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Full Length Research Paper

Pheno- and genotyping of *Pasteurella multocida* isolated from goat in Iran

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In this study, phenotype, capsular type and some virulence factors (pfha1, tbpA, and toxA) of caprine *Pasteurella multocida* were described in 10 isolates from goat. Two biochemical biovars were recognized among the isolates based on dulcitol and sorbitol fermentation. The isolates belonged to biovars *P. multocida* sub. multocida (80%) and *P. multocida* sub. septica (20%). Capsular typing of isolates by PCR demonstrated two capsular types A (90%) and D. Among 3 virulence genes detected by PCR, we found a remarkable high prevalence of tbpA (80%) and toxA (70%) genes among caprine isolates. The high prevalence of these genes owning association of disease status among healthy animals showed high potential of the strains in induction of disease. The high prevalence of toxA and tbpA among goat is very similar to the ones in sheep isolates. Comparison of virulence genes profile showed possibility of *P. multocida* transmission between sheep and goat.

Key words: *Pasteurella multocida*, goat, typing, Iran.

INTRODUCTION

Pasteurella multocida, a gram negative, non motile coccobacillus, has been considered a highly heterogenic bacterial species due to different antigenic specificity (Carter, 1987). This organism is classified into sero-groups A, B, D, E and F based on capsule antigens and subdivided into four subspecies that include multocida, gallicida, septica and recently described tigris (Harper et al., 2006). *P. multocida*, *Pasteurella trehalosi* (recently Bibersteinia trehalosi) and *Mannheimia haemolytica* are the three bacteria most commonly cultured from the lungs of domestic and wild sheep and goats. *P. multocida* is a potential cause of pneumonia in domestic sheep and goats, playing a role in the shipping fever complex (Garde et al., 2005).

In addition, it has been shown that some goats carry Mannheimia and Pasteurella species that have been identified in bighorn sheep disease events (Schommer and Woolever, 2008). DNA analysis revealed that feral goat and bighorn sheep shared a genetically identical *P. multocida* (Rudolph et al., 2003, Weiser et al., 2003).

Recently, Ewers et al. (2006) showed that some genes, including pfha1, tbpA and toxA, play vital role in the virulence of bacteria and also used epidemiological marker genes for characterizing *P. multocida* field strains. Among these genes, it was reported that toxA alone is associated with the disease status in swine and pfha1 and tbpA are associated with bovine diseases (Ewers et al., 2006). In our pervious study, we found a high prevalence of tbpA and toxA among ovine population (Shayegh et al., 2008).

This study aims to investigate the phenotyping and genotyping of caprine isolates and to ascertain the presence of the aforementioned virulence genes in isolates taken from goats in Iran.

MATERIALS AND METHODS

Sampling

About 300 nasal swabs taken from goats with nasal discharge were examined from different goat herds of East Azerbaijan, West Azerbaijan and Ardabil province, North West of Iran, covering spring and summer of 2008. All samples were transferred in Stuart's medium to laboratory, plated onto 10% sheep blood agar (SBA) and incubated at 37°C overnight.

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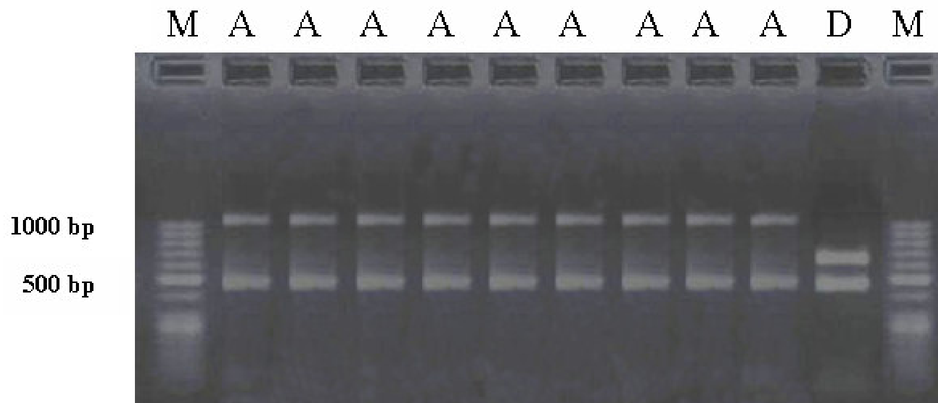

Figure. Capsular typing of pasteurella multocida isolates by multiplex PCR; A: Capsular type A, D: Capsular type D, M: marker

Bacterial isolation and identification

Culture and morphological identification of suspected *P. multocida* isolates were carried out according to standard biochemical tests (Songer and Post, 2005). The isolates were gram-negative cocco-bacilli and were indole, catalase and oxidase-positive. But, citrate, Methyl red (MR), Vogaes – Proskauer (VP), and gelatin liquefaction tests of all isolates were negative. Growth test on MacCankey agar was negative with no motile and non-hemolytic effects on blood agar.

Biotyping of isolates

All isolates of *P. multocida* were examined to phenotypical characterization based on dulcitol and sorbitol fermentation method in Phenol Red Base broth (Merk) with 1% of each sugar (Sigma Aldrich).

Capsular typing with multiplex PCR

Capsular genotyping was carried out on amplification of different capsular groups using multiplex polymerase chain reaction (PCR) in the presence of each capsular specific primers. A pair of specific primer was also added into the reaction for PCR confirmation of the isolates as a *P. multocida* (Townsend et al., 2001) (Figure 1).

The presence of a DNA band with about 460 bp size further established the identification of the isolates as *P. multocida.* All isolates were typed by PCR amplification (Townsend et al., 2001). The PCR amplification was conducted directly on bacterial colonies as temple. In addition, each 50 µl included 1 U Taq DNA polymerase (Fermentas), 3.2 mM from each primer (MWG), 200 µM of each dNTP (Fermentas), 1x PCR buffer, and 2 mM $MgCl_2$ (Fermentas). Amplification was carried out for 35 cycles, each cycle consisting of DNA denaturation at 94℃ for 30 s, annealing at 54℃ for 30 s, extension at 72℃ for 30 s. The cycles were preceded by an initial denaturation at 94℃ for 5 min and followed by final extension at 72℃ for 5 min. The resulting PCR products were electrophoresed in 2% agarose gel and finally stained with ethidium bromide and imaged.

Virulence genes detection

Virulence genes detection was conducted based on amplification of three virulence factor genes (pfha1, tbpA and toxA) using multiplex PCR in the presence of specific primers. Amplification of DNA bands with about 275, 728 and 846 bp sizes were addressed to the presence of pfha1, tbpA and toxA genes in these isolates, respectively (Ewers et al., 2006). The PCR amplification was conducted directly on bacterial colonies as template and added to the reaction mixture (50 ml) containing 3.2 mM of each primer pair, 200 µM from the four dNTPs, 5 µl of 10 x PCR buffer, 1.5 µl of 50 mM magnesium chloride, and 1U of Taq-Polymerase. The samples were subjected to 35 cycles of amplification in a thermal cycler. Each cycle consisted of DNA denaturation at 94℃ for 45 s, annealing at 54℃ for 50 s, extension at 72℃ for 50 s. The cycles were preceded and followed by an initial denaturation at 95℃ for 5 min and final extension at 72℃ for 10 min, respectively. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel, then stained and photographed as mentioned above. Statistic analysis were performed using software SPSS 12.0

RESULTS

Bacterial isolates

Of 300 samples collected from different caprine herds, 10 (3.3%) samples were positive. All isolates are biochemically confirmed.

Biotyping of isolates

Among 10 isolates phenotyped by dulcitol and sorbitol fermentation, 80% belong to *P. multocida* subsp. multocida, the remaining, 20%, were assigned as *P. multocida* subsp. septica. We did not found any sub-species as *P. multocida* subsp. gallicida.

Capsular typing with multiplex PCR

Amplified DNA products of ~1044, ~760 and ~657 bp corresponding to *P. multocida* capsular groups A, B an

d D, respectively, were observed. The band sizes ~511 and ~854 bp expected to be produced corresponding to *P. multocida* capsular serogroups E and F, respectively were not observed. Two genotypes (A and D) were found among both multocida and septica subspecies. Of the samples, 9 (90%) isolates were classified as capsular type A, 1 (10%) as type D.

Virulence genes detection

The results revealed that 70% of the isolates had toxA gene and 80% of them had tbpA gene. These findings are very similar to the characteristics of ovine isolates, which had been previously reported (Shayegh et al., 2008).

DISCUSSION

The present paper describes as a first report, phenotyping, capsular typing and virulence factor profile of Iranian caprine *P. multocida* isolates. *P. multocida* has on many occasions been isolated in many parts of world in both healthy and diseased goats (Ngatia, 1985). However, different *P. multocida* isolation rates have been reported in goats. According to the present study, *P. multocida* was not a highly prevalent agent among goats in this study and the frequencies of the bacteria in other studies were higher than our study (Megra et al., 2006; Ngatia et al., 1985). One of such previous studies reported that nasal swabs from sheep and goat were bereft of *P. multocida* isolates (Kapoor et al., 2004).

In this study, 90% of P. multocida isolates belonged to capsular type A, 10% was determined as capsular type D. The result of previous studies had shown that capsular type A and D are common capsular strains isolated from caprine pneumonia (Blanco-Viera et al., 1993; Martrenchar et al., 1995; Rudolf et al., 2003). Isolation of capsular type-B P multocida in goats has been reported, infection due to this type has not been observed even under experimental condition (Chandrasekaran et al., 1981; Dey et al., 2007; Heddleston et al., 1967). The result of this study indicated *P. multocida* type A and D participated in caprine pneumonic complex in Iran.

Phenotypical study of *P. multocida* showed high prevalence of *P. multocida* subsp. septica among isolates. Lower prevalence of this subspecies had previously been reported by other groups (Ekundayo et al., 2008). Recently, it has been asserted that biotyping methods based on dulcitol and sorbital fermentation do not always agree with genetic approach (Davies, 2004; Dey et al., 2007; Kuhnert et al., 2000). Hence, final confirmation of subspecies should be determined by genome analysis. On the other hand, since studies based on biochemical profile show high variation among different isolates (Blackall et al., 1997; Ekundayo et al., 2008; Fegan et al., 1995; Townsend et al., 1998), genetic-based investigations have been suggested to be used instead of phenotypic ones.

In the present research, we found high prevalence of toxA and tbpA genes among our isolates. This virulence gene profiles were similar to those reported in previous ovine studies (Shayegh et al., 2008). Since, sheep and goats have very close relationship; it may be that *P. multocida* is shared between domestic sheep and goat population through nose to nose contact in Iranian small ruminant herds. Similar transmission has already been reported in wild life (Rudolph, et al., 2003; Weiser et al., 2003). In this study, the resulted data confirmed the presence of a significant association between this gene and caprine disease ($P < 0.05$). Previous papers had shown a relationship between these virulence genes and diseased status. Ewers et al. (2006) reported the presence of a meaningful association between toxA and swine as well as tbpA and pfhA genes and bovine diseases. Recently, the high prevalence of toxA and tbpA genes was reported in our previous paper in sheep (Shayegh et al., 2008).

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