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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 pathogenicity. New vaccine formulation should consider multivariants of P. multocida in order to confer a wider protection.

Keywords: Pasteurella multocida; RFLP-PCR; ompH gene.
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 cattle, 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, Khuzestan 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 interstrain 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

| Primer | Gene | Name | Sequence | Amplicon size (bp) |
|--------|------|------|----------|-------------------|
| A11PASS | KMT1 | KMT1T7 | ATCCCGCATTTACCCAGTGG | 460 |
|        |      | KMTSP6 | GCTGTAACGAACTCGCCAC |       |
|        |      | ompH -FWD | ACTATGAAAAAGACAATGGTAG | 1200 |
|        |      | ompH -RWD | GATCCATTCTTGGCAACTTATT |       |

Table 1. Primer and Sequence used in the present study.
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: Fourtty 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).

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-chloroform- 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 (Fermentase) 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
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 types. 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 enzymes 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                 |
|                     | C               | 700-500                | 21                 |
|                     | D               | 900-300                | 14                 |
|                     | E               | 1200                   | 5                  |
| Hind III            |                |                        |                    |
|                     | A               | 1200                   | 27                 |
|                     | B               | 700-500                | 13                 |
|                     | 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 RFLP type | Restriction enzymes | Bacteria |
|-------------------|---------------------|----------|
| I                 | A(1200bp)           | C(500-700) | ShI1, 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 polymorphism (Jabbari et al., 2005). The phenotypic heterogeneity 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 pathogenicity 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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