

## SWINE HEALTH

**Title:** Development of a genus-specific virulence gene PCR panel for *Brachyspira* spp. –  
**NPB# 12-054**

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### Industry Summary:

In the latter part of 2008 and early 2009, strongly beta-hemolytic *Brachyspira* spp. not identified as *B. hyodysenteriae* by published polymerase chain reaction assays were recovered with increasing frequency from North American pigs with clinical swine dysentery (SD). Biochemical and molecular characterization of these atypical and untypable *Brachyspira* isolates revealed the emergence of a putatively novel species with two distinct phylogenetic clades (clade I and clade II) and the name “*Brachyspira hampsonii*” has been proposed. The ultimate goal of this study was to genetically characterize a representative isolate of each clade and compare these isolates with other known pathogenic and poorly or non-pathogenic *Brachyspira* in efforts to determine potential virulence genes that could be used as a target for a virulence gene PCR assay. This study addressed the overall goal in two ways: 1) whole genome sequencing of strains of both clades of “*B. hampsonii*” along with a strain of the pathogenic *B. hyodysenteriae* and the non-pathogenic *B. innocens* by the PacBio platform and 2) whole genome sequencing of these same 2 strains of “*B. hampsonii*” as well as an additional strain of “*B. hampsonii*” clade I and strains of the weakly beta-hemolytic *B. pilosicoli*, *B. intermedia*, and *B. murdochii* using the Ion Torrent platform. Using two separate platforms for whole genome sequencing of the proposed novel “*B. hampsonii*” provides greater confidence in the resultant sequences prior to comparison and analysis with other strains. Sequences of *B. hyodysenteriae*, *B. pilosicoli*, *B. intermedia* and *B. murdochii* are available in GenBank for comparison and validation of the sequences obtained in this project.

The isolation of adequate DNA and whole genome sequencing of these spirochetes proved highly challenging and took much longer than expected. As a result, sequence analysis remains ongoing and the development of a virulence gene PCR was not completed within the timeframe of this project. However, the ultimate result of this project was the completion of whole genome sequencing on more than double the number of isolates as originally planned and the obtained sequences will be extremely valuable as many of these isolates have been used in our prior animal inoculation experiments and have a known pathotype. Having a well-characterized virulence phenotype will be key in properly categorizing these genetic sequences as originating from either pathogenic or poorly/non-pathogenic spirochetes in future efforts to identify putative virulence genes and target sequences for a swine dysentery-specific PCR that would detect the presence of pathogens rather than specific *Brachyspira* spp.

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**Keywords:**

*Brachyspira*; *Brachyspira hyodysenteriae*; '*Brachyspira hampsonii*'; swine dysentery

**Scientific Abstract:**

In recent years the swine industry has observed a resurgence of swine dysentery and dysentery-like disease although the reason(s) for this increase in dysentery diagnoses is poorly understood. Classically, swine dysentery has been attributed to infection by *Brachyspira hyodysenteriae*, but recent research by this group and others has identified at least two potentially novel species of *Brachyspira*, "*Brachyspira hampsonii*" and "*Brachyspira suanatina*", with strong hemolytic potential that induce dysentery-like disease but are not positive by *B. hyodysenteriae*-specific PCR assays. These potentially novel species have proven to be diagnostically challenging. In this study, whole genome sequencing of multiple putatively novel pathogenic *Brachyspira* isolates along with several officially recognized species with a recognized virulence phenotype *in vivo* was completed. The obtained sequences can be used for ongoing analyses and investigations into genetic differences that may underlie the potential to induce dysentery in susceptible swine. This will provide critical insight into future diagnostic and prevention strategies.

**Introduction:**

Swine dysentery (SD) is classically associated with *Brachyspira hyodysenteriae* infection where susceptible growing and finishing age pigs develop severe mucohemorrhagic diarrhea which ultimately results in significant economic losses in affected systems.<sup>1</sup> Recently, however, multiple untypable strongly beta-hemolytic spirochetes have been recovered from pigs with clinical SD including the proposed novel species "*B. hampsonii*" and "*B. suanatina*".<sup>2,3</sup> Experimental reproduction of disease indistinguishable from SD has also been reported following inoculation with these proposed novel species.<sup>3,4,5</sup>

A clinical diagnosis of SD is based upon observation of characteristic bloody and mucoid diarrhea. Laboratory confirmation of SD is often based upon selective microbial culture with the isolation of a strongly beta-hemolytic spirochete followed by speciation through *nox*-based PCR methods;<sup>6</sup> however, these assays often take 6 days or more to complete resulting in a significant delay between receipt of clinical samples and the reporting of results. While direct sample PCR assays for *B. hyodysenteriae* have been described,<sup>6</sup> these assays are designed to detect known species and may therefore fail to detect novel pathogenic species such as "*B. hampsonii*" and "*B. suanatina*".

Investigation and understanding the genetic differences between *Brachyspira* spp. with the potential to induce SD and those that do not is of high priority as PCR assays focused on detecting common virulence factors may be more robust to detecting emerging pathogenic species than currently available species-based assays. Accordingly, whole genome sequencing was performed on *Brachyspira* spp. isolates with a known virulence phenotype based on prior inoculation studies in mice and pigs.<sup>4,7</sup>

**Objectives:**

1. Genetically characterize two potentially novel species of *Brachyspira*.
2. Develop a PCR-based *Brachyspira* virulence gene panel.

3. Determine optimal sampling strategies for diagnosis of clinical cases of *Brachyspira*-induced disease.

## **Materials & Methods:**

### *Bacterial propagation and microbial techniques*

Well-characterized *Brachyspira* spp. isolates that had been subpassaged for purity and confirmed to species by *nox* gene sequencing were obtained from the culture collection at the Iowa State University Veterinary Diagnostic Laboratory. The species and specific strains used in this analysis are listed in Table 1.

Briefly isolates were grown anaerobically in trypticase soy broth with 0.5% yeast extract, 0.05% L-cysteine, 1% VPI salt solution, and 5 to 10% heat-inactivated horse serum.<sup>8</sup> Eighteen to 24-hour-old cultures were harvested by centrifugation (9,000 x g for 10 minutes).

### *DNA extraction and whole genome sequencing*

DNA was extracted by Power Microbial Maxi DNA Isolation Kit (Mo Bio Laboratories, cat# 1226) followed by a phenol chloroform extraction or by Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories, cat #12224) followed by an ethanol precipitation. DNA for some isolates was further amplified using illustra Ready-To-Go Gemoni Phi V3 DNA Amplification Kit (GE Healthcare Life Sciences, cat #25-6601-24).

DNA from four isolates (EB102, EB106, EB107, and B204) was submitted to Expression Analysis (Durham, NC) for PacBio single molecule, real-time (SMRT<sup>®</sup>) sequencing. DNA was quantified using a Qubit ds DNA BR Assay Kit (Life Technologies, cat #Q32850) prior to submission. The advantages of this platform include longer read lengths compared to traditional next-generation sequencing with read lengths in the thousands of bases. This can resolve both SNPs and large-scale structural rearrangements and longer reads also simplify and improve genomic assembly.

DNA libraries for Ion Torrent PGM<sup>™</sup> (Person Genome Machine, Life Technologies) sequencing were made using NEB Next Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England BioLab Inc., cat # E62855). Libraries were barcoded using Nextflex DNA Barcodes for Ion Torrent, (Bio Scientific, cat#4010-01). DNA fragments were sized using Egel Size Select Agarose Gels (Invitrogen, cat# 661002). DNA libraries were templated onto the Ion Sphere Particles (ISP) using the Ion PGM<sup>™</sup>Template OT2 200 Kit (Life Technologies cat#4480974) and Ion PGM<sup>™</sup>Template OT2 400 Kit (Life Technologies cat#4479878) according to respective manual instructions (MAN0007220 and MAN0007218). Sequencing was done in-house on the Ion Torrent PGM<sup>™</sup> using Ion 316<sup>™</sup>chips (cat#4483188) with the Ion PGM<sup>™</sup> Sequencing 200 kit (Life Technologies cat #4482006) and the Ion PGM<sup>™</sup> Sequencing 400 kit (Life Technologies cat #4482002) according to respective manuals (MAN0007273 and MAN0007242).

## **Results:**

### *Bacterial propagation and microbial techniques*

There were many obstacles which had to be overcome in order to obtain the genomic DNA required for sequencing. Several of the isolates initially selected for sequencing either could not be recovered from frozen stock ("*B. hamptonii*" clade II KC58) or grew very slowly when first seeded from frozen stocks resulting in a delay of several months from initiation of bacterial propagation until sufficient growth was achieved for DNA extraction. Growth conditions and sera concentrations of the media had to be adjusted by trial and error to match the growth requirements of each isolate. Cultures were monitored daily to determine growth rate and viability which determined optimal times for subculturing and harvesting. *Brachyspira* spp. often do not grow to high numbers in broth cultures. Under optimal conditions, *Brachyspira* spp. will reach viable cell numbers of 10<sup>8</sup> cfu/mL, unlike *Escherichia coli* which will easily exceed cell numbers of 10<sup>9</sup> cfu/mL. Also, the genomes of *Brachyspira* spp. are approximately half of the size of the *E. coli* genome. For these reasons, isolates being sequenced using the

PacBio platform had to be grown in broth volumes of one liter in order to obtain sufficient cell mass.

#### *DNA extraction and whole genome sequencing*

DNA extractions from cell pellets (average wet weight of 137 mg) utilizing the Power Microbial Maxi DNA Isolation Kit yielded a 1 ml volume containing an average DNA concentration of 44 ng/ $\mu$ l. In order to meet Expression Analysis DNA submission requirements of 12,500 ng in a 250  $\mu$ l volume (50 ng/ $\mu$ l) for PacBio sequencing, these extractions had to be further purified and concentrated. This additional step required that multiple cell pellets be extracted and then pooled in order to obtain the required DNA. Because of the difference in platform, obtaining the genomic DNA template for Ion Torrent PGM™ sequencing was much easier. PacBio sequencing generates read lengths between 4,200 and 8,500 nucleotides so large segments of the genomes are required, while the Ion Torrent PGM™ sequences DNA fragments (libraries) that are 200 and 400 nucleotides long. Because of the short read lengths required by the PGM™, genomic DNA amplified using Illustra Ready-To-Go Gemoni Phi V3 DNA Amplification Kit could be used. This kit uniformly amplifies the whole genome using isothermal strand displacement producing numerous, short, overlapping, branched copies of the genome which is enzymatically fragmented during the library preparations. The Ready-To-Go Gemoni Phi V3 DNA Amplification Kit, starting with 10 ng of genomic DNA, yielded an average of 200 ng/ $\mu$ L multi-branched copies of the genomic DNA. PGM™ library preparation utilized 1  $\mu$ g of either the amplified genomic DNA or genomic DNA extracted with the Ultra Clean Microbial DNA Isolation Kit. The general quality of a PGM™ sequencing run was evaluated in terms of loading density of the ISP on the chip, percent usable reads, read length, and Q score. Results of sequencing by platform are listed in Table 1.

#### **Discussion:**

Significant challenges were encountered with this project during the bacterial propagation phase as *Brachyspira* DNA was difficult to obtain in sufficient quantity for sequencing due to issues with getting large volumes of spirochetes in broth culture. Due to these unforeseen delays and the transition of this project between PIs after the original PI, Dr. Erin Strait, left the university, the ultimate goal of creating a virulence gene PCR panel was not achieved during the period of this grant. While a virulence gene PCR panel for *Brachyspira* spp. has not yet been created, many more isolates were sequenced as part of this project than originally planned as we were able to sequence ten isolates rather than the original proposal of four, and we were able to perform sequencing on multiple platforms. As a result, the sequence data generated from this project will greatly expand the number of *Brachyspira* whole genomes available for comparison and these data will continue to be analyzed in the future with the ongoing goal of ultimately identifying virulence genes associated with the potential to induce swine dysentery.

An additional objective of this project was to determine optimal sampling strategies for diagnosis of *Brachyspira*-induced disease. Since the initiation of this project, much has been described regarding the various strengths and limitations of *Brachyspira* diagnostic tests. Selective anaerobic culture has traditionally been regarded as the most sensitive test for detection of *Brachyspira* spp., and multiple studies have shown that its diagnostic sensitivity exceeds PCR on spiked feces or surveillance samples.<sup>9,10</sup> However, in clinically affected pigs exhibiting typical mucohemorrhagic feces, the diagnostic sensitivity is often very similar between culture and PCR suggesting that either assay would be appropriate for pigs with overt disease where greater than 90% of such pigs will be positive by either assay.<sup>11</sup> Surveillance provides a much different situation where expected shedding rates in a subclinical herd may be less than 5%.<sup>12</sup> Accordingly, the sensitivity advantages of selective culture greatly outweigh the rapid turnaround and ease of use associated with PCR assays.

In summary, one or more unified genetic targets associated with *Brachyspira* virulence and the potential to induce swine dysentery remain to be determined and are the focus of ongoing genetic analysis of the whole genome sequences produced as part of this project. Once

such target sequences are identified, they may provide a more robust direct sample PCR assay to screen for virulent spirochetes in the feces of pigs with mucoid diarrhea. This project has more than doubled the number of whole genome sequences that were publically available for analysis prior to the initiation of this project and, given the well-characterized pathotype of many of these newly sequenced strains, will form a backbone for future comparison and analysis of pathogenic and non-pathogenic strains.

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**Tables:**

**Table 1.** *Brachyspira* isolates used for whole genome sequencing by various platforms

Species identification*	Strain ID	Cultural Phenotype	Sequencing Platform	
			PacBio	Ion Torrent (PGM™)
<i>B. hampsonii</i> clade I	EB106‡	Strong beta	complete	complete
<i>B. hampsonii</i> clade I	EB109	Strong beta	ND	complete
<i>B. hampsonii</i> clade II	EB107‡	Strong beta	complete	complete
<i>B. hampsonii</i> clade II	KC23†	Strong beta	ND	pending
<i>B. hampsonii</i> clade II	KC58†‡	Strong beta	insufficient growth	insufficient growth
<i>B. hyodysenteriae</i>	B204†‡	Strong beta	complete	complete
<i>B. pilosicoli</i>	BR2001	Weak beta	ND	complete
<i>B. intermedia</i>	BR2000	Weak beta	ND	complete
<i>B. murdochii</i>	KC60†‡	Weak beta	ND	complete
<i>B. murdochii</i>	EB104†‡	Weak beta	ND	pending
<i>B. innocens</i>	EB102†‡	Weak beta	complete	complete

\* Identification based upon PCR detection and *nox* gene sequencing.

† Virulence phenotype experimentally confirmed in mice.<sup>7</sup>

‡ Virulence phenotype experimentally confirmed in pigs.<sup>4</sup>

ND = not done