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Characterization, ribotyping and capsular PCR typing of *Pasteurella multocida* isolated from Iranian sheep and goats

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ABSTRACT

Pasteurella multocida is considered to be an important cause of ovine pneumonia and results causes considerable economic losses in Iran. Ribotyping PCR (Ribo-PCR) was used for investigating the diversity of ovine and caprine *P.multocida*. A total of 120 swab tonsil and nasal samples were obtained from sheep and goats. They were analyzed by biochemical tests and Ribo-PCR 16S-23S ribosomal RNA genes. Twenty samples representing *Pasteurella* phenotypes by Entero rapid kit and 17 out of 20 isolates were identified as *P.multocida*. Capsular type A was dominant among the isolates and was variable than serogroup D. Nine isolates including JF694004.1 and 8 isolates including JF681973.1 showed 100% and 94% similarity, respectively. Two minor cluster I and II was obtained with no significant diversity among the isolates. Cluster II was divided in four sub cluster including IIa, IIb, IIc and IId. Molecular approaches such as Ribo-PCR are necessary for determining and characterizing the diversity of *P. multocida* and understanding their interactions with host. The study has been the sensitive levels of Ribo-PCR in epidemiological studies of pasteurellosis, however, correlation among diversity of isolates and host origin is not fully understood.

1. Introduction

Pasteurella multocida is a gram-negative bacteria associated with a variety of diseases in animals including hemorrhagic septicaemia in cattle and buffaloes, fowl cholera in poultry, atrophic rhinitis in pigs, snuffles in rabbits, pneumonic and septicaemic pasteurellosis in sheep, goats, wild animals and humans (Stahel et al., 2009). They have been classified by different conventional capsule antigens into serogroups A, B, D, E and F (Harper et al., 2006).

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). Recently, approaches in molecular level have been used to assess the relatedness of bacterial isolates (Rastogi and Sani, 2011). The rRNA genetic locus is a genetic unit of broad evolutionary interest since it is found in both prokaryotic and eukaryotic organisms. There is sufficient conservation within the locus to be used in a universal organization of evolutionary relationships. In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species, 16S, 23S and 5S genes (Daffonchio et al., 2003). These genes are separated by spacer regions, which are transcribed together with the ribosomal genes and thus are named internal transcribed spacer(s) (ITS). These genomic

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regions show a high degree of variability between species, both in their base length and in their sequence (Osorio et al., 2005).
The molecular PCR basis is used to detect polymorphisms in the intergenic spacer regions of bacterial rRNA genes by primers from highly conserved flanking sequences (Osorio et al., 2005). In the present study PCR was used to explore the polymorphism of the genomic 16S-23S rRNA region from *P. multocida* local isolates from sheep and goats in the southern part of Iran.

2. Materials and Methods

2.1. Sampling

During a one year period from May 2013 to Jun 2014, 120 swab samples from the nasal and tonsil were collected from healthy and ailing sheep and goats in Shiraz, Iran. The ailing animals were clinically exhibited pneumonic symptoms, respiratory distress, profuse nasal discharge and sneezing. Heart blood and tissue pieces of spleen, liver and lungs were collected from dead animals. The samples were put in Stuart as a carrier media and then transferred to the laboratory in cold condition.

2.2. Biochemical tests

Biochemical tests were carried out using Entero rapid – 24 test kits as described by Tefera (2002). Entero rapid – 24 test kit is used for the identification of clinically important members of *P. multocida*.

2.3. Molecular assay

DNA extraction: Swab samples were inoculated in brain heart infusion (BHI) broth (Hi-media, India) and incubated overnight at 37°C and the culture was used directly for DNA extraction. Chromosomal DNA was prepared from clinical samples according to the study of Sahragard et al (2012) with some modifications. Briefly, 1.5-ml aliquots of the BHI were centrifuged at 13,000×g for 5 min. The supernatant was discarded and the pellets were washed twice in PBS at 13,000×g for 5 min. The bacterial cell pellets were re-suspended in a 600-µl natrium–Tris–EDTA buffer (pH=7.4). Three micro liters of a 20-mg/ml stock solution of proteinase K (Gibco BRL) was added to the bacterial suspensions to reach a final concentration of 100 µg/ml. After vortexing, the suspension was heated on a thermal Block at 98°C for 15 min. The cell debris was pelleted by centrifuge at 13000 g in a bench top microfuge for 5 min and 1 µl of the supernatant was used as DNA template.

***P. multocida* species specific PCR (PM-PCR):**

The species specific primers designed by Townsend et al., (1998) KMTIT7: ATC CGC GAT TTA CCC AGT GG and KMTISP6: GCT GTA AAC GAA CTC GCC AC were used to amplify the gene sequences in *P. multocida*. The PCR amplification mixture (25 µl) contained each primer at a concentration of 3.2 µM, each deoxynucleoside triphosphate at a concentration of 200 µM, 1 × PCR buffer, 2mM $MgCl_2$, and 0.5 U of Taq DNA polymerase (Fermentase, Germany). Twenty ng of template *P. multocida* genomic DNA was added to the mixture. All amplifications were performed on a Ependorf PCR system. Amplification was performed for 30 cycles. The cycles include an initial denaturation at 94°C for 30sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed by primary denaturation at 95°C for 5 min. The final cycle was followed by an extension at 72°C for 5 min. The amplified products were separated by electrophoresis (Cleaver System, UK) on 2% agarose gel and visualized by ethidium bromide staining in Gel Documentaion Systen (Kodak, USA).

16S-23S PCR: For PCR amplification, the following primers were used (described by Jabbari et al., 2008): 16S-23S FW: 5-TTG TAG ACA CCG CCC GTC A-3 and 16S RV: 5-GGT ACG TTA GAT GTT TCA GTT C-3.The primers were designed to correspond to rRNA gene of *P. multocida* by using the sequence data obtained from Gene Bank (National Institutes of Health).The reactions were performed in a final volume of 50 ml at the following reagent concentration: 10 mM Tris-HCL, pH: 8.3, 50mM KCL, 200µM dNTP, 0.5µM of each primer, 3mM $MgCl_2$, 2.5U Taq polymerase enzyme, and 1µl of template DNA.

All amplifications were performed on a Master Gradient Ependorf PCR system (Germany). Amplification was performed for 40 cycles. The cycles include an initial denaturation at 94°C for 1min, annealing at 55°C for 30 sec, and extension at 72°C for 2min, followed by

primary denaturation at 95°C for 5 min. The final cycle was followed by an extension at 72°C for 7 min. The amplified PCR products were run on 1.5% agarose gel by electrophoresis and visualized by gel documentation system (Kokak) after staining by ethidium bromide. The PCR products were purified and sequenced.

Sequencing: PCR products of 16S-23S for sequencing were purified using the PCR product purification kit (Roche, Germany).All purified PCR products were sequenced by MWG Laboratory, Germany.

Gene Bank accession numbers: The PCR product of sequences of Iranian *P. multocida* isolates from sheep and goats has been submitted to the Gene Bank. The Gene Bank accession numbers for 16S-23S spacer region nucleotide sequences determined in this investigation are listed as in Table I, JF694004.1, JF681973.1, JF694005.1, JF694003.1. The alignment of Iranian sheep isolates was compared with sequence of American reference strain 3480 with accession no. CP003313 (table 1).

Table 1. Standard *P. multocida* used in the 16S rRNA gene sequence.

Strain	Country	Gene bank accession no.	Source	Identity (%)
PMSHI-9	Iran	JF694004.1	Sheep	100
PMSHI-7	Iran	JF681973.1	Sheep	94
PMSHI-10	Iran	JF694005.1	Sheep	94
PMSHI-8	Iran	JF694003.1	Sheep	94
3480	USA	CP001409.1	Pig	

Mice bioassay: All identified *P. multocida* strains were inoculated separately into 5-ml BHI broth and incubated in a shaking incubator (120 rpm) O/N at 37ºC. Each group of balb/c mice (three mice in each group) was injected (0.2 ml) via intra-peritoneal route. The control group was injected with a fresh BHI broth alone. The infected mice were observed for possible fatality during the next 24 h. The heart blood smears and impression smears of the spleen, liver, and lung were collected from the dead mice. Liver and lung samples from dead mice were streaked onto 10% sheep blood agar and incubated at 37ºC. Aspirated heart blood was inoculated in BHI broth and incubated at 37ºC for 16h, and then the broth culture was streaked onto blood agar and MacConkey agar.

3. Results

3.1. Biochemical test

From 120 samples 20 (16.66%) Twenty of the 120 samples were susceptible to *Pasteurella* by biochemical tests. The isolates were indicated positive reaction for indole, nitrate reduction, oxidase, and catalase and negative for MR, VP, and simmons citrate tests. They fermented glucose, fructose, mannitol, terhalose, and sucrose as well. More information about healthy, respiratory distress and dead animals represented in table II.

3.2. PM-PCR analyses

P. multocida species specific PCR (PM-PCR) assay developed by Townsend *et al*., (1998) was used for this study to identify the subspecies of *P. multocida* by amplifying 460 bp DNA fragment within KMT1 gene using the primers KMTISP6 and KMTIT7 (Figure 1). 17 out of 20 susceptible isolates were identified as *P. multocida*.

3.3. 16s-23s PCR analyses

PCR amplification of 16s r-RNA genes of the isolates produced single fragments of 0.8 kbp (Figure 2). Fragment partial sequences were aligned using Megalign 4 software. A dendrogram of genetic relatedness is presented in Figure 3. It shows the alignment of the 16s-23s rRNA spacer sequences of 17 Iranian *P. multocida* isolates. Dendrogram of *P. multocida* data showed 100% similarity among 9 isolates including JF694004.1 and 94% similarity among 8 isolates including JF681973.1 ($p<0.05$). Two minor clusters (I and II) were found to have genetic similarity of 0.005. Cluster II was divided to four sub clusters (IIa, IIb, IIc, and IId). Strain Razi 10 that was isolated from healthy sheep was found in cluster I. Two isolates (Razi 5 and 6) were found in cluster II and were obtained from healthy animals. The remaining strains (both isolates

from healthy, distressed and dead) were found in cluster II.

The 17 isolates presented 9 unique patterns (Figure 3). Only four *P. multocida* isolates with the same patterns were found in cluster II in this study. *P. multocida* isolates with a similarity coefficient (SC) of < 0.001 were found in four out of five distressed respiratory animals (Razi 1, 9, 15 and 16). Although these four isolates were not closely related to the same origin, they were found in cluster II. Another cluster (11 identical organisms) was isolated from dead animal with SC between 0.001 and 0.035. All isolates had identical phylogeny patterns (except Razi 12, 17) and they were found in cluster II (except Razi 10). Overall, 14.16% (17/120) *P. multocida* was isolated from healthy, distressed or dead animals.

3.4. Mice bioassay

The MDT was confirmed in all 17 isolates, mainly in those with mean dead time between 8-12 h. (n=5). Control mice groups did not die even after 3 days post injection.

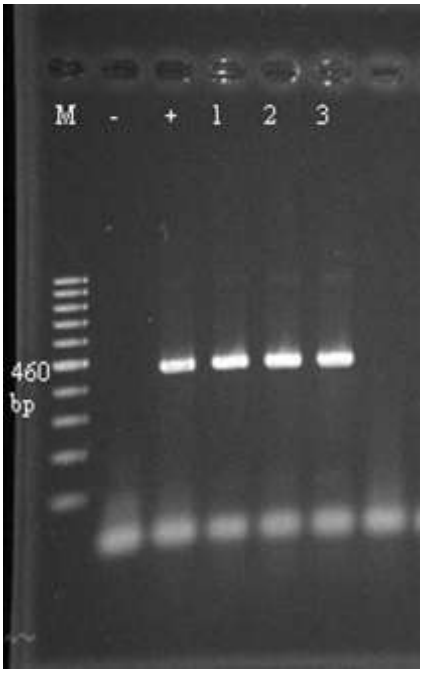

Figure 1. Polymerase chain reaction (REP-PCR) profiles of *P. multocida* isolates. Lane M: molecular weight standard 100 bp; lane +: positive control; lanes 1-4: isolated strain; lane-: negative control

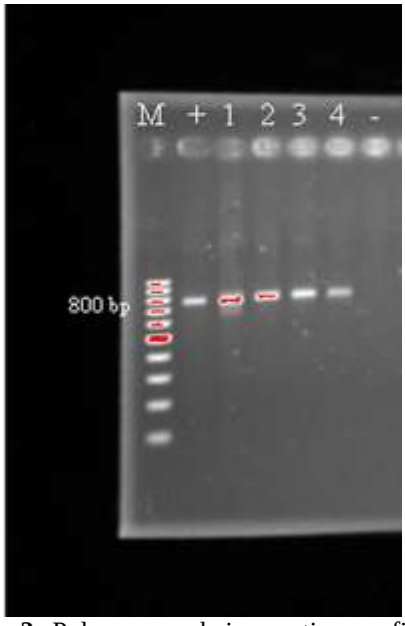

Figure 2. Polymerase chain reaction profiles of *P.multocida* isolates. Lane M: molecular weight standard 100 bp; lane -: negative control; lane +: positive control; lanes 1-3: isolated strain.

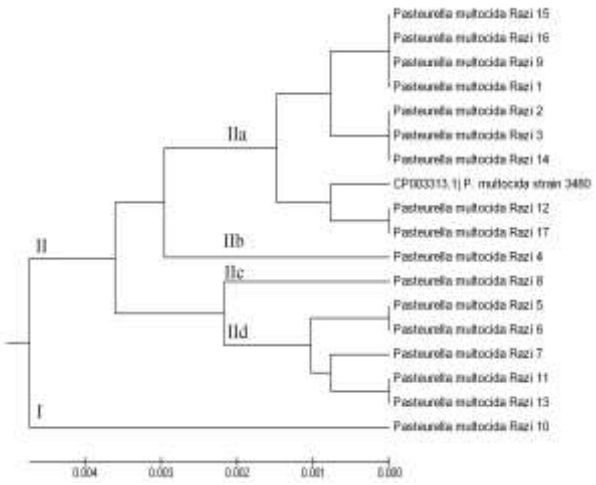

Figure 3. Dendrogram of genetic distance among 17 ribotypes clustered by Megalign 4 software. The two ribotype clusters (I and II) and four subclusters (IIa – IId) are marked.

4. Discussion

The study was examined for the isolation and identification of *P. multocida* from sheep and goats with regards to cultural identification, biochemical behaviors and molecular characteristics. The molecular identification was carried out by characterization of ribotyping-sequencing by repetitive sequence based PCR.

As it known, *Pasteurella* spp. is found as an upper respiratory normal flora in healthy domestic ruminants, and they may be isolated more frequently (Besser et al., 2012). According to dendrogram chart *P. multocida* isolates was classified into nine groups and no synergistic association was observed with reference strain (DQ157903.1, accession no). Five strains (Razi 4, 5, 6, 8 and 10) indicated the MDT less than 12 h and they were more virulent than others. These strains did not show synergistic phylogeny pattern. The same study was conducted by Stahel et al (2009), who presented *P. multocida* with MDT less than 12 h produced a dermonecrotic toxin. The animals were showed progressive pneumonia and finally died (Stahel et al., 2009).

Several studies have shown genetic and phenotypic variability between *P. multocida* isolates from sheep population (Stahel et al., 2009; Shayegh et al., 2011; Davies, 2004; Mohamed and Mageed, 2014). But, results from the present study indicate many unique patterns in the isolates from sheep and goats. The diversity of *P. multocida* strains in the current study indicated multiple origins among the isolates. Dendrogram revealed that isolates from distressed and dead animals belong to cluster II. However, relationship among individual clusters (sub clusters) and host origin was not completely known. There are no more studies about genetic diversity among *P. multocida* in Iran, but the comparison between different regions indicated phenotypic variability. In the study by Jabbari et al., (2008), according to phylogenic analysis based on ITS sequence alignment, the *P. multocida* isolates classified into 2 distinct clusters. The virulence of isolates in cluster II were higher than those in cluster I. They showed Ribotyping of *P. multocida* by using 16S-23S rRNA gene PCR sequencing could be used as a marker in epidemiologic studies. Jabbari et al (2005) and Ghanizadeh et al (2015) were found the PCR-RFLP of the ompH gene to be potentially a useful method for typing of *P.multocida* and therefore, for studying the epidemiology of *P. multocida* infections. The RFLP patterns of this gene exhibited extensive restriction site heterogeneity, which may be particularly suitable for fingerprinting of *P. multocida* isolates. Shayegh et al (2010) compared the 16S rRNA gene sequence and showed the role of goat as a reservoir for *P. multocida* to sheep independent of *toxA* genes transmission.

In this study significant diversity was not found among the isolates originating from different place, but based on the molecular characterization and comparison of the isolates, different *P. multocida* clones might be isolated from healthy, distressed and /or dead animals. The observation of current study was the first and most noticeable in the isolates of sheep origin in Iran. Furthermore, according to sequencing and dendrogram pattern and knowledge of all isolates belong to capsular type A (Sahragard et al., 2012), this serogroup might be more variable than serogroup D. Further study about other possible serogroups in different hosts is recommended.

In conclusion, much diversity was found among the *P. multocida* isolates. However, different strains were shown various pathogenesis in two hosts, sheep and goats. Therefore, infection and colonization of various hosts were caused by different *P. multocida* strains. This result was most considerable in the isolates with ovine and caprine origin. As is noted, serogroup A were more variable than serogroup D. The low MDT was much more common in the isolates with serogroup A.

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