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Virulence Genes Profile and Typing of Ovine *Pasteurella multocida*

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Abstract: To learn more about the virulence of *Pasteurella multocida* isolated from sheep, phenotype, capsular type and some virulence factors (*Pfha1*, *HgbB*, *TbPA* and *ToxA*) of ovine *Pasteurella multocida* is described in this study. Forty seven isolates of *Pasteurella multocida* were isolated from clinically healthy and diseased sheep. The isolates belonged to biovars 2(4.25%), 3(21.27%), 4(19.14%), 5(2.1%), 6(27.65%), 7(23.4%) and 11(2.1%). Capsular typing of isolates by PCR demonstrated two capsular types A (39), D (3) including 5 untyped with 83.3%, 6.3% and 10.6% prevalence, respectively. Among 4 virulence genes detected by PCR, we found a remarkable high prevalence of *TbPA* (69.4%) and *ToxA* (72.2%) genes in diseased animals. The high prevalence of *ToxA* and *TbPA* among diseased sheep may imply to important role of these genes in epidemiological and virulence of *P. multocida* isolates in sheep. Additionally, The high prevalence of *P. multocida* type A *ToxA*(+) in diseased sheep found in this study is noticeable and attribute to important role of *P. multocida* type A *ToxA*(+) in respiratory infection among sheep.

Key words: *Pasteurella multocida*, phenotype, capsular type, virulence factor, sheep

INTRODUCTION

Pasteurella multocida causes a wide range of important diseases in domestic animals, being responsible for pneumonia in cattle and sheep (Chanter and Rutter, 1989; Frank, 1989) and haemorrhagic septicemia in cattle and buffalo (Carter and de Alwis, 1989). Five serogroups (A, B, D, E and F), based on an capsular antigens were recognized in *P. multocida* (Harper *et al*., 2006). Townsend *et al*. (2001) developed a multiplex PCR system based on capsular genes loci amplification as the alternative way to the IHA test.

Mutters *et al*. (1985) classified *P. multocida* into three subspecies on DNA-DNA hybridization and named them as *P. multocida* subspecies *multocida*, *P. multocida* subspecies septica, *P. multocida* subspecies *galicida* and also demonstrated that dulcitol and sorbitol fermentation could be used to distinguish these three subspecies. An advanced method called microplate fermentation was described by Blackall *et al*. (1995) as an advanced technique for phenotyping of *P. multocida*.

Recently, a new method was suggested for epidemiological studies in *P. multocida* on the basis of a method that is called virulence genotyping by its advocators examined for capsular and 14 virulence associated genes (Ewers *et al*., 2006). This research group showed association between

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ToxA and swine diseases as well as *TbPA* and pftA and bovine diseases (Ewers *et al*., 2006). According to our knowledge association between *Pfha1*, *HgbB*, *TbPA* and *ToxA* genes and disease incidence in sheep till remains unclear. In order to achieve this goal, In this study we investigated the prevalence of *Pfha1*, *HgbB*, *TbPA* and *ToxA* genes along with genotype and phenotype of *P. multocida* in healthy and diseased sheep.

MATERIALS AND METHODS

Sampling

This study was carried out in North West of Iran covering East Azerbaijan, West Azerbaijan and Ardabil provinces. A total from 518 samples were collected from nasal discharge, pneumonic lung swab of diseased sheep (388) and nasal swab of clinically healthy ones (130), starting from January 2005 till February 2006 (Table 1). Specimens collected from diseased animals were fallen into two groups; group A: those collected from nasal discharge of sheep with respiratory infection (269) and group B: those collected from pneumonic lung of slaughtered sheep (119). All samples were plated onto 10% Sheep Blood Agar (SBA) and incubated at 37°C overnight.

Bacterial Isolation and Identification

Culture and morphological identification of suspected *P. multocida* isolates were carried out according to standard biochemical tests (Barrow and Feltham, 2006). Following incubation at 37°C for 24 h, small glistening mucoid dewdrop-like colonies were appeared on blood agar medium. Microscopic observations revealed that all isolates were gram-negative coccobacilli and biochemical analysis confirmed that the isolates were indol, catalase and oxidase positive but, citrate, MR, VP and gelatin liquefaction negative. Growth test on MacCankey agar was negative with no motile and non-hemolytic effects on blood agar. The cells were stored in Brain Heart Infusion (BHI) with 30% glycerol at -70°C.

Biochemical Characterization

All isolates were subjected to full biochemical characterization based on microplate fermentation method described by Blackall *et al*. (1995). Briefly, characterization was done using fermentation of ten carbohydrates and results of ornithine decarboxylase (ODC) and Ortho-nitrophenyl-b-D-

Table 1: Primers for detection of virulence genes and capsular types

Genes	Names	Sequence (5'-3')	References
ToxA	*ToxA*1F	TCT TAG ATG AGC GAC AAG G	Lichtensteiger *et al.* (1996)*
	*ToxA*1R	GAA TGC CAC ACC TCT ATA G	
TbPA	TbPA1F	TGG TTG GAA ACG GTA AAG C	Ewers *et al.* (2006)*
	TbPa1R	TAA CGT GTA CGG AAA AGC C	
HgbB	*HgbB* F	TCT TTG AGT ACG GCT TGA C	Present study
	HgbB R	CTT ACG TCA GTA ACA CTC G	
Pfha	*Pfha1*F	AGC TGA TCA AGT GGT GAA C	Ewers *et al.* (2006)*
	*Pfha1*R	TGG TAC ATT GGT GAA TGC TG	
KMT1(ALL)	KMT1T7	TCCGCTATTTACCCAGTGG	Townsend *et al.* (2001)*
	KMT1SP6	CTGTAAACGAACTCGCCAC	
hyaD-hyaC(A)	CAPA-FWD	TGCCAAAATCGCAGTCAG	Townsend *et al.* (2001)*
	CAPA-REV	TTGCCATCATTGTCAGTG	
bcbD (B)	CAPB-FWD	CATTTATCCAAGCTCCACC	Townsend *et al.* (2001)*
	CAPB-REV	GCCCGAGAGTTTCAATCC	
dcbF (D)	CAPD-FWD	TACAAAAGAAAGACTAGGAGC	Townsend *et al.* (2001)*
	CAPD-REV	TACCCACTCAACCATATCAG	
ecbJ(E)	CAPE-FWD	TCCGCAGAAAATTATTGACTC	Townsend *et al.* (2001)*
	CAPE-REV	GCTTGCTGCTTGATTTTGTC	
fcbD(f)	CAPF-FWD	CGGAGAACGCAGAAATCAG	Townsend *et al.* (2001)*
	CAPF-REV	TCCGCCGTCAATTACTCTG	

*: These primers were originally developed by mentioned references, but were modified in this study to improve PCR amplification

Table 2: Distribution and source of collected of *P. multocida* isolates

No. of samples	No. of positive sample (%)	Sample material	Clinical status
130	11 (8.46%)	Nasal swab	Clinically healthy
269	29 (10.70)	Nasal swab	Respiratory symptoms/Nasal discharge
119	7 (5.80)	Lung tissue	Pneumonia
518	47 (9.07)		

galactopyranoside (ONPG) tests. The carbohydrates used in this part included L-arabinose, dulcitol, D-glucose, D-lactose, maltose,D-mannitol, D-sorbitol, D-sucrose, D-trehalose and D-xylose.

Capsular Typing by Multiplex-PCR

Capsular typing was conducted with multiplex PCR (Townsend *et al*., 2001) using further modified primers designed on the basis of capsular gene sequences (Table 2). The PCR amplification was conducted directly on bacterial culture stock composed of BHI (70%) and glycerol (30%) without genomic DNA extraction step. Each 25 μL reaction contained 0.4 μL bacterial glycerol stock as DNA template, 1 U Taq DNA polymerase, 3.2 mM from each primer, 200 μM of each dNTP, 1 x PCR buffer and 2 mM $MgCl_2$. Amplification was carried out for 35 cycles, each cycle consisting of DNA denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 30 sec. The cycles were preceded by an initial denaturation at 94°C for 5 min and followed by a final extension at 72°C for 5 min. The resulting PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide and imaged. Distilled water without any DNA was used as negative control.

Virulence Genes Detection Using PCR Analyses

Virulence genes detection was carried out using a new multiplex PCR for *Pfha1*, *HgbB*, *TbPA* and *TonA* genes. For this multiplex PCR system, we designed new or modified previously developed primers for each gene to prepare suitable band size and PCR program (Table 2). This method comes in company with satisfied results in compare with PCR method for each gene separately. For all PCR reactions, 0.8 μL of bacterial culture stock composed of BHI (70%) and glycerol (30%) without genomic DNA extraction step were taken as template DNA and added to the reaction mixture (50 mL) containing 3.2 mM of each primer pair, 200 μM from the four dNTP, 5 μL of 10 × PCR buffer, 1.5 μL of 50 mM Magnesium chloride and 1 U of Taq-Polymerase. The samples were subjected to 35 cycles of amplification in a thermal cycler. The primers used in this study are listed in Table 2. Each cycle consisting of DNA denaturation at 94°C for 45 sec, annealing at 54°C for 50 sec, extension at 72°C for 50 sec. The cycles were preceded and followed by an initial denaturation at 95°C for 5 min and final extension at 72°C for 10 min, respectively. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and photographed at UV exposure. Statistical analyses were performed using software SPSS 12.0.

RESULTS

Bacterial Isolates

Of the 518 specimens originated from different healthy (130) and diseased (388) hosts and investigated for *P. multocida*, 47 (9.07%) samples were positive. Of 130 specimens collected from healthy sheep 11 (8.46%) and of 388 specimens collected from diseased hosts 36 (9.27%) were identified as positive samples (Table 1).

Biochemical Characterization

All positive samples (47 isolates) were biochemically phenotyped using microplate fermentation method. As shown in Table 3, 11 (23.4%) samples displayed biovar seven fermentation phenotype.

Table 3: Biochemical property of isolates of *P. multocida*

		Biovar							
Sample materials	Clinical status	2	3	4	5	6	7	11	Total
Nasal swab	Clinically healthy	1	6	-	1	1	2	-	11
Nasal swab	Respiratory symptoms/Nasal discharge	1	-	9	-	10	8	1	29
Lung tissue	Pneumonia	-	4	-	-	2	1	-	7
Total		2	10	9	1	13	11	1	47

Table 4: Capsular typing of isolates of *P. multocida*

		Results of cap-gene PCR						
Sample material	Clinical status	A	B	D	E	F	Neg	Total
Nasal swab	Clinically healthy	11	-	-	-	-	-	11
Nasal swab	Respiratory symptoms/Nasal discharge	23	-	2	-	-	4	29
Lung tissue	Pneumonia	5	-	1	-	-	1	7
Total		39	0	3	0	0	5	47

Consequently, these isolates were characterized as *P. multocida* subspecies *septica*. The remaining 36 (76.6%) samples were assigned as *P. multocida* subspecies *multocida*. According to fermentation results, *P. multocida* subspecies *multocida* isolates fell into six biovars. 2 (4.25%) isolate was classified as biovar two, 10 (21.27%) biovar three, 9 (19.14%) biovar four, 1 (2.1%) biovar five, 13 (27.65%) biovar six and 1 (2.1%) biovar eleven. This observation indicates that the majority of *P. multocida* isolates belong to biovar six and seven with 27.65 and 23.4% prevalence, respectively. Additionally, the high prevalence of biovars 4 (100%), 6 (92%) and 7 (81.8%) imply role of this biovars in disease status.

Capsular Typing by Multiplex-PCR

Capsular genotyping was conducted based on amplification of five different capsular groups using multiplex PCR in the presence of each capsule's specific primers. A pair of *P. multocida* specific primers was also added into the reaction for species confirmation of the isolates.

The presence of a DNA band with about 460 bp size further established the identification of the isolates as *P. multocida*. As seen, amplified DNA products of ~1044 and ~657 bp corresponding to *P. multocida* capsular groups A and D were observed, respectively. The band sizes of ~760, ~511 and ~854 bp expected to be produced corresponding to *P. multocida* capsular serogroups B, E and F, respectively were not observed. As shown in Table 4, two genotypes (A and D) were found among both *Multocida* and *Septica* subspecies. Of the samples, 39 (83%) isolates were classified as capsular type A and 3 (6.38%) as type D. No amplicon addressing to groups B, E and F was found. All two types (A and D) were found among healthy sheep and sheep with disease status. Five isolates (10.6%) from sheep with disease status were untyped.

Virulence Genes Detection Using PCR Analyses

Virulence genes detection was conducted based on amplification of four virulence factor genes using multiplex PCR in the presence of specific primers. Amplification of DNA bands with about 275, 540, 728 and 846 bp sizes was addressed to the presence of *Pfha1*, *HgbB*, *TbPA* and *ToxA* genes in the isolates, respectively.

As shown in Table 5, *TbPA* (69.4%) and *ToxA* (72.2%) genes have the highest prevalence rate among diseased cases. We did not detect any *ToxA*(+) isolate among healthy sheep and Phfa1(+) isolate among diseased samples sheep. Additionally, prevalence rate of *TbPA* among healthy isolates was low. The prevalence of *ToxA* and *TbPA* among biovars 4, 6 and 7 are higher than other biovar. Prevalence *ToxA* among biovars 4, 6 and 7 is 84.4, 80 and 80% and *TbPA* 84.6, 70 and 90%, respectively.

Table 5: Virulence factor detection of isolates of *P. multocida*

Virulence genes	Clinically healthy (%)	Respiratory symptoms/ Nasal discharge (%)	Pneumonia (%)	Diseased animal (%) (Nasal discharge, Pneumonia)
Pfha1	2 (18.20)	-	-	-
HgbB	4 (36.36)	-	4 (57.40)	4 (57.4)
TbPA	6 (54.50)	23 (79.3)	2 (28.40)	25 (69.4)
ToxA.	-	25 (86.2)	1 (14.28)	26 (72.2%)
Total	11	29	7	36

DISCUSSION

This study as the first report describes phenotyping, capsular typing and virulence factor profile of ovine *P. multocida*. The results of capsular typing except a few items are in accordance with some of previous studies described in the literature (Fussing *et al*., 1999; Weiser *et al*., 2003; Ewers *et al*., 2006). Two genotypes (A and D) were detected among the isolates. Type D was found only in diseased cases, while type A was found in diseased and healthy samples. Prevalence rate of type A (83%) was higher than type D (6.38%). The results suggest that type A strain are the most common in Iran independent of disease status.

In this study we show the remarkable high prevalence of *P. multocida ToxA*(+) (86.2%) in nasal swabs of sheep with respiratory infection that is noticeable. Other groups reported that dermonecrotic toxin encoded by *ToxA* gene, is expressed mainly by serogroup D strains responsible for the clinical and pathological signs of atrophic rhinitis (Harper *et al*., 2006) and with disease in goats (Baalsrud, 1987; Zamir-Saad *et al*., 1996). Although Ewers *et al*. (2006) found high prevalence of *ToxA*(+) strains among sheep population, but due to some limitation they did not identify the association of this gene with disease status. We found high prevalence of *ToxA*(+) strains among sheep with respiratory disorder (nasal discharge and pneumonia) ($p<0.05$) and absence of this gene in healthy sheep. This finding could address the important role of *P. multocida ToxA*(+) in respiratory infection in sheep. In addition, we found *ToxA*(+) strains among capsular type A(i.e., 24 out of 26 *ToxA*(+) isolate display capsular type A genotype). 2 out of 3 type D strains isolated from disease animals showed *ToxA*(+)(data not shown). However high prevalence of this capsular type A *ToxA*(+) suggested a more important role of this strain, in sheep diseases, which should be kept mind in future study.

On the other hand, some papers reported the isolation of *P. multocida* type D *ToxA*(+) from bighorn sheep introduced by the feral goats. it is suggested that goat may be served as reservoir of *Pasteurella* strains that is likely to be virulent in bighorn sheep (Rudolph *et al*., 2003; Weiser *et al*., 2003). Considering very close relation and nose to nose contact between sheep and goat in Iranian small ruminant herds; it is though that *P. multocida* type A *ToxA*(+) may be introduced to domestic sheep population through domestic goats similar to *P. multocida* type D, *ToxA*(+) to bighorn sheep through feral goats in the wild life. Comparative study based on genetic approach is necessary to clarify this relationship.

Two iron acquisition related genes, *TbPA* and *HgbB*, were studied in this study. The role of iron in pathogenesis of *P. multocida* is important, two independent non sidrophore-mediated acquisition of iron mechanism have been identified in *P. multocida*. The first mechanism involves iron-binding proteins expressed on the outer membrane of the bacterial cell, interacting directly with host iron binding glycoproteins. The second mechanism includes bacterial proteins that bind hemoglobin and hemoglobin complexed to the host glycoprotein (Cox *et al*., 2003). Two iron acquisition related genes, *TbPA* and *HgbB*, were studied in this paper associated to first and second mechanism of iron acquisition, respectively. Both of these genes were introduced as and epidemiological markers (Ewers *et al*., 2006).

A high prevalence of *TbPA* among *P. multocida* isolates from respiratory infection in sheep was found as well. Previous studies reported the presence of *TbPA* in bovine isolates of *P. multocida*

associated with pneumonia and hemorrghic septicemia (Veken *et al*., 1994; Ogunnariwo and Schryvers, 2001). Present results confirmed the presence of a significant association between this gene and ovine disease ($p<0.05$).

The total presence of *HgbB* gene in diseased sheep is lower than its prevalence rate in healthy animals. In contrast of bovine strain, this gene may be not important in ovine disease and it is also not valuable as epidemiological marker. However, present data show different results between animal with nasal discharges and pneumonic lungs. The absence of *HgbB* gene in isolates originated from animals with nasal discharge and high prevalence of that gene in pneumonic animals is noticeable and further studies are desired.

Additionally, despite of the report describing *Pfha1* as an epidemiological marker in cattle isolates, prevalence rate of *Pfha1* gene among *P. multocida* isolates from sheep was very low. This finding indicates that *Pfha1* is not important in virulence of sheep isolates and is not likely to be a suitable gene candidate in epidemiological studies in sheep. Probably the low rate of *Pfha1* gene among sheep population especially infected ones paled the importance of filamentous hemagglutinin encoded by this genes in colonization of ovine strain of *P. multocida* and there might be another factor such as type 4 fimbriae (PtfA) that high prevalence among ovine isolates has an important role in this processes (Ewers *et al*., 2006).

The PCR amplification using bacterial stockculture as the template DNA in this study provided satisfied results that has already been results obtained from colony touched PCR amplification used by Townsend *et al*. (2001) and DNA extraction used by Lichtensteiger *et al*. (1996) and Ewers *et al*. (2006). This experience shows that direct PCR capsular typing utilizing *P. multocida* glycerol stock is a reliable and applicable method for capsular typing that also significantly decreases the PCR typing time due to deletion of re-culture bacterial step.

Biochemical phenotyping, according to biotyping system of Blackall *et al*. (1995) allowed identification of nine biovar groups. This report is first experiment of microplate fermentation method about ovine isolates, showing the higher prevalence of biovars 3, 6 and 7. High percentage of biovars six and four ($p<0.05$) among disease cases showed the relation between these biovars and disease status. This finding is not in accordance with the results of other studies from Australia reported biovar 3 has the highest among avian and swine isolates (Fegan *et al*., 1995; Blackall *et al*., 1997).

No relevance was found between biochemical properties and capsular PCR typing. But high prevalence of *TbPA* and *ToxA* genes between biovars six and seven is higher than the other biovars which have association with ovine disease. Although the number of samples in this study was not too high, but according of the results outcome from this collection, in spite of the important role of capsular and epidemiological markers in swine and bovine disease (Ewers *et al*., 2006), it seems that in sheep the contemporary use of phenotyping and virulence epidemiological marker gene is useful method for epidemiological studies.

In summary, among four genes that is showed as epidemiological markers in swine and bovine diseases, only two genes, *TbPA* and *ToxA*, have significant association with diseases in sheep. Also, in attention to low variation in capsular typing system among ovine strains of *P. multocida*, it suggested that the use of alternative system with high variation among ovine isolates instead of capsular typing in epidemiological studies. Although phenotyping system is demonstrated as a suitable alternative system, more studies are necessary to introduce other systems on based molecular approach.

CONCLUSION

According to the result of this article, we can conclude that the role of *TbPA* and *ToxA* genes in epidemiological study of sheep respiratory disorder is significant. Additionally, *P. multocida* type A *ToxA*(+) plays an important role in sheep disease.

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