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Classification, capsular PCR typing and genetic diversity of *Pasteurella multocida* isolated from sheep and goats in Iran using Bam HI, Hind III restriction endonuclease enzymes by PCR-RFLP

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Abstract. The aim of this study was to differentiate*Pasteurella multocida*from ovine and caprine isolates in Iran by PCR-RFLP generated by restriction endonuclease enzymes *Bam HI* and *Hind III*. Among the isolates, 92.3% was identified as capsular type A and 7.7% as type D. All *P. multocida*isolates were found to be virulent except for two isolates that caused no death in mice bio-assay. Restriction endonuclease analysis revealed 7 distinct profiles by digestion with *Hind III*, and *Bam HI*. Strains of the same PCR-RFLP type were similar to each other, whereas strains with different PCR-RFLP types were diversified. The *HnidIII* was found more useful for differentiating between DNA fingerprints of *P. multocida* ovine and caprine isolates. The typing with PCR-RFLP and correspondence with MDT gave consistent results. The strains of type II were found to be more associated with diseases of animals. PCR-RFLP pattern could be used for identification, differentiation and classification of *P. multocida*.

INTRODUCTION

Pasteurella species are gram-negative spherical or rod-shaped and all are facultative anaerobic. Colonies of *Pasteurella* species are usually grey and viscous, with a strong mucinous odor (Advisory *et al.*, 2001). *P. multocida* is an important pathogen of domesticanimals (Arumugam *et al.*, 2011). *P. multocida* capsular serotype A and D, A1 and A3, B2, and A and D are the main cause of atrophic rhinitis in pig, fowl cholera, hemorrhagic septicemia in cattle, pneumonia in sheep and goats respectively (De Alwis 1992; Foged *et al.*, 1988; Glisson 2008). Capsular serotype A and D produce dermonecrotic toxin encoded by toxA gene(Glisson 2008; Yap *et al.*, 2013).

Various virulence factors are associated with pathogenesis of *P. multocida* (Fuller *et al.*, 2000; Harper *et al.*, 2006; Hunt *et al.*, 2001). Capsule and lipopolysaccharide, fimbriae, dermonecrotic toxin, hemoglobin binding protein and outer membrane proteins are the key virulence factors (VFs) for *P. multocida* (Adler *et al.*, 1999; Ewers *et al.*, 2006; Fuller *et al.*, 2000). Some researchers have identified more enhanced bacterial virulences (Chung *et al.*, 2001; Harper *et al.*, 2007). Host invasion, colonization, and tissue injury are facilitated by this VFs (Harper *et al.*, 2006; Hunt *et al.*, 2000). Therefore, to prevention and control of *P. multocida* infection requires information and knowledge about VFs. Besides, there was correlation between VFs and capsular serogroups, hemoglobin binding protein (encoded by

hgbA) and the dermonecrotic toxin (encoded by toxAgene) associated with serogroups A, B, and D, respectively (Ewers *et al.*, 2006).

Pathogenesis of *P. multocida* is a complex interaction between host specific factors and specific bacterial virulence factor; therefore, understanding the disease pathogenesis is complex and depends on the bacterial strain, the animal model and their interactions (Harper *et al.*, 2006). Before that, *P. multocida* was classified to capsular and somatic typing on the basis of capsule antigens and outer membrane protein (Vasfi Marandi *et al.*, 1997). But polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) has indicated information about genomic characteristic of bacteria (Jabbari and Esmaelizadeh 2005). Except for time consuming for digestion and electrophoresis, PCR-RFLP are novel and rapid method for classification of *P. multocida* (Tsai *et al.*, 2011). In the present study, PCR was used to amplify DNA fragments that contained the VFs genes and to find more information about molecular characteristics of sheep and goats *P. multocida*. PCR-RFLP analysis was carried out and the results compared with mean dead time (MDT). This is the first study was carried out in five epidemic pasteurellosis provinces in Iran during 2010 -2012.

MATERIALS AND METHODS

Bacteria. Twenty six isolates of *P. multocida* were used in this study. These obtained by previous our investigations (Daneshlari *et al.*, 2013; Sahragard *et al.*, 2012; Tahamtan *et al.*, 2013).

Mice bioassay. To examine the pathogenesis of the isolates, balb/C mice model was assessed using mean dead time (MDT) method. Mice were injected with different appropriate amount of *P. multocida* isolates via intra-peritoneal route. If more than 80% of treated mice in each group were died within 48h of inoculation, the isolates were indicated as pathogen (Harper *et al.*, 2006).

Capsule typing. Multiplex PCR was determined the capsular type of the isolates. PCR capsular typing (primers specific for *capA* and *capD*) was carried out described by Townsend *et al.* (2001). The oligonucleotides primer sequences used in the multiplex capsule PCR typing assay for *P. multocida* are listed in Table 1.

Table 1. Primers used for the detection of virulence-associated genes in strains of *P. multocida*

Primer	gene	Primer sequence 5'-3'	Amplicon size (bp)
Allpass	*KMT1*	ATCCGCGATTTACCCAGTGGGCTGTAAACGAACTCGCCAC	460
Capsular typeA	*hydD-hydC*	TGCCAAAATCGCAGTCAGTTGCCATCATTGTCAGTG	1044
Capsular typed	*dcbF*	TTACAAAAGAAAGACTAGGAGCCCCATCTACCCACTCAACCATATCAG	760
DNT	*toxA*	TACTCAATTAGAAAAAGCGCTTTATCTTCCTCCCAGTAATTT GTCTGTATTTTATCAAA	300
HgbA	*hgbA*	TCAACGGCAGATAATCAGGGGCGGGAATGCTGAAGATAAG	267 280
PtfA	*ptfA*	TGTGGAATTCAGCATTTTAGTGTGTCTCATGAATTCTTATGCGC AAAATCCTGCTGG	520
OmpH	*ompH*	CGCGTATGAAGGTTTAGGTTTTAGATTGTGCGTAGTCAAC	420

PCR assay

Virulence genes detection. DNA template for PCR was prepared by DNP kit (Sina gene). One ml overnight culture on brain heart infusion (BHI) (Sigma) broth was centrifuged and pellet was re-suspended homogeneously in 100 µl of sterile water, and follows the instruction. 25 µlPCR mixture including PCR mixturecontained 14.8 µl of water, 3 µl of 10 x buffer, 3µl of deoxyribonucleotide triphosphate (dNTP)(2.5mM each dNTP), 2 µl of primers (10 picomole each), 2 µl of template DNA, and 0.2 µl of high fidelity Taq DNA polymerase. The mixture was applied for PCR by PCR carried out with a PCR system (Master gradient Ependorf, Germany). The PCR conditions were: 1 cycle of 94ºC for 3 min, 35 cycles of 94ºC for 30 sec, 56ºC for 30 sec (based on different primers), and 68ºC for 6 min, and 1 cycle of 25ºC for 1 min. The amplified products were analyzed in 1.2% agarose gels by electrophoresis, and the results were recorded with a gel documentation system (Kodak gel Doc, USA). For validation and avoid missing positive samples, all tests were repeated two times in parallel. The PCR products were purified and send for sequencing.

PCR-RFLP analysis. Twenty six strains were initially selected for PCR-RFLP analysis. The procedure was carried out with *Bam HI* and *Hind III* restriction endonuclease enzymes. The PCR product (10 µl) was digested by the addition of 18 µl of free nuclease water, 2 µl of 10x enzyme buffer, and 1 µl (10 U) of restriction enzyme and incubated at 37°C for 16 hr. The product was then inactivated by incubation at 80°C for 20 min. Finally, the digested DNA was assayed in a 1.2% agarose gel (Fermantase) and then visualized under ultraviolet light after staining with ethidium bromide solution (0.5 pg/ml). The similarity of band patterns by PCR-RFLP was compared according to DNA fingerprinting. An alphabetic letter (A, B, C, D, E, F, G, H and R) was assigned to each pattern. A unique combination of restriction patterns of all enzymes was called an RFLP type (I, II, III... VII). Then, the RFLP pattern of isolates were analyzed and compared with their mortality rate resulted in MDT.

RESULTS

Mice assay. Except two, all isolates were found to be pathogen in mice with MDT less than 30 hrs.

Capsular type. A was identified in 24 (92.3%) of *P. multocida* isolates, while two isolates (7.7%) were indicated capsular type D. It was noted that the *toxA* gene, which is involved in the pathogenesis of progressive respiratory disease in sheep and goats, was found in 84.6% isolates, and it was strictly restricted to strains belonging to capsular serogroup A. Twenty six strains were carrying different virulence factors (Figures 1-6).

PCR-RFLP. ompH digestion with *Hind III* resulting in two patterns including 400, 20 bp (11 isolates), and 380, 40 bp (9 isolates) (Figure 7 patterns A and B). However three isolates have not cut with *Hind III* (Figure 7 pattern C) (Table 2). ptfA gene digestion was made two groups (Table 2). Nineteen of 23 isolates resulting in two fragments of 120, and 400 bp (Figure 7 pattern D). However ptfA gene from four isolates have no restriction sites for this enzyme (Figure 7 pattern E).

Analysis of hgbA gene showed all of the isolates divided into two groups (Table 2). hgbA from 21 isolates produced two fragment by *Bam HI* (Figure 8 pattern F). In another group, hgbA from two isolates have no restriction site for *Bam HI* (Figure 8 pattern G).

In Table 3 different patterns made by *Hind III* and *Bam HI* restriction enzymes were merged. Twenty three isolates of *P. multocida* were divided into 7 RFLP types. The table was shown relation between MDT and RFLP type. Isolates in RFLP type II with restriction patterns A, D and F have minimum MDT. In the other hand, the isolates with these patterns have more pathogencity in mice.

The comparison between MDT and PCR-RFLP showed that the MDT from isolates of the same RFLP type was similar ($p<0.05$), for example type II with MDT under 5 h in Table 3, whereas the MDT from isolates of different RFLP types were diversified (type I). Blast search showed the nucleotide

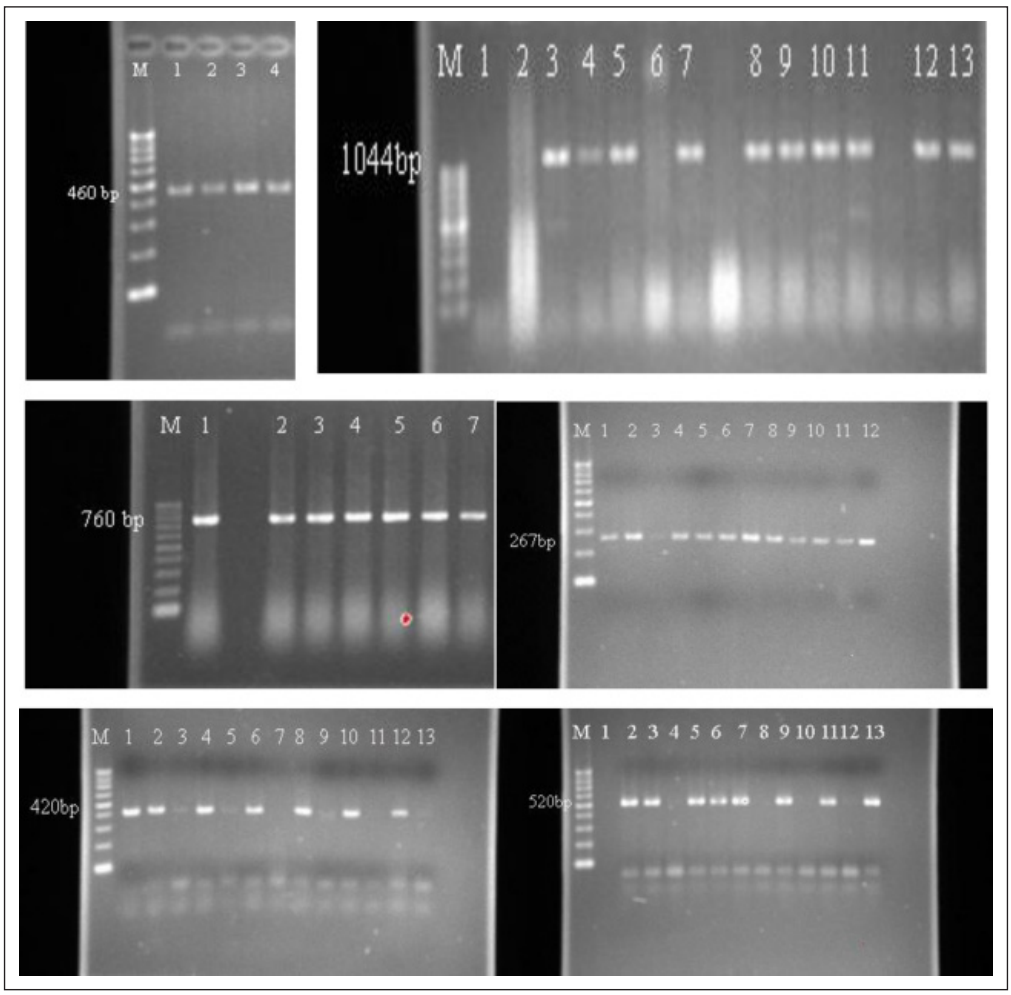

Figure 1-6. Polymerase chain reaction (PCR) profiles of *Pasteurella multocida* isolates detected by electrophoresis in agarose 1.2% gel and stained by ethidium bromide-. Lane M: molecular weight standard; other lanes representative all pass (460 bp), cap A (1044bp), cap D (760), hgbA (267 bp), oomph (420 bp), and ptfA (529 bp), respectively.

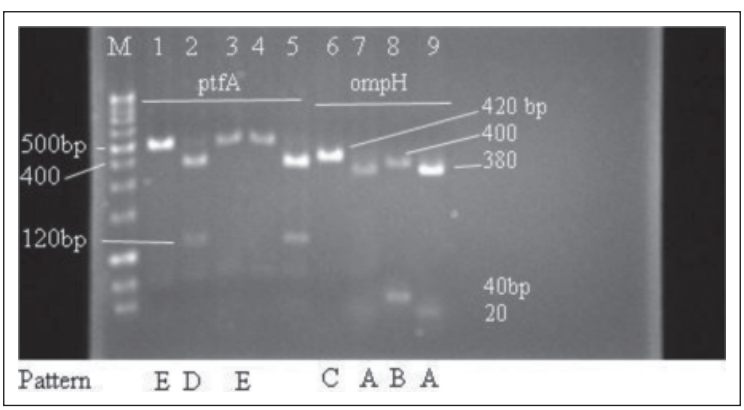

Figure 7. Agarose gel electrophoresis of representative of ptfA and ompH DNA from *Pasteurella multocida* after digestion with restriction endonuclease *Hind III*. Lanes 1–12 contain DNA from the representative isolates (patterns A-E).Fragment size given in base pairs (bp).

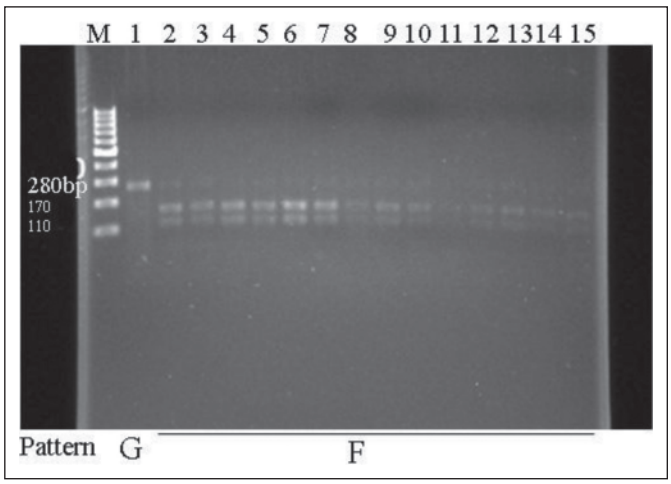

Figure 8. Agarose gel electrophoresis of representative of *hgbA* from *Pasteurella multocida* after digestion with restriction endonuclease with *BamHI*. Lanes 1–15 contain DNA from the representative isolates (patterns F and G). Fragment size given in base pairs (bp).

Table 2. Polymerase chain reaction foromph, ptfA and hgbA genes. They were digested by restriction endonuclease enzymes*Hind III* and *Bam HI*. The patterns produced by digestion were named by alphabetical letters

Restriction enzyme	HindIII					BamHI	
Gene		ompH		ptfA		hgbA	
Amplicon size (bp)		420		520		280	
No. isolate	11	9	3	19	4	21	2
Fragment (bp)	400-20	380-40	0	400-120	0	170-110	0
Pattern	A	B	C	D	E	F	G

Table 3. Classification of *P. multocida* strains according to PCR-RFLP types. Different restriction patterns made by *Hind III* and *Bam HI*

	HindIII pattern		BamHI pattern	Mean Dead Time (h)
RFLP type	ompH	ptfA	hgbA	
I	C	E	G	0 (No death)
II	A	D	F	Under 5
III	A	E	G	5–12
IV	B	E	G	12–18
V	A	D	G	18–24
VI	A	E	F	24–30
VII	B	E	F	After 30

Table 4. The frequency of the isolates in sheep and goats according to capsular type and compared with PCR-RFLP typing

Capsular group	Host	PCR-RFLP Type	Frequency %
A,D	Sheep, Goats	I	13
A	Sheep	II	73.91
A	Sheep	III	26
A, D	Sheep, Goats	IV	21.73
A	Sheep	V	47.82
A	Sheep	VI	52.17
A	Sheep	VII	52.17

sequences similarity among each type isolates. All isolates in type II were very similar (99–100% sequence identity). But the nucleotide sequences of the isolates of type III were similar (96.8–99.3% sequence identity). The nucleotide sequences of the isolates of types VI showed limited homology. The isolates in this type were showed MDT between 24-30 h. (Table 3).

The frequency of the isolates in sheep and goats according to capsular type RFLP patterns was shown in Table 4. The RFLP revealed that most of the ovine isolates belonged to type II, and all of the caprine isolates belonged to type I and IV. However, relationship between type pattern and history of isolates was not absolutely identified. But the morbidity rate was different in isolates originating from different type. The most commonly isolates detected in type II originating from distress animals.

Most of the toxigenic isolates (possess toxA gene), belonged to type II (22 out of 26), however, the remaining isolates were found to be non-toxigenic. While, a significant correlation among toxigenicity and RFLP typing was not observed ($p>0.05$). Because we have detected the *toxA* gene but not toxin production was assay.

DISCUSSION

The distribution and prevalence of serotypes and pathotypes of *P. multocida* can vary significantly from region to region. This is the first study that reports a collection of *P. multocida* isolates obtained from sheep and goats with clinical signs of respiratory infection in Iran. This study suggests that infections caused by *P. multocida* strains of serogroup A are more common than serogroup D. This is similar to results reported from United States (Pijoan *et al.*, 1983), and England and Wales (Davies *et al.*, 2003) and in contrast with Ewers *et al.* (2006) in Germany and Chan- Drasekaran & Yeap (1992) in Malaysia. They were reported the prevalence of strains of capsular serogroup A is lower than serogroup D. This finding in general is agreement with studies which reported serogroup A is the most common isolates in sheep and goats (Gautam *et al.*, 2004; Townsend *et al.*, 2001).

Several studies have reported that a number of virulence factors are correlated with the pathogenic mechanisms of *P. multocida* (Hunt *et al.*, 2000; Harper *et al.*, 2007; Sahragard *et al.*, 2012), but the molecular pathogenicity of *P. multocida* is well not understood. In general the isolates with type II RFLP showed more specificity than others, which was belong to capsular type A. Similar results have been also observed in host specificity of *P. multocida* isolates by Davies *et al.* (2004) and Dabo *et al.* (1999).

We have not observed close relation between toxigenicity and RFLP typing but others found this correlation in some geographical area (Davies *et al.*, 2003; Jaglic *et al.*, 2004). Nevertheless, correlation between toxA and toxin production with RFLP typing remains an opened question.

In conclusion, in the present study, we developed a rapid and multiple detection

method for *P. multocida* isolates in sheep and goats. The data presented *P. multocida* isolates could be classified into 7 PCR-RFLP types. Although isolates with type II pattern found more associated with distress animals isolates. These isolates had high mortality rate in mice with MDT under 5 hrs. PCR-RFLP typing explained the potential use for identification of isolates from sheep and goats.

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