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Original Article

Genotyping of *Pasteurella multocida* ovine and bovine isolates from Iran based on PCR-RFLP of *omp*H gene

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ABSTRACT

Pasteurella multocida (P. multocida), A Gram-negative facultative anaerobic bacterium, is a causative animal pathogen in porcine atrophic rhinitis and avian fowl cholera. The outer membrane of Gram-negative bacteria contains of many different protein in very high copy numbers. One of the major outer membrane, the H proteins have functional as high immunogenicity and antigenicity. In this study to increase information about epidemiology of ovine and bovine *P. multocida*, the 24 isolates from sheep and nine isolates from cattle were investigated by PCR-RFLP analysis of the *ompH* gene. In all 33 isolates, digestion of the amplified fragment of *ompH* gene by using *Eco*RI, *cfo*I and *Hind*III produced 3, 5 and 3 different restriction patterns respectively. Sixteen RFLP patterns were found among 33 investigated *P.multocida* isolates. This study showed that, the PCR RFLP based on *ompH* gene is potentially a useful method for typing of *P. multocida* isolates from sheep and cattle. The RFLP patterns of this gene exhibited extensive restriction site heterogeneity, which may be particularly suitable for fingerprinting of *P. multocida* isolates. Considering *ompH* protein as a protective immunogenic moiety of *P. multocida*, the results of this study showed a heterogenic bacteria and this means the possibility to produce a multivalent vaccine to be protective against diseases caused by this organism in sheep and cattle in Iran.

Keywords: *Pasteurella multocida, omp*H, PCR-RFLP

INTRODUCTION

P. multocida has been recognized as an important Veterinary pathogen for over a century. The bacteria is the etiologic agent of fowl cholera, a highly contagious and sever disease of poultry causing significant mortality, morbidity and commensal in the upper respiratory tract in both wild and domestic animals, including fowl cholera in poultry, haemorrhagic septicemia in cattle and buffalo, atrophic rhinitis in swine, and snuffles in rabbits (Abrahante *et al* 2013, Antony *et al* 2007, Lugtenberg *et al* 1984, Shayegh & Dolgari Sharaf 2009). The organism also causes a wide range of important diseases in domestic animals, being responsible for pneumonia in cattle and sheep (Chanter & Rutter 1989, Frank 1989). Pathogenesis is a result of complex interactions between specific host factors and specific bacterial virulence factors (Hatfaludi *et al* 2010). Some of virulence factors of *P. multocida* and antigens potentially served as vaccine candidates such

as outer membrane and porin proteins, type 4 fimbriae, a filamentous hemagglutinin, neuraminidases, iron acquisition related factors, dermonecrotoxin, and superoxide dismutase (Ewers *et al* 2006). Outer membrane protein H (OmpH), the major outer membrane protein has a role in immunogenicity and pathogenicity *of P. multocida* isolates (Luo *et al* 1999, Townsend *et al* 1998). Some studies have shown a high degree of heterogeneity among avian *P. multocida* isolates (Gunawardana *et al* 2000, Jabbari 2005). Jabbari (2005) suggested a classification method based on *omp*H gene used PCR-RFLP analysis. The result of mentioned study showed that the PCR-RFLP of the *omp*H gene is potentially a useful method for typing of *P. multocida* for studying the epidemiology of *P. multocida* infections in avian isolates. The aim of this study was to apply PCR-RFLP analysis of the *omp*H gene for discrimination and classification of ovine and bovine ones.

MATERIALS AND METHODS

Isolates. A total of 33 field isolates of *P. multocida* from sheep (N=24) and cattle (N=9) with respiratory diseases from enedemic areas were included in the study. The reference strains of *P.multocida* used in the study were PMI30 (capsular serogroupA) and PMI25 (capsular serogroup B) obtained from Aerobic Bacterial Vaccines Department, Razi Institute. All of the isolates have been already confirmed as *P. multocida* and their capsular types were determined by PCR (Rimler and Rhoades, 1987) (Table 1). The bacterial strains were grown at 37°C on blood agar containing 5% of sheep blood and Brain heart agar.

DNA extraction and PCR amplification of *omp*H gene. The isolates were confirmed as *P. multocida* by means of PCR using species-specific primers (Townsend *et al* 2001). All isolates were cultured in BHI broth at 37 °C for 24 to 48 h, then 500 µl of each samples were transferred to a clean micro-tube and centrifuged at 5,000 rpm for 15 min. The pellet was washed with PBS three times. In the next step, 50 µl PCR buffer was added and boiled for 20 min. All tubes were put in ice immediately and centrifuged at 13,000 rpm for 5 min. supernatant was stored as DNA template (Shayegh & Dolgari Sharaf 2009). Amplification of the *omp*H encoding gene (*omp*H) from the genomic DNA preparations of each isolate were performed with the *omp*H specific primers which were described by Luo et al. (1999) as follows:

FWD 5´- ACTATGAAAAAGACAATGGTAG -3

REV 5´- GATCCATTCCTTGCAACATATT -3´

For PCR reactions, 5 µl of extracted DNA were taken as template DNA and were added to the reaction mixture (50 µl), containing 1 µl of each primer, 200 µM from the four dNTPs, 5 µl of 10 x PCR buffer, 1.5 µl of 50 mM $MgCl_2$ and 1 µl of Taq DNA polymerase.

Table 1: The isolates used in this study, which have been already confirmed by multiplex PCR as *P. multocida* and their capsular types.

No.	Strain code	*Pasteurella multocida ssp*	Host	Capsular type
1	PM 087	*Septica*	Sheep	A
2	PM 078	*Multocida*	Sheep	Untyped
3	PM 084	*Septica*	Sheep	A
4	PM 0105	*Septica*	Sheep	A
5	PM 02	*Multocida*	Cattle	B
6	PM 08	*Multocida*	Cattle	A
7	PM 054	*Septica*	Sheep	A
8	PM 0101	*Septica*	Sheep	A
9	PM 010	*Multocida*	Sheep	A
10	PM 080	*Septica*	Sheep	A
11	PM 053	*Septica*	Sheep	A
12	PM 069	*Septica*	Sheep	A
13	PM 0103	*Septica*	Sheep	A
14	PM 0104	*Septica*	Sheep	A
15	PM 0102	*Septica*	Sheep	A
16	PM 02141	*Multocida*	Cattle	Untyped
17	PM 02139	*Multocida*	Cattle	B
18	PM 02134	*Multocida*	Cattle	B
19	PM 02136	*Multocida*	Cattle	B
20	PM 02142	*multocida*	Cattle	B
21	PM 0385	*multocida*	Sheep	A
22	PM 01307	*multocida*	Sheep	A
23	PM 0030	*multocida*	Sheep	A
24	PM 0317	*multocida*	Sheep	A
25	PM 051	*multocida*	Sheep	A
26	PM vaccine A	*multocida*	Sheep	A
27	PM vaccine B	*multocida*	Cattle	B
28	PM 098	*septica*	Sheep	A
29	PM 091	*multocida*	Sheep	B
30	PM 058	*multocida*	Sheep	A
31	PM 092	*multocida*	Sheep	A
32	PM 059	multocida	Sheep	A
33	PM 027	multocida	Cattle	B

The samples were subjected to 35 cycles in a thermal cycler. Each cycle consisted of DNA denaturation at 93

°C for 60 sec, annealing at 51°C for 60 sec, and extension at 72 °C for 60 sec. The cycles were preceded and followed by an initial denaturation at 93°C for 5 min and final extension at 72 °C for 10 min, respectively. Amplified products were analyzed by gel electrophoresis on a 1.5 % agarose gel, stained with ethidium bromide, and photographed at UV exposure (Tan *et al* 2010).

Restriction fragment length polymorphism (RFLP) analysis. RFLP was carried out on DNA fragments amplified with primer pairs for *omp*H. Target fragment was individually digested with the three restriction enzymes. 20 µl of PCR product with 500 ng/µl DNA concentration were used for restriction. The reaction mixture had a final volume of 30 µl containing 1µl of each enzymes included *Eco*RI, *Cfo*I and *Hind*III Tango Buffer. The reaction mixture was incubated at 37°C for overnight. Restriction fragments were separated by electrophoresis on 2% agarose gel in 1X TAE buffer at 80 V for 1 h. Restriction fragments were visualized and documented as described previously.

RESULTS

Results of PCR-RFLP typing of *P. multocida* isolates are presented in Table 2.

Table 2: Results of PCR-RFLP typing of *P. multocida* isolates

Endonuclease	Name of profile	Size of fragments
*Eco*RI	a	900bp,300 bp
	b	1200bp
	c	700bp,500 bp
*Hind*III	d	700bp,500 bp
	e	1200bp
	f	1100bp,100 bp
*Cfo*I	h	800bp,400 bp
	j	700bp,500 bp
	k	1000bp,200 bp
	l	600bp,400 bp,200 bp
	m	1200bp

The Polymerase chain reaction with *ompH* primers amplified a fragment of 1.2 kb. In all 33 isolates, digestion with *Eco*RI divided the isolates into 3 profiles including a, b and c (Figure 1).Three patterns of d, e and f were produced by HinIII digestion (Figure 2). *Cfo*I showed 5 profiles named h, j, k, l and m (Figure 3). Combination of different restriction patterns of EcoRI, HindIII and CfoI divided 33 P. multocida isolates into sixteen RFLP types (Table 3). The Combination of different restriction patterns discriminated 33 isolated of *P. multocida* into16 RFLP types according to Table 3.

Table 3. Combination of different restriction patterns of *Eco*RI, *Hind*III and *Cfo*I among 33 isolated of *P. multocida*.

RFLP Type	*Eco*RI	*Hind*III	*Cfo*I	Isolates
I	d	c	l	PM098- PM010- PM08
II	e	c	h	PM091- PM0317
III	d	a	l	PM054- PM084- PM078- PM0102- PM0103
IV	e	c	j	PM0101- PM051- PM0030- PM01307- PM0105
V	d	c	j	PM A- PM B
vI	e	b	h	PM092
VII	d	a	k	PM087
VIII	e	b	k	PM069
IX	d	b	h	PM0104
X	f	c	l	PM02- PM053
XI	d	a	m	PM059
XII	d	b	j	PM080
XIII	d	c	h	PM027
XIV	e	c	m	PM0385
XV	d	a	h	PM058- PM02141
XVI	d	a	j	PM02142- PM02134- PM02139- PM02136

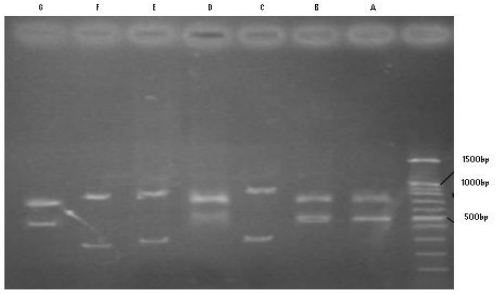

Figure 1. The DNA patterns results of isolates after digestion with *Eco*RI enzyme (The pattern A: C, E, F and The pattern C: G, A, B, D)

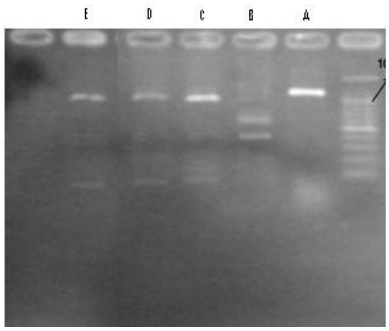

Figure 2. The DNA patterns results of isolates after digestion with *Hind*III enzymes The PatternF: E, D, C The patternD: B and patternE: Undigest

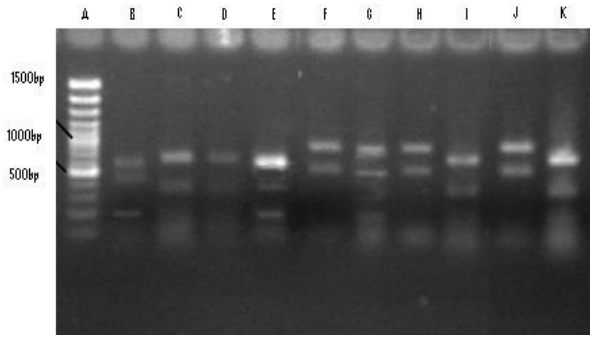

Figure 3. The DNA patterns results of isolates after digestion with *Cfo*I enzyme (The Patternl: B,E The Patternj: H, J The Pattern h:C,D,I,K The Pattern m:undigest)

Phylogenetic relationshio of the field isolates and vaccinal strains of *P. multocida* is shown in figure 4.

DISCUSSION

During the last decade several molecular techniques have been developed and supplemented for *P. multocida* typing such as restriction endonuclease analysis (REA), Ribotyping, Pulsed-field gel electrophoresis (PFGE) and PCR based fingerprinting (Hunt *et al* 2000). Among PCR based fingerprintings, PCR-RFLP based on *ompH* gene is more important for immunological or vaccinal studies, because *ompH* is one of the important protective proteins in these bacteria. Many gram-negative bacteria have one or more predominanat outer membrane proteins and these proteins have been shown to play essential role in host pathogen interaction and disease processes such as *ompA* and *ompH* (Davies *et al* 2003). In the previous studies *ompH* was known as H porin that possessed both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface (Shayegh & Dolgari Sharaf 2009). However role of *ompH* as a protective antigen has been identified against homologous infection. The immunogenicity and virulence activities of protein H has been demonstrated previously. (Lubke *et al* 1994) showed that monoclonal antibodies produced against protein H inhibited binding of P. multocida organisms to respiratory system (Lubke *et al* 1994, Townsend *et al* 1998). Additionally, variations in the molecular mass of *ompH* among different *P. multocida* strains have also been reported. PCR analysis based on RFLP in *ompH* gene was used successfully for genetic classification of avian *P. multocida* isolates by (Jabbari 2005). The RFLP patterns of this gene exhibited extensive restriction site heterogeneity, which may be particularly suitable for fingerprinting of *P. multocida* isolates. The present paper represents the first report of PCR RFLP based on ompH gene in ovine and bovine isolate. In this study, A high degree of heterogencity was shown among ovine and bovine *P. multocida* isolates compared with similar study among avian ones. In this study, 33 isolates of *P. multocida* was classified into 16 groups of RFLP based on *ompH* gene by using three restriction enzymes *EcoR*I, *Hind*III *and Cfo*I. Comparing with previous study (Jabbari 2005) on avian *P.multocida* strains. The ovine isolates showed more genetic diversity. It was found that the A and B vaccine strains were distinct from other species tested and formed a distinct RFLP group that reflected genetic differences among field isolates and vaccinal strains. Although there was a considerable genetic diversity among the strains examined with different host, the exact relationship between RFLP type and phenotype characters such as diversity diseases in animal, pathogenecity and immunogenecity remains to be determined. Considering *ompH* protein as a protective immunogenic moiety of *P. multocida*, the results of this study showed a heterogenic bacteria and this means the possibility to produce a multivalent vaccine to be a vast protective effect against disease caused by this agent in sheep and cattle in Iran. On the other hand so more

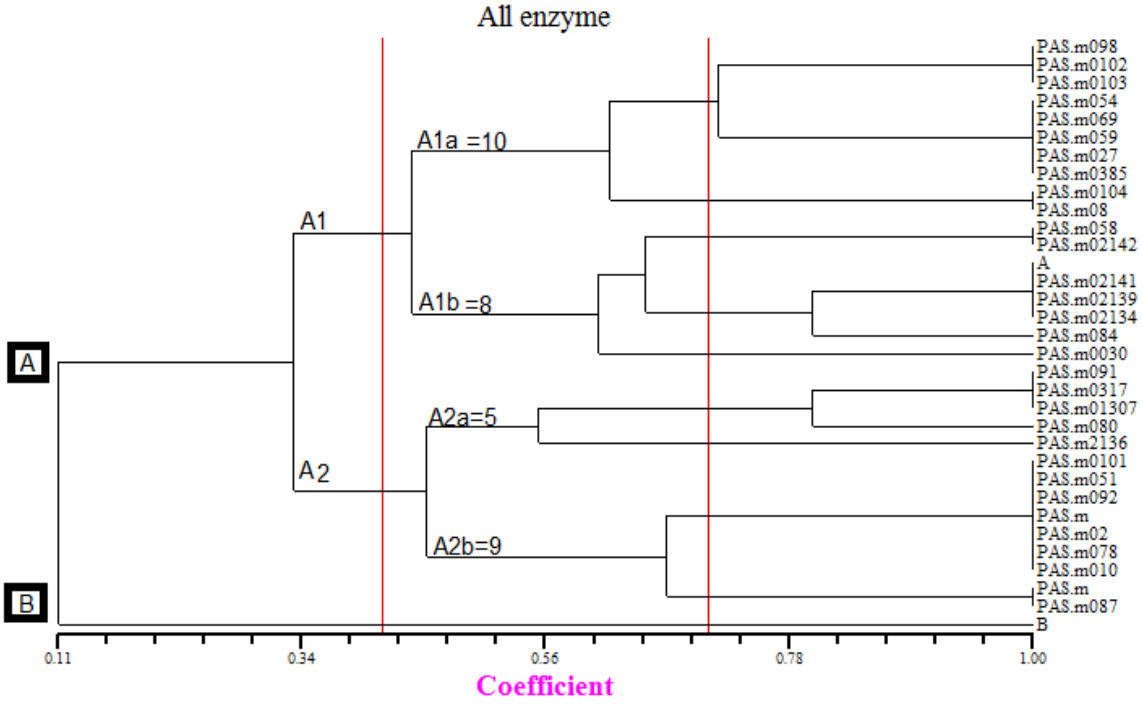

Figure 4. Dendrogram of genetic distance 16 RFLP_types for ovine and bovine *P. multocida* isolates.

studies involving *ompH* have been demonstrared to induce protective immunity in animal models against *p. multocida* and can be attractive vaccine candidates for heterologous immunity againts Gram-negative bacterial infection. However one of important notice that we follow this study, was development a subunit and recombinant vaccine (Lee *et al* 2007, Rhoades & Rimler 1992, Shayegh & Dolgari Sharaf 2009).

Our study showed that PCR-RFLP is useful epidemiological tool identifying of *p. multocida* serovars with using three different restriction endonuclease enzymes and concluded that PCR-RFLP was a rapid test and could be done for *p. multocida* species but until now no PCR-RFLP based techniques have been used for detection somatic serotyping of *p. multocida*. Hence one of the purpouse in present study was undertaken to develop a simple DNA based typing scheme.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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