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Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins

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Abstract

One hundred avian *Pasteurella multocida* isolates recovered from cases of fowl cholera and related infections in England and Wales over a 13-year period were characterised by capsular PCR typing and analysis of outer membrane protein (OMP) profiles. Sixty-eight percent of the strains were of capsular type A, 14% were type F, 5% were type D, 4% were type B and 9% were untypable. Nineteen distinct OMP profiles (OMP-types) were identified based mainly on molecular mass heterogeneity of the heat-modifiable (OmpA) and porin (OmpH) proteins. Fifty-six percent of the isolates were represented by 15 OMP-types, whereas 44% of the isolates were associated with four OMP-types. The extensive molecular mass heterogeneity of the OmpA and OmpH proteins supports previous findings that avian *P. multocida* strains are very diverse. Furthermore, the isolates studied were associated with different clinical symptoms and were recovered from a wide range of lesions and tissues. The high degree of strain diversity together with the wide variety of clinical symptoms suggest that certain avian strains of *P. multocida* are opportunistic pathogens of relatively low virulence. Strains of capsular types B, D and F, as well as the untypable isolates, were associated exclusively with specific OMP-types and represent distinct and widely disseminated clonal groups. These observations support the view that avian strains of *P. multocida* have a clonal population structure. Based on previous studies, the molecular mass heterogeneity of the OmpA and OmpH proteins might provide a selective advantage to *P. multocida* by generating antigenic variation.

Keywords: *Pasteurella multocida*; Avian isolates; Capsular PCR typing; OmpA; OmpH

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1. Introduction

Pasteurella multocida is the aetiological agent of fowl cholera, a widely distributed and economically important disease of poultry, particularly chickens, turkeys, ducks and geese (Rhoades and Rimler, 1989; Rimler and Glisson, 1997). The organism is also responsible for disease in wild birds, commercially raised game birds and caged birds (Rhoades and Rimler, 1989). Four capsular serogroups are recognised among avian strains of *P. multocida*, namely A, B, D and F (Rhoades and Rimler, 1987, 1989; Rimler and Rhoades, 1987). Strains of serogroup A are recognised as the primary cause of fowl cholera, whereas isolates of serogroups B, D and F are less frequently associated with disease (Rhoades and Rimler, 1987, 1989; Wilson et al., 1993). In addition, some avian strains of *P. multocida* are non-encapsulated and are not serogroupable (Rhoades and Rimler, 1987; Wilson et al., 1993). Sixteen somatic serotypes (1–16) are also recognised in *P. multocida* (Rhoades and Rimler, 1987, 1989, 1990a) and most of these have been demonstrated in avian capsular serogroup A strains (Rhoades and Rimler, 1987).

There is considerable evidence, based on a wide range of molecular studies (Snipes et al., 1989; Carpenter et al., 1991; Christiansen et al., 1992; Wilson et al., 1993, 1995; Blackall et al., 1995, 1998; Gunawardana et al., 2000; Petersen et al., 2001), that avian strains of *P. multocida* are extremely diverse. In particular, a study of the population genetics of Australian strains using multilocus enzyme electrophoresis (MLEE) identified 56 electrophoretic types among only 81 field isolates (Blackall et al., 1998). Based on DNA–DNA hybridisation and sugar fermentation patterns *P. multocida* has been subdivided into three subspecies, subsp. *multocida*, subsp. *gallicida*, and subsp. *septica* (Mutters et al., 1985) and all of these have been isolated from birds (Snipes et al., 1989; Hirsh et al., 1990; Fegan et al., 1995). However, conflicting results from ribotyping and 16S rRNA sequence data (Petersen et al., 2001) suggest that the precise phylogenetic relationships of strains representing each of these subspecies is complex and has yet to be satisfactorily resolved.

Control of fowl cholera is primarily by good management practice and vaccination in areas where the disease is endemic (Rimler and Glisson, 1997). Both whole-cell bacterins and live vaccines composed of attenuated strains are currently available but neither is entirely satisfactory. Bacterins only induce serotype-specific protection, whereas live vaccines sometimes cause disease (Bierer and Derieux, 1975; Schlink and Olson, 1987; Prantner et al., 1990) and there is increasing interest in the development of subunit vaccines (Kasten et al., 1995; Luo et al., 1999). Outer membrane antigens that might be considered as potential vaccine candidates include the heat-modifiable or OmpA and the porin or OmpH proteins (Vasfi Marandi and Mittal, 1996, 1997; Luo et al., 1997, 1999). Both of these proteins are expressed in high copy number, are surface exposed and immunogenic (Hancock, 1991; Tagawa et al., 1993; Yi and Murphy, 1997; Zeng et al., 1999; Neary et al., 2001). The OmpH protein has been shown to be heterogeneous in strains of *P. multocida* representing somatic serotypes 1–16 (Luo et al., 1999) and there is evidence that anti-OmpH antibodies are protective in chickens (Luo et al., 1999) and mice (Vasfi Marandi et al., 1996). There is less information available about the OmpA protein of *P. multocida*, but this protein also exhibits variation in other bacterial species (Duim et al., 1997; Webb and Cripps, 1998).

The aim of the study was to investigate capsular and outer membrane protein (OMP) diversity among avian *P. multocida* strains isolated from diseased poultry in England and Wales. In particular, heterogeneity of the OmpA and OmpH proteins was examined and used as the basis for an OMP classification scheme. Since OmpA and OmpH are important surface-exposed components of the outer membrane, analysis of their diversity in avian *P. multocida* strains will contribute to our understanding of host–pathogen interactions in fowl cholera, including the role of these proteins in immune evasion, and to the development of improved vaccines against this pathogen.

2. Materials and methods

2.1. Bacterial strains and growth conditions

One hundred avian field isolates of *P. multocida* were investigated. These were obtained from regional laboratories of the Veterinary Laboratories Agency (VLA) and originated from widespread geographic locations within England and Wales over a 13-year period (1987–1999). The isolates were recovered predominantly from cases of fowl cholera and related acute disease conditions such as septicaemia and pneumonia. However, some isolates were associated with chronic conditions such as conjunctivitis, sinusitis, swollen head, arthritis, etc. Properties of the isolates and details of the clinical symptoms of the birds of origin are provided in Table 1. The capsular reference strains X73 (A), M1404 (B), P3881 (D), P1235 (E) and P4679 (F) were kindly provided by Dr. R. Rimler, National Animal Disease Center, Ames, IA.

The isolates were stored at −85 °C in 50% (v/v) glycerol in brain heart infusion broth (BHIB). From −85 °C stock cultures, bacteria were streaked onto blood agar (brain heart infusion agar containing 5% (v/v) defibrinated sheep's blood) and incubated overnight at 37 °C. For preparation of DNA, a few colonies were inoculated into 10 ml volumes of BHIB and grown overnight at 37 °C at 120 rpm. For preparation of outer membranes, 0.4 ml of overnight growth in BHIB was inoculated into 400 ml volumes of BHIB in 2 l Erlenmeyer flasks and incubated for 7 h at 37 °C at 120 rpm.

2.2. Preparation of chromosomal DNA

Cells from 1.0 ml of overnight cultures were harvested by centrifugation for 1 min at 13,000 × *g* and washed once in sterile, distilled H_2O. DNA was prepared with the InstaGene Matrix (Bio-Rad) according to the manufacturers' instructions and stored at −20 °C.

2.3. Capsular PCR typing

The capsular types were determined by multiplex capsular PCR typing with the capsule-specific primer pairs (CAPA, CAPB, CAPD, CAPE and CAPF) described by Townsend et al. (2001). Isolates that were negative for all five capsular types were confirmed as *P. multocida* with a *P. multocida*-specific primer set (KMT1T7 and

Table 1
Properties of avian *P. multocida* isolates

OMP-type	Serotype	No. of isolates	Clinical symptoms
1.1	A	7	Septicaemia (3); arthritis (1); sinusitis (1); other (2)
1.2	A	9	Pneumonia (9)
1.3	F	2	Sinusitis (2)
2.1	A	5	Pneumonia (2); fowl cholera (1); scour (1); other (1)
2.2	A	4	Septicaemia (2); pericarditis (1); not known (1)
	F	11	Septicaemia (6); conjunctivitis (2); pneumonia (1); death (2)
3.1	A	7	Fowl cholera (2); septicaemia (2); death (2); pneumonia (1)
4.1	A	9	Septicaemia (3); oedema (1); peritonitis (1); conjunctivitis (1); pneumonia (1); other (2)
5.1	D	1	Septicaemia (1)
	UT	2	Septicaemia (1); fowl cholera (1)
6.1	A	4	Septicaemia (1); swollen joints (1); synovitis (1); swollen head (1)
	F	1	Conjunctivitis (1)
7.1	A	11	Swollen head (4); septicaemia (3); respiratory infection (2); arthritis (1); death (1)
8.1	A	3	Septicaemia (1); pneumonia (1); other (1)
9.1	A	3	Septicaemia (3)
10.1	UT	3	Septicaemia (2); fowl cholera (1)
10.2	UT	3	Septicaemia (1); fowl cholera (1); death (1)
10.3	UT	1	Respiratory infection (1)
11.1	A	3	Septicaemia (2); fowl cholera (1)
12.1	B	4	Septicaemia (1); respiratory infection (1); air saculitis (1); sinusitis (1)
13.1	D	4	Pneumonia (3); sinusitis (1)
14.1	A	3	Swollen head (3)

KMT1SP6) (Townsend et al., 2001) in separate PCR reactions (see Section 3) and classified as untypable. All primers were synthesised by Sigma-GenoSys (Cambridge, UK) and the capsular gene fragments were amplified with a TaqDNA polymerase kit (Boehringer Mannheim) according to the manufacturers instructions. PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler using the following amplification parameters: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. Thirty cycles were performed and a final elongation step of 72 °C for 10 min was used. Production of PCR amplicons of the expected size was confirmed by electrophoresis in 2% agarose gels. Pooled PCR amplicons of capsular type A, B, D, E and F reference strains were used as standards in each gel.

2.4. Preparation of OMPs

OMPs were prepared by Sarkosyl extraction as previously described (Davies et al., 1992; Davies and Donachie, 1996). Protein concentrations were determined by the modified Lowry procedure (Markwell et al., 1978) and adjusted to 2.0 mg/ml.

2.5. SDS-PAGE

OMPs were separated by SDS-PAGE in 12% (w/v) resolving gels (Hoefer SE600 electrophoresis apparatus) using the SDS discontinuous system of Laemmli (Laemmli, 1970) as previously described (Davies et al., 1992; Davies and Donachie, 1996). Unless otherwise stated all samples were heated at 100 °C for 5 min prior to electrophoresis. Twenty micrograms of protein were loaded per lane and the proteins were visualised by staining with Coomassie brilliant blue. Protein molecular mass standards (Pharmacia) consisted of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The molecular masses of individual proteins were calculated with the Labworks™ image acquisition and analysis computer software.

3. Results

3.1. Capsular PCR typing

The capsular types of the 100 avian *P. multocida* isolates were determined by capsular PCR typing and typical results are shown in Fig. 1. The distribution of capsular types among the 100 isolates is summarised in Table 1. Sixty-eight (68%) isolates were of capsular type A, 14 (14%) were of type F, five (5%) were of type D, four (4%) were of type B and nine (9%) isolates were untypable. Capsular type E was not detected among the population sampled. The *P. multocida*-specific primers were omitted from the capsular primer mixture because they interfered with the capsule-specific primers and resulted in a reduction of capsular PCR product (i.e. reduced band intensity). Therefore, isolates that were negative for capsular typing were confirmed as untypable *P. multocida* in separate PCR assays with the *P. multocida*-specific primers (Townsend et al., 2001). Microscopic examination of the untypable isolates after Indian ink staining (Hansen and Hirsh, 1989) indicated that they were non-encapsulated.

3.2. Analysis of OMP profiles

The stability of the OMP profiles was examined by comparing the profiles of two isolates after repeated subculture and at different stages of the growth cycle. The profiles of these isolates were identical after 5, 10, 15 and 20 rounds of subculture on blood agar and after 6, 8, 12 and 24 h of growth in BHIB (results not shown). The OMP profiles of the 100 isolates were analysed by SDS-PAGE and provisionally assigned to OMP-types based on profile similarity (described below). Isolates assigned to the same OMP-type were subsequently re-run on up to three or four occasions such that isolates of the same OMP-type were directly compared on the same gel. An OMP classification scheme was devised-based, firstly, on molecular mass variation of the two major proteins, OmpA and OmpH (OMP-type 1, 2, etc.), and, secondly, on variation of minor protein patterns (OMP-type 1.1, 1.2, etc.).

The OmpA and OmpH proteins have overlapping molecular mass ranges (33–39 kDa) and were distinguished on the basis of their different behaviours in SDS-PAGE gels after

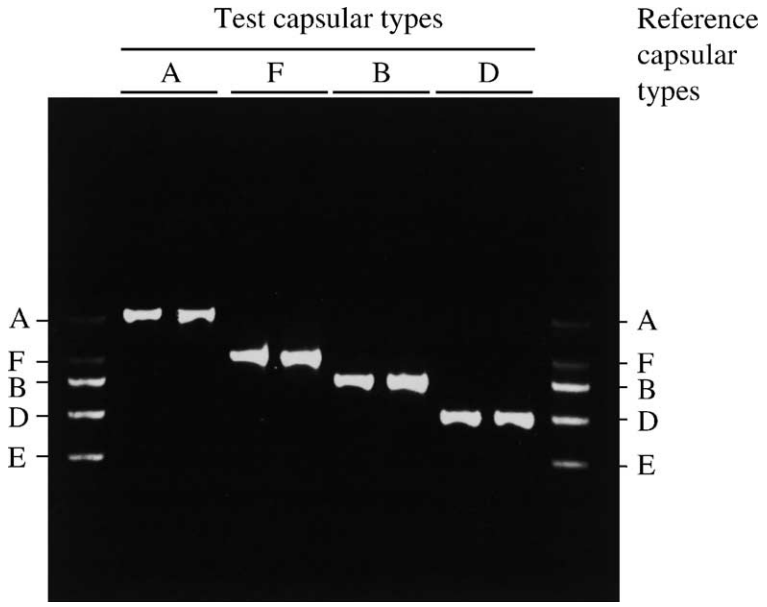

Fig. 1. Agarose gel showing results of capsular PCR typing for eight avian *P. multocida* isolates of serotypes A, F, B and D. Pooled amplification products representing reference capular types A, F, B, D and E are also shown.

heat-treatment. The OmpH porin protein is tightly associated with peptidoglycan and is not released unless heated at a temperature of approximately 60 °C or higher (Rosenbusch, 1974). Therefore, the OmpH protein does not migrate into the gel unless heated at 60 °C or higher prior to SDS-PAGE. In contrast, the OmpA protein is not associated with peptidoglycan and freely migrates into the gel after heat-treatment at temperatures below 60 °C prior to SDS-PAGE. However, the OmpA protein undergoes a characteristic conformational change when heated at 100 °C that results in an increase in its apparent molecular mass in SDS-PAGE gels (Beher et al., 1980). Therefore, to identify OmpA and OmpH, one isolate representing each OMP-type was subjected to heat-treatment at 50, 60, 70, 80, 90 and 100 °C prior to SDS-PAGE. The results for two isolates of OMP-types 3.1 and 10.2 are shown in Fig. 2. The OmpA (A) and OmpH (H) proteins for each OMP-type are indicated in Fig. 3 and the results are described below.

The 100 isolates consisted of 14 major OMP groups that were classified as OMP-types 1–14 based on variation of OmpA and OmpH (described above). Based on variation of minor proteins isolates of OMP-types 1, 2 and 10 could be further subdivided into OMP-types 1.1–1.3, 2.1 and 2.2, and 10.1–10.3, respectively. Profiles representing each of these OMP-types (with the exception of OMP-types 1.3 and 10.3) are shown in Fig. 3. The molecular mass of OmpA (A) varied from 36.9 to 37.9 kDa and that of OmpH (H) varied from 33.1 to 38.3 kDa. The distribution of OMP-types among the avian isolates is shown in Table 1. Isolates of OMP-types 2.2 (15%), 7.1 (11%), 1.2 (9%) and 4.1 (9%) were the most numerous and accounted for 44% of the total. A smaller number of isolates, ranging from 1

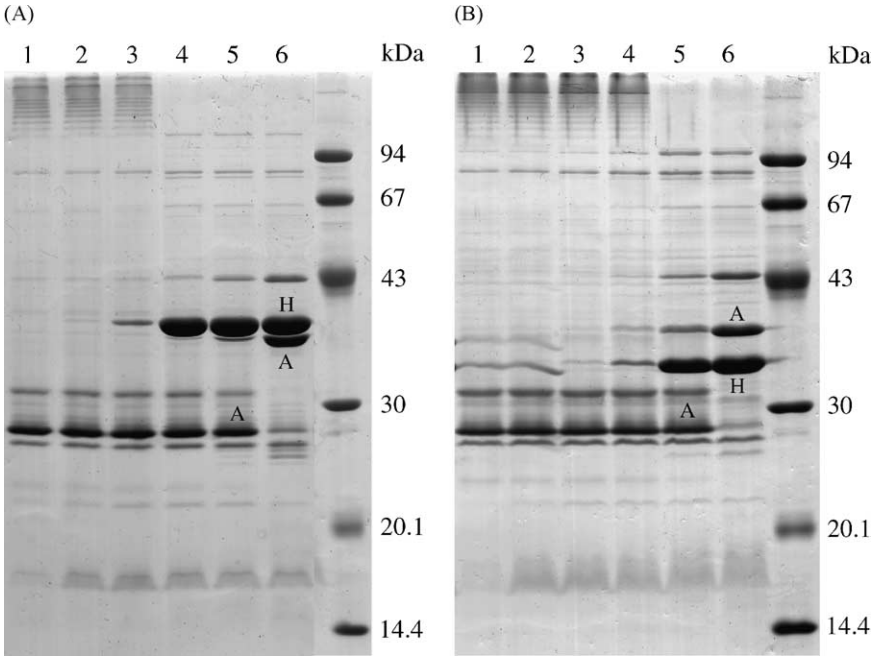

Fig. 2. Coomassie blue-stained SDS-PAGE gels showing the OMP profiles of two *P. multocida* isolates of OMP-types 3.1 (A) and 10.2 (B). The OMP samples were heated at 50, 60, 70, 80, 90 and 100 °C (lanes 1–6, respectively) prior to SDS-PAGE. The effect of heat-treatment on the major OmpA (A) and OmpH (H) proteins is clearly seen (see text).

to 7, were associated with each of the other 15 OMP-types but these accounted for 56% of the total number of isolates.

3.3. Relationship between capsular types and OMP-types

There was a strong correlation between certain capsular types and specific OMP-types (Table 1). The frequently occurring capsular type A was associated with 68 isolates representing 12 of the 19 OMP-types. In contrast, capsular type B was associated exclusively with the four isolates of OMP-type 12.1; these isolates originated from four different regional laboratories. Capsular type D was associated with 1/3 isolates of OMP-type 5.1 and with the four isolates of OMP-type 13.1; three of the four isolates of OMP-type 13.1 originated from different regional laboratories and the single OMP-type 5.1 isolate came from a fourth laboratory. Capsular type F was associated with the two isolates of OMP-type 1.3, with 11/15 isolates of OMP-type 2.2 and with 1/5 isolates of OMP-type 6.1. All seven of the isolates representing OMP-types 10.1–10.3 were untypable, as were 2/3 isolates of OMP-type 5.1. The seven untypable/OMP-type 10 isolates originated from six different regional laboratories; the two OMP-type 5.1 isolates also came from different laboratories. Overall, the majority of OMP-types were represented by a single capsular type, but isolates of OMP-types 2.2 and 6.1 were associated

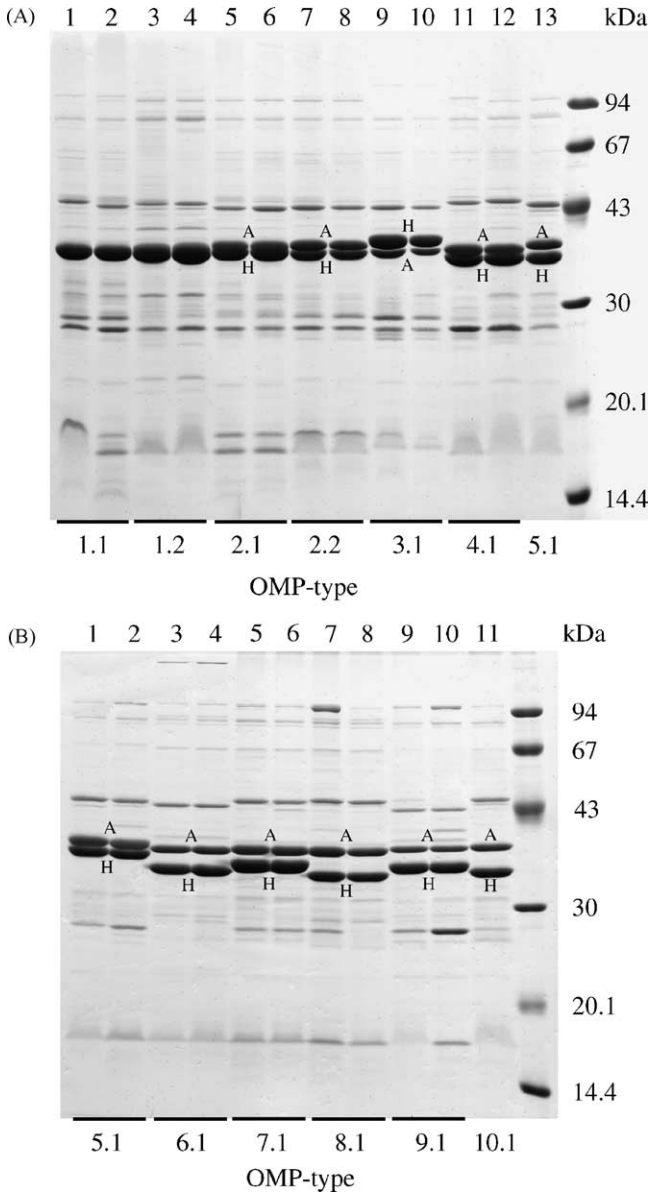

Fig. 3. Representative OMP profiles of avian *P. multocida* isolates in Coomassie blue-stained SDS-PAGE gels. The OMP-types are based on differences in the electrophoretic mobility of the major OmpA (A) and OmpH (H) proteins as well as differences in the banding patterns of the minor proteins. The OMP profiles of two isolates of each OMP-type are shown to demonstrate reproducibility. Molecular mass standards (kDa) are shown in the right-hand lane of each gel.

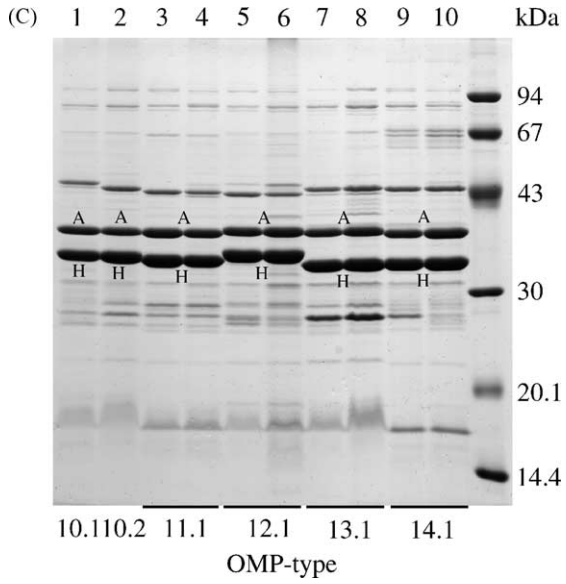

Fig. 3. (*Continued*).

with capsular types A and F, and isolates of OMP-type 5.1 were either untypable or possessed capsular type D.

4. Discussion

There are a number of difficulties associated with conventional capsular serotyping of *P. multocida* (Chengappa et al., 1986; Rimler and Rhoades, 1987, 1989). However, Townsend et al. (2001) described an alternative and highly specific multiplex capsular PCR assay that is based on nucleotide sequence variation within the five capsular biosynthetic loci. This PCR-based capsular typing method was used in the present study and found to be a reliable and rapid method for capsular typing large numbers of *P. multocida* isolates. Reference strains were used as internal standards and no cases of ambiguity occurred. The observed incidence of capsular serotypes in our sample was very similar to that described in the study of 246 isolates by Rhoades and Rimler (1987). In the latter investigation, capsular types A, F, B and D were associated with 67, 5, 2 and 2% of isolates, respectively, whereas 24% of strains were untypable. The significantly higher incidence of serotype A strains with respect to isolates of serotypes B, D and F in this and previous studies (Rhoades and Rimler, 1987; Wilson et al., 1993) suggests that the various serotypes differ in their virulence characteristics. Although virulence studies have shown that strains of serotypes B, D and F are potentially pathogenic (Rimler and Rhoades, 1987; Rhoades and Rimler, 1988, 1990b), there is very little information about the comparative virulence of strains representing the different serotypes.

The OMP profiles of the avian *P. multocida* isolates were very diverse. The isolates could be classified into 19 distinct OMP-types based on variation of OmpA and OmpH and, to a lesser extent, of the minor proteins. Fifty-six percent of the isolates were represented by 15 OMP-types, whereas 44% of the isolates were associated with four OMP-types. The high degree of heterogeneity observed in the OMP profiles, and of OmpA and OmpH in particular, was not unexpected because previous studies have shown that avian *P. multocida* strains are extremely diverse (Snipes et al., 1989; Christiansen et al., 1992; Wilson et al., 1993, 1995; Blackall et al., 1995, 1998; Petersen et al., 2001). In particular, Blackall et al. (1998) identified 56 electrophoretic types among only 81 *P. multocida* isolates from Australian poultry by MLEE. In a previous study of *Mannheimia haemolytica* (Davies and Donachie, 1996), 184 strains were sub-divided into three distinct groups based on their OMP profiles and these were subsequently shown to represent phylogenetically distinct lineages by MLEE (Davies et al., 1997). However, no such demarcation was apparent among the OMP profiles of the avian *P. multocida* isolates.

OMP patterns have been shown to be closely associated with electrophoretic types and clones identified by MLEE in other species (Achtman et al., 1983; Musser et al., 1985, 1988; Achtman and Pluschke, 1986; Kapur et al., 1992; Davies et al., 1997). The exclusive association of isolates of the less common capsular types with specific OMP-types provided evidence that OMP-types mark individual clonal groups of *P. multocida* (Achtman and Pluschke, 1986). For example, isolates of OMP-type 1.3 were associated with capsular type F, isolates of OMP-type 10 were untypable, isolates of OMP-type 12.1 were associated with capsular type B and isolates of OMP-type 13.1 were associated with capsular type D (Table 1). Furthermore, almost all of the isolates representing each of these groups originated from a different regional laboratory. This is significant because a characteristic feature of clonal bacterial populations is that strains representing the same clone originate from widespread geographic origins (Selander and Musser, 1990). The association of capsular types B and D and certain untypable isolates, with specific OMP-types is also important because it demonstrates for the first time that strains of these uncommon capsular types, together with untypable isolates, probably represent specific clones of *P. multocida*. In contrast, Dziva et al. (2001) were unable to demonstrate a relationship between RAPD patterns and capsular serogroups in their study of Zimbabwean isolates of *P. multocida* (Dziva et al., 2001). Isolates of OMP-types 2.2 and 6.1 were associated with capsular types A and F. This observation is probably due to the close relationships of these two capsular types (Townsend et al., 2001).

In many pathogenic bacterial species, the majority of cases of infectious disease are often caused by a small proportion of the total number of extant clones (Selander and Musser, 1990). In this respect, avian *P. multocida* strains differ from many other pathogens because the majority of cases of disease were associated with a relatively large number of OMP-types/clones. A possible reason for this is that the isolates were recovered from a diverse range of lesions and tissues and were associated with different types of infection ranging from pneumonia and septicaemia to sinusitis, conjunctivitis and swollen head. High levels of diversity were also observed among *Eschericha coli* strains isolated from chickens with swollen-head syndrome and from birds with colibacillosis (White et al., 1990). It was suggested that the large number of clonal genotypes associated with these avian diseases was due either to the opportunistic nature of the infections or to the

widespread occurrence of unknown virulence factors (White et al., 1990; Whittam, 1995). Swollen-head syndrome associated with *E. coli* is thought to be the result of a secondary infection subsequent to an initial viral infection caused by paramyxovirus, coronavirus, or pneumovirus. The high level of diversity observed among avian *P. multocida* isolates, together with the wide range of clinical symptoms and tissues of origin, similarly suggests that a high proportion of the isolates might represent opportunistic pathogens of relatively low virulence. In particular, isolates associated with conjunctivitis, sinusitis and swollen head could potentially be secondary pathogens following initial viral infection. Confirmation of this hypothesis will require the comparison of bacterial isolates from diseased birds with the normal avian flora.

The OmpA and OmpH proteins of avian isolates of *P. multocida* were shown to be heterogeneous since numerous molecular mass variants were identified (Fig. 3). However, the OmpH protein (33.1–38.3 kDa) is clearly more heterogeneous than the OmpA protein (36.9–37.9 kDa). Comparative nucleotide sequence analysis of the OmpH proteins representing the 16 somatic serotypes of *P. multocida* has shown that the molecular mass heterogeneity of this protein is due to variation in the number of amino acids (318–333) in the protein (Luo et al., 1999). However, most of this variation is confined to two discrete hypervariable regions (amino acids 60–80 and 200–220) which are thought to correspond to external surface-exposed loops (Luo et al., 1999). Similar heterogeneity occurs in the corresponding P2 (OmpH) and P5 (OmpA) proteins of *Haemophilus influenzae*, and has also been shown to be due to differences in the size of hypervariable surface-exposed loop regions (Forbes et al., 1992; Sikkema and Murphy, 1992; Duim et al., 1997; Webb and Cripps, 1998). These surface-exposed loops are thought to interact with the host immune system and, by undergoing antigenic variation, provide the bacterium with an important defence mechanism (Yi and Murphy, 1994, 1997; Neary et al., 2001). Furthering knowledge of the molecular basis of this diversity in *P. multocida* will lead to a better understanding of the role of these proteins in avian disease and contribute to the development of improved vaccines.

In summary, this investigation of capsule and OMP variation has confirmed the view that avian *P. multocida* isolates are very diverse. A possible explanation for the high level of strain diversity observed in the study is that many of the isolates were associated with chronic infections, were recovered from a wide range of lesions and tissues, and represent opportunistic pathogens. The association of certain capsular types with specific OMP-types suggests that OMP profiles mark individual clones of *P. multocida*. In particular, isolates of the uncommon capsular types B and D and certain untypable isolates, represent distinct clonal groups. The OmpA and OmpH proteins exhibit extensive molecular mass heterogeneity that might provide a selective advantage to the pathogen by generating antigenic variation.

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