Comparative analyses of *Pasteurella multocida* strains associated with the ovine respiratory and vaginal tracts

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Thirty-five isolates of *Pasteurella multocida* from the vagina and respiratory tract of sheep were compared by analysing their capsular polysaccharide types and outer membrane protein profiles. The phylogenetic relationships of selected isolates with respect to reference strains of *P multocida* were also determined by comparative 16S rRNA sequence analysis. Three capsular types, A, D and F, and three major outer membrane protein types were identified, and there were four different combinations of these characteristics which probably marked four individual clones of *P multocida*. Strains representing three of these clones were recovered from cases of ovine pneumonia, whereas isolates of the fourth clone were associated exclusively with the vagina of healthy ewes and the liver of a dead septicaemic lamb on the same farm. Analysis of the 16S rRNA sequences showed that there was 100 per cent identity between representative pneumonic isolates and reference strains of *P multocida* subspecies *galliseptica* and *P multocida* subspecies *multocida*. The 16S rRNA genes of representative vaginal and liver isolates from the same farm were identical but differed from the other strains at one nucleotide position, providing strong evidence that the vaginal and liver isolates represent a distinct subpopulation of *P multocida*.

Pasteurella multocida represents a diverse group of Gram-negative bacteria which are responsible for a wide range of economically important infections of domestic animals, including fowl cholera in chickens and turkeys (Rhoades and Rimler 1989), atrophic rhinitis in pigs (Chanter and Rutter 1989), pneumonia in cattle and pigs (Chanter and Rutter 1989, Frank 1989) and haemorrhagic septicaemia in cattle and water buffaloes in certain enzootic areas of Asia and Africa (Carter and de Alwis 1989). The bacterium also causes infections in deer (Rimler and others 1987, Aalbaek and others 1999) and rabbits (Rimler and Brogden 1986, Lu and others 1988), and is associated with human infections resulting from cat and dog bites (Holm and Tarnvik 2000, Westling and others 2000).

In temperate climates *P multocida* is not a common pathogen of sheep (Gilmour and Gilmour 1989), although it is isolated from sporadic cases of pneumonia at regional laboratories of the Veterinary Laboratories Agency (VLA). The bacterium has also been described in association with pneumonic pasteurellosis of sheep in Brazil (Hancock and others 1991), India (Umesh and others 1994), Malaysia (Chandrasekharan and others 1991), South Africa (Cameron and others 1978) and Turkey (Diker and others 1994). In addition, *P multocida* has been transmitted naturally by close contact from normal healthy sheep (*Ovis aries*) to desert bighorn sheep (*Ovis canadensis*) to cause a fatal pneumonia (Foreyt and Jessup 1982, Callan and others 1991). Recently, Watson and Davies (2002) described the isolation of *P multocida* in association with an unusual outbreak of septicaemia in neonatal lambs in a small pedigree flock of Suffolk ewes; the infection resulted in the sudden death of 11 of 26 lambs. A follow-up examination resulted in the isolation of *P multocida* from the vaginas of seven ewes in the affected flock; *P multocida* was also isolated from two of 30 vaginal swabs taken at three unconnected farms during the same period (Watson and Davies 2002).

This paper describes an investigation of strains of *P multocida* recovered from cases of ovine pneumonia and strains associated with the vaginal tracts of healthy ewes and cases of neonatal septicaemia. The aims of the investigation were to determine, first, whether the vaginal isolates were the same as an isolate recovered from the liver of one of the dead septicaemic lambs, and secondly, whether the vaginal/septicaemic isolates were the same as those recovered from cases of pneumonia. The strains were compared by an analysis of their capsular polysaccharide types and outer membrane protein profiles. The phylogenetic relationships of selected isolates with respect to reference strains of *P multocida* were also determined by comparative 16S rRNA sequence analysis.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Thirty-five ovine strains of *P multocida* were investigated, and their characteristics are shown in Table 1. The strains were obtained from various regional laboratories of the VLA and had been collected from 27 farms in widely separated areas of England and Wales between 1993 and 2000. With one or two exceptions, the strains were isolated from the lungs of sheep suffering from pneumonia or from the vaginas of healthy ewes. Isolate PM2 was recovered from the liver of a dead septicaemic lamb, and strains PM4 to PM18 were isolated from the vaginas of healthy ewes in the same affected flock (Watson and Davies 2002). Isolates PM26 and PM44 were isolated from the vaginas of healthy ewes at two other farms (Watson and Davies 2002). Reference strains of *P multocida* subspecies *multocida* (NCTC 10322), *P multocida* subspecies *galliseptica* (NCTC 10204) and *P multocida* subspecies *septica* (NCTC 11995) were obtained from the Public Health Laboratory Service's National Collection of Type Cultures. The isolates were received as transport swabs or on agar slopes, plated on to blood agar (brain heart infusion agar containing 5 per cent v/v defibrinated sheep's blood) and incubated overnight at 37°C. Single colonies were subcultured on blood agar overnight at 37°C and stored at –85°C in 50 percent v/v glycerol in brain heart infusion broth (BHIB). Bacteria from the –85°C stock culture were grown overnight at 37°C on blood agar. For the preparation of DNA, a few colonies were inoculated into 10 ml volumes of BHIB and grown overnight at 37°C, while being rotated at 120 rpm.

Capsular PCR typing and analysis of outer membrane protein profiles

The isolates' capsular polysaccharides were typed by the PCR-based method described by Townsend and others (2001). The isolation and sodium dodecylsulphate-polyacrylamide gel

electrophoresis (SDS-PAGE) analysis of the outer membrane proteins have been described by Davies and Donachie (1996).

Sequence analysis of the 16S rRNA gene

Chromosomal DNA was prepared from 1·0 ml volumes of overnight bacterial cultures in BHIB. The bacteria were harvested by centrifugation for one minute at 13,000 *g* and washed once in sterile, distilled water. The DNA was prepared with the InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions and stored at –20°C. A 1468 bp fragment of the 16S rRNA gene was amplified from the chromosomal DNA of each strain with the forward universal primer 5'-AGAGTTTGATYMTGGC-3' and the reverse universal primer 5'-GYTACCTTGTTACGACTT-3' (Davies and others 1996). The 16S rRNA gene was amplified with a *Taq* DNA polymerase kit (Boehringer Mannheim) according to the manufacturer's instructions. The PCRs were carried out in a DNA thermal cycler (480; Perkin Elmer) using the following amplification parameters: denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 45 seconds; 30 cycles were carried out and a final elongation step of 72°C for 10 minutes was used. The production of a PCR amplicon of the expected size was confirmed by agarose gel electrophoresis, and the DNA was purified with a QIAquick PCR purification kit (Qiagen). The DNA was finally eluted in 30 µl of sterile, distilled water and stored at –20°C. Sequence reactions were carried out with the ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems) in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler, and the sequences were analysed with an Applied Biosystems 377 DNA Sequencer (University of Glasgow Sequencing Service). Both strands of the gene were sequenced in three overlapping segments, using the following additional pairs of internal primers: 5'-AAGAAGCACCGGCTAACT-3' (forward/2), 5'-GCGAAG AACCTTACCTAC-3' (forward/3), 5'-GTAAGGTTCTTCGCGTTG-3' (reverse/2) and 5'-CCGGTGCTTCTTCTGTAA-3' (reverse/3). All the primers were synthesised by Sigma-GenoSys. The nucleotide sequence data were analysed and edited with SEQED (version 1.0.3; Applied Biosystems), aligned with CLUSTALX and phylogenetic analyses were carried out with MEGA (Kumar and others 1993). The GenBank accession numbers assigned to the 16S rRNA sequences of strains PM2, PM966, NCTC 10204, NCTC 10322 and NCTC 11995 are AY078996 to AY079000, respectively.

RESULTS

Capsular PCR typing

Three capsular PCR types were identified among the 35 isolates. Seven (20 per cent) of the strains were of capsular type A, 10 (29 per cent) were of capsular type D and 18 (51 per cent) were of capsular type F (Table 1). The isolates on seven of the 27 farms (26 per cent) were type A, on 10 (37 per cent) they were type D and on 10 (37 per cent) they were type F.

Analysis of outer membrane protein profiles

Three major outer membrane protein profiles were identified among the 35 isolates, and these were designated types 1, 2 and 3 on the basis of the variation of the two major proteins, OmpA and OmpH. Strains of types 1 and 3 were further subdivided into types 1·1 and 1·2, and 3·1 and 3·2, on the basis of variations of minor proteins. Profiles representing types 1·1 2·1 and 3·1 are shown in Fig 1. The distribution of the types of profile among the ovine strains is shown in Table 1. Fourteen (40 per cent) of the strains were type 1·1, two (6 per cent) were type 1·2, nine (26 per cent) were type 2·1, eight (23 per cent) were type 3·1 and two (6 per cent) were type 3·2. On 14 of the 27 farms the isolates were of type 1·1, on two they were type 1·2, on one they were type 2·1, on eight they were type 3·1 and on two they were type 3·2.

TABLE 1: Characteristics of the 35 strains of *Pasteurella multocida* analysed

Strain	Disease status	Site of origin	Geographical origin (VLA reference laboratory)	Serotype	Outer membrane protein type
PM976	Pneumonia	Lungs	Bristol	A	1·1
PM1000	Pneumonia	Lungs	Bristol	A	1·1
PM974*	Pneumonia	Lungs	Newcastle	A	1·1
PM978	Pneumonia	Lungs	Thirsk	A	1·1
PM984	Pneumonia	Lungs	Thirsk	A	1·1
PM966*	Pneumonia	Lungs	Winchester	A	1·1
PM492	Sudden death	Lungs	Thirsk	F	1·1
PM994	Pneumonia	Lungs	Penrith	F	1·1
PM996	Pneumonia	Lungs	Penrith	F	1·1
PM968	Pneumonia	Lungs	Reading	F	1·1
PM972	Pneumonia	Lungs	Starcross	F	1·1
PM992	Pneumonia	Nasal swab	Winchester	F	1·1
PM998	Pneumonia	Lungs	Shrewsbury	F	1·1
PM44*	Asymptomatic	Vagina	Penrith	F	1·1
PM26*	Asymptomatic	Vagina	Penrith	A	1·2
PM46	Pneumonia	Lungs	Penrith	F	1·2
PM2*†	Peritonitis	Liver	Penrith	F	2·1
PM4†	Asymptomatic	Vagina	Penrith	F	2·1
PM6†	Asymptomatic	Vagina	Penrith	F	2·1
PM8*†	Asymptomatic	Vagina	Penrith	F	2·1
PM10†	Asymptomatic	Vagina	Penrith	F	2·1
PM12†	Asymptomatic	Vagina	Penrith	F	2·1
PM14†	Asymptomatic	Vagina	Penrith	F	2·1
PM16†	Asymptomatic	Vagina	Penrith	F	2·1
PM18†	Asymptomatic	Vagina	Penrith	F	2·1
PM982*	Pneumonia	Lungs	Carmarthen	D	3·1
PM990	Pneumonia	Lungs	Carmarthen	D	3·1
PM986*	Pneumonia	Lungs	Luddington	D	3·1
PM970	Pneumonia	Lungs	Newcastle	D	3·1
PM122	Pneumonia	Lungs	Sutton Bonington	D	3·1
PM648	Pneumonia	Lungs	Sutton Bonington	D	3·1
PM964	Pneumonia	Lungs	Winchester	D	3·1
PM988	Pasteurellosis	Lungs	Winchester	D	3·1
PM1042	Pneumonia	Lungs	Penrith	D	3·2
PM980	Pneumonia	Lungs	Sutton Bonington	D	3·2

* Isolates in which the 16S rRNA gene was sequenced
† Isolates associated with an outbreak of neonatal septicaemia on one farm
VLA Veterinary Laboratories Agency

Comparative sequence analysis of the 16S rRNA gene

A 1468 bp fragment of the 16S rRNA gene was sequenced in eight of the field isolates of *P multocida* and in the three reference strains representing *P multocida* subspecies *gallisseptica*, *P multocida* subspecies *multocida* and *P multocida* subspecies *septica*. The phylogenetic relationships of the 16S rRNA sequences are shown in Fig 2. The sequences of the pneumonic isolates of outer membrane protein types 1·1 (PM966 and PM974) and 3·1 (PM982 and PM986) were identical. These sequences also showed 100 per cent homology with those of *P multocida* subspecies *galliseptica* and *P multocida* subspecies *multocida*. The liver and vaginal isolates of outer membrane protein type 2·1 (PM2 and PM8), which were recovered from the same farm, had identical 16S rRNA sequences that differed from those of the pneumonic strains at one nucleotide position (bp 1433). However, of the two vaginal isolates recovered from different farms, PM44 (type 1·1) had a sequence identical to that of PM2 and PM8, whereas PM26 (type 1·2) had a sequence identical to that of the pneumonic isolates. The reference strain of *P multocida* subspecies *septica* had a 16S rRNA sequence that differed at 21 nucleotide sites from the other two subspecies. This strain was originally isolated from a cat bite.

DISCUSSION

P multocida is responsible for sporadic cases of ovine pneumonia in the UK, but has recently been identified as a component of the vaginal flora of sheep and the cause of septicaemia and death in neonatal lambs (Watson and Davies

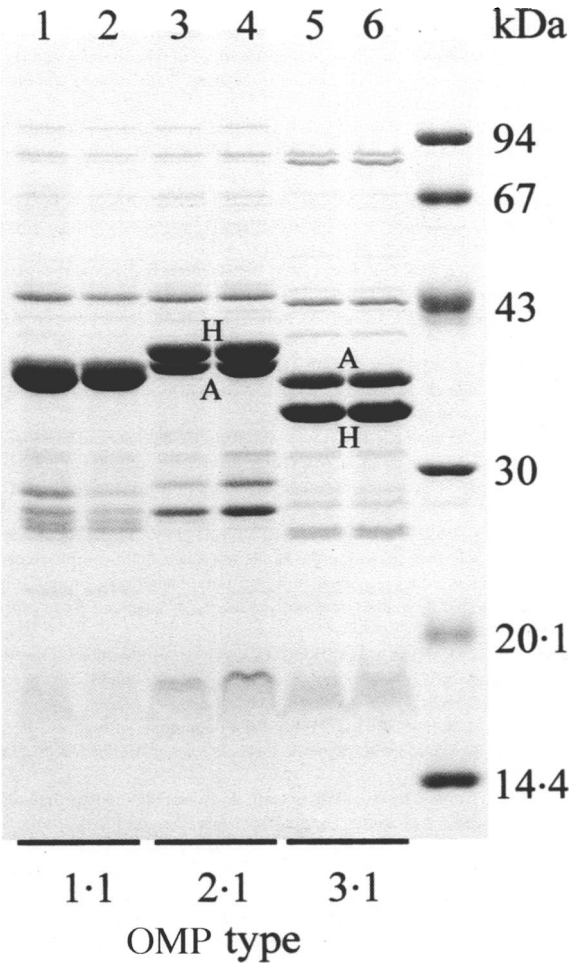

FIG 1: Representative outer membrane protein (OMP) profiles of ovine strains of *Pasteurella multocida* in Coomassie blue-stained SDS-PAGE gels. The three types are based on differences in the electrophoretic mobility of the major OmpA (A) and OmpH (H) proteins and the differences in the banding patterns of the minor proteins. Molecular mass standards (kDa) are shown in the right-hand lane

2002). This finding raises questions about the relationship between the pneumonic and vaginal isolates. The vaginal isolates might simply be pneumonic strains which have been transmitted to, and are occupying, a different ecological niche. Alternatively, they might represent a previously unidentified subpopulation of ovine *P multocida* strains that have become adapted to the vaginal habitat and may constitute an uncommon but potentially serious threat to newborn lambs.

To answer these questions, 35 strains of *P multocida* recovered from the lungs of pneumonic sheep and from vaginas of healthy ewes were compared by analysing the variations in their capsular polysaccharide types and outer membrane protein profiles. These variations have been used to identify distinct subpopulations of *Mannheimia (Pasteurella) haemolytica* associated with cattle and sheep (Davies and Donachie 1996), which were subsequently shown to represent genetically distinct clonal lineages by multilocus enzyme electrophoresis (Davies and other 1997). Three capsular polysaccharide types, A (20 per cent of strains), D (29 per cent) and F (51 per cent), were identified among the *P multocida* isolates. The distribution of capsular types among ovine isolates of *P multocida* is very different from that among strains isolated from other species. Avian strains consist mainly of capsular type A, although capsular types B, D and F occur less frequently (Rhoades and Rimler 1989). Bovine isolates are almost all capsular type A (Frank 1989) and porcine strains are predominantly capsular types A or D (Chanter and Rutter 1989). The presence of three capsular types, A, D and F, among ovine strains of *P multocida* is therefore unusual, particularly in comparison with cattle, and suggests that different strains may be associated with sheep.

Three major outer membrane protein types 1·1, 2·1 and 3·1, were identified among the isolates. However, there was a strong association between the capsular polysaccharide types and the outer membrane protein types. Strains of outer membrane protein types 1·1 and 1·2 were associated with capsular types A or F; isolates of type 2·1 were associated exclusively with capsular type F; and strains of types 3·1 and 3·2 were associated only with capsular type D (Table 1). The four different combinations probably mark individual clones (strains of the same clone are derived from the same ancestral cell) of *P multocida* (Achtman and Pluschke 1986, Kapur and others 1992). There are therefore at least four putative major clonal groups among ovine strains of *P multocida* which are marked by the following combinations of capsular and outer membrane proteins types: A/1·1, F/1·1, F/2·1 and D/3·1.

The results in Table 1 show that strains isolated from the lungs of pneumonic sheep were represented by the A/1·1 (six strains), F/1·1 (six), F/1·2 (one), D/3·1 (eight) and D/3·2 (two) clonal groups, whereas the strains recovered from the vaginas of healthy ewes were represented by the A/1·2 (one), F/1·1 (one) and F/2·1 (eight) clonal groups. These data suggest that different subpopulations of *P multocida* may be associated with the respiratory and vaginal tracts of sheep. In particular, the F/2·1 strains appear to be specifically adapted to the vaginal tract because they were isolated from the vaginas but not from nasal swabs of ewes (Watson and Davies 2002). This observation is not entirely unexpected because different strains of *P multocida* are thought to occupy different ecological niches and be responsible for pneumonia and atrophic rhinitis in pigs (Chanter and Rutter 1989). Different clonal groups of other bacterial species have also been shown to be associated with different disease syndromes within the same host (Selander and Musser 1990). Furthermore, since the vaginal and respiratory tracts provide very different environments, bacteria colonising the two habitats might be expected to have differentiated and evolved specific mechanisms of adaptation.

Isolate PM2 was recovered from the liver of a dead septicaemic lamb and appeared to be identical to isolates PM4 to PM18 (they were all F/2·1), which were isolated from the vaginas of healthy ewes in the same flock. This finding provided evidence (short of fulfilling Koch's postulates experimentally) that the strains of *P multocida* associated with the vaginas of healthy ewes may cause septicaemia and death in neonatal lambs (Watson and Davies 2002). However, isolates PM44 and PM26, which were also recovered from the vaginas of healthy ewes but on two other farms, were classified as F/1·1 and A/1·2, respectively. It is therefore likely that the diversity of vaginal isolates in the ovine population is greater than indicated by this study.

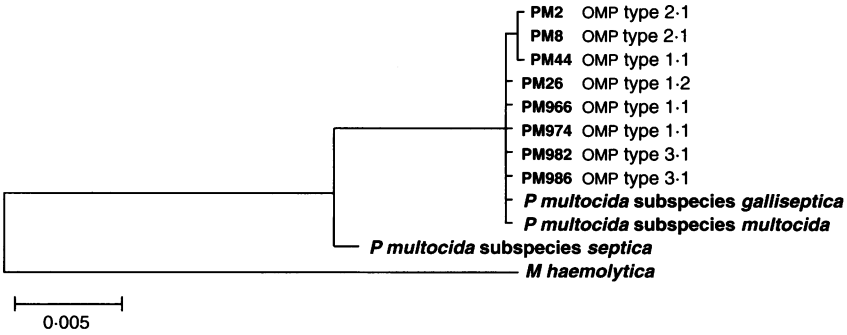

FIG 2: Neighbour joining tree for 16S rRNA sequences of field strains of *Pasterella multocida* and the reference strains of the three subspecies. The sequences represent a 1468 bp fragment of the 16S rRNA gene. The sequence of a *Mannheimia haemolytica* serotype A1 strain was used as an outgroup. OMP Outer membrane protein

Although the sample size was small and sheep movements are likely to be an influence, there was nevertheless some evidence of variations in the geographical distribution of the strains associated with pneumonia. For example, the two strains from Bristol, which were isolated in different years, were both A/1·1; the strains from Reading, Starcross and Shrewsbury were all of the the F/1·1 group; and the strains from Carmarthen, Luddington and Sutton Bonington were all of the D/3·1 group (the two strains from Carmarthen were also isolated in different years). Conversely, Winchester was associated with all three groups, and Newcastle, Penrith and Thirsk with two. These data demonstrate that capsular typing and outer membrane protein analysis, when used in combination, are capable of tracking regional variations in ovine strains of *P multocida* and can provide useful epidemiological markers.

The comparative 16S rRNA sequence analysis confirmed that the pneumonic isolates were *P multocida* because their sequences were identical with those of the *P multocida* subspecies *galliseptica* and *P multocida* subspecies *multocida* reference strains. The vaginal isolates were also clearly identified as *P multocida*, although strains PM2, PM8 and PM44 represent a distinct lineage because they differed from the pneumonic isolates at one nucleotide position. However, a difference of only one nucleotide in the slowly evolving 16S rRNA gene can be significant. For example, the 16S rRNA gene of the two species *M haemolytica* and *Mannheimia glucosida* differ at only four nucleotide sites (Davies and others 1996). The 16S rRNA sequence data supported the results of the capsular polysaccharide typing and outer membrane protein analysis and provided additional evidence that the vaginal and liver isolates associated with the outbreak of neonatal septicaemia represented a distinct subpopulation of *P multocida*.

The results of this study show that at least three different clonal groups (marked by specific combinations of capsular polysaccharide and outer membrane protein types) of *P multocida* were associated with ovine pneumonia. A fourth distinct clone was associated exclusively with the vagina of healthy ewes and with an unusual case of septicaemia and death in neonatal lambs at a single farm (Watson and Davies 2002), and was also shown to be distinct from the pneumonic clones by sequence analysis of the 16S rRNA gene. Capsular typing and outer membrane protein analysis, when used in combination, can identify these clonal variants and provide useful tools for the epidemiological discrimination of ovine strains of *P multocida*.

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