MOLECULAR DIAGNOSIS AND TYPING

PCR methods and plasmid profile analysis for characterisation of *Histophilus ovis* strains

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The value of polymerase chain reaction (PCR)-based DNA fingerprinting and plasmid profile analysis for differentiation of *Histophilus ovis* isolates was assessed. Nineteen isolates of *H. ovis* were typed by PCR-ribotyping, repetitive extragenic palindromic element (REP)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR. These methods distinguished five types by PCR-ribotyping, 11 types by REP-PCR and seven types by ERIC-PCR. The ribotyping method produced a relatively simple pattern and a small number of distinct types and was useful for differentiation of *H. ovis* from the phenotypically similar organism, *Haemophilus somnus*. REP- and ERIC-PCR both produced complex banding patterns, but increased the discrimination between strains. Plasmids were found in 12 of the 19 isolates and there were four different plasmid profiles. A combination of the PCR methods and plasmid profile analysis provided a high resolution typing method for *H. ovis*.

Introduction

The name *Histophilus ovis* has been applied to gram-negative pleomorphic coccobacilli isolated from sheep with a variety of conditions including mastitis, septicaemia, pyaemia, polyarthritis, metritis, abortion and most frequently epididymitis [1–3]. Its presumptive identification is generally dependent on cultural and biochemical methods [4], but these give variable results [5]. The taxonomic status of *H. ovis* is unclear, but it resembles *Haemophilus somnus*, an organism that causes similar diseases in cattle, in its cultural, biochemical and antigenic properties. Stephens *et al.* [6] suggested that these bacteria and an organism referred to as *Haem. agni* should be considered as a single *Haemophilus-Histophilus* group and they pointed out the problem of differentiation of strains within the group. Confusingly, there has been a trend to identify both bovine and ovine isolates of these organisms in North America as *Haem. somnus* [7] and in Australia as *H. ovis* [6, 8]. DNA–DNA hybridisation studies have shown that *Haem. somnus* and *H. ovis* are genetically homogeneous [9], but others have suggested, on the basis of restriction endonuclease analysis, biotyping and outer-membrane

protein profiles [7, 10], that bovine and ovine isolates should be considered as separate groups.

Polymerase chain reaction (PCR)-based fingerprinting methods have been applied recently to several bacterial genera as an aid to strain differentiation [11]. Some of these methods use primers for specific target sequences such as repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences which are dispersed throughout the genome in many bacterial species [12, 13]. Versalovic *et al.* [14] defined primers targeting these REP and ERIC sequences for the study of eubacterial species and revealed that inter-REP or inter-ERIC distances and patterns are specific for bacterial species and for strains within a species. PCR-ribotyping is another method that has been used to explore the bacterial genome and the rRNA genetic loci are frequently present in multiple copies. The genes within the rRNA loci are separated by spacer regions that exhibit a large degree of sequence and length variation at the level of genus and species. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacers [15].

In previous studies, a combination of PCR-ribotyping, REP-PCR and ERIC-PCR techniques was used for identification and fingerprinting of *Haem. somnus* isolates of bovine origin [16] and *Actinobacillus*

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seminis isolates of ovine and bovine origin [17]. These methods were also found to be applicable to the characterisation of *H. ovis* strains. Subgroups of *H. ovis* isolates of ovine origin have been identified by the presence of plasmids of different sizes [5], and so the utility of plasmid profiles in the differentiation of *H. ovis* strains was also examined.

Materials and methods

Bacterial isolates

For the purposes of this study, isolates from cattle and sheep, showing similar cultural and biochemical properties to those described by Humphrey and Stephens [1], are referred to as *Haem. somnus* and *H. ovis* respectively. Nineteen strains of *H. ovis* isolated by the Scottish Agricultural College Veterinary Science Division were available (Table 1). The first UK isolate – of Low and Graham [2], designated as SA24 – was used as a reference strain, as there is no type strain available in any of the major culture collections. Isolates were stored at -80°C in Brain Heart Infusion (BHI) Broth (Oxoid) supplemented with glycerol 10% v/v, Tris (BDH) 1% w/v, soluble starch (BDH) 1% w/v, sodium-L-aspartate (Sigma) 0.5% w/v and thiamine monophosphate (Sigma) 0.001% w/v, pH 7.8 [16]. They were propagated on BHI Agar (Oxoid) containing sheep blood 5% v/v and yeast extract (Oxoid) 0.5% w/v (BHIBYE) and plates were incubated at 37°C for 48 h in a candle jar. Their identity as *H. ovis* was confirmed by a panel of cultural and biochemical tests [6] and by the API ZYM system (bioMerieux, Marcy l'Etoile, France) [18].

PCR

The supernates from standardised bacterial suspensions which had been boiled then centrifuged served as the source of template DNA for PCR, as described previously [16]. Primers, optimised PCR conditions and electrophoresis of amplified products were also as described previously [16, 17]. The amplimers were photographed under UV illumination. Photographs were inspected visually and different band profiles were given a number or letter whenever a distinct pattern was observed. The reproducibility of the band patterns was assessed not only by repeat PCR experiments with the same template sample, but also with template samples derived from different cultures. Good reproducibility of PCR amplification products was obtained, although some minor, day-to-day variation in intensity was observed with the less intense amplimers.

Plasmid DNA extraction

Plasmid preparations were done with a QIAprep Spin Plasmid Kit (QIAGEN, West Sussex) according to the manufacturer's instructions. Plasmid DNA (10–15 μl) was analysed by electrophoresis in agarose type II-A (Sigma) 0.8% w/v in Tris-borate-EDTA buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) containing ethidium bromide 0.5 $\mu\text{g}/\text{ml}$.

Results and discussion

PCR-ribotyping of the *H. ovis* isolates produced simple banding patterns as shown in Fig. 1 and five distinct fingerprints were recognised for the 19 isolates, as

Fig. 1. Fingerprints obtained by PCR-ribotyping for *H. ovis* isolates. Lanes M, 1-kb DNA ladder. Lanes 1–19, *H. ovis* isolates, SA8, SA26, SA16, SA73, SA45, SA46, SA56, SA57, SA58, SA55, SA26, SA27, SA28, SA44, SA54, SA69, SA72, SA53 and SA24 respectively. The profiles have been arranged so that isolates of a similar type are grouped together. Lanes 1–6 (type a), lanes 7–9 (type c), lanes 10–15 (type b), lanes 16–18 (type d) and lane 19 (type e) (see Table 1).

noted in Table 1. The bands ranged from 215 to >1018 bp with three markers, of 400, 700 and 900 bp, common to all *H. ovis* isolates. The variation in patterns of amplimers generated by PCR-ribotyping indicated some diversity in number and structure of the ribosomal operons in *H. ovis* and such polymorphisms have been reported for other bacterial species [19].

The PCR-ribotyping patterns for the *H. ovis* isolates were similar to those of *Haem. somnus* as shown in Fig. 2 and as reported previously [16]. Nevertheless, the majority of the *H. ovis* isolates could be readily differentiated from *Haem. somnus* isolates by the presence of a dual band in the *H. ovis* isolates at c. 700 bp (arrow, Fig. 2). However, three *H. ovis* isolates (SA53, SA69 and SA72, lanes 16–18 in Fig. 1) from

Table 1. Distribution of *H. ovis* isolates among PCR and plasmid types

Isolate no.	Ovine source	Host status	Geographic origin	REP type	ERIC type	Ribotype	Plasmid type
SA29	Semen	Fertile	No record	1	A	a	β
SA45	Semen	No record	Southern Scotland	1	A	a	β
SA46	Semen	Fertile	Central Scotland	1	A	a	β
SA73	Semen	No record	Northern England	1	A	a	δ
SA16	Semen	No record	No record	2	B	a	α
SA27	Semen	Subfertile	Northern England	2	B	b	α
SA44	Semen	Fertile	No record	2	B	b	α
SA56*	Vagina	Subfertile	Central Scotland	3	C	c	ϵ
SA57*	Vagina	Subfertile	Central Scotland	3	C	c	ϵ
SA58*	Vagina	Subfertile	Central Scotland	3	C	c	ϵ
SA26	Semen	Fertile	Central Scotland	4	B	b	α
SA55	Semen	Infertile	Northern Scotland	4	B	b	α
SA28	Semen	Subfertile	Northern England	5	B	b	α
SA54	Semen	Subfertile	Central Scotland	6	B	b	α
SA69	Semen	Fertile	Southern Scotland	7	D	d	ϵ
SA72	Prepuce	Fertile	Central Scotland	8	D	d	γ
SA53	Semen	Infertile	Central Scotland	9	E	d	ϵ
SA24†	Semen	Infertile	Southern Scotland	10	F	e	ϵ
SA08	Semen	No record	No record	11	G	a	ϵ

*Isolated from different animals of the same flock that showed subnormal fertility.

†SA24: reference isolate [3].

Fig. 2. Differentiation of *Haem. somnus* and *H. ovis* by PCR-ribotyping. Lanes M, 1-kb DNA ladder. Lanes 1–6, different representative ribotypes of *Haem. somnus* isolates; 7–15, *H. ovis* isolates SA08, SA16, SA24, SA46, SA45, SA26, SA27, SA29 and SA44 respectively. The arrow indicates the main band difference between *Haem. somnus* and these *H. ovis* isolates.

different ovine sources did not show this dual band, but had ribotyping patterns very similar to those obtained with several strains of *Haem. somnus* of bovine origin (Fig. 3, lanes 1–6). Thus the host-specific relationship suggested for these organisms may not be absolute. The bovine and ovine strains showed similar but distinct banding patterns by REP- and ERIC-PCR (Fig. 3). Two of the ovine isolates (SA69 and SA72) showed similar profiles by ERIC-PCR (Fig. 3, lanes 17 and 18), but they had unique profiles by REP-PCR (Fig. 3, lanes 11 and 12).

REP-PCR produced 11 distinguishable patterns for the 19 *H. ovis* isolates and, therefore, the highest degree of discrimination between isolates (Table 1). The banding patterns were more complex than those seen with the ERIC-PCR and PCR-ribotyping methods, with bands ranging in size from 250 to 2500 bp and of various intensities. There were REP markers of 350, 400, 475, 520, 750, 950 and 1000 bp common to all *H. ovis* isolates. With ERIC-PCR, seven distinct patterns were seen, as indicated in Table 1. The amplified bands ranged from 75 to 950 bp with bands common to all isolates at 154 and 750 bp. Examples of REP-PCR and ERIC-PCR fingerprints for *H. ovis* and *Haem. somnus* isolates are shown in Fig. 3.

Twelve (63%) of the 19 *H. ovis* isolates showed the presence of plasmids. Seven isolates contained two plasmids, of 3.7 and 5.0 kb (Fig. 4, lanes 1–4, 6, 9, 10), and this profile was designated as type α (Table 1). Three isolates contained one plasmid of 2.9 kb (lanes 5, 7, 8, profile type β). One isolate contained two plasmids, of 2.3 and 2.9 kb (lane 11, type γ) and one contained plasmids of 2.5 and 3.0 kb (lane 12, type δ). The profile of those strains without plasmids was designated as type ϵ . There was some evidence for the presence of larger plasmids in certain strains, e.g., strains SA16 and SA72 (Fig. 4, lanes 1 and 11) but the procedure used for plasmid isolation was not ideal for larger plasmids and other procedures were not investigated. In general, plasmids of these *H. ovis* isolates were of low copy number when compared with plasmid pUC19 from *Escherichia coli* (Fig. 3, lane E). There were no obvious differences between these isolates in their antibiotic resistance profiles (tested by the disk diffusion method against a range of antibiotics commonly used for treatment of *H. ovis* infections) that could be related to the presence of a particular plasmid (data not shown). In another study, all *H. ovis* isolates tested, but none of the *Haem. somnus* isolates, contained plasmids [5] and the method was used to differentiate *H. ovis* from *Haem.*

Fig. 3. Comparison of the profiles of three ovine isolates (*H. ovis*, lanes 4–6, 10–12, 16–18) with those of three bovine isolates (*Haem. somnus*, lanes 1–3, 7–9, 13–15) by the three PCR fingerprinting methods. Lanes M, 1-kb DNA ladder. Lanes 1–6, PCR-ribotyping; 7–12, REP-PCR; 13–18, ERIC-PCR. *Haem. somnus*: strains SA12 (lanes 1, 7, 13); V3 (2, 8, 14); X4 (3, 9, 15). *H. ovis*: strains SA53 (lanes 4, 10, 16); SA69 (5, 11, 17); SA72 (6, 12, 18).

Fig. 4. Plasmid profiles of *H. ovis* isolates. Lanes M, supercoiled DNA ladder (GibcoBRL, Paisley); E, *E. coli* pUC19. Lanes 1–12, SA16, SA26, SA27, SA28, SA29, SA44, SA45, SA46, SA54, SA55, SA72 and SA73 respectively.

somnus. In contrast, Fussing and Wegener [20] found that 20% of Danish *Haem. somnus* isolates contained plasmids. In a parallel study to that reported here, four (13%) of 29 *Haem. somnus* contained single plasmids and their size ranged from 1.5 to 3.5 kb [21]. None of these plasmids was similar to those of the *H. ovis* isolates and the three *H. ovis* isolates that were similar to *Haem. somnus* by PCR-ribotyping did not have plasmids.

The first reported isolate of *H. ovis* in the UK, used as a reference strain for *H. ovis* in this study (SA24), appeared to be unique (Table 1) and, therefore, was perhaps not the best choice as reference. Three isolates (SA56, SA57 and SA58) from different animals of the same flock with subnormal fertility were indistinguishable by all four typing methods, suggesting the dissemination of a single virulent strain within the flock. Other isolates from different geographic locations also had identical fingerprints (e.g., SA45 and SA46, SA26 and SA55). These relationships among *H. ovis* isolates revealed by the typing methods indicated that there may be only a limited number of distinct strains in sheep flocks in Scotland and Northern England, although investigation of a larger number of isolates would be necessary to confirm this suggestion. Also, if isolates from different disease syndromes had been examined, as for *Haem. somnus* [16], a greater diversity of types might have been demonstrated.

Previously, PCR as a means of fingerprinting of *H. ovis* isolates had been applied only with random primers [22]. A single isolate of *H. ovis* was compared with a number of isolates of *Haem. somnus* and was clearly distinct. In the present study, PCR-ribotyping clearly differentiated all except three of the

H. ovis isolates from *Haem. somnus*. The relationship between these three sheep strains (*H. ovis*) and *Haem. somnus* requires further investigation, perhaps by 16S rRNA sequences analysis. REP-PCR produced the highest discrimination between *H. ovis* strains, whereas with *Haem. somnus* the highest discrimination was obtained with ERIC-PCR [16].

In conclusion, a combination of all three PCR primer sets provided a potentially useful typing system for *H. ovis*. Plasmid profile analysis added a further level of discrimination and was a useful supplement to the PCR typing methods.

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