Characterization of wild-type and laboratory attenuated "*Brachyspira hampsonii*"

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Abstract

This thesis aimed to characterize Western Canadian "*Brachyspira hampsonii*" clinical isolates and to develop and characterize an attenuated "*B. hampsonii*" vaccine candidate.

Clinical "*B. hampsonii*" isolates selected from different Western Canadian barns were described biochemically and by sequencing the NADH-oxidase (*nox*) gene to improve the information available about clinical isolates present in Western Canada. The biochemical profiles of two clade I and eight clade II isolates were compared and variability in some diagnostic biochemical markers (*β*-glucosidase and hippurate hydrolysis) was detected. Representative isolates of clade I and II "*B. hampsonii*" were also compared to other swine-associated *Brachyspira* spp. based on biochemical profiles and morphometric data. Cell dimensions, number of flagella, and select biochemical markers (hippurate hydrolysis, *α*-glucosidase, and *β*-glucosidase) were not sufficient to discern "*B. hampsonii*" from other *Brachyspira* species. The data highlighted that consistent biochemical characteristics (indole and *α*-galactosidase negative) of "*B. hampsonii*" and morphology were not sufficient to differentiate them from other *Brachyspira* spp. and that *nox* gene sequencing is a more reliable tool for identification and differentiation of *Brachyspira* species.

Development of a potential vaccine strain was also carried out by serial passage of a virulent "*B. hampsonii*" clade II strain (passage 13 (P13)) for 100 passages, resulting in strain P113. The virulent P13 and serially passaged P113 strains were phenotypically and genotypically compared to identify any changes in P113. Phenotypes of P13 and P113, including the biochemical profile, *β*-haemolysis, growth profile, and virulence in a murine model, were compared to identify

changes. More extensive β-haemolysis and more rapid growth of P113 over P13 was observed suggesting laboratory adaptation after serial passage. Two experiments were performed to assess the virulence of P113 and P13 in a murine model, which both demonstrated a decrease of P113 virulence, suggesting that partial attenuation was achieved. No difference between the virulent and attenuated strains was detected based on genomic profiling by random amplified polymorphic DNA (RAPD) or whole genome sequence comparison.

The results of this thesis demonstrate the complexity of phenotypic identification and discrimination of "*B. hampsonii*" and other swine-associated *Brachyspira* species. Serial passage of a virulent "*B. hampsonii*" resulted in a laboratory adapted, partially attenuated strain that will be attenuated further and used as a potential vaccine. This serially passaged strain also provides opportunities for further studies to identify virulence determinants, which can aid immensely with prevention and treatment of "*B. hampsonii*".

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1 Introduction and Literature review

1.1 Swine-associated *Brachyspira*

The genus *Brachyspira* consists of Gram-negative, aerotolerant, motile, and *β*-haemolytic spirochaetes. The progress of the formation of the genus originated with *Treponema*, then *Serpula*, *Serpulina*, and *Brachyspira* (combination of existing *Serpulina* and *Brachyspira* genera) (Harris *et al.*, 1972, Stanton *et al.*, 1991, Stanton, 1992, Ochiai *et al.*, 1997). Culture of *Brachyspira* spp. is performed anaerobically on variants of blood agar and in broth supplemented with fetal bovine serum or with fetal bovine serum and sheep blood (Joens and Kinyon, 1982, Jenkinson and Wingar, 1981, Kunkle and Kinyon, 1988). Electron microscopy of several *Brachyspira* spp. revealed cells 5 to 9.78 μm long and 0.27 to 0.36 μm wide with 8-16 periplasmic flagella per cell (Harris and Kinyon, 1972, Trott et al., 1996c, Stanton et al., 1997, Oxberry et al., 1998, Fellström et al., 1999, Mushtaq et al., 2015). *Brachyspira* spp. with standing in nomenclature and that are pathogenic in swine are *B. hyodysenteriae*, *B. pilosicoli*, and *B. murdochii*. "*B. suanatina*" is a proposed *Brachyspira* species that has only been found in parts of northern Europe where it has been isolated from pigs in Sweden and Denmark and mallards in Sweden (Råsbäck et al., 2006, Råsbäck et al., 2007a, Mushtaq et al., 2015). Conventional pigs experimentally infected with a clinical isolate of "*B. suanatina*" from pigs or isolated from mallards led to dysentery-like signs and mild diarrhea, respectively (Råsbäck et al., 2007a, Mushtaq et al., 2015). Another pathogenic *Brachyspira*, for which the name "*B. hampsonii*" has been proposed is described in detail in section 1.1.2.. *B. innocens* and *B. intermedia* are non-pathogenic species that are also found in swine.

Brachyspira hyodysenteriae is the causative agent of swine dysentery and was the first characterized species of the genus, isolated from swine in 1972 (Harris *et al.*, 1972). *B. hyodysenteriae* is strongly *β*-haemolytic, has 8 to 12 total periplasmic flagella, and is the most studied of the swine-pathogenic *Brachyspira* spp. (Trott *et al.*, 1996c). The history of *B. hyodysenteriae* is discussed in more detail below.

Milder disease (porcine intestinal colitis) is caused by *B. pilosicoli*, which can also cause intestinal colitis in humans, intestinal spirochaetosis in chickens, chronic diarrhea in weanling horses, and colonization of the intestines of dogs (Trott *et al.*, 1996c, Fellström *et al.*, 2001, Oxberry and Hampson, 2003, Hampson *et al.*, 2006, Bait-Merabet *et al.*, 2008). *B. pilosicoli* is weakly *β*-haemolytic, has 8 to 12 total periplasmic flagella, and was formerly known as "*Anguilina coli*", then *Serpulina pilosicoli* (Lee and Hampson, 1994). *B. pilosicoli* is associated with diarrhea in humans in developing nations and, in some rare cases, spirochaetemia (spirochaete invasion of the bloodstream) in immunocompromised patients, which is of great concern due to the potential zoonosis of *B. pilosicoli* (Mikosza and Hampson, 2001, Bait-Merabet *et al.*, 2008). In swine, *B. pilosicoli* infects the mucosal surface of the colonic epithelium, forming a dense layer of spirochaetes observed as a "false brush border" when viewed under microscopy (Trott *et al.*, 1996c). The colons of swine infected with *B. pilosicoli* exhibit gross lesions and watery, mucoid contents (Trott *et al.*, 1996b).

B. murdochii, a weakly *β*-haemolytic spirochaete, was originally considered to be non-pathogenic but has been reported as an agent of catarrhal colitis (Jensen *et al.*, 2010). While disease linked to *B. murdochii* has also been proposed to be due to high spirochaetal load (Weissenböck

et al., 2005, Komarek *et al.*, 2009). *B. murdochii*, on multiple occasions, has been the most prevalent *Brachyspira* spp. recovered from swine with diarrhea in Spain and Portugal (Osorio *et al.*, 2013) and in the USA (Warneke *et al.*, 2014). A study of *Brachyspira* spp. colonizing healthy pigs on a farm with a history of *Brachyspira* diarrhea based on culture and identification by partial NADH-oxidase (*nox*) gene sequencing, revealed a high prevalence of *B. murdochii*-like isolates (Patterson *et al.*, 2013). Among the isolates characterized in this study were strongly *β*-haemolytic *Brachyspira* highly similar to *B. murdochii* based on *nox* sequence, which suggests that there is variance in the haemolysis trait within *B. murdochii* (Patterson *et al.*, 2013).

Traditionally *B. innocens* is not thought to cause disease in pigs, but is able to colonize the colon of pigs (Kinyon and Harris, 1979). However, experimental colonization of gnotobiotic and conventional pigs with *B. innocens* led to mucoid faeces and typhlocolitis (inflammation of the colon and caecum) in the gnotobiotic pigs, but not conventional pigs, which suggests that *B. innocens* may have the potential to cause disease under certain conditions (Neef *et al.*, 1994).

Phylogenetic analysis of the genus has demonstrated that there is great diversity within the genus and even within species, and that distinct *Brachyspira*-like taxa with no obvious species affiliation can be isolated routinely from pigs (Råsbäck *et al.*, 2007b, Backhans *et al.*, 2010). Partial *nox*-sequencing of isolates cultured from a farrow-to-finish farm in western Canada indicated that a mixture of *Brachyspira* spp. were present at each production stage with more *B. murdochii* and unidentified *Brachyspira* taxa detected than other species (Patterson *et al.*, 2013). The presence of species mixtures in individual animals and the enormous diversity of *Brachyspira* are added challenges for understanding *Brachyspira* spp. disease in pigs. Weissenböck *et al.* (2005)

postulated that the spirochaete load is a key factor in the production of disease in pigs, regardless of the *Brachyspira* spp. present within the colon, supporting the idea that *Brachyspira* disease is not solely due to a single *Brachyspira* species. The potential importance of *Brachyspira* communities is also illustrated by an investigation of pigs from herds with chronic health problems including wasting and diarrhea where *Brachyspira* detection was common (13% of 202 animals tested) and 37% of *Brachyspira* infected pigs were colonized by two or more *Brachyspira* spp. (Komarek *et al.*, 2009).

1.1.1 *Brachyspira hyodysenteriae* and swine dysentery

Swine dysentery (mucohaemorrhagic diarrhea) was first described in 1921 and the aetiologic agent was subsequently identified in 1972 by electron microscopy to be a spirochaete and named *Treponema hyodysenteriae* (Harris and Kinyon, 1972, Harris *et al.*, 1972). In 1972, Harris and Glock (1972) showed that experimental infection with *T. hyodysenteriae* leads to swine dysentery in conventional swine. In the years following its initial isolation and characterization, *T. hyodysenteriae* was assigned to the genus *Serpula*, then *Serpulina*, and finally *Brachyspira* (Stanton *et al.*, 1991, Stanton, 1992, Ochiai *et al.*, 1997).

High morbidity and lower mortality of approximately 90% and 30%, respectively, have been reported for on-farm outbreaks of *B. hyodysenteriae* (Harris and Glock, 1973). The impact of swine-associated *Brachyspira* disease leads to poor feed conversion and growth rates, which negatively affects the economic return from the herd. Another problematic aspect of the disease is the cyclical pattern of disease where disease re-emerges in herds that have undergone treatment,

or affects replacement animals after depopulation of infected barns (Harris *et al.*, 1999). Prevention of recurrence and eradication of *Brachyspira* spp. from affected farms is also complicated by the fact that the agent survives in animal and environmental reservoirs including feral pigs, mice, waterfowl, soil, water, and faeces (Joens and Kinyon, 1982, Boye *et al.*, 1998, Oxberry *et al.*, 1998, Phillips *et al.*, 2009, Desrosiers, 2011, Martínez-Lobo *et al.*, 2013, Rubin *et al.*, 2013b). Subclinically infected pigs are another potential source of future outbreaks (Duff *et al.*, 2014).

In infected animals, spirochetes are found in close proximity to the luminal surface of the colon and within colonic crypts (Glock *et al.*, 1974). Putative virulence factors identified to date include haemolysis, cytolysis, motility, and endotoxins (Nibbelink and Wannemuehler, 1991, Muir *et al.*, 1992, Milner and Sellwood, 1994). However, specific genetic determinants and mechanisms for these putative virulence factors have not been elucidated. Complicating the understanding of *B. hyodysenteriae* virulence is that swine dysentery is a multi-factorial disease, requiring environmental stressors such as cold temperatures, transportation stress, poor hygiene, and overstocking (Brandenburg *et al.*, 1977, Burrough, 2016). It has also been suggested that availability of excess fermentable substrates in the diet reaching the colon can contribute to the expression of dysentery (Pluske *et al.*, 1996). Further support for the multi-factorial nature of swine dysentery is found in the results of studies demonstrating that singular infection of gnotobiotic pigs with *B. hyodysenteriae* does not lead to dysentery, but co-infection with other anaerobic bacteria does (Meyer *et al.*, 1975, Brandenburg *et al.*, 1977).

1.1.2 Re-emergence of mucohaemorrhagic diarrhea in Western Canada

A re-emergence of mucohaemorrhagic diarrhea and colitis was noted by producers and veterinarians in North America beginning in 2007 (Harding *et al.*, 2010, Burrough *et al.*, 2012b, Hampson *et al.*, 2015). The rise of antibiotic resistance has been suggested as a component of the re-emergence of mucohaemorrhagic diarrhea, with greater *Brachyspira* survival after antibiotic treatment (Hidalgo *et al.*, 2011, Rugna *et al.*, 2015). Subclinical disease may have also permitted persistent infections that led to outbreaks due to changes in the environmental conditions (temperature stress, poor hygiene, crowding, transportation), diet (type of carbohydrate, concentration of feed additives), or spirochaete load (O' Connor *et al.*, 1979, Pluske *et al.*, 1998, Durmic *et al.*, 2002, Weissenböck *et al.*, 2005, Hansen *et al.*, 2011, Hampson *et al.*, 2015, Burrough, 2016). The cessation of use of specific antimicrobials such as carbadox (sale stoppage in Canada in 2001) may also be linked to the re-emergence of mucohaemorrhagic diarrhea due to *Brachyspira* spp. (Hampson *et al.*, 2015). Reservoirs of *Brachyspira* spp. in feral pigs, mice, waterfowl, soil, and water may have also provided *Brachyspira* protection against eradication and enabled unknown changes to occur in these reservoirs in the spirochaetes that contributed to the re-emergence (Joens and Kinyon, 1982, Boye *et al.*, 1998, Oxberry *et al.*, 1998, Phillips *et al.*, 2009, Desrosiers, 2011, Martínez-Lobo *et al.*, 2013, Rubin *et al.*, 2013b). The re-emergence of mucohaemorrhagic diarrhea has been multi-faceted and complex, adding to the challenge of understanding *Brachyspira*-associated disease. Another major contributor to resurgence of mucohaemorrhagic diarrhea as a production-limiting disease has been the emergence of at least one new species of *Brachyspira.*

The agent responsible for the re-emergence of dysentery-like disease in North America, was identified in clinical material using NADH-oxidase (*nox*) gene sequencing (Harding *et al.*, 2010). Sequences of partial *nox* gene PCR amplicons from these cases were found to be only 92% identical to any known *Brachyspira* sp. (Harding *et al.*, 2010). Results of case-control studies supported a strong association between detection of this *nox* sequence and mucohaemorrhagic diarrhea in pigs free of *B. hyodysenteriae, B. pilosicoli,* and *Lawsonia intracellularis* (Harding *et al.*, 2012). Further investigation revealed two separate clades (clades I and II) within the novel species that could be resolved through *nox* and 16S rRNA gene sequencing (Chander *et al.*, 2012) and it was established that like *B. hyodysenteriae,* the new spirochete was strongly *β*-haemolytic. The name "*B. hampsonii*" has been proposed for this novel species (Chander *et al.*, 2012, Mirajkar *et al.*, 2016).

Burrough *et al.* (2012b) demonstrated that "*B. hampsonii*" fulfilled Koch's postulates when conventional pigs inoculated with clinical isolates of "*B. hampsonii*" developed mucohaemorrhagic diarrhea and spirochaetes from infected animals were isolated and identified to be "*B. hampsonii*". Subsequent experimental inoculations of conventional pigs with either tissue homogenate or a clinical isolate of clade II "*B. hampsonii*" (D09-30446) produced mucohaemorrhagic diarrhea and "*B. hampsonii*" D09-30446 was recovered from these experimentally inoculated pigs (Rubin *et al.*, 2013a). Similar experiments established the pathogenicity of *"B. hampsonii*" clade I (Burrough *et al.*, 2012b, Costa *et al.*, 2014b). The clinical and pathological presentation of disease following infection with either clade I or clade II *"B. hampsonii*" was indistinguishable from swine dysentery.

1.1.2.1 *Global* "B. hampsonii" *distribution*

The distribution of "*B. hampsonii*" is challenging to describe due to the many reservoirs (feral pigs, mice, waterfowl, soil, water, and fomites) and methods of transport (migrating waterfowl and pig transport) that have been identified. However, based on investigations to date, "*B. hampsonii*" is widely distributed across North America and has also been detected in Europe (Phillips *et al.*, 2007, Chander *et al.*, 2012, Martínez-Lobo *et al.*, 2013, Rubin *et al.*, 2013a, Rubin *et al.*, 2013b, Rohde *et al.*, 2014, Mirajkar *et al.*, 2015). Detection of "*B. hampsonii*" from Czech pigs in Belgium and Belgian pigs in Germany highlights that transport of pigs plays a potentially significant role in widening the distribution of the spirochaetes among farms (Mahu *et al.*, 2014, Rohde *et al.*, 2014). "*B. hampsonii*" has been detected in three species of waterfowl to date: lesser snow geese, greylag geese, and mallards (Martínez-Lobo *et al.*, 2013, Rubin *et al.*, 2013b). It is very likely that the distribution of "*B. hampsonii*" and other *Brachyspira* spp. is wider than currently acknowledged but information is lacking. Knowledge of distribution of *Brachyspira* spp. has been primarily through passive surveillance (voluntary reporting and diagnostic sample submission by producers and veterinarians), so its true prevalence remains unknown (Hampson *et al.*, 2015).

In North America, a difference in clade distribution has been noted for unknown reasons, with more clade I "*B. hampsonii*" detected in the United States of America and more clade II "*B. hampsonii*" in Western Canada (Mirajkar *et al.*, 2015, Perez *et al.*, 2016). In Europe, clade I "*B. hampsonii*" has been detected in pigs in Belgium and Germany, while the clade affiliation of isolates from waterfowl in Spain has not been specified (Mahu *et al.*, 2014, Rohde *et al.*, 2014,

Martínez-Lobo *et al.*, 2013). One particular isolate (P280/1) from the United Kingdom in the 1990s was labelled at the time as a non-*B. hyodysenteriae* strain, but was recently suggested to belong to "*B. hampsonii*" (Neef *et al.*, 1994, Mirajkar *et al.*, 2015). A recent retrospective study of clinical submissions in western Canada showed that the earliest cases of "*B. hampsonii*" clade I and II in western Canada were 2002 and 2006, respectively, and each involved a single farm (Fernando *et al.*, unpublished). More information on the distribution of "*B. hampsonii*" is needed to improve diagnosis and ensure that appropriate tools are available and utilized to control future outbreaks.

1.1.2.2 *Diagnostics of clinical material and tools for* Brachyspira *detection and identification*

Identification of the cause of mucohaemorrhagic diarrhea and other *Brachyspira*-related disease is important for appropriate treatment of herds. Diagnosis may be performed based on some combination of clinical signs, gross and histopathology, microscopic detection of spirochaetes, culture of spirochaetes from faeces or colonic tissue and differentiation of strength of *β*-haemolysis (Burrough, 2016). Histology has been useful for the detection of spirochaetes and determining their location within tissues after necropsy of pigs with disease, while lesions found during necropsy provides more information about the degree of illness and progress of disease (Hughes *et al.*, 1977, Kinyon *et al.*, 1977). Advances in laboratory detection and identification of *Brachyspira* have been made with the addition of biochemical assays, genome profiling tools, polymerase chain reaction (PCR), quantitative PCR, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, enzyme-immunoabsorbent assays (ELISA), and fluorescence *in situ* hybridization (FISH). Routine detection of "*B. hampsonii*" and other

Brachyspira spp. has shifted towards the use of tools such as PCR and MALDI-TOF, which provide differentiation capabilities that bolster traditional diagnostic methods (Burrough, 2016).

Microscopy has been useful for identifying isolated spirochaetes, although it is not useful for differentiating *Brachyspira* species due to similar sizes and morphologies (Hughes *et al.*, 1977, Perez *et al.*, 2016). Haemolysis strength is a characteristic used for differentiating cultured *Brachyspira* spp. from samples, as strong *β*-haemolysis suggests that an isolate may be *B. hyodysenteriae*, "*B. hampsonii*", or "*B. suanatina*" (Chander *et al.*, 2012, Råsbäck *et al.*, 2007a). However, variation in strength *β*-haemolysis has been observed among isolates of *B. hyodysenteriae* and *B. pilosicoli*, making haemolysis strength a less reliable option for classification of *Brachyspira* spp. as "strongly" or "weakly" *β*-haemolytic (Mahu *et al.*, 2016). Biochemical characterization and multi-locus sequence typing (MLST) have been used to characterize "*B. hampsonii*" isolates further and, in some cases, improve differentiation of strains, but are not used as the primary tools for detection and identification of "*B. hampsonii*" (Chander *et al.*, 2012). MLST has proven useful for investigations aimed at elucidating global distribution and population structure of "*B. hampsonii*" (Mirajkar *et al.*, 2015).

The NADH-oxidase (*nox*) gene is the preferred genetic marker for *Brachyspira* compared to the 16S rRNA gene as phylogenetic analysis with the *nox* gene sequence provides greater discrimination of *Brachyspira* spp. (Chander *et al.*, 2012). Genus-specific PCR primers facilitate the amplification of *nox* sequences from any *Brachyspira* spp. (Atyeo *et al.*, 1999), and so *nox* PCR followed by sequencing has become a mainstay in diagnostic laboratories. Various species-specific PCR, real-time quantitative PCR assays and restriction fragment length polymorphism

(RFLP) approaches have been developed based on the *nox* gene sequence (Rohde *et al.*, 2002, Song and Hampson, 2009, Rohde and Habighorst-Blome, 2012, Aller-Morán *et al.*, 2016).

MALDI-TOF generates spectra profiles for isolates that are compared to a reference database. While it has been shown to be effective for identification of *Brachyspira* species, it cannot reliably differentiate "*B. hampsonii*" clades (Calderaro *et al.*, 2013, Warneke *et al.*, 2014). The initial set-up costs for MALDI-TOF are also limiting, although analysis cost per sample once the equipment is in place is extremely low.

Serological ELISA has been developed to detect serum antibodies against *B. hyodysenteriae* with high specificity and sensitivity (Song *et al.*, 2015). ELISAs are useful for detecting pathogen exposure even in clinically healthy animals, but this method is not suited for detection of *B. hyodysenteriae* infection during an active outbreak since time is required for seropositivity to occur. Furthermore, "*B. hampsonii*" antigens still need to be identified in order to adapt the method for detection of serum response against "*B. hampsonii*" (Hampson *et al.*, 2016).

Fluorescence *in situ* hybridization has also been utilized to specifically detect "*B. hampsonii*" or other *Brachyspira* spp. in histologic sections of formalin-fixed colonic tissue from infected pigs (Burrough *et al.*, 2013), but this approach is also not practical for routine clinical diagnostics.

1.1.2.3 *"B. hampsonii" in swine and virulence determinants*

In swine, "*B. hampsonii*" causes mucohaemorrhagic colitis and has been observed in the crypts of the colon of infected animals (Rubin *et al.*, 2013a). The pathogenesis of *Brachyspira* including "*B. hampsonii*" is not yet well understood, as the determinants of virulence have not been elucidated. Some putative virulence determinants have been suggested for the genus, including haemolysis, motility, and lipopolysaccharides (Nuessen *et al.*, 1982, Halter and Joens, 1988, Greer and Wannemuehler, 1989, Bellgard *et al.*, 2009, Naresh and Hampson, 2010).

Haemolysis, as observed on agar containing blood, is suggestive of the capacity for cytotoxicity against enterocytes and other cell types (Muir *et al.*, 1992), and several putative haemolysins have been identified (ter Huurne *et al.*, 1994, Hsu *et al.*, 2001, Wanchanthuek *et al.*, 2010). Strong *β*-haemolysis has been generally associated with greater virulence (Hyatt *et al.*, 1994), and indeed the species that cause most severe disease in pigs (*B. hyodysenteriae, "B. hampsonii"* and "*B. suanatina*") are all defined as strongly haemolytic. However, identification of atypical isolates of *B. hyodysenteriae* and "*B. hampsonii*" with moderate or weak *β*-haemolysis has demonstrated that variance in the *β*-haemolysis trait exists and thus it may not always be reliable for indicating virulence of an isolate (Rubin *et al.*, 2013b, Mahu *et al.*, 2016).

Snake-like motility driven by periplasmic flagella allows *Brachyspira* spp. to navigate through viscous material and mucus, while chemotaxis guides the spirochaete to colonize the surface or crypts of the swine colon (Kennedy *et al.*, 1988). "*B. hampsonii*" have been observed in colonic crypts, where they are suspected to cause disease by some unknown mechanisms, and with snake-like motility under microscopy (Burrough *et al.*, 2013, Rubin *et al.*, 2013a).

Lipopolysaccharides are another group of virulence determinants that have been shown to be mostly homologously reactive with sera from rabbits exposed to whole cell extracts or pigs with swine dysentery due to *B. hyodysenteriae*, and some are toxic to mice and cultured murine cells (Nuessen *et al.*, 1983, Halter and Joens, 1988, Greer and Wannemuehler, 1989). However, variation of lipopolysaccharides between serotypes and species may permit colonization and disease if *Brachyspira* immunity is induced only by highly-specific antigens (Halter and Joens, 1988, Lee and Hampson, 1999, Hidalgo *et al.*, 2010). Panels of lipooligosaccharides have also been useful for detecting *B. hyodysenteriae* in herds by ELISA, although lipooligosaccharides from sera from pigs exposed to other *Brachyspira* do not react with the antibodies (Joens *et al.*, 1982).

In addition to specific features of *Brachyspira* pathogens, other extrinsic factors have been suggested to be important determinants of virulence of *Brachyspira* spp. in swine. Early experiments investigating *Brachyspira* pathogenesis showed that infection of gnotobiotic pigs with *B. hyodysenteriae* alone or co-infection with *B. hyodysenteriae* and *Vibrio coli* failed to cause disease (Brandenburg *et al.*, 1977). The presence of other anaerobic bacteria was later suggested as an important factor in the virulence of certain *Brachyspira* spp., since *B. hyodysenteriae* co-infection with select anaerobic bacteria led to disease in gnotobiotic pigs, which did not become ill after sole *B. hyodysenteriae* infection (Whipp *et al.*, 1979). These observations suggest that at least some of the pathogenicity of *Brachyspira* is due to secondary infection once the integrity of the colon epithelium is compromised. The role of the resident intestinal microbiota in *Brachyspira* pathogenesis remains unclear, and preliminary investigations of the relationship of colon microbiota population structure to development of clinical disease in experimentally infected pigs

did not reveal any obvious determinants of susceptibility (Costa *et al.*, 2014a). Others have suggested that the total spirochaete load within an animal is a possible determinant in the development of disease (Weissenböck *et al.*, 2005). The detection of only "non-pathogenic" *Brachyspira* spp. (*B. innocens, B. murdochii*, and *B. intermedia*) in pigs diagnosed as swine dysentery cases raises the possibility that high spirochaete load regardless of species, may cause disease (Weissenböck *et al.*, 2005). Although specific virulence factors are still not well understood due to the multi-factorial nature of *Brachyspira*-associated disease, progress is being made with advances in available tools and *in vivo* models to study disease.

1.2 *Brachyspira* disease models

Since "*B. hampsonii*" naturally infects and causes disease in swine, both conventional and gnotobiotic swine models are ideal models for studying its pathogenesis. Conventional pigs possess the wild-type gastrointestinal microbiota, whereas gnotobiotic pigs are germ-free. The models can be inoculated orally or less commonly via ligated colonic loops (Joens and Kinyon, 1982). High health weaner pigs (aged 3 to 10 weeks old) are commonly used as animal models since pigs at this age are most vulnerable, although older pigs may still become infected (Kinyon *et al.*, 1977, Hampson, 2012). Pigs with high health, being free of pathogens including *Lawsonia intracellularis*, porcine circovirus type 2 (PCV2) and porcine respiratory and reproductive syndrome virus (PRRSV), and with no history of swine dysentery, are required to avoid other illness and false positive results due to non-bloody diarrhea caused by *L. intracellularis*, PCV2, or

by other swine-virulent *Brachyspira* spp. (Møller *et al.*, 1998, Pogranichniy *et al.*, 2002, Segalés and Domingo, 2002, Rubin *et al.*, 2013a).

Diet fed to pigs during experimental inoculation experiments is another factor that affects disease development. A reduction in the expression of swine dysentery has been observed in pigs fed modified diets with cooked white rice or distillers dried grains with solubles (DGGS), while modified diets with soybean meal or resistant carbohydrates are associated with increases in swine dysentery expression (Siba *et al.*, 1996, Pluske *et al.*, 1998, Burrough, 2016). High levels of soybean meal have been suggested as necessary for the expression of clinical signs of swine dysentery, although other studies utilized commercial diets without soybean (Jacobson *et al.*, 2004, Rubin *et al.*, 2013b, Burrough, 2016). It has also been noted that other factors are important in the development of disease in the laboratory setting, such as cold ambient temperature, and inoculum dosage (Brandenburg and Wilson, 1974, Burrough, 2016).

Outcomes of interest in animal inoculation studies are changes in weight gain, faecal consistency changes, faecal shedding of spirochaetes, lesions in the colon, and presence of spirochaetes in colonic crypts (Kinyon *et al.*, 1977, Rubin *et al.*, 2013a). Swine models have been valuable in providing a realistic presentation of the disease for study of pathogenesis and the possible preventative effect of certain diets and vaccines, but achieving adequate statistical power may require large numbers of animals, which presents logistical and animal welfare challenges (Jacobson *et al.*, 2004, Burrough, 2016).

A desire to reduce animal use and study cost, and improve statistical power led to the development of other models to study *Brachyspira* spp., such as *in vitro* organ culture (IVOC) and

mouse models (Joens and Glock, 1979, Costa *et al.*, 2016b). Porcine IVOC has been developed to observe *Brachyspira* infection of and interaction with the colonic epithelium, but is limited as challenges with maintenance of viability of the IVOC requires experimental timelines of less than 5 days (Costa *et al.*, 2016b, Costa *et al.*, 2016a). Murine models have also been adapted for use in *Brachyspira* spp. infection studies due to the low cost of mice, ease of housing and care (Joens and Glock, 1979, Joens *et al.*, 1980, Jamshidian *et al.*, 2004). "*B. hampsonii*" has not been isolated from wild rodents yet, but other swine-pathogenic and non-pathogenic *Brachyspira* spp. have been isolated from wild rodents (Joens and Kinyon, 1982, Fellström *et al.*, 2004, Backhans *et al.*, 2010).

Two murine models (CF1 and C3H strains) have been experimentally infected with *B. hyodysenteriae* and "*B. hampsonii*", while C3H has also been used to model *B. pilosicoli* infection (Joens and Glock, 1979, Nibbelink and Wannemuehler, 1991, Jamshidian *et al.*, 2004, Burrough *et al.*, 2012a, Ek *et al.*, Submitted). For both *B. hyodysenteriae* and *B. pilosicoli* infection models, special diets were required for colonization and expression of some disease signs (decreased weight, colonic lesions, luminal mucus, and shedding), but blood and diarrhea have not been observed in these models (Joens and Glock, 1979, Nibbelink and Wannemuehler, 1992, Jamshidian *et al.*, 2004, Burrough *et al.*, 2012a). Assessments of disease in both CF1 and C3H models have been limited to detection of shedding by culture and/or PCR of faeces, weight changes, and identification of gross and microscopic lesions (Joens and Glock, 1979, Jamshidian *et al.*, 2004, Ek *et al.*, Submitted). Clinical signs have been overlooked (or at least not reported) in most studies that have utilized murine models, but mucoid faeces have been observed in CF1 mice experimentally infected with *B. hyodysenteriae* (Joens and Glock, 1979). Recent studies focused on detecting spirochaete shedding, weight changes, and histology have preferred utilization of the

C3H mouse strain, but the reasons for using this strain as a model have not been explained (Burrough *et al.*, 2012a). Regular commercial rodent diet and a low-zinc diet have been used for both murine model strains to enhance clinical signs due to *Brachyspira hyodysenteriae* infection (Joens and Glock, 1979, Suenaga and Yamazaki, 1984). A recent comparison of the regular commercial and low-zinc diets for mice experimentally infected with *B. hyodysenteriae* or "*B. hampsonii*" demonstrated that the low-zinc diet enhances disease in both CF1 and C3H mouse strains (Ek *et al.*, Submitted). However, clinical disease (occurrence of abnormal feces) was more evident in the CF1 mice, which suggests that it is a more sensitive model for demonstrating virulence of *Brachyspira* spp. (Ek *et al.*, Submitted). Another important factor to produce colonization in CF1 and C3H mice are inocula with greater than 1×10^2 CFU/mL and 1×10^6 CFU/mL, respectively (Suenaga and Yamazaki, 1984, Nibbelink and Wannemuehler, 1991).

1.3 Disease intervention

1.3.1 Current methods of prevention, treatment and control

Prevention of *Brachyspira* infection is done through various enhancements to standard biosecurity methods, which help prevent infection of herds and barns with *Brachyspira*. Enhanced biosecurity consists of limiting traffic into the farm and through aggressive rodent control (Burrough, 2016). Limitations on visitors based on how recently they have visited another farm can help reduce the likelihood of transmission of *Brachyspira* or other pathogens. The shower-in-shower-out practice can also help prevent introduction or export of pathogens via farm workers.

The removal of faecal matter by pressure washing, disinfection, and drying of vehicles can also help reduce the likelihood of transmission of *Brachyspira* and other pathogens in faeces in vehicles and trailers (Dewey *et al.*, 2014). If preventative measures are not able to prevent exposure and infection, other practices must be implemented to manage the infection and disease. Monitoring is key to detecting diarrhea and quick identification of the cause of disease using submitted live pigs or tissue samples for PCR, microbiological culture, and histopathology (Harding *et al.*, 2013, Burrough, 2016). Detection of "*B. hampsonii*" can then be followed up with treatment using antibiotics and enhancement of biosecurity to prevent future outbreaks.

Brachyspira-infection on a farm is managed by use of in-feed/in-water antibiotics and all-in-all-out herd movement. So far tiamulin has been shown to clear clinical signs within 24 hours and cease shedding of "*B. hampsonii*" after 72 hours using 0.006 and 0.018% (v/v) tiamulin in water (Wilberts *et al.*, 2014). In-feed medication has also been used as a vehicle for antibiotics and continuous treatment with antibiotics has been demonstrated to suppress pathogenic *Brachyspira* spp. populations, and thus reduce the number of cases of swine dysentery (Hampson *et al.*, 2015). The practice of all-in-all-out herd management can help prevent future outbreaks on the farm due to subclinically infected pigs mixed with replacement pigs by permitting a period of decontamination between herds, ensuring that the new incoming herds are not infected by pigs previously colonized by *Brachyspira*. All-in-all-out herds have been reported to perform better, with higher daily gain, lower mortality, and fewer cases of some illnesses (Heinonen *et al.*, 2001). When management practices are not able to suppress pathogen levels and prevent outbreaks, treatment with antibiotics becomes necessary.

Treatment of pigs exhibiting mucohaemorrhagic diarrhea is performed using antibiotics including pleuromutilins, lincosamides, macrolides, quinoxalines, and tetracyclines (Mirajkar and Gebhart, 2016). Tiamulin and valnemulin are most commonly used to treat swine dysentery due to *B. hyodysenteriae* and are also used to treat "*B. hampsonii*" infections (Wilberts *et al.*, 2014, Burrough, 2016). However, issues with antibiotic resistance have arisen due to overuse (Hampson *et al.*, 1993, Burch, 2005). Other challenges have been revealed through research on *Brachyspira*, such as the identification of prophage-like gene transfer agents that are transferred between *B. hyodysenteriae* strains when exposed to sub-inhibitory concentrations of carbadox and metronidazole (Stanton *et al.*, 2008). These gene transfer agents contain 7.5 kb DNA that could contain antibiotic resistance genes conferring resistance to tylosin and chloramphenicol (Stanton *et al.*, 2008). Resistance to macrolides and lincosamides is common, however tiamulin and valnemulin resistance has also observed in isolates from Germany (Rohde *et al.*, 2004, Aarestrup *et al.*, 2008).

Additional management practices for preventing recurrent disease, such as decontamination and depopulation, are employed to prevent future infection of new naïve herds. Decontamination of infected barns is challenging given the ability of *Brachyspira* to survive in feces, requiring pressure washing, disinfection, drying, repair of surfaces, movement of faeces to a different location, and treatment of manure with lime (Fossi *et al.*, 2001). The method of depopulation involves the removal of infected pigs, disinfection and cleaning of barns, followed by a period without pigs in the barns, then addition of new healthy pigs. Even partial depopulation has been shown to be effective in treating infected pigs and preventing recurrence, although it is costly to implement (Figi *et al.*, 2014). In addition to depopulation, movement of herds treated

with antibiotics to a clean barn has also been shown to prevent future outbreaks in the herd (Burrough, 2016).

Stringent biosecurity procedures and thorough disinfection of previously infected barns can help prevent future "*B. hampsonii*" outbreaks (Burrough, 2016). However, eradication is difficult due to the different reservoirs that exist around barns including clinical sign-free but colonized pigs (Diego *et al.*, 1995, Rubin *et al.*, 2013b). Vaccines are not yet available against "*B. hampsonii*", but would be a useful tool for aiding with control and eradication of the pathogen.

1.3.2 Potential for a vaccine

Vaccination against "*B. hampsonii*" has not been reported, while vaccination against *B. hyodysenteriae* has been attempted but has not been completely successful (Joens *et al.*, 1983, Hampson *et al.*, 1993). Cross-protection between serotypes of *B. hyodysenteriae* in swine was not observed when groups of swine were inoculated with different *B. hyodysenteriae* serotypes and followed by challenge with the same or different serotype by Joens *et al.* (1983), with protection only possible when re-infected with the same strain. Protection of 75-83% of pigs vaccinated against the same serotype of *B. hyodysenteriae* using an adjuvanted whole cell vaccine has been achieved, but protection against multiple serotypes still needs to be achieved to prevent disease at the herd level (Hampson *et al.*, 1993). *B. hyodysenteriae* vaccination has also been performed using recombinant proteins, such as *B. hyodysenteriae* outer membrane protein BmpB that induced protection against *B. hyodysenteriae* in some immunized pigs (La *et al.*, 2004, Witchell *et al.*, 2011). BmpB recombinant vaccines have been shown to reduce lesion formation, spirochaete

shedding, and appearance of clinical swine dysentery (La *et al.*, 2004). The Vsp outer membrane proteins of *B. hyodysenteriae* have also been shown to be antigenic, but vaccination using these proteins requires multiple Vsp variants to induce a sufficient level of immune response in pigs against *B. hyodysenteriae* as Witchell *et al.* (2011) has shown. Smp proteins are another family of outer membrane protein with some sequence similarity to Bmp proteins, which have been evaluated as candidates for vaccine development (Holden *et al.*, 2008). Experimental vaccination of mice with recombinant SmpB has led to 50% protection against *B. hyodysenteriae* (Holden *et al.*, 2008). However, variation in the expression of the Smp family of outer membrane proteins has been identified among global isolates of *B. hyodysenteriae*, with either SmpA or SmpB expressed in USA isolates of *B. hyodysenteriae* while only SmpA expression has been detected in Spanish isolates of *B. hyodysenteriae* (Holden *et al.*, 2006, Hidalgo *et al.*, 2010).

Progress on vaccine development and antigen research for the "*B. hampsonii*" has not yet advanced to the current state of *B. hyodysenteriae* research. Vaccine development for "*B. hampsonii*" is challenging since the species is quite distinct from *B. hyodysenteriae* and no specific antigenic epitopes have been identified for "*B. hampsonii*". A possible vaccine development strategy achievable for "*B. hampsonii*" is production of a live attenuated strain through serial passage of a virulent strain in the laboratory, which would not require knowledge of the specific determinants of virulence. Evidence from previous pathogenicity trials with *B. hyodysenteriae* highlighted that a serially *in vitro* passaged (35 times) *B. hyodysenteriae* strain B78 (ATCC 27164) was no longer able to cause disease, supporting the idea that serial passage can also lead to attenuation of "*B. hampsonii*" (Kinyon *et al.*, 1977).

Experience to date with *Brachyspira* spp. vaccine development suggests that identification of antigenic proteins and development of recombinant proteins for "*B. hampsonii*" may be challenging, especially given the lack of knowledge of infection mechanisms. However, based on previous work with *B. hyodysenteriae, in vitro* passaging of "*B. hampsonii*" to produce a live attenuated vaccine strain may present a viable alternative approach.

Objectives

The re-emergence of mucohaemorrhagic diarrhea as a production limiting disease of pigs presents a significant challenge to swine producers. Control and eradication of disease associated with *"B. hampsonii*" depends upon an improved understanding of pathogen biology and the development of tools for diagnosis, treatment and prevention of infection. To move toward achievement of these goals, the following objectives were addressed in this thesis:

1. To further characterize the phenotypic and genotypic traits of Western Canadian clinical isolates of "*B. hampsonii*".
2. To produce an attenuated strain of "*B. hampsonii*" by serial passage in laboratory culture.

2 Characterization of "*Brachyspira hampsonii*" clades I and II isolated from commercial swine in Western Canada

Citation

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Author Contributions

Conceived and designed the experiments: JER, JCS, JEH. Performed the experiments: JBP, JER, CF. Analyzed the data: JBP, JER, CF, JCH, JEH. Contributed reagents/materials/analysis tools: JER, JCH, JEH. Wrote the paper: JBP, JER, CF, JCH, JEH.

2.1 Abstract

Since 2009, a novel *Brachyspira* has emerged as a production limiting pathogen of pigs in North America. The name "*Brachyspira hampsonii"* has been proposed for this novel taxon. *"B. hampsonii"* is divided into two phylogenetically distinct clades based on sequence of the NADH oxidase gene, although the clinical disease associated with clades I and II is indistinguishable and phenotypic characteristics that discriminate the clades have not been determined. The objectives of the current study were to enhance the description of the provisional species "*B. hampsonii*" with biochemical profiles and morphometric data from isolates affecting Canadian swine, and to investigate potentially diagnostically informative characteristics for this emerging pathogen. Biochemical profiles of isolates from different commercial swine barns in Western Canada showed that biochemical profiles were insufficient to distinguish *"B. hampsonii"* clades I and II from each other or from other pathogenic *Brachyspira*. Hippurate hydrolysis, previously reported as uniformly negative in *"B. hampsonii*" was variable among Canadian isolates. Spirochete dimensions and flagella numbers for *"B. hampsonii*" overlapped with other *Brachyspira* species. Taken together, these results indicate that NADH oxidase gene sequencing remains a preferred method for identification and discrimination of *"B. hampsonii*" from other pig-associated *Brachyspira* spp.

Key words: "*Brachyspira hampsonii*", transmission electron microscopy, NADH oxidase, indole, hippurate

2.2 Introduction

The genus *Brachyspira* consists of Gram negative, motile, *β*-haemolytic, aerotolerant spirochaetes infecting the gastrointestinal tracts of wild and domestic animals, as well as humans. In swine, *Brachyspira hyodysenteriae* is the causative agent of swine dysentery (Harris *et al.*, 1972), while *B. pilosicoli* infection is associated with spirochaetal colitis (Trott *et al.*, 1996a). "*B. suanatina*", originally isolated from mallard ducks, has also been shown to cause dysentery-like disease in experimentally inoculated pigs (Råsbäck *et al.*, 2007a). Other species detected in pigs (*B. intermedia*, *B. innocens*, *B. murdochii*) are non-pathogenic or have been reported to cause mild catarrhal colitis under experimental conditions (Jensen et al., 2010, Stanton et al., 1997). Re-emergence of *Brachyspira*-associated mucohaemorrhagic diarrhea in swine in Western Canada and the US has been associated with a novel taxon (Harding *et al.*, 2010), provisionally named "*Brachyspira hampsonii*" (Chander *et al.*, 2012). "*B. hampsonii*" has also recently been detected in birds in Spain, in pigs in the Czech Republic and in pigs from Belgium transported to Germany (Martínez-Lobo *et al.*, 2013, Mahu *et al.*, 2014, Rohde *et al.*, 2014). Phylogenetic analysis of the NADH-oxidase (*nox*), 16S rRNA, and 23S rRNA genes revealed two distinct clades within the taxon (Chander *et al.*, 2012, Rubin *et al.*, 2013b, Burrough *et al.*, 2013). Replacement of clades I and II with genetic groups 1 to 4 has also been suggested based on multi-locus sequence typing of six targets for "*B. hampsonii*" (Mirajkar *et al.*, 2015). Although the clinical disease associated with clades I and II is indistinguishable (Costa *et al.*, 2014b, Rubin *et al.*, 2013a), the phenotypic properties distinguishing them have not been established.

Brachyspira do not form visible colonies on agar, so growth is detected on media containing blood by observing zones of *β*-haemolysis. Phenotypic characterization of *Brachyspira* is generally based on the evaluation of the strength of *β*-haemolysis on blood agar and activity of select biochemical markers (hippurate hydrolysis, indole production, *α*-galactosidase, *α*-glucosidase, and *β*-glucosidase). Spirochaete size and number of periplasmic flagella can also be used to discriminate species, although these approaches are not practical for the diagnostic laboratory. The phenotypic and genotypic characteristics of "*B. hampsonii*" isolates in the USA have been described (Chander *et al.*, 2012, Mirajkar *et al.*, 2015). Here, we describe phenotypic characteristics of "*B. hampsonii*" clade I and II isolates collected from clinical cases of mucohaemorrhagic diarrhea in Western Canadian swine between 2009 and 2013. The objectives of this study were to enhance the description of the provisional species "*B. hampsonii*" with information from isolates affecting Canadian swine, and to investigate potentially diagnostically informative characteristics for this emerging pathogen.

2.3 Materials and Methods

2.3.1 Selection of clinical isolates

"*B. hampsonii*" clade I isolate D11-30599 and clade II isolate D09-30446 were the first Canadian isolates of each clade derived from clinical cases of mucohaemorrhagic diarrhea in 2011 and 2009, respectively. The other isolates included in the study (one additional clade I and 7 additional clade II) were selected from a collection of isolates from 443 "*B. hampsonii*"-confirmed

clinical cases from different swine production sites in Alberta, Manitoba, or Saskatchewan between 2009 and 2013. The original identification of the isolates from these clinical cases was done using culture, *nox* sequencing, and clade-specific PCR with previously described assays (Costa *et al.*, 2014b, Rubin *et al.*, 2013a). While a number of clade I and II isolates were obtained from farms during this time period, isolates were selected for the current study only if they originated from different production systems (i.e. were epidemiologically distinct). Long-term storage of *Brachyspira* isolates from clinical cases was at -80°C in brain-heart infusion (BHI) broth containing 10% (v/v) glycerol.

2.3.2 *nox* gene sequencing and phylogenetic analysis

Genomic DNA was extracted and purified from JBS broth cultures ("Joe's *Brachyspira* Sask30446 Broth" is brain heart infusion (BHI, Becton Dickinson Canada, Mississauga, ON) with 1% (w/v) glucose, 5% (v/v) fetal bovine serum, 5% (v/v) sheep blood) using the DNeasy Blood & Tissue Kit (QIAGEN Inc., Mississauga, ON) following the manufacturer's instructions for pre-treatment for Gram negative bacteria and the Animal Tissue protocol. A 939 bp region of the *nox* gene was amplified using genus-specific primers Bnoxf (5'-TAG CYT GCG GTA TYG CWC TTT GG-3') and Bnoxr (5'-CTT CAG ACC AYC CAG TAG AAG CC-3') as previously described (Rubin *et al.*, 2013a, Rohde *et al.*, 2002). PCR products were purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Inc., Markham, ON) and sequenced with the amplification primers. Raw sequence data were trimmed to a uniform length of 810 bp prior to alignment with CLUSTALw and phylogenetic tree construction using PHYLIP (Felsenstein, 1989).

2.3.3 Biochemical tests

Study isolates were grown in BHIS (BHI with 1% (w/v) glucose, 10% (v/v) fetal bovine serum) broth to mid-log phase. Cultures were adjusted to McFarland 5 and enzymatic activities were determined using the API-zym kit (BioMerieux, St. Laurent, QC) according to the manufacturer's instructions. Spot indole and hippurate broth assays were performed as previously described (Fellström and Gunnarsson, 1995). *B. hyodysenteriae* ATCC 27164^T and *B. pilosicoli* ATCC 51139^T were included as controls in all biochemical tests.

2.3.4 Electron microscopy

Log phase *Brachyspira* cultures were prepared for TEM using the single droplet negative staining technique and carbon coated 200 mesh-200G-CP grids (Stanton *et al.*, 1998, Trott *et al.*, 1996a). Flagella counts, cell width (average of three locations along the length of the spirochaete not including the tapered region near both ends), and cell length were measured using ImagePro Plus (Media Cybernetics, Rockville, MD) on captured TEM images (**Figure 2.2**). Twenty cells of each isolate were measured. Images were considered suitable for width and length measurements if the cells were intact, isolated, and not twisted on themselves. Flagella counts were performed on untwisted cells with at least one end visible.

2.4 Results and Discussion

Clinical isolates of *"B. hampsonii"* were selected to represent distinct pig production sites, although most were related by a common source of replacement females. The number of isolates included in the study from either clade (two clade I and eight clade II) reflects

higher isolation rates of clade II isolates from clinical samples submitted to our laboratory, which to our knowledge was the only laboratory performing clade level identification of "*B. hampsonii*" in Western Canada during the period of study. This trend in relative occurrence of clade I and II "*B. hampsonii"* is opposite to that reported in a recent study of *"B. hampsonii"* diversity in the United States (Mirajkar *et al.*, 2015). Clade affiliation was confirmed, and relatedness of the selected clinical isolates to other *Brachyspira* species was investigated based on partial NADH oxidase (*nox*) gene sequences. The *nox* sequences of the two clade I isolates were identical, while the pairwise identities between clade II isolates ranged from 99-100% (**Figure 2.1**).

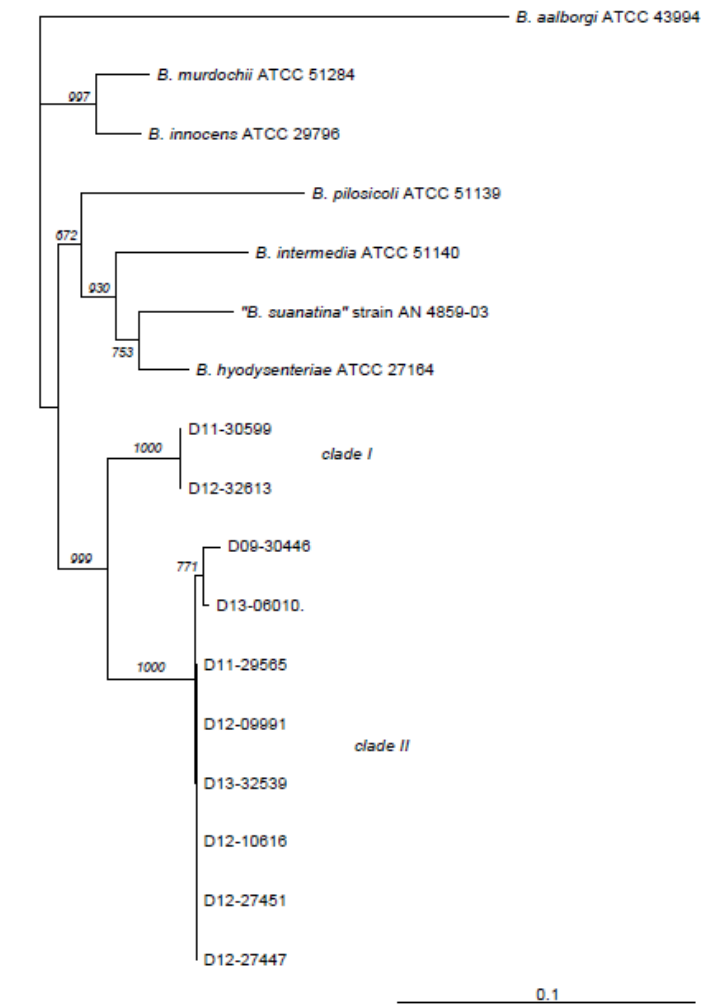

Figure 2.1. Phylogenetic relationships of "*B. hampsonii*" study isolates and reference species based on partial nox sequences. Bootstrap values (out of 1000) are indicated at the nodes. Reference sequences were included from *B. aalborgi* ATCC 43994^{T} (GenBank accession No. AF060816), *B. murdochii* ATCC 51284^{T} (KC984308), *B. innocens* ATCC 29796^{T} (KC984307), *B. pilosicoli* ATCC 51139^{T} (KC984310), *B. intermedia* ATCC 51140^{T} (KC984309), "*B. suanatina*" AN 4859-03 (DQ487119), and *B. hyodysenteriae* ATCC 27164^{T} (KC984311). Scale bar indicates 0.1 substitutions per site.

Results of the API-zym, indole production and hippurate hydrolysis tests for study isolates are summarized in Table 1. Activities of *α*-galactosidase, *α*-glucosidase, and *β*-glucosidase, and indole production and hippurate hydrolysis have been utilized as biochemical markers for differentiation of *Brachyspira* species (Achacha and Messier, 1991, Fellström and Gunnarsson, 1995). All clade I and II study isolates were negative for *α*-galactosidase activity, one clade II isolate was positive for *α*-glucosidase, and both clade I isolates and 6/8 clade II isolates were positive for *β*-glucosidase activity (**Table 2.1**). Although indole production was negative for all isolates as expected, hippurate hydrolysis activity was inconsistent among isolates in both clades (**Table 2.1**). These results are consistent with a previous phenotypic description of *"B. hampsonii"* from the USA (Chander *et al.*, 2012) in that none of these assays could distinguish clade I and clade II isolates. Interestingly, all isolates in the USA study (n=17 clade I, n=3 clade II) were negative for hippurate hydrolysis activity (Chander *et al.*, 2012). Variations in indole production, hippurate hydrolysis, *α*-galactosidase, and *α*-glucosidase activities of human isolates of *B. pilosicoli* have been previously observed (De Smet *et al.*, 1998), and variation in indole production of swine-isolated *B. hyodysenteriae* has also been reported (Fellström *et al.*, 1999). The greater consistency in hippurate hydrolysis activity in the USA study may reflect epidemiological relationships of isolates, but these relationships are not described in that report. Taken together, these results show that none of these biochemical assays can discriminate clade I and II or "*B. hampsonii*" from *B. hyodysenteriae* (**Table 2.2**). The lack of specificity of biochemical markers for differentiating *Brachyspira* species has inspired the development of MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) as a promising phenotypic method for differentiation of *Brachyspira.* This technology has been demonstrated to distinguish *Brachyspira*

species (Prohaska *et al.*, 2014, Calderaro *et al.*, 2013), but clades of *"B. hampsonii"* are not reliably distinguished (Warneke *et al.*, 2014). The initial capital costs for diagnostic MALDI-TOF have also limited its widespread adoption.

Table 2.1. Biochemical test results for Western Canadian clinical "*B. hampsonii*" isolates.

Clade[#]	Isolate	α-galactosidase	α-glucosidase	β-glucosidase	Indole Production	Hippurate Hydrolysis
I	D11-30599	–	–	+	–	–
	D12-32613	–	–	+	–	+
II	D09-30446	–	–	+	–	–
	D11-29565-A6P8	–	–	+	–	+
	D12-09991-28	–	–	–	–	–
	D12-10616-P8	–	–	+	–	+
	D12-27447	–	–	Weak +	–	+
	D12-27451	–	–	+	–	+
	D12-32539	–	–	+	–	+
	D13-06010	–	+	–	–	–

[#]Based on partial *nox* gene sequence

Table 2.2. Morphological and biochemical characteristics of "*B. hampsonii*" clade I and clade II and other swine-associated *Brachyspira* species.

Characteristic	**"*B. hampsonii*" clade I**	***"B. hampsonii"* clade II**	***B. hyodysenteriae*** (Trott *et al.*, 1996a)	***"B. suanatina"*** (Mushtaq et al., 2015, Råsbäck et al., 2007a)	***B. pilosicoli*** (Trott *et al.*, 1996a)	***B. innocens*** (Oxberry *et al.*, 1998)	***B. murdochii*** (Jensen et al., 2010, Stanton et al., 1997)	***B. intermedia*** (Stanton et al., 1997, Jensen et al., 2010)
Cell length (µm)	7.27 ± 1.30[#]	8.53 ± 2.19[#]	9.78 ± 1.87[§]	7.27 ± 1.00[$]	6.27 ± 0.98[§]	9.40 ± 1.85[§]	5-8*	7.5-10*
Cell diameter (µm)	0.26 ± 0.045[#]	0.46 ± 0.05[#]	0.35 ± 0.02[§]	0.3 ± 0.00[$]	0.27 ± 0.03[§]	0.36 ± 0.03[§]	0.35-0.40*	0.35-0.45*
No. flagella	11-16[¶]	12-15[¶]	8-12	14-16	4-6	10-13	11-13	12-14
β-haemolysis	Strong	Strong	Strong	Strong	Weak	Weak	Weak	Weak
Indole production	–	–	+	Weak +	–	–	–	+
Hippurate hydrolysis	Variable	Variable	–	–	+	–	–	–
α-galactosidase	–	–	–	–	+	+	–	–
α-glucosidase	–	Variable	+	Weak +	–	–	–	+
β-glucosidase	+	Variable	+	+	–	+	–	+

[#] Mean ± standard deviation for 20 spirochaetes

[¶] Range for 15 spirochaetes

[§] Mean ± standard deviation for 5 spirochaetes

*Authors reported range

[$] Mean ± standard deviation for 10+ spirochaetes

Transmission electron microscopy was performed on D11-30599 and D09-30446 to measure the dimensions of the spirochaetes and count the number of flagella for comparison to other *Brachyspira* species. Representative images are shown in Fig 2. Spirochaetes of clade I "*B. hampsonii*" isolate D11-30599 were 7.27 ± 1.30 μm and 0.26 ± 0.045 μm, with 11-16 periplasmic flagella (**Figure 2.2*a***, **Figure 2.2*b***, **Table 2.2**). For clade II isolate D09-30446, average length and width were 8.53 ± 2.19 μm and 0.46 ± 0.05 μm, with 12-15 periplasmic flagella (**Figure 2.2*c,* Figure 2.2*d,* Table 2.2**). Apparently dividing cells were observed (**Figure 2.2*e***), and tailed bacteriophages were occasionally detected on the surface D09-30446 spirochaetes under TEM (**Figure 2.2*f***). The presence of tailed bacteriophages is consistent with the type of bacteriophage detected with other *Brachyspira* (Ackermann, 2007, Calderaro *et al.,* 1998). The morphological measurements and flagella counts for "*B. hampsonii*" overlapped with other pig associated *Brachyspira* species and so do not on their own provide species identification (**Table 2.2**).

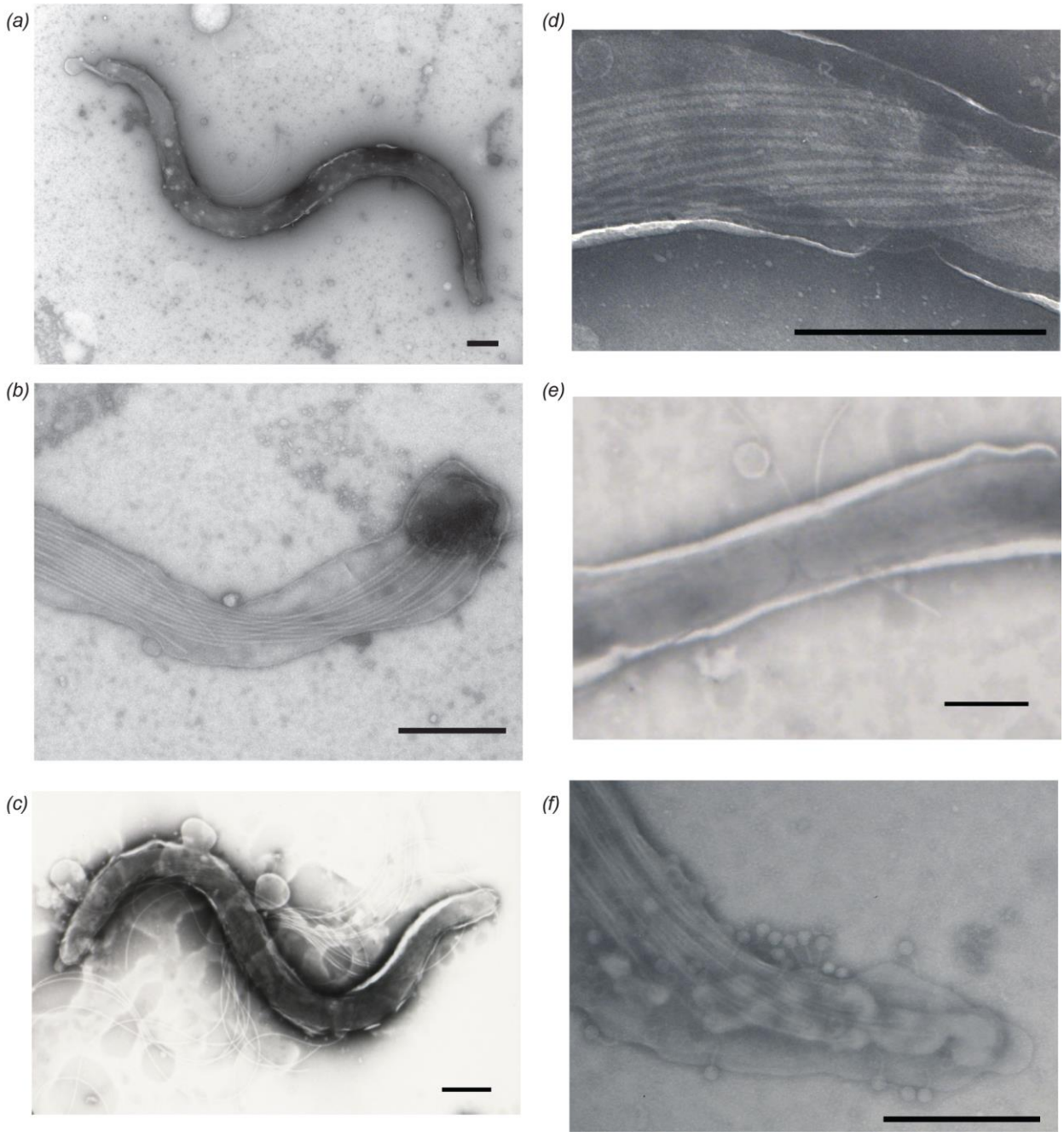

Figure 2.2. TEM images of clade I isolate D11-30599 (*a*, *b*) and clade II isolate D09-30446 (*c*, *d*, *e*, *f*). Images of entire spirochaete (*a*, *c*), periplasmic flagella (*b*, *d*), cell division (*e*), tip of D09-30446 spirochaete with tailed bacteriophages (*f*). Scale bars represent 0.5 μm.

2.5 Conclusions

The biochemical variation of "*B. hampsonii*" isolates from Western Canadian swine production systems highlights that biochemical characterization alone is not diagnostically informative. Although some traits of "*B. hampsonii*" isolates are consistent (indole and α-galactosidase negative), these characteristics are not solely sufficient to discern them from other *Brachyspira* species as previously observed in the USA study (Chander *et al.*, 2012). The morphology of selected isolates was also indistinguishable from other species of *Brachyspira*. Based on these results and previous observations, *nox* gene sequencing remains a useful, accessible and affordable tool for identification and discrimination of *Brachyspira* spp. affecting pigs.

2.6 Transition Statement

The characterization of Western Canadian "*B. hampsonii*" isolates showed that phenotypic variation exists amongst clinical isolates. Due to this variation, the only reliable diagnostic phenotypes for discriminating "*B. hampsonii*" from other *Brachyspira* spp. profiles are the combination of indole production, *α*-glucosidase activity and *β*-haemolysis. However, these characteristics do not distinguish clade I and II "*B. hampsonii*". Sequencing of the *nox* gene remains the best tool for identification and discrimination of different *Brachyspira* spp. and clades. The establishment of diagnostic characteristics for "*B. hampsonii*" is necessary to improve detection of the pathogen and to control the pathogen during outbreaks. Current methods for controlling infection are not effective due to the survival of *Brachyspira* spp. in the environment and wildlife. Depopulation, the removal of pigs from the barns and subsequent decontamination, is not sufficient in permanently removing the pathogen. Treatments against *Brachyspira* spp. are being challenged by the rising antibiotic resistance within the genus of *Brachyspira*. Better intervention methods, such as vaccination, are necessary for preventing future outbreaks, which may help with eradication of the pathogen from farms. Production of an attenuated vaccine strain, derived from a virulent strain can help control the pathogen and prevent future outbreaks of "*B. hampsonii*". In addition to producing a strain for vaccine development, comparison of an attenuated strain with its virulent precursor may lead to the identification of virulence factors.

3 *In vitro* attenuation of a virulent swine isolate of "*Brachyspira hampsonii*"

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Conceived and designed experiments: JBP, CF, JCH, JEH. Performed experiments: JBP, CF, RVN, AW. Analyzed samples: JBP, CF. Analyzed data: JBP, JCH, JEH. Wrote the paper: JBP

3.1 Abstract

The re-emergence of mucohaemorrhagic diarrhea and the novel taxon "*Brachyspira hampsonii*" is a major concern of swine producers. Prevention is limited to improved biosecurity procedures, but better methods for disease prevention are required, such as herd vaccination. The objectives of the study were to develop and characterize a "*B. hampsonii*" vaccine strain. A virulent clade II "*B. hampsonii*" passage 13 (P13) strain was serially passaged to produce a passage 113 (P113) strain that was phenotypically and genotypically characterized. The serially passaged strain was compared to the original virulent strain. No differences were identified in morphology or in selected biochemical markers of P13 and P113. Comparison of the β-haemolysis and growth profile of P13 and P113 demonstrated that P113 was better adapted to growth in laboratory medium. Comparison of P13 and P113 in a murine model fed with a zinc-deficient diet showed that mice infected with P113 produced less disease and that P113 has been partially attenuated. Genotypic comparison by random amplified polymorphic DNA (RAPD) and genome sequencing showed that there were no detectable differences in the genome profiles or sequences. Overall, a partially attenuated and laboratory adapted "*B. hampsonii*" strain was produced by serial passage.

3.2 Introduction

Swine dysentery, due to *B. hyodysenteriae*, was under control until the re-emergence of mucohaemorrhagic diarrhea started in the mid-2000s (Burrough, 2016). This re-emergence has been associated with "*Brachyspira hampsonii*", a Gram-negative, facultative anaerobic, and motile spirochaete. "*B. hampsonii*" was identified in 2009 as distinct from other *Brachyspira* spp. based on sequence differences in the NADH-oxidase (*nox*) gene (Harding *et al.*, 2010) and has subsequently been proposed as a novel species (Chander *et al.*, 2012, Mirajkar *et al.*, 2016). Clinical isolates of "*B. hampsonii*" have demonstrated a high degree of virulence in swine, leading to mucohaemorrhagic diarrhea (Rubin *et al.*, 2013a).

Control of "*B. hampsonii*" is possible through the use of biosecurity, antibiotics, and depopulation. The role of biosecurity is to prevent the exposure of swine to "*B. hampsonii*", while antibiotics are used to treat infected swine. Depopulation-repopulation is the removal of swine from the farm, decontamination of facilities, and eventual repopulation with a new herd. The method of depopulation-repopulation has been shown to be effective in removing *Brachyspira* spp. from the farm, but facilities remain at risk for reinfection due to environmental and wildlife reservoirs of *Brachyspira* spp. (Aarestrup *et al.*, 2008). A more effective tool that would help prevent outbreaks and possibly aid with regional eradication of "*B. hampsonii*" would be a vaccine. Several approaches to *Brachyspira* vaccine production have been pursued with varying success.

Recombinant protein *B. hyodysenteriae* vaccines have been composed of outer membrane proteins such as BmpB, which has been shown to be partially protective against at least one other serotype from the serotype the protein was originally isolated from (La *et al.*, 2004). Another outer

membrane protein, SmpB, was evaluated as a vaccine candidate and found to provide 50% protection against *B. hyodysenteriae* in mice (Holden *et al.*, 2008). However, vaccination with multiple outer membrane proteins is likely needed since only SmpA has been detected in Spanish isolates, while SmpA and SmpB are equally distributed in US isolates (Holden *et al.*, 2008, Hidalgo *et al.*, 2010). Further studies have been performed to improve the outer membrane-type vaccines through different vaccine strategies (DNA vaccine, BmpB protein in a carrier microparticle coated with M cell targeting peptide) or through modification of antigen proteins through fusion with unrelated domains (poly-histidine, maltose-binding protein, glutathione S-transferase) (La *et al.*, 2005, Holden *et al.*, 2008, Jiang *et al.*, 2014). Although sera from vaccinated pigs has been shown to recognize outer membrane extracts from multiple *B. hyodysenteriae* serotypes, *in vivo* serotype cross-protection has not yet been demonstrated (La *et al.*, 2004, Lee *et al.*, 2000).

Live attenuated *B. hyodysenteriae* strains have been developed by Hudson *et al.* (1974) and Kinyon *et al.* (1977) by serial passage of a swine-virulent *B. hyodysenteriae in vitro*. However, failure of heterologous protection between *B. hyodysenteriae* serotypes has also been raised as a concern based on attempts at live attenuated vaccination, which may be associated with the variable expression of the major outer membrane proteins (Bhmp) of *B. hyodysenteriae* (Joens *et al.*, 1983, Witchell *et al.*, 2006).

A challenge for live attenuated vaccine development for "*B. hampsonii*" is the lack of genetic systems for specific modification of genes and a lack of knowledge of virulence determinants to target, even if it were possible to perform targeted manipulations. Random mutagenesis of virulent

strains to produce live attenuated vaccine candidates is impractical due the large numbers of strains that would need to be screened *in vivo.* However, laboratory attenuation through serial passage has proven to be effective in reducing the virulence of *Brachyspira hyodysenteriae*, without knowledge of virulence determinants (Kinyon *et al.*, 1977). *In vitro* serial passage of *B. hyodysenteriae* has also led to the reduction of antibiotic resistance towards tylosin (Kitai *et al.*, 1987). The method of attenuation by serial passaging has also been effective for other bacteria, such as *Staphylococcus aureus* (Somerville *et al.*, 2002), and commercial live attenuated vaccines are marketed for control of pathogens such as *Lawsonia intracellularis* (Enterisol Ileitis, Boehringer Ingelheim) and *Erysipelothrix rhusiopathiae* (Ingelvac ERY-ALC, Boehringer Ingelheim) in pigs. Serial passage has been shown to lead to changes in the genome, such as changes in genome structure and sequence alterations in a wide range of genes (Fux *et al.*, 2005, Somerville *et al.*, 2002). Serial passage of *Staphylococcus aureus* resulted in reduced production of virulence factors, due to mutations in specific genes, such as *agr* which led to inactivation of quorum sensing, a decrease in secretion of α-toxins, and improved growth yield *in vitro* (Somerville *et al.*, 2002). Thus, in addition to producing a live attenuated vaccine candidate, attenuation by serial passage may also lead to changes in the virulence that would in turn lead to identification of genes linked to virulence determinants.

The objective of the current study was to produce an attenuated "*B. hampsonii*" strain by serial passage of an isolate previously demonstrated to be virulent in pigs. Changes in phenotypic and genotypic characteristics of the bacterium that occurred as a result of the attenuation process were assessed, and the effect of the attenuation process on virulence was determined in a mouse model.

3.3 Materials and Methods

Brachypsira **isolates and culture conditions**

The strain selected for serial passaging was "*B. hampsonii*" D09-30446 passage 13 (P13), a clade II strain of "*B. hampsonii*", which was originally isolated from intestinal contents from a 13-week old pig with mucohaemorrhagic diarrhea as described by Rubin *et al.* (2013a). "*B. hampsonii*" D11-30599 (clade I) was selected for genome profile comparisons as a representative of clade I "*B. hampsonii*". Type strains of other *Brachyspira* spp. were obtained from the American Type Culture Collection (ATCC). *B. hyodysenteriae* ATCC 27164^T and *B. pilosicoli* ATCC 51139^T were used as controls for the indole spot test, hippurate hydrolysis test, and genome profile comparisons. *B. murdochii* ATCC 51284^T was used for genome profile comparisons.

Brachyspira spp. were cultured in BHIS broth (brain heart infusion (BHI) broth + 10% (v/v) fetal bovine serum) or JBS broth (BHI + 5% (v/v) deactivated fetal bovine serum + 5% (v/v) defibrinated sheep blood) in glass vials containing magnetic stir bars. Broth cultures were incubated at 39°C under anaerobic conditions with constant stirring, using a commercial gas packet system (Anaerogen, Oxoid Limited, Basingstoke, UK) (Rubin *et al.*, 2013a). For growth on solid media, *Brachyspira* spp. were streaked on to BJ agar (trypticase soy agar + 5% (v/v) defibrinated sheep blood + 5% pig faeces extract + 25 µg/mL spiramycin + 12.5 µg/mL rifampin + 6.25 µg/mL vancomycin + 6.25 µg/mL colistin + 200 µg/mL spectinomycin) (Kunkle and Kinyon, 1988). Agar plates were incubated anaerobically at 42°C for 24 to 72 hours (Songer *et al.*, 1976, Rubin *et al.*, 2013a). Long-term storage of *Brachyspira* spp. isolates was in brain heart infusion (BHI) broth + 10% (v/v) glycerol at -80°C.

3.3.1 Serial passage

The initial culture of "*B. hampsonii*" D09-30446 P13 was grown in 10 mL JBS in duplicate. For serial passages, 1 mL of broth culture was transferred to fresh JBS broth (9 mL) once a dense culture had been achieved (usually 1-3 days, but occasionally up to 6 days). The presence of active spirochaetes in the donor culture was confirmed by phase contrast microscopy prior to each passage. Periodically, Gram-staining was performed on BHIS cultures grown from select JBS passage cultures, to check for obvious contaminants. The serial passage of P13 for 100 passages resulted in "*B. hampsonii*" D09-30446 passage 113 (P113). A minimum of four 1 mL aliquots of all passages, from P14 to P113 were stored at -80°C.

3.3.2 Biochemical characterization

"*B. hampsonii*" P13 and P113 cultures were grown in BHIS broth to the mid-log phase per the "*B. hampsonii*" clade II growth curve previously established in the lab. For the API-zym test, cultures were standardized to a turbidity of McFarland 5, per manufacturer's instructions (BioMérieux, St. Laurent, QC). Hippurate hydrolysis and spot indole tests were performed as previously described (Fellström and Gunnarsson, 1995). *B. hyodysenteriae* ATCC 27164^{T} and *B. pilosicoli* ATCC 51139^{T} were included as controls for the indole spot and hippurate hydrolysis tests.

3.3.3 *β*-haemolysis assays

Haemolysis activity of *Brachyspira* isolates was evaluated using two approaches. For qualitative assessment, BJ agar plates were streaked with isolates using a standard four-streak

pattern (3 replicate plates per isolate per time point; 12 total replicate plates per isolate). A 10µL inoculation loop was used for streaking to standardize the amount of inoculum. Plates were observed after 1, 2, 3 and 4 days of anaerobic incubation and haemolysis was scored according to the presence of visible β-haemolysis in quadrants 1-4. An average of scores from each of the three replicate plates for each isolate at each time point was calculated.

For quantitative evaluation of haemolysis, the wells of 96-well flat-bottomed plates (Greiner Bio-One, Kremsmünster, AT) were each filled with 150 µL of BJ agar. Active cultures of "*B. hampsonii*" P13 and P113, grown in BHIS, were adjusted to a McFarland 5 turbidity, using a densitometer (Grant-Bio, Kensington, UK). Wells containing BJ agar were inoculated with 5µL of "*B. hampsonii*" P13 or P113 (20 biological and 6 technical replicates each). Each plate also contained 12 wells inoculated with sterile BHIS, as a negative control. Plates were incubated at 42°C under anaerobic conditions for 4 days, under humid conditions. Absorbance measurements at 560 nm were made at hours 0, 24, 48, 72, and 96 using the Vmax plate reader (Molecular Devices, Sunnyvale, CA). A haemolysis score was calculated for each well at each time point using the following formula:$(OD_{600}\ test - OD_{600}\ control) \times -1 = haemolysis\ score$. For the haemolysis plate assay, raw data was compared by Repeated Measures ANOVA.

3.3.4 Growth study

The growth rates of "*B. hampsonii*" P13 and P113 were compared to detect differences in growth rates of the two strains. Three replicate cultures were prepared for each strain and each time point (0, 12, 24, 36, 48, and 72 hours). McFarland 5 standardized cultures (0.33 mL) were inoculated into 3.3 mL of BHIS broth, for incubation in smaller tubes (7 mL vials, 17mm × 60mm;

VWR, Radnor, USA). Broth cultures were incubated at 39°C with stirring, under anaerobic conditions. Optical density measurements at 600 nm were taken in polystyrene cuvettes using a spectrophotometer. The average optical density of each time point for both P13 and P113 were compared by Repeated Measures ANOVA.

3.3.5 Random amplified polymorphic DNA (RAPD) genome fingerprinting

RAPD fingerprinting was conducted as described by Quednau *et al.* (1998) and Jansson *et al.* (2004). "*B. hampsonii*" P13 and P113, "*B. hampsonii*" D11-30599, *B. hyodysenteriae* ATCC 27164^T, *B. pilosicoli* ATCC 51139^T, and *B. murdochii* ATCC 51284^T were grown in BHIS to a dense culture, then boiled for 10 minutes to release DNA. RAPD-PCR was performed on the boiled samples using 1.09× PCR buffer (0.2 M Tris-HCl (pH 8.4), 0.5 M KCl), 3.26 mM $MgCl_2$, 0.44 µM JH0724 (5'-ACGCGCCCT-3') or JH0725 (5'-CCGCAGCCAA-3'), 4.4 mM dNTPs, 0.0054 U Taq, and 34.5 µL ultra-pure water in a total volume of 50 µL. The RAPD-PCR was run on a Mastercycler (Eppendorf, Hamburg, Germany) using the following settings: 4 cycles (94°C for 45 sec, 30°C for 2 min, and 72°C for 1 min), 26 cycles (94°C for 5 sec, 36°C for 30 sec, and 72°C for 30 sec with one additional sec added per cycle), final extension at 72°C for 10 min, and a hold at 4°C (Quednau *et al.*, 1998).

PCR products (10 µL of each reaction) were loaded into a 1.5% agarose gel, then run for 2.5 hours at 100V. The gel was stained with ethidium bromide, visualized and photographed under UV light. The resulting image was analyzed using the Gel-Compar II software (Applied Maths, Austin, TX). Banding patterns were clustered using UPGMA clustering of Dice co-efficient with optimization and position tolerance settings of 0.00% and 1.00 % respectively.

3.3.6 Genome sequencing

3.3.6.1 *Preparation of DNA and genome libraries*

Genomic DNA was isolated from "*B. hampsonii*" P13 and P113 cultures grown in BHIS broth medium by modified salting out procedure (Martin-Platero *et al.*, 2007). Concentration of genomic DNA extracts was measured using a spectrophotometer and A260/A280 ratio was measured to confirm purity. PCR targeting and sequencing of the *nox* gene was performed to verify that the genomic DNA of both P13 and P113 belong to clade II, as previously described by Atyeo *et al.* (1999).

The library preparation was followed as outlined by the Illumina Nextera Mate Pair Sample Preparation Guide (Illumina, San Diego, CA). The index adapters utilized were AD002 (GCCAAT(A)) and AD012 (CTTGTA(A)). Average size of the final amplicon libraries in base pairs (bp) was calculated using the DNA High-Sensitivity chip on the 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) and concentration in ng/μL assessed using the Qubit fluorometer (Life Technologies, Inc., Burlington, ON). PhiX DNA (15% (v/v)) was added to the pooled indexed libraries prior to loading onto the flow cell. Sequencing was performed using the 500 cycle (2 × 250) V2 Nano platform.

3.3.6.2 *Sequence data processing and genome assembly*

Raw mate-pair sequence reads in fastq format were trimmed for quality using Trimmomatic (Bolger *et al.*, 2014) with a minimum read length of 50 and quality cut-off of Phred score >30. High quality paired reads were carried forward to adapter clipping. Mate-pair adapter

sequences were identified and reads trimmed to the adapter location using NextClip (Leggett *et al.*, 2014), which results in classification of reads into four categories: A, where the adapter is detected in both Read 1 and Read 2 of the mate-pair; B, adapter in Read 2 only; C, adapter in Read 1 only; D, adapter not detected in either read. Reads from categories A-C were included in further analysis and assembly.

To estimate genome coverage, and calculate average insert size, mate-pair reads were mapped on to the reference genome assembly for *"B. hampsonii*" strain 30446 (Genbank Accession NZ_ALNZ00000000) using Bowtie2 (Langmead and Salzberg, 2012) and the results were converted to BAM format for viewing in Qualimap v2.2.1 (Okonechnikov *et al.*, 2016). Genome assembly was done using all reads from NextClip categories A-C as input for SOAPdenovo2 (Luo *et al.*, 2012), using estimated average insert size from Qualimap analysis.

Overall genome sequence similarity between P13, P113 and additional reference genomes from other *Brachyspira* species was calculated using Average Nucleotide Identity by Mummer (ANIm) and tetranucleotide scores within JSpecies (Richter and Rossello-Mora, 2009).

3.3.7 Virulence assessment in mice

The experiments were designed and performed in accordance with the Canadian Council for Animal Care and all work was approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20150015).

3.3.7.1 *Experiment 1 – P13 and P113*

Twenty, six-week old, female CF1 mice (Charles River, Wilmington, MA) were randomly assigned into groups of 4 mice per cage and labelled using tail markings applied with black markers

(Ek *et al.*, Submitted). The mice were fed a low-zinc diet, TD8540 (Envigo, Indianapolis, IN) *ad libitum*. After one week of acclimatization, mice in each group were inoculated on three consecutive days with 0.3 mL (~10^8 genomic equivalents in JBS) of P13 (*n*=8), P113 (*n*=8), or sham (sterile JBS, *n*=8) by oral gavage using a 20 gauge blunt-ended feeding needle. Feed was removed six hours prior to inoculation. Health checks (hair condition, behaviour) were performed twice daily. Mice were weighed on Days -6, -2, 0 (prior to inoculation), 5, 7, 9, 12, 14, 16, and at termination (Day 19 post-inoculation (PI)) for the calculation of average daily gain (ADG = total weight gained/number of days). Faecal pellets were collected for selective *Brachyspira* culture on BJ agar on Days -2, 0 (prior to inoculation), 5, 7, 9, 12, 14, and 16. Caecal swabs were collected at termination (Day 19 PI) during necropsy. Faecal scoring was performed twice a day (morning and afternoon) throughout the experiment. Faecal consistency was scored as: 0 = normal/formed, 1 = formed with slight mucus, 2 = formed with abundant mucus, 3 mild (3m) = mucus only/soft faeces only, 3 severe (3s) = soft and very mucoid, 4 = soft and very mucoid with blood (**Figure 3.1**).

3.3.7.2 *Experiment 2 – P13, P113, and MP1*

Thirty, six-week old, female CF1 mice (Charles River, Wilmington, MA) were randomly assigned into pairs (2 mice per cage). The mice were fed a low-zinc diet, TD8540 *ad libitum*. After one week of acclimatization, each mouse group was inoculated by oral gavage on three consecutive days with 0.3 mL (~10^8 genomic equivalents in JBS) of P13 (*n*=8), P113 (*n*=8), MP1 (*n*=8), or sham (sterile JBS, *n*=6). MP1 (mouse passage 1) was an isolate recovered from the colon of the mouse infected with P113 from Experiment 1 with the fewest abnormal fecal scores (mouse P113-

7). Health checks, weight measurements, ADG calculations, and faecal pellet collection and culture were performed as done in Experiment 1. A modification to the faecal consistency scoring was made to add to the existing scoring system: score 0.5 = slightly soft, but formed (**Figure 3.1**). The second experiment ran for 19 days PI, however the diet was changed from TD8540 to regular rodent chow on Day 13 PI due to the exhaustion of TD8540 stocks. For this reason, only the data collected from Days -6 to 12 PI was used for analysis to avoid any potential effects of the diet change on health and faecal outcomes.

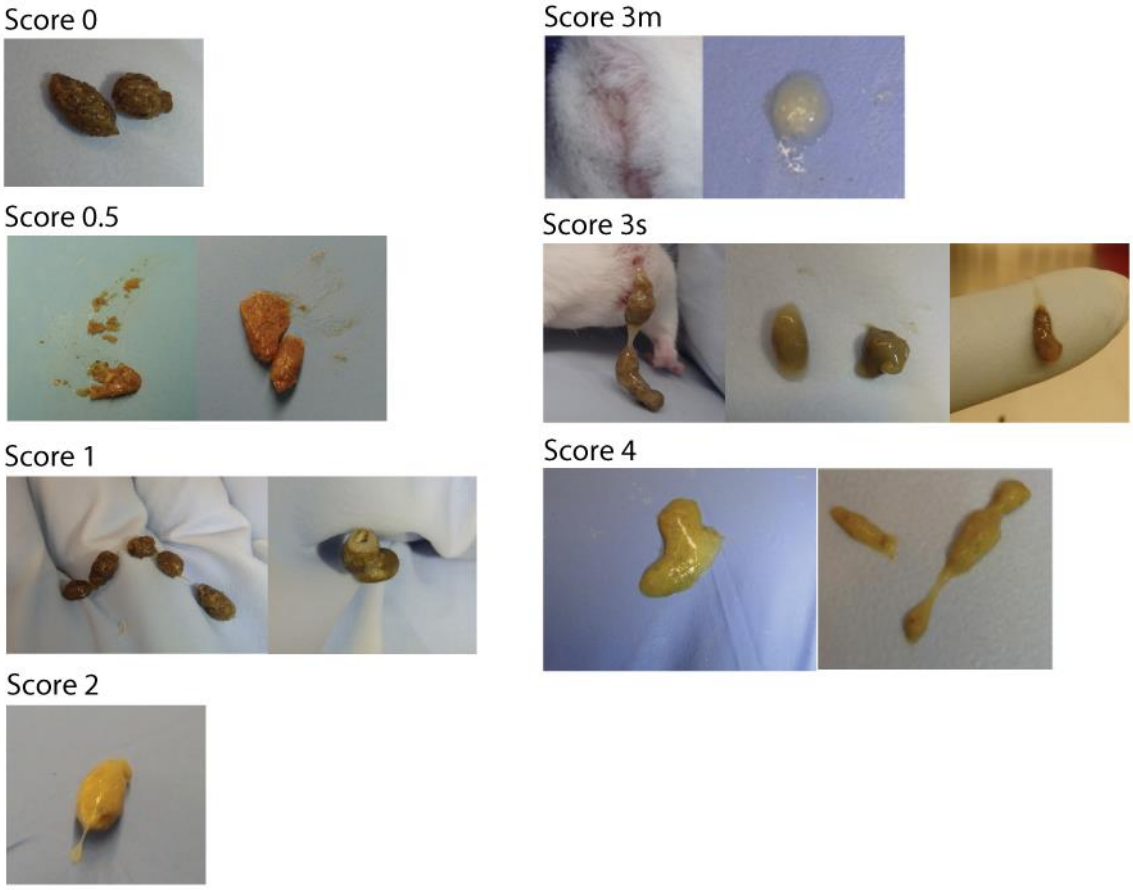


Figure 3.1. Faeces scoring sheet for Experiment 1 and 2. Score 0.5 was not used for Experiment 1. The scores represent the following faecal outcomes: 0 – formed pellet, 0.5 – slightly soft but formed pellet, 1 – formed pellet with a mucus tail or mucus on one end, 2 – formed pellet coated with mucus, 3m – mucus only/soft faeces only, 3s – mucus mixed with faeces (yellow mucus)/very soft faeces coated with copious amounts of mucus, 4 – mucus with faeces and some blood specks mixed in.

3.3.7.3 Statistics for Experiment 1 and 2

For Experiment 1, the mean ADG (a period from Day 0 to 19) for each group (P13, P113, or sham) were compared using the Kruskal-Wallis test. The average count of faecal scores ≥ 2 from Day 3 to 19 for each group (P13, P113, or sham) were also compared by Kruskal-Wallis.

For Experiment 2, the mean ADG (a period from Day 0 to 12) for each group (P13, P113, MP1, or sham) and the average count of faecal scores ≥ 2 from Day 3 to 12 for each group (P13, P113, MP1, and sham) were also compared by Kruskal-Wallis.

3.4 Results

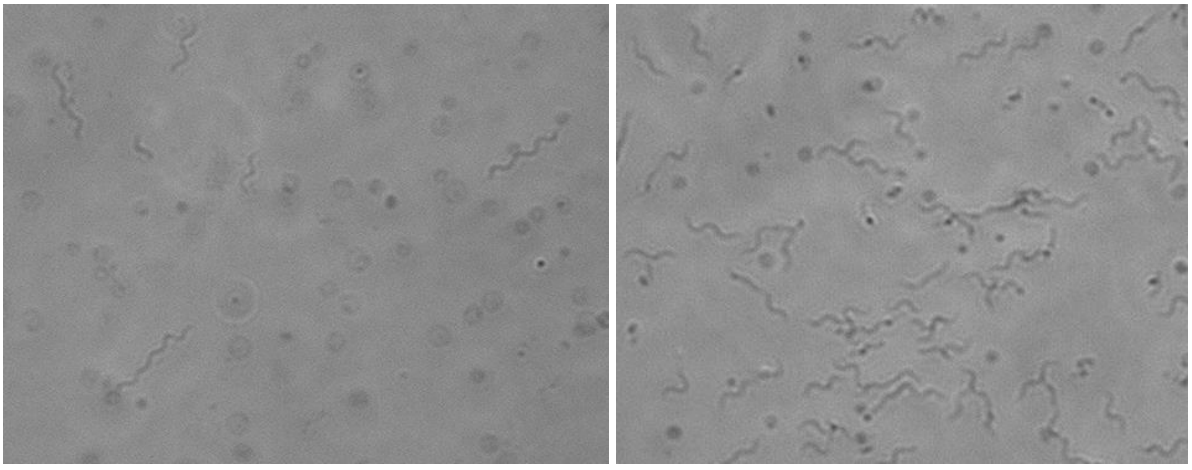
3.4.1 Phenotypic characteristics of P13 and P113

3.4.1.1 **Serial passage**

A total of 100 passages of P13 were done, resulting in strain P113. The time required to reach a dense culture under the growth conditions described was usually 1-3 days, although occasionally longer periods (up to 6 days) were required. No contamination was detected during serial passage. No changes to morphology were noted in Gram stains and phase-contrast images of P13 and P113 (**Figure 3.2**).

a)

P13 **P113**



b)

P13 **P113**

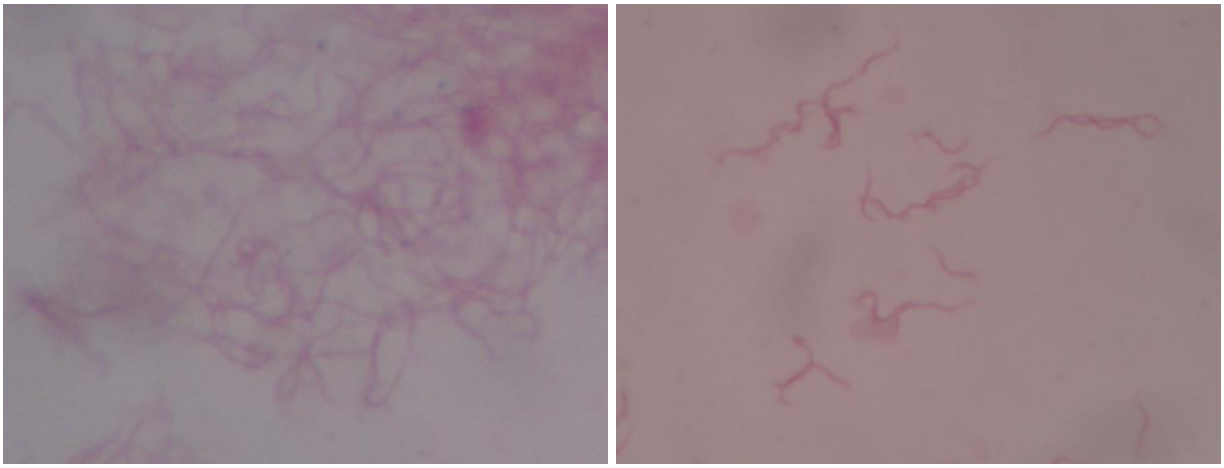


Figure 3.2. Phase-contrast **(a)** and Gram-stained **(b)** images of P13 and P113. Material present in the background of phase-contrast images were red blood cells, present in the JBS cultures used for phase-contrast imaging.

3.4.1.2 Biochemical characterization

To detect any changes to diagnostic biochemical characteristics, P13 and P113 were evaluated by API-zym, indole, and hippurate hydrolysis tests. *B. hyodysenteriae* ATCC 27164^T and *B. pilosicoli* ATCC 51139^T were included for the indole and hippurate assays to ensure that the assays were producing correct reactions. The API-zym assay resulted in positive β-glucosidase, negative α-galactosidase, and negative α-glucosidase activities for both P13 and P113 (**Table 3.1**). Negative hippurate hydrolysis and indole spot assay activities were also detected for both P13 and P113. The results for *B. hyodysenteriae* ATCC 27164^T (indole positive and hippurate negative) and *B. pilosicoli* ATCC 51139^T (indole negative and hippurate positive), were as expected. P13 was α-chymotrypsin negative, but a positive result was obtained for P113. Additionally, a difference was observed in β-glucuronidase results, with P113 giving a weak positive result compared to the negative result for P13.

Table 3.1. Biochemical profile of P13 and P113.

	D09-30446 P13	D09-30446 P113
Alkaline phosphatase	+	+
Esterase (C4)	+	+
Esterase Lipase (C8)	+	+
Lipase (C14)	-	-
Leucine arylamidase	-	-
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α -chymotrypsin	-	+
Acid phosphatase	+	+
Naphthol-AS-BI-phosphohydrolase	W+	W+
α-galactosidase	-	-
β -galactosidase	+	+
β -glucuronidase	-	W+
α-glucosidase	-	-
β -glucosidase	+	+
N-acetyl-β-glucosaminidase	-	-
α-mannosidase	-	-
α-fucosidase	-	-
Hippurate hydrolysis	-	-
Indole production	-	-

3.4.1.3 *β*-haemolysis assays

β-haemolysis was qualitatively assessed using a four-streak pattern on BJ agar. All P113 replicates reached quadrant 3 by Day 3 and one had reached quadrant 4 by Day 4. Only one of the P13 replicates reached quadrant 3 and only after 4 days of incubation (**Figure 3.3**).

The *β*-haemolysis activities of P13 and P113 were quantified to detect differences not detectable by qualitative methods. Results of the 96-well plate assay are shown in Figure 6. Haemolysis of BJ agar by P13 was greater than P113 at day 1 ($p<0.05$, Repeated Measures ANOVA), but remained at similar levels the following days (**Figure 3.4**). Whereas, the haemolysis performed by P113 continually increased over the 4 days and was greater than P13 on days 3 and 4 ($p<0.05$, Repeated Measures ANOVA) (**Figure 3.4**).

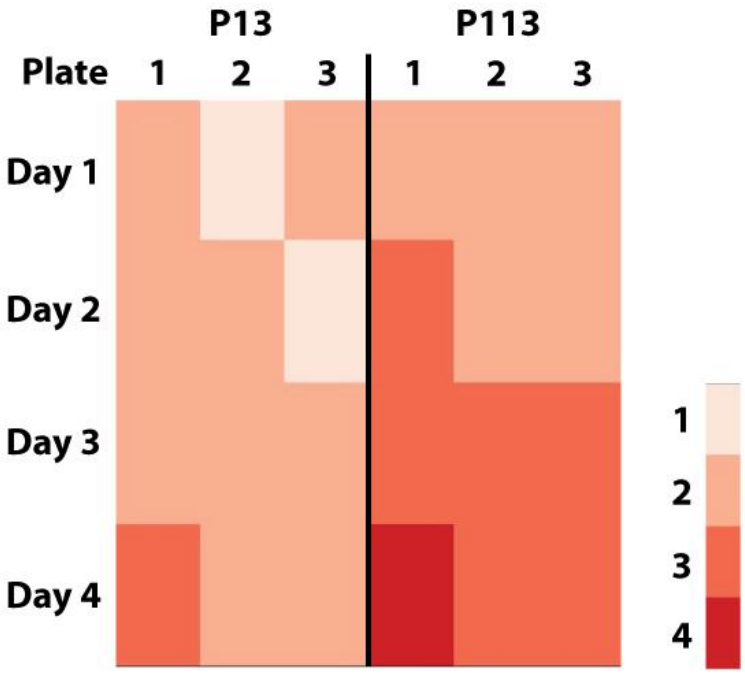


Figure 3.3. Qualitative assessment of *β*-haemolysis on BJ agar over 4 days. Each of three replicate plates for strains P13 and P113 were evaluated. Haemolysis score (quadrant number) is indicated by colour according to the legend.

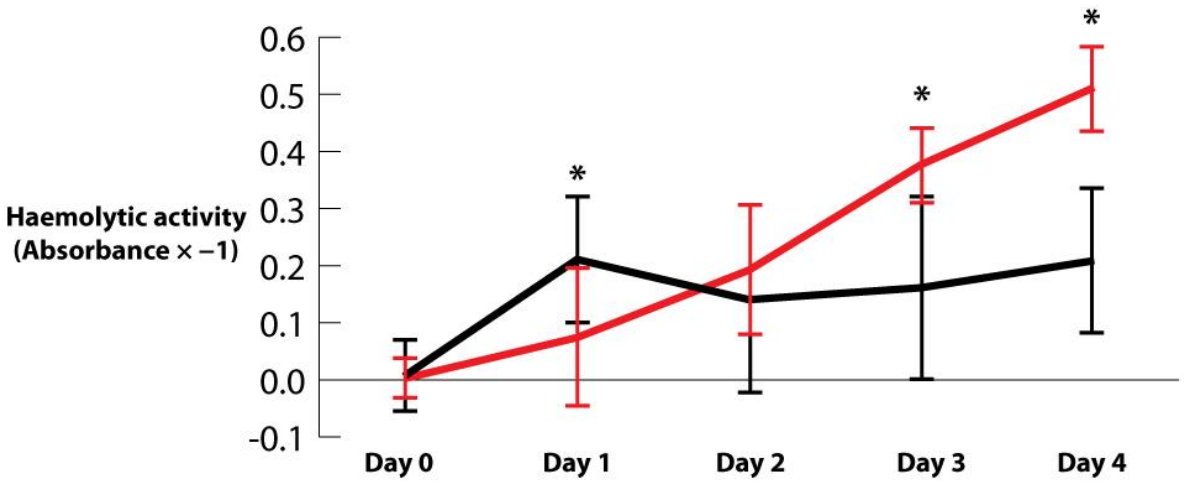


Figure 3.4. Quantitative assessment of P13 (black, *n*=120) and P113 (red, *n*=120) *β*-haemolytic activity (absorbance × –1) over 4 days. Error bars represent standard deviation. Significant differences ($p<0.05$, Repeated Measures ANOVA) are indicated by asterisks (*).

3.4.1.4 Growth study

To determine if the explanation for the unexpected observation of greater haemolysis activity for P113 compared to P13 was related to growth rate of the laboratory adapted P113 rather than a change in the specific activity of haemolytic factors, the growth rates of the two strains were compared. Triplicate cultures were prepared for each time point (hours 0, 12, 24, 36, 48, and 72). Logarithmic growth of P113 started at 12 hours and the optical density of P113 cultures were greater than P13 cultures over the 72-hour period, except for hours 36 and 72 (**Figure 3.5**). The average optical density for two of three replicates of P113 at hour 36 had very little growth resulting in an unexpectedly low average OD for that time point. Exponential growth of P13 did not begin until between hours 36 and 48. P113 reached the stationary phase between 48 and 72 hours, while a stationary phase was not observed for P13 during the experiment (**Figure 3.5**). There was significantly greater growth of P113 at 12, 24, and 48 hours and significantly more overall P113 growth (p=0.047, Mann-Whitney U).

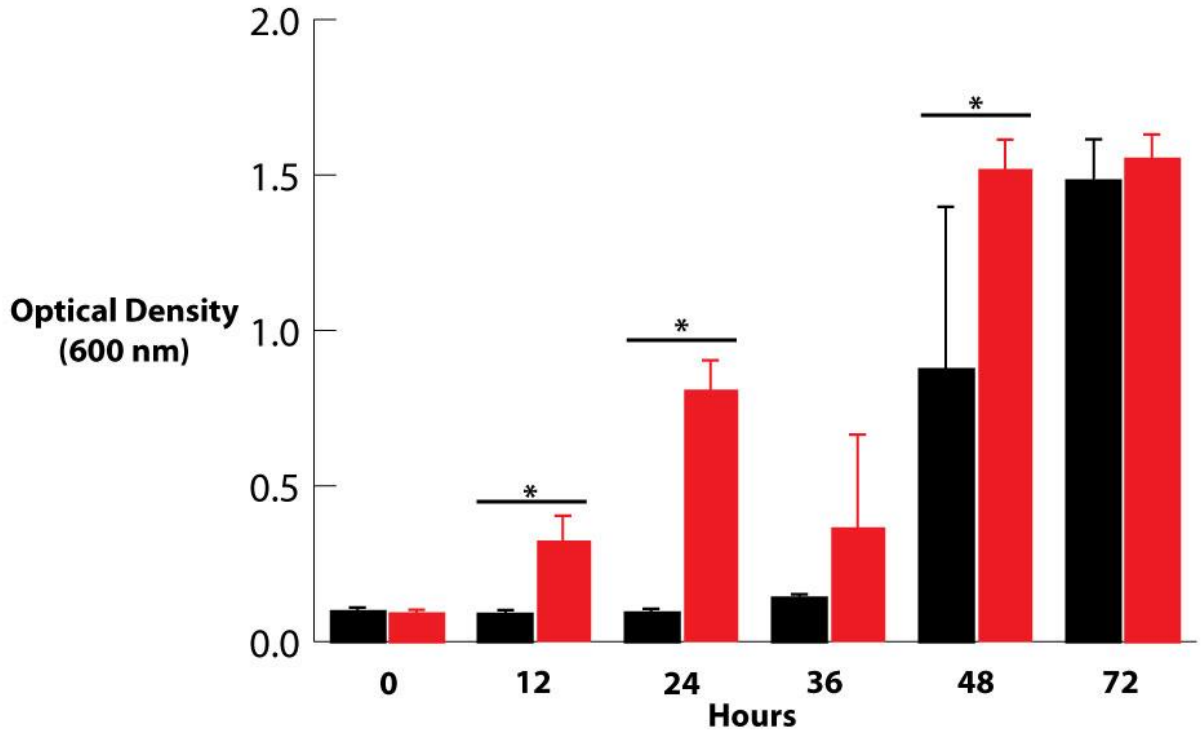


Figure 3.5. Growth study of P13 (black, *n*=3 per time point) and P113 (red, *n*=3 per time point) in broth medium over a 72-hour period. Error bars represent standard deviation. Significant differences ($p<0.05$, Mann Whitney U) are denoted by asterisks (*).

3.4.2 Genome fingerprinting by RAPD

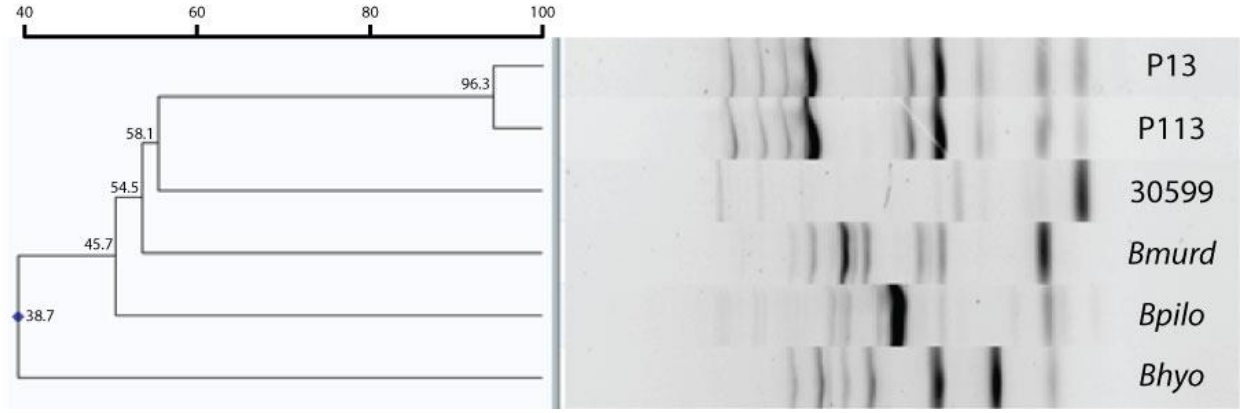
Visual inspection of banding patterns of replicate RAPD profiles from individual *Brachyspira* spp. indicated high reproducibility of banding patterns. RAPD profiling of P13 and P113 were 96.3% and 94.1% identical to each other based on primers JH0724 or JH0725, respectively (**Figure 3.6a** & **b**). *"B. hampsonii*" clade I strain 30599 was 62.5 & 58.1% and 37.5 & 50.0% identical to P13 and P113, respectively, based on RAPD with primers JH0724 or JH0725. RAPD patterns of other species of *Brachyspira* spp. were easily distinguished from *"B. hampsonii*" with only 47.1 – 76.1% similarity to P13 and P113 depending on the species and the primer used (**Figure 3.6**a & b).

3.4.3 Whole genome sequencing

Whole genome sequencing yielded a total of 816,716 and 909,529 raw reads for P13 and P113, respectively. Details of the numbers of reads surviving quality clipping, identification of paired reads and adapter trimming are shown in **Table 3.2**. A total of 520,555 and 545,614 reads were available for Bowtie2 mapping and genome assembly for P13 and P113, respectively. *De novo* genome assembly results are summarized in **Table 3.3**. Bowtie2 mapped >98% of reads from either genome to the *"B. hampsonii*" 30446 reference genome, and coverage was calculated as 24-25× for both sequenced genomes. The median insert size for the mate-pair fragments was 4,058 for P13 and 2,435 for P113. N50 values were low, indicating a highly fragmented genome assembly, although coverage was apparently complete with ~3 Mb of total contig length for both genomes (reference genome size: 3,071,124 bp). G+C content was ~27.5% for both P13 and P113.

Comparison of the P13 and P113 showed high similarity based on tetra scores (0.99675 – 0.99982 similar) and ANIm values (99.77 – 99.8% similar) (**Table 3.4**). Clade I "*B. hampsonii*" D11-30599 was highly similar by tetra scores to P13 (0.99675) and P113 (0.99717) and by ANIm to P13 (93.65-93.68%) and P113 (93.66-93.69%) (**Table 3.4**).

a)



b)

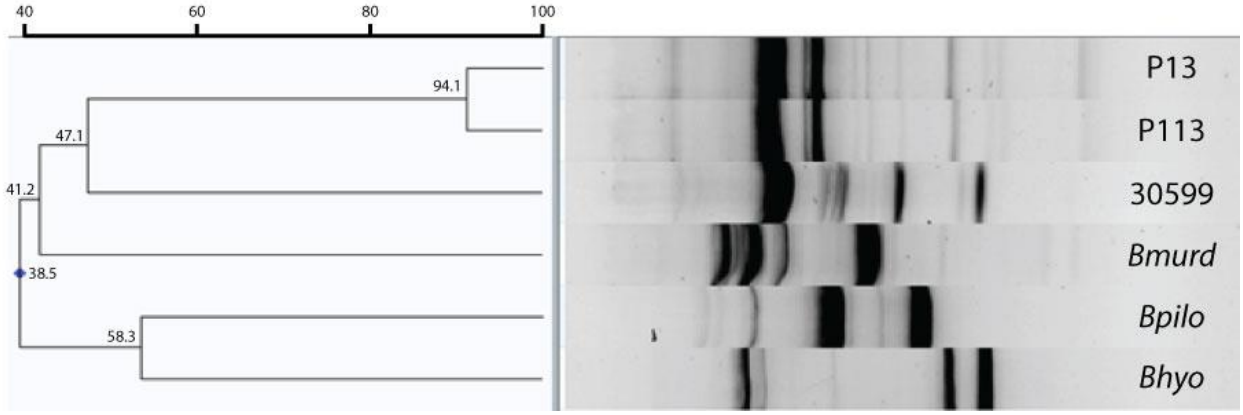


Figure 3.6. RAPD profile comparison of several *Brachyspira* spp. and strains using primers JH0724 **(a)** and JH0725 **(b)** by clustering of banding patterns using UPGMA clustering of Dice co-efficient with optimization and position tolerance settings of 0.00% and 1.00 % respectively.

Table 3.2. Raw read numbers and results of trimming for quality and adapter sequences.

	P13	**P113**
Number reads raw	816,716	909,529
Number paired (post-Trimmomatic)	720,154	754,209
NextClip A	208,466	229,096
NextClip B	137,894	143,343
NextClip C	174,195	173,175
Total post-NextClip	520,555	545,614

Table 3.3. *De novo* assembly results for P13 and P113.

	D09-30446 P13	**D09-30446 P113**
Sequencing coverage (×)	24.1	25.2
Mate pair insert size (median)	4 058 bp	2 435 bp
N50	1 562 bp	1 774 bp
Longest contig	12 764 bp	34 186 bp
Total contig length	3 032 560 bp	3 082 682 bp
No. of contigs (>500 bp)	2 093	1 939
%GC	27.56%	27.51%

Table 3.4. Tetra **(a)** and ANIm **(b)** scores of pairwise comparisons of P13, P113, and reference genomes.

a)

Isolate	P13	P113	D09-30446	D11-30599	*B. hyodysenteriae* ATCC 27164^T
P13	—	0.99675	0.99985	0.99717	0.9872
P113	0.99982	—	0.9999	0.99675	0.98697
D09-30446	0.99985	0.9999	—	0.99681	0.9872
D11-30599	0.99717	0.99675	0.99681	—	0.98703
B. hyodysenteriae ATCC 27164^T	0.9872	0.98697	0.9872	0.98703	—

b)

Isolate	P13	P113	D09-30446	D11-30599	*B. hyodysenteriae* ATCC 27164^T
P13	—	99.77	99.87	93.68	89.17
P113	99.8	—	99.89	93.69	89.15
D09-30446	99.84	99.84	—	93.54	88.7
D11-30599	93.65	93.66	93.55	—	89.18
B. hyodysenteriae ATCC 27164^T	89.17	89.13	88.73	93.54	—

3.4.4 Virulence in a mouse model

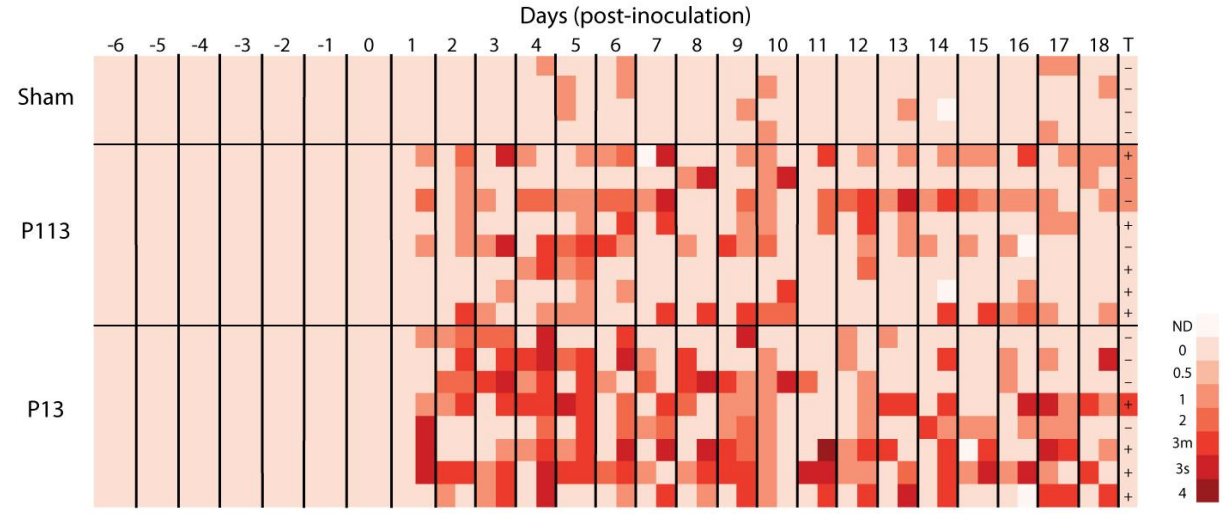
In Experiment 1, mouse behaviour did not differ between the sham, P13, and P113 groups. The calculated ADG also did not differ significantly between among groups (**Table 3.5**; NS, Kruskal-Wallis). There were no faecal scores ≥2 for the sham group, while the P113 group had fewer scores ≥2 than the P13 group. Differences in the number of daily faecal scores ≥2 were significant between all the groups (Kruskal-Wallis, $p<0.05$) and the number of faecal scores ≥2 in P13-inoculated mice was greater than P113- and sham-inoculated mice (**Figure 3.7a**; Kruskal-Wallis, $p<0.05$). On days when abnormal feces were observed, it was often observed in individual mice either in the morning or afternoon observation periods, but not both, which has also been observed by our group in swine trials (**Figure 3.7**). Mouse P113-7 had the fewest faecal scores ≥2 within the P113 group ($n=4$). To explore the possibility of further attenuation of P113 resulting from mouse passage or selection of a particularly avirulent subpopulation of P113 in this mouse, strain MP1 (mouse passage 1) was isolated from the colon of P113-7 upon termination for use as inoculum in Experiment 2.

In Experiment 2, mouse behaviour did not differ between the sham, P13, P113, and MP1 groups. The calculated ADG also did not differ significantly between all the groups (**Table 3.5**; NS, Kruskal-Wallis). The average count of faecal scores between all groups was significantly different, except between P113 and MP1 (Kruskal-Wallis, $p < 0.05$). The P13 group had the highest average count of faecal scores ≥2 (**Figure 3.7b**). Abnormal fecal scores were also observed in the sham group in this experiment. Due to low TD8540 feed stocks, less food was available 5 days PI, which may have acted as a stressor for all groups.

Table 3.5. Calculated average daily gain of mice for Experiment 1 and 2. No significant difference was detected by Kruskal-Wallis.

		Average ADG (g/day)	Standard Deviation
Experiment 1	Sham (*n*=4)	0.1641	0.1058
	P113 (*n*=8)	0.1869	0.0473
	P13 (*n*=8)	0.1589	0.1177
Experiment 2	Sham (*n*=6)	0.0450	0.1699
	MP1 (*n*=8)	0.2292	0.1677
	P113 (*n*=8)	0.2061	0.1034
	P13 (*n*=8)	0.1841	0.1200

a)



b)

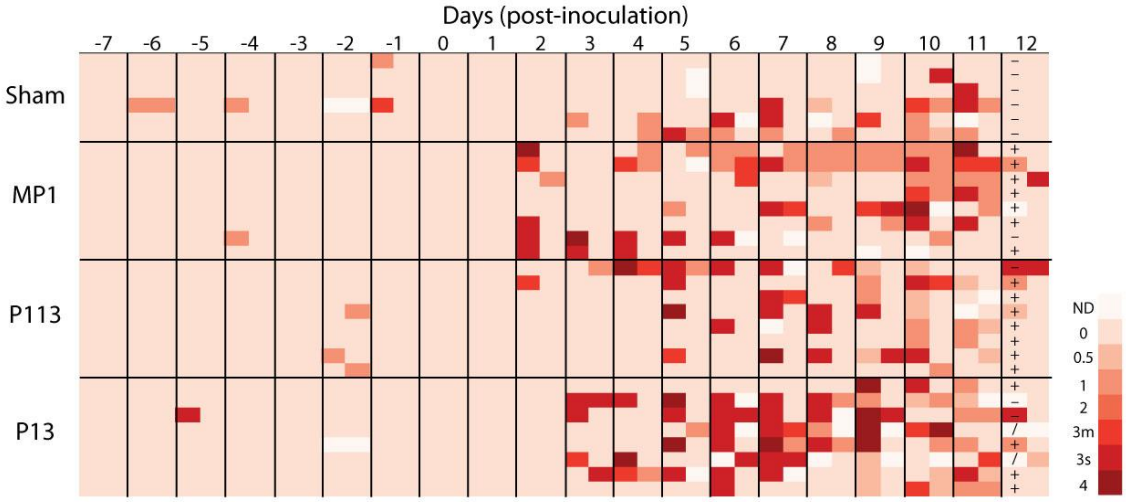
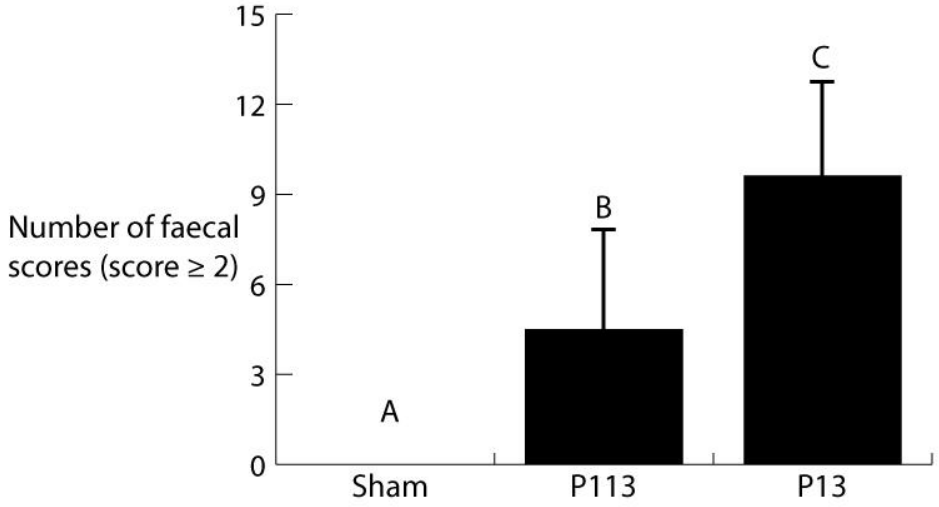


Figure 3.7. Heatmaps representing the daily faecal score (AM & PM) of each mouse in the different groups for Experiment 1 **(a)** and Experiment 2 **(b)**. The faecal scores are labelled by colour in the legend for each heatmap, ND indicates no data was collected. **T** indicates the termination day. For both Experiment 1 (termination - caecum) and 2 (last day of trial with low-zinc diet – faecal pellet), results of spirochaete recovery by culture are under the final day included as positive (+), negative (–), or no data (/). Feed rationing in Experiment 2 started 5 days PI.

a)



b)

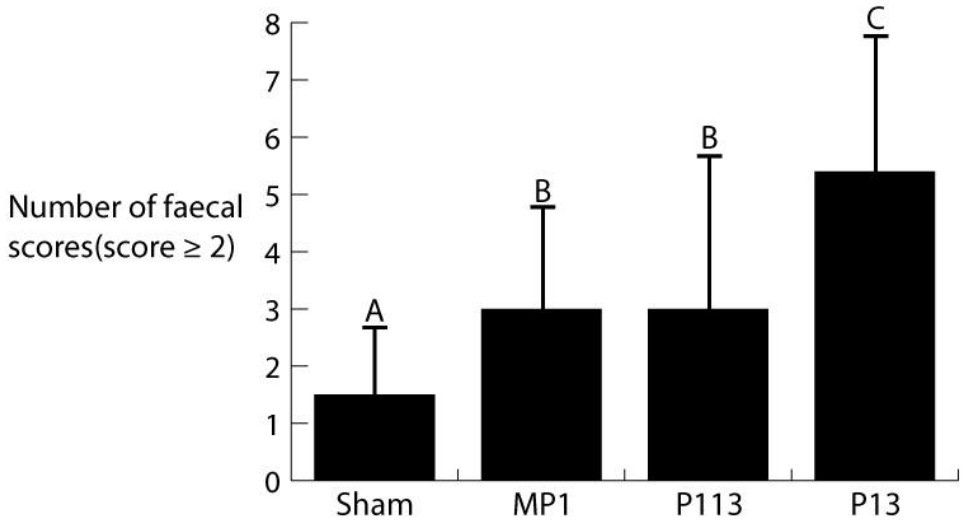


Figure 3.8. Average count of faecal scores ≥2 per group for Experiment 1 **(a)** (n_{Sham}=4, $n_{P13/P113}$=8) and Experiment 2 **(b)** (n_{Sham}=6, $n_{MP1/P13/P113}$=8). Error bars represent the standard deviation. Significantly different groups are denoted by different letters (p<0.05, Kruskal-Wallis).

3.5 Discussion

In vitro serial passage of a pathogenic "*B. hampsonii*" clade II strain D09-30446 was performed as a method of producing an attenuated vaccine candidate, based on previous experiences by other groups producing attenuated strains after several serial passages *in vitro* (Kinyon *et al.*, 1977). The resulting P113 strain was phenotypically (biochemical profile, *β*-haemolysis, growth curves, virulence in a mouse model) and genotypically (genome structure and sequence comparison) characterized, relative to the original, virulent P13 strain.

An attenuated "*B. hampsonii*" strain could be useful for vaccine production, but may also aid in the identification of virulence factors since comparison of virulent and attenuated strains may highlight changes linked to virulence in swine. In this study, we examined biochemical characteristics used to define *Brachyspira* species, *β*-haemolysis activity, genome structure, and genomic content. *Brachyspira* spp. were historically identified and differentiated based on a profile of biochemical markers (*α*-galactosidase, *α*-glucosidase, *β*-glucosidase, hippurate hydrolysis, and indole production) and strength of *β*-haemolysis, although variation in these biochemical markers has been reported in several *Brachyspira* spp. (De Smet *et al.*, 1998, Fellström *et al.*, 1999). Strong *β*-haemolysis has been associated with more severe illness in swine, while milder disease is associated with weakly *β*-haemolytic *Brachyspira* spp. (Burrough, 2016, Trott *et al.*, 1996c). The reliability of the strength of *β*-haemolysis as a marker for virulence is in question though as variability has been detected in the degree of *β*-haemolysis of *B. hyodysenteriae* on blood media, with some clinical isolates producing lower degrees of *β*-haemolysis (Mahu *et al.*, 2016).

Characterization of biochemical profiles of "*B. hampsonii*" P13 and P113 indicated no detectable changes in established diagnostic characteristics (**Table 3.1**). Differences in α-chymotrypsin and β-glucuronidase were observed, but cannot be interpreted since the degree of variation in these tests among "*B. hampsonii*" isolates has not been reported. Mutations of metabolic enzymes have been associated with attenuation in other systems, such as changes in glycerol dehydrogenase, an enzyme associated with virulence in *Mycoplasma gallisepticum*, leading to attenuation of strains (Szczepanek *et al.*, 2010). However, the lack of detectable changes in diagnostic biochemical markers in our study was not surprising, since these historically characterized markers have not been strongly linked to the level of pathogenicity of *Brachyspira* spp. (Achacha and Messier, 1991, De Smet *et al.*, 1998, Perez *et al.*, 2016).

The morphology of P113 did not differ from P13 based on a qualitative observation of Gram-stain and phase-contrast images (**Figure 3.2**). However, this is not a surprising result since major changes, such as greater sensitivity of *Brachyspira* to low pH that leads to the spherical morphotype (Bernardeau *et al.*, 2009) or changes in the membrane composition as observed in *Borrelia burgdorferi* (LaRocca *et al.*, 2013), would have to occur for bacterial cell morphology to change.

Attenuation of virulence has been attributed to modification of haemolysis pathway enzymes, such as *agr* in *S. aureus* (Somerville *et al.*, 2002). Given that strong β-haemolysis is considered a potential virulence factor of swine pathogenic *Brachyspira* spp., we anticipated that the putatively attenuated P113 would exhibit less haemolytic activity than P13. However, both the qualitative and quantitative assays suggested the opposite pattern with more haemolytic activity

observed for P113 (**Figure 3.3**, **Figure 3.4**). To determine if the difference was due to a modified haemolysin or due to an improvement in the growth of P113, we compared the growth rates of the two strains (**Figure 3.5**). The results highlight that the difference in haemolysis patterns is likely due to an improvement in the growth rate of P113 on BJ agar, rather than an increase in the haemolysis strength of P113. This is consistent with the culture adaptation of P113 through serial passage in the laboratory.

The genome profiling performed for P13 and P113 indicated no detectable difference between the two, based on RAPD profiling (**Figure 3.6**). The separation of other *Brachyspira* spp., a clade I "*B. hampsonii*" reference isolate, P13, and P113 by RAPD shows that the method is capable of differentiating clades within the species. These results also indicate that serial passage did not result in large-scale genomic changes such as major deletions or re-arrangements. To investigate genome changes at a more detailed level, we determined the whole genome sequences of P13 and P113. Mate-pair genome sequencing and *de novo* assembly of both P13 and P113 produced highly similar genomes based on the genome size, G+C content and sequence content determined by tetra scores (0.99675 – 0.99982 similar) and ANIm values (99.77 – 99.8% similar) (**Table 3.3**, **Table 3.4**). Comparison of the genome profile and sequence have so far have not produced any detectable differences, however ongoing supplementation of the current data with paired-end sequences will improve the assembly quality, permitting annotation and aiding with detection of smaller changes between the genomes resulting from the laboratory attenuation process.

Infection studies of swine-virulent *Brachyspira* spp. have primarily used swine models to study the effects of the spirochaete on the primary animal of concern. However, some studies have also utilized different mouse models to study the effects of *Brachyspira* spp. on the mouse intestine. A more recent study has shown that one particular strain of mice (CF1), fed with a low-zinc diet, produced more mucus and soft faeces when infected with *B. hyodysenteriae* and "*B. hampsonii*" compared to sham-inoculated mice (Ek *et al.*, Submitted). Mouse models are also appealing as they are more cost efficient and less sentient models. "*B. hampsonii*" P13 and P113 were both compared in a mouse model to assess the relative virulence of P113.

In Experiment 1, all mice inoculated with P113 had some abnormal fecal scores, but mice in this group had significantly fewer faecal scores ≥2 than controls or mice inoculated with P13. No difference was observed in ADG or mouse health (indicated by fur condition and behaviour) (**Table 3.5**). Similar results were obtained in Experiment 2 for controls, P13 and P113. Experiment 2 was complicated by the low availability of TD8540 feed and rationing of food 5 days PI. This stressor may be responsible for the observation of some higher faecal scores in sham group, which were not observed in Experiment 1 (**Figure 3.8**). Loss of body weight and higher mortality due to peritoneal infection after a period of fasting have been observed in mice, which may explain the higher number of abnormal faecal scores in all groups in Experiment 2 (Bermudes *et al.*, 2011).

To investigate the possibility that passage through mice might further attenuate virulence or select for less virulent subpopulations within P113, we isolated MP1 from feces of the mouse in Experiment 1 with the lowest overall fecal scores and used this isolate to challenge mice in

Experiment 2. However, no difference in the occurrence of abnormal fecal scores was detected between the MP1 and P113 groups.

Taken together, the results of the mouse infection studies show that partial attenuation has been achieved, however further experimental infection in swine will be required to determine if virulence is also attenuated in the natural host of *"B. hampsonii*". These trials are ongoing at the time of writing. Demonstration of a sufficiently low virulence in pigs will be critical to the success of P113 as a promising vaccine candidate, and further attenuation will likely be necessary. Based on previous experience with the lack of heterologous protection against different *B. hyodysenteriae* strains, other "*B. hampsonii*" strains should be used to challenge P113-vaccinated pigs in future studies (Joens *et al.*, 1983).

Further work identifying the "*B. hampsonii*" virulence determinants that have changed in P113 is still needed by detecting changes in the genome and putative virulence genes, such as *tly* cytolysins that have haemolytic activity and are linked to virulence (Muir *et al.*, 1992, Keith *et al.*, 2016). However, the availability of these two strains with demonstrably different virulence in mice (and perhaps pigs) provide powerful resources for these studies.

4 General discussion

4.1 Summary and limitations of the work

4.1.1 Biochemical characterization alone is not sufficient for differentiation of "*B. hampsonii*" clades or discrimination from other *Brachyspira* spp.

"*B. hampsonii*" is a novel *Brachyspira* taxon that has been demonstrated to cause mucohaemorrhagic disease in swine and is a contributor to the re-emergence mucohaemorrhagic disease (Chander *et al.*, 2012). US isolates of both clades have been phenotypically and genotypically described (Chander *et al.*, 2012), however Canadian isolates required further description to improve the knowledge of diagnostically informative characteristics.

The results of this chapter describe the diagnostic biochemical traits of Western Canadian isolates of clade I and II "*B. hampsonii*", indicating that biochemical traits cannot be relied upon for differentiation of clades due to variance of key biochemical traits in each clade. The chapter also includes descriptions of select biochemical traits (indole production, hippurate hydrolysis, α-galactosidase, α-glucosidase, and β-glucosidase), β-haemolysis strength, spirochaete dimensions, and count of flagella per cell of representative Canadian clade I and II "*B. hampsonii*" strains relative to previous descriptions of other swine-associated *Brachyspira* species. The work also provided the first published electron micrographs of *"B. hampsonii*". The results highlight that caution should be used when characterizing isolated *Brachyspira* spp. using phenotypic traits. The number of clade I isolates was small and was limited due to the apparent bias of more clade II "*B. hampsonii*" being present in Canada, and their corresponding rareness in our culture collection.

An additional constraint was that isolates were selected to be as epidemiologically independent as possible. Western Canadian swine production is dominated by a few large, interconnected systems, which limited the "independence" of many of the available clinical isolates. However, even the small number of isolates was sufficient to indicate that there is variance in the diagnostic biochemical traits within clade I. Analysis of additional isolates will help with identification of other variances within and between "*B. hampsonii*" clades. Genotypic profiling of these isolates, with techniques such as RAPD, MLST, or whole genome sequencing would help describe genetic variation among clinical *"B. hampsonii"* isolates, which was addressed only by *nox* sequence comparison in our study to differentiate the two clades.

4.1.2 *In vitro* attenuation produced a laboratory adapted and partially attenuated "*B. hampsonii*" clade II strain

Results described in Chapter 3 demonstrated that the serially passaged "*B. hampsonii*" strain (P113) has become laboratory adapted and partially attenuated. The results of the haemolysis assays and growth curves showed that P113 was better adapted to laboratory medium, with more rapid growth on BJ agar and in broth medium. Our results do not allow us to determine if there have been changes in haemolysis activity, due to the adaptation of P113 to laboratory medium, but this could be studied in more detail using the *in vitro* haemolysis assay described by Mahu *et al.* (2016) that evaluates the haemolytic capacity of supernatant (from filtered broth culture) on a red blood cell suspension. With this approach, the haemolytic activity could be expressed relative to cell number, eliminating the confounding factor of growth rate.

The virulence assessment in a murine model demonstrated the partial attenuation, based on the lower average faecal scores in P113-inoculated mice compared to P13 inoculated mice. However, despite the change in virulence, no difference between these strains was identified by the genomic profile and sequence comparisons done to date. The P13 and P113 whole genome sequences compared in Chapter 3 are not yet of sufficient quality for a greater depth of comparison, however the addition of more sequencing data from an alternate method (paired-end sequencing) and annotation of the resulting assembled genomes will permit detection of differences between the genomes.

4.2 Discussion of future prospects

The work described in Chapter 2 was focused on improving the information available about Western Canadian isolates and pointed to the variation in the biochemical traits of clinical isolates, which is important for differentiating "*B. hampsonii*" clinical isolates from other *Brachyspira* species. However, there are many other questions that remain unanswered about "*B. hampsonii*" due to the fastidiousness of *Brachyspira* and the existence of *Brachyspira* spp. in communities within swine. Improved diagnostic tools would facilitate surveillance to improve understanding of *Brachyspira* distribution and ecology and detection of *Brachyspira* in healthy herds, but tools such as ELISA have yet to be developed to detect "*B. hampsonii*" exposure and antigens specific to "*B. hampsonii*" still need to identified and assessed. Currently, amplification and sequencing of the *nox* gene, or species-specific PCR targeting this gene, remain the preferred methods for detection and identification of *Brachsypira*.

P113 was shown to be partially attenuated and laboratory adapted, although the specific changes in genes and/or expression that produced these observed changes have not yet been identified. The virulence determinants of *Brachyspira* spp. are still not clear and further work comparing virulent and attenuated strains, such as P113, could lead to detection of novel virulence-linked targets to study. Ongoing experiments will reveal the virulence of P113 in pigs, and determine if exposure to an attenuated strain provides protection from future challenge. Further studies are still required to characterize the mucosal immune response in swine to *"B. hampsonii*", to better understand the effect of P113 as a vaccine and the immune response after challenge. Further studies in swine models are also needed to determine whether heterologous protection is possible against other "*B. hampsonii*" isolates and to characterize the immune response after exposure of pigs to P113. Ultimately, the success of a live attenuated vaccine depends upon sufficient reduction of virulence coupled with stimulation of a protective immune response against a sufficient range of pathogen strains. The work described in Chapter 3 represents some important first steps toward this goal.

Future studies focused on "*B. hampsonii*" will improve diagnostics, prevention of disease, help with understanding the virulence of "*B. hampsonii*" and may provide clues about other swine-virulent *Brachyspira* species.

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