Characterization of avian *Pasteurella multocida* strains with PCR-RFLP analysis of the *omp*H gene

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ABSTRACT

The 16 somatic serotype type strains and 60 field isolates of *Pasteurella multocida*, representing various avian species and geographic regions in Hungary, were characterized by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *omp*H gene with *Dra*I restriction endonuclease. The type strains yielded eight different (I-VIII) profiles. Strains, which PCR fragment was uncut by *Dra*I (profile IV) could be differentiated with *Hind*III and *Pvu*II restriction endonucleases. Five of the eight PCR-RFLP profiles (I, III, V, VI and VII) were detected among the field strains. Only limited strength of correlation was found between the classical somatic serotypes and the PCR-RFLP profiles. However, our results confirmed that molecular methods could confidently distinguish serotype A:1 strains from the other serotypes and they suggested the existence of host-specific clones within the *P. multocida* population.

INTRODUCTION

Pasteurella multocida is the aetiological agent of fowl cholera (avian pasteurellosis), a widely distributed and economically important disease of poultry, particularly affecting chickens, turkeys, ducks and geese. Several factors may influence the outcome of the disease like susceptibility of the various avian hosts, presence of different predisposing factors in the flocks and pathogenicity of *P. multocida* strains.

Virulence of *P. multocida* is defined by various cell-surface expressed components. The most important of them are polysaccharide capsule and typical constituents of the outer membrane of the cell wall such as lipopolysaccharides (LPS), a limited number of major proteins, and several minor proteins presented in very high copy numbers. During the infection, these polysaccharides help the bacterium to avoid the innate immune mechanisms of the host like (phagocytosis or complement-mediated killing. On the other hand, as antigenic determinants, they stimulate antibody production of the adaptive immune system (Kubatzky, 2012). Based on their immunospecificity the strains can traditionally be classified into five capsular serogroups (A, B, D, E, and F) and sixteen somatic serotypes (1-16). The exact role of the outer membrane proteins (OMPs) in the infection is unclear but they may be mediators of bacterial interaction with the host environment (Lin et al., 2002). They could modulate the cytokine production of host's innate immune cells. Consequently, alteration in these surface structures may influence the immunogenicity of the strains through increasing antigenic variability.

The OMP patterns of the avian *P. multocida* strains are very diverse. The principal members of these profiles are the heat-modifiable OmpA and OmpH porin

proteins. Davies et al. (2003) demonstrated that the OmpH protein is more heterogeneous than OmpA. The sequence analysis of the *omp*H gene verified the increased diversity of the porin protein and revealed major variations in two discrete regions encoding large external loops that presumably interact with the host immune system (Luo et al., 1999). PCR-RFLP (restriction fragment length polymorphism) is widely applied for analysing polymorphism within a gene segment. Anthony et al. (2007) successfully used digestion of the amplified products of *omp*H-PCR with *Dra*I restriction endonuclease for the differentiation of *P. multocida* serotypes A:1, A:3, and B:2.

Our aim was to extend this technique to further *P. multocida* serotypes and apply it for the characterisation of our avian *P. multocida* strains.

MATERIAL and METHODS

Bacterial strains

Sixty strains of *P. multocida* were examined: 16 from geese, 20 from domesticated ducks (Anas species), 2 from Muscovy or Barbary ducks (*Cairina moschata*), 15 from turkeys, 4 from chickens and 3 from pheasants. The strains were collected from commercial poultry flocks affected by fowl cholera, and they represented various geographic regions in Hungary. The biovars, capsular serogroups and ERIC-PCR (enterobacterial repetitive intergenic consensus) analysis of the strains were described earlier (Sellyei et al., 2008). The reference strains of Heddleston serotypes 1-16 (X-73, M1404, P-1059, P-1662, P-1702, P-2192, P-1997, P-1581, P-2095, P-2100, P-903, P-1573, P-1591, P-2225, P-2237, and P-2723,

respectively) were kindly provided by Dr. R. B. Rimler (National Animal Disease Center, Ames, IA, USA).

Gel diffusion precipitin test

The somatic serotype of the strains was determined by the agar-gel immunodiffusion test (Heddleston, 1972).

DNA preparation

The strains were inoculated on Columbia agar (Nebotrade, Biatorbágy, Hungary) supplemented with 5% sheep blood and incubated under aerobic conditions at 37°C for 24 hours. Genomic DNA was extracted using the modified Chelex method as described previously (Sellyei et al., 2010). The DNA was stored at -20 °C until used.

The ompH gene polymorphism

The *ompH* gene polymorphism was examined by PCR-RFLP analysis. Approximately 1000 bp (base pair) fragment was amplified using primers: ompH forward 5'-GCG TTT CAT TCA AAG CAT CTC-3' and OmpH reverse 5'-ATG ACC GCG TAA CGA CTT TC -3' described by Antony et al. (2007). The 50µl PCR mixture containing: 5µl prepareted DNA, 1 x PCR puffer (with 2mM $MgCl_2$), 200µM dNTP-mix, 1U Dream Taq DNA polymerase (Fermentas Inc., Burlington, Canada), and 10 pmol of each primer. The PCR was performed on a Swift Mini Thermal Cycler (Esco Technologies, Inc., Hartboro, USA) with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 15 sec, annealing 56 °C

for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplified PCR products were purified with absolute ethanol and digested with either *Dra*I *or Pvu*II and *Hin*dIII restriction endonucleases in the supplied buffers according to the manufacturer's protocols (Fermentas Inc., Burlington, Canada). RFLP products were separated on a 2.0% agarose gels in 1XTBE buffer at 5 V/cm for 2 hours and 20 minutes, stained with ethidium bromide and then analysed under UV illumination by visual inspection of the band patterns. Lengths of PCR fragments were verified by a 100 bp DNA ladder (Fermentas Inc., Burlington, Canada).

RESULTS and DISCUSSION

Porin H is a surface-exposed major OMP in the envelope of *P. multocida* that is consequently presented in several serotypes (Luo et al., 1999). Its role in immunogenicity and pathogenicity was demonstrated both in mice (Tan et al., 2010) and in chickens (Shitmatee et al., 2008). The molecular mass and the sequence heterogeneity of the OmpH protein and its encoding gene suggest that it is a subject of diversifying antigenic variation generated by selection within the host playing an important role in host-pathogen interaction (Davies et al., 2004).

In the current study, using the formerly published PCR primers and conditions (Antony et al. 2007), we successfully amplified approximately 90% of the *omp*H gene from all *P. multocida* somatic serotype type strains. *Dra*I restriction endonuclease analysis of the PCR amplified products revealed eight distinct profiles designated I – VIII (Fig. 1). Table 1 shows the correlation between these profiles and the somatic serotypes. In three cases one PCR-RFLP profile corresponded to one distinct serotype (II=2, III=3, VIII=11) while in the case of four PCR-RFLP profiles

each pattern represented two serotypes (I=1,14, V=6,7, VI=8,13, VII=10,12). *Dra*I did not cut the amplicons of the type strains of five serotypes (4, 5, 9, 15 and 16) forming a separate group (profile IV). However, these serotypes could be divided into two groups digesting their amplicons with *Hin*dIII and *Pvu*II restriction endonucleases (Fig. 2). Fragments of serotype 4 and 9 could be differentiated with *Hind*III restriction endonuclease (app. 750bp, and 220bp = profile IVA) from serotype 5, 15 and 16 that possessed restriction site of *Pvu*II restriction endonuclease (app. 800bp, and 160bp = profile IVB).

Five of the eight PCR-RFLP profiles (I, III, V, VI, and VII) were detected in the 60 *P. multocida* strains isolated from poultry in Hungary and characterized phenotypically and genotypically in a previous study (Sellyei et al., 2010). Table 2 shows some details of the studied strains while Fig. 3 demonstrates the PCR-RFLP profiles on a number of representative strains. More than half of the strains (32) showed PCR-RFLP pattern I. All of them represented serotype A:1. One third of the strains (21) belonged to profile III. However, their antigenic features were remarkably diverse. This group contained mostly serotype A:3 strains (15) but serotype A:3,4 (1), A:4,5 (4) and F:1 (1) strains were also included indicating that other characteristics than the somatic serotype must be involved in the determination of the PCR-RFLP profile. Enlightening the possible effect of the presence of multiple serotypes on the PCR-RFLP profile needs further examinations. The strains in these two dominant groups had a rather wide host-range (duck, goose, turkey, chicken, pheasant, and Muscovy duck), however, no correlation was found between the profiles and the host origin in these groups. On the other hand, the other PCR-RFLP patterns (V, VI, and

VII) were only present in strains isolated from turkeys. Moreover, each profile represented only one serotype (V=A:3, VI=A:4,5, and VII=F:10).

Our results demonstrated, in agreement with the study of Jabbari and Esmaelizadeh (2005), that the PCR-RFLP on the *omp*H gene using *Dra*I restriction endonuclease could be useful for characterization of *P. multocida*. However, these RFLP profiles represent only limited strength of correlation with the somatic serotypes of the strains from the field. Nevertheless, our observations support the possibility of the recognition of serotype A:1 strains by molecular methods since the cross-reactive serotype 14 seems very rare in nature. Furthermore, the results with strains isolated from turkeys suggest the existence of host-specific clones within the *P. multocida* population. Concerning the future, we hypothesize that precise recognition of the diversity of surface-exposed proteins and LPS (Harper et a., 2011) is required for developing a practically feasible molecular serotyping of *P. multocida*.

Acknowledgement

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Table 1

PCR-RFLP profiles (I-VIII) on the studied *omp*H gene fragment of the 16 somatic serotype type strains using *Dra*I restriction endonuclease

PCR RFLP type	serotype	PCR size (bp)	profile (bp)
I	1, 14	998	337, 214, 216, 131
II	2	930	337, 306, 287
III	3	968	337, 317, 314
IV	4, 5, 9, 15, 16	968, 962, 972, 953, 962	—
V	6, 7	968	631, 337
VI	8, 13	986	649, 329, (8)
VII	10, 12	983	693, 290
VIII	11	956	337, 314, 305

Table 2

List of *P. multocida* strains used in the current study

RFLP profile	ID	Host	Place	Capsule	Serotype	ERIC
I	P61/1	goose	Fülöpszállás	A	1	III/4b
I	P131[(1)]	chicken	Gyöngyös	A	1	II
I	P236[(2)]	goose	Újléta	A	1	III/4a
I	P237[(3)]	turkey	Debrecen	A	1	IV
I	P239	goose	Debrecen	A	1	III/4a
I	P244	goose	Baktalórántháza	A	1	III/4a
I	P327	goose	Mikofalva	A	1	III/4a
I	P372	duck	Csólyospálos	A	1	III/4b
I	P373	duck	Csólyospálos	A	1	III/4b
I	P378	goose	Döge	A	1	III/1
I	P388	duck	Kiskunmajsa	A	1	III/1
I	P418	duck	Sükösd	A	1	III/4b
I	P473	goose	Kiskunmajsa	A	1	III/1
I	P485/9	duck	Szank	A	1	III/1
I	P486/1	duck	Petőfiszállás	A	1	III/4b
I	P487/1	duck	Petőfiszállás	A	1	III/4b
I	P487/2	duck	Petőfiszállás	A	1	III/1
I	P488	duck	Fülöpjakab	A	1	III/4b
I	P489	goose	Kiskunmajsa	A	1	III/4b
I	P492/4	duck	Kiskunmajsa	A	1	III/4b
I	P493/1	duck	Kecel	A	1	III/4b
I	P493/2	duck	Kecel	A	1	III/4b
I	P495	duck	Bócsa	A	1	III/1
I	P519/1	duck	Bócsa	A	1	III/1
I	P520/4[(4)]	duck	Kelebia	A	1	III/1
I	P522/1	duck	Tázlár	A	1	III/1
I	P538/1	duck	Pusztavacs	A	1	III/1
I	P547/1	duck	Budapest	A	1	III/1
I	P553/1	chicken	Ásotthalom	A	1	IV
I	P553/2	chicken	Ásotthalom	A	1	IV
I	P555/2	duck	Bócsa	A	1	III/4a
I	P559	duck	Gyula	A	1	III/4b

Table 2 (cont.)

III	P428[(10)]	Muskovy	Füzesgyarmat	F	1	I
III	P325	goose	Hortobágy	A	3	III/2
III	P331	goose	Hortobágy	A	3	III/2
III	P358	turkey	Hortobágy	A	3	IV
III	P359	turkey	Hortobágy	A	3	IV
III	P362	chicken	Hortobágy	A	3	IV
III	P391	goose	Balmazújváros	A	3	III/3
III	P392	goose	Nyírmártonfalva	A	3	III/3
III	P403	goose		A	3	III/3
III	P415[(5)]	turkey	Beled	A	3	II
III	P432[(6)]	goose	Tázlár	A	3	III/2
III	P440	goose	Balotaszállás	A	3	III/1
III	P471/1	turkey	Szombathely	A	3	IV
III	P471/3	turkey	Szombathely	A	3	II
III	P568/1	turkey	Rém	A	3	IV
III	P568/2	turkey	Rém	A	3	IV
III	P414[(9)]	Muskovy	Füzesgyarmat	A	3,4	I
III	P329[(7)]	goose	Hortobágy	A	4,5	III/2
III	P369	pheasant	Szombathely	A	4,5	II
III	P370[(8)]	pheasant	Szombathely	A	4,5	II
III	P371	pheasant	Szombathely	A	4,5	II
V	P531[(11)]	turkey	Dunatetétlen	A	3	II
V	P558/1[(12)]	turkey	Csólyospálos	A	3	IV
V	P558/2	turkey	Csólyospálos	A	3	IV
V	P558/3	turkey	Csólyospálos	A	3	IV
VI	P577[(13)]	turkey	Érsekcsanád	F	10	IV
VII	P339[(14)]	turkey	Bátya	F	4,5,(7)	IV
VII	P360[(15)]	turkey	Hortobágy	F	4,5,(7)	IV

Uppercase numbers in brackets shows the line number on Fig. 3

Figure 1

The RFLP patterns (I-VIII) on the app. 1000 bp long *omp*H gene PCR-fragment of *P. multocida* somatic serotype type strains (1-16) using *Dra*I restriction endonuclease. M – 100bp molecule marker (Fermentas Inc., Burlington, Canada)

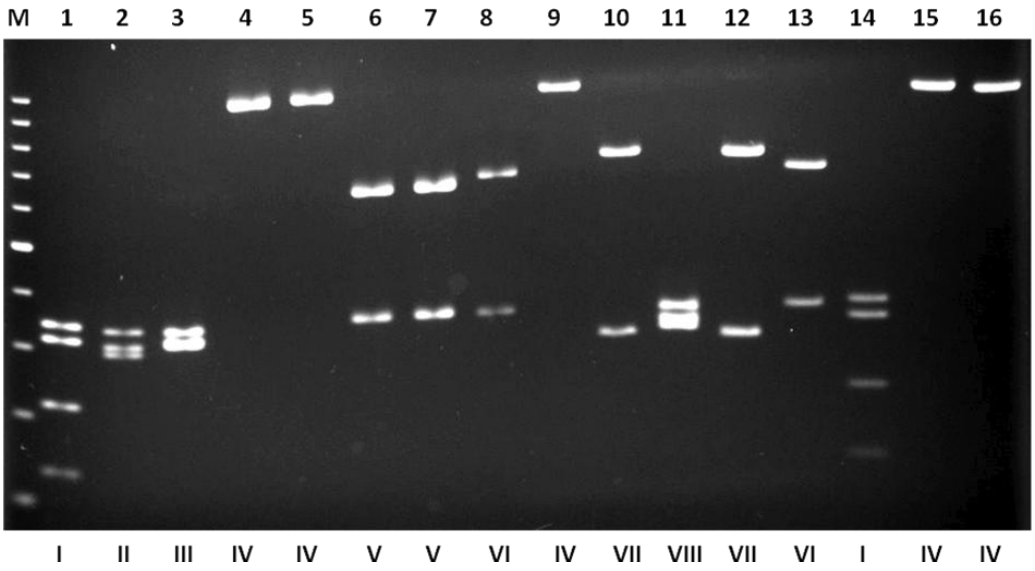

Figure 2

The RFLP profiles of the app. 1000 bp long *omp*H gene *Dra*I-uncut PCR-fragment of *P. multocida* serotype 4, 5, 9, 15, and 16 strains with *Hin*dIII and *Pvu*II restriction endonucleases. M – 100bp molecule marker (Fermentas Inc., Burlington, Canada)

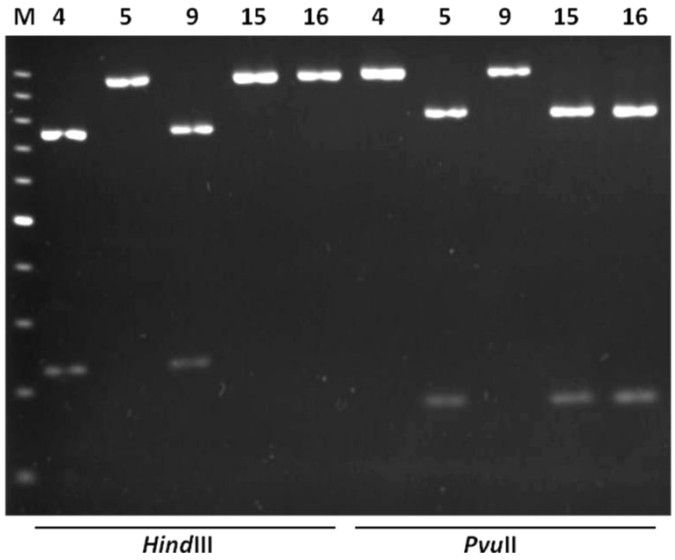

Figure 3

The RFLP patterns on the app. 1000 bp long *omp*H gene PCR-fragment of 15 representative avian *P. multocida* strains (Table 2) using *Dra*I restriction endonuclease. M – 100bp molecule marker (Fermentas Inc., Burlington, Canada)