

Characterization of “*Brachyspira hampsonii*” clades I and II isolated from commercial swine in Western Canada

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Abstract

A novel *Brachyspira* emerged in 2009 and has since become a production-limiting pathogen of pigs in North America. The name “*Brachyspira hampsonii*” has been proposed for this novel taxon. “*Brachyspira hampsonii*” is divided into two phylogenetically distinct clades based on the sequence of the NADH-oxidase (*nox*) gene, although the clinical disease associated with clades I and II is indistinguishable and phenotypic characteristics that discriminate the clades have not been determined. The objectives of the current study were to enhance the description of the provisional species “*B. hampsonii*” with biochemical profiles and morphometric data from isolates affecting Canadian swine and to investigate potentially diagnostically informative characteristics for this emerging pathogen. Biochemical profiles of isolates from different commercial swine barns in Western Canada showed that biochemical profiles were insufficient to distinguish “*B. hampsonii*” clades I and II from each other or from other pathogenic *Brachyspira*. Hippurate hydrolysis, previously reported as uniformly negative in “*B. hampsonii*,” was variable among Canadian isolates. Spirochete dimensions and flagella numbers for “*B. hampsonii*” overlapped with other *Brachyspira* species. Taken together, these results indicate that *nox* gene sequencing remains a preferred method for identification and discrimination of “*B. hampsonii*” from other pig-associated *Brachyspira* spp.

Key words: “*Brachyspira hampsonii*”, transmission electron microscopy, NADH oxidase, indole, hippurate

OPEN ACCESS

Citation: Perez JBDS, Rubin JE, Fernando C, Harding JCS, and Hill JE. 2016. Characterization of “*Brachyspira hampsonii*” clades I and II isolated from commercial swine in Western Canada. FACETS 1: 163–172. doi:[10.1139/facets-2016-0018](https://doi.org/10.1139/facets-2016-0018)

Editor: Alexandre Poulain

Received: April 27, 2016

Accepted: July 21, 2016

Published: October 19, 2016

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Published by: Canadian Science Publishing

Introduction

The genus *Brachyspira* consists of Gram-negative, motile, β -haemolytic, aerotolerant spirochaetes infecting the gastrointestinal tracts of wild and domestic animals, as well as humans. In swine, *Brachyspira hyodysenteriae* is the causative agent of swine dysentery (Harris et al. 1972), whereas *Brachyspira pilosicoli* infection is associated with spirochaetal colitis (Trott et al. 1996). “*Brachyspira suanatina*”, originally isolated from mallard ducks, has also been shown to cause dysentery-like disease in experimentally inoculated pigs (Råsbäck et al. 2007). Other species (*Brachyspira intermedia*, *Brachyspira innocens*, and *Brachyspira murdochii*) detected in pigs are non-pathogenic or have been reported to cause mild catarrhal colitis under experimental conditions (Stanton et al. 1997; Jensen et al. 2010). Re-emergence of *Brachyspira*-associated mucohaemorrhagic diarrhea in swine, in Western Canada and the USA, has been associated with a novel taxon

(Harding et al. 2010), provisionally named “*Brachyspira hampsonii*” (Chander et al. 2012). “*Brachyspira hampsonii*” has also recently been detected in birds in Spain, in pigs in the Czech Republic, and in pigs transported to Germany from Belgium (Martinez-Lobo et al. 2013; Mahu et al. 2014; Rohde et al. 2014). Phylogenetic analysis of the NADH-oxidase (*nox*), 16S rRNA, and 23S rRNA genes revealed two distinct clades within the taxon (Chander et al. 2012; Burrough et al. 2013; Rubin et al. 2013b). Replacement of clades I and II with genetic groups 1–4 has also been suggested based on multi-locus sequence typing of six targets for “*B. hampsonii*” (Mirajkar et al. 2015). Although the clinical disease associated with clades I and II is indistinguishable (Rubin et al. 2013a; Costa et al. 2014), the phenotypic properties distinguishing them have not been established.

Brachyspira do not form visible colonies on agar, so growth is detected on blood agar medium by observing zones of β -haemolysis. Phenotypic characterization of *Brachyspira* is generally based on the evaluation of the strength of β -haemolysis on blood agar and activity of select biochemical markers (hippurate hydrolysis, indole production, α -galactosidase, α -glucosidase, and β -glucosidase). Spirochaete size and number of periplasmic flagella can also be used to discriminate species, although these approaches are not practical for the diagnostic laboratory. The phenotypic and genotypic characteristics of “*B. hampsonii*” isolates in the USA have been described (Chander et al. 2012; Mirajkar et al. 2015). Here, we describe phenotypic characteristics of “*B. hampsonii*” clade I and clade II isolates collected from clinical cases of mucohaemorrhagic diarrhea in Western Canadian swine between 2009 and 2013. The objectives of this study were to enhance the description of the provisional species “*B. hampsonii*” with information from isolates affecting Canadian swine and to investigate potentially diagnostically informative characteristics for this emerging pathogen.

Materials and methods

Selection of clinical isolates

“*Brachyspira hampsonii*” clade I isolate D11-30599 and clade II isolate D09-30446 were the first Canadian isolates of each clade derived from clinical cases of mucohaemorrhagic diarrhea in 2011 and 2009, respectively. The other isolates included in the study (one additional clade I and seven additional clade II) were selected from a collection of isolates from 443 “*B. hampsonii*”-confirmed clinical cases from different swine production sites in Alberta, Manitoba, and Saskatchewan between 2009 and 2013. The original identification of the isolates from these clinical cases was done using culture, *nox* sequencing, and clade-specific PCR with previously described assays (Rubin et al. 2013a; Costa et al. 2014). Although a number of clade I and clade II isolates were obtained from farms during this time period, isolates were selected for the current study only if they originated from different production systems (i.e., were epidemiologically distinct). *Brachyspira* isolates from clinical cases were stored long-term at -80°C in brain-heart infusion (BHI) broth containing 10% (v/v) glycerol.

nox gene sequencing and phylogenetic analysis

Genomic DNA was extracted and purified from Joe’s *Brachyspira* Sask30446 (JBS) broth cultures (BHI with 1% (w/v) glucose, 5% (v/v) fetal bovine serum, 5% (v/v) sheep blood; Becton Dickinson Canada, Mississauga, Ont.) using DNeasy Blood & Tissue Kit (Qiagen Inc., Mississauga, Ont.) following the manufacturer’s instructions for pre-treatment for Gram-negative bacteria and the Animal Tissue protocol. A 939-bp region of the *nox* gene was amplified using genus-specific primers Bnox_f (5′-TAG CYT GCG GTA TYG CWC TTT GG-3′) and Bnox_r (5′-CTT CAG ACC AYC CAG TAG AAG CC-3′) as previously described (Rohde et al. 2002; Rubin et al. 2013a). PCR products were purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Inc., Markham, Ont.) and sequenced with the amplification primers. Raw sequence data were trimmed to a uniform length of 810 bp prior to alignment with ClustalW and phylogenetic tree construction using PHYLIP (Felsenstein 1989).

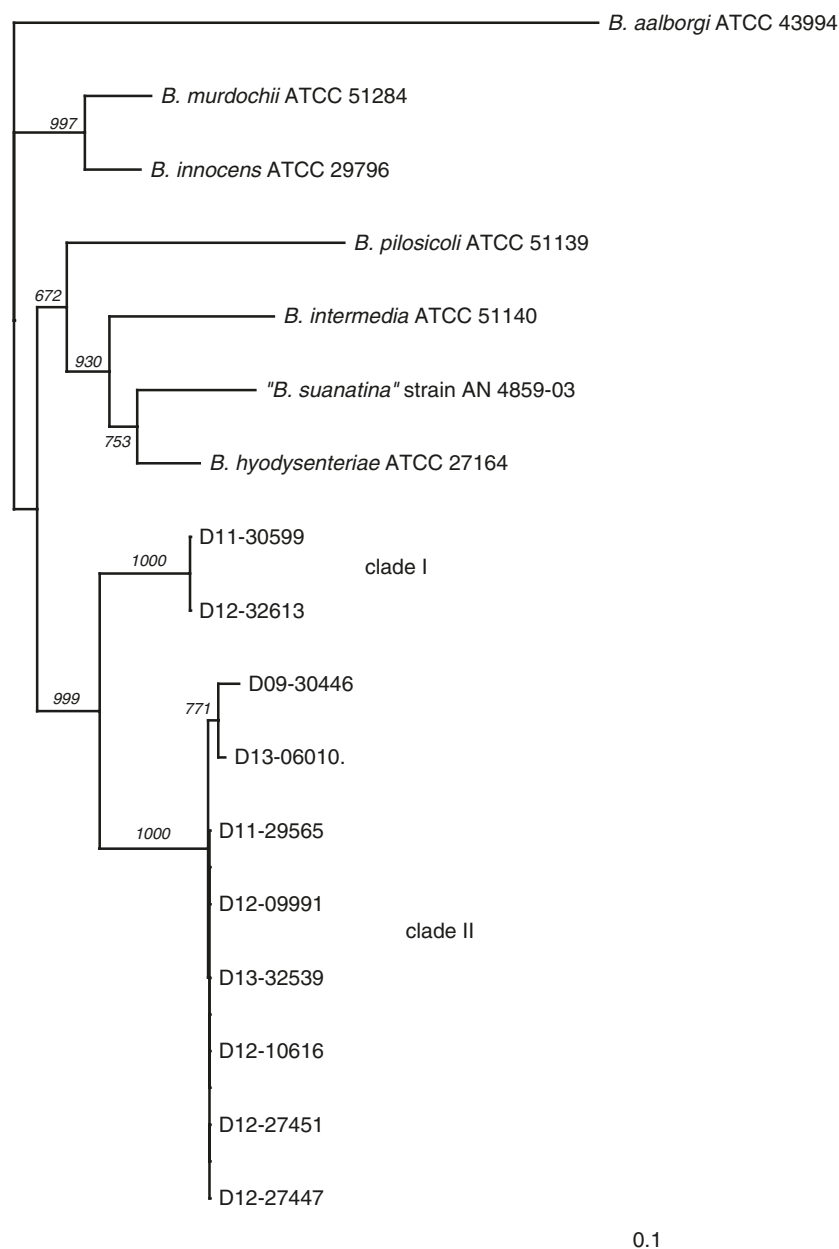


Fig. 1. Phylogenetic relationships of "*B. hampsonii*" study isolates and reference species based on partial *nox* sequences. Bootstrap values (out of 1000) are indicated at the nodes. Reference sequences were included from *B. aalborgi* ATCC 43994^T (GenBank accession No. AF060816), *B. murdochii* ATCC 51284^T (KC984308), *B. innocens* ATCC 29796^T (KC984307), *B. pilosicoli* ATCC 51139^T (KC984310), *B. intermedia* ATCC 51140^T (KC984309), "*B. suanatina*" AN 4859-03 (DQ487119), and *B. hyodysenteriae* ATCC 27164^T (KC984311). Scale bar indicates 0.1 substitutions per site.

Table 1. Biochemical test results for Western Canadian clinical “*B. hampsonii*” isolates.

	Clade I ^a		Clade II ^a							
	D11-30599	D12-32613	D09-30446	D11-29565-A6P8	D12-09991-28	D12-10616-P8	D12-27447	D12-27451	D12-32539	D13-06010
Alkaline phosphatase	+	W+ ^b	+	+	+	+	–	W+	+	–
Esterase (C4)	+	+	+	+	–	+	+	+	+	–
Esterase lipase (C8)	+	W+	+	+	–	+	+	W+	+	–
Lipase (C14)	–	–	–	–	–	–	–	–	–	–
Leucine arylamidase	–	–	–	–	–	–	–	–	–	–
Valine arylamidase	–	–	–	–	–	–	–	–	–	–
Cystine arylamidase	–	–	–	–	–	–	–	–	–	–
Trypsin	–	–	–	–	–	–	–	–	–	–
α-Chymotrypsin	–	–	W+	–	–	–	–	–	–	–
Acid phosphatase	+	+	+	+	W+	+	+	+	+	W+
Naphthol-AS-BI-phosphohydrolase	+	+	W+	+	W+	+	+	+	+	+
α-Galactosidase	–	–	–	–	–	–	–	–	–	–
β-Galactosidase	+	+	+	+	W+	+	+	+	+	–
β-Glucuronidase	–	–	–	–	–	–	–	+	–	–
α-Glucosidase	–	–	–	–	–	–	–	–	–	+
β-Glucosidase	+	+	+	+	–	+	W+	+	+	–
N-acetyl-β-glucosaminidase	–	–	–	–	–	–	–	–	–	–
α-Mannosidase	–	–	–	–	–	–	–	–	–	–
α-Fucosidase	–	–	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–	–	–
Hippurate hydrolysis	–	+	–	+	–	+	+	+	+	–

^aBased on the partial *nox* gene sequence.

^bW+ denotes a weak positive.

Biochemical tests

Study isolates were grown in BHIS (BHI supplemented with 1% (w/v) glucose and 10% (v/v) fetal bovine serum) broth to mid-log phase. Cultures were adjusted to McFarland 5 and enzymatic activities were determined using the API ZYM kit (bioMérieux, St. Laurent, QC) according to the manufacturer’s instructions. Spot indole and hippurate broth assays were performed as previously described (Fellström and Gunnarsson 1995). *Brachyspira hyodysenteriae* ATCC 27164^T and *B. pilosicoli* ATCC 51139^T were included as controls in all biochemical tests.

Electron microscopy

Log phase *Brachyspira* cultures were prepared for transmission electron microscopy (TEM) using the single droplet negative staining technique and carbon-coated 200 mesh-200G-CP grids (Trott et al. 1996; Stanton et al. 1998). Flagella counts, cell width (average of three locations along the length of

Table 2. Morphological and biochemical characteristics of “*B. hampsonii*” clade I and clade II and other swine-associated *Brachyspira* species.

Characteristic	“ <i>B. Hampsonii</i> ” clade I	“ <i>B. Hampsonii</i> ” clade II	<i>B. hyodysenteriae</i> (Trott et al. 1996; Fellström et al. 1999)	“ <i>B. suanatina</i> ” (Råsbäck et al. 2007; Mushtaq et al. 2015)	<i>B. pilosicoli</i> (Trott et al. 1996)	<i>B. innocens</i> (Oxberry et al. 1998)	<i>B. murdochii</i> (Stanton et al. 1997; Jensen et al. 2010)	<i>B. intermedia</i> (Stanton et al. 1997; Jensen et al. 2010)
Cell length (µm)	7.27 ± 1.30 ^a	8.53 ± 2.19 ^a	9.78 ± 1.87 ^c	7.27 ± 1.00 ^c	6.27 ± 0.98 ^c	9.40 ± 1.85 ^c	5–8 ^d	7.5–10 ^d
Cell diameter (µm)	0.26 ± 0.045 ^a	0.46 ± 0.05 ^a	0.35 ± 0.02 ^c	0.3 ± 0.00 ^e	0.27 ± 0.03 ^c	0.36 ± 0.03 ^c	0.35–0.40 ^d	0.35–0.45 ^d
No. of flagella	11–16 ^b	12–15 ^b	8–12	14–16	4–6	10–13	11–13	12–14
β-Haemolysis	Strong	Strong	Strong	Strong	Weak	Weak	Weak	Weak
Indole production	–	–	Variable	Weak+ / + ^f	–	–	–	+
Hippurate hydrolysis	Variable	Variable	–	–	+	–	–	–
α-Galactosidase	–	–	–	–	+	+	–	–
α-Glucosidase	–	Variable	+	Weak+	–	–	–	+
β-Glucosidase	+	Variable	+	+	–	+	–	+

^aMean ± standard deviation for 20 spirochaetes.
^bRange for 15 spirochaetes.
^cMean ± standard deviation for 5 spirochaetes.
^dAuthors’ reported range.
^eMean ± standard deviation for 10+ spirochaetes.
^fReported as positive by Mushtaq et al. (2015) and weak positive by Råsbäck et al. (2007).

the spirochaete not including the tapered region near both ends), and cell length were measured using Image-Pro Plus (Media Cybernetics, Rockville, Md.) on captured TEM images. Twenty cells of each isolate were measured. Images were considered suitable for width and length measurements if the cells were intact, isolated, and not twisted on themselves. Flagella counts were performed on untwisted cells with at least one end visible.

Results and discussion

Clinical isolates of “*B. hampsonii*” were selected to represent distinct pig production sites, although most were related by a common source of replacement females. The number of isolates included in the study from either clade (two clade I and eight clade II) reflects higher isolation rates of clade II isolates from clinical samples submitted to our laboratory, which to our knowledge was the only laboratory performing clade level identification of “*B. hampsonii*” in Western Canada during the period of study. This trend in relative occurrence of clades I and II “*B. hampsonii*” is opposite to that reported in a recent study of “*B. hampsonii*” diversity in the USA (Mirajkar et al. 2015). Clade affiliation was confirmed, and relatedness of the selected clinical isolates to other *Brachyspira* species was investigated based on partial *nox* gene sequences. The *nox* sequences of the two clade I isolates were identical, whereas the pairwise identities between clade II isolates ranged from 99% to 100% (Fig. 1).

Results of the API ZYM, indole production, and hippurate hydrolysis tests for study isolates are summarized in Table 1. Activities of α-galactosidase, α-glucosidase, and β-glucosidase, and indole production and hippurate hydrolysis have been utilized as biochemical markers for differentiation of

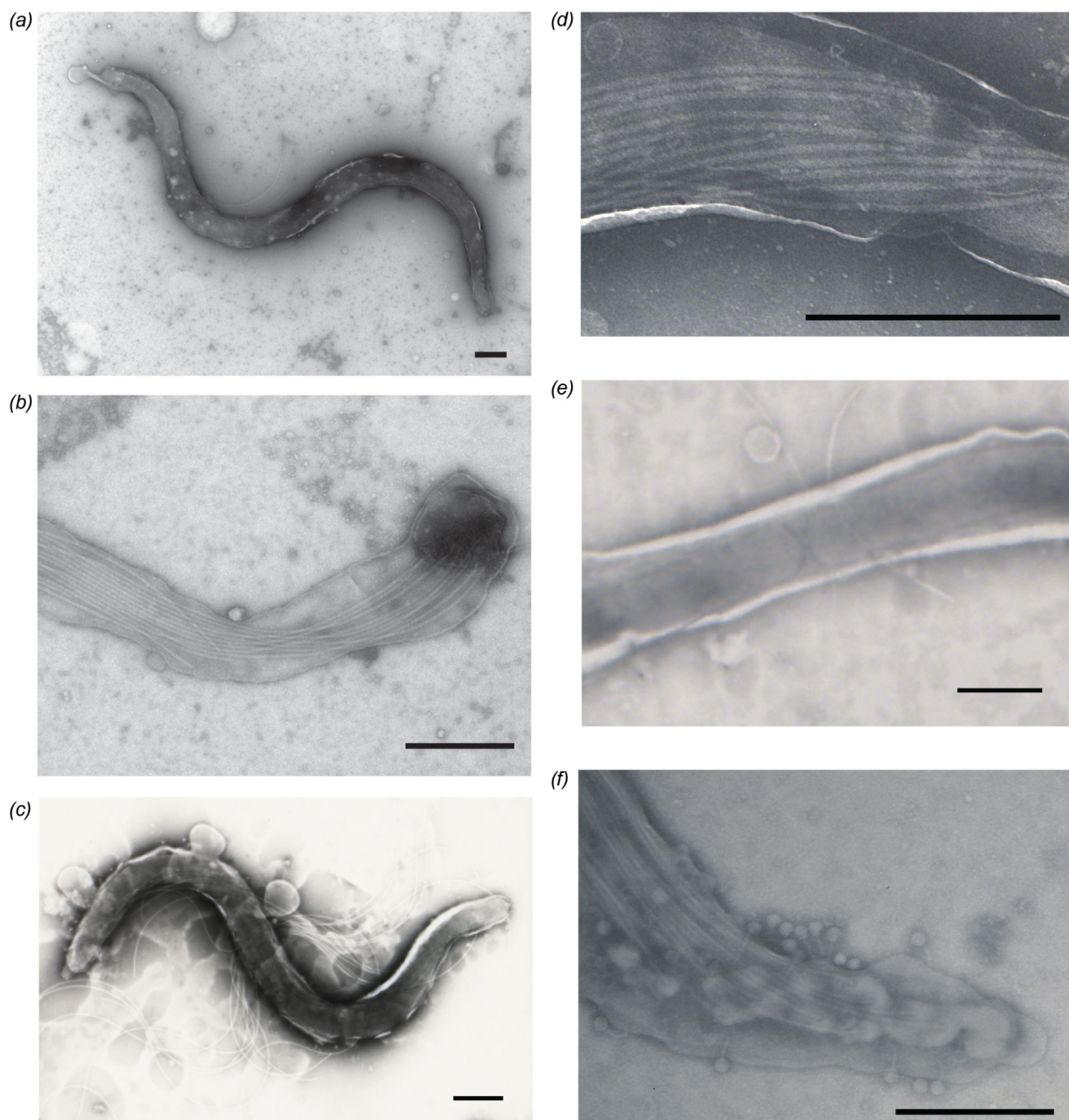


Fig. 2. TEM images of (a, b) clade I isolate D11-30599 and (c–f) clade II isolate D09-30446. (a, c) entire spirochaete, (b, d) periplasmic flagella, (e) cell division, and (f) tip of D09-30446 spirochaete with tailed bacteriophages. Scale bars represent 0.5 μm.

Brachyspira species (Achacha and Messier 1991; Fellström and Gunnarsson 1995). All clade I and clade II study isolates were negative for α -galactosidase activity, one clade II isolate was positive for α -glucosidase, and both clade I isolates and 6/8 clade II isolates were positive for β -glucosidase activity (Table 1). Although indole production was negative for all isolates as expected, hippurate hydrolysis activity was inconsistent among isolates in both clades (Table 1). These results are consistent with a previous phenotypic description of “*B. hampsonii*” from the USA (Chander et al. 2012) in that none of these assays could distinguish clade I and clade II isolates. Interestingly, all isolates in the USA study ($n = 17$ clade I, $n = 3$ clade II) were negative for hippurate hydrolysis activity (Chander et al. 2012). Variations in indole production, hippurate hydrolysis, α -galactosidase, and α -glucosidase activities of human isolates of *B. pilosicoli* have been observed in previous studies (De Smet et al. 1998), and variation in indole production of swine-isolated *B. hyodysenteriae* has also been reported (Fellström et al. 1999). The greater consistency in hippurate hydrolysis activity in the USA study may reflect epidemiological relationships of isolates, but these relationships are not described in that report. Taken together, these results show that none of these biochemical assays can discriminate clades I and II or “*B. hampsonii*” from *B. hyodysenteriae* (Table 2). The lack of specificity of biochemical markers for differentiating *Brachyspira* species has inspired the development of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) as a promising phenotypic method for differentiation of *Brachyspira*. This technology has been demonstrated to distinguish *Brachyspira* species (Calderaro et al. 2013; Prohaska et al. 2014), but clades of “*B. hampsonii*” are not reliably distinguished (Warneke et al. 2014). The initial capital costs for diagnostic MALDI-TOF have also limited its widespread adoption.

TEM was performed on D11-30599 and D09-30446 to measure the dimensions of the spirochaetes and count the number of flagella for comparison to other *Brachyspira* species. Representative images are shown in Fig. 2. Spirochaetes of clade I “*B. hampsonii*” isolate D11-30599 were $7.27 \pm 1.30 \mu\text{m}$ and $0.26 \pm 0.045 \mu\text{m}$, with 11–16 periplasmic flagella (Figs. 2a, 2b and Table 2). For clade II isolate D09-30446, average length and width were $8.53 \pm 2.19 \mu\text{m}$ and $0.46 \pm 0.05 \mu\text{m}$, with 12–15 periplasmic flagella (Figs. 2c, 2d and Table 2). Apparently dividing cells were observed (Fig. 2e), and tailed bacteriophages were occasionally detected on the surface D09-30446 spirochaetes under TEM (Fig. 2f). The presence of tailed bacteriophages is consistent with the type of bacteriophage detected with other *Brachyspira* (Calderaro et al. 1998; Ackermann 2007). The morphological measurements and flagella counts for “*B. hampsonii*” overlapped with other pig-associated *Brachyspira* species and so do not on their own provide species identification (Table 2).

Conclusions

The biochemical variation of “*B. hampsonii*” isolates from Western Canadian swine production systems highlights that biochemical characterization alone is not diagnostically informative. Although some traits of “*B. hampsonii*” isolates are consistent (indole and α -galactosidase negative), these characteristics are not solely sufficient to discern them from other *Brachyspira* species as previously observed in the USA study (Chander et al. 2012). The morphology of selected isolates was also indistinguishable from other species of *Brachyspira*. Based on these results and previous observations, *nox* gene sequencing remains a useful, accessible, and affordable tool for identification and discrimination of *Brachyspira* spp. affecting pigs.

Acknowledgements

The authors thank Ian Shirley, Chris Stuart, and Samantha Ekanayake (Western College of Veterinary Medicine) for their expertise in electron microscopy. This project was supported by funding from the Canadian Swine Health Board.

Author contributions

Conceived and designed the study: JER, JCSH, JEH. Performed the experiments/collected the data: JBDSP, JER, CF. Analyzed and interpreted the data: JBDSP, JER, CF, JCSH, JEH. Drafted or revised the manuscript: JBDSP, JER, CF, JCSH, JEH.

Competing interests

The University of Saskatchewan has filed patent applications pertaining to the “*B. hampsonii*” clade II strain (*Brachyspira* sp. SASK30446) titled “Isolated *Brachyspira* and methods and compositions for expanding and isolating *Brachyspira*” in Canada (serial number 2842068) and USA (serial number 14/233414) on 18 July 2012. The applications are published and available online at: <http://patentscope.wipo.int>.

Data accessibility statement

All relevant data are within the paper.

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