

SWINE HEALTH

Title: Investigation and characterization of pathogenic and molecular differences in atypical *Brachyspira* spp. clinical isolates versus classic strains - **NPB# 11-178**

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Industry Summary:

Swine dysentery (SD), or bloody scours, has historically been associated with the presence of the strongly beta-hemolytic spirochete *Brachyspira hyodysenteriae*; however, *Brachyspira* spp. not identified as *B. hyodysenteriae* by polymerase chain reaction assays (PCR) have recently been recovered from pigs with clinical SD. As several of these isolates were either undetectable by existing PCR assays or were identified as species typically regarded as poorly pathogenic, it is imperative that the potential virulence of these atypical isolates be determined for if these isolates are deemed pathogenic, the development of a diagnostic test that will correctly identify these strains is an urgent need. The ultimate goal of this study was to identify more consistent and accurate methods to confirm a clinical diagnosis of SD, and this study addressed the overall goal in three ways: 1) by utilizing a mouse model to assess the virulence potential of twenty-one *Brachyspira* strains including multiple atypical clinical isolates from pigs with gross and microscopic features consistent with SD as well as a group of nonpathogenic isolates and multiple isolates of *B. hyodysenteriae*, 2) based upon the results of the mouse experiment, eight isolates including both typical and atypical pathogenic strains as well as nonpathogenic strains were inoculated into pigs to confirm virulence and clinical manifestations in the target species, and 3) based upon the combined results of the mouse and pig experiments, the eight tested isolates were given a well-defined pathotype (virulent versus avirulent) and can be further compared by future genetic analyses in attempts to identify targets in the pathogenic strains which may form the basis for improved PCR assays for use in diagnostic laboratories.

In both the mouse and pig inoculation experiments, *Brachyspira* spp. that impart characteristic strong beta-hemolysis when cultured on blood agar were associated with the greatest degree of virulence as determined by the development of cecal and colonic inflammation in infected animals. Additionally, in pigs, the development of clinical SD was only observed following infection with strongly beta-hemolytic spirochetes with disease also occurring following infection by strongly beta-hemolytic isolates not identified as *B. hyodysenteriae* by PCR. These data suggest that the isolation of a strongly beta-hemolytic spirochete from feces or colonic tissues of pigs with bloody scours should be interpreted as compatible with a clinical diagnosis of SD even if the isolate is not identified as *B. hyodysenteriae*, and that the cultural characteristics of *Brachyspira* spp. are a more sensitive indicator of the potential to induce SD than the molecular identification of the isolate alone.

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Scientific Abstract:

Classical swine dysentery is associated with the presence of the strongly beta-hemolytic *Brachyspira hyodysenteriae*; however, multiple *Brachyspira* spp. can colonize the porcine colon. Recently, *Brachyspira* spp. not identified as *B. hyodysenteriae* by genotypic and/or phenotypic methods have been isolated from pigs with clinical swine dysentery. In this study, seventeen strains of *Brachyspira*, including multiple strongly beta-hemolytic and weakly beta-hemolytic isolates, were screened for virulence in mice to determine a proposed pathotype for each strain and to select the most appropriate isolates for inoculation into pigs. Following virulence screening, eight clinical isolates, including five strongly beta-hemolytic and three weakly beta-hemolytic *Brachyspira* strains, and a reference strain of *B. hyodysenteriae* (B204) were inoculated into pigs to compare pathogenic potential following oral inoculation. Results revealed that strongly beta-hemolytic isolates induced significantly greater typhlocolitis than those that were weakly beta-hemolytic, regardless of the genetic identification of the isolate, and that strongly beta-hemolytic isolates identified as “*Brachyspira* sp. SASK30446” and *B. intermedia* by polymerase chain reaction (PCR) produced lesions similar to those caused by *B. hyodysenteriae*. These results suggest that the microbial culture characteristics of *Brachyspira* spp. may be a more sensitive indicator of the potential to induce swine dysentery than the identification of the organism by currently available PCR assays alone.

Introduction:

In a diagnostic laboratory, molecular diagnostics, such as PCR, are highly specific methods for pathogen detection even in the presence of limited quantities of infectious agent; however, as these methods depend upon the stability of the genetic sequence targeted, there remains the potential for loss of both sensitivity and specificity of a given test if the genetic target is significantly altered or as novel related pathogens emerge. In the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL), we have recently observed a shift in the genotypic and phenotypic profiles of clinical isolates of *Brachyspira* spp. from pigs; whereby traditional PCR methods no longer appear to appropriately recognize and/or speciate clinically significant isolates, which in turn impedes intervention and elimination efforts on the farm. Additionally, at the ISU VDL we have observed clinical swine dysentery (SD) in pigs from which *B. hyodysenteriae*, the agent classically associated with SD, was not identified while an atypical *Brachyspira* spp. was isolated. It is imperative that the virulence of these atypical isolates be determined and, if these isolates are deemed pathogenic, the development of a diagnostic test that will correctly recognize these atypical strains is an urgent need. The ultimate goal of this study is to identify more consistent and accurate methods to confirm a clinical diagnosis of SD, and to provide the basis for molecular comparison of multiple *Brachyspira* spp. with defined virulence potential as it relates to the development of dysentery-like disease. Improved understanding of the virulence of emerging atypical *Brachyspira* isolates will improve detection and better facilitate eradication efforts for SD ultimately reducing costs associated with bringing pigs to market.

Objectives:

1. Utilize a mouse model to screen for virulence in a large number of atypical clinical isolates of *Brachyspira* spp. from pigs with gross and microscopic features consistent with swine dysentery as well as a group of expected nonpathogenic isolates.
2. Utilize results from the mouse study to select the most appropriate subset of virulent isolates representing atypical phenotypic and genotypic characteristics and inoculate them into pigs to confirm pathogenic versus nonpathogenic strains and to characterize lesions in the target species.
3. Utilize the results from these studies to appropriately categorize pathogenic versus nonpathogenic isolates for eventual genetic analysis to identify unique targets in the pathogenic strains that can be incorporated into a more accurate PCR assay for detecting pathogenic *Brachyspira* spp. for use in the ISU VDL and elsewhere.

Materials & Methods:

1. Mouse inoculation experiment:

a. Animals

- i. All procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University. Eighty-six C3H/HeN mice were obtained from a commercial source (Harlan Laboratories, Madison, WI). Mice were 8 weeks of age at the time of experimentation. All mice were fed Teklad diet TD 85420 (Harlan Laboratories) exclusively from the time of arrival three days prior to inoculation until termination of the study six days post-inoculation. Mice were housed 3 – 5 per cage and were randomly divided into 18 groups of 4 – 6 mice per group. The environmental temperature was maintained at 20.5 – 21.5°C throughout the experiment.

b. Bacterial strains, growth conditions, and preparation of challenge inocula

- i. Media used included trypticase soy agar with 5% defibrinated bovine blood (TSA); CVS selective agar containing colistin, vancomycin, and spectinomycin;¹ and BJ selective agar containing pig feces extract, spiramycin, rifampin, vancomycin, colistin, and spectinomycin.² Plate media used in this study were prepared in-house and passed quality assurance standards of the ISU VDL. An anaerobic environment was provided by BD GasPak EZ Anaerobe Container System (Becton Dickinson, Sparks, MD). Plates were incubated at $41 \pm 1^\circ\text{C}$.
- ii. Isolates used in this study (Table 1) were obtained from the culture collection of the ISU VDL with the exception of isolates 9, 16, and 17 which were reference strains of *B. hyodysenteriae* B204 (ATCC #31212), *B. innocens* (ATCC #29796), and *B. intermedia* (ATCC #51140). All isolates had been cloned on agar by 3x subculture from a single colony isolate to new plate media (TSA). Stock cultures of each cloned isolate were stored at -80°C until required for use in the study. The isolates were at 8 – 11 passages *in vitro* at the time of challenge, excepting the reference strains of *B. intermedia* and *B. innocens* for which passage was unknown. The *B. hyodysenteriae* B204 isolate had been previously recovered from an infected C3H/HeN mouse with severe typhlitis and was 11th passage at the time of inoculation.
- iii. To prepare the inocula, isolates were thawed and plated onto several TSA plates and incubated in an atmosphere of $< 1\% \text{O}_2$ and $\geq 13\% \text{CO}_2$ for 2 – 4 days at 41°C . The growth was harvested from the plates at a ratio of 1 ml of hemolyzed agar plugs per 5 ml of sterile saline. This preparation was vortexed for 1 minute, and the liquid phase was used for mouse inoculation. Counts were determined by serial dilution and plating.³ Single colonies

visible after 6 days incubation were counted and averaged to obtain the challenge titer in CFU/ml.

- iv. Mice received two doses of inoculum (1 ml each, Table 1) administered via gavage 24 hours apart and following a 6 hour fast. Immediately prior to inoculation, mice were briefly anesthetized in an anesthetic induction chamber using isoflurane gas. Anesthetized mice were held vertically by the scruff of the neck and an 18 gauge x 2 inch stainless steel feeding needle was passed into the stomach to allow administration of the inoculum. Mice then recovered in their original cage. All mice in each group received the same isolate on both days. Each group received the isolate corresponding to the group number (1 – 17) with group 18 receiving a sham inoculation consisting of non-inoculated medium processed identically to the inoculated media.
 - v. For isolation of *Brachyspira* spp. from necropsy samples, fresh cecal content from each mouse was plated on BJ and CVS agar.
- c. *Molecular identification*
- i. Each isolate was identified to species based upon sequencing of the *nox* gene as previously described.^{4,5} Additionally, a real-time PCR targeting the *nox* gene of 'B. sp. SASK30446' was developed based on the *nox* gene region previously described⁴ and the probe: 5'-/5Cy3/AGA AGG ATT AAA ACA AGA AGG TAC TG/3IAbRQSp/-3'. DNASTAR Lasergene version 8.0 (DNASTAR, Inc., Madison, WI) was used for sequence alignment and probe design. All isolates were tested against assays targeting: *B. hyodysenteriae*, 'B. sp. SASK30446', *B. intermedia*, *B. innocens*, *B. murdochii*, and *B. pilosicoli*. All isolates were tested by each assay prior to inoculation and again post-inoculation from cecal cultures of infected mice. Reference strains of *B. innocens* (ATCC #29796), *B. intermedia* (ATCC #51140), and *B. hyodysenteriae* B204 (ATCC #31212) served as quality controls. Sequencing of the *nox* gene of the 'B. sp. SASK30446' and strongly beta-hemolytic *B. intermedia* isolates was performed as described⁶ for comparison with previously published sequences of other *Brachyspira* spp. Sequences of the 16S rRNA gene were also compared.
- d. *Animal observations and necropsy*
- i. Following inoculation, animals were observed twice daily for feed and water consumption and signs of illness. Feed consumption was assessed based upon the number of pellets consumed per cage per day. Six days post-inoculation, mice were euthanized via CO₂ asphyxiation and necropsied immediately. Cecae were exteriorized and the length from apex to base was measured with a ruler and recorded before the lumen was opened to document the presence and distribution of luminal mucus. A sterile swab of cecal content was obtained for culture and tissue samples were placed in 10% neutral buffered formalin for histopathology.
- e. *Histopathology and histochemistry*
- i. Cecae were split longitudinally and placed in a cassette to allow maximal viewing including the cecal apex. Sections (4 µm) were stained routinely with hematoxylin and eosin. The pathologist responsible for scoring the lesions was blinded to the identity of the inoculum in each group at the time of evaluation. The number of neutrophils infiltrating the cecal lamina propria was determined in ten high power (40X) fields for each mouse and the average number of neutrophils per field was then calculated to establish the cecal inflammatory score for each mouse. Mucosal thickness was measured at 20X magnification using a standard eyepiece micrometer; the average of three measurements taken from areas with an intact epithelium

and where the crypts were perpendicular to the mucosal surface was used for statistical analysis. Sections were also evaluated for the presence of submucosal edema. Serial sections were stained with Warthin-Starry silver stain and evaluated for the presence of large spirochetes within cecal crypts and luminal contents.

f. *Statistical analyses*

- i. A commercial statistical software package was utilized to perform all analyses (JMP Pro 9, SAS Institute, Inc, Cary, NC). A 1-way ANOVA was used to detect differences in mean cecal length, mean cecal inflammatory scores, and mean cecal crypt depth between groups, with a Tukey adjustment for multiple comparisons. A Fisher exact test for binomial variables was used to compare the presence or absence of grossly observable cecal mucus and microscopic edema by culture phenotype. In all circumstances, *P* values of ≤ 0.05 were considered significant and *P* values of ≤ 0.01 were considered highly significant.

2. Pig inoculation experiment

a. *Animals*

- i. All procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University. Fifty-four 4-week-old crossbred pigs were obtained from a commercial source with no known previous history of *Brachyspira*-associated disease. Prior to arrival, multiple fecal samples from the source farm were negative for *Brachyspira* spp. by direct culture as described below. Upon arrival, pigs were ear tagged, randomly separated into groups of 6, and swabbed rectally to test for the presence of *Brachyspira* spp. by microbial culture. Each group of pigs was maintained in its own raised deck pen approximately 2 m x 4 m with a nipple watering system and single ad libitum feeder, and all pens were separated from one another to prevent any contact among groups. Pigs were acclimated to these groups and to the facility for one week prior to experimental inoculation and were fed a non-medicated, nutritionally complete, age-balanced diet for the duration of the study.

b. *Bacterial strains, growth conditions, and preparation of inocula*

- i. Media used included TSA and CVS and BJ selective agars as described in the previous mouse experiment. Plate media were prepared in-house and passed the quality assurance standards of the ISU VDL. An anaerobic environment was provided as described in the previous mouse experiment and plates were incubated at $41 \pm 1^\circ\text{C}$.
- ii. Isolates used in this study (Table 2) were obtained from the culture collection of the ISU VDL. Isolates administered to groups 2 – 8 were recent field isolates and the year and geographic location of origin are reported in Table 2. Each isolate was submitted by a different veterinarian and was representative of different groups of pigs with clinical diarrhea that varied from mild and nonspecific to mucohemorrhagic; however, it has not been definitively ruled out if any of these strains are epidemiologically connected. The *B. hyodysenteriae* isolate administered to group 9 had been previously recovered from an experimentally challenged C3H/HeN mouse with severe typhlitis and was at the 11th *in vitro* passage level at the time of inoculation in the present study. All isolates had been subcultured three times from a single colony isolate to new plate media (TSA). Stock cultures of each cloned isolate were stored at -80°C . The isolates were at 8 to 11 passages *in vitro* at the time of challenge.
- iii. To prepare the inocula, isolates were thawed and plated onto several TSA plates and incubated anaerobically for 2 – 4 days. All plates were examined

for contaminating colonies and were discarded and replaced if questionable. Thirty-five plates of each isolate were used to prepare the inoculum for each day. Agar was removed from plates with sterile wooden tongue depressors and placed in large plastic bags yielding approximately 630 ml of pooled agar per isolate. The agar was then homogenized by hand until a uniform consistency was obtained. Sterile saline (70 ml) was added to form a semiliquid slurry for inoculation. Inoculation was performed within 1.5 to 3 hours of preparation of the inocula. A 5 g sample of each slurry was retained for titration of the inoculum²² in which dilutions were plated and colonies counted after 6 days incubation to obtain the inoculum titer in colony-forming units (CFU) per ml. A sham inoculum, consisting of non-inoculated TSA plates processed in the same fashion as the inoculated media, was also prepared.

c. *Animal inoculation*

- i. Each pig received two doses of inoculum (100 ml / dose, Table 2) administered via gavage 24 hours apart with each administration preceded by a 6 hour fast. For gavage, pigs were restrained briefly by hand, a rubber speculum was placed between their incisors, and an 18 Fr rubber feeding tube was passed into the esophagus. A 60 ml feeding syringe was used to administer the inocula in two 50 ml boluses and the pigs were immediately returned to their respective pens. Feed was made available when all pigs in the group were inoculated. All pigs within each group received the same isolate prepared the same way on two consecutive days. Each group received the isolate corresponding to their group number (2 – 9), with group 1 receiving the sham inoculum.

d. *Molecular identification*

- i. Each isolate was identified to species by PCR assays targeting *nox* gene sequences as described in the previous mouse experiment. All isolates were tested against assays targeting: *B. hyodysenteriae*, 'B. sp. SASK30446,' *B. intermedia*, *B. innocens*, *B. murdochii*, and *B. pilosicoli*. All strongly beta-hemolytic isolates were tested in two separate assays described as specific for *B. hyodysenteriae*.^{4,7} All isolates were tested by each assay prior to inoculation and again post-inoculation against cultures isolated from rectal swabs or mucosal scrapings.
- ii. Sequencing of the *nox* gene of each isolate was performed as described in the mouse experiment and the sequences obtained were compared with those available in GenBank using a commercial software package (DNASTAR, Inc., Madison, WI); sequences of PCR amplicons generated from the 16S rRNA gene were also compared. BLAST analysis of the sequences obtained by PCR from strongly beta-hemolytic isolates identified as *B. intermedia* was performed to determine percent similarity with previously published sequences.

e. *Animal observations and necropsy*

- i. Following inoculation, animals were observed at least twice daily for feed and water consumption and signs of clinical illness. Each pen was evaluated daily for evidence of fecal blood and/or mucus, and pigs were weighed weekly. At seven and fourteen days post-inoculation, the fecal consistency of each pig was determined and each pig received a score based upon the following system: 0 if normal, 1 if soft but formed, 2 if unformed with a semisolid consistency, and 3 if severely liquid to watery with an additional 0.5 point added each for the presence of discernible mucus and/or blood. Animals were euthanized by barbiturate overdose 7 days after the first observation of fecal blood and/or mucus or at the termination

of the study 16 days post-inoculation. At necropsy, the entire intestinal tract was examined for gross lesions, and the cecum and colon were evaluated for the presence or absence of excessive luminal mucus, mucosal hemorrhage, and fibrinous exudate on the mucosal surface. Samples of liver, jejunum, ileum, cecum, spiral colon, and descending colon were placed immediately in 10% neutral buffered formalin for 24 hours and then processed routinely for histopathology. Samples, including a rectal swab, feces from the spiral colon, and colonic mucosal scrapings, were also collected from each pig for *Brachyspira* culture.

f. *Microbial culture*

- i. Pooled fecal content from each pen, individual rectal swabs from each pig, and fresh mucosal scrapings obtained at necropsy were plated on BJ and CVS agar within 6 hours of collection and incubated as described. Plates were observed for growth at 2, 4, and 6 days post inoculation. Pooled feces collected from each pen on the last day of the study were used to inoculate MacConkey agar and brilliant green agar to screen for *Salmonella* spp.

g. *Histopathology and histochemistry*

- i. Sections were cut to 4 μm and stained with hematoxylin and eosin. Sections of liver, jejunum, and ileum were evaluated for the presence of lesions unrelated to *Brachyspira* infection that might potentially have contributed to clinical diarrhea. Sections of cecum, spiral colon, and descending colon were evaluated for the presence of superficial necrosis, mucosal hemorrhage, goblet cell hyperplasia with excessive mucus production, crypt abscesses, and suppurative infiltration of the lamina propria. A suppurative inflammation score (SI) was determined for each section as follows: 0 if no neutrophilic infiltrates were identified, 1 for multifocal infiltrates of less than 10 neutrophils each, 2 for multifocal infiltrates of 10 – 20 neutrophils each, and 3 for multifocal infiltrates of greater than 20 neutrophils each. Superficial necrosis was scored as follows: 0 if no epithelial necrosis was observed, 1 for multifocal necrosis spanning less than 3 crypts, 2 for necrosis spanning 3 – 5 crypts, and 3 for necrosis spanning more than 5 crypts. A composite typhlocolitis score (TS) was then determined for each pig as the sum of cecal SI, spiral colon SI, descending colon SI, superficial necrosis score, one point for hemorrhage, one point for goblet cell hyperplasia, and one point for crypt abscesses if more than 15 crypts were affected. The maximum possible TS under this system was 15. Mucosal thickness was measured in each section using a standard eyepiece micrometer and measurements were taken from an area where the crypts were perpendicular to the mucosal surface and where the epithelium was intact. Serial sections of spiral colon were stained with Warthin-Starry silver stain and evaluated for the presence of characteristic large spirochetes within colonic crypts and luminal contents. All histologic sections were evaluated by two separate pathologists blinded to the treatment groups, and the average of these two scores was used in all analyses.

h. *Statistical analyses*

- i. A commercial statistical software packaged was utilized to perform all analyses. A 1-way ANOVA was used to detect differences in mean clinical diarrhea scores, average daily gain, mean typhlocolitis scores, and colonic crypt depth, with a Tukey adjustment for multiple comparisons. A Fisher exact test for binomial variables was used to compare the presence or absence of grossly observable colonic luminal mucus. In all circumstances, P values ≤ 0.05 were considered significant.

Results:

1. Objective 1: Seventeen strains of *Brachyspira* were inoculated into mice and results revealed that the strongly beta-hemolytic isolates were associated with significantly greater neutrophilic infiltration of the cecal mucosa ($P < 0.0001$), shorter cecal lengths ($P < 0.0001$), and increased cecal luminal mucus ($P < 0.0001$) versus weakly beta-hemolytic isolates and non-inoculated controls regardless of the molecular identification of the isolate. Additionally, no statistical differences were observed between any of these parameters in mice inoculated with weakly beta-hemolytic isolates versus control mice despite a high degree of colonization of the inoculated mice. Key findings:
 - a. The mouse is an effective model for comparing virulence of multiple *Brachyspira* spp.
 - b. Strongly beta-hemolytic *Brachyspira* spp. induce significantly more inflammation than weakly beta-hemolytic strains regardless of the molecular identity of the isolate, and strongly beta-hemolytic isolates identified as *B. intermedia* and “*B. sp. SASK30446*” by PCR appear to be highly virulent in this model.
 - c. **Complete results from this study were incorporated into a manuscript that was published in *Veterinary Microbiology*.⁸**
2. Objective 2: Based upon results of the mouse inoculation trial (Objective 1), 8 clinical isolates representing 5 different *Brachyspira* spp. (*B. innocens*, *B. murdochii*, *B. intermedia*, *B. hyodysenteriae*, and “*B. sp. SASK30446*”) were selected for inoculation into 5-week-old pigs. At 14-days-postinoculation, clinical diarrhea was significantly more severe in pigs receiving strongly beta-hemolytic isolates versus those groups receiving weakly beta-hemolytic isolates or a sham inoculum ($P = 0.0035$ and $P = 0.0351$, respectively). Gross lesions were limited to the cecum and large intestine and were most significant in pigs receiving strongly beta-hemolytic isolates. Microscopic lesions revealed that strongly beta-hemolytic isolates were associated with significantly greater neutrophilic infiltration of the colonic mucosa (Figure 1) and increased depth of the mucosal crypts versus weakly beta-hemolytic isolates ($P < 0.0001$ and $P = 0.0065$, respectively) and non-inoculated controls ($P < 0.0001$ and $P = 0.0183$, respectively) regardless of the molecular identification of the isolate. In contrast, weakly beta-hemolytic isolates were associated with greater neutrophilic infiltration of the colonic mucosa ($P = 0.0209$) but not mucosal crypt depth ($P = 0.8205$) versus sham-inoculated control pigs. A second smaller challenge study involving slightly older pigs (7-weeks-old) was also performed to more fully compare the lesions between typical isolates of *B. hyodysenteriae* and other the strongly beta-hemolytic strains of *B. intermedia* and “*B. sp. SASK30446*”. Results of this second study confirmed the highly pathogenic nature of the tested strongly beta-hemolytic isolates with no significant differences in clinical disease or gross lesions noted between strains. Key findings:
 - a. Clinical swine dysentery can occur following oral inoculation of pigs with strongly beta-hemolytic *Brachyspira* spp. other than *B. hyodysenteriae*; specifically those isolates further identified as *B. intermedia* and “*B. sp. SASK30446*” by PCR.
 - b. For *Brachyspira* spp., a strongly beta-hemolytic cultural phenotype is a more sensitive indicator or the potential to induce dysentery than the molecular identity alone.
 - c. Microbial culture of necropsy specimens is a more sensitive method for detection of *Brachyspira* spp. than direct PCR on the same samples using currently available PCR assays targeting *nox* gene sequences.
 - d. **Complete results from this study were incorporated into a manuscript that was published in the *Journal of Veterinary Diagnostic Investigation*.⁹**

3. Objective 3: The results of these animal inoculation experiments have provided a definitive pathotype for multiple clinical isolates at our disposal in the ISU VDL whereby strongly beta-hemolytic *Brachyspira* spp. are associated with the potential to induce dysentery-like disease while weakly beta-hemolytic isolates are not. We are currently preparing many of these isolates for full genome sequencing to allow direct comparison between virulent and avirulent strains with the goal of identifying genes may that underpin this pathogenic potential.

Discussion:

Results of both studies reveal that, for *Brachyspira* spp., a strongly beta-hemolytic cultural phenotype is a more sensitive indicator of potential virulence than the molecular identity of the isolate alone, and the pig inoculation studies have proven that clinical SD can occur following oral inoculation with strongly beta-hemolytic spirochetes other than *B. hyodysenteriae*. Molecular and biochemical analyses of clinical isolates of strongly beta-hemolytic spirochetes not identified as *B. hyodysenteriae* by PCR suggest that a novel species has emerged and is circulating in swine herds in the United States with the proposed name "*Brachyspira hampsonii*."¹⁰ This proposed new species is divided into two clades, "*B. hampsonii*" clade I and clade II. Analysis of the *nox* gene sequences of the type strains of these two clades further reveals that clade I isolates are analogous to those tested in the studies reported herein and identified as strongly beta-hemolytic *B. intermedia*, and that clade II isolates are analogous to those identified in the present report as "*B. sp. SASK30446*". Taken together, these findings reveal that the identification of "*B. hampsonii*" in clinical specimens from pigs with bloody scours should be interpreted as compatible with a diagnosis of SD.

Furthermore, these results support the use of culture as part of any initial screening tests for the presence of pathogenic *Brachyspira* spp. in diagnostic specimens and suggest that caution be applied when interpreting direct PCR results for *Brachyspira* spp. from clinical specimens without cultural confirmation of appropriate phenotypic traits. Sequencing of the *nox* gene of *Brachyspira* spp. is currently an effective means to speciate clinical isolates from culture and may be a useful diagnostic tool in epidemiologic investigations within production systems dealing with SD.

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Figures:

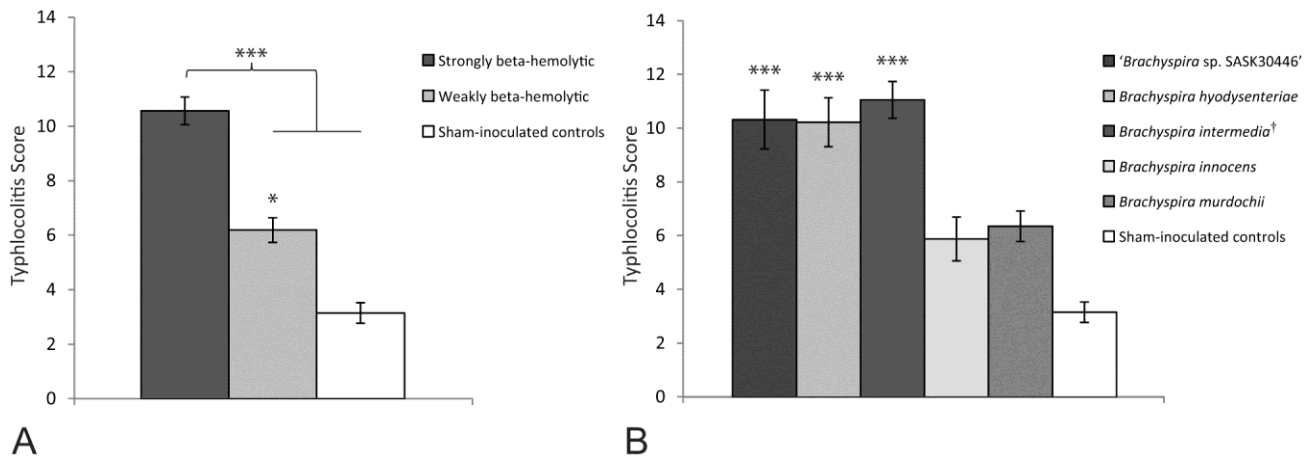


FIGURE 1. Typhlocolitis (TS) scores from pigs infected with various *Brachyspira* spp. **A**, mean TS for all pigs inoculated and grouped by phenotypic traits of the isolates on trypticase soy agar (TSA) with 5% bovine blood. **B**, mean TS for all pigs inoculated and grouped by molecular identification of the isolates. †Identified as *B. intermedia* by polymerase chain reaction assays described as specific for this species; however, these isolates have a strongly beta-hemolytic cultural phenotype which is atypical for this species.

Tables:

TABLE 1. *Brachyspira* spp. isolates used for mouse inoculation.

Group Number	Isolate Source and Year of Isolation	PCR Identification	Hemolysis Pattern	Ring Phenomenon	Inoculum concentration
1	Pig, Iowa, 2009	' <i>B. sp.</i> SASK30446'	Strong beta	Positive	1 x 10 ⁷ CFU/ml
2	Pig, Iowa, 2008	' <i>B. sp.</i> SASK30446'	Strong beta	Positive	9 x 10 ⁶ CFU/ml
3	Pig, Iowa, 2008	' <i>B. sp.</i> SASK30446'	Strong beta	Positive	9 x 10 ⁷ CFU/ml
4	Pig, Iowa, 2009	<i>B. intermedia</i>	Strong beta	Positive	9 x 10 ⁷ CFU/ml
5	Pig, Illinois, 2009	<i>B. intermedia</i>	Strong beta	Positive	9 x 10 ⁷ CFU/ml
6	Pig, South Dakota, 2009	<i>B. hyodysenteriae</i>	Strong beta	Positive	9 x 10 ⁷ CFU/ml
7	Pig, North Carolina, 2010	<i>B. hyodysenteriae</i>	Strong beta	Positive	4 x 10 ⁶ CFU/ml
8	Pig, Iowa, 2011 Lab passaged strain of <i>B. hyodysenteriae</i>	<i>B. hyodysenteriae</i>	Strong beta	Positive	2 x 10 ⁷ CFU/ml
9	B204 Pig, Missouri, 2010	<i>B. hyodysenteriae</i>	Strong beta	Positive	9 x 10 ⁷ CFU/ml
10	Pig, Virginia, 2011	<i>B. innocens</i>	Weak beta	Negative	6 x 10 ⁷ CFU/ml
11		<i>B. murdochii</i>	Weak beta	Negative	9 x 10 ⁷ CFU/ml
12	Pig, Iowa, 2011	<i>B. murdochii</i>	Weak beta	Negative	9 x 10 ⁷ CFU/ml
13	Pig, Iowa, 2009	<i>B. murdochii</i>	Weak beta	Negative	9 x 10 ⁷ CFU/ml
14	Pig, Nebraska, 2008	<i>B. murdochii</i>	Weak beta	Negative	9 x 10 ⁷ CFU/ml
15	Pig, Iowa, 2008 Lab passaged strain of <i>B. innocens</i>	<i>B. innocens</i>	Weak beta	Negative	9 x 10 ⁷ CFU/ml
16	B256 Lab passaged strain of <i>B. intermedia</i>	<i>B. innocens</i>	Weak beta	Negative	3 x 10 ⁷ CFU/ml
17	PWS/A	<i>B. intermedia</i>	Weak beta	Negative	4 x 10 ⁶ CFU/ml

TABLE 2. *Brachyspira* spp. isolates used for pig inoculation.

Group Number	Isolate source and year of isolation	PCR Identification	Hemolysis Pattern	Ring Phenomenon	Inoculum concentration
2	Pig*, Missouri, 2010	<i>B. innocens</i>	Weak beta	Negative	4 x 10 ⁷ CFU/ml
3	Pig*, Iowa, 2009	<i>B. murdochii</i>	Weak beta	Negative	1 x 10 ⁹ CFU/ml
4	Pig*, Iowa, 2011	<i>B. murdochii</i>	Weak beta	Negative	1 x 10 ⁹ CFU/ml
5	Pig†, Illinois, 2009	<i>B. intermedia</i> ‡	Strong beta	Positive	1 x 10 ⁹ CFU/ml
6	Pig†, Iowa, 2011	<i>B. intermedia</i> ‡	Strong beta	Positive	1 x 10 ⁸ CFU/ml
7	Pig†, Iowa, 2008	' <i>B. sp.</i> SASK30446'	Strong beta	Positive	1 x 10 ⁹ CFU/ml
8	Pig†, Iowa, 2011	' <i>B. sp.</i> SASK30446'	Strong beta	Positive	4 x 10 ⁶ CFU/ml
9	Lab passaged strain	<i>B. hyodysenteriae</i>	Strong beta	Positive	1 x 10 ⁷ CFU/ml

*Isolated from a pig with nonspecific diarrhea.

†Isolated from a pig with mucohemorrhagic diarrhea.

‡Identified as *B. intermedia* by PCR assays described as specific for this species; however, these isolates have a strongly beta-hemolytic cultural phenotype which is atypical for this species.