

SWINE HEALTH

Title: Fluorescent *in situ* hybridization for the detection of novel pathogenic *Brachyspira* spp. in porcine colonic tissues - **NPB# 12-192**

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Industry Summary:

Swine dysentery (SD) has historically been associated with the presence of *Brachyspira hyodysenteriae*, a strongly beta-hemolytic spirochete; however, beginning in the latter part of 2008 and early 2009 strongly beta-hemolytic *Brachyspira* spp. not identified as *B. hyodysenteriae* by polymerase chain reaction assays were recovered with increasing frequency from North American pigs with clinical SD. In 2012, biochemical and molecular characterization of these atypical and untypable *Brachyspira* revealed the emergence of a novel species and the name "*Brachyspira hampsonii*" has been proposed. The ultimate goal of this study was to develop a rapid, culture-independent assay to confirm the presence of virulent, non-*B. hyodysenteriae* spirochetes capable of producing SD within pig tissues. This study addressed the overall goal in two ways: 1) sequencing of the 23S rRNA gene was performed on numerous clinical isolates of "*B. hampsonii*" (clades I & II) along with multiple typical isolates of commonly recognized *Brachyspira* spp. infecting swine for comparison, and 2) based upon the results of 23S rDNA sequencing, an oligonucleotide probe specific for 23S rRNA of "*B. hampsonii*" was developed and evaluated for specificity and sensitivity in a fluorescent *in situ* hybridization (FISH) assay on formalin-fixed, paraffin-embedded colonic tissues from pigs experimental and naturally infected with a variety of *Brachyspira* spp.

The newly developed probe (Hamp1210) had a high degree of sensitivity on the tissues tested recognizing all tissues from which "*B. hampsonii*" was recovered by culture; however, a low percentage of *B. hyodysenteriae* infected samples had weak to moderate signal with this probe as well as a single case that was culture-positive for *Brachyspira intermedia*. The FISH assay in this study was designed to be performed with a 6 hour hybridization period making the turnaround time shorter than many previously described protocols. Additionally, a previously described probe for *B. hyodysenteriae* (Hyo1210) was validated under these same hybridization conditions and exhibited similar sensitivity. The observed cross-reactivity suggests that a second, more specific assay should be used to confirm positive results in index situations; however, this FISH assay, when incorporating both the Hamp1210 and Hyo1210 probes, can provide a rapid preliminary molecular identification of pathogenic spirochetes in cases with clinical signs of SD and reduce the time delay between sample submission, pathogen identification, and treatment initiation.

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Keywords:

Brachyspira; *Brachyspira hyodysenteriae*; '*Brachyspira hampsonii*'; swine dysentery; fluorescent *in situ* hybridization.

Scientific Abstract:

Swine dysentery is classically associated with infection by *Brachyspira hyodysenteriae*; however, the proposed novel species '*Brachyspira hampsonii*' has recently been isolated from clinical cases of dysentery in North America. Selective anaerobic culture is a highly sensitive method for detecting *Brachyspira* in clinical samples but requires several days for completion often followed by molecular testing for speciation. Alternatively, *in situ* hybridization applied to sections of formalin-fixed tissue can provide rapid, culture-independent identification of pathogens observed histologically. In this study, a fluorescent *in situ* hybridization assay was developed for confirmation of a clinical diagnosis of swine dysentery associated with '*B. hampsonii*' infection. An oligonucleotide probe (Hamp1210) targeting 23S rRNA of '*B. hampsonii*' was developed after sequence analysis and comparison of numerous *Brachyspira* spp. clinical isolates with reference sequences available in GenBank. Application of Hamp1210 and a previously published probe for *B. hyodysenteriae* (Hyo1210) to diseased colonic tissues from pigs successfully detected the target species in both experimentally infected pigs and naturally infected pigs, and the Hamp1210 probe consistently detected both clade I and clade II isolates of '*B. hampsonii*'. *In situ* hybridization incorporating these probes can reduce the delay from sample submission to pathogen identification in cases of swine dysentery where formalin-fixed tissues are available.

Introduction:

Swine dysentery (SD) is a severe mucohemorrhagic diarrheal disease of growing and finishing pigs and often results in significant economic losses in affected systems.¹ SD is classically associated with infection by the *Brachyspira hyodysenteriae*;² however, experimental reproduction of SD following inoculation with other strongly beta-hemolytic spirochetes that are not identified as *B. hyodysenteriae* by polymerase chain reaction (PCR) assays has also been described.^{3,4} Pigs infected with strongly beta-hemolytic spirochetes identified as *Brachyspira intermedia* and '*Brachyspira* sp. SASK30446' by PCR develop clinical disease and lesions typical of SD,³ and comparison of the reported *nox* gene sequences from those isolates with others available in GenBank reveals they are identical or nearly identical over the region compared to clade I and clade II isolates, respectively, of the proposed novel species '*Brachyspira hampsonii*'.⁵ Together these findings establish that SD can occur following infection with '*B. hampsonii*'.

A clinical diagnosis of SD is based upon observation of characteristic bloody to mucoid diarrhea. Gross lesions of SD are limited to large intestines and include mucosal thickening, hemorrhage, fibrinonecrotic exudation, and luminal mucus.¹ Laboratory confirmation of SD is often based upon microbial culture with the isolation of a strongly beta-hemolytic spirochete followed by speciation through *nox*-based PCR methods;⁶ 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. In cases of suspected dysentery where fixed tissues are available, spirochetes can often be visualized within affected tissue by silver staining. While observation of spirochetes by this method is supportive of a diagnosis of SD, the imprecise nature of the staining procedure does not allow differentiation of pathogenic from non-pathogenic species. Fluorescent *in situ* hybridization (FISH) using bacterial rRNA-targeted probes can be an effective assay for molecular identification of pathogens noted histologically, and FISH assays have been described for detection of *Brachyspira* spp. at the genus level as well as at the species level for identification of *Brachyspira pilosicoli*, *B. hyodysenteriae*, and *Brachyspira murdochii*.^{7,8} Given the emergence of '*B. hampsonii*' associated with SD in the United

States,⁵ and the experimental reproduction of SD following inoculation with clinical isolates,³ the development of rapid diagnostic tools for detection of this agent, such as FISH, are a pressing need.

Objectives:

1. Sequence the 23S rRNA genes of multiple clinical isolates representing two strongly beta-hemolytic, non-*B. hyodysenteriae* species identified by our laboratory that are consistent with '*B. hampsonii*' clades I and II as well as multiple typical clinical isolates and compare the sequences generated with reference sequences available in GenBank.
2. Develop FISH for the detection of '*B. hampsonii*' in porcine colonic tissues using an oligonucleotide probe specific for 23S rRNA of this species generated from the sequencing results obtained in objective 1.

Materials & Methods:

Bacterial isolates and microbial techniques

Clinical isolates of *Brachyspira* spp. were obtained from the culture collection of the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). Historical information, phenotypic characteristics, and species identification for the specific isolates used in this study are listed in Table 1. *Brachyspira* isolates were identified to species by *nox*-based PCR assays as previously described.^{9,10} For those strongly beta-hemolytic isolates not identified as *B. hyodysenteriae* by PCR, final identification as either clade I or clade II isolates of '*B. hampsonii*' was determined by PCR amplification and sequencing of the *nox* gene using previously described primers¹¹ followed by comparison with reference sequences available in GenBank. All clinical isolates had been stored at -80°C until use and were recovered on CVS and BJ selective agars. Plates were incubated in an anaerobic environment at 41 ± 1°C for up to 6 days with growth and strength of beta-hemolysis assessed every 48 hours. Once growth was deemed adequate, agar plugs were collected from hemolytic areas of each plate and then processed as described below for PCR.

23S rDNA sequencing and probe design

DNA was extracted by boiling the agar plugs in microcentrifuge tubes containing molecular biology-grade water followed by centrifugation at 20,000 x g for 5 minutes. The 23S rDNA of each isolate was PCR amplified and sequenced in three fragments using previously described¹² and modified primers. PCR amplicons were sequenced using a commercial analyzer and the sequences obtained were assembled and aligned using a commercial software package (Lasergene version 8.0, DNASTAR Inc., Madison, WI).

The 23S rRNA gene sequences obtained from clinical isolates in the present study were compared with reference sequences available in GenBank to determine a specific target sequence for probe design.

Fluorescent in situ hybridization

Descriptions of the three oligonucleotide probes used in this study are listed in Table 2. Custom oligonucleotide probes were purchased from a commercial source and were 5' labeled with either green-fluorescent (Hamp1210) or orange-fluorescent (SER1410 and Hyo1210) dye.

Blocks of paraffin-embedded colon tissue from pigs with clinical SD were obtained from ISU VDL clinical submissions and from a previous experimental study.³ Sections were selected where spirochetes with features typical of *Brachyspira* spp. were visible by Warthin-Starry silver staining, and in all cases samples were selected from tissues where a single *Brachyspira* sp. was previously isolated by culture. The following species were represented in this analysis and the number of individual samples tested for each species appears in parentheses: *B. hyodysenteriae* (4), '*B. hampsonii*' clade I (4), '*B. hampsonii*' clade II (4), *B. pilosicoli* (4), *B. intermedia* (1), and *B. murdochii* (4).

Sections of colon were mounted on glass slides, deparaffinized, and allowed to air dry. The area of interest on each slide was circumscribed with a hydrophobic pen prior to application of the hybridization solution. The labeled probes were reconstituted using DNase/RNase-free water and diluted to a working concentration of 5 ng/μl in hybridization buffer (20 mM Tris, 0.9 M NaCl, 20% SDS, 40% formamide, 10% dextran sulfate, and pH 7.2). Sections were covered with 150 μl of the hybridization solution containing the desired probe and were placed in a moisture chamber. Hybridization was carried out for 6 hours at 42°C for both the Hamp1210 and Hyo1210 probes or overnight at 45°C for the SER1410 probe. Following hybridization, slides were washed in a wash buffer (hybridization buffer without SDS or formamide and prewarmed to the appropriate hybridization temperature) for 20 minutes. After washing, the slides were rinsed with sterile water, allowed to air dry, and mounted using an antifade reagent.

A fluorescent microscope with filters specific for the fluorophores used in this study was used for all analyses. Sections were first screened using the SER1410 probe, and only those with positive signal by this probe were subjected to the species-specific probes.

Results:

23S rDNA sequencing and probe design

Comparison of the obtained 23S rDNA sequences from the clinical isolates in the present study with reference sequences available in GenBank revealed distinct clustering by species; however, the overall sequence similarity between '*B. hamptonii*', *B. intermedia*, and *B. hyodysenteriae* was very high with less than 1 nucleotide per 100 nucleotides difference. Analysis of the consensus sequence for *Brachyspira* spp. revealed a high degree of interspecies variability in the area of bases 1310 - 1328 of the 23S rRNA gene within the published whole genome sequence of *B. hyodysenteriae* WA1.¹³ This area also corresponds to the same region targeted by a previously described probe for *B. hyodysenteriae* (Hyo1210)⁸, and within this area the sequences of both clade I and clade II isolates of '*B. hamptonii*' were identical for all isolates evaluated. Given that this location provided 3 to 4 base pair mismatches between sequences of "*B. hamptonii*" and those of commonly isolated weakly beta-hemolytic *Brachyspira* spp., this location was selected for development of the Hamp1210 probe. Analysis of the target site corresponding to the SER1410 probe revealed 100% sequence homology for all strains evaluated.

Fluorescent in situ hybridization

In all sections tested, a moderate to strong signal was observed after hybridization with the SER1410 probe except in one clinical case from which *B. pilosicoli* was previously recovered by culture and one in which *B. murdochii* had been isolated.

For the Hamp1210 probe, moderate to strong signal was detected after hybridization in all cases where '*B. hamptonii*' was previously recovered by culture, and in none of the cases that were culture positive for *B. pilosicoli* or *B. murdochii*. Labeled spirochetes were visible within crypts, goblet cells, and enterocytes (Fig. 1). In the single case from which *B. intermedia* had been isolated, a strong positive signal was detected following hybridization with the Hamp1210 probe, and spirochetes could be visualized within crypt lumens, goblet cells, and luminal exudate. In one of four cases from which *B. hyodysenteriae* was previously recovered by culture, a weak signal was variably detected throughout the section following hybridization with the Hamp1210 probe.

The Hyo1210 probe produced a moderate to strong signal after hybridization in all cases where *B. hyodysenteriae* was recovered by culture, and in none of the cases that were culture positive for *B. pilosicoli*, *B. murdochii*, or *B. intermedia*. Labeled spirochetes were visible within crypt lumens, goblet cells, enterocytes, and luminal

exudate. Similarly to what was observed with the Hamp1210 probe, in one of the eight total cases where '*B. hampsonii*' was recovered by culture, a weak signal was variably detected following hybridization with the Hyo1210 probe revealing the potential for cross-reactivity with these probes.

Publication

The complete results of this study were incorporated into a manuscript that was published in the *Journal of Veterinary Diagnostic Investigation* 25(3):407-412.

Discussion:

These data reveal that FISH applied to formalin-fixed colon tissue from pigs with SD can serve as a rapid screening tool and provide a preliminary molecular identification of pathogenic spirochetes within 24 to 48 hours of sample receipt at the laboratory. The hybridization conditions described in this report allow for a shortened total time for assay completion as only six hours are required for hybridization with the species-specific probes versus the overnight hybridization protocols previously described for *Brachyspira* spp.⁸ Application of the Hamp1210 and Hyo1210 probes to serial sections of affected tissues will allow for the identification of the two most commonly identified, strongly beta-hemolytic spirochetes associated with SD in North America, and can provide rapid confirmation of a clinical diagnosis of SD ultimately shortening the delay between sample submission and therapeutic intervention. Given the potential for cross-reactivity with these two probes, however, additional testing and characterization of positive samples is indicated, particularly in index farm situations. Such additional testing would include, but is not limited to, cultural confirmation of hemolytic phenotype and *nox* gene sequencing of isolates for more definitive species identification.

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Figures:

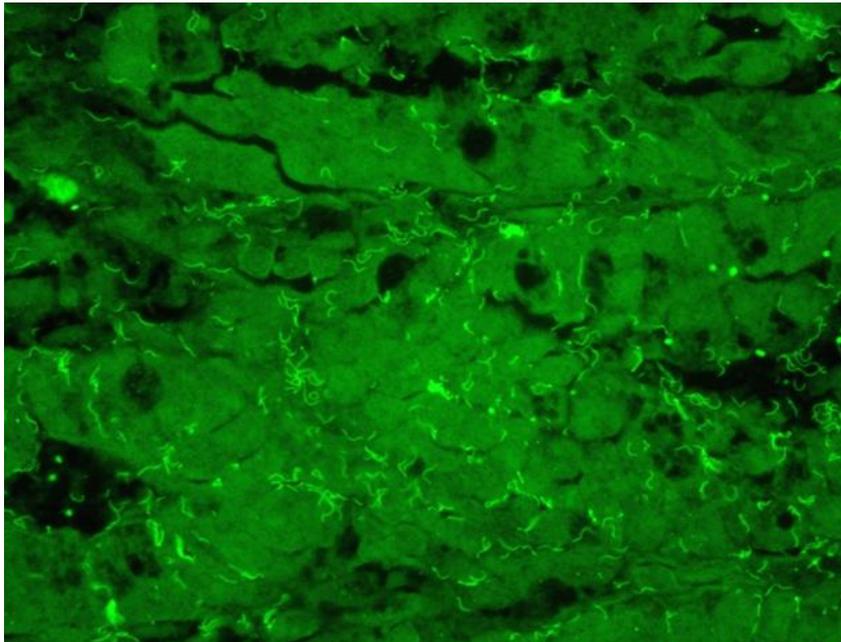


FIGURE 1. Fluorescent *in situ* hybridization using a green-fluorescent probe (Hamp1210) specific for '*B. hamptonii*'. In this photomicrograph of a section of colonic mucosa taken from a pig infected with '*Brachyspira hamptonii*', note the strong positive signal revealing numerous labeled spirochetes within crypt lumens and enterocytes.

Tables:

Table 1. Clinical isolates used for 23S sequencing and analysis

Species identification*	Strain ID	Isolate source, location, & year isolated	Hemolysis pattern
<i>B. hampsonii</i> clade I'	EB106	Pig†, Iowa, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade I'	BR2003	Pig†, Iowa, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade I'	BR2004	Pig‡, Iowa, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade I'	BR2005	Pig‡, Iowa, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade I'	BR2006	Pig†, North Carolina, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade I'	EB109	Pig†, North Carolina, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	EB107	Pig†, Iowa, 2011	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	KC9A	Pig†, Iowa, 2009	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	KC23	Pig†, Iowa, 2008	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	KC32	Pig†, Iowa, 2009	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	KC58	Pig†, Iowa, 2008	Strong beta; ring positive
<i>B. hampsonii</i> clade II'	EB100	Pig†, Iowa, 2010	Strong beta; ring positive
<i>B. hyodysenteriae</i>	B204	Pig†, Iowa, 1973	Strong beta; ring positive
<i>B. hyodysenteriae</i>	EB105	Pig†, Iowa, 2011	Strong beta; ring positive
<i>B. pilosicoli</i>	BR2001	Pig‡, Iowa, 2012	Weak beta; ring negative
<i>B. intermedia</i>	BR2000	Pig‡, New York, 2012	Weak beta; ring negative
<i>B. murdochii</i>	KC60	Pig‡, Iowa, 2009	Weak beta; ring negative
<i>B. murdochii</i>	KC61	Pig‡, Iowa, 2008	Weak beta; ring negative
<i>B. murdochii</i>	KC62	Pig‡, Iowa, 2008	Weak beta; ring negative
<i>B. murdochii</i>	EB104	Pig‡, Iowa, 2011	Weak beta; ring negative
<i>B. innocens</i>	EB102	Pig‡, Missouri, 2010	Weak beta; ring negative

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

† Isolated from a pig with mucohemorrhagic diarrhea.

‡ Isolated from a pig with nonspecific to mucoid diarrhea.

Table 2. Sequences, target sites, and specificities of rRNA-targeted oligonucleotide probes used in this study

Probe name	Sequence (5'-3') of probe	Target position*	Specificity
SER1410†	GTCATTCCATCGAAACATA	23S, 1511-1529	<i>Brachyspira</i> spp.
Hamp1210	CTCACGGTAAACCTTCGAC	23S, 1310-1328	' <i>Brachyspira hampsonii</i> '
Hyo1210†	CTCACGATGAACCTTCGAC	23S, 1310-1328	<i>Brachyspira hyodysenteriae</i>

* Based on the published whole genome sequence of *Brachyspira hyodysenteriae* WA1.¹³

† Previously published probes as described by Boye et al.⁸