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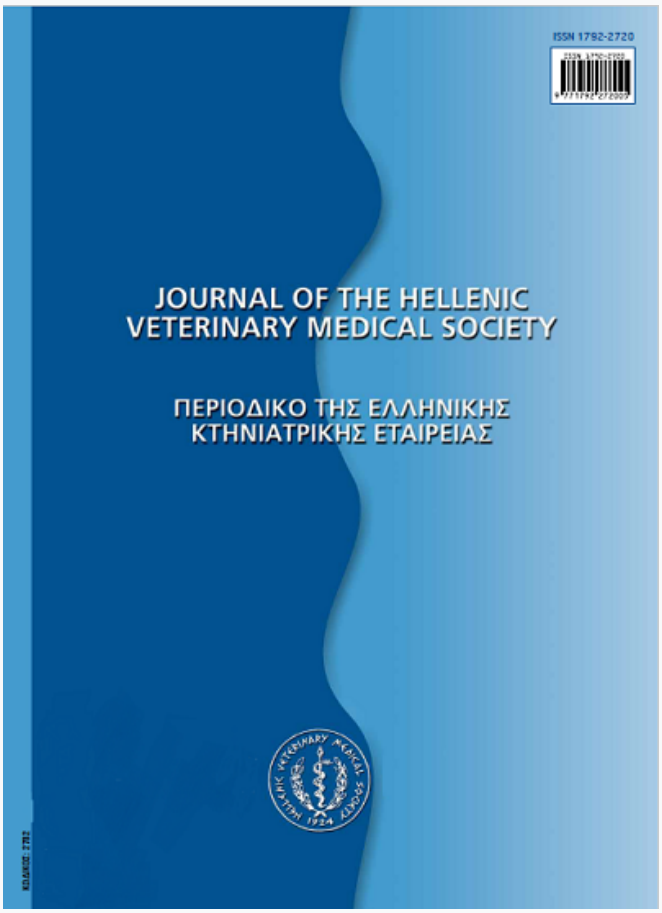

Polymorphism of ompH gene of Pasteurella multocida serotype A strains isolated in Iran

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Research article
Ερευνητικό άρθρο

Polymorphism of ompH gene of *Pasteurella multocida* serotype A strains isolated in Iran

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ABSTRACT. One of the most frequent causes of respiratory infection and death in sheep and goats is *Pasteurella multocida*. In humans, it has been associated with diseases of the respiratory tracts, arthritis, osteomyelitis and meningitis. Outer membrane protein H (OmpH) has a role in immunogenicity and pathogenicity of *P. multocida.* The aim of this study was to characterize the genetic diversity of ompH gene of a panel of *P. multocida* serotype A strains isolated in sheep. Forty *P. multocida* serotype A strains isolated in previous study were selected and analyzed by restriction fragment length polymorphism (RFLP) of a species-specific PCR assay. RFLP amplified fragment produced five different cleavage patterns. On the basis of combinations resulting from ompH gene digestion, the 40 *P. multocida* isolates were classified in six RFLP type. It seems that isolates with variants genetic profile represent different pathogenecity. New vaccine formulation should consider multivariants of *P. multocida* in order to confer a wider protection.

Keywords: *Pasteurella multocida*; RFLP-PCR; ompH gene.

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INTRODUCTION

Pasteurella multocida, a gram-negative facultative bacterium, is one of the notorious animal pathogen causing widespread infections in various domestic animals; snuffles in rabbits, pneumonia and haemorrhagic septicaemia in cattles, sheep and goats, fowl cholera in chickens and atrophic rhinitis in pigs (Lee et al., 2007). Sheep pasteurellosis is one of the most common infectious and economically important bacterial diseases which occur in temperate and subtropical areas (Prabhakar et al., 2010; Sahragard 2016). *P. multocida* is an endemic disease in Iran such as West Azarbaijan, Mazandaran, Gilan, Khozestan and Fars Provinces (Tabatabaei *et al* 2002, Shayegh *et al* 2009, Mirghafari 2016). In Iran, as in many other countries, strains of *P. multocida* have frequently been isolated from sheep and goats, and they represent a significant cause of outbreaks of respiratory infections (Danesh 2013).

Virulence factors of *P. multocida* are defined by various cell-surface expressed components. Among these, the most important 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 (Miguel et al., 2014). On the other hand, as antigenic determinants, they stimulate antibody production of the adaptive immune system (Sellyei et al., 2012). Studies utilizing outer membrane proteins (OMPs) of Gram negative bacteria indicated OMPs as protective immunogens that could play an important role in bacterial adherence and invasion (Singh et al., 2011). Protein H, or porin H, is the major outer membrane protein in the envelope of *P. multocida* (Luo et al., 1997). These proteins are at the interface between pathogen and host and are subject to various selective pressures depending on their function (Davies et al., 2003). Consequently, OMPs exhibit varying degrees of inter-strain heterogeneity and this can be used to assess intra-species diversity and determine epidemiological relationships (Chang et al., 2012). Outer membrane protein H (OmpH) gene encoded the major outer membrane protein that has a role in immunogenicity and pathogenicity of *P. multocida* isolates in mice, (Tan et al., 2010). ompH is one such major protein in the envelope of *P. multocida* that has been purified and characterized as a porin (Singh et al., 2011). In fact it is structurally and functionally related to the super family of porins of Gram negative bacteria (Luo et al., 1997). The sequence analysis of the ompH gene demonstrated 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 (Sellyei et al., 2012). OmpH as well as ompA proteins show considerable heterogeneity, and at least among avian *P. multocida* strains, a number of different variants appear to be associated with certain capsular serotypes (B, D, or F) (Davies et al., 2003; Williams et al., 1990).

The infectious serogroups of *P. multocida* associated with outbreaks of pneumonic pasteurellosis in

Table 1. Primer and Sequence used in the present study.

Primer	Gene	Name	Sequence	Amplicon size (bp)
A l lPASS	KMT1	KMT1T7	ATCCGCGATTTACCCAGTGG	460
		KMTSP6	GCTGTAAACGAACTCGCCAC	
Outer membrane protein	OMPH	ompH -FWD	ACTATGAAAAAGACAATGGTAG	1200
		ompH -RWD	GATCCATTCCTTGCAACTTATT	

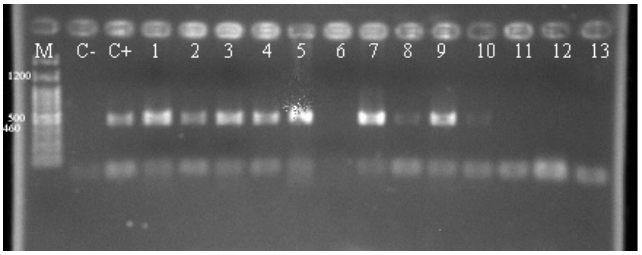
sheep and goats (A and D) have been incriminated as both primary and secondary agents of pneumonia (Zamrisaad et al., 1996; Sahragard et al., 2011; Danesh 2013). Our findings support the need for the development of a multivariant vaccine using the prevalent *P. multocida* serotype circulating in Iran as well as strategic deworming, and improved housing conditions for sheep and goats (Tahamtan 2014). But before that, classification and ranking of many *P. multocida* isolated is necessary to identify different variants.

There are several classifications of *P. multocida* based on the pathogenesis, mortality and genetic diversity in the animal models. But because of complex pathogenesis of *P. multocida* and interaction between host and bacterium, the past classification did not provide more information about that (Harper et al., 2006; Tahamtan and Mirghafari, 2016). Recent molecular techniques, especially restriction fragment length polymorphism (PCR –RFLP), are the most effective methods for identification and classification of genetic variation in bacterial animal isolates (Tshikhudo, 2013; La et al., 2006). PCR-RFLP is widely applied for analyzing polymorphism within a gene segment (Sellyei et al., 2012). Therefore, the aim of this study was to apply PCR-RFLP analysis to the ompH gene to characterize the genetic variation of local *Pasteurella* isolates from sheep.

MATERIALS AND METHODS

Sample: Fourty *P. multocida* serotype A strains collected in previous study were isolated from sheep with respiratory diseases, in different parts of Fars province, Iran (Tahamtan and Mirghafari, 2016).

Fig 1. PCR products of 460 bp resulting after amplification of all pass primers. M: molecular size marker; C-: negative control; C+: positive control; numbers (1-5 & 7-10): some positive samples; numbers (6, 11, 12 & 13): some negative samples.



PCR

DNA extraction and PCRs

DNA extraction was carried out according to previous study (Tahamtan et al., 2016; Jabbari et al., 2005). Briefly, bacterial cells were lysed by EDTA (0.5 M), SDS (0.5%) and proteinase K (20 ng/ml) followed by phenol-choloroform- isoamyl alcohol extraction, chloroform/ isoamyl alcohol (24:24:1) mixture. Genomic DNA was precipitated by addition of sodium acetate and absolute ethanol. Then ethanol 70% was added and after dried at room temperature, re-suspended in TE buffer (pH 8) (Jabbari et al., 2005). PCR was performed in a thermal cycler (Master Gradient Eppendorf, Germany). PCR mixture contained 3 µL of 1× PCR buffer, 3 µL of deoxyribonucleotide triphosphate (dNTP) blend (2.5 mM each dNTP), 2 µL of primers (10 picomole each) (Table 1). One µL of template DNA, and 0.2 µL of high fidelity Taq DNA polymerase were added with distilled water to reach 25 µL. One kb DNA marker (Fermantase) was used. The PCR conditions were: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 68°C for 6 min, and 1 cycle of 25°C for 1 min (Tsai et al, 2011). The isolates from sheep were identified as ShI 1 - ShI 40.

PCR-RFLP

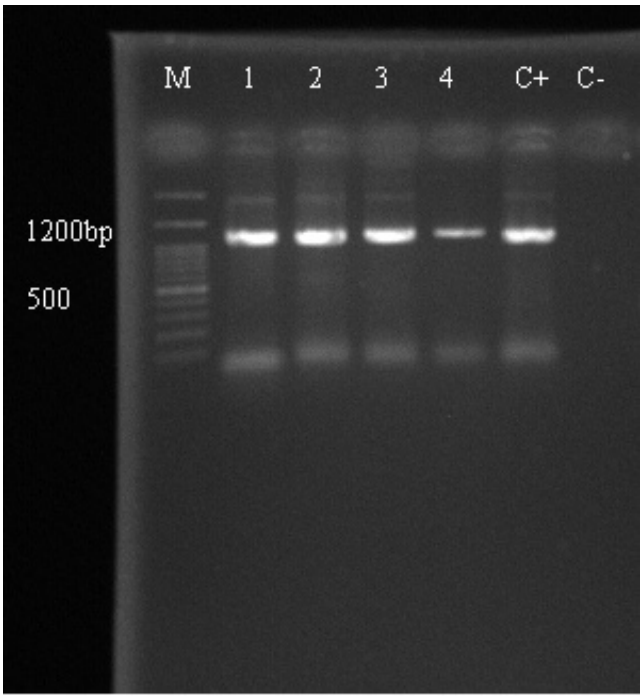
The RFLP procedure was carried out by digesting the amplified PCR products with *EcoRI* and *Hind III* endonucleasese enzymes in the supplied buffer according to the manufacturer's protocols. The PCR product (4 µl) was digested by the addition of 4 µl of water, 1 µl of 10X buffer, and 1 µl (10 U) of restriction enzyme, and incubated at 37°C for 2 hrs. The RFLP products were run on 2.0% agarose gels at 5 V/cm for 45 min, stained with ethidium bromide, and then visualized under ultraviolet illumination.

RESULTS

PCR amplification of the isolates using Pasteurella multocida species specific primers produced a single 460 bp band (figure 1). PCR product of ompH gene is shown in figure 2. PCR amplification of ompH- primers results in a 1.2 kb fragment.

PCR-RFLP typing of P. multocida isolates is presented in table 2. Twenty seven and 13 cloned EcoRI fragments indicate A and B patterns respectively. Twenty

Fig 2. PCR product of 1200 bp obtained with ompH primers. M: molecular size marker; C-: negative control; C+: positive control; numbers 1-4: some positive samples.



one, 14 and 5 cloned fragments representing C, D and E patterns to a different single restriction fragment in a genomic Hind III digestion. Figure 3 shows P. multocida isolates with 2 cleavage pattern when digested with EcoRI and 3 cleavage pattern when using Hind III restriction enzymes.

EcoRI and Hind III digestion resulted in fragments of 700-500, 900-300 and 1200 bp.

Combination of different restriction patterns grouped the 40 P. multocida strains into six RFLP types (table 3). On the basis of combinations, two to three bands in each digests are produced among the 40 isolates and identify six RFLP types.

DISCUSSION

Variations in the molecular mass of ompH among different P. multocida strains have also been reported (Hussaini et al., 2013). The role of ompH as a protective antigen has been identified against homologous infection by Ghanizadeh et al (2015). They have successfully used PCR analysis based on RFLP in ompH gene to investigate genetic heterogeneity and to classify avian P. multocida isolates. There was also considerable variation in the gene content of ompH (Prasannavadhana et al., 2014), but the genetic variation among P. multocida isolates is not well established in Iran. The present paper represents the first report of PCR-RFLP characterization based on ompH gene of P. multocida ovine strains isolated in Iran.

Although several studies have investigated the phylogeny of P. multocida species on the basis of 16S rDNA gene sequences (Michael and Abbott, 2007; Dousse et al., 2008; Bhimani et al., 2014) only few studies have used the ompH gene analysis (Tan et al., 2010).

In current research 40 P. multocida isolates were identified by genomic methods and divided into six different RFLP type. Similarly to what has been reported by Sellyei et al. (2012) our results demonstrated that a PCR-RFLP approach targeting the ompH gene using EcoR I and Hind III restriction endonucleases has been useful for characterization of P. multocida. The differentiation obtained by PCR-RFLP analysis enables a clear separation of the P. multocida group. ompH gene is more suitable for genetic differentiation of closely related species within the same cluster compared to 16S rRNA sequences because of its higher rate of nucleotide

Table 2. PCR-RFLP typing of *P. multocida* isolates with different fragment size.

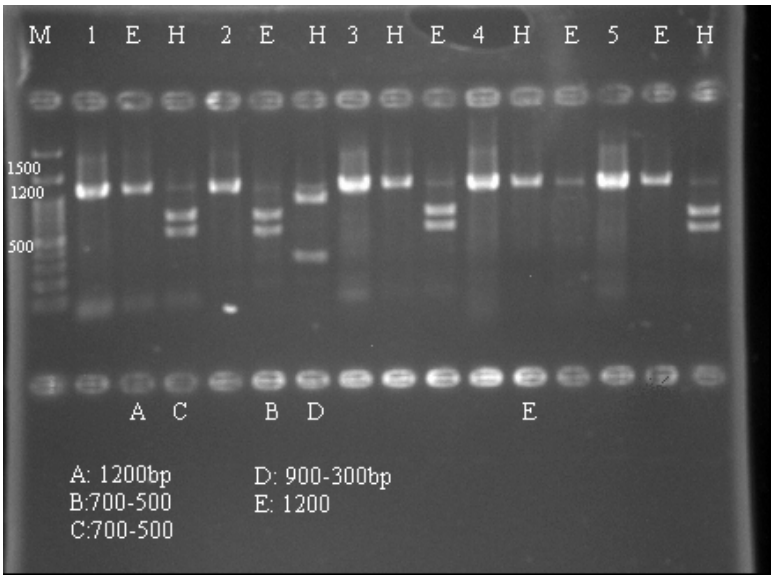
Endonuclease enzyme	Profile pattern	Size of fragments (bp)	Number of isolates
EcoR I	A	1200	27
	B	700-500	13
Hind III	C	700-500	21
	D	900-300	14
	E	1200	5

Table 3. Combination of different cleavage patterns of *P. multocida* isolated from sheep following EcoRI and Hind III restriction enzyme digestion.

Pattern	Restriction enzymes		Bacteria
RFLP type	EcoR I	Hind III	*P. multocida* Isolates
I	A(1200bp)	C(500-700)	ShI*1, ShI4,ShI6, ShI8, ShI10, ShI18
II	B(500-700)	D(900-300)	ShI2, ShI3, ShI5, ShI17, ShI20, ShI21
III	A(1200)	E(1200)	ShI7, ShI9, ShI11, ShI12, ShI14, ShI19, ShI22, ShI26, ShI27, ShI30, ShI33
IV	A(1200)	D(900-300)	ShI13, ShI15, ShI16, ShI23
V	B(500-700)	C(500-700)	ShI24, ShI25, ShI28, ShI29, ShI31, ShI32, ShI39, ShI40
VI	B(500-700)	E(1200)	ShI34, ShI35, ShI36, ShI37, ShI38

**ShI: sheep isolates*

Fig 3. The DNA patterns results of isolates after digestion with EcoR I and Hind III enzyme (The pattern A-E).Number1-5 is PCR products, E: EcoRI ,H:Hind III digestion.



polymorphism (Jabbari et al., 2005). The phenotypic heterogenecity of P. multocida isolates showed more genetic diversity among ovine isolates. We found that the sheep isolates were formed diverse RFLP type with various groups. This genetic diversity reflected the phenotype characteristic such as diseases in sheep, because they isolated from sheep with history of pasteurelosis. In current study, although the genetic diversity among the isolated strains was observed, the exact relationship between RFLP type and strain characteristics such as pathogenecity and immunogenecity remain to be determined.

CONCLUSION

In conclusion, in the present study, we applied two efficient, economic, and alternative molecular methods (single multiplex PCR and RFLP PCR) to characterize and to demonstrate genetic diversity of ovine *P. multocida* species. Both proposed methods accurately detected and distinguished six different types of heterogenic *P. multocida* in Iran. The possible effect of the presence of multiple serotypes on the PCR-RFLP profile needs further examinations. This genetic diversity among the isolates reflected the diseases in sheep, because they represented the history of pasteurelosis.

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