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Cessation of clinical disease and spirochete shedding after tiamulin treatment in pigs experimentally infected with "*Brachyspira hampsonii*"

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ABSTRACT

With the emergence of "*Brachyspira hampsonii*" associated with swine dysentery in North America, identification of effective treatments and interventions is a pressing need. Denagard® (tiamulin hydrogen fumarate) Liquid Concentrate 12.5% is approved in the United States for treatment of dysentery caused by *Brachyspira hyodysenteriae* at 0.006% in the water. In this study, the effectiveness of tiamulin in resolving clinical disease, eliminating viable spirochete shedding, and reducing neutrophilic colitis following infection with either "*B. hampsonii*" or *B. hyodysenteriae* was evaluated. Seventy-eight 7-week-old crossbred pigs were divided into three groups [sham-inoculated ($n = 18$), "*B. hampsonii*"-inoculated ($n = 30$), and *B. hyodysenteriae*-inoculated ($n = 30$)]. Each inoculum group was divided into three subgroups which received either 0.006% tiamulin, 0.018% tiamulin, or no medication. Both levels of tiamulin resolved clinical disease within 24 h of treatment initiation, eliminated spirochete shedding within 72 h of treatment initiation, and resolved and/or prevented histologic lesions in pigs infected with either *Brachyspira* spp.

1. Introduction

Since 2007, an increase in swine dysentery diagnoses has been observed in growing and finishing pigs in North America based upon case submissions to veterinary diagnostic laboratories (Burrough, 2013). Swine dysentery is characterized by a severe mucohemorrhagic diarrhea and typhlocolitis and is classically associated with infection with *Brachyspira hyodysenteriae*. Recently, a proposed novel *Brachyspira* species, "*Brachyspira hampsonii*" (Chander et al., 2012), has been isolated from pigs with mucohemorrhagic diarrhea and experimental infection with "*B. hampsonii*" strains has resulted in clinical disease and lesions that are similar to, if not indistinguishable from, *B. hyodysenteriae* infection (Burrough et al., 2012; Rubin et al., 2013; Wilberts et al., 2013). With the emergence of "*Brachyspira hampsonii*" associated with clinical swine dysentery in North America, there is a need to identify effective treatments and interventions (Burrough and Sexton, 2013). Tiamulin is a pleuromutilin antibiotic that is available in multiple formulations and is widely used for controlling swine dysentery worldwide. In the United States, tiamulin hydrogen fumarate is specifically approved for the treatment of swine dysentery caused by *Brachyspira hyodysenteriae* at 0.006% in the water; however, the effectiveness of this tiamulin formulation is unknown for the treatment of dysentery following infection with "*Brachyspira hampsonii*". A previous report found a 5-day treatment of 0.006% tiamulin in the water resulted in complete prevention of disease relapse with elimination of *B. hyodysenteriae* for at least 21 days following treatment (Taylor, 1980); however, more recent *in vitro* investigations have demonstrated discordant antimicrobial susceptibility to tiamulin among clinical isolates of *B. hyodysenteriae* suggesting resistance may be developing to this antibiotic in some geographic regions (Hidalgo et al., 2011; Zmudzki et al., 2012). The susceptibility of "*B. hampsonii*" isolates originating from wild waterfowl in Europe to tiamulin has also been recently described where all isolates were reported as susceptible to tiamulin (Martínez-Lobo et al., 2013). Accordingly, it is hypothesized that tiamulin should be effective in controlling swine dysentery associated with "*B. hampsonii*" infection. In the present study, the effectiveness of tiamulin treatment in the cessation of clinical disease, elimination of shedding of strongly beta-hemolytic spirochetes, and resolution of neutrophilic colonic inflammation in pigs was compared following infection with either "*B. hampsonii*" clade II (EB107) or *B. hyodysenteriae* (B204).

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2. Materials and methods

2.1. Animals

All procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University (10-12-7451-S). Seventy-eight 7-week-old crossbred pigs were obtained from a commercial source with no known previous history of *Brachyspira*-associated disease and tested negative for *Brachyspira* spp. and *Lawsonia intracellularis* prior to arrival by selective anaerobic culture and fecal PCR, respectively. Pigs were placed in one of three groups, with three separate pens per group (nine pens total), based upon the inoculum they were to receive [sham-inoculated ($n = 18$), "*B. hampsonii*"-inoculated ($n = 30$), and *B. hyodysenteriae*-inoculated ($n = 30$)]. All pigs were ear tagged for individual identification, maintained in separate rooms by inoculum to prevent any contact between groups, and were acclimated to these groups and to the facility for 1 week prior to inoculation. During the acclimatization period and throughout the study, pigs were fed a non-medicated, corn and soybean diet nutritionally complete for their age.

2.2. Bacterial strains, growth conditions, and preparation of inocula

Media, strains, and challenge inocula were prepared as previously described (Burrough et al., 2012; Wilberts et al., 2013). Strains used in this study were obtained from the culture collection at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). The "*B. hampsonii*" clade II strain (EB107) was previously used in two pig inoculation experiment (Burrough et al., 2012; Wilberts et al., 2013) after being recovered from a clinical case of SD in 2011 and was 8th–10th passage at the time of inoculation. The *B. hyodysenteriae* strain (B204) was originally recovered from a clinical case of SD in 1972 and was 8th–9th passage at the time of inoculation.

For isolation of *Brachyspira* spp., daily rectal swab samples were collected from each pig beginning 4 days post-inoculation through 5 days post-treatment to assess shedding of viable spirochetes and at necropsy. Swabs were plated onto selective agars within 6 h of collection and incubated as previously described (Burrough et al., 2012; Wilberts et al., 2013). Specifically, swabs were plated onto CVS selective agar containing colistin, vancomycin, and spectinomycin and BJ selective agar containing pig feces extract, spiramycin, rifampin, vancomycin, colistin, and spectinomycin. Plate media used in this study were prepared in-house and passed the quality assurance standards of the ISU VDL. An anaerobic environment was provided by a commercial system (BD GasPak EZ Anaerobe Container System, BD Diagnostic Systems, Sparks, MD) and plates were incubated at 41 ± 1 °C. Inoculated plates were observed for *Brachyspira* growth on days 2, 4, and 6. Mucosal scrapings collected at necropsy were plated and evaluated as described previously and were also plated onto MacConkey's agar and brilliant green agar with novobiocin in addition to tetrathionatebroth enrichment subcultured to brilliant green with novobiocin and XLT4 to test for the presence of *Salmonella* spp.

2.3. Animal inoculation

Pigs received three doses of an agar slurry containing the appropriate inoculum for their group (100 ml/dose) administered via gavage 24 h apart with each administration preceded by a 12–18 h fast. A 5-g sample of each inoculum was reserved from which 1 g was vortexed for 45 s in tubes with 9 ml of sterile PBS and a few glass beads. A standard plate count procedure was performed by titration of 1 ml of the vortexed sample into 9 ml and carried out to 10^{-9}. The dilution series was plated on trypticase soy agar with 5% defibrinated bovine blood and incubated anaerobically for 6 days with plates being observed on days 2, 4, and 6. *Brachyspira* spp. grew confluently at the more concentrated dilutions, but discrete colonies were observed from the more dilute plates. Colonies were counted after 6 days incubation to obtain an estimation of the inoculum titer in colony-forming units (CFU) per milliliter. Pigs in the *B. hyodysenteriae* group received in estimation 3×10^5 CFU/ml, 4×10^4 CFU/ml, and 5×10^5 CFU/ml on day post-inoculation (DPI) 0, 1, and 2, respectively. Pigs in the "*B. hampsonii*" clade II group received in estimation 3×10^5 CFU/ml, 2×10^5 CFU/ml, and 6×10^4 CFU/ml on DPI 0, 1, and 2, respectively. Pigs in the control group received a sham inoculum consisting of agar material from non-inoculated culture plates prepared in the same manner as for the *Brachyspira* inocula. The MIC values for tiamulin (agar dilution method) for the strains used in the inocula were 0.5 μg/ml for *B. hyodysenteriae* B204 and 1 μg/ml for "*B. hampsonii*" clade II EB107.

2.4. Molecular identification

The subpassaged strains used to prepare the inocula were verified to species by PCR assays targeting *nox* gene sequences as previously described for *B. hyodysenteriae* (Song and Hampson, 2009) and clade II isolates of "*B. hampsonii*" (Burrough et al., 2012). Challenge strains were further confirmed to species by PCR amplification of partial *nox* gene sequences using previously described primers (Rohde et al., 2002) followed by sequence comparison with sequences available in GenBank. All necropsy isolates were confirmed to species by the individual species PCR assays described earlier applied to extracts of colonic mucosal scrapings.

2.5. Treatment initiation

Tiamulin hydrogen fumarate (Denagard® Liquid Concentrate 12.5%, Novartis Animal Health, Greensboro, NC) was administered via an in-line commercial medication system (Dosatron D25, Clearwater, FL) with adjustable output. A common stock solution (12,000 ppm) was used for each inoculum group and an individual medicator, installed in a bypass loop, was used to deliver the target concentration to each subgroup. Prior to the arrival of pigs, medicator settings were evaluated to determine the appropriate setting necessary to achieve the desired concentration of tiamulin at the nipple. Once these levels were determined, medicators were bypassed and fresh water was run through the lines. Once at least 30% of pigs were shedding viable spirochetes in a given inoculum group and at least one pig had developed swine dysentery in that group, medicators were placed in-line and treatment was delivered continuously for 5 days with pens receiving either 0.006% tiamulin, 0.018% tiamulin, or no medication. After 5 days of treatment, the medicators were again bypassed and fresh water was delivered to pigs for 5 additional days.

2.6. Water analysis

A water sample was obtained from the nipple waterer in each pen at the beginning of the study, the day prior to initiation of water medication, at least once daily during the 5-day medication period, and once daily for 5 days after medication had ceased. Water samples were submitted to the ISU VDL for quantification of tiamulin in ppm.

2.7. Animal observations and necropsy

Following inoculation, animals were observed at least twice daily for feed consumption, availability of adequate water, and clinical illness. Fecal consistency was determined daily, and each pig received a score based upon the following system: 0 if normal, 1 if soft but formed, 2 if semisolid, and 3 if liquid to watery with an additional 0.5 point added each for the presence of discernible mucus