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Application of *nox*-restriction fragment length polymorphism for the differentiation of *Brachyspira* intestinal spirochetes isolated from pigs and poultry in Australia

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Abstract. Sixty-nine intestinal spirochetes isolated from pigs and poultry in eastern Australia were selected to evaluate the effectiveness of a species-specific PCR-based restriction fragment length polymorphism (RFLP) analysis of the *Brachyspira nox* gene. For comparative purposes, all isolates were subjected to species-specific PCRs for the pathogenic species *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*, and selected isolates were examined further by sequence analysis of the *nox* and 16S ribosomal RNA genes. Modifications to the original *nox*-RFLP method included direct inoculation of bacterial cells into the amplification mixture and purification of the PCR product, which further optimized the *nox*-RFLP for use in a veterinary diagnostic laboratory, producing sufficient product for both species identification and future comparisons. Although some novel profiles that prevented definitive identification were observed, the *nox*-RFLP method successfully classified 45 of 51 (88%) porcine and 15 of 18 (83%) avian isolates into 5 of the 6 recognized species of *Brachyspira*. This protocol represents a significant improvement over conventional methods currently used in veterinary diagnostic laboratories for rapid specific identification of *Brachyspira* spp. isolated from both pigs and poultry.

Key words: *Brachyspira;* nicotinamide adenine dinucleotide reduced oxidase; PCR; 16S ribosomal DNA sequencing.

Introduction

Species differentiation of the intestinal spirochetes within the genus *Brachyspira* has been largely dependent on accurate phenotypic and biochemical characterization of pure cultures, a process usually requiring a highly experienced reference laboratory. Preparation of each isolate for biochemical analysis is not only time consuming, but often the technique cannot distinguish unequivocally between *Brachyspira* spp.[1] Although the biochemical classification scheme of Fellström and Gunnarsson[2] appears to correlate with the recognized *Brachyspira* species isolated from pigs, the variable results and subjective nature of the test suggests that a genotypic method would be a beneficial alternative. This is particularly the case in veterinary diagnostic laboratories where rapid, sensitive, and specific differentiation of *Brachyspira* spp. is essential for accurate diagnosis.

Multilocus enzyme electrophoresis (MLEE) has proved more successful than 16S ribosomal DNA (rDNA) sequence analysis in the classification of intestinal spirochetes, with the assigned MLEE groupings reflecting phenotypic differentiation.[5,6,13] 16S Ribosomal DNA sequences of porcine *Brachyspira* spp. are highly conserved, with *Brachyspira pilosicoli* and the other weakly β-hemolytic *Brachyspira* spp. (*Brachyspira innocens*, *Brachyspira intermedia*, and *Brachyspira murdochii*) having 98.5% and >99.0% identity to *Brachyspira hyodysenteriae*, respectively.[11] However, both MLEE and 16S rDNA sequencing are not practical for routine use in a veterinary diagnostic laboratory. A number of species-specific PCR methods have been developed,[1,7,9,10,15] but to date there is no single PCR-based method capable of identifying all the major *Brachyspira* spp.

Genetic analysis of the nicotinamide adenine dinucleotide reduced oxidase (*nox*) gene in *Brachyspira* spp. allowed Rohde et al.[12] to identify restriction endonuclease sites that would produce species-specific restriction fragment length polymorphism (RFLP) patterns for the 5 species of *Brachyspira* isolated from pigs. The accuracy of this method was tested using a set of 132 field isolates from diseased and healthy pigs that had been identified using conventional biochemical methods. The novel *nox*-RFLP method was highly specific when compared with biochemical classification, although no comparison was made with current genetic methods of identification. In addition, because the method was only tested for porcine isolates and

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Table 1. List of oligonucleotide primers used for *nox*-specific RFLP, *nox*, and 16S rDNA sequencing, and species-specific PCRs for *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae*.

Primer	Sequence (5′ to 3′)	Size (bp)	
nox			
BnoxF	TAGCYTGCGGTATYGCWCTTTGG	939	
BnoxR	CTTCAGACCAYCCAGTAGAAGCC		
Bnox-F2	GAAAAAAGAATCAAAGAAGCTGGC	NA*	
Bnox-R2	CACCTATATAACCAGCACCAACTACC	NA	
Bnox322-F3	AAGAAATTTGAAGGGGAGGACAGAG	NA	
16S rDNA			*Escherichia coli* 16S rDNA position:
609	GTTTGATYCTGGCTYAGARCKAACG	1,480	11–35
594B	CCSSTACGGMTACCTTGTTACG		1517–1496
530f	GTGCCAGCMGCCGCGG	NA	515–530
545r	CCGCGGCTGCTGGCAC	NA	530–515
926f	AAACTYAAAKGAATTGACGG	NA	907–926
Bhyo-PCR		750	
BC4	CATCATTTGATGATTCTTGA	600	
BC5	GAATTAACAGAAGCAGAAGA	180	
Bpilo-PCR			
BpiloF	AGAGGAAAGTTTTTTCGCTTC	439	
BpiloR	CCCCTACAATATCCAAGACT		

* NA = not applicable.

the *nox* gene sequence of the avian species *Brachyspira alvinipulli* was not included in the reference data set, it is not known whether the specificity extends to *Brachyspira* spp. isolated from other animal hosts.

Avian intestinal spirochetosis (AIS) is a recently recognized disease of layer and meat breeder chickens and has been shown to be a common but currently underdiagnosed infection in the Australian poultry industry.[14] Intestinal spirochetes from commercial chicken flocks in Australia, the United States, the United Kingdom, and the Netherlands were analyzed by biochemical characterization and MLEE.[8] These isolates were shown to be genetically heterogeneous with the majority forming a novel electrophoretic group (provisionally designated "*Brachyspira pulli*"), distinct from that formed by *B. alvinipulli.* No consistent phenotypic characteristics were found within this heterogeneous genetic group; therefore, accurate identification of *Brachyspira* isolates from chickens remains extremely difficult. To date, the only avian intestinal spirochete to be analyzed by 16S rDNA sequencing is *B. alvinipulli.* By both MLEE and 16S rDNA sequence analysis, *B. alvinipulli* is phylogenetically distinct from the remaining *Brachyspira* species.[13]

This study was designed to determine whether the *nox*-RFLP method could accurately identify *Brachyspira* spp. isolated from pigs and poultry in eastern Australia, using comparisons with species-specific PCR for *B. hyodysenteriae* and *B. pilosicoli*, and 16S rDNA and *nox* amplicon sequencing for selected isolates. In addition, the method was further optimized to facilitate routine use and comparative analysis in a veterinary diagnostic laboratory.

Materials and methods

Bacterial strains and culture conditions. Reference cultures of *B. hyodysenteriae* B204^T and *B. pilosicoli* 95.1000 were obtained from the Reference Centre for Intestinal Spirochetes, Murdoch University, Perth, Australia. In addition, a total of 69 Australian *Brachyspira* field isolates (51 from pigs, 18 from poultry) were randomly selected from the isolate collection held at the Toowoomba Veterinary Laboratory, Animal and Plant Health Service, Department of Primary Industries, Toowoomba, Queensland, Australia (TVL). All isolates were originally obtained from fecal specimens or intestinal contents submitted to TVL for diagnosis. *Brachyspira* spp. were subcultured from frozen stock to 8% sheep blood agar plates and incubated at 42° C under anaerobic conditions for at least 3 days. Bacterial suspensions were then observed by phase contrast microscopy for characteristic spirochete morphology to confirm if the cultures were free from other bacterial contaminants.

nox-RFLP. The *nox*-RFLP method was performed essentially as described by Rohde et al.[12] with some minor modifications. Briefly, a 939-bp product was amplified from isolates of *Brachyspira* spp. using the oligonucleotide primers Bnoxf and Bnoxr (Table 1). For ease and rapidity, a small amount of bacterial growth removed from a pure culture on 8% sheep blood agar using a pipette tip was added directly to the amplification mixture (50 μl). All *nox* gene amplifications were performed using the GeneAmp™ PCR system 2400,[a] in a reaction mixture containing 3.2 pmol of each primer, 200 μM of each deoxynucleoside triphosphate (dNTP), 1× PCR buffer, 2 mM $MgCl_2$, and 0.25 U Red Hot®

Table 2. Corrected restriction fragment sizes determined by Clone Manager v4.0 for *Brachyspira* spp. reference strains, including *B. alvinipulli* and *B. aalborgi*. Novel restriction profiles identified by this study are also listed.

Species	*Bfm*I (bp)	*Dpn*II (bp)
B. aalborgi	453, 248, 238	939
B. alvinipulli	742, 197	898, 41
B. pilosicoli	742, 197	898, 41
B. hyodysenteriae	742, 197	684, 214, 41
B. intermedia	504, 238, 197	684, 214, 41
B. innocens	504, 211, 197, 27	684, 214, 41
B. murdochii	504, 211, 197, 27	684, 157, 57, 41
Isolates 320/322	701, 238	898, 41
Isolates 701/802	453, 238, 197 and 51	939
Isolate 805	504, 238, 182 and 15	684, 214, 41

DNA Polymerase[b] with an initial denaturation for 5 min at 95° C, then 30 cycles of denaturation for 30 sec at 94° C, annealing for 40 sec at 59° C, and extension for 54 sec at 72° C, followed by a final extension for 5 min at 72° C. Polymerase chain reaction products were observed after separation by electrophoresis in a 1.5% agarose gel stained with ethidium bromide, and then the remaining PCR products were purified using a QIAquick PCR purification kit[c] (50 μl final volume). Twenty microliters of the purified PCR product was digested separately with the restriction endonucleases *Bfm*I[d] (5 U) and *Dpn*II[e] (25 U) according to the manufacturer's instructions, incubating overnight at 37° C. Restriction fragments were separated in a 2% agarose gel and observed after staining with ethidium bromide. Provisional speciation based on the classification determined by Rohde et al.[12] was given to all field isolates analyzed.

Species-specific PCR for B. hyodysenteriae and B. pilosicoli. Previously developed PCR protocols specific for *B. hyodysenteriae*[1] and *B. pilosicoli*[9] were used for rapid screening of isolates, with the primers BC4/BC5 and BpiloF/BpiloR being used to identify *B. hyodysenteriae* and *B. pilosicoli*, respectively, in separate reactions. For ease and rapidity, whole cells were added to each amplification mixture (25 μl) that contained 3.2 pmol of each primer, 200 μM of each dNTP, 1× PCR buffer, 2 mM $MgCl_2$, and 0.25 U Red Hot® DNA Polymerase,[b] with an initial denaturation for 5 min at 95° C, then 30 cycles of denaturation for 30 sec at 94° C, annealing for 30 sec at 48° C, and extension for 30 sec at 72° C, followed by a final extension for 5 min at 72° C. Polymerase chain reaction products were separated by electrophoresis on a 1.5% agarose gel and observed after staining with ethidium bromide. The primers BC4/BC5 amplified 3 fragments of 750, 600, and 180 bp in isolates identified as *B. hyodysenteriae*, and BpiloF/BpiloR amplified a 439-bp product in those identified as *B. pilosicoli.*

Nucleotide sequence analysis of 16S ribosomal RNA and nox genes. Representative isolates from those subjected to the *nox*-RFLP method were chosen for DNA sequence analysis of both the *nox* and 16S ribosomal RNA (rRNA) genes (Table 2). In particular, isolates producing novel *nox*-RFLP profiles and those identified as *B. innocens, B. intermedia*, and *B. murdochii* were selected for sequence analysis. Randomly chosen isolates identified as *B. hyodysenteriae* and *B. pilosicoli* by *nox*-RFLP were also analyzed to support the species classifications. All primers used for amplification and sequencing analyses are listed in Table 1.

Long-template amplification of all products for sequence analysis was performed using the Expand High Fidelity PCR System[f] as directed by the manufacturer. The 16S rDNA genes were amplified directly using the primers 609 and 594B (~1,480 bp) with an annealing temperature of 48° C, and sequencing was completed using the primers 530f, 545r, and 926f. The *nox* genes were amplified using the primers BnoxF and BnoxR (939 bp), with sequencing being completed using the primers BnoxF2 and BnoxR2. Isolates identified as *B. pilosicoli* required the primer Bnox322-F3 instead of BnoxF2 to complete the sequence determination.

Amplified products were purified using the QIAquick PCR purification kit[c] and then sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit Version 3.1.[g] The reactions were analyzed with an ABI 3730xI 96-capillary automatic DNA Analyzer at the Australian Genome Research Facility, Brisbane, Australia. Neighbor-joining phylogenetic trees for both *nox* and 16S rDNA sequences were constructed using the Tamura–Nei model, with bootstrap analysis being performed by resampling the data 1,000 times. Sequences of equal length were used in the analysis: 893 nt for *nox* genes and approximately 1,380 nt for 16S rDNA gene sequences.

Results

nox-RFLP. Restriction endonuclease analysis of the 939-bp amplified *nox* sequence from GenBank *Brachyspira* reference strain submissions using the program Clone Manager v4.0 indicated that the predicted fragment sizes were not the same as those cited by Rohde et al.[12] The appropriate restriction fragment sizes for each *Brachyspira* species are listed in Table 2.

Of the 69 *Brachyspira* isolates analyzed by the *nox*-RFLP method, 63 were identified and allocated to known species (Table 3). Forty-three isolates were determined to be *B. pilosicoli* (34 porcine, 9 avian), 8 were *B. hyodysenteriae* (all porcine), 7 were *B. intermedia* (4 avian, 3 porcine), 4 were *B. murdochii* (3 avian, 1 porcine), and 1 was *B. innocens* (porcine). In addition, 2 isolates displayed *nox*-RFLP profiles that suggested the presence of a mixed culture because the sum of the restriction fragments was greater than that of the *nox* amplimer. A further 4 isolates demonstrated 2 distinct profiles that did not match those described by Rohde et al.[12] (Table 3).

nox and 16S rRNA gene sequence analysis. Representative isolates were chosen for sequencing analysis of both the *nox* and 16S rDNA genes (Table 2). This was done to support the identification made by the *nox*-RFLP method and to determine the phylogenetic position of the isolates that produced unique *nox*-RFLP profiles. Dendrograms indicating the phylogenetic relationships between *Brachyspira* reference strains and the isolates examined in this study based

Table 3. List of *Brachyspira* spp. isolates analysed by 16S rDNA sequencing, *nox*-specific RFLP, *nox* gene sequencing, and species-specific PCRs for *B. pilosicoli* and *B. hyodysenteriae*.

ID	Origin*	16S rDNA sequence homology	RFLP	*nox* Sequence homology	*B. pilosicoli* PCR	*B. hyodysenteriae* PCR
95-1000	P		*B. pilosicoli*		+	−
B204	P		*B. hyodysenteriae*	U19610†	−	+
320	P	99.7% P43/6 (Pilo)	?	95.2% P43/6/78 (Pilo)	+	−
322	P	99.7% P43/6 (Pilo)	?	95.2% P43/6/78 (Pilo)	+	−
802	A	99.5% R1 (Hyo)	?	92.5% R1 (Hyo)	−	−
701	A	99.2% R1 (Hyo)	?	92.8% R1 (Hyo)	−	−
98-0026	P	99.9% P43/6 (Pilo)	*B. pilosicoli*	99.9% P43/6/78 (Pilo)	+	−
903	A	99.5% P43/6 (Pilo)	*B. pilosicoli*	100% P43/6/78 (Pilo)	+	−
349	P	99.9% P43/6 (Pilo)	*B. pilosicoli*	99.1% P43/6/78 (Pilo)	+	−
255	P	99.9% R1 (Hyo)	*B. hyodysenteriae*	100% B169/B78 (Hyo)	−	+
328	P	99.9% R1 (Hyo)	*B. hyodysenteriae*	100% B169/B78 (Hyo)	−	+
819	P	100% B204 (Hyo)	*B. hyodysenteriae*	99.9% B169B78 (Hyo)	−	+
946	P	99.7% B204 (Hyo)	*B. murdochii*	99.3% 56-150 (Murd) 98.8% 155-20 (Murd)	−	−
788	A	99.5% B204 (Hyo) 99.3% 56-150 (Murd)	*B. murdochii*	100% 155-20 (Murd)	−	−
752	A	99.5% R1 (Hyo) 99.4% 56-150 (Murd)	*B. murdochii*	100% 155-20 (Murd)	−	−
532	A	99.5% R1 (Hyo) 99.4% 56-150 (Murd)	*B. murdochii*	100% 155-20 (Murd)	−	−
931	P	mixed	*B. intermedia*	98.1% PWS/A (Interm) 92.7% 2815.5 (Interm)	+	−
805	A	99.0% R1 (Hyo)	*B. intermedia*	93.3% *B. alvinipulli*	−	−
854	A	99.9% R1 (Hyo)	*B. intermedia*	97.8% PWS/A (Interm)	−	−
856	A	99.7% R1 (Hyo)	*B. intermedia*	97.7% PWS/A	−	−
872	P	99.8% B204 (Hyo)	*B. intermedia*	97.8% PWS/A (Interm)	−	−
876	A	99.8% R1 (Hyo)	*B. intermedia*	97.8% PWS/A (Interm)	−	−
878	P	mixed	*B. intermedia*	96.6% 2815.5 (Interm) 94.3% PWS/A (Interm)	−	−
269	P	99.4% B256 (Innoc)	*B. innocens*	99.8% B256 (Innoc)	−	−

* A = avian isolate; P = porcine isolate.
† GenBank accession number.

on their 16S rDNA and *nox* sequences are shown in Figs. 1, 2, respectively. The 16S rDNA dendrogram was divided into 3 major clusters, the first containing *B. hyodysenteriae* and *B. intermedia*, the second containing *B. innocens* and *B. murdochii*, and the third containing *B. pilosicoli* (Fig. 1).

The identification of isolates as *B. pilosicoli* and *B. hyodysenteriae* by *nox*-RFLP correlated well with the identity determined by both *nox* and 16S rDNA sequencing, with minor sequence variation demonstrated between strains. Although the remaining isolates also showed good correlation for identification deduced by the *nox*-RFLP method and *nox* sequence analysis, it was more difficult to determine significant relationships between the identification made by *nox*-RFLP and 16S rDNA sequencing. For example, 16S rDNA sequencing of isolates identified as *B. intermedia* and *B. murdochii* by *nox*-RFLP indicated the greatest similarity to *B. hyodysenteriae* rather than the type strain of the relevant species. In particular, isolate 854 (*B. intermedia* by *nox*-RFLP) showed 99.9% identity with *B. hyodysenteriae* R1 based on 16S rDNA sequence over 1,433 nt. This data confirms the conserved nature of *Brachyspira* 16S rDNA sequences and the close phylogenetic relationship between certain *B. hyodysenteriae* and *B. intermedia* isolates.

Interestingly, the *nox* sequence from 1 avian isolate (805) identified by *nox*-RFLP as *B. intermedia*, showed the greatest similarity (93.3%) to *B. alvinipulli*, with the 5 other *Brachyspira* spp. exhibiting less than 90% identity (e.g., 88.4% similarity with *B. intermedia*). Identification of the restriction sites within the *nox* amplimer DNA sequence indicated that there were minor fragment size differences between this strain and *B. intermedia*, with *Bfm*I restriction fragments of 504, 238, 182, and 15 bp, and *Dpn*II restriction fragments identical to *B. intermedia*. Therefore, strain 805 would be identified as *B. intermedia* by the *nox*-RFLP method, but sequencing and subsequent phylogenetic analysis demonstrated the misidentification. This strain clustered with *B. alvinipulli* in both the 16S rDNA and *nox* dendrograms (Figs. 1, 2), but detailed characterization of this isolate is required before definitive identification can be achieved.

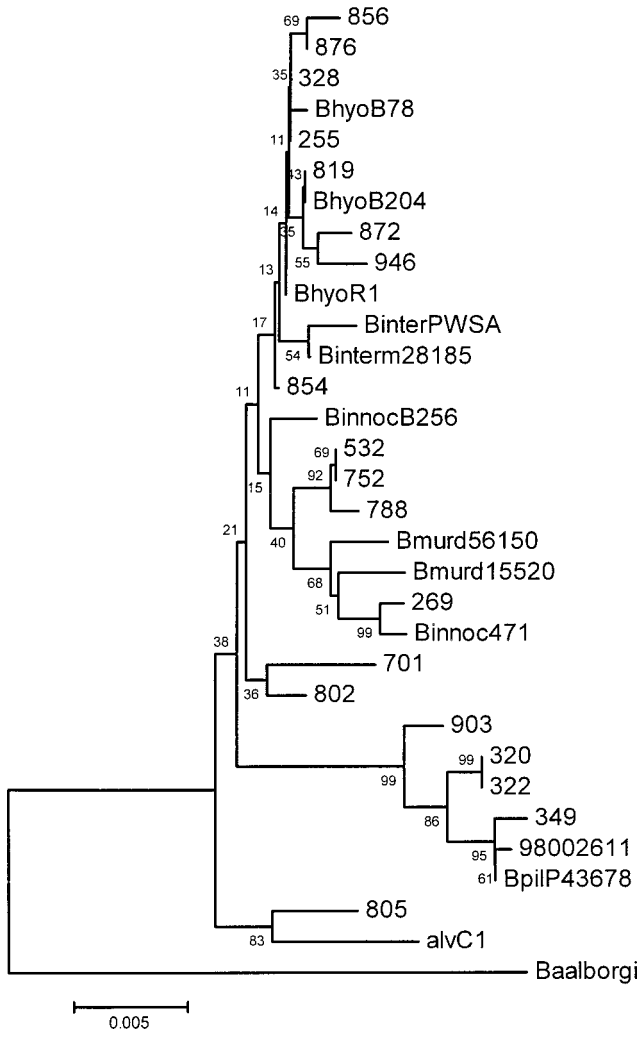

Figure 1. 16S rDNA dendrogram indicating the phylogenetic relationships among the *Brachyspira* spp. reference strains and the isolates examined in this study. The dendrogram cannot easily discriminate between *Brachyspira hyodysenteriae*, *Brachyspira intermedia*, *Brachyspira innocens*, and *Brachyspira murdochii* because there are very few informative nucleotides. This is also reflected in the low bootstrap values for the majority of branches.

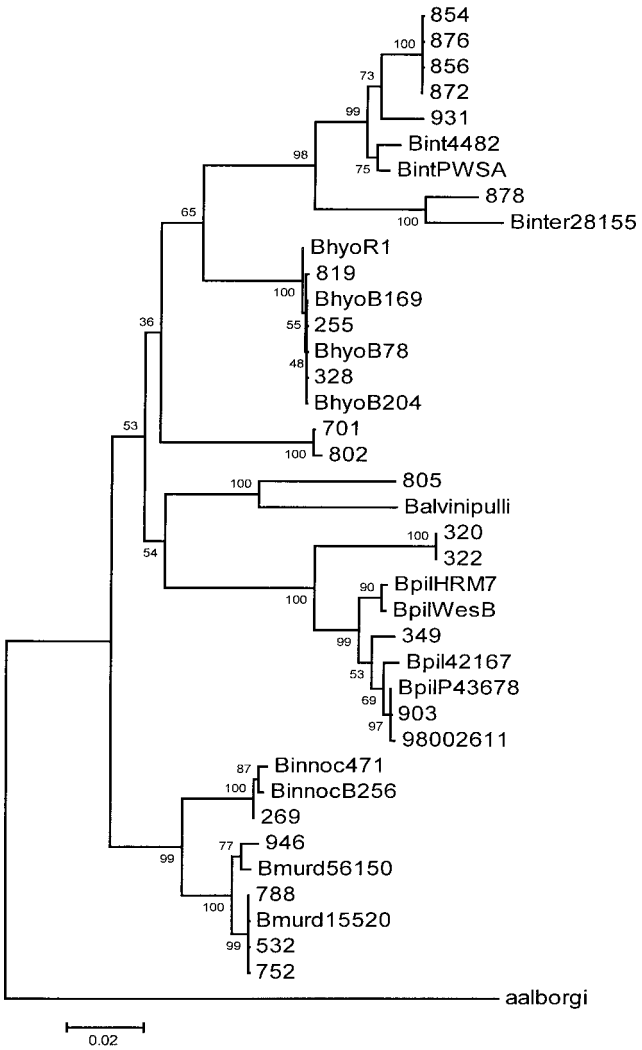

Figure 2. Dendrogram indicating the phylogenetic relationships among the *Brachyspira* spp. reference strains and the isolates examined in this study based on their *nox* sequences. Nucleotide sequences for the *nox* gene were more variable than the corresponding 16S rRNA gene, and there were more informative nucleotides. This allowed for the clearly differentiation of the major *Brachyspira* species, which was supported by high bootstrap values for most branches.

Sequence analysis of the *nox* and 16S rRNA genes amplified from the isolates with novel *nox*-RFLP profiles indicated that isolates 320 and 322 possessed identical sequences and that comparison of their *nox* and 16S rDNA sequences with the corresponding sequences from the reference strains identified them as *B. pilosicoli* (95.2% and 99.7% identity, respectively, with P43/6/78). The avian isolates 701 and 802 possessed nearly identical sequences for each gene and showed the highest sequence identity with *B. hyodysenteriae* R1 (92.5–92.8% and 99.2–99.5%, respectively, for *nox* and 16S rDNA). The percentage similarity and their phylogenetic position in the 16S rDNA and *nox* dendrograms suggest that these isolates do not belong to any of the 6 currently recognized *Brachyspira* spp. These strains could be representatives of the proposed species *B. pulli*, which is commonly isolated from poultry in Australia.[8]

Determination of restriction sites within the *nox* sequence from the 320 and 322 DNA sequence data indicated that these isolates would produce *Bfm*I restriction fragments of 701 and 238 bp, and *Dpn*II restriction fragments of 898 and 41 bp. The amplimer from isolates 701 and 802 would produce *Bfm*I restriction fragments of 453, 238, 197, and 51 bp, with no *Dpn*II restriction sites being present (Table 3).

Species-specific PCRs for B. hyodysenteriae and B. pilosicoli. All 69 field isolates were examined by species-specific PCR to validate the reliability of the *nox*-

RFLP method to identify the major pathogenic *Brachyspira* species. All 8 isolates identified as *B. hyodysenteriae* by *nox*-RFLP were positive with the *B. hyodysenteriae*–specific primers. Furthermore, none of *Brachyspira* isolates that were negative by the PCR showed a *B. hyodysenteriae*–specific *nox*-RFLP pattern, confirming that the *nox*-RFLP was 100% sensitive and specific for identifying *B. hyodysenteriae* strains in the isolate collection. A total of 48 of the field isolates were positive with the *B. pilosicoli*–specific primers, including all 43 identified as *B. pilosicoli* by the *nox*-RFLP method. The 5 additional isolates that were positive with the *B. pilosicoli*–specific PCR included isolates 320 and 322 (which showed a novel *nox*-RFLP profile), the 2 mixed cultures, and 1 isolate (931) that was identified as *B. intermedia* by *nox*-RFLP. Sequencing of the 16S rDNA amplimer from 931 also indicated a mixed culture. These results suggest that there was a predominance of *B. intermedia* in the stock culture of 931 providing a successful identification by *nox*-RFLP, with a small quantity of *B. pilosicoli* detectable by the specific PCR. If the 2 isolates showing the novel *nox*-RFLP pattern are included, these results indicate that *nox*-RFLP is also highly sensitive and specific for the identification of *B. pilosicoli* in the isolate collection. None of the isolates identified as *B. innocens*, *B. intermedia*, or *B. murdochii* by *nox*-RFLP produced amplimers with either species-specific PCR. Isolates 701 and 802 (unidentifiable by *nox*-RFLP) were negative by both the *B. hyodysenteriae*– and *B. pilosicoli*–specific PCRs.

Discussion

In this study, 69 intestinal spirochetes isolated from pigs and poultry from eastern Australia were examined by the novel *nox*-RFLP method of Rohde et al.[12] to assess the application of this technique for rapid identification of *Brachyspira* spp. in a veterinary diagnostic laboratory. Overall, the method was shown to be highly specific, with the identification of *B. hyodysenteriae* and *B. pilosicoli* being supported by the results of the species-specific PCRs. Confirmation of the *nox*-RFLP classifications obtained for the remaining *Brachyspira* spp. was more problematic because the only reliable method available to differentiate the selected isolates was 16S rDNA sequence analysis. Biochemical characterization could only provide limited information because not all isolates could be speciated, and the identification was mostly based on subjective phenotypic characteristics.

It is generally known that the 16S rRNA gene sequences of porcine *Brachyspira* spp. are highly conserved. PCR detection of *B. pilosicoli* is based on 16S rDNA sequence differences to other *Brachspira* spp., and, therefore, comparisons of this gene remain a useful taxonomic tool for some species. However, species identification of *B. hyodysenteriae* and the weakly β-hemolytic *Brachyspira* spp. using 16S rDNA sequence analysis is less discriminative with *B. hyodysenteriae* and *B. intermedia* forming 1 cluster and *B. innocens* and *B. murdochii* forming another.[3,4,13] The 16S rDNA dendrogram generated from this study supports these findings, with the formation of 3 major clusters (*B. hyodysenteriae*/*B. intermedia*, *B. innocens*/*B. murdochii*, and *B. pilosicoli*) and a separate branch formed by *B. alvinipulli*.

For the majority of isolates analyzed, the identifications made by the novel *nox*-RFLP method were further supported by sequence analysis of the entire *nox* amplicon. However, some anomalies were encountered in the identification of *Brachyspira* isolates after additional analysis that were not experienced by Rohde et al.[12] These anomalies were due either to the presence of novel restriction profiles or similar profiles generated by other *Brachyspira* spp. that were not considered by Rohde et al.[12] Two isolates identified as *B. pilosicoli* by species-specific PCR and 16S rDNA sequence analysis did not show the typical *nox*-RFLP banding pattern due to minor sequence variation within the *Bfm*I restriction site. Further analysis of 2 other isolates that could not be identified by *nox*-RFLP indicated that these isolates formed a distinct branch in both the *nox* and 16S rDNA dendrograms, and likely represent a new species of *Brachyspira*, most probably the proposed species *B. pulli*. If such cases are encountered in a veterinary diagnostic laboratory, further analysis of the isolates should be performed initially by species-specific PCR, and then the isolates should be sent to a reference laboratory for further identification if required. However, misidentification due to identical or similar restriction profiles between *Brachyspira* spp. would still be overlooked during this process. This is a potential complicating factor that needs to be addressed by laboratories that consider using the *nox*-RFLP method to identify *Brachyspira* spp. from hosts other than pigs.

The avian species *B. alvinipulli* was not analyzed by Rohde et al.[12] because the method was validated only for identification of porcine *Brachyspira* spp. Analysis of the *nox* sequence from *B. alvinipulli* (GenBank accession no. AF0608014) shows that this species would produce *nox*-RFLP profiles identical to that of *B. pilosicoli* using the restriction enzymes *Bfm*I and *Dpn*II. Interestingly, isolate 805 clustered with *B. alvinipulli* in both the *nox* and 16S rDNA dendrograms but produced *nox*-RFLP profiles that were indistinguishable from *B. intermedia*. Sequence analysis showed that minor restriction fragment size differences existed, once again demonstrating 1 of the disadvantages associated with species identification on a visual

assessment of the migration of DNA fragments. Although the majority of avian isolates in this small study were accurately identified as belonging to the 5 major *Brachyspira* spp., the level of unidentifiable isolates could be higher if more isolates were examined. In future studies, reevaluation of the restriction enzymes used or the addition of a third enzyme could be considered to improve identification of avian spirochetes to other than the 5 major *Brachyspira* spp.

The modifications described in this article further optimized the *nox*-RFLP technique to provide sufficient amplified product for restriction digestion as well as subsequent analysis. The original method used the PCR-amplified DNA product directly in the restriction digestion with no product available for further comparisons. With the inclusion of PCR product purification using a commercial kit, the *nox*-RFLP remains a rapid and relatively inexpensive method for identification of *Brachyspira* spp. and product is retained for further analysis, such as *nox* DNA sequencing, if required.

This study showed that the *nox*-RFLP method of Rohde et al.[12] was very useful for the identification of the major *Brachyspira* spp. in veterinary diagnostic laboratories with minimal molecular biology infrastructure. In addition, it was shown that although further modifications may be required to allow complete identification of *Brachyspira* spp. from all animal species, the benefits of this technique in providing rapid and accurate identification of the 5 major *Brachyspira* species far outweigh the disadvantages associated with currently unrecognized species. A significant improvement was clearly obtained in the speed and accuracy of diagnosis compared with conventional biochemical characterization. This technique represents a major advancement in laboratory identification of *Brachyspira* spp. of pigs and poultry and should provide a better understanding of the agents involved in AIS.

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Sources and manufacturers

a. Perkin Elmer, Boston, MA.
b. ABgene, Epsom, UK.
c. QIAGEN, Valencia, CA.
d. MBI Fermentas, Hanover, MD.
e. New England Biolabs, Beverley, MA.
f. F. Hoffmann-La Roche Ltd, Basel, Switzerland.
g. Applied Biosystems, Foster City, CA.

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