Genus *Brachyspira* in Birds:

Phenotypes, Phylogeny and Pathogenicity

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Abstract

Spirochaetes of genus *Brachyspira* colonize the large intestine of some mammals and birds, and cause intestinal disease and production losses in pigs and chickens. The precise significance of *Brachyspira* spp. colonization in birds, the bacterial species involved, and the epidemiology are incompletely understood. Possible transmission between birds and mammals, and the role of wildlife have previously received little attention. In this thesis intestinal spirochaetes were isolated from commercial laying hens and free-living wild mallards (*Anas platyrhynchos*), jackdaws (*Corvus monedula*), rooks (*C. frugilegus*) and hooded crows (*C. corone cornix*). The isolates were investigated by phenotypic tests, molecular methods (PCR, RAPD, PFGE, sequencing of 16S rRNA and *nox* genes) and phylogenetic analysis. Experimental animal models were applied in pigs and mallards to study colonization rates and enteropathogenicity. The results showed that *Brachyspira* spp. were commonly isolated from the investigated species. Phenotypic and molecular analyses showed considerable diversity, and simultaneous colonization by two or more species or genetic variants of the same species was commonly found. In laying hens, pathogenic species (*B. intermedia* and *B. alvinipulli*), presumed non-pathogenic species (*B. innocens*, *murdochii*, '*B. pulli*'), and isolates that could not be assigned to any presently known species were isolated. No association with disease or production losses was identified. The etiologic agent of swine dysentery, *B. hyodysenteriae*, was isolated from mallards, which is the first time from wild birds. A putative novel species, '*B. suanatina*', was isolated from mallards and Swedish and Danish pig herds. Isolates from both a pig and a mallard were shown to cause diarrhoea in pigs by experimental challenge. In mallards, focal epithelial changes were observed with *B. hyodysenteriae* and '*B. suanatina*'. Another novel and presumed non-pathogenic species, '*B. corvi*', from corvid birds, was characterized and provisionally described. The results of the thesis highlight the diagnostic difficulties, the genetic diversity, and suggest that birds may be important reservoirs of *Brachyspira* spp.

Keywords: Anas, *Brachyspira*, *Corvus*, experimental challenge, laying hen, phenotype, phylogeny, molecular characterization, spirochaete, swine dysentery

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Nothing is gayer than the persistence of memory.

Salvador Dalí, ca. 1961

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Jansson, D. S., Johansson, K.-E., Olofsson, T., Råsbäck, T., Vågsholm, I., Pettersson, B., Gunnarsson, A., & Fellström, C. (2004). *Brachyspira hyodysenteriae* and other strongly β-haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). *Journal of Medical Microbiology*, 53, 293-300.

II Råsbäck, T., Jansson, D. S., Johansson, K.-E. & Fellström, C. (2007). A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated '*Brachyspira suanatina*' sp. nov. *Environmental Microbiology* 9, 983-991.

III Jansson, D. S., Råsbäck, T., Fellström, C. & Feinstein, R. Experimental challenge of mallards (*Anas platyrhynchos*) with *Brachyspira hyodysenteriae* and "*Brachyspira suanatina*" isolated from pigs and mallards. *Submitted manuscript*

IV Jansson, D. S., Fellström, C., Råsbäck, T., Vågsholm, I., Gunnarsson, A., Ingermaa, F. & Johansson, K.-E. (2008). Phenotypic and molecular characterization of *Brachyspira* spp. isolated from laying hens in different housing systems. *Veterinary Microbiology*, 130, 348-362.

V Jansson, D. S., Fellström, C. & Johansson, K.-E. (2008). Intestinal spirochaetes isolated from free-living wild jackdaws, hooded crows and rooks (genus *Corvus*): Provisionally designated "*Brachyspira corvi*" sp. nov. *Anaerobe* 14, 287-295.

Abbreviations

AIS	Avian intestinal spirochaetosis
ATCC	American Type Culture Collection
BHI	Brain heart infusion broth
CFU	Colony forming units
DNA	Deoxyribonucleic acid
didNTP	Dideoxynucleotides
FAA	Fastidious anaerobe agar
FCS	Foetal calf serum
GTA	Gene transfer agent
H&E	Haematoxylin and eosin stain
HIS	Human intestinal spirochaetosis
Mbp	Mega base pairs
MEE	Multilocus enzyme electrophoresis
Mol% G+C	Genomic C+G content
Mya	Million years ago
nox	Nicotinamide adenine dinucleotide (NADH) oxidase gene
PFGE	Pulsed field gel electrophoresis
PCR	Polymerase chain reaction
PIS	Porcine intestinal spirochaetosis
^{R}	Reference strain of a species (superscript)
RAPD	Randomly amplified polymorphic DNA
rRNA	Ribosomal ribonucleic acid
S	Svedberg unit
SD	Swine dysentery
sp.	Species (singular)
sp. nov.	Species novum (new species)
spp.	Species (plural)
subsp.	Subspecies
SVA	National Veterinary Institute
^{T}	Type strain of a species (superscript)
TEM	Transmission electron microscopy
WS	Warthin-Starry silver stain

1 Introduction

1.1 Phylum *Spirochaetes*

1.1.1 Early history

Helically coiled and highly motile microorganisms have been known to exist in human faeces and the oral cavity since the early days of microscopy in the 17th century (Dobell, 1932). The first spirochaete (etymology Gr. speira 'coil' and chaite 'hair') to receive a name was *Spirochaeta plicatilis* (Ehrenberg, 1835). Spirochaetes were initially confused with protists, and their bacterial nature was not unequivocally proven until the 1960s-70s based on ultrastructural features (Ryter and Pillot, 1965; Holt, 1978).

1.1.2 Systematics and evolutionary aspects

The domain *Bacteria* is currently subdivided in 24 phyla, of which one (*Spirochaetes*) includes all spirochaetes (Table 1) (Garrity *et al.*, 2004). The spirochaetes form a coherent monophyletic lineage with deeply branching subclusters within the clade that correspond to different families (n=5) and genera (n=13) (Woese, 1987; Paster *et al.*, 1991; Paster and Dewhirst, 2000; Ludwig *et al.*, 2008). Genus *Spirochaeta* is the type genus, and *Spirochaeta plicatilis* is the type species, despite the lack of an isolate (Euzéby, 2008). More than half of the presently valid or proposed spirochaetal species (n>200) are yet to be cultured *in vitro* (Paster and Dewhirst, 2000). Spirochaetes have been suggested to be evolutionary ancient organisms (Canale-Parola, 1977; Margulis *et al.*, 1993). Several phylogenetic studies (Brown *et al.*, 2001; Daubin *et al.*, 2002; Griffiths and Gupta, 2004) have produced evolutionary trees where spirochaetes occupy a place closer to the root of the bacterial origin than inferred from small subunit ribosomal

sequence data (Woese, 1987). Further support is given by their presence in phototrophic bacterial mat communities, which are considered to be one of the oldest ecosystems on Earth (Margulis *et al.*, 1993). Other findings inferring ancient descent are the symbiotic relationship with the living fossil *Nautilus macromphalus* (genus *Nautilus* evolved during the Cambrian, 542-488 Mya [million years ago]), and with termites in 15-20 million year old amber (Wier *et al.*, 2002, Pernice *et al*, 2007).

Table 1. *Proposed taxonomic outline (families, genera) of phylum Spirochaetes. (Ludwig et al., 2008; Euzéby, 2008).*

Phylum *Spirochaetes*				
Family I	**Family II**	**Family III**	**Family IV**	**Family V**
Spirochaetaceae	***Brachyspiraceae***	***Brevinemataceae***	***Leptospiraceae***	***Incertae sedis*[a]**
Spirochaeta	*Brachyspira*	*Brevinema*	*Leptospira*	*Clevelandina*
Borrelia			*Leptonema*	*Diplocalyx*
Cristispira			*Turneriella*	*Hollandina*
Treponema				*Pillotina*

[a]Uncertain placement of genera because isolates and DNA sequences are not available.

1.1.3 Genetic characteristics

Most spirochaetes have a typical circular chromosome. One exception is *Borrelia burgdorferi*, which has a linear chromosome, and linear and circular plasmids (Kobryn and Chaconas, 2002). Two chromosomes of different sizes are present in some species (e.g. *Leptospira interrogans*). Public data of complete genome sequences (0.91 to 4.69 Mbp) are currently available from 16 strains (genera *Borrelia*, *Leptospira* and *Treponema*) (GOLD: Genomes OnLine Database v 2.0, 2008).

1.1.4 Ecological niches and metabolism

Spirochaetes are medically and ecologically important organisms that inhabit a diverse range of environments and embrace a wide variety of lifestyles. Many species are free-living in marine and limnic environments, soda lakes, hot springs, oil fields, soil, and intertidal microbial mat communities (Margulis *et al.*, 1993; Charon and Goldstein, 2002, Pernice *et al.*, 2007, Euzéby, 2008). Extremophilic (thermophile, alkaliphile, halophile) and mesophilic spirochaetes have been described (Euzéby, 2008). Others have adopted a commensal, symbiotic or parasitic relationship with eukaryotic hosts such as insects, molluscs, and vertebrates. Two genera (*Brachyspira* spp., some *Treponema* spp.) colonize the intestines of vertebrates. A

renowned example of a symbiotic relationship is that between spirochaetes of at least six different genera and wood-eating termites. These spirochaetes are consistently present in large numbers in the hindgut where they participate in bio-recycling of lignocellulose and nitrogen fixation (Warnecke *et al.*, 2007). Another example is the spirochetes that inhabit digestive or excretory organs of bivalve molluscs (Pernice *et al.*, 2007). Spirochaetes are metabolically diverse chemoorganoheterotrophic bacteria with complex nutrient demands, and they cover the entire spectrum of oxygen requirements.

1.1.5 Cell morphology and motility

Morphological characteristics distinguish spirochaetes from all other bacterial phyla, including other helically shaped bacteria. Most spirochaetes are helically coiled, but a flat wave and a coccoid cell shape have also been described (Dröge *et al.*, 2006; Pernice *et al.*, 2007; Charon *et al.*, 2009). A variant spirochaete morphology, i.e. spherical bodies, is known to occur *in vitro* in some species of the genera *Treponema*, *Borrelia*, *Leptospira*, and *Brachyspira*, and *in vivo* in spinal fluid of humans infected by *Borrelia burgdorferi* (Wood *et al.*, 2006). Aging cultures and adverse conditions have been suggested as possible causes (De Ciccio *et al.*, 1999; Wood *et al.*, 2006). The cell size among spirochaetes range from 0.1-3.0 μm in diameter and 2.0-180 (-500) μm in length depending on the species involved (Margulis *et al.*, 1993; Hovind-Hougen *et al.*, 1998; Charon and Goldstein, 2002). Outside the cytoplasmic membrane are a thin peptidoglycan layer and an outer bilayered membrane, which is often referred to as the outer membrane sheath in spirochaetes. Subterminally attached bipolar periplasmic flagella, also known as axial filaments or endoflagella, reside in the periplasmic space, i.e. between the cell membrane and the outer membrane sheath. Spirochaetal flagella possess a unique structure and contribute to the spirochaetal morphology and motility (Charon and Goldstein, 2002). They form symmetrically arranged, helical ribbons or bundles that overlap in the midsection of the cells in many species (Charon *et al.*, 2009). The numbers of periplasmic flagella per cell varies from 2-100s depending on the species (Charon and Goldstein, 2002). Asymmetrical flagellar rotation provides motility, and the cells penetrate and move efficiently in viscous media that would immobilize most other prokaryotes (Charon and Goldstein, 2002). A naturally occurring immotile spirochaete species has been described (Dröge *et al.*, 2006). Spirochaetes divide by binary fission.

1.1.6 Spirochaetal diseases

Spirochaetes cause a range of diseases of vertebrate hosts (Table 2). The epidemiology, host spectrum, tissue tropism and tissue invasiveness vary widely between spirochaetal species.

Table 2. *List of selected pathogenic spirochaetes and spirochaetal diseases of vertebrates.*

Species	Disease	Host animal
Treponema pallidum subsp. *pallidum*	Syphilis	Human
Treponema pallidum subsp. *endemicum*	Bejel (endemic syphilis)	Human
Treponema pallidum subsp. *pertenue*	Yaws	Human
Treponema carateum	Pinta	Human
Treponema denticola, and others	Gingivitis, periodontitis	Human, dog
Treponema paraluiscuniculi	Venereal rabbit spirochaetosis	Rabbit
Treponema spp.	Digital dermatitis	Cattle
Borrelia recurrentis	Epidemic recurrent fever	Human
Borrelia spp. (ca. 15 species)	Endemic recurrent fever	Human
Borrelia burgdorferi sensu latu (ca. 12 species)	Lyme disease/Lyme borreliosis/borreliosis	Human, dog, horse?
Borrelia anserina	Avian spirochaetosis	Chicken, turkey, pheasant, goose, duck
Leptospira interrogans serovar ichterohaemorrhagiae, and others	Leptospirosis	Human, dog, cattle, pig, sheep, horse
Brachyspira hyodysenteriae	Swine dysentery	Pig
Brachyspira hyodysenteriae	Necrotizing typhlocolitis	Common rhea
Brachyspira pilosicoli	Spirochaetal diarrhoea	Pig
Brachyspira pilosicoli, B. intermedia, B. alvinipulli	Avian intestinal spirochaetosis	Chicken

1.2 Genus Brachyspira

Bacteria of genus *Brachyspira* (etymology Gr. brachy 'short' and speira 'coil') are oxygen tolerant anaerobic spirochaetes that colonize the epithelial cell lining of the intestinal tract of some mammals and birds.

1.2.1 Taxonomy, systematics and genetics

Genus *Brachyspira* currently includes seven species that have standing in nomenclature, and six provisionally proposed species (Table 3). *B. aalborgi* is the type species of the genus.

Table 3. *List of valid and proposed Brachyspira spp.*[a]

Species	Published host range	Reference to species description or proposition[c]
B. aalborgi	Human, non-human primates	Hovind-Haugen *et al.*, 1982
B. hyodysenteriae	Pig, rat, mouse, common rhea, mallard (paper **I**), chicken, goose	Taylor and Alexander, 1971; Harris *et al.*, 1972
B. innocens	Pig, dog, horse, chicken	Kinyon and Harris, 1979 Stanton *et al.*, 1992
B. pilosicoli	Multiple species[b]	Trott *et al.*, 1996c
B. intermedia	Pig, chicken	Stanton *et al.*, 1997
B. murdochii	Pig, rat, chicken	Stanton *et al.*, 1997
B. alvinipulli	Chicken, domestic goose, Red-breasted merganser (*Mergus serrator*), dog	Stanton *et al.*, 1998
'*B. canis*'	Dog	Duhamel *et al.*, 1998
'*B. pulli*'	Chicken, dog (paper **IV**)	Stephens and Hampson, 1999
'*B. ibaraki*'	Human	Tachibana *et al.*, 2003
'*B. christiani*'	Human	Jensen *et al.*, 2001
'*B. suanatina*'	Pig, mallard	Paper **II**
'*B. corvi*'	Jackdaw (*C. monedula*), hooded crow (*C. corone cornix*), rook (*C. frugilegus*)	Paper **V**

[a]Species within quotation marks are not validated/recognized.; [b]Host range: pig, dog, horse, non-human primates, human, chicken, pheasant, grey partridge, feral water birds, common rhea.; [c]Additional references to host ranges: Joens and Kinion, 1982; Trott *et al.*, 1996a; Trott *et al.*, 1996c, Jensen *et al.*, 1996; Duhamel *et al.*, 1997; McLaren *et al.*, 1997; Webb *et al.*, 1997; Oxberry *et al.*, 1998; Trivett-Moore *et al.*, 1998, Duhamel, 2001; Munchi *et al.*, 2003; Johansson *et al.*, 2004; Nemes *et al.*, 2006; Hampson *et al.*, 2006b; Jansson *et al.*, 2007; Thomson *et al.*, 2007; Feberwee *et al.*, 2008; **I**; **IV**.

Over the years, there has been considerable taxonomic confusion. Originally, *B. hyodysenteriae* was described as a vibrio-like microorganism (*Vibrio coli*) (Lussier, 1962). A decade later, it was shown to fulfil Koch's postulates, and it was identified as a spirochaete and renamed *Treponema hyodysenteriae* (Taylor and Alexander, 1971; Harris *et al.*, 1972). Initially, this name was used for all intestinal spirochaetes isolated from pigs regardless of phenotypic properties and pathogenicity. Later, a weakly haemolytic, apparently non-pathogenic species present in pig faeces, was characterized and named *T. innocens* (Kinyon and Harris 1979). These species were then shown to be genetically closely related to each other, but rather distantly related to genus *Treponema*, and they were therefore reclassified in a new

genus *Serpula* (etymology Lat. 'little serpent') (Paster *et al.*, 1991; Stanton *et al.*, 1991), which was soon changed to *Serpulina* (Stanton, 1992). In 1996, another spirochete, previously proposed as *Anguillina coli* (Lee *et al.*, 1993), was added to genus *Serpulina* as *S. pilosicoli* (Trott *et al.*, 1996c), and it was soon followed by descriptions of *S. intermedia* and *S. murdochii* (Stanton *et al.*, 1997). *S. hyodysenteriae*, *S. innocens* and *S. pilosicoli* were later unified with a spirochaete previously isolated from a human patient, *B. aalborgi,* in a common genus (*Brachyspira*) based on the principle of priority of publication (Ochiai *et al.*, 1997). The new genus name was added as a footnote to the description of *S. alvinipulli* (Stanton *et al.*, 1998). In 2006, *S. intermedia* and *S. murdochii* were officially unified with genus *Brachyspira* (Hampson and La, 2006).

The chromosome of *Brachyspira* spp. is circular and has a low genomic mol% G+C content (Table 4). Three ongoing or drafted complete genome projects have been officially reported (*B. hyodysenteriae* WA1, *B. pilosicoli* 95/1000 and *B. murdochii* 56-150^{T}) (GOLD: Genomes OnLine Database v 2.0, 2008). Genome sizes of *B. hyodysenteriae* B78^{T}, *B. pilosicoli* P43/6/78^{T} and *B. murdochii* 56-150^{T} are 3.2, 2.45 and 3.2 Mbp, respectively (Zuerner *et al.*, 2004; GOLD: Genomes OnLine Database v 2.0, 2008). Prophage-like gene transfer agents (GTAs) have been detected in several *Brachyspira* spp. (Stanton *et al.*, 2003; Motro *et al.*, 2008).

Table 4. *Characteristics of type strains of Brachyspira spp.*

Species	Type strain	Mol% G+C	Cell diameter (μm)	Cell length (μm)	No. flagella/cell
B. hyodysenteriae	B78^{T}	25.8[b]	0.35±0.02[c]	9.78±1.87[x]	14-18[b]
		24.2[e]	0.3-0.4[b]	8-10[b]	16-24[c]
		25.9[c]	0.35-0.4[d]	6-8.5[d]	24-28[d]
B. intermedia	PWS/A^{T}	25[d]	0.35-0.45[d]	7.5-10[d]	24-28[d]
B. innocens	B256^{T}	25.6[b]	0.36±0.03[c]	9.40±1.85[c]	18-20[b]
			0.3-0.4[b]	7-10[b]	20-26[c]
B. murdochii	56-150^{T}	27[d], 27.7[f]	0.35-0.4[d]	5-8[d]	22-26[d]
B. alvinipulli	C1^{T}	24.6[e]	0.22-0.34[e]	8-11[e]	22-30[e]
B. pilosicoli	P43/6/78^{T}	24.6[c]	0.27± 0.03[c]	6.27±0.98[c]	8-12[c]
		24.9[b]	0.2-0.3[b]	4-8[b]	10-12[b]
B. aalborgi	513A^{T}	27.1[b]	0.2[a]	1.7-6[a]	8[a], 8-10[b]

References: [a]Hovind-Hougen *et al.*, 1982; [b]Ochiai *et al.*,1997; [c]Trott *et al.*, 1996c. [d]Stanton *et al.*, 1997; [e]Stanton *et al.*, 1998; [f]GOLD: Genomes OnLine Database v 2.0, 2008.

1.3 *Brachyspira* spp. in mammals

All presently known *Brachyspira* spp. colonize the intestinal tract of mammals and birds. The known host species ranges are shown in Table 3. A number of additional unidentified intestinal spirochaetes have been reported from various mammals such as guinea pigs, North American opossums, raccoons, house mice, field mice, nutria, rabbits, voles, cattle, and sika deer. *B. hyodysenteriae* and *B. pilosicoli* cause disease in pigs. In other mammals, a disease association has not been firmly established.

1.3.1 *Intestinal spirochaete diseases of swine*

Swine dysentery (SD), caused by *B. hyodysenteriae*, is a severe mucohaemorrhagic diarrhoeal disease that affects growing and finishing pigs in all major pig producing countries (Fig 1-4) (Hampson *et al.*, 2006a). The bacteria colonize the caecum, colon and rectum. Economic losses are caused by increased mortality, reduced growth, poor feed conversion, and medication expenses. Pigs become infected by ingestion of faeces from diseased animals or clinically healthy shedders.

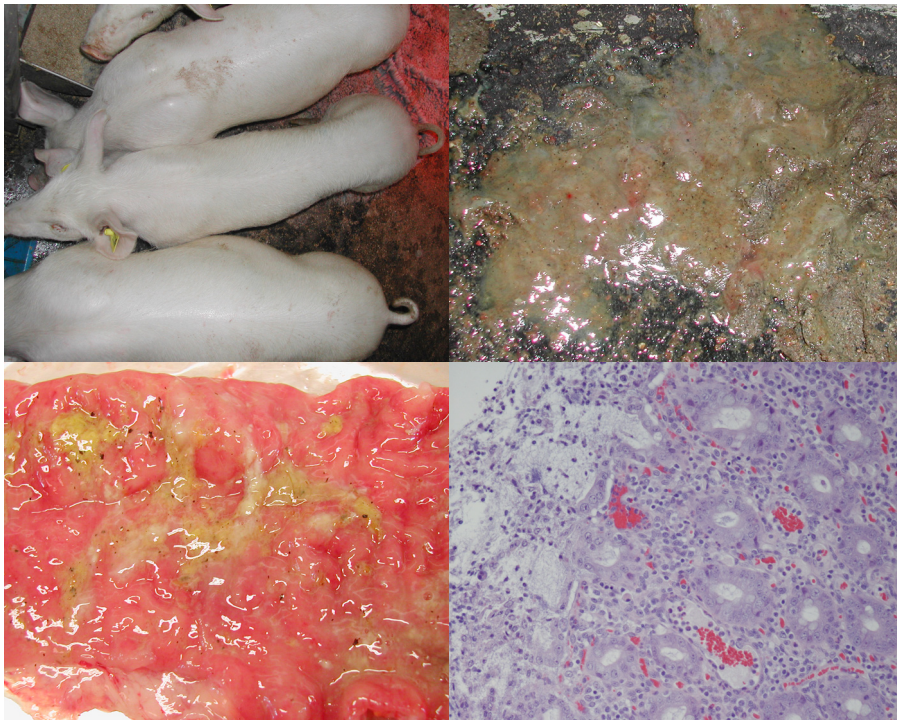

Figure 1-4. Swine dysentery. Affected pig between two healthy pigs of the same age (upper left). Mucoid diarrhoea with flecks of blood (upper right). Grossly, there is thickening and

congestion of the colonic mucosa and mucus adhering to the mucosal surface (lower left). Microscopic picture of superficial colonic epithelium (lower right) Mucous secretions, fibrin, desquamated leukocytes and cell debris are visible on the mucosal surface. Vessels are congested and focal necrosis and degeneration of epithelial cells is present. Spirochaetes are present in large numbers in dilated crypts in the adluminal epithelium but are poorly visualized by H&E staining. (H&E, original magnification ×200). Photo: DS Jansson.

B. pilosicoli causes mild to moderate typhlocolitis in pigs, i.e. spirochaetal diarrhoea, porcine colonic spirochaetosis or porcine intestinal spirochaetosis (PIS) (Hampson and Duhamel, 2006). It often affects pigs a few weeks after weaning. The infection causes watery or cement-like, sometimes mucoid or blood-flecked diarrhoea, unthriftiness, poor feed conversion and failure to reach market weight in due time (Hampson and Duhamel, 2006). *B. innocens* and *B. murdochii* are considered as non-pathogenic in pigs, whereas the enteropathogenic potential of *B. intermedia* is a somewhat contentious issue (Hampson *et al.*, 2006a).

1.3.2 Human intestinal spirochaetosis

B. pilosicoli, *B. aalborgi* and as yet uncharacterized spirochaetes colonize the human large intestine, which is known as human intestinal spirochaetosis (HIS). Intestinal spirochaetes have been implicated as causes of colitis with chronic diarrhoea, rectal bleeding, abdominal pain, and retarded growth. Spirochaetemia by *B. pilosicoli* is known to occur in debilitated patients (Trott *et al.*, 1997). A healthy human volunteer developed nausea, abdominal discomfort, bloating and headaches following experimental colonization by the human *B. pilosicoli* strain WesB (Oxberry *et al.*, 1998). *B. pilosicoli* shows a high prevalence (21-64%) in developing countries and in male homosexuals and HIV-positive persons in Western societies, whereas the incidence of *B. aalborgi* is lower (5.6-7.9% in Australian populations) and seems to be less affected by social structures and ethnicity (Mikosza and Hampson, 2001; Brooke *et al.*, 2006). However, a recent paper showed that the prevalence of *B. aalborgi* may be higher (on average 24.7%) than previously reported (Munshi *et al.*, 2008). Chickens and pigs have been successfully colonized by strains of *B. pilosicoli* originating from humans, pigs and dogs (section 1.6 and Trott *et al.*, 1996b). Results of multilocus enzyme electrophoresis (MEE) and pulsed field gel electrophoresis (PFGE) on isolates from a variety of animal species, including humans, show that *B. pilosicoli* seems to lack host specificity, which suggests the possibility of zoonotic transfer (Trott *et al.*, 1998; Hampson *et al.*, 2006c).

1.4 *Brachyspira* spp. in birds

1.4.1 Historical aspects

Early accounts of intestinal spirochaetes in birds involved morphological descriptions from red grouse (*Lagopus lagopus scoticus*), chickens (*Gallus gallus*), turkeys (*Meleagris gallopavo*) and pheasants (*Phasianus colchicus*) (Fantham, 1910; Harris, 1930; Mathey and Zander, 1955). In 1986, intestinal spirochaetes were isolated from chickens, and an association with enteric disease was suggested (Davelaar *et al.*, 1986).

1.4.2 Avian host range

The published avian host range includes orders *Galliformes*, *Anseriformes*, *Struthioniformes*, *Phoenicopteriformes*, and *Passeriformes* (Table 5).

Table 5. *Published avian host range of Brachyspira spp. detected by isolation or PCR.*

Bird category/species	Latin name	First reference
Poultry and farmed birds		
Chicken	*Gallus gallus*	Davelaar *et al.*, 1986
Turkey	*Meleagris gallopavo*	Shivaprasad *et al.*, 2005
Pheasant	*Phasianus colchicus*	Webb *et al.*, 1997
Grey partridge	*Perdix perdix*	Jansson *et al.*, 2001a
Mallard	*Anas platyrhynchos*	Jansson *et al.*, 2001a
Domestic geese	*Anser anser*	Nemes *et al.*, 2006
Common rhea	*Rhea americana*	Sagartz *et al.*, 1992
Ostrich	*Struthio camelus*	Stoutenburg and Swayne, 1992
Zoological gardens		
Pintail	*Anas acuta*	Stoutenburg *et al.*, 1995
Chiloe widgeon	*Anas sibilatrix*	Stoutenburg *et al.*, 1995
Black swan	*Cygnus atratus*	Stoutenburg *et al.*, 1995
Greater flamingo	*Phoenicopterus ruber*	Stoutenburg *et al.*, 1995
Common rhea	*Rhea americana*	Sagartz *et al.*, 1992
Free-living wild and/or feral birds[a]		
Mallard	*Anas platyrhynchos*	Oxberry *et al*, 1998
Red-breasted merganser	*Mergus serrator*	Jansson *et al.*, 2007b
Jackdaw	*Corvus monedula*	Paper **V**
Rook	*Corvus frugilegus*	Paper **V**
Hooded crow	*Corvus corone cornix*	Paper **V**

[a]Intestinal spirochaetes, (presumably *Brachyspira* spp.) were detected by indirect fluorescent antibody test in Ohio, USA in American widgeon (*Anas americana*), green winged teal (*Anas*

crecca), North American wood duck (*Anas sponsa*), American black duck (*Anas rubripes*), gadwall (*Anas strepera*), redhead (*Aythya americana*) and Canadian goose (*Branta canadensis*) (Swayne and McLaren, 1997).

1.4.3 Disease and production losses in chickens

The term avian intestinal spirochaetosis (AIS) (Swayne and McLaren, 1997) is often used in association with intestinal spirochaete colonization in birds. Since the definition of AIS varies, and as it may be mistaken for 'avian spirochaetosis' (acute septicaemic borreliosis, caused by *Borrelia anserina*) the term will henceforth not be used in this thesis.

Numerous case reports and field surveys jointly suggest an association between *Brachyspira* spp. colonization and intestinal disease and production losses in chickens (Davelaar *et al.*, 1986; Griffiths *et al.*, 1987; Swayne *et al.*, 1992, Trampel *et al.*, 1994; Stephens and Hampson, 1999; Burch *et al.* 2006; Bano *et al.*, 2008; Feberwee *et al.*, 2008). There is experimental support for the disease association regarding *B. alvinipulli* (Swayne *et al.*, 1995), *B. pilosicoli* (Stephens and Hampson, 2002a), and *B. intermedia* (Dwars *et al.*, 1990, 1991, 1992, 1993; Hampson and McLaren, 1999; Hampson *et al.*, 2002). Reported signs include diarrhoea or wet droppings, pasty vents, wet litter, faecal staining of eggshells (Fig. 5), increased faecal fat content, reduced feed conversion, approximately 5-10% reduced egg production, delayed start of egg laying, growth retardation, decreased egg weight, poor eggshell quality, and increased mortality. Reduced growth and poor feed digestion were reported in *Brachyspira* culture-negative broilers hatched from eggs produced by *Brachyspira* colonized parents (Smit *et al.*, 1998). By experimental challenge with strains of *B. intermedia* or *B. pilosicoli*, chicks developed diarrhoea within 7-9 days (Hampson and McLaren, 1997). In another experimental study in laying hen pullets, challenge with a *B. intermedia* strain of chicken origin (strain HB60) caused wet droppings, reduced growth, and significantly lower egg production and egg weights (Hampson and McLaren, 1999). Challenge of broiler breeder hens by *B. pilosicoli* (CPSp1) caused wet droppings and a severe drop in egg production during an 11 week trial (Stephens and Hampson, 2002a). Other causes of wet, sticky faeces and diarrhoea in chickens include some dietary components, excessive water consumption, and various infectious agents.

Figure 5-6. Faecal staining of eggshell (left). Caecal contents in a laying hen (right). The flock was colonized by *B. intermedia*, *B. innocens* and *B. murdochii*, and suffered losses from reduced egg production and faecal stains on 10% of laid eggs. Photo: DS Jansson.

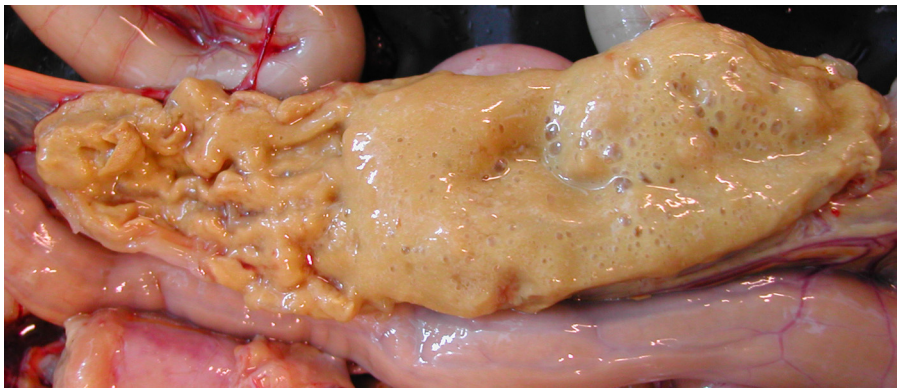

Figure 7. Luminal aspect of a caecum of a laying hen from another flock colonized by *B. intermedia* and *B. innocens*. Photo: DS Jansson.

Economic losses from *Brachyspira* colonization are mainly caused by the reduced egg production and downgrading of shell eggs to lower value eggs for processing. Annual losses from *B. pilosicoli* and *B. intermedia* colonization of laying hens alone were estimated in the United Kingdom in 2006 to £14 million or 1.5% of the production value (Burch *et al.*, 2006; Hampson and Swayne, 2008).

1.4.4 Pathologic findings in chickens

Chickens colonized by *B. intermedia*, *B. pilosicoli* and *B. alvinipulli* show unapparent to mild gross and microscopic changes. The caecal contents are normal or yellowish, gassy, wet, frothy or sticky (Fig. 6-7) (Dwars *et al.*, 1990. 1992, 1993; Swayne *et al.*, 1995; Hampson and McLaren, 1999; Stephens and Hampson, 2002a; Feberwee *et al.*, 2008). Histologically, a mild to moderately severe focal to diffuse infiltration in the lamina propria by lymphocytes and heterophilic granulocytes, distended crypts, crypt elongation, goblet cell hyperplasia, erosions and apical vacuolization of epithelial cells have been reported (Davelaar *et al.*, 1986; Griffiths *et al.*, 1987; Swayne *et al.*, 1995; Feberwee *et al.*, 2008). By Warthin-Starry staining (WS) spirochaetes appear brown to black (Fig. 7-8). *B. intermedia* and *B. alvinipulli* are randomly scattered in crypts (Fig. 7 & 9) and on the epithelial surface. Occasionally, spirochaetes are observed within and below the epithelial cell lining (Fig. 8). *B. pilosicoli* attach to enterocytes as a dense uniform layer on the mucosal surface, i.e. a false brush border.

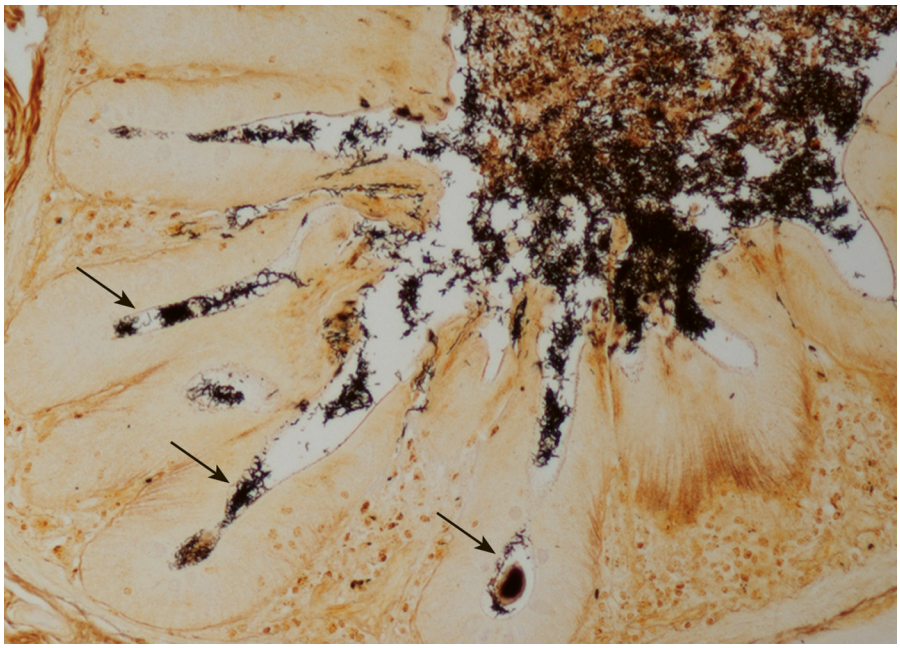

Figure 7. Caecal mucosa of a laying hen colonized by *Brachyspira* spp. (species not determined). Spirochaetes are located in the crypts (arrows). WS, original magnification ×200. Photo: DS Jansson.

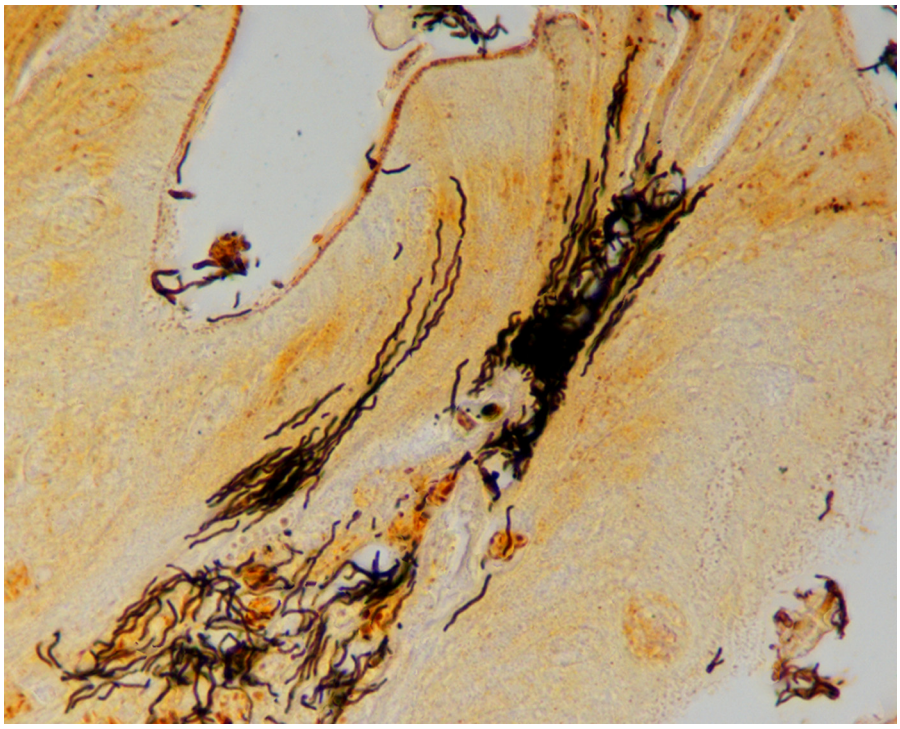

Figure 8. Caecal mucosa of a laying hen from a flock colonized by *B. intermedia, B. innocens* and *B. murdochii*. Spirochaetes are present between epithelial cells and in the lamina propria. WS, original magnification ×1000. Photo: DS Jansson.

1.4.5 Control options

Cleaning and disinfection between batches of birds, strict biosecurity routines and rodent control should be applied to avoid colonization and to prevent transmission between chicken flocks (Hampson and Swayne, 2008). In-feed zink bacitracin and dietary enzymes may reduce colonization with *B. intermedia*, whereas zinc bacitracin may enhance colonization with *B. pilosicoli* (Hampson *et al.*, 2002; Jamshidi and Hampson, 2002). No vaccines are currently available for use in poultry or other animals. Reports of antimicrobial treatment by dimetridazole, 5-nitroimidazole, lincomycin, lincomycin/spectinomycin, chlortetracycline, oxytetracyclin, and tiamulin have been published, but with variable results (Griffiths *et al.*, 1987; Smit *et al.*, 1998; Stephens and Hampson, 1999; Stephens and Hampson, 2002b; Burch *et al.*, 2006). Lack of appropriate licensed products and long withdrawal times for eggs for human consumption often restrict the use of antimicrobials in poultry.

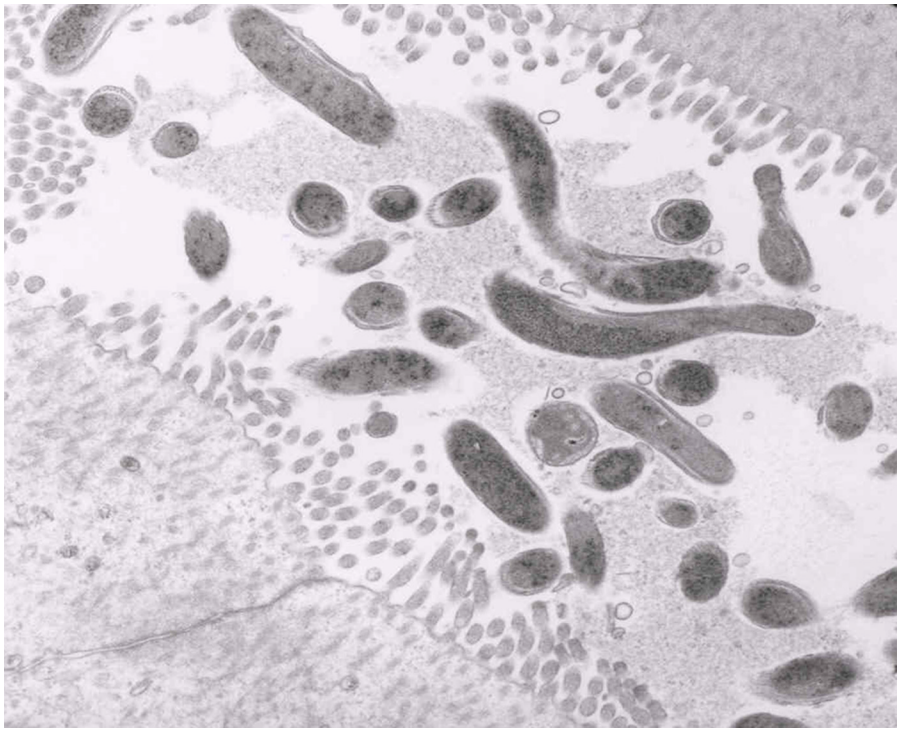

Figure 9. Transmission electron microscopic picture of a transversely cut caecal crypt containing intestinal spirochaetes. *B. intermedia* was isolated from a caecal scraping of this laying hen. Original magnification ×4100. Photo: T Nikkilä and DS Jansson.

1.4.6 Disease in other bird species

In the 1990s, intestinal spirochaetes were isolated from farmed common rheas (*Rhea americana*) in the United States in association with severe fibrinonecrotic typhlocolitis and 25-80% mortality rates (Sagartz *et al.*, 1992; Buckles *et al.*, 1997). *B. hyodysenteriae* was identified from affected birds (Jensen *et al.*, 1996). Gross and histologic lesions have been experimentally reproduced after challenge with rhea isolates in common rhea chicks, but not in pigs (Swayne, 1994; Stanton *et al.*, 1997).

Sporadic reports of spirochaete colonization and enteric disease have also been published from turkeys (*B. pilosicoli*), game birds (*B. pilosicoli* and uncharacterized species) and geese (*B. alvinipulli* and *B. hyodysenteriae*) (Webb *et al.*, 1997; Jansson *et al.*, 2001a; Shivaprasad and Duhamel, 2005; Nemes *et al.*, 2006). Experimental challenge of goslings with strains from goose field cases produced mild epithelial changes (Ivanics *et al.*, 2007).

1.4.7 Epidemiology

Colonization of chickens by *Brachyspira* spp. has been reported from many parts of the world, including Europe (Belgium, Finland, the Netherlands, Italy, Poland, Sweden, the United Kingdom, former Yugoslavia), the United States, Mexico, Iran and Australia (Davelaar *et al.*, 1986; Griffiths *et al.*, 1987; Swayne *et al.*, 1992; Trampel *et al.*, 1994; McLaren *et al.*, 1996; Stephens and Hampson, 1999; Jansson *et al.*, 2001b; Kizerwetter-Swida *et al.*, 2005; Thomson *et al.*, 2007; Razmyar *et al.*, 2007; Skrzypczak *et al.*, 2007; Corona-Barrera *et al.*, 2007; Bano *et al.*, 2008; **IV**). Field surveys (Table 6) indicate that colonization is widespread among laying hens and broiler breeder flocks, but is a rare event in broilers. Only one field case in broilers has been documented (Dwars *et al.*, 1990). Speculatively, young age, application of biosecurity routines, and use of ionophore coccidiostats and medicated feed for growth promotion may explain the results. The within-flock prevalence in chickens varies from 10-100% (McLaren *et al.*, 1996; Stephens and Hampson, 1999; Bano *et al.*, 2008).

The source of *Brachyspira* spp. on poultry farms has not been identified. Cross-infection between flocks on the same farm likely occurs by indirect transmission by animal care-takers or contaminated equipment, and possibly by flies or rodents (Joens and Kinyon, 1982; Phillips *et al.*, 2005). Survival times of avian strains (*B. intermedia* HB60, *B. pilosicoli* CPSp1) in chicken faeces under laboratory conditions varied from 41-84 hours at 4°C, and 17-74 hours at 25°C (Phillips *et al.*, 2003). Reported survival times in chicken faeces were shorter than those previously reported for *B. pilosicoli* ($P43/6/78^T$) in pig faeces and soil (Boye *et al.*, 2001). Little information on the influence of housing systems on *Brachyspira* colonization in laying hens has been published. No difference in prevalence between chickens housed in cages and in litter-based housing systems was observed in a field survey (Dwars *et al.*, 1989). In another study, free-range birds were at higher risk than birds housed indoors (Wagenaar *et al.*, 2003). A significant association between type of manure disposal and colonization was found, with hens in sheds with deep pits at higher risk than those in sheds with conveyor belts (Bano *et al.*, 2008). The flock size did not influence colonization (Bano *et al.*, 2008). An age-related difference in colonization of laying hen flocks has been reported, with old hens at higher risk than young birds (Stephens and Hampson, 1999; Jansson *et al.*, 2001b; Phillips *et al.*, 2005; Bano *et al.*, 2008). Prolonged colonization (23 weeks-9 months) has been reported from challenge trials (Dwars *et al.*, 1990, 1993).

Table 6. *Brachyspira spp. field surveys in commercial chickens.*

Country/ region	Study population	Selection method	No. farms, flocks	Detection method	No. samples/flock	Results (% positive)	Reference
The Netherlands	Laying hens Breeders[1]	Non-random	179 flocks	IFAT	3-20	21.8	Dwars *et al.*, 1989
Western Australia	Laying hens Broiler breeders[2]	NA	67 flocks	Culture	5-40	Laying hens: 31.5 Broiler breeders[2]: 53.3	McLaren *et al.*, 1996
Eastern Australia	Laying hens Broiler breeders[2] Broilers	Voluntary[3]	69 farms	Culture	Ca. 20	Laying hens: 68.2 Broiler breeders[2]: 42.9 Broilers: 0	Stephens and Hampson, 1999
Sweden	Laying hens Broilers	Non-random	64 flocks	Culture	20	Laying hens: 45.8 Broilers: 0	Jansson *et al.*, 2001b
UK	Laying hens	Non-random	96 submissions[4]	Culture	1-5	68.8	Thomson *et al.*, 2007
The Netherlands	Laying hens	Non-random	25 farms 25 flocks	IFAT, culture	1-30	100.0	Feberwee *et al.*, 2008
Italy, Treviso	Laying hens	Random	29 farms 45 flocks	Culture	10	Farms: 72.4 Sheds: 71.1	Bano *et al.*, 2008
Sweden	Laying hens	Random	92 farms 92 flocks	Culture	20	Non-organic: 27.3-34.8 Organic: 71.4	Paper **IV**

[1]'Breeders' refers to unspecified grandparent and/or parent birds.; [2]'Broiler breeders' refers to grandparents/parents of broilers.; [3]Reference: Bano *et al.*, 2008; [4]No. of farms and flocks not given.; Abbreviations: NA=information not available; IFAT: immunofluorescent antibody test.

1.4.8 Molecular studies of avian strains

The first attempts to characterize avian strains of *Brachyspira* spp. were made by MEE in the United States and Australia (Swayne *et al.*, 1995; Stanton *et al.*, 1996; Trott *et al.*, 1996a; McLaren *et al.*, 1997). MEE is a subtyping method by which selected cytoplasmic enzymes are electrophoretically separated according to their mass, charge and conformation (Boerlin, 1997). Detected strain variations reflect amino acid sequence differences in isoenzymes and allozymes. This technique has been widely used in microbiology for population genetics and epidemiology. In the early studies, isolates from common rheas, chickens and waterfowl were investigated. It was shown that isolates from common rheas were closely related to the type strain of *B. hyodysenteriae* (Trott *et al.*, 1996a), which was confirmed by other molecular methods (Jensen *et al.*, 1996). Isolates from chickens with diarrhoea on a farm in the United States were shown to differ from all known porcine isolates (Swayne *et al.*, 1995; Stanton *et al.*, 1996; Trott *et al.*, 1996a). These isolates were later confirmed by other methods as a new species, *B. alvinipulli* (Stanton *et al.*, 1998). One waterfowl isolate was identified as *B. pilosicoli* (Trott *et al.*, 1996a). In another study, chicken isolates originating from Australia, the United States and Europe were shown to be heterogenous, and by comparison with porcine strains, *B. intermedia* and *B. pilosicoli*, *B. innocens*, *B. murdochii* were detected (McLaren *et al.*, 1997). In a more resent study, MEE analysis of Australian isolates from chickens detected *B. intermedia*, *B. pilosicoli*, *B. murdochii*, and *B. innocens*, as well as several unidentified electrophoretic groups (Stephens *et al.*, 2005). One of these groups had previously been observed by MEE (McLaren *et al*, 1997) and had been proposed as '*B. pulli*' (Stephens and Hampson, 1999).

A range of single and duplex species-specific PCRs have been designed to detect *Brachyspira* spp. and have been applied to isolates from chickens and other birds (Stephens and Hampson, 1999; **I**; Kizerwetter-Swida *et al.*, 2005; Phillips *et al.*, 2005; Phillips *et al.*, 2006; Oxberry *et al.*, 1998; Stephens and Hampson, 1999; Råsbäck *et al.*, 2007; Bano *et al.*, 2008; Feberwee *et al.*, 2008; **IV**; **V**). PCRs may be used on cultures or on faeces. However, the sensitivity when applied to faecal samples is hampered because of presence of inhibitory factors (Lantz *et al.*, 2000; Phillips *et al.*, 2006). It has been suggested that the low pH and the presence of uric acid in chicken faeces are particularly problematic (Phillips *et al.*, 2006). The prevalence of the enteropathogens *B. intermedia* and *B. pilosicoli* in commercial chickens have been investigated by PCR (Stephens and

Hampson, 1999; Bano *et al.*, 2008). In an Italian study, *B. intermedia* and/or *B. pilosicoli* were detected on 24.4% of colonized farms (Bano *et al.*, 2008). In an Australian study, a selection of isolates from laying hens and broiler breeders were analyzed by PCR (Stephens and Hampson, 1999). Among colonized flocks, 56% were PCR positive for *B. intermedia* and/or *B. pilosicoli* with the latter species being almost twice as common.

Sequencing of the 16S rRNA gene and phylogenetic analysis were used to study avian *Brachyspira* spp. strains (Swayne *et al.*, 1995; Jensen *et al.*, 1996; Stanton *et al.*, 1996; **I**; Phillips *et al.*, 2005; Townsend *et al.*, 2005; Nemes *et al.*, 2006; **II;** Feberwee *et al.*, 2008; **IV**; **V**). The results showed good agreement with MEE, PCR and NADH oxidase (*nox*) gene sequencing, but also some limitations (see section 4.3) (Stanton *et al.*, 1996; Phillips *et al.*, 2005; Townsend *et al.*, 2005). Other molecular methods that have been applied to avian strains include protein electrophoresis and immunoblotting (Jensen *et al.*, 1996), ribotyping (Jensen *et al.*, 1996), RFLP-PCR (Wagenaar *et al.*, 2003; Townsend *et al.*, 2005; Bano *et al.*, 2008), PFGE (Trott *et al.*, 1996a; Suriyaarachchi *et al.*, 2000; **I**; Phillips *et al.*, 2005), randomly amplified polymorphic DNA (RAPD) (**I; II, III, V**), amplified fragment length polymorphism (AFLP) (van Bergen and Wagenaar *et al.*, 2003), multi-locus sequence typing (MLST) (Råsbäck *et al.*, 2007), and *nox* gene sequencing (Atyeo *et al.*, 1999; **I**; Townsend *et al.*, 2005; Råsbäck *et al.*, 2007; **III**; **V**).

1.5 Animal models

Animal models with pigs, guinea pigs, mice and chickens have been used to investigate colonization, host ranges, immune responses, and pathogenicity. Experimental challenge of pigs and mice are most commonly used to study SD (Hutto and Wannemuehler, 1999; Jacobsson *et al*, 2004). Many different *Brachyspira* strains colonize the intestines of chickens (Table 7). Most used strains (except 155-5, B256^T, 155-20, 27042-94B, and 513A^T) were shown to colonize chickens, and a majority (except CPSi1, SP16, 16242-94, Rosie 2299) were reported to produce mild intestinal signs and/or microscopic lesions. A few trials have been performed in other bird species, including geese (Ivanics *et al.*, 2007, section 1.4.5), common rheas (Swayne, 1994, Buckles, 1996, section 1.4.5) and mallards (Buckles, 1996). The mallards were challenged with a *B. hyodysenteriae* strain (R1) from a common rhea (Buckles, 1996). The birds were colonized, but lesions were not observed.

Table 7. *Challenge trials in chickens (uncharacterized/unnamed strains not included).*

Origin	*Brachyspira* spp.	Strain(s)	Reference
Chicken	*B. intermedia*	1380, HB60	Dwars *et al.*, 1990, 1991, 1993; Hampson and McLaren, 1999; Hampson *et al.*, 2002; Phillips *et al.*, 2004a, 2004b
	B. pilosicoli	CPSp1	Stephens and Hampson, 2002a, 2002b; Jamshidi and Hampson, 2002
	B. alvinipulli	C1	Swayne *et al.*, 1995
	B. innocens	CPSi1	Stephens and Hampson, 2002a
Common rhea	*B. hyodysenteriae*	R1	Swayne, 1994
Pig	*B. hyodysenteriae*	B78^{T}, B204$^{R;}$ WA15; SA3	Adachi *et al*, 1985; Sueyoshi *et al.*, 1986, 1987; Sueyoshi and Adachi, 1990 Trott *et al.*, 1995; Trott and Hampson, 1998
	B. intermedia	889	Trott and Hampson, 1998
	B. innocens	B256^{T}; 155-5	Muniappa *et al.*, 1997; Trott *et al.*, 1995; Muniappa *et al.*, 1997
	B. murdochii	155-20	Trott and Hampson, 1998
	B. pilosicoli	1648, 3295; UNL-3, UNL-5, UNL-8, D9201243A, T9300098, T9301604B	Trott *et al.*, 1995; Muniappa *et al.*, 1997; Trott and Hampson, 1998
Chiloe widgeon	*B. pilosicoli*	S76	Swayne *et al.*, 1993
Dog	*B. pilosicoli*	K9-12, 16242-94	Muniappa *et al.*, 1996
	B. innocens	27042-94B	Muniappa *et al.*, 1996
Human	*B. pilosicoli*	SP16, WesB, Kar, GAP 401, Rosie 2299, HIV3AB2	Muniappa *et al.*, 1996, 1998; Trott *et al.*, 1995; Trott and Hampson, 1998; Jamshidi and Hampson, 2003
	B. aalborgi	513A^{T}	Trott and Hampson, 1998
Rhesus monkey	*B. pilosicoli*	MMU27669, MMU26986, MMU26717	Muniappa *et al.*, 1998

1.6 Virulence attributes and pathogenesis

Virulence mechanisms and pathogenesis of *Brachyspira* spp. in mammals and birds are poorly understood and have focused on SD and PIS. SD is a multifactorial disease, with the aetiologic agent interacting with a range of other factors such as host immunity, intestinal microbiota and diet

(Jacobsson, 2004; Hampson *et al.*, 2006a). Suggested virulence mechanisms and factors include motility, chemotaxis, oxygen tolerance, lipooligosaccarides, lipopolysaccharides, and β-haemolysins (Hampson *et al.*, 2006a). Cells of *B. hyodysenteriae* are highly motile in viscous media, and are attracted to mucosal glycoproteins (Kennedy and Yancey, 1996). Targeted mutation of motility genes of *B. hyodysenteriae* produced strains that failed to colonize mice and showed reduced virulence (Rosey *et al.*, 1996; Kennedy *et al.*, 1997). The aerotolerance of *Brachyspira* spp. emanates from NADH oxidase activity (Stanton *et al.*, 1993, 1999). This enzyme has been suggested to protect bacteria when exposed to the oxygen respiring mucosa. A *nox*-defective mutant of *B. hyodysenteriae* lacked virulence (Stanton *et al.*, 1999). β-haemolytic activity has long been associated with virulence in *B. hyodysenteriae*. Extracted haemolysin from *B. hyodysenteriae* causes lysis of erythrocytes, cytotoxic effects in eukaryotic cell lines and epithelial lesions in a murine SD model (Hutto and Wannemuehler, 1999). The first putative β-haemolysin genes, *tly*A, *tly*B, and *tly*C, in *B. hyodysenteriae* strain B204^{R} (Muir *et al.*, 1992; ter Huurne *et al.*, 1994), were later suggested to be transcriptional regulators (Hsu *et al.*, 2001). The β-haemolysin gene *hly*A of *B. hyodysenteriae* has been identified (Hsu *et al.*, 2001), but interestingly, it is also present in the weakly β-haemolytic species *B. pilosicoli* (Zuerner *et al.*, 2004).

The pathogenesis of PIS (caused by *B. pilosicoli* in pigs) is even more poorly understood. In this case the bacterial cells attach to the apical cell membrane of enterocytes in large numbers, but also invade crypts, epithelium and the lamina propria (Hampson and Duhamel, 2006). Adhesins and invasins have, however, not yet been identified. DNA of *B. pilosicoli* did not hybridize with probes derived from attachment and invasin determinants of *Yersinia enterocolitica*, *Shigella flexneri* and enteropathogenic *Escherichia coli* (Hartland *et al.*, 1998). It has been suggested that the epithelial damage and loss of available epithelial surface may lead to reduced fluid and nutrient absorption (Gad *et al*, 1977; Muniappa *et al.*, 1998; Hampson and Duhamel, 2006).

2 Aims of the thesis

This thesis deals with various aspects of intestinal spirochaete colonization by members of genus *Brachyspira* among domestic and free-living wild birds. The thesis was based on the hypotheses that some bird species support a diverse population of *Brachyspira* spp., and that free-living wild birds are epidemiologically significant as reservoirs. The specific aims were:

- To study the prevalence of *Brachyspira* spp. in free-living wild mallards, and to characterize to species level a selection if isolates from farmed and free-living wild mallards that possess phenotypic characteristics of the swine dysentery agent (*B. hyodysenteriae*) (papers **I** and **II**).
- To investigate whether *Brachyspira* spp. that are genetically closely related to *B. hyodysenteriae* can colonize pigs and cause enteric disease (paper **III**).
- To modify and apply a chicken challenge model of SD in mallards to investigate colonization rates and assess morphological changes associated with *B. hyodysenteriae* and related bacteria in mallards (paper **III**).
- To study the occurrence of *Brachyspira* spp. in commercial laying hens in different housing systems in Sweden. To characterize a subset of the isolates to species level, with special focus on pathogenic species (*B. alvinipulli*, *B. intermedia* and *B. pilosicoli*) (paper **IV**).
- To collect and characterize intestinal spirochaetes from a free-living wild bird population where selection was based on a previous screening study (paper **V**).

3 Considerations on Materials and Methods

3.1 Study populations

The choice of avian study populations was based on the literature and screening data (Jansson *et al*, unpublished). Selection criteria for free-living wild birds included a favourable conservation status, availability of high-quality samples, and preferably an urban and/or farmland habitat.

3.1.1 Laying hens

The chicken (*Gallus gallus domesticus*) is the most numerous poultry species for egg and meat production around the world. It is descended from South Asian forest-dwelling junglefowl (Al-Nasser *et al.*, 2007; Eriksson *et al.*, 2008). The earliest archaeological findings of possible domesticated chickens date from 5400 B.C. in China, and from 2500-2100 B.C. in the Indus Valley (Al-Nasser *et al.*, 2007). The modern poultry industry is the result of selection for high-yielding genotypes, improved nutrition, housing and management, and control of infectious diseases by biosecurity, vaccination and antimicrobials. In 2005, the global number of laying hens was estimated to include 5,700 million birds and the egg production was approximately 60 million tons, with an annual growth of 1.5% (WATT90 Executive Guide to World Poultry Trend 2007/2008). In Europe, the number of laying hens has remained stable during the last decade, with 722 million birds in 2005. Approximately 5.3 million laying hens were kept in Sweden in June 2007 (Sveriges Officiella Statistik, 2008). The laying hen population in Sweden is based on imported grandparent or parent stock. Pullets, i.e. laying hens before start of lay, arrive to egg producing farms at about 16 weeks of age. The birds are kept until 70-80 weeks of age.

On a global basis, the majority of laying hens are housed in conventional (non-enriched) cages. In the member states of the European Union, three different types of housing systems are allowed; conventional cages (minimum area 550 cm^2/hen), enriched cages (minimum area 750 cm^2 per hen, cages contain perches, dust-bathing facilities and nest boxes), and non-cage systems with litter-area, perches and nest boxes (maximum number of hens is 9/m^2 usable area) (Council Directive 1999/74/EC, 1999). Conventional cages will be prohibited by 2012. The Swedish Animal Welfare Ordinance from 1988 prohibited conventional cages for laying hens from 1999, but time-limited individual exemptions were granted until January, 2004. When the sampling for paper **IV** was performed (May 2003-June 2004), the industry was in the process of exchanging conventional cages for other housing systems. At the beginning of the study, 33.3% of the eggs delivered to egg packing plants were produced from hens housed in conventional cages, 20.8% from hens in enriched cages and 45.9% from hens in litter-based housing systems (data from January-April, 2003; EuroEgg and Business AB, 2006). At the end of the sampling period (data from May-August, 2004) 13.5% of the eggs were produced by hens in conventional cages, 35.6% from hens in enriched cages and 59.3% by hens in litter-based housing systems (EuroEgg and Business AB, 2006).

3.1.2 Mallards

In paper **I**, mallards from a game farm, presumed free-living wild (non-ringed) mallards in a public park, and autumn-migrating free-living wild mallards were sampled for *Brachyspira* spp. The mallard is a Holarctic waterfowl species that is the ancestor of most domestic ducks. In Sweden, the guesstimated population size is 100,000-150,000 breeding pairs (Svensson *et al.*, 1999). Mallard habitats consist of shallow water, such as lakes, marshes, flooded fields, wetlands, park ponds, streams and rivers, where the birds dabble for aquatic vegetation, gastropods, insects, arthropods, small fish, fish eggs, tadpoles, snails and frogs. They may take advantage of other food resources such as crops and grass. Their adaptability often brings them in close contact with humans. The mallard is also an important game species, and large numbers of birds are raised on game farms for release and hunting in many countries, including Sweden. Analysis of recovery data of mallards ringed in southern Sweden has shown that most birds sampled in the autumn spend the breeding season in Finland, Russia and the Baltic States, and the winter season in southern Sweden, Denmark, and Western Europe (Wallensten *et al.*, 2007).

3.1.3 Corvid birds

In paper **V**, three species of corvid birds were sampled during spring and summer. The population sizes include approximately 300,000-800,000 jackdaws, 500,000-1,000,000 hooded crows, and 46,000 rooks (Svensson *et al.*, 1999). All three species are omnivorous and opportunistic feeders. They are found across Europe and Asia. Jackdaws breed in arable areas, in villages and cities in the southern half of Sweden, and along the coast northwards. Hooded crows breed in a wide variety of habitats all over the country, and rooks breed locally in colonies in southern Sweden (Fransson and Hall-Karlsson, 2008). Bird recovery data show that all three species migrate in late autumn in a south-western direction (Fransson and Hall-Karlsson, 2008). Most rooks migrate to Denmark. The majority of jackdaws and hooded crows stay in southern Sweden during the winter season, whereas a subset migrates to Denmark or north-western continental Europe (Fransson and Hall-Karlsson, 2008). The populations of jackdaws and rooks that were sampled for paper **V** probably intermingle to some extent at stopover sites in early spring on their return from over-wintering areas (Fransson, T, Naturhistoriska Riksmuseet, Stockholm, personal communication).

3.2 Laboratory diagnostics

3.2.1 Selective culture and phenotypic tests

Isolation of *Brachyspira* spp. requires at least 2-5 days of anaerobic incubation at 37-42°C in a selective culture medium supplemented with 5-10% blood (usually ovine or bovine blood). The diagnostic approach on solid media used in this thesis is outlined in Fig. 10-11. On agar plates, *Brachyspira* spp. produce colonies of varying size and appearance, and/or a diffuse haze on the agar surface (Fig. 12). Phenotypic tests are used for presumptive species determination (Fig. 10-11, Table 8). In paper **II** and **III**, broth culture was used to produce large numbers of viable cells for experimental challenge. The strains were obtained from storage, passaged once and incubated anaerobically in brain heart infusion broth (BHI) (Difco) with 10% foetal calf serum (FCS) at 37°C on a shaking platform.

3.2.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed in study **II** by a previously described broth dilution method (Karlsson *et al.*, 2001).

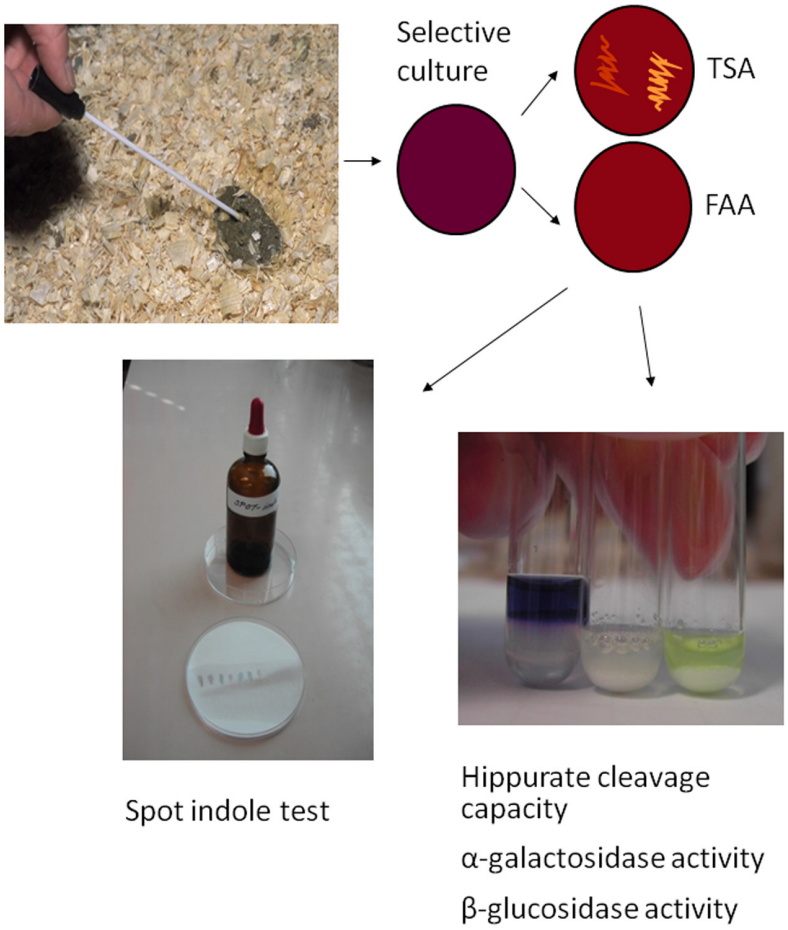

Figure 10. Diagnostic procedure of *Brachyspira* spp. Samples were transported in Amies medium (Venturi Transystem®, Copan Innovation, Italy). They were streaked on selective *Brachyspira* agar plates (blood agar base No. 2, 5% ovine blood, 1% sodium ribonucleate, spectinomycin (800 µg/ml), vancomycin (25 µg/ml), colistin (25 µg/ml), and were incubated at 42°C for 6 days. An appropriate atmosphere (90-95% H_2, 5-10% CO_2) was obtained by use of gas packs and anaerobic jars. The sensitivity of the isolation procedure used is 10^2 CFU/g faeces for *B. hyodysenteriae* (Fellström *et al.*, 2001). Isolates were subcultured on fastidious anaerobe agar plates (FAA) (LabM) supplemented with 10% equine blood. The isolates were tested for intensity of β-haemolysis on trypticase soy agar (TSA) supplemented with 5% bovine blood, indole spot test (left lower picture), hippurate cleavage capacity (right lower picture, blue tube), and α-galactosidase and β-glucosidase activity (right lower picture, middle and right tubes) as previously described (Fellström and Gunnarsson, 1995; Fellström *et al.*, 1999).

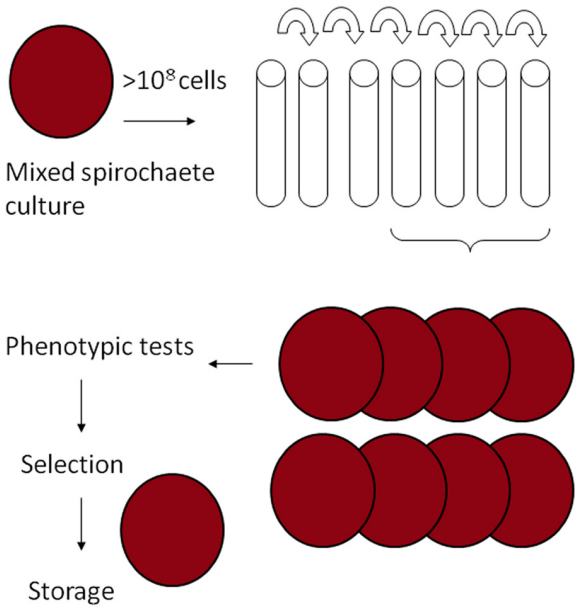

Figure 11. Subculture of *Brachyspira* spp. Isolates were subcultured to purity by broth dilution (**IV, V**). Bacteria were suspended in BHI (Difco), and a serial 10-fold dilution was performed. Samples calculated to contain 1-100 bacterial cells per 0.1 ml were seeded on separate FAA plates that were immediately subcultured anaerobically at 42°C for 48-72 h. Following incubation, all subcultures were subjected to phenotypic tests. Isolates were stored in liquid nitrogen in beef broth with 10% equine serum and 15% glycerol.

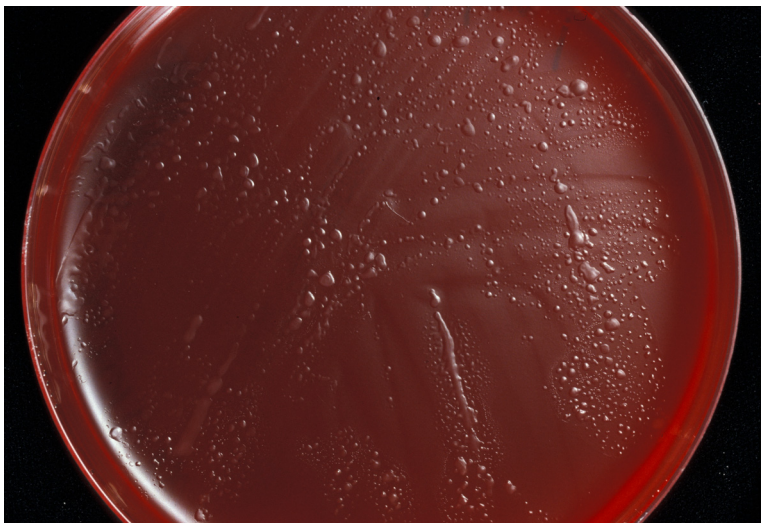

Figure 12. Morphology on a FAA plate of *B. intermedia* isolated from a lying hen. (The β-haemolysis is not visible in this picture.) Photo: B Ekberg & DS Jansson, SVA.

Table 8. *Phenotypic features for presumptive species identification of Brachyspira spp.*

Species	β-haem	IS	Hipp	α-gal	β-glu
B. hyodysenteriae	strong	pos (neg)	neg	neg	pos
'*B. suanatina*'	strong	pos	neg	neg	pos
B. intermedia	weak	pos	neg	neg	pos
B. innocens	weak	neg	neg	pos	pos
B. murdochii	weak	neg	neg	neg	pos
'*B. canis*'	weak	neg	neg	neg	pos
'*B. pulli*'	weak	neg	neg	pos	pos
B. pilosicoli	weak	neg (pos)	pos (neg)	pos	neg (pos)
B. alvinipulli	weak	neg	pos	neg	pos (neg)
B. aalborgi	weak	neg	neg	neg	neg
'*B. ibaraki*'	weak	neg	NA	NA	NA
'*B. christiani*'	NA	NA	NA	NA	NA
'*B. corvi*'	weak	neg	neg	pos (neg)	neg (pos)

Abbreviations: β-haem= β-haemolysis; IS=indole spot test, Hipp=Hippurate cleavage capacity; α-gal α-galactosidase; β-glu=β-glucosidase activity; pos=positive reaction; neg=negative reaction. NA=not available, less common reactions shown within parentheses. **References:** Trott *et al.*, 1996c; Duhamel *et al.*, 1998; Stanton *et al.*, 1997; Stanton *et al.*, 1998; Fellström *et al.*, 1995, 1999; Kraaz *et al.*, 2000; Jensen *et al.*, 2001; Tachibana *et al.*, 2003; Fossi *et al.*, 2004; Johansson *et al.*, 2004; Hampson and Duhamel, 2006; **II**; **IV; V**.

3.2.3 PCR, RAPD and PFGE

In this thesis ten different single or duplex PCRs were used targeting the *tly*A gene of *B. hyodysenteriae* (paper **I**, **II**, **IV**), the 16S rRNA gene of *B. pilosicoli* (paper **II, IV**, **V**), the *nox* gene of *B. hyodysenteriae* (paper **II**), the *nox* gene of *B. intermedia* (paper **II**, **IV**), the 23S rRNA gene of *B. hyodysenteriae* (paper **II**, **IV**), and the 23S rRNA gene of *B. intermedia* (paper **II**, **IV**).

Randomly amplified polymorphic DNA (RAPD) is a PCR-based molecular method that uses short non-specific primers to produce multiple random amplicons of various sizes that can be separated and visualized by electrophoresis on an agarose gel (Caetano-Anollés, 1993). No prior sequence information is needed, and the method is easy and inexpensive to perform. The most important disadvantage is that the results are not always reproducible between different runs. The results of the analysis can be visually compared or analyzed by a software package to generate a dendrogram that shows the degree of relatedness between investigated isolates. Both approaches were used in this thesis.

Pulsed field gel electrophoresis (PFGE) is often used for epidemiological studies in bacteriology. Bacterial DNA is digested by a rare cutting restriction enzyme *in situ* in agarose plugs, and the resulting large DNA fragments are separated on a gel by periodically switching the direction of the electrical field. PFGE was used in paper **I** to compare genetic relatedness among isolates from mallards with type, reference and field strains of *Brachyspira* spp. Data were analyzed by the GelCompar program, and a dendrogram was calculated by the UPGMA method.

3.2.4 Sequencing and phylogenetic analysis

Sequencing of the 16S rRNA gene and phylogenetic analysis (etymology: Gr. phyle 'tribe' or 'race' and genetikos 'relative to birth') was performed in papers **I**, **II**, **IV** and **V**. Molecular phylogeny stems from the seminal work of Zuckerkandl and Pauling, who showed that the evolutionary history of an organism is preserved in the sequences of macromolecules (Zuckerkandl and Pauling, 1965). The translational system has been highly conserved throughout evolution, and small subunit ribosomal RNA (16S rRNA in bacteria) has been used as a universal phylogenetic marker since the late 1970s (Woese *et al.*, 1977; Fox *et al.*, 1980; Ludwig and Klenk, 2001). One 16S rRNA gene is present per genome in *Brachyspira* spp., but the gene organization differs from most other bacteria, i.e. an assembled ribosomal rRNA operon is not present in *B. hyodysenteriae* and *B. pilosicoli* (data not available from other *Brachyspira* spp.) (Zuerner *et al.*, 2004).

Sequencing starts with PCR amplification by using a set of primers that targets the gene of interest (almost complete 16S rRNA gene in papers **I**, **II**, **IV** and **V**, and *nox* gene in papers **II** and **V**, see below). The amplicons are subsequently processed by automated cycle sequencing. The DNA, specific forward and reverse sequencing primers targeting conserved segments, DNA polymerase, nucleotides and fluorescent labelled reaction terminators (dideoxynucleotides, didNTP) (Big Dye, Applied Biosystems, Foster City, California, USA) are mixed. The DNA strands are separated by heating, and when the temperature is lowered the primers and the DNA polymerase bind to the template and a complementary strand is synthesized. The reaction stops every time a didNTP is incorporated in the growing polynucleotide chain. The mix, which will consist of millions of fragments of different sizes, is analyzed by capillary electrophoresis (ABI Prism 3100 genetic analyser, Applied Biosystems). The overlapping fragments are unified to a contig by software (Contig Express program included, Vector NTi Suite, InforMax, Bethesda, Md, USA). The frequent finding in papers **IV** and **V** of isolates containing several species or genotypes were visible as

double peaks in the electropherograms. The sequences were edited and aligned (GDE software, Smith, 1992), i.e. homologous nucleotides are arranged in columns. The alignment can be made by an alignment program or manually as in this thesis. For comparison, prealigned sequences can be obtained from the RDP database (Ribosomal Database Project, 2008). An evolutionary tree is constructed from the alignment by an algorithmic or tree-searching method. In this thesis, the distance matrix algorithmic method Neighbor Joining (Saitou and Nei, 1987) was used. The Kimura 2-parameter model was used as an evolutionary model to correct for multiple substitutions and different rates for transitions and transversions (Kimura, 1980).

Compared to the 16S rRNA gene, the *nox* gene possesses more sequence variation. A major drawback is the insufficient number of sequences available in databases. Trees based on the *nox* gene depict the phylogeny of this particular gene, not necessarily the evolution of the organisms.

The phylogenetic trees in papers **I**, **II**, **IV** and **V** are bifurcating and additive. Terminal nodes represent extant taxa i.e. the bacteria from which sequences were obtained, and internal nodes represent inferred ancestors. Branches between the nodes show the inferred evolutionary path. The added lengths of the branches between nodes show the calculated phylogenetic difference. The tree may be presented as an unrooted radial dendrogram (papers **II** and **V**), which gives information on the relationship between taxa, but not the evolutionary path, or as a rooted dendrogram (papers **I**, **II**, **IV** and **V**) which shows both. Trees should be considered as evolutionary hypotheses, i.e. they do not necessarily show the true historic evolutionary path.

3.3 Experimental challenge

In paper **II**, a previously described porcine SD infection model (Jacobsson *et al.*, 2004) was used to evaluate the enteropathogenic potential of a pig and a mallard isolate of '*B. suanatina*'. In paper **III**, a modified chicken model was applied to mallard ducklings to investigate pathogenicity of *B. hyodysenteriae* and '*B. suanatina*' of pig and mallard origin. The ducklings were housed in groups on litter in different rooms instead of in cages, and they had access to water baths and were allowed to acclimatize before challenge.

4 Results and Discussion

Since the first modern report on intestinal spirochaete colonization in birds was published in 1986, research activities in Australia, the US, and Europe, have brought us basic understanding of the clinical, epidemiological and bacteriological aspects. However, there are still many things to be learnt about these bacteria. Among the main causes of the lack of information and limited interest among poultry practitioners have been the non-specific nature of the clinical signs, and perhaps most importantly, diagnostic difficulties. It is only during the last 10-15 years that attempts have been made to identify avian intestinal spirochaetes to species level. In this thesis, two basic problems were addressed: first, the occurrence and species distribution of these bacteria in commercial chickens and in selected free-living wild bird species, and second, the diagnostics and classification of avian *Brachyspira* strains.

4.1 Synopsis of results

- Free-living wild and farmed mallards are regularly colonized by *Brachyspira* spp., including the swine dysentery agent *B. hyodysenteriae*, the proposed new species '*B. suanatina*' and other strongly β-haemolytic and indole-positive strains that cannot presently be classified to species level (**I**, **II**).
- *B. hyodysenteriae* from mallards were very closely related to isolates previously described as the cause of severe necrotizing typhlocolitis in common rheas on farms in the United States (**I**).
- Following experimental challenge of pigs with field isolates of the proposed new species '*B. suanatina*' of pig and mallard origin, clinical disease indistinguishable from SD (isolate from a pig) or a milder diarrhoea (isolate from a mallard) were observed (**II**).

- Mallards were readily colonized after experimental challenge by strains of '*B. suanatina*' of both mallard and pig origin, and by *B. hyodysenteriae* of mallard origin (**III**).
- No signs of clinical disease were noted in the challenged mallards, but focal intestinal changes coinciding with spirochaetal infiltration were observed on light and transmission electron microscopic levels in the caecal epithelium of the most heavily colonized birds (**III**).
- After challenge of mallard ducklings with '*B. suanatina*' by the cloacal route the birds were quickly colonized, which indicates that the cloacal infection route could be important under field conditions (**III**).
- The pathogenic species *B. intermedia* and *B. alvinipulli*, but not *B. pilosicoli*, were detected in randomly selected Swedish commercial laying hens. Also, the presumed nonpathogenic species *B. innocens*, *B. murdochii* and '*B. pulli*', as well as several isolates that could not be characterized to species level were found (**IV**).
- Organic laying hens were at higher risk for being colonized by *Brachyspira* spp. than hens in enriched cages or indoor litter-based housing systems (**IV**).
- No association with gastrointestinal disease or production losses were found in the colonized laying hen flocks (**IV**).
- Simultaneous colonization of more than one species and/or genetic variant of *Brachyspira* spp. were commonly found in laying hen flocks (**IV**).
- Results of phenotypic tests were reproducible and similar to previously reported results when applied to pure strains (**IV**).
- A new spirochaete species '*B. corvi*' was characterized and provisionally described (**V**).
- The hexa-T segment of the 16S rRNA gene equivalent to positions 176-181 in the type strain of *B. pilosicoli* can no longer be considered as species-specific for *B. pilosicoli* since it was identified in all investigated isolates of '*B. corvi*' (**V**).

4.2 Host range

To date all validated and proposed *Brachyspira* spp. except *B. aalborgi*, '*B. canis*', '*B. christiani*' and '*B. ibaraki*' have been isolated from at least one bird species. In this thesis, important new information on the host range of genus *Brachyspira* was gained. It was the first time a natural avian reservoir was found for the SD agent *B. hyodysenteriae* (**I**). Previously, the only non-domesticated animal species that have been shown to harbour *B.*

hyodysenteriae were rodents on pig-farms (Joens and Kinion, 1982; Hampson *et al.*, 1991; Duhamel *et al*, 2001), and feral pigs (Phillips *et al.*, 2008). Among birds, common rheas kept on commercial farms or in zoologic exhibits were the only known hosts before our finding in mallards (Jensen *et al.*, 1996). Available data indicate that a strain of *B. hyodysenteriae* from a mallard failed to colonize pigs by experimental challenge (**I**). If these preliminary results are confirmed, the strains from mallards could be used for comparative studies to identify putative pathogenicity determinants in porcine strains of *B. hyodysenteriae* from complete genome sequence data. Further findings on host range of this thesis include the proposed novel species '*B. suanatina*', the first avian strain shown to cause diarrhoea in pigs. This proposed novel species was identified from both pigs and free-living wild mallards (paper **I-II**). In paper **V**, the intestinal spirochaete proposed as '*B. corvi'* was isolated from corvid birds. We also showed that a previously described canine isolate was most closely related to '*B. pulli*', which had previously only been isolated from chickens (**IV**). Differences in host range of *Brachyspira* spp. in mammals and birds and between bird species could be caused by anatomic, physiologic, dietary or other unknown factors. Notably, the core body temperatures of many birds are above those of mammals (Dawson and Whittow, 2000). Reported core body temperatures of chickens and domestic ducks are 41.5°C and 42.1°C, respectively (Dawson and Whittow, 2000), and the mean body temperatures obtained from jackdaws and hooded crows were 42.4°C (Jansson DS, unpublished results). Further, caecal anatomy and physiology vary between avian species.

The known host distribution of *Brachyspira* spp. among birds is presently limited to a few orders, namely ratites, gallinaceous, anseriform, and single genera among phoenicopteriform and passerine birds (section 1.4.2, Table 5). This may be compared to what is known abut the evolution of birds. Based on palaeontological records, morphology and phylogenetic analyses, there is robust evidence for a basal split between palaeognathous birds (ratites and allies) and neognathous birds (all other birds), as well as for a split between galliform/anseriform birds (chickens, duck and allies) and all other neognathous birds (i.e. Neoaves) (Ericson *et al.*, 2006; Hackett *et al.*, 2008). The estimated timing of these events varies widely between studies. In a recent study, the neoavian branch, which is represented by the majority of extant species including all passerine birds, was shown to have undergone an explosive radiation at or soon after the Cretaceous-Tertiary (K/T) boundary 65 Mya (Ericson *et al.*, 2006). Corvid birds are a monophyletic group among the passerines of much more recent (mid or late Tertiary, 23 Mya or later) Southern Hemisphere origin (Feduccia, 1995; Ekman and

Ericson, 2006). These results first of all suggest that it should come as no surprise that *Brachyspira* spp. from corvid birds are phylogenetically distant to those of ratites, gallinaceous and anseriform birds. Secondly, one could speculate that *Brachyspira* spp. are likely colonizers of additional bird orders, having been detected in the most basal avian orders and the much more recent corvids.

4.3 Biodiversity among avian *Brachyspira* spp.

The biodiversity of naturally occurring bacterial populations can be studied to gain a better understanding of prokaryotic biology and evolution (Sikorski *et al.*, 2008). From such studies, hypotheses on historical events and the emergence of morphological structures, bacterial physiology and ecology may be put forward. Studies on biodiversity are also central to taxonomy. Bacteria are haploid organisms with clonal reproduction. Genetic variation stems from mutation and recombination, which produce intrataxon diversity and sometimes cladogenesis, i.e. the formation of new taxa, such as species. Characterization of *Brachyspira* spp. from birds in this thesis and by others has shown that these bacteria are in most cases genetically closely related to mammalian strains, although possessing a significantly higher degree of phenotypic and genomic diversity than in their mammalian counterparts. Several *Brachyspira* species seem to be able to cross species barriers between mammals, and between mammals and birds as shown by experimental challenge trials (Hampson *et al.*, 2006c; Trott *et al.*, 1996b; **II; III;** and Table 6). This higher level of spirochaete diversity in birds suggests that birds may be original reservoirs of ancestral *Brachyspira* spp. From ecological, evolutionary and veterinary medical perspectives, *Brachyspira* spp. in avian hosts seem to be excellent model microorganisms for future studies on microbial biodiversity. We have only just begun to reveal the biodiversity of this interesting bacterial group.

In several recently published studies on *Brachyspira* spp. from chickens (Stephens *et al.*, 2005; Phillips *et al.*, 2005; Bano *et al.*, 2008; Feberwee *et al.*, 2008), it was shown that some flocks may be colonized by more than one *Brachyspira* spp. simultaneously. This was confirmed in paper **IV**, and it was also shown that the occurrence of multiple species and even genotypes of the same species in the same flock has probably been significantly underestimated in the past. This is a most interesting result in view of the finding of prophage-like GTAs detected in *B. hyodysenteriae*, (VSH-1) and other *Brachyspira spp.* (Stanton *et al.*, 2003; Stanton, 2007). GTAs are the suggested causes of the recombinant genome structure detected by MEE in

B. hyodysenteriae and *B. pilosicoli* (Trott *et al.*, 1997; Zuerner *et al.*, 2004; Hampson *et al.*, 2006c). Speculatively, it could be responsible for random recombination events between strains of the same or possibly even between different *Brachyspira* spp. when present simultaneously in the same environment, such as in the avian intestine.

4.4 Diagnostic difficulties

The results of this thesis emphasize the previously reported diagnostic difficulties within genus *Brachyspira* (Råsbäck *et al.*, 2006). Paper **IV** clearly showed the difficulties in obtaining pure isolates for molecular characterization and the substantial problems in differentiating pathogenic from presumed non-pathogenic species. In papers **I** and **IV** some of the strains could not be identified to species level.

Phenotypic data have long been used for presumptive species determination of isolates of porcine origin (Fellström and Gunnarsson, 1995; Hommez *et al.*, 1998; Fellström *et al.*, 1999). Phenotypic and molecular tests used in conjunction can reliably differentiate most porcine isolates to species level. However, examples of porcine isolates with aberrant/atypical phenotypes are accumulating in literature (Hommez *et al.*, 1998; Fellström *et al.*, 1999, Thomson *et al.*, 2001; Fossi *et al.*, 2004). Biochemical data on avian *Brachyspira* spp. have, however, not been extensively published, or fully evaluated. Indeed, several authors have expressed doubts about the usefulness of phenotypic tests for avian *Brachyspira* spp. (Stephens *et al*, 2005; Townsend *et al.*, 2005; Feberwee *et al.*, 2008). It seems reasonable to assume that these previous reported inconsistencies are, at least partly, the results of analysis of impure isolates. Our results in paper **IV** (Tables 5-6) showed that biochemical tests as applied to isolates from chickens were consistent only when applied to pure isolates. Compared to *Brachyspira* spp. in pigs, the situation in chickens is more complicated. Pathogenic as well as presumed non-pathogenic avian *Brachyspira* spp. are (with rare exceptions, McLaren *et al.*, 1996, 1997; Feberwee *et al.*, 2008) weakly β-haemolytic and cannot be reliably differentiated and separated on agar plates, and birds host a larger number of different *Brachyspira* species than pigs. Further studies are clearly needed to evaluate the use of phenotypic tests for *Brachyspira* spp. isolated from chickens and other birds. Results of paper **IV** suggest that phenotypic tests can be useful as tools for screening and selection.

PCR has been applied in previous studies on primary cultures and faeces to detect various *Brachyspira* spp. in birds, but it has important limitations

because all species present in birds cannot be identified, and several studies indicate lack of sensitivity and/or specificity when applied to avian strains (Ateyo *et al.*, 1999; Suriyaarachchi *et al.*, 2000). Most PCRs have been designed to identify porcine or human *Brachyspira* strains. The lack of sensitivity was confirmed in paper **IV**, where the three applied *B. intermedia* specific PCRs based on 23S rRNA and *nox* genes produced different results, and two of the PCRs failed to detect most strains. The most promising PCR for detection of *B. intermedia* from chickens was the most recently developed, that had been specifically designed to identify chicken isolates (Phillips *et al.*, 2005). However, it was necessary to use another forward primer than presented in the original paper. This modification has also been suggested in another paper (Bano *et al.*, 2008). Furthermore, there is no PCR available for *B. alvinipulli*. Until very recently, this species had only been reported from a laying hen flock in the USA (Stanton *et al.*, 1998). Recent results from Sweden, the Netherlands and the UK (Feberwee *et al.*, 2007, Jansson *et al.*, 2007a; Thomson *et al.*, 2007; Feberwee *et al.*, 2008; **IV**) indicate that the occurrence of this species has been underestimated among chickens, and possibly also among other avian species (Jansson *et al.*, 2007b). Presently, the only way to screen for *B. alvinipulli*, is by use of biochemical tests (Table 6 and paper **IV**), and molecular analyses such as MEE or sequencing of the 16S rRNA gene or *nox* gene is required for identification. Clearly, more work needs to be invested for reliable routine diagnostics of potentially pathogenic species.

In 1995 a signature sequence in the 16S rRNA gene of *B. pilosicoli* was reported (Park *et al.*, 1995). This specific hexa-T segment has so far been shown to be present in every sequenced strain of *B. pilosicoli*, and several species-specific PCRs have been designed to target this particular segment (Park *et al.*, 1995; Fellström *et al.*, 1997; Råsbäck *et al.*, 2006). Interestingly, in paper **V**, it was shown that '*B. corvi*' also possesses this hexa-T segment, and consequently, PCRs previously regarded as specific for *B. pilosicoli* should give a false positive result. This hypothesis was confirmed by paper **V**. In view of these results, it is advisable to base the diagnosis of *B. pilosicoli* not on a positive PCR reaction alone, but also on phenotypic traits, such as the strong hippurate reaction that is characteristic for most strains of *B. pilosicoli*, but not for '*B. corvi*'.

Another advantage of applying traditional labour-intensive and time-consuming traditional laboratory methods i.e. selective culture, subculture to purity and biochemical tests, together with molecular tests is that new species and genotypes may be identified. Paper **II** illustrates what could happen if PCR had been used exclusively for confirmation of *B.*

hyodysenteriae from the diarrhoeic pigs. In this case the PCR based on the *tly*A gene of *B. hyodysenteriae* failed to confirm the suspicion of SD. Without selective culture and further characterization, these pathogenic isolates would not have been identified and could have been further spread among pig herds.

4.5 Molecular phylogeny

Throughout this thesis, phylogenetic analysis based on the 16S rRNA gene was used for species identification (papers **I**, **II**, **IV** and **V**). Phylogeny based on this gene provides the backbone for elucidating the natural relationships between prokaryotic taxa, and it has played a pivotal role for spirochaetal systematics (Paster *et al.*, 1991; Paster and Dewhirst, 2000). The phylogenetic results in this thesis agree with earlier studies on avian strains (Swayne *et al.*, 1995; Jensen *et al.*, 1996; Stanton *et al.*, 1996; Phillips and Hampson, 2005; Townsend *et al.*, 2005; Nemes *et al.*, 2006; Feberwee *et al.*, 2008). By phylogenetic analysis, avian *Brachyspira* spp. can be divided in four main clusters: 1) *B. hyodysenteriae*/*B. intermedia*/'*B. suanatina*', 2) *B. innocens*/*B. murdochii,* 3) *B. alvinipulli*, and 4) *B. pilosicoli*. The proposed species *'B. pulli'* forms a sister lineage to either *B. alvinipulli* or *B. innocens*/*B. murdochii*. Because of the mosaic pattern of highly conserved and moderately to highly varying sequence motives of the 16S rRNA gene there is not always enough sequence information within this gene to reliably differentiate strains on the subspecies level. The phylogenetic depths within the *Brachyspira* spp. cluster are shallow, and some species can therefore not be differentiated, i.e. *B. hyodysenteriae* and *B. intermedia* and *B. innocens* and *B. murdochii* (**I**, **IV**). This is particularly disconcerting in case of the *B. hyodysenteriae*/*B. intermedia* cluster because of the problems of sensitivity and specificity of PCRs discussed previously (section 4.2). The results of paper **I** and **IV** seem to indicate that there is a range of closely related strains within the *B. hyodysenteriae*/'*B. suanatina*'/*B. intermedia* cluster. Some strains of *B. intermedia* from chickens (AN3370/03 (**IV**), HB60 and MMM-06 from Australia, and 1380 from the Netherlands) were very closely related to avian strains from Swedish mallards and common rheas in the United States and porcine strains of *B. hyodysenteriae* over a segment of 1220 nucleotides (**IV**). Further, two Swedish chicken strains of unknown taxonomic status were closely related to '*B. suanatina*' (**IV**). Also, a number of strongly haemolytic mallard strains within this cluster could not be identified to species level (**I**). These results raise important questions on the accuracy of the current taxonomic classification of genus *Brachyspira* and

further sequencing of other genes and/or DNA-DNA reassociation assays are required. Further, the type strains of *B. innocens* (B256^T) and *B. murdochii* (56-150^T) of porcine origin do not seem to be as closely related to avian isolates of these species as to other porcine isolates, which is interesting in view of the failure to colonize chickens experimentally by *B. innocens* and *B. murdochii* of porcine and canine origin (section 1.5, Table 7).

The classification of the spirochaetal isolates from corvid birds in genus *Brachyspira* ('*B. corvi*', **V**) is provisional and requires further studies. Based on the deep branching and calculated genetic distances within phylum *Spirochaetes* it presently seems very unlikely that the isolates should be affiliated to any other recognized spirochaetal genus than *Brachyspira*. Further phylogenetic analysis with comparisons of signature sequences and genus specific segments within the 16S rRNA gene, and comparisons of sequences from additional genes will resolve this question. However, it cannot be excluded that the isolates should be classified into a new genus.

4.6 Disease association in chickens

The etiological association of some *Brachyspira* spp. with intestinal disturbances and production losses in chickens has been repeatedly suggested since the late 1980s, and experimental challenge studies have confirmed an association with disease and production losses. However, in paper **IV**, no association was found between colonization and reduced egg production, faecal staining of eggshells, increased mortality or wet litter. Failure to identify a disease association is however not unique to paper **IV**. Effects of *Brachyspira* spp. colonization on clinical parameters have varied between studies, and have sometimes failed to reach significance (McLaren *et al.*, 1996; Jamshidi and Hampson, 2002; Phillips *et al.*, 2004a, 2004b; Bano *et al.*, 2008). There are many possible confounding factors such as clinical inclusion criteria vs. randomization, short duration of experimental trials, variable or unknown enteropathogenicity of isolates used for challenge and in the field, and diagnostic difficulties. Further, a multifactorial pathogenesis with host factors and environment interacting with intestinal spirochaetes as previously shown in SD, cannot be excluded in chickens (Jacobsson, 2004; Hampson *et al.*, 2006c). In paper **IV**, the failure to identify a disease association may be related to the fact that the flocks were randomly selected within the study population without regard to flock health or production levels. Therefore, relatively few flocks were experiencing intestinal disturbances and/or production losses at the time of sampling. Several other studies that reported or suggested a disease association have specifically

targeted flocks with signs of intestinal disease or other health problems and/or production losses, (Dwars *et al.*, 1998; Stephens and Hampson, 1999; Feberwee *et al.*, 2008). Potentially pathogenic strains were relatively rarely found in paper **IV** compared to these other studies. Another possible confounding factor could have been the ongoing change in housing systems during the sampling period. Many of the flocks were housed in new barns or in old barns which had recently been emptied of birds while new equipment and furniture were being installed. The surprisingly short survival times reported for of avian strains of *B. intermedia* and *B. pilosicoli* in chicken faeces (Phillips *et al.*, 2003) decreases the likelihood of bacteria surviving in poultry barns if cleaning, disinfection and down time are applied between consecutive flocks. There could also be differences in biosecurity routines and/or climate that might have contributed to our findings. Additionally, detailed data on health and egg production for the duration of the production period of the sampled flocks were not available from all farmers. Therefore, only health status and percent egg production on the day of sampling were included in the analysis.

4.7 Novel bacterial species

In papers **II** and **V** novel *Brachyspira* spp. were officially proposed. '*B. suanatina*' (**II**) was shown to be closely related to *B. hyodysenteriae*. Most interestingly, the 16S rRNA and *nox* gene sequences of these strains were identical or very similar to the corresponding sequences of two strains from mallards that we had failed to identify to species level in paper **I**. The isolates from mallards were shown to cause diarrhoea in challenged pigs. The close genetic and epidemiological relationship of the strains from pigs and mallards has been confirmed by MLST (Råsbäck *et al.*, 2007). The other proposed new species was '*B. corvi*' in paper **V**. This species seems to colonize not only the large intestine but also the jejunum and ileum of its host species, which is an unusual feature among *Brachyspira* spp. that awaits further confirmation. Although the gross and histologic results failed to associate colonization with intestinal lesions, this proposed species is of evolutionary interest because it forms a basally divergent phylogenetic branch within genus *Brachyspira*.

Classification and naming of bacteria are practical necessities for diagnostic purposes and research. The first modern standard for identification and classification of bacteria, Bergey's Manual of Determinative Bacteriology, was published in 1923. Before the era of molecular genetics, the classification of prokaryotes relied on phenotypic

traits, such as cell morphology, staining characteristics, growth pattern on agar plates, serology, phage typing, biochemical tests, and pathogenicity. Classification based solely on phenotypic traits does not always reflect the true evolutionary relationships and easily becomes incomplete and distorted since its resolving power is insufficient. There are few distinguishing gross characters among bacteria that can be used for classification, and fossils that can provide information on evolution and natural kinship are rare.

The current systematic classification of bacterial taxa is a matter of general agreement among scientists and is based on a phylophenetic species concept. A collection of bacterial strains that possess a high degree of overall phenotypic and genomic similarity in combination with diagnostic characters by which they can be distinguished from other strains, are grouped together as a species (Roselló-Mora and Amann, 2001). The list of species and their taxonomy is continuously revised and improved when new results are being duly published. For validation of a bacterial species, phenotypic data, monophyletic genealogy, information on intraspecies variation, and genomic species delineation are necessary components (Wayne *et al.*, 1987; Rosselló-Mora and Amann, 2001). The final step after publication is the recognition by inclusion of the new species in the Notification list or Validation list that are regularly published by the International Journal of Systematic and Evolutionary Microbiology. The two proposed novel species in this thesis therefore need further exhaustive characterization.

5 Concluding remarks and future perspectives

Despite substantial advances in recent years, spirochaetes in general remain a poorly understood group of bacteria. More specifically, there is a long list of obvious problems to investigate relating to *Brachyspira* spp., such as virulence mechanisms and the epidemiology. For veterinarians dealing with everyday clinical problems, treatment strategies and prophylactic measures need to be improved. Among other important topics to clarify are the possible association between *B. pilosicoli* (and possibly other spirochaetes) and human colitis. A cause and effect may however, be very difficult to demonstrate in humans, and isolation of spirochaetes in association with intestinal disease may simply reflect spirochaetal overgrowth or even increased dislodging of spirochaetes in diarrhoeic patients. There are also questions concerning the pathogenicity of intestinal spirochaetes in other mammalian and avian species. The zoonotic potential of *B. pilosicoli* needs to be confirmed or refuted. Reliable diagnostic methods for detection of potentially pathogenic *Brachyspira* spp. in birds are needed. Final confirmation of novel species should be of high priority.

Phylogenetic research is highly dynamic and constantly produces new results that lead to identification of new species, and emendations, reclassifications or unifications of known bacterial taxa. One might expect that significant new findings within phylum *Spirochaetes*, including genus *Brachyspira*, will be introduced within the next decade, especially when multigene phylogenetic analysis will become more feasible. Another field of priority is to give public access to sequence data from ongoing and future *Brachyspira* spp. complete genome projects. Such data will most likely contribute substantially to the knowledge of these fascinating bacteria.

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