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Short Paper

Diversity of caprine and ovine *Pasteurella multocida* isolates based on 16S rRNA gene sequencing

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Summary

In this study, to increase information about the relationship between caprine and ovine isolates of *Pasteurella multocida*, 16S rRNA gene sequencing of 9 goats (5) and sheep (4) isolates were investigated. Also, capsular type and *toxA* gene presentation was studied in this paper. All isolates, except one, belong to capsular type A, and *toxA*+ strain distributed among strains were isolated from both species. Comparison of the 16S rRNA gene sequence showed the role of goat as a reservoir for *P. multocida* to sheep independent of *toxA* genes transmission.

Key words: *Pasteurella multocida*, 16S rRNA gene sequencing, *toxA*, Goat, Sheep

Introduction

Pasteurella multocida have been implicated as the causes of severe outbreaks of diseases in various hosts (Timoney *et al*., 1988; Harper *et al*., 2006). In sheep in temperate climates *P. multocida* rarely causes pneumonia and little is known of the epidemiology of the infection (Donachie, 2007). Also, it has been mentioned that, whereas much is known regarding the prevalence and pathogenesis of *Manhemia hemolytica* infection, *P. multocida* has been infrequently cited as the causal agent of researchers reporting ovine pneumonia outbreak (Gilmour and Gilmour, 1989; Odugbo *et al*., 2005).

Recently some papers have reported that goat may serve as a reservoir of *Pasteurella* strains that are likely to be virulent in bighorn sheep (Rudolph *et al*., 2003; Weiser *et al*., 2003). The research confirms that goat transmits capsular type D *toxA*+ of *P. multocida* to bighorn sheep (Rudolph *et al*., 2003). In addition, a high prevalence of *toxA*+ strains has been reported among domesticated sheep population (Ewers *et al*., 2006; Shayegh *et al*., 2008). Considering the very close relation and nose to nose contact between sheep and goat in Iranian herds, it is thought that *P. multocida* type *toxA*+ may be introduced to the domestic sheep population through domestic goats similar to bighorn sheep through feral goats in the wild.

A comparative study based on genetic approach is necessary to clarify this relationship. The 16S rRNA gene sequencing has been used as the gold standard for precise molecular identification and phylogenetic relationship studies of *P. multocida* and other species (Boerlin *et al*., 2000; Kuhnert *et al*., 2000; Frebourg *et al*., 2002; Davies *et al*., 2003; Davies, 2004; Dey *et al*., 2007).

In the present study, diversity of nine representative *P. multocida* isolates of sheep and goat were investigated by comparative sequence analysis of the 16S rRNA gene. Also, capsular genotyping and *toxA* gene presentation of *P. multocida* was studied.

Materials and Methods

Bacterial isolates

All of the *P. multocida* isolates were recovered from healthy goats, goats and sheep with nasal discharge (5 isolates from goat and 4 from sheep) in the northwestern provinces (west and east Azerbaijan) of Iran and were confirmed according to the biochemical standards described previously (Barrow and Feltham, 1993). All isolates studied in this study are listed in Table 1.

Table 1: List of *P. multocida* isolates studied in this research

Animal	Subspecies	Capsular type	*toxA* gene
Goat	*P. multocida* subsp. *multocida*	A	+
	P. multocida subsp. *multocida*	A	+
	P. multocida subsp. *multocida*	A	+
	P. multocida subsp. *multocida*	A	-
Sheep	*P. multocida* subsp. *multocida*	A	+
	P. multocida subsp. *multocida*	A	+
	P. multocida subsp. *multocida*	untyped	+
	P. multocida subsp. *multocida*	untyped	+
	P. multocida subsp. *multocida*	D	-

Table 2: List of primers used in this study

Gene	Name	Sequence (5'-3')
KMT1 (ALL)	*KMT1T7*	TCCGCTATTTACCCAGTGG
	KMT1SP6	CTGTAAACGAACTCGCCAC
hyaD-hyaC (A)	*CAPA-FWD*	TGCCAAAATCGCAGTCAG
	CAPA-REV	TTGCCATCATTGTCAGTG
bcbD (B)	*CAPB-FWD*	CATTTATCCAAGCTCCACC
	CAPB-REV	GCCCGAGAGTTTCAATCC
dcbF (D)	*CAPD-FWD*	TACAAAAGAAAGACTAGGAGC
	CAPD-REV	TACCCACTCAACCATATCAG
ecbJ (E)	*CAPE-FWD*	TCCGCAGAAAATTATTGACTC
	CAPE-REV	GCTTGCTGCTTGATTTTGTC
fcbD (F)	*CAPF-FWD*	CGGAGAACGCAGAAATCAG
	CAPF-REV	TCCGCCGTCAATTACTCTG
toxA	*ToxA1F*	TCT TAG ATG AGC GAC AAG G
	ToxA1R	GAA TGC CAC ACC TCT ATA G

Capsular typing

The capsular types were determined by multiplex PCR (Townsend *et al.*, 2001). The PCR amplification was conducted directly on bacterial stock composed of BHI (70%) and glycerol (30%) without the genomic DNA extraction step. Each 25 µl reaction contained 0.4 µl bacterial stock as the DNA template, 1 U Taq DNA polymerase, 3.2 mM from each primer (Table 2), 200 µM of each dNTP, 4 µl of 1 x PCR buffer, and 2 mM $MgCl_2$. The PCR reactions were intiated by an initial denaturation at 94°C for 5 min followed by 35 cycles, each cycle consisting of DNA denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 30 sec. The cycles were followed by a final extension at 72°C for 5 min. Amplified PCR products were separated by 2% agarose electrophoresis and finally stained with ethidium bromide and photographed (Fig. 1).

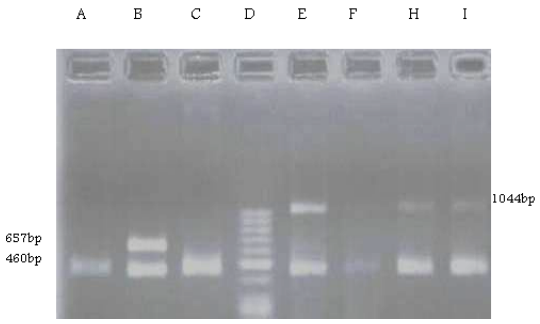

Fig. 1: Capsular typing by PCR of the isolates. Lane D: 250 bp DNA marker, Lane A and C: untyped isolates Lane B: type D, Lanes E to I: type A

Dermonecrotoxin *toxA* gene detection

Dermonecrotoxin gene (*toxA*) was conducted with PCR (Lichtensteiger *et al.*, 1996). For all PCR reactions, 0.8 µl of bacterial stock without the genomic DNA extraction step were taken as template DNA and added to the reaction mixture (50 µl) containing 3.2 mM of primer, 200 µM from the four dNTP, 5 µl of 10 x PCR buffer, 1.5 µl of 50 mM MCl_2, and 1 U of Taq DNA polymerase. The samples were subjected to 35 cycles of amplification in a thermal cycler (Eppendorf, Germany). The reactions were carried out according to the following programme: initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 45 sec for DNA denaturation, annealing at 54°C for 50 sec and extension at 72°C for 50 sec. The cycles were followed by a final extension at 72°C for 10 min. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed at UV exposure (Fig. 2).

Amplification and analysis of 16S rRNA gene

Amplification of the 16S rRNA gene from the genomic DNA preparations of each isolate were performed with the universal primers SR-FWD 5´- AGAGTT TGATCA

TGGCTCAG -3′ and SR-REV 5′-TACCTTGTTAGGACTTCACC -3′ as reported by Karlson *et al*. (1993). For PCR reactions, 0.8 µl of bacterial stock without the genomic DNA extraction step were taken as template DNA and added to the reaction mixture (50 µl) containing 1 µl of each primer , 200 µM from the four dNTP, 5 µl of 10 x PCR buffer, 1 µl of 50 mM MCl_2, and 1 µ of Taq DNA polymerase. The samples were subjected to 35 cycles of amplifie in a thermal cycler. Each cycle consisted of DNA denaturation at 95°C for 60 sec, annealing at 56°C for 50 sec, and extension at 72°C for 85 sec. The cycles were preceded and followed by an initial denaturation at 95°C for 5 min and final extension at 72°C for 5 min, respectively. Amplified products were analyzed by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed at UV exposure (Fig. 3).

The 16S rRNA gene amplification of ~1500 bp was confirmed with a standard molecular weight DNA marker (Fermentas, ready to use) in agarose gel electrophoresis and the PCR product was sent to Macrogen Co. (Seoul, South Korea) for sequencing. The strands of the gene were sequenced in three overlapping segments using the following additional internal primers: (forward/2; nucleotides 376-396), 5′- atgccg cgtgaatgaagaag - 3′ (forward/3; nucleotides 920-940), 5′- ggagcatgtggtttaattcg -3′ (reverse/2; nucleotides 980-963) and 5′-acttaaaagtccgcctgcgtgt -3′ (reverse/3; nucleotides 577-557).

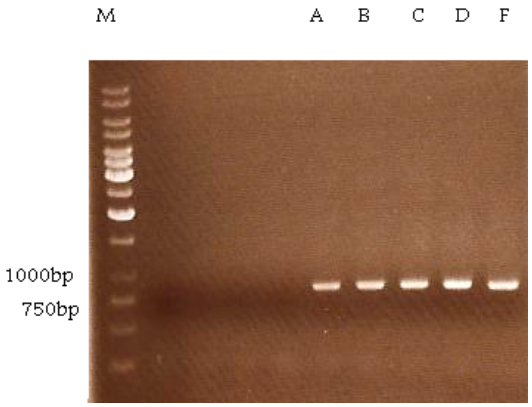

Fig. 2: PCR photograph on *toxA* gene products, M: 250 bp Ladder, A to F: *toxa*+ isolates

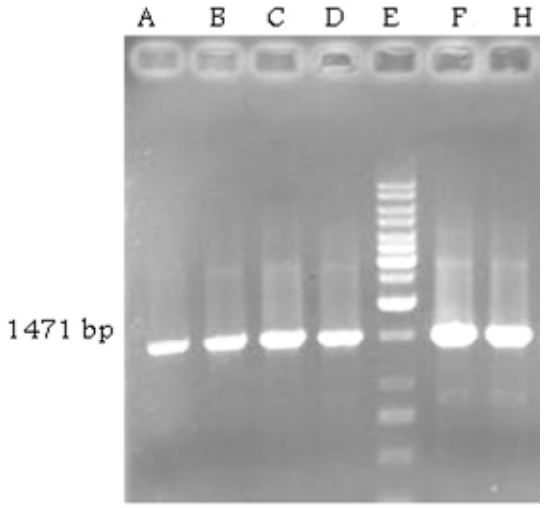

Fig. 3: PCR photograph on 16s rRNA gene products, A, B, C, D, F, and H: product of PCR image: 250 bp ladder

The data of the nucleotide sequence of the 16S rRNA gene of *P. multocida* isolates were aligned and compared using DNASTAR software. The phylogenetic analysis was conducted using MEGA version 3.1 (Kumar *et al*., 2004) with similar sequences of the 16S rRNA gene of the reference strains of *P. multocida* searched in the BLASTN programme of the NCBI website (http://www.ncbi.nlm.nih.gov/Blast). The 16S rRNA gene sequences of *P. multocida* reference strains NCTC 10322, type A (subsp. multocida), NCTC 11995, type A (subsp. septica) and outgroup sequence of *Haemophilus influenzae* (Accession No. M35019) were used.

Results

Capsular typing

Capsular genotyping was conducted based on 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 band of about 460 bp size further established the identification of the isolates as *P. multocida*. As seen, amplified DNA products of ~1044, ~760 and ~657 bp corresponding to *P. multocida* capsular groups A, B and D were observed, respectively. Six isolates investigated in this study carried the capsular type A gene, and the remainder were acapsular, with one

exception, which carried the capsular type D gene.

Dermonecrotoxin *toxA* gene detection

Dermonecrotoxin gene detection was conducted based on amplification of the *toxA* genes using PCR in the presence of specific primers. Amplification of DNA bands with about 846 bp size was addressed to the presence of the *toxA* gene.

This gene was present in seven isolates studied in this paper. Two isolates, one of sheep and another from goat, do not have the *toxA* gene in their genomes.

Nucleotide variation of 16S rRNA genes and phylogenetic relationships of ovine and caprine *P. multocida*

In this study, a 1471 bp fragment of 9 *P. multocida* 16S rRNA gene was sequenced. Our results showed 3 (0.2%) polymorphism sites among these sequences.

All goat isolates except one, with a variation site 99.9%, shared 100% homology with the 16S rRNA gene sequence of *P. multocida* reference strains NCTC 10322 type A (subsp. multocida). On the other hand, all of the ovine isolates showed 99.9% homology with the same reference strain.

A neighbour-joining dendrogram representing the phylogenetic relationships of 16S rRNA genes of *P. multocida* are shown in Fig. 4. The host origin, capsular type, and presence of *toxA* genes of the isolates are shown as well. According to this dendrogram, all of the isolates are divided into two branches, A and B. All of the ovine isolates fall into branch B, except one in branch A. Other members of branch A belong to caprine isolates. In other words, one of the ovine isolates is in branch A besides the isolates taken from goat (Fig. 4). Also, an exact review of the 16S rRNA gene sequences indicates that this gene has a special polymorphic site in branch A not available in branch B.

Discussion

This is a comparative study between caprine and ovine *P. multocida* isolates based on capsular genotyping, *toxA* gene presentation and 16S rRNA gene sequencing.

The results of the capsular type of caprine isolates are similar to the ovine ones reported in previous studies (Weiser *et al*., 2003; Ewers *et al*., 2006; Shayegh *et al*., 2008). We have no acapsular types among isolates taken from goat.

Previous studies have suggested feral goat as a reservoir to transmit *Pasteurella* sp. to bighorn sheep in the wild (Weiser *et al*., 2003). Some studies found a close proximity between dermonecrotic producing strains isolated from feral goat and bighorn sheep (Rudolph *et al*., 2003). These studies suggested the transmission of the *toxA*+

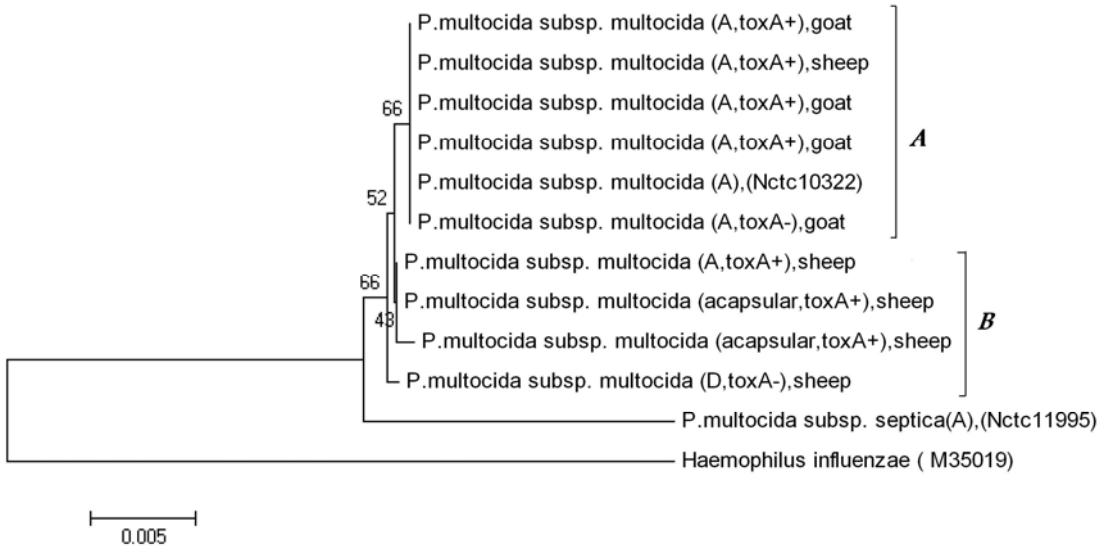

Fig. 4: Phylogenic relationship of ovine and caprine *P. multocida* isolates based on 16S rRNA using neighbour-joining dendegram

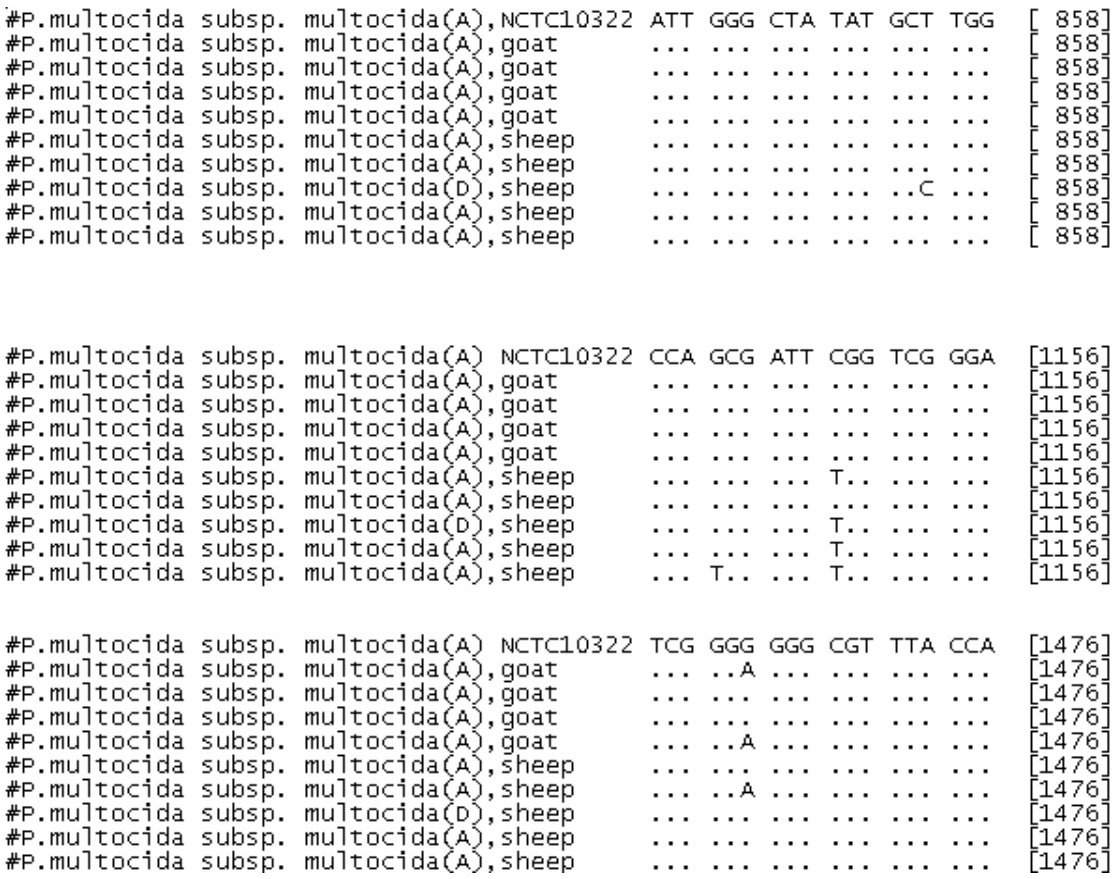

Fig. 5: Polymorphic sites of ovine and caprine *P. multocida* 16S rRNA genes in compare with *P. multocida* (NCTC10322)

strain from sheep to goat in the wild. Since some research groups have reported a high prevalence of *toxA*+ strains isolated from domestic sheep recently (Ewers *et al*., 2006; Shayegh *et al*., 2008), the phylogenetic relationship between *P. multocida* isolated from goat and sheep is significant for the investigation of similar transmission between domesticated sheep and goat. This subject is more significant in our studied field (East Azerbaijan, Iran) for the very close relation and nose to nose contact between sheep and goat in small ruminant herds.

In this study, although the similarity of 16S rRNA genes shows a close relationship between caprine and ovine isolates, we found a closer relationship between caprine *P. multocida* isolates and one of the isolates from domestic sheep based on the polymorphic site and phylogenetic denderogram in 16S rRNA genes sequencing (Fig. 5). Because a similar strain was not isolated from any of the other ovine isolates, it appears that the goat transmitted this strain to sheep. These transmissions have no association with dermonecotoxin producing ability, as the *toxA*+ strain is found among both goat and sheep isolates. This finding is not in agreement with some studies suggesting transmission of the *toxA*+ strain from sheep to goats in the wild. It is supposed that the *toxA* gene is independently transferred among various isolates from different hosts. Recently, the *toxA* gene has been found on lysogenic bacteriophage belonging to the siphovirde family (Pullinger *et al*., 2004). It seems that the *toxA*+ strains of *P. multocida* are capable of transducing the *toxA* gene to *toxA*$^-$ ones and other bacteria.

In comparison to other studies (Kuhnert *et al*., 2000; Dey *et al*., 2007), polymorphic sites are indicated in the last one third of the 16S rRNA gene instead of the first two thirds of it.

Our results showed there was no relation between the dulcitol and sorbitol fermentation pattern and 16S rRNA type. This result confirms the results of other

groups (Kuhnert *et al*., 2000; Davies, 2004; Dey *et al*., 2007) to support the suggestion that *P. multocida* subsp. *septica* might represent separate species.

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