was not detected in any sample during the 4.5-year follow-up.
List of original publications

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals. These articles were reprinted with the kind permission of each journal concerned.


Abbreviations

AFLP  amplified fragment length polymorphism
ATCC  American Type Culture Collection
CVSBA  colistin-, vancomycin- and spectinomycin-containing blood agar
FA  fastidious anaerobe
FCS  foetal calf serum
FISH  fluorescent in situ hybridization
MIC  minimal inhibitory concentration
MLEE  multilocus enzyme electrophoresis
MRP  macrorestriction profile
PCS  porcine colonic spirochaetosis
PFGE  pulsed-field gel electrophoresis
p.i.  post inoculation
PIS  porcine intestinal spirochaetosis
p.p.m.  parts per million
RFLP  restriction fragment length polymorphism
SD  swine dysentery
TEM  transmission electron microscopy
TS  trypticase-soy media
WBHIS  weakly β-haemolytic intestinal spirochaete
1 Introduction

*Brachyspira* spp. bacteria are anaerobic, motile spirochaetes that colonize the large intestine of various animal species and humans. *Brachyspira pilosicoli* belongs to the group of weakly β-haemolytic intestinal spirochaetes (WBHIS) that causes intestinal spirochaetosis in pigs, birds and humans (Hampson, 2000). Transmission of *B. pilosicoli* from animals to humans has been suggested (Trott et al., 1998), but these zoonotic aspects warrant further investigations. *B. pilosicoli* was fully characterized in 1996 (Trott et al., 1996c) but shown to be pathogenic to pigs much earlier by British researchers (Taylor et al., 1980). In growers and young fatteners aged 4–20 weeks, *B. pilosicoli* causes non-fatal, persistent diarrhoea in a disease known as porcine intestinal spirochaetosis (PIS) (Trott et al., 1996c) or porcine colonic spirochaetosis (PCS) (Girard et al., 1995). PIS has been recognized worldwide (Duhamel, 1998; Møller et al., 1998; Barcellos et al., 2000b; de Arriba et al., 2002; Choi et al., 2002), in Finland since the 1990s (Heinonen et al., 2000b).

A scheme for phenotypic differentiation of porcine intestinal spirochaetes was developed in Sweden by Fellström and Gunnarsson (1995). Hippurate hydrolysis capacity was deemed unique for *B. pilosicoli*, which made its detection easy. Diagnostic tools for *B. pilosicoli* soon expanded to include molecular methods such as polymerase chain reaction (PCR) (Park et al., 1995; Fellström et al., 1997; Leser et al., 1997), fluorescent *in situ* hybridization (FISH) (Boye et al., 1998) and PCR-based restriction fragment length polymorphism (RFLP) (Rohde et al., 2002).

In the late 1990s, *B. pilosicoli* was frequently isolated from Finnish pig herds. With the common use of antimicrobial feed additives, at least 30% of the sow herds were estimated to be carriers of *B. pilosicoli* (Heinonen et al., 2000b; EELA, unpublished data). The molecular epidemiological studies on *B. pilosicoli* performed elsewhere by pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) have shown genetic heterogeneity among *B. pilosicoli* strains (Atyeo et al., 1996; Trott et al., 1998; Oxberry and Hampson, 2003) between and within geographical areas as well as within individual herds. Similar research in Finland was needed to assess the population structure and potential infection routes of *B. pilosicoli* in the Finnish pork production chain.

Phenotypical determination of bacteria species should take into consideration single aberrations in biochemical traits. Biochemically atypical WBHIS bacteria have been isolated regularly from Finnish pigs since 1997; these isolates have been negative for hippurate hydrolysis, but according to PCR belong to the species *B. pilosicoli*, and thus, should be hippurate-positive. Biochemically aberrant WBHIS isolates have also been described in the UK (Thomson et al., 2001). Confirmation of phenotyping methods for *B. pilosicoli* is therefore desired. Utilization of D-ribose has been suggested to be unique to *B. pilosicoli* within the genus *Brachyspira* (Trott et al., 1996c, d). If this proves to be true for a high number of *Brachyspira* spp. strains, the test for D-ribose utilization could reinforce the phenotypical classification of *B. pilosicoli*.

The pathogenicity of *B. hyodysenteriae* strains can vary (Jensen and Stanton, 1993; Lee et al., 1993a; Milner and Sellwood, 1994; Thomson et al., 2003), a phenomenon also suggested apply to *B. pilosicoli* strains (Duhamel, 1997; Muniappa et al., 1997; Thomson et al.,
A few experiments have attempted to clarify the relationship between pathogenicity and naturally occurring biochemical or genetic abnormalities in B. hyodysenteriae and B. pilosicoli; these studies have not demonstrated reduced virulence (Thomson et al., 2001, 2003). The virulence of hippurate-negative B. pilosicoli isolates was unknown due to concomitant enteropathogens in the herds of origin. Hippurate-negative B. pilosicoli has been detected annually in several Finnish pig herds, stressing the need for studying its pathogenicity.

Chronic B. pilosicoli infection in a herd can be fairly silent, affecting only weight gain of growers (Duhamel, 1997; Heinonen et al., 2000b; Jacobson et al., 2003; Thomson et al., 2005). Thereby, B. pilosicoli can be spread by replacement animals and rearing pigs. The current trend of increased antimicrobial resistance among pathogenic Brachyspira species (Buller and Hampson, 1994; Molnár et al., 1996; Duhamel et al., 1998a; Karlsson et al., 2002; Karlsson et al., 2004a, b; Lobová et al., 2004) necessitates attempts to diminish the prevalence of B. pilosicoli in pig herds. Chronic B. hyodysenteriae infection can be eradicated from farrowing pig herds without total depopulation (Blaha et al., 1987; Christensen et al., 1987; Wallgren, 1988; Ala-Risku, 2005). Eradication of B. pilosicoli has been assumed, however, to be more difficult. Since B. pilosicoli has a broader host range and higher resistance to certain physical stress factors outside the host than B. hyodysenteriae (Trott et al., 1996c), the risk for re-infection has been regarded as high. An eradication strategy for infectious pig diseases has been widely employed in Finland (Heinonen et al., 1999, 2000a), and eradication of chronic B. pilosicoli infection was regarded worth of attempt.
2 Review of the literature

2.1 Taxonomy and general characteristics of intestinal spirochaetes

*Brachyspira* is the only genus in the family *Brachyspiraceae* to belong to the order *Spirochaetales*, together with the families *Leptospiraceae* and *Spirochaetaceae*. The order *Spirochaetales* belongs to the class *Spirochaetes*. The spirochaetes consist of over 200 species or phylotypes. Some of these are virulent while others are harmless to the host. Moreover, many spirochaetes are free-living in the environment. Many spirochaetes are uncultivable; thus, research of these bacteria is dependent on molecular methods (Paster and Dewhirst, 2000).

All spirochaetes are helical and motile by means of periplasmic flagella. The flagella, with a count varying from 2 to 100 per cell, are located between the outer sheet and the protoplasmic cylinder, enabling efficient motility of spirochaete cells in a high-viscosity environment. Some spirochaetes are anaerobic, like *Brachyspira* spp. and *Treponema* spp., and some microaerophilic, like *Borrelia* spp. The species in the genus *Leptospira* are obligately aerobic (Canale-Parola, 1984).

Spirochaetes can transform themselves into spheres, a phenomenon reported in certain species of *Borrelia*, *Leptospira* and *Brachyspira*. Sphere formation is regarded as a response to deteriorated environmental conditions (Stanton and Lebo, 1988; Faine, 1998; Murgia and Cinco, 2004).

![Figure 1](image-url)  
*Figure 1* Cells of *Brachyspora pilosicoli*, strain Br980 (hippurate-negative phenotype). a. Regularly coiled cell with a typical pointed end. b. Sphere-form of the cell. c. Partially sphere-formed, comma-shaped cell. Negatively stained with 1% phosphotungstic acid. Bar = 1.0 µm. (Photo: A. Sukura)
2.2 Characteristics of *Brachyspira* spp.

The ecological niche for all recognized *Brachyspira* species is the lower gastrointestinal tract, where these spirochaetes are situated close to the mucosal epithelium. Anaerobic *Brachyspira* bacteria can metabolize small amounts of oxygen by NADH oxidase, which is essential for thriving of *Brachyspira* despite oxygen diffusion from intestinal tissue (Stanton, 1997). This property eases the handling of *Brachyspira* bacteria in the laboratory, as well.

*Brachyspira* cells are loosely coiled; their length ranges between 2.0 µm and 14.0 µm, and their width between 0.19 µm and 0.40 µm. The cell end is blunt, pointed or tapered depending on the species. The number of periplasmic flagella varies between different species from 4 to 14; the figures are doubled by overlapping of the flagella in the middle of the bacterium (Sellwood and Bland, 1997).

*Brachyspira* bacteria are cultivable on solid and liquid media supplemented with blood or serum. In liquid media, continuous agitation is a prerequisite for their growth (Stanton and Lebo, 1988). Selective antimicrobials are needed for isolation of *Brachyspira* from faecal or intestinal samples. The colonies are weakly or strongly β-haemolytic depending on the species. The *Brachyspira* bacteria can be faintly visualized by Gram-staining. In practice, the characteristic shape and motility of *Brachyspira* bacteria are easily seen by, for example, phase contrast microscope.

2.3 Species in genus *Brachyspira*

Some *Brachyspira* species are pathogenic, some are commensals, and pathogenicity of a few species is unknown or controversial. Seven species of *Brachyspira* have been characterized to date, and two proposed species are waiting for adequate description. The *Brachyspira* species and their hosts are presented in Table 1.

Besides the species listed in Table 1, many partially characterized *Brachyspira* spp. isolates and *Brachyspira*-like bacteria in intestinal tissue samples from several animal species need further identification; *Brachyspira*-like spirochaetes have been observed in cats (Murray *et al*., 2003), opossums (Turek and Meyer, 1979), guinea pigs (Vanrobaeys *et al*., 1998), nutrias (Molnár, 1986), horses (Davies and Bingham, 1985; Shibahara *et al*., 2002), raccoons (Hamir *et al*., 2001) and Japanese wild deer (Shibahara *et al*., 2000). Recently, new phylotypes of *Brachyspira* spp. have been isolated from several species of wild birds, some of which can be confused with the well-known species of *Brachyspira* (Jansson *et al*., 2001, 2005; Råsbäck *et al*., 2005).

2.4 *Brachyspira pilosicoli* in pigs

2.4.1 Specific features of *Brachyspira pilosicoli*

The *B. pilosicoli* cell is one of the smallest within the genus *Brachyspira*; it is 5–12 µm in length and 0.19–0.30 µm in width. The number of periplasmic flagella varies from 4 to 7, and the flagella are inserted close to the end of bacteria in a single line. The cell end of
**B. pilosicoli** is distinctively pointed and covered by a unique lattice-like structure. This structure has been proposed to be important in pathogenesis (Sellwood and Bland, 1997).

**B. pilosicoli** is weakly β-haemolytic on blood agar. Like most of the other weakly β-haemolytic intestinal spirochaetes (WBHIS), **B. pilosicoli** grows well on the surface of agar, whereas the strongly β-haemolytic **B. hyodysenteriae**, the cause of swine dysentery (SD), tends to also grow below the agar surface. **B. pilosicoli** grows relatively fast in optimal conditions at 37–42°C; its doubling time, 1–2 h, is about half of that of other porcine **Brachyspira** species. **B. pilosicoli** tolerates oxygen slightly better (7% vs. 5% in headspace of broth).

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<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. hyodysenteriae</strong></td>
<td>Pig</td>
<td>Swine dysentery</td>
<td>Harris et al., 1972</td>
</tr>
<tr>
<td></td>
<td>Rhea</td>
<td>Typhlocolitis</td>
<td>Jensen et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Mallard</td>
<td>Not reported</td>
<td>Jansson et al., 2004</td>
</tr>
<tr>
<td><strong>B. intermedia</strong></td>
<td>Pig</td>
<td>Controversial</td>
<td>Stanton et al., 1997; Binek and Szynkiewicz, 1984</td>
</tr>
<tr>
<td></td>
<td>Poultry</td>
<td>Intestinal spirochaetosis</td>
<td>Griffiths et al., 1987; McLaren et al., 1997</td>
</tr>
<tr>
<td><strong>B. innocens</strong></td>
<td>Pig</td>
<td>Not reported</td>
<td>Kinyon and Harris, 1979; Trott et al., 1996a</td>
</tr>
<tr>
<td><strong>B. murdochii</strong></td>
<td>Pig</td>
<td>Controversial</td>
<td>Stanton et al., 1997; Jensen et al., 2005; Hampson et al., 1999</td>
</tr>
<tr>
<td><strong>B. pilosicoli</strong></td>
<td>Pig</td>
<td>Porcine intestinal spirochaetosis</td>
<td>Trott et al., 1996c</td>
</tr>
<tr>
<td></td>
<td>Poultry</td>
<td>Intestinal spirochaetosis</td>
<td>Stephens and Hampson, 2002; Shivaprasad and Duhamel, 2005</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>Intestinal spirochaetosis</td>
<td>Trivett-Moore et al., 1998; Jensen et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>Intestinal spirochaetosis</td>
<td>Duhamel et al., 1998b; Fellströmm et al., 2001a</td>
</tr>
<tr>
<td><strong>B. alvinipulli</strong></td>
<td>Poultry</td>
<td>Intestinal spirochaetosis</td>
<td>Swayne et al., 1995; Stanton et al., 1998</td>
</tr>
<tr>
<td><strong>B. aalborgi</strong></td>
<td>Man</td>
<td>Intestinal spirochaetosis</td>
<td>Hovind-Hougen et al., 1982</td>
</tr>
<tr>
<td></td>
<td>Non-human primate</td>
<td>Intestinal spirochaetosis</td>
<td>Duhamel et al., 1997; Munshi et al., 2003</td>
</tr>
<tr>
<td>“B. canis”</td>
<td>Dog</td>
<td>Not reported</td>
<td>Duhamel et al., 1998b; Johansson et al., 2004</td>
</tr>
<tr>
<td>“B. pulli”</td>
<td>Poultry</td>
<td>Not reported</td>
<td>McLaren et al., 1997; Phillips and Hampson, 2005</td>
</tr>
</tbody>
</table>

1 The proposed new species are indicated in quotation marks.
culture) and grows in a wider pH range (pH 5.6–8.0 vs. pH 6.0–8.0) than the other porcine Brachyspira species (Trott et al., 1996c). B. pilosicoli survives outside the host longer than B. hyodysenteriae. In laboratory conditions, B. pilosicoli has survived in faeces over 210 days and in soil or faeces-mixed soil over 119 days at 10°C (Boye et al., 2001). In lake water, B. pilosicoli has survived 66 days at 4°C and 4 days at 25°C (Oxberry et al., 1998).

The pattern of antibiotic susceptibility is important to consider when a selective medium for isolation is formulated. Like B. hyodysenteriae, B. pilosicoli is resistant to spectinomycin, colistin and vancomycin. However, spiramycin and rifampin retard the growth of B. pilosicoli, whereas B. hyodysenteriae is resistant to these antimicrobials (Trott et al., 1996c).

### 2.4.2 Porcine intestinal spirochaetosis

PIS has been recognized worldwide in all major pork-producing areas. In studies conducted in some European countries and Brazil, B. pilosicoli has been isolated from 1–52% of investigated herds (Møller et al., 1998; Thomson et al., 1998; Barcellos et al., 2000b; Heinonen et al., 2000b; de Arriba et al., 2002; Jacobson et al. 2005). The results of these studies are not, however, comparable because some of the surveys have targeted diarrhoeic herds, and some have examined both healthy and diarrhoeic herds. B. pilosicoli was detected in only one out of 125 Spanish herds with diarrhoea problems (de Arriba et al., 2002). The routine use of antimicrobials might have suppressed the growth of B. pilosicoli in some herds, thereby masking its presence. Additionally, the use of antimicrobial growth promoters in feed might have concealed B. pilosicoli positive herds. This was suggested by Heinonen et al. (2000b), who investigated the regional prevalence of intestinal spirochaetes in Finland by random sampling of the herds; B. pilosicoli was isolated from only one out of 20 herds using carbadox as a growth promoter, whereas B. pilosicoli was detected in three out of four herds not using antimicrobial feed additives.

PIS most commonly affects pigs aged 8–13 weeks, but symptoms are sometimes observed at 5–20 weeks of age, as well. The incubation time varies from 2 to 20 days. The infection causes typhlocolitis, which manifests as loose, cement-like faeces with occasional blood flecks. Pigs are non-febrile or their body temperature is slightly raised. Weight gain of pigs is markedly reduced during the diarrhoeic period. Recovery time varies, and the disease leads to uneven growth of pigs in the same group. Mortality is low unless intestinal co-infections aggravate the lesions (Taylor and Trott, 1997; Thomson et al. 1998; Hampson and Trott, 1999).

The gross lesions on the mucosa of the large intestine vary from almost normal to increased mucus excretion, oedema, haemorrhage and focal necrosis. Microscopically, a neutrophilic and lymphocytic typhlocolitis with microabscesses can be seen. B. pilosicoli cells attach onto the epithelial surface densely and side by side, which causes a phenomenon known as a false brush-border. The only other Brachyspira species capable of forming a similar fringe of densely packed spirochaetes on the intestinal epithelium is B. aalborgi, which is encountered in humans and non-human primates. This phenomenon is seemingly dependent on the stage of infection and the degree of post-mortem changes because it is rarely seen in routine necropsy material (Taylor and Trott, 1997; Thomson et al., 1997; Jensen et al., 2004). Beside epithelial attachment, B. pilosicoli can invade the intestinal lamina propria (Jensen et al., 2000; Duhamel, 2001).
Specific virulence factors of *B. pilosicoli* remain poorly understood. One explanation for the diarrhoea is that the densely packed spirochaetes on epithelial cells cause a physical blockage of absorption of fluids and disrupt the epithelial microvilli (Taylor and Trott, 1997). Recent studies have investigated proteolytic membrane proteins of *B. pilosicoli* that may interact with the host (Trott *et al.*, 2004, Dassanayake *et al.*, 2004).

The severity of PIS depends on several factors. Managemental measures for diminishing the amount of circulating bacteria are of great importance. Co-infections with other enteric pathogens, especially with *Lawsonia intracellularis*, *Yersinia* spp. or *Salmonella* spp., aggravate the symptoms (Thomson *et al.*, 1998; Jacobson *et al.*, 2003). Variability in pathogenicity of different *B. pilosicoli* strains (Thomson *et al.*, 1997; Jensen *et al.*, 2004) and individual differences in hosts’ cellular responses observed in conjunction with SD (Jonasson *et al.*, 2004), may also have an influence on manifestation of PIS.

### 2.4.3 Detection of *Brachyspira pilosicoli*

The diagnosis for PIS is based on detection of the organism by culture or molecular methods. Because *B. pilosicoli* strains are antigenically highly diverse (Lee and Hampson, 1999), no serological methods for diagnosis are yet available.

#### 2.4.3.1 Isolation methods

Rectal samples from live pigs should be placed in transportable media intended for an aerobes. For transport, the samples should be cooled but not frozen. Several combinations of antimicrobial agents are used for selective culture of *Brachyspira* spp. CVSBA is a commonly used solid medium containing colistin, vancomycin and spectinomycin, and it is suitable for searching for all porcine *Brachyspira* species (Jenkinson and Wingar, 1981). Some media that have been developed for identifying *B. hyodysenteriae* include spiramycin...
and rifampin, and therefore are suboptimal for isolation of *B. pilosicoli* (Kunkle and Kinyon, 1988). Enrichment methods have been developed for isolating *B. hyodysenteriae* (Fellström et al., 2001b) and *B. pilosicoli* (Calderaro et al., 2005), but in the case of *B. pilosicoli* further comparative studies between the methods are needed.

Isolation of *Brachyspira* spp. from faecal or intestinal samples is enhanced when the agar surface has been sliced before culturing. The spirochaetes glide along the cuts out of the cultivation site, and thus, a pure subculture is achieved faster (Olson, 1996). According to our unpublished studies, the speed of diverging growth of *Brachyspira* is enhanced when the cuts on the agar are very shallow. Further subcultures on nutrient-rich media, such as fastidious anaerobe agar (FA), provide a sufficient bacteria mass for subsequent analyses. The strains are stored at −70°C, or better yet in liquid nitrogen (−196°C), in a broth containing serum and glycerol.

2.4.3.2 Phenotypic differentiation

Differential diagnostics of porcine *B. pilosicoli* by biochemical methods is based on positivity for hippurate hydrolysis and negativity for β-glucosidase (Fellström et al., 1995). Hip-

![Figure 3](image_url) Growth of *B. pilosicoli* from porcine faecal sample (a) streaked transversally on presliced selective CVSBA plate. Three-day-old culture incubated anaerobically at 42°C. Diverging growth of *B. pilosicoli* has reached the ends of the cuts (b). (Photo: M. Fossi)
purate and glucosidase tests are simple 4-h or overnight tests, which record the indicative
colour change in test broths that have been incubated aerobically at 37°C. The hippurate
test has been proposed as a key test for *B. pilosicoli*, offering good species determination
(Fellström et al., 1997). To date, avian *B. alvinipulli* is the only other *Brachyspira* species
that has been shown to hydrolyse hippurate (Stanton et al., 1998). Glucosidase tests may
be overlooked because of the distinctive hippurate test for *B. pilosicoli*. In our experience,
the results from glucosidase tests are, however, sometimes poorly interpretable because of
a faint colour reaction.

Occasional biochemically aberrant strains, or mixed cultures of several *Brachyspira* strains
can confuse species determination. In such cases, the presence of *B. pilosicoli* can be sug-
gested by microscopy; the reference strains of *B. pilosicoli* and *B. hyodyssenteriae* as well
as the culture being studied are smeared side by side on the same slide for comparative
evaluation of bacteria cell sizes.

Tests for carbohydrate utilization are not in common use in *Brachyspira* diagnostics. Utiliza-
tion of various sugars has been studied only for a limited number of *Brachyspira* strains,
mainly in conjunction with characterization of the type strains of *Brachyspira* species (Trott
et al., 1996d; Stanton et al., 1997, 1998). Carbohydrate utilization tests last at least over-
night and require anaerobic conditions, which might explain their disfavour.

2.4.3.3 Molecular identification

Numerous DNA techniques have been applied for taxonomic and subspecies resolution
of *Brachyspira* bacteria. Sequence analysis of genes coding for ribosomal RNA (rDNA) has
been adopted as a powerful tool for phylogenetic studies of bacteria (Olsen and Woese,
1993). The bacterial 16S rDNA is highly conserved in general, and similarity of 16S rDNA
sequences among the *Brachyspira* species is reported to be ≥95.7% (Pettersson et al.,
1996). The sequencing of 16S rDNA in particular has resolved the taxonomy of *Brachyspira*
spp. from genus to subspecies level (Pettersson et al., 1996, 2000; Johansson et al., 2004).
In 16S rDNA, there are eight evolutionary, highly conserved universal sequences, which
are exploited as landmarks when the areas between the universal sequences are studied.
*B. pilosicoli* has a unique hexamere of consecutive thymines in 16S rDNA nucleotide posi-
tions 176–181 (consensus sequence of *Brachyspira spp.*, Pettersson et al., 1996), an area
commonly included in the forward primer for several *B. pilosicoli*-specific polymerase chain
reaction (PCR) applications (Park et al., 1995; Fellström et al., 1997; Mikosza et al., 2001).
However, a hexamere and even a heptamere of thymines have been identified in 16S rDNA
sequences of various avian *Brachyspira* isolates other than *B. pilosicoli* just recently (Jans-
son et al., 2005). These strains cause false diagnosis when only a 16S rDNA-based PCR is
used.

Leser et al. (1997) developed a *B. pilosicoli*-specific PCR based on 23S rDNA. Used in con-
junction with a 16S rDNA-based PCR, the risk of false results due to a single nucleotide
aberration can be avoided. The NADH oxidase gene (*nox*) has been shown to be a suit-
able target for PCR applications for several porcine *Brachyspira* species, but in the case of
*B. pilosicoli* its sensitivity is poor (Atyeo et al., 1999b). A PCR-based restriction fragment
length polymorphism (RFLP) has been applied for differentiation of *Brachyspira* species
from porcine (Rohde et al., 2002) or avian (Townsend et al., 2005) origin. The restriction patterns obtained with RFLP are species-specific, and the agreement between RFLP and species-specific PCR is quite good (Townsend et al., 2005).

A species of *Brachyspira* can be identified from fixed tissue samples by PCR when the specific target region in the DNA is relatively short, preferably less than 500 bp in length. Kraatz et al. (2001) identified *B. pilosicoli* and *B. aalborgi* from human colonic biopsies by sequencing the 207-bp amplicons from 16S rDNA obtained by primers designed for the genus *Brachyspira*. Mikosza et al. (1999) approached the same issue, but focused specifically on detection of *B. pilosicoli* and *B. aalborgi* from the biopsies; the species-specific primers designed for 196- to 472-bp sequences from both 16S rDNA and *nox* yielded the expected amplicons.

Fluorescent *in situ* hybridization (FISH) is a method in which an oligonucleotide probe is used for visualization of the target organism in tissue. This is advantageous when exact localization of the organism in tissue is needed or when the organism is unculturable. The specificity of designed probes can vary between domain bacteria and a single strain. For *Brachyspira* research, probes targeting 16S or 23S rDNA have been developed for *B. pilosicoli*, *B. hyodysenteriae* and *B. aalborgi*, as well as for the genus *Brachyspira* spp. (Boye et al., 1998; Jensen et al., 2001, 2005). In routine work, FISH is a faster method for *in situ* detection than e.g. immunohistochemical methods, but post-mortem autolysis of tissue samples can cause false-negative results. For *B. pilosicoli*, the sensitivity of FISH does not appear to exceed the sensitivity of cultivation, probably due to the localization of *B. pilosicoli* bacteria on the surface of the epithelium (Jensen, 2005).

### 2.4.3.4 Fingerprinting

For epidemiological purposes, subspecies differentiation of *Brachyspira* spp. is typically done by multilocus enzyme electrophoresis (MLEE) (Lee et al., 1993b; Stanton et al., 1996; Trott et al., 1996a, 1998; Oxberry et al., 2003), pulsed-field gel electrophoresis (PFGE) (Atyeo et al., 1996, 1999a; Trott et al., 1996a, 1998; Raymond et al., 1997; Atyeo et al., 1999a; Fellström et al., 1999; Suriyaarachchi et al., 2000), or by amplified fragment length polymorphism (AFLP) (Møller et al., 1999).

MLEE is one of the most frequently used methods for typing of intestinal spirochaetes. It has been used for identifying all of the currently recognized *Brachyspira* species, as well as for within-species subtyping. MLEE is, however, too time-consuming and laborious for daily use. PFGE is a popular method for subspecies typing, not only because of its high discrimination power but also because of its excellent reproducibility. The first comprehensive study of subspecies differentiation of *B. pilosicoli* strains was done by PFGE (Atyeo et al., 1996). The discriminatory power was found to be superior to MLEE, a finding confirmed in subsequent studies (Raymond et al., 1997; Trott et al., 1998). Recent technical improvements have decreased the drawbacks of PFGE, i.e. time-consuming protocol and occasional DNA degradation (Gautom, 1997; Koort et al., 2002). The third technique, AFLP, has been found to have a discriminatory power equal to that of PFGE, but its reproducibility is slightly lower (Møller et al., 1999; Savelkoul et al., 1999).
2.5 Transmission of infection

In a herd, PIS can spread by direct pig contact, by feed, by faeces-contaminated surfaces and fomites and by water. Replacement breeding animals and rearing pigs can transmit the infection between herds (Taylor and Trott, 1997; Oxberry et al., 1998, 2003). In addition, evidence has emerged that wild birds and mice can act as reservoirs and transmit B. pilosicoli to pigs (Oxberry et al., 1998; Fellström et al., 2004). A natural cross-species transmission of B. pilosicoli to pigs has not been confirmed, but an experimental infection of weaned pigs by human B. pilosicoli strains led to symptoms of PIS (Trott et al., 1996b). Moreover, an experimental infection of chickens by porcine and human B. pilosicoli strains caused avian intestinal spirochaetosis with symptoms (Trott et al., 1995).

Transmission of intestinal spirochaetes from nursing sows to offspring has not been reported. Young sucklings probably cannot be colonized by anaerobic spirochaetes because of the higher oxygen concentration in their intestines than in weaned pigs (Hillman et al., 1993) and because of protective antibodies in sow’s milk. The creep feed served before weaning might increase fermentation and subsequently decrease oxygen content in the colon, thus enabling colonization by intestinal spirochaetes.

2.6 Treatment and control of porcine intestinal spirochaetosis

Clinical PIS can be treated by antimicrobials. Macrolides, such as tylosine and lincomycin, and tiamulin and valnemulin, belonging to pleuromutilins, have been among the most popular agents used to treat spirochaetal diarrhoea in pigs. The medication should, however, be combined with improved hygiene in the pens and prevention of carriage by fomites. Reappearance of symptoms after medication is discontinued is common (Spearman et al., 1988), especially when improved cleaning measures are not permanently adopted.

Antimicrobial resistance of Brachyspira spp. has become common, especially among B. hyodysenteriae strains, but the trend has also been observed in B. pilosicoli strains (Duhamel et al., 1998a; Fossi et al., 1999; Karlsson et al., 2004b). This can be explained by the frequent co-occurrence of B. pilosicoli and B. hyodysenteriae, with B. hyodysenteriae being the principal initiator of therapy, as well as by continuous low-level medication on farms (Thomson et al., 1998; Stege et al., 2000). Trans-species spread of genes responsible for antimicrobial resistance among Brachyspira spp. has not been reported. Pleuromutilins have been effective against porcine spirochaetal diarrhoea (Blaha et al., 1987; Duhamel et al., 1998a; Messier et al. 1990). However, the minimal inhibitory concentration (MIC) values of pleuromutilins have been elevated for an increasing proportion of B. hyodysenteriae and B. pilosicoli isolates investigated over the last decade (Molnár, 1996; Karlsson et al., 2004a, b; Lobová et al., 2004).

Good management principles, segregation of pigs by age group, and sanitation measures, including rodent control, are crucial preventative measures for PIS. B. pilosicoli is sensitive to most of the common disinfectants (Corona-Barrera et al., 2004) and to drying. At a temperature of −70°C, B. pilosicoli survived in faeces for 14 days (Barcellos et al., 2000a). Similar studies at temperatures of 0°C to −20°C, simulating winter in northern areas, are
lacking. In our experience, recovery of *Brachyspira* spp. from faecal samples after a short freezing period at −18°C to −20°C is very poor.

The prevalence and severity of spirochaete-induced diarrhoea in pigs are feed-related (Durmic *et al.*, 1998; Pluske *et al.*, 1998; Stege *et al.*, 2001). Generally, fermentable carbohydrates in the large intestine promote thriving of intestinal spirochaetes. Experimentally, a highly digestible diet based on cooked rice and animal protein has prevented colonization of *B. hyodysenteriae* (Pluske *et al.*, 1996). In a corresponding study for *B. pilosicoli*, colonization of bacteria could not be prevented, but the symptoms of PIS were milder (Hampson *et al.*, 2000). From an economic point of view, highly digestible feed is not feasible in practice.

To date, no effective vaccine for control of PIS exists. One vaccination trial investigating parenteral *B. pilosicoli* bacterin has been reported. When infected with the homologous *B. pilosicoli* strain, vaccinated and unvaccinated pigs developed PIS with equal intensity (Hampson *et al.*, 2000). A recent immunization trial for SD using a subunit vaccine from recombinant outer-membrane lipoprotein BmpB of *B. hyodysenteriae* has been promising (La *et al.*, 2004). To our knowledge, no similar studies with *B. pilosicoli* have been published.
3 Aims of the study

The aims of the thesis were to investigate epidemiological characteristics of porcine \textit{B. pilosicoli} and to improve differential diagnostics of the infection. The specific aims were as follows:

1. to study the genetic diversity of \textit{B. pilosicoli} in Finnish pig herds (I, II);

2. to investigate the genetic relationship between hippurate-negative and -positive phenotypes of \textit{B. pilosicoli} (II), and to reinforce the scheme for phenotypical differentiation of porcine \textit{B. pilosicoli} by adding a discriminating carbohydrate utilization test (III);

3. to investigate the infectivity of hippurate-negative \textit{B. pilosicoli} by experimental infection (IV);

4. to study the possibility of eradicating \textit{B. pilosicoli} infection from a sow herd without total depopulation (V).
4 Materials and methods

4.1 Sampling (IV, V)

The faecal samples from live pigs were taken by swabs which were placed into transportable culture media intended for facultative and obligatory anaerobes (Probact transport swab, Technical Service Consultants Ltd., Heywood, UK). The swabs were streaked on culture media within 48 h (V) or 4 h (IV). At necropsy (IV), pieces 5–10 cm in length were dissected from the caecum, the proximal, mid-colon and distal colon, and from the rectum. The intestine samples were transected just prior to cultivation, and the mucosal scrapings were cultured within 4 h of necropsy.

4.2 Bacterial isolates and strains (I–V)

For the molecular epidemiological study (I), altogether 131 B. pilosicoli isolates were chosen from isolates obtained during 1997–2000 from 49 farrowing and farrow-to-finish herds located in the southern (S) and northern (N) pork production areas of Finland. Diarrhoea was a problem in 38 herds, no diarrhoea was observed in five herds and health status was unknown in six herds. In 41 herds, 1–10 B. pilosicoli isolates per herd were obtained from rectal samples of weaned pigs. In eight herds, a single B. pilosicoli isolate from necropsy samples was included.

For characterization of hippurate-negative B. pilosicoli (B. pilosicolihipp) (II), 11 B. pilosicolihipp isolates were studied. The isolates were obtained during 1997–2000 from seven unrelated farrowing and farrow-to-finish herds. In addition, nine biochemically common B. pilosicoli isolates, obtained concomitantly with B. pilosicolihipp isolates from three of these herds, were included.

For the study of D-ribose utilization (III), 53 B. pilosicoli isolates with a common biochemistry, seven hippurate-negative biovariants of B. pilosicoli, ten B. hyodysenteriae, eight B. intermedia and 14 Brachyspira spp. group III isolates were used. The isolates were obtained during 1997–2004. Thirty-six of the B. pilosicoli isolates had been shown to represent different genotypes (in Study I), and seven of the B. hyodysenteriae isolates to have dissimilar lipo-oligosaccharide profiles in their cell walls (Fossi et al., 2004). The remaining isolates were obtained from unrelated sources.

The B. pilosicolihipp strain Br1622 for the infection trial (IV) originated from a farrowing herd sampled in 2000. The 16S rDNA sequence of this strain possessed 12 nucleotide mismatches with the respective sequence from B. pilosicoli type strain P43/6/78T.

For the eradication study (V), weaners of the trial herd were sampled before eradication four times between January and May 1997. At least one B. pilosicoli isolate per sampling (altogether six isolates) was stored. Two and five B. innocens isolates, before and after eradication, respectively, were also stored.

The type and reference strains used in these works were purchased from the American Type Culture Collection (ATCC): B. pilosicoli P43/6/78T (ATCC 51139) (I–III), B. hyodysente-
riae B78T (ATCC 27164) (II), B. hyodysenteriae B204 (ATCC 31212) (II, III), B. intermedia PWS/A T (ATCC 51140) (III), B. innocens B256T (ATCC 29796) (III) and B. murdochii 155–20 (ATCC 700173) (III). All of the isolates were stored at −70°C in beef broth (Merck, Darmstadt, Germany) supplemented with 12% horse serum and 15% glycerol.

4.3 Isolation (IV, V) and phenotypic identification (I–V)

Faecal swabs and intestinal scrapings were streaked on pre-reduced and pre-sliced selective CVSBA plates transversally over the agar cuts. For selection, the agar base (Blood agar base no 2, Oxoid Ltd., Basingstoke, Hampshire, England) with 5% defibrinated sheep blood and 1% sodium ribonucleinate was supplemented with 0.4 mg ml\(^{-1}\) spectinomycin, 0.025 mg ml\(^{-1}\) colistin and 0.025 mg ml\(^{-1}\) vancomycin. The plates were incubated in anaerobic chambers at 42°C in an atmosphere of 90% N\(_2\) and 10% CO\(_2\) (Anaerogen\(\text{TM}\), Oxoid Unipath Ltd.). The plates were inspected every third or fourth day up to 14 days for Brachyspira-like growth. Suspected Brachyspira colonies were subcultivated on CVSBA until the culture was deemed pure by microscopy. For species determination by biochemical methods and for storage, the isolates were subcultured on fastidious anaerobe (FA) agar (Lab M\(\text{TM}\), Lancashire, UK), which was supplemented with 7–10% defibrinated horse or bovine blood.

The Brachyspira isolates were phenotyped according to the scheme of Fellström et al. (1999). Haemolysis was assessed as weak or strong by culturing the isolate, the B. hyodysenteriae reference strain B204, and the B. pilosicoli type strain P43/6/78\(\text{T}\) simultaneously on the same typticase-soy (TS) agar (BBL\(\text{TM}\), Becton, Dickinson and Co., Le Pont de Clai, France) plate supplemented with 5% defibrinated bovine blood.

Indole production was assessed by smearing the culture on a filter paper saturated with a reagent containing 1% p-dimethylaminocinnamaldehyde in 10% hydrochloric acid (Sutter and Carter, 1972). Appearance of a bluish-purple colour indicated positive reaction, and a pink tone a negative reaction for indole production. B. intermedia PWS/A\(\text{T}\) was used as a positive control. The test for hippurate hydrolysis was performed according to Rübsamen and Rübsamen (1986) and is described in detail in Paper III. The tests for α-galactosidase, α-glucosidase and β-glucosidase activities were performed by using the respective diagnostic tablets (Diatabs\(\text{TM}\), Rosco Diagnostica A/S, Taastrup, Denmark) according to the manufacturer’s instructions.

Isolates showing weak β-haemolysis, positive hippurate reaction, negative indole reaction and negative α-glucosidase and β-glucosidase reactions were classified as B. pilosicoli. The strains with the reactions listed above except for no positive hippurate reaction were preliminarily assessed as atypical isolates. For seven atypical isolates, which originated from different herds, the stability of the negative hippurate reaction was confirmed as follows: consecutive culture passages, which were incubated in parallel at 38°C and 42°C as doublets on FA agar, were tested for hippurate hydrolysis from seven to ten passages. All of the test results were negative for hippurate hydrolysis. All of these isolates were included in Study II, and one of the isolates in Study IV.

The protocol for the D-ribose test is described in detail in Paper IV. In brief, D-ribose utilization was demonstrated indirectly by recording pH reduction in the broth culture. Tryptone-peptone base broth contained 7% foetal calf serum (FCS). One per cent D-ribose was
added for testing D-ribose utilization. A sugar-free base broth was added on each testing occasion. The bacteria were suspended in the broths and incubated anaerobically on a planar shaker for 28–30 h. The difference in pH reduction between the D-ribose broth and the base broth was recorded. One per cent glucose or mannose replaced D-ribose when utilization of these sugars was tested. Reproducibility of the test and optimal cell density in broths were evaluated as follows: two *B. pilosicoli* field strains and the *B. pilosicoli* type strain P43/6/78^T were tested three times in D-ribose broth supplemented with FCS, in parallel in twofold cell densities of $2.3 \times 10^7$, $4.5 \times 10^7$ and $9.0 \times 10^7$ cells ml$^{-1}$, equivalent to McFarland units 2, 4 and 8, respectively. The reduction in pH was compared between the different cell densities in tests, and variation in pH reduction between test occasions in different cell densities was analysed.

### 4.4 Polymerase chain reaction (I–V)

All of the isolates were studied by two *B. pilosicoli*-specific PCR assays. The primers targeting 930 bp of ribosomal 16S rDNA have been described by Fellström et al. (1997), and those for 143 bp of 23S rDNA by Leser et al. (1997). The PCRs were performed as outlined by these authors.

A species-specific nested-PCR was used for detection of *L. intracellularis* in Study IV. The samples were prepared according to McOrist et al. (1994), and the primer pairs and PCR protocol described by Jones et al. (1993) were applied.

### 4.5 Sequencing of 16S rDNA (III)

The nucleotide sequence of 16S rDNA was almost completely sequenced for three *B. pilosicoli* hipp isolates. In addition, a partial 16S rDNA sequence of 877 bp was studied for five *B. pilosicoli* hipp isolates and two *B. pilosicoli* isolates. An almost complete 16S rDNA sequence (1435 bp out of 1485 bp) was obtained as described by L'Abee-Lund et al. (2003). All of the primers were universal (Weisburg et al., 1991). The nucleotide sequence obtained, with a length of 1435 bp, covered the *Brachyspira* consensus sequence (Pettersson et al., 1996) from position $\text{--8}$ to position 1431.

The partial 16S rDNA sequence of 877 bp, covering the *Brachyspira* consensus sequence from position 183 to position 1059, was obtained from the amplicon produced by *B. pilosicoli*-specific PCR. The PCR was performed as described by Fellström et al. (1997). The amplicon was sequenced directly or cloned with a PCR-Script Amp cloning kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The sequencing was purchased from AIV Institute, Kuopio, Finland.

### 4.6 Pulsed-field gel electrophoresis (I, II, IV, V)

Preparation of agar plugs, macrorestriction of bacterial DNA and electrophoresis are described in detail in Paper I. In brief, the washed bacteria in phosphate-buffered saline were mixed in agarose and hardened in a mould. The agarose plugs were placed in lysis solution
containing lysozyme and RNAase for 24 h at 37°C. Next, the plugs were placed in proteinase solution (proteinase K, sodium lauroyl sarcosine) for 24 h at 50°C. Prior to digestion, the plug slices were dialysed in TE buffer. Rare-cutting enzyme *Mlu*I was used for macro-restrictive digestion of all isolates and strains, and *Smal* was used for 70 out of 131 isolates or strains in Study I as well as for all 13 isolates in Study V.

The digested plugs were loaded on to a 1% agarose gel and subjected to electrophoresis for 22 h at 14°C with a linear ramp of 2–30 s and 9–55 s for plugs digested with *Mlu*I and *Smal*, respectively. The gels supplemented with ethidium bromide were photographed with UV illumination, and the images were scanned and analysed using GelCompar program (Applied Maths, Kortrijk, Belgium). Clustering analysis, based on the unweighted pair-group method with arithmetic averages (UPGMA), was performed in the first and second studies.

4.7 Fluorescent *in situ* hybridization (IV)

The colon and caecum sections of all 23 pigs in the infection trial were subjected to fluorescent *in situ* hybridization (FISH). The oligonucleotide probes used are described in Paper IV. A species-specific probe for *B. pilosicoli* hippoc strain Br1622 and genus-specific probes for *Treponema*, *Leptospira* and *Borrelia* were selected using ARB software (Strunk et al., 2000). Br1622 could be differentiated from *B. pilosicoli* type strain P43/6/78T because of differences in 16S rDNA nucleotide sequences. The probe designed for Br1622 had at least two mismatches to all other *Brachyspira* species in the database. The general bacterial probe EUB338, the genus-specific probe for *Brachyspira* and the species-specific probe for *B. pilosicoli* have been described previously by Boye et al. (1998).

Prior to hybridization, the sections were deparaffinized in xylene and transferred to ethanol for 10 min. The sections were circumscribed with a hydrophobic PAP pen (Daido Sangyo Co. Ltd., Tokyo, Japan). Hybridization was carried out at 37°C for 16 h in a moisture chamber with 30 µl of hybridization buffer (100 mM Tris, 0.9 M NaCl, 0.1% sodium dodecyl sulphate) containing 150 ng of probe. Washing was done in 100 ml of hybridization buffer for 15 min and then in 100 ml of washing solution (100 mM Tris, 0.9 M NaCl) for 15 min. The samples were rinsed in water, air-dried and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) prior to microscopy.

An Axioplan2 epifluorescent microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 75 W Xenon lamp and filter set XF53 (Omega Optical, Brattleboro, VT, USA) was used for simultaneous detection of red and green fluorescence.

4.8 Transmission electron microscopy (II, IV)

For ultrastructural study of cultured bacteria, *B. pilosicoli* hippoc isolates Br980 and Br1048, originating from different herds, *B. hyodysenteriae* type strain B-78T and *B. pilosicoli* type strain P43/6/78T were propagated on FA agar for three days at 37°C. The bacteria were suspended in 0.1 M phosphate buffer at a concentration of 1.0 × 10^8–9 cells ml^{-1}. The suspension was applied to carbon-coated grids and incubated for 1 min. The grid was then
rinsed with distilled water. Finally, the samples were negatively stained with 1% phosphotungstic acid for 10–20 min (Utriainen et al., 1997).

For ultrastructural study of unidentified bacteria in situ, 1.0 mm² samples of the colonic and caecal mucosa were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 4 h. The samples were embedded in Epon™, and thin sections were stained with 3% uranyl acetate for 40 min and 3% lead citrate for 1.5 min.

The samples were visualized and photographed using a Jeol Jem 100-s electron microscope. For the cultured Brachyspira bacteria, mean values for the cell dimensions were calculated from the measurements of 20 bacteria in the images.

4.9 Antimicrobial susceptibility testing (V)

Sensitivity of three B. pilosicoli and two B. innocens isolates to tiamulin before eradication, and three B. innocens isolates after eradication was studied. The MIC of tiamulin was determined by an agar dilution method using twofold dilutions of tiamulin-fumarate (Bio Cheme®) from 1.0 µg ml⁻¹ to 0.063 µg ml⁻¹ in TS agar supplemented with 5% defibrinated sheep blood (Rønne and Szancer, 1990; Fossi et al., 1999). Plastic plates of 9 cm diameter were used (Sterilin Ltd., code 101RT). TS agar without tiamulin was included in each assay for growth control.

The bacteria culture, which was grown on FA agar, was suspended in buffered saline, and the density of bacteria was adjusted with the barium sulphate standard of McFarland no.1 (bioMérieux®). Three droplets of bacteria suspension were spread on each test and control agar. The agar plates were incubated for six days anaerobically at 42°C. The lowest concentration of tiamulin that prevented haemolytic growth was interpreted as the MIC. A strain was assessed as sensitive to tiamulin when MIC was ≤ 1 µg ml⁻¹ (Rønne and Szancer, 1990).

4.10 Study design for hippurate-negative B. pilosicoli infection trial (IV)

Details of study design and measures during the trial are provided in Study V. In brief, the 24 piglets were separated from their sows at the age of 11 days and moved to barrier facilities, where they were raised together until being divided into three trial groups at the age of 45 days. During the next two days, the pigs in groups 1 and 2 were inoculated intragastrically with doses of 3.2–4.0 × 10⁹ cells of B. pilosicolihipp strain Br1622 and B. pilosicoli type strain P43/6/78 T, respectively. The third trial group was sham-inoculated with sterile broth. Faecal samples for enteric pathogens and blood samples for haematology were taken, and the body weights were recorded regularly. The pigs were necropsied within 7–23 days post inoculation (p.i.), and samples were taken for routine histopathology, TEM and microbiology. Antibodies for L. intracellularis were analysed by using an indirect fluorescent antibody test (IFAT) (McOrist et al., 1987; Lawson et al., 1988; Knittel et al., 1998) from the colostrum of sows and the sera of pigs at the age of 42 days and at necropsy.
4.11 Actions taken for eradication of *B. pilosicoli* (V)

The farrowing herd of 60 sows had suffered from post-weaning problems for many years. All four samplings between January and August 1997 had revealed *B. pilosicoli* in abundance. The MIC values of tiamulin for the three *B. pilosicoli* isolates analysed were ≤ 0.125 µg ml\(^{-1}\).

Eradication was undertaken during August-October 1997. Before moving the animals, in-feed medication with tiamulin (Tiamutin®) 200 p.p.m. was started. The sucklings were not medicated. The sows were medicated for 30 days, the weaners for 23 days and the finishing pigs for 18 days.

The old, non-segregated piggery had to be emptied at the same time; the farrowing sows and weaners were moved to a barn 20 m from the piggery, the dry sows and the boar to a paddock next to the piggery, and the finishing pigs to a shed located 100 m from the piggery. None of the growing pigs were returned to the piggery but were sold by December 1997.

The piggery was swept, washed, disinfected and left empty for 25 days. Old wooden materials were burned and worn surfaces repaired. The manure pit was disinfected and the manure was transported to remote fields. Rodents were controlled during and after the eradication by poison baits.

The sows and the boar were returned to the piggery between September 10 and October 3. All replacement animals bought after this were medicated with Tiamutin® 200 p.p.m. for three weeks, during which time they were quarantined. The post-control samples for intestinal spirochaetes were taken at 5- to 14-month intervals, with the last samples collected in February 2002.
5 Results

5.1 Subspecies analysis of Brachyspira isolates by PFGE (I, II, IV, V)

Digestion of *B. pilosicoli* DNA by *Mlu* I and *Sma* I restriction enzymes yielded 9–15 and 6–10 DNA fragments, respectively. For *B. innocens* strains, the corresponding figures were 12–15 and 8–10. The discriminatory power with *Mlu* I and *Sma* I restriction enzymes was similar for both *Brachyspira* species. Problems with DNA degradation were not encountered for *B. pilosicoli*. A distinct macrorestriction profile (MRP) was not achieved for one *B. innocens* strain.

In the study of *B. pilosicoli* isolates from 49 sow herds (I), the 131 field isolates were divided into 54 MRPs. The 38 strains from area N were divided into 20 MRPs, and the 93 strains from area S into 35 MRPs. From 21 herds, more than one *B. pilosicoli* isolate was studied; in 14 herds only one MRP, in six herds two MRPs, and in one herd five MRPs were observed among the isolates.

The genetic variation was slightly higher among isolates in area S (57–100%) than in area N (67–100%). Clustering of the MRPs according to the two geographical areas was not seen. Three MRPs in area S and one MRP in area N were shared by two herds. One MRP crossed the geographical areas and was shared by two herds. Persistence of a single genotype was observed in herds re-sampled within three months (eradication herd) or within three years (two herds). In one herd, where altogether five MRPs were found in two samplings spaced three years apart, one MRP from 1997 was recovered again in 2000 and had a high resemblance to the MRP of another isolate recovered in 2000.

Genotyping of *B. pilosicoli* hipp-isolates by PFGE did not reveal clustering of the strains according to hippurate hydrolysis capacity. The 11 *B. pilosicoli* hipp isolates and nine hippurate positive *B. pilosicoli* isolates were divided into six and four MRPs, respectively. Two of the MRPs were shared by hippurate-negative and -positive isolates obtained from same herds. *B. pilosicoli* hipp-strain Br1622, which was used for the infection trial, shared an MRP with a *B. pilosicoli* hipp-isolate from another herd, although these isolates had one different nucleotide position in their 16S rDNA sequences. The two *B. pilosicoli* hipp isolates from necropsied pigs in the infection trial had a common MRP with strain Br1622.

From the herd for *B. pilosicoli* eradication, eight *B. innocens* isolates were also studied by PFGE. The five *B. innocens* isolates obtained after eradication had a common MRP, which was shared by one of the two MRPs from the three isolates cultured before eradication.

5.2 16S rDNA sequences and ultrastructure of hippurate-negative Brachyspira pilosicoli (II)

Two out of eight *B. pilosicoli* hipp isolates shared the same 16S rDNA nucleotide sequence with *B. pilosicoli* type strain P43/6/78T (*B. pilosicoli* P43). In comparison with the sequence of P43, three *B. pilosicoli* hipp isolates had ten common mismatches between nucleotide positions 214–589. In addition, one isolate had an 11th mismatch in position 996, and two isolates a 12th mismatch in position 1025. The database search did not reveal spiro-
chaete strains of phylogenetic group IV (*B. pilosicoli*) (Fellström and Gunnarsson, 1995) to share any of the ten mismatching nucleotides between positions 214–589. One of the two sequenced hippurate-positive *B. pilosicoli* isolates had the same ten mismatches between positions 214–589 as the above-mentioned six hippurate-negative isolates.

In TEM images, the cell width of the two *B. pilosicoli* hipp- isolates was 0.27–0.28 µm, and cell length 6.26–6.53 µm. These dimensions were fairly concordant with those of *B. pilosicoli* P43, the respective mean cell dimensions of which were 0.27 µm and 6.83 µm. The cells of *B. pilosicoli* hipp- isolates had pointed ends and six periplasmic flagella, characters also seen in *B. pilosicoli* P43.

### 5.3 D-ribose utilization of porcine *Brachyspira* spp. (III)

A preliminary unpublished study ascertained that the broth formula was feasible and pre-reduction of broths was not necessary for any of the porcine *Brachyspira* species. Six non-*B. pilosicoli* and two *B. pilosicoli* isolates were studied for glucose utilization by the same protocol as used for the final D-ribose study (Table 2). Excluding *B. hyodysenteriae* reference strain B204 (S.h. B204), all strains showed glucose utilization by a notable reduction in pH

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sugar</th>
<th>Difference in pH reduction units between sugar broth and sugar-free control broth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Broths pre-reduced</td>
</tr>
<tr>
<td><em>B. innocens</em></td>
<td>Glucose</td>
<td>1.4</td>
</tr>
<tr>
<td>ATCC type strain B256&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. innocens</em></td>
<td>Glucose</td>
<td>1.3</td>
</tr>
<tr>
<td>field isolate Br2132</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. intermedia</em></td>
<td>Glucose</td>
<td>1.3</td>
</tr>
<tr>
<td>ATCC type stain PWS/A&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. murdochii</em></td>
<td>Glucose</td>
<td>1.2</td>
</tr>
<tr>
<td>ATCC reference strain 155-20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. murdochii</em></td>
<td>Glucose</td>
<td>1.1</td>
</tr>
<tr>
<td>field isolate Br2331</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>Glucose</td>
<td>0.3</td>
</tr>
<tr>
<td>ATCC reference strain B204</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>Mannose</td>
<td>1.0</td>
</tr>
<tr>
<td>ATCC reference strain B204</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>D-ribose</td>
<td>1.0</td>
</tr>
<tr>
<td>ATCC type strain P43</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>D-ribose</td>
<td>1.3</td>
</tr>
<tr>
<td>field isolate Br1622</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hippurate-negative biovariant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
regardless of the pre-reduction status of the broths. Due to an unexpectedly weak glucose utilization, strain B204 was tested for mannose utilization with the same formula; mannose utilization was clearly shown.

For the three *B. pilosicoli* isolates tested three times, the minimum pH reduction in D-ribose broth was 1.7, 1.6 and 1.3 units by using cell densities equivalent to 8, 4 and 2 McFarland units, respectively. The more dense the inocula used, the lower the variation in pH reduction between consecutive tests. Thus, cell densities representing ≥5.0 McFarland units were used in the final test protocol.

In the final tests for D-ribose utilization, among the 60 *B. pilosicoli* strains the pH reduction by D-ribose varied from 0.95 to 2.28 units (mean 1.72), whereas in the control broth, pH reduction varied from 0.10 to 0.49 units (mean 0.27) (Figure 4). The mean pH reduction among the seven *B. pilosicoli* hipp strains did not differ from the overall mean of the *B. pilosicoli* isolates. The lowest pH reduction observed in D-ribose broth did not make the results uncertain because in such cases the pH reduction in concomitant control broth was also below the mean. For the 35 non-*B. pilosicoli* strains, mean pH reduction was 0.37 units in both D-ribose broth and control broth.

**Figure 4** Reduction in pH units by *B. pilosicoli* and non-*B. pilosicoli* strains in TS-broth (control) and in TSR-broth (with 1% D-ribose). The box encloses the middle 50% of results and is bisected by the median value. The ranges are shown by the whiskers.
5.4 Experimental infection of pigs by *B. pilosicoli* strain Br1622 and type strain P43 (IV)

Mean daily weight gain of the pigs after challenge did not differ between the trial groups. Values of haematological parameters remained within the normal range, except for leucocyte count of one pig in both inoculated groups and two pigs in the control group; values for these animals exceeded reference values in one sampling of the first week p.i. Signs of PIS were absent during the course of the trial, other than in one pig inoculated with *B. pilosicoli* P43 that had loose faeces on days 9 and 10 p.i. *Brachyspira* spp. or other enteropathogens were not detected in faeces samples of this pig or in any of the other trial pigs. In colon tissue samples, however, *B. pilosicoli*Br1622 was re-isolated from two pigs necropsied on days 15 and 16 p.i.

At necropsy and upon histological examination, slight inflammatory changes were observed on the colonic and caecal mucosa for the majority of pigs in all trial groups. In silver-stained sections, spiral-shaped bacteria were seen invading the colonic mucosa of most pigs; in five, six and two of the pigs in groups challenged with Br1622, type strain and sterile broth, respectively. The degree of mild colitis and typhlitis observed in HE-stained sections was not completely concordant with observations of spiral-shaped bacteria. FISH with probes specific for genus *Leptospira* and domain bacteria revealed invasive spiral-shaped bacteria in colon and caecum samples of five, five and three pigs in the above-mentioned groups, respectively. These FISH-positive cases only partially matched, with the cases where spiral-shaped bacteria were seen with non-specific silver staining (Table 3). FISH studies with the three probes for *Brachyspira* were negative.

In TEM, endoflagellated bacteria were seen invading the colonic mucosa of two pigs: in one pig that had been inoculated with Br1622 and necropsied 17 days p.i., and in the other pig that had been sham-inoculated and necropsied 21 days p.i. The presence of endoflagella suggested spirochaetes. However, the number of endoflagella observed, 12–14, was not consistent with *B. pilosicoli* or *Leptospira* spp.

*L. intracellularis* was not detected in the trial pigs by PCR or serology. Four of the nine sows in the same farrowing group had antibodies to *L. intracellularis* in their colostrum. However, these four sows were not the dams of the pigs chosen for the trial.
Table 3  Pigs inoculated with *B. pilosicoli* \(^{\text{hipp}}\) strain Br1622, *B. pilosicoli* type strain P43 or sterile broth (control). Pathology, histology and fluorescent *in situ* hybridization results are shown.

<table>
<thead>
<tr>
<th>Trial group</th>
<th>Inoculum</th>
<th>Pig</th>
<th>Necropsy day post-inoculation</th>
<th>Gross pathology(^1)</th>
<th>Histology</th>
<th>Spiral-shaped bacteria(^2)</th>
<th>FISH(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Br1622</td>
<td>1</td>
<td>7</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Br1622</td>
<td>2</td>
<td>9</td>
<td>−</td>
<td>(+)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Br1622</td>
<td>3</td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Br1622(^5)</td>
<td>4</td>
<td>15</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Br1622(^5)</td>
<td>5</td>
<td>16</td>
<td>++</td>
<td>(+)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Br1622(^6)</td>
<td>6</td>
<td>17</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Br1622</td>
<td>7</td>
<td>21</td>
<td>+</td>
<td>(+)</td>
<td>++</td>
<td>−</td>
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<tr>
<td></td>
<td>Br1622</td>
<td>8</td>
<td>23</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>P43</td>
<td>9</td>
<td>7</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>10</td>
<td>9</td>
<td>−</td>
<td>(+)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>11</td>
<td>11</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
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</tr>
<tr>
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<td>P43</td>
<td>12</td>
<td>15</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>13</td>
<td>16</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>14</td>
<td>17</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>15</td>
<td>21</td>
<td>+</td>
<td>(+)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>P43</td>
<td>16</td>
<td>23</td>
<td>++</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
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<td>3</td>
<td>control</td>
<td>17</td>
<td>7</td>
<td>−</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>18</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>19</td>
<td>17</td>
<td>−</td>
<td>(+)</td>
<td>++</td>
<td>+</td>
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<td>control</td>
<td>20</td>
<td>23</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>21</td>
<td>9</td>
<td>+</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>22</td>
<td>11</td>
<td>−</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>control(^6)</td>
<td>23</td>
<td>21</td>
<td>+</td>
<td>(+)</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^1\) Gross finding in caecum and/or colon. − = normal; + = mucosa slightly hyperaemic; ++ = mucosa hyperaemic and slightly oedematous.

\(^2\) (+) = diffuse lymphocytic and plasmacytic infiltration in lamina propria, and multifocal accumulation of phagocytic macrophages beneath the epithelial cells of the caecum and/or colon; + = in addition, occasional microabsesses in crypts and/or mild exocytosis of neutrophils.

\(^3\) Spiral-shaped bacteria in caecum and/or colon in silver-stained sections. − = none; + = very rare or irregularly observed in crypts and/or near tips of villi; ++ = invading through epithelial lining into lamina propria and/or abundantly in crypts.

\(^4\) Fluorescent *in situ* hybridization. Positive (+) and negative (−) results with probes designed for domain bacteria and genus *Leptospira*.

\(^5\) Br1622 re-isolated from necropsy samples.

\(^6\) Endoflagellated bacteria observed in colonic mucosa by transmission electron microscopy.
5.5 Eradication of *B. pilosicoli* (V)

During the post-control period from April 1998 to February 2002 *B. pilosicoli* was not detected in any sample taken from weaners or growers (Table 4). *B. innocens* was isolated in each sampling, before and after eradication. Severe post-weaning diarrhoea disappeared, with only single litters occasionally having short periods of mild diarrhoea around weaning time. Exact data of production before and after eradication were unavailable. According to the owner’s opinion, the daily weight gain of growers had improved markedly.

The MICs for tiamulin of three *B. pilosicoli* isolates obtained before eradication were ≤0.125 µg ml⁻¹. The MICs for two *B. innocens* isolates before eradication were ≤0.063 µg ml⁻¹, whereas after eradication, the MICs for three *B. innocens* isolates were 0.25, 0.125 and ≤0.063 µg ml⁻¹.

**Table 4** Faecal samples taken from growers post-eradication, all with negative results for *B. pilosicoli*

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>20</td>
<td>49¹,²</td>
<td>42¹</td>
<td>45¹</td>
<td>37¹</td>
<td>31¹</td>
<td>38¹</td>
</tr>
</tbody>
</table>

¹ Primary cultures also studied by species-specific PCR.
² Ten samples were from colons of slaughtered pigs weighing 110 kg.
6 Discussion

Differential diagnostics for intestinal spirochaetes was undeveloped in Finland until the middle of the 1990s. Since the biochemical classification scheme for WBHIS was applied, *B. pilosicoli* has been identified in numerous faecal samples of diarrhoeic pigs. The regional prevalence studies conducted in the late 1990s showed high occurrence of *B. pilosicoli* in herds producing rearing pigs. Farmers and field veterinarians were highly concerned about the “new” pathogen. The subsequent ban on antimicrobial growth promoters gave a further push for investigations on PIS. There was a lack of knowledge of the epidemiology of *B. pilosicoli*, control methods for PIS in Finnish pig production and laboratory diagnostics for *B. pilosicoli*; the studies presented here focused on improving information in these three areas.

6.1 Subspecies analysis of *Brachyspira* isolates by PFGE

PFGE has proven to be an excellent technique for studying within-species genetic variation of *Brachyspira* spp. of porcine origin (Atyeo et al., 1996, 1999a; Fellström et al., 1999), and the genetic relationship between *B. pilosicoli* strains from different species of animals and humans (Atyeo et al., 1996; Rayment et al., 1997; Trott et al., 1998; Fellström et al., 2001a) The banding patterns of macrorestricted DNA by PFGE are highly reproducible, which enables comparison of MRPs from different studies, in and between laboratories. The current international database libraries of genomic profiles exploit this quality of PFGE (Swaminathan et al., 2001; Lukinmaa et al., 2004).

The DNA of all *B. pilosicoli* isolates of the both phenotypes could be smoothly restricted. For a few isolates, a mixed culture of two genotypes was suggested by the very high numbers of bands; such isolates were excluded from the study. We observed degradation of bacterial DNA in one *B. innocens* isolate. A similar problem with *B. hyodysenteriae* has been described by Atyeo et al. (1999a). Using HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid) as a running buffer during electrophoresis instead of Tris-containing buffer has prevented DNA degradation in problematic isolates of various species of foodborne enteric pathogens (Koort et al., 2002; Lukinmaa et al., 2004), and this technical improvement might work with *Brachyspira* spp., as well.

We found the discriminatory power of PFGE to be the same when either *MluI* or *SmaI* was used as the restriction enzyme. Due to higher number of fragments obtained with *MluI*, i.e. 9–15, a number of isolates were studied solely by using this enzyme. Atyeo et al. (1996) and Trott et al. (1998) examined 52 and 167 *B. pilosicoli* isolates, respectively, from various animal species and humans by PFGE using *MluI* as the restriction enzyme. The number of banding patterns obtained in these studies varied in the same range as in our study.

6.2 Molecular epidemiology of *B. pilosicoli* by PFGE

Nationwide molecular epidemiological studies of porcine *B. pilosicoli* exploiting PFGE are few. Atyeo et al. (1996) and Rayment et al. (1997) also used *SmaI* or *MluI* as restriction
enzymes in their studies. They observed that porcine *B. pilosicoli* strains obtained from different territories, countries and continents were genetically very diverse, and common MRPs were found only among isolates from the same herd. *B. pilosicoli* strains of human, canine, avian or porcine origin were not genetically related. Oxberry and Hampson (2003) investigated altogether 20 *B. pilosicoli* isolates from two Australian pig herds by PFGE with *Sma*I as a restriction enzyme. Both herds had several *B. pilosicoli* genotypes, one of which was shared by the herds. The common MRP could be explained by pig trade between the farms. Møller *et al.* (1999) examined Danish porcine *B. pilosicoli* genotypes by PFGE using restriction enzyme *Bln*I. The number of isolates per herd was quite similar to ours. Altogether 26 MRPs were found among the 35 *B. pilosicoli* isolates from 20 Danish pig herds, again consistent with our results, although the number of bands obtained by *Bln*I was not reported by the Danish authors. They found no common MRPs between herds. This can be explained by the lower number of herds in the Danish study, and by the volume of pork production in Denmark being more than tenfold that in Finland.

In our study of 131 *B. pilosicoli* isolates from 49 sow herds, the higher genetic variation between the isolates from area S can be explained simply by the 2.5-fold higher number of investigated isolates obtained there. Lack of genetic clustering according to geographic area and only five MRPs out of 54 being shared by two herds suggest that rodents, birds and fomites do not have a major role in transmitting *B. pilosicoli* in Finland.

We propose a genetic recombination or mutation of *B. pilosicoli* strains over time based on the close relatedness of two strains in one herd sampled three years’ apart. Recombination among *B. pilosicoli* strains has been suggested on the basis of molecular epidemiological studies of *B. pilosicoli* performed in villages of Papua New Guinea (Trott *et al.*, 1998) and in Australian piggeries (Oxberry and Hampson, 2003). The occurrence of bacteriophages has been demonstrated among *Brachyspira* spp. of animal and human origin (Humphrey *et al.*, 1997; Calderaro *et al.*, 1998; Stanton *et al.*, 2003). VHS-1, the prophage of *B. hyodysenteriae*, transduces genes between *B. hyodysenteriae* strains (Stanton *et al.*, 2001). Transduction of genes may occur among other *Brachyspira* species, as well (Stanton *et al.*, 2005).

Clustering analysis of the Finnish *B. pilosicoli* genotypes indicated that occurrence of *B. pilosicoli* in Finnish sow herds is endemic, and replacement animals are a likely source of new *B. pilosicoli* genotypes in a herd. The low mean density of pig farms (ca. one per 40 km² in pork production areas), the Finnish climate, the rarity of outdoor housing systems and the hygiene control measures adopted diminish the vector’s role in transmitting infection between herds.

In our work and in the above-mentioned Danish study, the MRPs obtained from non-diarrhoeic herds did not form clusters of their own. Concomitant enteric pathogens were not recorded. However, the MRPs obtained by PFGE probably do not possess characters predictive of the degree of pathogenicity among *B. pilosicoli* strains.

The MRPs of 11 hippurate-negative and nine hippurate-positive Finnish *B. pilosicoli* isolates in Study II did not form clusters of their own; a single MRP could include both hippurate-negative and hippurate-positive isolates. Furthermore, certain *B. pilosicoli*hipp strains shared a common MRP despite differences in their 16S rDNA nucleotide sequences. Thus, PFGE does not discriminate between hippurate-negative and -positive *B. pilosicoli* strains.
6.3 Characterization of hippurate-negative *Brachyspira pilosicoli*

Whether a single biochemical aberration of a *Brachyspira* species is related to the 16S rDNA nucleotide sequence has not been widely studied. Fellström and colleagues (1999) examined rare indole-negative *B. hyodysenteriae* isolates of Belgian and German origin. 16S rDNA sequences from six of these strains were identical, showing a unique nucleotide position not reported in any other *Brachyspira* strain. However, the uniform MRPs in their PFGE study of these isolates suggested a common source for these isolates, and as such, the unique 16S rDNA sequence could not be linked to indole-negativity of *B. hyodysenteriae*.

We showed that hippurate-negativity of *B. pilosicoli* is not related to any phylotype achieved by PFGE or 16S rDNA sequencing. We noted that hippurate-negative and -positive isolates can share a 16S rDNA sequence and possess the same MRP. These kinds of *B. pilosicoli* pairs were isolated from the same herds, strengthening the assumption of a common clone. Presumably, *B. pilosicoli* can downregulate the expression of hippurate hydrolase for some unknown reason. In our earlier studies, lack of expression for hippurate hydrolysis was irreversible *in vitro*.

*Campylobacter jejuni* (*C. jejuni*) is the only *Campylobacter* species with hippurate hydrolysis capacity, but all *C. jejuni* strains do not express hippurate hydrolysis despite the presence of the hippuricase gene (Chan et al., 2000). A specific PCR targeting the hippuricase gene of *C. jejuni* has been shown to be a useful tool for differential diagnostics of *Campylobacters* (Hani and Chan, 1995; Linton et al., 1997).

*B. pilosicoli*, as well as an avian spirochaete, *B. alvinipulli*, are the only characterized *Brachyspira* species with hippurate hydrolysis capacity. The genetic basis of their hippurate hydrolysis has not been studied. The diagnostic methods targeting the hippuricase gene for these *Brachyspira* species might be worth of exploring, particularly in view of the discrepancies observed in the current PCR applications for *B. pilosicoli* (Thomson et al., 2001; Jansson et al., 2005) and the lack of a species-specific PCR for *B. alvinipulli*.

The 16S rDNA sequences from both hippurate-negative and -positive *B. pilosicoli* isolates revealed strains possessing up to 12 nucleotide mismatches compared with the sequence from type strain P43. Species in the genus *Brachyspira* are phylogenetically more closely related than species in the other genera in the class *Spirochaetes* (Paster et al., 1991). Similarity in 16S rDNA nucleotide sequences within the genus *Brachyspira* and of strains of *B. pilosicoli* isolated from pigs has been reported as 98.1–99.6%, and 99.9%, respectively (Pettersson et al., 1996). In our study, the lowest similarity between *B. pilosicoli* isolates was 98.6% indicating that the genetic diversity among *B. pilosicoli* strains is higher than reported previously.

In TEM, the cell dimensions and other characteristics were similar between *B. pilosicoli*\textsuperscript{hipp} and type strain P43. The ultrastructural study was justified because of the chance of uncovering new intestinal spirochaetes in pigs. For example, wild birds have been shown to carry spirochaetes that mimic *B. pilosicoli* in routine PCR (Jansson et al., 2005), and transmission of intestinal spirochaetes between wild birds and pigs has been suggested (Råsbäck et al., 2005).
6.4 D-ribose test in differentiation of porcine *Brachyspira* spp.

The substrate consumption of a bacteria can be detected indirectly by comparing the growth rates in two broths where the requirements for minimum growth are fulfilled and the substrate under investigation is present in one broth. This method has been applied when several *Brachyspira* species have been characterized in detail (Trott et al., 1996c; Stanton et al., 1997, 1998). We applied the method tentatively for several *Brachyspira* isolates, but in daily use it is arbitrary and liable to failure (Mäkelä, 2005; unpublished data).

Utilization of a single sugar can also be detected by measuring the reduction in pH of a broth culture (Özcan and Miles, 1999) or a culture on solid agar (Phillips, 1976). In our laboratory, we found the method based on pH change in broth culture promising for detection of carbohydrate utilization in *Brachyspira* spp.; when *B. pilosicoli* inoculum in a test broth was dense, the reduction in pH was significant and rapid in the presence of D-ribose (Mäkelä, 2005). The pH reduction was not hampered by the sphere transformation of the spirochaetes, which often emerged at the end of the incubation period. Sphere formation leads to decreased turbidity in the broth, thus invalidating the detection of enhanced growth by optical methods.

Carbohydrate fermentation of *Brachyspira* bacteria can also be determined by using solid media (Phillips, 1976; Lemcke and Burrows, 1981). The growth of *Brachyspira* on solid agar media is, however, relatively slow, whereas our method yields a result within two days. In addition, the agar plates expire sooner than sealed broths when stored, an important consideration in routine use.

In our study, FCS supplement in the broth caused slightly higher background on pH reduction with non-*B. pilosicoli* isolates than with *B. pilosicoli*, as seen in the results of sugar-free control broths (Figure 4). This somewhat contradicts the observations of Trott et al. (1996), who observed higher background growth with *B. pilosicoli* type strain than with *B. hyodysenteriae* and *B. innocens* type strains in sugar-free heart infusion broth with 7% serum. Our method differed from that of Trott et al., and thus, other factors in addition to the undefined compounds in serum might cause background growth of spirochaetes in sugar-free media.

The 60 *B. pilosicoli* and 35 non-*B. pilosicoli* isolates and strains in this study were chosen to represent diverse clones as far as possible. According to the results, only *B. pilosicoli* among porcine intestinal spirochaetes used D-ribose. Because only three of the isolates originated from other species of animals, any conclusion must be limited to porcine intestinal spirochaetes.

*B. pilosicoli* has been isolated yearly in Finland from an average of 25 herds, and *B. pilosicoli* hipp from 2–7 herds. In this work, seven out of 60 *B. pilosicoli* isolates were of the hippurate-negative phenotype, which justifies the conclusion that D-ribose utilization is a more constant trait of *B. pilosicoli* than expression of hippurate reaction. The D-ribose test, based on recording of pH reduction, seemed to be fairly flexible with regard to culture conditions and timing. Consequently, the D-ribose test in routine practice would strengthen the current diagnostic scheme for porcine *Brachyspira* spp. infections (Table 5).
Applicability of carbohydrate utilization tests for identification of other *Brachyspira* species in the WBHIS group should be investigated. Trott *et al.* (1996d) and Stanton *et al.* (1997) showed *B. intermedia* type strain PWS/A\textsuperscript{T} to be negative for mannose utilization, differentiating it from type strains of *B. pilosicoli*, *B. innocens* and *B. murdochii*. *B. intermedia* is the only species in the WBHIS group with a positive indole reaction. However, Fellström *et al.* (2001a) found one indole-positive *B. pilosicoli* of canine origin. Furthermore, our unpublished studies of intestinal spirochaetes from poultry revealed indole-negative strains that were positive by *B. intermedia*-specific PCR, and *vice versa*, indole-positive WBHIS strains with negative PCR results for *B. intermedia*. Since *B. intermedia* is a poultry pathogen, and also isolated in pigs, further efforts should be directed at improving its identification.

### 6.5 Aspects of experimental infection of pigs with *B. pilosicoli* strains

Our preliminary hypothesis was that experimental infection with *B. pilosicoli* type strain P43 would cause at least mild typhlocolitis and softened faeces in pigs with a positive re-isolation of P43. For *B. pilosicoli*\textsuperscript{hipp} Br1622, our expectations were similar; our results from genetic and morphological investigations of hippurate-negative isolates, and observations of atypical *Brachyspira* spp. isolates elsewhere (Thomson *et al.*, 1998, Thomson *et al.* 2003), were not suggestive of decreased or lack of pathogenicity of Br1622. The design of our study had features that might explain the silence or absence of infection. Attenuation of the inocula strains must also be considered. Failure with the technical challenge procedure or pre-challenge death of the inocula was very unlikely. The volume and frequency of inocula should have been adequate since an inoculation of 10\textsuperscript{9} cells ml\textsuperscript{-1} of *B. pilosicoli* has been satisfactory for induction of PIS when given once (Thomson *et al.*, 1997, 2001), twice (Neef *et al.*, 1994) or thrice (Jensen *et al.*, 2004).

When actively growing bacteria are inoculated in an acid stomach as a broth culture, the number of viable cells entering the hind gut might not reach the threshold of an infective dose. In theory, a bacteria mass scraped from a solid agar culture for immediate inoculation might form cell clumps that protect some of the bacteria. At the active growth state the bacteria are most vulnerable to external stress. We can speculate that dormancy of spirochaetes in the sphere stage might be advantageous for successful peroral infection. In our experience, the comma and sphere formation of *Brachyspira* bacteria emerges fast.
when the culture has consumed the nutrients of the media and when the broth culture is left unagitated in a cool place. Our meticulous nursing of the challenge cultures, including strict heat control of bacteria containers until inoculation, may have turned against the success of infection.

We re-isolated only \textit{B. pilosicoli} Br1622, and merely from two pigs at necropsy. For subclinical infection, the sensitivity of the culture method might be inadequate. For \textit{B. pilosicoli}, the culture sensitivity is approximated as $5.4 \times 10^6$ CFU g$^{-1}$ (Stege et al., 2000) or $1.5 \times 10^2$ CFU g$^{-1}$ in faeces (Fellström et al., 1997), and according to our unpublished studies, the sensitivity lies between these figures. Jensen (2005) has shown FISH to be a sensitive method in general, especially when tissue samples have been taken prior to post-mortem autolysis. When he compared the sensitivity of FISH and a culture method for \textit{B. hyodysenteriae} and \textit{B. pilosicoli}, FISH proved to be superior for \textit{B. hyodysenteriae}, but for \textit{B. pilosicoli}, the culture method was better. In our study, FISH did not reveal \textit{Brachyspira} bacteria, not even from the two pigs with a positive re-isolation of Br1622. Possibly, neither the isolation method nor FISH can detect \textit{B. pilosicoli} when the infection is subclinical.

The strain for the \textit{B. pilosicoli} type strain P43 had first been reported in 1980 by Taylor et al., being deposited in the American Type Culture Collection far later by Trott et al. (1996c). The number of passages before its final deposition is unknown; however, some attenuation of the type strain is possible. The Finnish strain Br1622 had undergone 4–6 passages before its deposition at $-70^\circ$C, and a further four passages for the final inocula. It is unlikely that the Finnish strain was more attenuated than the type strain.

\textbf{L. intracellularis}-free experimental pigs were achieved by the early transfer of piglets, which was probably aided by the low infection pressure in the herd of origin. \textit{B. pilosicoli} together with particularly \textbf{L. intracellularis} seem to cause aggravated growers’ diarrhoea in the field (Thomson et al., 1998; Jacobson et al., 2003). In our study, temperature variation and draught were minimal in the barrier rooms, and pen space per pig increased gradually due to the necropsy schedule. The favourable environmental conditions and freedom from concomitant pathogens could have promoted the health of the experimental pigs.

The feed of the experimental pigs was granulated without heating. Heat pelleting of feed, which might alter the sites of digestion for certain nutrients, has been shown to increase the prevalence of WBHIS and non-specific colitis in pig herds (Smith and Nelson, 1987; Stege et al., 2001). Clinical SD is prevented by experimental feed with no insoluble carbohydrates (Pluske et al., 1998). According to Hampson et al. (2000), a similar experimental feed did not prevent PIS but did reduce shedding of \textit{B. pilosicoli}. However, a feed mainly digested in the fore gut might have some controlling impact on \textit{B. pilosicoli} infection.

In our experience, the histological sections from the large intestine of non-diarrhoeic weaners and growers frequently show mild inflammatory lesions, while no enteropathogenic microorganism is detected. The mild mucosal inflammation might be solely a response to the abundant, high-energy feed needed to meet the growth requirements of the modern pig. In our study, mild inflammatory changes were seen microscopically on colonic mucosa of each experimental pig, including the controls, but the degree did not clearly correlate with the observation of spiral-shaped bacteria. Thus, the slight mucosal inflammation could be non-specific in nature.
Separation of the piglets from the sow into a barrier at the age of 11 days did not prevent transmission of certain enteroinvasive spiral-shaped bacteria. The identity of the endoflagellated bacteria visualized by TEM remained unknown, but based on their high number of endoflagella, *B. pilosicoli* and the genus *Leptospira* could be excluded. If the invasive spirochaetes visualized by FISH using the *Leptospira* probe were true *Leptospira*, it would be concordant with the observation that suckling piglets can become infected with *Leptospira* via sow’s milk (Ellis, 1999).

The true virulence of *B. pilosicoli* *hipp* Br1622 strain remained uncertain in our infection trial. Further infection studies with *B. pilosicoli* *hipp* should be done with pigs raised in less favourable conditions than those here, and using a definitely unattenuated, hippurate-positive *B. pilosicoli* field isolate as a positive control.

### 6.6 Eradication of *B. pilosicoli*

Our attempt to eradicate *B. pilosicoli* from a 60-sow farrowing herd was successful. Due to the shelters available in the vicinity of the premises, none of the growing pigs had to be sold untimely, reducing costs to the farmer. All improvement in pigs' health can not be attributed solely to the disappearance of *B. pilosicoli*. Renovation measures undertaken on the premises improved the general hygiene on the pig farm, undoubtedly promoting the good health of the animals.

In Norway, one eradication attempt for *L. intracellularis* and *B. pilosicoli* was carried out in a small 35-sow farrow-to-finish unit in summer 1998 by methods comparable with ours (Flø et al., 2000). During the 20-month follow-up *B. pilosicoli* and *L. intracellularis* were not detected and the clinical diarrhoea among growers had disappeared. In our study, the presence of *L. intracellularis* before eradication was not examined. While the dosage of 200 p.p.m. tiamulin should be adequate to control clinical symptoms and prevent pathological lesions caused by *L. intracellularis* (McOrist et al., 1996), it is unclear whether this dose for 18–30 days is sufficient for its eradication. However, one can speculate that in fact *L. intracellularis* was the organism that had been eradicated, and the improved health was due to the disappearance of co-factors for intestinal spirochaetosis.

*L. intracellularis* survives in a terrestrial environment up to two weeks (McOrist, 1997; Collins et al., 2000) and is transmitted from sows to piglets rapidly (McOrist and Gebhart, 1999; Möller, 1999). The permanent eradication of *L. intracellularis* from a pig herd is considered to be very difficult (Waddilove, 1997). The risk of reinfection by fomites, rodents or avian carriers is high (Collins et al., 1999), and if reinfection occurs, an acute and more severe form of *Lawsonia*-induced disease, proliferative haemorrhagic enteritis (PHE), is to be expected (McOrist and Gebhart, 1999). Our eradicated herd was studied for *L. intracellularis* for the first time in September 2001 and again in May 2002. In both samplings, *L. intracellularis* was detected by nested-PCR in the faeces of weaners with mild diarrhoea. However, after eradication measures, no period of PHE-like severe diarrhoea had been observed. *L. intracellularis* had probably survived in the sows or returned to sows soon after the medication period of the eradication program. This assumption would strengthen the conclusion that *B. pilosicoli* had been an essential cause of health problems in the herd.
At least one *B. innocens* genotype persisted in the herd through eradication. A similar observation was made in Norway, where Flø et al. (2000) studied eradication of *Brachyspira* spp. and *L. intracellularis* from two small sow herds; *B. innocens* in the first herd and *B. murdochii* in the second herd were isolated from samples before and after the eradication program. Reinfection by *B. innocens* or *B. murdochii* from the environment can not be excluded, although the host range of these species and their survival in the environment has not been widely investigated. Trott et al. (1996a) studied the genetic relatedness amongst *Brachyspira* spp. isolates from pigs, rats and several avian species by using MLEE. They observed *B. murdochii* isolates from rats and pigs to cluster together. We have recently found *Brachyspira* spp. in abundance in Finnish free-range and indoor-housed laying hens as well as in captive pheasants, and a portion of the isolates have been positive by PCR targeting *nox* gene region specific for both *B. innocens* and *B. murdochii* (unpublished data). Thus, cross-species transmission of *B. innocens* and/or *B. murdochii* may be possible, and the *B. innocens* clone in our eradication study might have been silent in environmental animals.

Because the number of *B. pilosicoli* eradications attempted is low, determination of the success rate is unreliable. Today, the mean number of sows in herds in intensive pork production areas is higher than in the trial herds presented. A large herd size and the limited facilities available might pose a risk for failure of eradication. On the other hand, the cold climate and the low population density in Finland and Norway indirectly assist in the eradication of *B. pilosicoli*.

The selection of antimicrobial agents approved for treating pigs is limited in Finland; only tylosine, lincomycin or tiamulin can be used to treat enteric spirochaetoses (MAF, 2003). All Finnish *B. hyodysenteriae* isolates have been resistant to tylosine since 1997, and their resistance to lincomycin has increased to approximately 90%. All *B. pilosicoli* isolates have been resistant to tylosine since 1998 (MAF, 2000; EELA's unpublished data). In recent years, some *B. pilosicoli* and *B. hyodysenteriae* strains with intermediate sensitivity to tiamulin (MIC 1.0–2.0 µg ml$^{-1}$) have been observed in EELA (unpublished data). The emergence of multiresistant *B. pilosicoli* strains due to continuous usage of macrolides and pleuromutilins on certain pig farms is a significant concern.

The national herd health program for multipliers does not necessitate freedom from *B. pilosicoli*. Thus, *B. pilosicoli*-free herds have problems in purchasing clean replacement animals. Regular medication of purchased pigs in quarantine is an unsustainable strategy because of the threat of antimicrobial resistance. A nationwide eradication program for *B. pilosicoli* in the near future is not likely. However, preparation for the emergence of multi-resistant *Brachyspira* spp. should include encouraging the eradication of *B. pilosicoli*. 
7 Conclusions

1 Porcine \textit{B. pilosicoli} strains are genetically very diverse in Finland, and the genotypes obtained by PFGE do not cluster according to the major pork production areas. A single \textit{B. pilosicoli} genotype can persist in a herd for several years.

2 Finnish \textit{B. pilosicoli} strains possess from zero to 12 different nucleotide positions in the partial sequence of their 16S ribosomal DNA compared with the \textit{B. pilosicoli} type strain. This within-species genetic variation (\(\geq 98.6\%\)) is higher than that reported previously.

3 Hippurate-negative and -positive \textit{B. pilosicoli} strains are phylogenetically indistinguishable according to PFGE and 16S rDNA sequence analysis. \textit{B. pilosicoli} may downregulate the expression of the hippurate hydrolase, and consequently, the hippurate test is inadequate for differentiation of all \textit{B. pilosicoli} isolates.

4 D-ribose utilization differentiates porcine \textit{B. pilosicoli} from the other porcine \textit{Brachyspira} species. Supplementation of the differential diagnostic scheme with the D-ribose test strengthens the phenotype-based diagnostics of porcine \textit{B. pilosicoli}.

5 Hippurate-negative \textit{B. pilosicoli} can colonize the large intestine of a pig. Further research is needed to clarify its true pathogenicity.

6 Chronic \textit{B. pilosicoli} infection can be eradicated from a 60-sow farrowing herd without total depopulation and freedom from this infection maintained for at least four and a half years. Low MIC value of the \textit{B. pilosicoli} strain to the antimicrobial compound used and medication of replacement animals in quarantine are apparent preconditions for successful eradication.
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