2017

Methods for improving diagnostic techniques used for the identification and isolation of Brachyspira species from swine

Hallie Warneke
Iowa State University

Methods for improving diagnostic techniques used for the identification and isolation of *Brachyspira* species from swine

by

Hallie L Warneke

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Preventive Medicine

Program of Study Committee:
Eric R Burrough, Major Professor
Timothy S Frana
Annette M O'Connor

The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER 2 TABLES

CHAPTER 3 TABLES

ACKNOWLEDGMENTS

I would like to thank my committee chair, Eric R. Burrough, and my committee members, Timothy S. Frana, and Annette M O'Connor for their expertise, guidance, encouragement and support throughout the course of this research. I would especially like to thank Joann M. Kinyon for her mentorship and friendship. I would also like to thank my friends, colleagues, the department faculty and staff for making my time at Iowa State University a wonderful experience. Additionally, I would like to thank my family and friends for their continued support and encouragement through this process without which I would not have been able to complete this part of my education.

ABSTRACT

Brachyspira hyodysenteriae is the main etiological agent of swine dysentery and is an important species for ongoing research into the field. Swine dysentery (SD) is also caused by *Brachyspira hampsonii* and *Brachyspira suanatina*, which have recently been accepted as new species. These three species are all classified as strong beta species with a positive ring phenomenon. *Brachyspira hampsonii* includes 4 genetically distinguishable groups. Weak beta *Brachyspira* species include *Brachyspira pilosicoli* (the agent of porcine intestinal spirochetosis), *Brachyspira intermedia, Brachyspira murdochii,* and *Brachyspira innocens* that all have a negative ring phenomenon. Techniques used for diagnostic and research testing have been consistent but limited to PCR and sequencing that is expensive to producers and researchers. With the advent of MALDI-TOF MS systems in veterinary laboratories, adding *Brachyspira* species to the database would be beneficial. A total of 33 *Brachyspira* species were added to a MALDI-TOF MS database, which included the strains: *B. hyodysenteriae, B. hampsonii* clades I and II, *B. pilosicoli, B. intermedia, B. murdochii,* and *B. innocens*. After addition to the database, 176 field isolates were identified and compared using MALDI-TOF MS and *nox* sequencing, the gold standard for *Brachyspira* identification. From the field isolates, 98.9% matched species identification by both methods. Additionally, 92% of the *B. hampsonii* isolates matched clade designation by both methods.

In addition to improving identification techniques for *Brachyspira*, sampling techniques are another potential area for improvement. The gold standard for *Brachyspira* sampling is enteric samples. The prevalence of SD is low, therefore many animals have to be sampled for detection of *Brachyspira*. The introduction of oral fluids as an environmental sample has led to improvements in sampling for other organisms, and could be potentially

applied to detecting the agents of swine dysentery and other *Brachyspira* species. To establish if oral fluids could be used as a sample matrix for *Brachyspira* species, in vitro and in vivo investigations were completed. The first such investigation enlisted spiked samples of oral fluids in comparison to runny feces, solid feces, and a phosphate buffered saline control. In this study, *Brachyspira* was able to be isolated from oral fluids. Coincidentally, oral fluids allowed better survival of the organism over a 72 hour period when kept at refrigerated temperatures compared to runny feces and solid feces. Two additional investigations were performed to determine if *Brachyspira* could be recovered from animals that were experimentally infected with pre-determined species of *Brachyspira*. Again, all *Brachyspira* spp. inoculated were recovered from oral fluids. Finally, a field trial was completed to see if oral fluids could be used to isolate *Brachyspira* from animals of unknown infection status. A total of 20 pens were tested with feces and oral fluids by both culture and PCR. Five pens were culture positive for *B. hyodysenteriae* (4 via feces and 1 via oral fluid), and surprisingly, all 5 pens were positive for *B. hyodysenteriae* by PCR of the oral fluid.

The experiments described herein provide an expanded database for identification of porcine *Brachyspira* using MALDI TOF-MS and suggest that oral fluids are not suitable for culture of *Brachyspira* from field samples but could be used for PCR. Accordingly, fecal cultures can be supplemented with PCR of oral fluids for detection of *Brachyspira* from group-housed swine.

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

An abbreviated history of *Brachyspira* and diagnostic techniques

Swine dysentery (SD) was first described in 1921 as a mucohemorrhagic diarrhea leading to emaciation when prolonged disease occurs.[2] Animals with SD begin with soft feces with or without blood that then progresses to watery feces containing blood, mucus, and fibrinous exudate.[18] Through these materials, the agent of SD is shed. Since the first description of SD, it has gone through phases of dormancy and reemergence. Specifically, in the 1980s, an outbreak occurred in North America and during this period, the agent of SD was isolated, characterized and designated *Treponema hyodysenteriae*.[22] The etiological agent has undergone multiple name changes including *Serpula hyodysenteriae* in 1991,[50] *Serpulina hyodysenteriae* in 1992,[47] and finally to what it is known now as *Brachyspira hyodysenteriae* in 1997.[36] The next significant outbreak of SD occurred in 2008 and led to the discovery of a new species of *Brachyspira* as an additional etiologic agent. Through much investigation, the newly described agent was designated *Brachyspira hampsonii*.[33] It was originally thought that there were two clades within *B. hampsonii*;[33] however, with more thorough exploration it was determined that *B. hampsonii* includes four genetically distinguishable groups (I-IV).[31] A third agent of SD isolated in Europe, designated *Brachyspira suanatina,*[34] is thus far of little diagnostic significance in North America in pig rearing areas because *B. suanatina* has only been identified in Denmark and Sweden.[42] These three *Brachyspira* spp. are currently the only known agents of SD; however, the emergence of two novel agents in the last decade suggests testing methods for SD should be comprehensive enough to detect novel species and strains.[7] SD is manageable with treatment, but still causes significant financial loss to producers. Therefore, research into improved diagnostic

capabilities is relevant and ongoing with advances in technology and knowledge. Rapid diagnosis of disease reduces the losses producers incur from the disease.

Another lesser pathogenic *Brachyspira* species is *Brachyspira pilosicoli*,[18] the agent of porcine intestinal spirochetosis (PIS), which isn't as economically significant as SD in pigs. Pigs do not get as severe a disease reaction as with SD. *B. pilosicoli* is known to cause disease in other animal species including birds,[11,51] dogs,[14] rodents[4] and humans.[21] *B. pilosicoli* most commonly causes avian intestinal spirochetosis (AIS) in laying hens and affects egg production.[28] Other porcine *Brachyspira* species include: *B. murdochii, B. intermedia,* and *B. innocens*.[19] These species are considered to be commensals in pigs but are indistinguishable phenotypically when grown in culture. Other *Brachyspira* species include *B. alvinipulli*[35] and *B. pulli.*[30] These species are considered non-pathogenic in avian and other animal species. There has been some evidence that *B. murdochii* has the potential for being a pathogen in pigs[25] in addition to *B. intermedia*[53] in avian species.

Brachyspira culture characteristics

Brachyspira are oxygen tolerant anaerobic spirochetes that colonize the large intestines in the animal species listed above and a few others.[19] Isolation of *Brachyspira* from infected tissues is aided by the use of selective media. Typically tryptic soy agar containing 5% ovine or bovine blood is supplemented with the antibiotics colistin, vancomycin, spectinomycin, spiramycin, and rifampin.[1] The combination of colistin, vancomycin, and spectinomycin make up the commonly used CVS[24] that is the ideal choice when culturing *Brachyspira* species. The addition of spiramycin and spectinomycin to the CVS agar further enhance the selectivity and make up the medium commonly known as BJ agar.[26] Other agar mediums have been described including BAM-SR[8,9] and SR;[48] however, CVS and BJ are the most commonly used for isolation

of *Brachyspira* species from porcine diagnostic specimens. Culture on agar plates can take from 6-10 days for suspects to grow.[19] Suspects will appear as areas of hemolysis in the agar. There are two types of hemolysis that *Brachyspira* will produce: one of which is called "strong-beta hemolysis" and can be described as a bright hemolysis; the other type of hemolysis is "weak-beta hemolysis" and can be described as a duller less transparent hemolysis.[19] Another phenotypic characteristic of *Brachyspira* is the presence or absence of the "ring-phenomenon". The ring-phenomenon occurs when agar plugs are manipulated and removed from an area of hemolytic growth and the plates with the plugs removed are re-incubated. After 2 days of additional incubation, a positive ring-phenomenon will look like there has been additional uniform hemolysis occurring around the site of the agar plug removal. A negative ring-phenomenon will have no additional hemolysis around the agar plug. In some instances, a false positive ring-phenomenon can appear if the agar around the site where the plug was removed is moved and air is allowed under the agar. *B. hyodysenteriae, B. hampsonii,* and *B. suanatina* all have strong-beta hemolysis with a positive ring-phenomenon making differentiation impossible by phenotypic features alone.[14] *B. pilosicoli, B. murdochii, B. intermedia, B. innocens, B. alvinipulli,* and *B. pulli* all have weak-beta hemolysis with no ring-phenomenon making it impossible to differentiate between these species on growth characteristics alone as well.[14]

Brachyspira identification

Biochemical tests were developed to differentiate between the *Brachyspira* species,[14] but currently they are not as widely used due to inter-laboratory variability in methods. The biochemicals that can be used to differentiate *Brachyspira* into groups are indole production,[52] hippurate hydrolysis,[45] α-galactosidase activity, α-glucosidase activity, and ß-glucosidase

activity.[23] Biochemical tests sort *Brachyspira* into four groups (1-4) with group 3 including three subgroups (a-b). Group 1 includes only strong-beta hemolysis (*B. hyodysenteriae*) and the other groups are all weak-beta hemolysis species.[14] A more reliable method of identification of *Brachyspira* species is polymerase chain reaction (PCR) testing. PCR testing is a more specific method to differentiate between *Brachyspira* species. PCR testing can be limited to a single *Brachyspira* species,[13,20] or can include multiple species in a multiplex assay.[27,54] In an effort to screen diagnostic specimens for *Brachyspira* species, PCR testing was developed to differentiate between the pathogenic species of *Brachsypira*.[2,13,29] However, the problem with the specific pathogen PCR assays and other multiplex *Brachyspira* PCR assays is that they do not include all species of *Brachyspira* relevant to swine in one assay. Performing multiple PCR assays to determine the species of *Brachyspira* present in samples is not practical from a diagnostic laboratory standpoint.

Other molecular testing techniques for identification of *Brachyspira* species are available including *nox,*[43] 16S rRNA,[18] and 23S rRNA gene sequencing.[6] These sequencing techniques were only used if a consensus ID could not be reached by other methods, if isolates were to be completely characterized, or used only for research purposes. The current gold standard for determining the *Brachyspira* species is *nox* sequencing because the gene is well-conserved[3,49] among the genus *Brachyspira,* although the other methods are used when further characterization is required. More recently, multilocus sequence typing (MLST)[32] has been used to categorize and group *Brachyspira* species and to reveal interesting epidemiologic

relationships. A simpler, more practical method of identification has been utilized for other bacteria in the form of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).[5,46]

MALDI-TOF MS systems were originally developed for use in human health, but increasingly are being used more commonly in the veterinary field.[12,17] From a diagnostic standpoint, MALDI-TOF MS is less expensive than any molecular technique and therefore, more practical for producers. One of the advantages of MALDI-TOF MS systems is that the database can be customized and user-defined by the addition of mass-spectral profiles (MSP) to the library of organisms available. Once MSPs of characterized *Brachyspira* species are added to the library, then isolates can be tested against those MSPs. The main concern of MALDI-TOF MS would be the ability to differentiate between all species of *Brachyspira* and differentiate between the groups of *B. hampsonii*. One of the first groups to successfully add *Brachyspira* species to a MALDI-TOF MS database was in Switzerland.[40] This group added 30 *Brachyspira* strains to the database and tested 67 field isolates that were also identified by *nox* sequencing and biochemical tests. Genus grouping and species identification were homologous across all methods with 100% accuracy. The second group to successfully add *Brachyspira* strains and correctly identify field isolates was a group from Italy.[10] This group added 9 *Brachyspira* strains to the database and tested 56 field isolates. While both groups were successful in adding *Brachyspira* strains to the database they did not include *B. hampsonii.* The objective of this study would be to include this species as well and be able to distinguish between relevant groups within *B. hampsonii*. This would greatly increase the capabilities of the database and is the first objective in this masters thesis.

Brachyspira detection

The second goal of this thesis is to investigate a way to improve the efficiency of diagnostic sampling for group-housed pigs that are suspected to have SD or in surveillance screening for agents of SD. Also, from an epidemiological standpoint, knowing the prevalence of SD in the herd would be beneficial to understanding the incidence of disease in that herd or flow of animals. Because SD is a disease that affects the large intestine of pigs, samples are generally taken from the colon, cecum, or feces.[19] Fecal swabs are easy to collect from live animals without having to sacrifice a pig for sampling of the colonic mucosa; however, colonic tissue remains the ideal sample type allowing one to scrape away the luminal contents and sample the mucus layer and underlying, mucosa.[19] Because this is not a practical way to test if a herd is infected or colonized with agents of SD, rectal swabs are commonly collected. One of the problems with this is that only individual animals are tested. In theory, every animal in a herd should be tested to determine herd status, but as there are usually hundreds to thousands of animals in a herd, this is not practical. Culture of fecal samples is currently the gold standard for detection of *Brachyspira*; however, it is time consuming and labor intensive compared with molecular methods.

A more recent development in animal sampling is collection of oral fluids for population testing.[41] Oral fluids have been successfully used for detection of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Circovirus type 2 (PCV2), and Influenza A virus.[37,38,39,44] With the ease in which samples are collected, they may be useful for testing enteric pathogens, including *Brachyspira*. Oral fluids are collected by hanging a rope in a pen of pigs for approximately 20 minutes. The animals are allowed to chew on the rope and after the rope has hung for adequate time, the absorbed fluid is then wrung out into a clean plastic bag,

tube, or other sample collection device and sent to the laboratory for testing. It is assumed that for enteric pathogens, the pigs would root around in their feces then chew on the rope and consequently transfer the enteric organisms via their mouths. Essentially, this allows the animal to do their own "sampling." This would reduce the amount of time and people it would take to sample animals. In addition to being a more efficient sampling method, oral fluids have the benefit of being an environmental sample that is reflective of the pen rather than an individual animal. This is an important epidemiological benefit in tracking disease and prevalence of a pathogen in a herd. As stated earlier, testing methods for agents of SD and other *Brachyspira* spp. should be broad and able to detect differences in disease patterns, variability in phenotypical characteristics, and the potential atypical and novel species and strains. The advent of oral fluids as a composite sample for group-housed swine may make it easier to screen populations for agents of SD.

The work described herein will address potential areas of improvement for diagnostic testing related to agents of SD (MALDI-TOF MS and Oral Fluids) in chapters 2 and 3, respectively. These data are directly applicable to *Brachyspira* research and diagnostics and expand the array of diagnostic testing available for agents of SD.

CHAPTER 2

Brief Research Report

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for rapid identification of *Brachyspira* species isolated from swine, including the newly described "*Brachyspira hampsonii*"

Journal of Veterinary Diagnostic Investigation 2014, Vol. 26(5) 635–639
 DOI: 10.1177/1040638714541114
jvdi.sagepub.com

Hallie L. Warneke,[1] Joann M. Kinyon, Leslie P. Bower, Eric R. Burrough, Timothy S. Frana

From the Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA.

[1]Corresponding Author: Hallie L. Warneke, Veterinary Diagnostic Laboratory Iowa State University, 1600 South 16th Street, Ames, IA 50011. hwarneke@iastate.edu

Abstract

The *Brachyspira* species traditionally associated with swine dysentery and other diarrheal diseases in pigs are *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, and, to a lesser extent, *Brachyspira murdochii*. "*Brachyspira hampsonii*" is a recently proposed novel species that causes clinical disease similar to that caused by *B. hyodysenteriae*. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems are increasingly available in veterinary diagnostic laboratories, are less expensive, and are faster than traditional microbiological and molecular methods for identification. Thirty-three isolates associated with *Brachyspira* species of importance to swine were added to an existing MALDI-

TOF MS database library. In total, species included in the library were: *B. hyodysenteriae*, "*B. hampsonii*" clades I and II, *Brachyspira innocens*, *Brachyspira intermedia*, *B. murdochii*, and *B. pilosicoli*. A comparison between MALDI- TOF MS and *nox* sequencing was completed on 176 field isolates. Of the 176 field isolates, 174 (98.9%) matched species identification by both methods. Thirty field isolates were identified by both methods as "*B. hampsonii*". Twenty-seven of the 30 (90%) "*B. hampsonii*" field isolates matched clade designation in both assays. The *nox* sequencing identified 26 as "*B. hampsonii*" clade I and 4 as clade II. Comparatively, MALDI-TOF MS identified 25 of the 30 as "*B. hampsonii*" clade I and 5 as clade II. The current study indicates MALDI-TOF MS is a reliable tool for the identification of swine *Brachyspira* species; however, final clade designation for "*B. hampsonii*" may still require molecular techniques.

Key words: *Brachyspira*; matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Brachyspira spp. commonly recovered from swine feces include the agents of swine dysentery and other diarrheal diseases and species traditionally associated with disease in pigs. Such species include *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, and, to a lesser extent, *Brachyspira murdochii.*[11] "*Brachyspira hampsonii*" is a recently proposed novel species associated with mucohemorrhagic diarrhea in swine,[6] a disease indistinguishable from swine dysentery, which has been experimentally reproduced following infection with representative strains of this pathogen.[3,15]

Brachyspira are spiral-shaped, Gram-negative, obligate anaerobic bacteria.[9] Common methods used to identify *Brachyspira* spp. include phenotypic and biochemical analysis,[7] targeted polymerase chain reaction (PCR) assays, and *nox* sequencing.[1,14] Previously, it was

discovered that species identification via 16S ribosomal DNA (rDNA) gene sequencing was not as useful as *nox* sequencing because of the high homology of the 16S rDNA gene sequence between species.[14] However, *nox* sequencing revealed higher sequence diversity between species and thus greater species discrimination.[14]

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) bacterial identification systems are increasingly available and being used in veterinary diagnostic laboratories. Some advantages of these systems include their ability to identify bacterial isolates quickly, accurately, and inexpensively.[2,16] Another advantage is the ability to add mass spectral profiles (MSP) to a user-defined library increasing the available profiles for comparison and identification. The database library provided with one of the MALDI-TOF MS[a] systems used in the current study includes only 1 strain of *B. murdochii* and 2 strains of *B. pilosicoli*. Previous work has been done to create user-defined libraries which include *Brachyspira* species[5,13]; however, profiles of "*B. hampsonii*" have been omitted up to this point. The present report describes the creation of a user-defined MSP library that includes the major *Brachyspira* species commonly isolated from swine, including "*B. hampsonii*", and the use of this library to rapidly identify field isolates of *Brachyspira* from swine samples.

The 33 *Brachyspira* strains to be included in the user-defined library were selected based on phenotypic cultural characteristics followed by species identification using a combination of *nox*, 16S rDNA, and/or 23S rDNA sequencing. The strains are part of a culture collection maintained at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL; Ames, Iowa). The collection was started in 1974 and includes historical strains and more recent isolates from cases of swine diarrhea received for diagnostic microbiology. All isolates were cloned 3 times by single colony picks before inclusion in the culture collection. In addition, the

isolates were characterized by colony characteristics and molecular methods. At least 5 strains from each *Brachyspira* species were chosen except for *B. pilosicoli* and *B. intermedia* where 4 and 2 strains were used, respectively. The strains are detailed in Table 1. *Brachyspira* cultures were performed at 42°C in anaerobic jars[b] utilizing gas generators.[c] Surface growth from each strain was harvested from trypticase soy agar (TSA) containing 10% bovine blood after 2–6 days of anaerobic incubation into 0.85% physiological saline. One milliliter of the bacterial suspension was transferred to a centrifuge tube and centrifuged (17,968 × *g*, 2 min) to form a bacterial pellet. The pellet was resuspended in 300 µl of sterile water. Subsequently, 900 µl of 100% ethanol was added to the suspension and mixed thoroughly. The sample was then centrifuged (17,968 × *g*, 2 min), and the ethanol solution was decanted. The remaining ethanol was allowed to evaporate from the centrifuge tube. The pellet was then resuspended in 50 µl of 70% formic acid. Next, 50 µl of 100% acetonitrile was added to the solution and mixed completely. The suspension was centrifuged a final time (17,968 × *g*, 2 min). One microliter of the supernatant was then spotted onto the 96-well plate 12 times. Each time a database addition was performed, the MALDI-TOF MS was calibrated to a bacterial test standard according to the manufacturer's protocol. Thirty-six raw mass spectra were collected from each strain with at least 20 of these spectra compiled according to the manufacturer's protocol to create a MSP that became avail- able for use in a new user-defined *Brachyspira* library.

After creation of the *Brachyspira* library, all field isolates obtained at the ISU VDL between October 1, 2012 and December 31, 2013 (176 isolates) from swine samples and representing various *Brachyspira* species were compared using MALDI-TOF MS and *nox* sequencing. Isolates from this time frame were all from the United States with the exception of 1 isolate from Canada. Field isolates were cultured anaerobically on TSA plates containing 5% bovine

blood, and colistin, vancomycin, and spectinomycin (CVS[10]) and TSA plates containing 5% bovine blood, and colistin, vancomycin, spectinomycin, spiramycin, and rifampicin (BJ[12]). *Brachyspira* growth was subcultured from either CVS or BJ by at least 1 single colony pick for each field isolate. After 2, 4, or 6 days of incubation, cultures of *Brachyspira* spp. were spotted in duplicate onto a steel plate and allowed to dry. The CVS or BJ agar plates were used to spot onto the steel plate based on the amount of surface growth. One microliter of matrix solution was applied to each spot and allowed to dry. Next, the plate was processed according to the MALDI-TOF MS manufacturer's protocol for bacterial identification.

The generated spectra from these isolates were then com- pared against the combined manufacturer's bacterial library and the *Brachyspira* library. The resulting output is a score based bacterial identification ranging from 0 to 3.0. The manufacturer's guidelines suggest that scores ≥2.0 are considered reliable identifications at the species level and scores ≥1.7 are considered reliable at the genus level. For this evaluation, an isolate with an identification score ≥1.7 was considered acceptable at the species level and was used for *nox* sequencing comparison. The cutoff value of ≥1.7 was used to test the robustness of the new user-defined *Brachyspira* library. Multiple attempts of MALDI-TOF MS were performed on some isolates to obtain a score of ≥1.7. Two weakly beta-hemolytic isolates were further characterized by a species-specific PCR that targets portions of the *nox* gene to detect *B. pilosicoli*, *B. murdochii*, *B. intermedia*, and *B. innocens* as previously described.[17] Additional molecular techniques were not performed on any other isolates. Sanger sequencing of the *nox* gene was performed using previously published forward and reverse primers[14] and a DNA analyzer.[d] Primers amplify an approximate 939 base pair fragment of the central portion of

the *nox* gene. Sequencing of the 16S rDNA was performed using the following forward and reverse primers: F: 5′-TGGAGAGTTTGATCCTGGCT CAG-3′; R: 5′-TACCGCGGCTGCTGGCAC-3′. The primers amplify an approximate 500 nucleotide region at the 5'-end of the 16S rDNA gene.[8] Sequencing of the 23S rDNA was performed as previously described.[4] Used together, all 3 sets of primers were used to amplify and sequence approximately 2,400 nucleotides of the 23S rDNA. Consensus sequences were created and analyzed using a commercial software program.[e] Sequence identification was determined by alignment comparison with *nox* sequences available in GenBank from previously identified *Brachyspira* species. Consensus sequences were also subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare sequence similarity and to determine percent similarity to the entirety of the GenBank sequence database.

The results of identification using MALDI-TOF MS and *nox* sequencing performed on 176 *Brachyspira* field isolates are summarized in Table 2. All 176 field isolates had detectable peaks with scores ≥1.7. Of the 176 field isolates compared, 174 (98.9%) MALDI-TOF MS identification results matched speciation results obtained with *nox* sequencing. Of the 2 non-matched results, MALDI-TOF MS identified both as *B. innocens* whereas *nox* sequencing identified one as *B. pilosicoli* and the other as *B. murdochii*. These 2 weakly beta-hemolytic isolates were further characterized by PCR. The results of this additional work indicated that 1 isolate was likely *B. pilosicoli*, which matched the *nox* sequencing results and the other isolate was likely *B. innocens*, which matched the MALDI-TOF MS results. Of the 176 field isolates in the current study, 30 were identified as "*B. hampsonii*" by MALDI-TOF MS and *nox* sequencing. Twenty-six field isolates were identified by *nox* sequencing as "*B. hampsonii*" clade I, and 4 field isolates were identified by *nox* sequencing as clade II. Comparatively, 25

field isolates were identified by MALDI-TOF MS as "*B. hampsonii*" clade I, and 5 field isolates were identified by MALDI-TOF MS as clade II. Overall, of the 30 "*B. hampsonii*" isolates compared, 27 (90%) matched clade designation in both assays. One isolate was identified as both clades by MALDI-TOF MS and, in this case, the highest score was used for the final clade identification. A phylogenetic analysis was performed on the *nox* sequence of the "*B. hampsonii*" field isolates (Fig. 1). Two different groups were formed representing the 2 clades of "*B. hampsonii*." In addition to *nox* sequences, MALDI- TOF MS identities and scores were included in the phylogenetic dendrogram.

In conclusion, after creation of a *Brachyspira* library, MALDI-TOF MS identification compared favorably to results obtained by *nox* sequencing methods. A total of 98.9% of field isolates could be identified using MALDI-TOF MS with the same results as a more complex and expensive *nox* sequencing method, which has been considered the most reliable method to speciate swine *Brachyspira*.[14] The results support work presented previously.[5,13] In addition, the current study shows that MALDI-TOF MS is capable of differentiating between clades of "*B. hampsonii*" with a high degree of accuracy. Ninety percent (27/30) of the isolates matched clade designation by nox sequencing indicating that MALDI-TOF MS is a useful screening tool for "*B. hampsonii*" clade designation. The few anomalous results may be due to the potential for swine feces to contain multiple *Brachyspira species*. Resolution is possible through extensive and complicated cloning methods and may be warranted in certain clinical presentations. Therefore, the data from the current study shows that MALDI-TOF MS is a fast, cost-effective, reliable, and complete tool for the identification of swine *Brachyspira*.

Sources and manufacturers

a. MALDI biotyper, Bruker Daltonics, Bremen, Germany.

b. AnaeroPack system, Mitsubishi Gas Chemical Co. Inc., Japan.

c. GasPak EZ anaerobe container system, BD Diagnostic Systems, Sparks, MD.

d. Applied Biosystems 3730xl DNA analyzer, Life Technologies, Carlsbad, CA.

e. Lasergene, DNASTAR Inc., Madison, WI.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, or publication of this article.

References

1. Atyeo RF, Stanton TB, Jensen NS, et al.: 1999, Differentiation of *Serpulina* species by NADH oxidase gene (*nox*) sequence comparisons and *nox*-based polymerase chain reaction tests. Vet Microbiol 67:47–60.
2. Bizzini A, Greub G: 2010, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect 16:1614–1619.
3. Burrough ER, Strait EL, Kinyon JM, et al.: 2012, Comparative virulence of clinical *Brachyspira* spp. isolates in inoculated pigs. J Vet Diagn Invest 24:1025–1034.

4. Burrough ER, Wilberts BL, Bower LP, et al.: 2013, Fluorescent in situ hybridization for detection of "*Brachyspira hampsonii* " in porcine colonic tissues. J Vet Diagn Invest 25:407–412.

5. Calderaro A, Piccola G, Montecchini S, et al.: 2013, MALDI- TOF MS analysis of human and animal *Brachyspira* species and benefits of database extension. J Proteomics 78:273–280.

6. Chander Y, Primus A, Oliveira S, Gebhart CJ: 2012, Phenotypic and molecular characterization of a novel strongly haemolytic *Brachyspira* species, provisionally designated "*Brachyspira hampsonii* ". J Vet Diagn Invest 24:903–910.

7. Fellström D, Gunnarsson A: 1995, Phenotypical characterisation of intestinal spirochaetes isolated from pigs. Res Vet Sci 59:1–4.

8. Hall L, Doerr KA, Wohlfiel SL, Roberts GD: 2003, Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol 41:1447–1453.

9. Hampson DJ: 2012, Brachyspiral colitis. *In*: Diseases of swine, ed. Zimmerman JJ, Karriker LA, Ramirez A, et al., 10th ed., pp. 680–696. Wiley-Blackwell, Ames, IA.

10. Jenkinson SR, Wingar CR: 1981, Selective medium for the isolation of *Treponema hyodysenteriae*. Vet Rec 109:384–385.

11. Komarek V, Maderner A, Spergser J, Weissenböck H: 2009, Infection with weakly haemolytic *Brachyspira* species in pigs with miscellaneous chronic diseases. Vet Microbiol 134: 311–317.

12. Kunkle RA, Kinyon JM: 1988, Improved selective medium for the isolation of *Treponema hyodysenteriae*. J Clin Microbiol 26:2357–2360.

13. Prohaska S, Pflüger V, Ziegler D, et al.: 2014, MALDI-TOF MS for identification of porcine *Brachyspira* species. Lett Appl Microbiol 58:292–298.

14. Rohde J, Rothkamp A, Gerlach G: 2002, Differentiation of porcine *Brachyspira* species by a novel *nox* PCR-based restriction fragment length polymorphism analysis. J Clin Microbiol 40:2598–2600.

15. Rubin JE, Costa MO, Hill JE, et al.: 2013, Reproduction of mucohaemorrhagic diarrhea and colitis indistinguishable from swine dysentery following experimental inoculation with "*Brachyspira hampsonii*" strain 30446. PLoS One 8:e57146.

16. Seng P, Drancourt M, Gouriet F, et al.: 2009, Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551.

17. Weissenböck H, Maderner A, Herzog AM, et al.: 2005, Amplification and sequencing of *Brachyspira* spp. specific portions of *nox* using paraffin-embedded tissue samples from clinical colitis in Austrian pigs shows frequent solitary presence of *Brachyspira murdochii*. Vet Microbiol 111:67–75.

Table 1. *Brachyspira* strains used for the creation of the user-defined *Brachyspira* library and methods by which each strain was characterized.*

Species/Identifier	Year isolated	Source of isolate	Hemolysis type	Ring phenomenon	Sequencing target gene		
					nox	16S rDNA	23S rDNA
B. hyodysenteriae							
B204	1972	Iowa	Strong	Positive	×	×	×
WA1	1986	ATCC	Strong	Positive		×	×
KC42	2009	Iowa	Strong	Positive	×		
KC75	2009	South Dakota	Strong	Positive	×		
KC85	2010	North Carolina	Strong	Positive	×	×	×
"*B. hampsonii*" clade I							
KC35	2009	Illinois	Strong	Positive	×	×	×
BR2011	2012	North Carolina	Strong	Positive	×		
BR2010	2012	Iowa	Strong	Positive	×		
EB106	2011	Iowa	Strong	Positive	×	×	×
EB108	2011	Iowa	Strong	Positive	×	×	×
EB109	2011	North Carolina	Strong	Positive	×	×	×
B. pilosicoli							
P43/6/78	1980	ATCC	Weak	Negative	×	×	
KC45	2009	Iowa	Weak	Negative	×		
BR2001	2012	Iowa	Weak	Negative	×	×	×
BR2002	2012	Iowa	Weak	Negative	×	×	×
"*B. hampsonii*" clade II							
KC58	2008	Iowa	Strong	Positive	×	×	×
KC23	2008	Iowa	Strong	Positive	×	×	×
KC9A	2009	Iowa	Strong	Positive	×		×
EB100	2010	Iowa	Strong	Positive	×		×
EB107	2011	Iowa	Strong	Positive	×	×	×
B. intermedia							
BR2000	2012	Pennsylvania	Weak	Negative	×	×	×
BR2009	2012	Iowa	Weak	Negative	×		
B. murdochii							
KC21	2008	Iowa	Weak	Negative	×		
KC43	2009	North Dakota	Weak	Negative	×		
KC60	2009	Iowa	Weak	Negative	×		×
KC62	2008	Iowa	Weak	Negative	×		×
KC63	2008	Minnesota	Weak	Negative	×		
KC82	2010	North Carolina	Weak	Negative	×		
B. innocens							
BR2012	2012	North Carolina	Weak	Negative	×		
BR2013	2012	North Carolina	Weak	Negative	×		
BR2014	2013	Minnesota	Weak	Negative	×		
BR2008	2012	Illinois	Weak	Negative	×		
BR2015	2013	Ohio	Weak	Negative	×		

* ATCC = American Type Culture Collection, Manassas, VA; rDNA = ribosomal DNA.

Table 2. Number of field isolates identified by *nox* sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

	No. of isolates identified by:	
Species	*nox* sequencing	MALDI-TOF MS
Brachyspira hyodysenteriae	18	18
"*Brachyspira hampsonii*"	30	30
Brachyspira innocens	15	17
Brachyspira intermedia	4	4
Brachyspira murdochii	99	98
Brachyspira pilosicoli	9	9
Total	176	176

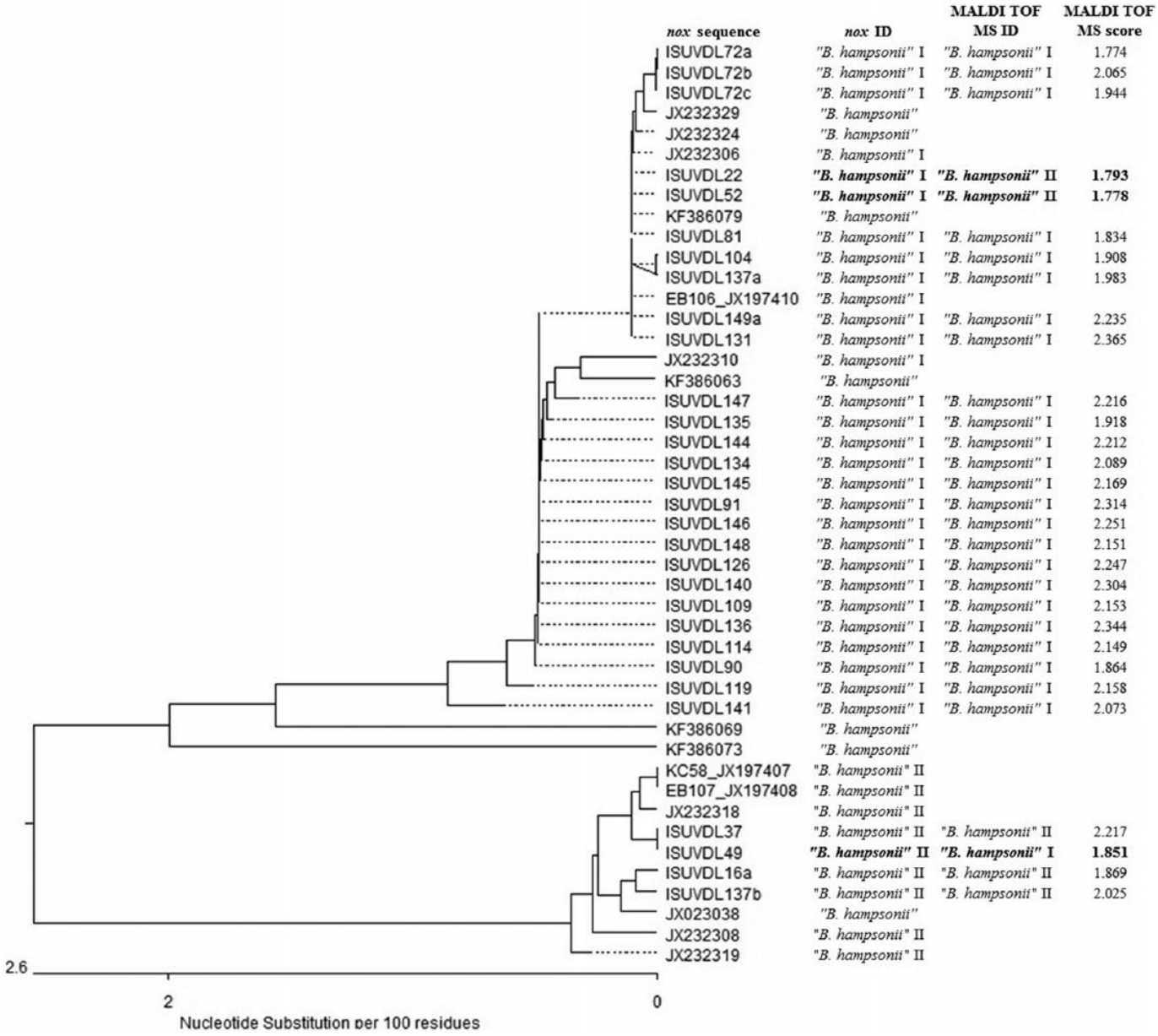

Figure 1. Phylogenetic analysis of the 30 "*Brachyspira hampsonii*" field isolates. Sequences starting with ISU VDL (Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa) are the field isolates compared, and other sequences were obtained from GenBank and are identified with accession numbers. The additional columns are the *nox* sequence ID, matrix-assisted laser desorption ionization time- of-flight mass spectrometry (MALDI-TOF MS) ID, and MALDI-TOF MS score. Bolded rows indicate differing clade designation by the 2 methods for the same isolate.

CHAPTER 3

A COMPARISON OF ORAL FLUIDS AND FECES FOR THE DETECTION OF *BRACHYSPIRA* SPP. IN SWINE

A manuscript prepared for publication in the Journal of Swine Health and Production

Hallie L. Warneke[1], Timothy S. Frana[2], Joann M. Kinyon[1], Eric R. Burrough[1]*

[1]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, United States of America

[2] Department of Pharmacovigilance, Boehringer Ingelheim Vetmedica, Inc., Saint Joseph, MO, United States of America

*Corresponding Author: Eric R. Burrough, Veterinary Diagnostic Laboratory Iowa State University, 1600 South 16th Street, Ames, IA 50011. burrough@iastate.edu

Introduction

Brachyspira spp. are Gram-negative, oxygen tolerant, anaerobic spirochetes that commonly colonize the pig colon and include both pathogenic and commensal species.[7] The most significant pathogens include the agents of swine dysentery (SD), *Brachyspira hyodysenteriae,*[8] *Brachyspira suanatina,*[14] and *Brachyspira hampsonii,*[13,17] and the etiologic agent of porcine intestinal spirochetosis, *Brachyspira pilosicoli.*[11] SD is a mucohemorrhagic diarrheal disease that results in dehydration, emaciation, and in severe cases, death.[5] *B. hyodysenteriae* can survive in carrier pigs, rodents,[3] waterfowl,[12] and lagoons[6] which then serve as a reservoir for re-emergence of SD at any site that has previously been diagnosed with the disease. Pathogen monitoring and surveillance is therefore important to detect infected farms early to prevent disease spread and for monitoring infected farms during treatment and elimination efforts. The most commonly used sample for surveillance is feces

and fecal swabs. The difficulty with these samples is that they only test a limited number of animals from a site or pen and may not reflect the entire site or pen from which they are derived. Accordingly, an alternative and potentially more efficient way to conduct surveillance would be to test the pens where the animals are housed. That way, the result from the test would reflect the pen level rather than the individual animal. Given that pigs are curious, cotton ropes hung within pens are readily chewed upon. Once the pigs have had sufficient time to chew on the rope, the rope can be wrung out to produce a composite sample of "oral fluid" which contains saliva, mucus, feces, and other environmental material. Oral fluids have been demonstrated as an effective sample type to test pens for the presence of respiratory viruses[15] by PCR presumably because a majority of animals in the pen will be sampled.

To determine if oral fluids are at least as sensitive as fecal samples for culture surveillance of *Brachyspira* spp., particularly the pathogenic species *B. hyodysenteriae* and *B. hampsonii, in vitro* and *in vivo* studies were completed. To replicate the typical transportation process and to determine if *B. hyodysenteriae* and *B. hampsonii* could survive transportation in oral fluids, an *in vitro* study was performed to compare survival in phosphate buffered saline (PBS), oral fluids, watery feces, and solid feces spiked with varying dilutions of the organisms of interest. To determine if *B. hyodysenteriae* and *B. hampsonii* could be recovered from oral fluids collected from pens of pigs with known infection status, samples were collected during two experimental inoculation trials where pigs were inoculated with these organisms. In both experimental infection trials, rectal swab and oral fluid samples were collected concurrently to compare rate of detection. Finally, to determine if *B. hyodysenteriae* and *B. hampsonii* could be cultured from animals that were

not known to be infected with either organism, reflecting a typical surveillance scenario, rectal swabs and oral fluids were collected and cultured from multiple field sites. The *a priori* hypothesis of this investigation was that oral fluid samples can provide an alternative surveillance sample for *Brachyspira* culture with sufficient diagnostic sensitivity to detect pens of pigs infected with agents of SD and other *Brachyspira* spp.

Materials and Methods

In vitro trial

To determine if *Brachyspira* spp. could be isolated from oral fluids, an *in vitro* trial was conducted comparing oral fluids to phosphate buffered saline (PBS) and two types of feces: manufactured watery feces (2 grams feces + 7 ml PBS) and solid feces (8 grams feces + 1 ml PBS). *B. hyodysenteriae* (B204; Iowa, 1973) and *B. hampsonii* (EB107; Iowa, 2011) strains were obtained from the culture collection of the Iowa State University Veterinary Diagnostic Laboratory and grown anaerobically in anaerobic jars[b] using gas paks[c] at 42°C on tryptic soy agar containing 5% bovine blood (TSA). A 0.5 McFarland Standard was prepared by harvesting the organism using a sterile swab rinsed in 9 mls PBS and subsequently 1:10 dilutions were made to 10^{-6} in PBS. One milliliter from each dilution was transferred to 9 mls of oral fluids, watery feces, and solid feces. Samples were mixed and 100 µl was plated and spread onto TSA; TSA with colistin, vancomycin, and spectinomycin (CVS);[1,9] and TSA with colistin, vancomycin, spectinomycin, spiramycin, rifampicin, and porcine fecal extract (BJ).[1,10] The PBS and sample suspensions were then refrigerated at 4°C and plating was repeated on the samples at 24, 48, and 72 hours. Inoculated plates were incubated anaerobically as stated above. Colonies were approximated at 2, 4, and 6 days post incubation and the highest approximation was used to calculate an estimate of colony

forming units (CFU)/ml. Average CFU estimates were calculated from two repetitions of each species and sample type. JMP software was used to analyze the data. A student's t-test was used to compare the difference in mean CFU estimates between time zero and the subsequent plating times. The CFU estimates for *B. hyodysenteriae* and *B. hampsonii* were combined for the student's t-test because similar patterns were seen in the level of organism being detected over time when CFUs were estimated.

In vivo trials

For the samples derived from animal inoculation experiments, the challenge isolates were prepared as previously described[4] and inocula were prepared as described here: Challenge isolates were grown anaerobically on TSA and an agar slurry was used for challenge doses where five (5) 18 ml TSA plates containing the challenge isolates were homogenized using a sterilized potato ricer with an additional 10 ml sterile PBS to prepare a 100 ml agar challenge dose. Control challenge doses were prepared as described using Sheep Blood Agar. All animals received 3 challenge doses on 3 consecutive days. Oral fluids were collected by hanging a rope in the pen and allowing animals approximately 20 minutes of exposure time to the rope. The oral fluid from the rope was wrung into a sterile plastic seal top bag (10 in x 12 in) and then transferred to a sterile 15 ml conical tube[h] and submitted for culture along with individual rectal swabs.

In vivo trial one

For the first experimental inoculation trial,[18] 6 control pigs received a sham inoculum as described above, 10 animals were inoculated with an agar slurry of *B. hyodysenteriae*, and 10 animals were inoculated with an agar slurry of *B. hampsonii.* Animals were housed in the same pen by inoculation group. Daily individual rectal swabs and pen-level oral fluids were

collected from day post-inoculation (dpi) 5 to dpi 20. Rectal swabs and oral fluids were plated onto CVS and BJ agar. Plates were incubated anaerobically as above. Plates were read at 2, 4, and, 6 days; suspect colonies were re-streaked to CVS agar for isolation and confirmation. Colonies with morphology typical of *Brachyspira* were confirmed to species using partial *nox* gene sequencing as previously described.[2,16]

In vivo trial two

In the second experimental inoculation trial,[20] five groups of 20 animals were divided into a control group, a group inoculated with *B. hyodysenteriae*, a group inoculated with *B. hampsonii*, a group inoculated with *B. pilosicoli*, and a group inoculated with *B. intermedia*. The 5 groups were then subdivided where half the group received a diet containing 30% distillers dried grains with solubles (DDGS) and the other half received a standard corn-soy diet. Inoculated animals were housed in pens of 10 animals. Rectal swabs and oral fluids were collected, plated, and evaluated as described in the first *in vivo* trail. Positive *Brachyspira* isolates were identified to species using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI TOF MS) as in chapter 2. For consistency in comparing with the first *in vivo* trial, only results from the pigs consuming the standard diet will be discussed herein.

In vivo field trial

For the field trial, 4 sites were tested with a total of 20 individual pens being sampled. There were 25-50 pigs per pen and in each pen, an oral fluid sample was collected in addition to rectal swabs or feces from 5 separate animals from that pen. Rectal swabs, feces, and oral fluids were plated and examined as described above. If multiple *Brachyspira* suspects were identified, one suspect per colony morphology was identified per pen as above using

MALDI-TOF MS. Both sample types were tested by PCR with primers and probes that have been previously described.[19] For PCR testing oral fluids were tested individually and the 5 fecal samples were pooled.

Results

In vitro trial

In table 1, approximate CFU counts are summarized for *B. hyodysenteriae*, *B. hampsonii,* and the combination of the two species. The individual results from *B. hyodysenteriae* and *B. hampsonii* were consistent across time points and were therefore combined for simplification of comparison. After 48 hours of refrigeration, no organisms were recovered from solid feces. After 24 hours, the only pair that was not statistically different was runny feces and solid feces. Oral fluids were statistically different compared to both solid feces and runny feces with a p-value of <0.0001 for both comparisons. Additionally, at 24 hours oral fluid average CFU estimates were higher than PBS, solid feces, and runny feces average CFU estimates with differences of $1.72x10^6$, $4.50x10^6$ and $4.06x10^6$ respectively. At 48 hours, the two pairs of oral fluids-PBS and runny feces-solid feces were not statistically different. Again, oral fluid average CFU estimates were higher than those of PBS, solid feces, and runny feces with differences of $7.25x10^5$, $2.22x10^6$, and $1.87x10^6$ respectively. At 72 hours, the same patterns were seen between pairs. There was statistical difference between the 3 pairs of oral fluids-solid feces, oral fluids-runny feces, and oral fluids-PBS with p-values of 0.0004, 0.0004, and 0.0211 respectively. Also, average CFU estimates of oral fluids were higher than average CFU estimates of solid feces, runny feces, and PBS with values of $1.44x10^6$, $1.44x10^6$, and $8.92x10^5$ respectively. Box plots depict

differences in average CFU estimates at the different refrigerated holding times in figures 1-4.

In vivo trial results

In vivo trial one

In the first inoculation trial, oral fluids from both infected groups were culture positive when at least one animal (1/10) was culture positive by rectal swab. The group inoculated with *B. hyodysenteriae* had a positive rectal swab starting on day 9 post-inoculation and continued to be positive through the end of the study with additional positive swabs. The oral fluid collected from the *B. hyodysenteriae* group was also positive starting day 9 post-inoculation and continued to be positive through the end of the study. The group inoculated with *B. hampsonii* had a positive rectal swab starting on day 13 and continued to be positive through the end of the study with additional positive swabs. However, the oral fluid collected from the *B. hampsonii* group was not positive until day 18 but then continued to be positive through the end of the study. Daily rectal swab and oral fluid results are summarized in Table 2.

In vivo trial two

In the second inoculation trial, oral fluids were positive from all groups at different days post-challenge, but there was always at least one positive rectal swab if the oral fluid was positive. However, the presence of positive rectal swabs did not associate with a positive oral fluid on all days post-challenge. Daily rectal swab and oral fluid culture results from each challenge group are summarized in Table 3.

In vivo field trial

Of the 20 pens that were tested, 9 pens were culture positive for at least one *Brachyspira* species by rectal swabs and 4 of those 9 pens were positive for *B. hyodysenteriae*. Three pens were culture positive for *Brachyspira* species by oral fluids and 1 of those 3 pens was positive for *B. hyodysenteriae*. The *B. hyodysenteriae* culture positive rectal swabs did not match the one pen with a positive oral fluid culture sample. *B. hampsonii* was not isolated from any of the samples collected. PCR detected *B. hyodysenteriae* in 2 of the 4 pooled rectal swab samples and in both cases these pools had positive individual cultures from 3 of the 5 swabs within the pool. The one oral fluid sample where *B. hyodysenteriae* was recovered by culture was also positive by PCR. PCR detected *B. hyodysenteriae* in four additional oral fluid samples and these samples reflected the same pens where *B. hyodysenteriae* was recovered by culture of rectal swabs. These results are summarized in Table 4. To estimate the diagnostic sensitivity and specificity of each individual sample type, the recovery of a strongly beta-hemolytic spirochete by culture with confirmation of species by MALDI-TOF MS from any sample type in a pen was considered the gold standard for detection of *B. hyodysenteriae.* Accordingly, PCR of oral fluids had a pen-level diagnostic sensitivity of 100% whereas culture of individual rectal swabs, PCR of rectal swab pools, and oral fluid culture had sensitivities of 80%, 40%, and 20%, respectively. All four sample types had pen-level diagnostic specificities of 100%.

Discussion

The primary objective of this investigation was to determine if culturing oral fluids would be an acceptable sample type for the surveillance of the organisms associated with SD. The initial *in vitro* quantitative study showed that *Brachyspira* cells could survive in oral

fluids at least 72 hours after collection in high numbers. In fact, *Brachyspira* cells survived better in oral fluids compared to runny feces and solid feces. Thus showing that if oral fluids were to be used for culture, the organism could survive transport from the field to the lab. The next step was to determine if *Brachyspira* cells could be recovered from oral fluids collected from groups of pigs shedding the organism in feces. The experimental inoculation studies verified that *Brachyspira* could be cultured from oral fluids collected from groups of animals showing clinical disease or known to have been infected with *Brachyspira*. Even if few of the rectal swabs were culture positive, indicating that few animals were likely shedding the organism, *Brachyspira* was recoverable from the oral fluid. In both inoculation studies, this was true for both *B. hyodysenteriae* and *B. hampsonii* revealing that if oral fluids were to be submitted for culture, these organisms could be successfully recovered from the sample. The final step in this investigation was to determine if *B. hyodysenteriae* and *B. hampsonii* could be cultured from animals that were not demonstrating clinical disease but could potentially be carriers of those organisms. In the field investigation, culture of rectal swabs detected *B. hyodysenteriae* positive pens more often than culture of the oral fluids; however, one of the 5 *B. hyodysenteriae* positive pens was only culture positive by oral fluid. Interestingly, PCR of the oral fluids detected all pens where *B. hyodysenteriae* was recovered by culture.

Results of this study reveal that when animals are exhibiting clinical SD, oral fluids can be used to effectively detect either *B. hyodysenteriae* or *B. hampsonii* by culture; however, using oral fluids seems to be a less effective option than feces for isolating *Brachyspira* species from animals of unknown clinical status. If isolation of the organism is desired for further testing or investigation, such as confirmation of hemolytic phenotype,

genotyping, or MIC determination, collecting a fecal sample is still the preferred option while oral fluids are an effective sample for PCR and detecting the organism within a population. One potential approach that provides high diagnostic sensitivity and specificity for detection of agents of SD would be parallel testing using both sample types. PCR on oral fluids improves sensitivity of detection and is robust to loss of organism viability while culture of individual fecal samples from the same pen will allow for confirmation of hemolytic phenotype as well as for the detection of novel species and atypical strains. Such a combined approach would be useful for disease diagnosis, pathogen monitoring, and surveillance purposes where oral fluid collection is applicable.

Sources and manufacturers

a. MALDI biotyper, Bruker Daltonics, Bremen, Germany.

b. AnaeroPack system, Mitsubishi Gas Chemical Co. Inc., Japan.

c. GasPak EZ anaerobe container system, BD Diagnostic Systems, Sparks, MD.

d. Applied Biosystems 3730xl DNA analyzer, Life Technologies, Carlsbad, CA.

e. Lasergene, DNASTAR Inc., Madison, WI.

f. Good Grips Stainless Steel Potato Ricer, Oxo, Chambersburg, PA.

g. TEGO™ Swine Oral Fluids Kit, ITL BioMedical, Melbourne, Australia.

h. 15 ml conical tube, VWR International, Radnor, PA.

i. Sheep Blood Agar, Remel, Lenexa, KS

References

1. Achacha M, Messier S: 1992, Comparison of six different culture medium for isolation of *Treponema hyodysenteriae*. J Clin Microbiol 30:249-251.

2. Atyeo RF, Stanton TB, Jensen NS, et al.: 1999, Differentiation of *Serpulina* species by NADH oxidase gene (*nox*) sequence comparisons and *nox*-based polymerase chain reaction tests. Vet Microbiol 67:47-60.

3. Backhans A, Jansson DS, Aspán A, Fellström C: 2011, Typing of *Brachyspira* spp. from rodents, pigs, chickens on Swedish farms. Vet Microbiol 153:156-162.

4. Burrough ER, Strait EL, Kinyon JM, et al.: 2012, Comparative virulence of clinical *Brachyspira* spp. isolates in inoculated pigs. J Vet Diagn Invest 24:1025-1034.

5. Burrough ER: 2017, Swine dysentery: etiopathogenesis and diagnosis of a reemerging disease. Vet Pathol 54:22-31.

6. Glock RD, Vanderloo KJ, Kinyon JM: 1975, Survival of certain pathogenic organisms in swine lagoon effluent. J Am Vet Med Assoc 166:273-275.

7. Hampson DJ. Brachyspiral colitis In: Zimmerman JJ, Karriker LA, Ramirez A, et al., eds. *Diseases of swine*. 10th ed. Ames, IA: Wiley-Blackwell, 2012;680 - 696.

8. Harris DL, Glock RD, Christensen CR, Kinyon JM: 1972, Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. Vet Med Small Anim Clin 67:61-64.

9. Jenkinson SR, Wingar CR; 1981, Selective medium for the isolation of *Treponema hyodysenteriae.* Vet Rec 109: 384-385.

10. Kunkle RA, Kinyon JM; 1988, Improved selective medium for the isolation of *Treponema hyodysenteriae*. J Clin Microbiol 26: 2357-2360.

11. Komarek V, Maderner A, Spergser J, Weissenböck H: 2009, Infection with weakly haemolytic *Brachyspira* species in pigs with miscellaneous chronic diseases. Vet Microbiol 134: 311-317.

12. Martinez-Lobo FJ, Hidalgo A, Garcia M, et al.: 2013, First identification of "*Brachyspira hampsonii*" in wild European waterfowl. PloS One 8:e82626.

13. Mirajkar NS, Phillips ND, La T, Hampson DJ, Gebhart CJ: 2016, Characterization and recognition of *Brachyspira hampsonii* sp. nov., a novel intestinal spirochete that is pathogenic to pigs. J Clin Microbiol 54: 2942-2949.

14. Mushtaq M, Zubair S, Råsbäck T, Bongcam-Rudloff E, Jansson DS: 2015, *Brachyspira suanatina* sp. nov., an enteropathogenic intestinal spirochaete isolated from pigs and mallards: genomic and phenotypic characteristics." BMC Microbiol 15: 208.

15. Prickett JR, Zimmerman JJ: 2010, The development of oral fluid-based diagnostics and application in veterinary medicine. Animal Health Research Reviews 11(2):207-216.

16. Rohde J, Rothkamp A, Gerlach G: 2002 Differentiation of porcine *Brachyspira* species by a novel *nox* PCR-based restriction fragment length polymorphism analysis. J Clin Microbiol 40: 2598-2600.

17. Rubin JE, Costa MO, Hill JE, et al.: 2013 Reproduction of mucohaemorrhagic diarrhea and colitis indistinguishable from swine dysentery following experimental inoculation with "*Brachyspira hampsonii*" strain 30446. PLoS One 8:e57146.

18. Wilberts BL, Arruda PH, Kinyon JM, et al.: 2014, Comparison of lesion severity, distribution, and colonic mucin expression in pigs with acute swine dysentery following oral inoculation with "*Brachyspira hampsonii*" or *Brachyspira hyodysenteriae*. Vet Pathol. 51:1096-1108.

19. Wilberts BL, Warneke HL, Bower LP, et al.: 2015, Comparison of culture, polymerase chain reaction, and fluorescent in situ hybridization for detection of *Brachyspira hyodysenteriae* and "*Brachyspira hampsonii*" in pig feces. J Vet Diagn Invest 27:41-46.

20. Wilberts BL, Arruda PH, Kinyon JM, et al.: 2014, Investigation of the impact of increased dietary insoluble fiber through the feeding of distillers dried grains with solubles (DDGS) on the incidence and severity of *Brachyspira*-associated colitis in pigs. PLoS One 9:114741.

Table 1. Averaged estimated CFU* counts for each medium at initial bacterial suspension and different refrigerated time points

		Refrigerated holding times (hours)							
		0		24		48		72	
	Medium	Mean‡	Std¥ Error	Mean	Std Error	Mean	Std Error	Mean	Std Error
Brachyspira hyodysenteriae	PBS†	$8.35x10^6$	$4.70x10^6$	$4.17x10^6$	$7.13x10^5$	$2.17x10^6$	$2.20x10^5$	$5.64x10^5$	$1.42x10^5$
	Oral Fluid	$1.54x10^7$	$4.70x10^6$	$4.44x10^6$	$7.13x10^5$	$1.79x10^6$	$2.20x10^5$	$7.69x10^5$	$1.42x10^5$
	Runny Feces	$1.38x10^6$	$4.70x10^6$	$9.68x10^5$	$7.13x10^5$	$6.92x10^5$	$2.20x10^5$	$1.20x10^3$	$1.42x10^5$
	Solid Feces	$2.58x10^5$	$4.70x10^6$	$3.96x10^4$	$7.13x10^5$	0	$2.20x10^5$	0	$1.42x10^5$
Brachyspira hampsonii	PBS	$1.13x10^7$	$1.24x10^6$	$1.50x10^6$	$8.15x10^5$	$8.09x10^5$	$7.40x10^5$	$5.26x10^5$	$4.91x10^5$
	Oral Fluid	$7.70x10^6$	$1.24x10^6$	$4.67x10^6$	$8.15x10^5$	$2.64x10^6$	$7.40x10^5$	$2.11x10^6$	$4.91x10^5$
	Runny Feces	$2.56x10^6$	$1.24x10^6$	$2.73x10^4$	$8.15x10^5$	0	$7.40x10^5$	0	$4.91x10^5$
	Solid Feces	$9.62\ x10^6$	$1.24x10^6$	$6.33x10^4$	$8.15x10^5$	0	$7.40x10^5$	0	$4.91x10^5$
Combined Species	PBS	$9.83x10^6$	$3.54x10^6$	$2.83x10^6$	$7.91x10^5$	$1.49x10^6$	$5.53x10^5$	$5.45x10^5$	$3.73x10^5$
	Oral Fluid	$1.15x10^7$	$3.54x10^6$	$4.56x10^6$	$7.91x10^5$	$2.22x10^6$	$5.53x10^5$	$1.44x10^6$	$3.73x10^5$
	Runny Feces	$1.97x10^6$	$3.54x10^6$	$4.98x10^5$	$7.91x10^5$	$3.46x10^5$	$5.53x10^5$	600	$3.73x10^5$
	Solid Feces	$4.94x10^6$	$3.54x10^6$	$5.14x10^4$	$7.91x10^5$	0	$5.53x10^5$	0	$3.73x10^5$

‡Note: Mean values represent averages of all 3 media types from 2 replicates.
*CFU – Colony forming units
†PBS – Phosphate buffered saline
¥Std – Standard

Table 2. Daily culture results of fecal swabs and oral fluids from the first inoculation trial

	Brachyspira hyodysenteriae			*Brachyspira hampsonii*		
Day Post-Inoculation	Number of Positive Rectal Swabs*	Number of Pigs Tested	Oral Fluid* (+/-)	Number of Positive Rectal Swabs*	Number of Pigs Tested	Oral Fluid* (+/-)
1-8	0	10	-	0	10	-
9	1	10	+	0	10	-
10	4	10	+	0	10	-
11	6	9	+	0	10	-
12	5	9	+	0	9	-
13	5	8	+	1	9	-
14	3	8	+	1	9	-
15	3	8	+	1	9	-
16	4	8	+	1	9	-
17	3	7	+	1	9	-
18	5	7	+	1	9	+
19	4	6	+	3	9	+
20	4	6	+	4	9	+
21	N/A	N/A	N/A	0	8	N/A

*Isolated *Brachyspira* species recovered by culture matched the inoculation species administered.

Table 3. Daily rectal swab and oral fluid culture results from second inoculation trial

Day Post-Inoculation	*Brachyspira intermedia* Number of Positive Rectal Swabs*	*Brachyspira intermedia* Oral Fluid* (+/-)	*Brachyspira intermedia* Number of Pigs Tested	*Brachyspira pilosicoli* Number of Positive Rectal Swabs*	*Brachyspira pilosicoli* Oral Fluid* (+/-)	*Brachyspira pilosicoli* Number of Pigs Tested	*Brachyspira hampsonii* Number of Positive Rectal Swabs*	*Brachyspira hampsonii* Oral Fluid* (+/-)	*Brachyspira hampsonii* Number of Pigs Tested	*Brachyspira hyodysenteriae* Number of Positive Rectal Swabs*	*Brachyspira hyodysenteriae* Oral Fluid* (+/-)	*Brachyspira hyodysenteriae* Number of Pigs Tested
5	2	-	10	7	-	10	3	+	10	3	-	10
6	3	-	10	9	+	10	5	+	10	5	-	10
7	4	-	10	10	+	10	6	+	10	6	-	10
8	2	-	10	9	+	10	7	+	10	4	+	10
9	3	+	10	9	+	10	7	+	9	8	+	10
10	3	-	10	9	+	10	7	+	8	6	+	10
11	3	-	10	9	+	10	5	+	8	5	+	7
12	4	-	10	8	+	10	4	+	7	4	+	6
13	8	+	10	9	+	10	5	+	7	4	+	6
14	6	+	10	7	+	10	3	+	7	4	+	6
15	6	+	10	4	+	10	0	-	4	1	-	4
16	6	+	10	1	-	10	0	-	4	2	+	4
17	6	+	10	1	-	10	1	+	4	2	-	4
18	7	+	10	2	-	10	1	+	4	1	+	4
19	5	-	10	3	-	10	3	+	4	1	+	4
20	3	+	10	2	-	10	4	+	4	1	-	4
21	4	-	10	2	-	10	4	+	4	1	+	4

* Isolated *Brachyspira* species recovered by culture matched the inoculation species administered.

Table 4. Individual pen culture and duplex PCR results from the field study

Pen Number	Rectal Swab Result (#/5)	Species Isolated	Pooled Rectal Swab PCR result	Oral Fluid Result	Species Isolated	Oral Fluid PCR result
1	-		-	-		-
2	-		-	-		-
3	2	*B. mur*	-	-		-
4	5	*B. mur*	-	-		-
5	-		-	-		-
6	1	*B. mur*	-	-		-
7	4	*B. mur*	-	+	*B. mur*	-
8	-		-	-		-
9	-		-	-		-
10	2	*B. mur*	-	-		-
11	1	*B. hyo*	-	-		+
12	2	*B. hyo*	-	-		+
13	3	*B. hyo* & *B. mur*	+	-		+
14	3	*B. hyo*	+	+	*B. mur*	+
15	-		-	+	*B. hyo*	+
16	-		-	-		-
17	-		-	-		-
18	-		-	-		-
19	-		-	-		-
20	-		-	-		-

B. hyo = Brachyspira hyodysenteriae
B. mur = Brachyspira murdochii

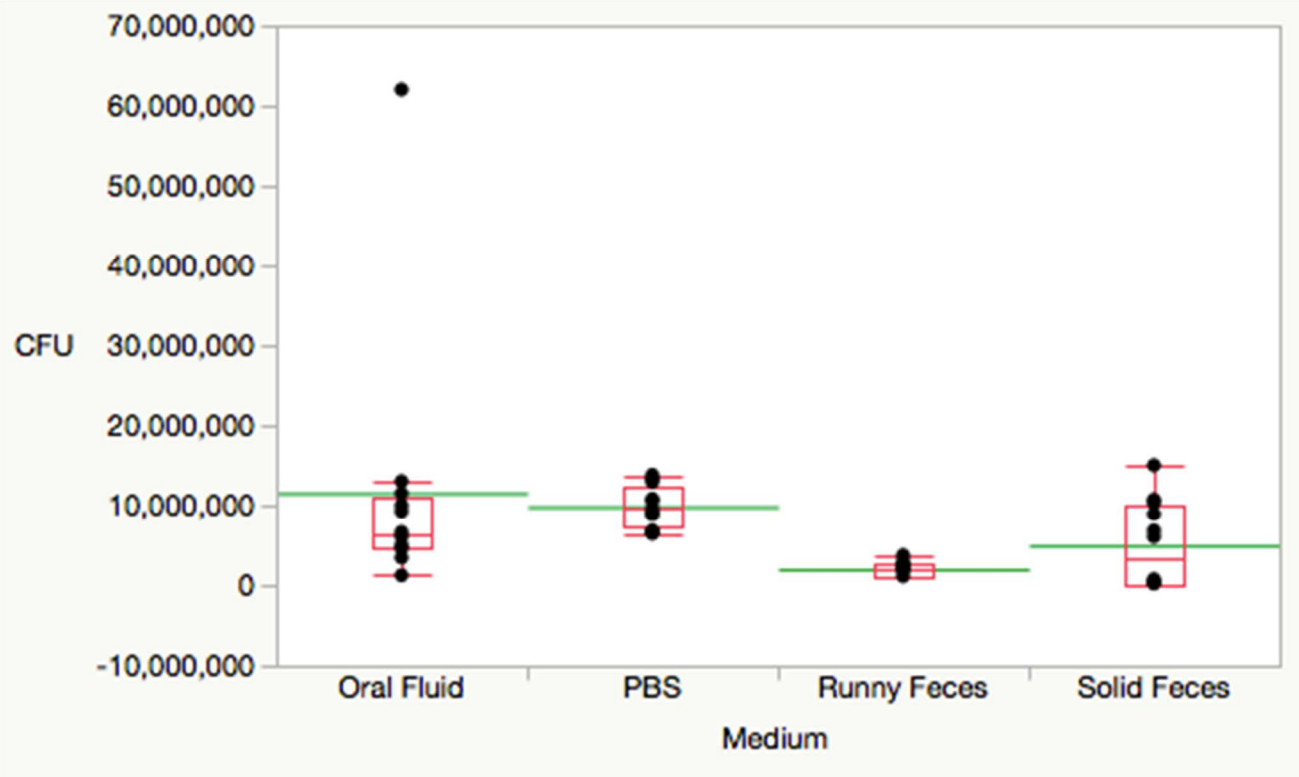

Figure 1: Boxplots representing estimated Colony Forming Units (CFU) by medium of both *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* combined at time 0

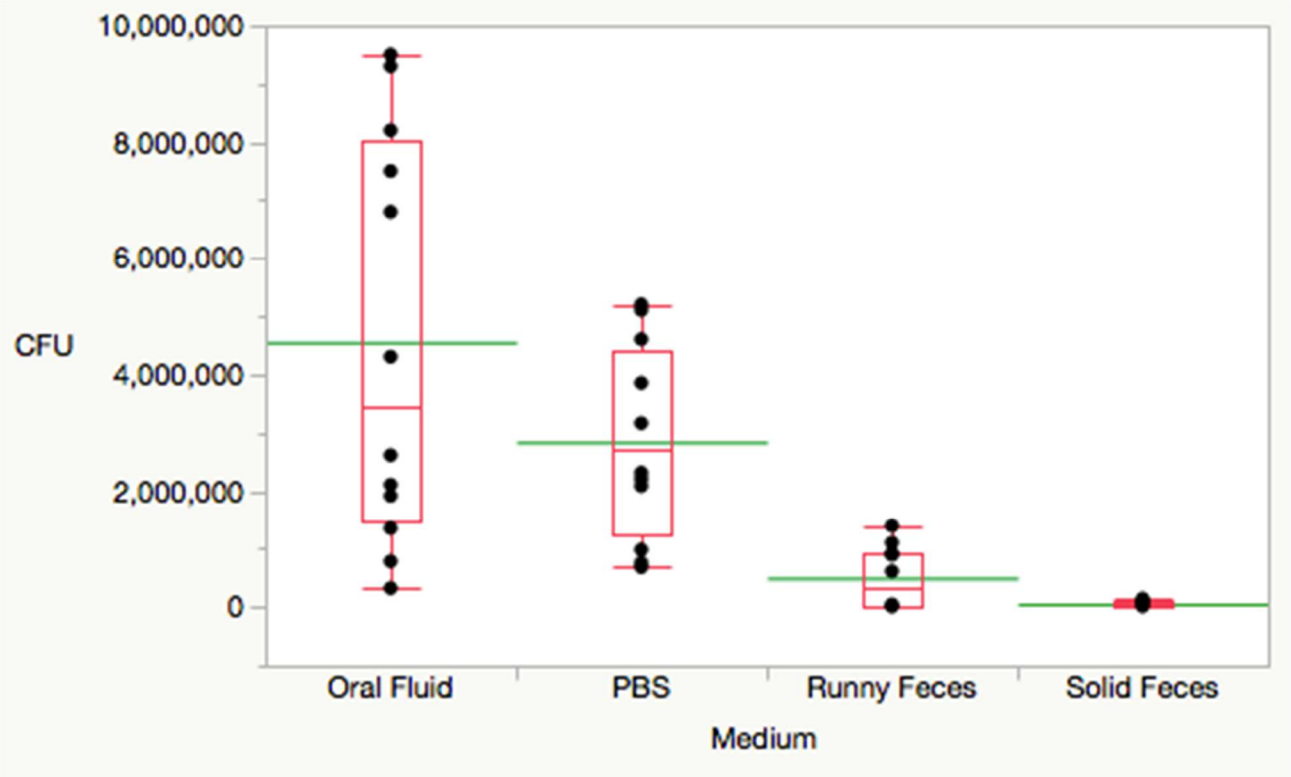

Figure 2: Boxplots representing estimated Colony Forming Units (CFU) by medium of both *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* combined at 24 hours

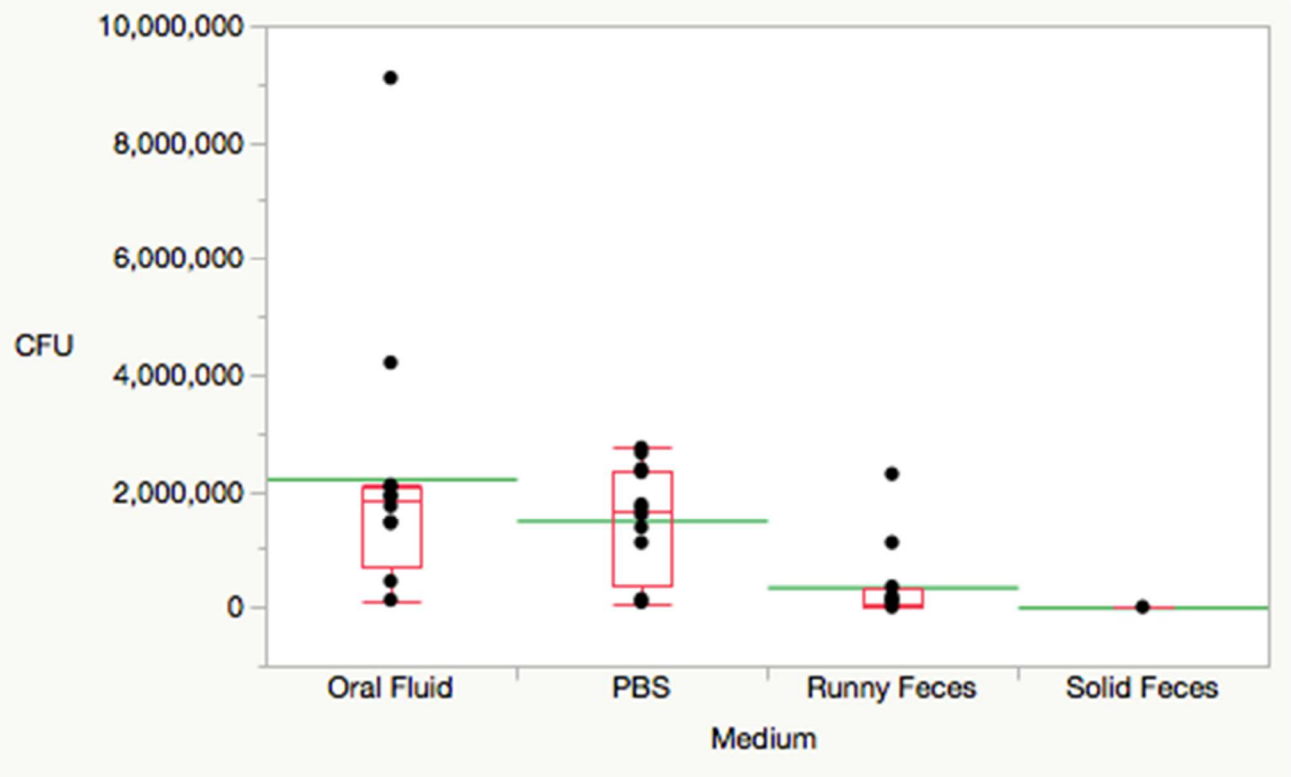

Figure 3: Boxplots representing estimated Colony Forming Units (CFU) by medium of both *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* combined at 48 hours

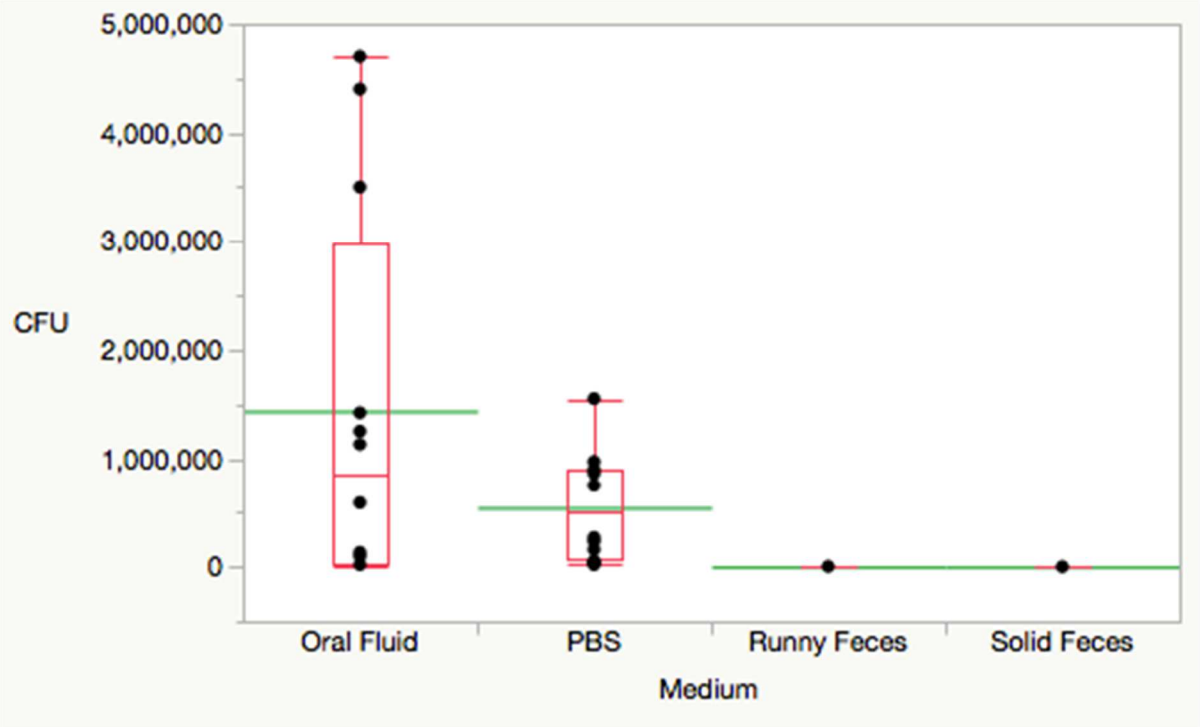

Figure 4: Boxplots representing estimated Colony Forming Units (CFU) by medium of both *Brachyspira hyodysenteriae* and *Brachyspira hampsonii* combined at 72 hours

CHAPTER 5

SUMMARY AND CONCLUSIONS

The knowledge gained from the studies discussed is highly impactful to the field of *Brachyspira* research and diagnostics. These studies reveal that identification of *Brachyspira* species can be simplified and more rapid using MALDI TOF MS. While this method is not ideal for conducting epidemiologic investigations and in depth research into these bacteria and their genetic factors, it is very useful for obtaining rapid, accurate identification of agents of SD thereby allowing informed treatment decisions in affected herds. It would be beneficial to continue to add porcine-origin *Brachyspira* species into the database to continually improve the accuracy of MALDI-TOF MS as new species are identified and officially recognized. Additionally, more strains could be added to the database to increase the robustness of the method. With the quantity of field isolates that have been compared using MALDI-TOF MS and *nox* sequencing, the confidence in MALDI-TOF MS identification methods is high. This is an important for confidence in determining if atypical or novel strains would be detected if isolated. The combination of phenotypic growth characteristics and speciation by MALDI-TOF MS help determine if field strains follow the typical pattern of the species identified. In the case that the isolate were to fall into the atypical or untypeable category, sequencing methods can be utilized to determine if a novel species has been isolated.

Population-level monitoring for *Brachyspira* spp. was also investigated through the use of oral fluids. Through these investigations, it was determined that oral fluids may be better suited for PCR testing than traditional culture techniques. Based upon the results of this thesis, the gold standard for isolating *Brachyspira* species remains selective anaerobic

culture of fecal samples, samples of colonic tissue, or fecal swabs. While oral fluids are reliable for PCR detection of many organisms and viruses, they appear unreliable for cultural isolation of *Brachyspira* spp. in diagnostic and surveillance specimens. In our initial evaluation of oral fluids for culture of *Brachyspira* spp. from experimentally infected pigs, oral fluids showed considerable promise; however, this may have been due to the capacity of the organism to survive better over a short period of time as those samples were set up for culture within a few hours post-collection compared with the 2 – 3 days post-collection that would be typical for field submissions. This difference in time between sample collection and plating and incubation could be an important factor in success in the isolation of *Brachyspira* spp. from oral fluids. For field situations, it is most likely going to be the case where samples are shipped overnight and the time from collection to incubation will be longer thus missing the optimal sampling window whereby culture is most effective. Another reason that oral fluids showed promise early on may have been that the amount of organism in the environment of both the spiked samples and inoculation trials was higher than in field conditions. The quantity of organism recovered from the spiked samples even after 72 hours of refrigeration was substantial. Also, in the inoculation trials, the amount of organism that was given to the animal to cause disease was very high and the animals then shed those organisms once infection occurred. Both of these circumstances do not likely reflect what would occur in the field. It could also be presumed that in the field, the bacterial load of *Brachyspira* spp. would be significantly lower because of how the animals are housed. In the inoculation trials, the pigs were housed on solid concrete floors and fecal material containing the inoculated *Brachyspira* species was consistently available for horizontal exposure. In contrast, animals in the field are typically housed on slatted floors, which allows waste

material to fall through the slats and into the pit. In addition to the environmental load of the organism being lower in the field, more animals of unknown status would be included in the analysis as compared to inoculation trials where all animals were known to have received a high dose of the inoculum. A third potential reason for the observed differences between the inoculation trials and the field sample data could be the unknown treatment status of animals in the field. It is possible that these animals were receiving in-feed antibiotics which may have impacted the viability of organisms when they reached the laboratory thereby predisposing to negative culture results.

A surprising finding of the evaluation of oral fluids was the ability of PCR to consistently detect *Brachyspira* in pens that were culture negative on PCR but positive for *B. hyodysenteriae* on culture of feces only. Therefore the benefit to using oral fluids for PCR would be a more rapid detection of agents of SD and therefore a quicker diagnosis of disease. Results of PCR testing can be available the same day that samples are received in the laboratory or at the latest, the following day, whereas culture results can take up to a week for confirmation and species identification. The advantage to the producer would be a quicker diagnosis so that treatment could be administered sooner or that decisions related to animal movement can be made. However, this is not to say that PCR should replace culture of fecal samples. While PCR is beneficial for identifying the agents of SD that are currently present in North America, many routine PCRs do not include *B. pilosicoli*, the agent of PIS, or any of the other swine *Brachyspira* spp. While PCR of oral fluids may be an excellent diagnostic tool for disease detection, they do not encompass all aspects of epidemiology and disease surveillance. Only 40% of pens were culture positive from a recent field study, which is not a high enough pen-level sensitivity to justify using oral fluids for culture when from

the same samples, fecal swabs had a 80% pen-level sensitivity. For surveillance purposes, sensitivity of culture needs to be high enough to detect the organism when it is in low numbers in the animal or environment.

As stated previously, growth characteristics and accurate species identification are important tools for identifying new species of *Brachyspira*. If PCR were to be used alone on diagnostic samples, new species or atypical species may be missed and the samples falsely reported as negative for agents of SD. Additionally, easier identification methods would be of value to confirm phenotypic properties of all species identified. Therefore, selective culture remains an essential part of diagnostic investigation in swine. Culture allows for nuances of the organism to appear. For example, if only PCR tests were performed and *B. hampsonii* was identified, the phenotypical characteristics wouldn't be able to confirm that. The isolate identified as *B. hampsonii* could potentially be an atypical isolate showing different patterns compared to other *B. hampsonii* isolates or it could be a different species entirely that picked up the genetic characteristics of *B. hampsonii*. Additionally, even new strains of varying pathogenicity could be missed if only PCR is performed on oral fluids. Any other number of situations could arise to prevent the accuracy of diagnosis if important aspects are left out. Therefore, a dual approach could be implemented to ensure accuracy and consistency of results. This method is beneficial for many reasons including: fewer total samples needed when considering oral fluids versus individual animal samples, earlier confirmation of disease diagnosis, high sensitivity of detection where culture can confirm the specificity of diagnosis, and ultimate isolation of the organism whereby further testing and characterization is possible. Using this combined sampling approach, diagnostic testing will maintain sufficient diagnostic sensitivity and specificity for confident surveillance and

detection of known agents of SD, potential changes occurring in disease expression patterns, and the emergence of novel species.

REFERENCES

1. Achacha M, Messier S; 1992, Comparison of six different culture medium for isolation of *Treponema hyodysenteriae*. J Clin Microbiol 30:249-251.

2. Atyeo RF, Oxberry SL, Combs BG, Hampson DJ; 1998, Development and evaluation of polymerase chain reaction tests as an aid to diagnosis of swine dysentery and intestinal spirochaetosis. Lett Appl Microbiol 26:126-130.

3. Atyeo RF, Stanton TB, Jensen NS, et al.; 1999, Differentiation of *Serpulina* species by NADH oxidase gene (*nox*) sequence comparisons and *nox*-based polymerase chain reaction tests. Vet Micro 67:47-60.

4. Backhans A, Jansson DS, Aspán A, Fellström C; 2011, Typing of *Brachyspira* spp. from rodents, pigs, chickens on Swedish farms. Vet Microbiol 153:156-162.

5. Bizzini A, Greub F; 2010, Matris-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect 16:1614-1619.

6. Burrough ER, Wilberts BL, Bower, LP, et al.; 2013, Fluorescent in situ hybridization for detection of "*Brachyspira hampsonii*" in porcine colonic tissues. J Vet Diagn Invest 25:407-412.

7. Burrough ER; 2017, Swine dysentery: etiopathogenesis and diagnosis of a reemerging disease. Vet Pathol 54:22-31.

8. Calderaro A, Dettori G, Grillo R, et al.; 1997, Comparative growth of pure cultures of human intestinal spirochaetes in six selective media. New Microbiol 20:47-54.

9. Calderaro A, Merialdi G, Perini S, et al.; 2001, A novel method for isolation of *Brachyspira (Serpulina) hydysenteriae* from pigs with swine dysentery in Italy. Vet Micro 80:47-52.

10. Calderaro A, Piccola G, Montecchini S, et al.; 2013, MALDI-TOF MS analysis of human and animal *Brachyspira* species and benefits of database extension. J Proteomics 78:273-280.

11. Davelaar FG, Smit HF, Hovind-Hougen K, et al.; 1986, Infectious typhlitis in chickens caused by spirochetes. Avian Pathol 15:247-258.

12. Decristophoris P, Fasola A, Benagli D, et al.; 2011, Identification of *Staphylococcus intermedius* group by MALDI-TOF MS. Syst Appl Microbiol 34:45-51.

13. Elder RO, Duhamel GE, Schafer RW, et al.; 1994, Rapid detection of *Serpulina hyodysenteriae* in diagnostic specimens by PCR. J Clin Microbiol 32:1497-1502.

14. Fellström C, Gunnarsson A; 1995, Phenotypical characterisation of intestinal spirochaetes isolated from pigs. Res Vet Sci 59:1–4.

15. Fellström C, Petterssen B, Zimmerman U, et al.; 2001, Classification of *Brachyspira* spp. isolated from Swedish dogs. Anim Health Res Rev 2:75-82.

16. Glock RD, Harris DL; 1972, Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. Vet Med Small Anim Clin 67:65-68.

17. Grosse-Herrenthey A, Maier T, Gessler F, et al.; 2008, Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Anaerobe 14:242-249.

18. Hall L, Doerr KA, Wohlfiel SL, Roberts GD; 2003, Evaluation for the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol 41:1447-1453.

19. Hampson DJ; 2012, Brachyspiral colitis. *In*: Diseases of swine, ed. Zimmerman JJ, Karriker LA, Ramirez A, et al., 10th ed., pp. 680–696. Wiley-Blackwell, Ames, IA.

20. Harel J, Forget C; 1995, DNA probe and polymerase chain reaction procedure for the specific detection of *Serpulina hyodysenteriae*. Mol Cell Probes 9:111-119.

21. Harland WA, Lee FD; 1967, Intestinal spirochaetosis, Br Med J 3:708-709.

22. Harris DL, Glock RD, Christensen CR, Kinyon JM;1972, Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. Vet Med Small Anim Clin 67:61-64.

23. Hunter D, Wood, T; 1979, An evaluation of the API ZYM system as a means of classifying spirochaetes associated with swine dysentery. Vet Rec 104:383-384.

24. Jenkinson SR, Wingar CR; 1981, Selective medium for the isolation of *Treponema hyodysenteriae.* Vet Rec 109: 384-385.

25. Jensen TK, Christensen AS, Boye M; 2010, *Brachyspira murdochii* colitis in pigs. Vet Pathol 47: 334-338.

26. Kunkle RA, Kinyon JM; 1988, Improved selective medium for the isolation of *Treponema hyodysenteriae*. J Clin Microbiol 26: 2357-2360.

27. La T, Phillips ND, Hampson DJ; 2003, Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. J Clin Microbiol 41:3372-3375.

28. LeRoy CI, Mappley LJ, La Ragione RM, et al.; 2015 *Brachyspira pilosicoli*-induced avian intestinal spirochaetosis. Microb Ecol Health Dis 26:28853.

29. Leser TD, Møller K, Jensen TK, Jorsal SE; 1997, Specific detection of *Serpulina hyodysenteriae* and potentially pathogenic weakly ß-haemolytic porcine intestinal spirochetes by polymerase chain reaction targeting 23S rDNA. Mol Cell Probes 11:363-372.

30. McLaren AJ, Hampson DJ, Wylie SL; 1996, The prevalence of intestinal spirochaetes in poultry flocks in Western Australia. Aust. Vet. J 74:319-230.

31. Mirajkar NS, Bekele AZ, Chander YY, Gebhart CJ; 2015, Molecular epidemiology of novel pathogen "*Brachyspira hampsonii*" reveals relationships between diverse genetic groups, regions, host species, and other pathogenic and commensal *Brachyspira* species. J Clin Microbiol 53:2908-2918.

32. Mirajkar NS, Gebhart CJ; 2014, Understanding the molecular epidemiology and global relationship of *Brachyspira hyodysenteriae* from swine herds in the United States: a multi-locus sequence typing approach. PLoS One 9:e107176.

33. Mirajkar NS, Phillips ND, La T, et al.; 2016, Characterization and recognition of *Brachyspira hampsonii* sp. nov., a novel intestinal spirochete that is pathogenic to pigs. J Clin Microbiol 54: 2942-2949.

34. Mushtaq M, Zubair S, Råsbäck T, et al.; 2015, *Brachyspira suanatina* sp. nov., an enteropathogenic intestinal spirochaete isolated from pigs and mallards: genomic and phenotypic characteristics. BMC Microbiol 15:208.

35. Nemes CS, Glávits R, Dobos-Kovács M, et al.; 2007, Typhlocolitis associated with spirochaetes in goose flocks. Avian Pathology 35:4-11.

36. 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. Microbiol Immunol 41:445-452.

37. Prickett JR, Kim W, Simer R, et al.; 2008 Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod 16:86-91.

38. Prickett J, Simer R, Christopher-Hennings J, et al.; 2008, Detection of *Porcine reproductive and respiratory syndrome virus* infection in porcine oral fluid samples: a longitudinal study under experimental conditions. J Vet Diagn Invest 20:156-163.

39. Prickett JR, Zimmerman JJ; 2010, The development of oral fluid-based diagnostics and application in veterinary medicine. Anim Health Res Rev 11:207-216.

40. Prohaska S, Plüger V, Ziegler D, et al.: 2014, MALDI-TOF MS for identification of porcine *Brachyspira* species. Lett Appl Microbiol 58:292-298.

41. Ramirez A, Wang C, Prickett JR, et al.; 2012, Efficient surveillance of pig populations using oral fluids. Prev Vet Med 104:292-300.

42. Råsbäck T, Jansson DS, Johansson K-E, Fellström C; 2007, A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated '*Brachyspira suanatina*' sp. nov. Environ Microbiol 9:983-991.

43. Rohde J, Rothkamp A, Gerlach G; 2002, Differentiation of porcine *Brachyspira* species by a novel *nox* PCR-based restriction fragment length polymorphism analysis. J Clin Microbiol 40:2598-2600.

44. Romagosa, A, Gramer M, Soo Joo H, Torremorell M; 2011, Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza Other Respir Viruses 6:110-118.

45. Rubsamen S, Rubsamen S; 1986, Rapid test for differentiation of *Treponema hyodysenteriae* and *Treponema innocens*. Tieraerztliche Umshau 41.9:673-677.

46. Seng P, Drancourt M, Gouriet F, et al.; 2009, Ongoing revolution in bacteriology: routing identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543-551.

47. Stanton TB: 1992, Proposal to change the genus designation *Serpula* to *Serpulina* gen. nov. containing the species *Serpulina hyodysenteriae* comb. nov. and *Serpulina innocencs* comb. nov. Int J Syst Bacteriol 42:189-190.

48. Stanton TB; 2011, Isolation and Maintenance of *Brachyspira* species. *In*: Current Protocols in Microbiology, Supplement 22, pp. 12D.1.1-12D.1.14. Wiley Online Library.

49. Stanton TB, Hanzelka BL, Jensen NS; 1995, Survey of intestinal spirochaetes for NADH oxidase by gene probe and by enzyme assay. Microb Ecol Health Dis 8:93-100.

50. Stanton TB, Jensen NS, Casey TA, et al.; 1991, Reclassification of *Treponema hyodysenteriae* and *Treponema innocens* in a new genus, *Serpula* gen. nov., as *Serpula hyodysenteriae* comb. nov. and *Serpula innocens* comb. nov. Int J Syst Bacteriol 41:50-58.

51. Stephens CP, Hampson DJ; 2002, Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli* causes reduced egg production. Avian Pathol 31:169-175.

52. Sutter VL, Carter WT; 1972, Evaluation of media and reagents for indole-spot tests in anaerobic bacteriology. Am J Clin Pathol 58:335-338.

53. Swayne DE: 2003, Avian intestinal spirochetosis. *In*: Diseases of Poultry, ed. Saif YM, Barnes HJ, Glisson JR, et al., 11th ed., pp826-836. Iowa State Press, Ames, IA.

54. Weissenböck H, Maderner A, Herzog AM, et al.; 2005, Amplification and sequencing of *Brachyspira* spp. Specific portions of *nox* using paraffin-embedded tissue samples from clinical colitis in Austrian pigs shows frequent solitary presence of *Brachyspira murdochii*. Vet Micro 111:67-75.

APPENDIX

NOMENCLATURE

SD	Swine Dysentery
CFU	Colony Forming Unit
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
AIS	Avian Intestinal Spirochetosis
MLST	Multi-locus Sequence Typing
MSP	Mass Spectral Profile
rDNA	Ribosomal Deoxyribonucleic Acid
TSA	Tryptic Soy Agar
PBS	Phosphate Buffered Saline
DNA	Deoxyribonucleic Acid
BB	Bovine Blood
BHI	Brain Heart Infusion
CVS	Colistin, Vancomycin, Spectinomycin
BJ	Colistin, Vancomycin, Spectinomycin, Spiramycin, Rifampicin
BAM-SR	Blood Agar Medium with Spectinomycin and Rifampicin
ATCC	American Type Culture Collection
NADC	National Animal Disease Center