Laboratory Diagnostics of *Brachyspira* species

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Cover illustration: *Brachyspira hyodysenteriae* cells. Photo: Märit Pingle
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Norman Vincent Peale

Abstract

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Bacteria of the genus *Brachyspira* are intestinal spirochaetes that can cause diarrhoea and mortality in pigs. Laboratory diagnostics of *Brachyspira* species is essential for confirming clinical diagnosis, for providing data for optimal treatment and for surveillance of the bacteria in individual animals or herds. The aims of the present thesis were to evaluate presently used, and develop new, laboratory diagnostic techniques for *Brachyspira* species, to describe the pathogenic and epidemiological features and antimicrobial susceptibility of selected *Brachyspira* isolates, and to evaluate DNA-based epidemiological and phylogenetic tools.

In study I, seeded samples were sent to laboratories in northern Europe to assess their ability to detect and identify *Brachyspira* species and test antimicrobial susceptibility. In study II, a PCR system was set up and compared with traditional culture and biochemical tests; and in study III, well-described *Brachyspira* isolates were analysed by five molecular typing methods. In the last two studies *Brachyspira* isolates from animals other than pigs were included. An atypical isolate detected in study II was further characterised together with identical or similar isolates (study IV) and a new genetic typing method for the entire genus of *Brachyspira* species was tested and evaluated (study V).

Overall, the results indicated that laboratory diagnostics of *Brachyspira* species is difficult. Of the tested genetic typing methods, the *nox* gene and MLST sequence analyses showed the highest taxonomic resolutions, and randomly amplified polymorphic DNA showed the highest discriminatory power for the isolates studied. A high genetic variability was observed within the *Brachyspira* genus. This genetic variability could make it difficult to rely solely on DNA-based methods for detection and identification of brachyspiras. A new unique group of isolates pathogenic to pigs was detectable only by the combined use of culture, biochemical tests and PCR. The provisional name *"B. suanatina"* was suggested for this type of isolates, of which some were found in mallards. By MLST data analysed by e-BURST, a close evolutionary relationship was identified for "*B. suanatina*" and *B. hyodysenteriae* isolates recovered from pigs and mallards.

In conclusion, these studies show that the most reliable method for laboratory diagnostics of *Brachyspira* species is culture and biochemical tests, used together with at least one DNA-based method.

Keywords: Spirochaetes, *Brachyspira*, anaerobe, culture, antimicrobial susceptibility, PCR, PFGE, RAPD, MLST, 16S rRNA, 23S rRNA, *nox*, *tly*A

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Sammanfattning

Intestinala spiroketer av bakteriesläktet *Brachyspira* är en vanlig orsak till diarré och dödsfall hos gris. *Brachyspira*-arter kan även påvisas hos andra djurslag, vilket inkluderar människan. *Brachyspira*-släktet utgör en grupp syretoleranta, anaeroba bakterier som växer långsamt och svärmande med svag eller stark hämolys. Den mest dramatiska sjukdomen hos gris som bakterier inom detta släkte kan orsaka är svindysenteri; en slemrik och blodig diarré som kan vara fatal. Svindysenteri orsakas av den starkt hämolyserande *B. hyodysenteriae*. En annan starkt hämolyserande spiroket är *”Brachyspira suanatina”* som här beskrivs för första gången och som hos gris också orsakar en dysenteriliknande diarré. En mildare form av *Brachyspira*-orsakad diarré hos gris kallas spiroketal diarré eller intestinal spiroketos, vilken orsakas av den svagt hämolyserande *B. pilosicoli*. Från gris kan även svagt hämolyserande *B. innocens*, *B. intermedia* och *B. murdochii* isoleras, vilka inte anses vara sjukdomsframkallande. Av Sveriges grisbesättningar uppskattas cirka 4 % vara infekterade med *B. hyodysenteriae* och 30 % med *B. piloscioli*. Syftet med denna avhandling var att utvärdera diagnostik och utveckla molekylärbiologiska metoder för påvisande och identifiering av bakterier inom *Brachyspira*-släktet.

De tre första studierna omfattade olika diagnostiska metoder för *Brachyspira*-arter isolerade från gris. I den första studien skickades bakterieisolat och faeces-prover med *Brachyspira*-celler till europeiska laboratorier för art- och antibiotika-resistensbestämning. I studie nummer två jämfördes en för studien utvecklad molekylärbaserad diagnostisk metod (PCR) med traditionell diagnostik (odling och biokemiska tester). I den tredje studien jämfördes resultat från fem molekylärbiologiska metoder (16S rRNA- och *nox*-gensekvensering, RAPD, PFGE och PCR) med traditionell diagnostik. I de två sista studierna ingick identifiering och klassificering av atypiska isolat, inklusive *Brachyspira*-isolat från andra djurslag än gris. I studie fyra beskrevs unika isolat som var identiska eller mycket lika det atypiska isolatet vilket identifierades i studie två. I den sista studien utvärderades en ny DNA-baserad diagnostik (MLST) avsedd för klassificering av *Brachyspira*-stammar. Denna metod kan komma att bli ”gold standard” för medlemmar av genus *Brachyspira* då MLST-data lätt kan jämföras mellan olika laboratorier i världen.

Resultaten visar att laboratoriediagnostik av bakteriesläktet *Brachyspira* kan vara en utmaning. PCR-resultaten och DNA-sekvensanalyserna för somliga gener visar på en hög genetisk variabilitet inom arterna. Detta kan försvåra utformningen av tillförlitliga molekylärbiologiska metoder, men kan utnyttjas i smittspårning. Jämförelser mellan olika molekylärbiologiska metoder och traditionell diagnostik visar att vissa DNA-baserade metoder inte är tillförlitliga för klassificering av alla *Brachyspira*-arter. De atypiska isolaten, som även fanns hos gräsand, visade sig vara sjukdomsframkallande hos gris. En ny art, *”B. suanatina”*, föreslogs, vilken kräver odling i kombination med PCR för identifiering. Sammanfattningsvis bör odling och biokemiska tester användas tillsammans med minst en DNA-baserad metod för en säker och tillförlitlig laboratoriediagnostik av släktet *Brachyspira*.

Supervisors, international exchange, faculty opponent and board of examiners

A public oral defence of this doctoral thesis has been timetabled for 9.15 a.m. Friday 14th September, 2007, in lecture hall "Ettan", KC (Clinical Centrum; The University Animal Hospital) at the Swedish University of Agricultural Sciences (SLU), Ultuna, Uppsala, Sweden.

Supervisors

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International exchange

An international visit was undertaken for three and half months during 2006, to Murdoch University, Perth, Australia. My host was Prof. *David J. Hampson*, DVM, PhD, Professor in veterinary microbiology, and his group.

Faculty opponent and board of examiners

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Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Råsbäck, T., Fellström, C., Bergsjø, B., Cizek, A., Collin, K., Gunnarsson, A., Jensen, S.M., Mars, A., Thomson, J., Vyt, P. & Pringle, M. 2005. Assessment of diagnostics and antimicrobial susceptibility testing of *Brachyspira* species using a ring test. *Veterinary Microbiology* 109, 229-243.

II. Råsbäck, T., Fellström, C., Gunnarsson, A. & Aspán, A. 2006. Comparison of culture and biochemical tests with PCR for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*. *Journal of Microbiological Method*s 66, 347-353.

III. Fellström, C., Råsbäck, T., Olofsson, T., Johansson, K.-E. & Aspán, A. Identification and genetic fingerprinting of *Brachyspira* species. Submitted for publication.

IV. 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.

V. Råsbäck, T., Johansson, K.-E., Jansson, D.S., Fellström, C., Alikhanie, M. Y., La, T., Dunn, D. S. & Hampson, D. J. Evaluation of a multilocus sequence typing scheme for intestinal spirochaetes of the genus *Brachyspira*. Submitted for publication.

Abbreviations

A	adenine
ATCC	American Type Culture Collection
B. species	*Brachyspira* species
"B. suanatina"	*"Brachyspira suanatina"* (the quotation marks indicate that the species is provisionally named)
BHI	brain heart infusion
BHIS	brain heart infusion with serum
bp	base pair
Brachyspira spp.	*Brachyspira* species (plural)
Brachyspira sp.	*Brachyspira* species (singular)
C	cytosine
CBT	culture and biochemical tests
CCUG	culture collection, University of Gothenburg, Sweden
CFU	colony-forming unit
DNA	deoxyribonucleic acid
dNTP	all four deoxynucleoside triphosphates (A, C, T, G)
DVM	doctor of veterinary medicine (Legitimerad Veterinär)
FAA	fastidious anaerobe agar
G	guanine
MIC	minimum inhibiting concentration
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
NADH	reduced form of nicotinamide adenine dinucleotide
NCCLS	National Committee for Clinical Laboratory Standards
nox	NADH oxidase
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
rDNA	the DNA coding for a ribosomal RNA gene
RNA	ribonucleic acid
rRNA	ribosomal RNA
S	the Svedberg unit
SLU	Sveriges Lantbruksuniversitet (Swedish University of Agricultural Sciences)
sp. nov.	species novum (new species)
Superscript T	type strain for a species (held at an international strain collection)
Superscript R	reference strain for a species (held at an international strain collection)
SVA	Statens Veterinärmedicinska Anstalt (National Veterinary Institute)
T	thymine
TSA	tryptone soya agar
U	uracil

Hypothesis and objectives

This thesis is based upon the hypothesis that laboratory diagnostics of *Brachyspira* species can be improved and that it is in constant need of adjustment to allow for the ever-changing bacterial population. The general objectives of the studies were to develop and evaluate suitable methods for laboratory diagnostics of *Brachyspira* species and to study the genetic diversity within the genus. The main group of *Brachyspira* species studied were those detected in pigs.

The specific objectives of the studies were to:

- Assess laboratory diagnostics and antimicrobial susceptibility testing of *Brachyspira* species in northern European laboratories through a ring test (Study I).

- Develop and/or evaluate different laboratory diagnostic techniques for detection and identification of *Brachyspira* species (Studies II, III and V).

- Evaluate epidemiological and phylogenetic tools for *Brachyspira* species (Studies III and V).

- Describe selected *Brachyspira* isolates, their pathogenic, antimicrobial susceptibility and epidemiological features (Study IV).

Introduction

The Roman philosopher Lucretius suspected the existence of microorganisms already between 98-55 before Christ (Prescott, Harley & Klein, 1996), but it was not until 1674 that the first observations and descriptions were made by Leeuwenhoek. Some of the organisms were described as very nimble with serpentwise bodies, which presumably were spirochaetes. Leeuwenhoek used a single-lens microscope of his own design to study what he called “animalcules” (Dobell, 1932). The name *bacterium*, which means small staff (stick, rod), was introduced in 1828 by Ehrenberg (http://www.etymonline.com/, 4-July-2007). In the development of medical microbiology, Louis Pasteur (1822-1895) and Robert Koch (1843-1910) are considered the two most influential founders. Pasteur confirmed the germ theory of disease, and created the first vaccine for rabies. Robert Koch became famous for isolating the tuberculosis bacillus (1882), for which he was awarded the Nobel Prize in 1905. He also stipulated Koch's postulate, which says that to establish an organism as the cause of a disease, it must be: found in all cases of the disease examined; prepared and maintained in a pure culture; capable of producing the original infection, even after several generations in culture; be retrievable from an inoculated animal and cultured again.

Traditionally, bacteria have been described by identifying what they lack in comparison to the mammalian (eukaryotic) cells. The bacteria (formerly eubacteria) are prokaryotic cells (i.e. without a cell nucleus). They do not have mitochondria or most other organelles such as the Golgi apparatus and the endoplasmic reticulum. Bacteria are often smaller than eukaryotic cells. The morphology of bacteria differs widely and they can be found practically everywhere on earth, and have been estimated to a number of 4-6 x 10^{30} (Whitman, Coleman & Wiebe, 1998). However, only half of approximately 50 bacterial phyla (major lines of descent) have representatives that can be cultured (Rappé & Giovannoni, 2003), which corresponds to only approximately 1% of all bacteria (Hugenholtz, Goebel & Pace, 1998).

The intestinal spirochaetes of the genus *Brachyspira* are a common cause of diarrhoea in pigs. Other animals, including humans, can also harbour these bacteria in their intestines. The brachyspiras are fastidious, oxygen tolerant, anaerobes, and cause haemolysis on blood agar and have a slow confluent growth. The genus comprises seven officially named species and four provisionally named species. Six of these can be found in the pig intestine, of which only three are considered enteropathogenic to the pig. Traditional laboratory diagnostics of *Brachyspira* species is based on anaerobic culture for detection and biochemical tests for identification. Antimicrobial susceptibility tests are performed on pure sub-cultured bacterial isolates. Many DNA based techniques have been introduced for detection and identification of microorganisms. These techniques are often quick and easy, and have precise targets. However, for antimicrobial susceptibility testing, epidemiological investigations and research, culture and long-term storage of isolates are needed. For clinicians, correct laboratory diagnosis is essential for optimal treatment and surveillance of the bacteria in individual animals or herds.

Phylum *Spirochaetes*

The *Spirochaetes* represent one phylum in the domain bacteria (Fig. 1). The morphology (outward appearance), phenotype (detectable characteristic of an organism) and genotype (DNA) are main components for phylogeny. The morphologic characteristics and ultrastructure of the members of the phylum *Spirochaetes* are unique (Woese, 1987). They are helical-shaped, motile, slender and long with characteristic periplasmic flagella hooked to the cell membrane but enclosed by an outer sheath. The outer sheath is similar to the Gram-negative cell wall in many aspects, however, it is very loosely connected to the thin peptidoglycan layer that closely surrounds the cell membrane (Holt, 1978; Trott *et al.*, 2001). This loose connection between the outer sheath and the peptidoglycans is assumed to contribute to the motility of the cell.

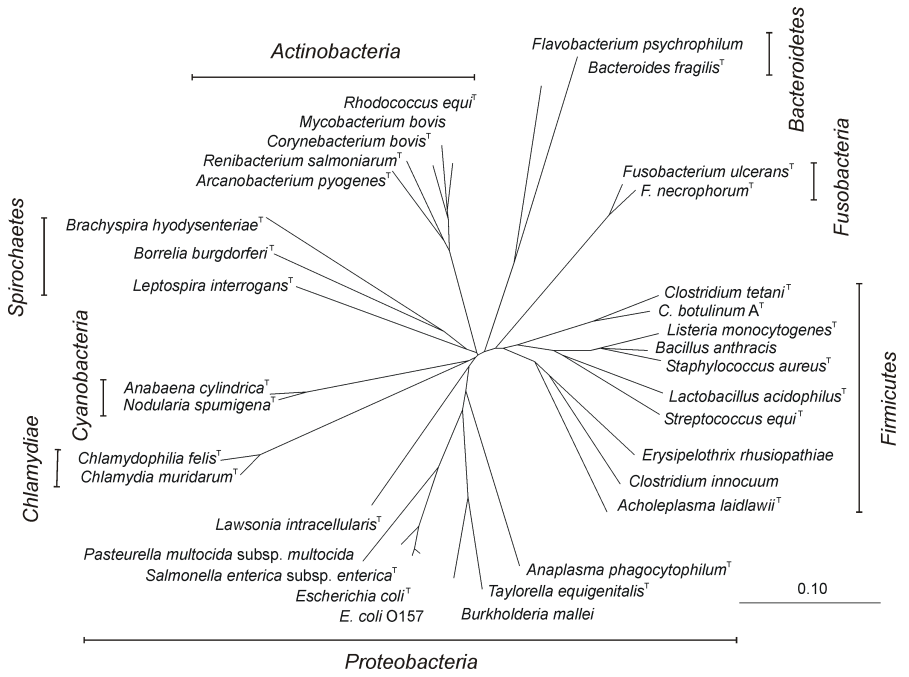

Fig. 1. A radial tree illustrating bacterial phylogeny based on 16S rDNA. The origin and evolution of a set of organisms, usually a set of species, are the phylogeny (or phylogenesis). Only some phyla are represented and each phylum is based on a restricted selection of genera and strains. The majority of the isolates are pathogens of veterinary interest, but strict human pathogens as well as some non-pathogenic strains are also included. Approximately 1,250 nucleotides have been used for calculation by the distance matrix method of Neighbor Joining (Saitou & Nei, 1987). The evolutionary distance between two organisms is proportional to the sum of corresponding horizontal branch lengths. The scale bar shows the distance equivalent to 10 substitutions per 100 nucleotide positions, which corresponds to approximately 120 substitutions in the sequenced gene fragment. Members of the genus *Anabaena* and *Nodularis* were used as out-group.

The phylum contains one class and one order, which includes three families: *Spirochaetaceae*, *Brachyspiraceae*, and *Leptospiraceae*. The *Spirochaetaceae*

family includes the genera *Spirochaeta*, *Borrelia*, *Brevinema*, *Clevelandina*, *Cristispira*, *Diplocalyx*, *Hollandia*, *Pillotina* and *Treponema*. The *Brachyspiraceae* family includes only the genus *Brachyspira*, and the *Leptospiraceae* family includes the genera *Leptospira* and *Leptonema* (http://dx.doi.org/10.1007/bergeysoutline200310, 4-July-2007; Paster & Dewhirst, 2000).

Taxonomy

The practice and science of classification according to degree of related evolution, i.e. taxonomy, includes nomenclature and identification criteria. To begin with, all intestinal porcine spirochaetes were included into the genus *Treponema*. However, after comparing treponemes by DNA-DNA reassociation and 16S rDNA sequencing *inter alia*, a new genus for the haemolytic porcine intestinal spirochetes was proposed. The new genus was named *Serpula*, which had to be changed to *Serpulina* due to the existence of a fungal genus *Serpula* (Stanton, 1992; Stanton *et al.*, 1991).

Later, a unification of the genus *Brachyspira*, which included a haemolytic intestinal spirochaete recovered from humans (*B. aalborgi*, see below), and *Serpulina* (the porcine haemolytic intestinal spirochaetes) was later proposed based on DNA-DNA reassociation experiments (Ochiai, Adachi & Mori, 1997). In the unification of the two genera, the *Brachyspira* genus preceded the latter genus because it was the first published of the two. Two *Serpulina* species were described at the time of the unification of the genera, which were not renamed as *Brachyspira* until 2006 (Hampson & La, 2006). Today, all haemolytic intestinal spirochaetes that can be cultured are included in the genus *Brachyspira*.

The scientific classification system for *Brachyspira* species, exemplified by the species *Brachyspira hyodysenteriae*, is outlined below. The brachyspiras have no subspecies. A phylogenetic tree for the phylum *Spirochaetes* is shown in Fig. 2.

Domain: Bacteria
Phylum: *Spirochaetes*
Class: *Spirochaetes*
Order: *Spirochaetales*
Family: *Brachyspiraceae*
Genus: *Brachyspira*
Species: *B. hyodysenteriae*

Three non-haemolytic intestinal spirochaetes have been cultured from pig faeces: *Treponema porcinum*, *Treponema berlinense* (Nordhoff *et al.*, 2005) and *Treponema succinifaciens* (Cwyk & Canale-Parola, 1979; Harris *et al.*, 1972). These species require culture techniques other than those for brachyspiras. Undoubtedly, there are other species of faecal spirochaetes that have not been cultivated or characterised (Stanton *et al.*, 1997).

Genus *Brachyspira*

A large haemolytic spirochaete was almost concurrently identified in the UK and the USA as the agent of swine dysentery (Harris, *et al.*, 1972; Taylor & Alexander, 1971). This spirochaete was named *Treponema hyodysenteriae* (Harris, *et al.*, 1972) and was distinguished from the small non-haemolytic spirochete, *Treponema succinifaciens* (Harris *et al.*, 1971). Further, weakly haemolytic spirochaetes similar to *Treponema hyodysenteriae* were identified from healthy pigs (Taylor, 1970; Taylor & Alexander, 1971) and were named *Treponema innocens* (Kinyon & Harris, 1979). However, some researchers suspected, and later showed, that not all weakly haemolytic large spirochaetes were nonpathogenic (Hudson, Alexander & Lysons, 1976; Taylor, 1977). *Serpulina pilosicoli* was identified as causing mild diarrhoea in pigs (Taylor, Simmons & Laird, 1980; Trott *et al.*, 1996b). These spirochaetes, according to multilocus enzyme electrophoresis (MLEE) studies, were thought to represent a new genus: *"Anguillina coli"* (Lee & Hampson, 1994; Lee *et al.*, 1993b), but 16S rDNA analysis showed that they should be considered as belonging to the same genus as *Serpulina* (*Treponema*) *hyodysenteriae* (Fellström *et al.*, 1995; Stanton *et al.*, 1996). Two new species were described shortly after: *Serpulina intermedia* and *Serpulina murdochii* (Stanton, *et al.*, 1997), which were considered to be non-pathogenic. Nevertheless, two Swedish studies (Fellström & Gunnarsson, 1995; Fellström, *et al.*, 1995) and one Polish study (Binek & Szynkiewicz, 1984) indicated that isolates with the phenotype of *Serpulina intermedia* were related to diarrhoea. However, in the latter study, the isolates had a strong haemolysis identical to that of *B. hyodysenteriae* after 72 h of incubation, and their species classification can therefore be debated. Recently, a new porcine pathogenic *Brachyspira* species was identified and given the provisional name of *"B. suanatina"* (Paper IV).

Birds are colonised by many different intestinal spirochaetes, some of which have not yet been characterised (Jansson *et al.*, 2007a; Jansson *et al.*, 2005). Hitherto, in comparison with the porcine *Brachyspira*, two additional species have been described. These have been isolated from chickens; *Brachyspira alvinipulli* (Stanton, Postic & Jensen, 1998; Swayne *et al.*, 1995), and *"B. pulli"* the name assigned to a distinct group of chicken isolates based on data from MLEE (Stephens & Hampson, 1999; Stephens *et al.*, 2005) and 16S rDNA sequence data (Phillips, La & Hampson, 2005). A unique *Brachyspira* species has also been identified from dogs, *"Brachyspira* (*Serpulina*) *canis"* (Duhamel *et al.*, 1998; Johansson *et al.*, 2004).

The first isolated spirochaetes from humans were shorter and thinner than the porcine spirochaetes, had a slower growth and different metabolism. As human spirochaetes do not possess any cytoplasmic tubules, a characteristic distinguishing treponemes, a new genus was proposed; *Brachyspira*, the species being *B. aalborgi* (Hovind-Hougen *et al.*, 1982). This species has been detected in humans, monkeys and opossums (Duhamel, 2001). New species of human spirochaetes have been proposed, *"B. ibaraki"*, based on DNA-DNA hybridisation (Tachibana, Nakamura & Adachi, 2003), and *"B. christiania"* based on *in situ* hybridisation with a probe for *Brachyspira* (Jensen *et al.*, 2001).

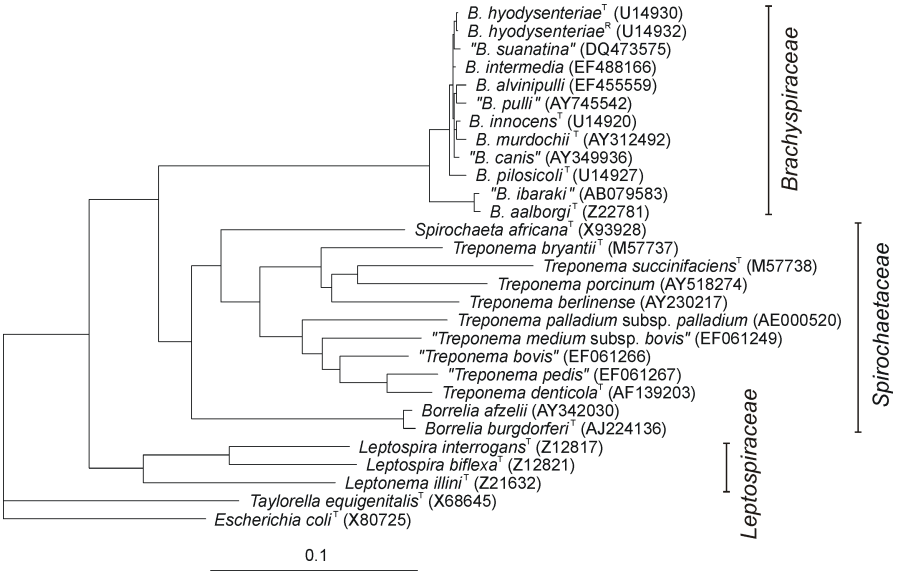

Fig. 2. Phylogenetic tree for the phylum *Spirochaetes* based on 16S rDNA to elucidate evolutionary relatedness. Included are all named and provisionally named *Brachyspira* species with determined 16S rDNA sequences, strains from three of the nine genera of the *Spirochaetaceae* family and strains from the two genera in the *Leptospiraceae* family. The tree is based on a distance matrix of approximately 1,250 nucleotides and calculated by Neighbor Joining (Saitou & Nei, 1987). The evolutionary distance between two organisms is the sum of corresponding horizontal branch lengths. The scale bar shows the distance equivalent to 10 substitutions per 100 nucleotide positions, which corresponds to approximately 120 substitutions in the sequenced gene fragment. Members of the genera *Taylorella* and *Escherichia* were used as out-group.

Characteristics of *Brachyspira* species

Brachyspira species are loosely coiled spirochaetes (Fig. 3). The number of periplasmatic flagella differs between 8-28 for the species in the following ascending order: *B. aalborgi*, *B. pilosicoli* and *B. hyodysenteriae* (Hampson & Stanton, 1997). The size varies between 2-12.9 µm in length and 0.2-0.4 µm in width, with *B. aalborgi* being the smallest, followed by *B. pilosicoli* and *B. hyodysenteriae*. The two latter species can be of the same length but *B. pilosicoli* is thinner than *B. hyodysenteriae*. Non-optimal growth results in longer and straighter cells and unfavourable conditions can result in spherical bodies.

The genome of *Brachyspira* sp. consists of a circular chromosome with a size of approximately 3.2 Mbp for *B. hyodysenteriae* and 2.45 Mbp for *B. pilosicoli* (Zuerner & Stanton, 1994; Zuerner *et al.*, 2004). Genetic rearrangement and sequence drift between *B. hyodysenteriae* and *B. pilosicoli* were identified when comparing gene maps for the two species (Zuerner, *et al.*, 2004). No extrachromosomal DNA was detected in these studies. However, other researchers have reported the occurrence of plasmids (Adachi, Hara & Hirano, 1994; Joens, Margolin & Hewlett, 1986) and bands of extrachromosomal DNA (Combs,

Hampson & Harders, 1992). These bands of extrachromosomal DNA were later shown to be of chromosomal origin, and most likely random fragments packaged by a bacteriophage (VSH-1) (Humphrey, Stanton & Jensen, 1995; Ritchie *et al.*, 1978; Turner & Sellwood, 1997). The VHS-1 phages can transfer gene material between *B. hyodysenteriae* strains when co-cultured (Humphrey *et al.*, 1997; Matson, Zuerner & Stanton, 2007; Stanton, 2007; Stanton, Matson & Humphrey, 2001). This phage transfer mechanism, together with spontaneous point mutation and recombination events, are most likely to be responsible for the high genetic diversity of *B. hyodysenteriae* and *B. pilosicoli*, as indicated from MLEE studies (Stanton, 2007; Trott *et al.*, 1998; Trott, Oxberry & Hampson, 1997). At present, no complete *Brachyspira* genome sequence is available, but at least one commercial genomic project of *B. hyodysenteriae* and *B. pilosicoli* is ongoing (Hampson, 2005).

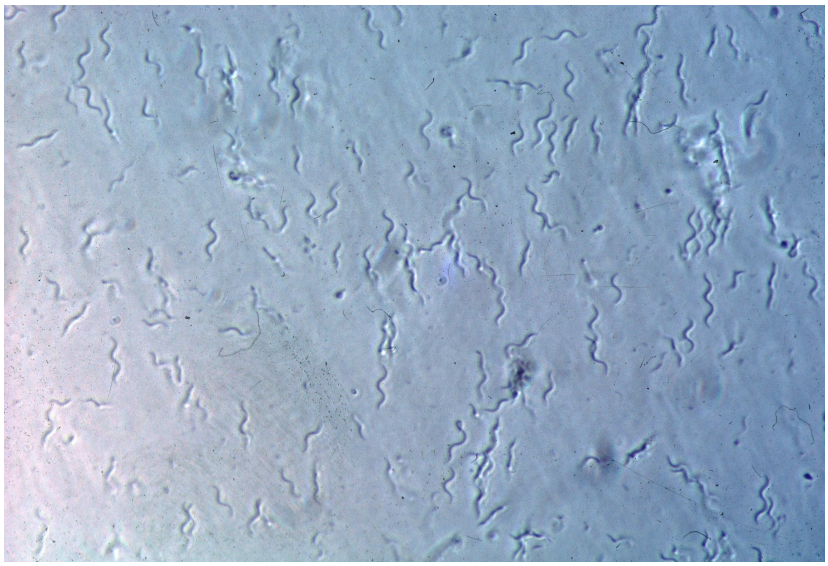

Fig. 3. Brachyspira hyodysenteriae cells (6-13 µm in length) in phase-contrast microscope. In comparison a red blood cell is 7 µm and a solitary coccus (e.g. *Staphylococcus*/ *Streptococcus*) is 1 µm in diameter. Photo: Claes Fellström.

Clinical aspects

Pigs (*Sus scrofa domestica*) were domesticated 4,000 to 6,000 years ago (Johansson, 1953). In 2004, there were well over one billion domestic pigs in the world, and half of them were reared in China (http://www.fao.org/, 31-July-2007). The slaughter age for a finishing pig is around 4-7 months. General hazard times for diarrhoea can be categorised for the pig, which can be divided into time periods according to the production system: sucklings, weaners and growers (Jacobson, 2003). Diarrhoeal diseases are often viewed as one-microbe diseases. However, the composition of the microflora in the intestine may influence the ability of a microbe to colonise and cause diarrhoea. The amount and composition of bacteria in the intestine are dependent on age, food, and herd health status (Melin, 2001).

Swine dysentery

Brachyspira hyodysenteriae causes severe diarrhoea with blood, mucus and white fibrin-debris grains, which is referred to as swine dysentery (Harris, *et al.*, 1972; Taylor & Alexander, 1971). The disease was first described in 1921 in the USA (Whiting, 1924; Whiting, Doyle & Spray, 1921). In Europe, swine dysentery became an increasing problem in the early 1960's (Ronéus, 1960). The severity of the disease varies depending on the virulence of the strain and the immunity to *B. hyodysenteriae* in the herd (Lee *et al.*, 1993a; Lysons *et al.*, 1982; Thomson *et al.*, 2001).

The incubation period can be as short as 5 days, but varies generally between 10-16 days (Fisher & Olander, 1981; Jacobson *et al.*, 2004b; Olson, 1974). However, it has been shown to be as long as up to 51 days when the bacterium is transmitted by an asymptomatic carrier pig that shed the bacteria (Songer & Harris, 1978). When first introduced in a herd, all age groups are often affected with a morbidity up to 90% and a mortality that can be over 50%. In chronically infected herds, the disease is typically seen in growers, affecting up to 30% of the animals in a cyclic manner. Some animals may die from the diarrhoea even if treated, but most pigs recover within two weeks, even though their growth may be depressed for a much longer period. Untreated pigs that recover from swine dysentery can transmit the disease up to 90 days after recovery, and mice have been shown to be able to spread the microbe for up to 180 days (Joens, 1980; Songer & Harris, 1978). The bacterium can survive up to 112 days in porcine faeces at 10 °C (Boye *et al.*, 2001).

Brachyspira hyodysenteriae colonises the large intestine. The spirochaetes can be found on the luminal surface and within the crypts of the caecum, colon and rectum. Spirochaetes have been detected invading the enterocytes, goblet cells and the lamina propria (Glock, Harris & Kluge, 1974; Taylor & Blakemore, 1971). Although the pathogenesis of the disease is not fully understood, the motility of the bacteria and their strong chemotaxis to mucus appears of particular importance (Kennedy *et al.*, 1988; Milner & Sellwood, 1994; Rosey, Kennedy & Yancey, 1996). Further, toxins such as haemolysin(s) (Hsu *et al.*, 2001; Hyatt *et al.*, 1994; Lysons *et al.*, 1991) and lipooligosaccarids (Nibbelink & Wannemuehler, 1991; Nuessen, Birmingham & Joens, 1982; Wannemuehler, Hubbard & Greer, 1988) may be involved in, or contribute to, damages in the mucosa of the intestine. Protection from oxygen toxicity by NADH oxidase is an important virulence factor through enhancing the ability to colonise the oxygen-respiring tissue of the intestine (Stanton *et al.*, 1999). Repeated infection provides protection to varying degrees; between 7-53% of the pigs can be reinfected with *B. hyodysenteriae* (Joens, Harris & Baum, 1979; Joens *et al.*, 1983; Olson, 1974; Rees *et al.*, 1989).

The severity of swine dysentery disease appears to increase if other anaerobic bacteria that normally form part of the intestinal flora, such as *Bacteroides fragilis*, *Bacteroides vulgatus* and *Fusobacterium necrophorum*, are present in the gut (Harris *et al.*, 1978; Joens *et al.*, 1981; Whipp *et al.*, 1979). Changes in diet can alter the microbial flora in the intestine, which may contribute alone or together

with other mechanism to decrease the severity of the diarrhoea (Durmic *et al.*, 2002; Leser *et al.*, 2000; Siba, Pethick & Hampson, 1996; Zhang *et al.*, 2001). Changes in the diet can also be used to obtain the reverse effect, i.e. to enhance the diarrhoea, by the use of for example soybean meal (Jacobson, *et al.*, 2004b).

Spirochaetal diarrhoea

Brachyspira pilosicoli causes a milder form of diarrhoea, which is referred to as spirochaetal diarrhoea (Taylor, 2005a; Taylor, Simmons & Laird, 1980), porcine intestinal spirochaetosis (Trott, *et al.*, 1996b), or colonic spirochaetosis (Duhamel, 2001). The diarrhoea is grey-wet, sometimes with mucus and occasionally with blood. This disease is often detected amongst younger pigs, approximately two weeks after weaning. There is little or no mortality, but colonisation results in loss of condition, which is regained after one or two weeks. Some pigs do not develop diarrhoea even though colonised by the bacteria, and if diarrhoea occurs, changing the dietary management may reduce clinical signs (Hampson *et al.*, 2000; Lindecrona, Jensen & Møller, 2004; Thomson *et al.*, 2007). A characteristic fringe of *Brachyspira* cells often attaches apically to the enterocytes of the caecum and colon, often called "false brush border" (Taylor, Simmons & Laird, 1980; Trott, McLaren & Hampson, 1995). Some spirochaetes invade the epithelium of the intestine and the lamina propria. Diarrhoea may be due to physical blockage of passive absorption by the brachyspiras (Gad *et al.*, 1977; Trott, McLaren & Hampson, 1995), but other mechanisms may also be involved (Thomson *et al.*, 1997; Trott, Huxtable & Hampson, 1996). *Brachyspira pilosicoli* is not attracted to porcine mucus (Milner & Sellwood, 1994), but the efficiency of motion is improved for *B. pilosicoli* in viscous environments (Nakamura *et al.*, 2006).

Intestinal spirochaetosis in hosts other than pigs

In chickens, *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* are considered as potential pathogenic species causing reduced egg production, delayed start of lay, increased water content in faeces and faecal staining of egg shells; *B. innocens*, *B. murdochii* and *"B. pulli"* are presumed to be non-pathogenic species (Jansson *et al.*, 2001; Stephens & Hampson, 2001). In rheas, turkeys and geese, naturally acquired infections have been reported to cause necrotising typhlocolitis (Jensen, Stanton & Swayne, 1996; Nemes *et al.*, 2006; Shivaprasad & Duhamel, 2005). *Brachyspira* species have been isolated from dogs and cats, and it is suggested that *B. pilosicoli* can induce diarrhoea in puppies (Duhamel, 2001; Lee & Hampson, 1994; Songer *et al.*, 1978). Mice and guinea pigs develop intestinal lesions from *Brachyspira* species colonisation (Joens & Glock, 1979; Joens *et al.*, 1978). The few naturally infected rats investigated have not shown any intestinal lesions (Backhans, Råsbäck & Fellström, 2007; Blaha *et al.*, 1984; Hampson *et al.*, 1991; Joens & Kinyon, 1982). In addition, intestinal spirochaetes have been isolated from opossums, non-human primates, guinea pigs, horses and humans (Duhamel, 2001; Hampson *et al.*, 2006; Hovind-Hougen, *et al.*, 1982). The clinical significance of human intestinal spirochaetosis is uncertain (Kraaz *et al.*, 2000; Mikosza & Hampson, 2001), although zoonotic transmission of *B. pilosicoli* to humans may be considered (Hampson, Oxberry & La, 2006; Trott, *et al.*, 1998).

Virulence assessments

Considering the expensive and laborious eradication of the brachyspiras from affected herds, determination of the virulence of the isolates is of great concern. Some isolates within pathogenic *Brachyspira* species are reported as not inducing diarrhoea or lesions in the intestine (Brown *et al.*, 2007; Kinyon, Harris & Glock, 1977; Lee, *et al.*, 1993a; Lysons, *et al.*, 1982; Neef *et al.*, 1994; Thomson *et al.*, 2003). The virulence is further of importance when new genotypes or species are identified.

When the agent of swine dysentery was identified in the early seventies, experimental challenge with host animals was used to fulfil Koch's postulate (Harris, *et al.*, 1972; Taylor & Alexander, 1971). This pig model was later supplemented with other animal models involving mice (Jamshidian *et al.*, 2004; Joens & Glock, 1979; Nibbelink & Wannemuehler, 1992), chickens (Adachi *et al.*, 1985; Sueyoshi & Adachi, 1990; Trott & Hampson, 1998) guinea pigs (Joens, *et al.*, 1978), and mallards (Jansson *et al.*, 2007b). However, some degree of animal species specificity is observed in animal models resulting in lower or no colonisation and no intestinal lesions in other animals than the natural host species (Achacha, Messier & Mittal, 1996; Jansson, *et al.*, 2007b; Trott & Hampson, 1998). As an alternative to animal models, porcine blood cells and intestinal epithelial cell-cultures have been used (Binek *et al.*, 1995; Bowden, Joens & Kelley, 1989; Knoop, Schrank & Ferraro, 1979; Wilcock & Olander, 1979; Witters & Duhamel, 1997). It would be more convenient to identify genes suitable as PCR markers of virulence and which play a role in colonising and inducing lesions in the intestine. However, to the author's knowledge, no such genes have been identified.

Today, the main method available to assess reliable virulence capacity of *Brachyspira* isolates is by animal model, preferable with the host animal. Therefore, a refined pig model has been developed that enables pathogenesis studies during the infection (Jacobson, *et al.*, 2004b; Jacobson *et al.*, 2007), which will result in the use of less animals. However, further development is needed in this field to refine the methods, and to reduce and finally replace animals for virulence assessments.

Clinical importance

Brachyspira species can be found worldwide. In Brazil, 35% of herds with diarrhoea are infected with *B. hyodysenteriae* and 41% with *B. pilosicoli* (Barcellos *et al.*, 2000a). In South Korea, 37% of the herds with diarrhoea or a history of diarrhoea are PCR positive for *B. hyodysenteriae* (Suh & Song, 2005a). In Denmark, 2.5% of randomly selected herds are infected by *B. hyodysenteriae*, and 19% by *B. pilosicoli* (Stege *et al.*, 2000). In Hungary, amongst diarrhoeic herds, *B. hyodysenteriae* could be detected in 45%, and *B. pilosicoli* in 61% (Biksi *et al.*, 2007). Among diarrhoeic outbreaks in the UK, the primary agent or concomitant with other pathogens in herds are *B. hyodysenteriae* (29%) or *B. pilosicoli* (42%) (Thomson, *et al.*, 2001). In randomly selected herds in Sweden,

none were infected with *B. hyodysenteriae*, but 32% were infected by *B. pilosicoli* (Jacobson *et al.*, 2005). In the same study, *Brachyspira* species were not detected in the wild boar population. However, another study showed that 26% of domestic pig herds with suspected *Brachyspira*-caused diarrhoea were infected with *B. hyodysenteriae*, which corresponds to approximately 4% of the pig herds in Sweden (Råsbäck *et al.*, 2004). Only old prevalence data exists for Australia, in which a serological survey predicted that 33% of the herds were infected with *B. hyodysenteriae* (Mhoma, Hampson & Robertson, 1992). A recent study indicated that 7% of the feral pigs in Western Australia are infected with *B. hyodysenteriae* (Phillips *et al.*, 2007).

Epidemiology

The most common cause of spreading *Brachyspira* species is through carrier pigs, but the bacteria can be transmitted through mice, rats, dogs, and cats (Fellström & Holmgren, 2005; Hampson, *et al.*, 1991; Songer, *et al.*, 1978; Trott *et al.*, 1996a; Weber & Schramm, 1989). In addition, faecal contaminated transport vehicles, clothing, farm equipment, soil, lagoon water, or housing can also spread the bacteria (Boye, *et al.*, 2001; Chia & Taylor, 1978; Olson, 1995). Lately, mallards have been suspected of being involved in the transmission of the bacterium (Råsbäck *et al.*, 2005). The efficiency of disinfectants on *B. pilosicoli* and total inhibition of viability of the bacteria is difficult to achieve if the disinfectant is mixed with organic matter such as faeces (Corona-Barrera *et al.*, 2004b). Differential diagnoses to *Brachyspira* infection in pigs are infections with *Lawsonia intracellularis*, *Salmonella enterica* subspecies *enterica* serotypes, *Escherichia coli*, and the parasite *Trichuris suis* (Taylor, 2005b).

Laboratory diagnostics of *Brachyspira*

A pig suffering from diarrhoea caused by brachyspiras excretes 10^7-10^{10} CFU/g faeces (Neef, *et al.*, 1994). *Brachyspira* species that do not cause diarrhoea are present only in low numbers (10^2 –10^4 CFU/g faeces) (Kinyon *et al.*, 1976). However, pigs that have recovered from *Brachyspira* infection can infect susceptible pigs even though the bacteria cannot be detected by culture (Songer & Harris, 1978); furthermore, the pigs may excrete the bacteria intermittently. Historically, faecal smear observations were used for detection of the *Brachyspira* cells, but today, anaerobic culture with selective media is used. For antimicrobial susceptibility testing and differentiation below species level, pure isolates are crucial.

Culture and biochemical tests

The porcine *Brachyspira* species need 2-7 days in a temperature between 37-43 °C to flourish on blood agar base or similar media supplemented with 5-10% blood (Fig. 4). However, on selective media, the growth can often bee see only as a thin haze. *Brachyspira aalborgi* will only grow at 37-38.5 °C (Hovind-Hougen, *et al.*, 1982). The *Brachyspira* species grow under anaerobic conditions; although low amounts of oxygen are important to enable efficient growth (Stanton & Lebo, 1988). Selective agar for primary isolation will suppress general faecal flora or

enhance the growth of *Brachyspira* species, or both, and antimicrobial substances are used to suppress bacteria other than *Brachyspira* species. Several studies have been performed to compare selective agars for *Brachyspira* growth (Achacha & Messier, 1992; Calderaro *et al.*, 2005; Jenkinson & Wingar, 1981; Kunkle & Kinyon, 1988). Some *Brachyspira* species grow very slowly or are inhibited by the use of excessive amounts or numbers of antimicrobial substances (Duhamel *et al.*, 1995; Kraaz, *et al.*, 2000). Elevated incubation temperature (approximately 42 °C) can also be used to suppress general faecal flora and mould (Corona-Barrera *et al.*, 2004a; Lemcke *et al.*, 1979; Songer, Kinyon & Harris, 1976).

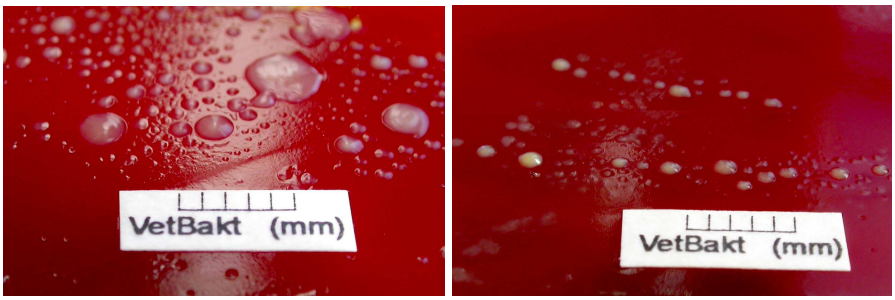

Fig. 4. Growth of the type strains of *B. pilosicoli* P43/6/78^T (left) and *B. hyodysenteriae* B78^T (right) on fastidious anaerobe agar (FAA) plates incubated 3 days at 42 °C. Photo: Karl-Erik Johansson (http://www.vetbakt.se/, Accessed 7-July-2007).

Broth culture is useful, especially if large amounts of spirochaetes are needed (Kunkle, Harris & Kinyon, 1986; Stanton & Lebo, 1988). Classification of brachyspiras can be based on biochemical tests to identify the phenotypical features (Fellström & Gunnarsson, 1995; Taylor & Alexander, 1971). The reported sensitivity for detection by culture and biochemical tests is 10^2-10^6 CFU/g faeces for *B. hyodysenteriae* (Fellström *et al.*, 2001; Stege, *et al.*, 2000).

Antimicrobial susceptibility testing

The most commonly used drugs for treatment of *Brachyspira* infections are macrolides, lincosamides and pleuromutilins. Point mutation(s) in the 23S rRNA gene in *B. hyodysenteriae* and *B. pilosicoli* cause resistance to both macrolides (e.g. tylosin) and lincosamides (e.g. lincomycin) (Karlsson *et al.*, 1999; Karlsson *et al.*, 2004b). The two pleuromutilins used are tiamulin and valnemulin. In some countries, tetracyclines are used (Čížek, Prášek & Smola, 2007).

Between 85-100% of *B. hyodysenteriae* isolates, and only slightly fewer *B. pilosicoli* isolates, are resistant to tylosin (Buller & Hampson, 1994; Herbst *et al.*, 2007; http://www.sva.se/, 8-July-2007; Karlsson *et al.*, 2003; Rønne & Szancer, 1990; Uezato, Kinjo & Adachi, 2004). Tiamulin resistant *B. hyodysenteriae* exist (Gresham, Hunt & Dalziel, 1998; Karlsson *et al.*, 2004a; Molnar, 1996) and are steady in number, if not increasing (Čížek, Prášek & Smola, 2007; Hidalgo *et al.*, 2007; Lobová, Smola & Cizek, 2004). In Sweden, all *B. hyodysenteriae* isolates tested have so far been susceptible to tiamulin; however, *B. pilosicoli* isolates resistant to tiamulin have been detected in Sweden and

Finland (Fossi, Saranpää & Rautiainen, 1999; http://www.sva.se/, 8-July-2007). Isolates resistant to both tylosin and tiamulin are an increasing problem (Čížek, Prášek & Smola, 2007; http://www.sva.se/, 8-July-2007). In Europe, stamping out due to multi-resistant *B. hyodysenteriae*, may be the only method to clear a farm from the infection (Merialdi & Sandri, 2007). The situation is further complicated in that no standard method is available for antimicrobial susceptibility testing and that different methods yield different minimum inhibiting concentrations (MICs) (Pringle *et al.*, 2006; Rohde *et al.*, 2004; Råsbäck *et al.*, 2003b).

DNA-based methods

Several DNA-based methods have been used for analysis of *Brachyspira* species, e.g. polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD), DNA restriction endonuclease analysis, restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), DNA probes, DNA-DNA homology, ribotype analysis and gene sequencing. These methods measure the DNA sequence variation in different ways, and can be used for species or strain identification, or both.

The most extensively used DNA-based typing method for laboratory diagnostics is PCR, which targets a specific region on the DNA. PCR is usually designed to be species specific, but some genus specific systems have also been developed. The products obtained from a genus specific PCR can be digested by enzymes before analysis by electrophoresis, which may result in species-specific banding patterns (PCR-RFLP). The genes that have been used for diagnostics of *Brachyspira* species include: the *nox* gene (Atyeo *et al.*, 1999; La, Phillips & Hampson, 2003; Rohde, Rothkamp & Gerlach, 2002); the 23S rRNA gene (Barcellos *et al.*, 2000b; Leser *et al.*, 1997; Suriyaarachchi *et al.*, 2000; Thomson, *et al.*, 2001); the *tly*A gene (Fellström, *et al.*, 2001); the 16S rRNA gene (Fellström *et al.*, 1997; La, Phillips & Hampson, 2003; Muniappa, Mathiesen & Duhamel, 1997; Park *et al.*, 1995); and two genes of unknown function (Elder *et al.*, 1994; Harel & Forget, 1995). PCR applied on pure cultures has an extremely high sensitivity (one DNA molecular per reaction), however, when applied directly on faecal samples the sensitivity is reported to be decreased by two or three orders of magnitude.

RAPD, a PCR-based system with a short primer that binds to unknown segments of the DNA and yields many different fragments sizes for each genome, is a method that has lately been used for *Brachyspira* species identification and subtyping (Dugourd *et al.*, 1996; Jansson *et al.*, 2004; Råsbäck, *et al.*, 2005).

PFGE is a method that has been used extensively for typing and subtyping of *Brachyspira* species. Genomic DNA is cleaved with a rare cutting enzyme and the banding pattern is analysed in a gel by pulsed-field electrophoresis. The method has been used for gene mapping (Zuerner & Stanton, 1994; Zuerner, *et al.*, 2004), epidemiological studies (Atyeo, Oxberry & Hampson, 1996; Fossi, Pohjanvirta & Pelkonen, 2003; Karlsson, *et al.*, 2004a), and for typing of isolates (Fellström *et al.*, 1999; Rayment, Barrett & Livesley, 1997).

Sequence analysis of the 16S rRNA gene is the backbone for bacterial phylogeny (Ludwig & Klenk, 2001; Olsen & Woese, 1993). However, for some of the species in the *Brachyspira* genus, the 16S rRNA gene analysis is insufficient for species identification because the gene is too conserved (Pettersson *et al.*, 1996; Stanton, *et al.*, 1996). The *nox* gene that is relatively conserved amongst *Brachyspira* species, but to a lesser degree than the 16S rRNA gene, has been investigated for its suitability as a phylogenetic marker for *Brachyspira* species (Atyeo, *et al.*, 1999; Stanton, Hanzelka & Jensen, 1995). The 23S rRNA gene has also been used for phylogenetic studies of members of the genus *Brachyspira*, but only few sequences are available. This gene has been shown to contains hypervariable regions and it has more sequence divergences than the 16S rRNA gene, but less than the *nox* genes (Atyeo, *et al.*, 1999; Leser, *et al.*, 1997; Olsen & Woese, 1993).

Other characterisation techniques

Proteins of the *Brachyspira* cells have been studied by electrophoresis in sodium dodecyl sulphate containing polyacrylamide gels (SDS-PAGE). The proteins can afterwards be transferred from the gel to a membrane for further analysis. In a Western blot, antibodies are used to bind to the proteins, for which substantial cross-reactivity exists amongst the *Brachyspira* species (Joens & Marquez, 1986). Based on lipooligopolysaccarides, *Brachyspira* species have been divided into several serogroups and later also serovars (Lau & Hampson, 1992). Although, there are no commercial methods available for serology, several potential proteins have been identified that may be useful as future serologic tools. Another protein-based technique that has been used is the MLEE, by which enzymes can be detected with an artificial substrate that can undergo a colour reaction in the presence of enzyme activity. Potentially new *Brachyspira* species have been identified by this technique (Duhamel, *et al.*, 1998; Lee, *et al.*, 1993b; Swayne, *et al.*, 1995). However, MLEE is a time-consuming method and is not available in most diagnostic laboratories: it is also less discriminatory than PFGE (Suriyaarachchi, *et al.*, 2000; Trott, *et al.*, 1998).

Aspects on materials and methods

A general introduction and some additional information to materials and methods used are given below. Further details are presented in the publications included in this thesis.

Bacterial isolates

All isolates used (studies I, III and IV, and partially study II) were held at the strain collection at the Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden. The isolates were carefully selected to fulfil criteria such as being type or reference strains of *Brachyspira* species, possessing low or high MICs for antimicrobial substances, exhibiting a known deviating genotype or phenotype, or being isolated from different animal host species. Type and

reference strains were obtained from the ATCC Bacteriology Collection (Manassas, VA, USA). The Swedish isolates were recovered from porcine faecal samples submitted from field veterinarians or collected from previous research projects at the department. Field isolates were kindly sent by colleagues at foreign laboratories. The isolates were stored in beef broth with 10% horse serum and 15% glycerol in liquid nitrogen (-196 °C).

The Swedish field isolates used in study II were recovered from clinical samples from diarrhoeic herds that were submitted by field veterinarians for *Brachyspira* diagnostics. The samples consisted of rectal swabs transported in Amies Charcoal medium (Copan, Italy), which is the standard method of submitting clinical samples in Sweden, and this medium has proved reliable for obtaining viable spirochaetes for culture after one or even after three weeks of storage (Fellström, *et al.*, 2001; Taylor *et al.*, 1985). Routinely, one *B. hyodysenteriae* and/or one *B. pilosicoli* subcultured isolate from each submission was randomly chosen and stored at the National Veterinary Institute's strain collection.

Culture and biochemical tests

Traditionally, culture and biochemical test (CBT) (studies I-II) can be divided into primary isolation on a selective agar plate from faecal swabs followed by sub-culture on a non-selective agar plate. The selective agar plate consisted of blood agar base no 2 (Oxoid), 5% sheep blood and 1% sodium ribonucleate, to enhance haemolysis (Picard, Massicotte & Saheb, 1979), and three antibiotics: spectinomycin 800 µg/ml, vancomycin 25 µg/ml and colistin 25 µg/ml. Anaerobic incubation time was 6-7 days at 42 °C. *Brachyspira* species was subcultured to a non-selective agar plate consisting of fastidious anaerobe agar (Lab M) with 10% horse blood (FAA) and a tryptone soya agar (Oxoid) with 5% ox blood (TSA), before identification by biochemical analysis (study I-IV) (Fellström, *et al.*, 1995; Fossi *et al.*, 2004). Type of haemolysis was read on the TSA agar after 3 and 6 days of incubation under the same conditions as described above (Fig 5). Bacteria from the FAA plate incubated for 6 days were used for detection of indole production, α-galactosidase and β-glucosidase activities and hippurate hydrolysis. The type strains of *B. hyodysenteriae* and *B. pilosicoli* were included as controls for the biochemical tests.

This culture method was used as a standard method (studies I-II), as a previous study showed high sensitivity for *B. hyodysenteriae* culture for the method used (Fellström, *et al.*, 2001). However, for extraction of bacterial DNA in study II, the incubation period for primary plates was reduced to half of the routine incubation time (i.e. 3 days) for half of the samples analysed, to evaluate whether the time needed for a laboratory diagnosis could be shortened without impaired sensitivity. Thawed bacterial strains or isolates were subcultured onto agar plates at least once before biochemical testing (studies I-IV).

Broth culture (study IV) obtained large numbers of bacteria for virulence assessment. The cells were cultured in 40 ml BHIS broth (Brain Heart Infusion,

Difco, supplemented with 10% foetal calf serum). From an agar subculture incubated anaerobically for 3 days at 42 °C, bacteria were picked with a cotton swab and transferred to the broth. The inoculated broth was incubated anaerobically at 37 °C on a shaker for 2 days, after which viability was checked with a phase-contrast microscope and optical density with a spectrophotometer. By this method, sufficient amounts of bacteria were obtained for inoculation of *B. hyodysenteriae* in pigs, in accordance with a challenge model previously described (Jacobson, *et al.*, 2004b).

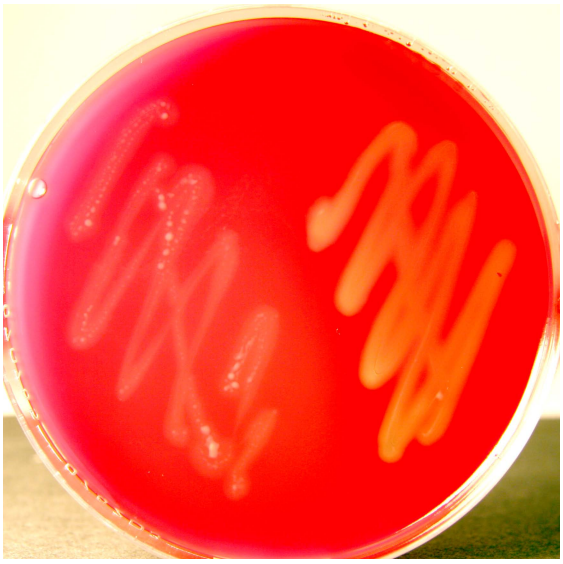

Fig. 5 Haemolysis after 3 days culture on tryptone soya agar (TSA) plate supplemented with 5% ox blood for *B. pilosicoli* P43/6/78^T (left side) and *B. hyodysenteriae* B78^T (right side), incubation at 42 °C. Photo: Märit Pringle.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (studies I and III) at the National Veterinary Institute in Sweden is routinely performed by broth dilution (Karlsson, Oxberry & Hampson, 2002). In brief, two 1-µl loops of bacteria from a 3 day-old agar culture were mixed in 2 ml of BHIS, before diluted by transferring 300 µl of the bacterial suspension to 30 ml BHIS broth. Of the final dilution, 0.5 ml was dispensed into each well in a panel and incubated anaerobically at 37 °C on a shaker (60-80 rpm) for 4 days. The MICs were read with a special devise containing a mirror to obtain indirect light. Broth dilution generally shows one twofold-dilution step lower MICs than agar dilution (Karlsson, *et al.*, 2003; Rohde, *et al.*, 2004).

Transportation and distribution

In study I, pure cultures were transported in semi-solid transport medium (PortaGerm, bioMérieux, Göteborg, Sweden). This medium did not contain any charcoal, which has previously been shown to have an inhibiting effect on the survival of pure cultures of *B. hyodysenteriae* (Duhamel *et al.*, 1992). The bacteria

were picked with a sterile cotton swab, dipped in BHI broth, from fastidious agar plates that had been incubated for 3 days at 42 °C. Seeded faeces (study I) was transported in a modified Amies medium with 0.6% agar (National Veterinary Institute, Uppsala, Sweden). This transport medium was chosen mainly for practical reasons, because the size of the tube it was provided with fitted well in the plastic container that was used for courier transportation. Further, the medium contained charcoal as well as high water content which have been shown to be beneficial for viability of *B. hyodysenteriae* in faeces during transportation (Chia & Taylor, 1978; Duhamel, *et al.*, 1992; Taylor, *et al.*, 1985). Courier or postal services were used for distribution due to country regulations.

DNA extraction and faecal seeding

For all DNA-based methods (studies I-V) except PFGE, DNA was extracted from subcultured *Brachyspira* strains or isolates by boiling bacteria, picked with a 1-µl plastic loop, in 50 µl SuperQ-water for 10 min. The DNA was then separated from the cell debris by centrifugation at 6,000 rpm for 4 min and the supernatant was transferred to a new tube for storage. The DNA used for analysis was diluted 1/10. DNA from faeces with seeded *Brachyspira* cells (study II) was extracted with QIAampDNA® Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) or by robot extraction, based on magnetic beads (MagAttract DNA Mini M48 Kit, QIAGEN GmbH), according to manufacture's instructions. These two easily available methods for DNA extraction are used at the National Veterinary Institute for other bacteria (Jacobson *et al.*, 2004a).

Faecal seeding (studies I and II) was achieved by dilution of *Brachyspira* cells in BHI broth, before mixing equivalent amounts of the broth with porcine faeces. Cultures had been subcultured and incubated for 3 days at 42 °C. In brief, 10 µl of bacterial cells (picked with a 1-µl plastic loops) were inoculated into 10 ml BHI broth followed by thorough mixing for 2 min with a vortex machine. A dilution series was made by transferring 1 ml of mixed-bacterial broth to 9 ml fresh BHI broth, and so on. The concentration of cells in the first mixture (the highest concentration) was estimated to $1.5x10^8$ CFU per ml of broth. The dilution series was obtained to –8 (approximately 1.5 CFU per ml of broth). The CFU per ml of broth was estimated by viable counts. In brief, 100 µl of broth mixture were streaked onto an agar plate without antimicrobial substances, followed by anaerobic incubation at 42 °C for 3-6 days, before counting visible colonies. If less amount of the broth mixture was needed, only a tenth of the above-mentioned amounts was used, i.e. bacterial cells picked with a 1-µl plastic loop and 1 ml broth (study II) were mixed. Faeces were collected from pigs (weight approximately 30 kg) from the Swedish University of Agricultural Sciences' pig-research herd, which is known to be free from brachyspiras. The herd is a one-site, closed, integrated and age separated herd. To ensure that the faeces did not contain any spirochaetes, culture was performed on randomly selected pigs two weeks before faecal collection, and on faeces collected for use in studies I-II. Broth and faeces were mixed in a stomacher bag and homogenised for 5 min. This seeding method has previously been satisfactory (Fellström, *et al.*, 2001).

DNA-based methods

The PCR (studies I-IV) is based on a primer pair (approximate length of 20 nucleotides) that is designed to bind to flanking regions of a known gene or segment of the DNA. A PCR usually consists of a series of 20 to 35 cycles, where each cycle consists of three different steps at different temperatures. The first step separates the DNA strands (usually around 95 °C), the second step allow the primers to bind to each of the respective DNA strands (at a low temperature specific for the primers; around 50-60 °C), in the third step a DNA polymerase adds the nucleotides to the 3'-end of the primer (optimal temperature normally around 72 °C) (Fig 6). This reaction results in a new strand of DNA, which has been synthesised with the old strand as template. The length of the fragment can be estimated by electrophoresis in an agarose gel before visualisation under UV-light, where the migration distance is compared with a standard; a DNA ladder (Fig. 6). This method is widely used in laboratory diagnostics.

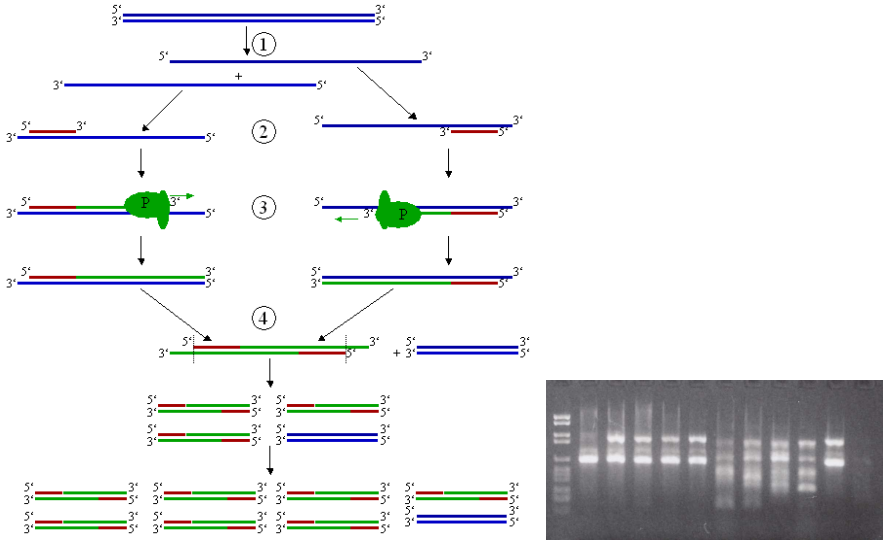

Fig. 6. Schematic drawing of how a PCR system works (left; image created by Madeleine Price Ball). Gel with RAPD products analysed by electrophoresis (right). The gel was stained with ethidium bromide and viewed under UV-light. First lane is loaded with a DNA ladder, followed by DNA products from single isolates for each well.

At the onset of the studies, two systems designed for *Brachyspira* species were available at the National Veterinary Institute. These were systems targeting the *tly*A gene for *B. hyodysenteriae* (Fellström, *et al.*, 2001), and the 16S rRNA gene for *B. pilosicoli* (Fellström, *et al.*, 1997). These systems were combined into a duplex system (Råsbäck *et al.*, 2003a) for convenience in the laboratory. In study II this duplex system was tested for sensitivity and specificity. Studies III and IV included PCR systems other than the one used in-house, either because species other than *B. hyodysenteriae* and *B. pilosicoli* were analysed (study III), and/or because of diverging PCR results from isolates tested in the in-house system (studies III-IV). The supplementary PCR systems were species-specific for

B. hyodysenteriae and *B. intermedia* and targeted portions of the 23S rRNA gene (Leser, *et al.*, 1997), or the *nox* gene (Atyeo, *et al.*, 1999; La, Phillips & Hampson, 2003). These systems were chosen because they have been extensively used for DNA detection in *Brachyspira* research and routine diagnostics worldwide.

RAPD (studies III-V) is based on short primers (approximate length of nine nucleotides). The principal of RAPD is similar to PCR, but only one primer is used in the reaction, which binds to unspecified segments of the DNA. The resultant products will be amplicons of different sizes. If analysed by electrophoresis, a banding pattern is observed when viewed under UV-light. The banding pattern for each isolate is discriminatory at species and strain level, and can be used for epidemiological studies (Dugourd, *et al.*, 1996). The technique is easy and fast to carry out; however, it is sensitive because small differences in batches of the mixture or involuntarily altered volume in the reaction tubes can result in different band intensities. This can sometimes be interpreted as two separate banding patterns for the same isolate analysed at different occasions. Primers used in studies III-IV have previously been described briefly (Jansson, *et al.*, 2004). In study III, the method was used to evaluate its usefulness for *Brachyspira* species, and in study IV, this method was used because no banding patterns were obtained by PFGE on the strongly haemolytic *Brachyspira* isolates.

PFGE was used to separate long DNA fragments (studies III-IV). These fragments are normally too long to migrate in an agarose gel under a constant electric field, but the DNA fragments can be forced to migrate in the gel by alternating the electric field. To obtain long DNA fragments, genomic DNA is cleaved with rare cutting enzymes. In study III, two rare cutting enzymes were used: *Mlu*I and *Sal*I, as previously used for *B. hyodysenteriae* isolates (Fellström, *et al.*, 1999). In study IV, *Mlu*I was used alone since other studies had shown that one enzyme could accurately discriminate between *Brachyspira* isolates (Atyeo, Oxberry & Hampson, 1996; Karlsson, *et al.*, 2004a; Trott, *et al.*, 1996a). Washed intact bacterial cells were moulded in an agarose plug before the DNA was released to ensure that the genomic DNA was not degraded by shearing before or during the analysis. PFGE is especially useful for epidemiological studies, in particular for *B. pilosicoli*. However, certain strongly haemolytic *Brachyspira* isolates are difficult or unfeasible to analyse by PFGE (Atyeo, Oxberry & Hampson, 1999; Fossi, Pohjanvirta & Pelkonen, 2003; Suriyaarachchi, *et al.*, 2000).

Sequencing, aligning of sequences and constructing dendrograms are used for phylogenetic analysis and species identification (studies III-V). The procedure for sequencing starts with a PCR amplification of the gene or fragment of interest, which in studies III-V were the almost complete 16S rRNA gene (Johansson, *et al.*, 2004; Pettersson, *et al.*, 1996) and in studies III-IV, the partial *nox* gene (Rohde, Rothkamp & Gerlach, 2002; Townsend *et al.*, 2005). The DNA fragments obtained from the PCR-reaction were purified before the strands were labelled by cycle sequencing, in which BigDye (dNTP in combination with labelled terminators) was used in a similar reaction as for the PCR, but with only one primer that bound to the amplicons in either a forward or reversed direction. This resulted in copied

fragments of all lengths from the original amplicons obtained by PCR. The labelled DNA was then analysed by electrophoresis in a DNA analyser.

The raw sequence data appeared as coloured peaks representing the respective type of base. Large gene fragments need to be sequenced into shorter segments that overlap, so that the whole gene could be unified into a contig. When aligning, the sequences of one gene for several isolates were organised into a matrix. The rows contained the sequences and the columns consisted of nucleotides in homologous positions (Fig. 7). This arrangement facilitated computerised comparison. The differences were identified and the relatedness among the isolates calculated. Several computer programmes can be used for this purpose. The distances in the dendrogram correspond to the number of substitutions separating two isolates: the fewer substitutions, the closer the isolates are related. The Neighbor joining method, which is based on a distance matrix, was used for calculating phylogenetic trees.

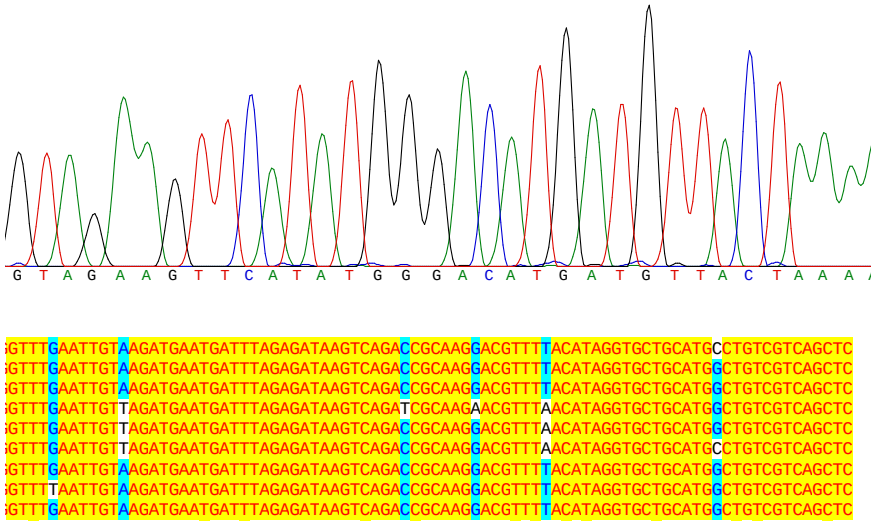

Fig. 7. Raw sequence data (above). A short alignment for the 16S rRNA gene for some *Brachyspira* isolates (below).

Brachyspira species have only one single copy of the ribosomal RNA genes (*rrn*) 16S, 23S and 5S (Zuerner & Stanton, 1994; Zuerner, *et al.*, 2004). Therefore, mixed isolates can be identified by the presence of double peaks when analysing these genes by sequencing. The *nox* gene was analysed as this gene is well distributed amongst *Brachyspira* species and has higher sequence divergences than the 16S rRNA gene (Atyeo, *et al.*, 1999; Stanton, Hanzelka & Jensen, 1995).

Multilocus sequence typing (MLST) (study V) is a method where several segments of genes, or loci, on the genome are analysed by sequencing. The majority of loci included were portions of genes that encode for previously published MLEE enzymes for *Brachyspira* species. The MLST data were analysed by constructing a dendrogram, and by using e-BURST (http://eburst.mlst.net) to

identify epidemiological connections and infer closely related isolates. The eBURST is a computer programme that groups isolates with identical locus for all loci in one dot if seven loci or more are analysed. The size of the dot will be proportional to the number of isolates incorporated. The grouped isolates may represent a clone, which can be interpreted as an epidemiological connection between the isolates within that group. Further, the programme connects isolates with only one diverging locus. The isolates (or groups) that are connected with a line (i.e. which have six out of seven identical loci) indicate that a close evolutionary relation exists between these isolates or groups.

Results and discussion

Paper I

This study assessed laboratory diagnostics for porcine intestinal spirochaetes of the genus *Brachyspira*. Implementing a ring test for bacterial laboratory diagnostics proved to be time-consuming and troublesome. This was partly due to the strict regulations for transporting biological matter, with special packages, labelling and documents. Delivery time of the consignments to the consignees could not be monitored, which resulted in a delay of up to 17 days in some cases. However, this did not affect the outcome of laboratory analyses, which confirmed good viability of the cultures and a suitable transport medium for this purpose.

The majority of laboratories used culture and biochemical tests in conjunction with PCR. The lowest concentration of *B. hyodysenteriae* that could be detected, in seven of nine laboratories, was 10^3 CFU per ml faeces. However, when two species were mixed, the majority of laboratories identified only one of the two. The different growth rates for *Brachyspira* species, which has been described as slightly faster for *B. pilosicoli* than for *B. hyodysenteriae* (Trott, *et al.*, 1996b), may have influenced the results for the mixed samples. The preparation of the seeded samples was uncomplicated, but detection rates obtained by the laboratories could still be related to uneven distribution of the *Brachyspira* cells in the mixture. This speculation may be especially relevant for samples with mixed *Brachyspira* species or low concentration of *Brachyspira* cells.

The detection of *Brachyspira* species in samples not containing any *Brachyspira* cells and the vast disparity in MICs reported was alarming. Large differences in detection rate have previously been reported for culture with a span of 10^2-10^6 bacterial cells for *B. hyodysenteriae* (Fellström, *et al.*, 2001; Stege, *et al.*, 2000). This study clearly illustrated that a ring test for *Brachyspira* laboratory diagnostics is crucial for monitoring the standard of laboratory diagnostics of these microbes. *Brachyspira* species proved to be difficult to detect, identify and to test for antimicrobial susceptibility. The outcome of the study emphasised the importance of using methods with similar sensitivity and specificity in order to be able to compare prevalence of these bacteria in pig herds and the resistance to antimicrobial substances.

Paper II

Traditional laboratory diagnostics based on culture and biochemical tests (CBT) is time consuming (2-3 weeks) and the sensitivity have sometimes been reported to be insufficient for the detection of carrier pigs excreting very low numbers of *Brachyspira* cells (Songer & Harris, 1978). A duplex PCR system was developed in this study for easier and quicker analysis than by the excisting separate PCR systems for *Brachyspira* species at National Veterinary Institute (Fellström, *et al.*, 1997; Fellström, *et al.*, 2001). The sensitivities of traditional laboratory methods and the newly developed duplex PCR system were tested for the detection of *B. hyodysenteriae* and *B. pilosicoli* in seeded porcine faeces. The results revealed a very high sensitivity for detection by culture, in agreement with study I. The sensitivity for the PCR system was similar to traditional diagnostics based on culture (approximately 100 cells for *B. hyodysenteriae* and *B. pilosicoli*), if DNA from pure *Brachyspira* isolates was analysed. However, with DNA extracted from seeded faeces, the sensitivity of the PCR system decreased substantially, which probably was due to PCR inhibitors in the faeces (Jacobson, *et al.*, 2004a; Lantz *et al.*, 2000; Phillips, La & Hampson, 2006), or extensive loss of *Brachyspira* DNA or cells with the DNA extraction kit, previously observed for other kits (Corona-Barrera, *et al.*, 2004a). However, this observation contradicted other studies that revealed no differences, or even superior sensitivity, for PCR applied on faecal samples after DNA extraction compared to culture and PCR (La, Phillips & Hampson, 2003; Suh & Song, 2005b). In an unpublished study, seeded faecal samples used for sensitivity testing at the National Veterinary Institute were applied to a previously published duplex PCR protocol, with a reported sensitivity on seeded faeces of 10^3-10^4 cells per gram faeces (La, Phillips & Hampson, 2003). The sensitivity with Swedish porcine faeces was lower than that previously reported; the lowest detection was 10^6 CFU per ml faeces with the same extraction kit. This further supported the hypothesis that porcine faecal samples include varying levels of PCR inhibiting factors.

The duplex PCR technique was simple to use and results were obtained within a day. However, due to the high number of DNA fragments from the amplification procedure, DNA contamination can occur when the tubes containing PCR products are opened for gel electrophoresis. To avoid false positive PCR reactions, separate rooms for preparation and gel electrophoresis, in combination with non-contaminated personnel, are vital. Personnel authorised to prepare PCR mixtures for PCR amplification at the National Veterinary Institute must not have opened a tube with a PCR product or visited the gel electrophoresis room that day. Real-time PCR, by which amplification is registered directly while amplifying the DNA, could solve these problems. Nevertheless, the PCR system is still rather sensitive to small alterations of the composition of the PCR mixture, which may occur from different pipettes or users. In routine diagnostics this can be solved by pre-mixed batches of PCR mixture that are allotted and frozen pending usage, or through a pre-mixed PCR mixture from the manufacturer (although more expensive).

The study showed that the PCR system applied to DNA, extracted from suspected *Brachyspira* growth on primary cultures and incubated for 6 days at 42 °C, was as sensitive as traditional CBT. However, when the PCR system was applied parallel to traditional CBT, on routine samples submitted from field veterinarians for *Brachyspira* diagnostics of diarrhoeic pigs, one strongly haemolytic *Brachyspira* isolate was detected only by CBT. Furthermore, the results showed that PCR applied on suspected *Brachyspira* growth from primary cultures incubated a week had detection rates similar to traditional CBT for *B. hyodysenteriae* and *B. pilosicoli*. This application resulted in a faster laboratory diagnostics for *Brachyspira* species, although, isolates still need to be cultured in parallel for biochemical tests, antimicrobial susceptibility tests and to be stored for future studies.

Paper III

In this study, *Brachyspira* isolates from all recognised porcine *Brachyspira* species were included and analysed by RAPD, PFGE, sequencing of 16S rRNA and *nox* genes, and species-specific PCRs. The RAPD and PFGE analysis did not separate the *Brachyspira* species into clear distinct clusters for each species. For example, isolates with phenotypes of *B. hyodysenteriae* and *B. intermedia* formed one cluster when analysed by PFGE. RAPD proved to be a much easier technique to use and slightly more discriminating than PFGE.

The analysis of the 16S rRNA gene sequences confirmed that *B. hyodysenteriae* and *B. intermedia* or *B. innocens* and *B. murdochii* could not be differentiated by this method (Pettersson, *et al.*, 1996; Stanton, *et al.*, 1996). The *nox* gene (Stanton & Jensen, 1993; Stanton & Sellwood, 1999) showed a high taxonomic resolution with a clear distinction for the *Brachyspira* species tested. These results indicated that the *nox* gene was useful for phylogeny and species identification for porcine *Brachyspira* species. However, unpublished data revealed that not all isolates included in study V could be sequenced with the protocols used (Rohde, Rothkamp & Gerlach, 2002; Townsend, *et al.*, 2005). The isolates that could not be amplified were mainly avian in origin, although also other isolates could not be sequenced with the sequencing primers. This was in agreement with a previous study in which not all isolates could be probed with a degenerated probe for the N-terminal end of the NADH oxidase gene (Stanton, Hanzelka & Jensen, 1995). Interestingly, some of these isolates were porcine isolates of Swedish origin. However, all 45 strains analysed, except for *Treponema succinifaciens* and *Treponema bryantii*, possessed NADH oxidase activity. This indicated that the *nox* gene is divergent within the genus.

The use of PCR systems for identification of isolates with the phenotype of *B. intermedia* targeting portions of the *nox* and 23S rRNA genes gave unforeseen results. Of the twenty isolates analysed initially in this study, the 23S rRNA gene fragment of one isolate was unexpectedly amplified by the 23S rRNA PCR (Leser, *et al.*, 1997) and the *nox* gene fragment of another was not amplified as predicted by the *nox* gene PCR (Atyeo, *et al.*, 1999) specific for *B. intermedia*. Of the tested porcine isolates presumed to be *B. intermedia* in study III, 13 isolates did not react

by the *nox* gene PCR. The *nox* gene PCR have previously been shown to correctly identify all tested porcine isolates, but not all tested isolates recovered from chickens (Atyeo, *et al.*, 1999). However, a *nox* gene based PCR-RFLP was recently tested that did not amplify all porcine *Brachyspira* isolates within the genus, and which also gave diverging results for some of the isolates (Calderaro *et al.*, 2006). The false positive reaction for one isolate by a 23S rRNA based PCR system was in agreement with a previous study on *Brachyspira* species where 11 of 195 isolates gave a false-positive reaction (Suriyaarachchi, *et al.*, 2000). In the extended analysis in study III, three isolates with the phenotype of *B. intermedia* gave false-negative reactions with the *B. intermedia* species-specific 23S rRNA system. The long hypervariable regions in the 23S rRNA gene (Leser, *et al.*, 1997) and general higher rate of sequence variations compared with the 16S rRNA gene (Olsen & Woese, 1993) can make this gene difficult to use in *Brachyspira* diagnostics. The PFGE analysis further indicated that isolates with the phenotype of *B. intermedia* were genetically heterogeneous, as previously suggested (Suriyaarachchi, *et al.*, 2000), and this genotypic variability might be the reasons for the unexpected PCR results. New primers for PCR identification of *B. intermedia* have been tested on isolates recovered from chicken (Phillips, La & Hampson, 2006), and even though these were not tested on porcine isolates, they might show a higher specificity for all isolates with the phenotype of *B. intermedia*.

Paper IV

In this study, a previously described group of strongly haemolytic intestinal spirochaetes, with a genotype different from *B. hyodysenteriae* was further characterised. These isolates were detected because they tested negative in a PCR system detecting *B. hyodysenteriae*, even though they were identified as *B. hyodysenteriae* by traditional CBT (study II).

The isolates were initially analysed by DNA-based methods to investigate whether any of the most commonly used PCR systems would detect these isolates as *B. hyodysenteriae*. None of the PCR systems tested gave a positive reaction for *B. hyodysenteriae*. False negative reactions have previously been obtained for *B. hyodysenteriae* isolates when analysed by the 23S rRNA gene PCR-RFLP developed for the genus *Brachyspira* (Thomson, *et al.*, 2001). However, analysis of the 16S rRNA gene showed that the porcine isolates characterised were identical, or very similar to, two mallard isolates, but different from *B. hyodysenteriae*. In contrast, a Danish isolate of this group of diverging spirochaetes gave a positive reaction with a 23S rRNA gene based PCR system (Leser, *et al.*, 1997) developed for detection of *B. intermedia*. The tendency of this PCR system for giving false-positive results has been discussed above (study III). Analysis of *nox* gene sequences revealed that these unique isolates grouped together in a separate cluster, indicating that they comprised a new species within the *Brachyspira* genus. In this study, the genomic DNA from these isolates could not be digested with the rare cutting restriction enzyme *Mlu*I: the reason for this was not investigated further. Similarly, other studies have reported difficulties in analysing strongly haemolytic isolates by PFGE (Atyeo, Oxberry & Hampson, 1999; Fossi, Pohjanvirta & Pelkonen, 2003). Further, *Mlu*I has been reported to

give many fragments that are difficult to resolve when applied to the type strain of *B. hyodysenteriae* (B78^T) (Zuerner & Stanton, 1994), and difficulties in digesting some *B. intermedia* isolates with the same enzyme have been reported (Suriyaarachchi, *et al.*, 2000). The usefulness of PFGE for typing of *B. hyodysenteriae* has been shown in other studies (Fellström & Holmgren, 2005; Fellström, *et al.*, 1999; Karlsson, *et al.*, 2004a). For subtyping of the new group of isolates, RAPD was useful.

The isolates characterised were recovered from one Danish and three Swedish pig herds with diarrhoea, and from mallards. An epidemiological link between the three pig herds in Sweden was identified from herd and transportation data, together with RAPD analysis of the isolates. Diarrhoea in pigs similar to swine dysentery was induced by one of the porcine isolates in a challenge study with the new genotype, and diarrhoea without mucus and blood was induced by an avian isolate. In a subsequent study, mallards inoculated with porcine isolates, were colonised (Jansson, *et al.*, 2007b). To the author's knowledge, this is the first study of experimental transmission of the bacteria from mallards to pigs resulting in cross infection. The provisional name *"Brachyspira suanatina"* was proposed, specifying that the isolates had been recovered from both pigs and mallards; Latin noun: suus (pig/swine) and Latin adjective: anatinus (from/of ducks) mallard; added together, shortened and used with the pertaining feminine genus of *Brachyspira* will be Neo-Latin feminine adjective: suanatina (from pigs/swine and ducks/mallard).

Paper V

In this study, *Brachyspira* isolates from different animal origins were included, of which almost one third consisted of provisionally named species or atypical isolates. Some DNA was from extractions stored at –20 °C over 15 years, before diluted 1/10 and transported three weeks without temperature control. All these templates yielded distinct PCR bands and sequence results where expected. The primers for the MLST system that were used for analysis were designed at Murdoch University. This was the first study to develop and evaluate an MLST system for analysis of isolates of the genus *Brachyspira*.

Five of the loci included were segments of genes coding for previously used MLEE enzymes of *Brachyspira* species. These enzymes were present in all named *Brachyspira* species, as well as in two provisionally proposed species. However, only two loci could be amplified and sequenced for all isolates with these primers. The sequences obtained showed superimposed peaks for particular isolates and segments, which could be interpreted as mixed isolates or multiple gene copies. The existence of repeated gene sequences or repetitive DNA could not be excluded in a previous study (Zuerner & Stanton, 1994); however, the genes included in the MLST study had been confirmed as existing in only one gene copy in the *B. hyodysenteriae* and *B. pilosicoli* genome-sequencing project ongoing at Murdoch University (Hampson, 2005). The sequences sometimes differed greatly within the genus, and some species sometimes appeared to have a similar deviation in comparison with other species within the genus. Many single mutations were

observed for some of the genes, others had long fragments of insertions/deletions. These changes could be a result of DNA rearrangement or gene transfer by a phage-like VHS-1 virus (Humphrey, *et al.*, 1997; Matson, Zuerner & Stanton, 2007; Stanton, 2007). This study indicated that the entire genus of *Brachyspira* exhibit a high genetic recombination structure; however, only a few bacteria are well known for their promiscuous exchange of DNA, e.g. members in the genus *Neisseria* (Smith, Feil & Smith, 2000).

Phylogenetic and eBURST analyses of the sequence data were performed. The dendrograms for the entire genus, based on the MLST data, showed high taxonomic resolution, even though only two loci were included. However, the positions of the species clusters within the genus differed compared to a phylogenetic tree, based on the 16S rRNA gene. Epidemiological analysis by using eBURST agreed with results obtained by PFGE and RAPD, i.e. those isolates that represented the same PFGE type and RAPD type were grouped together in the eBURST analysis. Further, a close evolutionary relation was identified for *B. hyodysenteriae* isolates and *"B. suanatina"* isolates. There was a close evolutionary connection between isolates recovered from pigs and mallards within two *Brachyspira* species, and these results further strengthened the hypothesis that mallards could be a natural source for transmitting *Brachyspira* isolates to pigs, as suggested in study IV.

Conclusions

The overall conclusions from studies I-IV were that laboratory diagnostics of *Brachyspira* species needs to be surveyed and adjusted regularly to meet the changing bacterial population. Of prime importance would be to initiate a regularly performed ring test for laboratory diagnostics and antimicrobial susceptibility testing of *Brachyspira* species. In addition, standardised methods for antimicrobial susceptibility testing are needed. A workshop on antimicrobial susceptibility testing procedures and techniques should preferably be carried out before strains are sent out for validation.

The two developed and evaluated DNA-based systems, duplex PCR and MLST systems, were shown to be useful for detection, identification and analysis of *Brachyspira* species. However, not all *Brachyspira* isolates could be analysed by the existing MLST system, and the system needs further development. The results obtained from all tested DNA-based systems showed that the genus *Brachyspira* is genetically heterogeneous. For the isolates studied, sequences analysis of the *nox* gene and the MLST system were shown to give the highest taxonomic resolution and the RAPD analysis were shown to be the most discriminatory.

A new genetic type, pathogenic to pigs, was identified, which could easily be missed if DNA-based methods had been the only methods used for identification. Laboratory diagnostics based on traditional culture and biochemical tests were the

most reliable methods for detecting strongly haemolytic isolates. DNA-based methods could be combined with traditional CBT to shorten the time needed for laboratory diagnosis, for surveillance of new genotypes of the bacterial population, and to increase the specificity of the diagnostics. Pure isolates is a necessary prerequisite for biochemical tests, antimicrobial susceptibility tests, epidemiological studies, and research. Therefore culture should always be performed.

The results further showed that pigs can get infected with *Brachyspira* isolates originating from mallards. Mallards were suspected as the infection source for the provisionally named *"B. suanatina"* and a close evolutionary relationship was identified for *"B. suanatina"* and *B. hyodysenteriae* isolates recovered from pigs and mallards.

The specific conclusions for the separate studies were: Study I emphasised the difficulties with laboratory diagnostics and antimicrobial susceptibility testing of *Brachyspira* species. Studies II-IV showed that the use of PCR solely may result in false negative results in *Brachyspira* diagnostics, if not used together with traditional CBT. The erroneous PCR results seem to be due to the high DNA sequence variability particularly within *B. intermedia* (study III) and within the whole genus (study V). If the divergences are too high within the genus, it may be difficult to identify conserved regions suitable for development of primers covering the entire genus for, e.g. the *nox* gene, or loci for a MLST system, or to find suitable species-specific primers for specific genes, such as the 23S rRNA gene. RAPD was shown to be a useful method for epidemiological studies, and to be more discriminatory than PFGE with the respective primers and enzymes used.

Future studies

The MLST systems should be further developed, and this should involve the identification of suitable regions conserved within the genus to include more genes that are not too variable. With the high genetic variability within the genus, such genes might be difficult to find, and therefore species-specific MLST systems could be an alternative. However, a method that covers the entire genus would be desirable especially since isolates with diverging biochemical reactions may represent a new species that may not amplify in a species-specific MLST system. From the MLST system data used for identification, epidemiological links can be deduced. The drawback for such a system is that sequencing facilities, as well as additional analysis capacities in the laboratories, would be required, which is expensive. Furthermore, several loci have to be analysed for each isolate, which is time-consuming, even if the existing online MLST database will simplify analysis for species identification and epidemiological links.

A thorough study with information from pig herds tested positive for *B. intermedia* by CBT should be performed, to search for correlations between presence of different genotypes of spirochaetes with the phenotype of *B. intermedia* and diarrhoea. Virulence studies could thereafter be performed with field isolates with suspected pathogenicity. In addition, *B. intermedia* isolates recovered from birds should be tested to evaluate the possibility of transmission of the bacteria between birds and pigs. This is important since we showed that isolates within the phenotype of *B. intermedia* are heterogeneous, and some researchers have reported a correlation between diarrhoea and *B. intermedia*. Hence, some of the genotypes of *B. intermedia* may be pathogenic to pigs.

Reliable *in-vitro* methods for studying virulence should be developed. Such techniques would be useful for screening *Brachyspira* isolates, and for searching for virulence genes that could be used as PCR markers to elucidate the pathogenesis, or for development of an ELISA for serologic surveillance of pig herds.

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