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Characterization of *Brachyspira* communities from clinical cases of swine mucohaemorrhagic diarrhea through deep sequencing of the NADH oxidase (*nox*) gene

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ABSTRACT

Swine dysentery is traditionally associated with *Brachyspira hyodysenteriae,* but the re-emergence of *Brachyspira*-associated disease in North America associated with a novel causative species, *B. hampsonii,* is now a concern for swine producers. The pathogenesis of *Brachyspira*-associated disease is not completely understood, and it is not known whether mixed infections of *Brachyspira* spp. are important in disease development. Deep sequencing of partial sequences of the *nox* gene amplified with genus-specific primers was used to detect *Brachyspira* spp. in 55 fecal samples from clinical cases of mucohaemorrhagic diarrhea in pigs from Western Canada that had been identified as positive for one or more *Brachyspira* species using established diagnostic tests. Synthetic mixtures of *Brachyspira* genomic DNA were included in the study to define detection limits for the technique and identify biases in detection of different species. Multiple species were detected in all clinical cases for which sufficient *nox* sequence data were generated (n = 47), indicating that mixed species *Brachyspira* infections are common, although in most cases, one species accounted for at least half of the sequences identified. In all cases, the species detected in the original diagnostic investigation of each case was also detected by *nox* sequencing. Results from synthetic communities indicated that the method was highly reproducible, but also indicated potential PCR bias against *B. hampsonii* genomovar I. Deep sequencing of the *nox* gene target is a suitable method for simultaneous detection of multiple *Brachyspira* species in clinical case material that may offer advantages over current, more targeted diagnostic approaches for investigating the significance of mixed infections in disease development.

1. Introduction

Brachyspira is associated with swine dysentery, or mucohaemorrhagic diarrhea and colitis, in pigs. Swine dysentery was traditionally associated with *Brachyspira hyodysenteriae* (Harris et al., 1972), and had been mostly eliminated from Canadian swine production systems by the early 1990s. Of growing concern is the re-emergence of disease in the late 2000s that has been associated with a novel emergent species (Chander et al., 2012; Harding et al., 2010), now known as *Brachyspira hampsonii* (Mirajkar et al., 2016; Oren and Garrity, 2017). While there is renewed interest in developing methods to treat and control swine dysentery, there are major challenges in mitigating this production-limiting disease including a lack of rapid, inexpensive clinical diagnostic methods for application in developing a complete understanding of the complex ecology of *Brachyspira* species. The genus *Brachyspira* currently includes nine species and several proposed species. In addition to *B. hyodysenteriae* and *B. hampsonii*, several other species of *Brachyspira* have been isolated from pigs: *B. pilosicoli, B. murdochii, B. intermedia, B. suanatina, "B. pulli",* and *B. innocens*. Weakly haemolytic *B. pilosicoli* is the agent of porcine intestinal spirochetosis (Trott et al., 1996), while *B. intermedia* and *B. murdochii* have been inconsistently associated with disease in pigs (Jensen et al., 2010; Komarek et al., 2009; Weissenböck et al., 2005). *B. innocens* is generally considered non-pathogenic. In addition to these well-characterized taxa, *Brachyspira*-like organisms distinct from named species continue to be detected in studies of healthy and diarrheic pigs (Osorio et al., 2013; Patterson et al., 2013).

Diagnostic methods currently include selective culture for *Brachyspira* spp., gross and histopathology, serology, microscopic detection, biochemical assays of isolates, and PCR (Burrough, 2017). Limitations exist with different methods, for example, although selective culture has better analytical sensitivity than PCR on fecal samples

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(Patterson et al., 2013), its success depends on the nature of the clinical sample and proper transportation and storage. Culture is also slow, requiring incubation periods of more than a week, and requires selective media containing a cocktail of antibiotics. Methods such as microscopy, strength of haemolysis demonstrated on agar plates, and biochemical tests of cultured isolates are useful to detect the presence of *Brachyspira*, but do not differentiate all *Brachyspira* species (Chander et al., 2012; Mahu et al., 2016; Perez et al., 2016). Species-specific PCR methods may lack analytical sensitivity, and since they are focused on detection of individual species they are likely to fail to detect novel pathogenic species or mixed infections. Genus-specific PCR targeting the NADH oxidase gene (*nox*) has been widely employed for *Brachyspira* detection and identification (Atyeo et al., 1999; Burrough et al., 2012; Costa et al., 2014b; Patterson et al., 2013; Råsbäck et al., 2007; Rohde et al., 2002), but species identification requires post-PCR analysis using methods (restriction digestion or more commonly, sequencing) that are not conducive to detection of mixtures of PCR products. Due to these limitations, diagnostic protocols may employ a combination of methods to detect and/or identify *Brachyspira* spp. in clinical specimens, which increases both the cost and the time required for a thorough investigation.

We and others have observed that application of multiple diagnostic methods often results in detection of multiple species from individual clinical samples. A study describing the prevalence of weakly haemolytic *Brachyspira* in pigs reported that concurrent infections with two or more *Brachyspira* spp. was common, and suggested that weakly haemolytic species may contribute to colonic pathology (Komarek et al., 2009). In diagnostic samples from diarrheic grow-finish pigs analysed from western Canada by our laboratory (2009–2017), two, three or four *Brachyspira* species were identified in 12.9%, 1.8% and 0.3% of samples using conventional diagnostic methods. The traditionally causative and pathogenic agent, *B. hyodysenteriae*, has also been isolated from pigs without clinical disease (La et al., 2016a), demonstrating that *B. hyodysenteriae* alone may not always be pathogenic. Results of experimental inoculation experiments with *B. hampsonii* also support the hypothesis that presence of the pathogen alone is often insufficient to cause disease in some animals (Costa et al., 2014b; Rubin et al., 2013a). It is not known whether mixed infections are of clinical significance in regards to production limiting *Brachyspira* colitis, or if multiple *Brachyspira* species may contribute to the pathogenesis of the disease, specifically mucohaemorrhagic diarrhea.

Due to the gaps in our knowledge about mixed species infections and our lack of methods appropriate for their detection, this study aimed to develop a method for simultaneous detection of multiple *Brachyspira* species in clinical samples using deep sequencing of PCR amplicons from the NADH oxidase (*nox*) gene. This method was then used to characterize mixed infections from pigs with swine dysentery, and aid in our understanding of the distribution of *Brachyspira* spp.

2. Methods

2.1. Brachyspira isolates, culture and DNA extraction

The *Brachyspira* strains used in this study included *B. hyodysenteriae* ATCC 27164^T, *B. pilosicoli* ATCC 51139^T, *B. hampsonii* genomovar II strain 30446 (IDAC 161111-01), and *B. hampsonii* genomovar I strain 30599. The type strains of *B. hyodysenteriae* and *B. pilosicoli* were obtained from the American Type Culture Collection. *B. hampsonii* 30446 and 30599 were originally isolated from diarrheic pigs in Western Canada (Costa et al., 2014b; Rubin et al., 2013a). These *Brachyspira* isolates have been stored at −80 °C in brain-heart infusion (BHI) broth (BHI, Becton Dickinson Canada, Mississauga, ON) containing 10% (v/v) glycerol. Strains from storage were cultured in JBS broth (BHI with 1% (w/v) glucose, 5% (v/v) fetal bovine serum, 5% (v/v) sheep blood), and cultures were incubated anaerobically at 37 °C with stirring.

Genomic DNA was extracted from broth cultures of *Brachyspira* by pelleting 1 ml of culture and following a modified salting-out procedure, as previously described (Martin-Platero et al., 2007). Concentration of the extracted DNA and A_{260}/A_{280} ratios were determined using a Nanodrop spectrophotometer, and the DNA was stored at −20 °C.

2.2. Clinical samples

Porcine fecal samples were from clinical cases of diarrhea and colitis that were submitted to our laboratory for diagnostics. Since 2009, clinical cases have been submitted from farms in Western Canada to determine if *Brachyspira* is present, and if so, to identify species using selective culture, PCR and sequencing methods. Cultured *Brachyspira* is identified by genus-specific *nox* PCR directly on growth picked from haemolytic zones on the culture plate followed by DNA sequencing. Genus-specific *nox* PCR and sequencing are also performed on DNA extracts from the clinical specimen. Species-specific real-time PCR assays for *B. hampsonii* genomovars I and II, *B. hyodysenteriae* and/or *B. pilosicoli* may also be applied directly to specimen DNA extracts. This has resulted in a collection of tissues, *Brachyspira* isolates, DNA extracts and *nox* gene sequence data from nearly 1000 clinical cases. From this collection, we chose samples to include in this study based on whether they were *Brachyspira* positive by any diagnostic method, and were epidemiologically distinct (from different farms). Cases were included that had diagnostic results suggesting infections by a single *Brachyspira* species (n = 41), as well as possible mixed species infections (n = 14). Upon original submission of fecal samples, total genomic DNA was extracted using the QIAamp DNA Stool Mini Kit, and stored at −20 °C.

The suitability of genomic DNA extracts from clinical samples for PCR was confirmed using PCR targeting the pig cytochrome oxidase subunit 1 (*cox*1) gene using primers listed in Table 1. Each PCR reaction contained 1 × PCR reaction buffer (0.2 M Tris-HCl pH 8.4, 0.5 M KCl), 2.5 mM $MgCl_2$, 200 µM dNTPs, 400 nM each primer, 2.5 U Taq DNA Polymerase and 2 µl of template DNA, in a final volume of 50 µl. PCR was performed in a thermocycler (Eppendorf Mastercycler) with the following program: 94 °C for 5 min, 40 cycles of 95 °C for 30 s, annealing at 60 °C for 30 s, and final extension of 72 °C for 2 min. A no template control and positive amplification control (pig fecal DNA extract from an unrelated study) were included. PCR products were visualized on a 1% agarose gel by ethidium bromide staining. Only samples producing the expected PCR product were included in the study.

2.3. Construction of synthetic Brachyspira communities and quantitative real-time PCR

Synthetic microbial communities were created by spiking known amounts of *Brachyspira* genomic DNA into a background of fecal DNA known to be *Brachyspira*-free. The *Brachyspira*-free fecal samples used as a source of this DNA were obtained from control pigs in an unrelated study. Samples were screened with PCR targeting the *nox* gene as previously described (Rohde et al., 2002); no PCR product was produced. All negative samples were pooled together to create a uniform fecal DNA background.

Quantification of genomic DNA from four *Brachyspira* isolates (*B. hyodysenteriae* ATCC 27164^T, *B. pilosicoli* ATCC 51139^T, *B. hampsonii* strain 30446, and *B. hampsonii* strain 30599), was achieved by qPCR and known amounts of genomic DNA from each species were added to the synthetic communities. These four taxa were chosen for synthetic community construction since they are the most frequently observed pathogenic species in Western Canada. Quantitative real-time PCR (qPCR) was performed using the primers described in Table 1, and as previously described (Costa et al., 2014b; Rohde et al., 2002; Rubin et al., 2013a). Briefly, qPCR was performed using the Bio-Rad MyiQ thermocycler with iQ SYBR green supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). All samples were tested in duplicate, and no template controls were included with each run.