IMPROVING THE DIAGNOSTIC METHODS AND PROCESSES FOR THE IDENTIFICATION AND CHARACTERIZATION OF *BRACHYSPIRA*

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements For the

Degree of Doctor of Philosophy

In the Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

By

DHARMASIRI GAMAGE RUWINI SULOCHANA KULATHUNGA

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor who supervised my thesis work, or in their absence, permission may be granted by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Veterinary Microbiology
Western College of Veterinary Medicine
University of Saskatchewan
52 Campus Drive
Saskatoon, Saskatchewan S7N 5B4
Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9
Canada

ABSTRACT

There are no standardized antimicrobial susceptibility test methods or interpretative criteria for *Brachyspira* spp. and laboratories rely heavily on in-house methods that yield highly variable test results. Despite advances in diagnostic technologies, treatment choices for swine dysentery are most often empiric, which can lead to increasing levels of antimicrobial resistance. The objectives of this study were (1) to develop a standardized agar dilution method and describe the antimicrobial susceptibility of a collection of *Brachyspira* isolates, (2) to develop a feasible methodology for antimicrobial susceptibility testing to include in the diagnostic workflow; including an analysis of accuracy and cost for different diagnostic approaches, (3) to develop assay selection decision trees based on the diagnostic objectives and (4) to investigate the genetic determinants of resistance to protein synthesis inhibitory drugs. To obtain consistent minimum inhibitory concentrations (MICs) between replicates in susceptibility testing for all *Brachyspira* spp. the media (TSA based), temperature (42°C), and the starting inoculum size (1-2 × 10^8 CFU/ ml) were determined. A standard antibiotic susceptibility test (agar dilution) with good reproducibility (80-100%) was developed. According to the test results *B. hyodysenteriae* and *B. hampsonii* isolates were profoundly susceptible to pleuromutilin drugs while some *of B. pilosicoli* isolates indicated high MICs (MIC > 8 µg/ml) for pleuromutilin drugs. Moreover, highly variable susceptibility test results for tylosin, tylvalosin, and lincomycin were observed amongst all species. The material and technical cost for all the diagnostic tests performed in the WCVM diagnostic laboratory were calculated, including a modified (miniaturized) agar dilution test. Further, decision trees were developed to select the most cost-effective test bundle based on the diagnostic objective. Using whole genome sequencing, isolates with higher MICs to macrolides were found to carry either a resistant gene called *lnu*C or have SNPs (A2058G or A2059G both) while isolates with higher

MICs for pleuromutilin drugs were found to have the *tva*B gene. None of these resistance genes or SNPs were identified in isolates with lower MICs for those drugs. A multiplex PCR was developed to screen the two resistance genes (*lnu*C and *tva*B) and predict the phenotypic resistance of clinical isolates in future. In conclusion, overall findings and novel test methods that were developed will help to improve the diagnostic workflow for the identification of *Brachyspira*-associated diseases including swine dysentery and support veterinarians for rapid diagnosis and evidence-based selection of antimicrobials to improve the treatment outcome.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor Dr. Joe Rubin, for his continued guidance, dedication, and support throughout my PhD program. His dynamism, vision, sincerity, and motivation have deeply inspired me, and it was a great privilege and honour to work and study under his guidance. I would also like to thank all my graduate committee members Dr. J. Harding, Dr. S. Gow, Dr. M. Links and my graduate committee chair Dr. J. Hill for their support and assistance during my research study and writing of this thesis. Also, recognition is extended to Dr. S. Parker for her support in statistical data analysis.

Very special thanks go to Champika and Michelle for all their support in my research. I would also like to acknowledge my lab mates (Roshan, Kazal, Dongyun, Bev, and Rachel), office mates (Rajnish, Emilie, Tami and Kela), the staff at GMP, Linda, Lana and all my other friends for their help and kind friendship during my PhD program. Most importantly, I dedicate this thesis to my parents D.G. Kulathunga (father), I. Dissanayake (Mother), husband (Amila) and my loving two sisters for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Finally, I wish to thank my two wonderful daughters, Prabashi and Methushi who provide unending love and patience during my studies. Finally, gratitude is extended to the funding agencies Swine Innovation Porc., Elanco Animal Health, NSERC, Devolved Scholarship, Department of Veterinary Microbiology, SK Innovation for financial support during this study.

.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP	Ampicillin
ATM	Antimicrobials
AUG	Amoxicillin + clavulanic acid
BH	*B. hyodysenteriae*
BHIS	Brain heart infusion broth
BHM	*B. hampsonii*
BI	*B. innocens*
BM	*B. murdochii*
BP	*B. pilosicoli*
CARD	Comprehensive Antibiotic Resistance Database
CFU	Colony forming unit
CHO	Chloramphenicol
CIPARS	Canadian Integrated Program for Antimicrobial Resistance
CLSI	Clinical and laboratory standard institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FISH	Fluorescent in-situ hybridization
FISH	Fluorescent in-situ hybridization
GFU	Growth forming unit
LIN	Lincomycin
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
NAL	Nalidixic acid
NC	Non-clustering

NGS	Next generation sequencing
OD	Optical density
PABA	Para-aminobenzoic acid
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PIS	Porcine intestinal colitis
PTC	Peptidyl transferase center
RAPD	Randomly amplified polymorphic DNA
RELP	Restriction fragment length polymorphism
SD	Swine dysentery
SNP	Single nucleotide polymorphisms
TET	Tetracycline
TIA	Tiamulin
TSA	Trypticase soy agar
TYL	Tylosin
TYV	Tylvalosin
VAL	Valnemulin
WGS	Whole genome sequencing

1. INTRODUCTION AND LITERATURE REVIEW

1.1 *Brachyspira*-associated diseases of pigs

1.1.1 *Brachyspira* taxonomy

Brachyspira is a genus of spirochetes with helically shaped bodies, regular coiling patterns, internal flagella, and cell ends that are either blunt or pointed (Stanton 2006; Mirajkar et al. 2016). These Gram-negative cells consist of an outer phospholipid by layer (outer membrane), a thin peptidoglycan layer in the periplasmic space, a periplasmic flagellum, an inner cell membrane or cytoplasmic membrane and cytoplasm (Stanton 2006; Ivanics et al. 2007; Blair et al. 2014). These spirochetes have adapted to specialized niches in the large intestine of various birds, humans and other mammals (swine, rats, dogs, and non-human primates) (Table 1.1).

Some pathogenic species of *Brachyspira* are associated with swine dysentery (SD) in grower-finisher pigs. Swine dysentery was first reported in 1921 in the USA (Whiting, Doyle, and Spray 1921). Initially, this disease was simply referred to as “bloody diarrhoea” because of the typical clinical signs being a bloody mucoid intestinal discharge. This initial report described in detail not only the clinical signs of the affected animals but also both the macroscopic and microscopic lesions found. Whiting et al., did a challenge trial to reproduce the disease in hog cholera-immune pigs by exposing them to pigs from a field outbreak of swine dysentery (Whiting, Doyle, and Spray 1921). In this study, they concluded that dysentery is distinct from hog cholera, and that the etiological agent was being secreted in the bloody intestinal discharge of sick animals (Whiting, Doyle, and Spray 1921).

Table 1. 1 Taxonomy of *Brachyspira* species, and their associated host.

Species[a]	Intestinal origin/host	Reference
B. innocens	Pigs	(Ochiai, Adachi, and Mori 1997)
B. intermedia	Pigs and chicken	(Hampson and La 2006)
B. pilosicoli	Pigs, birds, dogs, humans and nonhuman primates	(Ochiai, Adachi, and Mori 1997)
B. murdochii	Pigs and rats	(Hampson and La 2006)
B. aalborgi	Humans	(Hovind-Hougen et al. 1997)
B. alvinipulii	Chickens	(Stanton, Postic, & Jensenl, 1998)
B. hyodysenteriae	Pigs, bird species (rheas, chickens, ducks, and geese), mice, rats, dogs and feral birds	(Ochiai, Adachi, and Mori 1997; Hampson 2012)
B. hampsonii (Genomovars I, II and III)	Pigs and Lesser Snow Geese	(Rubin, Harms, et al. 2013; Mirajkar et al. 2016)
B. suanatina	Pigs and mallards	(Mushtaq et al. 2015)

[a]All species with standing in nomenclature are listed.

In the 1970s, spirochetes were observed when the colonic epithelium of SD affected pigs was examined using an electron microscope (Taylor and Alexander 1971). This discovery led researchers to hypothesize that spirochetes were playing a major role in the development of SD. The hypothesis was later proven as researchers were able to reproduce the disease in pigs by experimental infection (oral feeding) with spirochete cultures (Taylor and Alexander 1971). In these trials, they observed that affected pigs shed spirochetes in their diarrhea, and that these spirochetes were morphologically similar to those in the inoculum (Taylor and Alexander 1971). These intestinal spirochetes were initially described as *Treponema hyodysenteriae* (Stanton 2006). This genus included both pathogenic (strongly hemolytic) and non-pathogenic (weakly hemolytic) species (Stanton 2006). Further studies revealed that these two groups (pathogenic and non-pathogenic) had very low DNA sequence similarity, the non-pathogenic (weakly hemolytic) group were therefore re-classified as *T. innocens* while the pathogenic organisms (strongly hemolytic) were named *T. hyodysenteriae* (Kinyon and Harris 1979). In 1991, both *T. hyodysenteriae* and *T. innocens* were reclassified under the genus "*Serpula*" based on 16S rRNA nucleotide sequence analysis (Stanton et al. 1991). As the name *Serpula* was already used for a fungi genus, the genus *Serpula* was changed to "*Serpulina*" (Stanton 1982). The genus name *Brachyspira* was first assigned to *Brachyspira aalborgi* which was isolated from rectal biopsies of patients with intestinal spirochetosis in 1982 (Hovind-Hougen et al. 1997). In 1997 the two genera (*Serpulina* and *Brachyspira*) were finally united within the genus *Brachyspira* (Ochiai, Adachi, and Mori 1997). At present, there are nine species within the genus *Brachyspira* (Table 1.1) (Kulathunga and Rubin 2017).

1.1.2 Re-emergence of *Brachyspira*-associated diseases in Canada

In North America clinical disease associated with swine dysentery dropped to a low level by the mid-1990s. It is hypothesized that the decrease in SD may have been associated with the implementation of successful biosecurity practices and effective treatment protocols (Mirajkar and Gebhart 2014). However, the disease has re-emerged in Canada since 2009 and may be due to the appearance of a novel pathogenic species of *Brachyspira* which causes mucohemorrhagic diarrhoea indistinguishable from SD in grower-finisher pigs, or the emergence of antimicrobial resistant strains (Harding et al., 2010; Harding et al. 2013; Hill et al., 2018). Around the same time as the novel *Brachyspira* strain was identified in Canada in 2009, American researchers from Iowa and Minnesota also identified the novel species on their pig farms and provisionally named as "*Brachyspira hampsonii*" and according to the phylogenetic analysis of nucleotide sequence of *nox* and 16S ribosomal RNA genes isolates were initially grouped in to 2 clades (clade I and II) (Schwartz 2011; Chander et al. 2012). Later the novel species was characterized and formally named as *B. hampsonii* (Mirajkar et al. 2016). Based on the comprehensive genotypic, phenotypic, and genomic evaluation, it was proposed that *B. hampsonii* is a single species with multiple genomovars (Genomovars I, II and III) (Mirajkar et al. 2016). The term genomovar (phenotypically similar but genetically distinct species) was proposed for the first time for genomic groups identified by DNA-DNA hybridization in *Pseudomonas stutzeri* (Rossellb et al. 1991). A retrospective study in Canada revealed that even before the first identification of the novel species in 2009, *B. hampsonii* genomovar I was identified in formalin-fixed colon tissue samples collected in 2002 and genomovar II existed in samples collected in 2006 (Hill et al. 2018).

From clinical samples submitted from western Canadian pigs since 2009, the most frequently detected *Brachyspira* species has been *B. hampsonii* (Harding et al. 2013). *B. hampsonii* has an

approximately three-fold higher frequency of detection than that observed for *B. hyodysenteriae* in Canada (Harding et al. 2013). Between 2009 and 2013, *B. hampsonii* was detected in 34% of 380 samples (feces, tissues or carcasses) submitted from 70 commercial Canadian swine farms with suspected SD (Harding et al. 2013). While *B. hampsonii* is the primary species detected in Canada, in the Midwest United States *B. hyodysenteriae* is still the predominant species reported from such submissions (Burrough 2013; Harding et al. 2013; Mirajkar et al. 2016).

With the identification of the novel species both in the American Midwest and western Canada, researchers were interested in investigating whether there was any relationship between wildlife hosts and in the emergence of novel species. Therefore, a research study was conducted in western Canada, and *B. hampsonii* was detected in Lesser Snow Geese (*Chen Caerulescens Caerulescen*) in the Canadian Arctic. The study described that these birds, which migrate through the major pork-producing areas of the United States and Canada, possibly contribute to the spread of *Brachyspira* species among North American swine farms (Rubin, et al. 2013).

1.1.3 Pathogenesis and clinical disease in pigs

B. hyodysenteriae, B. hampsonii and *B. suanatina* are highly pathogenic species that affect grower-finisher pigs and cause muco-hemorrhagic diarrhea (Burrough 2017). In SD outbreaks, morbidity can reach 90% and mortality can reach 30% (Hampson, 2012). *B. suanatina* was collected from pigs and mallards and geographically limited to Northern Europe (Råsbäck et al., 2007; Mushtaq et al., 2015). The enteropathogenicity of *B. suanatina* was confirmed with type strain (AN4859/03) which cause swine-dysentery like symptoms in pigs (Råsbäck et al., 2005). In North America, only *B. hyodysenteriae* and *B. hampsonii* have been identified in porcine clinical samples (Harding et al., 2013a; Rubin, et al., 2013). Interestingly, novel atypical strains of *B. hyodysenteriae* with weak

haemolysis and reduced virulence emerged and their distinct genetic properties were described (Card et al. 2019).

B. pilosicoli, a less pathogenic species can cause milder disease called porcine intestinal colitis (PIS) or colonic spirochetosis in pigs. Although *B. murdochii* was identified as a normal intestinal commensal, some studies found it to be associated with mild catarrhal colitis in pigs (Komarek et al. 2009; Jensen, Christensen, and Boye 2010).

The primary pathophysiological mechanism of *Brachyspira*-associated diarrhea was described as malabsorption and inflammation in the colon of affected animals (Thomson and Friendship 2012). Most enteric bacterial pathogens cause cellular lysis, villous atrophy, and eventually reduce absorption from the epithelial cells (Moon 1978). *Brachyspira* infections have been shown to predominately cause malabsorptive diarrhea due to reducing intake of water, and sodium (Na^+) from damaged epithelial cells and these damaged cells are replaced by immature cells which lack absorptive capabilities (Argenzio, Whipp, and Glock 1980; Suh and Song 2005). Further a recent study established that *Brachyspira* cells can down regulate the mRNA expression of Na^+/H^+ exchanger in the colon and lead to loss of electroneutral Na^+ absorption and the development of diarrhoea (Enns et al. 2019).

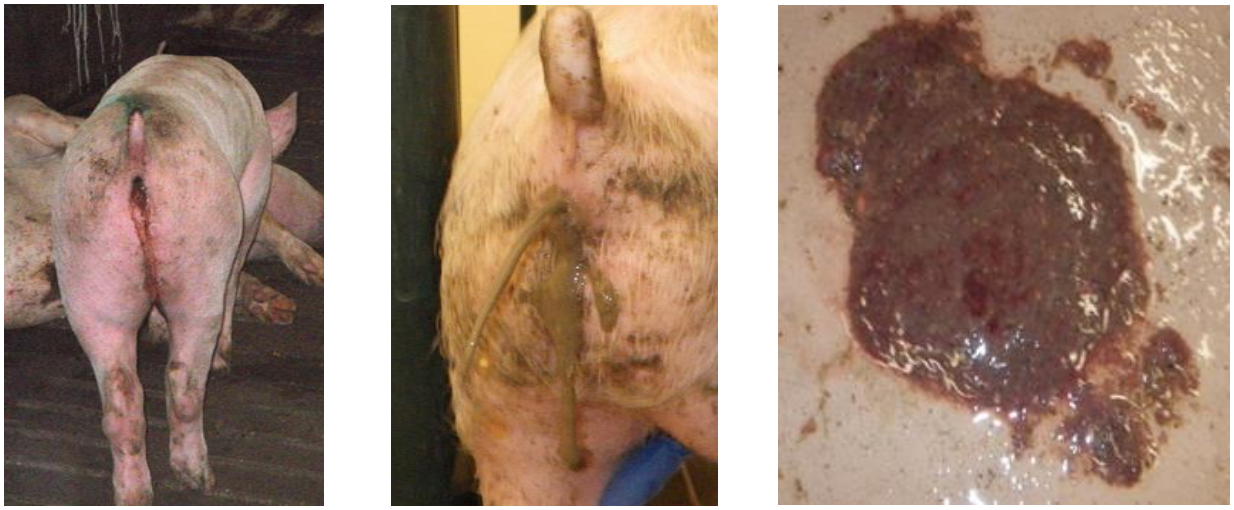
The presence of flagella and chemotactic factors facilitate movement (chemotaxis) of spirochetes through the thick mucus layer and colonization in the colonic epithelium (Hampson, 2012). The presence of the NADH oxidase gene in spirochetes also facilitates the survival and colonization in the colon, even with the presence of oxygen in that environment (Hampson, 2012). In swine dysentery, spirochetes colonize deep inside the crypts of epithelium and clinical signs usually develop within 7-10 days in a susceptible host (Hampson, 2012; Burrough, 2017). Experimental

inoculation followed by endoscopic examinations revealed that goblet cells undergo hyperplasia deep within the crypts and the expulsion of mucins occurs in the deeper crypts (Jacobson et al. 2007). An increase in the production of specific types of mucins (e.g., mucin 5AC and mucin 2) is associated with both *B. hyodysenteriae* and *B. hampsonii* infections. Microscopic examination of tissues from SD affected pigs has identified numerous pathological lesions in the large intestine such as necrosis, hemorrhage, and abundant mucus secretion (Wilcoc and Oland 1979). The hemolysins and lipopolysaccharides present in *Brachyspira* may also play a role in damaging the superficial epithelial cells leading to malabsorption which reduces feed efficiency in affected animals (Argenzio, Whipp and Glock, 1980; Hampson, 2012).

1.1.4 Diagnostic methods of *Brachyspira*-associated diseases

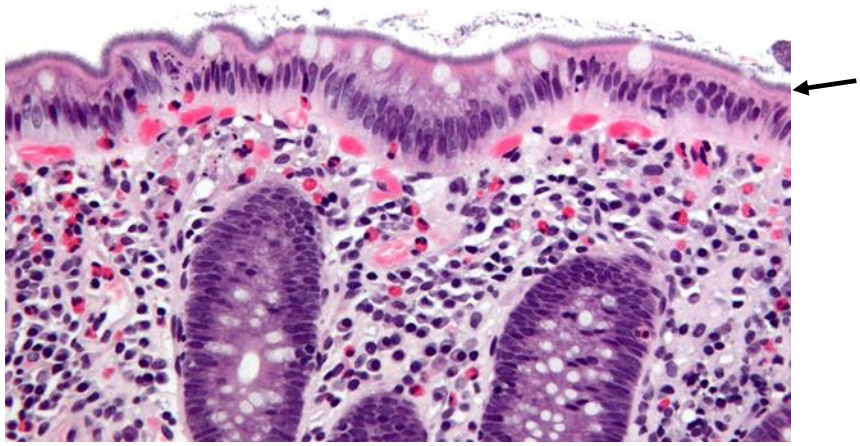
At present, the diagnosis of *Brachyspira*-associated diseases is based on the presence of clinical signs, pathological lesions (macroscopic and microscopic) in colon and/or caecum, bacterial culture and confirmatory molecular diagnostics (Hampson, 2012; Burrough, 2017). Swine dysentery primarily affects growing and finishing pigs (approximately 8–26 weeks of age) and is characterized by clinical signs including: profuse mucohemorrhagic diarrhea, loss of body condition, increased rectal temperature, dehydration, and emaciation (Figure 1.1). Porcine intestinal spirochetosis (PIS) leads watery diarrhea described as having the consistency of wet cement that sometimes contains blood and mucus. With PIS there is fecal staining of the perineum, pigs are occasionally febrile and will often have a reduced feed conversion (Hampson, 2012).

Figure 1.1 Clinical signs in swine dysentery.



From left to right debilitated animal, profuse hemorrhagic diarrhea any typical muco-hemorrhagic diarrhea (blood and mucus in feces).

Figure 1.2 “False brush border” appearance of the colonic epithelium.



B. pilosicoli attached to the columnar epithelium surface in the colon and appeared as a false brush border. This image is reproduced under a creative commons license (https://upload.wikimedia.org/wikipedia/commons/f/fa/Intestinal_spirochetosis_-_very_high_mag.jpg). An arrow was added to the image to indicate the false brush border.

The gross and microscopic lesions due to *Brachyspira* colitis can aid in making a confirmatory diagnosis of SD. In SD, gross lesions are characterized by colitis with inflammation observed on the mucosal and serosal surfaces (Hampson, 2012; Burrough, 2017). The consistency of colonic content varies, but may be watery, contain frank blood and possibly mucus. The lesions in PIS are milder than SD, and the cecum and colon may be flaccid, fluid-filled with edematous serosal surfaces and enlarged mesenteric and colonic lymph nodes can be observed (Hampson 2018). Microscopic lesions can be seen in cecum, colon, and rectum in both SD and PIS and the mucosa and submucosa become thickened due to vascular congestion, extracellular fluid accumulation and infiltration of leucocytes (Hampson 2018). Histologically, spirochetes can be demonstrated by silver staining or in-situ hybridization within colonic crypts or between intracellular gaps in the epithelium of post-mortem samples (Hampson, 2012; Burrough, 2017). In PIS, *B. pilosicoli* forms a "false brush border" due to attachment to the columnar epithelium in most of the clinical cases (Figure 1.2) (Girard, Lemarchand, and Higgins 1995). Further, the accumulation of fibrin, mucus, cellular debris and necrosis of mucosa can be microscopically visible at the later stage of the disease.

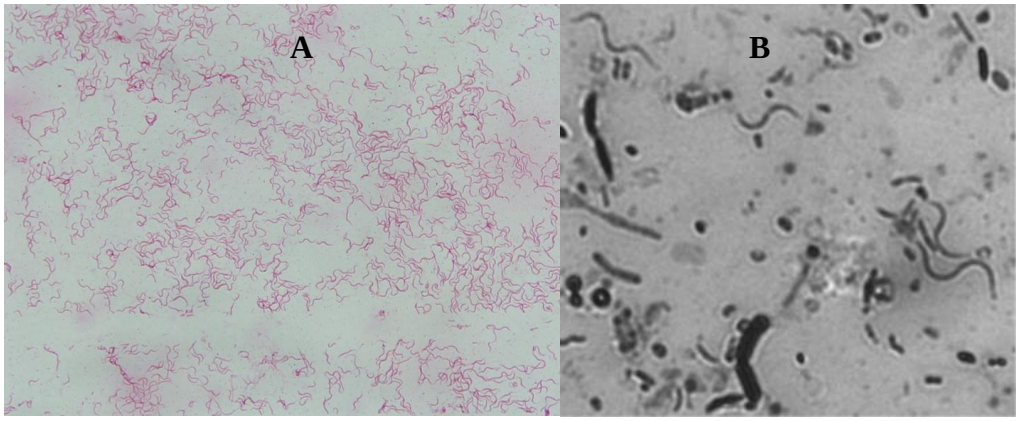
For the diagnosis of enteric disease in pigs, the suggested antemortem samples are fresh feces or rectal swabs (Torrison 2012; Gardner 2012). In *Brachyspira* infection, affected pigs excrete spirochetes in the feces, and fecal or rectal swabs are usually submitted by producers or veterinarians for diagnosis (Neef et al. 1994). At least one fresh fecal sample should be collected from each affected pen before treating the pigs with antimicrobials. Pooling of samples is possible if multiple samples are collected from the same pen; samples should not be pooled if from different pens. Around 20g of the feces should be collected into a leak-proof container and transported chilled to the lab. Alternatively, anaerobic swabs, which are the preferred antemortem sample for

Brachyspira, can be collected. Gram-stained smears or phase contrast microscopic examinations can be performed by making smears from fresh fecal samples from animals suspected of having either SD or *Brachyspira* infections (Figure 1.3).

Several studies suggested that optimal methods for the detection of *B. hyodysenteriae* in feces are a combination of direct microscopy examination and selective culture (Olsoni and Fales 1983; Stanton and Jensen 1993; Ovotná and Kardová 2002). However, direct microscopic examination or Gram stain does not allow for species identification and can lead to a false diagnosis of SD (Ovotná and Kardová 2002). At present antemortem tests for *Brachyspira* can be categorized into two groups (a) culture-based methods, (b) molecular-based methods. Diagnostic laboratories use anaerobic cultures with selective media for isolation of *Brachyspira* spp. Selective media are required to inhibit the growth of other fecal microbial communities sufficiently to identify *Brachyspira* spp. if they are present.

A number of selective media for the isolation of *Brachyspira* have been developed; the first attempt described in a 1976 publication was TSA-S400 which was comprised of trypticase soy agar (TSA) with 400 µg of spectinomycin per ml (TSA-S400) (Songer, Kinyon, and Harris 1976). Subsequently blood agar with colistin (25 µg/ml), vancomycin (25 µg/ml), and spectinomycin (400 µg/ml) (CVSBA) was developed (Jenkinson and Wingar 1981). However, compared to these media, BJ agar (TSA + 5% bovine blood, 5% pig feces extract with spectinomycin (200 µg/ml), spiramycin (25 µg/ml), rifampin (12.5 µg/ml), vancomycin (6.25 µg/ml), and colistin (6.25 µg/ml)), was better able to inhibit other microbial communities and isolate *B. hyodysenteriae* (Kunkle and Kinyon 1988).

Figure 1.3 Microscopic examination of *Brachyspira* spp.



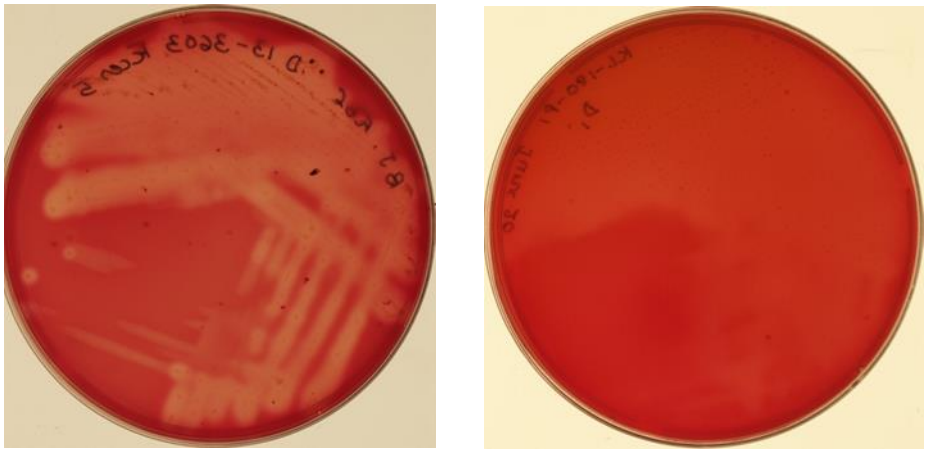
(A) Gram-negative spirochetes on a smear (100x with oil-immersion)

(B) Spirochetes were seen in a fecal sample under the phase-contrast microscope (Image courtesy of Dr. Janet Hill)

At present diagnostic laboratories use either BJ or other selective media derived from BJ which contain different combinations and concentrations of antimicrobials to inhibit growth of other bacteria (Råsbäck et al. 2005). Although *Brachyspira* spp. are anaerobes, they require low oxygen (<1%) levels for sufficient growth (Stanton and Lebo 1988). To culture *Brachyspira*, the use of a range of incubation temperatures (37-43°C) and incubation times (2-7 days) have been reported (Råsbäck et al. 2005). On blood agar *Brachyspira* spp. produce strong or weak β- hemolysis with or without surface growth or colonies (Figure 1.4). However, this observation is very subjective and there is not a standard definition to differentiate strong vs. weak hemolysis and atypical strains have been identified (Table 1.2). A recent study reported a strain of *B. hyodysenteriae* which was weakly hemolytic on blood agar. (Card et al. 2019). A variety of biochemical tests have been used to describe *Brachyspira* spp., including individual tests and commercially available kits such as API ZYM (bioMérieux, St. Laurent, QC) (Fellstrom and Gunnarsson 1995; Hampson 2012; Perez et al. 2016) (Table 1.2). These tests have been useful for identifying unusual phenotypes such as variable hippurate hydrolysis and β-glucosidase production among North American isolates of *B. hampsonii* genomovar I and II (Perez *et al.*, 2016). Several nucleic acid amplification methods or tools are used in the diagnosis of *Brachyspira* spp. and molecular epidemiological studies; polymerase chain reaction (PCR), real time PCRs, randomly amplified polymorphic DNA (RAPD), DNA restriction endonuclease analysis, restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST), fluorescence in situ hybridization (FISH), pulsed-field gel electrophoresis (PFGE), DNA-DNA homology, ribotype analysis, gene or whole genome sequencing (Atyeo et al. 1999; Rohde, Rothkamp, and Gerlach 2002; Fellstrom 2011; Mirajkar et al. 2015).

Figure 1.4 Hemolysis on BJ media.

(a) (b)



(a) a clinical isolate of *B. hyodysenteriae* on BJ media with strong β- hemolysis

(b) a clinical isolate of *B. pilosicoli* on BJ media with weak β- hemolysis

Table 1.2 Biochemical test results and different hemolysis pattern on TSA by *Brachyspira* spp. (Hampson, 2012; Perez et al., 2016).

Species	Hemolysis	Indole	Hippurate hydrolysis	α-glucosidase	β-glucosidase
B. hyodysenteriae	Strong[a]	+	-	+	+
B. intermedia	Weak	+	-	+	+
B. innocens	Weak	-	-	-	+
B. murdochii	Weak	-	-	-	-
B. pilosicoli	Weak	+	+	-	-
B. suanatina	Strong	+	-		
B. hampsonii Clade I	Strong	-	+/-[b]	-	+
B. hampsonii Clade II	Strong	-	+/-[b]	-	+/-[b]

[a]There is a genetically distinct variant of *B. hyodysenteriae* group which produces weak hemolysis on agar (Card et al. 2019).
[b]Variable results were observed (Perez et al. 2016). Such as negative indole strains of *B. hyodysenteriae* and positive indole strains of *B. pilosicoli* (Hampson, 2012).

The *nox* gene, which encodes the enzyme NADH oxidase, protects anaerobic *Brachyspira* spp. against oxygen toxicity and is a common gene target used for the identification of *Brachyspira* spp. (Stanton et al. 1999; Rohde, Rothkamp, and Gerlach 2002). This gene is well conserved within the genus of *Brachyspira*, there is a minimum of 86.3% sequence identity between species (Atyeo et al. 1999; Rohde, Rothkamp, and Gerlach 2002). Other gene targets used for *Brachyspira* spp. identification include 16S rRNA, 23S rRNA, *cpn*60, and *tly*A (Leser et al. 1997; Fellström, Zimmerman, and Aspan 2001; Rohde et al. 2019). Compared to *nox*, the 16S rRNA and 23S rRNA genes are much more conserved amongst members of the genus, but have lower divergence (2-3%) between species making theme less desirable targets for species differentiation (Atyeo et al. 1999). A recent study compared *Brachyspira* spp. identifications by *cpn*60 and *nox* sequencing concluded that *cpn*60 is a good alternative to *nox* owing to multiple copies of *nox* gene in the genome and potential lateral *nox* gene transfer by phage VSH-1 (Rohde et al. 2019). The authors also noted that *cpn*60 is well standardized and was shown to be able to resolve weakly vs. strongly hemolytic isolates of *B. hyodysenteriae* (Rohde et al. 2019).

Several typing methods have been used to compare the relatedness of *Brachyspira* strains. Restriction fragment length polymorphism (RFLP) analysis involves amplification of gene targets (16S rRNA, 23S rRNA and *nox* gene) followed by restriction enzyme digestion and visualization of species-specific banding patterns by gel electrophoresis (Stanton et al. 1997; Barcellos et al. 2000; Rohde, Rothkamp, and Gerlach 2002). Multilocus enzyme electrophoresis analysis (MLEE) was previously used for strain identification of *Brachyspira*, but has been replaced by multilocus sequence typing (MLST) which is instead based on the nucleotide sequence of the seven loci which were used in MLEE (Trott et al. 1996; Rasback et al. 2007).

Fluorescence in-situ hybridization (FISH) allows the visualization and identification of single bacteria within tissue sections. In *Brachyspira* diagnostics, intestinal tissue sections or formalin fixed feces can be used for FISH (Wilberts et al. 2015). Other staining techniques such as the Warthin-Starry method allow the visualization of spirochetes in affected tissues but are not able to differentiate pathogenic spp. from non-pathogenic spp. or commensal spp. By using bacterial ribosomal RNA (rRNA)- targeted oligonucleotide probes FISH can differentially detect *Brachyspira* at both genus and species level (Boye et al. 1998; Jensen et al. 2001; Schmiedel et al. 2009; Jensen, Christensen, and Boye 2010; Burrough et al. 2013; Wilberts et al. 2015). A recent study utilizing oligonucleotide probes that targeted the specific regions of the 23S rRNA of *B. hampsonii* for culture-independent identification of the pathogen demonstrated that this test can provide organism identification within 24-48 hrs of sample submission (Burrough et al. 2013). However, this study also reported cross-reactivity between species-specific probes requiring additional testing such as PCR and sequencing for species confirmation (Burrough et al. 2013; Wilberts et al. 2015).

Pulsed-field gel electrophoresis (PFGE) is a DNA fingerprinting method, which uses restriction endonucleases to digest the bacterial genome that are then subjected to electrophoresis for separation of large fragments of DNA (Prior and Fegan 2005). Epidemiological studies on *B. pilosicoli* and *B. hyodysenteriae* have used PFGE and have observed that genotypes of those spp. were highly variable between farms and/or production systems from which the samples originated (Atyeo, Oxberry, and Hampson 1996; Rfaty 1999; Fossi, Pohjanvirta, and Pelkonen 2003).

With the advancement of next-generation technologies, whole-genome sequencing has become a rapid diagnostic tool in diagnostic laboratories (Hasman et al. 2014; Kwong et al. 2015; Deurenberg et al. 2017). However, WGS is not routinely used for species identification in

Brachyspira but it has been used in studies on species characterization, comparative genomic analysis, and identification of genetic determinant of antimicrobial resistance in *Brachyspira* spp. (Mappley et al. 2012; Mirajkar et al. 2016; Hampson and Wang 2017; Card et al. 2018; De Luca et al. 2018).

1.1.5 Prevention and control of swine dysentery

Swine dysentery can lead to substantial economic losses due to reduced feed efficiency, slower weight gain, treatment costs, labour costs associated with additional animal care, and death or depopulation (Hampson, 2012). Therefore, prevention and control measures are important to minimize the devastating effect of this disease on the swine industry.

At present, producers use antimicrobials and management practices (biosecurity, dietary interventions, reduced stressors, and sometimes herd elimination) to control and prevent the disease (Hampson, 2012). Prophylactic antimicrobial therapy has been used in the past to control and prevent the *Brachyspira* infection while maintaining the productivity of the pigs (Burch 2013). There are several antimicrobial classes (macrolides, lincosamides, and pleuromutilins) and different antimicrobial regimes utilized globally to prevent and control SD (Kulathunga and Rubin 2017). The development of antimicrobial resistance in *Brachyspira* isolates has been reported (Karlsson, Gunnarsson, and Franklin 2001; Lobova 2004; Clothier et al. 2011; Mirajkar, Davies, and Gebhart 2016; Kulathunga and Rubin 2017). In addition to antimicrobial use, biosecurity measures and good management practices can reduce disease occurrence or control *Brachyspira* diseases in pig herds (Alvarez-Ordóñez et al. 2013). All-in/all-out management system is practiced in the grower-finisher pig farms followed by thorough cleaning and disinfecting of the pens, feeders, waterers, and floors to control the spread of any enteric infection including *Brachyspira*

infection between batches (Alvarez-Ordóñez et al. 2013). This strategy can help break the cycle of enteric infections in pigs and prevent the premises from becoming a source of infection (Jayaraman and Nyachoti 2017). It is important to note that *B. hyodysenteriae* can survive in manure pits for an extended period and therefore frequent manure removal, adequate draining, drying and lime application can be used for removal of spirochetes from the manure pit environment (McOrist and Bennett 2006; Pico et al. 2008). Reservoir hosts such as rats, mice, wild birds, some insects, domestic animals such as dogs and wild boars are potential sources of *Brachyspira* infection and can lead to spread and maintenance of the disease within and between farms (Songer et al. 1978; Trott et al. 1996; Jensen, Stanton, and Swayne 1996; Phillips et al. 2009; Rubin et al. 2013). Therefore, additional measures such as double fences to prevent the entry of large wild animals and frequent control of rodents and insects to prevent contamination of the pigs or the environment have been implemented outside of North America (Jensen, Stanton, and Swayne 1996).

Several vaccines have been developed to control SD, but these tools have been experimental and have had limited success (Song et al. 2009; Mahu et al. 2017). These vaccines can be categorized into whole-cell bacterins, attenuated strains, or recombinant vaccines (Parizek et al. 1985; Diego, Ck-menes, and Carvajal 1995; Song et al. 2009). Bacterin vaccines are lipopolysaccharides (LOS) specific, require autogenous preparations and don't provide cross-protection against different strains. These tools are therefore very expensive and not a very feasible method of SD control (Song et al. 2009). Further, although bacterin vaccines were capable of inducing systemic antibody titers, this vaccine did not result in sterile immunity (Hampson et al. 2000). In addition, vaccination with recombinant proteins also failed to give full protection from the disease and to prevent colonization in experimentally infected pigs (Gabe et al. 1995; La et al. 2004).

Dietary management have been reported as a measure which can be used to control SD. Diet which are highly digestible or contain high inulin concentrations can protect the animals from the disease by changing the bacterial population in the colon, which can inhibit the spirochetes' growth (Hampson, 2012). Supplementing the feed with probiotics or beneficial bacteria such as lactobacilli, enterococci, bacilli, bifidobacterial have also been shown to inhibit *B. hyodysenteriae* colonization protecting susceptible animals (Bernardeau et al. 2009; Klose et al. 2010). The exact mechanisms by which the diet's compositions predispose to or protect against SD have not been adequately explained, and the results of these investigations were poorly reproducible suggesting that these interventions may not be effective (Alvarez-Ordóñez et al. 2013).

1.1.6 Treatment (Kulathunga and Rubin 2017)

In pigs, *Brachyspira*-associated disease has been effectively treated with pleuromutilins, macrolides, lincosamides, and carbadox, while metronidazole has been effectively used in people with intestinal spirochetosis (Hampson, 2012; Helbling *et al.*, 2012). However, the ability to use some of these agents in food-producing animals is restricted in many jurisdictions; metronidazole is banned for use in food animals in North America and the European Union, and carbadox is banned in Canada, the European Union, the United Kingdom and recently had its approval rescinded in the United States (Food and Drug Admistration 2016). Pleuromutilins (tiamulin and valnemulin), macrolides and lincosamides (tylosin, lincomycin. and tylvalosin) are the mainstay of anti-*Brachyspira* therapy in swine medicine, although a number of other antimicrobials including bacitracin, virginiamycin, and gentamicin are labeled for the treatment or prevention of *Brachyspira*-associated diseases in pigs (Hampson, 2012). The heavy reliance on mechanistically similar drugs, to which resistance in other organisms has been shown to develop by a common mechanism, highlights the potential impact of resistance emergence and the clinical value of

laboratory test-guided therapeutic selection. Although there is no consensus method for determining the antimicrobial susceptibility of *Brachyspira*, trends of decreasing susceptibility among *B. hyodysenteriae* for the macrolides and pleuromutilins have been described in Europe and the United States (Lobova 2004; Aarestrup, Oliver, and Burch 2008; Prášek et al. 2014; Rugna et al. 2015; Mirajkar, Davies, and Gebhart 2016).

1.2 Antimicrobial drug action and resistance

1.2.1 General introduction to antimicrobial therapy

Antimicrobials are defined as any substance which can have a natural, synthetic, or semi-synthetic origin and able to kill or inhibit the growth of microorganisms. Penicillin was considered a miracle drug which was first discovered in 1929 and introduced to the market in the 1940s (Giguere 2013a). Since then, new antimicrobial drug classes were discovered, and natural, synthetic and semi-synthetic drugs were developed and have been widely used to prevent and treat infectious diseases in both humans and animals (Giguere 2013a). At present, very few new antibiotic classes are in the drug pipeline because of scientific difficulties and financial and regulatory hurdles (Coates, Halls, and Hu 2011).

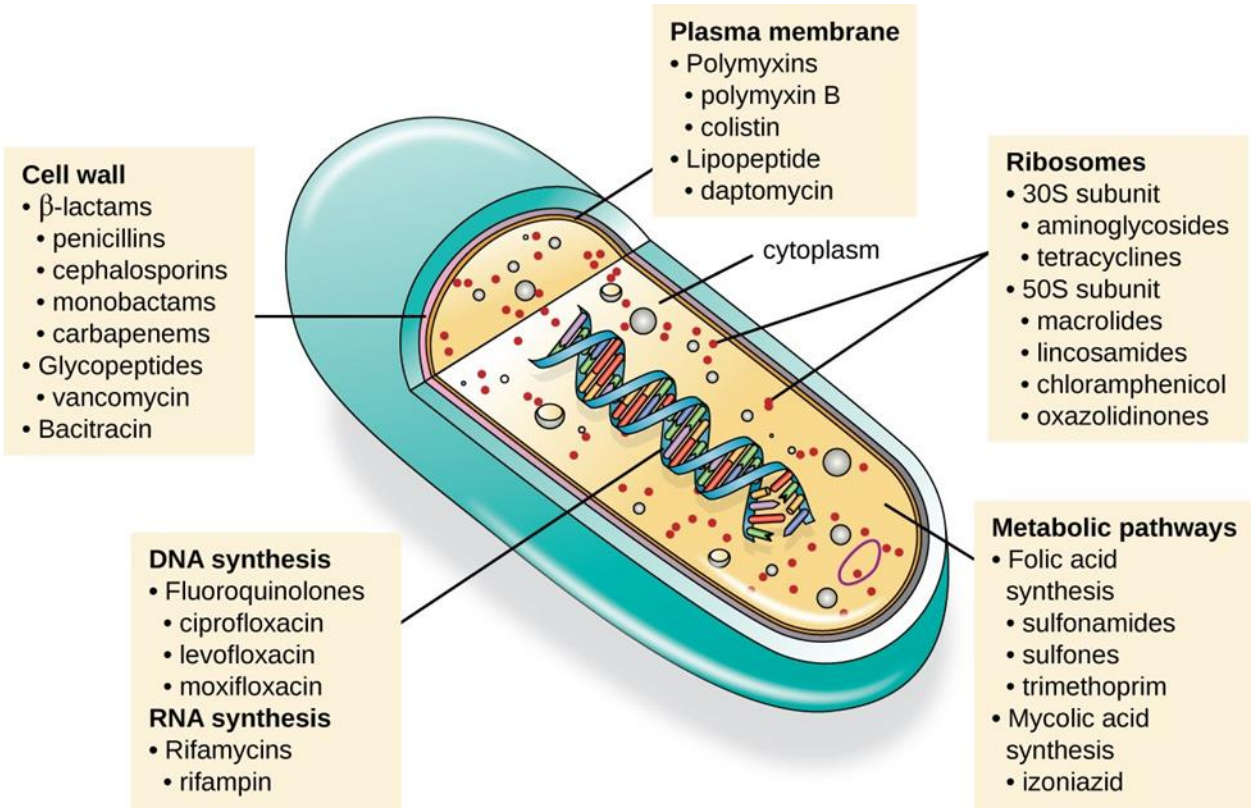
Antimicrobials can be generally categorized into four groups based on their mechanism of action: (i) inhibitors of cell wall synthesis, (ii) damage to the cell membrane function, (iii) inhibition of nucleic acid synthesis or function, (iv) inhibition of protein synthesis (Figure 1.5).

The cell membrane of Gram-positive bacteria is different from that of Gram-negative bacteria. Gram-negative bacteria have two cell membranes; a cytoplasmic cell membrane, and outer membrane, while Gram-positive bacteria have an only cytoplasmic membrane (Guntram and Holst 2013). Gram-negative bacteria have an additional component, lipopolysaccharide (LPS), in their

outer membranes. Both Gram-positive and Gram-negative bacteria have a cell wall that is made of a peptidoglycan layer. Compared to Gram-negative bacteria, Gram positive bacteria have a thick cell wall (Guntram and Holst 2013). The bacterial cell wall plays a significant role in maintaining structural integrity, cell shape and osmolarity (Guntram and Holst 2013). However, β-lactam and glycopeptide antimicrobials can inhibit or interfere with the cell wall synthesis of target bacteria (Prescott 2013a; Dowling 2013b). β-lactam antimicrobials include the penicillins, cephalosporins, monobactams, and carbapenems and have the affinity for penicillin-binding proteins (PBP) on bacterial cell wall (Prescott 2013a). Once these drugs bind to the PBP, the transpeptidation process of cell wall synthesis is inhibited leading to cell lysis. Glycopeptides can inhibit the peptidoglycan synthesis by binding to the d-Ala-d-Ala C-terminus of peptidoglycan precursors, thereby blocking their addition to the growing peptidoglycan chain and preventing the subsequent transglycosylation and transpeptidation steps of cell wall biosynthesis (Dowling 2013b). Some examples of glycopeptide antimicrobials are vancomycin, avoparcin, and teicoplanin (Dowling 2013b).

There are some antimicrobials (e.g. polymyxin B and colistin) which bind to lipopolysaccharides in the cell membrane and thereby inhibit the permeability of the cell membrane and lead to bacterial cell toxicity and cell death (Dowling 2013a; Epand et al. 2016).

Figure 1.5 Summary of Antimicrobial Mechanisms of Action.



The antimicrobial drug classes were categorized based on their mechanism of action: (i) inhibitors of cell wall synthesis, (ii) damage to the cell membrane function, (iii) inhibition of nucleic acid synthesis or function, (iv) inhibition of protein synthesis. This image is reproduced under a creative commons license. This image is reproduced under a creative commons license (https://courses.lumenlearning.com/microbiology/chapter/mechanisms-of-antibacterial-drugs/).

Polymyxins have a narrow antibacterial spectrum, they are active against many Gram-negative bacteria while some species such as *Brucella, Burkholderia cepacian, Campylobacteria* spp., *Morganella morganii, Pseudomonas mallei, Providencia* spp., *Proteus* spp., *Serratia marcescens* and *Vibrio cholerae* are intrinsically resistant (Poirel, Jayol, and Nordmann 2017). In veterinary medicine colistin has been used extensively for the treatment and prevention of infectious diseases, such as *E. coli* infections in poultry and pigs and for growth promotion in animals (Catry et al. 2015). However, investigations of plasmid mediated colistin resistance determinants in animals and food products have led to ban colistin as a feed additive in some countries (Olaitan et al. 2015; Liu and Liu 2018; Kumar et al. 2020).

The ribosome is the site of protein synthesis in bacteria, and this complex unit has two major subunits, small and large (Schmeing and Ramakrishnan 2009). In bacteria, the small subunit (30S) comprises ribosomal proteins and 16S rRNA while the large subunit (50S) comprises ribosomal proteins and 23S rRNA and 5S rRNA. There are different antimicrobial drug classes that can inhibit any of the main protein synthesis steps of the ribosome; initiation, elongation, termination and ribosomal recycling (Table 1.3) (Giguere 2013b; Dowling 2013a; Epand et al. 2016). Some antimicrobials bind to 30S subunit (tetracycline, doxycycline and aminoglycoside antimicrobials such as streptomycin) whereas others to bind to 50S ribosomal subunit and inhibit the protein synthesis (macrolides, lincosamides, pleuromutilin, and chloramphenicol) (Table 1.3). The phenicol drugs, pleuromutilins and lincosamides interfere with the peptide bond formation by preventing the accurate placement of aminoacylated-CCA-end of the A-tRNA at the peptidyl transference center of the ribosome and eventually inhibit protein synthesis (Arenz and Wilson 2016).

Table 1.3 Major classes of protein synthesis inhibiting antimicrobials and their mechanism of action.

Binding site	Class	Mechanism of action
50S subunit	Chloramphenicol	Prevent peptide bond formation and prevent protein synthesis
	Macrolide	
	Lincosamide	
	Pleuromutilin	
30S subunit	Aminoglycoside	Impaired proofreading, resulting in the production of faulty proteins
	Tetracycline	Block the binding of tRNA and thereby inhibit protein synthesis

(Giguere 2013c, 2013b; Dowling 2013a; Castillo 2013)

Natural tetracyclines, produced by *Streptomyces* spp. were first discovered in 1945, later semisynthetic tetracyclines (e.g., methacyclines, doxycyclines and minocyclines) were developed (Duggar 1948; Michalova, Novotna, and Schlegelova 2004). Tetracyclines are broad-spectrum antimicrobials which have been extensively used in human and animal infections, prophylactic purposes, and growth promotion in food animals. In veterinary medicine, tetracyclines are used for the treatment of gastrointestinal, respiratory, skin infections, infectious diseases of locomotive organs, genito-urinary tract infections and systemic infections (Michalova, Novotna, and Schlegelova 2004; Castillo 2013). Tetracyclines bind to the 30S bacterial ribosome and allosterically inhibit the binding of aminoacylated transfer RNA (AA-tRNA) to the A-site on the ribosome and reversibly inhibit the protein synthesis of bacteria (Castillo 2013).

The antimicrobial fluoroquinolones, rifamycins and sulfonamides act by inhibiting nucleic acid synthesis or function. The first antimicrobial discovered in the synthetic quinolone class of drugs was nalidixic acid. Modification of nalidixic acid (addition of both a fluorine molecule at the 6 position of the basic quinolone structure and a piperazine substitution at the 7 position) improved the antimicrobial properties and pharmacokinetics properties of this drug and led to the development of the fluoroquinolones. Fluoroquinolones (e.g., ciprofloxacin and levofloxacin) drugs are active against a wide range of Gram-negative and Gram-positive pathogens and in veterinary medicine extensively used for dermal infections, respiratory and urinary tract infections (Giguere and Dowling 2013). Quinolones target type II topoisomerases (DNA gyrase) and topoisomerase IV enzymes which are involved in the modulation of the chromosomal supercoiling required for DNA synthesis, transcription and cell division in bacteria (Hawkey 2003). These drugs non-covalently bind to complexes that form between DNA and gyrase or topoisomerase IV and induce conformational changes in those enzymes. Eventually ligase activity of the enzyme is

inhibited, leading to disruption of supercoiling, release of DNA strands and cell death (Hawkey 2003; Giguere and Dowling 2013).

Rifampin is a semisynthetic antimicrobial belonging to the rifamycin drug class. It is broad-spectrum and can be used against Gram-positive and select Gram-negative bacteria. This drug is well recognized as an important component of combination therapy to treat human tuberculosis (Dowling 2013c; Goldstein 2014). Rifampin is lipid soluble and can therefore be used against both intra- and extra-cellular bacteria. Rifampin inhibits RNA polymerase, thereby inhibiting transcription and bacterial cell death (Goldstein 2014). Sulfonamides and trimethoprim competitively prevent the activity of bacterial metabolic enzymes in the folic acid synthesis pathway, thereby inhibiting nucleic acid synthesis (Prescott 2013b). Sulfonamides compete with the enzyme dihydropteroate synthase for the substrate para-aminobenzoic acid (PABA) and prevent the synthesis of dihydrofolic acid in folic acid synthesis pathway. Trimethoprim inhibits the synthesis of tetrahydro folic acid from dihydrofolate by combining with the enzyme dihydrofolate reductase and eventually prevents the synthesis of purines (Prescott 2013b). The combination of diaminopyrimidines with sulfonamides ("potentiated sulfonamides") results in a synergistic, bactericidal effect due to the inhibition of folic acid synthesis.

1.2.2 Antimicrobial drugs use in swine medicine

Historically, antimicrobials were used in the swine industry for disease treatment, prevention, control, and growth promotion/feed efficiency (Viola and Devincent 2006). The responsible use of antimicrobials by veterinarians and producers is a critical step for reducing the AMR development in pathogens (Burch 2013). However, with increased interest in the development of antimicrobial resistance development and the associated zoonotic risks, antimicrobial growth promotion claims were removed in the European Union in 2006. The Public Health Agency of

Canada (PHAC) removed the growth promotion claims from antimicrobial labels by 2018 (PHAC 2017). Antimicrobial use for prevention or treatment of animal disease is still permitted as long as there are the appropriate corresponding label claims (PHAC 2017). At present, it is mandatory to have veterinary prescriptions for all medical important antimicrobials use in animal agriculture and these agents are prohibited to use prophylactically or as growth promoters in animal feed (Chekabab et al. 2020). Health Canada has categorized antimicrobial drugs used in human medicine into four groups (category I - very high importance, II - high importance, III - medium importance and IV-low importance) based on their indication and availability of alternative antimicrobial drugs for use in humans. For disease prevention and treatment, the Canadian swine industry mostly uses category II or III drugs such as tetracyclines, macrolides, lincosamide, pleuromutilins, and penicillin drug classes (CIPARS 2015, 2016). The most frequently reported antimicrobials used were parenteral penicillin G (43%, 39/91 herds), lincomycin (38%, 35/91 herds) and tylosin (24%, 22/91 herds) primarily through feed, and chlortetracycline (24%, 22/91 herds) all in feed (CIPARS 2016). Comparing 2016 to 2015, the number of herds reporting the use of penicillin G (43% in 2016 and 44% in 2015) and lincomycin (38% in 2016 and 39% in 2015) were similar. The use of tylosin decreased from 27% (23/85) in 2015 to 24% (22/91) in 2016, and the use of chlortetracycline also decreased from 35% (30/85) in 2015 to 24% (22/91) in 2016.

1.3 Antimicrobial susceptibility testing

1.3.1 Classification of antimicrobial susceptibility tests

Antimicrobial susceptibility testing is essential to detect resistance and to guide clinicians in choosing the most appropriate antimicrobial therapy. The inhibition of bacterial growth by antimicrobial substances is the underlining mechanism of any susceptibility test, and was first

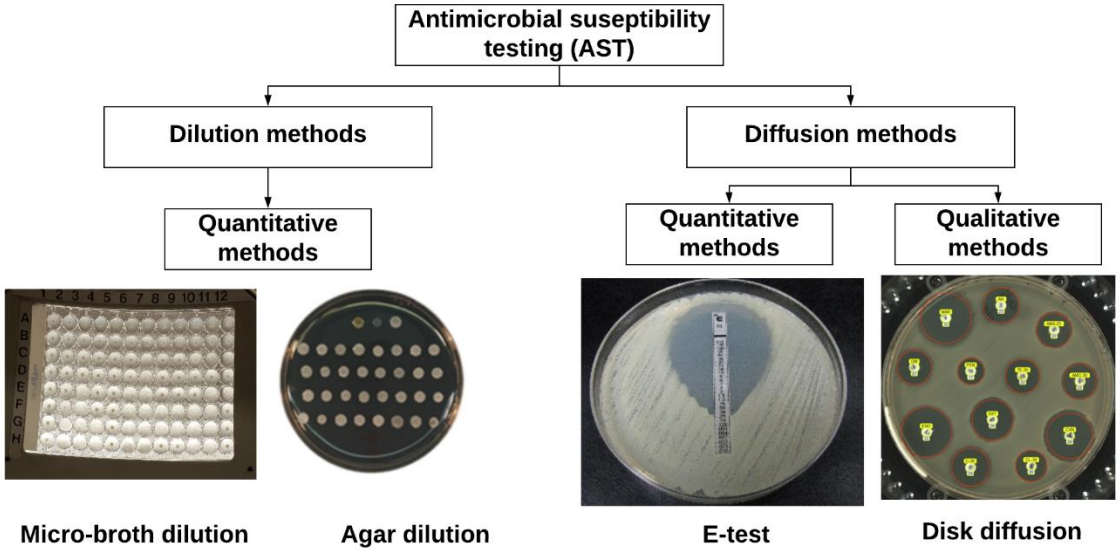
discovered by Alexander Fleming (Fleming 1929). Fleming developed the world's first susceptibility test methods; the primordial ditch plate technique and the broth dilution method. These methods were used to evaluate antimicrobial qualities of antiseptic solutions and cultures of *Penicillium* and their effects on bacterial growth (Fleming 1923, 1929). In the ditch-plate technique, the antimicrobial solution is instilled into a ditch cut in nutrient agar contained in a petri dish. Thereafter, the test organisms are streaked up to the ditch and plates are incubated. The width of the inhibition zone is an indication of the relative activity of antimicrobial substance against various test organisms. The measurement of this inhibitory zone represents an early precursor to the disk diffusion technique which was first reported to be used in the 1940s (Vincent 1942; Heatley 1943).

The first agar dilution method, which incorporated antimicrobials into agar media, was described by a research study on susceptibility testing for gonococci (Schmith and Reymann 1940). Later researchers observed that the results of susceptibility testing could be affected by variables such as inoculum size, and incubation conditions emphasizing the need for standardization of susceptibility testing (WHO 1961). In 1966 Bauer, Kirby, and fellow researchers described the disk diffusion in a detailed method to use in clinical laboratories (Bauer et al. 1966). The first attempt at experimentally standardizing susceptibility testing was described and introduced in a 1971 publication (Ericsson and Sherris 1971). Thereafter, several countries established national bodies to develop, update and publish standard guidelines and clinical breakpoints for various drug-organism combinations (CLSI 2013; EUCAST 2017; CLSI 2017). At present, two sets of guidelines are used; the Clinical and Laboratory Standard Institute Guidelines (CLSI), which can be purchased, while the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines are available free on their website (CLSI 2017; EUCAST 2017).

Antimicrobial susceptibility tests can be divided into two categories: diffusion-based methods and dilution-based methods. Each category can be divided in to two groups based on the type of results obtained, (a) categorical (susceptible, intermediate, or resistant) or (b) quantitative (minimum inhibitory concentration (MIC)) (Figure. 1.6) (Rubin 2013). Examples of diffusion-based tests are disk diffusion and gradient strips (E-test). In the disk-diffusion test, antimicrobial impregnated disks are placed on bacterial organism inoculated agar media and after incubation, the diameter of zones of growth inhibition are measured and compared to a standard to categorize an organism as susceptible or resistant. In contrast, the gradient test method yields an MIC (Jorgensen and Ferraro 2009). In this test, a test-strip with a concentration gradient is applied to an agar plate inoculated with the test organism and incubated overnight. The intersection of the ellipse shaped inhibitory zone with the test strip indicates the MIC value (Figure 1.6) (Jorgensen and Ferraro 2009).

Quantitative susceptibility testing by exposing an organism to a series of antimicrobial dilutions was an early concept on which pioneer scientists in the field of bacteriology worked frequently (Poupard, Walsh, and Kleger 1994). In the broth macrodilution method, 1 ml of antimicrobial containing broth is diluted in test tubes and each tube is inoculated with a known number of test organisms (Jorgensen and Ferraro 2009; Tenover 2009). Tubes are incubated overnight, and the concentrations at which the organism is able to grow, including the MIC, is recorded.

Figure 1.6 Classification of antimicrobial susceptibility test



Antimicrobial susceptibility tests can be divided into two distinct categories, diffusion and dilution based. These tests can give either categorical (susceptible, intermediate, or resistant) or quantitative (minimum inhibitory concentration (MIC)) data.

This method has also been miniaturized; broth microdilution utilizes disposable 96 well plates, allowing multiple antibiotic to be to be tested simultaneously (Jorgensen and Ferraro 2009; Sandle 2016). This test is the most practical and popular dilution-based susceptibility testing in clinical laboratories (Jorgensen and Ferraro 2009).

In agar dilution, a series of agar plates containing a doubling dilution series of each antimicrobial (e.g., 1, 2, 4, 8, 16, 32 µg/ml, etc.) are prepared (Rubin 2013). These media are then inoculated with a standardized quantity of the test organism; a suspension equal the turbidity of a 0.5 McFarland standard ($1\text{-}10^8$ colony forming units (CFU)/ml) is prepared and 1-5 µl of this suspension is spotted onto the plate (Rubin 2013; CLSI 2013). For non-fastidious organisms plates are incubated for 16-18 hrs at 35-37°C in ambient temperature while some fastidious organisms may be incubated in a CO_2-enriched atmosphere for 18-24 hrs (Jorgensen and Ferraro 2009; Sandle 2016; CLSI 2013, 2017, 2015b). Due to the requirement to prepare media in-house, the agar dilution method is very laborious and infrequently use in diagnostic settings.

Automated instrument systems, including the MicroScan WalkAway (Beckman Coulter, Inc. Atlanta, Georgia, USA), Micronaut (Merlin, berlin, Germany), Avantage test (Abbott Laboratories, Irving, Texas, USA), Vitek 2 (bioMe'rieux, Marcy-l'Étoile, France), Phoenix (BD Diagnostics, Franklin Lakes, New Jersey, USA), and Sensititre ARIS 2X (Trek Diagnostic Systems, Oakwood Village, Ohio, USA) were developed to improve laboratory throughput and reduce labour requirements (Ligozzi et al. 2002; Puttaswamy et al. 2018). These systems allow for the automated inoculation of MIC panels, computer assisted incubation and use optical systems (camera or a scanner) for detecting bacterial growth and determining MICs (Jorgensen and Ferraro 2009). The average time for performing these tests is 9-20 hrs; a spectrophotometer or fluorometer to determine the pathogen growth development (Khan, Siddiqui, and Park 2019). Similarly,

automated zone readers can read disk diffusion tests and incorporate the results into the laboratory information management systems, improving the efficiency of data handing, storage and retrieval of data (Felmingham and Brown 2001). Automated systems have the advantage of produce susceptibility test results with less hands on time, reducing labour costs and variability (Ligozzi et al. 2002; Jorgensen and Ferraro 2009). However, because all of these systems require pure cultures, these methods can still take 1-5 days to yield results, similar to manually conducted assays may have the lengthy diagnostic workflow(Puttaswamy et al. 2018).

Special susceptibility tests are performed to detect specific phenotypes such as inducible resistance, one of the examples for such test is the "D-test" in which the inducible resistance of Streptococci or Staphylococci is investigated (Rubin 2013). In this test a flattened inhibitory zone around the clindamycin disc (positive 'D-zone test') can be observed when erythromycin and clindamycin disks are placed adjacently on an agar plate used for disc diffusion testing.

For bacteria like *Brachyspira*, there are no standard guidelines for conducting or interpreting susceptibility tests published by EUCAST or the CLSI. In these cases, laboratories practice their in-house susceptibility test methodologies, which result in variability among test results and interpretations. The discrepancies in test results due to non-standardized susceptibility testing methods emphasizes the importance of developing standard testing (Råsbäck et al. 2005).

1.3.2 Specific challenges to susceptibility testing of *Brachyspira* (Kulathunga and Rubin 2017)

The ability to assess the antimicrobial susceptibility of *Brachyspira* is hindered by two proximate causes: (*i*) the growth conditions required for *Brachyspira* are different than those standardized for susceptibility testing and (*ii*) the inability to use typical susceptibility test endpoints. Although

members of *Brachyspira* spp. are reported to grow within the range of conditions prescribed by the CLSI for antimicrobial susceptibility testing, no single set of conditions have been standardized for testing these organisms.

The unusual growth characteristics of *Brachyspira* are at the root of many of these challenges. On solid media *Brachyspira* does not form distinct colonies; growth is typically inferred by the presence of hemolytic zones on blood-containing agar. This property complicates and (or) precludes the application of the concept of the colony-forming unit, a foundational bacteriological principle that allows viable cells to be enumerated. The inability to differentiate whether apparently distinct hemolytic zones represent unique founding organisms or multiple individuals is a critical limitation. Pure cultures are a pre-requisite for antimicrobial susceptibility testing; tests of mixed cultures yield cumulative results, which may not reflect the susceptibility of either organism individually. Furthermore, the lack of colony formation characteristic potentially precludes a reliable test endpoint definition; whereas an antimicrobial inhibiting the formation of a colony is taken to indicate growth inhibition, failure to observe hemolysis in the presence of an antimicrobial may simply reflect inhibition of hemolysin production with or without effects on microbial growth.

This is particularly germane for *Brachyspira*, for which protein synthesis inhibiting drugs are the primary agents of interest. Creative approaches to address endpoint validity, including microscopic evaluation or subculture of media, may prove useful in identifying viable organisms in media containing supra-MIC drug concentrations. Broth culture of *Brachyspira* spp. also presents some unique challenges. Anecdotal observations suggest that broth cultures require a high starting inoculum, and so failure to grow an organism in broth may simply reflect an insufficient initial inoculum density. This complicates 2 steps of the susceptibility testing process: (*i*) growing an

initial broth culture used to inoculate test media, and (*ii*) interpreting broth-based susceptibility tests, and leads to questions such as: "Did the culture not grow because it was inhibited by the drug or because the culture was insufficiently dense?"

1.3.3 Summary of susceptibility test methods that have been used for *Brachyspira* (Kulathunga and Rubin 2017)

A literature review (up to 2017) was performed and studies describing antimicrobial susceptibility testing of *Brachyspira* were reviewed. Both agar and broth dilution techniques were used, although there was substantial variability in the methods reported (Table 1.4).

Incubation temperatures ranged from 37 to 42°C and incubation times ranged from 2 to 5 days for agar dilution, and from 37 to 38°C and 3 to 5 days for broth dilution. Where reported, highly variable inoculum densities were described: 1×10^5–5×10^6 CFU/mL for broth dilution and 1×10^4–1×10^6 CFU/spot for agar dilution. Finally, where reported, atmospheres with varying composition were used. According to the manufacturers, all anaerobic systems yield a final O_2 concentration of ≤1% but yield highly variable CO_2 concentrations (7%–>15%). In addition to agar and broth dilution, the use of doxycycline gradient strips has also been described, however this method is not widely used (Mirajkar and Gebhart 2016), further complicating the interpretation of susceptibility test results is the lack of agreed upon interpretive criteria. With the lack of CLSI and EUCAST resistance breakpoints, investigators have relied upon divergent sets of researcher proposed breakpoints (Ronne and Szancer 1990; Duhamel et al. 1998; Pringle et al. 2012; Burch 2013; SVARM 2013).

Table 1.4 Chronological summary of antimicrobial susceptibility test methodologies (agar dilution and broth dilution) utilized in published investigations.

Method	Country	Year	Organism	Incubation Temp	Incubation Time	Final Inoculum	Atmosphere	Media	References
Agar Dilution	Sweden	2003	Various	37ºC	96 hours	1 -5 x 10^5 / spot	GENbox	TSA5OXW C5SB	(Karlsson et al. 2003)
	Japan	2004	BH	Unclear, 37-38ºC	72 hours	10^4 - 10^5 / spot	BBD GasPak	TSA5SB	(Uezato, Kinjo, and Adachi 2004)
	Czech Republic	2004	BH	37ºC	72 - 96 hours	10^4 / spot	Anaerobic jar using gas generating kit (BR38, Oxoid)	WC5SB	(Lobova 2004)
	Germany	2004	BH	42ºC	48 hours	3X 10^5 / spot	No details reported	TSA10BB	(Rohde et al. 2004)
	Japan	2010	BH	37ºC	72 hours	10^5 / spot	No details reported	TSA5SB	(Ohya and Sueyoshi 2010)
	USA	2011	Various	42ºC	48-96 hours	1.6 - 4 x 10^4 / spot	GasPak	TSA5SB	(Clothier et al. 2011)
	Korea	2012	Various	37ºC	72 - 120 hours	1.5 x 10^6 / spot	No details reported	MHA5SB	(Lim et al. 2012)
	Czech Republic	2011	BH	37ºC	72-96 hours	Undefined	AnaeroGen	WC5SB	(Sperling, Smola, and Cízek 2011)
	Czech Republic	2014	BH	37ºC	72-96 hours	10^5 / spot	AnaeroGen	WC5SB	(Prášek et al. 2014)
	Japan	2015	BH	37ºC	72 hours	10^4 - 10^5 / spot	No details reported	TSA5SB	(Kajiwara et al. 2016)
	USA	2016	Various	37ºC	96 hours	1 x 10^5 / spot	No details reported	TSA5SB0	(Mirajkar, Davies, and Gebhart 2016)
Broth Dilution	Sweden	1999	BH	37ºC	up to 96 hours	5 x 10^5 - 10^6 CFU/ml	No details reported	BHIS10	(Karlsson et al. 1999)
	Sweden	2001	BH	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	No details reported	BHIS10	(Karlsson, Gunnarsson, and Franklin 2001)
	Australia	2002	BH	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	GENbox	BHIS10	(Märit Karlsson, Oxberry, and Hampson 2002)
	Sweden	2003	Various	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	GENbox	BHIS10	(Karlsson et al. 2003)
	Sweden	2004	BP	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	GENbox	BHIS10	(Karlsson *et al.*, 2004a)
	Germany	2004	BH	37ºC	96 hours	3 × 10^6 CFU/ml	GENbox	BHIS10	(Rohde et al. 2004)
	European	2004	BH	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	GENbox	BHIS10	(Karlsson *et al.*, 2004b)
	Sweden	2006	BP	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	BBD GasPak	BHIS10	(Pringle, Landén, and Franklin 2006)
	Spain	2009	BH	38ºC	72-120 hours	1 - 5 x 10^6 CFU/ml	GENbox	BHIS10	(Hidalgo et al. 2009)
	Spain	2011	BH	37ºC	96 hours	1 - 5 x 10^6 CFU/ml	No details reported	BHIS10	(Hidalgo et al. 2011)
	Sweden	2012	BH, BP	37ºC	96 hours	1 - 5 × 10^6 CFU/ml	No details reported	BHIS10	(Pringle et al. 2012)
	Poland	2012	BH	37ºC	96 hours	10^6 CFU/ml	GENbox	BHIS10	(Zmudzki et al. 2012)
	Germany	2014	BH	37ºC	120 hours	10^5 GFU_{50}/ml*	GENbox	BHIS20	(Herbst et al. 2014)
	Italy	2015	BH	-	-	No details reported	-	-	(Rugna et al. 2015)
	USA	2016	Various	37ºC	96 hours	10^6 CFU/ml	No details reported	BHIS10	(Mirajkar, Davies, and Gebhart 2016)

BH - *B. hyodysenteriae*; BP - *B. pilosicoli*
Media: Tryptic soy agar + 5% sheep blood: TSA 5 SB; Tryptic soy agar + 10% sheep blood: TSA 10 SB; Tryptic soy agar + 5% ox blood: TSA 5 OX; Tryptic soy agar + 10% bovine blood: TSA10BB; Wilkins-Charlgren + 5% sheep blood: WC 5SB; Mueller-Hinton agar + 5% sheep blood: MHA 5 SB; Brain heart infusion + 10% fetal calf serum: BHIS 10; Brain heart infusion + 20% fetal calf serum: BHIS 20;
Atmosphere: AnaeroGen Sachets Oxoid: AnaeroGen; Anaerobic gas pack GasPak BD: GasPak; anaerobic atmosphere generator biomerieux GENbox: GENbox; Anaerobic gas generator envelopes BBL gas pak: BBD GasPak
*GFU - authors report "growth forming units" as opposed to CFU Studies which reported the use of the VetMIC Brachy panels.

The introduction of the VetMIC *Brachyspira* broth microdilution system, first described in 2001, has led to substantial progress towards test standardization (Karlsson, Gunnarsson, and Franklin 2001; Karlsson et al. 2004). This product includes serial 2-fold dilutions of dried antimicrobials in tissue culture trays. Testing is done by inoculating wells with 500 µL of broth culture and incubating anaerobically at 37°C for 4 days with agitation (SVA 2011). Antimicrobial MICs as determined using this method have been shown to be reproducible, although interestingly typically yield a doubling dilution lower MIC than agar dilution tests (Rohde et al. 2004).

1.3.4 Challenges in comparing susceptibility data generated using nonstandard tests (Kulathunga and Rubin 2017)

Because there are no established standard test methods, comparison of results generated by different laboratories must only be done with extreme caution. The inability to compare data was exemplified by a 2005 study in which 8 European laboratories participated in a ring test of *Brachyspira* diagnostics (Råsbäck et al. 2005). In this study, each laboratory received samples of pig feces seeded with specific concentrations of a previously identified *Brachyspira* isolate and 2 pure cultures of known *Brachyspira* for susceptibility testing. Antibiotic susceptibility test methods differed by media composition and type, incubation temperature and time. Only a very limited description of the methods used by each laboratory for susceptibility testing and interpretation was presented. Not surprisingly, results were highly variable; errors including failure to detect or identify isolates or discordant susceptibility test results occurred 32% of the time (Råsbäck et al. 2005). The varying concentrations of antimicrobials tested made comparison of MICs between laboratories impossible even when the results were not inconsistent (e.g., different laboratories reported lincomycin MICs of ≤4, ≤2, and ≤0.5 for the same isolate). The lack of validated *Brachyspira*-specific susceptibility test methods has resulted in the development of a

variety of “in-house” techniques; consequently, susceptibility data are not reliably comparable between laboratories. Further confounding susceptibility data portability is the lack of a consensus scheme for determining species level identification (Råsbäck et al. 2005). Researchers have used combinations of conventional methods (e.g., culture and biochemical tests) and a variety of molecular methods (e.g., *nox* and 16S rDNA sequencing and *nox*-RFLP), which are recognized to have varying levels of sensitivity and specificity.

1.4 Antimicrobial resistance and mechanism

The World Health Organization defines antimicrobial resistance (AMR) as:

> The ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals, and antimalarials) from working against it

(http://www.who.int/antimicrobial-resistance/en/). Antimicrobial resistance in bacteria can be either intrinsic or acquired. Antimicrobials are widely used to prevent and treat infectious diseases in both humans and animals. However, their use has led to the development of antimicrobial resistance in bacteria which were previously used effectively to control infectious diseases. With the emergence of multi-drug resistance or pan drug-resistant bacteria and very slow development of novel antimicrobials in the pipeline, AMR has become a global threat which is discussed widely all over the world (Schwarz, Loeffler, and Kadlec 2017).

1.4.1 Intrinsic resistance

Intrinsic resistance is a natural or inherent mechanism of bacteria, independent of antibiotic selective pressure, that occurs due to the structural, biochemical or functional nature of bacteria (Boerlin and White 2013; Olivares et al. 2013; Blair et al. 2014; Munita and Arias 2016). Intrinsic resistance is an ancient phenomenon and probably a survival mechanism present in most of the

environmental bacteria such as soil bacteria (Ghysels, Fajardo, and Martı 2008; Cox and Wright 2013). Intrinsic resistance in bacteria has been a widely discussed the topic in both human and veterinary medicine. It is more difficult to treat Gram-negative bacterial infections than Gram-positive infections because of their natural multidrug resistance phenotype (Iredell, Brown, and Tagg 2015). The simplest example is Gram-negative cells are naturally resistant to large molecular size antimicrobial drugs because their outer membrane acts as a barrier to large molecules (Boerlin and White 2013). Intrinsic resistance can be divided into three mechanistic categories: (1) lack of target, (2) production of inactivating enzymes encoded by chromosomal genes, (3) impermeable cell structures (cell wall and membrane) and efflux pump (Olivares et al. 2013; Munita and Arias 2016). Other mechanisms conferred by genes involved in various cellular pathways in bacteria, have been unexpectedly shown contribute to antibiotic resistance (Gomez and Neyfakh 2006). These intrinsic mechanisms were discovered by performing gene-knockout studies (Cox and Wright 2013). For example, normally Ciprofloxacin traps the DNA as a DNA-protein complex and prevents it from dissociating which leads to blocking DNA synthesis and therefore the DNA breaks. By knocking out the gene *rec*D, which is responsible for DNA recombination and repair in *Acinetobacter baylyi* the intrinsic mechanism of resistance was removed and the organism became hypersusceptible to ciprofloxacin (Gomez and Neyfakh 2006). These studies have lead to a novel area of research where antimicrobials can inhibit the targets that are involved in intrinsic resistance mechanisms of different bacteria (Cox and Wright 2013).

1.4.2 Acquired resistance

Acquired resistance, is dependent on antimicrobial selection pressure and develops in bacteria due to some genetic modification; genetic mutations in the chromosome or horizontal resistance gene transfer (Tenover 2006). Because of this, a previously susceptible bacterial population can become

resistant to an antimicrobial drug (Boerlin and White 2013; Munita and Arias 2016). Acquired mechanisms can include reduced permeability, antimicrobial modifying enzymes, target modifications or active efflux (Boerlin and White 2013). If acquired resistance determinants are associated with mobile genetic elements such as plasmids, transposons or integrons, resistance can be transferred to other bacteria in the same species or between different species or genera (Tenover 2006; Schwarz, Loeffler, and Kadlec 2017). The acquisition of foreign genetic material occurs in bacteria by three different mechanisms: transformation: uptake of naked DNA which is released in to the surrounding environment when bacteria cells burst, transduction: transfer DNA to a bacteria through bacteriophages, conjugation: transfer of plasmids through a sex pilus formed between two adjacent bacterial cells (mating like process) (Schwarz, Loeffler, and Kadlec 2017).

1.4.3 Resistance mechanisms present in *Brachyspira* spp.

The current literature describes several acquired resistance mechanisms found in *Brachyspira* spp., (Table 1.5) (Pringle et al. 2004; Pringle, Fellström, and Johansson 2007; Mortimer-Jones et al. 2008; Card et al. 2018; De Luca et al. 2018). Protein synthesis inhibitory drugs (macrolides, lincosamides, and pleuromutilins) bind to the peptidyl transferase center (PTC) of 23S rRNA in *Brachyspira* 50S ribosome. Once bound to these antimicrobials protein synthesis and cell growth is inhibited in *Brachyspira* (Karlsson et al. 1999; Karlsson, Gunnarsson, and Franklin 2001; Giguère 2013). However, detailed studies on binding sites of those antimicrobials in PTC revealed that any mutation of nucleotides can lead to inhibition of drug binding and resistance to these drugs in *Brachyspira* isolates (Karlsson et al. 1999; Karlsson, Gunnarsson, and Franklin 2001; Hidalgo et al. 2011; Hillen et al. 2014; Kulathunga and Rubin 2017).

Table 1.5 Genetic associations with decreased antimicrobial susceptibility in *Brachyspira*.

Organisms Investigated	Phenotypic Resistance	Genetic Mechanism	References
B. hyodysenteriae ***B. pilosicoli***	macrolides, lincosamides, streptogramins, tiamulin	Single nucleotide polymorphisms at positions 2032 and 2058 of the 23S ribosomal subunit	(Hidalgo et al. 2011; Karlsson et al. 1999; Karlsson et al. 2004)
B. hyodysenteriae	tiamulin	Mutation in ribosomal protein L2, L3, L4, L22 and 23S rRNA in the peptidyl transferase region	(Hillen et al. 2014; Pringle et al. 2004)
B. hyodysenteriae ***B. intermedia***	doxycycline	Single nucleotide polymorphism at position 1058 of the 16S ribosomal subunit	(Pringle, Fellström, and Johansson 2007; Verlinden et al. 2011)
B. pilosicoli	penicillin, ampicillin and oxacillin	β-lactamases (OXA-63, OXA-136, OXA-137 and OXA-470 - OXA-479)	(La et al. 2015; Meziane-Cherif et al. 2008)

This table was taken from the review article (Kulathunga and Rubin 2017)

To number the position of SNPs in the 23S rRNA researchers use the *E. coli* numbering system which facilitates comparison between different organisms and avoids discrepancies in other notations (Pringle et al. 2004; Vester and Douthwaite 2014). The mutations A2058G or T and A2059G or T were reported to increase the MIC of macrolide drugs in *B. hyodysenteriae* and *B. pilosicoli* (Hillen et al. 2014). A number of mutations (G2032A, A2058G or T, A2059G or T) have been shown to led to increased lincomycin MICs in *B. hyodysenteriae* and *B. pilosicoli*

Resistance to pleuromutilins has also been associated with mutations in PTC (G2032A, C2055A, G2447T, C2499A and T2504G) and L3 ribosomal protein (Asn148Ser and Ser149IIe) in both *B. hyodysenteriae and B. pilosicoli* (Pringle et al. 2004; Hidalgo et al. 2011; Hillen et al. 2014). Further, mutations of PTC at positions T2504G and G2447T was associated with increased MICs of chloramphenicol in *B. hyodysenteriae* (Pringle et al. 2004).

In addition to mutations, there are also genes which have been shown to confer antimicrobial in *Brachyspira* spp. A study published in 2018 described *lnu*C, a gene associated with elevated lincomycin MICs in *B. hyodysenteriae* strains from Italy, this was the first report a gene that encoding resistance to protein synthesis inhibitor in *Brachyspira* spp. (DeLuca et al. 2018). This gene was found in a collection of *B. hyodysenteriae* isolates collected from a swine dysentery outbreak from 2013-2015 with very high lincomycin MIC's (≥64 μg/ml). This gene was first identified in *S. agalactiae* and encoded for the production of an enzyme called lincosamide nucleotidyl transferase which can catalyze the lincomycin drugs thereby inactivating them (Achard et al. 2005). Later a second resistance gene called *tva* in *Brachyspira* was described and published by a group of researches from Europe (Card et al. 2018). This gene was identified as responsible for pleuromutilin drug resistance in *B. hyodysenteriae* and *B. pilosicoli*. This gene encodes a

hypothetical ribosomal protection protein which inhibits the pleuromutilin drug binding making *Brachyspira* resistance to these antimicrobials.

β-lactamase production in *B. pilosicoli* isolates from humans was first identified in 2003 in western Australia using the nitrocefin test; 50% of the isolates were positive (Brooke, Hampson, and Riley 2003). Later multiple oxacillinases related to OXA-63 were discovered in *B. pilosicoli* isolates from both humans and animals (Mortimer-Jones et al. 2008; Meziane-Cherif et al. 2008).

To date there has not been a systematic description of intrinsic resistance mechanisms found in *Brachyspira*. However, the ability of *Brachyspira* to grow on selective medias such as BJ (spectinomycin, vancomycin, colistin, spiramycin and rifampin with swine fecal extract) or CVS (colistin, vancomycin, and spectinomycin) indicates that *Brachyspira* may be intrinsically resistant to these antimicrobials (Calderaro et al. 2005; Rubin, Harms, et al. 2013; Hampson et al. 2019).

1.5 Next-generation sequencing (NGS) as a diagnostic tool in clinical microbiology

1.5.1 Introduction to NGS technologies and application in infectious disease diagnosis

The discovery of the three-dimensional structure of deoxyribonucleic acid (DNA) by James Watson, Francis Crick, Rosalind Franklin contributed enormously to the field of research in the DNA replication process and encoding proteins in nucleic acid (Watson and Crick 1953). This discovery opened the door to the development of sequencing technologies to describe the DNA and RNA make up of given individuals (e.g., microbial ribosomal RNA, tRNA, bacteriophages with RNA or DNA, etc.). In 1977, sequencing technology was revolutionized by Fredrick Sanger and the most successful sequencing method (Sanger's 'chain termination" or dideoxy technique) was developed (Sanger, Nicklen, and Coulson 1977). The method required the sequencing process to be split into four individual DNA synthesis reactions that included the DNA template strand,

DNA primer, normal A,G,C, and T deoxynucleotide triphosphates (dNTPs), DNA polymerase and each reaction contains a low level of one of four dideoxynucleotide triphosphates (ddNTPs): ddATP, ddGTP, ddCTP, or ddTTP, which were chemically altered bases (Sanger, Nicklen, and Coulson 1977; Heather and Chain 2016). When a ddNTP is incorporated into a chain of nucleotides, synthesis terminates, as ddNTP molecule lacks a 3' hydroxyl group, which is required to form a link with the next nucleotide in the chain. Since only a small proportion of ddNTPs are included in the reaction, one may be randomly incorporated in place of a dNTPs thereby terminating strand elongation at each location along the sequence resulting in truncated fragments. Upon the completion of four parallel reactions, partial replication DNA products will occur for every nucleotide in the template. Therefore, each reaction has now stet of DNA fragments that have same starting points but different end points terminating with a defined base, reaction products were then loaded into four lanes of a single gel and separated by size electrophoretically (Sanger, Nicklen, and Coulson 1977). The smallest fragments migrate to the bottom of the gel while largest fragments migrate a minimum distance and remain on the top of the gel. As the primers are radiolabelled in all four-reaction autoradiography of the gel will be obtained and sequence will be read from bottom (short fragment) to top (large fragments) according to their increasing size. Sanger sequencing is an expensive and labor intensive method and to increase the parallelization, reproducibility, throughput and reduce the cost several modifications were done to the original methods, such as labeling the chain-terminating nucleotides with different dyes to run the reaction in a single tube, eliminating the need to cast gels by using capillary gel electrophoresis and automation of the process (Smith et al. 1986; Prober et al. 1987; Luckey et al. 1990; Lander et al. 2001; Karger and Guttman 2009; Stranneheim and Lundeberg 2012; Alfaroa, Sepulveda, and Lyonc 2019).

Subsequently, the commercial launch of massively parallel DNA sequencing (next-generation sequencing or second generation sequencing) platforms have reduced the cost and time of WGS drastically (Mardis 2008). These next-generation sequencing platforms share some common features with Sanger-sequencing such as use of polymerases for synthesis, modified nucleotides, and fluorescence detection. However, NGS platforms differ from Sanger's method in their ability to perform high throughput sequencing (Mardis 2008; Metzker 2009). There are several companies (e.g., Illumina, Thermo Fisher Scientific, Oxford Nanopore etc.) which have developed different NGS technologies such as sequencing by synthesis, pyrosequencing, sequencing by ligation or real-time sequencing (Metzker 2009). Currently, Illumina has Miseq, NextSeq 500, HiSeq 2500 platforms producing 15 Gb, 120 Gb and 1000 Gb sequencing data per run have a maximum 2 × 300 bp, 2 × 150 bp and 2 × 125 bp read length, respectively. The MiSeq platform is convenient for sequencing small microbial genomes such as bacteria and viruses, while the larger-output Illumina platforms are more appropriate for eukaryotic genomes or very large studies, due to the balance of system/reagent cost and required sequencing depth (Glenn 2011; Loman et al. 2012; Vincent et al. 2017). The process generally consists of three main steps: library preparation, clonal amplification, and sequencing by synthesis. Illumina uses bridge amplification to generate clusters of identical DNA fragments which are subsequently sequence by synthesis (Adessi et al. 2000; Bennett 2004). In Illumina all the sequencing process take place in a flow cell and depending on the platform type it is partitioned into separate lanes (e.g., 1 lane in MiSeq, 2 in HiSeq2500 or 8 in HiSeq2000, HiSeq2500) (Adessi et al. 2000; Bennett 2004; Buermans and den Dunnen 2014). The advantage of this technology is that it is technically simple to perform, and high enough throughput to allow entire genomes to be sequenced in a single run. The major disadvantage associated with the Illumina platform is the high equipment cost and less therefore less cost-effective when

sequencing a few numbers of targets (Mardis 2008; Ginsburg, Willard, and Mardis 2013; Levy and Myers 2016).

The first Ion Torrent platform (Ion Torrent Personal Genome Machine (PGM)) was launched in early 2011 and uses both emulsion PCR and sequencing-by-synthesis approach (Rothberg 2011; Buermans and den Dunnen 2014). However, this technique differs from Illumina ant it uses native dNTP chemistry and reliance on a modified silicon chip to detect hydrogen ion release by the enzyme DNA polymerase during the addition of a base. Several instruments using the Ion Torrent technology are available (e.g. Ion Torrent S5, S5 XL, and GenStudio S5, S5 Plus and S5 Prime) and can produce data outputs from 30 megabases (Mb) to 25 gigabases (Gb) per chip (Rothberg 2011; Buermans and den Dunnen 2014). The Ion Torrent sequencers can be used mainly for targeted, exome, transcriptome, small RNA sequencing, and viral and bacterial typing studies (Rothberg 2011; Quail et al. 2012; Marine et al. 2020). Nevertheless, high error rate for specific regions is still an important obstacle for Ion Torrent technology.

Most of the dominant NGS technologies yield relatively short-read lengths (e.g. 50-400bp reads) compared to third generation technologies (Stranneheim and Lundeberg 2012). The presence of repetitive regions in the genome can lead to errors in read mapping in *de novo* assembly (Alkan, Sajjadian, and Eichler 2011; Treangen and Salzberg 2012; Firtina and Alkan 2016). In some instances, repetitive regions can be even longer than the short-reads generated by some platforms, leading to fragmented and incompletely assembled genomes (Alkan, Sajjadian, and Eichler 2011; Lu, Giordano, and Ning 2016; Magi et al. 2017).

Third generation sequencing developed by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies, are real-time single-molecule DNA sequencing platforms in which the direct

measurement of long-read DNA sequences is performed (Levy and Myers 2016; Xiao and Zhou 2020). The use of biological nanopore for DNA sequencing was first proposed in 1990s but the first commercially available sequencer, MinION was developed in 2014 by Oxford Nanopore Technologies (ONT) (MinION 2017). The Oxford Nanopore system translocates single-stranded DNA molecules through a nanopore across which a current is applied. As each nucleotide passes through the pore there is a change in normal ionic current which can be measured and the identity of nucleotide in the DNA strand determined. Advantages of this technology include the requirement for relatively little sample preparation and minimal reagents (no nucleotides, polymerases or ligases are not required) as no amplification step is performed prior to sequencing. Nanopore sequences are also recognized to have long read lengths (>1-5 × 10^4 nt) with high mapability, portability and high throughput (Branton et al. 2009; Bayley 2015). One of the primary disadvantage of nanopore technology is the high error rate in identifying individual bases (Branton et al. 2009; Mikheyev and Mandy 2014; Bayley 2015; Senol Cali et al. 2018).

Whole genome sequencing is currently used to identify a genetic basis for complex diseases, infectious disease diagnosis, identification of resistomes (all the possible antimicrobial-resistant determinates of pathogens), new diagnostic test development, new drug development, drug target determination studies and to address pharmacogenomics questions (Green and Guyer 2011; Manolio and Green 2011; Eyre et al. 2017). The very small amount of DNA (< 5 ng) required in some of the newly developed sequence platforms (e.g., Oxford Nanopore technology) combined with improved test result turnaround time has increased the utilization of these methods (Mikheyev and Mandy 2014; Didelot et al. 2016).

In any diagnostic laboratory which is following the conventional culture-based diagnosis there are three main objectives: (a) species identification (b) determine the antimicrobial susceptibility of

the identified pathogen and (c) the identification of any disease outbreaks (Didelot et al. 2016). With the advancement of technology, WGS has become a possible alternative approach to achieve all these objectives more successfully (Chrystoja and Diamandis 2014). Even though many pathogenic bacteria are culturable, it is estimate that 10% of the population may be extremely fastidious or unculturable (Koser et al. 2012; Didelot et al. 2016). The time for culturing pathogens can vary from a day (e.g., Isolation of *E.coli* from a urine sample) to months, especially for bacteria like *Mycobacterium tuberculosis* and some fungal spp.; WGS offers a possible alternative for these challenging microbes (Didelot et al. 2016). At the beginning, WGS was used to identify the isolated colonies (culture-based) whereas at present with the metagenomic approach WGS can be completed directly from the clinical samples, and is therefore a very rapid method of identification (Hasman et al. 2014; Forbes et al. 2017). Metagenomics is the sequencing of all DNA extracted from a sample followed by assembly of sequence reads or mapping them to a reference database followed by annotation of the genes (Hoyles and Swann 2019). These approaches have been successfully applied not only to bacteria but also to viral disease outbreaks (Gire et al. 2015). Two metagenomic analytical strategies are (a) shotgun sequencing and (b) amplicon sequencing. In shotgun sequencing, DNA fragments originating from genomes of a complex community are extracted and sequenced while in amplicon sequencing specific portions or marker genes (e.g., 16S rRNA, *rpo*B, *cpn*60, etc.) are first amplified by PCR prior to sequencing (Mande, Mohammed, and Ghosh 2012). Shotgun metagenomics is usually recommended for novel microbial identification and characterization studies as targeted-amplicon sequencing relies on a curated database of known microbes and may not be able to adequately analyze novel microbes (Forbes et al. 2017).

In diagnostic laboratories, bacterial serotyping has historically been performed by submitting samples to reference laboratories. However, the highly specialized nature of these assays means that there are relatively few reference centres performing these tests and turn-around-time can be unacceptably long in clinical settings; the increasing availability of WGS technologies shows promise for improving access to high resolution, rapid typing data (Didelot et al. 2016). Whole genome sequencing also shows promise in improving the turn-around-time and depth of information gleaned from antimicrobial susceptibility testing (CLSI 2013). The resistomes of either individual organisms, or entire microbial communities can be rapidly identified by performing WGS, and querying the sequence data using reliable resistance gene databases such as the Comprehensive Antibiotic Database (CARD) and Antibiotic-Resistant Database (ARDB) (Mcadam et al. 2011; Mutreja et al. 2013). However, these results can only be used to predict resistance genes and phenotypic tests are required to confirm susceptibility. Furthermore, the possibility that isolates carry novel resistance genes or mutations which are not included in databases leading to an insensitivity of this approach for detecting emerging resistance must be considered (Sch and Schaik 2017). Although genome sequencing has been demonstrated to yield data predictive of categorical susceptibility (susceptible or resistant), more recent studies have also demonstrated its potential to predict MICs, highlighting the roll of this technology in resistance surveillance (Eyre et al. 2017).

1.6 Summary

Brachyspira-associated disease has re-emerged in western Canada resulting in economic losses (Harding et al. 2010). At present no standardized method for conducting or interpreting antimicrobial susceptibility tests for *Brachyspira* and no published report describing the antimicrobial susceptibility of western Canadian *Brachyspira* isolates leaving Canadian swine

veterinarians without any invitro evidence to guide therapeutic selection. Several attempts have been made to validate susceptibility test methodologies, unfortunately these attempts have been hindered by poor reproducibility (Stubberfield et al. 2020). Despite these problems, there is compelling evidence that antimicrobial resistance is increasing in some *Brachyspira* spp. . (Karlsson, Landén, et al. 2004; Mirajkar, Davies, and Gebhart 2016; Hampson et al. 2019). These findings highlight the importance of standardizing susceptibility test methods and characterizing isolates causing disease locally.

OBJECTIVES

1) To develop and standardize an agar dilution method and describe the antimicrobial susceptibility of a collection of *Brachyspira* isolates
2) To modify and miniaturize the agar dilution method developed to improve the feasibility and including it in routine diagnostic workflows
3) To perform an analysis of the accuracy and cost of different diagnostic approaches and develop analytical decision trees to improve the efficiency of laboratory workflows
4) To identify the genetic determinants of resistance to macrolide and pleuromutilin drugs in *Brachyspira* spp.

TRANSITION STATEMENT

The methods used to detect antimicrobial susceptibility of *Brachyspira* spp. have not been standardized, and laboratories frequently rely heavily on in-house techniques. This chapter primarily focused on developing a standardized agar dilution test which gave high reproducible (89-100%) results for most of the antimicrobials tested. The developed method was applied to investigate the antimicrobial susceptibility of *Brachyspira* isolates collected from porcine samples (fecal or colon) submitted from western Canadian swine farms. As the first study in western Canada, our results indicated that all isolates of *B. hyodysenteriae* and *B. hampsonii* were profoundly susceptible to the pleuromutilin drugs while some *B. pilosicoli* isolates had high MICs. Highly variable macrolide MICs were found among all species. The results of this study emphasize the need for continuous monitoring and susceptibility testing and modification of agar dilution test to make it a feasible test to include in the routine diagnostic workflow of *Brachyspira.*

2. STANDARDIZATION AND APPLICATION OF AN AGAR DILUTION METHOD FOR DETERMINING THE ANTIMICROBIAL SUSCEPTIBILITY OF CANADIAN *BRACHYSPIRA* ISOLATES

Kulathunga D.G.R.S and Rubin J.E

Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Saskatchewan

Author contributions

Conceived and designed experiment: DGRSK, JER. Performed the experiment DGRSK, JER. Analysed the Data: DGRSK, JER. Wrote the paper: DGRSK, JER

2.1 Abstract

The re-emergence of *Brachyspira*-associated disease in pigs since the late 2000s in western Canada has illuminated some of the diagnostic challenges associated with this genus. Unlike other organisms, there is no standardized antimicrobial susceptibility test method for *Brachyspira* spp., and laboratories rely heavily on in-house methods that yield highly variable test results. To date, regardless of the testing method, antimicrobial susceptibility of western Canadian *Brachyspira* isolates have not been reported. The objectives of this study were, therefore, to standardize an agar dilution test method for *Brachyspira* spp. and to determine the antimicrobial susceptibility of a collection of western Canadian *Brachyspira* isolates to a range of clinically important antimicrobials. An agar dilution test was standardized in terms of starting inoculum (1-2 × 10^8 CFU/ml) media, incubation temperature and time and was assessed for repeatability. This method was used to determine the antimicrobial susceptibility of a collection of (n=87) clinical porcine *Brachyspira* isolates collected between 2009-2016. The method developed was found to be highly reproducible; overall, repeat susceptibility testing of the same isolate gave an identical result 92% of the time. Most of the isolates had very low MICs to the commonly used antimicrobials to treat swine dysentery in western Canada. However, exceptional isolates with elevated MICs (>32 µg/ml) for tiamulin, valnemulin, tylosin, tylvalosin, and lincomycin were identified. Overall, this study further emphasizes the importance of establishing CLSI approved clinical breakpoints for *Brachyspira* to improve the interpretation of test results and support the evidence-based selection of antimicrobials in swine industry.

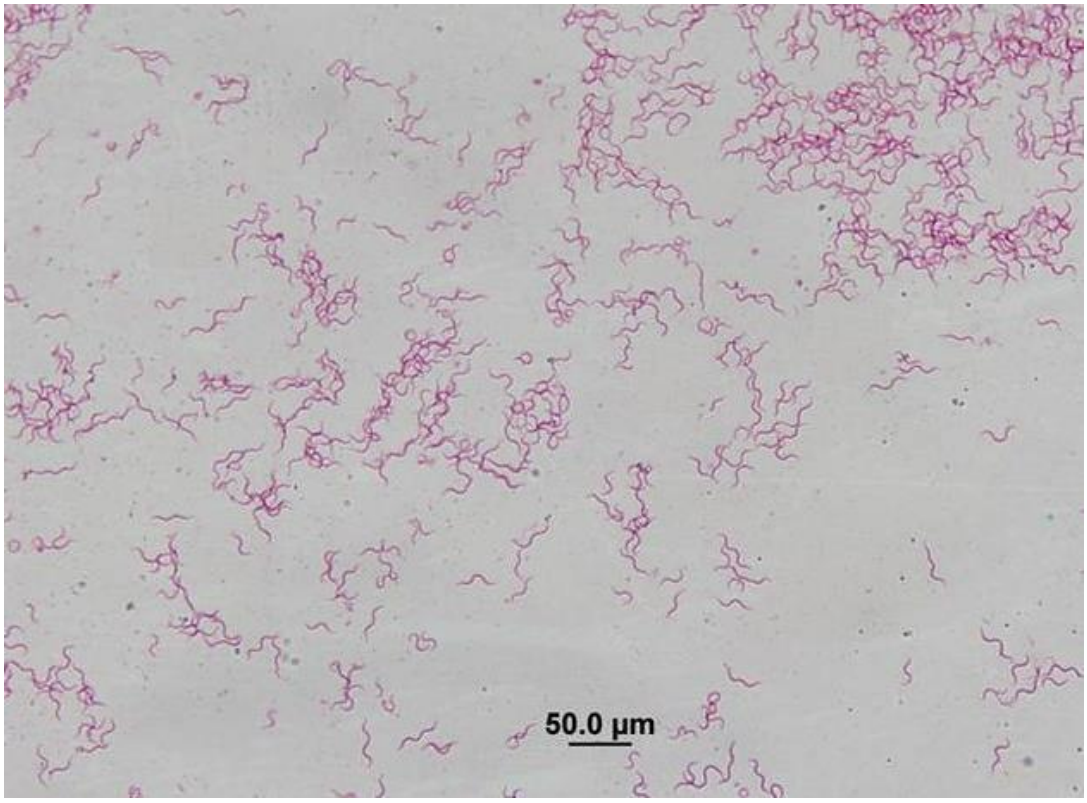
2.2 Introduction

Swine dysentery, which is characterized by profuse muco-hemorrhagic colitis, was first described in 1921 in the United States, although the causative agent (*B. hyodysenteriae*) was not isolated until the early 1970s (Whiting, Doyle, and Spray 1921; Harris and Kinyon 1972). Swine dysentery, which primarily affects grower-finisher pigs, is one of the most economically damaging diseases in terms of mortality and production limiting sub-optimal feed conversion associated with *Brachyspira* (Alvarez-Ordóñez et al. 2013).

Brachyspira are Gram-negative (Figure 2.1), fastidious, aerotolerant, anaerobes, which grow at high incubation temperatures (39-42ºC) and typically only produce hemolytic zones without visible colonies. At present there are nine species included in the genus of *Brachyspira* (Parte et al. 2020). Swine dysentery, or dysentery like disease is classically caused by *B. hyodysenteriae* although this syndrome has more recently been associated with *B. hampsonii* or *B. suanatina* infections (Burrough, 2017). *B. pilosicoli* has a broader host spectrum including pigs, other domestic animals, wildlife, and humans and can cause porcine intestinal spirochetosis in pigs within 7 to 14 days post-weaning (Trivett-Moore et al. 1998; Hampson 2012).

Since the mid 1990's the occurrence of swine dysentery has been rare however it re-emerged in the late 2000s and in 2009 *B. hampsonii* was detected for the first time in Saskatchewan (Harding et al. 2010). The return of swine dysentery was concurrently identified in the mid-western United States; reports from Iowa and Minnesota also identified *B. hampsonii*. Ultimately, the recurrence of swine dysentery led to *B. hampsonii* being divided into three genomovars (I, II and III) (Schwartz 2011; Chander et al. 2012; Mirajkar et al. 2016).

Figure 2.1 Gram stain of *Brachyspira*.



The image shows gram-negative (pink stain) anaerobic bacteria with a spiral-shaped body with tapered ends. The length of the porcine *Brachyspira* spp. can vary from 5 to 11 µm in length and from 0.2 to 0.4 µm in width.

The re-emergence of *Brachyspira*-associated disease has illuminated the diagnostic challenges of this genus, particularly for antimicrobial susceptibility testing which has not been standardized for the conditions under which *Brachyspira* spp. grow (Kulathunga and Rubin 2017). The Clinical and Laboratory Standards Institute (CLSI) prescribes a standardized set of test conditions for both aerobic and anaerobic bacteria including: media composition (pH, ionic composition and presence or absence of blood), incubation time, temperature and atmosphere, starting inoculum size and test endpoints (CLSI 2017, 2016, 2015b). The effects of alteration of these parameters on the results of susceptibility tests are well recognized and were perhaps best described in a 1971 report which formed the basis for an international effort to develop test standards (Ericsson and Sherris 1971). Particularly germane for *Brachyspira* are the effects of inoculum on MIC because these organisms don't form colonies and enumeration of live cells/ml (CFU/ml) is difficult and makes using a consistent culture density challenging. Furthermore, *Brachyspira* spp. do not reliably grow in liquid media (Kulathunga and Rubin 2017). When conducting a broth micro-dilution test it can be difficult to differentiate whether a culture didn't grow because it was inhibited by the antimicrobial or because of insufficient organism density to grow.

Determining the antimicrobial susceptibility of *Brachyspira* relies heavily on in-house methods yielding highly variable test results thereby making comparisons between labs impossible. This was exemplified by a 2005 study where eight European laboratories participated in a ring test of *Brachyspira* diagnostics and the results between labs were inconsistent (Råsbäck et al. 2005; Kulathunga and Rubin 2017). A follow up ring trial reported that between labs there was overall agreement 90% for the assays detected, and that between 79% and 97% of MICs determined were within the pre-determined ranges (Stubberfield et al. 2020). In this study differences in assay repeatability were observed between species, ≥ 80% of MICs within the expected range for *B.*

pilosicoli while for *B. hyodysenteriae* inter-laboratory results were less consistent(Stubberfield et al. 2020). While this investigation represents an advance in the field, the authors did not provide a method for quantitatively measuring the starting inoculum. Furthermore, the reference strain ($B78^T$) used in the study has acceptable quality control MIC ranges for five of the six antimicrobial agents tested below proposed ECOFF values (Stubberfield et al. 2020). Finally, as this investigation did not include *B. hampsonii*, the pathogenic species that emerged in western Canada, further study is required to validate this method for this newly emerged pathogen (Stubberfield et al. 2020). Although a number of studies have proposed interpretive criteria for describing the results of in vitro-susceptibility tests, no standard resistance breakpoints have been approved by either the CLSI or EUCAST (Aarestrup, Oliver, and Burch 2008; CLSI 2017; 2016). The lack of both test methods and interpretive criteria is a critical barrier to the evidence-based use of antimicrobials (Kulathunga and Rubin 2017).

In pigs, *Brachyspira*-associated disease has been treated with pleuromutilin and macrolide/lincosamide type drugs while metronidazole has been effectively used in people with intestinal spirochaetosis (Karriker et al. 2012; Helbling et al. 2012). Although inter-laboratory comparisons of *Brachyspira* MICs is speculative, there is compelling evidence from within individual labs suggesting that resistance is emerging to pleuromutilin, macrolide and lincosamide drugs in pathogenic species of *Brachyspira* (Hidalgo et al. 2011; Cogliani, Goossens, and Greko 2011; Mirajkar, Davies, and Gebhart 2016). The antimicrobial susceptibility pattern of Canadian *Brachyspira* isolates including novel species *B. hampsonii* has not been described. Therefore, the purpose of this study was to develop a standard agar dilution test for determining the antimicrobial MICs of *Brachyspira* spp. and to describe the antimicrobial susceptibility of a collection of western Canadian isolates.

2.3 Materials and methods

2.3.1 Standardized antimicrobial susceptibility test development

2.3.1.1 Development of a standard curve relating organism concentration to an optical density

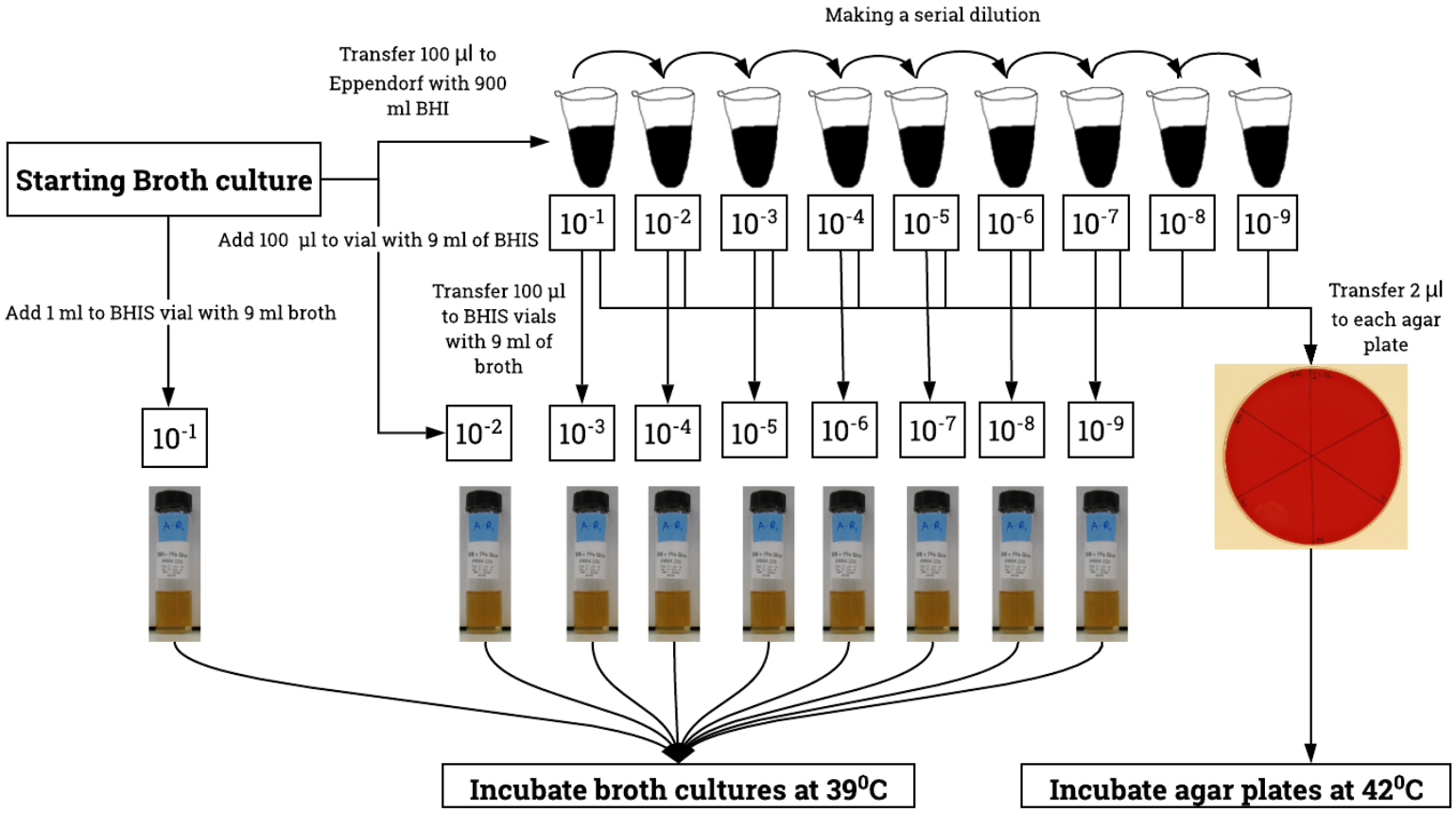
An equation to relate a *Brachyspira* culture density to the measured optical density (OD_{600nm}) was developed using 3 type strains: *B. pilosicoli* (ATCC 51139*), B. hyodysenteriae (*JXNI00000000) and *B. hampsonii* genomovar II (IDAC No 161111-01, ALNZ00000000) (Rubin, Harms, et al. 2013; Black et al. 2015). Frozen isolates were sub cultured on agar medium (BJ) before being transferred into brain heart infusion (BHI) broth which was supplemented with 10% fetal calf serum (Rubin, et al. 2013). Broth cultures were incubated for 24-48 hours at 39°C in an anaerobic jar (Anerogen TM 2.5 L, Thermo Scientific Oxide Sachet) on a magnetic stirrer to obtain fresh bacterial cultures. Several dilutions (1:1.1, 1:1.2 and 1:1.3 and 1:2-1:512) were prepared from fresh cultures to better define the OD curve and OD readings of those dilutions were recorded. Thereafter, serial 1:10 dilutions were prepared from each initial dilution. 100 µl of each broth culture were taken from all the final dilutions and plated on blood agar and incubated for 42 hours at 42°C and inspected for hemolytic zones. Thereafter, haemolytic zones were counted, and Microsoft Excel was used to generate scatter plots relating OD_{600nm} to CFU/ml, and to determine an equation describing this relationship. The relationship between OD_{600nm}, CFU/ml and genome equivalents/ml as measured by qRT PCR was previously determined and found to be consistent (data not shown).

2.3.1.2 Determination of the minimum inoculum required to start a culture

Brachyspira pilosicoli, *B. hyodysenteriae*, and *B. hampsonii* were grown anaerobically at 42°C for 48 hrs on BJ agar (Kunkle and Kinyon 1988). Isolates were then sub-cultured into brain heart infusion broth (BHIS) + 10% fetal calf serum and incubated at 39°C in an anaerobic jar (Anerogen TM 2.5 L, Thermo Scientific Oxoide Sachet), with constant stirring, for 24-48 hrs (usually *B. pilosicoli* and *B. hyodysenteriae* isolates grew within 24 hrs whereas *B. hampsonii* required 48 hrs of incubation). After incubation, a drop of each culture was examined under the phase-contrast at 400x to confirm the presence of live, motile spirochetes. The optical density (at 600 nm) of cultures was then measured to determine the bacterial concentration.

A 1:10 dilution series (10^{-1} to 10^{-9}) was made of each culture and inoculated onto agar in 2 µl spots. This culture was also sub-cultured, in triplicate, into fresh broth (1 ml into 9 ml fresh BHIS) (Figure 2.2). Cultures were incubated anaerobically at 39°C for 24 hrs (broths) or at 42°C for 48 hrs (agar). Following incubation, the media were inspected for growth. In the case of broth, visible turbidity compared to an uninoculated control was considered to indicate growth, while the presence of hemolytic zone was considered growth (positive culture) on agar. The concentration of organisms in the most diluted starting inoculum which resulted in visible growth was then calculated using the equation derived to determine the minimum inoculum required to obtain a positive culture.

Figure 2.2 Preparation of different starting inoculum sizes.



To check the effect of starting inoculum size on the growth of different *Brachyspira* spp. in both agar and liquid media, serial dilutions (10^{-1} to 10^{-9}) of starting inoculum sizes were prepared. Then broth cultures with different inoculum sizes were inoculated by adding 100 µl of starting inoculum to brain heart infusion (BHIS) vial and by adding 2 µl to the agar media.

2.3.1.3 Determining the effect of inoculum size on MIC determined by agar dilution

In this experiment, trypticase soy agar (TSA) + 5% sheep's blood containing antimicrobials representing four drug classes (pleuromutilin (tiamulin), macrolide (tylosin), β-lactam (ampicillin), and quinolone (nalidixic acid)) were prepared. For each antimicrobial, plates containing a series of dilutions from 0.25-128 µg/ml were made and inoculated with *B. pilosicoli*, *B. hyodysenteriae* and *B. hampsonii*. The organism concentration of each BHIS broth culture was determined by measuring (Thermo Scientific ND-2000 UV-Vis Spectrophotometer) the OD_{600nm}. A 1:5 dilution series (starting culture, 1:5, 1:25, 1:625, 1:3125) was made of each broth culture, and 2 µl was spotted onto the antimicrobial-containing plates in triplicate. Plates were incubated anaerobically for 2 days at 42°C and the lowest concentrations at which no hemolysis was observed were recorded.

By considering several factors, (a) how MIC changes in different species with different inoculum concentrations, (b) the repeatability of MICs between replicates when inoculum size changes for different antimicrobials and (c) standard conventions of the field (CLSI standards) an optimal starting inoculum concentration was selected.

2.3.2 Antimicrobial susceptibility testing

2.3.2.1 Isolate selection and species identification

A total of 93 samples including 87 clinical porcine isolates collected between 2009 and 2016 and four ATCC strains (*B. pilosicoli* ATCC 51139, *B. innocens* ATCC 29796, *B. murdochii* ATCC 51284, *B. hyodysenteriae* ATCC 27164) and two in-house type strains (*B. hampsonii* 30446 and *B. hampsonii* 30599) were included in the study.

Porcine clinical isolates originated from fecal or colonic samples submitted to the diagnostic laboratory at the Western College of Veterinary Medicine, University of Saskatchewan. Primary culture for *Brachyspira* spp. was begun by plating fecal samples on BJ agar, a previously described selective media based on trypticase soy agar containing: pig feces extract, spiramycin, rifampin, vancomycin, spectinomycin, colistin, and 5% sheep's blood (Kunkle & Kinyon, 1988). Inoculated plates were placed in an anaerobic jar (AnaeroGen TM 2.5 L, Thermo Scientific Oxide Sachet) and incubated at 42°C for 48 hours and inspected for the presence of β-hemolysis indicating the growth of *Brachyspira*. When hemolysis was observed, $1mm^2$ of agar was scraped from an isolated zone of hemolysis using a sterile bacteriological loop, macerating and then streaking it out on a sterile BJ plate. These plates were then similarly incubated at 42°C for 48 hours and visually inspected. For each sample where *Brachyspira* grew, a total of 2-3 subcultures were performed to ensure that a pure culture was obtained. Thereafter, an approximately $2cm^2$ of agar was sliced from an isolated β-haemolytic zone and transferred into a vial containing 10ml of brain heart infusion broth + 1% glucose + fetal calf serum (10% v:v) (BHIS) and incubated anaerobically at 39°C for 24 hrs with stirring. Broth cultures were then pelleted by centrifugation, the supernatant was removed, and the pellet resuspended in 1ml of BHI + 10% glycerol. The re-suspended pellet was stored at -80°C.

The clinical isolates originated from 39 different swine farms belonging to eight epidemiologically distinct production systems located in the provinces of Saskatchewan, Alberta, and Manitoba. A production system was defined as an umbrella company comprised of one or more swine farms which are independent of other companies (Mirajkar, Davies, and Gebhart 2016). The pigs' genotype, environmental conditions, and other natural resources (feed, manipulative materials) used in one production system may be different from other production systems (Lukovic, Skorput,

and Karolyi 2017). The farms within a production system follow similar management practices and have pigs from a common source. The number of isolates from each production system are listed in table 2.1.

The isolates were identified based on *nox* phylogeny (Atyeo et al. 1999; Rohde and Habighorst-blome 2013). Briefly DNA was purified from cell pellets of isolates grown in BHIS using a DNeasy Blood and Tissue Kit (QIAGEN). Partial NADH oxidase (*nox*) sequences were amplified with genus-specific primers as described in previous studies. PCR amplicons were purified using a commercial kit (BS664-250 REP, EZ-10 Spin Column PCR purification Kit, Bio Basic Canada Inc., Ontario, Canada) and were sequenced using the amplification primers. Sequences were assembled and edited using the pregap4 and gap4, and sequence alignments were performed in CLC Sequence Viewer (Version 7.7) using the ClustalW algorithm. A phylogenetic tree was constructed from the aligned sequences using maximum likelihood method in MEGA (version 7.0.26) (Hall 2013). Isolates were categorized as: *B. hyodysenteriae*, *B. hampsonii*, *B. pilosicoli*, *B. murdochii*, *B. innocens*, or non-clustering based on similarity with type strains.

2.3.2.2 Agar dilution

Ten antimicrobials, tiamulin, valnemulin, tylosin, lincomycin, tylvalosin, tetracycline, chloramphenicol, nalidixic acid, ampicillin, and amoxicillin + clavulanic acid (2:1), were selected to represent the breadth of products used to treat swine bacterial diseases and a multiple mechanism of action. For each antimicrobial, concentrations from 0.25-128 µg/ml were prepared in TSA with 5% sheep blood as per the CLSI guidelines and were used within 1-3 days (CLSI 2017).

Table 2.1 The number of isolates from each production system.

Production systems	Total number of isolates	Number of isolates from each groups of species					
		BH	BHM	BP	BM	BI	NC
A	3				2		1
B	39	15	8	3	4	6	3
C	16		2		8		6
D	5		2	1			
E	13	1		6	3		3
F	5			4	1		
G	4	1		2		1	
H	2		2				

The table indicated total number of isolates from each production system and the number of different species isolated from each production system. BH=*B. hyodysenteriae*, BHM=*B. hampsonii*, BP=*B. pilosicoli*, BM=*B. murdochii*, BI=*B. innocens*, NC=non-clustering.

Prior to susceptibility testing, isolates were transferred from freezer stocks into BHIS and incubated at anaerobically at 39°C for 2–3 days until turbidity was observed. Concurrently, a drop of each culture was spread on an antibiotic free TSA agar plates and incubated at 42°C anaerobically for 48 hours to ensure purity of the culture. Gram-stains were prepared from each broth to confirm the presence of Gram-negative spirochetes (Figure 2.1) and the absence of contaminating organisms. When the turbidity was observed in BHI broth cultures, ODs were measured, and bacterial density was adjusted to 1-2 × 10^8 CFU/ml. A 2 µl aliquot (2-4 × 10^5 CFU) of each isolate was spotted onto the antibiotic-containing (number of antimicrobials = 10, number of concentrations = 10, number of replicates from each concentration = 3, total number of plates = (10 ×10 ×3) = 300)) and antimicrobial free plates (n=3). Each isolate was spotted in triplicate onto separate plates which were incubated in separate jars at 42°C for 48 hours. Hemolysis at the inoculum site was used as the indicator of growth, and a lightbox was used to aid in the visualization of hemolysis. The MIC was defined as the lowest antimicrobial concentration, where hemolysis was not observed. In cases where MICs differed between replicates, and the difference was by no more than a single doubling dilution, the modal value was selected. In cases where greater variability was seen, the isolate was retested. The effect of the protein synthesis inhibitors on the production of hemolysin in *Brachyspira* is unknown. Therefore, we were interested to know whether production of hemolysins is inhibited at a lower antimicrobial concentration than is required to inhibit the growth. To test this, the inoculation site was sub-cultured from each for plates representing the MIC for each isolate. Both MICs and growth (Yes/No) on sub-cultured plates were recorded. Thirty-five isolates were randomly selected, using a random number generator, for re-testing at a later date to assess the reproducibility of the assay.

2.3.3 Statistical analysis

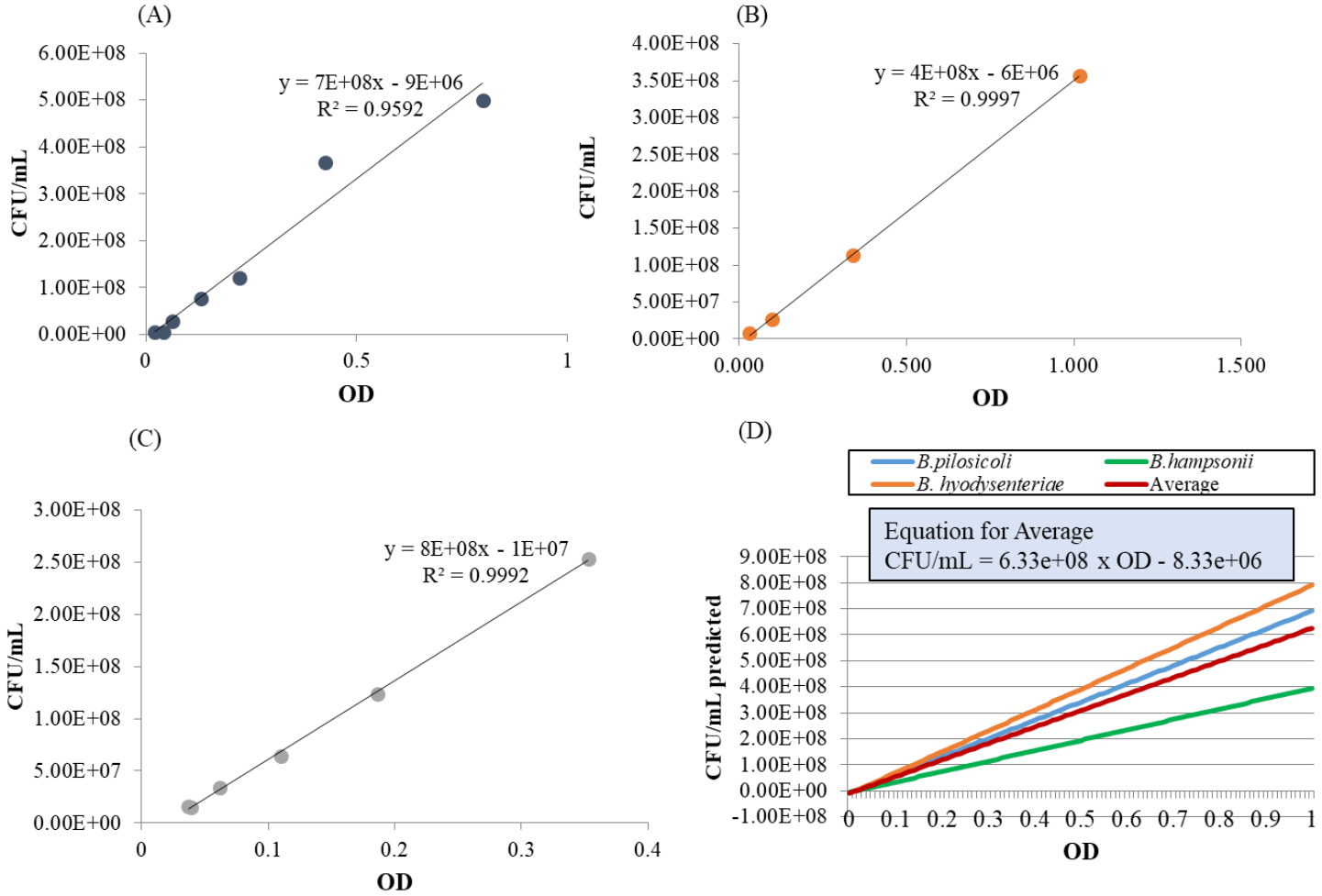
For all analyses, STATA (version 14) was used. As MIC data were not normally distributed, non-parametric statistical tests were used. Although the isolates included in this study originated from 8 different production systems, the species distribution between systems was non-uniform, and therefore not possible to compare MICs between production systems. Differences in susceptibility between species within a production system were assessed in cases where five isolates of multiple species were present. The Kruskal-Wallis (K-W) test followed by Dunn's multiple comparisons test (Dunn's test) was used to test for significant differences in the MIC distributions between species. When the Dunn test was performed in STATA the p-values were adjusted with Bonferroni corrections in which the family-wise error rate was controlled by multiplying the p-value in each pairwise test by the total number of pairwise comparisons. The α-level for significance was set at 0.05 in all tests. Reproducibility of the assay was statistically evaluated between first observation of MIC and the second observation of MIC using a parameter of correlation (Kendall's tau-b) and a measurement of agreement (Kappa).

2.4 Results

2.4.1 Development of an equation relating organism concentration to an optical density

The linear relationship of bacterial concentration and absorbance is observed when OD ranged from 0-1 (Lawrence and Maier 1977; Sutton 2011). Therefore, the equation we derived is applicable when the broth culture OD is between 0-1. The equation (equation: CFU/ml = (6.33×10^{8}) OD-8.33×10^{6}, OD = optical density of the given broth culture measured at 600nm wavelength of spectrophotometer)) allows for the number of viable CFU from a broth culture (Figure 2.3).

Figure 2.3 The effect of optical density on determination of bacterial density (CFU/ml) in a broth culture of *Brachyspira* spp.



(A) Relationship between optical density and colony forming unit of *B. pilosicoli* (OD) (B) Relationship between optical density and colony forming unit of *B. hampsonii* (C) Relationship between optical density and colony forming unit of *B. hyodysenteriae* (D) Plotted equations determined from experimental data and averaged into have an equation which can predict the bacterial density (CFU/ml) in a broth culture

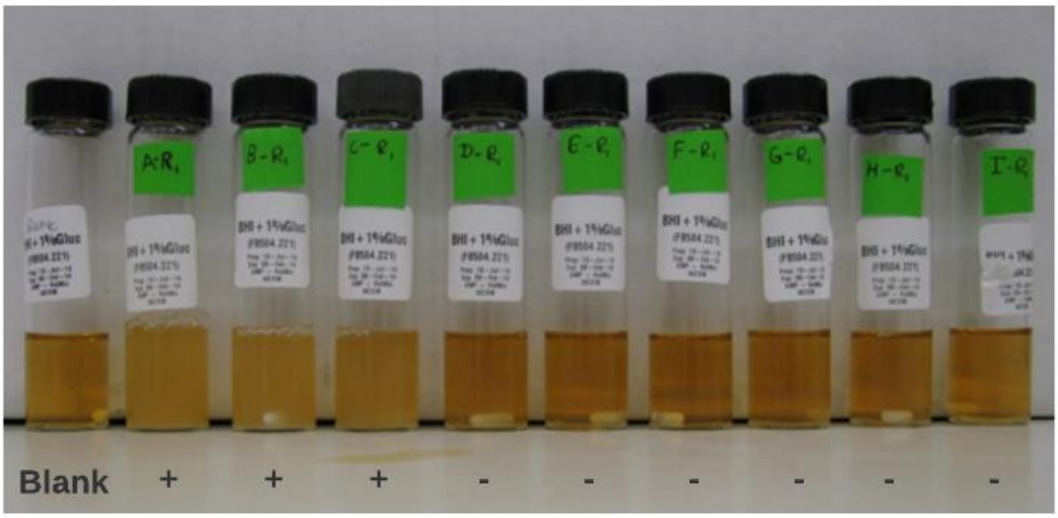
2.4.2 Determination of minimum inoculum size to grow *Brachyspira* on solid and liquid media

Differences in *Brachyspira* growth when media were inoculated with different initial culture densities were observed (Figures 2.4 and 2.5). Both in liquid and solid media visible growth (turbidity or haemolysis respectively) was observed only with a sufficient starting inoculum size which was designated as the cut-off bacterial concentration. Further, we found that the starting bacterial concentration required to obtain visible growth varied by species and media state (solid or liquid) (Table 2.2-2.4). When broth cultures were inoculated with starting inoculum size of *B. pilosicoli* >8.7×10^7 CFU/ml and incubated overnight turbidity was observed, whereas when starting inoculum size is <8.7×10^7 CFU/ml no turbidity/visible growth was observed in broth cultures (Figure 2.4). *B. pilosicoli* was found to requires the lowest and *B. hyodysenteriae* the highest starting bacterial concentration to obtain growth on both media types. These results demonstrate that viable broth cultures require a higher starting inoculum compared to cultures on agar. Further, anecdotally it is difficult to grow *Brachyspira* in agar media than broth cultures. Moreover, agar dilution is the recommended antimicrobial susceptibility test methodology for other fastidious anerobic bacteria (CLSI 2016).

2.4.3 Effect of inoculum size on observed minimum inhibitory concentration (MIC)

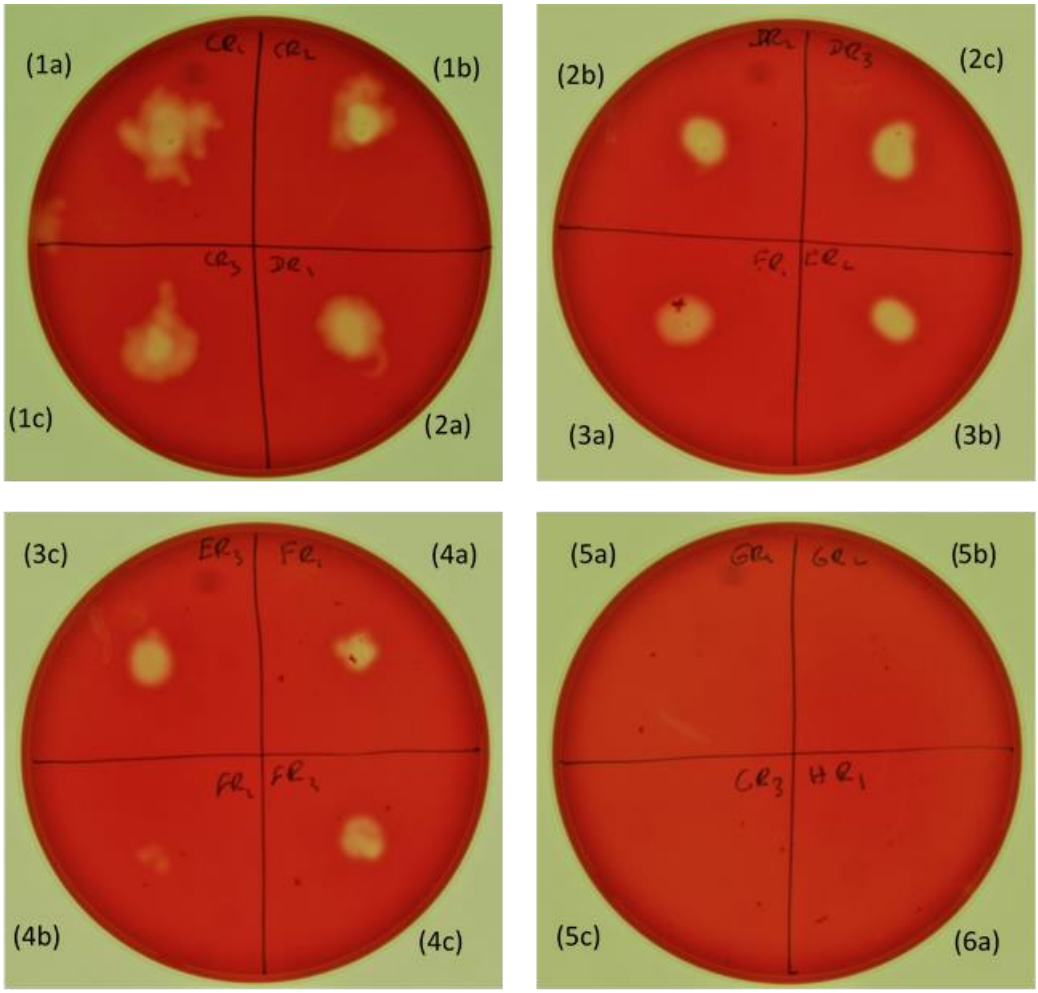
For all species, increased MICs were observed when test media was inoculated with an increasing inoculum size (Table 2.5). For all three species, consistent MICs were observed with concentrations higher than or equal to 4.4×10^7 CFU/ml for *B. hyodysenteriae*, 7.2×10^7 for *B. hampsonii* and 3.6×10^7 CFU/ml for *B. pilosicoli*. Inoculum concentrations less than this yielded inconsistent MICs between replicates (Table 2.5).

Figure 2.4 The growth of *B. pilosicoli* in brain heart infusion (BHI) broth with different starting inoculum sizes.



Visible growth (turbidity = (+) growth, no turbidity = (-) growth) in brain heart infusion broth (supplemented with 1% defibrinated serum) with decreasing starting inoculum size (from left to right: blank, (A) 8.7×10^{8}, (B) 8.7×10^{7}, (C) 8.7×10^{6}, (D) 8.7×10^{5}, (E) 8.7×10^{4}, (F) 8.7×10^{3}, (G) 8.7×10^{2}, (H) 8.7×10^{1}, (I) 8.7 CFU/ml).

Figure 2.5 Visible growth of *B. hampsonii* on trypticase soy agar (TSA) with different starting inoculum sizes.



Three replicates were included for each inoculum size. (1a-1c) 4.1×10^{7}, (2a-2c) 4.1×10^{6}, (3a-3c) 4.1×10^{5}, (4a-4c) 4.1×10^{4}, (5a-5c) 4.1×10^{3}, (6a) 4.1×10^{2}. Haemolysis was used as an indicator of visible growth.

Table 2.2 Inoculum concentrations of different *Brachyspira* spp. in liquid media.

Species	Starting inoculum size CFU/ml	Visible growth (turbidity observed (+) or not (-))									Lowest starting inoculum size (CFU) produce visible growth
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
B. pilosicoli	8.75×10^8	+	+	+	-	-	-	-	-	-	8.75×10^5
B. hyodysenteriae	5.40×10^8	+	-	-	-	-	-	-	-	-	5.4×10^7
B. hampsonii	4.10×10^8	+	+	-	-	-	-	-	-	-	4.1×10^6

The table summarizes if there was or was not visible growth on broth culture of the different *Brachyspira* species when the starting inoculum size was changed. The second column indicates the starting inoculum size while the 11^{th} column indicates the lowest starting inoculum which had visible growth on media after incubation.

Table 2.3 Inoculum concentrations of different *Brachyspira* spp. on solid media.

Species	Starting inoculum size (CFU)	Visible growth (hemolysis (+) or no haemolysis (-)) 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Lowest starting inoculum size produce hemolysis
B. pilosicoli	8.75×10^8	+	+	+	+	-	-	-	>1 CFU
B. hyodysenteriae	5.43×10^8	+	+	+	-	-	-	-	>11 CFU
B. hampsonii	4.11×10^8	+	+	+	-	-	-	-	>8 CFU

The table summarizes if there was or was not visible growth on solid/agar media the different *Brachyspira* species when the starting inoculum size was changed. The second column indicates the starting inoculum size while the 10^{th} column indicates the lowest starting inoculum, which was able to have visible growth on media after incubation.

Table 2.4 Summary of the lowest inoculum concentrations of different *Brachyspira* spp. on solid and liquid media.

Species	Lowest inoculum size which can give positive results	
	Liquid media (CFU/ml)	**Solid media (CFU in 2 µl)**
B. pilosicoli	8.75×10^5	1
B. hyodysenteriae	5.43×10^7	11
B. hampsonii	4.11×10^6	8

The table summarizes the lowest starting inoculum needed for different *Brachyspira* spp. to have visible growth after incubation based on either liquid or solid media type. *B. pilosicoli* required the lowest starting inoculum size for both types of media.

Table 2.5 Effect of inoculum size on minimum inhibitory concentrations (MICs) observation for four antimicrobials on agar.

Inoculum dilutions	Minimum Inhibitory Concentration (MIC) (µg/ml)											
	Tiamulin			Tylosin			Ampicillin			Nalidixic acid		
	R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3
B. hyodysenteriae (G44)												
0:0, 1.1×10^9	8	8	8	16	16	16	>128	>128	>128	>128	>128	>128
1:5, 2.2×10^8	8	8	8	8	8	8	>128	>128	>128	>128	>128	>128
1:25, 4.4×10^7	8	8	8	8	8	8	>128	>128	>128	>128	>128	>128
1:125, 8.8×10^6	8	8	4	8	8	4	4	32	64	4	4	64
1:625, 1.8×10^6	4	4	8	2	4	8	1	1	4	0.5	≤0.25	≤0.25
1:3125, 3.5×10^5	1	1	2	1	4	4	1	1	0.5	≤0.25	≤0.25	≤0.25
B. hampsonii (30446)												
0:0, 3.6×10^8	1	1	1	>128	>128	>128	>128	>128	>128	>128	>128	>128
1:5, 7.2×10^7	0.5	0.5	0.5	64	64	64	8	8	8	16	16	16
1:25, 1.4×10^7	≤0.25	≤0.25	0.5	8	8	4	≤0.25	≤0.25	0.5	2	2	4
1:125, 2.4×10^6	≤0.25	≤0.25	≤0.25	2	2	1	≤0.25	≤0.25	0.5	2	2	1
1:625, 5.7×10^5	≤0.25	≤0.25	≤0.25	1	1	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5
1:3125, 1.1×10^5	NGC[a]	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC
B. pilosicoli												
0:0, 9×10^8	≤0.25	≤0.25	≤0.25	8	8	8	2	2	2	32	32	32
1:5, 1.8×10^8	≤0.25	≤0.25	≤0.25	4	4	4	2	2	2	8	8	8
1:25, 3.6×10^7	≤0.25	≤0.25	≤0.25	4	4	4	1	1	1	4	4	4
1:125, 7.2×10^6	≤0.25	≤0.25	≤0.25	1	0.5	0.5	0.5	0.5	≤0.25	≤0.25	4	2
1:625, 1.2×10^6	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	0.5	≤0.25	≤0.25	≤0.25	≤0.25	2
1:3125, 2.5×10^5	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC	NGC

The table indicates how the minimum inhibitory concentrations (MICs) changed with different starting inoculum sizes for different *Brachyspira* species. For each antimicrobial and starting inoculum size, three replicates were tested. In grey areas the MICs observed for all three replicates in each antimicrobial were consistent. The lowest starting inoculum size which gave the consistent MICs was defined as the cut-off and all concentrations above this are indicated by grey shading. In white area we can observed that the MICs became variable between replicates in each antimicrobial when the starting inoculum was lower than the cut-off concentration. NGC = no growth in control plates.

Based on these results, a concentration of 1-2×10^8 CFU/ml (final inoculum of 2-4 × 10^5 CFU per spot on agar) was chosen to optimize assay repeatability. Furthermore, as this inoculum is consistent with the CLSI guidelines, it will be relatively easy to integrate into current standard diagnostic procedures (CLSI 2012).

2.4.4 Reproducibility of the standardized agar dilution method

Randomly selected isolates (n=35) were tested a second time and the observed MICs were compared (Table 2.6). Results were categorized as: complete agreement (when two observations were identical), one doubling dilution difference (when the two observations were different by a single doubling dilutions), > one doubling dilution difference. The precision of the MICs observed in susceptibility testing is considered to be plus or minus 1 two-fold concentration (CLSI 2015a). Therefore, both complete agreement and one doubling dilution differences were categorized as "agreement" for the calculation of reproducibility.

2.4.5 Species identification

Based on *nox* phylogeny (over 765 base pairs) clinical isolates were identified as: *B. hampsonii* (n=14), *B. hyodysenteriae* (n=17), *B. pilosicoli* (n=16), *B. murdochii* (n=18), *B. innocens* (n=9) and non-clustering (n=13) (Figure 2.6 and Table 2.7). Non-clustering isolates were dissimilar to type strains and could therefore not be reliably classified as one of the recognized species; furthermore, the diversity of these strains precluded grouping them in the statistical analyses.

2.4.6 Antimicrobial susceptibility test results

A wide range of susceptibility to all drugs tested were observed among our collection of *Brachyspira* spp. (Table 2.8). The majority of isolates were inhibited by low concentrations of the

pleuromutilins; the MIC_{90} for all isolates except *B. pilosicoli* (MIC_{90} for tiamulin = 64 µg/ml, valnemulin = 32 µg/ml), *B. murdochii* (MIC_{90} for tiamulin 8 µg/ml, valnemulin = 0.5 µg/ml) and *B. hampsonii* (MIC_{90} for tiamulin = 1 µg/ml) was ≤0.25 µg/ml.

Tiamulin inhibited the growth of 94% of the *B. hyodysenteriae* and 86% of the *B. hampsonii* isolates at the lowest drug concentration (0.25 µg/ml). For valnemulin, all isolates of *B. hampsonii* and *B. hyodysenteriae* were inhibited at ≤0.25 µg/ml (Table 2.8).

B. hampsonii, *B. hyodysenteriae*, *B. murdochii*, and non-clustering spp. had heterogeneous MIC distributions for tylosin while bi-modal MIC distribution was observed for *B. pilosicoli*. For all spp. the MIC_{90} for tylosin was > 128 µg/ml. A heterogeneous MIC distribution was observed in *B. hampsonii*, *B. murdochii*, *B. pilosicoli*, and *B. innocens* for tylvalosin. For tylvalosin, MIC_{90} was observed to be lower in *B. hyodysenteriae* and non-clustering isolates (4 µg/ml) than for *B. pilosicoli* and *B. innocens* (≥128 µg/ml). In our collection there were six isolates with very high MICs (≥128 µg/ml) for tylvalosin drugs, including a single *B. hampsonii* (Table 2.9).

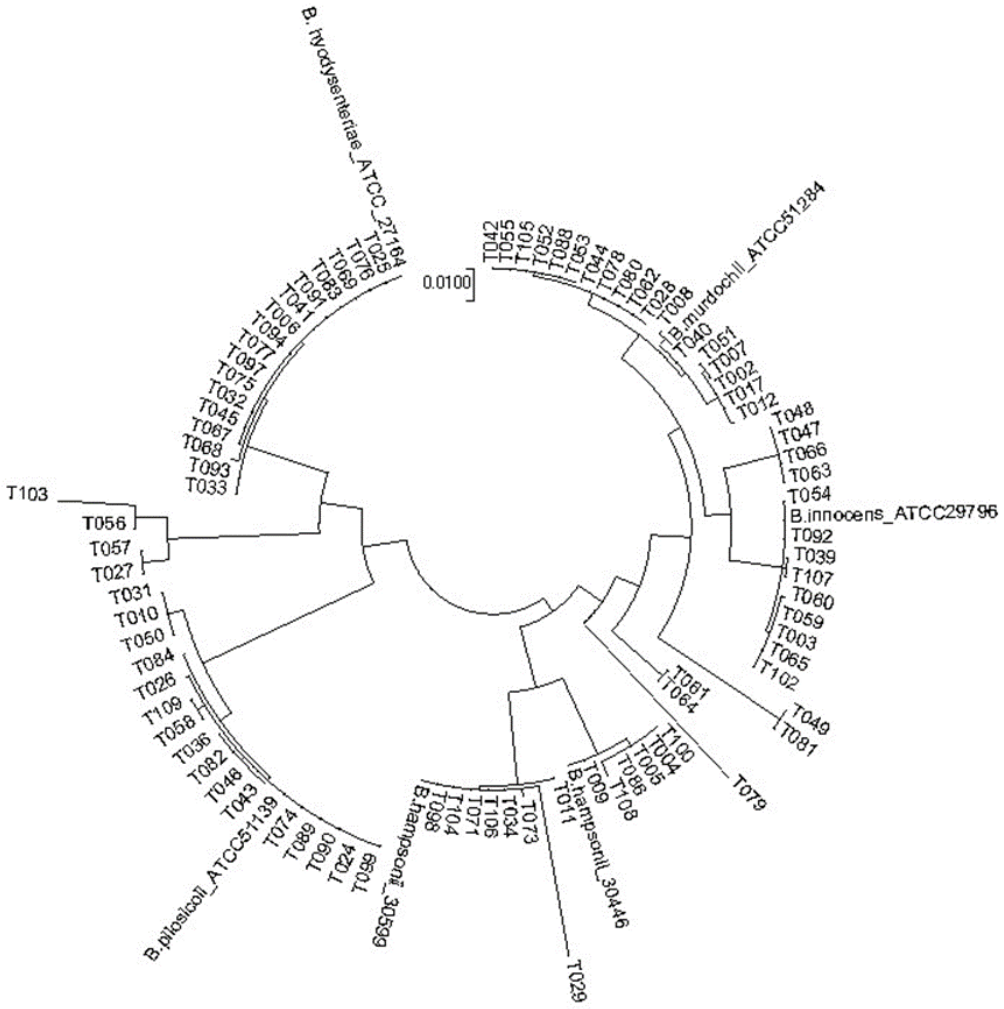
A bimodal lincomycin MIC distribution was observed for *B. murdochii*, while all other organisms displayed multimodal distributions. There were 8 isolates, including representatives from each recognized species with lincomycin MICs of ≥128 µg/ml. Except for two isolates, when MICs of tylosin and lincomycin was > 8µg/ml all other isolates had high MICs for tylvalosin (≥ 4 µg/ml) (Table 2.9).

Table 2.6 Reproducibility of the standard agar dilution method.

ATM	Observations with 100% agreement %, (n)	One doubling dilution difference observation (± 1) %, (n)	Reproducible %, (n)	Number of observations with MIC≤0.25 µg/ml %, (n)	More than one doubling dilution difference observations %, (n)	Correlation (Kendall's tau-b)	The measure of agreement (Kappa)
TIA	91, (n=32)	6, (n=2)	97, (n=34)	74, (n=26)	3 (n=1)	0.921	0.795 (*p = 0.000*)
VAL	80, (n=28)	20, (n=7)	100, (n=35)	77, (n=27)	0 (n=0)	0.793	0.443 (*p = 0.000*)
TYL	63, (n=22)	17, (n=6)	80, (n=28)	3, (n=1)	20 (n=7)	0.722	0.517 (*p = 0.000*)
TYV	69, (n=24)	20, (n=7)	89, (n=31)	26, (n=9)	11 (n=4)	0.888	0.631 (*p = 0.000*)
LIN	63, (n=22)	17, (n=6)	80, (n=28)	6, (n=2)	20 (n=7)	0.722	0.517 (*p = 0.000*)
TET	71, (n=25)	23, (n=8)	94, (n=33)	49, (n=17)	6 (n=2)	0.835	0.575 (*p = 0.000*)
CHO	80, (n=28)	17, (n=6)	97, (n=34)	0, (n=0)	3 (n=1)	0.816	0.694 (*p = 0.000*)
NAL	48, (n=17)	46, (n=16)	94, (n=33)	0, (n=0)	6 (n=2)	0.549	0.297 (*p = 0.010*)
AMP	80, (n=28)	14, (n=5)	94, (n=33)	26, (n=9)	6 (n=2)	0.913	0.774 (*p = 0.000*)
AUG	69, (n=24)	23, (n=8)	92, (n=32)	29, (n=10)	8 (n=3)	0.880	0.606 (*p = 0.000*)

Test results were considered to be consistent (reproducible) if MICs between replicates were identical or different by no more than a single doubling dilution. Total number of isolates = 35. The fifth column included the number of isolates with MIC ≤ 0.25 µg/ml. ATM = antimicrobials, TIA = tiamulin, VAL= valnemulin, TYL = tylosin, TYV = tylvalosin, LIN = lincomycin, CHO = chloramphenicol, TET = tetracycline, NAL = nalidixic acid, AMP = ampicillin, AUG = amoxicillin + clavulanic acid.

Figure 2.6 The phylogenetic tree of *Brachyspira* spp. tested.



Phylogenetic tree was drawn based on the NADH oxidase gene (product size is 765 bp).

Table 2.7 Species identification.

Species identification	***Number of isolates tested***
Brachyspira hampsonii	14
Brachyspira hyodysenteriae	17
Brachyspira pilosicoli	16
Brachyspira murdochii	18
Brachyspira innocens	9
Non-clustering	13
Total number of isolates	87

The total number of isolates belonging to each species category.

Table 2.8 MIC distributions of *Brachyspira* spp.

Antimicrobial	Species[a]	≤0.25	0.5	1	2	4	8	16	32	64	128	>128	MIC_{50}	MIC_{90}
Tiamulin	BHM	12		2									≤0.25	1
	BH	16			1								≤0.25	≤0.25
	BP	5	2	2			2	1	2	2			1	64
	BM	15		1			2						≤0.25	8
	BI	9											≤0.25	≤0.25
	NC	13											≤0.25	≤0.25
Valnemulin	BHM	14											≤0.25	≤0.25
	BH	17											≤0.25	≤0.25
	BP	9	1		2	1			2	1			≤0.25	32
	BM	16	1		1								≤0.25	0.5
	BI	9											≤0.25	≤0.25
	NC	13											≤0.25	≤0.25
Tylosin	BHM	3		2	1	2	4					2	4	>128
	BH	2		1	1	4	3	3	1			2	8	>128
	BP					5	1				1	9	>128	>128
	BM	3				6	4		1			4	4	>128
	BI			1	3	2	1					2	4	>128
	NC	3		1	3	1		1		1		3	4	>128
Tylvalosin	BHM	8		1	2	1		1				1	≤0.25	16
	BH	7	2	4	2	1		1					0.5	4
	BP	1	4		1	4	2			1		3	2	>128
	BM	4	7	2	1	1		1		1	1		0.5	64
	BI	6		1				1				1	≤0.25	>128
	NC	8	1	2		1	1						≤0.25	4

Antimicrobial	Species[b]	≤0.25	0.5	1	2	4	8	16	32	64	128	>128	MIC_{50}	MIC_{90}
Lincomycin	BHM	6	1	3		1				1		2	0.5	>128
	BH	6	4	4	1	1					1		0.5	4
	BP	1		2	2	2			2	4	2	1	32	128
	BM	5	1	2	6				2	1	1		2	64
	BI	4	1	1		1				1	1		0.5	128
	NC	1	1	6				2		3			1	64
Chloramphenicol	BHM		1	4	7	1		1					2	4
	BH	2		2	7	3	3						2	8
	BP			1	6	5	2	2					4	16
	BM	2		2	1	3							2	4
	BI		1	1	6	1							2	4
	NC		1	4	7	1							2	2
Tetracycline	BHM	8	1	3			1	1					≤0.25	8
	BH	9	4	2	1	1							≤0.25	2
	BP	7	3	3	1	1	1						0.5	4
	BM	13	2	1	2								≤0.25	2
	BI	6		1	2								≤0.25	2
	NC	10	1		2								≤0.25	2
Nalidixic acid	BHM	1			1		1		1	1	4	5	128	>128
	BH								3	4	5	5	128	>128
	BP								3		9	4	128	>128
	BM	1							7	4	6		64	128
	BI								1		7	1	128	>128
	NC							1	1	6	5		64	128

Antimicrobial	Species[a]	≤0.25	0.5	1	2	4	8	16	32	64	128	>128	MIC_{50}	MIC_{90}
Ampicillin	BHM	3	1	2					1			4	32	>128
	BH	3		4				2		1		7	16	>128
	BP		2	5			1		1			7	8	>128
	BM	15	2									1	≤0.25	0.5
	BI	4	4									1	≤0.25	0.5
	NC	11	2										≤0.25	0.5
Amoxicillin + clavulanic acid	BHM	3	1	2						1		7	32	>128
	BH	1	4		1			1	2		1	7	32	>128
	BP	1		2	1	2	3		1		2	4	8	>128
	BM	17										1	≤0.25	≤0.25
	BI	8										1	≤0.25	>128
	NC	13											≤0.25	≤0.25

[a]BHM = *B. hampsonii* (n = 14), BH = *B. hyodysenteriae* (n = 17), BP = *B. pilosicoli* (n = 16), BM= *B. murdochii* (n = 18), BI = *B. innocens* (n= 9), NC = non-clustering (n=13). This table indicated the minimum inhibitory concentrations (MICs) µg/ml observed for each spp. in agar dilution test. The ten antimicrobials tested are listed in the first column and the *Brachyspira* spp. are listed in the second column. The column 3-13 included the number of isolates were inhibited at each MIC listed on the top row. The last two columns have the MIC_{50} (50% of the isolates have an MIC that is ≤ this MIC) and MIC_{90} (90% of the isolates have an MIC that is ≤ this MIC) which were calculated based on the MICs distributions.

Table 2.9 The macrolide and lincosamides MICs in *Brachyspira* isolates.

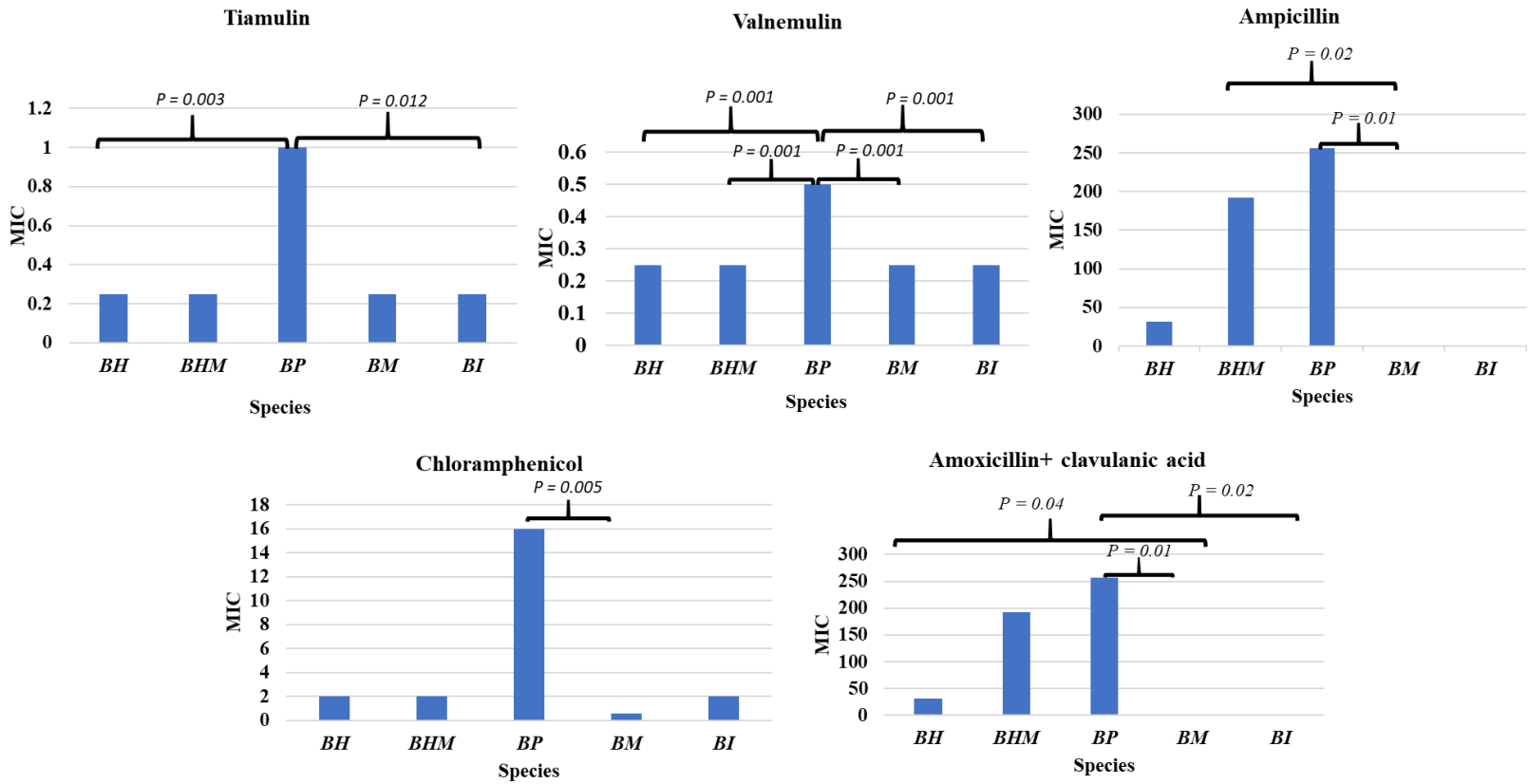
Isolate ID	Species Identification	MIC		
		Tylosin	Lincomycin	Tylvalosin
T009	*B. hampsonii*	>128	64	>128
T010	*B. pilosicoli*	>128	32	>128
T024	*B. pilosicoli*	>128	128	>128
T020	*B. murdochii*	>128	64	8
T026	*B. pilosicoli*	>128	>128	8
T040	*B. murdochii*	>128	128	>128
T042	*B. murdochii*	>128	32	64
T047	Non-clustering	>128	64	8
T050	*B. pilosicoli*	>128	64	>128
T053	*B. murdochii*	>128	32	16
T054	Non-clustering	>128	128	>128
T058	*B. pilosicoli*	>128	64	4
T063	Non-clustering	>128	16	32
T066	Non-clustering	>128	64	1
T074	*B. pilosicoli*	>128	32	8
T077	*B. hyodysenteriae*	>128	128	16
T082	*B. pilosicoli*	16	64	4
T084	*B. pilosicoli*	>128	128	8
T085	Non-clustering	>128	128	32
T086	*B. hampsonii*	>128	>128	16
T088	*B. murdochii*	>128	64	4
T092	Non-clustering	>128	64	4
T103	Non-clustering	16	16	≤0.25
T109	*B. pilosicoli*	>128	64	4

Isolates with tylosin and lincomycin MIC > 8 µg/ml were included in the table

Except for *B. pilosicoli* all isolates displayed low chloramphenicol MICs (≤ 16 µg/ml) with an MIC_{50} of 2 µg/ml. Similarly, tetracycline MICs were low with an MIC_{50} of ≤ 0.25 µg/ml for all species except *B. pilosicoli* where an MIC_{50} of 0.5 µg/ml was found. The MICs of nalidixic acid were nearly uniformly high (MIC_{50} of ≥ 64 µg/ml with MIC_{90} ≥ 128 µg/ml) of all species. Finally, although the MIC_{90} for the β-lactam drugs was >128 µg/ml for *B. hampsonii*, *B. hyodysenteriae* and *B. pilosicoli*, isolates with MICs across the range of concentrations tested were identified. Conversely, the MICs of ampicillin and amoxicillin+ clavulanic acid in *B. murdochii*, *B. innocens* and non-clustering, skewed toward the low end of the MIC range and MIC_{50} was ≤ 0.25 µg/ml.

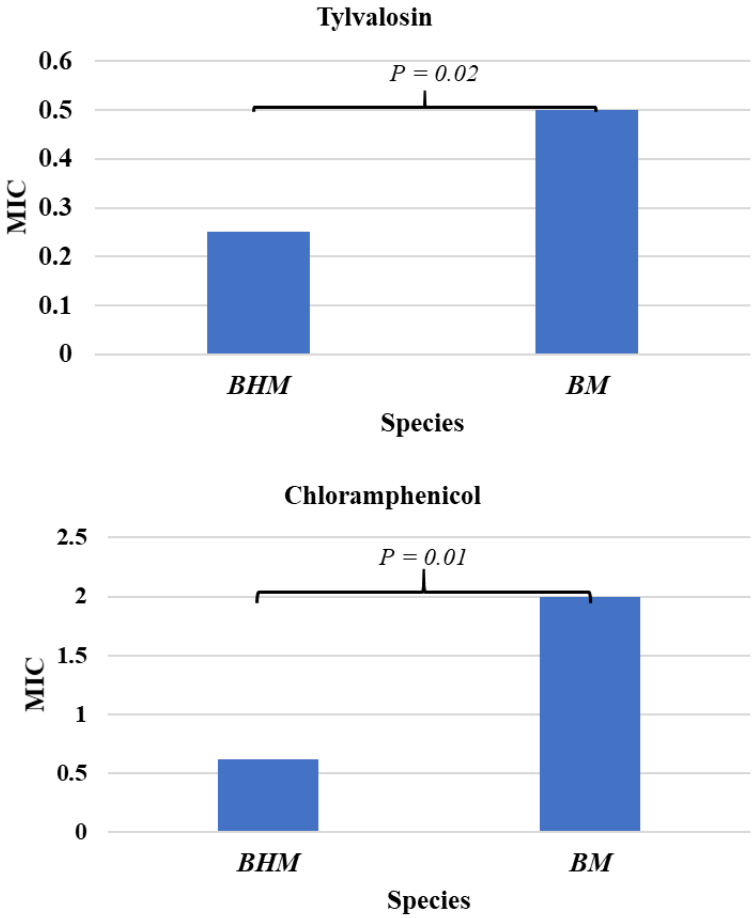
The results of multiple comparisons of median MICs between different species within production systems B, C, and E were presented in Figure 2.7-2.9. In each production system, significant differences between species were only observed for several antimicrobials (for production system B: tiamulin, valnemulin, ampicillin, chloramphenicol, and amoxicillin+ clavulanic acid; C: tylosin and chloramphenicol; and E: ampicillin and amoxicillin+ clavulanic acid). In production system B, the median MICs of *B. pilosicoli* was significantly higher than that of other species in all antimicrobials listed in Figure 2.7. In production system C, the median MICs (tylvalosin ($p= 0.02$) and chloramphenicol ($p = 0.01$) of *B. murdochii* was significantly higher than that of *B. hampsonii* (Figure 2.8). Among isolates from production system E, *B. hyodysenteriae* had significantly higher median MICs to ampicillin and amoxicillin + clavulanic acid compared to *B. pilosicoli* and *B. murdochii*; and the MICs of *B. murdochii* to ampicillin were significantly lower than that of *B. hyodysenteriae* ($p = 0.03$) and *B. pilosicoli* ($p = 0.03$) (Figure 2.9). Due to low number of isolates collected from each system, statistical comparisons between species in production systems A, D, F, G and H were not performed.

Figure 2.7 Pair wise comparison of median tiamulin, valnemulin, ampicillin, chloramphenicol and amoxicillin + clavulanic acid MICs between *Brachyspira* spp. from production system B.



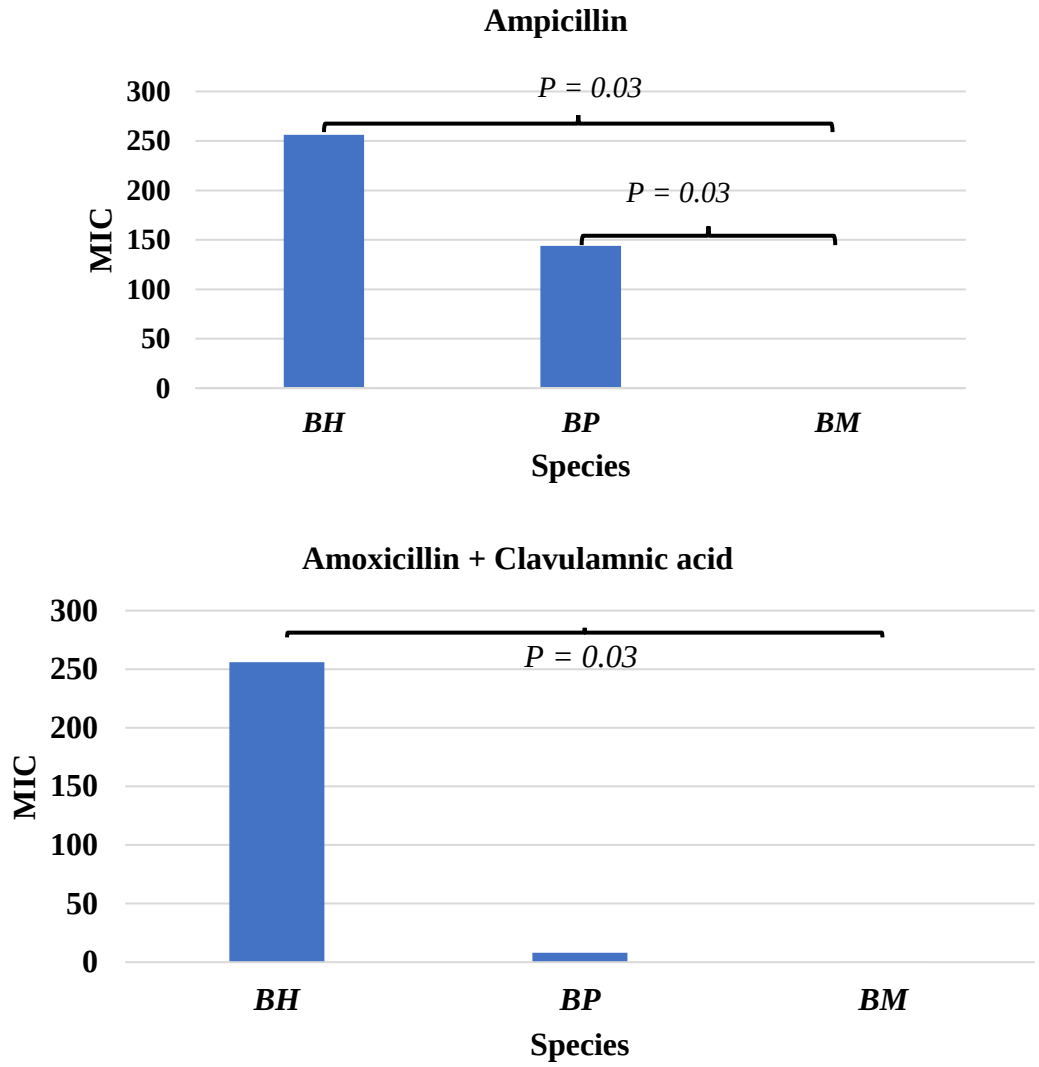
The figure shows a pairwise comparison of the median MICs of antimicrobials between species within the production system B. Bonferroni-adjusted p-values were included and when $P < 0.05$ we have considered the differences to be statistically significant. BH=*B. hyodysenteriae* (n=15), BHM=*B. hampsonii* (n=8), BP=*B. pilosicoli* (n=3), BM=*B. murdochii* (n=4), BI=*B. innocens* (n=6).

Figure 2.8 Pair wise comparison of median tylvalosin and chloramphenicol MICs between *Brachyspira* spp. from production system. C.



The figure shows a pairwise comparison of the median MICs of antimicrobials between species within the production system C. Bonferroni-adjusted p-values were included and when P < 0.05 we have considered the differences to be statistically significant. BHM=*B. hampsonii* (n=2), BM=*B. murdochii* (n=8).

Figure 2.9 Pair wise comparison of median ampicillin and amoxicillin + clavulanic acid MICs between *Brachyspira* spp. from production system E.



The figure shows a pairwise comparison of the median MICs of antimicrobials between species within the production system E. Bonferroni-adjusted p-values were included and when $P < 0.05$ we have considered the differences to be statistically significant. BH= *B. hyodysenteriae* (n=1), BP= *B. pilosicoli* (n=6), BM= *B. murdochii* (n=3).

2.5 Discussion

The current lack of standardized antimicrobial susceptibility tests for *Brachyspira* species is a serious diagnostic limitation that prevents the evidence-based application of the test for therapeutic guidance, and the detection of the emergence of resistance. The development of a standardized susceptibility test method is therefore crucial for the control of *Brachyspira*-associated disease and ensuring that the use of antimicrobials is based on evidence. Previous studies have failed to describe a standard protocol for conducting antimicrobial susceptibility testing for *Brachyspira*; including a standardized starting inoculum (Märit Karlsson, Oxberry, and Hampson 2002; Kulathunga and Rubin 2017; Stubberfield et al. 2020). The use of inocula varying by up to two orders of magnitude in previous investigations (from $1 \times 10^5 – 5 \times 10^5$ CFU/ml for broth dilution and $1 \times 10^4 – 1 \times 10^6$ CFU/spot for agar dilution) has been reported and likely affected the MICs observed in those studies (Egerva *et al.*, 2007; Kulathunga and Rubin, 2017). This inconsistency makes it impossible to reliably compare data between laboratories. In this study we determined that there is minimum inoculum size require to obtain a visible growth, and that observed MIC is also affected by the starting bacterial concentration. Although this is a well-recognized phenomenon in other bacterial taxa it has not been systematically investigated in *Brachyspira* spp. (Eley and Greenwood 1981; Borobio et al. 1986; Brown 1988; Egerva et al. 2007; Bidlas, Du, and Lambert 2008). The development of a standard curve defining the relationship between colony count (hemolysis forming unit) and the optical density for *B. hampsonii*, *B. hyodysenteriae* and *B. pilosicoli* was a critical first step. Optical density is a rapid method of determining the density of a bacterial culture which allows test inoculum to be standardized in susceptibility testing.

The results of this investigation confirmed that the starting inoculum was a limiting factor for the growth of *Brachyspira* spp. on both liquid and solid media. Furthermore, the results of

susceptibility testing indicated that consistent results (observed MIC between replicates) were achieved using the bacterial concentrations within the range of prescribed by the CLSI standard. For agar dilution a concentration of 2-4 × 10^5 CFU/spot was chosen in this investigation, standardizing our method using this concentration has the advantage of being familiar to clinical diagnosticians, which should facilitate the incorporation of this assay methodology into diagnostic workflows. In the current study, highly reproducible (80-100%) results were observed for most of the antimicrobials tested using our agar dilution method. This is in contrast to the poor reproducibility which has been reported for the broth micro-dilution test; uninterpretable results due to "skipped wells" are recognized when testing lincosamide and macrolides in *B. pilosicoli* (Mirajkar and Gebhart 2016; Stubberfield et al. 2020). Interestingly, a recent study aiming to validate the a broth microdilution test, also failed to standardize the starting inoculum (Stubberfield et al. 2020).

Consistent with previous studies, a wide variety of species including isolates which didn't cluster with recognized species were identified (Patterson et al. 2013; Rohde et al. 2019). The MIC distribution of bacterial populations can be described as homogenous, bi-modal, or multi-modal distributions (Boerlin and White 2013). The MIC distribution may provide clues to the mechanism of resistance acquisition. When bacteria acquire a resistance gene for example, there may be a distinct jump in the MIC indicated by a bi-modal MIC distribution in which wildtype and 'resistant' organisms form distinct populations. Conversely, step-wise MIC increases may be seen with the acquisition of successive resistance conferring mutations, as is observed with fluoroquinolone MICs following successive topoisomerase gene mutations (Boerlin and White 2013).

Anecdotally tiamulin is the most commonly used drug to treat *Brachyspira*-associated diseases in western Canada. In the Czech Republic, one study found that the MICs to tiamulin and valnemulin increased significantly between the periods 1997-98 and 1999-2001 among a collection of *B. hyodysenteriae* isolates (Lobova 2004). Similar findings were reported in a Japanese study among *B. hyodysenteriae* isolates between 1985 and 2009 (Ohya and Sueyoshi 2010). Although the lack of a standardized methodology prevents comparing MIC results between labs, these longitudinal studies were conducted within single laboratories, suggesting that pleuromutilin MICs are increasing among (Ohya and Sueyoshi 2010). In contrast, isolates originating from the United States were observed to have lower pleuromutilin MICs (Mirajkar, Davies, and Gebhart 2016). Our results suggest that perhaps not surprisingly, the situation in western Canada is more similar to the United States where *B. hyodysenteriae* and *B. hampsonii* isolates have very low MICs (≤ 1 µg/ml) to pleuromutilin drugs. One study from the United States used three interpretive criteria to described isolates with MICs >8 µg/ml as having decreased susceptibility or resistance to tiamulin (Mirajkar, Davies, and Gebhart 2016). In our study, 44% *B. pilosicoli* isolates had similarly high pleuromutilin MICs, possibly indicating resistance. Previous *in vitro* studies have demonstrated the tendency of *B. pilosicoli* to develop resistance to macrolides, lincosamides and pleuromutilins more rapidly than other *Brachyspira* species possibly explaining the MIC distribution of *B. pilosicoli* in our study (Mirajkar et al. 2015; Mirajkar, Davies, and Gebhart 2016). Moreover, *in vitro* studies have described cross-resistance between valnemulin and tiamulin in *B. pilosicoli* (Karlsson, Gunnarsson, and Franklin 2001). This phenomenon may explain the relatively similar MIC distributions we observed for both tiamulin and valnemulin in *B. pilosicoli*.

A recent study from the United States described high MICs to lincomycin (MIC_{50}=8 µg/ml and MIC_{90}=32 µg/ml) and tylosin (MIC_{50}>128 µg/ml and MIC_{90}>128 µg/ml) among *Brachyspira*

isolates (Mirajkar, Davies, and Gebhart 2016). Interestingly, in our study the MIC distributions observed were more heterogeneous, including isolates with both high and low MICs for both antimicrobials. This wide-ranging MIC distribution suggests that the isolates at the higher end of the MIC distribution were likely to have some acquired resistance to these drugs due to the selective pressure exerted on the spirochetes from the use of macrolide drugs, both as therapeutic agents and as a growth promoter in swine production. Previous studies have identified single nucleotide polymorphisms in the 23S ribosomal RNA gene and the ribosomal protein (L3), as well as the acquisition of the *lnu*C and *tva*A that are associated with resistance to protein synthesis inhibitors (Karlsson *et al.*, 1999; Pringle *et al.*, 2004; De Luca *et al.*, 2018; Card *et al.*, 2018). Those studies observed bimodal distribution of MICs to tylosin in both *B. hyodysenteriae* and *B. pilosicoli*, with isolates possessing 23S rRNA single nucleotide polymorphisms having higher MICs than those isolates without SNPs (Karlsson *et al.*, 2004; Giguere, 2013). In our study, *B. pilosicoli* had bimodal MIC distributions for tylosin and the highest MIC_{50} (>128 µg/ml) compared to other species. This observation suggests the emergence of a resistant population in the *B. pilosicoli*. It has previously been suggested that the strongly recombinant population structure and the substantial amount of genomic variation in *B. pilosicoli* may contribute to the emergence of antimicrobial resistance (Neo et al. 2013; Mirajkar, Davies, and Gebhart 2016).

Interestingly, MICs for tylvalosin tended to be elevated in isolates with high tylosin and lincomycin MICs (Table 2.9). Macrolides, lincosamides, and pleuromutilins bind to the peptidyl transferase center (PTC) of 23S rRNA of the 50S ribosome and prevent the peptide bond formation and thereby prevent the protein synthesis of bacteria (Karlsson et al. 2004). Nucleotide mutations or methylations which could occur in the highly conserved main loop of domain V in PTC has been shown to lead to resistance to macrolides, lincosamide, streptogramins and pleuromutilin

drugs (Karlsson et al. 1999). Those mutations were studied in depth for *Brachyspira* spp. and the relationship between these mutations and phenotypic susceptibility results has been documented (Jacoby 2005; Hidalgo et al. 2011). The common target of lincomycin, tylosin and tylvalosin (the 23S rRNA) may be responsible for the cross resistance observed in our study.

Although chloramphenicol is banned in food animals in Canada, it is commonly included in surveillance programs (Masterton 2008; Burch 2013). Because it is related to florfenicol, a drug that is used in swine industry, and the identification of elevated MICs for chloramphenicol, it is useful for the purposes of resistance surveillance (Masterton 2008; Burch 2013). The fluoroquinolones are used in both veterinary and human medicine, and resistance to these drugs can occur by target modifications, decreased permeability, efflux and target protection (Conrads, Citron, and Goldstein 2005; Jacoby 2005). There is evidence of intrinsic quinolone resistance among Gram-negative anaerobes, although it has not been determined if this is the case for *Brachyspira* spp. (Giguere and Dowling 2013). Most of the isolates tested in this study among all species had very high nalidixic acid MICs (>16 µg/ml), possibly suggesting intrinsic resistance.

High MICs to ampicillin and amoxicillin + clavulanic acid was observed in *B. hyodysenteriae*, *B. hampsonii* and *B. pilosicoli*. While β-lactams are not used to treat *Brachyspira*-associated infections, the use of penicillin to treat other infections in pigs may have contributed to the selection of resistance to these drugs in *Brachyspira* (Mortimer-Jones et al. 2008; Prescott 2013a; La et al. 2015). Previous studies found that *B. pilosicoli* with high β-lactam drugs MICs possessed the OXA-63 gene, a class D β-lactamase (Mortimer-Jones et al. 2008).

An individual production system includes multiple swine farms, which follow similar management practices including antimicrobial usage for disease control and prevention (Mirajkar, Davies, and

Gebhart 2016). We therefore expect to find similar antimicrobial susceptibility profiles of *Brachyspira* isolates collected within the same production system. In production systems B, C and E there were no significant differences observed for most of the antimicrobials tested. The median MICs of *B. pilosicoli* isolates were most frequently significantly higher than other species (Figure 2.7 and 2.9). The highly recombinant nature of *B. pilosicoli* may have facilitated the development of resistance (higher MICs) compared to other species (Neo et al. 2013; Mirajkar, Davies, and Gebhart 2016). Further, finding significant differences in the susceptibility of different species within a production system emphasizes the need for diagnostic tests to identify the species present before medicating affected animals. Further, identification of isolates with higher MICs for commonly used antimicrobials to treat swine dysentery in western Canada is important to control the rapid spread of resistant strains as resistance can be disseminate within production system through affected pigs, fomites and vectors.

2.6 Conclusions

In this study we developed a standard agar dilution method with high reproducibility. This method reduces the variability of the susceptibility test results and will allow results to be compared between laboratories. Since the emergence of the species, *B. hampsonii,* in Canada no surveillance study has been conducted. For the first time the antimicrobial susceptibilities of western Canadian *Brachyspira* isolates have been described. Low pleuromutilin MICs were found for *B. hampsonii* and *B. hyodysenteriae* isolates while few *B. pilosicoli* isolates with higher MIC (>8 µg/ml) were found. All isolates demonstrated heterogeneous distributions to other antimicrobials (macrolide, lincosamide, quinolones, tetracycline and β-lactam drugs) tested, indicating reduced susceptibilities and the emergence of resistance. Antimicrobial susceptibilities varied by species suggesting that swine veterinarians should perform diagnostic tests to confirm the identification of

Brachyspira species and antimicrobial susceptibility to assist with selecting evidence-based therapeutics. There is a need for continuous monitoring and susceptibility testing to identify any emerging resistance. Finally, the feasibility of implementing this assay into routine diagnostic practice could be improved by refining the process and reducing the technical time required to perform this test.

2.7 Transition statement

In the previous study we determined that the consistent starting inoculum size is critical for generating reproducible MICs, a starting inoculum size (1-2 × 10^8 CFU/ml) was selected to give consistent and reproducible results. The antimicrobial susceptibility of a collection of western Canadian isolates were tested with this method and the results indicated the presence of acquired resistance to some of the most commonly used antimicrobials for treating *Brachyspira*-associated disease in pigs. Although agar dilution is described as the gold standard for susceptibility testing there are many challenges with its implementation; it is labor-intensive, and time consuming. Antimicrobial susceptibility tests are not currently included in the current diagnostic workflow to identify *Brachyspira* spp. at the WCVM. It is clear that an economic analysis was needed to determine the most efficient diagnostic strategy for *Brachyspira* including the addition of agar-dilution based susceptibility testing method, would be useful for improving current laboratory practices.

3. ECONOMIC ANALYSIS OF DIAGNOSTIC WORKFLOWS FOR THE IDENTIFICATION AND CHARACTERIZATION OF *BRACHYSPIRA*

Kulathunga D.G.R.S, Fernando C., Harding J.C.S, Rubin J.E. and Hill J.E.

Author contributions: Conceived and designed experiment: DGRSK, JCSH, JER, CF, JEH. Performed the experiment DGRSK, JER. Analysed the Data: DGRSK, JER. Wrote the paper: DGRSK, JER

3.1 Abstract

Swine dysentery affects grow-finisher pigs leading to mortality, sub-optimal performance, and economic losses. In western Canada, swine dysentery in pigs has re-emerged since the late 2000s. When selecting diagnostic tests in agricultural settings it is important to maximize diagnostic value while minimizing costs. Despite advances in diagnostic technologies, in western Canada susceptibility testing is not performed on *Brachyspira* isolates, and therapeutic selection is most often empiric. The purpose of this study was to determine the cost for all the diagnostic tests available for *Brachyspira* at Western College of Veterinary Medicine with the inclusion of a feasible agar dilution test to the current diagnostic workflow, retrospectively determine the measures of diagnostic test accuracy and finally incorporate all this data to build decision trees to guide diagnosticians to select the most efficient diagnostic strategy to identify and characterize *Brachyspira*. In susceptibility testing quadrant plates were used to minimize media, consumables, antimicrobials, and technical time needed to create the agar dilution plates. Tiamulin (0.25-1 μg/ml), valnemulin (0.25-1 μg/ml), tylosin (1-16 μg/ml), tylvalosin (1-16 μg/ml), and salinomycin (0.25-1 μg/ml) were included in the agar dilution test and five concentrations were tested from each antimicrobial drug. To determine the labour and consumable cost for each diagnostic test, a total of 5 batches of field samples including 20 individual specimens (1-7 samples per batch) submitted between December 2017 and March 2018 for the identification of *Brachyspira* spp. were evaluated. Analyses included species-specific quantitative PCR, conventional PCR identifying a genus-specific target followed by amplicon sequencing, conventional culture on solid and liquid media followed by agar-dilution susceptibility testing. For each step of the diagnostic process, the consumables utilized, and technical time required to perform each assay were recorded and the per sample cost/time was determined by dividing the total cost/time by the number of samples tested

in each batch. A retrospective study was performed to determine the clinical sensitivity and specificity, positive and negative predictive values of each diagnostic test using the test results recorded in an in-house *Brachyspira* database of diagnostic cases (n=500) submitted from 2013 to 2018. The final diagnosis, determined by a swine veterinarian (JCSH) and co-director of the *Brachyspira* diagnostic service at Western College of Veterinary Medicine based on overall results of all diagnostic tests, was considered the gold standard for the purposes of determining the clinical sensitivity and specificity. These results were incorporated into decision trees by associating the consumable costs, technical time and experimental time (the days spent from sample receiving to confirmatory diagnosis) for each diagnostic test with existing workflows, each with a specific diagnostic objective. For the identification of any *Brachyspira* spp. in a given sample, culture had the lowest clinical sensitivity (62.3-64.6%) which might be explained by the fastidious nature of the organism and long transportation time of samples. By contrast, identification of *B. hyodysenteriae* or *B. hampsonii* performed by qPCRs had high clinical sensitivities (100%) but fail to detect other less pathogenic *Brachyspira*. This study generated the data needed to develop more efficient diagnostic workflows which maximized the clinical sensitivity while minimizing the cost thereby providing an important decision-making tool for both diagnosticians and veterinarians.

3.2 Introduction

Swine dysentery (SD) is a severe contagious disease characterized by severe muco-hemorrhagic colitis associated with substantial economic losses due to mortality and poor performance of surviving animals (Burrough 2017; Alvarez-Ordóñez et al. 2013). In North America, the incidence of *Brachyspira*-associated disease decreased in the 1990s, before re-emerging in the late 2000's

possibly due to the development of antimicrobial resistance (Harding et al. 2010; Chander et al. 2012; Alvarez-Ordóñez et al. 2013; Burrough 2017).

There has been increasing interest in susceptibility testing for *Brachyspira* spp.; while one study from the United States demonstrated that *Brachyspira* species had low MIC's for tiamulin and valnemulin there is a paucity of Canadian data (Mirajkar, Davies, and Gebhart 2016). In previous studies we demonstrated that while *B. hyodysenteriae* and *B. hampsonii* isolates from western Canada were profoundly susceptible to pleuromutilin, 44% of *B. pilosicoli* isolates had high MICs possibly indicating the emergence of resistance. Conversely, highly variable MICs were observed amongst all species for tylosin, tylvalosin, and lincomycin amongst all species.

Both antemortem and post-mortem collected samples can be used in the diagnosis of *Brachyspira*-associated diarrhea by a variety of culture-based or molecular test methods. However, there has not been a systematic evaluation of the advantages and disadvantages of various analytical strategies. Affected intestinal tissue, fecal samples and/or rectal swabs are typically submitted from animals suspected to have SD. Pathological lesions, both gross and microscopic lesions in colonic tissues have been used in the diagnosis of this disease (Costa et al. 2014; Burrough 2017). Culture for *Brachyspira* is typically done on selective media which contains antimicrobials that prevent the growth of other bacteria and facilitate the growth of *Brachyspira* (Songer, Kinyon and Harris, 1976; Jenkinson and Wingar, 1981). Some laboratories use biochemical tests (e.g., indole, hippurate hydrolysis, α-glucosidase and β-glucosidase) for the identification of *Brachyspira* spp., although atypical phenotypes and variable results are possible limitations to these assays (Hampson 2012; Perez et al. 2016). Molecular techniques (PCR and DNA sequencing based assays) have been increasingly used for the identification and further characterization of

Brachyspira spp. (Rohde, Rothkamp, and Gerlach 2002; Bellgard et al. 2009; Fellstrom 2011; Mirajkar et al. 2015; Rohde et al. 2019).

In the late 2000's, a novel species called B. hampsonii emerged as the most common cause OF swine dysentery in western Canada (Harding *et al.*, 2010; Chander *et al.*, 2012). Three genomovars of *B. hampsonii* have since been reported (Mirajkar et al. 2015). The *Brachyspira* research group at the Western College of Veterinary Medicine (WCVM), University of Saskatchewan have established improved diagnostic tools for the identification of *Brachyspira* spp., including quantification of genomovar I and II of *B. hampsonii* by RT-PCR (Harding et al. 2010; Rubin et al. 2013; Costa et al. 2016). However, there are still many challenges that producers and veterinarians face in dealing with outbreaks of bloody diarrhea in pigs, such as a lack of therapeutic guidance, inefficient application of resources and lack of knowledge on realistic costs for diagnostic testing.

Brachyspira is a fastidious aero-tolerant anaerobic organism which does not form colonies on solid media (Hampson 2012). Therefore, unlike colony forming organisms, viable growth of *Brachyspira* is indirectly assessed by the production of a hemolytic zone on solid media. Furthermore, unlike other anaerobic organisms, there are no standard guidelines to perform antimicrobial susceptibility tests and no interpretative criteria or clinical breakpoints for *Brachyspira* spp. (CLSI 2013, 2012, 2016). The pleuromutilins, macrolide/lincosamides are the most commonly used antibiotics to treat, control or prevent infections caused by *Brachyspira* spp. (Karlsson et al. 2003; Aarestrup, Oliver, and Burch 2008). However, the availability of effective treatment options varies between countries as the result of rules and regulations implemented in countries regarding antimicrobial use (Kulathunga and Rubin 2017). The pleuromutilins are currently the mainstay of therapy in Canada and Europe for treating *Brachyspira*-associated

disease in pigs. Anecdotal information, and a limited number of studies have described the emergence of resistance among *Brachyspira* spp. to some of the most commonly used antibiotic agents for treating swine dysentery. *B. hyodysenteriae* isolates with decreased antibiotic susceptibility to tiamulin and valnemulin have reported in Australia, Japan, Sweden, and Germany (Molnar 1996; Karlsson et al. 2003; Lobova 2004). Decreased susceptibility to tiamulin has also been identified in *B. pilosicoli* (Pringle, Landen, and Franklin 2006). However, there are no published data on the antimicrobial susceptibility of Canadian *Brachyspira* isolates including *B. hampsonii*. Consequently, due to the lack of antimicrobial susceptibility testing in the routine diagnostic workflows for *Brachyspira* in Canada, veterinarians rely on empirical antimicrobial treatment without laboratory guidance. In our previous study we standardized an agar dilution method, which was laborious due to the time required to prepare agar plates with different concentrations of each antimicrobial agent was tested. Therefore, it was important to modify the agar dilution method to make it a more feasible test which can be adopted by any diagnostic laboratory.

Regardless of the diagnostic objective, the diagnostic laboratory at the WCVM performs both culture-based and PCR-based (conventional PCR, qPCRs, and sequencing) testing for the identification of *Brachyspira* spp. in samples. However, the clinical sensitivities and specificities of these diagnostic assays have not been determined. Furthermore, the diagnostic targets of certain assays are such that certain species are not detected (e.g., no qPCR specific to *B. murdochii*). Therefore, it is often necessary to run multiple assays in parallel on all samples before making a final diagnosis, a process which is expensive and lengthy. The expense and lack of a timely result is a barrier to sample submission and ultimately the laboratory data required for evidence-based therapy; however, these costs have not been systematically evaluated. Determining the reagent

cost and technical time for various testing algorithms would allow diagnosticians to optimize their procedures while making their service more affordable to the end users of the data. This study was designed to perform an economic analysis and create a decision tree to guide diagnosticians to select the most cost-effective strategy to determine the antimicrobial susceptibility profiles of clinical *Brachyspira* isolates utilizing a modified agar dilution method.

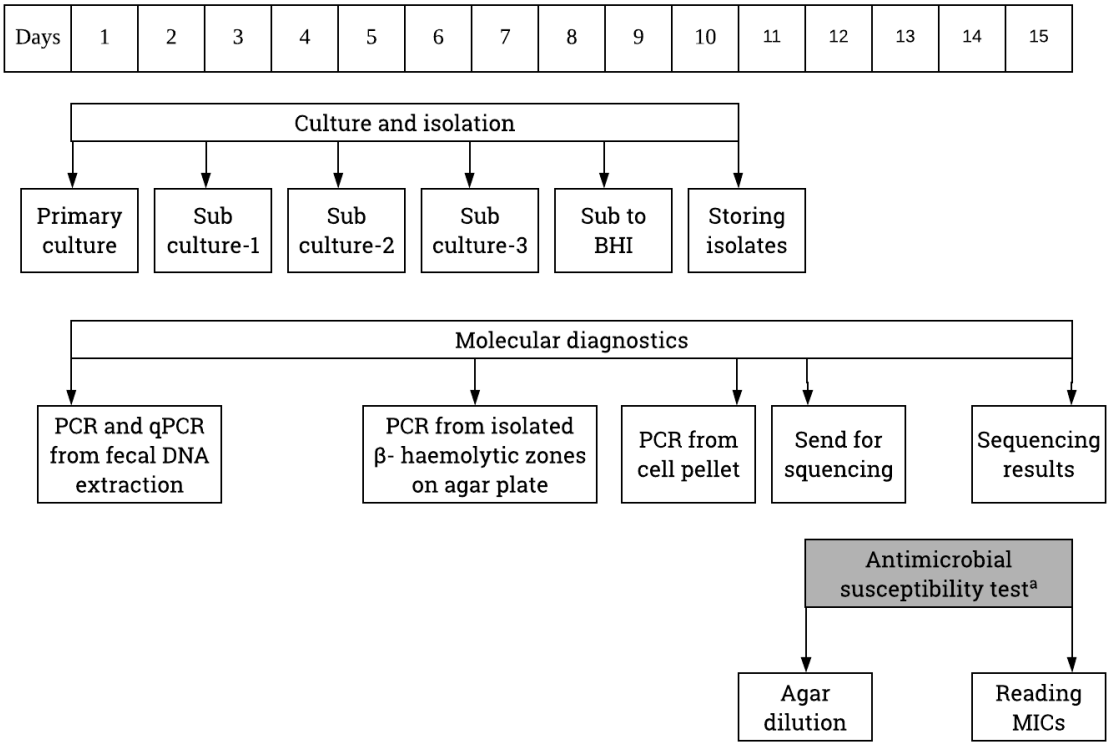
3.1 Materials and methods

3.1.1 Economic analysis

3.1.1.1 Sample selection

Between December 2017 and March 2018, 5 batches of samples including 20 individual fecal samples (1-7 samples per batch) were included. Submissions were provided by western Canadian veterinarians who provided samples from clinical cases of diarrhea. All samples were processed using a workflow existing since 2011 with the addition of antimicrobial susceptibility testing, (Figure 3.1) the method developed and described in Chapter 2. For each step, the consumables utilized, and the technical time required to perform each assay were recorded. A master price list was prepared according to the 2017-2018 invoices and overall cost for consumables were calculated. For each batch of samples, the per sample cost/time was determined by dividing the total cost/time by the number of samples tested. Analytical steps were grouped into three categories: culture and isolation, molecular diagnostics, and antimicrobial susceptibility testing.

Figure 3. 1 Timeline of diagnostic workflow.



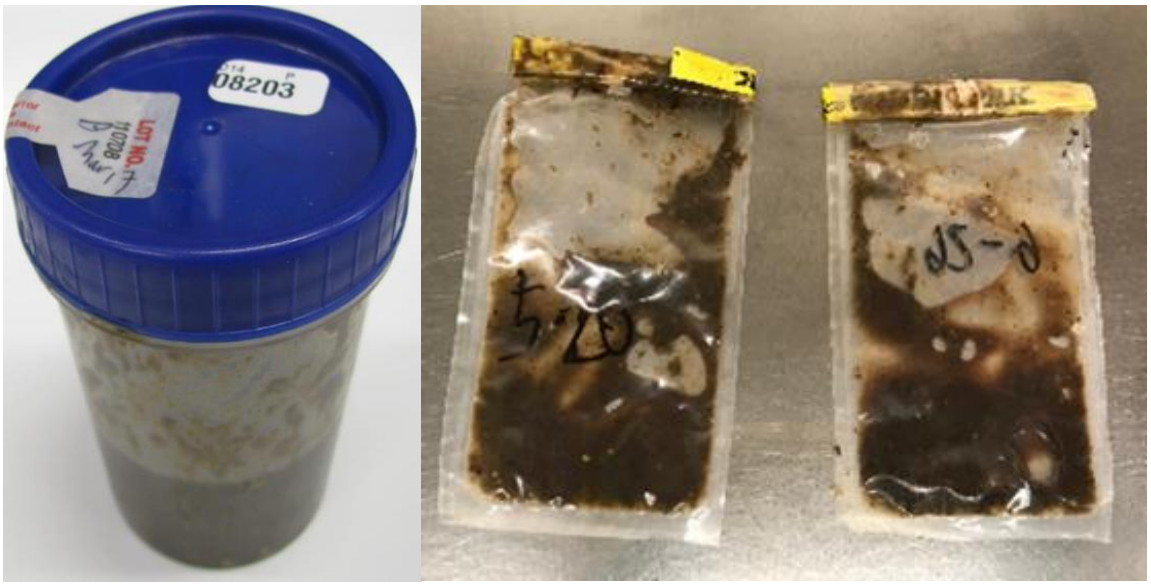
[a]Antimicrobial susceptibility testing is not included in the current process at the diagnostic laboratory of the Western College of Veterinary Medicine, University of Saskatchewan but has been incorporated into this study. The current diagnostic workflow includes only primary isolation and molecular diagnostic methods. BHI = brain heart infusion broth, MIC = minimum inhibitory concentration

3.1.1.2 Culture and isolation

Culture was performed on BJ agar, a selective media with trypticase soy agar, pig feces extract, spiramycin, rifampin, vancomycin, spectinomycin, colistin, and 5% sheep's blood (Kunkle and Kinyon 1988). For primary culture, fecal matter was taken from the homogenized sample (Figure 3.2) and inoculated on BJ agar plates using the quadrant streaking method (4 streak method). Thereafter, plates were incubated at 42°C for 48 hours in an anaerobic jar (Anerogen TM 2.5 L, Thermo Scientific Oxide Sachet) (Figure 3.3). Following incubation, plates were inspected for the presence of β-hemolysis indicating the growth of *Brachyspira*. When hemolysis was observed 1-2 mm^3 volume of agar was scraped from an isolated zone of hemolysis using a sterile bacteriological loop, the agar was macerated and then streaked it out on a sterile BJ plate. These plates were then similarly incubated at 42°C for 48 hours and visually inspected.

If no β-haemolysis was observed, BJ agar plates were re-incubated for up to 10 days, checking every 48 hours, before samples were reported as Brachyspira negative. For each sample where *Brachyspira* grew, a total of 2-3 subcultures were performed to ensure that a pure culture was obtained. Subsequently, approximately 2 cm^3 of agar was sliced from an isolated β-haemolytic zone and transferred into a vial containing10 ml of brain heart infusion broth + 1% glucose + fetal calf serum (10% v:v) (BHIS) and incubated anaerobically at 39°C for 24 hrs with stirring (Figure 3.4). For broth cultures where turbidity was observed, a Gram-stain was made to ensure the presence of spirochetes and to rule out other bacterial contaminants. Finally, around 1-1.5 ml of each broth culture were aliquoted into 2 ml micro-centrifuge tubes and pelleted by centrifugation for 10 minutes at 11200 g. The supernatant was then removed, and the pellet was used for PCR based identification, as described below.

Figure 3. 2 Fecal samples submitted to be analyzed for the presence of *Brachyspira* spp.



Fecal samples were submitted in labeled sterile containers with screwcap or sealed bags.

Figure 3. 3 Materials used for anaerobic incubation of BJ agar plates.

(1) BJ agar plates, (2) indicator strips (Fisher Scientific, Catalog No: B71051), (3) anaerobic jar, (4) anerobic gas pack (Anerogen TM 2.5 L, Thermo Scientific Oxide)

Figure 3. 4 Broth culture of *Brachyspira* spp.

(1) Broth culture vials incubating anaerobically in anaerobic jars at 39°C (2) The growth (turbidity) of *Brachyspira* spp. in BHI + 1% glucose + 10% fetal calf serum media after incubation.

Another 1 ml of broth culture was allocated to new BHIS vials and incubated anaerobically for 24-48 hrs to prepare a fresh inoculum for susceptibility testing.

3.1.1.3 Molecular diagnostic tests

DNA extraction were made from fecal extracts using the QIAMP Fast DNA Stool Mini Kit (Qiagen, Toronto, Canada). The DNA extracts were used as templates for SYBR Green real-time PCR to identify *B. pilosicoli*, *B. hyodysenteriae*, *B. hampsonii* genomovar I and genomovar II, and genus-specific PCR (fecal *nox* PCR) which targeted the *nox* gene (Rohde, Rothkamp, and Gerlach 2002). Another PCR (culture *nox* PCR) was performed from isolated hemolytic zones observed on subculture plates to confirm the presence of *Brachyspira* spp. DNA was also extracted from pure culture cell pellets; a commercial kit (Qiagen blood and Tissue kit, Qiagen, Toronto, Canada) and PCR was performed targeting the *nox* gene (isolate *nox* PCR). All PCR (fecal *nox* PCR, culture *nox* PCR or isolate *nox* PCR) positive samples were sequenced. Amplicons were purified (EZ-10 Spin Column PCR Product Puri Kit, Bio Basic, Ontario, Canada) and sent to a commercial service for sequencing (Macrogen Inc., Korea) to allow species identification.

3.1.1.3 Antimicrobial susceptibility tests

Five antimicrobials (tiamulin, valnemulin, tylosin, tylvalosin and salinomycin) selected to reflect those compounds of greatest interest to local swine veterinarians were included. Antimicrobial suspensions were prepared and stored at -80°C for use in susceptibility testing. When preparing media, stock solutions were diluted to yield following final concentrations in agar of: tiamulin (0.25-1 µg/ml), valnemulin 0.25-1 µg/ml), tylosin (1-16 µg/ml), tylvalosin (1-16 µg/ml), and salinomycin (0.25-1 µg/ml). Media were prepared in quadrant plates (Fisher Scientific, Ottawa, Ontario). For each quadrant, trypticase soy (TS) agar (4.5 ml), ovine blood (0.25 ml) and the

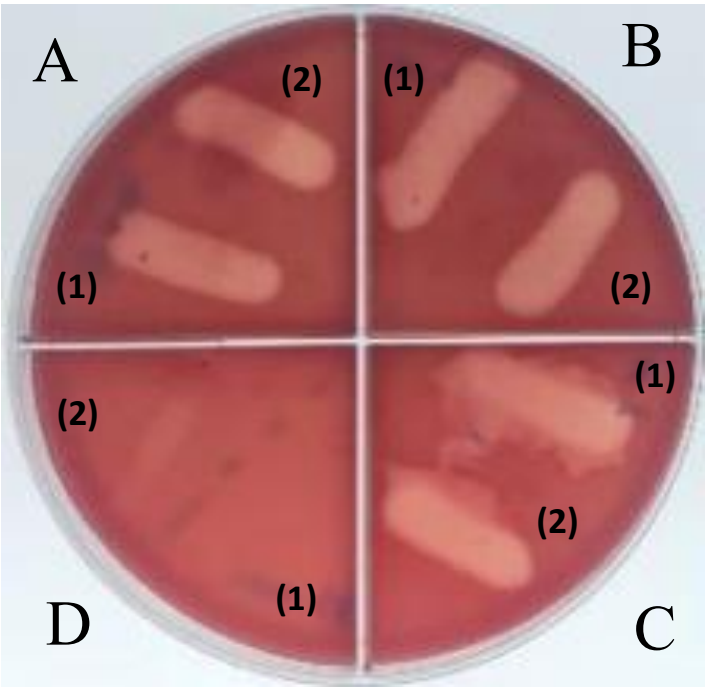
antimicrobial drug suspension (0.25 ml) were aliquoted. Briefly, TS agar (4.5 ml) containing vials were incubated in a water bath (100°C) until the agar melted, tubes were then transferred into a 50-55°C water bath to cool prior to adding antimicrobial suspensions and blood. Following the addition of antimicrobials and blood, the agar was poured to each quadrant and allowed to solidify in a biosafety cabinet; susceptibility testing was done the same day the media was prepared. Drug free control plates were also prepared. BHI broth cultures grown anaerobically for 24-48 hrs and the optical density (OD) was measured using a spectrophotometer as described in Chapter 2. The OD was adjusted using BHI blank solution to make a bacterial culture with inoculum concentration of 1-2 × 10^8 CFU/ml. From that broth culture, 2µl was inoculated on to the quadrant plates (Figure 3.5), allowed to dry for up to 10-15 minutes before inverting and placing in an anerobic jar for incubation. The inoculated plates were incubated anaerobically for 48 hrs and minimum inhibitory concentrations (MICs) were recorded.

3.1.2 Post-hoc analysis of diagnostic test effectiveness and accuracy

3.1.2.1 Cases selection for the retrospective study

From 2009 through 2018, diagnostic test results and sample metadata for over 1,000 cases submitted for *Brachyspira* testing at WCVM have been recorded in an in-house *Brachyspira* diagnostic database. Available metadata included the identification number, submission date, the number of samples submitted per batch, the production system, the farm ID and the province, clinical signs of affected animals, and results of all *Brachyspira* related culture and molecular diagnostic test and final diagnosis.

Figure 3. 5 Quarter plate including tylosin at 4 different concentrations.



Each quadrant of the plate contained a different concentration of tylosin: A–1µg/ml, B–2µg/ml, C–4µg/ml and D–8µg/ml. *Brachyspira* growth, indicated by hemolytic zones. Two isolates (1 and 2) were able to inoculate in one quadrant. The isolate (1) grew in panels A, B and C but not in D, indicating that this isolate had an MIC of 8 µg/ml while isolate (2) grew in all panels and MIC > 8µg/ml.

Out of 1000 cases submissions, 500 submissions submitted between 2013-18 with a complete set of diagnostic test results (culture, conventional PCRs (target NADH oxidase gene), sequencing and qPCRs for *B. pilosicoli*, *B. hyodysenteriae* and *B. hampsonii*) were selected for inclusion (Table 3.1) (Rubin et al. 2013).All tests were done in parallel, and results were combined to make the final diagnosis. The final diagnosis, determined by a swine veterinarian (JCSH) and co-director of the *Brachyspira* diagnostic service at WCVM based on overall results of all diagnostic tests, was considered the gold standard for the purposes of determining the clinical sensitivity and specificity of individual diagnostic test (Table 3.2).

3.1.2.2 Statistical analysis

Sensitivity, specificity, positive and negative predictive values for different diagnostic tests were calculated using previously defined approaches (Altman 1990; Campbell, Machin, and Walters 2010; Fletcher 2012) (Figure 3.6). The clinical sensitivity is to identify true positives as defined by the final clinical diagnosis whereas specificity is the ability of the test to correctly identify the absence of an organism compared to the final clinical diagnosis (Smith and Slenning 2000; Shaikh 2011). Positive predictive value is the probability that a sample which gives positive test result is truly positive for *Brachyspira* and while negative predictive value is the probability that a sample which gives a negative test result is truly negative for *Brachyspira* (Trevethan 2018).

Table 3.1 Number of cases from database used in post-hoc analyses.

Year	Number of cases
2013	106
2014	116
2015	136
2016	58
2017	41
2018	43
Total number of cases	500

The table includes the number of cases based on their submitted year to the diagnostic laboratory. For these (n=500) cases culture and molecular tests were performed at the time of submission and the results were recorded in an in-house database. These data were used in the post-hoc analysis to test clinical sensitivity, specificity and positive and negative predictivity.

Table 3.2 Number of clinical cases with different final diagnosis recorded in an in-house database.

Final diagnosis	**Number of clinical cases (n=500)**
Any *Brachyspira* spp.	271
B. hyodysenteriae	70
B. hampsonii	115
B. pilosicoli	60
Negative for all *Brachyspira* spp.	229

The final diagnosis determined by a swine veterinarian (JCSH) and co-director of the *Brachyspira* diagnostic service at WCVM based on overall results of all diagnostic tests.

Figure 3. 6 A template of a 2 ×2 table.

		Final diagnosis for *Brachyspira* spp. according to the gold standard		
		Positive for *Brachyspira* spp.	Negative for *Brachyspira* spp.	
Diagnostic test	Positive	True-Positives (TP)	False-Positives (FP)	PPV: Positive Predictive Value = TP/ (TP + FP)
	Negative	False-Negatives (FN)	True-Negatives (TN)	NPV: Negative Predictive Value = TN/ (TN + FN)
		Sensitivity = TP / (TP + FN)	Specificity = TN / (TN + FP)	

The 2×2 tables were used to compare results (positive or negative) of each diagnostic test with the final diagnosis (gold standard), which was determined by a swine veterinarian (JCSH) and co-director of the *Brachyspira* diagnostic service at WCVM based on overall results of all diagnostic tests. The clinical sensitivity, clinical specificity, positive predictive value and negative predictive value of individual diagnostic test were determined.

3.1.3 Development of workflow decision trees

Decision trees were developed to display the relative cost of laboratory materials, technical time, laboratory time and the sensitivity and specificity along four hypothetical diagnostic objectives: (1) identification of *B. pilosicoli, B. hampsonii* (genomovar specific ID) or *B. hyodysenteriae* in a sample; (2) identification of *B. pilosicoli, B. hampsonii* genomovars or *B. hyodysenteriae* spp. in the sample in addition to antimicrobial susceptibility testing; (3) identification of any *Brachyspira* spp. present in the sample; (4) identification of any *Brachyspira* spp. in the sample in addition to antimicrobial susceptibility testing. For each branch of the decision tree, days from submission to reporting of final results (laboratory time), total material (consumable) costs and technical time and the numbers of true and false-positives and true and false-negatives of each diagnostic test, which were calculated based on data from the in-house database were indicated.

3.2 Results

3.2.1 The overall cost and technical time for conducting all tests

The starting number of fecal samples per submission varied from 3-7. For each diagnostic step and each individual batch of samples, the material cost (CAD) and technical time (minutes) per sample were calculated per sample (Table 3.3). Per sample material cost and technical time decreased as sample batch size increased. However, the number of samples tested with each diagnostic test varied, because *Brachyspira* spp. did not always grow in broth or liquid media so downstream analyses were not performed (Table 3.3). The inclusion of susceptibility testing increased the per sample material cost and technical time by $8.81 to $20.33 CAD and 17.5 to 84.3 minutes respectively over culture and molecular diagnostic tests (Table 3.3).

Table 3. 3 Cost and technical time calculations per batch of samples tested.

Batch No (Starting fecal sample number)	Analysis (No of samples, cost ($) per sample and the time (min) for per sample	*Brachyspira* diagnostic step categories												
		Culture							Molecular					AST
		P	S-1	S-2	S-3	BHI	STO	EX	PCR[a]	qPCR[b]	Plate[c]	Isolate[d]	SQ[e]	Agar
1 (6)	No: samples	6	6	6	6	6	5	6	6	6	6	5	12	3
	$/sample	2.66	2.55	2.57	2.57	3.36	0.59	6.86	2.16	4.24	2.16	6.73	12.67	11.05
	min/sample	3.3	2.5	2.5	2.5	2.8	4	22.8	8.3	16.8	9.1	16	18.8	27.5
2 (3)	No: samples	3	1	1	1	1	1	3	3	3	1	1	5	1
	$/sample	3.82	8.41	2.57	2.57	9.19	0.80	7.22	3.09	6.57	6.93	10.58	12.85	20.33
	min/sample	4	5	2.5	2.5	5	15	30	11.6	28.3	40	52	16.6	84.3
3 (4)	No: samples	4	4	4	4	4	3	4	4	4	4	3	11	3
	$/sample	3.24	3.16	3.16	3.3	3.94	0.63	7.11	2.71	4.38	2.65	7.82	12.74	12.36
	min/sample	3.7	4	3.75	3.65	3.7	5	26.2	9.75	30	15.7	18.2	17	26
4 (7)	No: samples	7	4	4	4	4	4	7	7	7	4	4	12	4
	$/sample	2.49	3.10	3.16	3.16	3.7	0.60	6.80	1.92	3.04	2.69	7.16	12.62	8.81
	min/sample	2.1	3.5	3.5	3.4	4.0	5.1	20	6.4	13.5	16.5	17	18	17.5
5 (5)	No: samples	5	4	4	4	4	4	5	5	5	5	4	12	4
	$/sample	2.89	3.2	3.16	3.16	3.16	0.60	6.91	2.38	3.24	2.69	7.07	12.70	8.93
	min/sample	3.0	3.5	3.75	4.1	3.8	5.0	25	9	18	15	16.8	19	18
Cost per sample	Mean	3.02	4.08	2.92	2.95	4.67	0.64	6.98	2.45	4.29	3.42	7.87	12.72	12.30
	Range	2.49-3.82	2.55-8.41	2.57-3.16	2.57-3.30	3.16-9.19	0.59-0.80	6.80-7.22	1.92-3.09	3.04-6.57	2.16-6.93	6.73-10.58	12.62-12.85	8.81-20.33
Time per sample	Mean	3.23	3.7	3.2	3.23	3.86	6.8	24.8	9.01	21.3	19.3	24	17.9	34.7
	Range	2.1-4	2.5-5	2.5-3.7	2.5-4.1	2.8-5	4-15	20-30	8.3-11.6	13.5-30	9.1-40	16-52	16.6-19	17.5-84.3

[a]PCR= PCR which target the gene *nox* was done from the fecal DNA extraction (Rohde, Rothkamp, and Gerlach 2002).
[b]the cost per sample includes material cost for all qPCRs that target *B. hyodysenteriae*, *B. pilosicoli* and both clades (I and II) of *B. hampsonii*
[c]a PCR target the gene *nox* was done from the isolated hemolytic zones observed on sub-culture plates of BJ.
[d]once isolates were collected in BHI broth cultures DNA was extracted and PCR (*nox*) was done and the cost for all these steps were included.
P= primary culture, S-1, S-2 and S-3= sub-cultures, STO = after isolation isolates can be stored at -80°C freezer (optional step), EX = DNA extraction, BHI= brain heart infusion broth and AST= antimicrobial susceptibility test, Agar = agar dilution test.
Primary and sub-cultures were done on BJ agar. The table indicated the individual material cost (CAD) and technical time (minutes) per sample calculated for each batch for different diagnostic steps involved in *Brachyspira* diagnosis. Even in the same batch, the number of samples tested with different tests were varied as no *Brachyspira* spp. were grown on media.

3.2.2 Sensitivity, specificity of individual and bundled *Brachyspira* assays and workflow development

The clinical sensitivity and specificity of each test, and bundle of tests for predicting the final diagnosis was determined (Table 3.4 and Figure 3.7 -3.10). The clinical sensitivity of individual tests used for diagnosis of any *Brachyspira* spp. (culture, culture *nox* PCR, fecal *nox* PCR, and qPCRs) ranged from 62.3–79.9%, whereas the specificity ranged from 88.4-100% (Table 3.4). Compared to doing all qPCRs (65.2%), fecal *nox* PCR had higher clinical sensitivity (79.9%), when the diagnostic target was any *Brachyspira* spp. Culture *nox* PCR had the lowest clinical sensitivity (62.3%) compared with other diagnostic tests. Moreover, performing all test (all qPCRs + fecal *nox* + culture *nox*) had the highest (100%) clinical sensitivity and lowest clinical specificity (85.3%) compared to other test options available (Figure 3.7).

If the diagnostic objective was to identify agents of swine dysentery (*B. hyodysenteriae* or *B hampsonii*), species specific qPCR assays had a stand alone clinical sensitivity of 97.1 and 95.7 % and clinical specificity of 99.3% respectively (Table 3.4). Culture *nox* PCR was the test with the lowest clinical sensitivity (52. 9% and 21.7% respectively) compared to other individual tests or test bundles. For the identification of both *B. hyodysenteriae* and *B. hampsonii* the final test option (qPCRs + fecal *nox* PCR + culture *nox* PCR) had the highest clinical sensitivity (100%) and negative predictive value (100%) compared to other test bundles. When compared with the individual qPCRs for *B. hyodysenteriae* and *B. hampsonii*, the qPCR of *B. pilosicoli* had the lowest clinical sensitivity (72.5%). Surprisingly, when all tests were included in a bundle, the clinical sensitivity of diagnosing *B. pilosicoli* only reached 52.5%, much lower than for either *B. hyodysenteriae* or *B. hampsonii* (Table 3.4 and Figures 3.7-3.10).

Table 3. 4 Clinical sensitivity, specificity and ability of diagnostic tests to predict the final outcome.

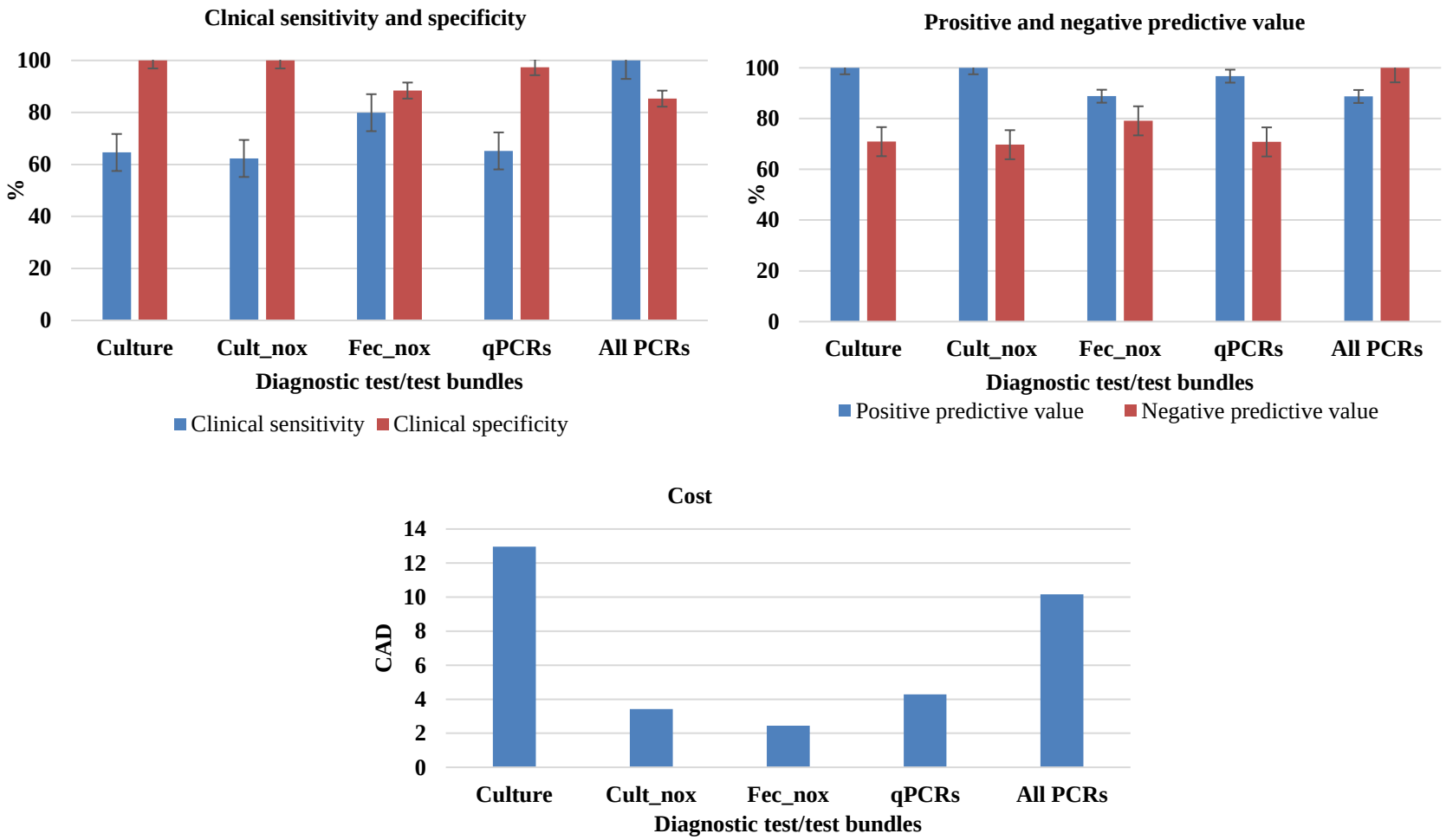
Diagnostic target	Test bundles	Clinical sensitivity (CI 95%)	Clinical specificity (CI 95%)	Predictive value (CI 95%)		Cost (CAD)[a]
				Positive	Negative	
Any *Brachyspira*	Culture	64.6 (64.4 -64.7)	100	100	70.9 (70.8-71.1)	12.97
	Culture *nox*[a]	62.3 (62.1-62.4)	100	100	69.7 (69.5-69.8)	3.42
	Fecal *nox*[b]	79.9 (79.7-80)	88.4 (88.2-88.5)	88.8 (88.7-88.9)	79.1 (79.0-79.3)	2.45
	All qPCRs	65.2 (65.1-65.4)	97.4 (97.3-97.5)	96.7 (96.6-96.8)	70.8 (70.6-71.0)	4.29
	All qPCRs + fecal *nox* + culture *nox*	100	85.3 (85.1-85.4)	88.7 (88.6-88.8)	100	10.16
BH	Culture	72.9 (61.5-81.9)	71.6 (67.2-75.7)	29.5 (23.2-36.7)	94.2 (91.1-96.2)	12.97
	Culture *nox*	52.9 (40.5-64.9)	99.5 (98.3-99.9)	94.8 (82.0-98.7)	92.8 (91.0-94.3)	3.42
	Fecal *nox*	67.1 (54.9-77.9)	98.3 (96.7-99.3)	87.0 (76.0-93.4)	94.8 (92.9-96.3)	2.45
	qPCR	97.1 (90.1-99.7)	99.3 (98.0-99.9)	95.8 (88-98.6)	99.5 (98.2-99.9)	1.07
	qPCR + fecal *nox* + culture *nox*	100	99.1 (99.04-99.10)	94.5 (94.4-94.7)	100	6.94
BHM	Culture	55.2 (46.1-63.9)	71.6 (66.9-75.9)	37.0 (30.2-44.4)	84.1 (79.7-87.7)	12.97
	Culture *nox*	21.7 (14.6-30.4)	99.7 (98.6-99.9)	96.2 (77.4-82.4)	81.0 (79.5-82.4)	3.42
	Fecal *nox*	50.4 (41.0-59.9)	99.7 (98.6-99.9)	98.3 (89.0-99.8)	87.0 (84.9-89.0)	2.45
	qPCR	95.7 (95.6-95.8)	97.1 (97.0-97.2)	91.0 (90.9-91.2)	98.7 (98.6-98.7)	2.14
	qPCR + fecal *nox* + culture *nox*	100	96.9 (96.81-96.9)	90.6 (90.46-90.79)	100	8.01
BP	Culture	73.0 (59.1-82.9)	70.0 (65.5-74.0)	21.5 (18.0-25.5)	95.7 (93.5-97.2)	12.97
	Culture *nox*	37.2 (25.0-50.8)	100.0 (99.2-100)	100	92.2 (90.7-93.5)	3.42
	Fecal *nox*	25.5 (25.1-25.9)	99.3 (99.30-99.35)	81.2 (56.1-93.6)	92.1(90.9-93.2)	2.45
	qPCR	72.5 (72.1-72.9)	98.7 (98.63-98.69)	86.0 (85.7-86.3)	96.9 (96.8-96.9)	1.07
	qPCR + fecal *nox* + culture *nox*	52.5 (39.1-65.7)	100 (99.1-100)	100	94.0 (92.3-95.4)	6.94

The clinical sensitivity, specificity and predictive values were calculated based on the final diagnosis. BH= *B. hyodysenteriae*, BHM= *B. hampsonii*, BP= *B. pilosicoli*.

[a]a PCR (target *nox* gene) was performed from the isolated haemolytic zones on BJ plates.

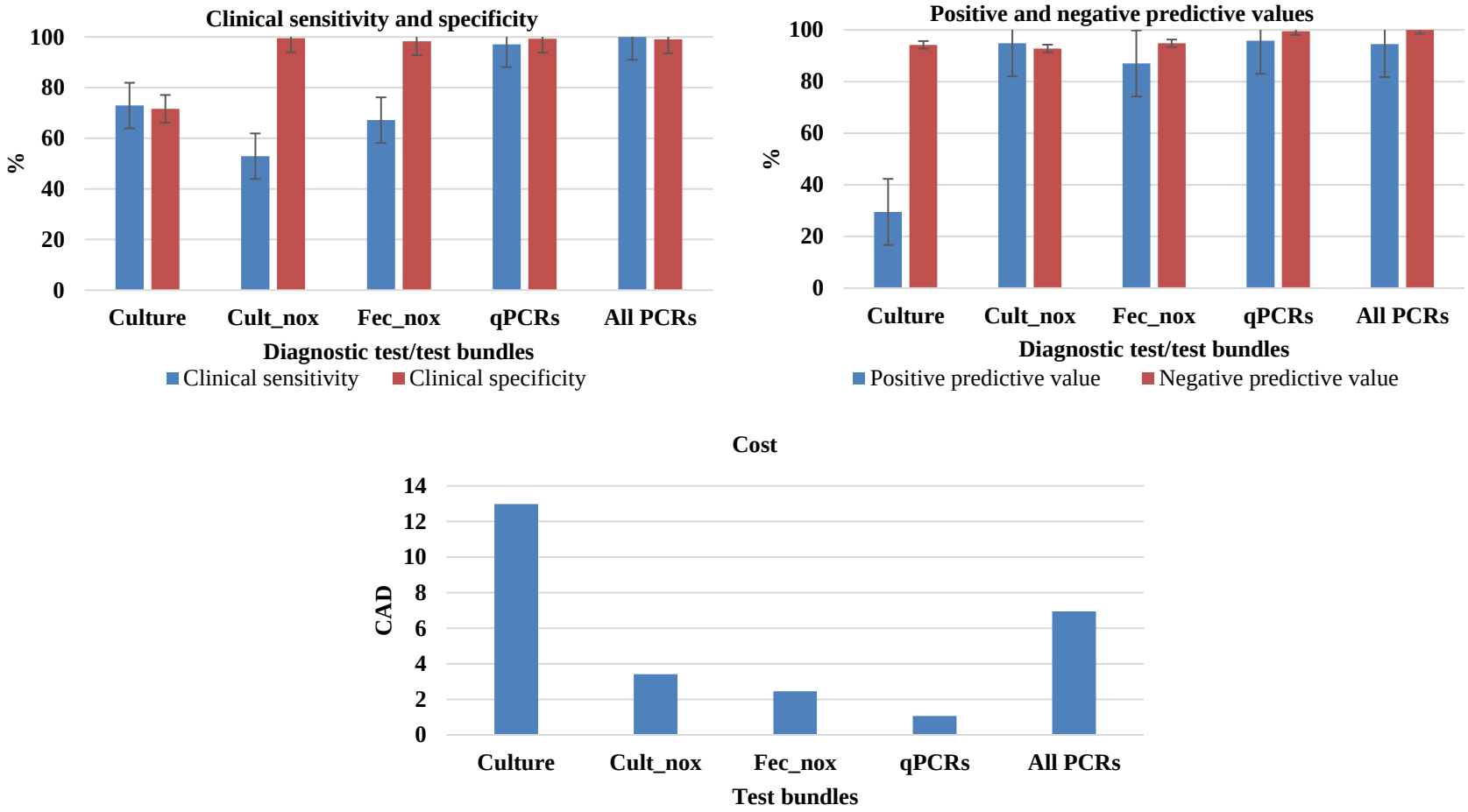
[b]a PCR (target *nox* gene) was performed from the fecal DNA extract

Figure 3. 7 Diagnostic target: Any *Brachyspira* spp.



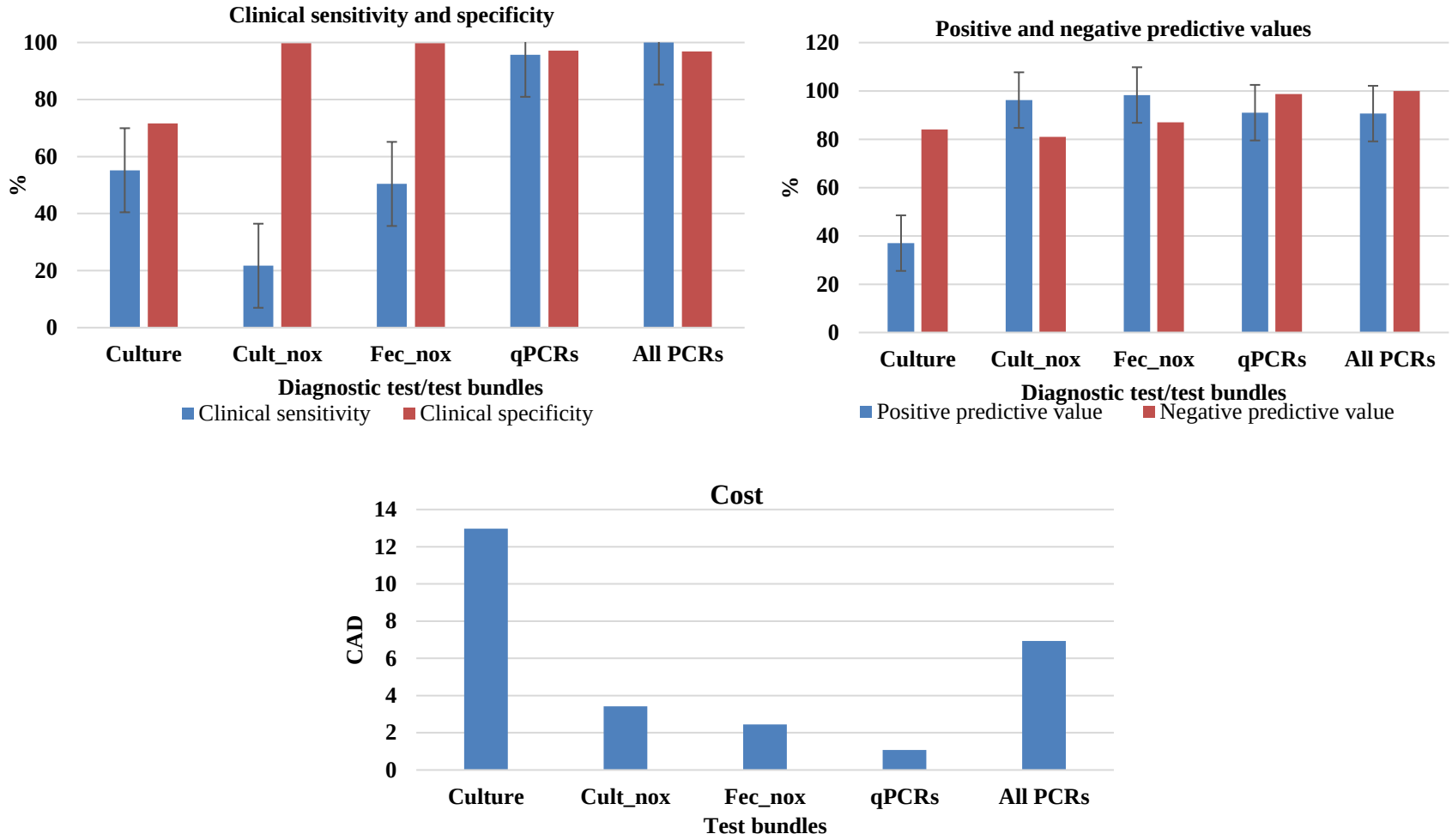
The clinical sensitivity, specificity and predictive values were of each diagnostic test/test bundles (Cult = culture, Cult_nox = culture *nox* PCR, Fec_nox = fecal *nox* PCR, qPCRs = qPCRs for *B. hyodysenteriae*, *B. hampsonii* genomovar I and II and *B. pilosicoli*, All PCRs = culture *nox* PCR + fecal *nox* PCR and all qPCRs) were indicated in graph.

Figure 3. 8 Diagnostic target: *B. hyodysenteriae.*



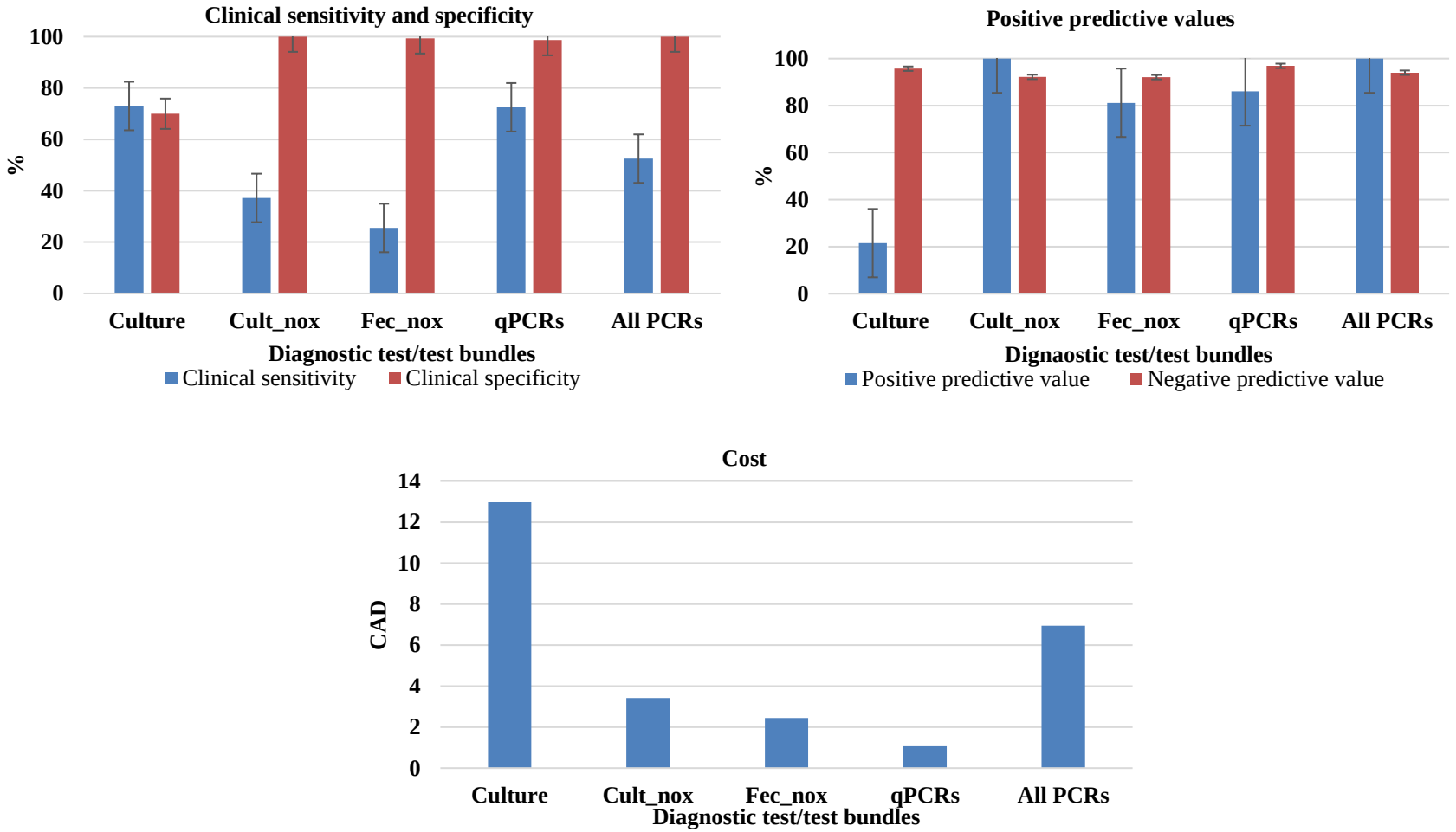
The clinical sensitivity, specificity and predictive values were of each diagnostic test/test bundles (Cult = culture, Cult_nox = culture *nox* PCR, Fec_nox = fecal *nox* PCR, qPCRs = qPCRs for *B. hyodysenteriae*, *B. hampsonii* genomovar I and II and *B. pilosicoli*, All PCRs = culture *nox* PCR + fecal *nox* PCR and all qPCRs) were indicated in graph.

Figure 3. 9 Diagnostic target: *B. hampsonii*.



The clinical sensitivity, specificity and predictive values were of each diagnostic test/test bundles (Cult = culture, Cult_nox = culture *nox* PCR, Fec_nox = fecal *nox* PCR, qPCRs = qPCRs for *B. hyodysenteriae*, *B. hampsonii* genomovar I and II and *B. pilosicoli*, All PCRs = culture *nox* PCR + fecal *nox* PCR and all qPCRs) were indicated in graph.

Figure 3. 10 Diagnostic target: *B. pilosicoli*.



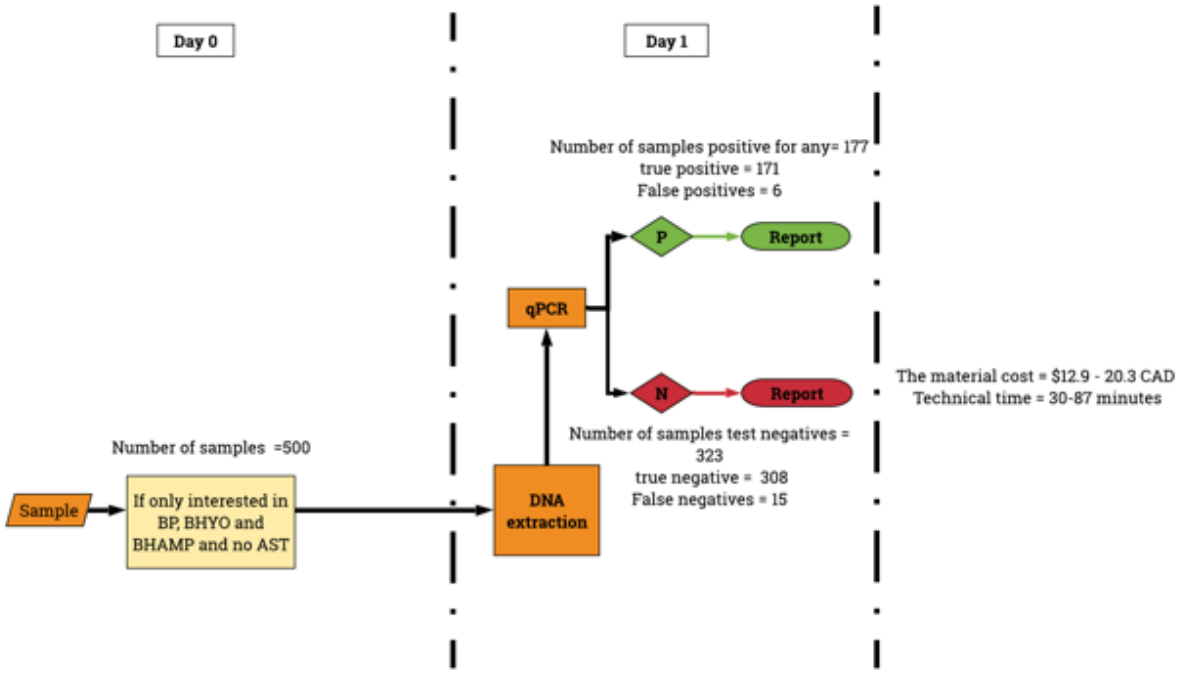
The clinical sensitivity, specificity and predictive values were of each diagnostic test/test bundles (Cult = culture, Cult_nox = culture *nox* PCR, Fec_nox = fecal *nox* PCR, qPCRs = qPCRs for *B. hyodysenteriae*, *B. hampsonii* genomovar I and II and *B. pilosicoli*, All PCRs = culture *nox* PCR + fecal *nox* PCR and all qPCRs) were indicated in graph.

Except for *B. hampsonii* (genomovar I and II), qPCR was the least expensive ($2.14 CAD) test available for diagnosis of *Brachyspira*. Moreover, the culture ($12.97 CAD) was the most expensive test used for diagnosis of any diagnostic target.

To achieve different diagnostic end points three decision trees were developed (Figures 3.11-3.13). The estimated experimental time required from receipt of samples until the generation of the final diagnostic report of *Brachyspira*-associated diseases varied from 1-13 days (Figure. 3.11-3.13) based on the diagnostic workflow selected. In the first workflow (Figure 3.11) no culture-based diagnosis was completed, and species-specific qPCRs (*B. hyodysenteriae*, *B. hampsonii* and *B. pilosicoli*) provides results within one day.

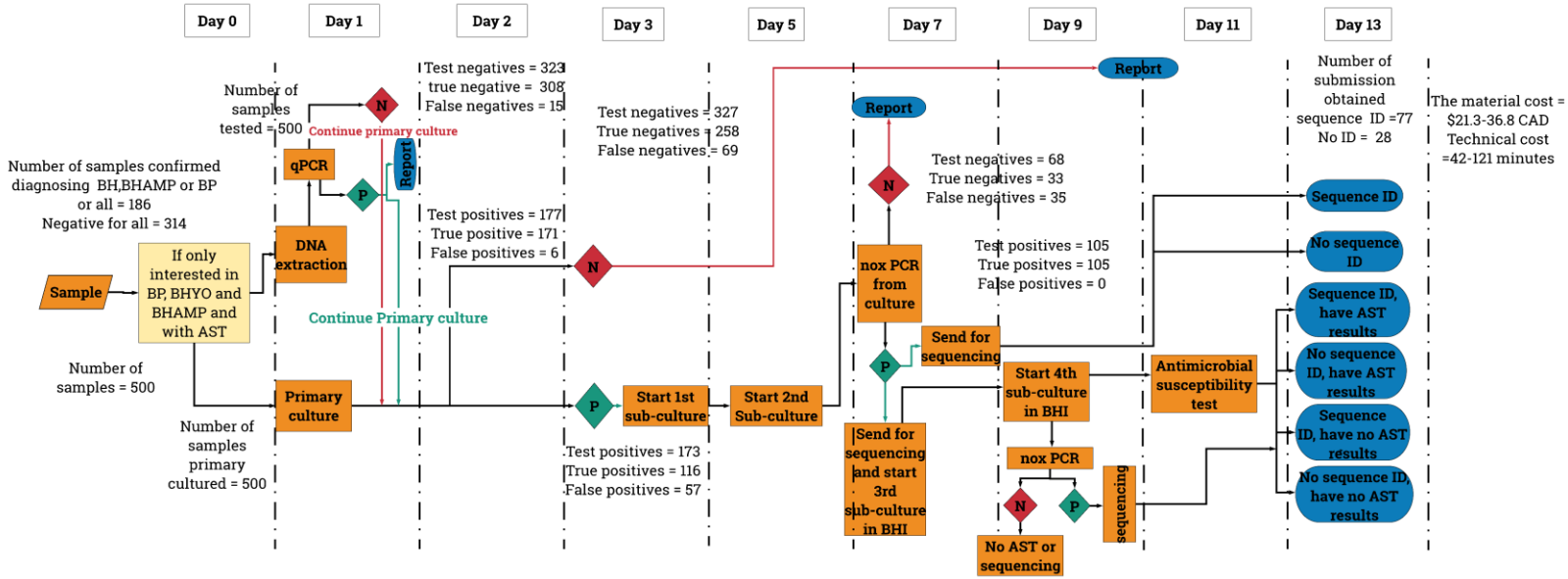
When the diagnostic objective was to identify *B. hampsonii*, *B. hyodysenteriae* and *B. pilosicoli* (Figure 3.11) results could be obtained in one day using qPCR. This approach was also the least expensive (material cost was $12.9-20.3 CAD and technical time was 30-87 minutes) compared to the other two workflows (Figure 3.12-3.13); although no susceptibility data would be generated. Compared to the first workflow the second workflow included additional culture and susceptibility testing which provide species identification and antimicrobial susceptibility profiles. The material cost to perform all the diagnostic tests included in the 2nd workflow (Figure 3.12) was $21.30-$36.80 CAD and the technical time spent on those tests was 42-121 minutes. In workflows two and three (Figure 3.12-3.13) culture-based diagnosis required 13 days to achieve the final diagnosis. Starting from primary culture (2 days) the 2nd and 3rd workflows included two sub-cultures (2×2 days) on agar and if susceptibility testing was requested, two additional sub-cultures in BHI (2×2 days) were required before processing of the sample was complete. However, if no β-haemolytic zones were isolated, additional steps of sub-culturing on agar was required and may extend the diagnosis process further.

Figure 3. 11 Workflow (1) targeted diagnosis of *B. pilosicoli*, *B. hyodysenteriae* and *B. hampsonii* by species specific qPCR.



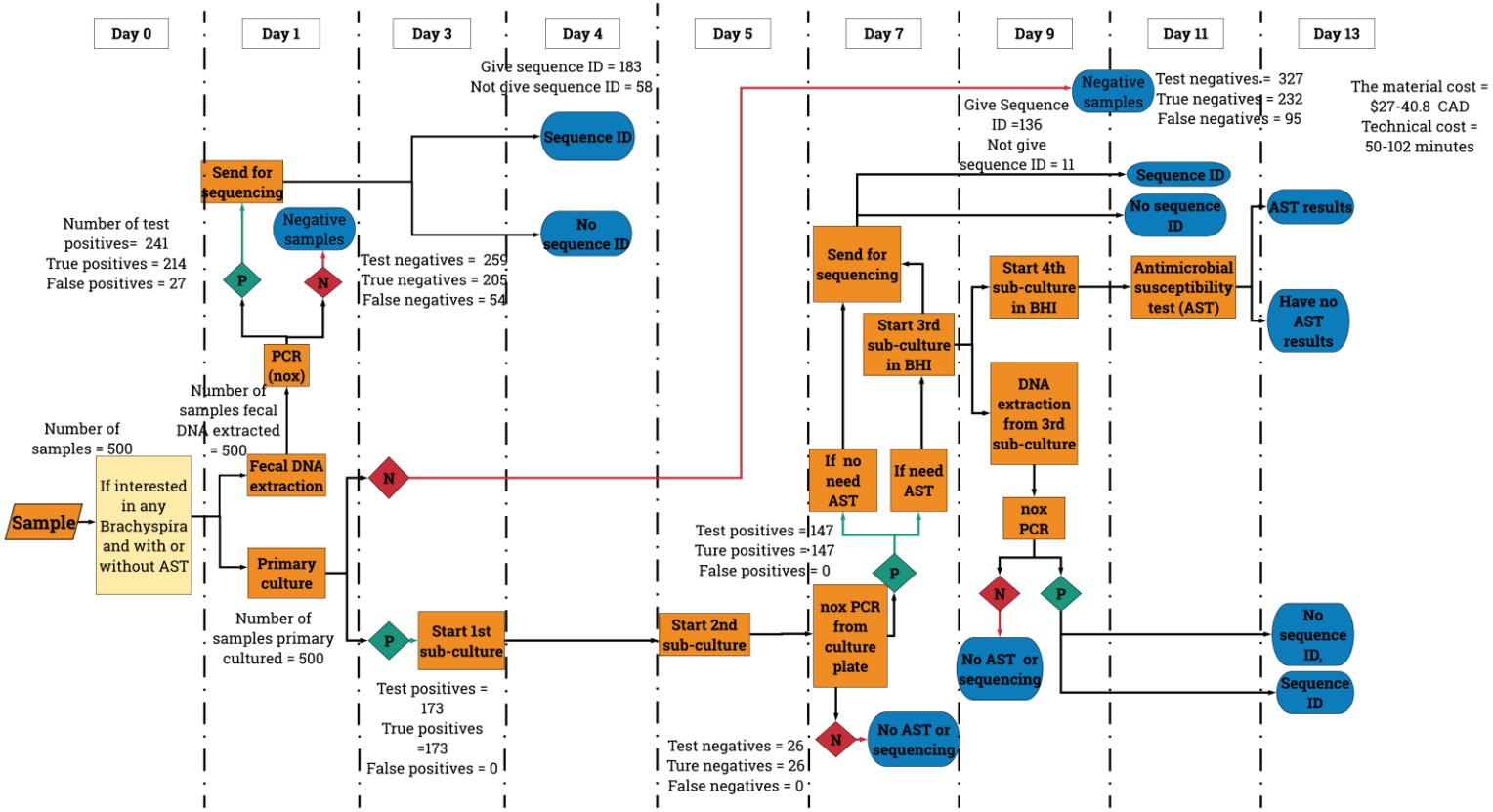
The experimental time (days) is indicated at the top of the figure and each day is separated by a dotted line. The true positives, false-positives, true-negatives, false-negatives, specificity, and sensitivity were calculated by a retrospective analysis of our in-house database. The total material cost and technical time for all the diagnostic tests listed on the tree was indicated on the right. The parallelogram represents the input (sample) to the process while oval shapes represent the end points (results) of the process. All the rectangles represent the process/diagnostic test while arrows make the connection between the representative shapes. The diamond indicates a decision or branching point in the tree.

Figure 3. 12 Workflow (2) targeted diagnosis of *B. pilosicoli*, *B. hyodysenteriae* and *B. hampsonii* + antimicrobial susceptibility test.



The experimental time (days) is indicated at the top of the figure and each day is separated by a dotted line. The true positives, false-positives, true-negatives, false-negatives, specificity and sensitivity were calculated by a retrospective analysis of our in-house database. The total material cost and technical time for all the diagnostic tests listed on the tree was indicated on the right. The parallelogram represents the input (sample) to the process while oval shapes represent the end points (results) of the process. All the rectangles represent the process/diagnostic test while arrows make the connection between the representative shapes. The diamond indicates a decision or branching point in the tree.

Figure 3. 13 Workflow (3) to diagnosis of any *Brachyspira* spp. + antimicrobial susceptibility testing.



The experimental time (days) is indicated at the top of the figure and each day is separated by a dotted line. The true-positives, false-positives, true-negatives, false-negatives, specificity, and sensitivity were calculated by a retrospective analysis of our in-house database. The total material cost and technical time for all the diagnostic tests listed on the tree was indicated on the right. The parallelogram represents the input (sample) to the process while oval shapes represent the end points (results) of the process. All the rectangles represent the process/diagnostic test while arrows make the connection between the representative shapes. The diamond indicates a decision or branching point in the tree.

Based on the 2nd workflow if any sample was found to be qPCR negative, the culture was re-incubated to confirm that the sample was negative for *Brachyspira*. However, if qPCR's results were positive, qPCR results can be reported, and culture could be continued in order to perform the susceptibility test. The objective of 3rd workflow (Figure 3.13) was to diagnose any *Brachyspira* spp. and a *nox* PCR was performed for identifying of species. Fecal *nox* PCR had a higher clinical sensitivity (79.4%) compared to performing all qPCRs (clinical sensitivity = 65.2%), when the diagnostic objective was any *Brachyspira* spp. (Table 3.4). There was no qPCR test developed for identification of *B. murdochii*. However, in this workflow (Figure 3.13) cultures were also included to improve the clinical sensitivity and to permit susceptibility test if the clients elect for this test later. However, the material cost and turnaround time was increased (Figure 3.12-13).

According to our analysis we observed that performing all qPCRs (Figure 3.11) for *B. hyodysenteriae, B. hampsonii* and *B. pilosicoli* had higher specificity (true-negatives = 5, false-positives = 1, specificity = 83.3%) and it took only one day to report the negative results. However, culture had lower specificity (true-negatives= 308, false-positives= 6, specificity= 98.0%) and when primary culture was negative, usually inoculated plates were anaerobically incubated for 10 days before it was confirmed as a *Brachyspira* negative culture (Figure 3.12). This lengthy process can increase the time and cost of the diagnostic process. Discussion

The diagnostic workflow currently used to identify *Brachyspira* at the University of Saskatchewan is time consuming and laborious requiring at least 15 days from initial receipt of samples until the generation of a final report (Figure 3.1). The lack of susceptibility testing is another major limitation which needs to be filled to give evidence-based guidance for veterinarians to select the appropriate antimicrobial therapy. We previously developed a standard agar dilution test for

Brachyspira spp. however as it was very laborious and time-consuming, modifications were done to enhance the feasibility of incorporating it into the existing diagnostic workflow. Modifications included reducing the number of antimicrobials tested to five (tiamulin, valnemulin, tylosin, tylvalosin and salinomycin) and testing a more limited range of concentrations. The antibiotic concentrations included in this study were selected to highlight the diversity of MICs detected in our previous investigation. Although salinomycin was not included in our previous study we have included that in modified agar dilution test, as anecdotally it is an effective medicine used for treatment and prevention of swine dysentery in Canada. Additionally, salinomycin was included based on previous literature (Karlsson, et al. 2004). Finally, full 100 mm agar plates (20 ml volume) were replaced with quadrant plates to reduce the total material cost for susceptibility test including ovine blood, trypticase soy agar and antimicrobial by 75% and gas packs (Figure. 3.4).

Although quadrant plates are associated with a reduced testing capacity (two isolates can be tested simultaneously vs. ten), this is consistent with normal sample volumes and eliminates wasteful additional testing capacity of the full plates (Figure 3.4). The development of this new less expensive susceptibility test method will allow diagnostic labs to provide therapeutic guidance and help veterinarians to reduce unnecessary selection pressure for antimicrobial resistance.

Usually culture (for any *Brachyspira* spp. (64.6%) and *B. hampsonii* (55.2%)) and culture *nox* PCR (any *Brachyspira* spp. (62.3%), *B. hyodysenteriae* (52.9%) *B. hampsonii* (21.7%)) had low sensitivities compared to qPCRs. *Brachyspira* require special conditions (aerotolerant anaerobes) to grow in media and lack of those conditions may lead to negative culture results (Stanton and Lebo 1988). Moreover, some of the cases with negative culture might have undergone antimicrobial therapy leading to loss of viability of the bacteria (Nathues et al. 2007). The fastidious nature of *Brachyspira* may explain the low clinical sensitivities observed for culture.

Interestingly, this finding is in contrast to one recently published study which compared culture, qPCR assays and fluorescent in-situ hybridization (FISH) for detection of *B. hyodysenteriae* and *B. hampsonii*. Those authors reported that culture was the most sensitive method for detecting both species if tested immediately after sample preparation (Wilberts et al. 2015). Interestingly, this study also demonstrated that long transport time (>48 hrs) can reduce the number of culturable spirochetes in pig feces and can increase the likelihood of false-negative culture (Wilberts et al. 2015). In our study the in-house diagnostic database which was used to calculate the clinical sensitivities of the tests included real diagnostic sample submission data and therefore prolonged transport time may have increased the number of false-negative cultures. Further, *B. hampsonii* had the lowest clinical sensitivities for culture and culture *nox* PCR test compared with other *Brachyspira* spp. Anecdotally, *B. hampsonii* is more fastidious compared with other *Brachyspira* spp. possibly explaining this lower sensitivity. Further, if animal is exposed to antimicrobial therapy prior to sampling that also can negatively affect the isolation of *Brachyspira* spp. from culture and reduce the clinical sensitivity. Culture *nox* PCR was performed directly by taking samples from the isolated haemolytic zones and lack of DNA extraction step may have led to the very low clinical sensitivities (21.7-62.3%) in all diagnostic targets.

The use of qPCR for *B. pilosicoli* had a low clinical sensitivity compared to that of *B. hyodysenteriae* and *B. hampsonii*. The qPCR (SYBR green qPCR) for both *B. hampsonii* and *B. hyodysenteriae* was developed by WCVM *Brachyspira* diagnostic team and validated earlier (Harding et al. 2013b; Costa et al. 2016), while the qPCR (probe based qPCR) used for *B. pilosicoli* was developed by other diagnostic laboratory and have limitations in the methodology. With the exception of *B. pilosicoli*, the clinical sensitivity and specificity was highest when all diagnostic tests (culture *nox* PCR + fecal *nox* PCR+ qPCRs) were performed than when only individual tests

were used (Table 3.4). Individual diagnostic tests of *B. pilosicoli* had relatively low clinical sensitivities and therefore even those tests were combined (all) clinical sensitivity (52.5%) was low (Table 3.4)

Culture or culture coupled with PCR to detect *Brachyspira* species requires eleven days or longer (Figure 3.1) to be completed and can be tedious compared to qPCR assays which can provide results within one day. The rapid diagnosis of *Brachyspira*-associated disease using qPCRs is important for controlling the disease outbreaks and treating sick animals. These finding were consistent with previous studies which compare species-specific qPCR assays with other diagnostic tests and suggested that species-specific qPCR assays (due to their quick turn-around-time, sensitivity and specificity) were useful for the diagnosis of swine dysentery (Akase et al. 2009; Borgström et al. 2017). However, if clients are interested in antimicrobial susceptibility testing qPCR assays need to be coupled with culture methods to isolate the bacteria. Further, at present there are no qPCRs or any species-specific assays developed for some *Brachyspira* spp. (e.g., *B. murdochii*). Other than species specific qPCRs, MALDI-TOF has also been shown to be a fast and robust method for the identification of swine *Brachyspira* species following culture (Prohaska et al. 2014). In future, as MALDI-TOF does not need any DNA extraction steps, sample preparation or sequencing it can be included in any of the diagnostic workflows we developed.

In this study, we calculated the material cost and technical time for all the *Brachyspira* diagnostic tests performed in the WCVM *Brachyspira* diagnostic laboratory including the new modified agar dilution test. To date, no study systematically analyzed the diagnostic tests used for *Brachyspira* spp. and our study which describes both the economics of each test and the ability of each test to predict diagnostic outcomes is an important contribution to the literature. The overall findings of the investigation are likely generalizable and can be used by other diagnostic laboratories, we

suggest that laboratory specific technical costs may be calculated by multiplying the technical time by the local hourly wage.

Based on the decision trees presented, diagnosticians can select the most fitting test method to meet their diagnostic objectives. For instance, to identify the species present in the sample and find optimal antimicrobial therapy, the most cost-effective step is to perform culture followed by sequencing and antibiotic susceptibility testing. By contrast, if the diagnostic objective is acquiring species identification only, this is best accomplished by either fecal *nox* PCR followed by sequencing or performing species specific qPCRs. Further, sensitivity and specificity vary based on the test method and combination of diagnostic tests can improve the clinical sensitivity and specificity. The decision points in the workflows presented herein will help optimize the use of laboratory resources while reducing turn-around-time. Culture (primary and secondary) and isolation requires most of the experimental time as *Brachyspira* is a slow-growing organism that can be difficult to isolate. Furthermore, to confirm negative cultures, plates were incubated for 10 days and this was a major limitation of the diagnostic process (Figure 3.12 and 3.13). Some antimicrobials (spiramycin, rifampin, vancomycin, spectinomycin and colistin) are included into the selective media (BJ) to prevent growth of normal intestinal microbiota while permitting growth of *Brachyspira* spp. However, several studies have reported that some *Brachyspira* can be inhibited by those antimicrobials and may be difficult to grow and isolate using the BJ media (Lugsomya et al. 2012; Hampson et al. 2019). Furthermore, it is possible that increasing antimicrobial resistance among the normal intestinal microbiota can allow overgrowth of those bacteria, and make the isolation of *Brachyspira* spp. more difficult (Lugsomya et al. 2012). This can eventually lead to increased time for isolation of *Brachyspira*. Therefore, it is important to investigate new selective media or enrichment media that will facilitate the growth of *Brachyspira* and aid in rapid diagnosis.

Finally, the inclusion of an antimicrobial susceptibility test will provide laboratory data to help veterinarians select the most appropriate therapy for diseased pigs and can ultimately improve the clinical outcome, provide economic benefits for producers, and reduce the selection pressure for the development of the antimicrobial resistance.

3.3 Conclusion

In this study we have miniaturized the standardized agar dilution test to reduce the cost and labour intensity and can be included in the routine diagnostic testing of *Brachyspira*-associated diseases in Canada and other countries in the near future. The availability of a feasible test will encourage veterinarian to make evidence-based selection of therapeutics, improve clinical outcomes and reduce the selection pressure for the development of antimicrobial resistance in *Brachyspira* spp. The workflows which we developed will guide diagnosticians and veterinarians to select the most cost-effective test bundle based on their diagnostic objective and can be adopted by any diagnostic laboratory. According to the analysis of diagnostic test accuracies species-specific qPCRs had higher specificities and sensitivities compared to other diagnostic tests and results can be obtained within one day. However, no species-specific qPCR available for some *Brachyspira* spp. and there is a need of developing those molecular tests. Further, according to the workflows *Brachyspira* spp. isolation was the lengthiest process (2-7 days) compared to both species' identification and antimicrobial susceptibility test. Therefore, it is important to develop novel selective media which will aid in rapid isolation of any *Brachyspira* spp.

3.4 Transition statement

Although there are no criteria to interpret antimicrobial MICs for *Brachyspira*, we have identified isolates with high pleuromutilin and macrolide MICs indicating the acquisition of resistance mechanisms. Previous studies have reported the presence of genes or SNPs associated with resistance to these drugs among porcine *Brachyspira* isolates. We used conventional PCR and whole genome sequencing to identify resistance genotypes which can explain the resistance phenotypes we observed in some isolates. Further, we developed a duplex PCR to quickly screen *Brachyspira* for resistance which could be incorporated into a diagnostic workflow. The results of these tests will help to improve the interpretation of our in-vitro antimicrobial susceptibility test results.

4 INVESTIGATION OF THE GENETIC DETERMINANTS OF RESISTANCE TO PROTEIN SYNTHESIS INHIBITORY DRUGS IN *BRACHYSPIRA* SPP.

Kulathunga DGRS., Fernando C., Rubin JE., Hill JE.

Author Contributions: Conceived and design experiment: DGRSK, JER, JEH. Performed the experiment: DGRSK, CF, JER. Analysed the data: DGRSK, JER. Wrote the paper: DGRSK, JER.

4.1 Abstract

Diagnostic laboratories perform and interpret phenotypic antimicrobial susceptibility tests using standardized clinical breakpoints. Determining the phenotypic antimicrobial susceptibility of Brachyspira is confounded by the lack of standardized methods and interpretive criteria, the difficulty of isolating these organisms and their slow growth present additional challenges. There has not yet been a study which describes the presence of antimicrobial resistance genes found in western Canadian *Brachyspira* isolates. In our previous study, we observed that some *Brachyspira* isolates had wide range of minimum inhibitory concentrations for the pleuromutilins, macrolides and lincosamides suggesting the presence of acquired resistance mechanisms. Therefore, the objectives of this study were to identify genetic determinants associated with resistance to pleuromutilin, macrolide and lincosamide drugs and to develop a genotypic test to screen for resistance genes. Nine isolates with high and low MICs were selected for whole genome sequencing. The sequence data were analyzed to identify genetic determinants of resistance and differences in the presence of these genes in isolates with high and low MICs to the pleuromutilins and macrolides. Genomes were queried in the Comprehensive Antimicrobial Resistance Database and searched for the presence of possible resistance genes. Single nucleotide polymorphisms in the 23S rRNA can be responsible for resistance to drugs which inhibit protein synthesis. Potential mutations were investigated by aligning 23S rRNA nucleotide sequences of all isolates with that of *E. coli*. Isolates with high macrolide MICs were found to carry the *lnu*C gene or to have 23S rRNA single nucleotide polymorphisms (A2058G or A2059G, or both) while isolates with elevated pleuromutilin MICs possessed the *tva*B gene. No resistance genes or single nucleotide polymorphisms were found in isolates with low MICs. These results indicate that clinically relevant resistance genes and resistance conferring mutations are present in *Brachyspira* in western

Canada. Finally, a duplex PCR to screen *Brachyspira* isolates for the presence of the identified resistance determinants was developed.

4.2 Introduction

Brachyspira is a genus of Gram-negative, oxygen-tolerant, anaerobic spirochetes. These bacteria colonize the intestinal tracts of wild and domestic animals and humans (Hampson and Ahmed 2009). The genus *Brachyspira* includes nine species with standing in nomenclature (Kulathunga and Rubin 2017). Most importantly, *B. pilosicoli* and *B. hyodysenteriae* have long been known as important gut pathogens of pigs (Hampson 2012). Swine dysentery characterized by mucohemorrhagic colitis and primarily affects grower-finisher pigs, and it is the most economically damaging disease associated with *Brachyspira.* The disease causes mortality and production limiting, sub-optimal growth and feed conversion in pigs (Hampson 2012). In North America *Brachyspira*-associated diseases re-emerged in the late 2000s and a novel species *B. hampsonii,* which can cause swine dysentery-like disease in pigs, was identified and characterized (Chander et al. 2012; Harding et al. 2013; Rubin, et al. 2013; Mirajkar et al. 2016).

Protein synthesis inhibitory antimicrobials (pleuromutilins, macrolides and lincosamides) have been used to treat and prevent *Brachyspira*-associated diseases in North America and other parts of the globe for decades (Kulathunga and Rubin 2017). The pleuromutilins are derived from a natural product produced by the fungus *Clitopilus scyphoides*, these drugs are active against anaerobic bacteria and mycoplasma. Tiamulin and valnemulin are examples of pleuromutilins used in veterinary medicine. Both tiamulin and valnemulin are semisynthetic derivatives of naturally occurring pleuromutilins (Giguere 2013b). The macrolides are an important antimicrobial class, which is widely used in veterinary medicine. These drugs consist of 12-,14-,16- or 17-membered

macrocyclic lactones to which sugar moieties (amino and deoxy sugars) are attached (Giguere 2013c). The lincosamides (lincomycin, clindamycin and pirlimycin) are monoglycosides with an amino acid chain (Giguere 2013b). The macrolides inhibit protein biosynthesis by binding reversibly to the 23S rRNA in bacterial ribosome and preventing translocation of peptidyl-tRNA (Hansen et al. 2002). The binding site of macrolide overlap with that of lincosamides and both antimicrobial classes have similar mechanism of action (Giguere 2013b, 2013c).

Several studies have reported that the minimum inhibitory concentrations (MICs) of the pleuromutilins, macrolides and lincosamides have increased suggesting the emergence of resistant *Brachyspira* spp. in North America and globally (Karlsson et al. 2003; Ohya and Sueyoshi 2010; Clothier et al. 2011; Duijkeren et al. 2014; Mirajkar and Gebhart 2016; Hampson et al. 2019). Both single nucleotide pleomorphisms (SNPs) and specific resistance genes associated with macrolide, lincosamides and pleuromutilin resistance in *Brachyspira* has been compellingly described (Karlsson et al. 1999; Karlsson et al. 2004; Pringle et al. 2004). Single nucleotide polymorphisms in the 23S rRNA can alter the drug binding site (peptidyl transferase center (PTC)) preventing the drug from binding thereby conferring resistance (Pringle et al. 2004). Mutations of the ribosomal protein L3, which is in very close contact with PTC, can also affect the structure of the PTC and prevent drug binding (Pringle et al. 2004). One study found that in *B. hyodysenteriae,* a single rRNA operon and one mutation is sufficient to confer resistance to macrolides, lincosamides and streptogramin antimicrobials (Zuernerl and Stanton 1994). The previously described SNPs in the 23S rRNA and amino acid substitution in ribosomal proteins are listed in Table 4.1.

Table 4.1 The mutations published for *Brachyspira* spp. associated with increased MICs to drugs which inhibit protein synthesis.

Gene with mutation	Position	Resistance phenotype	Reported spp. [a]	Reference
23S rRNA	G2032A	Lincomycin Tiamulin Valnemulin	BH	(Hidalgo *et al.*, 2011; Pringle *et al.*, 2004)
	C2055A	Tiamulin	BH	(Pringle et al. 2004)
	A2057G	Can ameliorate the fitness cost of A2058G	BH	(Hidalgo et al. 2011)
	A2058G	Macrolides Lincomycin Streptogramin	BH	(Hillen *et al.*, 2014; Hidalgo *et al.*, 2011)
	A2058T	Macrolide Lincosamide Streptogramin	BP	(Karlsson et al. 2004)
	A2059G	Macrolide Lincosamide	BP	(Karlsson et al. 2004)
	A2059T	Macrolide Lincosamide	BP	(Karlsson et al. 2004)
	G2447T	Tiamulin Chloramphenicol	BH	(Pringle et al. 2004)
	C2499A	Tiamulin	BH	(Pringle et al. 2004)
	T2504G	Tiamulin Chloramphenicol	BH BP	(Pringle et al. 2004)
	G2535A	Orthosomycin (avilamycin and evernimicin)	BH	(Hillen *et al.*, 2014; Hidalgo *et al.*, 2011)
	A2572T with C2055A	Together disturb the environment of 2504	BH	(Pringle et al. 2004)
	C2611T	Can ameliorate the fitness cost of A2058G	BH	(Hidalgo et al. 2011)
Ribosomal protein L3	Asn148Ser	Tiamulin Valnemulin	*Brachyspira* spp.	(Pringle *et al.*, 2004; Hidalgo *et al.*, 2011), (Hillen et al. 2014)
	Ser149IIe	Tiamulin	BH	(Pringle et al. 2004)

[a]BH = *B. hyodysenteriae*, BP = *B. pilosicoli*

These SNP positions are labeled according to the *E. coli* numbering system (Hidalgo et al. 2009). Macrolide and lincosamide resistance in *Brachyspira* has been shown to occur through both chromosomal mutations and the acquisition of resistance genes (Pringle et al. 2004; Hidalgo et al. 2011; Card et al. 2018; De Luca et al. 2018).

A gene (*lnu*C) conferring resistance to lincosamides in *Brachyspira* spp. was identified, that was originally found on a transposon in *Streptococcus agalactiae* (Achard et al. 2005). This gene encodes lincosamide nucleotidyl transferase, an enzyme which has been shown to inactivate lincosamides and reduce the drug susceptibility (DeLuca et al. 2018). A novel gene was identified in *B. hyodysenteriae* (*tva*A: tiamulin valnemulin antibiotic resistance) and a variant (*tva*B) of that gene was recently described in *B. pilosicoli* (Card et al. 2018). This gene encodes ABC-F ribosomal protection protein and prevents pleuromutilin binding to the target, thereby reducing drug susceptibility (Card et al. 2018).

Genotypic tests can provide additional information to describe the antimicrobial susceptibility of bacteria. The major reasons to use genetic testing are; (i) to improve the speed of detection through PCR-based assays (iii) predict resistance (iii) to evaluate the accuracy of new susceptibility tests and (iv) to validate cut-off values differentiating wild type organisms from those with acquired resistance (Bard and Lee 2018; Mensah et al. 2019). Whole genome sequencing techniques are able to generate massive quantities which allow for the interrogation of an organisms resistomes (Green and Guyer 2011; Manolio and Green 2011; Eyre et al. 2017). Previous studies targeting *Brachyspira* spp. have employed whole genome sequencing to identify resistance genotypes, virulence factors and for comparative genomic analysis (Bellgard et al. 2009; Mappley et al. 2012; Card et al. 2018; De Luca et al. 2018). The first objective of this study was therefore to describe the association between phenotypic results and resistance genotypes in *Brachyspira* spp. using

next-generation sequencing technology. The second objective was to develop a diagnostic PCR, using the data generated in the first objective, to facilitate screening for the presence of resistance genotypes in *Brachyspira* spp. isolated from clinical samples.

4.3 Materials and methods

4.3.1 Whole genome sequencing and analysis

4.3.1.1 Sample selection

A total of 87 porcine *Brachyspira* isolates collected from fecal or colonic clinical samples from pig farms in western Canada were tested using an agar dilution method developed in a previous study. These clinical samples were submitted to a diagnostic laboratory at the Western College of Veterinary Medicine, University of Saskatchewan, and isolates were identified based on *nox* phylogeny (Atyeo et al. 1999; Rohde and Habighorst-blome 2013). Five antimicrobials including tiamulin, valnemulin, tylosin, lincomycin, and tylvalosin were selected to represent the protein synthesis inhibiting antimicrobial drugs which are used to treat swine dysentery. For each antimicrobial, concentrations from 0.25-128 µg/ml were prepared and tested. The lowest antimicrobial concentration where hemolysis was not observed was defined as minimum inhibitory concentration (MIC) and recorded for each isolate. There are no clinical breakpoints for the interpretation of phenotypic results (MICs) of *Brachyspira* (CLSI 2013, 2016), therefore, isolates were classified as having high or low MICs, with the high MIC isolates being identified as possible carriers of resistance genes or chromosomal mutations (Figure 4.1).

Figure 4. 1 The MIC distribution of a collection of *Brachyspira* isolates (n=87).

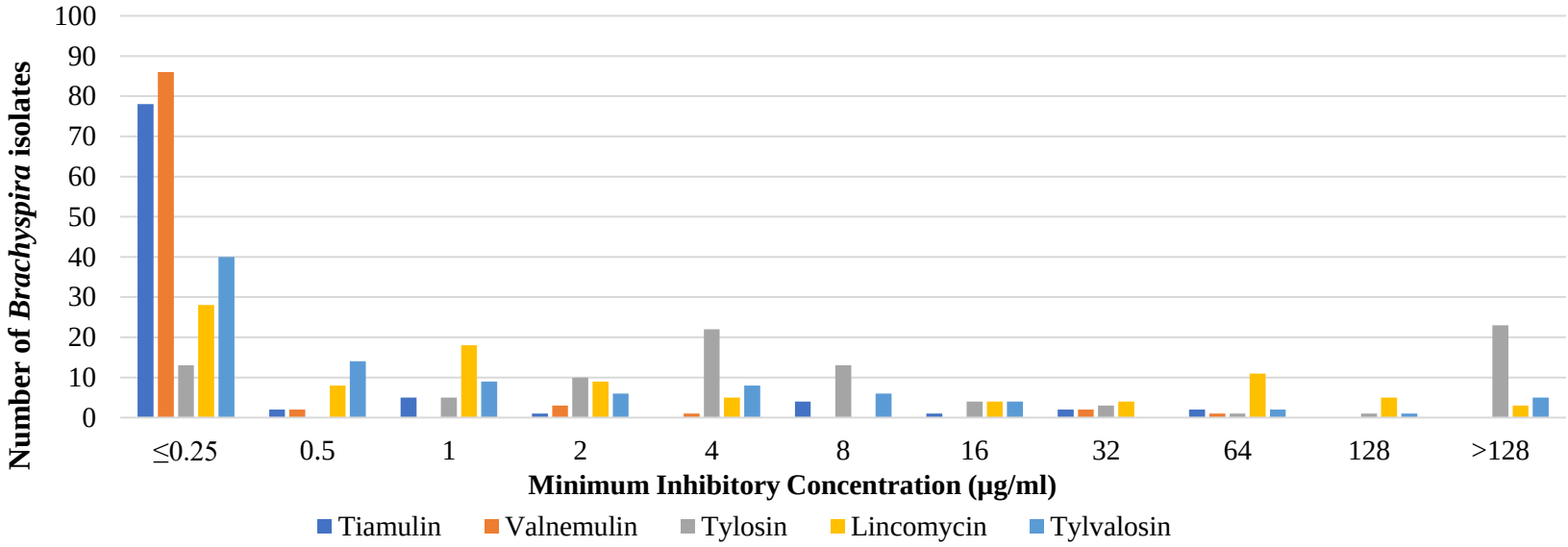


Figure of the MIC distribution observed in agar dilution for tiamulin, valnemulin, tylosin, lincomycin and tylvalosin. For all antimicrobials, multi-modal type distribution was observed. There are isolates with low/high MICs to either pleuromutilins (low MIC < 4 µg/ml, high MIC ≥ 4 µg/ml) or macrolide (low MIC < 4 µg/ml, high MIC ≥ 8 µg/ml).

According to the MIC observed, nine isolates (ID - 1, 2, 4, 5, 6, 7, 8, 9, 12) which had low/high MICs to either pleuromutilins (low MIC < 4 µg/ml, high MIC ≥ 4 µg/ml) or macrolides (low MIC < 4 µg/ml, high < 8 µg/ml) were selected (Figure. 4.1) for WGS.

4.3.1.2 Whole genome sequencing

Whole genome sequencing was performed using the Illumina platform; the analytical and bioinformatic strategy used is summarized in Figure 4.2. Genomic DNA (gDNA) was extracted from overnight cultures of each isolate grown in brain heart infusion (BHI) broth. The cell pellets were collected by centrifugation for 10-15 min at 11,200g. Thereafter, a modified salting out procedure was followed for gDNA extraction from collected cell pellet (Valdivia 2007).

To ensure that the gDNA was intact, samples were run on a 1% agarose gel. If smearing of samples were seen (fragmentation of the DNA) those samples were of too low quality to sequence, while samples with a distinct high molecular weight band on the gel were of sufficient quality (Figure 4.3). Further, to evaluate the purity of gDNA sample absorbance was measured using a spectrophotometer. The absorbance at 260 nm was divided by the reading at 280nm to evaluate the gDNA samples, A_{260}:A_{280} ratios 1.8-2 samples considered high quality while lower rations (<1.8) samples were considered contaminated and extraction was repeated (Haupt, Lea, and Griffiths 2012). The DNA concentration of each extract was measured fluorometrically (Qubit dsDNA BR kit, Life Technologies, Inc., Burlington, ON). Finally, a PCR targeting the NADH oxidase gene using genus-specific primers was performed, and products were sequenced to confirm the purity of the samples and the identify of the isolate (Rohde, Rothkamp, and Gerlach 2002).

Figure 4.2 Summary of workflow employed for whole genome sequencing.

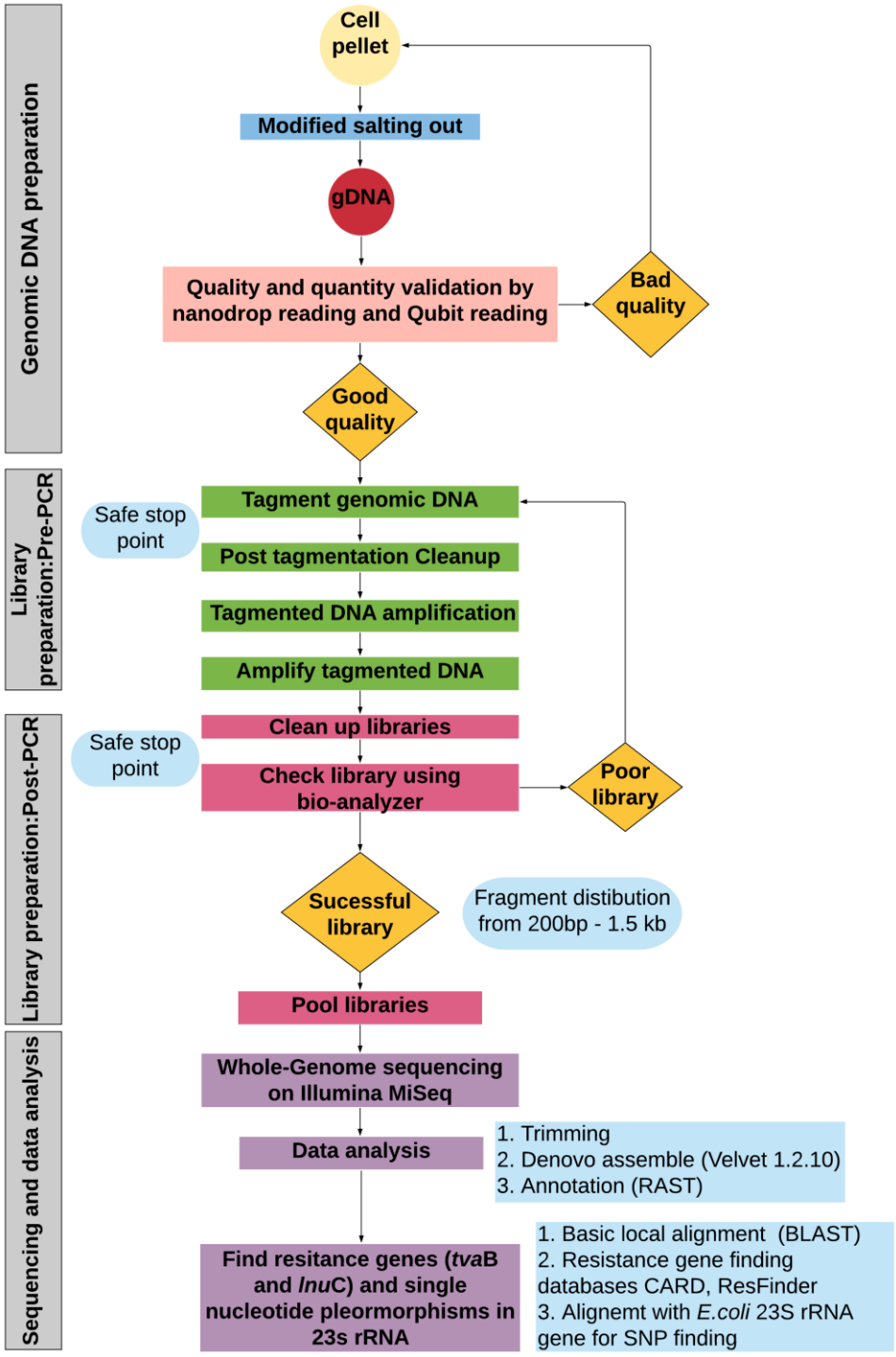
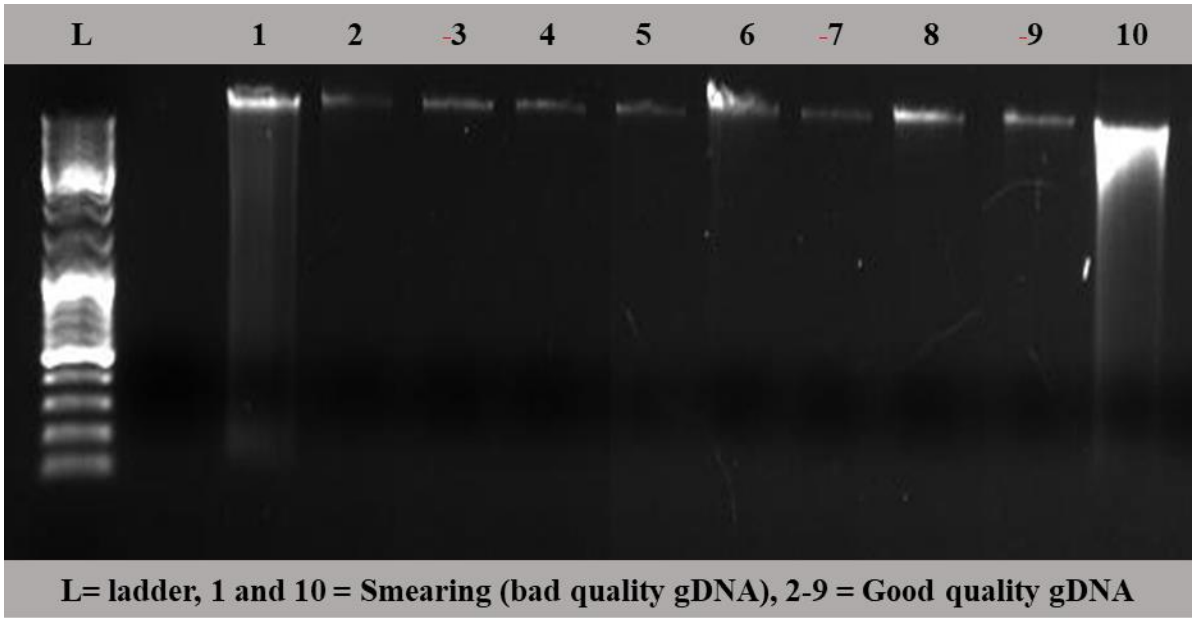


Figure 4.3 Quality analysis of the genomic DNA by running on 1% agarose gel.



Genomic DNA fragmentation assessed by gel electrophoresis. Unfragmented genomic DNA, indicated by a high molecular weight band at the top of the gel is shown in lanes 2-9. Fragmentation indicated by a smear across the gel lane was seen for the samples in lanes 1 and 10.

From high quality extracts, DNA libraries were prepared using the Nextera XT DNA library preparation kit (24 sample indexes) according to the manufacturer's instructions (Document number: 15031942 v03). The average size of the final fragment libraries in base pairs (bp) was measured using the DNA High-Sensitivity chip on the 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). One sequencing run was performed on the Illumina MiSeq platform (500 cycles (2×250)) using MiSeq V2 reagent kits. The FastQC quality control tool (Version 0.11.8) was used to identify and remove low-quality sequences and contaminants. Analysis of whole genome sequences was done using the Geneious software platform (Version 11.1.5). After trimming/removal of low-quality reads from the ends of each read, they were assembled de-novo using Velvet (Version 1.2.10) short sequence alignment method to build contigs. Assembled genomes were annotated using the SEED-based automated annotation system provided by the RAST-OMGE (Rapid Annotation using Subsystem Technology) server (Aziz et al. 2008; Kearse et al. 2012). The assembled genomes were uploaded to NCBI (Accession number SAMN10690269-277). To identify resistance genes, genomes were interrogated using the online resistant gene databases ResFinder (genomicepidemilogy.org) and CARD (Comprehensive Antimicrobial Resistance Database – card.macmaster.ca) (McArthur et al. 2013; Jia et al. 2017). Each assembled genome was also investigated for the presence of resistance genes sequences (gene *lnu*C and *tva*B) by basic local alignment tool (BLAST) in Geneious.

4.3.2 Polymerase chain reaction to investigate the resistance gene *lnu*C

Primers were designed for the *lnu*C gene sequence of *B. pilosicoli* (CP002025.1) using Primer3 (V.0.4.0) (Table 4.2).

Table 4.2 Primers designed for *lnu*C gene detection in *Brachyspira* isolates by singleplex PCR.

Gene	Gene size	Product size	Forward	Reverse
*lnu*C	495 bp	387 bp	AATTTGCAATAGATGCGGAGA	CAAACTCGTATCCCAGATGGA

Primer sequences were designed based on the *lnu*C gene found in *B. pilosicoli* (CP002025.1) genome.

The PCR was optimized with the following conditions: 6 min at 94°C of initial denaturing followed by 30 cycles of: 30 sec at 94°C, 30 sec at 48.5°C and extension for 1 min at 72°C and finally for 10 min, 72°C with 4°C hold. Thereafter, the collection of *Brachyspira* isolates (n=87) were tested with this assay. PCR products were run on an agarose gel, and amplicons from all positive samples were purified and sent for sequencing. The nucleotide sequences were translated and aligned with the amino acid sequence of first reported *lnu*C gene (*S. agalactiae* GenBank accession number AY928180) using the tool CLC sequence viewer (Version 8.0).

4.3.3 Development of multiplex PCR as a diagnostic test for identification of resistance genes

Primer pairs were designed for both *tva*A (Accession number CP003490; locus lag B2904_orf1849) and *lnu*C (*Brachyspira pilosicoli* 95/1000 (CP002025.1)) genes using the tool PrimerPlex tool. This tool considers the melting temperature of primers during the design phase to ensure the best primer pairs and to avoid binding of primers to each other in PCR cycle. Four samples in which these genes were identified by WGS and PCR (T087 - positive for both *tva* and *lnu*C, T024 - positive for *tva*, T067 - positive for *lnu*C, T022 - negative for both genes) were selected as controls in the initial assessment of primer activity. PCR reactions on a temperature gradient were first carried out on isolates that were carrying only one gene (T024 and T067) to determine the optimal individual annealing temperatures. By running the PCR products on the 1% agarose gel and analyzing the bands the best annealing temperature (48.5°C) for both types of primers was selected. Thereafter, duplex reactions with different primer concentrations (both *tva* and *lnu* genes) were tested to optimize the thermocycler program of duplex PCR. The optimized master mixture for 25 µl volume/per reaction was reported with the optimized thermocycler program.

4.4 Results

4.4.1 Whole genome sequencing and findings

The summary of the whole genome sequence data was listed in Table 4.3 and NCBI accession numbers were included. Five *B. pilosicoli* (ID 1,2,8,9 and 12) isolates and four *B. hampsonii* (ID 4,5,6 and 7) isolates were included in the analysis. The guanine + cytosine (GC) content ranged from 27.7-28.9% for both *B. pilosicoli* and *B. hampsonii* species. The genomes were assembled to the contigs level and the number of contigs assembled are listed in Table 4.3. The genome coverage was calculated to be 50–117 fold.

Four isolates with high macrolide and lincosamide MICs (>8 µg/ml) were found to be carrying previously described SNPs in peptidyl transferase center at position 2058 and 2059 (Table 4.4). Another isolate possessing *lnu*C had a very high lincomycin MIC (128 µg/ml) (Table 4.4). The SNPs which have association with pleuromutilin resistance were not identified in isolates with high tiamulin or valnemulin MICs (>4 µg/ml), although all of these possessed the *tva*B gene (Table 4.5).

4.4.2 Polymerase chain reaction to screen for *lnu*C gene

When our entire collection (n=87 isolates) was screened for *lnu*C, 7 isolates: *B. pilosicoli* (n=4), *B. hyodysenteriae* (n=1), *B. murdochii* (n=1), *B. innocens* (n=1) were found to be positive (Table 4.6). All of these isolates had high MICs for lincomycin (≥32 µg/ml) (Table 4.6).

Table 4.3 Summary of whole genome sequencing and analysis

ID	Species	Genome size	Contigs	Contigs N50 (bp)	GC%	Genome coverage	Genome Accession[a]
1	*B. pilosicoli*	2422372	356	11891	28.1	*117x*	SRZT00000000
2	*B. pilosicoli*	2481785	333	12912	28.1	70x	SRZS00000000
4	*B. hampsonii* 30446	2877583	872	5699	27.7	217x	SRZR00000000
5	*B. hampsonii* 30599	2625429	1162	3525	28.5	91x	SRZQ00000000
6	*B. hampsonii* 30446	2699081	621	8009	27.9	63x	SRZP00000000
7	*B. hampsonii* 30599	2683276	784	6152	28.2	50x	SRZO00000000
8	*B. pilosicoli*	2118647	588	5479	28.9	66x	SRZN00000000
9	*B. pilosicoli*	2465798	463	8702	28.1	60x	SRZM00000000
12	*B. pilosicoli*	2282838	450	8486	28.5	72x	SRZL00000000

[a]Genome sequences were deposited in the NCBI database and accession numbers were included.

Species identification and genome assembled data (genome size, contigs, GC% and coverage) were summarized in the table.

Table 4.4 Macrolide and lincosamide MICs and corresponding presence of resistance determinants.

ID	Species	Minimum inhibitory concentration (µg/ml)			*lnu*C gene	23S rRNA SNP[a]	
		Tylosin	Tylvalosin	Lincomycin		2058	2059
1	*B. pilosicoli*	>128	>128	128	-	-	A→G
2	*B. pilosicoli*	>128	8	>128	-	A→G	-
4	*B. hampsonii* 30446	>128	>128	64	-	A→T	-
5	*B. hampsonii* 30599	<0.25	<0.25	<0.25	-	-	-
6	*B. hampsonii* 30446	<0.25	<0.25	<0.25	-	-	-
7	*B. hampsonii* 30599	<0.25	<0.25	<0.25	-	-	-
8	*B. pilosicoli*	4	0.5	2	-	-	-
9	*B. pilosicoli*	>128	>128	32	-	-	A→G
12	*B. pilosicoli*	128	8	128	+	-	-

[a]Different SNPs found in peptidyl transferase center of 23S rRNA (Table 4.1) were checked by aligning 23S rRNA of isolates with both *B. pilosicoli* and *B. hampsonii* reference genes. Only SNPs identified among the isolate collection are included. The location of SNPs named according to the *E. coli* numbering system.

Table 4.5 The MICs of pleuromutilin drugs and WGS findings of resistance determinants.

ID	Species	Minimum inhibitory concentration (μg/ml)		
		Tiamulin	**Valnemulin**	***tva*B gene**
1	*B. pilosicoli*	64	32	+
2	*B. pilosicoli*	32	32	+
4	*B. hampsonii* 30446	≤0.25	≤0.25	-
5	*B. hampsonii* 30599	≤0.25	≤0.25	-
6	*B. hampsonii* 30446	≤0.25	≤0.25	-
7	*B. hampsonii* 30599	≤0.25	≤0.25	-
8	*B. pilosicoli*	64	16	+
9	*B. pilosicoli*	≤0.25	≤0.25	-
12	*B. pilosicoli*	8	4	+

B. pilosicoli samples with high MICs for both tiamulin (≥8 μg/ml) and valnemulin (≥4 μg/ml) were also positive for the gene *tva*B, while none of the isolates that had low MICs (≤0.25 μg/ml) were positive for the gene *tva*B.

Table 4.6 The MIC distribution of lincomycin in both *lnu*C gene positive and negative isolates.

Minimum inhibitory concentration (µg/ml)	Number of *lnu*C gene positive isolates (n=7)	Number of *lnu*C gene negative isolates (n=80)
≤0.25		5
0.5		4
1		13
2		15
4		6
16		3
32	3	3
64	3	7
128	1	4
>128		3

All *lnu*C gene positive isolates had high lincomycin MICs.

When the sequence of *lnu*C amplicons were compared between positive isolates, it was found that all seven of these genes were 100% identical to each other. Furthermore, when compared to the *lnu*C gene previously described from *Streptococcus agalactiae (*AY928180), the translated amino acid sequence was 99.2% identical, differing by a single residue over 118 amino acids (Figure 4.4).

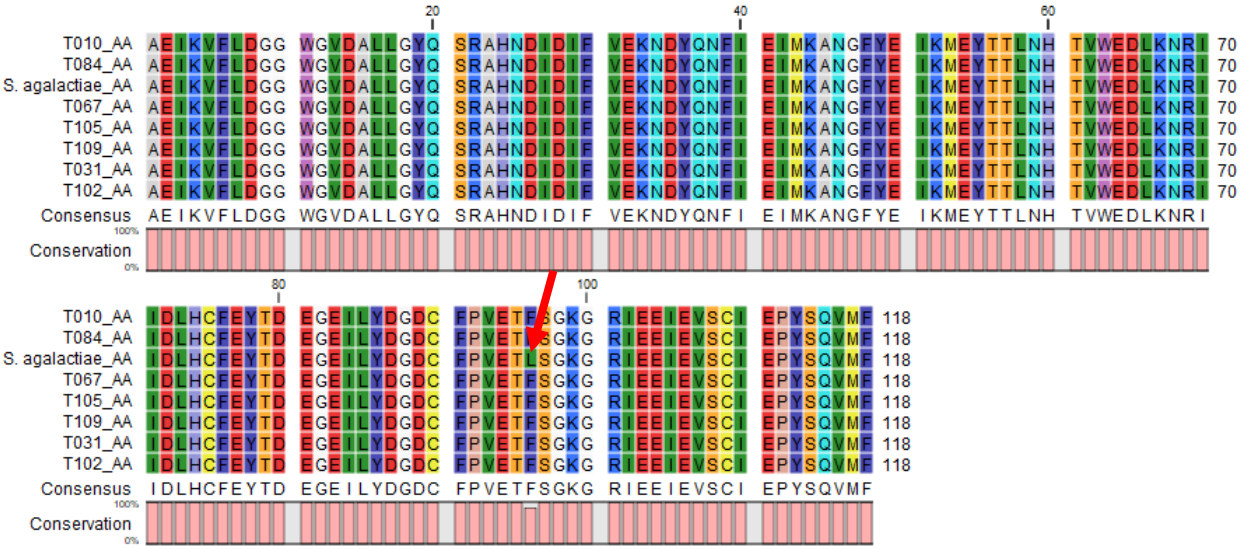
4.4.3 Multiplex PCR and results

The primer pairs for the duplex PCR, the master mixture and the thermocycler program were selected in the optimization process (Tables 4.7 and 4.8). The optimization process determined that the volume of primer pair of *tva*B and *lnu*C needs to be mixed at the ratio of 1:4 to achieve relatively equal signal strength for all targets when a mixture of template DNA was used. The duplex PCR was able to identify the *lnu*C and *tva*B genes in isolates possessing one or both of these genes (Figure.4.5). The duplex PCR performed well for isolates carrying both genes (T087), one of the resistance genes (T024 and T067) and also negative controls (T022).

4.5 Discussion

This study identified antimicrobial resistance genes and SNPs in *Brachyspira* isolates from western Canada. While this investigation included a small number of isolates these data are important first step towards understanding the mechanisms of resistance in our region. Consistent with previous reports, a low GC content (27.7-28.9%) for both *B. pilosicoli* and *B. hampsonii* was identified (Hampson and Wang 2017). Resistance to tylosin, tylvalosin, and lincomycin was associated with mutations at both 2058 (A→T or A→G) and 2059 (A→G) in *B. pilosicoli* and *B. hampsonii* isolates.

Figure 4.4 Comparison of amino acid sequences of *lnu*C amplicons detected in *Brachyspira* and *S. agalactiae.*



The amino acid sequence of *lnu*C genes of *Brachyspira* and *S. agalactiae* (reference gene) were aligned and viewed in CLC Sequence Viewer 8.0 application. The amino acid sequences of the seven isolates were 100% identical to each other, and 99.2% similar or one amino acid difference (indicated by the arrow) to the *lnu*C gene previously described from *Streptococcus agalactiae (*AY928180).

Table 4.7 Optimized master mixture for duplex PCR.

Component	Primer sequences	Volume per reaction (µl)	Gene size (bp)	Product size (bp)
Water		17.05		
10X buffer		2.5		
dNTP		1		
MgCl2		1.25		
*tva*B primer	Forward: ACAGCAGAATAGGTATTGAA	0.8	1518	404
	Reverse: AATCAAAGCACAATTAAAAG	0.8		
*lnu*C primer	Forward: GTGTAGATGCTCTTCTTGGA	0.2	495	212
	Reverse: ACCTTCGTCCGTATATTCAA	0.2		
Taq		0.2		
Template		1.0		

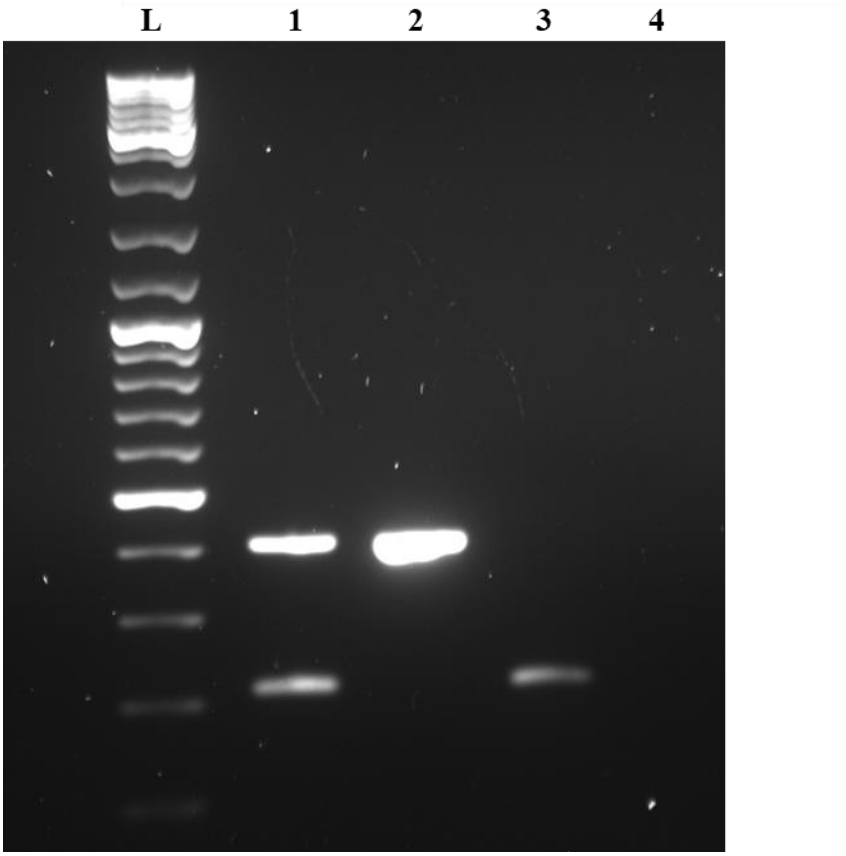
The table includes a master mixture (25 µl) for a duplex PCR. The PCR mixture needs *tva*B and *lnu*C primers to mix at 4:1 ratio.

Table 4.8 The optimized thermal cycler program for duplex PCR.

Step	Temperature	Time
Initial denaturation	94°C	6 min
30 cycles	95°C	1 min
	48.5°C	1 min
	72°C	0.5 min
Final extension	72°C	10 min
Hold	20°C	

Above table describe the thermal cycler program for screening resistance gene *lnu*C and *tva*B genes in a given DNA extract of *Brachyspira* isolate.

Figure 4.5 Gel image of optimized duplex PCR for resistance gene *tva*B and *lnu*C gene.



L = ladder, 1 = isolate positive for both *tva*B and *lnu*C (T087), 2 = isolate positive for only *tva*B gene (T024), 3 = isolate positive for *lnu*C gene (T067), 4 = isolate negative for both genes (T022). The IDs of the isolates were indicated within brackets.

Consistent with previous reports, these mutations were not found in any isolates with low MICs; these findings add strength to the hypothesis that these SNPs are in fact causally associated with elevated MICs. Macrolides, lincosamides, and pleuromutilins are the most commonly used antimicrobials to treat *Brachyspira* infections in pigs in the North American swine industry (Hampson 2012; Burch 2013; Kulathunga and Rubin 2017).

Although these antimicrobials are structurally distinct they share a common binding site on the 50S ribosomal subunit, the peptidyl transferase center (PTC) (Giguere 2013b, 2013c). Lincomycin and tylosin for example share the same drug binding site at 23S rRNA, a single nucleotide mutation at this binding site can therefore confer resistance to both drugs (Schlünzen et al. 2004). The peptide bond formation on the large ribosomal subunit (PTC) is associated with the central loop in domain V of 23S rRNA. The correlation of phenotypic resistance to macrolide and lincosamide drugs with specific SNPs present in the highly conserved main loop of domain V of 23S rRNA were investigated both in *Brachyspira* spp. and other bacteria (Schlünzen et al. 2004; Vester and Douthwaite 2014). The interaction of MLS_B antimicrobials with PTC was mapped using the chemical foot-printing method (Douthwaite 1992; Rodriguez-fonseca, Amils, and Garrett 1995; Hansen, Mauvais, and Douthwaite 1999). The 16-member-ring macrolides (tylosin and tylvalosin) were found to have more extensive interactions with the PTC than the 14-member-ring macrolides (erythromycin and clarithromycin). The chemical foot-printing studies identified that erythromycin and ketolide derivatives not only bind to domain V but also binds to the hairpin 35 in domain II of 23S rRNA simultaneously (Hansen, Mauvais, and Douthwaite 1999; Xiong, Shah, and Mauvais 1999). The highest level of resistance for macrolide drugs was reported in bacteria with mutations at position 2058 and 2059. In *Helicobacter pylori* resistance was reported to macrolide and lincosamide drugs when mutations (A→G or A→C) occur at the position of 2058

or 2059 (Wang and Taylor 1998). A previous study in Sweden reported that A→T mutation was associated with resistance to tylosin, erythromycin, and clindamycin in *B. hyodysenteriae* (Karlsson et al. 1999). Earlier studies described that in *Brachyspira* various combination of two or three mutations can cause a higher level of resistance for tiamulin than one single mutation (Karlsson, Gunnarsson, and Franklin 2001; Pringle et al. 2004). Mutations (A→G or A→C) at position 2059 were associated with resistance for macrolide and lincosamide drugs in *Helicobacter pylori*, Propionibacteria, *Streptococcus pneumonia* and *Mycoplasma pneumoniae* (Bush, Jacoby, and Medeiros 1995; Lucier, Heitzman, and Liu 1995; Ross et al. 1997; Davies et al. 2000). Some previous studies have employed allele-specific primer for the detection of mutations without the requirement for sequencing (Domingo, Prieto, and Alarco 2000; Nakamura et al. 2007; Wangkumhang et al. 2007). In this technique SNPs can be identified using allele-specific primers based on the 3' terminal nucleotide of a primer that corresponds to a specific SNP site. Extension of the primer only occurs when its' 3′-end is a perfectly complemented to the template and results can be observed by simply comparing the length of PCR products (Nakamura et al. 2007). This methodology can be used in the diagnostic workflow to screen SNPs (at position 2058 and 2059) we identified in 23S rRNA of *Brachyspira* spp.

Using the CARD and ResFinder databases, *lnu*C was identified in one of *B. pilosicoli* isolates (ID 12) which had very high MIC (128 µg/ml) for lincomycin. The *lnu*C gene encodes lincosamide nucleotidyltransferase, an enzyme which inactivates lincosamide drugs by adenylation (Giguere 2013b). This gene was identified for the first time in *S. agalactiae* isolated from people in France (Achard et al. 2005). The *lnu*C gene was described for the first time in *Brachyspira* spp. by in-silico analysis of the *B. pilosicoli* 95/1000 (CP002025.1) genome using the resistance gene database ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) (DeLuca et al. 2018). After this

finding the researchers performed the whole genome sequencing of *B. hyodysenteriae* strain and described that the *lnu*C gene was on a small transposon which was also present in *S. suis* (GenBank acc. no. CP011419) from pigs in Canada (DeLuca et al. 2018). The amino acid sequence alignment confirmed that the *lnu*C of *S. agalactiae* was highly similar to *lnu*C gene sequences from *Brachyspira* spp. suggesting that *Brachyspira* may have acquired this gene from Streptococci. However, additional studies need to confirm this supposition. Our study demonstrates the presence of *lnu*C gene in *B. pilosicoli*, *B. innocens* and *B. murdochii*, in which it has not been previously reported.

Recent studies done in Europe identified a novel resistance gene (*tva)* in *B. hyodysenteriae* and *B. pilosicoli* which confers resistance to tiamulin and valnemulin (Card et al. 2018). This gene encodes a predicted ATP-binding cassette subfamily (ABC-F) ribosomal protection protein, which can prevent pleuromutilin binding to the PTC. Two variants of the gene, *tva*A (*B. hyodysenteriae*) and *tva*B (*B. pilosicoli*) have so far been identified (Card et al. 2018). Similar to the Card et al. 2018 study, the current study identified *B. pilosicoli* isolates which had high MICs (≥ 4 μg/ml) for pleuromutilin drugs (tiamulin and valnemulin) were also positive for the *tva*B gene while the gene was not found in any isolates with low MICs. This finding supports the correlation between the presence of resistance gene *tva* and high MICs for pleuromutilin drugs which was previously described (Card et al. 2018).

The duplex PCR targeting *lnu*C and *tva* can now be incorporated into diagnostic workflows. This assay is a rapid and cost-effective diagnostic test and can also enhance resistance surveillance activities. As *Brachyspira* do not have published clinical breakpoints, this assay can provide useful context for the interpretation of phenotypic results. The results of the test can be used to guide veterinarians for evidence-based judgments in treating *Brachyspira*-associated diseases and can

have a positive impact on clinical outcomes. However, the duplex PCR we developed still requires the isolation of *Brachyspira* spp. using selective culture methods, which can be a lengthy and expensive process. It is not possible to identify the presence of the *lnu*C or *tva*B resistance genes directly from a fecal samples as other bacteria (e.g., *Streptococcus* spp.) have been shown to also possess them. One of the limitations of this PCR is its specificity and inability to detect other resistance genes which may be present; this assay will therefore be useful for identifying isolates which are likely resistant but should not be relied upon as an indicator of susceptibility. Furthermore, this assay would fail to detect resistance which is due to the changes in gene expression.

At present for *Brachyspira* spp. only very few complete genomes have been published (Hampson and Wang 2017). Our study included only *B. pilosicoli* and *B. hampsonii* and to determine if these observations are generalizable across the genus additional studies including more species of *Brachyspira* are needed to investigate possible additional resistance genotypes. Previous studies described plasmids (~ 36kb) and transposons in *B. hyodysenteriae*, however these mobile genetic elements have not been described in other species (Bellgard et al. 2009; DeLuca et al. 2018). No sequences consistent with plasmids were identified in our study.

4.6 Conclusion

This is the first study to describe the genetic determinants of resistance to protein synthesis inhibitors in western Canadian *Brachyspira* isolates. A strong relationship between genotype and phenotypic susceptibility test results was identified but further investigations are required to confirm these observations. Finding both resistance genes (*lnu*C and *tva*) and SNPs indicates that antimicrobial resistance is emerging in *Brachyspira* isolates in western Canada and emphasizes the importance of laboratory guidance in the selection of antimicrobials. Furthermore, the duplex

PCR is a simple, rapid and cost-effective genotypic test method which can be adapted by any clinical diagnostics laboratory to forecast lincosamide and pleuromutilin resistance in *Brachyspira* spp.

5. GENERAL DISCUSSION AND CONCLUSION

5.1 Limitation of the study

5.1.1 Summary of the development of standardized agar dilution method and antimicrobial susceptibility of western Canadian *Brachyspira* isolates

Brachyspira is a very fastidious anaerobic spirochete which lacks any published standardized antimicrobial susceptibility test guidelines (CLSI 2012, 2016, 2018). At present most laboratories utilize in-house test methodologies or a commercially available micro-broth plate technique. However, the methodologies have been inadequately standardized and have likely led to poor reproducibility of the tests preventing the comparison of results between laboratories (Råsbäck et al. 2005).

In this investigation we developed an agar dilution method and described a standardized inoculum size (1-2 × 10^8 CFU/ml) which can be easily quantified by diagnostic laboratories using the spectrophotometer method and the equation we developed. This method yielded consistent results between replicates ranging between 80-100% for the antimicrobials were tested. We used this method to test the antimicrobial susceptibility of a collection of *Brachyspira* isolates to ten antimicrobials (tiamulin, valnemulin, tylosin, tylvalosin, lincomycin, chloramphenicol, tetracycline, nalidixic acid, ampicillin, and amoxicillin + clavulanic acid). However, we were most interested in the pleuromutilins (tiamulin and valnemulin), macrolides (tylosin and tylvalosin) and the lincosamides (lincomycin), as those are the most commonly used antimicrobials to treat *Brachyspira*-associated disease (Kulathunga and Rubin 2017). With the exception of *B. pilosicoli* and *B. murdochii*, all other species were inhibited at very low concentration (≤0.25 μg/ml) of the pleuromutilin antimicrobials. All species had heterogenous MIC distributions for macrolide and

lincosamide antimicrobials. Antimicrobial susceptibilities varied by species suggesting that swine veterinarians should perform diagnostic tests to confirm the identification of *Brachyspira* species and antimicrobial susceptibility testing to assist with selecting evidence-based therapeutics. This is the first study which developed a standardized agar dilution method for *Brachyspira* and also the first study which described the antimicrobial susceptibility of *Brachyspira* isolates from Canada.

Although the agar dilution method is recommended for testing fastidious bacteria, it is a very laborious and time-consuming method requiring a great deal of technical time. Therefore, to make it a feasible diagnostic tool it was essential to adjust or modify the developed method. Another challenge is that no quality control organism was identified for agar dilution for *Brachyspira* spp. Although a study published quality control ranges for *B. hyodysenteriae* ATCC 27164 for micro-broth dilution test, the MIC ranges for five of the six antimicrobials in the test were at or above ECOFF values (Pringle et al. 2006; Stubberfield et al. 2020).

Although producers were advised to collect and submit samples (fecal or colon) before giving any antimicrobial therapy we do not have any confirmatory data on antimicrobial use at the farm level. Therefore, it is possible that some of the resistance profiles identified may not be representative of samples from untreated. Moreover, we do not have any published clinical breakpoints for *Brachyspira* in CLSI or EUCAST guidelines. Therefore, based on the MIC data we observed it was not possible to categorize isolates as susceptible, intermediate or resistant and only can group as high MIC or low MIC group.

5.1.1 Summary of the economic analysis of *Brachyspira* diagnostics: from the sample receiving to antimicrobial susceptibility test results

The original susceptibility test method developed was modified to improve the feasibility of incorporating it into diagnostic workflows. We selected five antimicrobials (tiamulin, valnemulin, tylosin, tylvalosin, and salinomycin) of greatest importance to treating *Brachyspira* in pigs and included five concentrations of each in an agar dilution method. Moreover, we used quarter plates in which we were able to inoculate two isolates in one quarter. By using quadrant plates, the volume of agar components including ovine blood, trypticase soy agar and antimicrobial were reduced by 75%, considerably lowering the reagent costs of this assay. The modified agar dilution method can be included in the current diagnostic workflow and offered as a service at the University of Saskatchewan.

Currently both culture and molecular tests are used to isolate and identify *Brachyspira* spp. at the *Brachyspira* diagnostic laboratory, WCVM, University of Saskatchewan. This is a very time consuming and expensive diagnostic process. Therefore, our second objective was to determine the sensitivity, specificity and calculate the cost for all available diagnostic tests and create decision trees which would support diagnosticians to make more appropriate and cost-effective decisions in *Brachyspira* diagnosis. We calculated the material and technical time/cost for different diagnostic tools that we have used at the *Brachyspira* diagnostic laboratory, WCVM. These findings can be adopted to any diagnostic laboratory and technical cost can be adjusted based on the technical cost per hour which can be different from one laboratory to other.

One of the limitations of the study was there was no gold standard test for *Brachyspira*, in this study the final diagnosis made by a board-certified veterinarian incorporating all laboratory data

was used as a gold standard to calculate sensitivity and specificity. Further, there are several species of *Brachyspira* and not all the tests in use can diagnose all relevant *Brachyspira* species (e.g., there is no qPCR develop to test *B. murdochii*). While we were successful in reducing the time and cost for the agar dilution test, the overall turn-around-time remains lengthy; the slow growth of this organism is a rate, limiting critical step required prior to susceptibility testing. This is a disadvantage for veterinarians to make evidence-based decisions in control and prevention of the disease and may discourage veterinarians in selecting these diagnostic tests. However, the presence of antimicrobial resistance in *Brachyspira* spp. in western Canada emphasizes the importance of performing susceptibility testing. The material cost and technical time was calculated and reported as a range because the number of samples tested varied from one batch to another. Therefore, laboratories need to come up with strategies to make an estimation of the cost based on the number of samples going to be submitted. However, we observed that when the number of samples were submitted was low, the average cost per sample increases while the number of samples submitted was high the average cost per samples decreases.

5.1.2 Summary of the investigation of the genetic determinants of resistant to protein synthesis inhibitory drugs in *Brachyspira* spp.

In our first study we observed that some of the isolates of *Brachyspira* in our collection have higher MICs for pleuromutilins, macrolides and lincosamides and we were interested to determine whether these isolates were carrying any resistance genes or any single nucleotide pleomorphisms (SNPs) associated with resistance.

By performing whole-genome sequencing and conventional PCRs, we were able to identify a strong association between high MICs for protein synthesis inhibitory drugs of isolates and

resistance genes (*tva* and *lnu*C) or single nucleotide polymorphisms of 23S rRNA gene. Isolates that carry the *tva* gene had very high MICs for pleuromutilin drugs while *lnu*C carriers had high MICs for lincosamide drugs. Further, we found that resistance to tylosin, tylvalosin and lincomycin were associated with mutations at both 2058 (A → T or A → G) and 2059 (A → G) positions of the 23S rRNA in *B. pilosicoli* and *B. hampsonii* isolates. No prior study has described the genetic determinants of antimicrobial resistance phenotypes observed in *Brachyspira* isolates from western Canada. A duplex PCR was developed which targets the resistance genes identified, this test can be incorporated into the diagnostic workflow in future to contextualize phenotypic susceptibility test. By improving the ability of diagnosticians to identify resistance, these results will support the prudent use of antimicrobials by veterinarians working to control *Brachyspira*-associated infections.

One of the limitations in this study was, there are no published clinical breakpoints for *Brachyspira* to describe the isolates as resistant or susceptible; the presence or absence of these genes can therefore be used to predict clinical outcome without additional investigations. Furthermore, this study included a small collection (n = 9) *B. hampsonii* and *B. pilosicoli* isolates. The role of the resistance genes and resistance associated SNPs in other species of *Brachyspira* is still unknown and requires study.

5.2 Future directions

Although our study was the first in western Canada to determine the antimicrobial susceptibility of *Brachyspira* isolates, only used a small number of isolates collected between 2009 and 2016 were included. As we have observed the emergence of resistance in this study it is important to continue surveillance on the antimicrobial susceptibility of *Brachyspira* isolates.

In addition, according to the antimicrobial susceptibility profiles observed in our study we saw that there were isolates with higher MICs for β-lactam drugs. The β-lactam drugs have been extensively used in swine industry to treat other bacterial disease and there is a possibility of the dissemination of genetic determinants from other colonic bacteria to *Brachyspira* spp. In the future, it would be interesting to investigate the determinants of β-lactam resistance in *Brachyspira* to better understand the selection pressures which led to its emergence.

Moreover, in the future, we are planning to take the results of the study to producers and encourage them to perform susceptibility testing for the *Brachyspira* diagnosis to support the evidence-based selection of antimicrobial therapy in the swine industry. One of the most important future directions is to conduct a well-planned animal trial to determine clinical breakpoints for the most used antimicrobials and submit the proposed clinical breakpoints to CLSI or EUCAST for publication. This will allow the diagnostic laboratories to interpret the susceptibility testing results and provide more consistent results.

With the advancement of the next-generation technologies, metagenomic approaches have the potential to reduce diagnostic turn-around time to 2-3 days. In addition to improved turn-around-time, such approaches would be invaluable for identifying mixed *Brachyspira* infections and larger perturbations to the intestinal microbial community. The technical time and cost of next-generation sequencing technologies is rapidly decreasing, making these platforms an attractive alternative for *Brachyspira* diagnostics in the near future.

While this investigation identified genetic markers of resistance which are easily identifiable using a simple PCR (*lun*C and *tva*B), mutation associated resistance is more challenging to identify quickly and inexpensively. In the future, diagnostic tests not requiring sequencing should be

developed to screen for SNPs in the 23S rRNA gene (Wang and Taylor 1998; Nakamura et al. 2007; Wangkumhang et al. 2007). One possible method would be to design primers specific to the site of mutation and perform the PCR and check for the primer product presence or absent to find out the SNPs, without sending the PCR product for sequencing, which is expensive.

None of the studies confirmed the relationship between resistance genes present in *Brachyspira* sp. and the observed MICs by cloning the genes to a gene expression vector and check for increased MICs. Therefore, in future it is important to perform studies to confirm the association between *tva* and *lnu*C gene and the MICs.

While the investigations presented in this thesis advance the field of *Brachyspira* diagnostics, there are still many areas requiring work. *Brachyspira* is a very fastidious organism and investigation of selective and enriching media or protocol which can expedite the isolation process is a necessity. Moreover, it is important to develop qPCR test for *B. murdochii* to increase the clinical sensitivity and specificity of *Brachyspira* diagnostics. Further, MALDI-TOF has been used for the identification of the *Brachyspira* spp. in other laboratories; although this method requires a pure culture, it will allow for identification of *Brachyspira* spp. without PCR and sequencing. Therefore, MALDI-TOF spectra for different *Brachyspira* spp. including *B. hampsonii* (genomovars I, II and III) should be added to the databases used by veterinary diagnostic labs and validated.

References

Aarestrup, F.M., Duran, C.O., and David, G.S.B., 2008. "Antimicrobial Resistance in Swine Production." *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases* 9 (2): 135–48. https://doi.org/10.1017/S1466252308001503.

Achard, A., Corinne V., Vianney P., Roland L., 2005. "New Lnu (C) Gene Conferring Resistance to Lincomycin by Nucleotidylation in *Streptococcus agalactiae* UCN36" 49 (7): 2716–19. https://doi.org/10.1128/AAC.49.7.2716.

Adessi, C., Gilles M., Guidon A., Gerardo T., Jean J.M., Pascal M., Eric K., 2000. "Solid Phase DNA Amplification: Characterisation of Primer Attachment and Amplification Mechanisms." *Nucleic Acids Research* 28 (20): 87. https://doi.org/10.1093/nar/28.20.e87.

Akase, S., Yumi U., Yoshiko S., Keikichi T., Kenji S., Yoshikazu A., 2009. "Application of Real Time PCR for Diagnosis of Swine Dysentery." *Journal of Veterinary Medical Science* 71 (3): 359–62. https://doi.org/10.1292/jvms.71.359.

Alfaroa, M.P., Jorge L.S., Elaine L., 2019. "Molecular Testing for Targeted Therapies and Pharmacogenomics." In *Accurate Results in the Cloinal Laboratory*, edited by Amitava Dasgupta and L Sepulveda, Jorge, 2nd ed., 349–63.

Alkan, C., Saba S., Evan E.E., 2011. "Limitations of Next-Generation Genome Sequence Assembly." *Nature Methods* 8 (1): 61–65. https://doi.org/10.1038/nmeth.1527.

Altman, D.G., 1990. *Practical Statistics for Medical Research. CRC Press*.

Alvarez-Ordóñez, A., Francisco J.M., Héctor A., Ana C., Pedro R., 2013. "Swine Dysentery: Aetiology, Pathogenicity, Determinants of Transmission and the Fight against the Disease." *International Journal of Environmental Research and Public Health* 10 (5): 1927–47. https://doi.org/10.3390/ijerph10051927.

Andrusch, A,, Piotr W.D., Jeanette K., Simon H.T., Claudia K., Abdalla A.O., Bernhard Y.R., Andreas N., 2018. "PAIPline : Pathogen Identification in Metagenomic and Clinical next Generation Sequencing Samples." *Bioinformatics*, 715–21. https://doi.org/10.1093/bioinformatics/bty595.

Arenz, S., Daniel N.W., 2016. "Bacterial Protein Synthesis as a Target for Antibiotic Inhibition." *Cold Spring Harb Perspect Med*, 1–14.

Argenzio, R.A., Shannon C.W., Robert D.G., 1980. "Pathophysiology of Swine Dysentery : Colonic Transport and Permeability Studies." *Oxford Journals* 142 (5): 676–84.

Atyeo, R.F., Stanton, T.B., Jensen, N.S., Suriyaarachichi, D.S., Hampson, D.J., 1999. "Differentiation of *Serpulina* Species by NADH Oxidase Gene (Nox) Sequence Comparisons and Nox-Based Polymerase Chain Reaction Tests." *Veterinary Microbiology* 67 (1): 47–60. https://doi.org/10.1016/S0378-1135(99)00030-9.

Atyeo, R.F., Oxberry, S.L., Hampson, D.J., 1996. "Pulsed-Field Gel Electrophoresis for Sub-Specific Differentiation *Serpulina pilosicoli* (Formerly ' Anguillina Coli ')." *FEMS Microbiology Letters* 141: 77–81.

Aziz, R.K., Daniela B., Aaron A.B., Matthew D., Terrence D., Robert A.E., Kevin F., 2008. "The RAST Server : Rapid Annotations Using Subsystems Technology." *BioMed Central* 15: 1–15. https://doi.org/10.1186/1471-2164-9-75.

Barcellos, D.E.S.N., Uzeda, M.De., Ikuta, N., Lunge, V.R., Fonseca, A.S.K ., Kader, I.I.T.A., G Duhamel. E., 2000. "Identi ® Cation of Porcine Intestinal Spirochetes by PCR-Restriction Fragment Length Polymorphism Analysis of Ribosomal DNA Encoding 23S RRNA."

Veterinary Microbiology 75: 189–98.
Bard, J.D., Francesca L., 2018. "Clinical Microbiology." *Why Can't We Just Use PCR? The Role of Genotypic versus Phenotypic Testing for Antimicrobial Resistance Testing* 40 (January): 87–95. https://doi.org/10.1136/jcp.41.2.239-e.
Bauer, A.W., Kirby, W.M.M.J., Sherris, C., Turck, A.M., 1966. "Antibiotic Susceptibility Testing by a Standardized Single Disk Method." *The American Journal Of Clinical Pathology* 45 (4): 493–96.
Bayley, H., 2015. "Reflections Nanopore Sequencing : From Imagination to Reality." *Clinical Chemistry* 61:1: 25–31. https://doi.org/10.1373/clinchem.2014.223016.
Bellgard, M.I., Wanchanthuek, P., La, T., Ryan, K., Moolhuijzen, P., Albertyn, Z., Babak S., 2009. "Genome Sequence of the Pathogenic Intestinal Spirochete *Brachyspira hyodysenteriae* Reveals Adaptations to Its Lifestyle in the Porcine Large Intestine." *PloS One* 4 (3). https://doi.org/10.1371/journal.pone.0004641.
Bennett, S., 2004. "Solexa Ltd." *Pharmacogenomics* 5 (4): 433–38.
Bernardeau, M., Gueguen, M., Smith, D.G.E., Corona-barrera, E., Paul, J., 2009. "In Vitro Antagonistic Activities of *Lactobacillus* Spp . against *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*." *Veterinary Microbiology* 138: 184–90. https://doi.org/10.1016/j.vetmic.2009.03.020.
Bidlas, E., Du, T., Lambert, R.J.W., 2008. "An Explanation for the Effect of Inoculum Size on MIC and the Growth / No Growth Interface." *International Journal of Food Microbiology* 126: 140–52. https://doi.org/10.1016/j.ijfoodmicro.2008.05.023.
Black, M., Moolhuijzen, P., Barrero, R., La, T., Phillips, N., Hampson, D., Herbst, W., Barth, S., Matthew, B., 2015. "Analysis of Multiple *Brachyspira hyodysenteriae* Genomes Confirms That the Species Is Relatively Conserved but Has Potentially Important Strain Variation." *PLoS ONE* 10 (6): 1–16. https://doi.org/10.1371/journal.pone.0131050.
Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock. L.J.V., 2014. "Molecular Mechanisms of Antibiotic Resistance." *Nature Publishing Group* 13 (1): 42–51. https://doi.org/10.1038/nrmicro3380.
Boerlin, P., White D.G., 2013. "Antimicrobial Resistance and Its Epidemiology." In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère and John F. Prescott, 5th ed., 21–40.
Borgström, A., Scherrer, S., Kirchgässner, C., Schmitt, S., Frei, D., Wittenbrink, M.M., 2017. "A Novel Multiplex QPCR Targeting 23S RDNA for Diagnosis of Swine Dysentery and Porcine Intestinal Spirochaetosis." *BMC Veterinary Research* 13 (1): 1–8. https://doi.org/10.1186/s12917-016-0939-6.
Borobio, M.V., Pascual, A., Dominguez, M.C., Perea, E.J., 1986. "Effect of Medium , PH , and Inoculum Size on Activity of Ceftizoxime and Sch-34343 against Anaerobic Bacteria." *Antimicrobial Agents and Chemotherapy* 30 (4): 626–27.
Boye, M., Jensen, T.K., Møller, K., Leser, T.D., Jorsal, S.E., 1998. "Specific Detection of the Genus *Serpulina* , *S . hyodysenteriae* and *S . pilosicoli* in Porcine Intestines by Fluorescent RRNA in Situ Hybridization." *Molecular and Cellular Probes*, 323–30.
Branton, D., Deamer, D.W., Marziali, A., Bayley, H., Benner, S.A., Butler, T., Di M.V., 2009. "The Potential and Challenges of Nanopore Sequencing." *Nature Biotechnology* 26 (10): 1146–53. https://doi.org/10.1038/nbt.1495.The.
Brooke, C.J., Hampson, D.J., Riley, T.V., 2003. "In Vitro Antimicrobial Susceptibility of *Brachyspira pilosicoli* Isolates from Humans." *Antimicrobial Agents and Chemotherapy* 47

(7): 2354–57. https://doi.org/10.1128/AAC.47.7.2354.
Brown, W.J., 1988. “National Committee for Clinical Laboratory Standards Agar Dilution Susceptibility Testing of Anaerobic Gram-Negative Bacteria.” *Antimicrobial Agents and Chemotherapy* 32 (3): 385–90.
Buermans, H.P.J., Dunnen, J.T.D., 2014. “Next Generation Sequencing Technology: Advances and Applications.” *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1842 (10): 1932–41. https://doi.org/10.1016/j.bbadis.2014.06.015.
Burch, D.G.S., 2013. “Antimicrobial Drug Use in Swine.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 5th ed., 553–58.
Burrough, E.R., 2017. “Swine Dysentery: Etiopathogenesis and Diagnosis of a Reemerging Disease.” *Veterinary Pathology* 54 (1): 22–31. https://doi.org/10.1177/0300985816653795.
Burrough, E.R., 2013. “Swine Dysentery—Re-Emergence in the United States and Canada.” In *Proceedings of 6th International Conference on Colonic Spirochaetal Infections in Animals and Humans.*, 55–56. Guildford, UK: University of Surrey.
Burrough, E.R., Wilberts, B.L., Bower, L.P., Jergens, A.E., Schwartz, K.J., 2013. “Fluorescent in Situ Hybridization for Detection of ‘ *Brachyspira hampsonii* ’ in Porcine Colonic Tissues.” *Journal of Veterinary Diagnostic Investigation* 25 (3): 407–12. https://doi.org/10.1177/1040638713485228.
Bush, K., Jacoby, G.A., Medeiros, A.A., 1995. “MINIREVIEW A Functional Classification Scheme for -Lactamases and Its Correlation with Molecular Structure.” *Antimicrobial Agents and Chemotherapy* 39 (6): 1211–33.
Calderaro, A, Bommezzadri, S., Piccolo, G., Zuelli, C., Dettori, G., Chezzi, C., 2005. “Rapid Isolation of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* from Pigs.” *Veterinary Microbiology* 105: 229–34. https://doi.org/10.1016/j.vetmic.2004.10.021.
Campbell, M.J., Machin, D., Walters, S.J., 2010. *Medical Statistics: A Textbook for the Health Sciences. John Wiley & Sons.*
Card, R.M., Stubberfield, E., Rogers, J., Nunez-Garcia, J., Ellis, R., AbuOun, M., Strugnell, B., Teale, C., Williamson, S., Anjum. M.F., 2018. “Identification of a New Antimicrobial Resistance Gene Provides Fresh Insights Into Pleuromutilin Resistance in *Brachyspira hyodysenteriae*, Aetiological Agent of Swine Dysentery.” *Frontiers in Microbiology* 9 (June). https://doi.org/10.3389/fmicb.2018.01183.
Card, R.M., La, T., Burrough, E.R., Ellis, R.J., Garcia, J.N., Thomson, J.R., Mahu, M., 2019. “Weakly Haemolytic Variants of *Brachyspira hyodysenteriae* Newly Emerged in Europe Belong to a Distinct Subclade with Unique Genetic Properties.” *Veterinary Research*, 1–13. https://doi.org/10.1186/s13567-019-0639-x.
Castillo, J.R.E., 2013. “Tetracycline.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 5th ed., 257–68.
Catry, B., Cavaleri, M., Baptiste, K., Grave, K., Grein, K., Holm, A., Jukes, H., 2015. “Use of Colistin-Containing Products within the European Union and European Economic Area (EU/EEA): Development of Resistance in Animals and Possible Impact on Human and Animal Health.” *International Journal of Antimicrobial Agents* 46 (3): 297–306. https://doi.org/10.1016/j.ijantimicag.2015.06.005.
Chander, Y., Primus, A., Oliveira, S., Connie J.G., 2012. “Phenotypic and Molecular Characterization of a Novel Strongly Hemolytic *Brachyspira* Species, Provisionally Designated ‘*Brachyspira hampsonii*’.” *Journal of Veterinary Diagnostic Investigation :*

Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 24 (5): 903–10. https://doi.org/10.1177/1040638712456975.
Chekabab, S.M., Lawrence, J.R., Alvarado, A., Predicala, B., Korber. D.R., 2020. "A Health Metadata-Based Management Approach for Comparative Analysis of High-Throughput Genetic Sequences for Quantifying Antimicrobial Resistance Reduction in Canadian Hog Barns." *Computational and Structural Biotechnology Journal* 18: 2629–38. https://doi.org/10.1016/j.csbj.2020.09.012.
Chrystoja, C.C., Diamandis, E.P., 2014. "Whole Genome Sequencing as a Diagnostic Test : Challenges and Opportunities." *Clinical Chemistry* 733. https://doi.org/10.1373/clinchem.2013.209213.
CIPARS. 2015. "Canadian Integrated Program For Antimicrobial Survalance, 2015.Www.Phac-Aspc.Gc.ca/Cipars-Picra/Index-Eng.Php."
CIPARS. 2016. "Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)." http://publications.gc.ca/collections/collection_2018/aspc-phac/HP2-4-2016-eng.pdf.
Clothier, K.A., Kinyon, J.M., Frana, T.S., Naberhaus, N., Bower, L., Strait, E.L., Schwartz, K., 2011. "Species Characterization and Minimum Inhibitory Concentration Patterns of *Brachyspira* Species Isolates from Swine with Clinical Diseas." *J Vet Diagnost Invest* 23 (6): 1140–45. https://doi.org/10.1177/1040638711425580.
CLSI. 2012. "Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition. (Clinical and Laboratory Standards Institute)" 32 (5): 1–38.
CLSI. 2013. "VET01-A4: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard—Fourth Edition (Clinical and Laboratory Standards Institute)" 33 (7): 94.
CLSI. 2015a. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard.10th Ed. Wayne, PA:10th Ed. Wayne, PA: Clinical and Laboratory Standard Institute Document No.: M07-A10. 10th ed.
CLSI. 2015b. VET01S Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals (Wayne, PA, Clinical and Laboratory Standards Institute).
CLSI. 2016. M45-Ed3 Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Clinical and Laboratory Standards Institute, Wayne, Penn., USA.
CLSI. 2017. M100-S27 Performance Standards for Antimicrobial Susceptibility Testing (Clinical and Laboratory Standards Institute).
CLSI. 2018. M11 Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; 9 Th Edition. https://doi.org/10.1016/0738-1751(90)90024-7.
Coates, A.R., Halls, G., Hu, Y., 2011. "Novel Classes of Antibiotics or More of the Same?" *British Journal of Pharmacology* 163 (1): 184–94. https://doi.org/10.1111/j.1476-5381.2011.01250.x.
Cogliani, C., Goossens, H., Greko, C., 2011. "Restricting Antimicrobial Use in Food Animals: Lessons from Europe." *Microbe* 6 (6): 274–79. https://doi.org/10.1128/microbe.6.274.1.
Conrads, G., Citron, D.M., Goldstein, E.J.C., 2005. "Genetic Determinant of Intrinsic Quinolone Resistance in *Fusobacterium canifelinum*." *Antimicrobial Agents and Chemotherapy* 49 (1): 434–37. https://doi.org/10.1128/AAC.49.1.434.
Costa, M.O., Hill,J.E., Fernando, C., Lemieux, H.L., Detmer, S.E., Rubin, J.E., Harding, J.C.S.,

2014. “Confirmation That ‘*Brachyspira hampsonii*’ Clade I (Canadian Strain 30599) Causes Mucohemorrhagic Diarrhea and Colitis in Experimentally Infected Pigs.” *BMC Veterinary Research* 10 (1). https://doi.org/10.1186/1746-6148-10-129.

Cox, G., Wright, G.D., 2013. “Intrinsic Antibiotic Resistance : Mechanisms , Origins , Challenges and Solutions.” *International Journal of Medical Microbiology* 303 (6–7): 287–92. https://doi.org/10.1016/j.ijmm.2013.02.009.

Davies, T., Cronan, M., Jacobs, M.R., Appelbaum, P.C., Fu, W., Retsema, J., Rainville, J., Smyth, D., Su, W., 2000. “Mutations in 23S RRNA and Ribosomal Protein L4 Account for Resistance in *Pneumococcal* Strains Selected In Vitro by Macrolide Passage.” *Antimicrobial Agents and Chemotherapy* 44 (8): 2118–25.

DeLuca, S., Nicholson, P., Chiara, F.M., García-Martín, A.B., Rychener, L., Zeeh, F., Frey, J., Perreten, V., 2018. “Transposon-Associated Lincosamide Resistance Lnu(C) Gene Identified in *Brachyspira hyodysenteriae* ST83.” *Veterinary Microbiology* 214 (October 2017): 51–55. https://doi.org/10.1016/j.vetmic.2017.12.003.

Deurenberg, R.H., Bathoorn, E., Chlebowicz, M.A., Couto, N., Ferdous, M., García-cobos, S., Kooistra-smid, A.M.D., 2017. “Application of next Generation Sequencing in Clinical Microbiology and Infection Prevention.” *Journal of Biotechnology* 243: 16–24.

Didelot, X., Bowden, R., Wilson, D.J., Peto, T.E.A., Derrick, W., 2016. “Transforming Clinical Microbiology with Bacterial Genome Sequencing.” *Nature Reviews Genetics* 13 (9): 601–12. https://doi.org/10.1038/nrg3226.

Diego, R, Ck-menes, P., Carvajal, A., 1995. “*Serpulina hyodysenteriae* Challenge of Fattening Pigs Vaccinated with an Adjuvanted Bivalent Bacterin against Swine Dysermtery.” *Vaccine* 13 (7): 663–67.

Domingo, D., Prieto, N., Alarco, T., 2000. “PCR Using 3 ′ -Mismatched Primers To Detect A2142C Mutation in 23S RRNA Conferring Resistance to Clarithromycin in *Helicobacter pylori* Clinical PCR Using 3 J -Mismatched Primers To Detect A2142C Mutation in 23S RRNA Conferring Resistance to Clarithromycin.” *Journal of Clinical Microbiology* 38 (May 2014): 4–7.

Douthwaite, S., 1992. “Interaction of the Antibiotics Clindamycin.” *Nucleic Acids Research* 20 (18): 4717–20.

Dowling, P.M., 2013a. “Aminoglycosides and Aminocyclitols.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 5th ed., 233–53.

Dowling, P.M., 2013b. “Peptide Antibiotics:Polymyxins, Glycopeptides, Bacitacin and Fosfomycin.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 189–210.

Dowling, P.M., 2013c. “Miscellaneous Antimicrobials: Ionophores, Nitrofurans, Nitroimidazoles, Rifamycins, and Others.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 315–32.

Duggar, B.M., 1948. “Aureomycin: A Product of the Continuing Search for New Antibiotics.” *Annals of the New York Academy of Sciences* 1241 (Antimicrobial Therapeutics Reviews): 163–69. https://doi.org/10.1111/j.1749-6632.2011.06254.x.

Duhamel, G.E., Kinyon, J.M., Mathiesen, M.R., Murphy, D.P., Walter, D., 1998. “In Vitro Activity of Four Antimicrobial Agents against North American Isolates of Porcine *Serpulina pilosicoli*.” *Journal of Veterinary Diagnostic Investigation* 356: 350–56.

Duijkeren, E.V., Greko, C., Moreno, M.A., Constanc, M., Pomba, F., Pyo, S., Sanders, P., 2014.

"Pleuromutilins: Use in Food-Producing Animals in the European Union, Development of Resistance and Impact on Human and Animal Health ¨." *Journal of Antimicrobial Chemotherapy* 69 (May): 2022–31. https://doi.org/10.1093/jac/dku123.

Egerva, M., Lindmark, H., Roos, S., Huys, G., Lindgren, S., 2007. "Effects of Inoculum Size and Incubation Time on Broth Microdilution Susceptibility Testing of Lactic Acid Bacteria □." *Antimicrobial Agents and Chemotherapy* 51 (1): 394–96. https://doi.org/10.1128/AAC.00637-06.

Eley, A., Greenwood, D., 1981. "In Vitro Activity of Ceftizoxime Against Bacteroides Fragilis : Comparison with Benzylpenicillin , Cephalothin , and Cefoxitin." *Antimicrobial Agents and Chemotherapy* 20 (3): 332–35.

Enns, C.B., Keith, B.A., Challa, N., Harding, J.C.S., Loewen, M.E., 2019. "Impairment of Electroneutral Na + Transport and Associated Down-Regulation of NHE3 Contributes to Diarrhea Following in Vivo Challenge with *Brachyspira* Spp." *American Journal of Physiology-Gastrointestinal and Liver Physiology*, ajpgi.00011.2019. https://doi.org/10.1152/ajpgi.00011.2019.

Epand, R.M., Walker, C., Epand, R.F., Magarvey, N.A., Hilpert, K., 2016. "Molecular Mechanisms of Membrane Targeting Antibiotics." *BBA - Biomembranes* 1858 (5): 980–87. https://doi.org/10.1016/j.bbamem.2015.10.018.

Ericsson, H.M., Sherris. C., 1971. "Antibiotic Sensitivity Testing. Report of an International Collaborative Study.Acta Pathologica et Microbiologica Scandinavica Suppl. 217."

EUCAST. 2017. "Standard Operating Procedure MIC Distributions and the Setting of Epidemiological Cut-off (ECOFF). EUCAST SOP 10.0," no. November: 1–17.

Eyre, D.W., De Silva, D., Cole, J.P., Cole, M.J., Grad, Y.H., Demczuk, W., 2017. "WGS to Predict Antibiotic MICs for *Neisseria gonorrhoeae*." *Journal of Antimicrobial Chemotherapy* 72 (7) (March): 1937–47. https://doi.org/10.1093/jac/dkx067.

Fellstrom, C., 2011. "Typing of *Brachyspira* Spp . from Rodents , Pigs and Chickens on Swedish Farms." *Veterinary Microbiology* 153: 156–62.https://doi.org/10.1016/j.vetmic.2011.03.023

Fellstrom, C., Gunnarsson, A., 1995. "Phenotypical Characterisation of Intestinal Spirochaetes Isolated from Pigs." *Research in Veterinary Science*, 1–4.

Fellström, C., Zimmerman, U., Aspan, A., 2001. "The Use of Culture , Pooled Samples and PCR for Identification of Herds Infected with *Brachyspira hyodysenteriae*." *Animal Health Research Reviews* 2 (1): 37–43. https://doi.org/10.1079/AHRR200120.

Firtina, C., Alkan, C., 2016. "On Genomic Repeats and Reproducibility." *Bioinformatics* 32 (15): 2243–47. https://doi.org/10.1093/bioinformatics/btw139.

Fleming, A., 1923. "A Comparison o f the Activities Antiseptics on Bacteria and on Leucocytes." In *Proceeding of the Royal Society of London*, 171–80. London.

Fleming, A., 1929. "On the Antibacterial Action of a Penicillium, with Special Reference to Their Use in the Isolation of *B. influenza*." *British Journal of Experimental Pathology* 10.3 (1923): 226–36.

Felmingham, D., Brown, D.F., 2001. Instrumentation in antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy*, *48*(suppl_1), 81-85.

Fletcher, R.H., Fletche, S.W., Fletcher, G.S., 2012. *Clinical Epidemiology:The Essentials.Lippincott Williams & Wilkins*.

Food and Drug Admistration, Center for Veterinary Medicine. 2016. "FDA Takes Steps to Withdraw Approval of the Swine Drug Carbadox Due to Safety Concerns.," 2016.

Forbes, J.D., Natalie C.K., Jennifer R., Pagotto, F., Reimer, A., 2017. "Metagenomics: The next

Culture-Independent Game Changer." *Frontiers in Microbiology* 8 (JUL): 1–21. https://doi.org/10.3389/fmicb.2017.01069.
Fossi, M., Pohjanvirta, T., Pelkonen, S., 2003. "Molecular Epidemiological Study of *Brachyspira pilosicoli* in Finnish Sow Herds." *Epidemiology Infection*, no. 2003: 967–73.
Gabe, J.D., Chang, R.J., Slomiany, R., Andrews, W.H., McCaman, M.T., 1995. "Isolation of Extracytoplasmic Proteins from *Serpulina hyodysenteriae* B204 and Molecular Cloning of the FlaB1 Gene Encoding a 38-Kilodalton Flagellar Protein." *Infection and Immunity* 63 (1): 142–48.
Gardner, I.A., 2012. "Analysis and Use of Diagnostic Data." In *Diseases of Swine*, edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, and Gregory W. Stevenson, 10th ed., 94–105.
Ghysels, B., Fajardo, A., Martı, N., 2008. "The Neglected Intrinsic Resistome of Bacterial Pathogens." *PLoS ONE* 3 (2): 1–6. https://doi.org/10.1371/journal.pone.0001619.
Giguere, S., 2013a. "Antimicrobial Drug Action and Interaction: An Introduction." In *Antimicrobial Therapy in Veterinary Medicine*, 5th ed., 3–11.
Giguere, S., 2013b. "Lincosamides, Pleuromutilins and Steptogramins." In *Antimicrobial Therapy in Veterinary Medicine*, 199–210.
Giguere, S., 2013c. "Macrolides, Azalides, and Ketolides." In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 5th ed., 211–31.
Giguere, S., Dowling, P.M., 2013. "Fluoroquinolones." In *Antimicrobial Therapy in Veterinary Medicine*, 295–314.
Ginsburg, G.S., Willard, H.F., Mardis, E.R., 2013. "Whole-Genome Sequencing: New Technologies, Approaches, and Applications." *Genomic and Personalized Medicine*, January, 87–93. https://doi.org/10.1016/B978-0-12-382227-7.00007-0.
Girard, C., Lemarchand, T., Higgins, R., 1995. "Porcine Colonic Spirochetosis : A Retrospective Study of Eleven Cases." *Canadian Veterinary Journal* 36 (May): 291–94.
Gire, S.K., Goba, A., Andersen, K.G., Sealfon, R.S.G., Park, D.J., Kanneh, L., Jalloh, S., 2015. "Genomic Surveillance Elucidates Ebola Virus Origin and Transmission during the 2014 Outbreak A." *Science* 345 (6202): 1369–72. https://doi.org/10.1126/science.1259657.Genomic.
Glenn, T.C., 2011. "Field Guide to Next-Generation DNA Sequencers." *Molecular Ecology Resources* 11 (5): 759–69. https://doi.org/10.1111/j.1755-0998.2011.03024.x.
Goldstein, B.P., 2014. "Resistance to Rifampicin : A Review." *The Journal of Antibiotics* 67 (9): 625–30. https://doi.org/10.1038/ja.2014.107.
Gomez, M.J, Neyfakh, A.A., 2006. "Genes Involved in Intrinsic Antibiotic Resistance of *Acinetobacter baylyi*." *Antimicrobial* 50 (11): 3562–67. https://doi.org/10.1128/AAC.00579-06.
Green, E.D, Guyer, M.S., 2011. "From Base Pairs to Bedside Genomic Achievements since the Human Genome Project." *Nature*, no. 470: 204–13. https://doi.org/10.1038/nature09764.
Gullapalli, R.R., Lyons-Weiler, M., Petrosko, P., Dhir1, R., Becich, M.J., LaFramboise1, W.A., 2013. "Clinical Integration of Next Generation Sequencing Technology." *Clinical Laboratory Medicine* 32 (4): 585–99. https://doi.org/10.1016/j.cll.2012.07.005.Clinical.
Guntram, S., Holst, O., 2013. *The Bacterial Cell Wall*. Springer Science and Business Media.
Hall, B.G., 2013. "Building Phylogenetic Trees from Molecular Data with MEGA." *Molecular Biology and Evolution* 30 (5): 1229–35. https://doi.org/10.1093/molbev/mst012.

Hampson, D.J., 2012. “Brachyspiral Colitis.” In *Diseases of Swine*, edited by Ramirez A Zimmerman JJ, Karriker LA, 10th ed., 680–96. Ames, IO: John Wiley & Sons.

Hampson, D.J., 2018. “The Spriochete Brachyspira Pilosicoli, Enteric Pathogen of Animals and Humans.” *Clinical Microbiology Review* 31 (1): 1–32.

Hampson, D.J., Robertson, I.D., La, T., Oxberry, S.L., Pethick, D.W., 2000. “Influences of Diet and Vaccination on Colonisation of Pigs by the Intestinal Spirochaete *Brachyspira* (Serpulina) *pilosicoli*.” *Veterinary Microbiology* 73: 75–84. https://doi.org/10.1016/S0378-1135(99)00200-X.

Hampson, D.J., La, T., 2006. “Reclassification of Serpulina Intermedia and *Serpulina murdochii* in the Genus *Brachyspira* as *Brachyspira intermedia* Comb. Nov. and *Brachyspira murdochii* Comb. Nov.” *International Journal of Systematic and Evolutionary Microbiology* 56 (Pt 5): 1009–12. https://doi.org/10.1099/ijs.0.64004-0.

Hampson, D.J., 2012. “Brachyspira Colitis.” In *Diseases of Swine*, edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, and Gregory W. Stevenson, 10th ed., 680–891. Ames, IO: John Wiley & Sons.

Hampson, D.J., and Niyaz Ahmed. 2009. “Spirochaetes as Intestinal Pathogens: Lessons from a *Brachyspira* Genome.” *Gut Pathogens* 1 (1): 10. https://doi.org/10.1186/1757-4749-1-10.

Hampson, D.J., Lugsomya, K., La, T., Dale, N., Trott, D.J., Abraham, S., 2019. “Antimicrobial Resistance in *Brachyspira* – An Increasing Problem for Disease Control.” *Veterinary Microbiology* 229 (December 2018): 59–71. https://doi.org/10.1016/j.vetmic.2018.12.019.

Hampson, D.J., Wang, P., 2017. “Colonic Spirochetes: What Has Genomics Taught Us?” In *Spirochete Biology:The Post Genomic Era.*, edited by Adler B., 273–94.

Hansen, J.L., Ippolito, J.A., Ban, N., Nissen, P., Moore, P.B., Steitz. T.A., 2002. “The Structures of Four Macrolide Antibiotics Bound to the Large Ribosomal Subunit.” *Molecular Cell* 10 (1): 117–28. https://doi.org/10.1016/S1097-2765(02)00570-1.

Hansen, L.H., Mauvais, P., Douthwaite, S., 1999. “The Macrolide – Ketolide Antibiotic Binding Site Is Formed by Structures in Domains II and V of 23S Ribosomal RNA.” *Molecular Microbiology* 31 (2): 623–31.

Harding, J.C.S., Fernando, C., Rubin, J.E., Hill, J.E., 2013a. “Emeegence of Mucohaemorrhagic Diarrhoea Associated with ‘*B. hampsonii*’ in Wetsern Canada.” In *Proceeding of 6th International Conference on Colonic Spirochettal Infection In Animals and Humans*, 61. Guildford, UK: University of Surrey.

Harding, J.C.S., Chirino-Trejo, M., Fernando, C., Jacobson, M., Forster, Z., Hill, J.E., 2010. “Detection of a Novel *Brachyspira* Species Associated with Haemorrhagic and Necrotizing Colitis.” In *In International Pig Veterinary Society Congress: July 18–21 2010. Vancouver, Canada: International Pig Veterinary Society*, 740.

Harding, J.C.S., Fernando, C., Rubin, J.E., Hill, J.E., 2013b. “Emergence of Mucohaemorrhagic Diarrhoea Associated with ‘*B. hampsonii’* in Western Canada.” In *Spirochaete Conference Proceedings*, 61. Guildford, UK: University of Surrey.

Harris, D.L., Kinyon, .M., 1972. “Isolation and Propagation of Spirochetes from the Colon of Swine Dysentery Affected Pigs.” *Canadian Journal of Comparative Medicine*, 74–76. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1319661/.

Hasman, H., Saputra, D., Sicheritz-ponten, T., Lund, O., Svendsen, A., Frimodt-møller, N., 2014. “Rapid Whole-Genome Sequencing for Detection and Characterization of Microorganisms Directly from Clinical Samples.” *Journal of Clinical Microbiology* 52 (1): 139–46. https://doi.org/10.1128/JCM.02452-13.

Haupt, D.C., Larisa, M., Rod, A.L., Griffiths, L.R., 2012. “Comparison of Genomic DNA Extraction Techniques from Whole Blood Samples : A Time , Cost and Quality Evaluation Study.” *Molecular Biology Reports* 39 (5): 5961–66. https://doi.org/10.1007/s11033-011-1408-8.

Hawkey, P.M., 2003. “Mechanisms of Quinolone Action and Microbial Response.” *Journal of Antimicrobial Chemotherapy* 51 (SUPPL. 1): 29–35. https://doi.org/10.1093/jac/dkg207.

Heather, J.M., Chain, B., 2016. “The Sequence of Sequencers: The History of Sequencing DNA.” *Genomics* 107 (1): 1–8. https://doi.org/10.1016/j.ygeno.2015.11.003.

Heatley, N.G., 1943. “A Method for the Assay of Penicillin.” *Biochemical Journal* 38 (December): 61–65.

Helbling, R., Osterheld, M., Vaudaux, B., Jaton, K., Nydegger, A., 2012. “Intestinal Spirochetosis Mimicking Inflammatory Bowel Disease in Children.” *BMC Pediatrics* 12 (1): 163. https://doi.org/10.1186/1471-2431-12-163.

Herbst, W., Schlez, K., Heuser, J., Baljer, G., 2014. “Antimicrobial Susceptibility of *Brachyspira hyodysenteriae* Determined by a Broth Microdilution Method.” *Veterinary Record* 174 (15): 382. https://doi.org/10.1136/vr.102169.

Hidalgo, A., Carvajal, A., García-Feliz, C., Osorio, J., Rubio, P., 2009. “Antimicrobial Susceptibility Testing of Spanish Field Isolates of *Brachyspira hyodysenteriae*.” *Research in Veterinary Science* 87 (1): 7–12. https://doi.org/10.1016/j.rvsc.2008.10.017.

Hidalgo, Á., Carvajal, A., Vester, B., Pringle, M., Naharro, G., Rubio, P., 2011. “Trends towards Lower Antimicrobial Susceptibility and Characterization of Acquired Resistance among Clinical Isolates of *Brachyspira hyodysenteriae* in Spain.” *Antimicrobial Agents and Chemotherapy* 55 (7): 3330–37. https://doi.org/10.1128/AAC.01749-10.

Hill, J.E., Detmer, S.E., Fernando, C., Derbawka, SA., Nosach, R., Harding, J.C.S., 2018. “Retrospective Detection of *Brachyspira hampsonii* in Archived Colitis Cases from Western Canadian Swine.” *Transboundary and Emerging Diseases*, 0–2. https://doi.org/10.1111/tbed.13032.

Hillen, S., Willems, H., Herbst, W., Rohde, J., Reiner, G., 2014. “Mutations in the 50S Ribosomal Subunit of *Brachyspira hyodysenteriae* Associated with Altered Minimum Inhibitory Concentrations of Pleuromutilins.” *Veterinary Microbiology* 172 (1–2): 223–29. https://doi.org/10.1016/j.vetmic.2014.04.021.

Hovind-Hougen, K, Birch-Andersen, A., Henrik-Nielsen, R., Orholm, M, Pedersen, J.O., Teglbjaerg, P.S., Thaysen, E.H., 1997. “Intestinal Spirochetosis : Morphological Characterization and Cultivation of the Spirochete *Brachyspira* ntestinal Spirochetosis : Morphological Characterization and Cultivation of the Spirochete Brachyspira Aalborgi Gen . Nov.,.” *J. Clin. Microbiol.* 16 (6): 1127.

Hoyles, L., Swann, J., 2019. “Influence of the Human Gut Microbiome on the Metabolic Phenotype.” *The Handbook of Metabolic Phenotyping*, January, 535–60. https://doi.org/10.1016/B978-0-12-812293-8.00018-9.

Iredell, J., Brown, J., Tagg, K., 2015. “Antibiotic Resistance in Enterobacteriaceae : Mechanisms and Clinical Implications.” *State of The Art Review*, 1–19. https://doi.org/10.1136/bmj.h6420.

Ivanics, E., Dobos-Kovács, M., Glávits, R., Kaszanyitzky, E., Nemes, C., Szeredi, L., Beregszászi, A., Dencso, L., 2007. “Experimental Study on the Role of *Brachyspira alvinipulli* in Intestinal Spirochaetosis of Geese.” *Acta Veterinaria Hungarica* 55 (3): 315–26. https://doi.org/10.1556/AVet.55.2007.3.6.

Jacobson, M., Lindberg, R., Jonasson, R., Fellström, C., Jensen, M., 2007. "Consecutive Pathological and Immunological Alterations during Experimentally Induced Swine Dysentery - A Study Performed by Repeated Endoscopy and Biopsy Samplings through an Intestinal Cannula." *Research in Veterinary Science* 82 (3): 287–98. https://doi.org/10.1016/j.rvsc.2006.07.012.

Jacoby, G.A., 2005. "Mechanisms of Resistance to Quinolones." *Clinical Infectious Diseases*, no. 41 no. Supplement_2: S120-126.

Jayaraman, B., Nyachoti, C.M., 2017. "Husbandry Practices and Gut Health Outcomes in Weaned Piglets : A Review." *Animal Nutrition* 3: 205–11. https://doi.org/10.1016/j.aninu.2017.06.002.

Jenkinson, S. R., Wingar, C.R., 1981. "Selective Medium for the Isolation of *Treponema hyodysenteriae*." *Veterinary Record* 109 (17): 384–85.

Jensen, N.S., Stanton, T.B., Swayne, D.E., 1996. "Identification of the Swine Pathogen *Serpulina hyodysenteriae* in Rheas (Rhea Americana)." *Veterinary Microbiology* 52: 259–69.

Jensen, T.K, Boye, M., Ahrens, P., Korsager, B., Teglbjærg, P.S., Lindboe, C.F., Møller, K., 2001. "Diagnostic Examination of Human Intestinal Spirochetosis by Fluorescent In Situ Hybridization for *Brachyspira aalborgi* , *Brachyspira pilosicoli* , and Other Species of the Genus *Brachyspira* (Serpulina)." *Journal of Clinical Microbiology* 39 (11): 4111–18. https://doi.org/10.1128/JCM.39.11.4111.

Jensen, T.K., Christensen, A.S., Boye, M., 2010. "Brachyspira Murdochii Colitis in Pigs." *Veterinary Pathology* 47 (2): 334–38. https://doi.org/10.1177/0300985809359054.

Jia, B., Raphenya, A.B., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., 2017. "CARD 2017 : Expansion and Model-Centric Curation of the Comprehensive Antibiotic Resistance Database." *Nucleic Acids Research* 45 (October 2016): 566–73. https://doi.org/10.1093/nar/gkw1004.

Jorgensen, J.H., Ferraro, M.J., 2009. "Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices." *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 49 (11): 1749–55. https://doi.org/10.1086/647952.

Kajiwara, K., Kozawa, M., Kanazawa, T., Uerskuka, K., Nakajima, H., Adachi, Y., 2016. "Drug-Susceptibility of Isolates of *Brachyspira hyodysenteriae* Isolated from Colonic Mucosal Specimens of Pigs Collected from Slaughter Houses in Japan in 2009." *Journal of Veterinary Medical Science* 78 (3): 517–19. https://doi.org/10.1292/jvms.15-0608.

Karger, B.L., Guttman, A., 2009. "DNA Sequencing by Capillary Electrophoresis." *Electrophoresis* 30 (S1): 196–202. https://doi.org/10.1002/elps.200900218.DNA.

Karlsson, M., Fellström, C., Johansson, K.E., Franklin, A., 2004. "Antimicrobial Resistance in *Brachyspira pilosicoli* with Special Reference to Point Mutations in the 23S RRNA Gene Associated with Macrolide and Lincosamide Resistance." *Microbial Drug Resistance* 10 (3): 204–8. https://doi.org/10.1089/mdr.2004.10.204.

Karlsson, M., Landén, A., Aspán, A., Franklin, A., 2004. "Further Characterization of Porcine *Brachyspira hyodysenteriae* Isolates with Decreased Susceptibility to Tiamulin." *Journal of Medical Microbiology* 53 (4): 281–85. https://doi.org/10.1099/jmm.0.05395-0.

Karlsson, M., Fellström, C., Gunnarsson, A., Landén, A., Fellstro, C., Lande, A., 2003. "Antimicrobial Susceptibility Testing of Porcine *Brachyspira* (Serpulina) Species Isolates." *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.41.6.2596.

Karlsson, M, Fellström, C., Heldtander, M.U., Johansson, K.E., Franklin, A., 1999. "Genetic Basis of Macrolide and Lincosamide Resistance in *Brachyspira* (Serpulina) *hyodysenteriae*" *FEMS Microbiology Letters* 172 (2): 255–60. http://www.ncbi.nlm.nih.gov/pubmed/10188254.

Karlsson, M, Gunnarsson, A., Franklin, A., 2001. "Susceptibility to Pleuromutilins in *Brachyspira* (Serpulina) *hyodysenteriae*." *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases* 2 (1): 59–66. https://doi.org/10.1079/AHRR200118.

Karlsson, M., Oxberry, S.L., Hampson, D.J., 2002. "Antimicrobial Susceptibility Testing of Australian Isolates of *Brachyspira hyodysenteriae* Using a New Broth Dilution Method." *Veterinary Microbiology* 84 (1–2): 123–33. https://doi.org/10.1016/S0378-1135(01)00444-8.

Karriker, L.A., Johann, C., Friendship, R.M., Prescott, J.F., 2012. "Drug Pharmacology, Therapy, and Prophylaxis." In *Diseases of Swine*, edited by Ramirez A Zimmerman JJ, Karriker LA. Ames, IO: John Wiley & Sons.

Kearse, M., Moir, R., Wilson, A., Stones-havas, S., Sturrock, S., Buxton, S., Cooper, A., 2012. "Geneious Basic : An Integrated and Extendable Desktop Software Platform for the Organization and Analysis of Sequence Data." *Bioinformatics* 28 (12): 1647–49. https://doi.org/10.1093/bioinformatics/bts199.

Khan, Z.A, Siddiqui, M.F., Park, S., 2019. "Current and Emerging Mehods of Antibiotic Susceptibility Testing." *Diagnostics* 9 (49): 268–91. https://doi.org/10.1002/0471748897.ch11.

Kinyon, J.M., Harris, D.L., 1979. "Treponema Innocens, a New Species of Intestinal Bacteria, and Amended Description of the Type Strain of *Treponema hyodysenteriae*." *Int. J. Syst. Bacteriol* 29: 102–9.

Klose, V., Bruckbeck, R., Henikl, S., Schatzmayr, G., Loibner, A.P.P., 2010. "Identification and Antimicrobial Susceptibility of Porcine Bacteria That Inhibit the Growth of *Brachyspira hyodysenteriae* in Vitro." *Journal of Applied Microbiology* 108 (4): 1271–80. https://doi.org/10.1111/j.1365-2672.2009.04521.x.

Komarek, V., Maderner, A., Spergser, J., Weissenböck, H., 2009. "Infections with Weakly Haemolytic *Brachyspira* Species in Pigs with Miscellaneous Chronic Diseases." *Veterinary Microbiology* 134 (3–4): 311–17. https://doi.org/10.1016/j.vetmic.2008.08.017.

Koser, C.U., Ellington, M.J., Cartwright, E.J.P., Gillespie, S.H., Ko, C.U., Brown, N.M., Farrington, M., 2012. "Routine Use of Microbial Whole Genome Sequencing in Diagnostic and Public Health Microbiology." *PloS Pathogens* 8 (8). https://doi.org/10.1371/journal.ppat.1002824.

Kulathunga, D.G.R.S., Rubin, J.E., 2017. "REVIEW A Review of the Current State of Antimicrobial Susceptibility Test Methods for *Brachyspira*." *Canadian Journal of Microbilogy* 474 (December 2016): 465–74.

Kunkle, R.A., Kinyon, J.M., 1988. "Improved Selective Medium for the Isolation of *Treponema hyodysenteriae*." *Journal of Clinical Microbiology* 26 (11): 2357–60.

Kwong, J.C., Mccallum, N., Sintchenko, V., Howden, B.P., 2015. "Whole Genome Sequencing in Clinical and Public Health Microbiology." *Pathology* 47 (3): 199–210. https://doi.org/10.1097/PAT.0000000000000235.

La, T., Neo, E., Phillips, N.D., Hampson, D.J., 2015. "Genes Encoding Ten Newly Designated OXA-63 Group Class D b -Lactamases Identified in Strains of the Pathogenic Intestinal

Spirochaete *Brachyspira pilosicoli*." *Journal of Medical Microbiology*, no. 2015: 1425–35. https://doi.org/10.1099/jmm.0.000162.
La, T., Phillips, N.D., Reichel, M.P., Hampson, D.J., 2004. "Protection of Pigs from Swine Dysentery by Vaccination with Recombinant BmpB, a 29.7 KDa Outer-Membrane Lipoprotein of *Brachyspira hyodysenteriae*." *Veterinary Microbiology* 102 (1–2): 97–109. https://doi.org/10.1016/j.vetmic.2004.06.004.
Lander, S., Waterston, R.H., Sulston, J., Collins, F.S., 2001. "Initial Sequencing and Analysis of the Human Genome." *Nature* 409 (February): 565–66.
Lawrence, J.V., Maier, S., 1977. "Correction for the Inherent Error in Optical Density Readings." *Applied and Environmental Microbiology* 33 (2): 482–84.
Lefterova, M.I., Suarez, C.I., Banaei, N., Pinsky, B.A., 2015. "Next-Generation Sequencing for Infectious Disease Diagnosis and Management A Report of the Association for Molecular Pathology." *The Journal of Molecular Diagnostics* 17 (6): 623–34. https://doi.org/10.1016/j.jmoldx.2015.07.004.
Leser, T.D., Møller, K., Jensen, T.K., Jorsal, S.E., 1997. "Specific Detection of *Serpulina hyodysenteriae* and Potentially Pathogenic Weakly -Haemolytic Porcine Intestinal Spirochetes by Polymerase Chain Reaction Targeting 23S RDNA." *Molecular and Cellular Probes* 11: 363–72.
Levy, S.E., Myers, R.M., 2016. "Advancements in Next-Generation Sequencing." *Annual Review of Genomics and Human Genetic*, 95–115. https://doi.org/10.1146/annurev-genom-083115-022413.
Ligozzi, M., Bernini, C., Bonora, M.G., Fatima, M.D., Zuliani, J., Fontana, R., 2002. "Rapid Identification and Antimicrobial Susceptibility Profiling of Gram-Positive Cocci in Blood Cultures with the Vitek 2 System." *European Journal of Clinical Microbiology Infectious Diseases* 40 (1): 89–95. https://doi.org/10.1128/JCM.40.5.1681.
Lim, S.K., Lee, H.S., Nam, H., Cho, Y.S., Jung, J.C., Joo, Y.S., 2012. "Prevalence and Antimicrobial Susceptibility of Brachyspira Species in Pigs in Korea." *Korean Journal of Veterinary Research* 52 (4): 253–57.
Lobova, D., 2004. "Decreased Susceptibility to Tiamulin and Valnemulin among Czech Isolates of *Brachyspira hyodysenteriae*." *Journal of Medical Microbiology* 53 (4): 287–91. https://doi.org/10.1099/jmm.0.05407-0.
Loman, N.J., Misra, R.V., Dallman, T.V., Constantinidou, C., Gharbia, S.E., Wain, J., Pallen, M.J., 2012. "Performance Comparison of Benchtop High-Throughput Sequencing Platforms." *Nature Biotechnology* 30 (5): 434–39. https://doi.org/10.1038/nbt.2198.
Lu, H., Giordano, F., Ning, Z., 2016. "Oxford Nanopore MinION Sequencing and Genome Assembly." *Genomics, Proteomics and Bioinformatics* 14 (5): 265–79. https://doi.org/10.1016/j.gpb.2016.05.004.
Lucier, T.S., Heitzman, K., Liu, S., 1995. "Transition Mutations in the 23S RRNA of Erythromycin- Resistant Isolates of Mycoplasma Pneumoniae." *Antimicrobial Agents and Chemotherapy* 39 (12): 2770–73.
Luckey, J.A., Drossman, H., Kostichka, A.J., Mead, D.A., D'cunha, J., Norris, T.B., Smith, L.M., 1990. "High Speed DNA Sequencing by Capillary Electrophoresis." *Nucleic Acids Research* 18 (15): 4417–21. https://doi.org/10.1093/nar/18.15.4417.
Lugsomya, K., Tummaruk, P., Hampson, D.J., Prapasarakul, N., 2012. "Development of a Modified Selective Medium to Enhance the Recovery Rate of *Brachyspira hyodysenteriae* and Other Porcine Intestinal Spirochaetes from Faeces." *Letters in Applied Microbiology* 54

(4): 330–35. https://doi.org/10.1111/j.1472-765X.2012.03213.x.
Lukovic, Z., Skorput, D., Karolyi, D., 2017. “Pig Welfare at Different Production Systems.” *11th International Symposium - Modern Trends in Livestock Production*, no. October: 169–74.
Magi, A., Semeraro, R., Mingrino, A., Giusti, B., D’Aurizio, R., 2017. “Nanopore Sequencing Data Analysis: State of the Art, Applications and Challenges.” *Briefings in Bioinformatics* 19 (6): 1256–72. https://doi.org/10.1093/bib/bbx062.
Mahu, M., Boyen, F., Canessa, S., Marchan, J.Z., Haesebrouck, F., Martel, A., Pasmans, F., 2017. “An Avirulent *Brachyspira hyodysenteriae* Strain Elicits Intestinal IgA and Slows down Spread of Swine Dysentery.” *Veterinary Research* 48 (1): 1–13. https://doi.org/10.1186/s13567-017-0465-y.
Mande, S.S., Mohammed, M.H., Ghosh, .S., 2012. “Classification of Metagenomic Sequences : Methods and Challenges.” *BRIEFINGS IN BIOINFORMATICS* 13 (6). https://doi.org/10.1093/bib/bbs054.
Manolio, T.A., Green, E.D., 2011. “Commentary Genomics Reaches the Clinic : From Basic Discoveries to Clinical Impact.” *Cell* 147 (1): 14–16. https://doi.org/10.1016/j.cell.2011.09.012.
Mappley, L.J., Black, M.L., AbuOun, M., Darby, A.C., Woodward, M.J., Parkhill, J., Turner, A.K., 2012. “Comparative Genomics of *Brachyspira pilosicoli* Strains: Genome Rearrangements, Reductions and Correlation of Genetic Compliment with Phenotypic Diversity.” *BMC Genomics* 13 (January): 454. https://doi.org/10.1186/1471-2164-13-454.
Mardis, E.R., 2008. “Next-Generation DNA Sequencing Methods.” *Annual Review of Genomics and Human Genetics* 9(1): 387–402. https://doi.org/10.1146/annurev.genom.9.081307.164359.
Marine, R.L., Magaña, L.C., Castro, C.J., Zhao, K., Montmayeur, A.M., Schmidt, A., Diez-Valcarce, M., 2020. “Comparison of Illumina MiSeq and the Ion Torrent PGM and S5 Platforms for Whole-Genome Sequencing of Picornaviruses and Caliciviruses.” *Journal of Virological Methods* 280: 1–36. https://doi.org/10.1016/j.jviromet.2020.113865.
Masterton, R., 2008. “The Importance and Future of Antimicrobial Surveillance Studies.” *Clinical Infectious Diseases* 47 (S1): S21–31. https://doi.org/10.1086/590063.
Mcadam, P.R., Holmes, A., Templeton, K.E., Fitzgerald. J.R., 2011. “Adaptive Evolution of *Staphylococcus aureus* during Chronic Endobronchial Infection of a Cystic Fibrosis Patient.” *PLoS ONE* 6 (9): 1–5. https://doi.org/10.1371/journal.pone.0024301.
McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., 2013. “The Comprehensive Antibiotic Resistance Database.” *Antimicrobial Agents and Chemotherapy* 57 (7): 3348–57. https://doi.org/10.1128/AAC.00419-13.
McOrist, S., Bennett, C., 2006. “Eradication of Swine Dysentery on Large-Scale Breeder Farms by Partial Depopulation/Medication.” In *20th International Pig Veterinary Society Congress*, 319. Durban, South Africa.
Mensah, N., Tang, Y., Cawthraw, S., Abuoun, M., Fenner, J., Thomson, N.R., Mather, A.E., Petrovska-Holmes, L., 2019. “Determining Antimicrobial Susceptibility in *Salmonella* Enterica Serovar Typhimurium through Whole Genome Sequencing: A Comparison against Multiple Phenotypic Susceptibility Testing Methods.” *BMC Microbiology* 19 (1): 1–10. https://doi.org/10.1186/s12866-019-1520-9.
Metzker, M.L., 2009. “Sequencing Technologies — the next Generation.” *Nature Reviews Genetics* 11 (1): 31–46. https://doi.org/10.1038/nrg2626.
Meziane-Cherif, D., Lambert, T., Dupêchez, M., Courvalin, P., Galimand, M., 2008. “Genetic

and Biochemical Characterization of OXA-63, a New Class D β-Lactamase from *Brachyspira pilosicoli* BM4442 .” *Antimicrobial Agents and Chemotherapy* 52 (4): 1264–68. https://doi.org/10.1128/AAC.00684-07.

Michalova, E., Novotna, P., Schlegelova, J., 2004. “Tetracyclines in Veterinary Medicine and Bacterial Resistance to Them.” *Veterinarni Medicina* 49 (3): 79–100. https://doi.org/10.17221/5681-VETMED.

Mikheyev, A.S., Mandy, T.M.Y., 2014. “A First Look at the Oxford Nanopore MinION Sequencer.” *Molecular Ecology Resources*, 1097–1102. https://doi.org/10.1111/1755-0998.12324.

MinION. 2017. “Oxford Nanopore Technologies.” 2017.

Mirajkar, N.S., Davies, P.R., Gebhart, C.J., 2016. “Antimicrobial Susceptibility Patterns of *Brachyspira* Species Isolated from Swine Herds in the United States.” *Journal of Clinical Microbiology* 54 (8): 2109–19. https://doi.org/10.1128/JCM.00834-16.

Mirajkar, N.S., Gebhart, C.J., 2014. “Understanding the Molecular Epidemiology and Global Relationships of *Brachyspira hyodysenteriae f*rom Swine Herds in the United States: A Multi-Locus Sequence Typing Approach.” *PLoS ONE* 9 (9). https://doi.org/10.1371/journal.pone.0107176.

Mirajkar, N.S., Phillips, N.D., La, T., Hampson, D.J., Gebhart, C.J., 2016. “Characterization and Recognition of *Brachyspira hampsonii* Sp. Nov., a Novel Intestinal Spirochete That Is Pathogenic to Pigs.” *Journal of Clinical Microbiology* 54 (12): 2942–49. https://doi.org/10.1128/JCM.01717-16.Editor.

Mirajkar, N.S., Bekele, A.Z., Chander, Y.Y., Gebhart, C.J., 2015. “Molecular Epidemiology of Novel Pathogen ‘ *Brachyspira hampsonii* ’ Reveals Relationships between Diverse Genetic Groups , Regions , Host Species , and Other Pathogenic and Commensal *Brachyspira* Species.” *Journal of Clinical Microbiology* 53 (9): 2908–18. https://doi.org/10.1128/JCM.01236-15.

Mirajkar, N.S., Gebhart,C.J., 2016. “Comparison of Agar Dilution and Antibiotic Gradient Strip Test with Broth Microdilution for Susceptibility Testing of Swine Brachyspira Species.” *Journal of Veterinary Diagnostic Investigation* 28 (2): 133–43. https://doi.org/10.1177/1040638716629154.

Molnar, L., 1996. “Sensitivity of Strains of *Serpulina hyodysenteriae* Isolated in Hungary to Chemotherapeutic Drugs.” *The Veterinary Record*. http://europepmc.org/abstract/med/8677605.

Moon, H.W., 1978. “Mechanisms in the Pathogenesis of Diarrhea: A Review.” *Journal of Veterinary Medicine Association* 172 (4): 443–48.

Mortimer-Jones, S.M., Phillips, N.D., La, T., Naresh, A., Hampson, D.J., 2008. “Penicillin Resistance in the Intestinal Spirochaete *Brachyspira pilosicoli* Associated with OXA-136 and OXA-137 , Two New Variants of the Class D b -Lactamase OXA-63.” *Journal of Medical Microbiology* 57 (2008): 1122–28. https://doi.org/10.1099/jmm.0.2008/001552-0.

Munita, J.M., Arias,C.A., 2016. “Mechanisms of Antibiotic Resistance.” *Microbiology Spectrum* 4 (2): 1–37. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.Mechanisms.

Mushtaq, M., Zubair, S., Råsbäck, T., Bongcam-rudloff, E., Jansson, D.S., 2015. “*Brachyspira suanatina* Sp. Nov., an Enteropathogenic Intestinal Spirochaete Isolated from Pigs and Mallards : Genomic and Phenotypic Characteristics.” *BMC Microbiology*, 1–13. https://doi.org/10.1186/s12866-015-0537-y.

Mutreja, A., Kim, D.W., Thomson, N., Connor, T.R., Hee, J., Lebens, M., Niyogi, S.K., 2013.

"Europe PMC Funders Group Evidence for Multiple Waves of Global Transmission within the Seventh Cholera Pandemic." *Nature* 477 (7365): 462–65. https://doi.org/10.1038/nature10392.Evidence.

Nakamura, A., Furuta, T., Shirai, N., Sugimoto, M., Kajimura, M., 2007. "Determination of Mutations of the 23S RRNA Gene of *Helicobacter pylori* by Allele Specific Primer-Polymerase Chain." *Gastroenterology* 22: 1057–63. https://doi.org/10.1111/j.1440-1746.2006.04546.x.

Nathues, H., Oliveira, C.J.B., Wurm, M., Beilage, E.G., Givisiez, P.E.N., 2007. "Simultaneous Detection of *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Lawsonia intracellularis* in Porcine Faeces and Tissue Samples by Multiplex-PCR." *Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine* 54 (9): 532–38. https://doi.org/10.1111/j.1439-0442.2007.00995.x.

Neef, N.A., Lysons, R.J., Troyt, D.J., Hampson, D.J., Jones, P.W., Morgan, J.H., 1994. "Pathogenicity of Porcine Intestinal Spirochetes in Gnotobiotic Pigs." *Infection and Immunity* 62 (6): 2395–2403.

Neo, E., La, T., Phillips, N.D., Alikani, M.Y., Hampson, D.J., 2013. "The Pathogenic Intestinal Spirochaete *Brachyspira pilosicoli* Forms a Diverse Recombinant Species Demonstrating Some Local Clustering of Related Strains and Potential for Zoonotic Spread." *Gut Pathogens* 11 (1): 1–8. https://doi.org/10.1186/s13099-019-0328-3.

Ochiai, S., Adachi, Y., Mori, K., 1997. "Unification of the Genera Serpulina and Brachyspira, and Proposals of *Brachyspira hyodysenteriae* Comb. Nov., *Brachyspira innocens* Comb. Nov. and *Brachyspira pilosicoli* Comb. Nov." *Microbiology and Immunology* 41 (6): 445–52. http://www.ncbi.nlm.nih.gov/pubmed/9251055.

Ohya, T., Sueyoshi, M., 2010. "In Vitro Antimicrobial Susceptibility of *Brachyspira hyodysenteriae* Strains Isolated in Japan from 1985 to 2009." *Bacteriolgy*, 2–4.

Olaitan, A.O.L., Thongmalayvong, B., Akkhavong, K., Somphavong, S., Paboriboune, P., Khounsy, S., Morand, S., Rolain, J.M., 2015. "Clonal Transmission of a Colistin-Resistant *Escherichia coli* from a Domesticated Pig to a Human in Laos." *The Journal of Antimicrobial Chemotherapy* 70 (12): 3402–4. https://doi.org/10.1093/jac/dkv252.

Olivares, J., Bernardini, A., Garcia-leon, G., Corona, F., Sanchez, M.B., Martinez, J.L., 2013. "The Intrinsic Resistome of Bacterial Pathogens." *Frontiers in Microbiology* 4 (April): 1–15. https://doi.org/10.3389/fmicb.2013.00103.

Olsoni, L.D., Fales, W.H., 1983. "Comparison of Stained Smears and Culturing for Identification of *Treponema hyodysenteriae*." *Journal of Clinical Microbiology* 18 (4): 950–55.

Ovotná, M.N,. Kardová,O.S., 2002. "*Brachyspira hyodysenteriae* : Detection , Identification and Antibiotic Susceptibility." *Veterinary Medicine* 2002 (4): 104–9.

Parizek, R., Stewart, R., Brown, K., Blevins, D., 1985. "Protection against Swine Dysentery with an Inactivated Treponema Hyodysentariae Bacterin." *Vet. Med.* 80: 80–86.

Parte, A.C., Sardà Carbasse, J., Meier-Kolthoff, L.C., Reimer, J.P., Göker, M., 2020. "List of Prokaryotic Names with Standing in Nomenclature (LPSN) Moves to the DSMZ." *International Journal of Systematic and Evolutionary Microbiology*. ijsem004332.

Patterson, A.H., Rubin, J.E., Fernando, C., Costa, M.O., Harding, J.C., Hill, J.E., 2013. "Fecal Shedding of *Brachyspira* Spp . on a Farrow-to-Finish Swine Farm with a Clinical History of ' *Brachyspira hampsonii* ' -Associated Colitis." *BMC Veterinary Research* 9 (1): 1. https://doi.org/10.1186/1746-6148-9-137.

Perez, J.B.D.S., Rubin, J.E., Fernando, C., Harding, J.C.S., Hill, J.E., 2016. "Characterization of

‘ *Brachyspira hampsonii* ’ Clades I and II Isolated from Commercial Swine in Western Canada.” *FACETS*, 163–72. https://doi.org/10.1139/facets-2016-0018.
PHAC. 2017. “Responsible Use of Medically Important Antimicrobials in Animals. from Https://Www.Canada.ca/En/Public-Health/Services/Antibiotic-Antimicrobial-Resistance/Animals/Actions/Responsible-Use-Antimicrobials.Html.” 2017.
Phillips, N.D., La, T., Adams, P.D., Harland, B.L., Fenwick, S.G., Hampson, D.J., 2009. “Detection of *Brachyspira hyodysenteriae* , *Lawsonia intracellularis* and *Brachyspira pilosicoli* in Feral Pigs.” *Veterinary Microbiology* 134: 294–99. https://doi.org/10.1016/j.vetmic.2008.08.006.
Pico, L., Szancer, J., Pique, J., Domeneque, A., Rodriguez-Sierra, A., Vidal, E., 2008. “Swine Dysentery Eradication Programme in a Large Farm with Three Site Production by Strategic Management and Medication.” In *In Proceedings of the 20th International Pig Veterinary Society Congress*, 131. Durban, South Africa,.
Poirel, L., Jayol, A., Nordmann, P., 2017. “Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes.” *Clinical Microbiology Reviews* 30 (2): 557–96.
Poupard, J.A., Walsh, L.R., Kleger, B., 1994. *Antimicrobial Susceptibility Testing: Critical Issues for the 90s*. Springer Science & Business Media,.
Prášek, J., Šperling, D., Lobová, D., Smola, J., Čížek, A., 2014. “Antibiotic Susceptibility of *Brachyspira hyodysenteriae* Isolates from Czech Swine Farms: A 10-Year Follow-up Study.” *Acta Veterinaria Brno* 83: 3–7. https://doi.org/10.2754/avb201382040003.
Prescott, J.F., 2013a. “Beta-Lactam Antibiotics: Penam Penicillins.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguere, John F Prescott, and Patricia M. Dowling, 5th ed., 135–52.
Prescott, J.F., 2013b. “Sulfonamides, Diaminopyrimidines, and Their Combinations.” In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F. Prescott, and Patricia M. Dowling, 5th ed., 279–94.
Pringle, M., Aarestrup, F.M., Bergsjø, B., Fossi, M., Jouy, E., Landén, A., Mevius, D., 2006. “Quality-Control Ranges for Antimicrobial Susceptibility Testing by Broth Dilution of the *Brachyspira hyodysenteriae* Type Strain (ATCC 27164T).” *Microbial Drug Resistance* 12 (3): 219–21.
Pringle, M., Landen, A., Franklin, A., 2006. “Tiamulin Resistance in Porcine *Brachyspira pilosicoli* Isolates.” *Research in Veterinary Science*. http://www.sciencedirect.com/science/article/pii/S0034528805000445.
Pringle, M., Landén, A., Franklin, A., 2006. “Tiamulin Resistance in Porcine *Brachyspira pilosicoli* Isolates.” *Research in Veterinary Science* 80 (1): 1–4. https://doi.org/10.1016/j.rvsc.2005.02.004.
Pringle, M., Poehlsgaard, J., Vester, B., Long, K.S., 2004. “Mutations in Ribosomal Protein L3 and 23S Ribosomal RNA at the Peptidyl Transferase Centre Are Associated with Reduced Susceptibility to Tiamulin in *Brachyspira* Spp. Isolates.” *Molecular Microbiology* 54 (5): 1295–1306. https://doi.org/10.1111/j.1365-2958.2004.04373.x.
Pringle, M., Fellström, C., Johansson, K., 2007. “Decreased Susceptibility to Doxycycline Associated with a 16S RRNA Gene Mutation in *Brachyspira hyodysenteriae*.” *Veterinary Microbiology* 123 (1–3): 245–48. https://doi.org/10.1016/j.vetmic.2007.02.019.
Pringle, M., Landén, A., Ericsson H., Molander, B., Bengtsson, B., 2012. “Antimicrobial Susceptibility of Porcine *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* Isolated in

Sweden between 1990 and 2010." *Acta Veterinaria Scandinavica* 54 (January): 54. https://doi.org/10.1186/1751-0147-54-54.

Prior, P., Fegan, M., 2005. "Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex." In *Bacterial Wilt Disease and the Ralstonia Solanacearum Species Complex*, edited by and A.C. Hayward C. Allen, P. Prior, 449–61. https://espace.library.uq.edu.au/view/UQ:71427.

Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Charles, W., Zagursky, R.J., Cocuzza, A.J., 1987. "Linked References Are Available on JSTOR for This Article : A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynuclcotides." *Science* 238 (4825): 336–41.

Prohaska, S., Pflüger, V., Ziegler, D., Scherrer, S., Frei, D., Lehmann, A., Wittenbrink, M.M., Huber, H., 2014. "MALDI-TOF MS for Identification of Porcine *Brachyspira* Species." *Letters in Applied Microbiology* 58 (3): 292–98. https://doi.org/10.1111/lam.12189.

Puttaswamy, S., Gupta, S.K., Regunath, H., Smith, L.P., Sengupta, S., 2018. "A Comprehensive Review of the Present and Future Antibiotic Susceptibility Testing (AST) Systems." *Archives of Clinical Microbiology* 09 (03): 1–9. https://doi.org/10.4172/1989-8436.100083.

Quail, M.A., Smith, M., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., Bertoni, A., Swerdlow, H.P., Gu, Y., 2012. "A Tale of Three next Generation Sequencing Platforms : Comparison of Ion Torrent , Pacific Biosciences and Illumina MiSeq Sequencers."

Rasback, T., Johansson, K.E., Jansson, D.S., Fellstro, C., Alikhani, M.Y., La, T., Dunn, D.S., Hampson, D.J., 2007. "Development of a Multilocus Sequence Typing Scheme for Intestinal Spirochaetes within the Genus *Brachyspira*." *Microbiology*, no. 2007: 4074–87. https://doi.org/10.1099/mic.0.2007/008540-0.

Råsbäck, T, Fellström, C., Bergsjø, B., Cizek, A., Collin, K., Gunnarsson, A., Jensen, S.M., 2005. "Assessment of Diagnostics and Antimicrobial Susceptibility Testing of *Brachyspira* Species Using a Ring Test." *Veterinary Microbiology* 109 (3–4): 229–43. https://doi.org/10.1016/j.vetmic.2005.05.009.

Råsbäck, T., Jansson, D.S., Johansson, K., Fellström, C., 2007. "A Novel Enteropathogenic , Strongly Haemolytic Spirochaete Isolated from Pig and Mallard , Provisionally Designated ' *Brachyspira suanatina* ' Sp . Nov ." *Environmental Microbiology* 9: 983–91. https://doi.org/10.1111/j.1462-2920.2006.01220.x.

Rfaty, E.O.,1999. "Analysis of Serpulina Hyodysenteriae Strain Variation and Its Molecular Epidemiology Using Pulsed-Field Gel Electrophoresis." *Epidemiology Infection* 123: 133–38.

Rodriguez-fonseca, C., Amils, R., Garrett, R.A., 1995. "Fine Structure of the Peptidyl Transferase Centre on 23 S-like RRNAs Deduced from Chemical Probing of Antibiotic-Ribosome Complexes." *Journal of Molecular Biology* 247: 224–35.

Rohde, J., Habighorst-blome, K., 2013. "Corrigendum to '" An up-Date on the Differentiation of *Brachyspira* Species from Pigs with Nox -PCR-Based Restriction Fragment Length Polymorphism "' [Veterinary Microbiology 158 (2012) 211 – 215]." *Veterinary Microbiology* 162 (1): 303. https://doi.org/10.1016/j.vetmic.2012.08.027.

Rohde, J., Kessler, M., Baums, C.G., Amtsberg, G., 2004. "Comparison of Methods for Antimicrobial Susceptibility Testing and MIC Values for Pleuromutilin Drugs for *Brachyspira hyodysenteriae* Isolated in Germany." *Veterinary Microbiology* 102 (1–2): 25–32. https://doi.org/10.1016/j.vetmic.2004.03.023.

Rohde, J., Rothkamp, A., Gerlach, G.F., 2002. "Differentiation of Porcine *Brachyspira* Species

by a Novel Nox PCR-Based Restriction Fragment Length Polymorphism Analysis." *Journal of Clinical Microbiology* 40 (7): 2598–2600. https://doi.org/10.1128/JCM.40.7.2598.

Rohde, J., Rubin, J.E., Kulathunga, D.G.R.S., Hill, J.E., Habighorst-Blome, K., Hampson, D.J., La, T., 2019. "Identification of *Brachyspira* Species by Cpn60 Universal Target Sequencing Is Superior to NADH Oxidase Gene Sequencing." *Veterinary Microbiology* 239 (June): 108454. https://doi.org/10.1016/j.vetmic.2019.108454.

Ronne, H., Szancer, J.,1990. "In Vitro Susceptibility of Danish Field Isolates of T*reponema hyodysenteriae* to Chemotherapeutics in Swine Dysentery (SD) Therapy. Interpretation of MIC Results Based on the Pharmacokinetic Properties of the Antimicrobial Agents." In *International Pig Veterinary Society, 11th Congress*, 129. Lausanne, Switzerland.

Ross, J.I., Eady, E.A., Cove, J.H., Jones, C.E., Ratyal, A.H., Miller, Y.W., Vyakrnam, S., 1997. "Clinical Resistance to Erythromycin and Clindamycin in Cutaneous Propionibacteria Isolated from Acne Patients Is Associated with Mutations in 23S RRNA." *Antimicrobial Agents and Chemotherapy* 41 (5): 1162–65.

Rossellb, R., Garcia-Valdks, E., Lalucat, J., Ursing, J., 1991. "Genotypic and Phenotypic Diversity of *Pseudomonas stutzer*." *Systematic Applied Microbiology* 8: 124–27.

Rothberg, J., 2011. "An Intergrated Semiconductor Device Enabling Non-Optical Genome Sequencing." *Nature* 475 (7456): 348–52. https://doi.org/10.3969/j.issn.1009-0002.2013.04.034.

Rubin, J.E., 2013. "Antimicrobial Susceptibility Testing Methods and Interpretation of Results." In *Antimicrobial Therapy in Veterinary Medicine*, edited by Steeve Giguère, John F Prescott, and Patricia M. Dowling, 5th ed., 11–20.

Rubin, J.E., Costa, M.O., Hill, J.E., Kittrell, Fernando, C., Huang, Y., Connor, O.,.Harding, J.C.S., 2013. "Reproduction of Mucohaemorrhagic Diarrhea and Colitis Indistinguishable from Swine Dysentery Following Experimental Inoculation with '*Brachyspira hampsonii*' Strain 30446." *PLoS ONE* 8 (2). https://doi.org/10.1371/journal.pone.0057146.

Rubin, J.E., Harms, J.N., Fernando, C., Soos, C., Detmer, S.E., Harding, J.C.S., Hill, J.E., 2013. "Isolation and Characterization of *Brachyspira* Spp. Including '*Brachyspira hampsonii*' from Lesser Snow Geese (Chen Caerulescens Caerulescens) in the Canadian Arctic." *Microbial Ecology* 66 (4): 813–22. https://doi.org/10.1007/s00248-013-0273-5.

Rugna, G., Bonilauri, P., Carra, E., Bergamini, F., Luppi, A., Gherpelli, Y., Magistrali, C.F., 2015. "Sequence Types and Pleuromutilin Susceptibility of *Brachyspira hyodysenteriae* Isolates from Italian Pigs with Swine Dysentery: 2003-2012." *Veterinary Journal* 203 (1). https://doi.org/10.1016/j.tvjl.2014.10.033.

Sandle, T., 2016. "Antibiotics and Preservatives." In *Pharmaceutical Microbiology, Essentials for Quality Asurance and Quality Control*, 171–83.

Sanger, F., Nicklen, S., Coulson. A.R., 1977. "DNA Sequencing with Chain-Terminating Inhibitors." *Proceedings of the National Academy of Sciences of the United States of America* 74 (4): 5463–67. https://doi.org/10.1097/00006250-199004001-00013.

Sch, A.C., Schaik, W.V., 2017. "Challenges and Opportunities for Whole-Genome Sequencing – Based Surveillance of Antibiotic Resistance." *Annals of the New York Academy of Sciences* 1388: 108–20. https://doi.org/10.1111/nyas.13310.

Schlünzen, F., Pyetan, E., Fucini, P., Yonath, A., Harms, J.M., 2004. "Inhibition of Peptide Bond Formation by Pleuromutilins : The Structure of the 50S Ribosomal Subunit from Deinococcus Radiodurans in Complex with Tiamulin." *Molecular Microbiology* 54 (5):

1287–94. https://doi.org/10.1111/j.1365-2958.2004.04346.x.
Schmeing, T.M., Ramakrishnan, V., 2009. “What Recent Ribosome Structures Have Revealed about the Mechanism of Translation.” *Nature* 461 (7268): 1234–42. https://doi.org/10.1038/nature08403.
Schmiedel, D., Loddenkemper, C., Ignatius, R., Wagner, J., Hammer, B., Petrich, A., Stein, H., Go, U.B., Schneider, T., 2009. “Rapid and Accurate Diagnosis of Human Intestinal Spirochetosis by Fluorescence In Situ Hybridization □.” *Journal of Clinical Microbiology* 47 (5): 1393–1401. https://doi.org/10.1128/JCM.02469-08.
Schmith, K., Reymann, F.E., 1940. “Expeimentelle Og Kliniske Undersogelser over Gonococcers Folsomhed Overfor Sulfapyridin.” *Nordisk Medicin* 8: 2493–99.
Schwartz, K., 2011. “*Brachyspira*: What’s Happening in Iowa and Why.” In *The Western Canadian Association of Swine Veterinarians Conference*, Vol 25,P 33.
Schwarz, S., Loeffler, A., Kadlec, K., 2017. “Bacterial Resistance to Antimicrobial Agents and Its Impact on Veterinary and Human Medicine.” *Advances in Veterinary Dermatology* 28(1): 82-e19. https://doi.org/10.1111/vde.12362.
Senol C., Damla, J.S.K., Ghose, S., Alkan, C., Mutlu, O., 2018. “Nanopore Sequencing Technology and Tools for Genome Assembly: Computational Analysis of the Current State, Bottlenecks and Future Directions.” *Briefings in Bioinformatics* 20 (4): 1542–59. https://doi.org/10.1093/bib/bby017.
Shaikh, S.A, 2011. “Measures Derived from a 2 x 2 Table for an Accuracy of a Diagnostic Test.” *Journal of Biometrics & Biostatistics* 02 (05): 2–5. https://doi.org/10.4172/2155-6180.1000128.
Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S,.B.H., Hood, L.E., 1986. “Fluorescence Detection in Automated DNA Sequence Analysis.” *Nature* 321: 674–79.
Smith, R.D., Slenning, B.D., 2000. “Decision Analysis: Dealing with Uncertainty in Diagnostic Testing.” *Preventive Veterinary Medicine* 45 (1–2): 139–62. https://doi.org/10.1016/S0167-5877(00)00121-5.
Song, Y., La, T., Phillips, N.D., Bellgard, M.I., Hampson, D.J., 2009. “A Reverse Vaccinology Approach to Swine Dysentery Vaccine Development.” *Veterinary Microbiology* 137: 111–19. https://doi.org/10.1016/j.vetmic.2008.12.018.
Songer, J.G., Glock, R.D., Schwartz, K.J., Harris, D.L., 1978. “Isolation of Treponema Hyodysenteriae from Sources Other than Swine.” *J. Am. Vet. Med. Assoc.* 172: 464–66.
Songer, J.G., Kinyon, K., Harris, D.L., 1976. “Selective Medium for Isolation of *Treponema hyodysenteriae*.” *Journal of Clinical Microbiology* 4 (1): 57–60.
Sperling, D., Smola, J., Cízek, A., 2011. “Characterisation of Multiresistant *Brachyspira hyodysenteriae* Isolates from Czech Pig Farms.” *The Veterinary Record* 168 (8): 215. https://doi.org/10.1136/vr.c4247.
Stanton, T.B., Jensen, N.S., Casey, T.A., Tordoff, L.A., Dewhirst, F.E., Paster, B.J., 1991. “Reclassification of *Treponema hyodysenteriae* and *Treponerna innocens* in a New Genus, Serpula Gen. Nov., as *Serpula hyodysenteriae* Comb. Nov. and *Serpula innocens* Comb. Nov.” *Int. J. Syst. Bacteriol.* 41: 51–58.
Stanton, T.B., Lebo, D.F., 1988. “*Treponema hyodysenteriae* Growth under Various Culture Conditions.” *Veterinary Microbiology* 18: 177–90.
Stanton, T.B, Postic, D., Girons, I.S., Stanton E.T., 1997. “Recognition of Two New Species of Intestinal and *Serpulina murdochii* Sp . Nov .” *International Jornal of Systemic*

Bacteriology 47 (4): 1007–12.
Stanton, T.B., Postic, D., Jensenl, N.S., 1998. "*Serpulina alvinipulli* Sp. Nov., a New *Serpulina* Species That Is Enteropathogenic for Chickens." *International Journal of Systematic Bacteriology* 1 (1 998): 669–76.
Stanton, T.B., 2006. "The Genus *Brachyspira*." In *The Prokaryotes*, 330–56. Springer, New York, NY,.
Stanton, T.B., 1982. "Proposal To Change the Genus Designation Serpula to *Serpulina* Gen . Nov . Containing the Species *Serpulina hyodysenteriae* Comb . Nov . and *Serpulina innocens* Comb . Nov ." *International Journal Oif Systematic Bacteriology*, 189–90.
Stanton, T.B., Jensen, N.S., 1993. "Monitoring Experimental Swine Dysentery : Rectal Swab Blood Test and *Serpulina* (Treponema) *hyodysenteriae* Detection." *Veterinary Microbiology* 34: 389–96.
Stanton, T.B., Rosey, E.L., Kennedy, M.J., Jensen, N.S., Bosworth, T.B., 1999. "Isolation , Oxygen Sensitivity , and Virulence of NADH Oxidase Mutants of the Anaerobic Spirochete *Brachyspira* (Serpulina) *hyodysenteriae* , Etiologic Agent of Swine Dysentery" 65 (11): 5028–34.
Stranneheim, H., Lundeberg, S., 2012. "Stepping Stones in DNA Sequencing." *Biotechnology Journal* 7 (9): 1063–73. https://doi.org/10.1002/biot.201200153.
Stubberfield, E., Pringle, M., Landén, A., Veldman, K.T., Geurts, Y., Jouy, E., Devendec, L.L., 2020. "Validation of an Antimicrobial Susceptibility Testing Protocol for *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in an International Ring Trial." *Veterinary Microbiology* 244 (February): 0–6. https://doi.org/10.1016/j.vetmic.2020.108645.
Suh, D.K., Song, J.C., 2005. "Prevalence of Lawsonia Intracellularis, *Brachyspira hyodysenteriae* and *Salmonella* in Swine Herds." *Journal of Veterinary Science* 4: 289–93.
Sutton, S., 2011. "Measurement of Microbial Cells by Optical Density." *Journal of Validation Technology* 17(1): 46–49.
SVA. 2011. "VetMIC Brachy for Antimicrobial Susceptibility Testing of *Brachyspira* Spp. [Product Insert]." Available from.
SVARM. 2013. "Swedish Veterinary Antimicrobial Resistance Monitoring."
Taylor, D.J., Alexander, T.A., 1971. "The Production of Dysentery in Swine by Feeding Cultures Containing a Spirochaete." *The British Veterinary Journal* 127 (11): 58–61. https://doi.org/10.1016/S0007-1935(17)37282-2.
Tenover, F.C., 2009. "Antibiotic Susceptibility Testing." In *Encyclopedia of Microbiology*, edited by Moselio Schaechter, Third Edit, 67–77.
Tenover, F.C., 2006. "Mechanisms of Antimicrobial Resistance in Bacteria." *American Journal of Infection Control* 119. https://doi.org/10.1016/j.amjmed.2006.03.011.
Thomson, J.R., Friendship, R.M., 2012. "Digestive System." In *Diseases of Swine*, edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, and Gregory W. Stevenson, 10th ed., 199–226.
Torrison, L.J., 2012. "Optimizing Diagnostic Value and Sample Collection." In *Diseases of Swine*, edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, and Gregory W. Stevenson, 10th ed., 67–76.
Treangen, T.J., Salzberg,S.T., 2012. "Erratum: Repetitive DNA and next-Generation Sequencing: Computational Challenges and Solutions (Nature Reviews Genetics (2012) 13 (36-46))." *Nature Reviews Genetics* 13 (2): 146. https://doi.org/10.1038/nrg3164.
Trevethan, R., 2018. "Commentary: Sensitivity, Specificity, and Predictive Values: Foundations,

Pliabilities, and Pitfalls in Research and Practice." *Frontiers in Public Health* 6 (November): 1–7. https://doi.org/10.3389/fpubh.2018.00256.
Trivett-moore, N.L., Gilbert, G.L., Law, C.L.H., Trott, D.J., Hampson,D.J., 1998. "Isolation of *Serpulina pilosicoli* from Rectal Biopsy Specimens Showing Evidence of Intestinal Spirochetosis." *Journal of Clinical Microbiology* 36 (1): 261–65.
Trott, D.J., Atyeo, R.F., Lee, J.I., Swayne, D.A., Stoutenburg, J.W., Hampson, D.J., 1996. "Genetic Relatedness amongst Intestinal Spirochetes Isolated from Rats and Birds." *Letters in Applied Microbiology*, 431–36.
Uezato, K., Kinjo, E., Adachi, Y., 2004. "In Vitro Susceptibility of 21 Antimicrobial Agents to 37 Isolates of *Brachyspira hyodysenteriae* Isolated from Pigs in Okinawa Prefecture." *Journal of Veterinary Medical Science* 66 (3): 307–9. https://doi.org/10.1292/jvms.66.307.
Valdivia, E., 2007. "Fast , Convenient , and Economical Method for Isolating Genomic DNA from Lactic Acid Bacteria Using a Modification of the Protein '" Salting-out "' Procedure." *Analytical Biochemistry* 366: 102–4. https://doi.org/10.1016/j.ab.2007.03.010.
Verlinden, M., Boyen, F., Pasmans, F., Garmyn, A., Haesebrouck, F., Martel, A., 2011. "Antimicrobial Susceptibility Pattern of *Brachyspira intermedia* Isolates from European Layers." *Microbial Drug Resistance (Larchmont, N.Y.)* 17 (3): 485–88. https://doi.org/10.1089/mdr.2011.0082.
Vester, B., Douthwaite, S., 2014. "MINIREVIEW : Macrolide Resistance Conferred by Base Substitutions in 23S RRNA Macrolide Resistance Conferred by Base Substitutions in 23S RRNA." *Antimicrobial Agents and Chemotherapy*, no. June: 1–12. https://doi.org/10.1128/AAC.45.1.1.
Vincent, A.T., Derome, N., Boyle, B., Culley, A.I., Charette, S.J., 2017. "Next-Generation Sequencing (NGS) in the Microbiological World: How to Make the Most of Your Money." *Journal of Microbiological Methods* 138: 60–71. https://doi.org/10.1016/j.mimet.2016.02.016.
Vincent, G., 1942. "Filter Paper Disk Modification of the Oxford Cup Penicillin Determination." In *Proceeding of the Society of Experimental Biology and Medicine*, 162–64.
Viola, C., Devincent, S.J., 2006. "Overview of Issues Pertaining to the Manufacture , Distribution , and Use of Antimicrobials in Animals and Other Information Relevant to Animal Antimicrobial Use Data Collection in the United States" 73: 111–31. https://doi.org/10.1016/j.prevetmed.2005.09.020.
Wang, G.E., Taylor, D.E., 1998. "Site-Specific Mutations in the 23S RRNA Gene of *Helicobacter pylori* Confer Two Types of Resistance to Macrolide-Lincosamide-Streptogramin B Antibiotics" 42 (8): 1952–58.
Wangkumhang, P., Chaichoompu, K., Ngamphiw, C., Ruangrit, U., Chanprasert, J., Assawamakin, A., Tongsima, S., 2007. "WASP : A Web-Based Allele-Specific PCR Assay Designing Tool for Detecting SNPs and Mutations" 9: 1–9. https://doi.org/10.1186/1471-2164-8-275.
Watson, J.D., Crick, F.H.C., 1953. "Molecular Structure of Nucliec Acids." *Nature* 171: 737–38.
Whiting, R.A., Doyle, L.P.,Spray, R.S., 1921. "Swine Dysentery. Purdue University Agricultural Experimental Station." https://doi.org/10.1177/030098587901600409.
WHO. 1961. "Standerdization of Methods for Conducting Microbic Sensitivity Test. Second Report of the Expert Committee on Antibiotics. World Health Organization Technical Report Series, No 210." WHO, Geneva.
Wilberts, B.L., Warneke, H.L., Bower, L.P., Kinyon, J.M., Burrough, E.R., 2015. "Comparison

of Culture, Polymerase Chain Reaction, and Fluorescent in Situ Hybridization for Detection of *Brachyspira hyodysenteriae* and '*Brachyspira hampsonii*' in Pig Feces." *Journal of Veterinary Diagnostic Investigation* 27 (1): 41–46. https://doi.org/10.1177/1040638714563064.

Wilcoc, B.P., Oland, H.J., 1979. "Studies on the Pathogenesis of Swine Dysentery." *Veterinary Pathology* 16: 450–65.

Xiao, T., Zhou, W., 2020. "The Third Generation Sequencing: The Advanced Approach to Genetic Diseases." *Translational Pediatrics* 9 (2): 163–73. https://doi.org/10.21037/TP.2020.03.06.

Xiong, L., Shah, S., Mauvais, P., 1999. "A Ketolide Resistance Mutation in Domain II of 23S RRNA Reveals the Proximity of Hairpin 35 to the Peptidyl Transferase Centre." *Molecular Microbiology* 31 (2): 633–39.

Zmudzki, J., Szczotka, A., Nowak, A., Strzelecka, H., Grzesiak, A., Pejsak, Z., 2012. "Antimicrobial Susceptibility of *Brachyspira hyodysenteriae* Isolated from 21 Polish Farms." *Polish Journal of Veterinary Sciences* 15 (2): 259–65. https://doi.org/10.2478/v10181-011-0143-3.

Zuernerl, R.L., Stanton, T.B., 1994. "Physical and Genetic Map of the *Serpulina hyodysenteriae* B78T Chromosome." *Journal of Bacteriology* 176 (4): 1087–92.