Title: Enhanced Antimicrobial Susceptibility Testing and Molecular Diagnostic Methods for Brachyspira in Swine NPB #10-050

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Industry Summary:

Brachyspira infection(s) appear to growing in importance in today’s swine industry. While largely disappearing from U.S. swine herds between the late 1990’s and the early 2000’s, Brachyspira-associated disease and Brachyspira spp. isolation from swine with clinical disease has increased in the last several years with non-\textit{B. hyodysenteriae} isolates being more commonly identified. Antimicrobial resistance may have a role in this resurgence. Seventy-nine clinical isolates identified at the Iowa State University Veterinary Diagnostic Lab (ISU VDL) were tested with multiple PCR assays that were evaluated and/or developed as part of this study to establish species identity. Isolates were then tested for antibiotic sensitivity to lincomycin, gentamicin, valnemulin, tiamulin, salinomycin, and carbadox. Only 38.0 \% of isolates could be confirmed as the known pathogens \textit{B. hyodysenteriae} (30.4\%) or \textit{B. pilosicoli} (7.6\%). Twenty of the 79 isolates (25.3\%) were identified as \textit{B. murdochii} and 13.9\% could not initially be identified to species. Subsequently, a new PCR assay was developed targeting a potentially novel species of Brachyspira identified by Dr. John Harding’s group as ‘\textit{Brachyspira spp. SASK30446}’. This new PCR test positively identified these remaining isolates. The antibiotic testing indicated resistance to lincomycin for all Brachyspira; and moderately high resistance against gentamicin. The \textit{Brachyspira} tested appeared to be more susceptible to the remaining antimicrobials. \textit{B. murdochii} and \textit{Brachyspira} spp. SASK30446 appeared to be more resistant to several of these drugs than the other \textit{Brachyspira} examined. The increased incidence of these less definitively characterized \textit{Brachyspira} species with increased resistance to commonly-prescribed antimicrobials may, at least in part, explain the increased prevalence and severity of this disease complex in recent years. Further research is necessary to better understand these changes. For any questions regarding these finding, please contact the Iowa State University Veterinary Diagnostic Laboratory.

Keywords: Brachyspira, swine dysentery, MIC, PCR, antibiotic sensitivity
Scientific Abstract:

Brachyspira infection(s) appear to growing in importance in today’s swine industry. While largely disappearing from U.S. swine herds between the late 1990’s and the early 2000’s, Brachyspira-associated disease and Brachyspira spp. isolation from swine with clinical disease has increased in the last several years with non-\textit{B. hyodysenteriae} isolates being more commonly identified.

Antimicrobial resistance may have a role in this resurgence. Seventy-nine clinical isolates identified at the Iowa State University Veterinary Diagnostic Lab (ISU VDL) were tested with multiple PCR assays that were evaluated and/or developed as part of this study to establish species identity. Isolates were then evaluated for minimum inhibitory concentrations (MIC) using an agar dilution method against lincomycin, gentamicin, valnemulin, tiamulin, salinomycin, and carbadox. Only 38.0% of isolates could be confirmed as the known pathogens \textit{B. hyodysenteriae} (30.4%) or \textit{B. pilosicoli} (7.6%). Twenty of the 79 isolates (25.3%) were identified as \textit{B. murdochii} and 13.9% could not initially be identified to species. Subsequently, a new PCR assay was developed targeting a potentially novel species of Brachyspira identified by Dr. John Harding’s group as ‘\textit{Brachyspira} spp. SASK30446’. This new PCR test positively identified these remaining isolates. MIC values were consistently high against lincomycin for all Brachyspira; and moderately high against gentamicin. The remaining antimicrobials had MIC’s that were at the low end of the test ranges. \textit{B. murdochii} and \textit{Brachyspira} spp. SASK30446 had significantly greater MIC values against several of these drugs than other Brachyspira examined. The increased incidence of these less definitively characterized Brachyspira species with increased MIC values to commonly-prescribed antimicrobials may, at least in part, explain the increased prevalence and severity of this disease complex in recent years. Further research is necessary to better understand these changes.

Introduction:

Swine dysentery (SD) is an economically important disease in grow/finish pigs worldwide. \textit{Brachyspira hyodysenteriae} is the spirochete bacterium that produces this disease, resulting in severe damage to the large intestine which leads to profuse watery diarrhea containing mucus and blood (dysentery). The colitis causes defective absorption of fluids and nutrients from the large intestine, resulting in loss of condition and poor feed conversion. In addition, animals that recover from SD can have persistently poor growth and difficulty reaching market weight. \textit{Brachyspira pilosicoli} has been associated with a less severe diarrhea but does contribute to production losses in growing pigs. During the late 1990’s and early 2000’s, the incidence of SD decreased, presumably due the changes in production by high health management systems. Recently, however, SD has re-emerged as a health concern in swine production. From 2004-2007, the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) performed an average of 1400 Brachyspira cultures per year. In 2008, that number jumped to over 3100 cultures for this pathogen. Additionally, clinical presentation and pathogenic behavior of Brachyspira appears to have changed, and other species of Brachyspira are frequently identified in cases with colitis. These changes may be the result of virulence-gene transfer to previously non-pathogenic species, or may indicate the emergence of a new Brachyspira species.

Confirmation of diagnosis of SD by culture requires special conditions, media, extended incubation times to complete; Brachyspira is not identified with routine bacteriological tests. Currently, there is no validated antimicrobial susceptibility test for this bacterial species. Standard minimum inhibitory concentration (MIC) methods used for testing other bacteria do not meet the specific conditions necessary to support growth of and cannot be used for Brachyspira. Carbadox, lincomycin, tiamulin, and tylosin have been used in the past to successfully treat \textit{B. hyodysenteriae} infections; however, there are reports of resistance to all of these drugs. Therefore, a consistent and
validated method for determining antibiotic sensitivities to Brachyspira species is needed to provide guidance on treatment options and to reliably monitor development of resistance to these drugs.

This study sought to further validate antimicrobial susceptibility testing for Brachyspira strains identified at the ISU VDL. Additionally, an improved panel of PCR tests is needed to better identify multiple Brachyspira species. An examination of virulence genes in Brachyspira spp. is also needed in order more easily survey isolates for their capacity to cause clinical disease.

Objectives:

- a. To validate a broth method for MIC testing in *Brachyspira* species and perform antibiotic sensitivity assessments on a representative sample of these isolates

- b. To determine if *Brachyspira* species can be more accurately identified using a discriminatory multiplex PCR assay than by phenotypic method

- c. To determine the relationship between *B. hyodysenteriae* and other *Brachyspira* species that may have expanded virulence by screening isolates for known virulence genes

Materials & Methods:

Bacterial isolates

Seventy-nine Brachyspira spp. isolates collected from cases of pigs with clinical disease submitted to the ISU VDL between 2008 and 2010 were identified for testing. Swine farm locations included NC (36), IA (23), MN (9), NE (3), MI (2), IL (2), MO (1), ND (1), SD (1), and OH (1). Isolates were subcultured onto tripticase soy agar with 5% sheep blood containing 0.008% colistin, 0.008% vancomycin, and 0.5% spectinomycin (CVS) under anaerobic conditions using a commercial system at 42˚ C and examined for growth at 48 and 96 hours. Isolated colonies were classified and subcultured onto CVS agar three times to obtain a single genetic clone of Brachyspira from each case. Isolates were stored at -80 C until testing was performed.

MIC Testing

Agar antimicrobial susceptibility testing was performed on each isolate against lincomycin (1.0-64.0 µg/ml); gentamicin (2.0-64.0 µg/ml); valnemulin (0.125-8 µg/ml); tiamulin (0.125-16 µg/ml); salinomycin (0.126-16.0 µg/ml); and carbadox (0.004-0.5 µg/ml). Stock solutions of each drug were made by mixing the antimicrobial powders with sterile distilled water (lincomycin, gentamicin, tiamulin, valnemulin); ethanol followed by sterile distilled water (carbadox) or ethanol followed by saline (salinomycin); solutions were kept refrigerated and made fresh every two weeks. MIC agar plates were made by adding the antimicrobial solution of appropriate concentration in 0.5ml volume to 4.5 ml of molten TSA 5% sheep blood and poured into culture plates. Plates were used within 24 hours of assembly.

Each isolate was cultured on tripticase soy agar with 5% sheep blood (TSA) at 42˚ C for 48 hours to remove exposure of the bacteria to antimicrobials prior to susceptibility testing. One ml of agar was added to a vial containing 3ml of sterile brain heart infusion (BHI) broth and sterile glass beads. The mixture was homogenized to liberate bacteria and 20 µl of the resultant solution containing 8.0 X 105-2.0 X 106 cfu/ml was used to inoculate MIC plates. Plates were air dried, incubated anaerobically at 42˚ C, and examined for the presence of growth associated with strong or weak hemolysis at 48 and 96 hours. MIC was defined as the lowest concentration in which no
bacterial growth was detected. Additionally, each bacterial suspension was cultured for purity to ensure that no aerobic or anaerobic contaminants were present in the sample. ATTC strains *B. hyodysenteriae* B204 (31212) and *B. pilosicoli* P43/6/78 (#51139) were used as quality control organisms. Isolates were tested in duplicate; any isolate with MIC values that were greater than two antibiotic dilutions different from one another in replicates were tested a third time to obtain a consensus MIC value.

PCR testing

PCR testing was performed using both real-time and gel-based previously described techniques to identify each isolate to species. ATCC isolates *B. hyodysenteriae* B204 (# 31212), *B. pilosicoli* P43/6/78 (#51139), *B. intermedia* PWS/A (#51140), *B. murdochii* 155-20 (#700173), and *B. innocens* B256 (#29796), were used as quality control organisms. A new assay was developed against 'Brachyspira spp. SASK30446' as well and is in use at the ISU-VDL.

VIII. Results:

Objective a.

Although proposed, the broth method for MIC has not yet been validated due to difficulties in obtaining pure cultures in liquid media. Work is continuing towards optimizing the broth method. As an alternative, an agar MIC method was validated. Seventy-nine *Brachyspira spp*. isolates collected from cases of pigs with clinical disease submitted to the ISU VDL between 2008 and 2010 were identified for testing. Swine farm locations included: NC (36), IA (23), MN (9), NE (3), MI (2), IL (2), MO (1), ND (1), SD (1), and OH. Isolates were subcultured onto trippticase soy agar with 5% sheep blood containing 0.008% colistin, 0.008% vancomycin, and 0.5% spectinomycin (CVS) under anaerobic conditions using a commercial system at 42˚ C and examined for growth at 48 and 96 hours. Isolated colonies identified by hemolysis were subcultured onto CVS agar three times to obtain a single genetic clone of Brachyspira from each case. Isolates were stored at -80˚ C until testing was performed.

Isolates were tested by multiple PCR assays to establish species identity. Only 38.0 % of isolates could be confirmed as the known pathogens *B. hyodysenteriae* (30.4%) or *B. pilosicoli* (7.6%). Twenty of the 79 isolates (25.3%) were identified as *B. murdochii* and 13.9% could not be identified to species by initial PCR testing, but were later identified as a potentially novel species currently being identified as: 'Brachyspira sp. SASK30446'.

MIC Testing

Agar antimicrobial susceptibility testing was performed on each isolate against lincomycin (1.0-64.0 µg/ml); gentamicin (2.0-64.0 µg.ml); valnemulin (0.125-8 µg/ml); tiamulin (0.125-16 µg/ml); salinomycin (0.126-16.0 µg.ml); and carbadox (0.004-0.5 µg/ml). Stock solutions of each drug were made by mixing the antimicrobial powders with sterile distilled water (lincomycin, gentamicin, tiamulin, valnemulin); ethanol followed by sterile distilled water (carbadox) or ethanol followed by saline (salinomycin); solutions were kept refrigerated and made fresh every two weeks. MIC agar plates were made by adding the antimicrobial solution of appropriate concentration in 0.5ml volume to 4.5 ml of molten TSA 5% sheep blood and poured into culture plates. Plates were used within 24 hours of assembly.

Each isolate was cultured on trypticase soy agar with 5% sheep blood (TSA) at 42˚ C for 48 hours to remove exposure of the bacteria to antimicrobials prior to susceptibility testing. One ml of agar was added to a vial containing 3ml of sterile brain heart infusion (BHI) broth and sterile glass beads. The mixture was homogenized to liberate bacteria and 20 µl of the resultant solution containing 8.0 X 10⁵-2.0 X 10⁶ cfu/ml was used to inoculate MIC plates. Plates were air dried, incubated anaerobically at 42˚ C, and examined for the presence of growth associated with strong or weak hemolysis at 48 and 96 hours. MIC was defined as the lowest concentration in which no bacterial growth was detected. Additionally, each bacterial suspension was cultured for purity to
ensure that no aerobic or anaerobic contaminants were present in the sample. ATTC strains \textit{B. hyodysenteriae} B204 (#31212) and \textit{B. pilosicoli} P43/6/78 (#51139) were used as quality control organisms. Isolates were tested in duplicate; any isolate with MIC values that were greater than two antibiotic dilutions different from one another in replicates were tested a third time to obtain a consensus MIC value.

A manuscript describing the results of this objective as been published:

Objective b.

Several previously published PCR assays were performed using both real-time and gel-based techniques as previously described in an attempt to identify each isolate to species. ATCC isolates \textit{B. hyodysenteriae} B204 (#31212), \textit{B. pilosicoli} P43/6/78 (#51139), \textit{B. intermedia} PWS/A (#51140), \textit{B. murdochii} 155-20 (#700173), and \textit{B. innocens} B256 (#29796), were used as positive controls. Initially, seventy-nine clinical isolates from the Iowa State University Veterinary Diagnostic Lab (ISU VDL) were tested with multiple PCR assays (referenced below) to establish species identity. From this, only 38.0 \% of isolates could be confirmed as the known pathogens \textit{B. hyodysenteriae} (30.4\%) or \textit{B. pilosicoli} (7.6\%). Twenty of the 79 isolates (25.3\%) were identified as \textit{B. murdochii} and eleven isolates (13.9\%) could not initially be identified to species. An assay targeting the \textit{nox} gene of the proposed new species, \textit{Brachyspira} sp. SASK30446 was developed. Using this assay, these eleven isolates were (tentatively) identified as this new species. Further work to evaluate these assays against additional isolates is ongoing and sequencing of select genes from some isolates for confirmation of their identity is also ongoing.


Objective c.

A list of virulence genes has been identified through literature searches. One target, which has been investigated and implemented in diagnostic assays, is the NADH oxidase (\textit{nox}) gene. The \textit{nox} gene has been shown to play a role in protecting \textit{Brachyspira} cells from the lethal effects of oxygen, thus enabling colonization and virulence in the intestinal environment (Stanton et al., 1999). Mutant \textit{B. hyodysenteriae} strains deficient of \textit{nox} were shown to be less virulent than their wild-type strain (Stanton, 2006). All currently identified \textit{Brachyspira} species possess the \textit{nox} gene (Stanton et al., 1999) (Barth et al., 2012). Several PCR assays targeting the \textit{nox} gene were used to identify the 79 \textit{Brachyspira spp}. isolates in this study (Song and Hampson, 2009; Weissenböck et al., 2005). All isolates amplified with the combination of these assays, including a novel \textit{Brachyspira sp}. that was tentatively identified. Upon sequencing many of these isolates, it was found that \textit{nox} possesses
much greater sequence diversity among species than does the 16S rRNA sequence, which enables greater specificity for species identification. Thus, the nox gene is a useful target for identifying *Brachyspira* spp. The ISU-VDL had implemented sequencing of the nox gene for *Brachyspira* spp. identification as a test offering.

Another potential target includes the hemolysin gene *hlyA*, which encodes an acyl-carrier protein (ACP) that is directly linked to the expression of the hemolytic phenotype (Hsu et al., 2001). This gene is present in the ATCC type strains of *B. hyodysenteriae* and *B. intermedia*, and a positive association has been found between the presence of *hlyA* and pathogenicity in *Brachyspira* isolates (Hsu et al., 2001). Primers were designed in-house to amplify the entire *hlyA* gene based on sequences available from GenBank. PCR was run on one type strain and one field isolate of each *Brachyspira* species (*B. hyodysenteriae, B. pilosicoli, B. intermedia, B. murdochii, B. innocens*). PCR was also run on two field isolates of the previously identified novel *B. sp.*, which were characterized as strongly beta-hemolytic. Amplification occurred in all isolates except the field isolate of *B. pilosicoli*. Very weak amplification occurred in the type strain of *B. murdochii* as well as both *B. innocens* isolates. PCR products were sequenced to confirm *hlyA* amplification. Results demonstrate that all *Brachyspira* species contain the *hlyA* gene. The lack of or weak amplification of isolates suggests sequence diversity in the primer regions located upstream and downstream of *hlyA*. Upon sequence alignment, it was confirmed that very little sequence diversity is present in the *hlyA* gene of all the isolates sequenced. Alignment of the *hlyA* gene sequences available from GenBank also showed considerable sequence homology among species, which suggests this gene would not be an ideal target to differentiate species in a diagnostic test.

The pseudo-hemolysin genes *tlyA, tlyB, and tlyC* have also been suggested as virulence genes. These genes have been shown to induce a hemolytic phenotype in *E. coli*, and *tlyA* was detected in the pathogenic *B. hyodysenteriae* but not nonpathogenic *B. innocens* (Hsu et al., 2001; Hyatt et al., 1994). To investigate *tlyA* as a potential PCR target, gel-based PCR was designed using *tlyA* forward and reverse primers published by Råsbäck, et al. (2006). PCR was run on type strains and field isolates of *B. hyodysenteriae, B. pilosicoli, B. intermedia, B. murdochii, B. innocens*, as well as field isolates of the novel *B. sp*. Results showed only amplification in the *B. hyodysenteriae* type strain and all but one field isolate. No isolates of the novel *B. sp.* amplified. These results demonstrate that the *tlyA* target is not specific to strongly beta-hemolytic isolates as five isolates with strongly beta-hemolytic phenotypes failed to amplify. The other two *tly* genes, *tlyB* and *tlyC*, have yet to be investigated.

Other potential targets yet to be investigated include a putative hemolysin gene first identified in *B. hyodysenteriae* (BHWA1_00962), which has been identified in the type strains of *B. intermedia* and *B. murdochii* but not *B. pilosicoli* (Wanchanthuek et al., 2010). Other genes of interest include genes involved in the biosynthesis of the lipooligosaccharide (LOS) contained in the outer envelope of the species (La et al., 2011; Wanchanthuek et al, 2010). In addition, genes involved in motility and chemotaxis are also potential genetic targets for virulence as are genes affecting iron metabolism (Wanchanthuek et al., 2010; Barth et al., 2012).

Currently, work is being done to design PCR assays from conserved regions in the virulence genes identified. The most informative and accurate PCR assays will likely be designed to amplify multiple virulence targets. Developed PCRs will be tested against a panel of isolates already assembled that includes multiple species of *Brachyspira* to ensure specificity and sensitivity of the assays.
References


Discussion:

The results of this study have yielded an antibiotic sensitivity assay that can be used for Brachyspira spp. and demonstrated a range of sensitivity to a variety of antibiotics in the isolates tested here. PCR tests were compared and evaluated for their diagnostic ability and led to updated test being used at the ISU-VDL, including the development a new assay targeting a potential new species, Brachyspira sp. SASK30446. Initial work on evaluation of PCR assays targeting virulence genes was also begun. This work is ongoing as more sequence data becomes available for the various species.