Prevalence of Mycoplasma bovis in Respiratory Tract of Cattle Slaughtered in Balochistan, Pakistan

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ABSTRACT

Cattle lungs (n=1200) obtained from abattoir of 10 districts of Balochistan were processed for isolation and identification of Mycoplasma species. A total of 156 isolates produced typical fried egg colonies on Modified Hayflick’s agar medium and 87.8% were preliminarily identified as Mycoplasma species, 12.2% species were Acholeplasmas. All the digitonin sensitive isolates were further subjected to different biochemical and PCR tests for further identification. Overall prevalence of M. bovis lungs samples obtained from slaughter house samples was 9%. Among the Mycoplasma isolates; 108 M. bovis, 29 Mycoplasma mycoides subsp. capri (Mmc) and 16 M. arginini were identified through the biochemical tests. M. bovis and Mycoplasma mycoides subcluster members were further validated through PCR and RFLP. Mycoplasma mycoides subspecies mycoides small colony type (Mmm SC) was not isolated from any of the lung samples. Among the Mycoplasma bovis species isolated, the highest number was observed from Quetta district (16%) followed by Pishin (15%), Zhob (11%) and Kalat (10%). Conversely the lowest number of M. bovis isolates was found in Bolan (2%) district followed by Jaffarabad (3%), 4%, each from Khuzdar, Mustung, Killasaifullah and 7% in Sibi district. Statistical analysis using chi square test, showed a significance difference ($\chi^2=33.38$) in the recovery of Mycoplasma bovis from the lungs of cattle slaughtered in 10 districts of Balochistan.

INTRODUCTION

Mycoplasmas are the smallest, self-replicating diverse group of wall-less Prokaryotes belonging to class Mollicutes (Ayling et al., 2000; Amin et al., 2013). They can colonize the respiratory tract and other sites of bovine (Marouf et al., 2011) and lead to pneumonia and many other disorders. In Ruminant Mycoplasma are responsible for causing many important diseases and some are listed by the World Organization (OIE) for Animal Health (Chazel et al., 2010). Among these, Mycoplasma mycoides subspecies mycoides small colony (Mmm SC) the causative agent of contagious bovine pleuropneumonia (CBPP) is the most pathogenic Mycoplasma (Nicholas and Ayling, 2003).

Mycoplasma bovis was first isolated in USA in 1962 and now it is present worldwide with growing prevalence day by day (Fu et al., 2011; Mustafa et al., 2013). M. bovis is the second most pathogenic Mycoplasma worldwide and inflicts considerable financial losses in cattle in the form of disease and mortality (Maunsell et al., 2011). It causes pneumonia and arthritis (Hewicker-Trautwein et al., 2003), mastitis (Wilson et al., 2007; Khan et al., 2013) in cattle and also pneumonia and arthritis in calves (Hermeyer et al., 2012).

Mycoplasma agalactiae and Mycoplasma bovis are phenotypically and genotypically closely related and both share considerable number of related proteins and common epitopes. Their diagnosis is problematic when only serological and biochemical test are used (Flitman-Tene et al., 1997). By using the PCR based on primers derived from 16SrRNA gene sequence for distinction between these two species is rather difficult as sequence similarity is high and the two rRNA operons present in...
each species possess several polymorphic sites. Recently PCR based on the uvrC (A gene for deoxyribopyrimidine photolase) were used to differentiate M. bovis and M. agalactiae with two separate PCR systems (Subramaniam et al., 1998) and were found more sensitive and specific. Mycoplasma mycoides subspecies capri is generally considered pathogenic in small ruminants (Singh et al., 2004) but its pathogenicity in cattle is a question mark (Cottew, 1979). This organism has been reported from cattle (Pitches and Nicholas, 2005; Chazel et al., 2010). Recently Mmc has also been identified by PCR and RFLP from goats in Balochistan, Pakistan (Awon et al., 2012). The aim of this study was to investigate prevailing Mycoplasma especially Mmm SC and M. bovis in cattle lungs through PCR and RFLP. This study is reported for the very first time in Balochistan, Pakistan.

**MATERIALS AND METHODS**

**Study area and sample size:** A Total of 1200 cattle lungs samples without considering age, sex, breed and origin of animal were collected from abattoirs of 10 districts of Balochistan in sterile plastic bags and were transported in cold chain to Center for Advance Studies in Vaccinology Balochistan in sterile plastic bags and were transported in cold chain to Center for Advance Studies in Vaccinology (CASVAB), UoB, Quetta.

**Mycoplasma isolation and biochemical tests:** For the isolation of *Mycoplasma* the method described by Allen et al. (1991) was followed. Briefly a piece (one gram) of lung was triturated in 5ml of Modified Hayflick’s broth medium (Rosendale, 1994). After one day incubation at 37°C, 1ml of this inoculated culture was shifted in another test tube containing 9 ml of above *Mycoplasma* broth for making three 10 fold serial dilutions (from 10^-1 to 10^-5) and 0.2 ml from each dilution was inoculated in Modified Hayflick’s agar plate. All the inoculated broth tubes and agar plates were incubated at 37°C in 5% CO2 for 7 to 10 days and monitored daily for color change and appearance of fried egg colonies. The recovered *Mycoplasmas* were triple cloned for obtaining pure growth and all the purified isolates were lyophilized and stored at – 80°C for further use.

Series of biochemical tests; including digitonin sensitivity test, glucose fermentation, arginine decarboxylation, phosphatase activity, casein digestion and reduction of 2,3,5-triphenyltetrazolium hydrochloride were performed for the preliminarily identification of *Mycoplasma* species (Poveda, 1998).

**DNA extraction and PCR:** The DNA was extracted from each of the isolated *Mycoplasma* by using PUREGENE genomic DNA extraction kit (Genta System, USA). The purified genomic DNA samples were stored in micro tubes (1.5 ml) at -20°C until used in specific PCR for the *Mycoplasma* species.

*Mycoplasma mycoides* sub cluster (MmmSC and Mmc) members were identified following the method described by Bashiruddin et al. (1994). Primer pair MM450-F (5'-GTA TTT TCC TTT CTA ATT TG-3') and MM451 - R (5'-AAA TCA AAT TAA TAA GTT TG-3') synthesized from Gene-Link USA.

*M. bovis* *uvrC* sequence (A gene for deoxyribopyrimidine photolase) was amplified using primer (synthesized from Gene-Link USA) pair MBOUVRC2-L (5'-TTACGCAAAGAATGCTTCA-3') and MBOUVRC2-R (5'-TAGGAAAGCACC TATTGT A-3') by following the method as described previously (Subramaniam et al., 1998).

**Restriction Fragment Length Polymorphism (RFLP):** The method used by Bashiruddin et al. (1994) was used for conducting RFLP. The entire sub-cluster PCR positive samples were subjected to RFLP.

**Data analysis:** Chi-square (χ²) value was calculated by using the Graph pad prism software.

**RESULTS AND DISCUSSION**

From 1200 infected cattle lungs, 156 (13.8%) isolates produced typical fried egg colonies on modified Hayflick’s agar medium. On the basis of biochemical tests, a total of 12.1% *Acholeplasma*, 62.7% *M. bovis*, 16.8% *Mmc* and 9.3% *M. arginini* was identified (Table 1). By PCR, 29 isolates were found positive for *Mycoplasma mycoides* cluster and subcluster PCR (Fig. 1) and were identified as Mmc when the PCR product (amplicon) of *Mycoplasma mycoides* sub cluster members was digested with *Vsp* during RFLP. It yielded three band (230bps, 178bps, and 153bps) and *MmmSC* was not identified from any sample as its two characteristic bands of 379bps and 178bps (Fig. 2) could not observed.

Table 1: Results of comparative identification of *Mycoplasma* species through Biochemical test, and confirmation through polymerase chain reaction and restriction fragment length polymorphism.

<table>
<thead>
<tr>
<th>District</th>
<th>M. bovis</th>
<th>Mmc</th>
<th>M. arginini</th>
<th>Acholeplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitonin sensitivity test</td>
<td>Sen</td>
<td>Sen</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>Serum dependence</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Arginine decarboxylation</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NP</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NP</td>
</tr>
<tr>
<td>Phosphatase production</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
</tr>
<tr>
<td>TZ-2 reduction aerobic</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NP</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NP</td>
</tr>
<tr>
<td>Casein digestion</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NP</td>
</tr>
<tr>
<td>Identified Through PCR</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>RFLP</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>29</td>
<td>16</td>
<td>19</td>
</tr>
</tbody>
</table>

* = Number of isolates; **S** = Sensitive; *M. mycoides* subspecies capri (Mmc); *M. mycoides* subspecies capri (Mmc); *M. mycoides* subspecies capri (Mmc); *M. mycoides* subspecies capri (Mmc) | *M. arginini* (Subramanium et al., 1998). When the PCR product (amplicon) of *Mycoplasma mycoides* sub cluster members was digested with *Vsp* during RFLP. It yielded three band (230bps, 178bps, and 153bps) and *MmmSC* was not identified from any sample as its two characteristic bands of 379bps and 178bps (Fig. 2) could not observed.

A total of 108 isolates were identified as *M. bovis* (Fig. 3). The highest number of *M. bovis* isolates (16%) was obtained from Quetta district followed by Pishin (15%), Zhob (11%) and Kalat (10%). The lowest number of *M. bovis* isolates from Bolon (2%) followed by Jaffarabad (3%), (4%) each from Khuzdar, Mustang, Killasaifullah and Sibi (7%). Staticky, significance difference in recovery of *Mycoplasma bovis* in lungs of cattle slaughtered in 10 districts of Balochistan (χ²=33.38; P=0.0001) was observed. This is in agreement with others who also isolated *M. bovis* with variable % age (Burnens et al., 1999; Brice et al., 2000; Le Grand et al., 2001) from cattle lungs.

In the present study, *Mycoplasma mycoides* subsp. mycoides small colony (SC) the causative agent of CBPP was not detected from any of the lungs. Presence of *Mmc* is interesting as there are several reports about the cross transmission of *Mycoplasma* between small and large
ruminants (Taylor et al., 1992) and recently the strict host-specificity of several Mycoplasmas, has been increasingly questioned and notably cattle were found hosting Mmc and M. agalactiae (Chazel et al., 2010). Comparatively high isolation rate of Mycoplasma bovis as compared to other Mycoplasma spp is another indicator of an important bovine respiratory pathogen as already been documented (Caswell and Archambault, 2007) whereas, M. bovis is being reported first time in Balochistan.

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REFERENCES


