Towards improved diagnostics and control of swine dysentery

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A	adenine
adh	alcohol dehydrogenase
alp	alkaline phosphatase
ATCC	American type culture collection
BHI	brain heart infusion
BLAST	basic local alignment search tool
C	cytosine
CGH	comparative genomic hybridisation
CVSBA	colistin-vancomycin-spectinomycin-blood-agar
DDGS	distillers dried grains with solubles
CLSI	Clinical and Laboratory Standards Institute
DGV	dextrose glucose veronal
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immuno sorbent assay
est	esterase
FBS	foetal bovine serum
FISH	fluorescent in situ hybridization
G	guanine
gdh	glutamate dehydrogenase
glpK	glucose kinase
HPLC	high performance liquid chromatography
hrs	hours
IFNγ	interferon γ
Ig	immunoglobuline
IL	interleukine

kb	kilobase
kDa	kiloDalton
LOS	lipo oligosaccharides
LPS	lipopolysaccharides
MALDI-TOF MS	matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
MEE	multilocus enzyme electrophoresis
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MLVA	multiple locus variable number tandem repeat analysis
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NSH	novel strongly haemolytic
PAS	periodic acid schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCS	porcine colonic spirochetosis
pgm	phosphoglucomutase
PIS	porcine intestinal spirochetosis
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RFLP	random fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SD	swine dysentery
SNP	single-nucleotide polymorphism

sp.	species
ST	sequence type
SAM	S-adenosylmethionine
SEM	scanning electron microscopy
T	thiamine
^{T}	typestrain
thi	thiolase or acetyl-CoA acetyltransferase
TSA	tryptic soy agar
UK	United Kingdom
USA	United States of America
VNTR	variable number of tandem repeat
WBHIS	weakly beta-haemolytic intestinal spirochetes

PART I

General Introduction

1 Genus *Brachyspira*

Brachyspira is the sole genus in the *Brachyspiraceae* family and together with the families of Spirochaetaceae and Leptospiraceae they are assigned to the Order Spirochaetales, Class Spirochaetes and Phylum Spirochaetae. All *Brachyspira* species have a helical cell shape (figure 1) and two sets of opposing internal periplasmic flagella. The number of flagella varies from 4-14 inserted at each end. The *Brachyspira* sp. cell size varies from 5-11 µm in length and 0.2-0.4 µm in width [144,66,132,51,137]. They are anaerobic but, partly due to high levels of NADH oxidase, aerotolerant. *Brachyspira* sp. use soluble sugars as carbon and energy source [182].

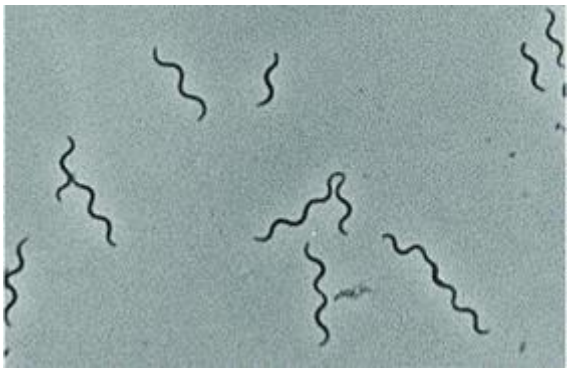

Figure 1: Phase contrast microscopic image of *B. hyodysenteriae* (from Hampson et al. [53])

Brachyspira sp. grow slowly *in vitro* and do not form colonies. Instead they show a low flat film of growth after 3-5 days at 37-42°C. On ovine or bovine blood containing agar plates, growth is surrounded by a weak or strong zone of beta-haemolysis, dependent on the species. *B. hyodysenteriae, B. suanatina* and *B hampsonii* are strongly haemolytic, *B. pilosicoli, B. intermedia, B. innocens* and *B. murdochii* are weakly haemolytic and the latter are sometimes referred to as 'weakly beta-haemolytic intestinal spirochetes' (WBHIS) [34,136].

Nine *Brachyspira* species have been officially recognized: *B. hyodysenteriae* (pig, rhea) [17,77], *B. pilosicoli* (various mammals and birds) [201], *B. intermedia* (pig, chicken) [184], *B. murdochii* (pig) [184], *B. innocens* (pig) [186], *B. hampsonii* (pig) [132], *B. suanatina* (pig) [137], *B. aalborgi* (human) [66] and *B. alvinipulli* (chicken) [187]. Many other proposed species exist: *"B. pulli"* (chicken) [190], *"B. canis"* (dog) [35], *"B. corvi"* (corvid birds) [73], *"B. rattus"* (rat) [7], *"B. muridarum"* (mouse) [7], *"B. muris"* (mouse) [7], *"B. ibaraki"* (human) [191], *"B. christiani"* (human) [78], *"B. hominis"* (human) [209]. The majority of *Brachyspira* species are associated with

one host, some species can cause disease in several different hosts. Some characteristics of the pig associated *Brachyspira* species are given in table 1. Except for *B. pilosicoli,* the 16S rRNA sequences between *Brachyspira* species are highly conserved, which renders the sequence analysis of solely 16S rRNA insufficient to identify an isolate to the species level [39]. The marginal differences between the 16S rRNA of some *Brachyspira* sp. raises the question if all described species are truly distinct species or that perhaps some species should be regarded as biovars of another species instead [148].

Brachyspira has a circular genome of varying size, which is accompanied by a plasmid in some species. The G+C content is typically about 27% [147]. For most of the porcine related species one or more whole genome sequences are available: 21 for *B. hyodysenteriae,* 5 for *B. hampsonii,* 4 for *B. pilosicoli,* 2 for *B. intermedia,* 1 for *B. murdochii* and 1 for *B. suanatina* [49,137].

Table 1: Pig associated *Brachyspira* spp., type strain, host(s) and reservoirs.

Species	Type strain	Host	Reservoirs
B. hyodysenteriae	B78^T	Pig, rhea	Pig, mouse, rat
B. intermedia	PWS/A^T	Pig, chicken	Pig, chicken, mouse, rat
B. murdochii	51-150^T	Pig	Pig, mouse, rat
B. innocens	B256^T	Pig	Pig, mouse rat
B. pilosicoli	P43/6/78^T	Pig, birds, dogs, non-human primates, human	Pig, birds, dogs, non-human primates, human
B. suanatina	AN4859/03 ^{T}	Pig	Mallard
B. hampsonii	NSH-16^T (clade I) NSH-24 (clade II)	Pig	Waterfowl

2 Porcine *Brachyspira* infections: introduction

Brachyspira hyodysenteriae and *B. pilosicoli* are the two swine related *Brachyspira* species that are clearly pathogenic and are the causative agents of swine dysentery (SD) and Porcine Intestinal Spirochaetosis (PIS) respectively [193,201]. *B. innocens* is considered to be a non-pathogenic commensal [96]. For *B. intermedia* and *B. murdochii* the pathogenic potential is less clear-cut [80,139,20,208,100]. Two species, more recently recovered from swine, are *B. hampsonii* and *B. suanatina.* These two species can cause disease signs in pigs that are indistinguishable from swine dysentery [132,137,158].

Since there are now three strongly haemolytic *Brachyspira* species that can cause dysentery-like disease, it has been suggested to change the name swine dysentery, since not only *B. hyodysenteriae* can act as the causative agent of SD. For now, Hampson has introduced the more general name 'Brachyspiral colitis' to 'describe the situation where colitis, diarrhoea and/or dysentery occurs in pigs infected with one or more pathogenic *Brachyspira* species' [48].

3 Porcine *Brachyspira* infections other than *Brachyspira hyodysenteriae*

3.1 *Brachyspira pilosicoli*

Brachyspira pilosicoli is the causative agent of Porcine Intestinal Spirochetosis (PIS), sometimes also referred to as Porcine Colonic Spirochetosis (PCS) [193,201]. PIS is most often seen shortly after weaning and is characterised by diarrhoea with a consistency of wet cement, in some cases evolving to watery or mucoid diarrhoea. It can be accompanied by reduction of growth and increased feed conversion.

A pathognomonic feature of PIS is the attachment of *B. pilosicoli* by one cell end to the enterocytes in the colon, forming a so called "false brush-border" [193]. *B. pilosicoli* is the only *Brachyspira* sp. in which such obvious attachment to epithelium is apparent. Other *Brachyspira* sp. seem to be associated only with the mucus layer, covering the colonic epithelium. *B. pilosicoli* is also the only *Brachyspira* sp. which has a broad host-range; it can cause infections in various bird species, dogs, and humans [35,111,200,122]. Other marked differences between *B. pilosicoli* and the other pig related *Brachyspira* sp. are its more slender form and its lower number of flagella. This might also be reflected in *B. pilosicoli* being the most genetically distinct species with regard to the other pig related *Brachyspira* sp. [112]. This is depicted in figure 2, a

radial tree based on the concatenated sequences of four MLST genes (*est, glpK, thi, pgm)* [129].

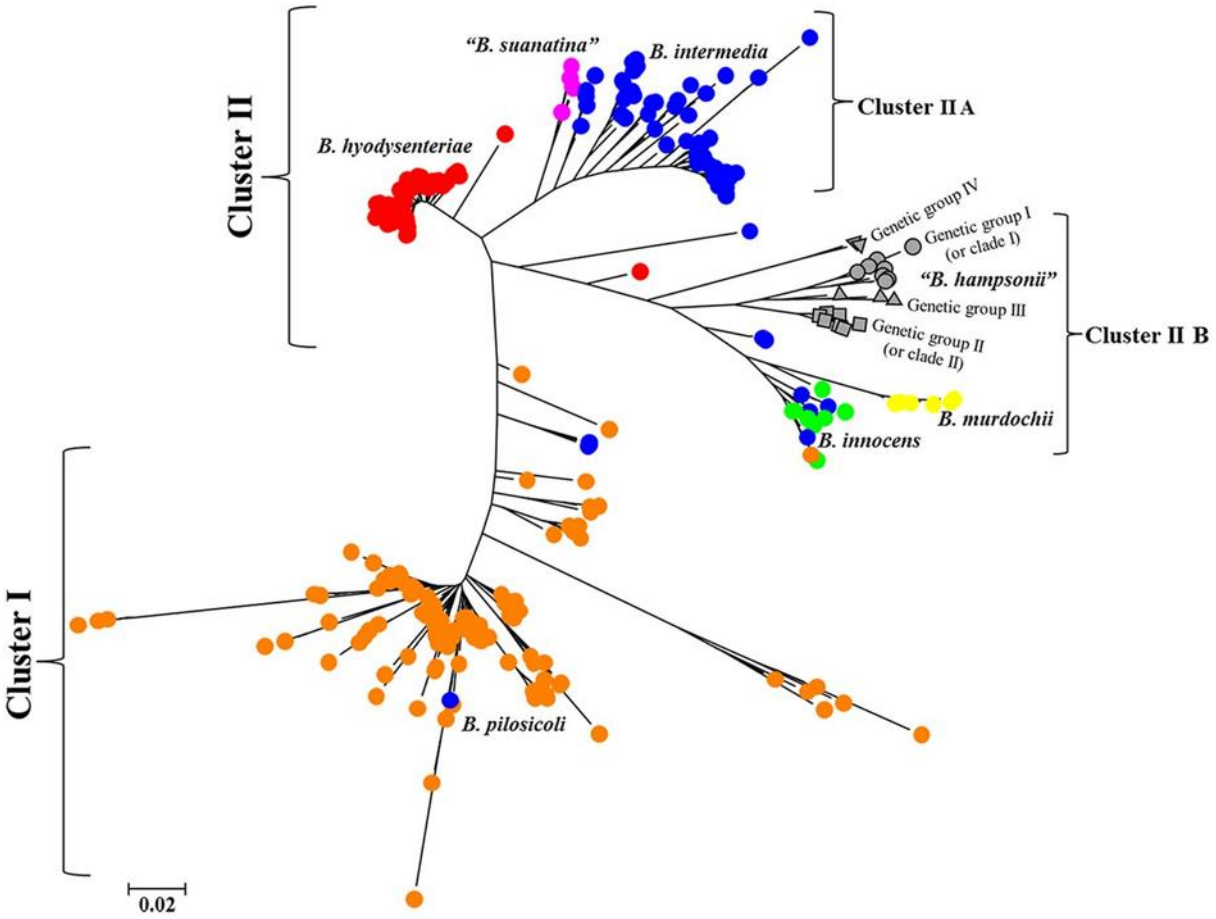

Figure 2: Radial tree of maximum likelihood analysis portraying the clustering of 430 isolates representing seven *Brachyspira* species. Genetic relatedness determined using nucleotide differences among four loci (*est, glpK, thi* and *pgm*). The scale unit represents 2 substitutions per 100 nucleotide positions. From Mirajkar et al. [129].

3.2 *Brachyspira intermedia* and *Brachyspira murdochii*

The pathogenic potential of *B. intermedia* and *B. murdochii* for pigs is less clear. Both species have been isolated from clinical cases of mild diarrhoea or catharral colitis [39,64,12,28,80]. However, experimental infection using field isolates of *B. intermedia* and *B. murdochii* does either not result in clinical signs or in very mild diarrhoea [20,80,81,139]. Furthermore, there is no correlation between the presence of diarrhoea and the presence of *B. murdochii* or *B. intermedia* in pig herds [59]. Vögely et al. noted only a slight increase of diarrhoea problems on farms where *B. intermedia* could be detected, and no increase on farms where *B. murdochii* was detected [203].

Komarek et al. report an association between the prevalence of *B. intermedia, B. murdochii* and *B. innocens,* and chronic wasting of pigs [100]. No association with diarrhoea was present and it should be noted that in a number of *Brachyspira* positive herds other pathogens (Porcine circovirus 2 (PCV-2), *Lawsonia intracellularis*), were detected as well. Weissenböck et al. also suggested co-infections with PCV-2 or *L. intracellularis* as conditions enhancing the risk of abundant growth of weakly haemolytic *Brachyspira* species and concurrent development of clinical signs [208].
With regard to *B. intermedia,* there might be an additional explanation for the seemingly variable pathogenic potential of this species. MLST analysis of this species revealed a large strain diversity and indicates that this species is paraphyletic. The genetic distances between clusters of *B. intermedia* strain are so large that some clusters might actually be other species, not yet identified [148]. This is also demonstrated in figure 2: the blue dots represent clusters of *B. intermedia* strains and are found dispersed along clusters of other *Brachyspira* species [129]. Some of these distinct clusters of *B. intermedia* strains, might have different biological properties or different pathogenic potential compared to the strains that have been used to experimentally infect pigs.

3.3 *Brachyspira suanatina*

Brachyspira suanatina isolates were first noticed in Sweden where they were recovered from pigs and mallards. The pig associated isolates derived from herds suffering from mild to mucohaemorrhagic diarrhoea [158]. Mallard isolates came from a gamebird farm and from wild mallards that were sampled in a public park or in a bird observatory. None of the mallards showed clinical signs of disease [74].

The isolates were phenotypically indistinguishable from *B. hyodysenteriae,* showing strong haemolysis on agar plates supplemented with ovine blood. Remarkably, these isolates were PCR-negative for the *tlyA* gene of *B. hyodysenteriae* [157]. Analysis of the 16S rRNA and NADH oxidase (*nox)* genes of these atypical isolates showed that they formed a distinct cluster, apart from *B. hyodysenteriae.* These isolates were proposed to form a new species. DNA-DNA hybridization of *B. suanatina* strain AN4859/03 ^{T} (ATCC BAA-2592) with the seven previously recognized *Brachyspira* species confirmed that *B. suanatina* forms a separate species [137].

Experimental infection demonstrated that the new species *B. suanatina* could induce diarrhoea or dysentery in pigs. Moreover, pigs developed diarrhoea after infection with an isolate recovered from a bird, demonstrating the possible risk of transmission from mallards to pigs [158]. To date *B. suanatina* infections in pigs have been exclusively reported in Sweden and Denmark.

3.4 *Brachyspira hampsonii*

Since the mid 2000's the number of *Brachyspira* infections has increased in the USA and Canada. Remarkably, since the late 2000's, over 50% of strongly haemolytic isolates from outbreaks of mucohemorrhagic diarrhoea, tested negative for *B. hyodysenteriae* by PCR [25,129,169]. Phylogenetic analyses of the *nox* and 16S rRNA genes of these atypical isolates, revealed such a large genetic distance between those isolates and all other known *Brachyspira* sp. that these isolates likely represented a novel species, for which the name *Brachyspira hampsonii* was proposed [25,132].

B. hampsonii isolates group in two distinct clades: clade I and clade II. Within each clades *nox* sequences showed a similarity of >99%, between clades the sequence similarity was 96% [25]. Further genetic analyses of this species by MLST suggested that the *B. hampsonii* species rather encompassed four genetic groups, instead of two clades. This is depicted in figure 2 [129].

Rubin et al. proved that experimental infection of pigs with a *B. hampsonii* clade II isolate results in mucohaemorrhagic diarrhoea, undistinguishable from diarrhoea seen in a *B. hyodysenteriae* infection [169]. Furthermore, gross lesions and histological changes in the colonic tissue of pigs infected with *B. hyodysenteriae* or pigs infected with *B. hampsonii* were similar [213]. Costa et al. showed that these similarities in clinical signs and gross and microscopic lesions are also seen after experimental infection with *B. hampsonii* clade I [29].

4 Infections with *Brachyspira hyodysenteriae*

Taylor and Alexander in the UK and Harris et al. in the USA quite simultaneously described the isolation of a spirochete from clinical cases of swine dysentery [57,192]. The spirochete was named *Treponema hyodysenteriae* by Harris et al. and both groups fulfilled Koch's postulates for this organism [57,192]. DNA-DNA hybridization and 16S rRNA analysis showed that the organism was not closely related to other

Treponema species or other spirochaetal species. Hence the organism was renamed into *Serpula hyodysenteriae,* which was later corrected into *Serpulina hyodysenteriae* [183,186]. In 1997, the genera *Serpulina* and *Brachyspira* were synonymised, leading to the current name *Brachyspira hyodysenteriae* [144]. Although *B. suanatina* and *B. hampsonii* are able to induce clinical symptoms similar to these typically associated with swine dysentery (SD) caused by *B. hyodysenteriae*, SD will be considered as the disease complex caused by *B. hyodysenteriae* in this introduction.

4.1 Epidemiology

4.1.1 Prevalence

Swine dysentery was first reported in 1920 in the USA, and has been described worldwide since 1950-1960 [124,165]. In most pig producing countries, the incidence of SD increased during 1960-1970 due to intensification of pig production. Since the 1980's a general decline in SD incidence was noted in North America and Europe, probably due to increased attention to biosafety measures and the use of antimicrobial growth promoters. Since the 2000's, an increase of SD incidence is noted, most likely due to the restriction of antimicrobial growth promoters in Europe [5]. Prevalence reported in Europe varies between countries: 18% of herds in Italy [123], 7-18% of herds in the UK [199,198], 2.5-14% of herds in Denmark [133,189], 32-40% of herds in Spain [5,24]. It is however difficult to directly compare these percentages since sample size and the sampled population differ between countries: some sample only herds which suffer from diarrhoea [24,123,198,199], some sample only healthy herds [189] and some sample healthy herds as well as herds with diarrhoea [133].

Although the use of antimicrobial growth promoters is not prohibited in North America, a substantial increase in SD cases is seen since the late 2000's as well. For example IOWA state diagnostic laboratory recovers 3 isolates from 15 cases/farms in 2005 and 466 isolates from 3465 cases/farms in 2010 [19]. Partly, this increase can be attributed to the emergence of the novel species *B. hampsonii.* The concurrent increase of *B. hyodysenteriae* related cases of SD is not fully understood. Probably one or more influencing factors such as environment, management, diet, microbiota of the colon, and/or host susceptibility have changed [19].

4.1.2 Transmission

Transmission of *B. hyodysenteriae* occurs mainly through direct contact. The pathogen is often introduced into a previously uninfected herd by the introduction of sub-clinically infected carrier animals [5]. Once endemic, infection remains established in a herd through contact with faeces or with clothes, boots or equipment contaminated with faecal material. *B. hyodysenteriae* can survive up to 78 days in soil contaminated with pig faeces, and up to 112 days in pure pig faeces, at 10°C [15].

Rodents on farms have been identified as carrier animals of several *Brachyspira* sp, including *B. hyodysenteriae* [7,50,83]. Moreover, Joens and Kinyon were able to induce dysentery in experimentally infected pigs with three of four *B. hyodysenteriae* isolates recovered from wild mice (*Mus musculus)* [83]. Insects, in particular cockroaches (*Blatta orientalis),* have been identified as possible carriers of *B. hyodysenteriae* as well. After experimental infection, cockroaches excrete viable *B. hyodysenteriae* for up to three days [14].

Birds have also been identified as carriers of several *Brachyspira* sp.. *B. hyodysenteriae* was isolated from wild and farmed mallards, sampled in Sweden [74]. It has not been determined if these isolates can experimentally infect pigs. Lesser snow geese (*Chen caerulescens caerulescens)* sampled in the Canadian Arctic region were positive (8.8% of animals) for *Brachyspira* sp. including *B. hampsonii* clade I. No *B. hyodysenteriae* could be isolated from birds sampled in this region. In an experimental infection the goose isolate of *B. hampsonii* clade I did not cause clinical signs in colonized pigs [170]. In Spain, *B. hampsonii* clade I and II were isolated from waterfowl. Birds were sampled in a known wintering area visited by Northern European waterfowl. Over 20% of geese and 50% of mallards were positive for *Brachyspira* sp., and 20% of all isolates belonged to the *B. hampsonii* species, while no *B. hyodysenteriae* or *B. suanatina* could be detected [121]. An experimental infection study with one of these Spanish *B. hampsonii* isolates recovered from migrating waterfowl, showed that such isolates can colonize pigs, be transmitted along pigs and cause clinical signs of swine dysentery in a number of animals [3].

B. hyodysenteriae has also been detected in farms with rheas where it caused severe necrotizing typhlocolitis [77,172] and from chickens from a laying flock with above average number of eggs with faecal staining [38]. It was hypothesised that the poultry

infection might be coming from a nearby swine farm. Apart from those reports, there has been no further mention of *B. hyodysenteriae* isolation from poultry or rheas.

4.1.3 Risk factors

Since transmission of SD mainly occurs through contact with carrier animals, contact with contaminated clothes or equipment or contact with vectors, it is not surprising that frequent purchasing of breeders or growing pigs from different sources, the presence of rodents and the free allowance of visitors onto a farm were identified as risk factors for the presence of SD [162]. Next to these management or husbandry related risks of being infected by *B. hyodysenteriae*, the outcome of infection by *B. hyodysenteriae* might be affected by several other factors as well.

The virulence of a particular *B. hyodysenteriae* strain may play a role in the clinical outcome. Differences in virulence between strains have been described. Jensen and Stanton described type strain B78 (ATCC 27164) as low pathogenic, probably due to the large number of subculturing before the strain was deposited in the culture collection [76]. Achacha et al. compared virulence of *B. hyodysenteriae* strains belonging to different serotypes and found strain A1 and B234 to be avirulent [1]. Recently La et al. provided evidence for lack of plasmid encoded genes in these less virulent strains [108]. A common factor in these less virulent strains is that they colonize fewer animals compared to virulent strains such as B204. However, for most of these strains, colonization is still concurrent with the development of SD. In contrast with this, Lysons et al. isolated three strains of *B. hyodysenteriae* from herds free of SD and could not induce disease signs using two of these strains in an *in vivo* experiment, even when animals were colonized by the strain [120].

The outcome of an infection by *B. hyodysenteriae* can substantially be influenced by the diet of the pigs. Diet can influence the pH in the colon and/or cecum and can influence the composition of the microbiota in the colon. Some diet components can have direct antibacterial effects as well. Although impact of diet on the development of clinical SD has been extensively studied, a general consensus has not been reached. Several studies describe that feeding highly digestible diets based on cooked white rice can reduce the severity of SD symptoms in an experimental infection study [150,176]. Other studies describe a symptom reducing effect of a highly digestible pre-fermented liquid diet, but fail to reproduce the effect of cooked rice [113]. Both types

of diet are supposed to reduce the amount of fermentable substrate that enters the large intestine, which would alter pH. However, alkalinity could not always be demonstrated for the colon and/or the cecum when administering these diets. Improved digestibility of diets was achieved by extrusion of seeds and the addition of exogenous enzymes as well. However, no reduction in SD was achieved feeding those diets [36].

Somewhat in contrast to the beneficial effect of some highly digestible feeds, the addition of highly fermentable carbohydrates protected pigs against development of SD [197]. Protective fermentable carbohydrates were derived from the addition of dried chicory root and sweet lupins to a barley and triticale based feed. Hansen et al. demonstrated that the protective aspect came from inulin in the chicory root rather than from lupins [56]. However, the beneficial effect of inulin could only be confirmed at high concentrations [55]. Recently, Wilberts et al. demonstrated that increasing the percentage of insoluble dietary fiber of a feed by adding distillers dried grains with solubles (DDGS), a common practice in the USA, led to a much faster development of SD in pigs after experimental infection with *B. hyodysenteriae* [212].

In conclusion it can be stated that swine dysentery is a multifactorial disease in which virulence properties of the *B. hyodysenteriae* strain, management and husbandry factors play an important role [5]. Nutritional factors are also described as an important factor but its influence is less clear-cut as demonstrated by the contradictory findings.

4.2 Pathogenesis

Mostly, swine dysentery is instigated by ingestion of faecal material containing *B. hyodysenteriae*. Survival of the acidic environment of the stomach is presumably supported by the presence of mucus in infectious faecal material [48,185], although this has not been demonstrated as such. After passing the stomach, *B. hyodysenteriae* reaches the large intestine and predominantly resides in the colon and sometimes in the cecum. Most often, no other organs are involved, although sporadically involvement of the stomach, ileum and rectum has been mentioned [69]. Clinical signs of dysentery appear after a relatively long incubation time, on average 17 days (2-31) after exposure in experimental infections.

In the large intestine the number of *B. hyodysenteriae* gradually increases and Wilcock and Olander established that once the number of viable *B. hyodysenteriae* approximately reaches 10^5 cfu/g mucosa, lesions start to develop [215].

B. hyodysenteriae can be found in faecal samples 1-4 days before the onset of SD. However, experimentally inoculated pigs often show no signs of dysentery or detectable amounts of *B. hyodysenteriae* in their faeces, before the sudden onset of SD [69].

B. hyodysenteriae requires the presence of other anaerobic bacteria in the large intestine. Several authors [125,139,211] describe the impossibility to establish a *B. hyodysenteriae* infection in gnotobiotic pigs. However if gnotobiotic pigs are inoculated with colonic scrapings of pigs suffering from SD [125], with a mixture of *B. hyodysenteriae* and five enteric anaerobes [126] or with a culture of *B. hyodysenteriae* and one other anaerobe (*Fusobacterium necrophorum, Bacteroides vulgatus, Clostridium* sp, *Listeria denitrificans*), inoculation is followed by the development of clinical swine dysentery [211].

B. hyodysenteriae is mainly localised in the crypts of the colon, specifically at the base. Crypts elongate and at the base mucus is depleted from the Goblet cells [215]. Ligated colonic loops have been used in swine to determine the order and time-frame of lesion development following inoculation with *B. hyodysenteriae* cultures or with preparations from minced colons of pigs with active SD [68,210]. Gross lesions started to develop after 72 hrs and consisted of hyperaemia of the mucosa, which is thickened and shows prominent rugae. The relative number of Goblet cells starts to increase and patchy focal necrosis of epithelial cells can be visible. Thereafter (24-48 hrs later), the mucosal surface starts to be covered with a layer of mucus and fibrin while the colonic mucosae further thickens and becomes oedematous. Due to epithelial necrosis, blood vessels of the underlying lamina propria are exposed and damaged, leading to blood leakage into the colonic lumen [69].

Some aspects of the pathogenesis of *B. hyodysenteriae* are still unclear. The development of the diarrhoea is not completely understood and is debated to be caused by malabsorption and/or by active fluid secretion. The extensive mucus outpouring may considerably attribute to the fluid loss and loss of electrolytes, although it is not clear how significant this contribution is [215]. Active fluid secretion would suggest the involvement of enterotoxins. However, filtered culture supernatant could not induce fluid secretion or any lesions in colonic loops [210].

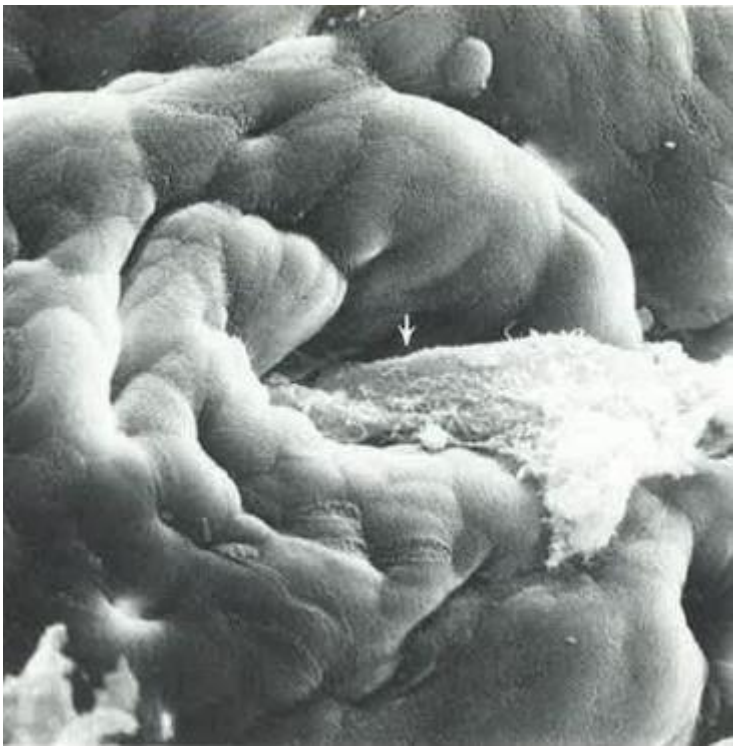

Figure 3: SEM of pig in early stage of swine dysentery showing spirochetes and mucus in a crypt orifice (arrow). From Kennedy and Strafuss [90].

4.3 Virulence factors of *B. hyodysenteriae*

Several virulence factors involved in the pathogenesis in *B. hyodysenteriae* infections in pigs have been investigated. Virulence factors include colonisation factors such as motility or chemotaxis and toxins such as haemolysins [194].

4.3.1 Motility and chemotaxis

In order to occupy the mucus dominated environment in the large intestine, *B. hyodysenteriae* greatly relies on its motility and chemotactic capacity. It has been demonstrated that *B. hyodysenteriae* is chemotactic towards hog gastric mucin (1% (w/v)) and porcine colonic mucin (1% (w/v)) and not towards bovine submaxillary mucin (1% (w/v)) [128]. Moreover, the less or non-pathogenic species *B. intermedia* and *B. innocens* showed reduced chemo attraction compared to virulent *B. hyodysenteriae* strains. Kennedy and Yancey (1996) further demonstrated that the chemo attractive nature of mucus could mainly be attributed to fucose, a terminal sugar of mucins, and L-serine, an amino acid largely present in the protein core structure of mucins [93]. Other terminal sugars such as lactose, galactose and other amino acids like threonine and L-cysteine also act as chemoattractant. It has also been demonstrated that

B. hyodysenteriae is not only chemically attracted to mucus, but that *B. hyodysenteriae* is also attracted *in vitro* to solutions with increasing viscosity [138].

B. hyodysenteriae, like all spirochetes, has a spiral morphology, ideal for moving through mucus [92]. Besides their morphology, spirochetes share a motility system in which flagellar filaments are positioned within the outer membrane sheath, as shown in figure 4. The periplasmic flagella are inserted at each end of the protoplasmic cylinder, and overlap in the middle of the protoplasmic body [26,27]. Koopman et al. described the protein composition of the periplasmic flagella of *B. hyodysenteriae*: three different proteins can be found in the core of the flagella (FlaB1, FlaB2, FlaB3), two different proteins can be found in the sheath of the flagella (FlaA1, FlaA2) [101].

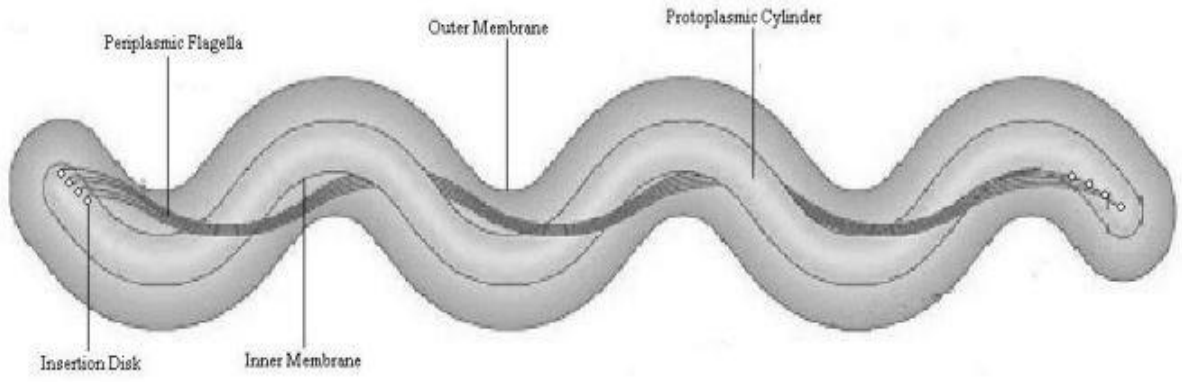

Figure 4: schematic view of the position of periplasmatic flagella in *Brachyspira*. From Neo [140].

Although *flaA1* mutants, *flaB1* mutants, and *flaA1 flaB1* dual mutants are still able to assemble periplasmatic flagella, the *in vitro* motility of these mutants is impaired [91,167]. Of mice experimentally inoculated with the *flaA1 flaB1* dual mutant, only 2% was colonised, compared to 75% colonisation following inoculation with the wild type strain [168].

4.3.2 Adhesion

B. hyodysenteriae is mainly found in the mucus in the crypts of Lieberkühn and in the mucus layer lining the epithelium of the colon. Although attachment of *B. hyodysenteriae* to epithelial cell cultures has been described *in vitro* [46,99], direct attachment of *B. hyodysenteriae* to the colonic epithelial cells does not seem to play an important role in colonisation [92]. SEM images of colonic and caecal mucosal

surfaces of experimentally infected pigs, show that *B. hyodysenteriae* is almost exclusively associated with mucus, and that the epithelium itself is relatively free of spirochetes.

4.3.3 Haemolysin

In the first decade after the description of the ethological agent of SD, only two species of *Brachyspira* (then *Treponema)* were described that could be detected in the faeces of swine: *Treponema hyodysenteriae*, which is strongly haemolytic and enteropathogenic for swine, and *Treponema innocens,* which is weakly haemolytic and not pathogenic. Obviously this led to an interest in the haemolysin produced by *B. hyodysenteriae.*

Several authors describe the purification and characterization of haemolysin produced by *B. hyodysenteriae.* The precipitation and filtration of haemolysin from *B. hyodysenteriae* culture supernatant led to the description of haemolysins with different molecular weights. Saheb et al. found a protein of 74 kDa, Knoop a protein of 68 kDa, and Kent et al. described a protein of 19 kDa [94,98,174]. The protein of 19 kDa was used in an ileo-colonic loop model in swine and loops injected with this purified protein showed extensive lesions, comparable to lesions seen in cases of SD [119].

Muir et al. constructed a *B. hyodysenteriae* genomic library in an *Escherichia coli (E. coli)* strain [135]. The DNA inserts of haemolytic recombinant *E. coli* clones were sequenced. This led to the description of *tlyA* [135], *tlyB,* and *tlyC* [195]. A *tlyA*$^{-}$ deletion mutant strain of *B. hyodysenteriae* does not provoke clinical signs of SD in experimentally infected mice or swine [71,196]. The recombinant *E. coli* clones harbouring the *tlyB* or *tlyC* insert were less haemolytic on blood containing agar plate compared to the clone with the *tlyA* insert. However, the supernatant of the *tlyC* and *tlyA* inserted clones showed an equally haemolytic activity *in vitro*, while the haemolytic activity of the *tlyB* inserted clone was even stronger compared to that of the *tlyA* inserted clone. Deletion mutants of *tlyB* and *tlyC* have not been constructed and their role in *in vivo* pathogenesis is not quite clear [195].

Hsu et al. extracted a protein from the supernatant of a *B. hyodysenteriae* culture and revealed the N-terminal amino acid sequence [67]. The gene coding for this haemolysin was named *hlyA.* Comparing the molecular weight, the *hlyA* gene probably codes for the 19kDa protein first described by Kent et al. that causes lesions in an ileo-

colonic loop model in swine [94,119]. Unlike the *tlyA* gene, *hlyA* can be found in weakly haemolytic *Brachyspira* species as well. However, only in *B. hyodysenteriae* the *hlyA* gene is correctly placed between *fabG,* coding for an ACP-reductase, and *fabF,* coding for an ACP-synthase. The ACP-reductase and –synthase probably influence the chemical moiety of the lipid that is attached to the HlyA protein. This may affect the haemolytic activity of the HlyA protein [219]. Barth et al. confirmed that in German *Brachyspira* isolates, *tlyA* was exclusively detected in *B. hyodysenteriae.* The *hlyA* gene could be found in weakly haemolytic *Brachyspira* species. However, *hlyA* was then either not accompanied by *fabF* and *fabG,* or *hlyA, fabF* and *fabG* showed substantial sequence variation [8].

The mechanism of haemolysis induced by *B. hyodysenteriae* haemolysins is not fully elucidated. The 74 kDa haemolytic protein described by Saheb et al. appears to lyse red blood cells through colloid osmotic lysis, since lysis is associated with swelling of the erythrocytes [174]. However part of the haemoglobin release seems to appear before swelling of the erythrocytes [173]. For the *tlyA* encoded haemolytic protein, Muir et al. find no evidence for haemolysis through pore formation since the haemolysis is not blocked by adding sugars of different sizes [135]. Hyatt and Joens provide evidence that cell lysis by the *tlyA* encoded haemolytic protein can be blocked by sugars of 2.0 to 2.3 nm diameter, suggesting that haemolysis by this protein is caused by pore formation in the erythrocyte membrane [70].

The whole genome sequencing of *B. hyodysenteriae* strain WA1 by Bellgard et al. revealed three other haemolysis associated genes encoding haemolysin III, haemolysin activation protein and haemolysin III channel protein. The exact function of these genes is unknown [10].

4.3.4 Plasmid associated virulence factors

The whole genome sequencing of *B. hyodysenteriae* strain WA1 by Bellgard et al. has revealed the presence of a 36 kilobase (kb) plasmid [10]. Six *rfb* genes on this plasmid were predicted to play a role in pathogenesis and virulence. La et al. demonstrated that a *B. hyodysenteriae* strain (WA400) lacking the 36kb plasmid colonised significantly less pigs after experimental challenge, and therefore caused SD in fewer animals compared to *B. hyodysenteriae* strain WA1 containing the plasmid [109].

Microarray-based comparative genomic hybridisation (CGH) analysis of six *B. hyodysenteriae* strains reported to be virulent and eight strains defined as ‘avirulent’

or at least as having reduced pathogenic potential, identified four genes that were absent in all 'avirulent' strains and present in all virulent strains. These four genes form an adjacent block on the 36 kb plasmid and encode a radical S-adenosylmethionine (SAM) protein, a glucosyltransferase, an NAD-dependent epimerase and an dTDP-4-dehydrorhamnose 3,5 epimerase. Proteins encoded for by these four genes are catalytic enzymes that are probably involved in LOS biosynthesis or glycosylation [108].

Although the microarray CGH analysis showed a convincing correlation between the absence of the depicted four plasmid genes and reduced pathogenic potential, *B. hyodysenteriae* isolates lacking some of these genes were retrieved from six different pig herds suffering from SD in Germany [110].

4.3.5 Other virulence factors

Like for other Gram-negative bacteria, lipopolysaccharides (LPS) are present in the outer envelope of *Brachyspira* sp. [9]. LPS extracted from *B. hyodysenteriae* seems to play a role in the pathogenesis of SD, since the LPS showed biological activity comparable to LPS of *E.coli in vitro*. Furthermore, a LPS-resistant strain of mice did not develop lesions after experimental inoculation with *B. hyodysenteriae,* whereas a LPS-sensitive strain of mice showed obvious gross and microscopic lesions after the same experimental inoculation [141-143]. However, Greer and Wannemuehler found LPS extracted from *B. hyodysenteriae* less active *in vitro.* Endotoxin preparations of *B hyodysenteriae,* which contain LPS and lipid-A associated proteins, were more active but endotoxin preparations of *B. hyodysenteriae* and *B. innocens* were equally active *in vitro*, suggesting that the difference in pathogenic potential of these two species cannot be attributed to the biological activity of their endotoxins [47].

The enzyme NADH oxidase is also regarded as a virulence factor of *B. hyodysenteriae*. This enzyme is active in the four electron reduction of oxygen, and its presence aids to the survival of *B. hyodysenteriae* in atmospheres containing oxygen. NADH oxidase negative mutants of *B. hyodysenteriae* are over a 100 fold more sensitive to dying due to oxygen exposure. In an *in vivo* experimental inoculation fewer pigs were colonised by the NADH oxidase negative mutant strain and animals that were colonised, showed milder, transient clinical signs compared to animals inoculated with the wild-type strain [188].

4.4 Clinical signs

Clinical signs are most frequently seen in pigs during growth and finishing period, mostly in animals of 10-16 weeks old [2,5]. The incubation period can be substantial and may vary considerably [145]. Ranges from 2 days to three months have been described, with a 10-14 day period being average [48]. Severity of disease signs may vary as well, ranging from mild diarrhoea with no apparent changes in general condition of the pig to severe haemorrhagic diarrhoea, accompanied by anorexia, depression and death.

Most commonly an outbreak starts gradually, by affecting a small number of growers/finishers, showing minor loss of appetite and softer, discoloured faeces. The appearance of the faeces evolves from looking like wet cement with specs of blood and/or mucus, to watery deep chocolate red faeces with a large amount of blood. The perineum and thighs of the pigs are stained with faeces and the pigs can look severely debilitated by now and the loss of appetite can be more pronounced. Clinical SD can be accompanied by fever up to 40°C, but mostly no fever is recorded [2].

In endemically affected herds symptoms typically resurface every 3-4 weeks. Cessation of the use of antimicrobials, other infections, or environmental stressors can cause a re-emergence of clinical SD [5,48]. Pigs that recover from SD have a reduced weight gain and feed conversion can dramatically increase.

4.5 Lesions

4.5.1 Macroscopic lesions

Macroscopic lesions of *B. hyodysenteriae* infection are initially noted in the large intestine as thickened mucosae with prominent rugae. In the first phase, lesions are most pronounced near the apex of the colon. This progresses into thickening of the entire colonic mucosa with marked hyperaemia and oedema. A mucoid and/or haemorrhagic exudate is present in the colonic lumen. Mucosal erosions can be seen covering large areas or can be more localized, sometimes covered with fibrino-necrotic material. The cecum can show comparable lesions and mesenteric lymph nodes appear enlarged and congested [68,69,72,145]. In experimentally infected pigs, hyperaemic mucosae in the fundic portion of the stomach have been described in a small number of animals [68].

4.5.2 Microscopic lesions

Microscopic changes during a *B. hyodysenteriae* infection are hallmarked by several changes in the colonic crypts [68,69]. During the first days of clinical signs, mucus glands of infected animals are dilated, followed by depletion of mucus in the Goblet cells at the base of the colonic crypts. In a later stage the number of Goblet cells increases rapidly [20,65], markedly increasing the depth of the colonic crypts as shown in figure 5.

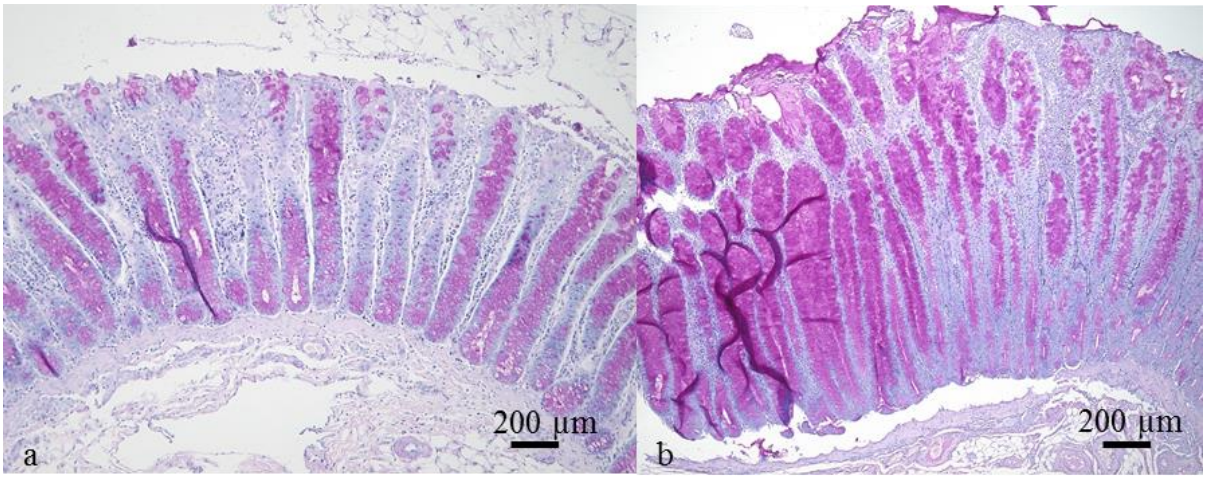

Figure 5: HE staining of a colonic tissue from a healthy pig (left panel) and from a pig experimentally infected with *B. hyodysenteriae* (right panel)

At the surface epithelium of the colon, necrosis is first restricted in shallow erosions, evolving into larger plaques of erosion which are covered with mucus and fibrin, sometimes forming thick diphteric membranes [68]. Capillaries lying beneath eroded epithelium can be dilated or ruptured, initiating streams of free blood into the colonic lumen [69]. The lamina propria of the mucosa appears congested and oedematous and infiltrates of mononuclear leukocytes and some neutrophils can be found in the lamina propria and submucosa.

B. hyodysenteriae can be visualised using a Warthin-Starry stain or Fluorescent In Situ Hybridisation (FISH) [16]. Although the Warthin-Starry stain is not specific for *B. hyodysenteriae*, the bacteria can be recognized due to their characteristic morphology (figure 6).

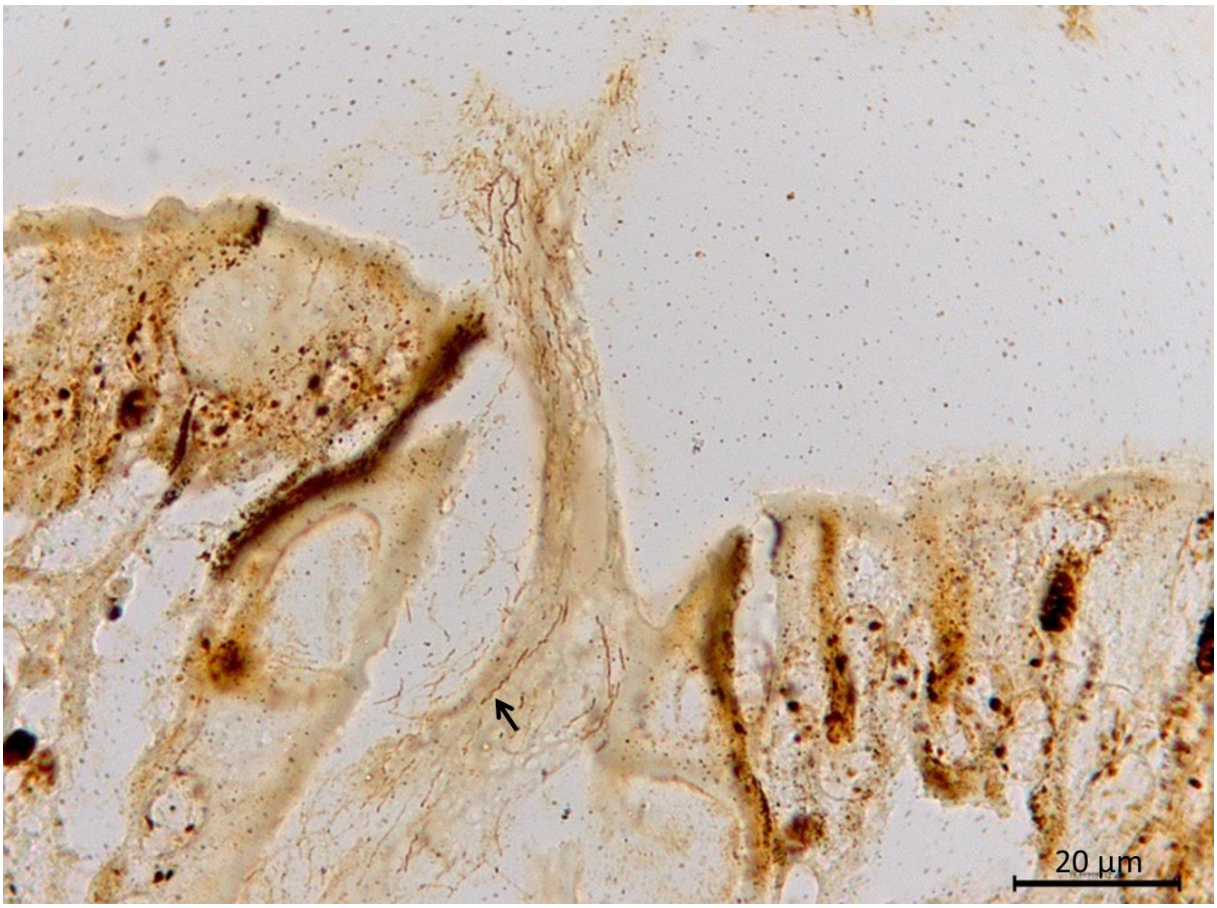

Figure 6: *B. hyodysenteriae* in colonic crypt of pig with SD, Warthin-Starry stain. Arrow: spirillic form of *B. hyodysenteriae*

4.6. Host response

Several changes in the immune system of the host are detected during a *B. hyodysenteriae* infection. Changes in the structural components of the mucus lining the colonic epithelium can be observed, as well as systemic and local changes in cellular and humoral immunity.

4.6.1 Colonic mucus layer

The mucus lining the colonic epithelium forms a barrier between potential pathogens in the gut lumen and the tissue of the host. The proteins in the mucus layers are composed of mucins. Mucins are large glycoproteins that can be divided in two categories: cell surface mucins that are anchored in the epithelial cells of the colon, and secreted, gel-forming mucins [213].

The mucins present in the porcine colon mucus layer are the cell-surface mucins mucin 1 (MUC1) and mucin 4 (MUC4), and the gel-forming mucin 2 (MUC2) that is secreted by Goblet-cells. It has been demonstrated that the colon of pigs infected with *B. hyodysenteriae* shows a decrease in MUC4-expression, and an increase in MUC2 and mucin 5ac (MUC5AC) expression [154,213]. MUC5AC is a gel-forming mucin that is absent in the colon mucus layer of healthy pigs but present in the small intestine and gastric mucus layer [95]. It should be noted that in the study describing MUC5AC in the small intestine antibodies directed against human and not porcine MUC5AC were used in the staining methods. An upregulation of MUC5AC is also seen in response to *Shigella dysenteriae* infections in a rabbit ileal-loop model and in human cell lines *in vitro* [155,156], and in mice as a response to infection with the nematode *Trichuris muris* [58]. The role of the MUC4 down-regulation or MUC2 and MUC5AC up-regulation during a *B. hyodysenteriae* infection is not known.

4.6.2 Immune response

During a *B. hyodysenteriae* infection in swine several changes can be detected in the peripheral blood. Coinciding with the onset of clinical signs of SD, elevated levels of circulating monocytes and T-cells are observed. Numbers of one specific subtype of T-cells are elevated in particular; $CD4^+CD8^+$ T-cells [86]. This subset of T-cells are regarded as memory/effector T-cells in swine, and during *B. hyodysenteriae* infection this subset is found also in large clusters in the lamina propria of the colon [65]. It has been demonstrated that these $CD4^+CD8^+$ T-cells can proliferate or produce IFNγ in reaction to antigen recall [205]. By producing IFNγ, $CD4^+CD8^+$ T-cells stimulate macrophage phagocytosis. The $CD4^+CD8^+$ T-cells also produce IL-10 which enhances growth, activation and differentiation of B-cells and thus stimulates local antibody production [86].

Local IgA and IgG antibodies can be detected in colonic washings and in faeces of experimentally infected pigs. It has been demonstrated that the IgG present in the

intestine is serum derived, while intestinal IgA is actively secreted locally [161,160]. Serum antibodies can be detected from 2-4 weeks after experimental inoculation with *B. hyodysenteriae,* which implies they generally appear after clinical symptoms have developed. Serum antibodies can be detected up to 8-10 weeks post infection with a peak at 4-7 weeks post infection [82,160]. In the serum IgG, IgM and IgA are present and the levels of antibodies present in the serum vary substantially between individual pigs. High levels of serum antibodies however, do not seem to be related to protection against subsequent infection. This indicates that humoral immunity, at least solely, is not enough to confer protection in the colon against development of SD [82,161].

4.7 Diagnosis

4.7.1 Clinical presentation and differential diagnosis

In cases of acute clinical outbreaks with severe bloody and/or mucoid diarrhoea, a *B. hyodysenteriae* infection might seem obvious. In endemically affected farms less typical disease signs may be present, such as non-bloody diarrhoea, weight loss, and poor growth. When these less typical clinical signs are present differential diagnosis should include *Lawsonia intracellularis, Brachyspira pilosicoli, Salmonella,* haemolytic *Escherichia coli*, and in some regions *Trichuris suis* infections [48,133,198].

4.7.2 Histopathology

On necropsy, a diffuse or patchy mucohaemorrhagic colitis can be indicative of a *B. hyodysenteriae* infection. Spirochetes can morphologically be suspected on Warthin-Starry staining as described above. *B. hyodysenteriae* can be identified more definitively *in situ* using specific probes for FISH [79,214].

4.7.3. Sampling

Colonic content, faecal samples or rectal swabs can be used for detection of *B. hyodysenteriae*. Samples for culture are preferably processed within 48 hrs after collection and should be held at 4°C until processing. Swabs in Amies medium were reported to produce good results without decrease in sensitivity for culturing up to 3 weeks after sampling when kept at 4°C [41]. Pooling of faecal samples was reported to have no negative influence on sensitivity of culturing [41]. It should be noted that this was only demonstrated in one study, and only for samples of clinically ill animals.

An optimal sampling protocol to detect carrier animals has not been indisputably described. The within-herd prevalence of *B. hyodysenteriae* in clinically healthy animals has been demonstrated to vary substantially ranging from 0-5% [32] in a multiplier herd in the USA to 25% in a multiplier herd in Switzerland [115]. Due to the lack of a gold standard method of detection of *B. hyodysenteriae* in carrier animals, and the low number of herd investigated for within-herd prevalence, the question of how many animals in a herd should be sampled to obtain a reliable result remains difficult to answer. Since the number of *B. hyodysenteriae* is higher in samples of colonic contents compared to faecal samples, colonic sampling at the abattoir could increase the probability of detection to some extent [52].

4.7.4 Culture conditions

For culturing of *B. hyodysenteriae* selective media are required, in which antimicrobial compounds inhibit the growth of bacteria that are part of the colonic or faecal flora. Several selective media have been described. Jenkinson and Wingar described a colistin-vancomycin-spectinomycin-blood-agar (CVSBA) which consists of tryptone soy agar supplemented with 5% sheep blood, 25 µg/ml colistin, 25 µg/ml vancomycin, and 400 µg/ml spectinomycin [75]. Kunkle and Kinyon described a selective medium named BJ consisting of tryptone soy agar, 5% pig faeces extract, 5% bovine blood, 25 µg/ml spiramycin, 12.5 µg/ml rifampin, 6.25 µg/ml vancomycin, 6.25 µg/ml colistin and 200 µg/ml spectinomycin [103]. Several variations of the described media have been used [22]. For example the addition of flavomycin to the CVSBA medium could be useful in regions where a high incidence of resistance is reported for the normal intestinal microbiota [118]. Cultures are incubated at 37°C–42°C for 2 to 10 days under anaerobic conditions. If *B. hyodysenteriae* is present a thin smear of growth surrounded by haemolysis can be observed. As soon as a haemolytic pattern is observed the thin smear can be subcultured for purification.

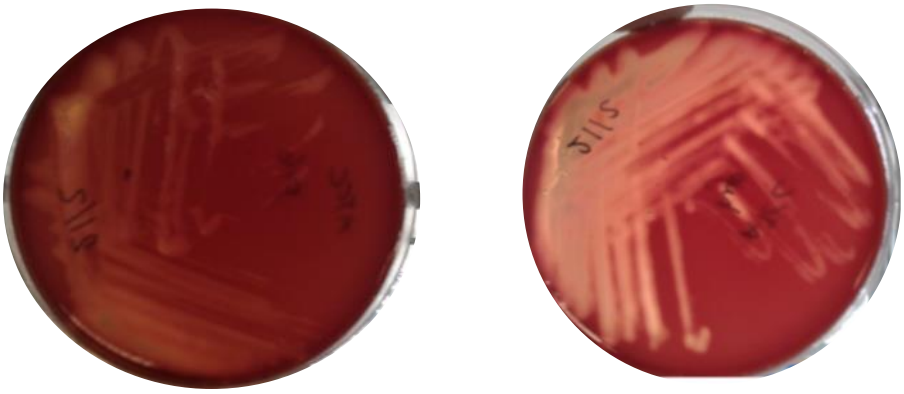

Figure 7: weak haemolysis (left panel) and strong haemolysis (right panel) on TSA agar supplemented with 5% sheep blood.

4.7.5 Phenotypic characterization

Pure cultures of *Brachyspira* can be further identified up to species level by phenotypic characterization. Phenotypic characterization is based on the strength of haemolysis as shown on blood containing agar plates, indole production and the presence or absence of several enzymes: hippurate hydrolysis, α-galactosidase, α-glucosidase and β-glucosidase. Based on these phenotypic characteristics, *Brachyspira* isolates have been allocated into four biochemical groups [39,64]. The four biochemical groups and characteristics are given in Table 2. The biochemical classification was linked to species delineation, based on differences in 16S rRNA sequences [40].

It must be noted that the discovery of the novel strongly haemolytic *Brachyspira* species *B. suanatina* and *B. hampsonii* has hampered the species identification by phenotypic characterization, since both species share most phenotypic characteristics with *B. hyodysenteriae.* Numerous strains of *B. murdochii* and *B. pilosicoli* with exceptional phenotypic characteristics have been reported, rendering the phenotypic species determination far less conclusive [43,199].

Table 2: Original phenotypic characterization scheme of *Brachyspira* sp.

Bio-chemi-cal group	β-hemo-lysis	Indole produc-tion	Hippur-ate hydro-lysis	α-galactosi-dase	α-glucosi-dase	β-glucosi-dase	Proposed species
I	strong	+/-*	-	-	+	+	*B. hyodys-enteriae*
II	weak	+	-	-	+	+	*B. intermedia*
IIIa	weak	-	-	-	-	+	*B. murdochii*
IIIb	weak	-	-	+	-	+	*B. innocens*
IIIc	weak	-	-	+	+	+	*B. innocens*
IV	weak	-	+	+	-	-	*B. pilosicoli*

* isolates positive for indole as well as isolates negative for indole have been described

4.7.6 Genotypic identification

Genetic based identification methods have been developed for the identification of *Brachyspira* species. For most pig related *Brachyspira* species there is substantial sequence homology of 16s rRNA, making this gene inappropriate for species differentiation. *B. pilosicoli* forms an exception since this species is more genetically distant from the other species. Two genes proved to be more genetically different between the *Brachyspira* species: 23S rRNA and the NADH oxidase gene (*nox).* Polymerase chain reaction (PCR) assays based on the detection of these two genes have been described [114,149]. For specific identification of *B. hyodysenteriae,* a PCR based on *tlyA,* a haemolysis associated gene, has been described as well [41,157].
Several duplex or multiplex (q)PCRs have been described for simultaneous detection of multiple species: La et al. described a duplex PCR detecting *B. pilosicoli* and *B. hyodysenteriae* [104]. Song et al. described a multiplex qPCR detecting *B. intermedia, B. pilosicoli,* and *B. hyodysenteriae* [179]. Willems et al. described a multiplex qPCR detecting *Lawsonia intracellularis, B. pilosicoli* and *B. hyodysenteriae* [216]. Several multiplex qPCR assays are also commercially available.
Rohde et al. described a Restriction Fragment Length Polymorphism (RFLP) assay based on the *nox* gene [164]. Using two restriction endonucleases, DpnII and BfmI, distinct restriction patterns can be obtained for *B. hyodysenteriae, B. pilosicoli, B. intermedia, B. murdochii, B. suanatina* and *B. innocens.* However, on a yearly basis

6-9% of isolates submitted for diagnostic purposes gave atypical restriction patterns and could not be identified to species level using this method [163].
Fluorescent *in situ* hybridization (FISH) has been described for detection of *B. hampsonii* and *B. hyodysenteriae* in formalin-fixed tissues. Probes for both species are based on the 23S rRNA gene. FISH using these probes can also be applied to detect both species in formalin fixed faeces, which shortens the time of detection in comparison with the application on tissues [16,21].

4.7.7 Matrix–assisted laser desorption/ionization time-of-flight mass spectrometry

The last few years matrix–assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has made a new contribution in the identification of bacterial and fungal pathogens in human and veterinary medicine [13,175]. This technique has been explored to identify *Brachyspira* isolates to species level, both of animal and human origin. Using reference strains and strains from collections at diagnostic facilities that had previously been identified to species level using a combination of phenotypical characterization and *nox* based genetic characterization (RFLP and sequencing), databases for MALDI-TOF MS identification of *Brachyspira* sp. have been described. In general, high level of agreement was shown between MALDI-TOF MS identification and *nox* based identification for *B. hyodysenteriae, B. pilosicoli, B. intermedia, B. innocens, B. murdochii*, *B. aalborgi,* and *B. alvinipulli* [23]. However, some discrepancy was recorded for *B. murdochii* isolates (as specified by *nox* sequencing) that were ambiguously identified as *B. murdochii/B. innocens* by MALDI TOF MS [153]. *B. suanatina* has not been included in any database so far and although *B. hampsonii* could be identified, there was no agreement between the designation to clade I or II between MALDI-TOF MS analysis and *nox* sequencing [204].

4.7.8 Strain typing methods

To help understand epidemiological connections and to determine routes of transmission, strain typing methods for *B. hyodysenteriae* have been developed. Multi locus sequence typing (MLST) for *B. hyodysenteriae* is based on seven house-keeping genes; alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glp*), acetyl-CoA acetyltransferase or thiolase (*thi*), and phosphoglucomutase (*pgm*) [159]. This MLST scheme has a high

discriminatory power and has provided evidence that two or three distinct strains, with different sequencetypes, can be present at one farm. The presence of different strains on one farm could influence the outcome of control measurements, since these strains may differ in their biological properties, such as their antimicrobial resistance [106]. A database containing MLST data from *B. hyodysenteriae, B. intermedia* and "*B. hampsonii"* has been installed: http://pubmlst.org/brachyspira/ [85].

Since MLST is relatively expensive and not routinely used in diagnostic veterinary laboratories, a more economical strain typing method has been pursued as well. Based on the presence of multiple loci in *B. hyodysenteriae* with variable number of tandem repeats (VNTRs), a multiple-locus variable-number tandem-repeat analysis (MLVA) scheme has been established. The MLVA scheme uses eight polymorphic loci and proved to show a high discriminatory power. In contrast to the strains typed by MLST, MLVA profiles seem to be stable within one farm. This could be due to the VNTRs being less prone to minor changes or by coincidence the strain collection used for MLVA analysis may not harbour different strains isolated from the same farm [60].

4.7.9 Serology

Identification of infected animals by detection of serum antibodies has been of interest, especially to identify carrier animals. Carrier animals are the most important source of infection for herds free of SD. Carrier animals are difficult to identify by demonstrating the presence of *B. hyodysenteriae*, either by culture of faecal samples or by PCR on DNA extracted from faecal samples, since both methods require a minimum amount of *B. hyodysenteriae* or its DNA to be present in the faeces. In herds without clinical SD there is not only a low prevalence of animals shedding *B. hyodysenteriae* but shedding of *B. hyodysenteriae* occurs intermittent and in low numbers [37,32]. Detection of circulating antibodies directed to *B. hyodysenteriae* would be a better strategy to determine the SD status of animals. It has been demonstrated that elevated antibody levels can maintain up to 150 days after experimental infection. This implies that collection of sera at slaughter could be used to determine the SD status of herds [178].

Enzyme-linked immunosorbent assays (ELISA) for detection of serum antibodies based on different coating substrates have been described. ELISA's based on LPS extracts from *B. hyodysenteriae* strains are of limited use since they only detect antibodies directed to the homologue serotype [84,127,177]. When hyperimmune sera

are tested, cross reactivity between serotypes is noted, but sera of orally infected animals do not show the same cross reactivity. However, LPS based ELISA's have been proved to be quite useful in regions where one specific serotype of *B. hyodysenteriae* is dominant [102]. To circumvent serotype specificity, ELISA's based on whole cell sonicates of *B. hyodysenteriae* strains have been described. Nevertheless, these ELISA's show a positive reaction with sera of animals that have been infected or colonised by other *Brachyspira* sp. such as the non-pathogenic *B. innocens* [218].

The use of surface expressed conserved proteins of *B. hyodysenteriae* as ELISA antigen could resolve the issues with low sensitivity for the LPS based ELISA's and with false positive reactions in whole cell sonicate based ELISA's. Up till now the surface protein Bhlp29.7 was postulated as a suitable antigen but was later shown to be cross-reactive with *B. innocens* [105]. Besides, the *Bhlp29.7* gene was proven to be present in only 58% of the strains in a German *B. hyodysenteriae* collection [8]. Recently Song et al. described a reverse vaccinology approach to identify several membrane associated proteins that could be suitable as ELISA antigen [181]. Eventually one of these predicted proteins proved to reach complete specificity, and sustain an acceptable sensitivity (91.7%). Still, in order to reach 100% specificity, a cut-off value of five standard deviations above the mean of SD negative herds was necessary. This in fact might indicate that the SD negative herds that were used to determine the cut-off, were not truly negative which is a concern for the accuracy of this ELISA [181].

4.8 Treatment and prevention

4.8.1 Antimicrobial therapy

Several antimicrobial products are registered for the treatment of SD. The most commonly used products are macrolides, lincosamides and pleuromutilins, which all interact with the protein synthesis of the bacteria. The most currently used antimicrobial products, their dosage and administration route are summarized in Table 3 [5].

Before 1980, tylosin, lincomycin and carbadox were most frequently used to treat or prevent SD. *B. hyodysenteriae* strains resistant to tylosin and lincomycin were first reported in the seventies [134] and the percentage of resistant strains has increased up to 100% for lincomycin and >90% for tylosin in most countries. The pleuromutilins

tiamulin and valnemulin (available since 1979 and 1999 respectively) became the drugs of choice to treat SD in the early 2000's due to increasing resistance against lincomycin and tylosin, and the ban of carbadox in general, and the ban of tylosin and virginiamycin as feed additives in 1999. During the last decade decreased susceptibility has been reported for tiamulin and valnemulin in several swine producing countries worldwide [61,117,130,151,171].

Acquired antimicrobial resistance against pleuromutilins, lincosamides and macrolides in *B. hyodysenteriae* is based on vertical transmission of certain mutations in the 50S ribosomal subunit or so called peptidyl transferase centre. Tylosin and lincomycin resistance is initiated by a single point mutation in the 23S rRNA gene [87]. This mechanism of resistance has not been described for tylvalosin, and fewer isolates with acquired resistance against tylvalosin have been reported [61]. For the pleuromutilins decreased susceptibility has been linked to several point mutations in domain V of the 23S rRNA gene, sometimes in combination with mutations in the L3 protein [62,152].

Table 3: antimicrobial products for treatment of SD

Antimicrobial class	Drug	Dosage	Duration	Administration
		10 mg/kg	1-3 days	intramuscular
		8 mg/kg	5-7 days	drinking water
		100 ppm	7-10 days	feed
	Valnemulin	3-4 mg/kg	1-4 weeks	feed
		10 mg/kg	3-5 days	intramuscular
		5-10 mg/kg	5-7 days	drinking water
	Tylvalosin	4.25 mg/kg	10-14 days	feed
		8 mg/kg	1-10 days	drinking water
		100 ppm until disappearance of clinical symptoms, followed by 40 ppm		feed

For determination of Minimal Inhibitory Concentrations (MIC), agar dilution and broth dilution methods have been described [89,166]. In general, the broth microdilution method generates MIC values 1-2 twofold dilutions lower compared to agar dilution methods [131]. With regard to breakpoints several different criteria have been reported [130,33,151,166,18]. The need for internationally harmonized MIC determination and Clinical and Laboratory Standards Institute (CLSI) approved clinical breakpoints has been expressed by numerous authors [61,88,132]. Given the limited number of antimicrobial compounds that can be used to treat SD and the high prevalence of (multi-)resistant isolates, prudent use of the remaining antimicrobial compounds is warranted.

4.8.2 Alternative treatments

Due to decreasing susceptibility of *B. hyodysenteriae* against antimicrobial products, the use of several alternative treatments has been reported. Vande Maele et al. demonstrated the *in vitro* antibacterial effects for *B. hyodysenteriae* of several essential oil components and organic acids [202]. Lowest MIC values were reported for cinnamaldehyde and lauric acid. Feed supplements containing extracts of citrus fruits

have also been reported to have an antibacterial activity *in vitro*. Bactericidal concentrations of the products ranged from 0.05% [116] to 20-40 ppm [4], depending on the product.

Probiotic bacteria have also been investigated for their antagonistic activities against *B. hyodysenteriae.* Porcine isolated strains of *Enterococcus faecium, Bifidobacterium thermophilum* and *Bacillus subtilis* were able to inhibit the growth of *B. hyodysenteriae in vitro* [97]. Lactobacilli *L. rhamnosus* and *L. farciminis* coaggregate with *B. hyodysenteriae in vitro,* trapping them in a physical network [11].

4.8.3 Elimination protocols

To eliminate SD from a herd several strategies can be applied, depending on the infrastructure of a farm. If the *B. hyodysenteriae* strain isolated from the farm is still susceptible to antimicrobial products, a medicated approach can be used in which specific units of a farm are emptied, cleaned and disinfected before medicated pigs are housed in those units [217]. However, careful selection of appropriate farms is crucial and financial benefits should be studied for each individual farm. If the *B. hyodysenteriae* strain isolated from the farm is resistant to antimicrobial products, complete depopulation/repopulation can be considered.

4.8.4 Prevention

Prevention should include management factors such as all-in/all-out management with adequate cleaning and disinfection. Since SD outbreaks can be associated with stressful conditions, management practices should be designed to minimize stressful circumstances. Introduction of SD through the purchase of carrier animals is a genuine risk. Therefore, replacement stock should be purchased from a herd with an ascertained history and should be kept in quarantine for at least three weeks [48]. During this time period faecal samples can be examined for the presence of *B. hyodysenteriae.* Although it has been demonstrated that the incubation period of *B. hyodysenteriae* can surpass this three week time-frame [48], a quarantine of three weeks is regarded as a good consensus. Next to implementation of adequate biosafety measures, the presence of rats and mice on a farm should be kept to an absolute minimum [6].

4.9 Vaccination

Animals that have recovered from SD seem to have established an immunological response, since these animals can be protected from re-infection [82]. Therefore, a number of different vaccine approaches have been explored with regard to swine dysentery.

4.9.1 Inactivated vaccines

Several reports describe the use of whole cell bacterins [31,42,45,54,146] or protein digests of whole cell bacterins [205-207]. Some of these bacterins, administered intramuscularly or intravenously, induced partial protection, demonstrated by less animals developing clinical SD, or animals developing less severe symptoms of SD [31,42,45]. In contrast with this, Olson et al. (1994) described animals developing a more severe form of SD, with an earlier onset, after vaccination with an inactivated *B. hyodysenteriae* vaccine [146]. For most of these studies serum antibody response was the only immune response that was monitored, and serum antibody response was never correlated with the level of protection a certain vaccine could induce [31,45]. Waters et al. describe the increase of $CD4^+CD8^+$ T-cells and their ability to proliferate and produce IFNγ upon antigen-recall after vaccination with a pepsin-digested bacterin. However none of these immune response parameters of this study are correlated with the level of protection [207].
A major downside of the use of inactivated whole cell bacterins is that they can only evoke protection against infection with a homologous serotype of *B. hyodysenteriae*. Autogenous vaccines are reported to have a beneficial effect on farm level (personal communication J. Osorio, 2016). However, no experimental data on autogenous vaccines for *B. hyodysenteriae* are available so far.

Vaccination with recombinant proteins has been reported to induce variable levels of protection, depending on the selected protein. The use of a recombinant *flaB1* flagellar protein could not reduce the number of pigs developing SD after challenge with a virulent *B. hyodysenteriae* strain [44]. A preparation of *BmpB,* an outer membrane lipoprotein, resulted in a 50% reduction in clinical SD [107]. Song et al. describe a reverse vaccinology approach to select proteins for use in a subunit vaccine [180]. They also report a reduction in number of animals developing clinical SD, albeit not significant. In the studies of La et al. [107] and Song et al. [180] serum antibody

response is monitored and the level of colonic IgA is determined once using colonic epithelial scrapings collected at necropsy. Neither serum antibody response nor colonic IgA would be correlated with the level of protection each vaccine induced.

DNA vaccines based on *ftnA,* encoding a putative ferritin protein, or *SmpB*, encoding a protein with unknown function, failed to protect mice against challenge with a virulent *B. hyodysenteriae* strain [30,63]. The use of DNA vaccines for SD has not been investigated in pigs.

4.9.2 Live attenuated vaccines

A *tlyA* mutant strain of *B. hyodysenteriae* has been examined for its use as a live attenuated vaccine. The mutant strain was intragastrically delivered to pigs and a 50% reduction in the number of animals developing clinical SD upon homologues challenge was demonstrated. However, there was no reduction in the number of animals that was colonised by the challenge strain [71]. In the same study a heterologous challenge was carried out as well. No reduction in the number of animals that was colonized and/or showed clinical signs could be demonstrated for this heterologous challenge. The presence of serum or colonic antibodies was not determined in this study.

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PART II

Scientific Aims

Swine dysentery caused by *Brachyspira hyodysenteriae*, is associated with substantial economic losses. Besides the economic impact of a clinical outbreak, the occurrence of *B. hyodysenteriae* in a herd affects pig trade, even in the absence of overt clinical signs. The definitive *Brachyspira* species identification, which is crucial in such cases, is hampered by the close genetic relatedness of pig associated *Brachyspira* sp., which can differ greatly in their pathogenic potential. Even within the species of *B. hyodysenteriae*, major differences have been reported with regard to pathogenicity between strains. The recent rise of new, pathogenic, initially non-typeable isolates and species of *Brachyspira* have further increased the need for better diagnostics of porcine *Brachyspira* species. From a clinical point of view, it would be even more helpful to identify the pathogenic potential of a clinical isolate, regardless of species identification.

The classical treatment of swine dysentery consists of antimicrobial agents. Since acquired antimicrobial resistance is increasing in *B. hyodysenteriae*, the sole use of antibiotics can result in therapeutic failure. Moreover, the use of antimicrobials is of growing public concern as it favors the development and spread of antimicrobial resistance in pathogenic and commensal bacteria. However, there are currently no efficient alternatives to antimicrobials available to treat or prevent swine dysentery. An efficient vaccine would be an important tool to manage this disease.

Therefore, the general scientific aims of this thesis were to identify virulence factors of *Brachyspira spp.* and their genetic background, which could be used to predict the pathogenic potential of strains or species using molecular techniques, and to evaluate the protective capacity of a *B. hyodysenteriae* strain with mutations for one of these virulence factors when used as a live attenuated vaccine.

The specific scientific aims were

1. To establish a strain collection of pig associated *Brachyspira* sp. and evaluate the robustness of routinely used diagnostic procedures to identify *Brachyspira* isolates to the species level.
2. To evaluate the minimum inhibitory concentration (MIC) patterns of this strain collection, associate MIC phenotypes with relevant gene mutations, and to estimate strain diversity for this population.

3. To evaluate *in vitro* virulence factor differences between strains, identify the underlying molecular differences, and correlate this to the *in vivo* pathogenic potential of these strains.
4. To determine the effect of immunization of pigs with an avirulent *B. hyodysenteriae* strain, on the spread of swine dysentery after challenge with a virulent *B. hyodysenteriae* strain.

Part III

Experimental Studies

1: First isolation of *Brachyspira hampsonii* from pigs in Europe

2: Presence and mechanisms of acquired antimicrobial resistance in Belgian *Brachyspira hyodysenteriae* isolates belonging to different clonal complexes

3: Variation in hemolytic activity of *Brachyspira hyodysenteriae* strains from pigs

4: An avirulent *Brachyspira hyodysenteriae* strain elicits intestinal IgA and slows down spread of swine dysentery

1

First isolation of *Brachyspira hampsonii* from pigs in Europe

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Abstract

Swine dysentery in Europe is classically attributed to *Brachyspira hyodysenteriae.* However, other *Brachyspira* species have been increasingly associated with intestinal disorders in pigs. This case report describes the first diagnosis of a *Brachyspira hampsonii* infection in European pigs. In a routine quarantine monitoring protocol, two gilts were presented for necropsy, in which soft watery non-haemorrhagic colonic content was found. Microbial culture from the colonic content and from faecal samples revealed the presence of strongly haemolytic, ring-phenomenon positive spirochetes indicative for *Brachyspira hyodysenteriae*. A diagnostic commercial PCR could not confirm the presence of *B. hyodysenteriae*. Phenotypic characterisation and PCRs targeting the 16S rRNA, 23S rRNA, *nox, hlyA* and *tlyA* genes of different swine-related *Brachyspira* spp*.* were performed. Phylogenetic analysis of sequences of the partial *nox* and 16S rRNA genes and multi locus sequence typing demonstrated that the isolates in this case were *B. hampsonii* isolates*.* This case report shows that the diagnosis of infections caused by new, emerging *Brachyspira* species is not self-evident and that the combination of microbial culture and PCR is recommended, completed with more extensive genotyping if necessary.

Key words: *Brachyspira*, swine dysentery, haemolysis, *Brachyspira hampsonii*

Case report: first isolation of *Brachyspira hampsonii* from pigs in Europe

Introduction

Infections with *Brachyspira* spp. in swine occur in most swine-rearing countries and can result in substantial economic losses. Of all swine-related *Brachyspira* spp. infections classical swine dysentery, caused by *Brachyspira hyodysenteriae,* results in the most severe clinical symptoms (eg. mucohaemorrhagic diarrhoea, weight loss, poor feed conversion). *B. hyodysenteriae* was first recognized as the cause of swine dysentery in 1971 [28]. At that time, the strong haemolysis of *B. hyodysenteriae* appeared indicative for pathogenicity since other, weakly haemolytic *Brachyspira* (formerly *Serpulina, Serpula* and *Treponema*) appeared to be commensal and were therefore named *Brachyspira innocens* [15]. Several reports of clinical disease caused by weakly haemolytic *Brachyspira* indicated that not all weakly haemolytic *Brachyspira* spp. were non-pathogenic for pigs [19,29]. Further research of these weakly haemolytic isolates including DNA-DNA hybridisation, resulted in the designation of three more weakly haemolytic species namely *B. intermedia, B. murdochii* and *B. pilosicoli* [27,30].

These weakly haemolytic species of *Brachyspira* diverge in the severity of clinical symptoms they cause. *B. pilosicoli* is pathogenic and causes spirochaetal colitis in pigs, which is marked by non-haemorrhagic diarrhoea and a poor feed conversion. For *B. intermedia* and *B. murdochii* the pathogenic potential is less clear-cut. Although both species have been isolated from clinical cases of diarrhoea, the clinical symptoms are mild or absent in experimental infections and yet high numbers of spirochetes are necessary to cause an effect [12,13].

Recently, a new type of *Brachyspira* infection has been described. Outbreaks of mucohaemorrhagic diarrhoea, caused by strongly haemolytic *Brachyspira* strains inconsistent with *B. hyodysenteriae,* were reported in the USA and Canada. Phylogenetic analysis of these strains showed such a large genetic divergence between those isolates and all other *Brachyspira* spp. that these isolates likely represent a novel species, for which the name *Brachyspira hampsonii* has been proposed [5]. The current case report describes, to the best of our knowledge, the first confirmed *B. hampsonii* infection in pigs outside North-America.

Materials and methods

Two gilts, imported from the Czech Republic, were presented for necropsy in a routine quarantine monitoring protocol. General macroscopic findings consisted of a low body weight and dilated large intestines in which soft watery non-haemorrhagic colonic content was present. Histological examination of these large intestines was not performed. Microbial culture of the colonic content was performed on Tryptic Soy Agar (BD, Heidelberg, Germany) supplemented with 5% sheep blood (IMP, Brussels, Belgium), 0.1% yeast extract (Oxoid, Aalst, Belgium) and following antimicrobials: spectinomycin (200 µg/ml), spiramycin (25 µg/ml), rifampin (12.5 µg/ml), colistin (6.25 µg/ml), and vancomycin (6.25 µg/ml) [9]. The microbial cultures revealed strongly haemolytic, ring phenomenon-positive spirochetes, indicative for *B. hyodysenteriae* [7,9]. Some of the pigs, housed in the same group as the two gilts presented for necropsy, showed mild semi-solid non-bloody and non-mucoid diarrhoea. From the next batch of gilts from the same origin, additional faecal samples were taken in the quarantine. Strongly haemolytic *Brachyspira* isolates, with ring phenomenon, were again found on microbial culture, whereas commercial diagnostic PCR analysis (Adiavet Brachy, Paris, France) did not confirm the presence of *B. hyodysenteriae* in these samples. All faecal samples were negative for *Salmonella.*

Phenotypic characterisation tests were performed on pure cultures which were obtained by at least three subcultures on Tryptic Soy Agar (TSA) plates supplemented with 5% defibrinated sheep blood and 1% yeast extract [11]. Phenotypic characterisation was performed on 4-day old cultures and was based on beta haemolysis, indole production, hippurate hydrolysis and the presence or absence of α-galactosidase, α-glucosidase and β-glucosidase [7]. Indole production was determined using a spot-indole test (Remel BactiDrop, Dartford, UK) and for the other biochemical characteristics commercial discs were used according to the manufacturer's instructions (Rosco Diatabs, Taastrup, Denmark). Type strains of *B. hyodysenteriae* (ATCC 27164)*, B. pilosicoli* (ATCC 51139) and *B. innocens* (ATCC 29796) were included to provide positive controls for all the phenotypic characteristics that were examined.

Several species-specific PCRs were performed, based on the following genes: *tlyA* [22], 23S rRNA [17] and *nox* [16] for *B. hyodysenteriae, nox* [21] and 23s rRNA [17] for *B. intermedia*, 16S rRNA for *B. pilosicoli* [16] and *nox* for *B. murdochii/B. innocens* [1].

Additionally, PCR's were performed for the haemolysis related genes *hlyA* and *hlyA*-ACP [2].

Forward primer 5' TAGCYTGCGGTATYGCWCTTT 3' and reverse primer 5' GCMTGWATAGCTTCRGCATGRT 3' were used to partially sequence the *nox* gene [32]. A product of 1014 base pairs was obtained. Forward primer 5' GTTTGATYCTGGCTCAGARCKAACG 3' and reverse primer 5' CTTCCGGTACGGMTGCCTTGTTACG 3' were used to partially sequence the 16S rRNA gene of which a 1044 base pair product was obtained [14]. Sequencing reactions were performed on purified PCR-product with the same primers as for PCR. *Nox* and 16S rRNA sequences from other *Brachyspira* isolates were retrieved from GenBank and compared with the sequences of the described field case isolate (D52) by BLAST analysis.

The sequences of the *nox* gene of the strain retrieved in this case report (D52), of *B. hyodysenteriae, B. intermedia, B. murdochii* and *B. innocens* ATCC type strains and of 42 additional strains of several *Brachyspira* spp. retrieved from GenBank were aligned using ClustalW. Sequences of clade I strain 30599 and clade II strain 30446 of *B. hampsonii* were also included [26]. Phylogenetic analysis was performed with an alignment sequence fragment of 540 bp and Kimura distance calculation and neighbour-joining method were used.

For multilocus sequence typing (MLST) primers and PCR conditions as described by Råsbäck and others [24] were used to analyse genes encoding alcohol dehydrogenase (*adh)*, esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and acetyl-coA acetyltransferase (*thi*). For each locus the sequence obtained from the D52 isolate was matched with the online MLSTdatabase (www.pubmlst.org/brachyspira).

Results

The phenotypic characteristics of isolate D52 corresponded to those of *B. hampsonii* as described by Chander and others [5]. The isolate was strongly beta haemolytic, indole negative, hippurate negative, negative for α-galactosidase and α-glucosidase, and positive for β-glucosidase. Although not exclusively, most isolates of clade I are positive for β-glucosidase as compared to clade II, in which most isolates are negative for β-glucosidase.

Table 1 shows the PCR results. Isolate D52 generated a positive result in the two species-specific PCRs for *B. intermedia* based on the 23S rRNA and *nox* gene respectively. Interestingly, the PCR targeting *tlyA*, presumed typical for *B. hyodysenteriae,* also generated a positive result. The PCRs for several haemolysis associated genes, *hlyA* and ACP(fabF-fabG), were positive as well.

The *nox* sequence of isolate D52 (GenBank accession nr KF202498) showed a similarity of 100% over 547 basepairs with *B. hampsonii* type strain NSH-16^T (ATCC BAA-2463 = NCTC 13792) [18], Besides, the *nox* sequence of isolate D52 showed a similarity of more than 99% over 874 basepairs with previously described isolates KC35 en EB106 (JX197410.1 and JX197409.1) [4]. These isolates, originally described as strongly haemolytic *B. intermedia* are recently referred to as *B. hampsonii* clade I (GenBank). With strain *B. hampsonii* 30599 (clade I, NZ_AOMM01000255.1) as described by Rubin and others [26], the *nox* sequence of our isolate showed a similarity of 99% over 1014 bp (difference of 2 nucleotides). The 16S rRNA sequence of isolate D52 (GenBank accession nr KF586484) showed a sequence similarity of 99% over 1044 bp with *B. hampsonii* type strain NSH-16^T.

Table 1*:* Primers used in PCRs and results for *B. hyodysenteriae* reference strain ATCC 27164, *B. intermedia* reference strain ATCC 51140 and the field case isolate D52.

Target Gene	**Species-specificity**	**Primer name**	**Primer Sequence (5'-3')**	**Result** D 52	**Result** ATCC 27164 *B.hyodys-enteriae*	**Result** ATCC 51140 *B. intermedia*
hlyA	Non- specific	hlyAFo	TCG ATG AAA TTA AAG ATG TTG TT	positive	positive	positive
		hlyARe	TTT TTC TTG ATC TTC TTG AGG A			
ACP(fabF -fabG)	Non-specific	ACPFo	AGG IGA AGT IAT AGC IGT TGA CG	positive	positive	positive
		ACPRe	GAA ACA CCA TTA AGI AIA TTA TCC CA			
23S	*B. hyodysenteriae*	Hyo23SFo	CGG TAA GTG ATG TAC TTG	negative	positive	negative
		Hyo23SRe	AGC CTC AAC CTT AAA GA			
nox	*B. hyodysenteriae*	HyonoxFo	ACT AAA GAT CCT GAT GTA TTT G	negative	positive	negative
		HyonoxRe	CTA ATA AAC GTC TGC TGC			
tlyA	*B. hyodysenteriae*	tlyAFo	GCA GAT CTA AAG CAC AGG AT	positive	positive	negative
		tlyARe	GCC TTT TGA AAC ATC ACC TC			
nox	*B. intermedia*	IntnoxFo	AGA GTT TGA AGA CAC TTA TGA C	positive	negative	positive
		IntnoxRe	ATA AAC ATC AGG ATC TTT GC			
23S	*B. intermedia*	Int23SFo	CCG TTG AAG GTT TAC CGT G	positive	negative	positive
		Int23SRe	CGC CTG ACA ATG TCC GG			
16S	*B. pilosicoli*	Pilo16SFo	AGA GGA AAG TTT TTT CGC TTC	negative	negative	negative
		Pilo16SRe	GCA CCT ATG TTA AAC GTC CTT G			
nox	*B. innocens/ B. murdochii*	InnmurdnoxFo	CCT GAA AGT TTA AAA GCT G	negative	negative	negative
		InnmurdnoxRe	CGA TGT ATT CTT CTT TTC C			

Phylogenetic analysis of the *nox* sequence of isolate D52 and *nox* sequences of other *Brachyspira* spp. clearly place isolate D52 in the cluster of isolates comprising clade I of *B. hampsonii* (figure 1)

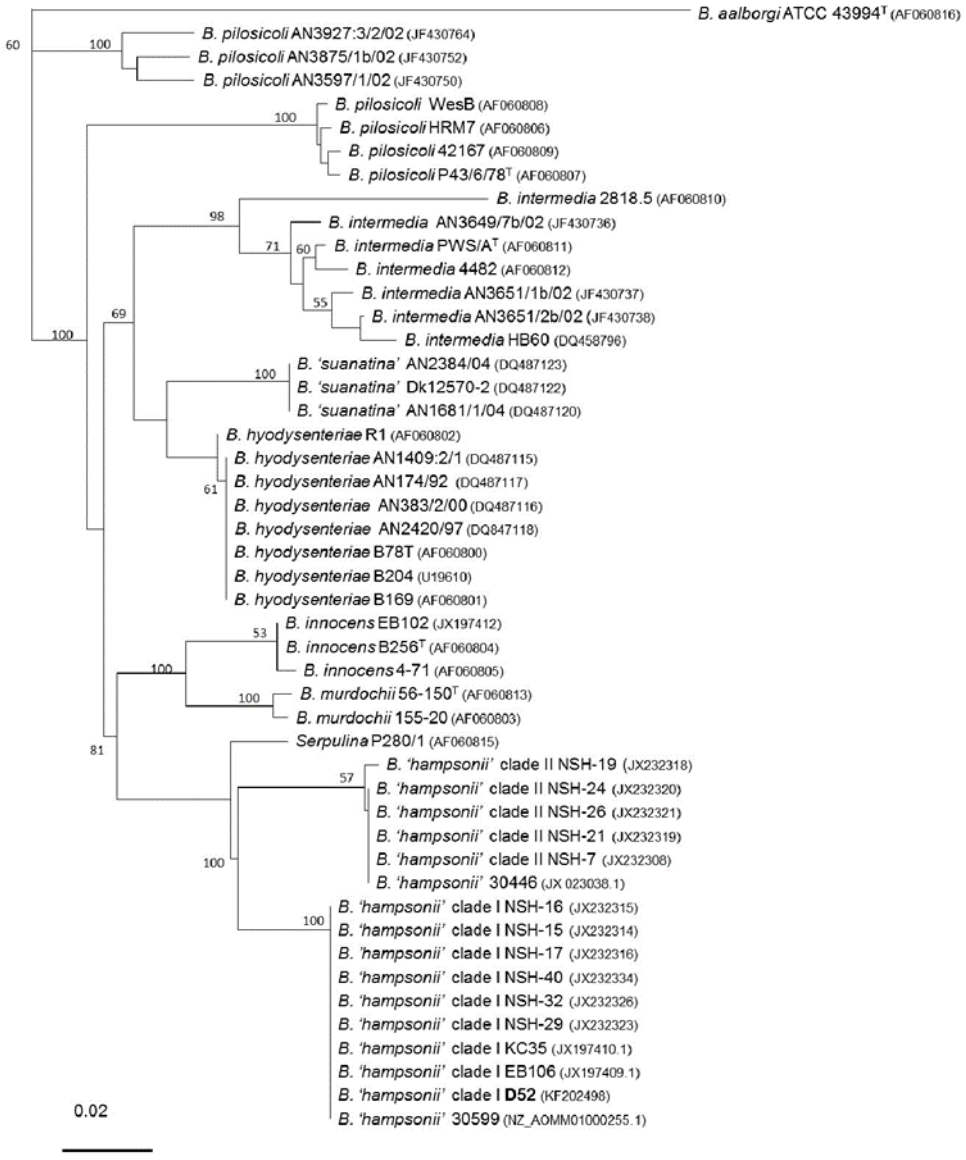

Figure 1*:* Phylogenetic tree based on the alignment (540bp) of the *nox* gene of *Brachyspira* spp. The alignment was created using CLUSTALW, distance calculation (Kimura) and neighbour joining using PHYLIP. Bootstrap values are indicated. Scale bar indicates 0,02 substitutions per site.

As described for *B. hampsonii* in previous studies three of the seven loci for MLST could not be amplified [5]. From the sequences of the 4 loci that could be amplified (*est, pgm, glp* and *thi*), none of them gave an exact match with known alleles in the MLST database. The *thi* sequence matched closest with allele 24 of "*Serpulina* sp. P280/1" (difference of 21 nucleotides) in accordance with the findings of Chander and others for *B. hampsonii* [5].

Discussion

The results of the phenotypic characteristics, sequence comparisons, MLST and phylogenetic analysis based on the *nox* sequence, identify the D52 isolate as *B. hampsonii* clade I. To the best of our knowledge it is the first time that *B. hampsonii* isolates from porcine origin are described in Europe, although the isolate *Serpulina* sp. P280/1 in retrospect also may belong to *B. hampsonii* [19].

The isolates of strain D52 obtained from the current field case, all contained the *hlyA*, *tlyA* and ACP (*fabF,fabG*) genes. HlyA is the protein responsible for the strong haemolysis in *B. hyodysenteriae* [10]. In order to adequately perform its actions, the *hlyA* gene has to be correctly placed between the accompanying *fab*-F and *fab*-G genes, coding for an ACP-reductase and –synthetase [34]. Although the presence of *hlyA* has been reported in some weakly haemolytic *Brachysira* spp. isolates, the *fabF* and *fabG* genes were in those cases absent, probably rendering the *hlyA* gene functionally inactive [2]. Another haemolysin, namely *tlyA* is consistently found in *B. hyodysenteriae*. Although it has also been twice reported in weakly haemolytic species [20,31], these sequences show low sequence similarity (82-83%) with *tlyA* of *B. hyodysenteriae* [2]. The presence of both these haemolysin encoding genes in the isolates in the current field case may be responsible for the strong haemolysis displayed by these isolates.

Rubin and others could experimentally induce mucohaemorrhagic diarrhoea in swine when infected with a *B. hampsonii* strain 30446 [25]. The clinical signs were indistinguishable from swine dysentery. It should, however, be noted that the strain 30446 clearly falls into the cluster II isolates of *B. hampsonii* whereas the strain from this case falls into cluster I as shown in the phylogenetic tree in figure 1. Although experimentally *B. hampsonii* strain 30599, which belongs to clade I, can induce severe clinical symptoms [6], the symptoms in this case report were rather mild. This could be

due to difference in pathogenic potential between strains of clade I or be related to *Brachyspira* colitis being a multifactorial disease. Environmental or nutritional factors may alter the severity of clinical signs. For example soy feed may deteriorate faecal consistency [8] and addition of distillers dried grains with solubles may lead to a faster development of swine dysentery [33].

This case report and the recent case reports from Canada and USA indicate that new, emerging species of *Brachyspira* can be important in swine-rearing countries [3,25]. The results of the species-specific PCRs show that diagnosis of infections caused by these emerging species can be confusing. When diagnosis is solely based on microbial culture, all strongly haemolytic isolates will be reported as *B. hyodysenteriae*, whereas they could belong to *B. hampsonii*, *B. suanatina* or even other emerging *Brachyspira* species [3,5,23,25]. On the other hand, when diagnosis is entirely based on PCR, strongly haemolytic isolates inconsistent with *B. hyodysenteriae* could easily be missed. For now, the combination of microbial culture and PCR, complemented with sequencing if necessary, is presumably the most complete method for diagnosis of *Brachyspira* spp. infections.

Declaration of conflicting interests

Sources of financial support have been acknowledged and the authors declare that they have no competing interests.

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2

Presence and mechanisms of acquired antimicrobial resistance in Belgian *Brachyspira hyodysenteriae* isolates belonging to different clonal complexes

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Abstract

Swine dysentery (SD) is an economically important disease for which antimicrobial treatment still occupies an important place to control outbreaks. However, acquired antimicrobial resistance is increasingly observed in *Brachyspira hyodysenteriae*. In this study, the Minimal Inhibitory Concentrations (MIC) of six antimicrobial compounds for 30 recent Belgian *B. hyodysenteriae* isolates were determined using a broth microdilution method. In addition, relevant regions of the 16S rRNA, 23S rRNA and the L3 protein encoding genes were sequenced to reveal mutations associated with acquired resistance. Finally, a phylogeny was reconstructed using minimal spanning tree analysis of multi locus sequence typing of the isolates.

For lincomycin, doxycycline, tylosin and tylvalosin, at least 70% of the isolates did not belong to the wild-type population and were considered to have acquired resistance. For valnemulin and tiamulin, this was over 50%. In all isolates with acquired resistance to doxycycline, the G1058C mutation was present in their 16S rRNA gene. All isolates showing acquired resistance to lincomycin and both macrolides displayed the A2058T mutation in their 23S rRNA gene. Other mutations in this gene and the N148S mutation in the L3 protein were present in both wild-type isolates and isolates considered to have acquired resistance. Multi locus sequence analysis revealed a previously undescribed clonal complex, with 4 novel sequence types in which the majority of isolates showed acquired resistance to all tested antimicrobial products.

In conclusion, acquired antimicrobial resistance is widespread among Belgian *B. hyodysenteriae* isolates. The emergence of multi-resistant clonal complexes can pose a threat to swine industry.

Keywords: Swine dysentery, *Brachyspira hyodysenteriae*, Minimal Inhibitory Concentration, antimicrobial resistance, Multi Locus Sequence Typing

Presence and mechanisms of acquired antimicrobial resistance in Belgian *Brachyspira hyodysenteriae* isolates belonging to different clonal complexes

Introduction

Swine dysentery (SD) is an economically important disease in swine producing countries worldwide. The causative agent, *Brachyspira hyodysenteriae* (*B. hyodysenteriae*), was first described in the early 1970s [2, 33]. Before 1980, tylosin, lincomycin and carbadox were most frequently used to treat or prevent SD. *B. hyodysenteriae* strains resistant to tylosin and lincomycin were reported in the 1970s [22] and the percentage of resistant strain has gradually increased since then in most countries. In Europe, the pleuromutilins tiamulin and valnemulin (available since 1979 and 1999, respectively) became the drugs of choice to treat SD in the early 2000's due to increasing resistance against lincomycin and tylosin, the ban of carbadox in general, and the ban of tylosin and virginiamycin as feed additives in 1999. During the last decade, acquired resistance has been reported for tiamulin and valnemulin as well in several swine producing countries worldwide [4, 17, 20, 27, 31].

Acquired antimicrobial resistance against pleuromutilins, lincosamides and macrolides in *B. hyodysenteriae* is caused by mutations in the 50S ribosomal subunit or so called peptidyl transferase centre [4, 5, 10, 28]. Tylosin and lincomycin resistance are initiated by a single point mutation in the 23S rRNA gene at a position corresponding to position 2058 in *Escherichia coli* [10]. Since tylvalosin is generated by refermentation of tylosin, cross-resistance between those two antimicrobial agents is expected [9]. However, *B. hyodysenteriae* isolates showing high MICs for tylosin but belonging to the wild-type population for tylvalosin, have been described [4, 27]. For the pleuromutilins, acquired resistance has been linked to several point mutations in the V domain of the 23S rRNA gene, sometimes in combination with mutations in the L3 protein [4, 5, 28]. Acquired resistance for doxycycline is associated with a 16S rRNA gene mutation at a position corresponding to position 1058 in *Escherichia coli* [26]. This specific mutation has been associated with tetracycline resistance in other bacteria as well [24].

Acquired resistance in *B. hyodysenteriae* strains has been reported for strains isolated in Belgium in 1995-1996 and 2003 [6, 37]. The aim of this study was to determine the antimicrobial susceptibility pattern of more recent Belgian *B. hyodysenteriae* isolates. The possible gene mutations responsible for reduced susceptibility were investigated

and Multilocus Sequence Typing (MLST) was performed to determine any possible sequence type or clonal complex related MIC patterns.

Materials and methods

Brachyspira hyodysenteriae isolates and growth conditions

A collection of *Brachyspira hyodysenteriae* isolates (n=30) was assembled at our facilities during 2010-2015. Fresh faecal samples were collected by swine veterinarians on farms with SD-like clinical signs, apart from strains D28 and 10cI which were isolated from pigs with mild diarrhoea as previously described [19]. Participating swine veterinarians collected two or three pooled faecal samples on each farm which were cultured within 24 hours after sampling on selective plates consisting of Trypticase Soy Agar (TSA) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 % sheep blood (E&O Laboratories, Bonnybridge, UK), 1% yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), 25 µg/ml vancomycin, 400 µg/ml spectinomycin and 25 µg/ml colistin (all antimicrobial compounds from Sigma-Aldrich,St. Louis, MO, USA). Plates were anaerobically incubated at 38°C for 3-10 days. Isolates were purified by three to five subcultures on Trypticase Soy Agar (TSA) plates supplemented with 5% sheep blood and 1% yeast extract [8] and stored at -70°C in 300 µl of a medium consisting of 75ml horse serum (Thermo Fisher Scientific, Carlsbad CA, USA) and 25ml Brain Heart Infusion (BHI) broth (Bio-Rad, Hercules CA, USA) supplemented with 10% (w/v) glucose (Merck, Darmstadt, Germany) until further use.

All isolates were phenotypically characterized by determination of beta haemolysis, indole production, hippurate hydrolysis and the presence of α-galactosidase, α-glucosidase and β-glucosidase as described previously [1]. Three *B. hyodysenteriae*-specific PCRs were performed, based on the: *tlyA* [29], 23S rRNA [16] and *nox* [13] genes.

All isolates originated from different, non-related farms, except isolates M1 and M2, which originated from the same farm. M1 was isolated from faecal samples of finisher pigs, M2 was isolated from faecal samples of growing pigs.

Antimicrobial susceptibility testing

Six antimicrobial compounds were used in following concentration ranges: lincomycin (0.063-128 µg/ml), doxycycline (0.016-16 µg/ml), valnemulin (0.002-32 µg/ml), tiamulin

(0.002-16 µg/ml), tylosin (0.25-1024 µg/ml), and tylvalosin (0.016-128 µg/ml). Stock solutions of each compound were prepared according to the Clinical and Laboratory Standards Institute [7]. For determination of the Minimal Inhibitory Concentration (MIC) the broth dilution method as described by Karlsson et al. [11], was used with some modifications [35]. Serial dilutions of the antimicrobial products in BHI broth supplemented with 10% foetal bovine serum (FBS) were freshly prepared and 200 µl of each dilution was placed in wells of a 48-well cell culture plate (Greiner Bio-One, Frickenhausen, Germany). Plates filled with antimicrobial serial dilutions were transferred to an anaerobic cabinet (84%N2, 8% H2, 8% CO2). To each well 200 µl of *B. hyodysenteriae* inoculum was added. The inoculum for each strain was prepared by harvesting a 4-day old TSA plate into BHI broth supplemented with 10% FBS. Using a spectrophotometer, inoculum density was adjusted to contain 1 x 10^6 to 5 x 10^6 Brachyspira/ml. The inoculated 48-well plates were incubated anaerobically at 37°C on a rotary shaker for 3 days. The MIC was determined as the lowest antimicrobial concentration at which visible growth was not observed.

For each strain the MIC of all antimicrobial products was determined in three independent experiments, and when a difference of one dilution was found, the value that was obtained twice was retained. In each experiment, the MIC for type strain *B. hyodysenteriae* B78 (ATCC 27164) was determined as well. The MICs for this strain were compared with the quality-control range as proposed by Pringle et al. and MIC values were recorded only if the MICs of strain B78 were within those quality-control ranges [25]. For analysis of all MIC values, epidemiological cut-off values were used as proposed by Pringle et al.: doxycyline > 0.5 µg/ml, lincomycin > 1 µg/ml, tylosin > 16 µg/ml, tylvalosin > 1 µg/ml, valnemulin > 0.125 µg/ml, tiamulin > 0.25 µg/ml [27]. Isolates were classified as multi-resistant if acquired resistance was demonstrated for at least one antimicrobial compound of all following classes: macrolides, lincosamides, tetracyclines, pleuromutilins. MIC_{50} and MIC_{90} were determined as the lowest concentration that inhibited the growth of 50% and 90% of the isolates.

Point mutations possibly involved in resistance mechanisms

In order to determine the presence of a G1058C mutation in the 16S rRNA gene, associated with acquired resistance for doxycycline, a 644 bp fragment was sequenced for all isolates. Primers were previously described by Verlinden et al. [35]. Additionally a 910 bp fragment of the 23S rRNA gene was sequenced using following

primers: Fo 5'-GAGAGGTTAGCGTAAGCGAAGC-3' and Re 5'-GCTTCCCACTTAGATGCTTTCAG-3' as described by Hillen et al. (2014) to detect mutations that could be associated with acquired resistance for lincomycin, pleuromutilins or macrolide antimicrobial agents [5]. A portion of the L3 ribosomal protein encoding gene was sequenced as well, using the primers Fo 5'-GGGTATGACAACAGTTTTCG-3' and Re 5'-GCTCCAGGTATAGAACCYTT-3' as described by Pringle et al., to detect mutations associated with acquired resistance for pleuromutilins [28].

Multilocus sequence typing

Multilocus sequence typing was based on the scheme developed earlier by Råsbäck et al. and performed with some modifications [30, 36]. Briefly, the following genes were sequenced: alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and thiolase (*thi*). All sequences were matched with the online MLST database (http://pubmlst.org/brachyspira/). A minimal spanning tree was constructed using Bionumerics Software 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Clonal clusters were identified as sequence types sharing six or more loci using Bionumerics [31].

Results

Antimicrobial susceptibility

The MICs of all tested antimicrobial agents are given in table 1. Using the epidemiological cut-off values proposed by Pringle et al., 56.7% of the tested isolates were considered to have acquired resistance for valnemulin and 53.3% for tiamulin [27]. For lincomycin 86.7% of isolates was classified as displaying acquired resistance, and for tylosin 80% of the tested isolates were considered to have acquired resistance. For tylvalosin this was 70% and for doxycycline 83.3%.

For tylosin, a bimodal distribution of MICs was observed, in which the majority of isolates could either only be inhibited at the highest tested concentration, or could not be inhibited at all. Doxycycline exhibited a bimodal distribution as well, with the majority of isolates showing acquired resistance at a MIC of 1µg/ml, and a group of isolates without acquired resistance showing MIC values reaching from 0.032 µg/ml to

0.25 µg/ml. For the other tested antimicrobial compounds no clearly distinct populations were observed.

The MIC_{50}, MIC_{90} and the MIC range for each tested antimicrobial agent are given in table 2. For all the tested antimicrobial agents the MIC_{50} is above the wild type cut-off values as proposed by Pringle et al. [27].

Table 1. Minimal inhibitory concentration distribution for six antimicrobial compounds on 30 *B. hyodysenteriae* isolates. Isolates with acquired resistance according to epidemiological cut-offs are given in bold. Grey shading: concentration test range, light grey: below epidemiological cut-off, dark grey: above epidemiological cut-off.

Anti-microbial agent	number of isolates with a given MIC (µg/ml)																			
	≤0.002	0.002	0.004	0.008	0.016	0.032	0.064	0.128	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1024
Tiamulin			2	1	5	1	2	3		**6**	**5**	**1**	**3**	**1**							
Valnemulin	5		1		4	1		2	**3**	**4**	**3**	**4**	**2**	**1**							
Tylosin												3		3			**2**	**3**	**3**	**3**	**13**
Tylvalosin								2		2	5	**5**	**10**	**2**	**2**	**2**					
Lincomycin									1		3	**1**	**4**	**5**	**7**	**8**	**1**				
Doxycycline						1	1	2	1		**21**	**2**	**2**								

Table 2: MIC_{50}, MIC_{90}, and MIC range of *B. hyodysenteriae* isolates of this study.

Antimicrobial compound	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC range (µg/ml)
Tiamulin	0.5	4	0.004-8
Valnemulin	0.25	2	≤0.002-8
Tylosin	>128	>128	2-≥1024
Tylvalosin	4	16	≤0.128-32
Lincomycin	16	32	0.25-64
Doxycycline	1	2	0.032-4

Molecular mechanisms of resistance

For doxycycline, all isolates with MICs higher than the cut-off value displayed the G1058C mutation in their 16S rRNA gene. Accordingly, four of five isolates with MIC values below the cut-off, displayed the wild-type sequence. The isolate that had a MIC of 0.25 µg/ml, surprisingly also harboured the G1058C mutation.

The results of both MIC tests and molecular typing of the associated resistance mechanism for lincomycin, tylosin and tylvalosin are given in table 3. All isolates considered to have acquired resistance for lincomycin and both macrolides (n=20) displayed the A2058T mutation in their 23S rRNA gene. Seven isolates were considered to have acquired resistance against either lincomycin or tylosin, against tylosin and tylvalosin, or against lincomycin alone. Six of those isolates harboured the A2058T or A2058G mutation in their 23S rRNA gene. Isolate M7 harboured the wild-type 23S rRNA gene sequence and had the N148S mutation in its L3 protein. Three isolates were fully susceptible for tylosin, tylvalosin and lincomycin. Two of those isolates showed the wild type sequence for the 23S rRNA gene and the L3 protein coding gene, while one isolate (10cI) possessed the A2058G mutation in its 23S rRNA gene.

Table 3: MIC values for lincomycin and macrolides, 23S rRNA and L3 mutations

	MIC (µg/ml)			23S rRNA mutations					
isolates	lincomycin	tylosin	tylvalosin	G2032	A2058	T2528	G2535	Other positions	Sequence L3
Isolates with acquired resistance for tylosin, tylvalosin and lincomycin									
M3	32	1024	8		T	C			
M5	32	>1024	2		T	C			
M6	32	>1024	2		T	C			
M8	32	1024	4		T	C			
M12	16	64	4		T	C			
M13	4	1024	4		T		A		
M14	16	1024	4		T	C			
D1	8	128	4		T			G2201T	
D2	16	512	4		T		A		
D3	32	1024	8		T		A		
D4	32	128	4		T			G2116A G2165T	
D5	8	256	2		T		A		
3bIII	16	512	4		T	C			
4cI	8	1024	4		T	C			
6bI	32	256	2		T				
8dII	64	>1024	16	A	T			C2146T	
14bII	16	1024	4		T	C			N148S
22cI	16	>1024	32		T		C	G2201C	
25cI	4	256	2		T				
62	32	>1024	32		T			G2116A	N148S
Isolates with acquired resistance for lincomycin and tylosin									
5aI	8	64	1		G				N148S
15bI	16	512	1		T				
M11	8	128	0,5		T				
Isolates with acquired resistance for tylosin and tylvalosin									
M9	1	>1024	16		T				N148S
Isolates with acquired resistance for lincomycin only									
M1	4	8	1		T		A		
M2	4	8	1		T		A		
M7	2	8	1						N148S
Isolates susceptible for lincomycin, tylosin and tylvalosin									
10cI	1	2	0,5	A	G				
49	1	2	<0,125						
D28	0,25	2	<0,125				A	T2402C	

For the pleuromutilins, the MIC values and 23S rRNA gene and L3 protein coding gene mutations are given in table 4. Eighteen isolates were considered to have acquired resistance for both pleuromutilins. In four of these isolates (M9, M7, 14, and 62) there was an Asparagine – Serine amino-acid change at position 148 of the L3 protein (according to *B. pilosicoli* numbering). In another four isolates either no additional mutations were detected in the 23S rRNA gene (15bI, 49) or mutations occurred (D1, D4) at positions quite distant from the peptidyl transferase centre. Seven resistant isolates harboured a T2528C mutation in their 23S rRNA gene. A G2535A/C mutation could be detected in susceptible as well as resistant isolates. These nucleotides at position 2528 and 2535 form an adjacent base-pair as depicted in figure 1.

Twelve isolates showed MICs below the epidemiological cut-off value of 0.125 µg/ml for valnemulin and 0.25 µg/ml for tiamulin [27]. Isolate 5aI showed the Asparagine – Serine amino-acid change at position 148 of the L3 protein. One isolate harboured the T2528C mutation (M14). Three isolates showed no additional mutations, two harboured the G2032A mutation, and two isolates showed mutations in positions quite distant from the peptidyl transferase centre (larger central loop in figure 1).

Table 4: MIC values for pleuromutilins, 23S rRNA and L3 mutations

	MIC (µg/ml)		23S rRNA mutations					
isolate	tiamulin	valnemulin	G2032	A2058	T2528	G2535	other positions	Sequence L3
Isolates with acquired resistance for tiamulin and valnemulin								
22cI	8	8		T		C	G2201C	
M5	4	4		T	C			
M6	4	4		T	C			
M8	1	2		T	C			
M3	1	1		T	C			
D3	1	0.5		T		A		
15bI	4	0.5		T				
M7	1	2						N148S
49	2	2						
M9	0.5	2		T				N148S
3bIII	0.5	1		T	C			
4cI	0.5	1		T	C			
M12	0.5	0.5		T	C			
D5	0.5	0.25		T		A		
Isolates with acquired resistance for valnemulin only								
14	0.125	0.5		T				N148S
D1	0.125	0.25		T			G2201T	
Isolates with acquired resistance for tiamulin only								
D4	1	<0.002		T			G2116A G2165T	
62	0.5	0.125		T			G2116A	N148S
Isolates susceptible for valnemulin and tiamulin								
M11	0.125	0.125		T				
M13	0.064	0.25		T		A		
8dII	0.063	0.016	A	T			C2146T	
10cI	0.031	0.032	A	G				
M1	0.016	0.016		T		A		
M2	0.016	0.016		T		A		
M14	0.016	0.004		T	C			
D2	0.016	0.002		T		A		
6bI	0.016	<0.002		T				
5aI	0.008	<0.002		G				N148S
D28	0.004	0.016				A	T2402C	
25cI	0.004	<0.002		T				

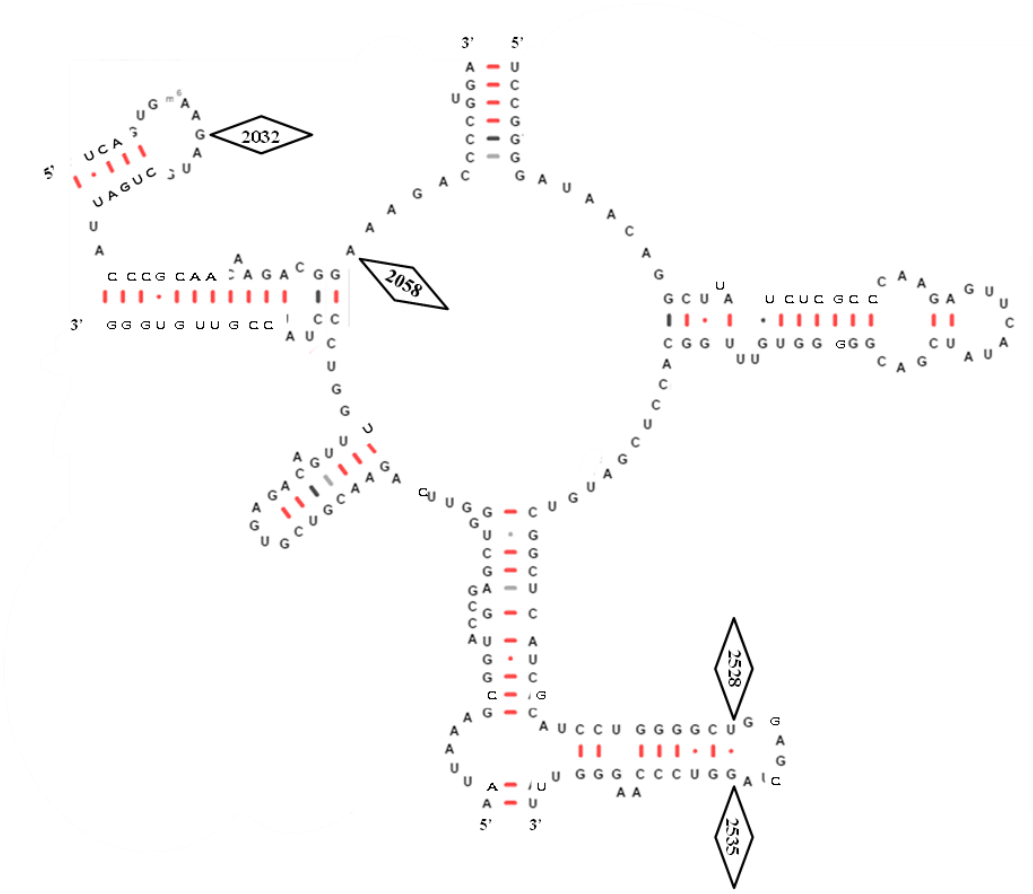

Figure 1:. *B. hyodysenteriae* 23S rRNA secondary structure, including peptidyl transferase loop (central larger loop). Numbering according to *E. coli.* Structure modified from *E. coli* structures from the comparative RNA website: http://www.rna.ccbb.utexas.edu/. The rhombi indicate positions where mutations are demonstrated in multiple isolates.

Multilocus sequence typing

All seven genes could be sequenced for the isolates of the current collection. Ten isolates showed previously deposited sequence types (ST). Seven of these isolates shared ST8. Sequence types ST 60, ST 112, ST 132 were each detected once. Those sequence types have all been described in European countries. Sequence type 8 has been reported to be a dominant isolate in Spain [23] and it has also been found in the UK, Italy, Serbia and Germany [14, 31, 32]. Sequence types 132 and 112 have been found in Germany [15]. Sequence type 60 has been reported from a mallard in Sweden [14].

All other profiles detected here represented new sequence types but had four to six loci in common with already existing profiles in the pubMLST database. All isolates

have been deposited in the pubMLST database and sequence type numbers have been assigned (supplementary table S1, some were described in Mahu et al. (2016) [19]).

A minimum spanning tree based on MLST typing showing isolates and their pleuromutilin susceptibility pattern is given in figure 2. The same tree, colour coded for the total number of antimicrobial compounds an isolate shows acquired resistance for, is given in figure 3.

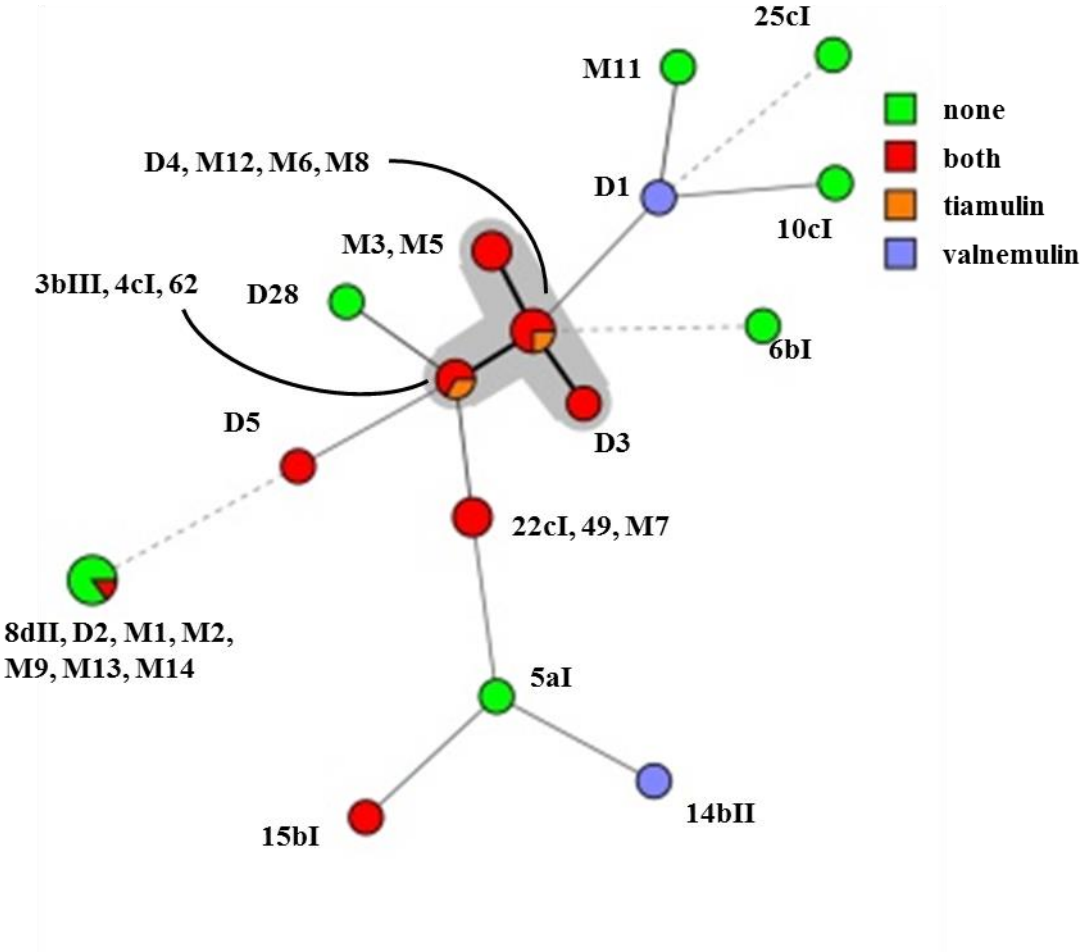

Figure 2: Minimal spanning tree analysis based on MLST typing showing strains and their susceptibility pattern for pleuromutilins. Green isolates are susceptible for both of the tested pleuromutilins, red isolates were considered to have acquired resistance for both pleuromutilins, orange for tiamulin, and purple for valnemulin. Grey shading defines a clonal cluster in which isolates have at least six loci in common with another isolate in the complex. Single locus variants are connected by dark bold lines, double locus variants by thin lines, and variants with three or more different loci with dotted lines.

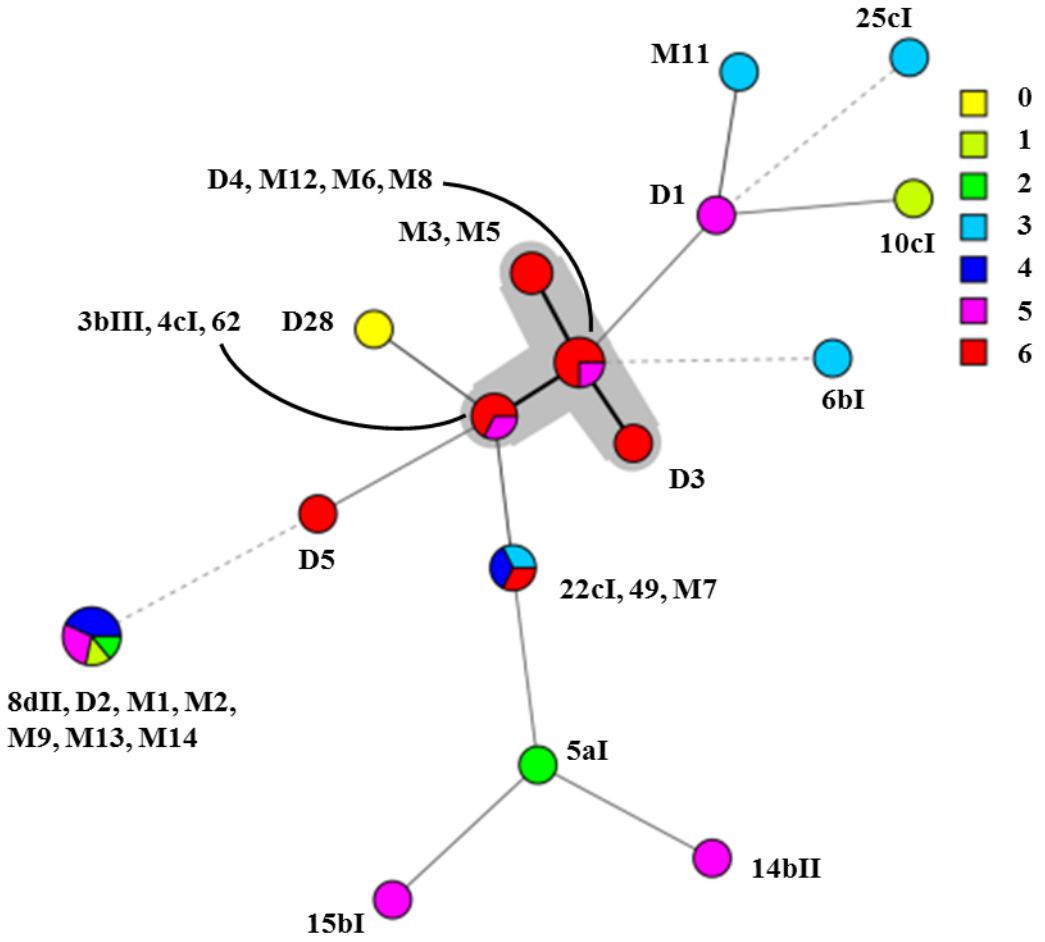

Figure 3: Minimal spanning tree analysis based on MLST typing. Colour codes for the number of antimicrobial substances an isolate shows acquired resistance for. Grey shading defines a clonal cluster in which isolates have at least six loci in common with another isolate in the complex. Single locus variants are connected by dark bold lines, double locus variants by thin lines, and variants with three or more different loci with dotted lines.

Ten isolates formed a clonal complex, depicted in grey shading in figure 2 and 3. Eight of these isolates were considered to have acquired resistance for both pleuromutilins. Two isolates were considered to have acquired resistance for tiamulin only, but the MIC value for valnemulin of isolate 62 was 0.125 µg/ml, thereby reaching the epidemiological cut-off value. All isolates in this cluster also showed acquired resistance for doxycyline, lincomycin and the macrolides, designating the isolates in this cluster multi-resistant (figure 3).

Discussion

The MIC_{50} and MIC_{90} values recorded for lincomycin, tylosin and tylvalosin of this set of Belgian field isolates are comparable to those recently recorded in most other European countries (supplementary table S1). Earlier MIC studies regarding Belgian isolates have been conducted on field isolates from 1995-1996 [6] and field isolates from 2003 [37]. Those earlier Belgian studies used agar dilution to determine MIC concentrations instead of broth microdilution methods. Due to the different methods used for MIC determination, results from earlier Belgian studies and our results were not statistically compared. However, taking into consideration that on average MIC values are one dilution lower in broth microdilution procedures compared to agar dilution procedures [21], a trend towards increased pleuromutilin MICs can be observed, as MIC_{50} and MIC_{90} for the pleuromutilins have noticeably increased since the earlier studies (supplementary table S2). Tylosin MIC_{50} and MIC_{90} were already high in the nineties, and remain on that level. Lincomycin MIC_{50} and MIC_{90} seem slightly lower for the isolates of this study, but are still above the epidemiological cut-off value, proposed by Pringle et al. [27].

Interpretation of MICs against *B. hyodysenteriae* is hampered by the lack of Clinical and Laboratory Standards Institutes (CLSI) approved clinical breakpoints. Here, the microbiological or epidemiological criterion was used and the epidemiological cut-off values as proposed by Pringle et al. were applied [27]. This criterion allows distinguishing wild-type populations of bacteria from those with acquired resistance [34]. For tylosin and, to a lesser extend for doxycycline, a clear bimodal distribution of MICs was observed, indicating acquired resistance in isolates in the higher range of MIC values. For all these isolates, MICs of tylosin or doxycycline were higher than the epidemiological cut-off values proposed by Pringle et al. [27], providing further evidence that they do not belong to the wild-type population. No clear bimodal but rather an extended frequency distribution range was seen against the other antibiotics tested here, making interpretation of the results more difficult.

For doxycycline the G1058C 16S rRNA gene mutation is a well described causal mutation for acquired resistance. The isolates investigated here corroborate this, except one isolate (M2) harbouring the mutation while it was considered to belong to the wild-type population for doxycycline. Isolate M2 originated from a farm where M1 was isolated as well. These two isolates share the same sequence type, and have

identical 23S rRNA gene and 16S rRNA gene sequences. Presumably these isolates represent one strain. A possible explanation for the discrepancy between the MIC value of M2 for doxycycline and the G1058C mutation in its 16S rRNA gene, could be that this isolate developed acquired resistance on the farm and that the isolate was a mixed culture of susceptible and resistant *B. hyodysenteriae*.

For the macrolides and lincomycin, acquired resistance has been described to be caused by a single mutation at the 2058 position of the 23S rRNA gene. In accordance, all isolates in this study with increased MIC values for both macrolides and lincomycin harboured the A2058T mutation. Three isolates harboured the A2058T/G mutation, showed acquired resistance for lincomycin and tylosin, but had MIC values one or two dilutions below the cut-off value for tylvalosin. Such isolates have previously also been described in Spain [4]. This might indicate that there is no complete cross-resistance between tylosin and tylvalosin, which are both 16-membered ring macrolides. Alternatively, the epidemiological cut-off value for tylvalosin used here might have been set too high.

For the pleuromutilins, the genetic mechanism of acquired resistance is less clear. Pringle et al. identified that laboratory derived mutants with an increased MIC of tiamulin, possessed mutations in their ribosomal L3 protein coding gene, or in the V domain of their 23S rRNA gene [28]. The nucleotide mutation in the L3 protein coding gene, causing a Asparagine–Serine amino acid substitution at position 148 (N148S) has been linked with pleuromutilin resistance by several authors [4, 5, 28]. In our isolates this amino acid substitution was found in five isolates. Four of those had MICs above the epidemiological cut-off values, but one isolate (5aI) was considered to belong to the wild-type population for both pleuromutilins.

For the 23S rRNA gene, the mutation G2535A was first described to have no influence on the susceptibility for pleuromutilins due to its position, quite distant from the peptidyl transferase centre on the one hand (fig 2), and its presence in both resistant and susceptible isolates on the other hand [4]. In contrast with this, Hillen et al. described a significant correlation between the presence of the G2535A mutation and lower MICs for pleuromutilins [5]. In particular in isolates which were expected to have a high MIC due to the presence of an Asparagine – Serine amino-acid change at position 148 of the L3 protein, the G2535A mutation would subsequently lower the MIC value. In the isolate collection of our study, the G2535A mutation is present in susceptible and

resistant isolates, and never simultaneously with a N148S mutation in the L3 protein. Therefore the results for the isolates in our collection neither confirmed nor contradicted these findings.

In our isolate collection, the adjacent nucleotide of 2535, T2528 (figure 1) was often mutated into a C, mainly in isolates considered to have acquired resistance for lincomycin, macrolides and pleuromutilins. This mutation has not been linked to pleuromutilin resistance in *B. hyodysenteriae* before. Since it has been stipulated that a mutation in G2535 influences susceptibility for pleuromutilins, it seems likely that a mutation in the adjacent nucleotide (figure 2) may influence pleuromutilins susceptibility as well. It might be of interest to conduct chemical footprinting experiments for mutations in these two adjacent nucleotides to investigate the influence of these nucleotides on the binding of pleuromutilins to the peptidyl transferase centre [28].

The G2032A mutation was found in the majority of Spanish field isolates with high tiamulin MICs and was also observed in laboratory derived resistant strains [4, 28]. In our subset of strains however, this G2032A mutation only occurred in pleuromutilin susceptible strains (8dII, 10cI). In our isolates, the C2146T mutation was found in an isolate susceptible for pleuromutilins. This is in accordance with the findings of Hidalgo et al. who found this mutation in susceptible and resistant isolates and considered this mutation to be located too far from the peptidyl transferase centre to influence susceptibility for pleuromutilins [4]. Mutations in the 23S rRNA gene at positions 2055, 2447, 2504 and 2572 were demonstrated in laboratory selected isolates with high pleuromutilin MICs and it has been demonstrated by antibiotic footprinting experiments on mutant ribosomes that these mutated ribosomes have a reduced binding affinity for pleuromutilins [18, 27]. None of these mutations were present in our isolates, and have not yet been demonstrated in any field isolates to the best of our knowledge.

The sequence types that were found in the current Belgian isolates mainly consist of sequence types that have not been previously described. Sequence type 8 however, is found in a variety of European countries, and was present among our isolates 7 times as well. Minimal spanning tree analysis of the multilocus sequence typing of the isolates of this study revealed the presence of a clonal complex in which the vast majority of the isolates showed acquired resistance for at least one of the pleuromutilins. Since the isolates in this cluster also showed acquired resistance for

doxycyline, lincomycin and the macrolides, this cluster harboured multi-resistant isolates. The existence of multi-resistant clonal complexes can pose a threat to the swine industry.

Conclusions

A high frequency of acquired resistance was demonstrated in Belgian field isolates of *B. hyodysenteriae*. For doxycyline, lincomycin and tylosin there was a quite clear link between acquired resistance and mutations in the 16S rRNA and 23S rRNA genes. For the pleuromutilins, the presence or absence of certain mutations in the 23S RNA gene and L3 protein coding gene were less clearly linked with acquired resistance. The current Belgian isolates mainly belonged to new sequence types of which four of them formed a newly described clonal complex. Ten of these isolates showed acquired resistance to the majority of the tested antimicrobial compounds, including the pleuromutilins.

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Supplementary data

Table S1: isolates used in this study, with their sequence types and alleles for the seven housekeeping genes of the MLST

strain	ST	adh	alp	est	gdh	glpK	pgm	thi
D1	171	2	11	8	5	10	2	6
D2	8	2	2	3	12	11	1	3
D3	210	2	11	28	1	11	2	21
D4	211	2	11	28	1	10	2	21
D5	112	2	11	3	10	10	11	3
M1	8	2	2	3	12	11	1	3
M2	8	2	2	3	12	11	1	3
M3	212	2	11	28	12	10	2	21
M5	212	2	11	28	12	10	2	21
M6	211	2	11	28	1	10	2	21
M7	173	2	13	3	6	10	2	21
M8	211	2	11	28	1	10	2	21
M9	8	2	2	3	12	11	1	3
M11	215	2	11	7	5	10	2	3
M12	211	2	11	28	1	10	2	21
M13	8	2	2	3	12	11	1	3
M14	8	2	2	3	12	11	1	3
3bIII	167	2	11	3	1	10	2	21
4cI	167	2	11	3	1	10	2	21
5aI	60	2	7	3	6	12	2	3
6bI	216	2	8	17	1	22	2	3
8dII	8	2	2	3	12	11	1	3
10cI	168	2	11	8	4	9	2	3
14bII	217	2	7	3	10	9	2	6
15bI	132	2	6	3	4	12	2	7
22cI	173	2	13	3	6	10	2	21
25cI	170	2	18	8	5	6	1	11
49	173	2	13	3	6	10	2	21
62	167	2	11	3	1	10	2	21
D28	172	2	11	3	20	6	2	21

Table S2: MIC_{50}, MIC_{90}, and MIC range (all in µg/ml) of *B. hyodysenteriae* isolates from the last decade (upper half of the table), and of isolates previously isolated in Belgium (lower half of the table).

Country **Period of isolation** **Reference**	**MIC_{50} (µg/ml)** **MIC_{90} (µg/ml)** **MIC range (µg/ml)** **for following antimicrobial agents**					
	Lincomycin	Tylosin	Tylvalosin	Tiamulin	Valnemulin	Doxycycline
Germany **2009-2012** **[3]**	16 32 1->128	>128 >128 >128	NA*	4 >16 0.125->128	2 >4 ≤0.004->128	NA NA
Switzerland **(2009-2015)** **[12]**	16 32 ≤0.5-32	128 >128 ≤2->128	4 8 ≤0.125-8	≤0.063 0.125 ≤0.063-0.125	≤0.031 ≤0.031 ≤0.031-0.063	0.25 1 ≤0.125-2
Spain **2008-2009** **[4]**	16 >64 1->64	>128 >28 16->128	4 16 0.5->32	1 8 ≤0.063->8	1 4 ≤0.031->4	NA
USA **2009-2014** **[20]**	16 32 ≤0.5->64	4 >128 ≤2->128	NA	≤0.063 0.25 ≤0.063->8	≤0.031 ≤0.031 ≤0.031-2	0.25 1 ≤0.125-4
Belgium **2010-2015** **This study**	16 32 0.25-64	>128 >128 2->128	4 16 ≤0.125-32	0.5 4 ≤0.004-8	0.25 2 ≤0.002-8	1 2 0.031-4
Belgium **1995-1996** **[6]**	64 >256 ≤1->256	128 128 4->256	NA	0.063 0.5 ≤0.031-2	NA	NA
Belgium **2003** **[37]**	64 128 ≤2->128	>128 >128 4->128	NA	0.125 0.5 ≤0.031-2	≤0.031 0.125 ≤0.031-0.25	NA

*NA: not available

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3

Variation in haemolytic activity of *Brachyspira hyodysenteriae* strains from pigs

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Abstract

Brachyspira hyodysenteriae is the primary cause of swine dysentery, which is responsible for major economic losses to the pig industry worldwide. The haemolytic activity of 10 *B. hyodysenteriae* strains isolated from stools of pigs with mild to mucohaemorrhagic diarrhoea was compared and seven haemolysis associated genes were sequenced. Haemolysis induced by these strains varied from strong to near absent. One weakly haemolytic *B. hyodysenteriae* strain showed sequence changes in five haemolysis associated genes (*tlyA, tlyB, haemolysin III, haemolysin activation protein* and *haemolysin III channel protein*) resulting in amino acid substitutions. The occurrence of weakly haemolytic strains identifiable as *B. hyodysenteriae* should be taken into account in swine dysentery diagnostics. The presence of these strains may affect herd dysentery status, with great impact on a farms trading opportunities.

Keywords: *Brachyspira hyodysenteriae,* swine dysentery, haemolysis, haemolysis associated genes

Variation in haemolytic activity of *Brachyspira hyodysenteriae* strains from pigs

Introduction

Swine dysentery (SD) is caused by the anaerobic spirochete *Brachyspira hyodysenteriae* and is an important intestinal disease in swine rearing countries. Clinical signs typically consist of mucohaemorrhagic diarrhoea. The economic losses on farm level can be substantial due to mortality, diminished growth rates, deterioration of feed conversion and costs of medical treatment [3]. The occurrence of *B. hyodysenteriae* in a herd may affect the trading of pigs with economic consequences, even in the absence of overt clinical signs. Diagnostics of herds for the presence of *B. hyodysenteriae* is usually done by examining pooled faecal samples for the presence of this micro-organism by microbial culture and/or PCR tests [36].

Besides *B. hyodysenteriae,* other *Brachyspira* species of varying virulence have been described in pigs. There has been an interest in putative virulence factors to help explain the differential pathogenic potential of these different *Brachyspira* spp. Possible virulence factors include motility, chemotactic capacities, lipopolysaccharide, haemolysin(s) and enzymes such as NADH oxidase [3, 41].

The pronounced haemolysis of *B. hyodysenteriae* that is displayed by growth on blood agar plates has been considered a hallmark of its pathogenicity [20, 21]. Apart from *B. pilosicoli,* weakly haemolytic porcine *Brachyspira* spp. such as *B. murdochii* and *B. intermedia* are indeed regarded mildly or non-pathogenic. Although they have been isolated from clinical cases of diarrhoea [18], their pathogenic potential is less clear-cut since experimental infections require large numbers of spirochetes and clinical symptoms are either mild or absent [30]. On the other hand, virulence of the recently described strongly haemolytic *B. suanatina* and *B. hampsonii* is considered to be similar to that of *B. hyodysenteriae* [8, 10, 29, 38, 40, 46].

Several reports describe the purification of haemolysin produced by *B. hyodysenteriae* [20, 21, 23]. Using purified haemolysin in an ileal-colonic loop model, microscopic lesions similar to those seen in natural cases of swine dysentery have been reproduced [25]. Four haemolysis associated genes have been defined: *tlyA, tlyB*, *tlyC* and *hlyA* [15, 28, 43]. The protein encoded by *tlyA,* haemolysin A, shows homology with pore forming haemolysins of several bacteria such as *Mycobacterium tuberculosis* [34, 47], and *Treponema denticola* [9]. These homologues and Haemolysin A also encompass

a conserved domain which is predicted to function as a rRNA methyltransferase [9]. *TlyA* negative *B. hyodysenteriae* mutants are less haemolytic and induce less severe lesions in mice and pigs compared to their wildtype [16, 42]. The *TlyB* gene encodes a Clp protease, and *tlyC* encodes haemolysin C. Both recombinant proteins were proven to show haemolytic and cytotoxic activity *in vitro* [43]. Bellgard et al. [6] describe that, in order to display a haemolytic phenotype, *B. hyodysenteriae* could need an acyl carrier protein (ACP) for acylation of toxins. Such an ACP is encoded for by *hlyA. The fabF* and *fabG* genes encode an ACP-reductase and synthase that presumably play a role in the lipidation of the HlyA protein [48]. Even though some weakly haemolytic *Brachyspira* spp. strains also contain the *hlyA* gene, it is probably not functional due to incorrect localization between the *fab* genes [5].

In addition to previously described haemolysis related genes *tlyA, tlyB, tlyC* and *hlyA*-ACP Bellgard et al. [6] found three possibly important additional genes when the whole genome sequence of reference strain WA1 was described: *haemolysin III, haemolysin activation protein* and *haemolysin III channel protein* genes. Haemolysin III (BHWA1_RS02195) [6], encompasses a conserved domain *yqfA*, a predicted channel-forming protein of the haemolysin III family. Homologues of haemolysin III are found in several bacteria such as *Bacillus cereus* [35]. The haemolysin III related channel protein (BHWA1_RS09085) [6], has a conserved domain composing an integral membrane protein. The haemolysin activation protein (BHWA1_ RS02885) [6], shares conserved domains with haemolysin C.

We previously mentioned the existence of *B. hyodysenteriae* strains with an aberrant haemolytic phenotype [27]. In 1982, Lysons et al. [26] isolated three strains of *B. hyodysenteriae* that were reported to appear slightly less haemolytic on blood-containing agar plate than virulent strains of *B. hyodysenteriae*, though considerably more haemolytic than avirulent *B. innocens*. Disease signs could not be induced using two of these strains in an *in vivo* experiment, even when animals were colonized by the strain. Recently, Hampson et al., described the existence of weakly haemolytic *B. hyodysenteriae* strains in Australia as well [13]. The current study aims to quantify the haemolytic capacity of a selection of *B. hyodysenteriae* strains and to identify the underlying molecular differences.

Materials and methods

Brachyspira isolate collection and selection

A collection of isolates of different *Brachyspira* species was composed during a six month period (Oct 2011-March 2012) at our facilities. *B. hyodysenteriae* isolates were collected by the participation of swine veterinarians who were asked to share fresh faecal samples, if *B. hyodysenteriae* infection was suspected on a farm based on clinical symptoms. Furthermore two diagnostic laboratories (Animal Healthcare Flanders, Drongen, Belgium and Mediclab, Aalst, Belgium) donated isolates of *B. hyodysenteriae* and other porcine associated *Brachyspira* species that they had collected during 2010-2011.

Participating swine veterinarians collected two or three pooled faecal samples (3 pigs per pooled sample) on each farm which were cultured within 24 hours after sampling on selective plates consisting of Trypticase Soy Agar (TSA) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 % sheep blood (E&O Laboratories, Bonnybridge, UK), 1% yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), 25 µg/ml vancomycin, 400 µg/ml spectinomycin and 25 µg/ml colistin (all antimicrobial compounds from Sigma-Aldrich). Plates were anaerobically incubated at 38°C. Isolates were purified by three to five subcultures on Trypticase Soy Agar (TSA) plates supplemented with 5% sheep blood and 1% yeast extract [17] and eventually stored at -70°C in 300µl of a medium consisting of 75ml horse serum (Thermo Fisher Scientific, Carlsbad CA, USA) and 25ml Brain Heart Infusion broth (Bio-Rad, Hercules CA, USA) supplemented with 10% (w/v) glucose (Merck, Darmstadt, Germany) until further use. Isolates donated by diagnostic laboratories were delivered on agar plates. All isolates were subcultured once after which they were also stored at -70°C. All donated isolates were accompanied by a brief description of clinical symptoms on the farm of origin.

On all collected isolates phenotypic characterization and species-specific PCRs were performed. Phenotypic characterization was performed on pure 4-day old cultures and was based on beta-haemolysis, indole production, hippurate hydrolysis and the presence or absence of α-galactosidase and β-glucosidase [12, 14]. Indole production was determined using a spot-indole test (Remel BactiDrop, Dartford, UK). For the other biochemical characteristics, commercial discs were used according to the manufacturer’s instructions (Rosco Diatabs, Taastrup, Denmark). Type strains of

B. hyodysenteriae (ATCC 27164)*, B. pilosicoli* (ATCC 51139) and *B. innocens* (ATCC 29796) were included to provide positive controls for all the phenotypic characteristics that were examined.

Three *B. hyodysenteriae*-specific PCRs were performed, based on the following genes: *tlyA* [37], 23S rRNA [24] and *nox* [22]. Species-specific PCR for the other species were based on *nox* [33] and 23S rRNA [24] for *B. intermedia*, 16S rRNA [22] for *B. pilosicoli* and *nox [4]* for *B. murdochii/B. innocens.*

Out of the complete collection of *B. hyodysenteriae* isolates, 8 were selected at random to be evaluated in an *in vitro* assay for haemolytic capacity, MLST profiling, and sequence analysis of 16S rRNA, the NADH oxidase gene, and haemolysis associated genes. Two more isolates were specifically selected for the same assays, since they showed an aberrant phenotype when grown on blood containing agar plates: M2 showed only moderate haemolysis and isolate D28 showed weak haemolysis. Strain B204 (ATCC 31212) was included as a positive control. These 10 selected isolates (and the positive control), the age group of the sampled pigs, and the clinical symptoms on the farm of origin are given in table 1. All isolates originated from different, non-related farms, except isolates M1 and M2, which originated from the same farm. M1 was isolated from faecal samples of finisher pigs, M2 was isolated from faecal samples of growing pigs. Both age groups suffered from mucohaemorrhagic diarrhoea.

Sequencing of 16S rRNA, NADH oxidase and Multi Locus Sequence Typing genes

The NADH oxidase (*nox*) gene and 16S rRNA gene were partially sequenced as previously described [19, 45]. The sequences retrieved from the isolates used in this study were compared by BLAST analysis to known sequences of *B. hyodysenteriae* type- and reference strains [1].

Multilocus sequence typing (MLST) of the *B. hyodysenteriae* isolates was based on the MLST scheme as previously published [39] and performed with modifications [44]. For all strains, sequences for genes encoding alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and thiolase (*thi*) were determined and matched with the online MLST database [2]. The concatenated sequences of the described isolates, a previously typed Belgian *B. hyodysenteriae* strain Be45 [31], *B. hyodysenteriae* reference strains B204 (ATCC 31212) and WA1 (ATCC 49526) and

four *B. intermedia* strains were aligned using ClustalW. The *B. intermedia* strains included the type strain PWS/A^T (ATCC 51140), and three strains isolated from pigs previously described by Råsbäck et al. [39]. A dendrogram was constructed using Kimura distance calculation and unweighed pair group method with averages (UPGMA).

Table 1: Clinical signs on the farm of origin, phenotypic characteristics, MLST profile and sequence type, 16S rRNA and *nox* sequence lengths and accession numbers.

Strain ID*	Age group pigs	Clinical signs on the farm of origin[a]	Haemolysis on agar plate[b]	Enzymatic profile[c]	MLST profile (sequence type)[d]	*Nox* sequence accession number and sequence length (bp)	16S rRNA sequence accession number and sequence length (bp)
3bIII	growing	MH diarrhoea	++	1001	2-11-3-1-10-2-21 (ST167)	KM052166990	KM1120831286
4cI	finishing	MH diarrhoea	++	1001	2-11-3-1-10-2-21 (ST167)	KM052167975	KM1120821286
8dII	sows	MH diarrhoea	++	1001	2-2-3-12-11-1-3 (ST8)	KM0521681000	KM1120811319
10cI	finishing	mild diarrhoea	++	1001	2-11-8-4-9-2-3 (ST168)	KM052169971	KM1120801299t
21bI	growing	MH diarrhoea	++	0001	2-13-3-6-10-2-3 (ST169)	KM052170977	KM1120791299
25cI	finishing	MH diarrhoea	++	1001	2-18-8-5-6-1-11 (ST170)	KM0521711005	KM1120781350
M1*	finishing	MH diarrhoea	++	1001	2-2-3-12-11-1-3 (ST8)	KM052172933	KM1120771175
M2*	growing	MH diarrhoea	+	1001	2-2-3-12-11-1-3 (ST8)	KM052173983	KM1120761175
D1	finishing	MH diarrhoea	++	1001	2-11-8-5-10-2-6 (ST171)	KM052174986	KM1120751300
D28	weaners	mild diarrhoea	+/-	0001	2-11-3-20-6-2-21 (ST172)	KM0521751005	KM1120741182
B204 (ATCC 31212)	positive control strain	MH diarrhoea	++	1001	1-16-3-4-2-3-6 (ST54)	U19610.11705	U14932.11433

*strains M1 and M2 were isolated on one farm, all other isolates originated from different, non-related farms

a: Clinical signs on the farm from which each strain was isolated, MH = mucohaemorrhagic

b : ++ = strong, + = moderate, +/- = weak

c : indole production, hippurate hydrolysis, α-galactosidase, β-glucosidase (1 present, 0 absent)

d : allele numbers for adh-alp-est-gdh-glpK-pgm-thi. Between brackets: sequence type as assigned by Pub MLST database

In vitro haemolysis assay

The haemolysis assay was based on the assays described by Fedorka-Cray et al. [11] and ter Huurne et al. [43] with some modifications. Fresh blood was collected from 8 week old pigs and a volume of blood was immediately mixed with an equal volume of Alsever's solution (Sigma-Aldrich) (50/50 v/v). This blood-Alsever's mixture was washed three times with Dextrose-Glucose-Veronal (DGV) (Lonza, Walkersville MD, USA) buffered solution by centrifugation for 10 minutes at 500 g. The haematocrit of the suspension was determined using a micro-haematocrit centrifuge and reader. DGV buffered solution was added until a 10%-suspension of red blood cells was obtained.

Fresh cultures of the different *B. hyodysenteriae* isolates were prepared by harvesting a 4-day old culture plate with a sterile cotton swab and stirring the cotton swab in an anaerobic Brain Heart Infusion (BHI) broth (Bio-Rad), supplemented with 10% of Foetal Bovine Serum (FBS) (Thermo Fisher Scientific). These cultures were incubated for 24 hours under anaerobic conditions at 37°C on a rocking platform and for each strain three cultures were made. After incubation, cultures were microscopically examined for purity and the Optical Density at 620 nm (OD_{620}) was measured. Cultures were only retained if their OD_{620} measured between 0.30 and 0.35. Supernatant was collected by centrifugation at 500 g for 20 minutes and was sterilized by filtration (Millipore, 0.2 µm). The strongly haemolytic strain B204 (ATCC 31212) served as a reference strain in the *in vitro* haemolysis assay.

The haemolysis assay was performed in 96-well U-bottom microtiter plates. After pipetting 100 µl of the 10% red blood cell suspension in each well, 100 µl of the filtered *B. hyodysenteriae* culture supernatant was added. Triton-X 2% served as a positive control (complete haemolysis) and DGV served as a negative control (no haemolysis). Plates were incubated for two hours at 37°C in a 5% CO_2 atmosphere after which the 96-well plate was centrifuged for 10 minutes at 500 g. The supernatant of the incubated fluid was transferred to a 96-well IWAKI-plate and the absorption at 450 nm was determined using an ELISA-reader. All assays were performed in triplicate and repeated three times.

Sequencing of haemolysis associated genes

Complete sequences of the *hlyA, tlyA, tlyB*, *tlyC, haemolysin III (BHWA1_RS02195), haemolysin activation protein (BHWA1_RS02885),* and *haemolysin III channel protein (BHWA1_RS09085)* genes were determined for all *B. hyodysenteriae* strains. For *hlyA*, the ACP1-Fo and ACP1-Re primers were used as described by Barth et al [5]. For *tlyA,* the primers were designed based on the sequences of *tly* (GenBank: X61684.1) (*tlyA* was originally named *tly* as it was presumed to be the only haemolysin of *Brachyspira*) as deposited by Muir et al. [28] and the whole genome sequence of *B. hyodysenteriae* WA1 (GenBank: NC_012225.1) [24]. The *tlyB* and *tlyC* primers were based on the sequences (GenBank: X73140.1) (*tlyB*), (GenBank: X73141.1) (*tlyC*) [28] and their alignment with the whole genome sequence of WA1 respectively. In order to obtain a full length sequence for *tlyB* several amplicons were sequenced using the four described primer pairs. The generated sequences were concatenated to obtain the full length sequence. Primers for *haemolysin III, haemolysin activation protein* and *haemolysin III channel protein* genes were designed based on the whole genome sequence of *B. hyodysenteriae* strain WA1 (GenBank: NC_012225.1) [26] Primers, position as given in the whole genome sequence of WA1 (GenBank: NC_012225.1), product length and annealing temperature are shown in Table 2.

PCR was performed under standard conditions in a 25 µl reaction volume with *Taq* polymerase (Bioline, Taunton, USA). The PCR program started with 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 30 seconds, 1 minute at the primer specific annealing temperature and 72 °C for 1 minute. The final extension step was 72 °C for 2 minutes after which samples were cooled to 4 °C. Optimal annealing temperatures are given for each primer pair in table 2. For all strains, the sequences were compared to each other and to the whole genome sequence of *B. hyodysenteriae* strain WA1 [26]. Furthermore, all sequences were compared to the whole genome sequences of 18 additional *B. hyodysenteriae* strains, including type strain B78 and reference strains B204 and FM88.90. These whole genome sequences were recently described by Black et al. [47].

Statistical analysis

The *in vitro* haemolysis test results were analysed by a one-way ANOVA, with Bonferroni corrections. A P-value of <0.05 was considered significant and all statistical

analysis was performed with the SPSS Statistics 22.0 software (SPSS Inc., Chicago, USA).

Table 2: Primers, position, product size and annealing conditions for detection of haemolysis related genes *tlyA, tlyB,tlyC, haemolysin III, haemolysin activation protein* and *haemolysin III channel protein*

Target gene : primer names	Nucleotide sequence (5'→3')	Position (NC_012225.1)	Product size (bp)	Temperature annealing (°C)
tlyA: haemolysin A				
tlyAS1Fo	GGTATTGGAGATG AATATAC	267034-267054	956	58
tlyAS1Re	TGATGTAGAAGGC TTCTATA	267969-267989		
tlyB : haemolysin B				
tlyBS3Fo	GGAGTGGAGAGAA AGTATTA	1414613-1414633	974	57
tlyBS3Re	TGCTGTAAGCAGA CTTATAG	1415566-1415586		
tlyBS4Fo	AGCTGTCCTTCTTC AAGTAC	1415413-1415433	390	63
tlyBS4Re	AGTCGTAGGACAG AAAGAAG	1415782-1415802		
tlyBS2Fo	CCCTCTTCATAACC AACATA	1415533-1415553	1062	65
tlyBS2Re	AGGGACTTGCTGA AAAGATA	1416653-1416673		
tlyBS1Fo	TTGTACCAGCAAC AACTGAA	1416575-1416595	1082	54
tlyBS1Re	AGCTCTATCTACAG CAATAC	1417635-1417655		
tlyC : haemolysin C				
tlyCFo	TTACGAATGCCTG CTATTTG	1644915-1644935	1131	50
tlyCRe	CTATTTTTAGGCGA GGCTTT	1646025-1646045		
BHWA1_RS02195: haemolysin III				
HlysCBSFo	GGAAAAAGGGATC CTGGAAC	704725-704745	1570	54
HlysCBSRe	TCCTGCTTGTTATC AGCACA	706278-706298		
BHWA1_RS02885: haemolysin activation protein				
Hlys3-1Fo	CTATTGGAGAGCG TACATCT	503577-503597	1014	58
Hlys3-1Re	TACCCTGTACCTAC AGAACA	504571-504591		
BHWA1_RS09085: haemolysin III channel protein				
Hlys3-2Fo	CTCCTCCCGTTCA ATATGTA	2156200-2156220	974	58
Hlys3-2Re	AATCCGCCATGTA AAACTGC	2157154-2157174		

Results

Phenotypic and molecular identification of *B. hyodysenteriae* isolates

A collection of 35 *B. hyodysenteriae,* 15 *B. intermedia,* 7 *B. pilosicoli,* 12 *B. murdochii,* 10 *B. innocens* isolates, and 1 *B. hampsonii* isolate has been assembled. During the characterization of the strain collection it was noted that one isolate, M2, that was donated by a diagnostic laboratory, showed only moderate haemolysis on TSA plates supplemented with 5% sheep blood, although it had been presented as a *B. hyodysenteriae* isolate. Another isolate, D28, had been presented as *B. murdochii* by a diagnostic laboratory. This isolate was phenotypically identifiable as *B. murdochii*, but was positive in all *B. hyodysenteriae* specific PCR's and negative in all species-specific PCR's for other *Brachyspira* sp..

For the final selection of ten isolates supplemented with the positive control strain B204, results of the phenotypic characterization are shown in Table 1. Most isolates showed strong haemolysis after growth for four days on TSA plates supplemented with 5% sheep blood. However, as mentioned previously, isolate M2 showed only moderate haemolysis and isolate D28 showed weak haemolysis. Eight out of ten isolates were indole positive and two were negative.

The ten selected strains tested positive in the *B. hyodysenteriae* specific PCRs based on 23S rRNA, *nox* and *tlyA* genes. Sequences of the *nox* genes of all the isolates showed 100% similarity to previously described *B. hyodysenteriae* strains retrieved from GenBank. For the ten selected strains the *nox* gene sequences were identical, except for strain 25cI. The 16S rRNA gene sequence of these strains also showed 100% similarity to previously described *B. hyodysenteriae* strains retrieved from GenBank. All sequences were deposited in GenBank, accession numbers and sequence length are given in table 1.

MLST results are given in table 1. All 7 genes could be amplified and sequenced for the described isolates. The MLST profiles of isolates 8dII, M1 and M2 are identical and have previously been deposited as sequence type 8. All other profiles represent new sequence types but have 4 or 5 loci in common with already existing profiles in the pubMLST database. A dendrogram based on the concatenated sequence (4086bp) of the 7 MLST genes of *B. hyodysenteriae* and *B. intermedia* is given in figure 1.

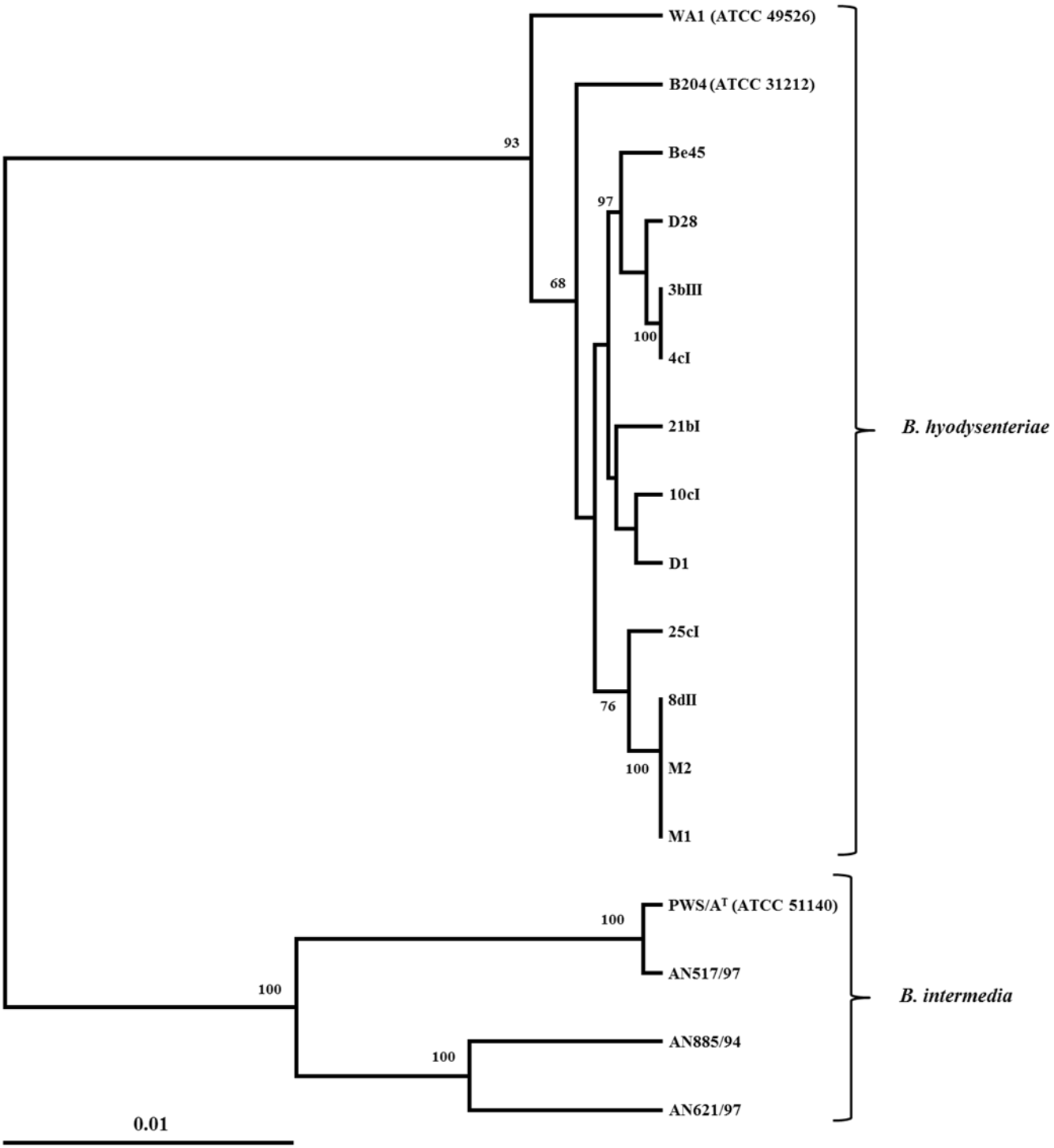

Figure 1: Dendrogram based on the concatenated sequence (4086bp) of the 7 MLST genes of *B. hyodysenteriae* and *B. intermedia*

The alignment was created using clustalw, for the dendrogram distance calculation (Kimura) and UPGMA were used (PHYLIP). Bootstrap values greater than 60 are shown in the nodes. Scale bar indicates a distance of 1 substitution in 100 nt.

In vitro haemolysis of *B. hyodysenteriae* strains shows gradual variation

Figure 2 displays the *in vitro* haemolysis of the described *B. hyodysenteriae* strains. The strength of haemolysis showed gradual variation, nevertheless most strains showed a strength of haemolysis in the same range as the B204 reference strain. For strain D28 and M2 the haemolysis was significantly lower than for the B204 reference strain (P<0.01).

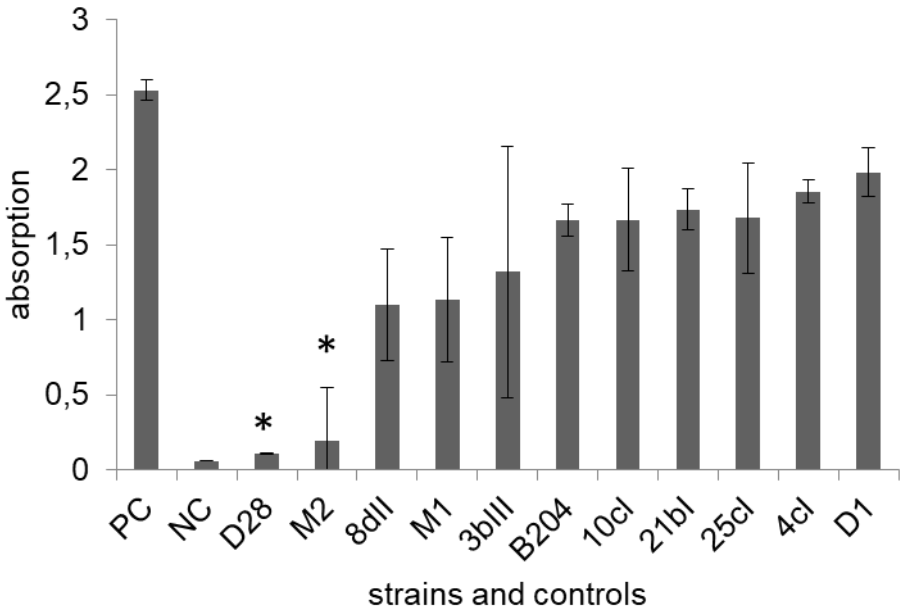

Figure 2: In vitro haemolytic capacity of *B. hyodysenteriae* strains used in this study.
Haemolysis is represented by the mean value of absorption at 450 nm after incubation of red blood cell suspension with supernatant of the different *B. hyodysenteriae* identifiable strains. PC: positive control, NC: negative control. Significant differences between *B. hyodysenteriae* identifiable strains and reference strain B204 are indicated,* P< 0.01.

Nucleic acid and amino acid substitutions in haemolysis associated genes

The sequences for *hlyA* were identical to the whole genome sequences of WA1 and the 18 additional strains [7], except strain 3bIII and 4cI, which differed with regard to 2 nucleotides. However, these nucleotide differences were synonymous and did not translate into a different amino acid sequence. The positive result for all strains in the *hlyA*-ACP PCR also showed that the *hlyA* gene was placed as expected between the accompanying *fab*-F and *fab*-G genes, coding for an ACP-reductase and –synthetase [48].

Weakly haemolytic strain D28 was the only strain with a nucleic acid substitution in the *tlyA* gene. The substitution was located at position 501 (G→T) as given in Tly (GenBank: X61684.1) by Muir et al. [28] or position 267228 as in the genome sequence of WA1 (GenBank: NC_012225.1) [6]. This non-synonymous nucleic acid substitution translated into a different amino acid at position 10 in the amino acid sequence (Glycine → Cysteine). In all other whole genome sequences the sequence of *tlyA* was identical to WA1, except for strain ST195 were there was a synonymous substitution in one nucleotide at position 938 (A→C) as given in Tly (GenBank: X61684.1) by Muir et al. [28] or position 267725 as in the genome sequence of WA1 (GenBank: NC_012225.1) [6].

The sequence of the *tlyB* gene showed differences between the isolates and the number of nucleotide changes varied from 1 to 7 as given in table 3. For all strains, except the weakly haemolytic strain D28, these nucleotide differences were synonymous. The sequence of strain D28 differed at 2 positions of which the nucleotide change at position 1416206 (C→T) translates into an amino acid substitution at position 384 in the amino acid sequence (Alanine→ Threonine). In all other whole genome sequences only one strain (ST195) was reported to have a synonymous substitution [7].

With regard to the *tlyC* gene, all strains were identical to WA1 and all other whole genome sequences except for weakly haemolytic strain D28 of which the *tlyC* sequence differed in four nucleotides. Nonetheless this altered nucleotide sequence consisted of synonymous substitutions only.

The *haemolysin III* gene sequence (*BHWA1_RS02195*) showed no nucleotide differences for seven of the strains. The strains 8dII, M1 and M2 shared an identical sequence which diverged 10 nucleotides compared to the sequence of *B. hyodysenteriae* reference strain WA1. However, these nucleotide differences did not translate into a different amino acid sequence. The weakly haemolytic strain D28 showed 68 nucleotide differences compared to the sequence of *B. hyodysenteriae* reference strain WA1. These nucleotide differences resulted in 5 amino acid substitutions at following positions: 81 (Valine → Isoleucine), 113 (Methionine → Valine), 164 (Glutamic acid → Aspartic acid), 227 (Threonine → Serine), 264 (Valine → Isoleucine). The majority of the other whole genome sequences showed a *haemolysin III* gene sequence identical to WA1, 6 strains showed synonymous

nucleotide substitutions and strain B6933 had two amino acid substitutions at position 241(Methionine → Isoleucine) and 335 (Valine → Isoleucine).

With regard to the *haemolysin activation protein* gene (*BHWA1_RS02885*) all strains showed a difference of 14 or 15 nucleotides with the sequence of *B. hyodysenteriae* reference strain WA1 (table 3). These sequences translated in 5 amino acid sequence differences at following positions: 51 (Proline → Serine), 56 (Valine → Isoleucine), 59 (Valine → Leucine), 82 (Leucine → Isoleucine), 93 (Valine → Isoleucine). Strain D28 showed 41 nucleotide differences compared to the sequence of *B. hyodysenteriae* reference strain WA1 (table 3), which translates into an amino acid sequence different from that of strain WA1 by 8 amino acids: 47 (Threonine → Isoleucine), 49 (Valine → Methionine), 56 (Valine → Isoleucine), 79 (Valine → Isoleucine), 82 (Leucine → Isoleucine), 111 (Valine → Isoleucine), 114 (Leucine → Proline), 133 (Methionine → Isoleucine). The whole genome sequences of the 18 additional *B. hyodysenteriae* strains showed various amino acid substitutions compared to WA1. Six strains shared the 5 amino substitutions as seen in most of the strains of this study, strains B204, B6933 and B78 showed one additional amino acid substitution at position 157 (Lysine → Glutamic acid). One strain (NSW15) showed three amino acid substitutions compared to WA1 at positions 19 (Lysine → Arginine), 133 (Methionine → Isoleucine), 180 (Isoleucine → Methionine), and strains Q17, B8044 and 865 showed four amino acid substitutions compared to WA1 at positions 54 (Isoleucine → Methionine), 82 (Leucine → Isoleucine), 93 (Valine → Isoleucine) and 157 (Glutamic acid → Lysine).

The sequences for *haemolysin III channel protein* gene (BHWA1_RS09085) of the strains in this study were either identical to that of *B. hyodysenteriae* reference strain WA1, differed by 1 or 2 nucleotides, or differed by 12 (strain D28). For strains 3bIII and 4cI this resulted in an amino acid substitution at position 217 (Arginine → Isoleucine), and for strain D28 at position 209 (Valine → Isoleucine). For the other whole genome sequences 7 strains showed an identical *haemolysin III channel protein* gene sequence to WA1, 9 strains shared a synonymous nucleotide substitution at position 2156792 as given in the genome sequence of WA1. Strain B78 showed one amino acid substitution at position 120 (Alanine → Threonine).

Table 3 displays the number of nucleotide and amino acid differences for the sequences of the *hlyA, tlyA, tlyB*, *tlyC, haemolysin III, haemolysin activation protein* and *haemolysin III channel protein* genes between the *B. hyodysenteriae* strains in

comparison with the genome sequence of *B. hyodysenteriae* reference strain WA1. All sequences have been deposited in GenBank (accession numbers KM112034-KM112073, KU215622-KU215658).

Table 3: Nucleotide and amino acid differences for haemolysis related genes of *B. hyodysenteriae* identifiable strains used in this study. Differences compared with the genome sequence of *B. hyodysenteriae* strain WA1. Number of amino acid changes are given in brackets.

Strain	*In vitro* haemo -lysis	*tlyA* 723 nt	*hlyA* 237 nt	*tlyB* 2487 nt	*tlyC* 807 nt	*haemolysin III* 1335 nt	*haemolysin activation protein* 675 nt	*haemolysin III channel protein* 672 nt
3bIII	++	0	2 (0)	7 (0)	0	0	15(5)	2(1)
4cI	++	0	2 (0)	7 (0)	0	0	15(5)	2(1)
8dII	++	0	0	0	0	10(0)	15(5)	0
10cI	++	0	0	5 (0)	0	0	14(5)	1(0)
21bI	++	0	0	1 (0)	0	0	15(5)	1(0)
25cI	++	0	0	7 (0)	0	0	14(5)	1(0)
D1	++	0	0	5 (0)	0	0	14(5)	1(0)
D28	+/-	1 (1)	0	2 (1)	4 (0)	63(5)	44(8)	12(1)
M1	++	0	0	0	0	10(0)	15(5)	0
M2	+	0	0	0	0	10(0)	15(5)	0

Discussion

This study describes quantification of haemolytic capacity of *B. hyodysenteriae* strains, and provides evidence that the degree of haemolysis can vary within the species *B. hyodysenteriae.* The phenotypic characterization tests, species-specific PCR, and sequences of the *nox* and 16S rRNA genes of moderately or weakly haemolytic strains show that these strains belong to the species *B. hyodysenteriae.* The dendrogram based on the MLST results (Figure 1) shows that the weakly haemolytic *B. hyodysenteriae* strains are nested within clades containing strongly haemolytic *B. hyodysenteriae* strains. Even if only DNA/DNA hybridization might be considered sufficiently accurate enough to effectively identify a strain, the strains described here would undoubtedly be identified as *B. hyodysenteriae* in all currently used methods for genetic identification (PCR, *nox* and 16S rRNA sequencing, MLST).

The comparative sequence analysis of the haemolysis associated genes leads to a hypothesis with regard to the underlying mechanism of the weak haemolysis. The

weakly haemolytic *B. hyodysenteriae* strain D28 possesses nucleotide sequence differences in the *tlyA, tlyB, haemolysin III, haemolysin activation protein* and *haemolysin III channel protein* genes resulting in amino acid substitutions. These sequences differ from those of all other strains in the study and from that of reference strain WA1. Whether the amino acid substitutions reported here are the sole reason for the weak haemolysis of this strain needs further studies. In our opinion the most important genes involved in the strong haemolytic phenotype of *B. hyodysenteriae* are *tlyA, hlyA* and probably *haemolysin III.* Deletion mutants for *tlyA* have been reported to be weakly haemolytic on blood containing agar plate [16]. The role of ACP in acylation of toxins has been demonstrated for other toxins, such as RTX toxins [6], which makes it likely that *hlyA* encoding an ACP plays a role in the haemolytic capacity of *B. hyodysenteriae.* Haemolysin III harbours a conservative domain yqfA, a predicted channel-forming protein of the haemolysin III family, which might indicate its role in *B. hyodysenteriae* haemolysis. Whether this reduced haemolytic capacity can be attributed to one of the amino acid changes in one of the haemolysis associated genes, remains to be determined. In order to completely elucidate this, the construction of specific mutants of *B. hyodysenteriae* which harbour one of the divergent haemolysis associated genes is a prerequisite. This might be hampered by the fact that is difficult to genetically manipulate *B. hyodysenteriae.*

Not only a difference in amino acid sequence, which can affect the function of a protein, might influence the gradation in haemolytic capacity but there might also occur a more distant variance such as altered activity of promoter regions or altered transcription of genes under specific circumstances *in vitro* as well as *in vivo.* Although repeated subculturing can result in phenotypical changes such as loss of haemolysis [32], this has, to our knowledge, not been described for *B. hyodysenteriae.* Besides, already during primary isolation of strains D28 and M2, haemolysis was always weak and moderate, respectively.

B. hyodysenteriae strain M2 is only moderately haemolytic. However, the nucleotide sequence differences observed for strain M2 did not result in amino acid changes except for the *haemolysin activation protein* gene. However, for this gene amino acid substitutions were observed for all investigated strains compared to WA1. It should be mentioned that unlike D28, which originated from a farm where only mild diarrhoea was present, M2 originated from a farm where pigs were suffering from

mucohaemorrhagic diarrhoea. Alongside M2, another isolate M1, originated from the same farm. The presence of different strains with divergent biological properties on one farm could influence the outcome of control measurements, since these strains may differ in other biological properties as well, such as their antimicrobial resistance.

Strain D28 originated from a farm were only mild diarrhoea was present. In preliminary trials, in which pigs were inoculated with this strain, no symptoms of SD were observed, even if the strain was shed in the faeces of the inoculated pigs at 10^7copies/ g faeces. Even though the significance of the presence of weakly haemolytic strains of *B. hyodysenteriae* in a herd as a hazard for porcine health is not clear at the moment, the mere occurrence of weakly haemolytic strains of *B. hyodysenteriae* poses problems for the diagnosis of swine dysentery. When diagnosis is primarily based on microbial culture procedures, these strains could be mistaken for *B. intermedia* or *B. murdochii,* since the phenotypic profile of weakly haemolytic, indole positive *B. hyodysenteriae* is equal to that of *B. intermedia* and the phenotypic profile of weakly haemolytic, indole negative *B. hyodysenteriae* is equal to that of *B. murdochii.* When diagnosis is primarily based on the current PCR tests, the degree of haemolysis of the specific strain cannot be estimated. If a herd tests positive for *B. hyodysenteriae*, this may influence the trading possibilities of the farm in question, because of the possible risk of *B. hyodysenteriae* carrier animals. In order to avoid misdiagnosis, the combination of phenotypic characterization and PCR, complemented with sequencing of *nox, haemolysin III* or *haemolysin activation protein* genes if necessary, is presumably the most complete method for species identification of *Brachyspira* sp. for now.

Although in our collection of 35 isolates, spanning a time-period of two years, only two *B. hyodysenteriae* strains were found with an aberrant haemolytic phenotype, appearance of weakly haemolytic, possibly low virulent strains of *B. hyodysenteriae* may affect herd dysentery status, with great impact on a farms trading opportunities. The prevalence of weakly haemolytic *B. hyodysenteriae* could be underestimated since it has not been regularly looked for or could go unnoticed if PCR and microbial culture are not combined.

Declaration of conflicting interests

Sources of financial support have been acknowledged and the authors declare that they have no competing interests.

Authors' contributions

MM participated in the design of the study, performed the experiments, analysed the data and drafted the manuscript. NDP, LVM and MV participated in the experiments. FB participated in the design of the study and edited the manuscript. RD helped to interpret the results and edited the manuscript. FP, AM and FH coordinated the study, participated in the design of the study, helped to interpret the results and edited the manuscript. All authors read and approved the final manuscript.

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An avirulent *Brachyspira hyodysenteriae* strain elicits intestinal IgA and slows down spread of swine dysentery

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Abstract

Swine dysentery caused by *Brachyspira hyodysenteriae*, results in substantial economic losses in swine producing countries worldwide. Although a number of different vaccine approaches have been explored with regard to this disease, they show limitations and none of them have reached the market. We here determine the vaccine potential of a weakly haemolytic *B. hyodysenteriae* strain. The virulence of this strain was assessed in experimental infection trials and its protection against swine dysentery was quantified in a vaccination-challenge experiment using a seeder infection model. Systemic IgG production and local IgA production were monitored in serum and faeces respectively.

Across all trials, pigs that were colonized by virulent, strongly haemolytic *B. hyodysenteriae* strains consistently developed swine dysentery, in contrast to none of the pigs colonized by the weakly haemolytic *B. hyodysenteriae* vaccine strain. In the seeder vaccination trial the velocity of spread of swine dysentery was significantly reduced in animals immunised with the weakly haemolytic strain compared to sham-immunised animals. Furthermore, faecal scores, estimating severity of diarrhoea, were significantly lower in immunised animals compared to sham-immunised animals. The IgA response of immunised animals upon challenge with a virulent *B. hyodysenteriae* strain significantly correlated to a later onset of disease. The correlation between intestinal IgA production and protection induced by a non-haemolytic *B. hyodysenteriae* strain provides leads for future vaccine development against swine dysentery.

Keywords: *Brachyspira hyodysenteriae,* vaccination, IgA, transmission, swine dysentery, ELISA

An avirulent strain of *B. hyodysenteriae* elicits intestinal IgA and slows down spread of swine dysentery

Introduction

Swine dysentery (SD) caused by *Brachyspira hyodysenteriae*, results in substantial economic losses in swine producing countries worldwide. Major costs associated with SD comprise medical treatment, retarded growth and increased feed conversion [1]. For infected herds costs/kg live weight have been estimated to increase with 15% [36]. Treatment with antimicrobial compounds is hampered due to increasing resistance against pleuromutilins and macrolides which are the most widely used compounds against SD [10,23]. Besides instigating therapeutic failure, there is growing public concern against the use of antibiotics in animal production in general because it may favour spread of antimicrobial resistance in different bacterial species, including zoonotic agents [26,32]. The impact of SD on swine health and production, increasing therapeutic failure of antimicrobial treatment and the need for a reduction of the use of antimicrobial compounds urge for alternative control measures against SD.

The immunological response in pigs that recovered from SD has been shown to protect against subsequent challenge with *B. hyodysenteriae* [15]. Therefore, a number of different vaccination approaches have been explored with regard to SD. Several reports describe the use of whole cell bacterins [4,5,7,9,24] or protein digests of whole cell bacterins [33-35]. Some of these bacterins, administered intramuscularly or intravenously, induce partial protection, demonstrated by a lower proportion of animals developing clinical SD, or animals developing less severe disease signs of SD [4,5,7,8]. In contrast, Olson et al. [24] described animals developing a more severe form of SD with an earlier onset after vaccination with an inactivated *B. hyodysenteriae* vaccine. A major downside of the use of inactivated whole cell bacterins is that they usually only evoke protection against infection with a homologous serotype of *B. hyodysenteriae* [1].

Vaccination with recombinant proteins has been reported to induce variable levels of protection, depending on the selected protein. The use of a recombinant flaB1 flagellar protein could not reduce the number of pigs developing SD after challenge with a virulent *B. hyodysenteriae* strain [6]. A preparation of BmpB*,* an outer membrane lipoprotein, resulted in a 50% reduction in clinical SD [18]. Song et al. [31] described a

reverse vaccinology approach to select proteins for use in a subunit vaccine. They also reported a reduction in number of animals developing clinical SD, albeit not significant. DNA vaccines based on *ftnA,* encoding a putative ferritin protein, or *SmpB*, encoding a protein with unknown function, failed to protect mice against challenge with a virulent *B. hyodysenteriae* strain [3,11]. The use of DNA vaccines for SD has not been investigated in pigs. A *tlyA* mutant strain of *B. hyodysenteriae* has been examined for its use as a live attenuated vaccine. A 50% reduction in the number of animals developing clinical SD upon challenge with a virulent *B. hyodysenteriae* strain was demonstrated. However, there was no reduction in the number of animals that was colonised by the challenge strain [13].

Despite all these efforts, an efficient vaccine against *B. hyodysenteriae* is currently not available. Recently, we isolated a weakly haemolytic *B. hyodysenteriae* strain which appeared to be less virulent than strongly haemolytic *B. hyodysenteriae* strains [22]. In this study we explore this strain's vaccination potential by verifying its virulence in pigs and determining the extent of protection it provides against SD in an experimental infection trial.

Materials and methods

The animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2012/01, EC 2013/147, EC2014/130, EC2015/22, EC2015/134) and complied with all ethical and husbandry regulations.

B. hyodysenteriae strains and growth conditions

Three *B. hyodysenteriae* field strains and the strongly haemolytic reference strain B204 (ATCC32121) were used in the experimental infection trials: weakly haemolytic strain D28 and strongly haemolytic strain 8dII are two field strains which have been described previously [22]. Strongly haemolytic strain 49 was isolated in this study from seeder animals that were purchased from a commercial source suffering an acute outbreak of SD. Strains and their strength of haemolysis are given in table 1. Strength of haemolysis was determined as visible haemolysis of growth on blood supplemented culture plates and by *in vitro* quantification as described in a previous study [22].

For the virulence trials, strains were obtained from frozen stocks, thawed and grown on Tryptic Soy Agar (BD, Heidelberg, Germany), supplemented with 5% sheep blood (IMP, Brussels, Belgium) and 1% yeast extract (Oxoid, Aalst, Belgium) [14]. Strains

were subcultured twice and suspensions were prepared by harvesting a 4-day-old culture plate with a sterile cotton swab and stirring the cotton swab in 50 ml of an anaerobic Brain Heart Infusion (BHI) broth, supplemented with 10% of Foetal Bovine Serum (FBS). The broth was incubated during 40 hrs on a rocking platform at 37°C. After incubation, cultures were microscopically examined for purity and each animal was administered 40 ml of *B. hyodysenteriae* culture which contained approximately $1x10^8$ colony forming units per ml. For the seeder vaccination trial, cultures were obtained in grossly the same way as for the virulence trials except that bacteria were grown in BHI broth with 10% FBS for 30 hrs, after which the broth was anaerobically centrifuged at 1500 g for 20 minutes and the pellet was suspended in a volume leading to a final concentration of approximately $1x\ 10^9$ *B. hyodysenteriae* per ml.

Virulence trials

In order to determine the *in vivo* virulence of different *B. hyodysenteriae* strains, several experimental infection trials were conducted. The correlation between faecal shedding, as a proxy of intestinal colonization, and faecal score, as a measure for the development of SD, was determined independently for the four different *B. hyodysenteriae* strains in five experimental infection trials. Experiments were conducted separately in different time periods. In each experiment a single strain was used. Strain D28 was used in two independent experiments.

Experimental set-up: the five experimental set-ups are presented in table 1. In all set-ups, experimental animals were purchased from commercial sources with no prior history of SD. Animals were 5-6 weeks old on arrival, except for trial 3 in which animals were 11 weeks old at the start of the trial. On arrival, faeces were collected from all individual animals and examined for the presence of *Salmonella* sp. by microbial culture and for the presence of *B. hyodysenteriae* by microbial culture and qPCR [30]. All animals were fed a commercial starter feed ad libitum.

Experimental procedures: inoculation was performed on three consecutive days and was preceded by a 12h fast. Inoculation was performed orally or intragastrically as given in table 1. For intragastric inoculation, animals were anaesthetized by intramuscular injection with a combination of xylazin at 4.4 mg/kg (Xyl-M 2%®, VMD, Arendonk, Belgium) and zolazepam/tiletamin at 2.2 mg/kg (Zoletil® 100, Virbac, Carros, France). All intragastrically inoculated animals were pretreated 90 minutes

before inoculation with 0.75 mg/kg ranitidine (Zantac™, GlaxoSmithKline, Genval, Belgium) to reduce stomach acid production.

In experiment 3 instead of direct inoculation, the contact animals (receivers) were placed in the same unit with animals that were shedding *B. hyodysenteriae* and had been confirmed to have SD (seeders). These seeder animals were purchased from a commercial source suffering an acute outbreak of SD. Strain 49 was isolated from faecal samples of those pigs.

Follow-up: In all trials, animals were observed daily for the presence of diarrhoea and other disease signs. Two to three times a week, faeces were scored and stool samples were collected. Faecal scores were determined as 0: normal, 1: softer but formed, 2: unformed semi-wet, 3: runny, 4: runny with mucus and blood. Scores 2 and 3 were supplemented with 0.5 if blood or mucus were present. DNA was extracted from the stool samples using a Qiagen Stool Mini Kit (Qiagen, Hilden, Germany) and the extracted DNA was used to determine the quantity of *B. hyodysenteriae* DNA with qPCR [30]. Correlation between faecal excretion of the strain used for inoculation and faecal score was determined for each experiment.

At the end of the trial (3-5 weeks after inoculation) or 24 hrs (trial 2) after the first signs of swine dysentery, animals were euthanized. During necropsy, tissue samples of the apex of the colon were collected in 10% buffered formalin for histology. Animals were anaesthetized with a combination of xylazin at 4.4 mg/kg (Xyl-M 2%®, VMD, Arendonk, Belgium) and zolazepam/tiletamin at 2.2 mg/kg (Zoletil® 100, Virbac, Carros, France). They were euthanized by administering an overdose of pentobarbital (Release®, 45mg/kg; Ecuphar, Oostkamp, Belgium) by intracardial injection. Fixed samples were paraffin embedded, sectioned at 5–8 µm and stained with hematoxylin and eosin or with Periodic Acid Schiff reagent (PAS).

Vaccination trials

In the seeder vaccination trial, non-virulent *B. hyodysenteriae* strain D28 was used as the immunising strain, and virulent *B. hyodysenteriae* strain B204 as the challenge strain. Sixty, six-week-old male and female piglets were purchased from a commercial source with no previous history of SD. On arrival, animals were weighed and randomly assigned to six groups; three immunisation groups (10 animals each) and three non-immunisation groups (10 animals each). Individual faecal samples were taken to

confirm absence of *B. hyodysenteriae* by microbial culture and qPCR. All animals were fed a commercial starter feed ad libitum.

After a nine-day acclimatization period, animals in the immunisation groups were orally inoculated as described for the virulence trials on three consecutive days (d-2, d-1, d0) with 20 ml of a culture containing approximately 10^9 colony forming units (cfu)/ml of nonvirulent strain D28. Correspondingly, animals in the non-immunisation groups were orally inoculated with 20 ml of BHI broth supplemented with 10% FBS. All animals were pre-treated 90 minutes before inoculation with 0.75 mg/kg ranitidine (Zantac™, GlaxoSmithKline, Genval, Belgium) to reduce stomach acid production.

Three weeks after (sham-)immunisation, five animals of each group were challenged with virulent *B. hyodysenteriae* strain B204 on three consecutive days by oral inoculation (d19, d20, d21) as described above for the immunising strain. These challenged animals served as seeder animals for the remaining five animals (receivers) in each group.

Animals were observed daily for the presence of diarrhoea and other disease signs. During the period post-immunisation until challenge, faecal samples were collected three times a week from immunised animals. From these faecal samples DNA was extracted as described above to determine excretion of the immunising strain and faeces were scored as described for the virulence trials. These faecal samples were also used to determine the presence of faecal IgA against *B. hyodysenteriae*. Faecal samples from non-immunised animals were collected once during this period to confirm the absence of *B. hyodysenteriae*. After challenge, faecal samples were taken two times a week from all animals. These samples were scored and DNA was extracted as described above to determine the excretion of the immunising and/or challenge *B. hyodysenteriae* strain. These faecal samples were used to determine the presence of faecal IgA against *B. hyodysenteriae* as well.

Animals were weighed at the start of the trial (before immunisation), after immunisation at day 17 and at necropsy. Average daily weight gain was calculated for each individual animal. Blood samples for determination of the presence of *B. hyodysenteriae* reactive serum IgG were taken before immunization (d-13) and before challenge (d17).

Animals were euthanized at day 50-52 (30-32 days post challenge) or sooner if apathy or depression was noted. Euthanasia was performed as described for the virulence trials.

qPCR differentiating between the immunising and the challenge strain

In order to specifically determine the quantity of *B. hyodysenteriae* DNA of the immunising strain and the challenge strain in faecal and intestinal samples, primers were designed to specifically anneal with DNA of either strain. Primers were based on the haemolysin III gene from both strains: D28 (GenBank KU215635) and B204 (GenBank JXND01000108) [2,22]. Following primers were used for specific detection of the immunising strain D28: HlyVacFo 5'TGGTGAAATACTGCCAAAA3' and HlyVacRe 5'TGTTGTTATATCGTCCATAC3'. Following primers were used to specifically detect the challenge strain: HlyInfFo 5'GTTAATGCTGAAAAAATGATG3' and HlyInfRe 5'AAGCTCTTGTATGGAATATAC3'. For both strains, following primer pair was used to generate an amplicon to be used as a standard: HlySTFo 5'CAAGTTCTATGATACCTAC3' and HlySTRe 5'GCCGCCTTTAACATAYTCTTT3'. The quantitative PCR was performed on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules CA, USA). Two µL of DNA was suspended in a 10µL reaction mixture consisting of SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, UK), HPLC water and primers at 1.5 µM for the challenge strain, and at 0.5 µM for the immunising strain. The PCR program consisted of denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30s, and 60 °C for 30s. Standards and samples were run in duplicate. Reactions for both strains were performed separately, since both amplicons generated a melt temperature of 74.5°C and could not be distinguished based on their melt temperatures. The Bio-Rad CFX Manager (version 1.6) software was used for calculation of threshold cycles (Ct)-values and melting curve analysis of amplified DNA.

Enzyme-Linked Immuno Sorbent Assay (ELISA) for specific detection of serum IgG and faecal IgA against *B. hyodysenteriae*

For detection of antibodies against *B. hyodysenteriae* strains D28 or B204, in-house whole cell ELISAs were prepared as described previously for *Salmonella enterica* [20]. Each strain was grown in BHI with 10% FBS for 48hrs on a rocking platform at 37°C. Cultures were inactivated by adding 0.18% (v/v) formalin. The inactivated *B. hyodysenteriae* suspensions were washed with Phosphate Buffered Saline (PBS) with 0.18% formalin (v/v) and finally resuspended in coating buffer (1.08g $Na_2CO_3.10H_2O$, 0.968g $NaHCO_3$, 0.25 l aqua ad injectabilia 100% w/v). F96 Nunc-

immuno plates (Nunc International, Roskilde, Denmark) were coated with 140 µl of inactivated *B. hyodysenteriae* in coating buffer, diluted to an optical density of 0.3 at 660 nm. After a 24hr-incubation period at 4°C, plates were washed three times with 100 µl of wash buffer (0.6g $NaH_2PO_4.2H_2O$, 5.6g $NaH_2PO_4.12H_2O$, 0.5ml Tween 20, 12.5g NaCl). Plates were kept at 4°C until further use.

Wells were pre-incubated with 1% skim milk powder solution in distilled water for 15 minutes to block non-specific binding. For detection of IgG in serum samples, 100 µl of 1/200 diluted sera were added to the wells and incubated for 30 minutes at room temperature. After incubation, wells were washed 5 times with wash buffer after which 100 µl of a 1/20 000 dilution of a horseradish peroxidase conjugated anti-porcine IgG (Sigma-Aldrich, St. Louis, MO, USA) was added. After 30 minutes of incubation wells were washed 5 times with wash buffer and 100 µl 3, 3′,5 ,5′-Tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO, USA) reagent was added. The enzymatic reaction was stopped after 10 minutes by adding 100 µl of 1N HCl. Optical densities were measured with a spectrophotometer (Multiskan MS, Thermofisher Scientific, Waltham, MA, USA) at 450 nm.

For detection of IgA in faecal samples, extracts were prepared as described by Peeters et al. [25]. One gram of frozen faeces was weighed and placed on ice. Three ml of extraction buffer (PBS, 0.5% Tween 20%, 0.05% NaN_3) was added and the suspension was centrifuged at 4°C for 20 minutes at 1500 g. The supernatant was collected in a 2ml Eppendorf tube (Eppendorf, Hamburg, Germany). Twenty µl of proteinase inhibitor (Sigma-Aldrich, St. Louis, MO, USA) was added before centrifugation for 10 minutes at 3000 g at 4°C. Supernatant was collected and stored at -20°C until further use. For detection of IgA reactive with *B. hyodysenteriae* in these faecal extracts, ELISA was carried out as for IgG detection in serum with following changes: faecal extracts were used undiluted and were incubated for 60 minutes, the secondary antibody, goat anti-porcine IgA (Bio-rad, Kidlington, UK) was used in a 1/5 000 dilution.

Statistical analysis

Correlation between faecal excretion and faecal score was determined by Spearman's rank order correlation (r) and was performed with SPSS 22.0 software (SPSS Inc, Chicago, USA). In the seeder vaccination model, faecal scores were analysed using cumulative logit link regression. Interactions between time, type and treatment were included, as well as random effects at the individual level, to control for pseudo-

replication in the individual time series, and at the pen effect to account for clustering. The analysis was performed using package “ordinal” in R. Analysis was repeated for faecal excretion, this time using a linear mixed model (package “lme4” in R), with the same predictors as above.

The effect of treatment on average daily weight gain was analysed using linear regression. The effect of faecal IgA response on the time of onset of SD was analysed in two steps. First, measurements for individual animals, collected at regular intervals (3, 6, 10, 13, and 17 days post challenge), were analysed using a survival model, estimating whether the probability of an individual developing SD was delayed by a stronger faecal IgA response. The use of the survival model enabled us to account for censoring (some individuals had not developed disease by the time the experiment was terminated). To capture several possibilities, the analysis of the survival model was repeated using as response variable alternatively the maximum IgA value, the arithmetic mean and the geometric mean across all days before the first notice of disease signs for a given individual. The geometric mean was used to better reflect the possible dependency between successive measures of IgA in the same individual. All three survival models included a fixed effect for individual type (seeder/receiver) and a random effect at the pen level to account for clustering. All analyses of faecal IgA responses were carried out using the “survival” package in R. For all analyses, the statistical significance level was set at $\alpha=0.05$.

3 Results

The low haemolytic *B. hyodysenteriae* strain D28 is avirulent in pigs.

In all virulence trials, faeces of all animals were negative for *B. hyodysenteriae* on arrival, although some animals of each trial tested positive for *B. innocens.* All animals tested negative for *Salmonella* sp.. Across all infection trials, out of 12 pigs that were colonized by strongly haemolytic *B. hyodysenteriae* strains, 11 consistently developed SD, in contrast to none of the 34 pigs colonized by the weakly haemolytic strain D28 (table 1). One receiver animal in experiment 3 shed *B. hyodysenteriae* in its faeces on two occasions at the end of the trial. At the same time the animal had a faecal score of 2. It is possible that this animal would have developed SD in the following days. However, since the trial ended simultaneously for all animals in that trial, the animal was euthanized and the development of SD could not be confirmed.

In the seeder vaccination trial, during the period between immunisation with strain D28 and the challenge with the virulent strain, no clinical signs of dysentery were noticed in any animal. On all sampling occasions, maximum faecal score was 1. Score 1 was noted in immunised as well as in non-immunised animals.

Table 1: *B. hyodysenteriae* strains, experimental set-up and results for faecal excretion and clinical signs of SD

Virulence trial number	*B. hyodysenteriae* strain	Strength of haemolysis	Model and inoculation route	Number of pigs positive for faecal excretion*	Number of pigs with SD*
1	8dII	strong	direct oral inoculation	2/6	2/6
2	B204	strong	direct gastric inoculation	5/9	5/9
3	49	strong	seeder model	5/14	4/14
4	D28	weak	direct oral inoculation	5/8	0/8
5	D28	weak	direct gastric inoculation	4/12	0/12
vaccination experiment	D28	weak	direct oral inoculation	25/30	0/30

* given as proportion of total number of inoculated pigs

Figure 1 shows the correlation between faecal excretion and faecal scores for weakly haemolytic strain D28 (panels a-c) and for strongly haemolytic strains 8dII (panel d), 49 (panel e) and B204 (panel f).The correlation between faecal excretion and faecal scores was significant ($p<0.01$) for the strongly haemolytic strains and the correlation coefficients (r) were 0.67 (strain 8dII), 0.40 (strain 49) and 0.64 (strain B204) respectively. For weakly haemolytic strain D28 there was no correlation between faecal excretion and faecal score in any of the experiments; experiment 4 $r = 0.049$, $p = 0.684$, experiment 5 $r = -0.064$, $p = 0.536$, seeder vaccination experiment $r = -0.007$ $p = 0.919$.

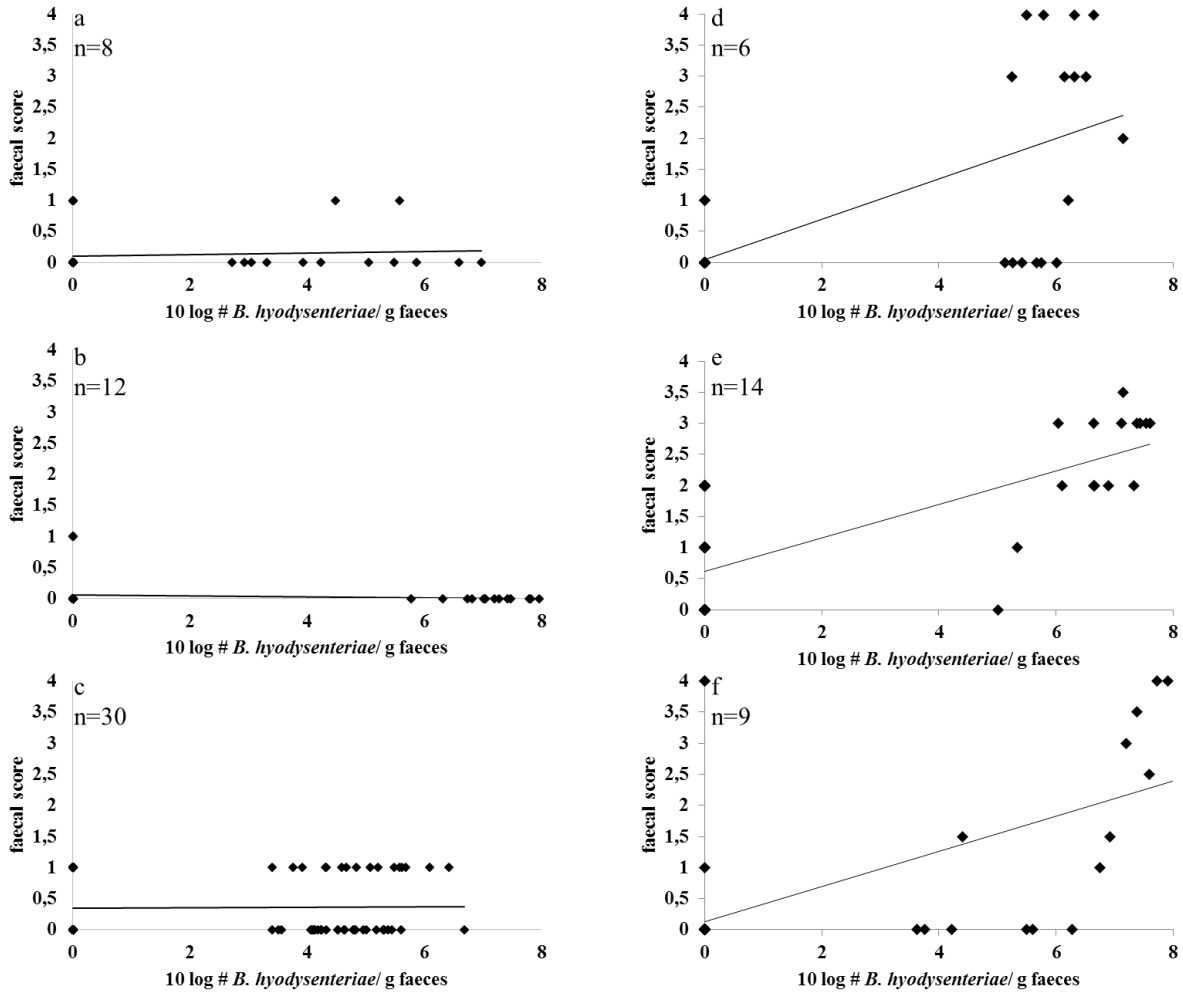

Figure 1: Correlation between faecal excretion of the different *B. hyodysenteriae* strains used for inoculation and faecal scores of pigs inoculated with these strains. Panels a and b: weakly haemolytic *B. hyodysenteriae* strain D28 in experiments nr 4 and 5, panel c: weakly haemolytic *B. hyodysenteriae* strain D28 in the seeder vaccination experiment, panel d: strongly haemolytic *B. hyodysenteriae* strain 8dII in experiment nr 1, panel e: strongly haemolytic *B. hyodysenteriae* strain 49 in experiment nr 3, panel f: strongly haemolytic *B. hyodysenteriae* strain B204 in experiment nr 2. n= total number of inoculated pigs in each experiment.

All animals, inoculated with one of the strongly haemolytic *B. hyodysenteriae* strains, that showed clinical signs of SD had various lesions in large parts of the colon. Contents of the colon were liquid and macroscopic lesions consisted of serosal hyperaemia, fibrinous colitis, enlarged mesenteric lymph nodes and the presence of excessive mucus at the colonic mucosa. Histologically, elongation of the colonic crypts

and presence of a large amount of mucoid material in the lumen of the infected animals were remarkable. The lamina propria mucosae was infiltrated by lymphocytes, plasma cells, and neutrophils.

Of the animals that shed weakly haemolytic *B. hyodysenteriae* strain D28, some showed slight hyperaemia of the colonic mucosa but no other apparent macroscopic lesions were observed. Histologically, no elongation of the colonic crypts was observed and no infiltration of inflammatory cells could be observed in the lamina propria.

PAS staining showed elongation of the colonic crypts (reflected by the size of the brackets) and a high number of Goblet cells in animals colonized with a strongly haemolytic strain (figure 2b; animal colonized with strongly haemolytic strain 8dII as an example), but not in those colonized with the weakly haemolytic strain D28 (figure 2c) or in negative control animals (figure 2a).

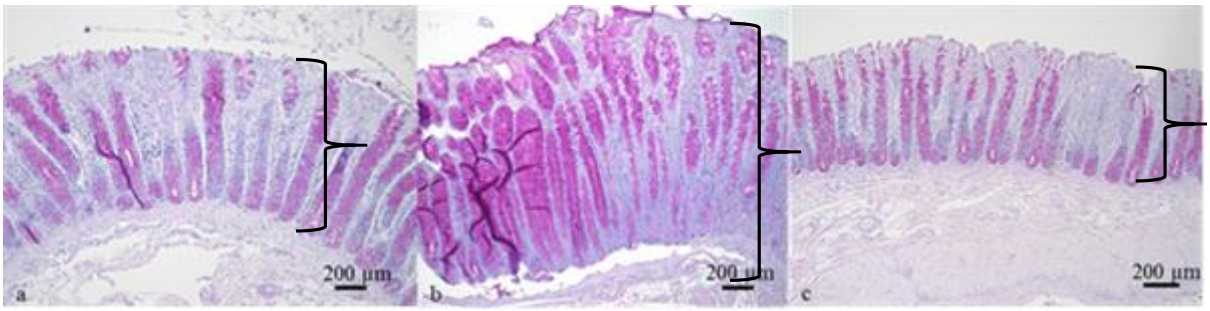

Figure 2. PAS staining of formalin fixed colonic tissue samples of pigs infected with different *B. hyodysenteriae* strains. Colonic mucosa from a: sham inoculated animal, b: animal infected by strongly haemolytic *B. hyodysenteriae* strain 8dII (33 days post inoculation), c: animal infected by weakly haemolytic *B. hyodysenteriae* strain D28 (32 days post inoculation).

Vaccination with the avirulent strain delays the spread of SD

In the seeder vaccination trial, of the 30 animals inoculated with immunising strain D28, the majority shed the strain in their faeces for less than one week (12 animals) or less than two weeks (10 animals). Three animals shed the strain for more than two weeks and for five animals, strain D28 could not be detected with qPCR. Most animals (18 out of 25) started shedding strain D28 in their faeces within the first week after inoculation, six animals in the second week, and one animal at 17 days post inoculation. During the period post vaccination until challenge, vaccinated animals had a significantly lower average daily weight gain (507 g/day) compared to sham-

vaccinated animals (650 g/day) (linear regression coefficient for treatment: β=-0.52±0.2 s.e., *p*=0.010).

The total number of animals that developed SD after challenge with the virulent *B. hyodysenteriae* strain is given in figure 3. The onset of SD, defined as the first day on which a faecal score of 2.5 or more was reached and mucus and/or blood were present, was postponed in immunised animals compared to non-immunised animals. There was a significant interaction between immunisation and number of days from challenge until onset of SD (regression estimate β=0.065±0.022 s.e., *p*=0.004). Seeder animals in the immunisation groups developed SD on average after 11.1 (10-11.8) days while seeder animals in the non-immunisation groups developed SD on average after 9.2 days (7.6-12). Receiver animals developed SD on average after 21.2 days (18.8-22.4) in the immunised groups, and after 17.3 days (15.6-20.25) in the non-immunised groups.

The average cumulative faecal score of pigs after challenge with virulent *B. hyodysenteriae* strain B204 is given in figure 4. The main effects in the regression showed that the probability of having a higher faecal score decreased with immunisation (regression coefficient: β= -1.398±0.384 s.e., *p*=0.0002), indicating that immunisation significantly reduced the probability of having a higher faecal score.

Immunisation was not significantly correlated with a decrease in faecal excretion of the *B. hyodysenteriae* challenge strain (all *p*>0.3). Faecal excretion of the challenge strain was correlated with faecal scores, and equally strong for vaccinated and non-vaccinated animals (vaccinated animals r= 0.79, p<0.001, non-vaccinated animals r=0.74, p<0.001). In all weight gain analyses, no significant differences were found between seeders and receiver individuals. Although a trend could be observed for immunised animals, most noticeable for receiver animals, to have a higher weight gain in the post challenge period compared to non-immunised animals, this difference was not significant (*p*=0.17). Plots of average daily weight gain are given in figure 5.

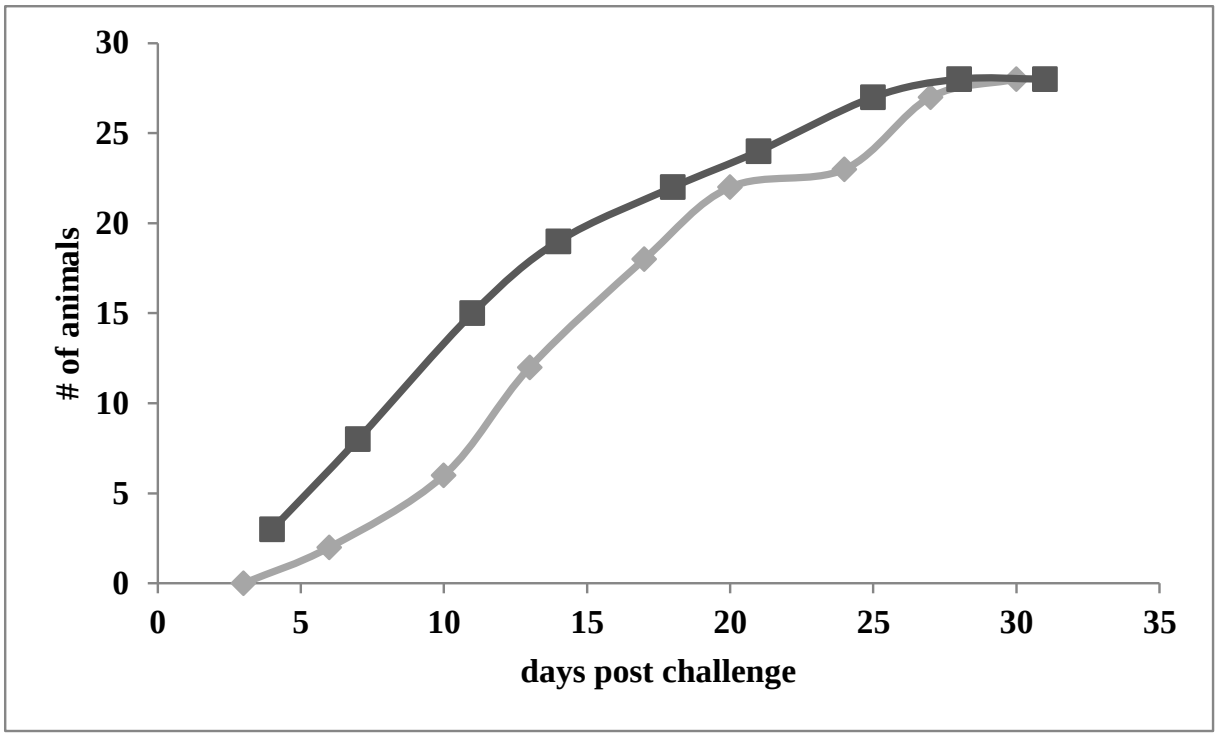

Figure 3: Cumulative number of pigs that developed SD after challenge with virulent *B. hyodysenteriae* strain B204, preceded either (rhombi) or not (squares) by exposure to non-virulent *B. hyodysenteriae* strain D28.

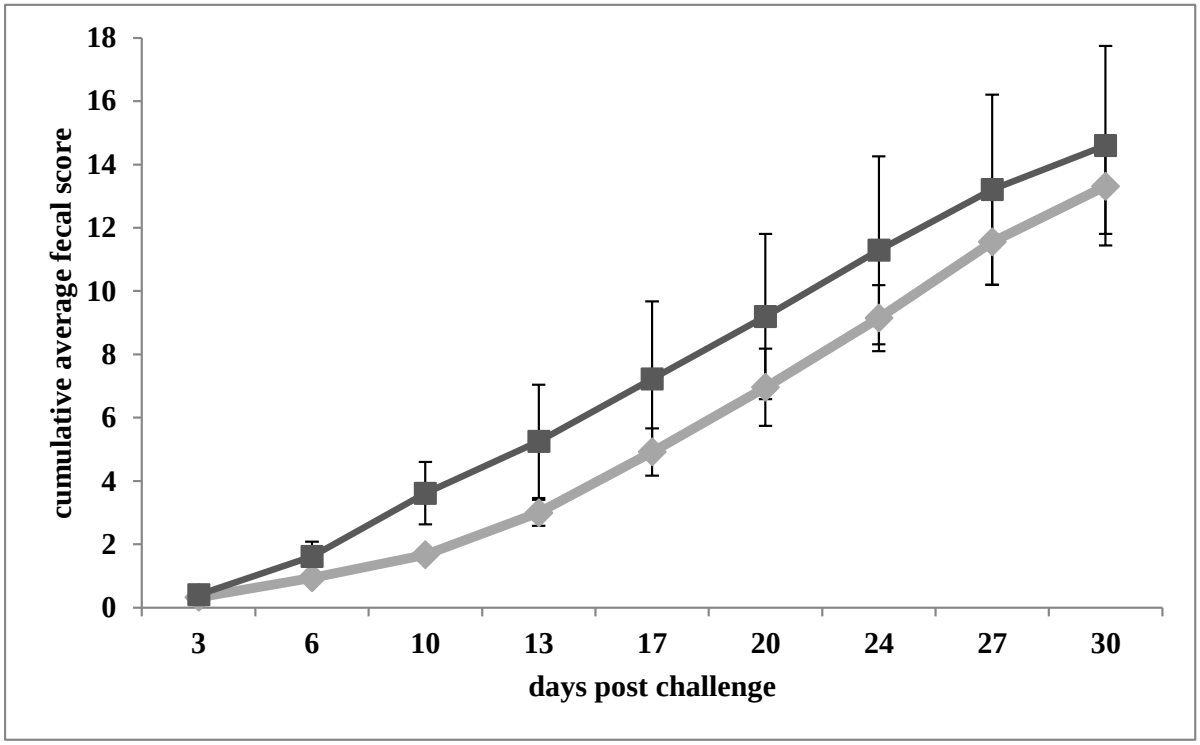

Figure 4: Average cumulative faecal score of pigs after challenge with virulent *B. hyodysenteriae* strain B204, preceded either (rhombi) or not (squares) by exposure to non-virulent *B. hyodysenteriae* strain D28.

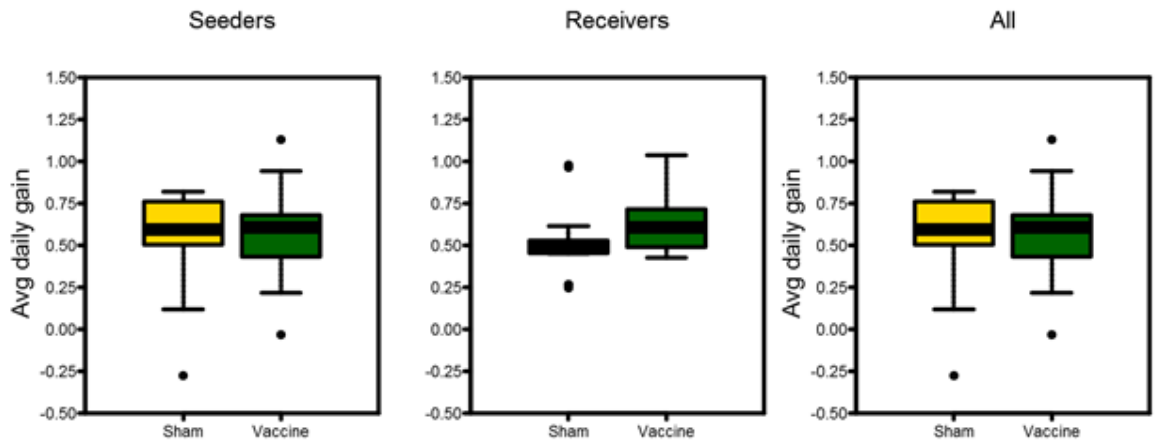

Figure 5: Plots of average daily weight gain (period: post-vaccination to necropsy), of pigs after challenge with virulent *B. hyodysenteriae* strain B204, preceded either (green) or not (yellow) by exposure to non-virulent *B. hyodysenteriae* strain D28, grouped by individual type (seeder, receiver and both) and by vaccination treatment. Boxes indicate 25% and 75% quantiles: bars indicate 2.5% and 97.5% quantiles. Average daily weight gain given in kg.

The avirulent strain does not induce a fast IgG response

Serum IgG ELISA responses against *B. hyodysenteriae* are given in table 2. There was no significant increase of IgG post-vaccination either for vaccinated or non-vaccinated animals, regardless of which strain was used to coat the whole cell ELISA. There were no significant differences between vaccinated and non-vaccinated animals, before or after vaccination.

Table 2: Production of serum IgG against *B. hyodysenteriae* before and after vaccination with the avirulent strain D28. The reaction of IgG with both the vaccination strain (D28) and the challenge strain (B204) are shown and presented as OD_{450} values with standard deviation.

Group	Whole cell ELISA, D28 coated		Whole cell ELISA, B204 coated	
	Pre vaccination (d-13)	Post vaccination (d17)	Pre vaccination (d-13)	Post vaccination (d17)
Vaccinated animals	0.27 ± 0.17	0.31 ± 0.13	0.25 ± 0.16	0.23 ± 0.06
Non-vaccinated animals	0.19 ± 0.14	0.23 ± 0.17	0.21 ± 0.14	0.21 ± 0.09

The avirulent strain induces a local yet variable IgA response

During the period post-vaccination and before challenge with the virulent *B. hyodysenteriae* strain, faecal IgA increased in 11 of 30 vaccinated animals. The faecal IgA response was measured 7 times during this period and there were large individual differences. The faecal IgA response was measured in non-vaccinated animals once at the end of this period. For none of those non-vaccinated animals there was an increase in IgA. Faecal IgA responses are given in table 3.

Table 3: Faecal IgA production before and after vaccination with the avirulent *B. hyodysenteriae* strain D28. The reaction of IgA with both the vaccination strain (D28) and the challenge strain (B204) are shown and presented as OD_{450} values with standard deviation.

	Whole cell ELISA, D28 coated		**Whole cell ELISA, B204 coated**	
Days post vaccination	Vaccinated animals	Non-vaccinated animals	Vaccinated animals	Non-vaccinated animals
Day -13	0.12 ± 0.14	0.12 ± 0.28	0.11 ± 0.13	0.14 ± 0.41
Day 3	0.08 ± 0.04	NA*	0.07 ± 0.03	NA
Day 5	0.15 ± 0.28	NA	0.11 ± 0.13	NA
Day 7	0.14 ± 0.15	NA	0.09 ± 0.06	NA
Day 10	0.34 ± 0.70	NA	0.13 ± 0.18	NA
Day 12	0.39 ± 0.55	NA	0.12 ± 0.09	NA
Day 14	0.66 ± 0.99	NA	0.25 ± 0.47	NA
Day 17	0.62 ± 0.96	0.11 ± 0.06	0.18 ±0.21	0.10 0.06

*NA= not applicable

Faecal IgA responses were also determined in the period after challenge on days 3, 6, 10, 13, and 17 post challenge. To assess the effect of IgA on the delay of development of clinical SD, maximum IgA response and geometric mean IgA response of individual animals were determined and correlated to the time of onset of SD for that specific animal. Since delay in onset of SD was to be determined, only IgA values before the actual onset of SD for each animal were retained. Furthermore, we only considered seeder animals, since for receiver animals the time of first exposure to *B. hyodysenteriae* is unknown.

Non-vaccinated animals showed low levels of faecal IgA until onset of SD. For vaccinated seeder animals there was greater variability between individuals. The maximum IgA response and the geometric mean IgA response of those vaccinated seeder animals were significantly correlated to a later onset of disease (regression coefficient for geometric mean IgA: $\beta=7.26\pm3.59$ s.e., $p=0.043$, for maximum IgA

response: β=7.59±2.58 s.e., *p*=0.003). The maximum IgA and geometric mean IgA response, correlated with the time of onset of SD are shown in figure 6 and figure 7.

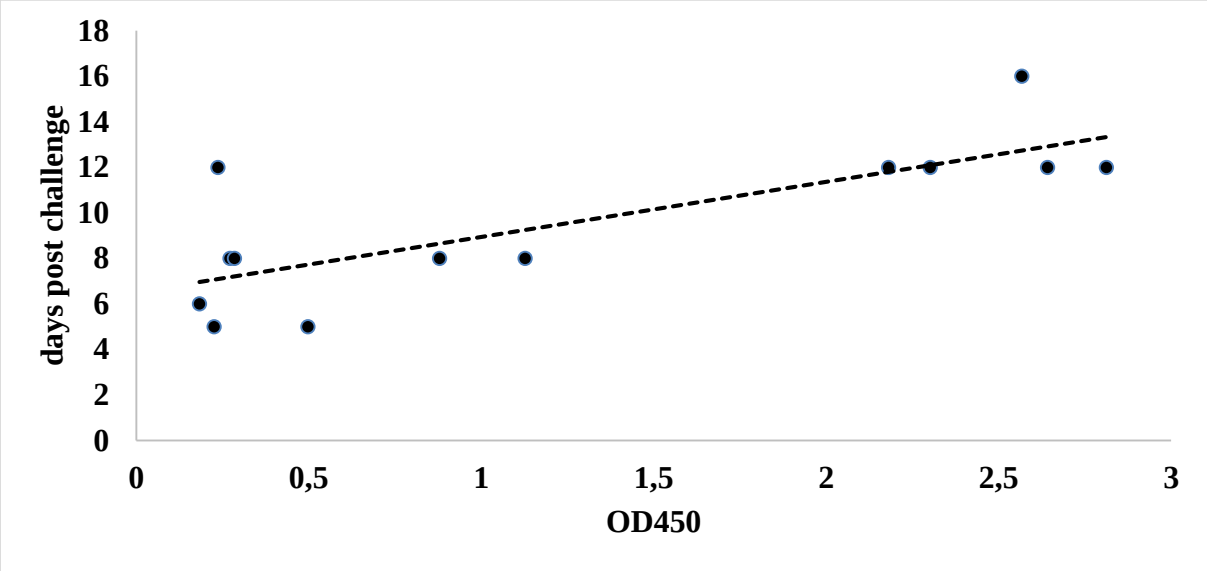

Figure 6: IgA max correlated with time of onset of SD (given in days post challenge with the virulent strain) for vaccinated seeder animals. X-axis presents OD450 values as measured in the ELISA.

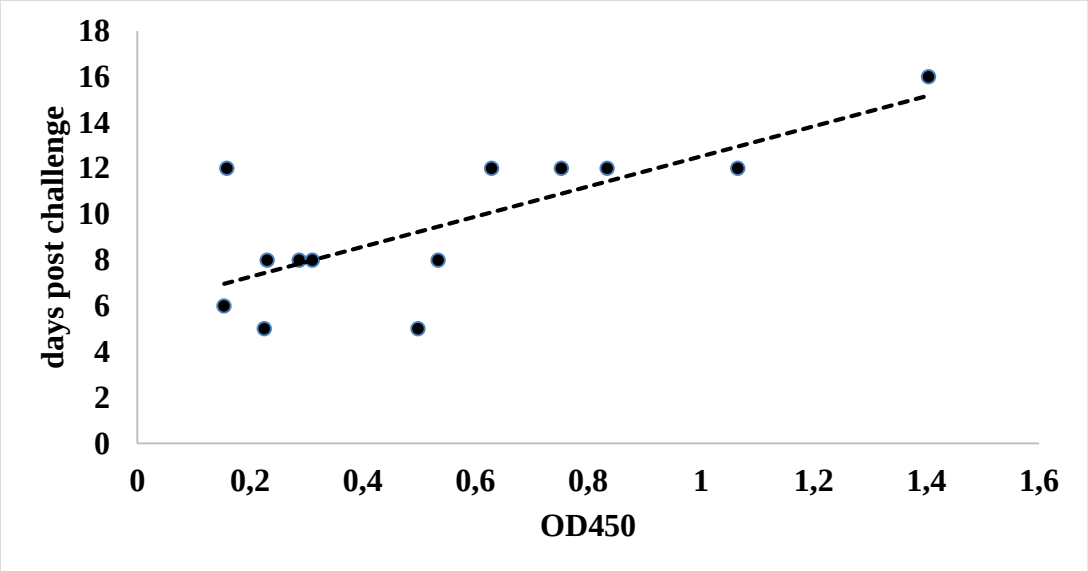

Figure 7: Geometric mean IgA correlated with time of onset of SD (given in days post challenge with the virulent strain) for vaccinated seeder animals. X-axis presents OD450 values as measured in the ELISA.

Discussion

In the pigs inoculated with the three strongly haemolytic strains, a significant correlation between faecal excretion of *B. hyodysenteriae* and faecal scores was observed. In the two virulence trials using the weakly haemolytic strain D28, however, none of the nine animals that shed the strain in their faeces developed disease signs or lesions associated with SD. Moreover a faecal score of more than one was never observed and there was no correlation between shedding of the weakly haemolytic *B. hyodysenteriae* strain and an elevated faecal score. This lack of correlation was independently confirmed in the seeder vaccination trial, in which none of the 25 pigs that shed strain D28 in their faeces developed disease signs of SD, and no correlation between shedding of this strain and elevated faecal scores could be observed. All these results strongly indicate that the weakly haemolytic *B. hyodysenteriae* strain is avirulent in pigs.

La et al. [19] described reduced virulence in a weakly haemolytic *B. hyodysenteriae* strain obtained from a herd with no clinical signs of SD. In this herd, sows were present with substantial growth of *B. hyodysenteriae* strain JR11 in their colon, without any clinical sign of SD. Although true virulence potential has not yet been verified under experimental conditions, authors described strain JR11 as particularly innocuous [19]. Interestingly, strains D28 and JR11 share almost identical differences in their haemolysin III and haemolysin activation protein, as given for strain D28 earlier [22]. Compared to the whole genome sequence of reference strain WA1 (accession number NC_012225), both weakly haemolytic strains JR11 and D28 show five identical amino acid substitutions (positions 81, 113, 164, 227, 265) in the haemolysin activation protein BHWA1_RS02885 and eight identical amino acid substitutions in the haemolysin III protein BHWA1_RS02195 (positions 47, 49, 56, 79, 82, 111, 114, 133). For strain JR11 two additional amino acid substitutions were identified in the haemolysin III protein at positions 30 and 213. It should be noted that unintentionally, for the haemolysin III protein table 3 in La et al [19], comparing the nucleotide and amino acid differences of strain D28 and JR11, gives the amino acid differences from other *B. hyodysenteriae* strains compared to WA1 and not the amino acid differences found in D28 as given in Mahu et al. [22]. As determined by MLST strain JR11 and D28 do not share the same sequencetype

Since structural studies or deletion mutant studies are not available for those haemolysis associated genes, it is uncertain if the amino acid changes in these two strains indeed alter the functionality of their proteins. However, the unique similarity of the amino acid changes in two, otherwise genetically unrelated strains, that share the same aberrant phenotype and presumably are both low or avirulent, is striking.

The seeder vaccination trial described in this study shows that the velocity of spread of SD is significantly reduced in immunised animals compared to sham-immunised animals. Although faecal shedding after challenge was not significantly decreased, pre-immunised animals showed significantly lower faecal scores compared to sham-immunised animals. The absence of a clear IgG response in serum after immunisation is not surprising since it has been demonstrated that after experimental infection, serum antibody levels start to rise after two to four weeks and reach their maximum after 4 to 7 weeks. [15]. In this experiment serum samples were taken 17 days post inoculation with the immunizing strain, which might have been too early to see a clear response. Furthermore, since this strain lacks virulence, epithelial damage in the colon can be expected to be absent. Disruption of the colonic mucosa during SD probably permits further penetration of the underlying tissues and blood vessels by bacterial antigen, which could enhance serum antibody response more intense compared to local mucosal stimulation alone [28].

The production of intestinal IgA may play a role in protection against SD. The delay of the onset of SD in immunised pigs coincided with the presence of a substantial local IgA response at the moment of challenge. Earlier, Rees et al. [29,28] described the presence of colonic IgA and IgA memory cells in gut associated lymphoid tissue in pigs who were re-challenged 14 weeks after recovering from a first or second challenge with *B. hyodysenteriae* strain B204. In those studies the IgA levels in colonic washings or faeces were correlated with a recent exposure, but not with protection against development of clinical SD. This discrepancy with our findings is partially explained by the definition of protection in those earlier studies as the presence or absence of SD, rather than time to development of SD. Most importantly in those studies, samples were taken only once, at the time of necropsy, which was several weeks after the last exposure to *B. hyodysenteriae.* Indeed IgA in seeder vaccinated animals rose shortly after the exposure to the challenge strain, but in those seeder vaccinated animals

where this rise in IgA upon challenge was absent, the development of SD was not delayed.

For other bacterial enteropathogens like *Shigella flexneri*, a rise in colonic IgA plays a role in induction of protection by immunization with live attenuated strains [16]. It was shown there that in order to elicit a high and optimal mucosal immune response, multiple doses of the live strain were necessary. Obviously there are differences between the pathogenesis of *Shigella flexneri* infections and SD*,* as well as between the responses of their respective hosts. However, both bacteria share the niche of the colon and for both bacteria, serum antibody responses are not linked with protection. It has been demonstrated on several occasions that the number of animals that is protected against SD is significantly higher after three or four exposures to experimental challenge [15,24] which would be in line with having a higher and prolonged mucosal immune response after multiple exposures.

The link between local IgA and delayed onset of SD opens perspectives for future vaccine development against *B. hyodysenteriae.* Some earlier studies of immunity induced by experimental infection of *B. hyodysenteriae* point to the importance of mucosal IgA. Recently there has been a developing interest in Th17 cells and interleukin-17A (IL-17) as critical host defence against extracellular pathogens through upregulation of intestinal IgA [21]. In pigs, IL-17 is exclusively produced by $CD4^+$ and $\gamma\delta TCR^+$ T-cells. The importance of CD4+ cells in the immune response following *B. hyodysenteriae* infection has been described by several authors [12,35]. We have demonstrated a 9-fold increase in mRNA levels of IL-17A in SD infected pigs [27]. Taken together, it would be of most interest to determine if CD4+ cells and upregulation of IL-17A are present in the colonic mucosae of pigs immunised with our strain D28 and to explore ways to further optimise the intestinal IgA response. For example, combined parenteral and oral immunisation has been demonstrated to significantly enhance mucosal IgA response for *S. flexneri* [17] and might be useful in a *B. hyodysenteriae* immunisation regime as well.

In conclusion, we describe the lack of virulence of weakly haemolytic *B. hyodysenteriae* strain D28. Immunisation of pigs by oral inoculation of this strain, significantly slows down the spread of SD compared to sham-immunised animals in a

seeder challenge model. This protection was associated with a strong IgA response upon challenge, providing directions for future vaccine development.

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Author contributions

MM participated in the design of the study, performed the experiments, analysed the data and drafted the manuscript. SC performed statistical analyses and helped to interpret the results. FB participated in the design of the study, helped to interpret the results and edited the manuscript. JZM performed the experiments. FP, AM and FH coordinated the study, participated in the design of the study, helped to interpret the results and edited the manuscript. All authors read and approved the final manuscript.

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I

PART IV

General Discussion

Since the first description of swine dysentery (SD) in 1920, the specific needs for diagnostics, treatment and prophylaxis have evolved and changed during this nearly 100 year period. Substantial advances in the fight against SD have been made since the causative agent "*Treponema hyodysenteriae"* (as *Brachyspira hyodysenteriae* was originally named), was identified [20, 58]. In those early days of SD, diagnosis and treatment might have seemed straightforward: clinical outbreaks were unambiguously related to the colonic and faecal presence of the strongly haemolytic spirochete, *"Treponema hyodysenteriae",* and treatment consisted of lincomycin, carbadox or tylosin. If weakly haemolytic spirochetes were present in faeces, these were considered non-pathogenic and grouped under *"Treponema innocens"* [30]. Recent taxonomic insights and increasing issues with SD treatment, have resulted in new challenges for SD diagnosis and treatment. For consistency, the species name *Brachyspira* will be used throughout this discussion, regardless the species name at a given time-point *(Treponema = Serpula = Serpulina = Brachyspira).*

SPECIES DELINEATION: WHAT'S IN A NAME?

Brachyspira taxonomy was once based on haemolytic properties, with on the one hand the weakly haemolytic, non-pathogenic *Brachyspira innocens* [31], and on the other hand the strongly haemolytic, pathogenic *Brachyspira hyodysenteriae* [20]. The discovery of several weakly haemolytic *Brachyspira* isolates not compliant with the description given for *B. innocens,* called for a new taxonomy [5,61]. Phenotypic and genotypic analyses using multilocus enzyme electrophoresis (MEE) of *B. hyodysenteriae* isolates and a variety of weakly beta-haemolytic intestinal spirochetes (WBHIS), suggested the existence of five subgroups later identified as: *B. hyodysenteriae, B. innocens, B. intermedia*, *B. murdochii* and *B. pilosicoli* [5,36-38]. For diagnostic purposes phenotypic identification schemes based on strength of haemolysis and enzymatic profiles were provided by Fellström and Gunnarsson [12] and Hommez et al. [25] who divided the genus *Brachyspira* in six biochemical groups (I, II, IIIA, IIIB, IIIC, IV).

However, 16S rRNA sequence analysis of isolates in these biochemical groups, only discriminated between three groups unambiguously [13,48]. Based on 16S rRNA sequence analysis there was one genetic group comprising biochemical groups I and II, supposedly representing *B. hyodysenteriae* and *B. intermedia*, a second genetic

group comprising biochemical groups IIIa, IIIb, and IIIc, supposedly representing *B. innocens* and *B. murdochii,* and a third genetic group with biochemical group IV, representing *B. pilosicoli.*

One could wonder, as demonstrated by the existence of multiple isolates that do not comply with the biological or pathogenic properties of a species, if current species delineation is perhaps too narrow and a division in only three species, with further subspecific division, might have been more appropriate. That would also explain why 16S rRNA of *Brachyspira* is said to be unspecific to delineate the existing species. Conversely, the 16S rRNA analysis might mirror a more appropriate species division. This is somewhat corroborated by DNA-DNA hybridisation between *B. innocens*-*B. murdochii*, which show a similarity of 64-66% and *B. intermedia-B. hyodysenteriae* which show a similarity of 57-68%, thereby approaching the threshold of being accepted within species diversity (70%) [59].

In contrast to this, for the recently named *Brachyspira hampsonii* a single species has been maintained, subdivided over several genomovars [42]. The genome-to-genome distances between these genomovars of *B. hampsonii* are larger than the genetic distances between *B. innocens* and *B. murdochii*, or between *B. hyodysenteriae*–*B. intermedia-B. suanatina*. The rationale for maintaining all *B. hampsonii* genomovars within a single same species was based on the lack of clearly distinguishable properties such as phenotypic characteristics or pathogenicity [42,63]. This rationale would evenly apply to *B. murdochii-B. innocens,* which are phenotypically only different in the presence of α-galactosidase, or to *B. hyodysenteriae-B. suanatina,* for which there are no phenotypic differences [45,52].

Following figure represents a phylogenetic tree based on core genomes (adapted from Mushtaq et al.) [45]. The figure shows that the species *B. innocens* and *B. murdochii*, and the species *B. hyodysenteriae, B. intermedia, B. suanatina* are separated from each other by a distance similar to that separating genomovars *B. hampsonii* I and *B. hampsonii* II, represented in the figure by *B. hampsonii* 30599 and *B. hampsonii* 30446 respectively.

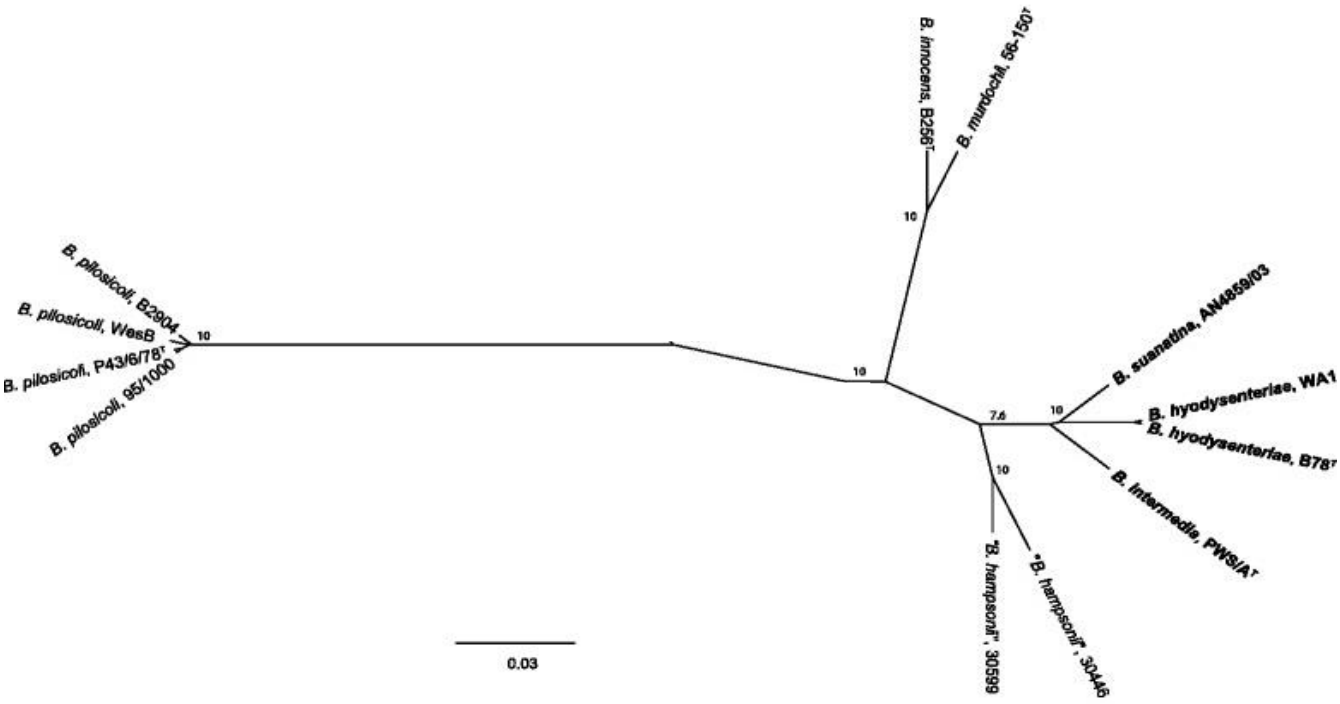

Figure 1: radial unrooted tree based on concatenated amino acid sequence of 1309 core genes [45]

Furthermore, species delineations have sometimes been based on information of a few isolates, which were later shown not to be representative of a certain taxon. For example, the type strain of *B. intermedia* shows a large genetic distance from most other *B. intermedia* isolates [49], a bias of the large strain diversity found in this species. Since most diagnostic PCRs have also been based on genetic information retrieved from those first few isolates of a species, it is quite common to find weakly haemolytic *Brachyspira* isolates for which there is an inconsistency between the phenotypic species identification and the identification by PCR, substantially confounding species determination [8].

The last decade the diagnostic identification of *Brachyspira* sp. has further been complicated due to the appearance of new strongly haemolytic *Brachyspira* species, *Brachyspira hampsonii* and *Brachyspira suanatina* [7,44,45,52,54]. The strong haemolysis of these two species had always been considered pathognomonic for *B. hyodysenteriae*. Diagnostic laboratories using culture methods would misidentify these new species as *B. hyodysenteriae* when culture methods were not accompanied by PCR, which would alert laboratories that the isolate in question was aberrant since species-specific PCRs for *B. hyodysenteriae* will be negative for these isolates. More

worrying, diagnostic laboratories relying on PCR instead of culture methods would identify *B. hampsonii* or *B. suanatina* as 'a weakly haemolytic *Brachyspira sp.',* since most diagnostic PCRs only distinct *B. hyodysenteriae, B. pilosicoli, B. intermedia,* and 'others'. This kind of misidentification would lead to false conclusions regarding the clinical relevance of an isolate.

Additionally, our identification of weakly haemolytic *B. hyodysenteriae* isolates [40], further extended by the description of weakly haemolytic *B. hyodysenteriae* isolates in Germany and Australia [35,17], complicates diagnostics further. These strains will be identified as *B. hyodysenteriae* by PCR, but PCR will not provide the nuance of the weak haemolysis which would be detected if culture methods were used as well. Laboratories relying on culture methods and biochemical tests would identify such isolates as *B. intermedia* or *B. murdochii* depending on the presence or absence of indole production.

VIRULENT: TO BE OR NOT TO BE?

The fluidity of *Brachyspira* taxonomy and pronounced virulence differences within currently recognized *Brachyspira* species render attribution of virulence to a field isolate unreliable when merely based on species identification. Instead of focusing on proper identification as main indicator for virulence, a pragmatic approach may be more appropriate, one focused on virulence characteristics. Such approach might have important consequences for the pig industry, for example for pig trade. Currently, when a herd tests positive for *B. hyodysenteriae*, this compromises the trading possibilities of the farm regardless of the strain's virulence. The presence of avirulent strains should not compromise trading opportunities.

Reliable prediction of the pathogenic potential of a *B. hyodysenteriae* field isolate depends on a thorough knowledge of the pathogen's major virulence traits. However, since the mechanisms underpinning the pathogenesis of swine dysentery are largely unknown, few virulence traits have been unambiguously identified. Lipoproteins, haemolysins and genes involved in motility and chemotaxis have been linked with virulence in the past [62]. Although motility and chemotaxis undoubtedly play a role in effective colonisation of pigs, and therefore in a strain's virulence [30,53], *B. hyodysenteriae* strains with insufficient colonisation capacity would probably remain largely undetected in a farm due to their low presence in the colon. Besides, recent

analysis of whole genome sequences of *B. hyodysenteriae* isolates with different pathogenic potential has revealed very limited variation in genes associated with motility and chemotaxis [6], making it unlikely that genes involved in chemotaxis and motility are useful in distinguishing virulent and avirulent strains.

Although Black et al. demonstrated some genetic variation for lipoproteins and surface proteins, this genetic variation could not be linked with variation in virulence, which makes these genes unfavourable for distinguishing virulent and avirulent strains as well [6]. The presence of a plasmid and of six *rfb* genes on that plasmid was linked with virulence [34]. Complete absence of the plasmid has been shown in avirulent strains, but so far this has only been described for one laboratory strain (B78) and for one Australian field strain. Since the description of the specific genes on the plasmid, several field strains have been identified that either are virulent and lack some of these genes, or are less virulent and possess all the plasmid genes [35]. This makes the *rfb* plasmid genes unsuitable as virulence markers.

The haemolytic capacity of *B. hyodysenteriae* has been regarded a hallmark of its pathogenic properties ever since the first description of the species. Our research into the weakly haemolytic *B. hyodysenteriae* strain D28 [40] and the research of La et al. [35] into a weakly haemolytic *B. hyodysenteriae* strain isolated in Germany have reinforced the importance of haemolysis as a virulence trait, and have presented further genetic analysis of this virulence trait.

Substantial nucleotide and amino acid differences in the haemolysin III and haemolysin activation protein were revealed when haemolysis associated genes of our weakly haemolytic strain D28 and the German weakly haemolytic strain JR11 were compared to haemolysis associated genes of 20 *B. hyodysenteriae* strains. Additionally, strain JR11 showed an insertion mutation in the promoter region of *hlyA*. Although these three haemolysis associated genes are assumed to be a key factor in virulence of *B. hyodysenteriae,* due to the avirulence of the described strains on one side, and the unique nucleotide differences in these otherwise strongly conserved genes on the other side, definitive proof is lacking. This would require function tests of laboratory derived specific mutant strains which are very difficult to construct for *B. hyodysenteriae.*

Whole genome sequencing and the increasingly easier accessibility of this tool will undoubtedly promote the identification of virulence traits and their genetic background. Ideally the whole genome sequence of a large number of *B. hyodysenteriae* isolates, for which pathogenic potential has been decisively proven in experimentally infected pigs, should be analysed and compared, to identify virulence traits that predict a strain's virulence. Genetic information of these traits could be used in further design of diagnostic (q)PCRs or even serological tests.

WILL ANTIMICROBIAL THERAPY SURVIVE?

Worldwide, antimicrobial resistance against doxycyline, lincomycin and macrolides is high for *B. hyodysenteriae* [22,32,43,50]. For many swine producing countries, pleuromutilins are the only valid treatment option that is left. With the exception of the USA and Switzerland, levels of antimicrobial resistance for the pleuromutilins valnemulin and tiamulin are increasing as well [32,43]. In the USA and Switzerland, the lack of any such increase can probably be explained by the limited usage of these products as therapeutic agent against swine dysentery. In Switzerland, SD has only emerged after 2008 and in the USA, carbadox can still be used, although since 1 January 2017 it has been withdrawn for growth promoting purposes, and the FDA has recommended to prohibit the use of carbadox therapeutically for swine as well [10,11]. Antimicrobial resistance against pleuromutilins is expected to increase when pleuromutilin products are used more often, as exposure of *B. hyodysenteriae* to these antimicrobials will induce SNPs responsible for acquired antimicrobial resistance [22,24,51].

In Belgium, due to relatively high levels of acquired resistance against lincomycin and macrolides, pleuromutilins are the drugs of choice to treat SD. The existence of Belgian *B. hyodysenteriae* strains with acquired resistance against pleuromutilins, renders swine dysentery untreatable in some cases. If multi-resistant clonal complexes as we described further spread, this poses a genuine threat to swine industry [41].

It would be very helpful to install a monitoring program in which the epidemiology of acquired resistance of *B. hyodysenteriae* isolates is surveyed. The limited number of antimicrobial products that are available, should be used only in confirmed cases of SD, and after determining the MIC of the antimicrobial to be used.

The lack of CLSI-approved methods for susceptibility testing of *B. hyodysenteriae*, combined with a lack of standardized clinical breakpoints greatly impair the set-up for such control programs. Although there is a general consensus among researchers in the field of SD that broth dilution method is the most reliable technique for susceptibility testing, there seems to be no real prospect for CLSI authorisation in the near future (7th Conference on Colonic Spirochaetosis in Animals and Humans, Hannover, 2016). The use of antimicrobial products in food producing animals in general is under severe pressure, due to potential consequences for public health. Although currently there are few products using pleuromutilins in human medicine, veterinary use of pleuromutilins may select for resistance against other antibiotics in bacteria that are of zoonotic importance. Treatment of swine with pleuromutilins for instance may favour spread of *vga* or *cfr* genes in methicillin resistant *Staphylococcus aureus* (MRSA) or enterococci. These genes are often localized on plasmids or transposons that may spread horizontally. *Vga* genes encode cross resistance against pleuromutilins, streptogramin A and lincosamides, while *cfr* genes code for cross resistance against phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A. Some of those antimicrobial compounds are of critical importance in human medicine [64].

Undoubtedly, this continuing pressure on the use and on the effectiveness of the currently available antimicrobials for SD, strongly urges for the development of alternative measures to tackle this disease. As shown in figure 2 in the lower section, depopulation/repopulation and alternative treatments are additional options to manage pigs with swine dysentery. Although alternative treatments such as plant derived feed additives [1,39], may be beneficial for certain more chronically infected farms, it's unlikely that such additives will replace antimicrobial therapy in general. Depopulation/repopulation demands large financial efforts and as long as the purchase of carrier animals is difficult to avoid, it is unlikely that depopulation/repopulation will be used on a large scale in swine industry.

In the upper section of figure 2 the most important routes of infection are given. In some farms enhanced biosecurity or improved rodent control might still decrease the risk of *B. hyodysenteriae* infection [3,16,29]. However, in modern swine industry where biosecurity including rodent control is assumed to be adequate, more substantial improvement can be expected from either avoiding the purchase of carrier animals or from vaccines that prevent development of SD in animals.

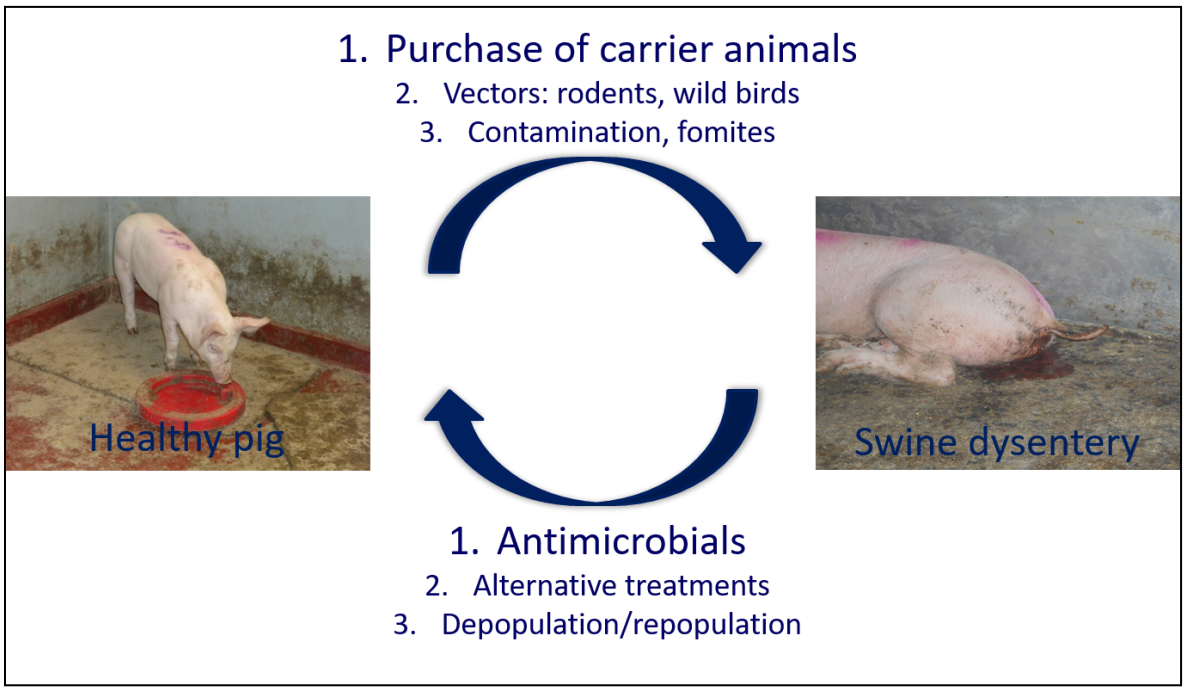

Figure 2: Swine dysentery: main routes of infection in the upper section, therapeutic options in the lower section.

ELISA: HOW TO BUY PIGS FREE OF SD?

Given that purchase of asymptomatic *B. hyodysenteriae* carriers is a major infection risk for a pig farm, the availability of a specific and sensitive screening tool for entry control, would be extremely helpful in the fight against SD [2,58]. As detection of *B. hyodysenteriae* in a farm will have substantial implications on the trading abilities of that farm, false positive results should be reduced to a minimal. Taking this into consideration, specificity might be more important for SD than sensitivity. Since *B. hyodysenteriae* is shed intermittently in low numbers, which do not always surpass the detection limit, detection systems based on presence of *B. hyodysenteriae* in faeces, are less effective and require a very large sampling number to reach some sensitivity. The number of *B. hyodysenteriae* is higher in samples of colonic contents compared to faecal samples. Therefore, colonic sampling at the abattoir would somewhat increase detection probability compared to faecal sampling on farm [18].

Serological detection of a *B. hyodysenteriae* infection at farm level would be a far more practical tool. As mentioned earlier, genetic differences between *Brachyspira* species are quite small. This is translated into a limited number of species-specific proteins,

which could serve as antigens for serological detection of *B. hyodysenteriae* infections using e.g. ELISA [57]. The wide spread presence of *B. innocens* in swine farms, and *B. murdochii* to a lesser extent, renders non-species specific *Brachyspira* antibodies to be quite present in pig herds [65].

An ELISA based on protein H114 has been developed [18,57]. In this ELISA the cut-off had to be set at five standard deviations above the mean, to maintain a specificity of 100%, seriously impeding the sensitivity. When using a lower cut-off, several farms which were considered negative for SD, were positive in the ELISA. Later it was shown that a number of farms considered to be healthy and free of SD which were positive in the Priocheck® *Brachyspira* porcine Ab ELISA, based on the protein H114, eventually proved to have *B. hyodysenteriae* carrier animals by culturing faeces or colonic abattoir samples [18]. However, this was true for 6 of 14 herds that were considered free of SD, but were positive on ELISA. In five other farms, carrier animals could not be detected, and three farms were not tested. Since there is no gold standard method for determining whether a herd is truly negative for *B. hyodysenteriae,* it is problematic to use these pig herds as a negative control in validation of ELISA under field conditions.

In conclusion, the screening of breeding farms for *B. hyodysenteriae* on a herd level using a specific ELISA, would be tremendously useful and desired by swine industry, but might proof to be exceptionally difficult to construct.

VACCINATION: HOW TO KEEP PIGS FREE OF SD?

A vaccine would be most welcome as an alternative or additional tool for swine veterinarians trying to obtain or maintain a SD free production unit. The most straightforward way of vaccine development is the production of whole cell bacterins. However, these induce little protection and are serotype specific [9,14,15,46]. Although in most parts of the world like the USA, Europe and Australia large serotype or serogroup diversity has been reported [4,19,21], serotype specific vaccines may have some use in areas where most field strains share the same serotype, for example in South-East-Asia. The on farm use of autogenous vaccines, which are generally speaking also whole cell bacterins, has been reported to have beneficial effects [23,47]. To justify a wider use of autogenous vaccines in swine industry, these vaccines should be evaluated experimentally more thoroughly.

Oral administration of live attenuated strains has the potential to reduce clinical signs or delay the spread of SD in a herd as demonstrated in our seeder vaccination experimental trial using a naturally attenuated *B. hyodysenteriae* strain and by Hyatt et al. (1994) who used a *tlyA* mutant strain of *B. hyodysenteriae* [26]. The drawbacks of using live attenuated strains as a commercial vaccine include the presumed difficulties of growing these strains on large scale. If they were to be developed into a commercial vaccine, methods for freeze-drying and preserving viability would certainly be the first concerns.

Alternatively, the use of recombinant proteins as demonstrated for *BmpB* [33] or a number of proteins identified by reverse vaccinology [56] could further be developed into a commercial vaccine. For *BmpB* a significant reduction of clinical SD has been demonstrated. Although in the study using *BmpB* as a vaccine no rise in colonic IgA was shown, subsequent research has been published about methods increasing local IgA production for this protein. Jiang et al. described the use of an M cell homing peptide fused with the BmpB protein to enhance antigen uptake by M-cells [28]. Furthermore different protein carriers have been described to enhance bioavailability of the protein [27,28,55]. For example the fusion of BmpB with M-cell homing peptide carried by microparticles induced a 24.7 fold increase in local IgA production in mice compared to using the BmpB protein alone [27].

The lack of extensive knowledge on parts of the pathogenesis of *B. hyodysenteriae* infections and the host immune responses, has hampered a more targeted development of efficient vaccines. Current growing availability of whole genome sequences of virulent and avirulent strains of *B. hyodysenteriae* and their comparison might enhance knowledge on detailed pathogenesis mechanisms by identification of proteins essential for virulence. Extended knowledge on the regulation of IgA production and its exact significance with regard to protection can further aid in the development of a vaccine for SD, regardless if it is based on live attenuated strains or on recombinant proteins.

CONCLUSION

Brachsypira hyodysenteriae infections will remain a challenge in the future. Newly emerging *Brachyspira* sp. that are pathogenic for swine ask for awareness and carefulness of diagnostic laboratories when they determine *Brachyspira* species identity. Diversity in pathogenic potential, not only between pig associated *Brachyspira* sp., but also within the species *B. hyodysenteriae* appeals for a diagnostic tool to detect virulence markers. This would allow swine veterinarians to estimate the pathogenic potential of a *B. hyodysenteriae* field isolate and the need for treatment or trading restrictions on a farm. Undoubtedly, increasing antimicrobial resistance will further push research towards vaccination and alternative treatments. Ideally, those newer treatments would be complemented with a reliable method for entry control such as ELISA.

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Summary

Summary

Swine dysentery (SD) is an economically important disease in swine producing countries worldwide, caused by the spirochete *Brachyspira hyodysenteriae.* A typical SD clinical outbreak is characterized by a bloody, mucoid diarrhea. Swine farms are often chronically infected, which leads to recurrent periods of diarrhea, mainly in growing pigs. The economic impact of SD arises from mortality, costs for treatment and from retarded growth and increased feed conversion. Furthermore, the mere presence of *B. hyodysenteriae* on a farm, even in the absence of overt clinical signs, negatively influences the trading opportunities for the farm in question.

Treatment of swine dysentery primarily exists of the administration of antimicrobial compounds. Lincomycin, doxycycline, macrolides such as tylosin or tylvalosin, or pleuromutilins such as valnemulin and tiamulin have been registered for treatment of SD. Unfortunately, increasing acquired resistance of *B. hyodysenteriae* for those compounds has been reported from different parts of the world. The use of antimicrobial compounds in livestock in general is under pressure, due to potential consequences for public health. A vaccine would be most welcome as an alternative or additional tool for swine veterinarians trying to obtain or maintain a swine dysentery free production unit.

The aims of this thesis were to establish a collection of *B. hyodysenteriae* strains, supplemented with strains from other swine associated *Brachyspira* spp, and to characterise this collection of strains. The minimal inhibitory concentrations (MIC) of six antimicrobial compounds against the *B. hyodysenteriae* strains were determined. One of the most important virulence factors, haemolysis, was phenotypically and genotypically further studied and the pathogenic potential of a weakly haemolytic *B. hyodysenteriae* strain was determined *in vivo.* Finally, the extent of protection conferred by this weakly haemolytic *B. hyodysenteriae* strain against infection with a virulent *B. hyodysenteriae* strain was determined.

In chapter 1, 50 *Brachyspira* isolates were collected and identified. For identification, the strong haemolysis that is shown when *B. hyodysenteriae* is grown on agar plates, was considered a hallmark for this *Brachyspira* species. The last decade two additional strongly haemolytic *Brachyspira* species have been identified: *B. suanatina,* reported from Scandinavia, and *B. hampsonii*, reported from USA and Canada. The description

of the first isolation of *B. hampsonii* in Europe in chapter 1, indicates that *B. hampsonii* should be included in diagnostic protocols in Europe as well.

To establish the presence of acquired antimicrobial resistance in the recent Belgian *B. hyodysenteriae* isolates from the first study, the minimal inhibitory concentration (MIC) of 6 antimicrobial compounds against these field isolates was determined in chapter 2. We show that over 50% of the field isolates harbour acquired resistance against pleuromutilins and over 70% of the field isolates harbour acquired resistance against doxycycline, lincomycin or macrolides. Sequence type analysis as determined by Multi Locus Sequence typing of the field isolates, revealed a novel clonal complex, containing multi-resistant isolates.

In chapter 3 we reveal that strong haemolysis, assumed to be a hallmark for *B. hyodysenteriae,* is not always present in *B. hyodysenteriae* strains. Quantitative *in vitro* analysis of the haemolytic capacity of field strains, shows that the degree of haemolysis can vary between *B. hyodysenteriae* strains. Sequence analysis of seven haemolysis associated genes of weakly and strongly haemolytic strains was performed. One weakly haemolytic strain, D28, showed marked differences in five of these haemolysis associated genes, mainly resulting in amino acid substitutions in tlyA, haemolysin III and the haemolysin activation protein.

To further investigate the importance of haemolytic capacity as a virulence trait of *B. hyodysenteriae,* this unique weakly haemolytic *B. hyodysenteriae* strain D28 was used in *in vivo* virulence trials, as described in chapter 4. Pigs colonised by strain D28 showed no clinical signs of SD, even if they shed strain D28 in high numbers. In contrast, for strongly haemolytic *B. hyodysenteriae* strains, faecal excretion as a proxy for intestinal colonisation, was shown to be consistently accompanied by clinical symptoms. Furthermore, pigs colonised by strain D28 showed no macroscopic or histological lesions on necropsy.

Given that strain D28 proved to colonize pigs, without causing clinical symptoms and that this strain is phenotypically and genotypically distinguishable from virulent field strains, this strain shows potential for use as a vaccine strain. Therefore the protective capacity of strain D28 was investigated in chapter 4. In a seeder vaccination model, three groups of animals were vaccinated by oral inoculation with *B. hyodysenteriae* strain D28 and three groups of animals were sham vaccinated by inoculation with culture medium without *Brachyspira.* Three weeks later, 50% of animals (seeder

animals) in each group were challenged by inoculation with a virulent *B. hyodysenteriae* strain. These seeder animals could infect the remaining animals (receiver animals) by faecal excretion of the challenge strain. Vaccination with strain D28 significantly slowed down the spread of swine dysentery in vaccinated animals compared to non-vaccinated animals. For vaccinated seeder animals there was a significant correlation between the presence of a strong local IgA response and the delay of onset of swine dysentery.

In conclusion, diagnosis of infections with *Brachyspira* spp. in swine has become less straightforward. For accurate species identification one should be aware of the possible presence of 'other' strong haemolytic *Brachyspira* spp. such as *B. hampsonii*, and it should be kept in mind that not all *B. hyodysenteriae* isolates will show strong haemolysis.

For swine veterinarians, in order to provide accurate advise on clinical importance of a strain, or on the trading implications of any given strain, a diagnostic tool differentiating between virulent and avirulent strains might become necessary. This diagnostic tool could be based in the haemolysis associated genes as described in chapter 3.

As antimicrobial resistance in *B. hyodysenteriae* is increasing worldwide, and in Belgium as seen in chapter 2, future treatment options for SD will probably evolve towards vaccination and alternative treatments. The significant correlation between local IgA response and delay in the development of SD as seen in chapter 4 provides directions for future vaccine development.

Samenvatting

Samenvatting

Varkensdysenterie is een wereldwijd verspreide ziekte die een grote economische impact heeft op de varkensindustrie. De aandoening wordt veroorzaakt door de spirocheet *Brachyspira hyodysenteriae* en de typische symptomen bij een uitbraak zijn bloederige, slijmerige diarree. Vaak zijn bedrijven chronisch besmet en treedt een cyclisch terugkerende periode van diarree op, voornamelijk bij de vleesvarkens. De economische gevolgen van een *B. hyodysenteriae* infectie zijn te wijten aan sterfte, kosten voor medicatie en vooral aan de vertraagde groei van aangetaste dieren en de gestegen voederconversie. Bovendien kan de aanwezigheid van *B. hyodysenteriae* op een bedrijf impact hebben op de handelsmogelijkheden van dit bedrijf, zelfs in afwezigheid van duidelijke symptomen.

Behandeling van dysenterie bestaat hoofdzakelijk uit toediening van antimicrobiële middelen. Naast lincomycine en doxycycline, kunnen macroliden zoals tylosine of tylvalosine, of pleuromutilinen zoals valnemuline en tiamuline worden ingezet. Helaas wordt uit verschillende delen van de wereld resistentie van *B. hyodysenteriae* tegen de meeste van deze middelen gemeld. Door de toenemende resistentie, maar ook door de toenemende maatschappelijke druk op het gebruik van antimicrobiële middelen bij voedselproducerende dieren in het algemeen, staat de traditionele behandeling van dysenterie onder druk. De beschikbaarheid van een vaccin zou een zeer welkome aanvulling op of alternatief voor de traditionele behandeling vormen.

Het doel van deze thesis was om een verzameling aan te leggen van *B. hyodysenteriae* stammen, aangevuld met stammen van andere, bij varkens voorkomende *Brachyspira* sp. en deze te karakteriseren. De minimale inhibitorische concentratie (MIC) van zes antimicrobiële middelen werd bepaald voor de *B. hyodysenteriae* stammen. Eén van de belangrijkste virulentiefactoren, hemolyse, werd fenotypisch en genotypisch onderzocht. Het pathogeen effect van een *B. hyodysenteriae* stam met zwakke hemolyse werd nagegaan *in vivo.* Tenslotte werd ook onderzocht of deze stam beschermende immuniteit kon induceren in een “seeder”-vaccinatie model.

In hoofdstuk 1 werden 50 *Brachyspira* isolaten verzameld en geïdentificeerd. Van oudsher werd de sterke hemolyse bij groei op cultuurplaat van *B. hyodysenteriae* aanzien als kenmerkend voor deze pathogene species. De laatste jaren zijn er bijkomend twee sterk hemolytische, pathogene species geïdentificeerd: *B. suanatina*,

die voornamelijk in Scandinavië wordt gerapporteerd en *B. hampsonii* die wordt gerapporteerd in de VSA en Canada. De beschrijving van het voorkomen van *B. hampsonii* in België in hoofdstuk 1 toont aan dat er ook in Europa aan deze species aandacht moet besteed worden.

Om het voorkomen van verworven antibioticaresistentie na te gaan bij Belgische *B. hyodysenteriae* isolaten uit de eerder aangelegde collectie, werd voor elk van deze isolaten de Minimum Inhibitorische Concentratie (MIC) van 6 antimicrobiële middelen bepaald zoals beschreven in hoofdstuk 2. Uit de analyse van de MICs van deze isolaten blijkt dat meer dan 50% van de isolaten verworven resistentie vertoont tegen pleuromutilinen en meer dan 70% van de isolaten tegen doxycyline, lincomycine of macroliden. Uit analyse van de aanwezige sequentietypes zoals bepaald met behulp van Multi Locus Sequence Typing, blijkt dat sommige Belgische stammen klonale complexen vormen die bestaan uit multiresistente isolaten.

In hoofdstuk 3 wordt aangetoond dat de sterke hemolyse die onlosmakelijk verbonden leek met *B. hyodysenteriae*, niet altijd aanwezig is. Door de hemolytische capaciteit van verschillende stammen te bepalen en te vergelijken, hebben we kwantitatief kunnen aantonen dat de hemolyse, die bij groei op plaat eerder zwak lijkt voor sommige *B. hyodysenteriae* stammen, inderdaad significant lager kan zijn.

Sequentie analyse van zeven met hemolyse geassocieerde genen van sterk en zwak hemolytische *B. hyodysenteriae* stammen toonde aan dat één van de zwak hemolytische stammen, D28, nucleotideverschillen had in vijf van deze genen. Dit resulteerde in aminozuur substituties in tlyA, hemolysine III en in het hemolysine activatie eiwit.

Om het belang van hemolyse als virulentiefactor verder te onderzoeken werd in hoofdstuk 4 de virulentie van de zwak hemolytische *B. hyodysenteriae* stam D28 getest. Bij varkens die gekoloniseerd werden door deze stam werden geen ziektetekens waargenomen, ondanks uitscheiding van hoge aantallen van deze stam in de mest. Dit in tegenstelling tot virulente, sterk hemolytische stammen waarbij kolonisatie en uitscheiding in de mest steeds gepaard gingen met het ontwikkelen van dysenterie. Ook vertoonden varkens, gekoloniseerd door zwak hemolytische stam D28, geen macroscopische of histologische letsels ter hoogte van het colon.

Omdat stam D28 dus varkens koloniseert zonder symptomen te veroorzaken en omdat deze stam zowel genotypisch als fenotypisch te onderscheiden is van virulente veldstammen, zou deze stam geschikt kunnen zijn als vaccinstam. Daarom wordt in hoofdstuk 4 ook de beschermende capaciteit van stam D28 nagegaan. In een seeder vaccinatie model werden drie groepen varkens gevaccineerd door orale toediening van een cultuur van D28. Drie placebo groepen werden oraal geïnoculeerd met cultuurmedium zonder stam D28. Drie weken later werd de helft van alle dieren ("seeder" dieren) in een groep geïnoculeerd met een virulente *B. hyodysenteriae* stam. Deze "seeder" dieren zorgden voor besmetting van de andere dieren in de groep ("receiver dieren") door uitscheiding van de virulente stam in de mest.
Vaccinatie met stam D28 reduceerde de spreiding van dysenterie binnen een groep dieren. In vergelijking met de groepen waar placebo was toegediend, verliep de spreiding van dysenterie trager in de groepen gevaccineerde dieren. Bij de gevaccineerde "seeder" dieren trad dysenterie significant later op bij die dieren, die na contact met de challengestam een sterke lokale IgA respons vertoonden.

Als conclusie kan gesteld worden dat de diagnose van *Brachyspira* infecties bemoeilijkt wordt door de variabele mate van hemolyse die *B. hyodysenteriae* stammen vertonen en door de aanwezigheid van andere, sterk hemolytische species zoals *B. hampsonii*.

Opdat varkens dierenartsen juiste adviezen kunnen formuleren aangaande het klinisch belang van een *B. hyodysenteriae* isolaat, en daarmee gepaard gaande de handelsmogelijkheden van een bedrijf, is een diagnostische test die het onderscheid maakt tussen virulente en niet-virulente *B. hyodysenteriae* stammen noodzakelijk. Zo een test kan bijvoorbeeld gebaseerd zijn op de met hemolyse geassocieerde genen, zoals beschreven in hoofdstuk 3.
Omdat antimicrobiële resistentie van *B. hyodysenteriae* wereldwijd toeneemt en ook in België een probleem vormt (hoofdstuk 2), zullen in de toekomst allicht alternatieve maatregelen moeten aangewend worden voor de zal de bestrijding van dysenterie, zoals vaccinatie. Voor vaccinontwikkeling kan de bevinding dat vertraging van het ontwikkelen van dysenterie significant gecorreleerd is met een lokale IgA respons, zoals besproken in hoofdstuk 4, een goede basis vormen voor verder onderzoek.

Curriculum vitae

Curriculum Vitae

Maxime Mahu was born on August 9, 1981 in Sint Niklaas. In 2005 she obtained her Master's degree in Veterinary Medicine with distinction at Ghent University Gent. As from 2006 she worked in different small animal clinics in Belgium and the Netherlands.

In 2011 she started her PhD research at the department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University on an IWT Agriculture project. This research focused on the optimisation of the control of *Brachyspira* infections in pigs. This was extended into further research on vaccine development for swine dysentery funded by the Industrial Research Fund of Ghent University.

Maxime Mahu is (co-)author of 11 publications in international peer-reviewed journals and has given multiple oral/poster presentations at (inter-)national conferences.

Curriculum Vitae

Maxime Mahu werd geboren op 9 augustus 1981 te Sint Niklaas. Na het behalen van het diploma hoger secundair onderwijs aan het Jansenius college in Hulst, Nederland, behaalde zij in 2005 het diploma van Master in de Diergeneeskunde aan de Universiteit Gent met onderscheiding.
In datzelfde jaar trad zij korte tijd in dienst als doctoraatstudent aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde, Universiteit Gent. In 2006 begon zij te werken als dierenarts kleine huisdieren in verschillende praktijken in België in Nederland om uiteindelijk 4 jaar verbonden te blijven als dierenarts aan dierenkliniek WHG Bron in Alkmaar. Gebeten door onderzoek keerde zij in 2011 terug naar de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde Universiteit Gent om een doctoraatstudie te starten over *Brachyspira hyodysenteriae* bij varkens. De doctoraatsstudie werd gefinancierd door het Agentschap voor Innovatie door Wetenschap en Technologie (IWT LO 100850) en het Industrieel Onderzoeks Fonds (F2014/ConcepTT/272 , F2015/Advanced/159)
Maxime Mahu is (co-)auteur van 11 publicaties in internationale tijdschriften en gaf meermaals presentaties op (inter)nationale congressen.

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SOMS HEB JE VAN DIE DROMEN
DIE JE WAKKER HOUDEN

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