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RESEARCH ARTICLE

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 (χ^2=33.38) in the recovery of *Mycoplasma bovis* from the lungs of cattle slaughtered in 10 districts of Balochistan.

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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 photolyase) 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 (Awan *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 and Biotechnology (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 (Rosendal, 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^{-3}) 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% CO_2 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 (Gentra 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 photolyase) was amplified using primer (synthesized from Gene-Link USA) pair MBOUVRC2-L (5'-TTACGCAAGAGAATGCTTCA-3') and MBOUVRC2-R (5'-TAGGAAAGCACCC TATTGT A-3') by following the method as described previously (Subramanium *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 (χ^2) 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 *Vsp1*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.

District	*M. bovis*	*Mmc*	*M. arginini*	*Acholeplasma*
Digitonin sensitivity test	Sen**	Sen	Sen	Resistant
Serum dependence	Yes	Yes	Yes	No
Arginin decarboxylation	-	-	+	NP
Glucose fermentation	-	+	-	NP
Phosphatase production	+	-	-	NP
TZ [2] reduction aerobic	+	+	-	NP
Anaerobic	+	+	+	
Casein digestion	-	+	-	NP
Identified Through PCR	Yes	Yes	No	No
RFLP	No	Yes	No	No
Total*	108	29	16	19

*= Number of isolates; **= Sensitive; 1= *Mycoplasma mycoides subspecie capri (Mmc);* 2= Tetrazolium; RFLP= Restriction Fragment Length Polymorphism; NP= Not performed

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%). Statically, significance difference in recovery of *Mycoplasma bovis* in lungs of cattle slaughtered in 10 districts of Balochistan (χ^2=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

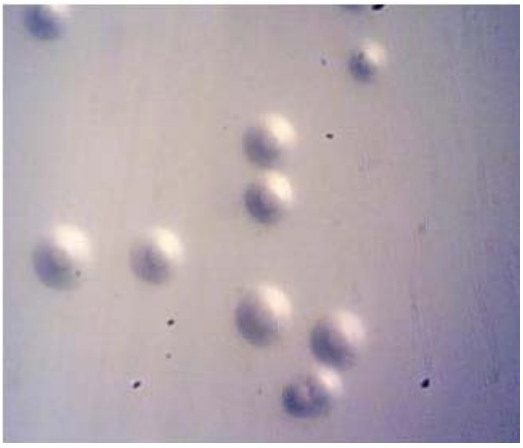

Plate 1: Fried egg colonies of *Mycoplasma bovis* on modified Hayflick's agar medium isolated from cattle lungs (35x)

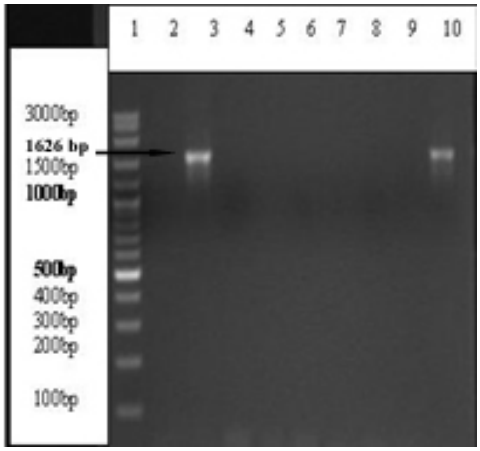

Plate 2: Gel electrophoresis of the PCR product showing amplification of 1626bp fragments of *M. bovis*. Lane 1: molecular marker (3000bp DNA ladder) (Vivantis) Lane 2, 4, 5, 6, 7 and 8 negative sample for *M. bovis*. Lane 3: positive *M. bovis* isolate. Lane9: negative control. Lane 10: *M. bovis* positive control

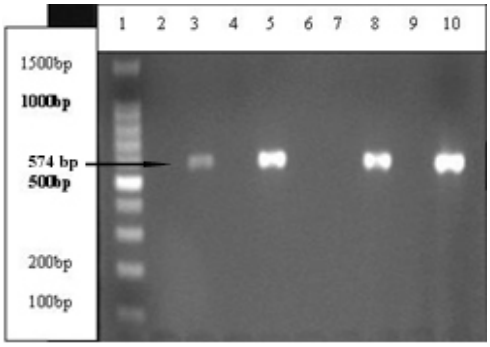

Plate 3: Gel electrophoresis of the PCR product showing amplification of 574bp fragment of *Mycoplasma mycoides sub cluster* members. Lane 1: Molecular marker (1500 bp DNA ladder) (Vivantis) Lane: 2, 4, 6 and 7 are negative samples. Lane: 3, 5, 8 are positive samples for *Mycoplasma mycoides sub cluster* members. Lane 9: Negative control Lane 10: Positive control (Mmc PG 3 strain)

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

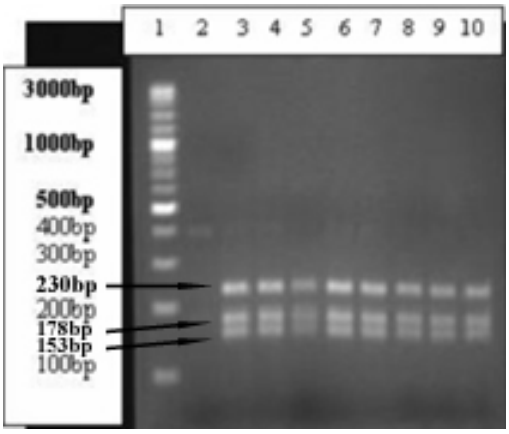

Plate 4: RFLP performed on *Mycoplasma mycoides sub cluster* (574bp) PCR product. Lane 1: Molecular marker (3000bp). Lane: 2 negative control. Lane 3: positive control of (*Mmc* PG 3 strain). Lane 4, 5, 6, 7, 8, 9 and 10 are positive samples of *Mmc* (yielding 3 bands with sizes of 230, 178 and 153bps specific for Mmc).

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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