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Genomic Fingerprinting of *Haemophilus somnus* by a Combination of PCR Methods

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Twenty-three isolates of *Haemophilus somnus* were typed by repetitive extragenic palindromic (REP) element-based PCR, enterobacterial repetitive intergenic consensus (ERIC)-based PCR, and PCR ribotyping. A total of 11 types were distinguished by REP-PCR, 13 types were distinguished by ERIC-PCR, and 5 types were distinguished by PCR ribotyping. PCR ribotyping produced a relatively simple pattern and a small number of distinct types, whereas REP- and ERIC-PCR both produced more complex banding patterns but increased the discrimination between strains. Clearly distinguishable profiles were obtained for respiratory and genital isolates of *H. somnus* by all three typing methods. The results suggest that a combination of all three primer sets provides a high-resolution fingerprinting method for epidemiological studies of *H. somnus* and for its differentiation from related species.

Haemophilus somnus is the causative agent of infectious thromboembolic meningoencephalitis and other disease syndromes in cattle, including pneumonia, septicemia, reproductive failure, abortion, and arthritis, and economic losses due to *H. somnus* infection are high. Asymptomatic reproductive and respiratory carrier states also occur (5). *H. somnus*-like organisms have been isolated from sheep with a range of disease syndromes similar to that in cattle, but such isolates are generally referred to as *Histophilus ovis* or *Haemophilus agni* (5). Traditional phenotypic methods have been used for identification and typing of *H. somnus* isolates (1, 2, 12), but these methods generally lack the discriminatory power of recently developed molecular fingerprinting methods. For example, the randomly amplified polymorphic DNA PCR assay revealed genetic polymorphisms within *H. somnus* isolates (8). However, the reproducibility of randomly amplified polymorphic DNA has been questioned, and for this reason more specific target sequences were used in the present study. Repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences are dispersed throughout the prokaryotic genome, and PCR studies of eubacterial species revealed that inter-REP or inter-ERIC distances and patterns are specific for bacterial species and strains within species (11). PCR ribotyping is another method that has been used to explore the bacterial genome. The genes within the rRNA loci are separated by spacer regions which exhibit a large degree of sequence and length variation at the level of genus and species (6).

Bacterial isolates. Twenty-two *H. somnus* isolates and two *Histophilus ovis* isolates were obtained from different sources within Scotland (Table 1). *H. somnus* type strain ATCC 43626 was obtained from the American Type Culture Collection. Isolates were stored at −80°C in brain heart infusion broth (Oxoid) supplemented with 10% glycerol, 1% Tris (BDH), 1% soluble starch (BDH), 0.5% sodium L-aspartate (Sigma), and 0.001% thiamine monophosphate (Sigma) pH 7.8 (10). All isolates were propagated on brain heart infusion agar (Oxoid) containing 5% sheep blood and 0.5% yeast extract (Oxoid) and incubated at 37°C for 48 h in a candle jar. Their identities were confirmed by conventional biochemical reactions (2, 10) and by the API ZYM system (BioMerieux, Marcy l'Etoile, France) (3).

PCR amplification. Bacteria were suspended in 1-ml volumes of sterile distilled water to a turbidity equivalent to a McFarland no. 5 standard (BioMerieux), heated to 100°C for 20 min, and centrifuged at 15,000 × *g* for 10 min; the supernatants were used as sources of template DNA for PCR. The primers for PCR were obtained from Genosys (Cambridge, United Kingdom). The primers for REP-PCR were REP-IRDT (IIINCGNCGNCATCNGGC) and REP2-DT (NCG NCTTATCNGGCCTAC) (11), those for ERIC-PCR were ERIC-IR (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAGTGACTGGGGTGAGCG) (11), and those used for PCR ribotyping were GIRRN (GAAGTCGTAACAAGG) and LIRRN (CAAGGCATCCACCGT) (6). The reaction mixture (25 μl) contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (Boehringer Mannheim, Lewes, United Kingdom), 100 pM each primer, 0.625 U of *Taq* DNA polymerase (Life Technologies Ltd., Paisley, United Kingdom), and 2.5 μl of template DNA preparation. Twenty-five microliters of liquid paraffin was used to overlay each reaction mixture. Amplification was done in a thermocycler (Techne Ltd., Cambridge, United Kingdom) by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 6 min, with a final extension at 72°C for 6 min. These reaction conditions were found to give the best resolution and intensity of bands with REP-PCR. They were also suitable for ERIC-PCR and PCR ribotyping and thus enabled simultaneous amplification with all three primer sets. The amplified products were electrophoresed in 2.0% agarose type II-A (Sigma) in Tris-borate-EDTA buffer containing ethidium bromide (0.5 μg/ml), and the amplimers were visualized and photographed under UV light. Whenever a distinct PCR profile, in terms of the number and position of the clearly visible bands, was observed, the corresponding strain was given a unique number or letter designation.

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TABLE 1. *H. somnus* and *H. ovis* strains and their types

Designation of isolate[a]	Species	Host animal	Site, disease status	REP type	ERIC type	Ribotype
T	*H. somnus*	NA[b]	NA	1	A	a
SA06	*H. somnus*	Bovine	Lung	1	A	a
SA05	*H. somnus*	Bovine	Lung, pneumonic	1	A	a
SA14	*H. somnus*	Bovine	Lung, pneumonic	1	A	a
SA15	*H. somnus*	Bovine	Lung, pneumonic	1	A	a
SA19	*H. somnus*	Bovine	Lung, pneumonic	1	A	a
SA20	*H. somnus*	Bovine	Lung, pneumonic	1	A	a
SA17	*H. somnus*	Bovine	Lung	1	L	a
SA07	*H. somnus*	Bovine	Lung, pneumonic	1	D	d
SA04	*H. somnus*	Bovine	Lung, pneumonic	11	G	b
SA13	*H. somnus*	Bovine	Lung, pneumonic	6	E	c
SA01	*H. somnus*	NA	NA	2	B	b
SA02	*H. somnus*	NA	NA	2	B	b
SA03	*H. somnus*	NA	NA	2	B	b
SA21	*H. somnus*	Bovine	Prepuce	3	C	b
SA22	*H. somnus*	Bovine	Prepuce	3	C	b
SA23	*H. somnus*	Bovine	Prepuce	3	C	b
SA12	*H. somnus*	Bovine	Semen	5	M	c
SA11	*H. somnus*	Bovine	Semen	4	O	d
X1	*H. somnus*	Bovine	Cervix	7	N	f
V8	*H. somnus*	Bovine	Cervix	12	J	c
X4	*H. somnus*	Bovine	Vagina	13	K	d
V3	*H. somnus*	Bovine	Vestibular opening	8	H	c
SA08	*Histophilus ovis*	Ovine	Semen	9	I	e
SA16	*Histophilus ovis*	Ovine	Semen	10	F	e

[a] Sources of the isolates: T, American Type Culture Collection (ATCC 43626); SA01, SA02, SA03, SA21, SA22, and SA23, D. J. Taylor, Glasgow University Veterinary School; SA04 and SA05, Scottish Agricultural College (SAC) Veterinary Investigation Centre, Dumfries; SA06, SA08, SA15, and SA16, SAC Veterinary Investigation Centre, Ayr; SA07, SA13, SA14, SA17, SA19, and SA20, SAC Veterinary Investigation Centre, Aberdeen; SA11 and SA12, SAC Veterinary Investigation Centre, St. Boswells; X1, X4, V3, and V8, isolated by us from slaughterhouse materials.

[b] NA, not available.

Typing of strains. With the REP-PCR method, profiles of *H. somnus* and *Histophilus ovis* revealed amplified bands ranging from 1.4 to <0.28 kb with various intensities (Fig. 1). The 25 isolates showed 13 distinct fingerprints, each of which was assigned a number (Table 1). Group one, which included the type strain of *H. somnus* and eight other isolates with identical patterns, was the largest group. Groups two and three each comprised three strains with similar patterns. The remainder of the *H. somnus* isolates and the two *Histophilus ovis* isolates each produced unique patterns. ERIC-PCR produced 15 distinguishable patterns for the 25 isolates (Table 1). The fragment sizes ranged from 2.2 to <0.1 kb with various band intensities (Fig. 2). This method produced a higher degree of discrimination between isolates, but the complex banding patterns were more difficult to interpret. Again the type strain fell into the largest group (group A), which also contained six other isolates. Groups B and C each contained three isolates, and the remainder of the isolates had unique patterns. PCR ribotyping of the isolates gave fingerprints with bands ranging from 1.0 to <0.4 kb (Fig. 3). This method produced the simplest patterns, which were easy to interpret. Six groups were recognized for the 25 isolates (Table 1). Group a included the type strain and seven other isolates, group b included seven isolates, and group c included four isolates. Groups d and e comprised three and two isolates, respectively, and the remaining isolate showed a unique banding pattern.

For each typing method, many of the *H. somnus* isolates produced the same pattern as the type strain, and all of these were lung isolates (Table 1). However, not all of the lung isolates fell into the same group; indeed, isolates SA04, SA07, and SA13 produced different fingerprints with each typing method. Genital and respiratory isolates were clearly separated by all three PCR typing methods, which suggests that distinct strains inhabit these different sites. Strains SA21, SA22, and SA23 were genital isolates from different animals within the same herd which had a history of subnormal fertility. They were indistinguishable by all three methods, suggesting dissemination of a single strain within the herd. Genital isolates were also examined from slaughterhouse samples taken from individual animals which were not from the same herd. Isolates from different sites (e.g., cervix and vestibular opening) in any one animal gave the same profile with each primer set, but the profiles of isolates from different animals differed (data not shown).

Species differentiation. With other, related bacteria from the family *Pasteurellaceae*, *Actinobacillus seminis*, *Actinobacillus pleuropneumoniae*, *Pasteurella haemolytica*, *Pasteurella trehalosi*, and *Pasteurella multocida*, REP-PCR, ERIC-PCR, and PCR ribotyping produced patterns that were completely different from those of *H. somnus* and *Histophilus ovis*. This is illustrated for REP-PCR in Fig. 4. The sheep isolates (*Histophilus ovis*) SA08 and SA16 were distinguishable from *H. somnus* by all three methods (Fig. 1 to 3).

The presumptive identification of *H. somnus* is generally dependent on culture and biochemical methods, but these techniques give varying results (5). Such phenotypic methods have also been used for typing of *H. somnus*. Fussing and Wegener (1) identified 21 different biotypes among 105 *H.*

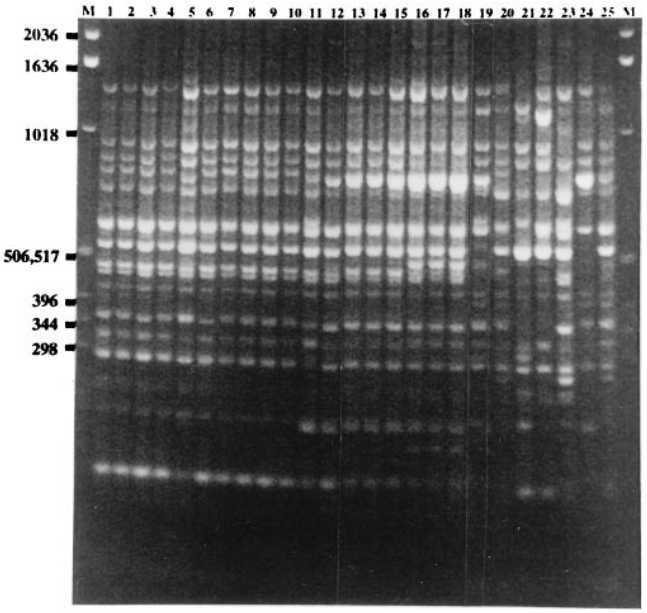

FIG. 1. Fingerprints obtained by REP-PCR. Lanes M, 1-kb DNA ladder (Gibco BRL, Paisley, United Kingdom); lanes 1 to 20 and 23 to 25, *H. somnus* type strain and isolates SA05, SA06, SA07, SA12, SA14, SA15, SA17, SA19, SA20, V3, X1, SA21, SA22, SA23, SA01, SA02, SA03, SA13, SA11, SA04, V8, and X4, respectively; lanes 21 and 22, *Histophilus ovis* SA08 and SA16, respectively. The profiles have been arranged so that, in general, isolates of a similar type are grouped together. Lanes 1 to 4 and 6 to 10, type 1; lane 5, type 5; lane 11, type 8; lane 12, type 7; lanes 13 to 15, type 3; lanes 16 to 18, type 2; lane 19, type 6; lane 20, type 4; lane 21, type 9; lane 22, type 10; lane 23, type 11; lane 24, type 12; lane 25, type 13 (see Table 1).

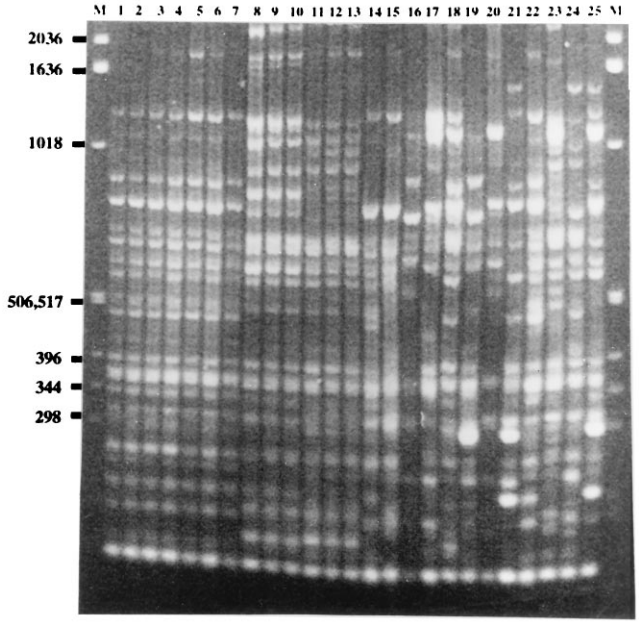

FIG. 2. Fingerprints obtained by ERIC-PCR. Lanes M, 1-kb DNA ladder; lanes 1 to 15, 17, 18, and 20 to 25, *H. somnus* type strain and isolates SA05, SA06, SA14, SA15, SA19, SA20, SA01, SA02, SA03, SA21, SA22, SA23, SA07, SA13, SA04, V3, V8, X4, SA17, SA12, X1, and SA11, respectively; lanes 16 and 19, *Histophilus ovis* SA16 and SA08, respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1 to 7, type A; lanes 8 to 10, type B; lanes 11 to 13, type C; lane 14, type D; lane 15, type E; lane 16, type F; lane 17, type G; lane 18, type H; lane 19, type I; lane 20, type J; lane 21, type K; lane 22, type L; lane 23, type M; lane 24, type N; lane 25, type O (see Table 1).

somnus isolates, and Ward et al. (12) identified 6 types among 18 isolates. The API ZYM system has also been used for identification of *H. somnus* and related organisms (3), but it is not a reliable method for differentiation of *H. somnus* from *Histophilus ovis*, *H. agni*, or *A. seminis*. At least 15 different serotypes of *H. somnus* have been reported by researchers using immunodiffusion tests, enzyme-linked immunosorbent assays, and agglutination tests (9), but these methods depend on the availability of cross-absorbed antisera. The limitations of traditional phenotypic methods for typing of bacterial isolates have stimulated the development of genotyping methods (7), and these techniques have recently been applied to *H. somnus*. Fussing and Wegener (1) fingerprinted 100 isolates of *H. somnus* by restriction enzyme assay and distinguished 14 different DNA types from 80 pneumonic isolates (of which 64 isolates were grouped into the same type) and 17 different groups from 20 genital isolates. PCR as a means of fingerprinting *H. somnus* isolates has previously been applied only with random primers; as in the present study, *H. somnus* was differentiated from *Histophilus ovis* (8). In our study of 23 isolates of *H. somnus*, REP-PCR fingerprinting produced 11 distinct profiles, ERIC-PCR produced 13 types, and PCR ribotyping produced five types, and they were distinct from those of two *Histophilus ovis* strains. The results indicate that *H. somnus* and *Histophilus ovis* could be identified and typed by any one of these PCR methods, but if the data from each PCR method were combined, *H. somnus* could be further subtyped. The variation in patterns of amplimers generated by PCR ribotyping indicates some diversity in number and structure of ribosomal operons in *H. somnus*. Such polymorphisms have been reported for other bacterial species (4).

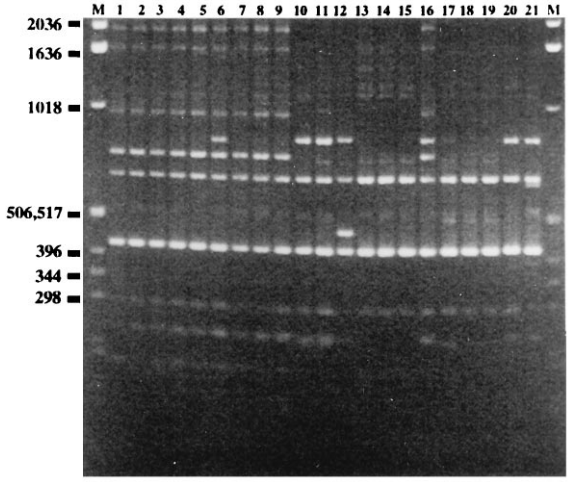

FIG. 3. Fingerprints obtained by PCR ribotyping. Lanes M, 1-kb DNA ladder; lanes 1 to 20, *H. somnus* type strain and isolates SA05, SA06, SA14, SA15, SA07, SA17, SA19, SA20, SA12, V3, X1, SA01, SA02, SA03, SA11, SA21, SA22, SA23, and SA13, respectively; lane 21, *Histophilus ovis* SA08. Isolates SA04, SA16, V8, and X4 are not included in this figure. The profiles have been arranged so that, in general, isolates of a similar type are grouped together. Lanes 1 to 5 and 7 to 9, type a; lanes 6 and 16, type d; lanes 10 and 11, type c; lane 12, type f; lanes 13 to 15 and 17 to 19, type b; lane 21, type e (see Table 1).

PCR-based fingerprinting is simple and rapid and can be performed with very small quantities of bacterial cultures. Reproducibility was excellent with all three methods, although some day-to-day variation in the intensity of amplified fragments, particularly the minor bands, was observed. Boiled cell extracts as template DNA gave the same results as extracted chromosomal DNA for all three PCR methods, as reported

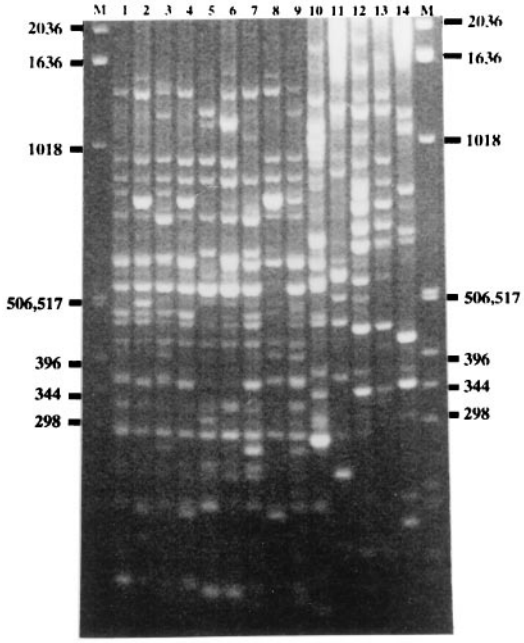

FIG. 4. Comparison of REP-PCR fingerprints of *H. somnus* with those of some other members of the family *Pasteurellaceae*. Lanes M, 1-kb DNA ladder; lanes 1 to 9, representatives of the different REP types of *H. somnus*; lanes 10 to 14: *A. seminis*, *A. pleuropneumoniae*, *P. haemolytica*, *P. trehalosi*, and *P. multocida*, respectively.

previously (7). Rapid and reproducible fingerprint production by REP-PCR, ERIC-PCR, and PCR ribotyping was achieved without any knowledge of the genomes of *H. somnus* and *Histophilus ovis*. The banding profiles were relatively complex for REP- and ERIC-PCR, and the significance of less-intense band differences needs to be established, but PCR ribotyping produced a simpler pattern and a smaller number of distinct types. A combination of all three primer sets provided a powerful discriminatory technique and may be useful as a reproducible typing system for *H. somnus.*

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