Short Communication

Isolation and Identification of Pasteurella multocida by PCR from sheep and goats in Fars province, Iran

Tahamtan*, Y., Hayati, M., Namavari, M.M.
1. Department of Bacteriology, Razi Vaccine and Serum Research Institute, Shiraz, Iran

ABSTRACT
During one year period from 2010 to 2011 the samples from pneumonic animals were taken and transported to the laboratory. Pasteurella multocida were identified in 16.6% of animal by biochemical test. The high incidence of P. multocida was obtained in the south of Fars province, where the area was warm region. The Mean Death Time between the isolates was 12-18 and 19-24 hours. Only the capsular type A was identified in all the isolates and it is agreement with the finding by others, they indicated type A is the dominant type of Pasteurella multocia in tropical and sub tropical climate.

Keywords: Pasteurella, PCR, Toxigenic, Type, Iran

INTRODUCTION
Pasteurella (P) multocida causing pneumatic and septicaemic pasteurellosis in sheep, goats, and humans (Alwis 1996). It has been implicated in a number of diseases that manifest differently in various hosts and is also restricted to a specific geographical region (Blackall & Miflin 2000). According to polysaccharide capsules on their surface, they were differentiated into five serogroups including A, B, D, E and F (Chung et al 1998). Serogroup A and D of P. multocida have been incriminated agents of pneumatic Pasteurellosis in sheep and goats (Chandrasekaran et al 1991, Zamrisaad et al 1996). These isolates synthesize a 145-kDa toxin encoded by the chromosomal toxA gene. The toxA protein is an essential virulence factor for progression of Pasteurellosis in sheep and goats; in particular those of capsular serotypes A and D. Non-toxigenic P. multocida isolates do not cause the disease (Lichtensteiger et al 1996). Toxigenic P. multocida from an infected herd is distributed to clean herds by asymptomatic carriers, however many epidemiological and ecological aspects of disease and the pathogen remain unknown. P. multocida is part of normal flora of the upper respiratory tract, therefore predisposing factors such as overcrowding and bad ventilation, causes pasteurellosis (Mustafa et al 1978, Shayegh et al 2008, Hawari et al 2008). P. multocida is an endemic disease in Iran such as West Azarbaijan, Mazandaran, Gilan, Khuzistan (Tabatabaei et al 2002, Shayegh et al 2009). But no information about the disease and prevalence in Fars province is available. There are several methods for
isolation and identification of Pasteurella sp. They include bacterial culture, mice bio assay and antiserum base methods (Carter 1955, Heddleston et al 1972), meanwhile some of these such as culturing bacterial isolation are time consuming and give false negative results. Mice inoculation is not practical for identification (de Jong 1992). Therefore, the purpose of this study was to isolation and identification of Pasteurella sp by routine and molecular methods from sheep and goats in Shiraz Iran.

MATERIALS AND METHODS

Sample. One hundred and twenty nasals and tonsil swab samples were collected from ailing sheep and goats during 2010 to 2011 in cold and warm region of Fars province, Iran. Lung and liver lymph node, and tissue samples of the spleen, liver and lungs were collected from dead animals. The samples were then transported to the laboratory in cold condition. Biochemical tests were carried out on all 120 sample isolates using Entero rapid 24 test kits as described by Tefera (2002).

Mice assay. Twenty isolates identified as Pasteurella strains by biochemical test were inoculated separately in brain heart infusion (BHI) broth and incubated in a shaking incubator (100 rpm) at 37 °C overnight. Twenty groups of balb/C mice (3 mice in each group) were injected (0.2ml) via the intra peritoneum method. Three mice were left as control group and injected with only fresh BHI broth. Injected mice were observed for 24 hours for the fatality rates. The impression smears of the spleen, liver and lung were collected from the dead mice. Liver and lung samples from the dead mice were streaked onto sheep blood agar and incubated as the same above.

DNA extraction. DNA extraction was carried out according to described by Ozbey et al (2004) with minor modifications. Briefly, overnight BHI culture was centrifuged. The pellets were washed twice in PBS. The washed pallets were resuspended in trice EDTA (ethylenediaminetetraacetate) (pH, 7.3). The cell was extracted with phenol previously saturated with trice EDTA (pH, 7.3) and centrifuged. The upper phase centrifuged solution was collected and transferred to clean micro tube. The content was mixed with phenol/chloroform/isoamyl and centrifuged as well. After addition sodium acetate and absolute ethanol, DNA was precipitated. DNA was dried and resuspended in EDTA and reserved in freezer until used. Identification of P. multocida was confirmed by PCR using primers set in table 1. The kmnt gene was amplified by all pass primer. This gene indicated the species of P. multocida among the isolates. The PCR reaction was performed in a total volume 25 μL containing 0.4 μM from each primer, 0.2 mM dNTPs, 1X Taq reaction buffer, 2.5 mM MgCl₂, 1.25 U from Taq DNA polymerase and1 μL from the isolated DNA (all reagents made from Sina-gene, Iran). The DNA was amplified under the following conditions in a thermo cycler (gradient master cycler Eppendorf, Germany) include: primary denaturation step at 94 °C for 4 min, 35 cycles using the following settings: initial denaturation at 94 °C for 45 sec, annealing at 45 °C for 45 sec and extension at 72°C for 45 sec, followed by 5 min at 72 °C. The PCR product was analyzed on 1.3% agarose gel stained with ethidium bromide. Two DNA ladders 1 kb and 100 bp (Fermentas) were used to determine the size of the amplified fragments. The results were then analyzed under SPSS 12.5 with ANOVA software.

RESULTS AND DISCUSSION

The isolates subjected to biochemical tests were positive for indole, nitrate reduction, oxidase and catalase and negative for MR VP and simmons citrate test. The isolates fermented glucose, fructose, mannitol, trehalose and sucrose. According to the biochemical results, twenty (16.6%) of the 120 samples were identified as Pasteurella. On the basis of these results, P. multocida are the most common in sheep and goats in the warm region of Fars province (P<0.50%).
Table 1. Sequences of the oligonucleotides used for detection of *P. multocida*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Name</th>
<th>Sequence Amplimer size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Pass</td>
<td>KMT1</td>
<td>KMT1T7</td>
<td>ATCCCGCGATTACCCAGGTG        460</td>
</tr>
<tr>
<td>KMTSP6</td>
<td></td>
<td></td>
<td>GCTGTAAACGAACCTGCGCA</td>
</tr>
<tr>
<td>CAPA-REW</td>
<td></td>
<td></td>
<td>TTGCCATATTGTCAG</td>
</tr>
<tr>
<td>Capsular type A</td>
<td>hydD-hydC</td>
<td>CAPA-FWD</td>
<td>TGCCAAAATCGCAGTCA           1044</td>
</tr>
<tr>
<td>Capsular type D</td>
<td>dcbF</td>
<td>CAPD-FWD</td>
<td>CATCTACCCACTCAAACCATACAG    760</td>
</tr>
<tr>
<td>Dermo necrotic toxin</td>
<td>toxA</td>
<td>TOXA-FWD</td>
<td>TACTCAATTAGAAAAAGCGCTTTATCTTCC 300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TOXA-REV</td>
</tr>
</tbody>
</table>

Table 2. Biochemical, mice assay and PCR results of isolates from sheep and goats

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Biochemical test</th>
<th>Dead mice</th>
<th>P. m (kmt1)</th>
<th>Type A Cap (hydD-hydC)</th>
<th>DNT (toxA)</th>
<th>Type D Cap (dcbF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>20(16.6%)</td>
<td>16(13.3%)</td>
<td>10 (8.33)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Capsular typing and *toxA* frequency of *P. multocida* isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>All pass</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Capsular A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Capsular D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>toxA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The serotypes of the isolates were determined due to PCR analysis. The serotypes of the isolates were determined due to PCR analysis. The 12 mice groups injected with isolates identified by biochemical test were found to be virulent and died within Mean Death Time (MDT) between 12-18 hrs and four isolates had a low virulence with MDT between 19 and 24 hours. Whereas, all three mice injected with BHI broth culture as control did not die even after two days post injection (P<0.05%) (Table 2). Liver and spleen impression smear evidenced characteristic of *Pasteurella* on gram staining. The isolates showed typical morphological and cultural characteristics of dew drop, mucoid, non haemolytic colonies in blood agar. No growth was observed in MacConkey agar. Culture smears revealed characteristic gram negative coccobacillary organisms. PCR assay for *P. multocida* species specific developed by Townsend et al. (1998) with some modification. The method identifies the subspecies of *P. multocida* by amplifying 460 bp DNA fragment within KMTI gene using the primers KMTISP6 and KMTIT7. This indicated whether the species is *P. multocida* or not (figure 1). Table 3 was shown the results of multiplex PCR (mPCR) based on primer set as in table 1.

According to amplification of hya C – hyaD gene of *P. multocida* by PCR, all the isolates were identified as capsular type ‘A’ and the amplicon size was found to be 1044bp (figure 1). *P. multocida* has also been reported as a cause of pneumonic pasteurellosis in sheep and goats (Jaglic et al 2005, Xibiao et al 2009).

![Figure 1](image.png)

**Figure 1.** 1.4% agarose gel electrophoresis for specific factors (kmt1), capsule (capA) and toxigenic (toxA) primers for Pasteurella. Lane M: 100 bp and 1 kb ladder, line 1-10: virulent factors of *P. multocida* isolates, C+: positive and C-: negative controls.

Sheep and goats pneumonia is especially common in newborn and in feedlot lambs, it can also occurs in the
mature ewe flock with milder clinical signs. Pneumonia occurs in all ages of sheep and goats, in all breeds, in every country of the world (Ozbey et al 2004). The high incidence of disease in warm region, exhibition a positive correlation with area, suggesting the climate condition plays a role in respiratory problems in that area. Factors such as crowding, dust, damp humid weather, or stress all can increase the disease (Weiser et al 2003). The mouse bio assay findings were comparable with the findings of Mustafa et al (1978) and Diallo et al (1995). These results were also in accordance with observation of Holmes (1998) and OIE manual (2004). They were reported 50% of the isolates with minimum MDT. The finding of biochemical results are in agreement with Tefera (2002) and OIE manual (2004). They found 17.5% according to biochemical test. The P. multocida isolates that were positive by biochemical reactions subjected also for possible positive results by PCR. No amplifications were produced from the negative control. These results were in agreement with Ozbey and Muz (2006), who mentioned that some P. multocida isolates that were positive by culture were also detected to be positive by PCR. Methods like biochemical tests using API 20E, and mice bio assay were not detected any of the virulence factors. (McAvin et al 2001). Oligonucleotide primers designed for these isolates have formed the basis for mPCR assays that specifically identify P. multocida and, in particular, capsular type and toxigenic species isolates that cause severe disease (Chandrasekaran et al 1991; Zamrisaad et al 1996). Similar results were not obtained with biochemical test and mice assay. It also indicated that molecular typing methods can provide a stable and highly useful analysis of bacterial isolates and have proved to be beneficial in reducing the limitations of the biochemical tests (Hawari et al 2008). The findings of this study are in agreement with reports that P. multocida type A are the most prevalent in Iran (Tehrani et al 2004, Shayegh et al 2008). Our findings support the need for the development of a vaccine using the most prevalent P. multocida serotypes as well as strategic deworming, and improved housing conditions for sheep and goats in the Fars province and totally in Iran.

Acknowledgment

We thank to Dr. MH Hosseini and all of the people who collaborated with the authors, particularly the Veterinary Organization of Fars province. The authors also thank Dr. M Mansoorian for edition.

References


