SEROLOGICAL AND MOLECULAR DIAGNOSIS

Identification by monoclonal antibodies of serotype D strains of *Pasteurella multocida* representing various geographic origins and host species

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Two outer-membrane proteins (OMPs) of *Pasteurella multocida* serotype D, designated H and W, possess potentially important serotype D-specific antigens. Antigenicity as well as toxigenicity of 55 strains of *P. multocida* representing various serotypes, geographic origins and host species were studied by SDS-PAGE, enzyme-linked immunosorbent assay (ELISA), immunoblot and polymerase chain reaction (PCR) assays. Based on the electrophoretic mobility of protein H, different OMP patterns were observed within different capsular serotypes. Three monoclonal antibodies (MAbs) designated MT1, MT2 and MT3 were produced against H and W proteins of *P. multocida* in BALB/c mice. MAbs MT2 and MT3 reacted with two distinct epitopes on W protein of serotype D in competitive ELISA. MAb MT1 reacted with all serotype D-I strains but not with D-II strains, whereas MAb MT2 reacted with both serotype D-I and D-II strains in dot-ELISA and immunoblot assay. MAb MT3 reacted with all *P. multocida* strains belonging to different capsular serotypes in dot-ELISA. None of the MAbs reacted with other gram-negative bacteria tested, indicating that protein H has a serotype D-I specific epitope and protein W has both serotype and species-specific epitopes. PCR assay was used to identify toxigenic strains of *P. multocida*; 92% of *P. multocida* strains possess both *toxA* gene and MAb MT2 reacting epitope, suggesting a strong association between MAb MT2 reacting epitopes and *toxA* gene. Rapid dot-ELISA with MAb was found to be specific, sensitive and easy to perform and thus suitable for routine serotyping of *P. multocida* serotype D strains which might be potentially pathogenic.

Introduction

Pasteurella multocida serotype D strains have been isolated from cattle, goats, rabbits, pigs, turkeys and man [1, 2]. Some of the serotype D strains are toxigenic, causing atrophic rhinitis in calves, goats and pigs [3–6] and dermal lesions in poultry [7]. Several tests have been used to detect toxigenic *P. multocida* strains, including the guinea-pig skin test [4], cell culture assay [8], enzyme-linked immunosorbent assay (ELISA) [6, 9] and polymerase chain reaction (PCR) assay [10, 11]. The gene that encodes the toxin of *P. multocida*, *toxA*, has been isolated and sequenced [12]. PCR assay based on *toxA* gene has been shown to be a highly sensitive and specific assay for identification of toxigenic strains of *P. multocida* [10, 11].

Indirect haemagglutination (IHA) [13], mouse passive protection [14], gel diffusion precipitin (GDP) [15], co-agglutination [16] and counter-immunoelectrophor-

esis [17] have been used for serotyping *P. multocida* strains. There are five capsular serotypes, recognised by the IHA test [13, 18], and 16 somatic serotypes, recognised by the GDP test [15]. Non-serological tests such as mucopolysaccharidase [2] and acriflavine [19] tests have also been developed for rapid identification of serotypes A, D and F strains of *P. multocida*. Serotyping of *P. multocida* strains by current techniques produces ambiguous results [20]. It is difficult to prepare high-titre antisera against serotypes A and D specific antigens [18]. Numerous strains which remain untypable have also been reported [20–23]. Moreover, these tests are also known to give false-negative results with non-capsulate or less capsulate variants of *P. multocida* [17]. Thus, a more reliable method for serotyping of *P. multocida* isolates needs to be developed.

P. multocida serotype D strains were recently classified into two types (D-I and D-II) based on the electrophoretic mobility of two outer-membrane proteins (OMPs) (proteins H and W) and their reactivities with monoclonal antibodies (MAbs) [24, 25]. The purpose of this investigation was to develop a more

precise and specific tool for routine typing of serotype D strains and to find out if there is any relationship between the presence of MAb-reacting epitopes and the *toxA* gene among *P. multocida* strains.

Materials and methods

Bacterial strains and culture conditions

The 55 strains of *P. multocida* representing various serotypes, geographic origins and animal species used in this study are listed in Table 1. *P. aerogenes* ATCC 27883, *P. gallinarum* ATCC 13360, *P. canis* NCTC 11622, *P. sp. B* SSI P683 and *P. SP-g* SSI P603, were obtained from R. Mutter, Institut für Medizinische Mikrobiologie Klinikum der Philipps-Universität Marburg (Lahn), Germany. *Haemophilus influenzae* ATCC 9006, *Actinobacillus lignieresii* ATCC 19393, *A. pleuropneumoniae*, *A. suis*, *Bordetella bronchiseptica*, *Klebsiella pneumoniae*, *Escherichia coli* K12 143, *Salmonella arizona* ATCC 13314, *Yersinia enterocolitica* ATCC 23715, *Pseudomonas aeruginosa* ATCC 27853, *Shigella sonnei* ATCC 29930 and *Campylobacter jejuni* were obtained from the stock culture collection of the Faculté de Médecine Vétérinaire, Université de Montréal, Canada.

A. pleuropneumoniae and *H. influenzae* were grown on chocolate blood agar plates in air with CO₂ 7%. *C. jejuni* was cultured on blood agar plates in a micro-aerophilic environment (O₂ 5%). *P. multocida* strains and all the other gram-negative bacteria were cultured aerobically on blood agar plates at 37°C for 24 h.

Preparation of OMPs

P. multocida strains of serotype A (1662, 2100, 4062), serotype B (656, 5325), serotype D (4065, 2153), serotype E (4096) and serotype F (4679 and 4218) were disrupted in a French press and Sarkosyl-insoluble OMPs were prepared as described previously [25, 26]. Sarkosyl-soluble OMPs of *P. multocida* serotypes B 656 and D 2153 were dialysed against Tris-HCl (pH 7.8) and were treated with Triton X-100 (Sigma) 1% v/v in the presence of 10 mM MgCl₂ at room temperature for 1 h. After centrifugation, the Triton-insoluble OMP preparation was resuspended in distilled water and stored at -20°C. The protein concentration was determined by the method of Bradford [27].

Production of monoclonal and polyclonal antibodies

Three monoclonal antibodies (MAbs) designated MT1, MT2 and MT3 were produced in BALB/c mice, with OMPs of serotypes D P210 or B 656 of *P. multocida* as described previously [24]. A MAb produced against *Mycoplasma gallisepticum* (MAb K1) was used as a negative control in competitive ELISA [28]. Polyclonal antibodies against the 32-kDa OMP (porin H) of *P.*

multocida were produced in BALB/c mice, as described previously [25]. MAbs were purified from ascitic fluid with caprylic acid and ammonium sulphate as described by Ogden and Leung [29]. Proteinase-K and sodium periodate treatments of OMPs were performed as described previously [24].

Epitope analysis by competitive ELISA

Competitive ELISA was performed as described previously [28], in 96-well microtitration plates coated with Triton-insoluble OMPs of *P. multocida* serotype D 2153 as antigen, to analyse the epitope specificity of MAbs MT2 and MT3. Purified MAb MT2, at a protein concentration of 1 mg/ml, was biotinylated according to the manufacturer's instructions (Biotinylation kit, Amersham). It was used in a reciprocal competitive ELISA at a dilution of 1 in 1000 corresponding to an optical density (OD) of 1.0 at 405 nm. All incubations were at 37°C for 90 min, and dilutions and washings were performed in PBS-Tween (PBS-T). Ten-fold serial dilutions of the unlabelled competing homologous and heterologous MAbs were prepared. MAb against *M. gallisepticum* was used as a negative control. One hundred µl of MAbs in PBS-T were added to each well and incubated. After three washings and a further incubation with biotinylated MAb MT2, the plates were washed and incubated with an optimal dilution of streptavidin-peroxidase conjugate (BioRad). Finally, the plates were washed and developed for 30 min at room temperature in the dark with *o*-phenylenediamine (Sigma). The percentage of competition was calculated by the formula $[100 (A - n)/(A - B)]$. *A* is the OD in the absence of competitor MAb (negative control MAb), *n* is the OD in the presence of heterologous competitor, and *B* is OD in the presence of homologous competitor. For the interpretation of results, < 30% competition was considered non-competitive, 30–70% was considered partially competitive and > 70% was considered fully competitive.

Rapid dot-ELISA

The spectrum of activity of MAbs with different *P. multocida* strains and other gram-negative organisms was tested by rapid dot-ELISA as described by Achacha and Mittal [30]. A 10-µl volume of optimally diluted boiled cell suspension was spotted on to a nitrocellulose membrane and allowed to dry at room temperature. The membrane was incubated with skim milk 5% v/v in PBS-T before incubation with MAb supernates for 1 h at room temperature. The blots were washed and incubated with goat anti-mouse horseradish peroxidase conjugate (BioRad) for 1 h at room temperature and washed. Membranes were developed in 4-chloro-1-naphthol substrate (BioRad) for 5–10 min, and the colour reaction was terminated by flooding the membrane with distilled water. Very strong binding, strong binding, distinct binding, weak binding and no binding of MAbs with antigens were recorded as 4+, 3+, 2+, 1+ and – respectively.

SDS-PAGE and immunoblot assay

SDS-PAGE was performed by the method of Laemmli [31] with acrylamide 12% resolving gels. Immunoblot assay was performed by the method of Towbin [32], as described previously by Vasfi Marandi *et al.* [25].

Preparation of genomic DNA

Genomic DNA of 55 strains of *P. multocida* was prepared by the method of Daigle *et al.* [33]. *P. multocida* strains were grown in brain-heart infusion broth at 37°C overnight. One ml of overnight culture was centrifuged at 18 000 *g* for 10 min. The sediment was suspended in 200 µl of sterile water and boiled for 10 min at 100°C. After centrifugation of the lysate, a 150-µl sample of the supernate was stored at -20°C as a template DNA stock.

PCR amplification of *toxA* gene

Two oligonucleotide primers with the following sequences were synthesised on a Gene Assembler (Pharmacia LKB Biotechnology Inc.): oligonucleotide 1; 5'-TACTCAATTAGAAAAAGCGCTTTATCTTCC-3'; oligonucleotide 2, 5'-TCTACTACAGTTGCTGG-TATTTTAAATAT-3'. Oligonucleotide 1 corresponds to the sense strand of nucleotide positions 1983–2012 and oligonucleotide 2 corresponds to the anti-sense strand of nucleotide position 3212–3183 of the *toxA* gene of *P. multocida* [11, 12].

Amplification of DNA was performed in a total volume of 50 µl by the method of Nagai *et al.* [11]. The reaction mixture consisted of 1 µM of each oligonucleotide primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 unit of Taq DNA polymerase (Gibco BRL, Burlington, Canada), 5 µl of 10 × reaction buffer (1.5 mM MgCl₂, 10 mM Tris HCl, pH 8.4, 50 mM KCl, glycerol 4%) and 10 µl of sample DNA. The reaction mixture was covered with a drop of mineral oil and was subjected to 25 cycles of 1 min at 92°C, 1 min at 55°C, and 2 min at 70°C in a Thermal Cycler (Perkin Elmer Cetus, Canada). A toxigenic serotype D strain 4-4056 and non-toxicogenic serotype A strain X-73 were used as controls. A negative control without DNA was also included in each run. Ten µl of the amplification products were separated by electrophoresis in agarose 1.2% gels (Gibco BRL), and the DNA fragment was visualised by UV fluorescence after staining with ethidium bromide 0.5 µg/ml. A 1-kb DNA ladder (Gibco BRL) was used as a size marker in all gels.

Results

W protein of *P. multocida*

Two MABs designated MT2 and MT3 were produced with OMP preparations of *P. multocida* serotype D P210 and B 656 respectively. In immunoblot assay, both MABs MT2 and MT3 reacted with a 32-kDa OMP

of *P. multocida* serotype D 2153 (Fig. 1). The epitopes reactive with MAB MT2 were sensitive to treatment with both proteinase K and sodium periodate whereas those reactive with MAB MT3 were sensitive to treatment with proteinase K but not sodium periodate. The results of competitive ELISA showed that there was no competition (< 10%) between the two MABs MT2 and MT3 (Fig. 2).

SDS-PAGE analysis of *H* protein of *P. multocida*

Sarkosyl-insoluble OMPs or whole-cell proteins of 55 strains of *P. multocida* were analysed by SDS-PAGE. Based on the migration of the major OMP (porin H), three OMP patterns within serotype A (A-I, A-II and A-III), two within serotype B (B-I and B-II), two within serotype D (D-I and D-II) and two within serotype F (F-I and F-II) were observed. No heterogeneity was found among three strains of serotype E tested. Polyclonal antibodies directed against the 32-kDa porin H of *P. multocida* reacted with the major OMPs of all *P. multocida* strains tested in immunoblot assay (Fig. 3, Table 1).

Serospecificity of MABs

The 55 strains of *P. multocida* and 17 other gram-negative bacteria were tested for reactivity with MABs MT1, MT2 and MT3 by dot-ELISA and immunoblot assay. MAB MT1 directed against the 32-kDa porin of *P. multocida* reacted only with serotype D-I strains; 90% of serotype D strains (D-I and D-II) reacted with MAB MT2. However, two serotype A strains (1702, 2305A) and some of the untypable strains also reacted with MAB MT2. None of the serotype B, E and F strains reacted with MABs MT1 and MT2. There was 100% correlation between results obtained with dot-ELISA and immunoblot assay. MAB MT3 reacted with all 55 strains of *P. multocida* in dot-ELISA. However, three strains (4399, 4096 and 4679) failed to react in immunoblot assay. MAB MT3 reacted more strongly with serotype D strains than with other serotypes of *P. multocida* strains in dot-ELISA (Table 1).

MABs MT1, MT2 and MT3 did not react with other gram-negative bacteria including *P. aerogenes* ATCC 27883, *P. gallinarum* ATCC 13360, *P. canis* NCTC 11622, *P. sp. B* SSI P683, *P. SP-g* SSI P603, *H. influenzae* ATCC 9006, *A. lignieresii* ATCC 19393, *A. pleuropneumoniae*, *A. suis*, *B. bronchiseptica*, *K. pneumoniae*, *E. coli* K12 HFR, *S. arizona* ATCC 13314, *Y. enterocolitica* ATCC 23715, *Ps. aeruginosa*, ATCC 27853, *Sh. sonnei*, ATCC 29930 and *C. jejuni*.

PCR amplification of *toxA* gene

PCR assay was used to discriminate between toxigenic and non-toxicogenic *P. multocida* strains tested. For PCR amplification, two oligonucleotide primers were chosen on the basis of the sensitivity and specificity obtained when used in PCR. These primers were located around the 1.5-kb *Hind*III–*Hind*III fragment of the *toxA* gene

Table 1. Classification of *P. multocida* strains, based on the electrophoretic mobility of porin H and presence of MABs MT1-, MT2- and MT3-reacting epitopes as well as *tox4* gene among 55 strains of *P. multocida*, representing various serotypes, geographic origins and host species, by SDS-PAGE, dot-ELISA, immunoblot and PCR assays

Serotype (strain)*	Origin	Received from†	Host	Mr of porin H [‡]	Reactivity of MABs in immunoblot [§]			Reactivity of MABs in dot-ELISA			Toxicogenicity in PCR**
					MT1,	MT2,	MT3	MT1,	MT2,	MT3	
A: 14(P-2225)	Iowa	K. R. Rhoades	Cattle	39 kDa	—	—	+	—	—	2+	—
A:-(Q-1322)	Canada	Collection	Cattle	37	—	—	—	—	—	—	—
A: 1(X-73)	Maryland	K. R. Rhoades	Chicken	39	—	—	+	—	—	1+	—
A: 12(P-1573)	Iowa	R. B. Rimler	Human	38	—	—	+	—	—	1+	—
A: 15(P-3017)	South America	R. B. Rimler	Pig	37	—	—	+	—	—	1+	—
A: 4,7(2305A)	Canada	Collection	Pig	38	—	—	+	—	—	1+	—
A:-(Q-1321)	Canada	Collection	Pig	32	+	+	+	4+	4+	4+	+
A:-(Q-1323)	Canada	Collection	Rabbit	39	—	—	+	—	—	1+	—
A: 1(P-4062)	Belgium	R. B. Rimler	Rabbit	39	—	—	+	—	—	1+	—
A: 11(P-4059)	Belgium	R. B. Rimler	Rabbit	37	—	—	+	—	—	1+	—
A: 12(P-5310)	Zimbabwe	R. B. Rimler	Rabbit	37	—	—	+	—	—	1+	—
A: 3(P-1059)	West Virginia	K. R. Rhoades	Turkey	37	—	—	+	—	—	1+	—
A: 16(P-2723)	Indiana	K. R. Rhoades	Turkey	38	—	—	+	—	—	1+	—
A: 4(P-1662)	South Carolina	K. R. Rhoades	Turkey	37	—	—	+	—	—	1+	—
A: 10(P-2100)	Indiana	K. R. Rhoades	Turkey	38	—	—	+	—	—	1+	—
A: 16(P-2723)	Indiana	K. R. Rhoades	Turkey	38	—	—	+	—	—	1+	—
A: 9(P-2095)	Minnesota	K. R. Rhoades	Turkey	38	—	—	+	—	—	1+	—
A: 5(P-1702)	Virginia	K. R. Rhoades	Turkey	37	—	+	+	—	4+	4+	—
B:-(656)	—	R. Carter	Bison	32	—	—	+	—	—	1+	—
B: 2(M-1404)	Wyoming	K. R. Rhoades	Bison	32	—	—	+	—	—	1+	—
B: 4(P-5325)	Canada	R. B. Rimler	Bison	38	—	—	+	—	—	1+	—
B: 2(P-5120)	UK	R. B. Rimler	Cattle	32	—	—	+	—	—	1+	—
B: 2(P-4992)	India	R. B. Rimler	Pig	32	—	—	+	—	—	1+	—
B: 1(P-3269)	California	R. B. Rimler	Swan	38	—	—	+	—	—	1+	—
D: 10(P-5152)	The Netherlands	R. B. Rimler	Chicken	32	+	+	+	4+	4+	4+	—
D:-(4-4056)	The Netherlands	M. F. de Jong	—	37	—	—	+	—	—	4+	+
D:-(P-4533,4533B)	Sweden	R. B. Rimler	—	37	—	+	+	—	4+	4+	+
D:-(P-4884,4884B)	USA	R. B. Rimler	—	37	—	+	+	—	4+	4+	+
D:-(P-4275,4275B)	Arkansas	R. B. Rimler	—	37	—	+	+	—	4+	4+	+
D: 3(P-4991)	Iowa	R. B. Rimler	Pig	32	+	+	+	4+	4+	4+	+
D: 12(P-3018)	South America	R. B. Rimler	Pig	32	+	+	+	4+	4+	4+	—
D: 3,16(30-1)	Canada	R. B. Rimler	Pig	32	+	+	+	4+	4+	4+	+
D: 4,7(23-3)	Canada	Collection	Pig	32	+	+	+	4+	4+	4+	+
D:-(Q-1352)	Canada	Collection	Pig	37	—	+	+	—	4+	4+	+
D:-(Q-1326)	Canada	Collection	Rabbit	32	+	+	+	4+	4+	4+	+
D: 12(3023)	South America	R. B. Rimler	Rabbit	37	—	+	+	—	4+	4+	+
D: 3(P-4065)	Belgium	R. B. Rimler	Rabbit	37	—	+	+	—	4+	4+	+
D: 12(P-4673)	Belgium	R. B. Rimler	Rabbit	32	+	+	+	4+	4+	4+	—
D: 14(P-4399)	Michigan	R. B. Rimler	Rabbit	37	—	—	+	—	4+	4+	—
D: 11(P-2153)	Minnesota	R. B. Rimler	Turkey	39	—	—	+	—	—	1+	—
				37	—	+	+	—	4+	4+	+

D: 3(P-4886)	Malaysia	R. B. Rimler	Goat	36	-	-	+	-	-	1+	+
E: (Bunia II)	—	R. Carter	Cattle	38	-	-	+	-	-	1+	-
E: 5(P-4096)	Michigan	R. B. Rimler	Cattle	38	-	-	-	-	-	1+	-
E: 5(P-4097)	Michigan	R. B. Rimler	Pig	38	-	-	+	-	-	1+	-
F: (P-1435)	USA	R. B. Rimler	Turkey	36	-	-	+	-	-	1+	-
F: 1(P-4679)	Arkansas	R. B. Rimler	Turkey	36	-	-	-	-	-	1+	-
F: 3(P-4218)	California	R. B. Rimler	Turkey	38	-	-	+	-	-	1+	-
NT: 13(P-1591)	Iowa	K. R. Rhoades	Human	37	-	-	+	-	-	2+	-
NT: 8(P-1581)	Massachusetts	K. R. Rhoades	Pine siskin	37	-	-	+	-	-	3+	-
NT: 15(P-2237)	Iowa	K. R. Rhoades	Turkey	37	-	-	+	-	-	4+	-
NT: 6(P-2192)	Texas	K. R. Rhoades	Turkey	37	-	-	+	-	-	1+	-
NT: (P-5234)	Arkansas	R. B. Rimler	Chicken	37	-	-	+	-	-	4+	-

*The letter denotes the capsular serotype; the number denotes the somatic serotype; NT, non-typable strain; —, non-determined somatic serotype; strains 4533B, 4884B and 4275B are non-capsulated variants of capsule strains 4533, 4884 and 4275, respectively.

†K. R. Rhoades and R. B. Rimler, National Animal Disease Center, Ames, Iowa, G. R. Carter, Virginia-Maryland Regional College of Veterinary Medicine, USA; collection, stock culture collection, Faculté de Médecine Vétérinaire, Université de Montréal, Canada.

‡OMP patterns with capsular serotype strains were detected in SDS-PAGE, based on the migration of porin H.

§The presence and absence of MABs MT1, MT2- and MT3-reacting antigens in immunoblot are indicated by — or +.

||The intensity of reaction of MABs MT1, MT2 and MT3 with boiled cell suspension antigen is indicated by —, 1+, 2+, 3+ and 4+.

***tox*A gene was amplified with specific primers and 1.5-kb amplified product was electrophoresed in agarose 1.2% gel.

Fig. 1. Reactivities of MAbs MT2 (lane 1) and MT3 (2) with 32-kDa Triton insoluble OMP preparation of *P. multocida* serotype D 2153 in immunoblot assay.

Fig. 2. Epitope analysis of W protein of *P. multocida* serotype D 2153 by competitive ELISA. Biotinylated MAb MT2 (□) was tested in competition with homologous MAb MT3 (◆) and a negative control MAb (K1) (■) against *M. gallisepticum* with Triton-insoluble OMP preparation as antigen.

because the amplified product of this fragment has been clearly detected by agarose gel electrophoresis [11]. The *toxA* gene was detected in 13 (65%) of 20 serotype D, 1 (5.6%) of 18 serotype A and 1 (20%) of five untypable strains of *P. multocida*. The *toxA* gene was not detected among any of the serotype B, E and F strains of *P. multocida* (Fig. 4). No relationship was found between serotype D-I or D-II strains and presence of *toxA* gene. However, 92% of *P. multocida* strains with the *toxA* gene possessed a MAb MT2-reacting epitope (Table 1).

Discussion

Serological diagnosis and vaccination programmes have been used in attempts to control various animal

Fig. 3. Electrophoretic mobility of major OMP (porin H) of selected *P. multocida* strains. Approximately 15 µg of sarkosyl-insoluble OMPs were dissolved in sample buffer by boiling and were separated by SDS-PAGE with a 12% separating gel. The lanes contain samples of the following strains: 1, serotype A 1662 (type A-I); 2, serotype A 2100 (type A-II); 3, serotype A 4062 (type A-III); 4, serotype B 656 (type B-I); 5, serotype B 5325 (type B-II); 6, serotype D 4065 (type D-I); 7, serotype D 2153 (type D-II); 8, serotype E 4096; 9, serotype F 4679 (type F-I); 10, serotype F 4218 (type F-II).

Fig. 4. PCR-amplified *toxA* gene of selected *P. multocida* strains detected by electrophoresis in agarose 1.2% gel. The lanes contain samples of the following strains: 1, molecular size markers; 2, serotype A 1059; 3, serotype A 2305A; 4, serotype A 656; 5, serotype D 4275; 6, serotype D 2153; 7, serotype D 4886; 8, serotype D 3001; 9, serotype D 4673; 10, serotype D 5152; 11, serotype E 4096; 12, serotype F 4679; 13, untypable 2237 strain; 14, serotype A X-73 (negative control); 15, serotype D 4-4056 (positive control); 16, negative control sample without DNA; 17, molecular size markers.

diseases. All these programmes require an extensive knowledge of different serotypes prevalent in the particular region. *P. multocida* is known to be an antigenically heterogeneous pathogen that causes various septicaemic or respiratory diseases in domestic and wild animals and man [1, 2]. The heterogeneity of this species is evident from the fact that five distinct capsular serotypes [13, 18] and 16 somatic serotypes [15] have been identified. Serotyping of *P. multocida* strains by current techniques may produce ambiguous results [20].

It was reported earlier that a MAb (MT2) directed against the 32-kDa OMP (W protein) of *P. multocida* serotype D P210 may be potentially useful for

identifying serotype D strains [24]. In the present study, another MAb (MT3) was produced against the W protein of *P. multocida* 656. Results obtained with competitive ELISA and immunoblot assay showed that both MABs MT2 and MT3 were directed against two distinct epitopes of the W protein. MAB MT2 reacted with 90% of serotype D and 80% of untypable strains in dot-ELISA and immunoblot assay, whereas MAB MT3 reacted with *P. multocida* strains belonging to all serotypes. MABs MT2 and MT3 did not react with other gram-negative bacteria, suggesting that MABs MT2 and MT3 were directed against serotype and species-specific epitopes on W protein, respectively. Both MABs reacted more intensely with serotype D strains in dot-ELISA than with strains belonging to other serotypes. Seemingly, this may be due to the high expression of the 32-kDa OMP (W protein) in serotype D strains. MAB MT2 recognised both capsulate strains (4275, 4533 and 4884) and their non-capsulate variants (4275B, 4533B and 4884B) of serotype D of *P. multocida*. This observation may be potentially useful especially considering that non-capsulate or less capsulate strains cannot be identified by either IHA or the acriflavine test [17]. MAB-based rapid dot-ELISA was found to be specific, sensitive and easy to perform, for routine serotyping and epidemiological study of both capsulate and non capsulate variants of serotype D strains.

In a previous study, based on the migration of proteins H and W in SDS-PAGE and their reactivities with MABs MT1 and MT2, serotype D strains of *P. multocida* were classified into two groups (D-I and D-II). In this study, two serotype A strains reacted with MAB MT1 or MT2; however, two serotype D strains did not react with MAB MT2. To explain such discrepancy, porin H of all 55 *P. multocida* strains was compared in SDS-PAGE as well as in immunoblot assay with monoclonal and polyclonal antibodies. In view of the fact that serotype A strain 1702 bearing 37-kDa porin H reacted with MAB MT2 and serotype A strain 2305A bearing 32-kDa porin H reacted with both MABs MT1 and MT2, and the serotype D strains 4886 and 4399 bearing 36- and 39-kDa porin H, respectively, failed to react with both MABs MT1 and MT2, it is suggested that in this serotyping system based on OMP patterns, MAB MT2-reacting serotype A strains may belong to the same serotype as MAB MT2-reacting serotype D strains, whereas serotype D strains which failed to react with MAB MT2 may belong to another serotype.

Some gram-negative bacterial porins are involved in protection against infection [34]. As different OMP patterns, based on the electrophoretic mobility of porin H, were detected among *P. multocida* strains belonging to the same capsular serotype (Fig. 3), it is suggested that the strains with different OMP patterns should be incorporated in a multivalent vaccine for effective protection against pasteurellosis.

To find an eventual relationship between toxigenic strains and those carrying MABs MT1- or MT2-reacting epitopes, the present study proceeded to differentiate toxigenic and non-toxigenic strains by PCR to select the *toxA* sequence. The *toxA* gene was detected among serotype D strains, in one serotype A strain and in one untypable strain but not in strains belonging to other serotypes. As 92% of *P. multocida* strains possessing the *toxA* gene expressed the MAB MT2-reacting epitope, there seems to be a strong association between the presence of MAB MT2-reacting antigen and *toxA* gene. Kamps *et al.* [35] reported that some non-toxigenic strains of *P. multocida* might have an incomplete or highly mutated *toxA* gene. Harel *et al.* [5] showed a similarity between DNA fingerprinting of toxigenic and some non-toxigenic strains and suggested that the inability of some of the non-toxigenic strains to produce toxin may be related to subtle differences at the chromosomal DNA level.

Although serotype-specific somatic antigens of *P. multocida* have been well characterised and are considered to be lipopolysaccharide (LPS) components of the heat-stable antigens [36], serotype-specific capsular antigens of *P. multocida* have not yet been well characterised. Some investigators have demonstrated that the major antigen involved in the IHA of capsular serotyping was LPS [37], and other workers have shown that capsular polysaccharides, rather than LPS, confer serotype specificity [38]. Rimler *et al.* [39] showed that the capsule of *P. multocida* serotype A, D and F strains contain mucopolysaccharides that block antigenic determinants involved in IHA test. Recently it was shown that serotype D specific antigen of *P. multocida* may be a glycoprotein which is masked by capsule [24]. As 90% of capsular serotype D strains possessed MAB MT2-reacting epitopes which are of glycoprotein nature, it is possible that the antigen involved in the capsular serotyping system may be glycoprotein rather than LPS or capsular polysaccharide. The biological role of serotype D specific antigen has not been studied. However, the α -complex antigen of *P. multocida* serotype B described by Prince and Smith [40] was immunogenic for cattle and closely bound to the cell envelope. It probably consists of protein-polysaccharide complex [41]. A protective antigen extracted from the turkey pathogenic strain of *P. multocida* serotype A contains three protein subunits, as well as one carbohydrate band [42], or a glycoprotein-like antigen [43]. More recently, Rimler [44] reported that the membrane associated protein-carbohydrate antigens of the turkey isolate of *P. multocida* may contribute to immune cross-protection. Further studies are needed to demonstrate antigenic relationships between 32-kDa serotype D specific glycoprotein (W protein) and protective protein-polysaccharide antigens described for serotypes A and B strains of *P. multocida*.

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