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Differentiation of Porcine *Brachyspira* Species by a Novel *nox* PCR-Based Restriction Fragment Length Polymorphism Analysis

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A novel PCR-based restriction fragment length polymorphism analysis of the *Brachyspira nox* gene was developed. The restriction patterns for *Brachyspira hyodysenteriae*, *B. pilosicoli*, *B. intermedia*, *B. murdochii*, and *B. innocens* were highly distinct with two restriction endonucleases only. The assay proved to be user-friendly and robust.

The porcine intestinal tract is frequently colonized by different *Brachyspira* species. *Brachyspira hyodysenteriae* is the causative agent of swine dysentery, and *B. pilosicoli* causes intestinal spirochetosis (9). The other species, namely, *B. intermedia*, *B. murdochii*, and *B. innocens*, are considered nonpathogenic (9). Due to differences in pathogenicity (9) and the zoonotic potential of *B. pilosicoli* (15), rapid and user-friendly differentiation between species is important with respect to the economic use of medication, preharvest food safety, and animal trade.

Currently, porcine *Brachyspira* isolates are differentiated based on phenotypic criteria, including intensity of hemolysis, indole production, hippurate hydrolysis, and activities of α-galactosidase, α-glucosidase, and β-glucosidase (6, 14). In addition, a positive ring phenomenon is indicative of *B. hyodysenteriae* (11). Due to variable results in these tests and difficulty in correctly assessing the intensity of hemolysis, it seems desirable to replace phenotypic identification schemes with genotypic methods. The currently used sequencing analyses of 16S or 23S ribosomal DNA (rDNA), the *nox* gene, or unidentified DNA segments or PCR methods based on these data (1, 4, 5, 10, 12, 13) either are not practical for routine veterinary diagnostic purposes or are confined to the identification of selected species only. Therefore, the purpose of the present study was to develop a robust and user-friendly method for the identification of all porcine *Brachyspira* species and to evaluate its performance in routine diagnostic work.

Based on an analysis of sequencing data derived from GenBank for the *nox* genes of 15 *Brachyspira* strains (Table 1), we designed a *nox* gene-specific PCR with subsequent evaluation of restriction fragment length polymorphisms (RFLPs) of the PCR products. A forward primer [Bnoxf; 5′-TAG C(CT)T GCG GTA T(CT)G C(AT)C TTT GG-3′] and a reverse primer [Bnoxr; 5′-CTT CAG ACC A(CT)C CAG TAG AAG CC-3′] specific for the *Brachyspira nox* gene were designed to include a 939-bp fragment encompassing positions 345 to 1283 of the *B. hyodysenteriae* strain B204 *nox* gene. To perform the PCR, bacterial growth was removed from a 2-day-old pure culture on Columbia blood agar (Oxoid, Wesel, Germany), and a bacterial suspension was prepared with 1 mM Tris (pH 8.0)–0.1 mM EDTA. To achieve bacterial lysis, the suspension was boiled for 8 min in a microwave. Ten microliters of lysate was added to 40 μl of a PCR premixture to yield final concentrations of 1.5 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), PCR buffer (Gibco BRL), a 0.2 mM concentration of each deoxynucleoside triphosphate (Roth, Karlsruhe, Germany), 0.5 μM Bnoxf, 0.5 μM Bnoxr, and 2.5 U of *Taq* polymerase (Gibco BRL). The mixture was subjected to 30 cycles of amplification in a Crocodile III cycler (Quantum Appligene, Illkirch, France). After initial denaturation at 94°C for 3 min, each cycle involved denaturation at 94°C for 30 s, annealing at 59°C for 40 s, and extension at 72°C for 54 s. The amplification was finished after a final extension step at 72°C for 10 min. An aliquot of 10 μl of the PCR product was visualized after separation by electrophoresis in a 1.5% agarose gel and staining with ethidium bromide.

The buffer composition of the remaining PCR product was modified to meet the conditions for restriction digestion with *Dpn*II (New England Biolabs, Frankfurt, Germany) and *Bfm*I (MBI Fermentas, St. Leon-Rot, Germany). We prepared 10× PCR restriction buffer (230 mM Tris acetate [pH 7.6], 41 mM potassium acetate, 9.25 mM magnesium acetate, 30 mM spermidine [Sigma, Taufkirchen, Germany], 1 mg of bovine serum albumin/ml). For restriction digestion, 12.5 μl of the PCR product, 2.5 μl of 10× PCR restriction buffer, and 2.5 μl of dithiothreitol (10 mM; Roth) were mixed; the volume was adjusted to 25 μl with distilled H_2O; and 3 or 1.5 U of *Dpn*II or *Bfm*I, respectively, was added. Digestion was carried out at 37°C for 2 h. The restriction fragments were separated in a 2% agarose gel and visualized after staining with ethidium bromide.

The predicted fragment lengths (Table 2) were confirmed by using the reference strains *B. hyodysenteriae* B204 (kindly provided by D. L. Harris and S. C. Whipp, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames) and *B. pilosicoli* P43/6/78, *B. intermedia* AN26:93, *B. innocens* C336, and *B. murdochii* C301 (kindly provided

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TABLE 1. GenBank accession numbers of *nox* gene sequences of *Brachyspira* spp.

Strain	GenBank accession no.
B. hyodysenteriae B204	U19610
B. hyodysenteriae R1	AF060802
B. hyodysenteriae B169	AF060801
B. hyodysenteriae B78	AF060800
B. pilosicoli WesB	AF060808
B. pilosicoli 42167	AF060809
B. pilosicoli P43/6/78	AF060807
B. pilosicoli HRM7	AF060806
B. intermedia 2818.5	AF060810
B. intermedia PWS/A	AF060811
B. intermedia 4482	AF060812
B. murdochii 155-20	AF060803
B. murdochii 56-150	AF060813
B. innocens B256	AF060804
B. innocens 4/71	AF060805

TABLE 2. Predicted fragment sizes after digestion of the *nox*-specific PCR product with *Dpn*II and *Bfm*I for the five porcine *Brachyspira* spp.

Species	Predicted restriction fragments (bp) obtained with:	
	*Dpn*II	*Bfm*I
B. hyodysenteriae	684, 209, **24**	741, 197
B. pilosicoli	896, **24**	741, 197
B. intermedia	684, 209, **24**	504, 238, 197
B. innocens	684, 209, **24**	504, 210, 197, **25**
B. murdochii	684, 157, **24**	504, 210, 197, **25**

[a] Values in bold are fragments not visualized in ethidium bromide-stained gels.

by C. Fellström, Department of Medicine and Surgery, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden) (Fig. 1). In addition, 132 field strains isolated from clinical specimens from diseased and healthy pigs were identified conventionally as described by Stanton et al. (14) and Fellström et al. (6). Hemolysis was judged based on the appearance of growth on Trypticase soy agar (BD, Heidelberg, Germany) with 10% bovine blood (WDT, Hoyerhagen, Germany) and on the ring phenomenon occurring upon incision of the inoculated agar (11). Hippurate hydrolysis and α-galactosidase, α-glucosidase, and β-glucosidase activities were determined by using Rosco diagnostic tablets (Rosco Diagnostic, Taastrup, Denmark). To determine indole production, growth was collected with a swab from a 2-day-old pure culture on Columbia blood agar, and 1 or 2 drops of DMACA reagent (BD) was added; a blue-green color appearing within 5 min was considered a positive result.

According to these criteria, 80 isolates belonged to the species *B. hyodysenteriae*, 7 were *B. pilosicoli*, 4 were *B. intermedia*, 24 were *B. innocens*, and 17 were *B. murdochii*. By *nox*-specific RFLP typing, 121 of these isolates were classified accordingly, while 11 isolates showed different results (Table 3). Five isolates showed a banding pattern that appeared to be the result of a mixture of fragments from two *Brachyspira* spp. In order to confirm this result, a bacterial suspension was serially diluted and plated to obtain the growth of single colonies. Single colonies were retested and proven to be a pure culture of one

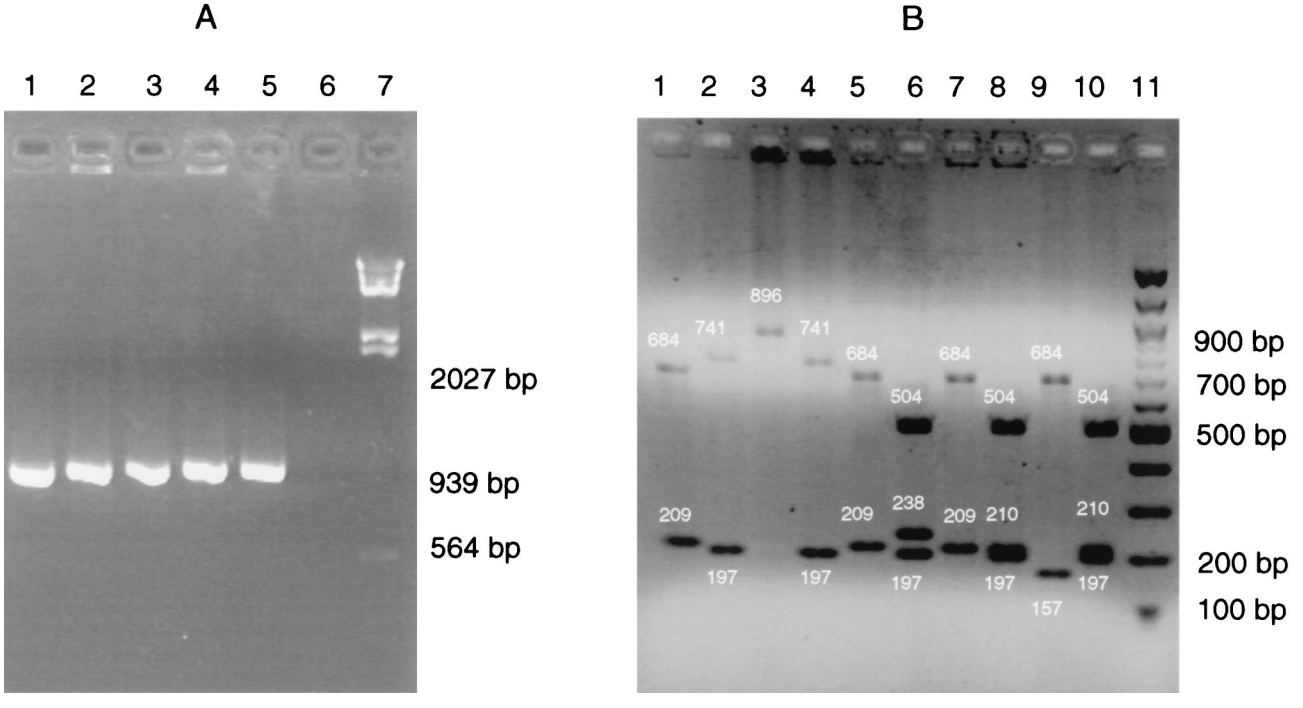

FIG. 1. *nox*-specific PCR (A) and RFLP (B) analyses of the five *Brachyspira* reference strains. (A) Lanes 1 to 5, *B. hyodysenteriae* B204, *B. pilosicoli* P43/6/78, *B. intermedia* AN26:93, *B. innocens* C336, and *B. murdochii* C301; lane 6, distilled H_2O as a template; lane 7, *Hin*dIII-digested λ DNA. (B) Lanes 1 and 2, *B. hyodysenteriae* B204; lanes 3 and 4, *B. pilosicoli* P43/6/78; lanes 5 and 6, *B. intermedia* AN26:93; lanes 7 and 8, *B. innocens* C336; lanes 9 and 10, *B. murdochii* C301. Digestion was done with *Dpn*II (odd-numbered lanes) and *Bfm*I (even-numbered lanes). Lane 11, 100-bp DNA ladder. The small white numbers are fragment sizes (in base pairs).

TABLE 3. Comparison of the results of identification by conventional testing and *nox*-specific RFLP typing

Phenotypic identification (no. of isolates)	Identification by *nox*-specific RFLP typing		
	No. of identical isolates	Different (*n*)	Mixed culture of two species (*n*)
B. hyodysenteriae (80)	77	*B. innocens* (1)	*B. hyodysenteriae* + *B. innocens* (2)
B. pilosicoli (7)	5	*B. intermedia* (1)	*B. pilosicoli* + *B. murdochii* (1)
B. intermedia (4)	3		*B. intermedia* + *B. murdochii* (1)
B. innocens (24)	20	*B. intermedia* (1) *B. murdochii* (1); no identification (1)	*B. innocens* + *B. intermedia* (1)
B. murdochii (17)	16	*B. intermedia* (1)	

of the formerly identified species. For another five isolates, the two methods yielded different identifications (Table 3). One indole-negative, apparently strongly hemolytic *B. hyodysenteriae* isolate with a weakly positive α-galactosidase reaction was identified as *B. innocens* by the *nox*-specific RFLP method. One hippurate-positive, weakly indole-positive, and α-galactosidase-negative *B. pilosicoli* isolate showed the banding pattern of *B. intermedia*. Two indole-negative, α-galactosidase-positive *B. innocens* isolates were identified as *B. intermedia* and *B. murdochii*. Finally, one weakly hemolytic isolate with only β-glucosidase activity (*B. murdochii*) showed the restriction fragment pattern of *B. intermedia*. Since there are known variations and even discrepancies in species descriptions based on biochemical reactions (6, 7, 14), the genotypic identification is to be considered the correct one. This conclusion is supported by the finding that only a single isolate biochemically classified as *B. innocens* could not be assigned to one of the five relevant species with the proposed *nox*-specific RFLP method.

Comparable attempts to separate *B. hyodysenteriae*, *B. pilosicoli*, *B. intermedia*, *B. murdochii*, and *B. innocens* by DNA-based methods had been made previously, also with *nox*-specific RFLP or 23S rDNA-based RFLP analysis. Both methods established by Barcellos et al. (2, 3) did not allow the differentiation of all weakly hemolytic spirochetes due to overlapping banding patterns, and the *nox*-based method also showed strain variations of RFLP patterns for *B. intermedia*, *B. pilosicoli*, and *B. innocens*. A *nox*-based PCR recently developed by Ateyo et al. (1) required four different primers and a complex PCR protocol not easily established in a routine diagnostic laboratory. In addition, it also did not allow the differentiation of all five relevant *Brachyspira* species. Another method, based on Southern blotting analysis of *Eco*RV chromosomal DNA digests with a PCR-amplified and digoxigenin-labeled fragment of the *flaA1* gene, was described by Fisher et al. (8). It corresponded well to the known classification of all five relevant *Brachyspira* species but is not a practical method for a routine diagnostic laboratory. The 23S rDNA-based PCR developed by Leser et al. (10) needed different sets of primers and different annealing temperatures and could not identify all biochemical groups.

To our knowledge, the *nox*-specific RFLP method described here is the first PCR-based method developed that facilitates the complete discrimination of all five porcine *Brachyspira* spp. of veterinary interest without the help of sequencing or hybridization technology. It is user-friendly and robust enough to be performed in clinical veterinary laboratories with basic equipment for molecular biology work.

A. Rothkamp is a fellow of Niedersächsische Tierseuchenkasse.

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