Identification of Pasteurella multocida and molecular diagnosis of haemorrhagic septicaemia in Iranian camels

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ABSTRACT

Pasteurella multocida, a gram-negative coccobacillus in the Pasteurellaceae family is associated with hemorrhagic septicaemia in camels, also known as ‘Pneumonic Head’. Hemorrhagic septicaemia is a disease with unclear etiology which is very probably caused by P. multocida. Sudden climatic changes, poor management practices, exposure to various diseases, over traveling and low grade nutrition are the most important predisposing factors for hemorrhagic septicaemia. Although P. multocida serotype B is known as a causal agent of respiratory disease in camels, its role as the causative agent for hemorrhagic septicaemia in camels remains unclear and needs further investigation. This paper looks at the isolation and molecular aspects of pasteurellosis in camels from October 2010 to April 2012 in the southern part of Iran. Sixty nine nasal swabs and blood specimens from clinical cases and One hundred and fifteen nasal discharge swabs from apparently healthy camels, and a lung sample from a 10 dead camel were taken for bacterial isolation and PCR assay. Pasteurella multocida was isolated from 80% of the internal organs of the dead camel. The isolation rate of P. multocida in the clinical cases was 68% while 7% of healthy camels were shown to be infected with P. multocida. Bacteria species and P. multocida serotype were identified in Pasteurella isolates by amplification of a 460 pb PCR product using KMT1SP6 -KMT1T7 primers for all pass. Serotype B represented the main P. multocida serotype. It was concluded from this investigation that P. multocida infection can be diagnosed with different methods such as biochemical and molecular assays and may be the primary organism associated with severe respiratory signs and septicaemia in camels.

Keywords: Pasteurella multocida, Haemorrhagic septicaemia, Camel, Iran.

Introduction

Amongst domestic ruminants, camel has been for a long time a neglected species (22). The camel is an important domestic animal in many parts of Iran because of its adaptation to adverse climatic conditions and to shortage of water and forage. Camels are living in arid and semi-arid areas particularly eastern, middle and southern parts of Iran. Globally, Except for some parasitic diseases, there is only few information on camel diseases [1, 2, 7, 24]. Furthermore camel respiratory infections have received little attention in Iran. According to author’s knowledge, no studies in Iranian camels have been extensively communicated in comparison with other livestock species. Further studies have been advocated on etiology and identification of causing pathogens including a variety of viruses, fungi, bacteriae and parasites (3) in camel respiratory outbreaks. In recent years, camel research received more attention worldwide. His ability to adapt to varying harsh environmental conditions has made it breeding possible especially in the arid and semi arid areas, especially in areas where a moisture deficit exists. It provides meat, means of transport and plays an important role for the socio-cultural structure of the community (3, 33).

Haemorrhagic septicaemia also called pasteurellosis is a disease caused by Pasteurella multocida (22). P. multocida in camels was considered a hazardous disease that drastically affects productivity of camels and may even cause death (8, 21, 41). Symptoms of the disease include very high temperature, increased respiratory and heart pulse rates and general...
depression. (18). In camels subcutaneous tissue, mandibular and/or cervical lymph nodes are usually enlarged (34). Affected animals seldom recover and usually die in the next 24 to 48 hours (31). Signs of dyspnea such as dilated nostrils or open mouth breathing and cyanotic mucous membranes are currently seen (11).

Respiratory diseases remain a major threat to the camel population in the southern parts of Iran where more than 5000 camels are living. Respiratory disease and sudden death occurred in this region during 2009-2011. Some Veterinary Institutions have tried to isolate and isolate the etiological agent of the disease. Before that, a respiratory disease outbreak associated with sudden death has occurred in the Afar and Oromia regions during 2005/2006 (9, 17, 30). In a recent study conducted in Lapur division of Turkana district, hemorrhagic septicaemia was ranked as the fourth most important disease in camels (25). Carter (1959) recorded Pasteurellaceae associated with disease in Sudan and in a snow leopard in the Himalayas (5, 10, 12, 14, 47).

This study described for the first time isolation and molecular epidemiology of respiratory disease in camels according to capsular types of bacterial species in central region of the South of Iran.

**Material and Methods**

The study was carried out from October 2010 to April 2012 in 194 camels the Fars province, a middle region of southern part of Iran. The climate of this area is semi-arid. It is located at about 250 km south of Shiraz, the capital of the Fars province.

**SAMPLING AND PROTOCOL**

The sampling was made by Fars Veterinary organization notification. Three different types of samples were collected in the presence of respiratory signs after camel outbreaks notifications: 1) Tissues of pneumonic lungs, draining lymph nodes, liver, spleen, heart, kidney, and other affected organs from 10 dead camels less than 5 years of age with natural history and course of severe respiratory disease with symptoms such as distress, pyrexia, and nasal mucopurulent discharge were aseptically obtained from different apparently healthy camels as controls. They were inoculated directly on Brain Heart Infusion (BHI) broth and incubated O/N at 37°C. After that, O/N culture was streaked onto BA plates. According to Muters et al (1985), suspected colonies were identified by morphologically and biochemically current methods (26).

**BACTERIA COLONIES**

Bacteria colonies were identified by colony shape, Gram staining, and further characterized by biochemical testing. Biochemical tests were carried out using Entero rapid – 24 test kits as described by Tefera (2002) for the identification of clinically important members of P. multocida (42).

**PCR ASSAY**

Suspected colonies were cultured in BHI broth and incubated O/N at 37°C for additional identification by PCR method. DNA was extracted as described in Jabbari et al (2005) with minor modifications (20). Briefly, one ml O/N BHI culture was centrifuged. The supernatant was discarded and the pellets were washed twice and centrifuged again. Bacteria pellets were re-suspended in sodium tris-EDTA (NTE) buffer (pH = 7.4). Then proteinase K (Gibco BRL) was added to the bacterial suspensions. The cell lysate was extracted using ultrapure phenol (Gibco BRL) previously saturated with tris (pH: 8.3). The mixture was rotated vigorously for 5 min at room temperature and then centrifuged again. The upper phase was transferred to a clean 1.5-ml microtube. This phase was mixed with one volume of a phenol/chloroform mixture and centrifuged again. DNA was precipitated by the addition of sodium acetate and absolute ethanol. The DNA was then washed with 70% ethanol, dried at room temperature and re-suspended in TE buffer (pH: 8) and stored at -20°C until used.

PCR was performed using previously described primers as indicated in table I (32, 44). These include KMTI for species identification, hydD-hydC and bcbD for capsular typing. The master mix PCR was prepared by mixing deoxynucleoside triphosphate, PCR buffer, MgCl₂, Taq DNA polymerase, primers, DNA template and distilled water. PCR procedure

<table>
<thead>
<tr>
<th>Primer</th>
<th>gene</th>
<th>Primer sequence 5’-3’</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allpass</td>
<td>KMT1</td>
<td>ATCCGGCGATTTACCCAGTGGA</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTGTTAACGAACTCGCCAC</td>
<td></td>
</tr>
<tr>
<td>Capsular type A</td>
<td>hydD-hydC</td>
<td>TGCCAAAATCGCAGTCAG</td>
<td>1044</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTGCGATCATTTGTCAGTGA</td>
<td></td>
</tr>
<tr>
<td>Cap B</td>
<td>bcbD</td>
<td>CATTATCAGAAGCTCCACC</td>
<td>760</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCCCGAGAGTTTCCATCC</td>
<td></td>
</tr>
</tbody>
</table>

**Table I:** Primers used for the detection of virulence-associated genes in strains of *P. multocida*.

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was conducted on a Eppendorf PCR system (master gradient Eppendorf, Germany). Amplified PCR products were run on 1.2% agarose gel by electrophoresis and visualized by gel documentation system (Kodak) after staining by ethidium bromide.

**MICE BIOASSAY**

To determine pathogenicity of the isolates, a mice bioassay was applied. Identified Bacteria were inoculated into BHI broth and incubated in a shaking incubator (120 rpm) O/N at 37°C. Inoculum was injected (0.2ml) into the peritoneal cavity of Balb/c mice and fatality rates were recorded during the next 24 hrs.

**Results**

Rate of infection. Report on the investigation of bacteriological examination was summarized in table II. *P. multocida* was isolated from 80% (8/10) of dead camels. Lungs from 10 dead camels were showing various types of lesions. Pneumonia, bronchitis, emphysema, pulmonary edema, congestion, and serous secretion were observed in affected lungs. Sixty-nine clinically ill camels with clinical signs including increased temperature up to 40°C, dyspnea, bad mouth odor, swelling of the lips and around the neck and mandible, anorexia and pneumonia (figure 1). The isolation rate of *P. multocida* in the clinical cases was 68% (47/69). Subcutaneous serous edema around the neck and fibrin clots around nostril (figure 2) were also noticed. Of 115 apparently healthy camels, *P. multocida* was isolated from almost 7% of nasal swabs, indicating that nasal carriage is not frequent. *P. multocida* was not isolated from blood samples collected on apparently healthy camels. Although no *P. multocida* isolation was possible from blood samples, bacteriological examination from nasal swabs from the clinical cases and apparently healthy camels elucidated similar isolation pattern.

**PCR analysis.** The isolates were tested by PCR. An amplification product of 460 pb is expected when KMT1SP6 -KMT1T7 are used amplifying a broad range of bacterial species. *P. multocida* serotypes A and B were identified by the specific primers for capsular types A and B amplifying 1044 and 760 bp respectively.

In the PCR assay, an amplicon size of 460 bp was observed from all the isolates in agarose gel electrophoresis (figure 3), while two isolates from distressed camels were negative with

<table>
<thead>
<tr>
<th>Camel condition</th>
<th>No. of isolates</th>
<th><em>P. multocida</em> isolates (%)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead</td>
<td>10</td>
<td>8(80)</td>
<td>80</td>
</tr>
<tr>
<td>Clinical sign</td>
<td>69</td>
<td>47(68.11)</td>
<td>65.21 (45/69)</td>
</tr>
<tr>
<td>Healthy</td>
<td>115</td>
<td>8(6.95)</td>
<td>6.34</td>
</tr>
<tr>
<td>Organs</td>
<td>60</td>
<td>52(86.66)</td>
<td>87</td>
</tr>
</tbody>
</table>

**Table II:** Frequency of dead, clinical sign and healthy camel and affected organs due to *P. multocida*.  

**Figure 1:** A camel clinically affected with haemorrhagic septicaemia. Note the dull appearance and rough coat and the prominent submandibular swelling and extended neck.

**Figure 2:** Fibrinous nasal discharge in a camel suffering from haemorrhagic septicaemia.
this assay. In the capsular PCR assay, 80% (8/10) isolates from dead camels and 65.21% (45/69) from camels with respiratory distress gave a product of 760 bp and correspond to capsular type B (figure 4). No capsular type A except one was detected (figure 5).

Discussion

A variety of viruses, fungi, bacteriae and parasites may possibly cause respiratory diseases in camels, but the definitive etiology has not yet been determined (21, 35). All test results whether biochemical or morphological suggested the involvement of *P. multocida*. *P. multocida* infection in camels was accompanied most of the time by heavy economic losses (23, 39, 40).

Species determination reported by Townsend et al. (2001) revealed PCR amplification of 460 bp band for *P. multocida* (44), and similar assay has also been used by others. Dutta et al. (2001) carried out all pass primers for identification of *P. multocida* (16, 19). Dey et al. (2007) presented the usefulness of PCR amplifying KMT1 gene for the identification of *P. multocida* isolates (15). A 760 bp amplicon size corresponds to capsular type B. This finding has also been reported before by Vidhya et al. (2007) for the B serogroups of *P. multocida* (45). These finding demonstrate that a majority of camel isolates belong to serogroup B. Kebede and Gelaye (2010) has reported that this serogroup is mainly responsible for haemorrhagic septicaemia in camels (21).
Although respiratory diseases usually pertaining to various factors, it is due to a secondary infection by bacteria following parainfluenza virus infection (4, 37). In this study the actual reason of the respiratory disease in camel in the current area was identified. P. multocida has been found as the primary agent responsible for the camel respiratory disease. This finding is in disagreement with Kebede and Gelaye (2010) who reported pasteurellosis as a bacterial complication following parainfluenza exposure (21). In Iran, no studies were carried out before that with the aim of identifying respiratory problems in camels compared to other livestock species.

Camels infected with P. multocida showed symptoms of pneumonia which might be accompanied by septicaemia, pyrexia and mucopurulent nasal discharge (8, 29). The pleurisy, observed at post-mortem, and clots of fibrin around the nostrils and mouth in distressed camels might be due to the interaction of P. multocida with mononuclear leukocytes and alveolar macrophages. These dying cells release histamine, prostaglandin and fibrinolytic elements, all of which can cause inflammation and deposition of fibrin (27, 28).

Schwartz (2003) indicated that the most important predisposing factors are sudden climatic changes, poor management practices, exposure to various diseases, over traveling and low grade nutrition (34). According to Seifert (1996), change in climatic conditions and stress in combination with a viral infection like parafluenza increase the incidence of Pasteurellosis (36).

The isolation rate of P. multocida in this study was compatible with the results of other studies [2, 3, 6, 8, 11]. In the current study, P. multocida species were isolated from the lungs of all dead camels. The variation rate of P. multocida in camel may result in different sample size, method of sample collection and differences amongst geographical areas (6). In the present study, the lack of bacteriological isolation in pneumatic lung samples might be due to involvement of other organisms commonly involved in respiratory problems such as Mycoplasma, viruses, parasite, fungi or other anaerobic bacteria.

Although P. multocida serotype B has been isolated before from affected camels, its role as the causative agent for hemorrhagic septicaemia in camels remains unclear (47). Yet, there is an urgent need to identify the causes of respiratory diseases in camels in order to design better control strategies (43).

In conclusion, up to now, P. multocida Carter serotype B has been isolated once from affected camels, and was associated with respiratory disease in camel, but the detailed epidemiological information and identification of a primary agent, if any, and its role as the causative agent for hemorrhagic septicaemia in camel remain unclear and need further investigation.

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