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Identification and Molecular Characterization of Mycoplasma Species from Bovine Lungs Samples Collected from Slaughter House, Quetta, Balochistan, Pakistan

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Abstract

Bovine respiratory mycoplsamosis is a world wide problem. The aim of this study was to highlight the presence of Mycoplasma species in the lung samples from bovine. Five hundred (n=500) lungs samples of cattle slaughtered at abattoir in district Quetta, Balochistan were collected and processed for the isolation and identification of *Mycoplasma* species using biochemical tests and molecular (Polymerase Chain Reaction-PCR; Restriction Fragment Length Polymorphism-RFLP) techniques. Two types of *Mycoplasma* species were identified. Eleven (2.2%) samples were found positive for *Mycoplasma mycoides subsp.capri* and two samples (0.4%) were positive for *Mycoplasma arginini*. *Acholeplasmas* (1%). All the isolates were confirmed as members of *Mycoplasma* genus on the basis of digitonin sensitivity test. All the isolates showing the zone of inhibition less than 2mm were considered as digitonin resistant. PCR products (Amplicon) of 1500 and 574 bp were obtained for *Mycoplasma "mycoides* cluster and *Mycoplasma "mycoides* sub cluster" respectively. PCR results for the *Mycoplasma mycoides* sub-cluster was further validated by using RFLP which yielded three bands of 230bps, 178bps and 153bps specific for *Mmc* when digested with *Vsp1*. The results of the study suggested the possibility of strains crossing the species barriers.

Keywords: Mycoplasma, Bovine, Pneumonia, Identification, PCR

Introduction

Pakistan is endowed with a large livestock population. There are 24.2 million cattle, 26.3 million buffaloes in Pakistan (Anonymous, 2008 - 009). Balochistan is the largest province of Pakistan, about 44% of the total geographical area of the country and Quetta, being the provincial capital is located at North West of Balochistan. Its bordering districts are Pishin, Sibi, Mastung and Chaghai. There are 11244 cattle and 25247 buffalos in Quetta District (Anonymous, 2006).

Mycoplasma belongs to the class *Mollicutes* and is known as the smallest self replicating bacteria (Razin et al., 1998). These are widely distributed in nature and live in animals, plants, insects and humans (Rottem and Naot, 1998). Only few of several Mycoplasma species colonizing the bovine respiratory mucous membrane are considered to be pathogenic, whereas others are ubiquitous, part of normal flora (ter Laak et al., 1992). Among ruminant mycoplasmosis causing important diseases world, several are listed by the world health organization for animal health to be major economic significances (Chazek et al., 2010).

There is a possibility of cross transmission of Mycoplasma between small and large ruminant (Taylor et al., 1992). The agent for CBPP had also been isolated from small ruminants (goat and sheep) in Africa, Asia and Europe (De Santis et al., 1999; Kusiluka et al., 2000; Yaya et al., 2008). Small ruminant may be potential reservoirs and also included in the surveillance strategy and some isolates have been shown to be pathogenic to cattle (Hudson et al., 1967).

Classical methods for identifying and detecting *Mycoplasmas* are laborious and time consuming and are complicated by serological cross reaction between the closely related organisms. Introduction of molecular tools in the field of diagnosis has improved the detection and identification of microorganisms. A rapid diagnostic test based on the implication of DNA by PCR and further restriction fragment length polymorphism (RFLP) is very valuable to differentiate between M mycoides subsp mycoides SC (MmmSC) and Mmc (Bashiruddin et al., 1994). The recently introduced PCR and

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denaturing gradient gel electrophoresis (DGGE) method are also important diagnostic tools (McAuliffe et al., 2005).

Keeping in view the above scenario, the following study was conducted with the objective to identify and confirm *Mycoplasma* species in bovine lungs samples using biochemical tests and molecular (PCR-RFLP) techniques.

Materials and Methods

Study Area and Sample Collection

The study was conducted on cattle lung samples collected from government slaughter house located in Quetta, Balochistan, Pakistan. Five hundred lung samples were randomly collected from cattle slaughter house Quetta during 2008-2009. Samples were initially kept in ice box and immediately transported to the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta for the isolation and identification of *Mycoplasma* species.

Isolation and identification of Mycoplasma species

Samples for bacteriological isolation was taken at the interface between the consolidated and normal parts of the lung using sterile scalpels and was then minced using scissors. Suspension was made and serially diluted tenfold step in modified Hayflick's broth. Dilutions were also plated on to solid Hayflick's medium. All the cultures were incubated at 37°C in a humid atmosphere with 5% CO_2. Both the broth and agar media were observed daily for any color change and presence of fried egg colonies respectively. Stereomicroscope (35x) was used for examination of fried egg colonies. Three to four blind passages were carried out before discarding cultures as being negative for mycoplasma (ter Laak et al., 1992). Both broth and agar were observed up to 14 days before discarding any culture.

Biochemical tests

All the isolates were triple cloned to obtain the pure growth before performing ant biochemical test. To confirm genus *Mycoplasma*, digitonin sensitivity test was performed. All digitonin negative isolates were considered as Acholeplasmas. Series of biochemical tests such as arginine decarboxylation, glucose fermentation, and liquefaction of inspissated serum, phosphatase activity, casein digestion and reduction of 2, 3, 5, triphenyl tetrazolium hydrochloride were performed on the isolates for identification of Mycoplasma species (Poveda, 1998).

Polymerase Chain Reaction

All the Mycoplasma isolates were also verified by PCR. DNA was extracted with the help of DNA purification kit (PUREGENE– Gentra System, USA) and stored in 1.5 ml micro tubes at -20^{0}C for further use. The primer was synthesized from Gene-Link, USA. Polymerase chain reaction (PCR) tests was performed for detection of *Mycoplasma "mycoides* cluster" members,*Mycoplasma mycoides* sub cluster (*Mmm*SC, *Mmc*,*Mmm*LC) members (Bashiruddin et al., 1994). 38 ul PCR grade water, 5ul (10X) reaction buffer, 1.5ul (50 mM) MgCl2, 2ul (10mM dNTPs), 1ul of each primer (50 pmols/ul) and 0.5ul of Taq polymerase was used to make master mix. Total reaction of 50 ul by adding 2 ul of DNA was placed in thermal cycler and cycling condition were set as set 94^{o}C for 4 min, followed by 33 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30s, 72^{o}C for 30 second, followed by 72^{o}C for 7 min, and 4^{o}C until further used.

PCR products (Amplicon) of 1500 and 574 bp were obtained in 2% agarose gel (Vivantis, USA) for *Mycoplasma "mycoides* cluster and *Mycoplasma "mycoides* sub cluster" respectively. RFLP was performed according to the method described by Bashiruddin et al. (1994).

Results

Isolation and identification

Two types of *Mollicutes* namely *Mycoplasmas* and *Acholeplasmas* were isolated from bovine lungs samples. All the isolates were confirmed as members of *Mycoplasma* genus on the basis of digitonin sensitivity test. All the isolates showing the zone of inhibition less than 2mm were considered as digitonin resistant and were also inoculated on modified hayflick agar media with out serum and were considered as *Acholeplasmas*.

Biochemical test

Eleven isolates (2.2%) were digitonin sensitive; glucose fermenters, reduced tetrazolium both aerobically and an aerobically, hydrolyzed casein and were positive for liquification on insipissated serum and were identified as *Mycoplasma mycoides subsp.capri* (Mmc) they grew with in 24-48 hours. Two (0.4%) isolate were found hydrolyzing arginine were considered as *M.arginini*. Five isolates (1%) were resistant to digitonin and were considered as Acholeplasma (Table 1).

Polymerase Chain Reaction

All the isolate identified through biochemical test as *Mmc* were further confirmed through PCR- RFLP. PCR profile of *Mycoplasma mycoides* cluster is shown in Fig 1 and subcluster in Fig 2. The PCR results for the *Mycoplasma mycoides* sub-cluster was further validated by RFLP which yielded three bands of 230bps, 178bps, and 153bps specific for *Mmc* (Figure 3) when digested with *Vsp1*. None of the PCR product was observed with two bands of 379bps and 178bps specific for *MmmSC* in RFLP.

Table 1 Biochemical characteristics of *Mycoplasma* species isolated from bovine

No of isolate	A	b	c	d	e		F	Mycoplasma spp
					1	2		
11 (2.2 %)	Sensitive	+	+	+	+	+	+	*Mmc*
2 (0.4 %)	Sensitive	-	-	+	-	+		*M.arginini*
5 (1.0 %)	Resistant	NT	NT	NT	NT		NT	*Acholeplasma*
Total 18 (3.6%)								

a- Digitonin, b -Casein hydrolysis, c -Glucose fermentation, d- Arginine hydrolysis, e - Tetrazolium reduction (1-aerobic, 2-anerobic), f- liquefaction of inspissated serum. NT- Not tested (identified as digitonin resistant)

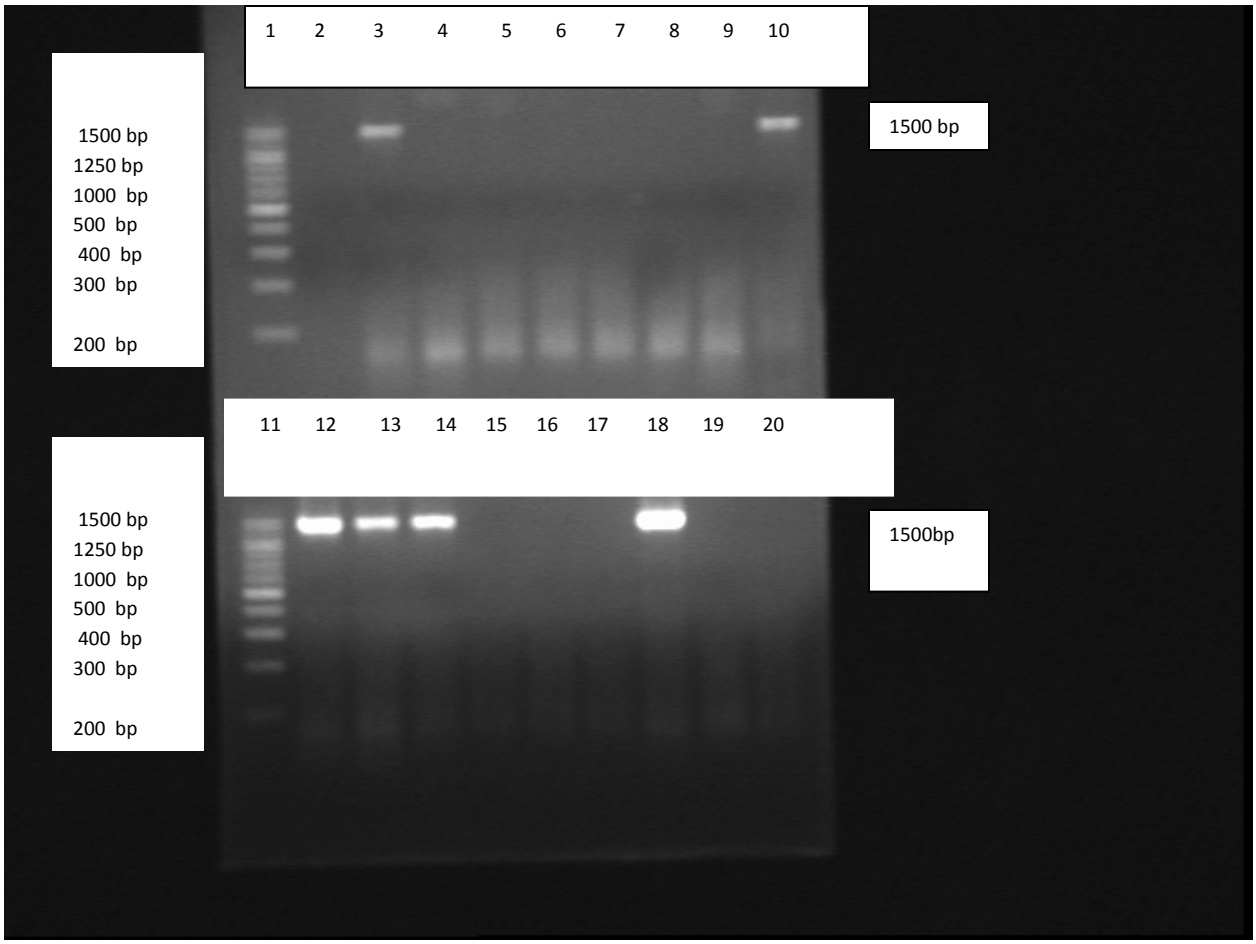

Fig 1 A PCR band of 1500bp size indicating the members of Mycoplasma mycoides cluster. Line 1 & line 11 in above fig representing molecular ladder. Lane 10, 12, 13, 14, and 18: are positive samples for *Mycoplasma mycoides* cluster members; 4, 5, 6, 7, 8, 9, 15, 16, 17, 19 and 20 lanes representing –ve samples for Mycoplasma mycoides clusters. lane 2 is negative and Lane 3 is positive control.

Discussion

In the present study, the isolation and identification of *mycoplasma* from bovine pneumonic lungs were carried out in Quetta district of Balochistan. Another study was conducted earlier in which molecular tools such as PCR-RFLP along with DGGE were used for the identification of caprine respiratory myco-plasmosis (Awan et al., 2009a; Awan et al., 2009b).

Overall 18 (3.6 %) isolates were recovered and identified in the present study. The highest isolation rate of *Mycoplasma mycoides capri* (*Mmc*) species (2.2 %), *Acholeplasma* (1%) and *M.arginini* (0.4%) from lungs was recorded. A single name of "*Mycoplasma mycoides subspecies capri*" has been proposed for *Mmc* and *Mmm LC* due to close similarities (Vilei et al., 2006). All the mycoplasma isolates were preliminary identified by the digitonin

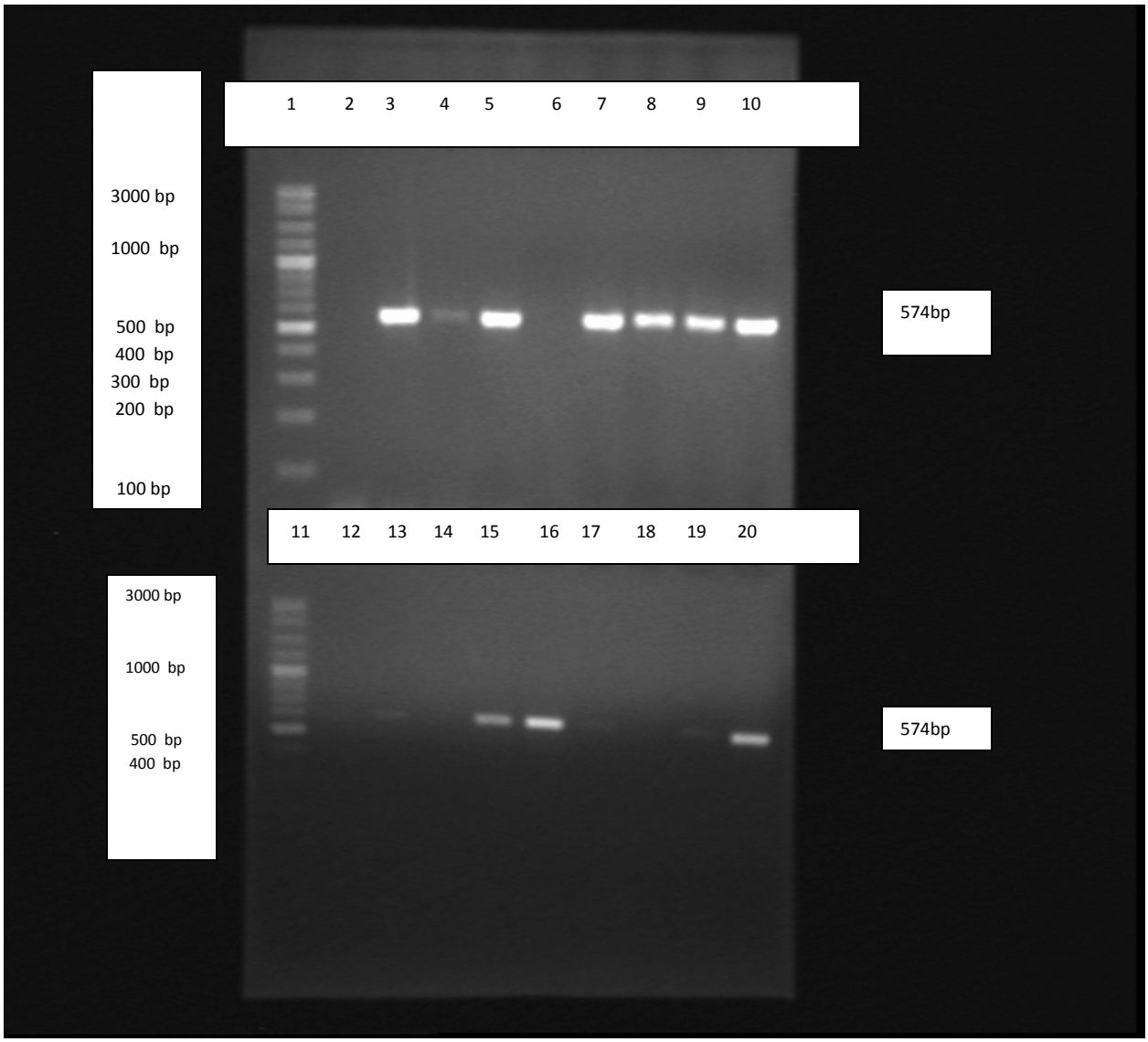

Fig 2 A PCR band of 574 bp size indicating the members of Mycoplasma mycoides sub cluster. Line 1 & line 11 in above fig representing molecular ladder of 3000bp. Lane 2 **is a negative control. Lane, 3, 4, 5, 7, 8, 9, 10, 13, 15, 16, 17, 19 and 20 are positive samples for *Mycoplasma mycoides* sub cluster members. While 6, 12, 14, and 18 lanes representing –ve samples for Mycoplasma mycoides clusters. Lane 10 is positive control.**

sensitivity and growth on serum non-enriched modified hayflicks growth medium (Al-Momani et digitonin sensitivity, glucose fermentation, tetrazolium reduction and arginine hydrolysis; and GIT. Aluotto et al. (1970) has also used these test for biochemical identification of *Mycoplasma*.

The mycoplasma confirmed by biochemical tests were further validated by PCR-RFLP method. Among these isolates eleven isolate were identified as member on *Mycoplasma mycoides* cluster and subcluster as *Mm*c. These results were further validated by the RFLP in which three bands (230bps, 178bps, and 153 bps) specific for *Mmc* have been al. 2006). Isolated *mycoplasmas* were identified by a number of classical biochemical tests such as observed similar to those as reported by Bashiruddin et al. (1994).

Although Mmc produce pneumonia arthirits and mastitis in goats (Singh et al., 2004) and are pathogenic for sheep as well, but apparently not for cattle (Cottew, 1979. Recently, the strict host-specificity of several mycoplasmas, such as *MmmSC, M. agalactiae, M. bovis* and *Mcc* (Pitcher and Nicholas, 2005: Taoudi & Kirchhoff, 1986) has been increasingly questioned & notably cattle were found hosting *Mmc* and *M. agalactiae* (Chazek et al.,2010).

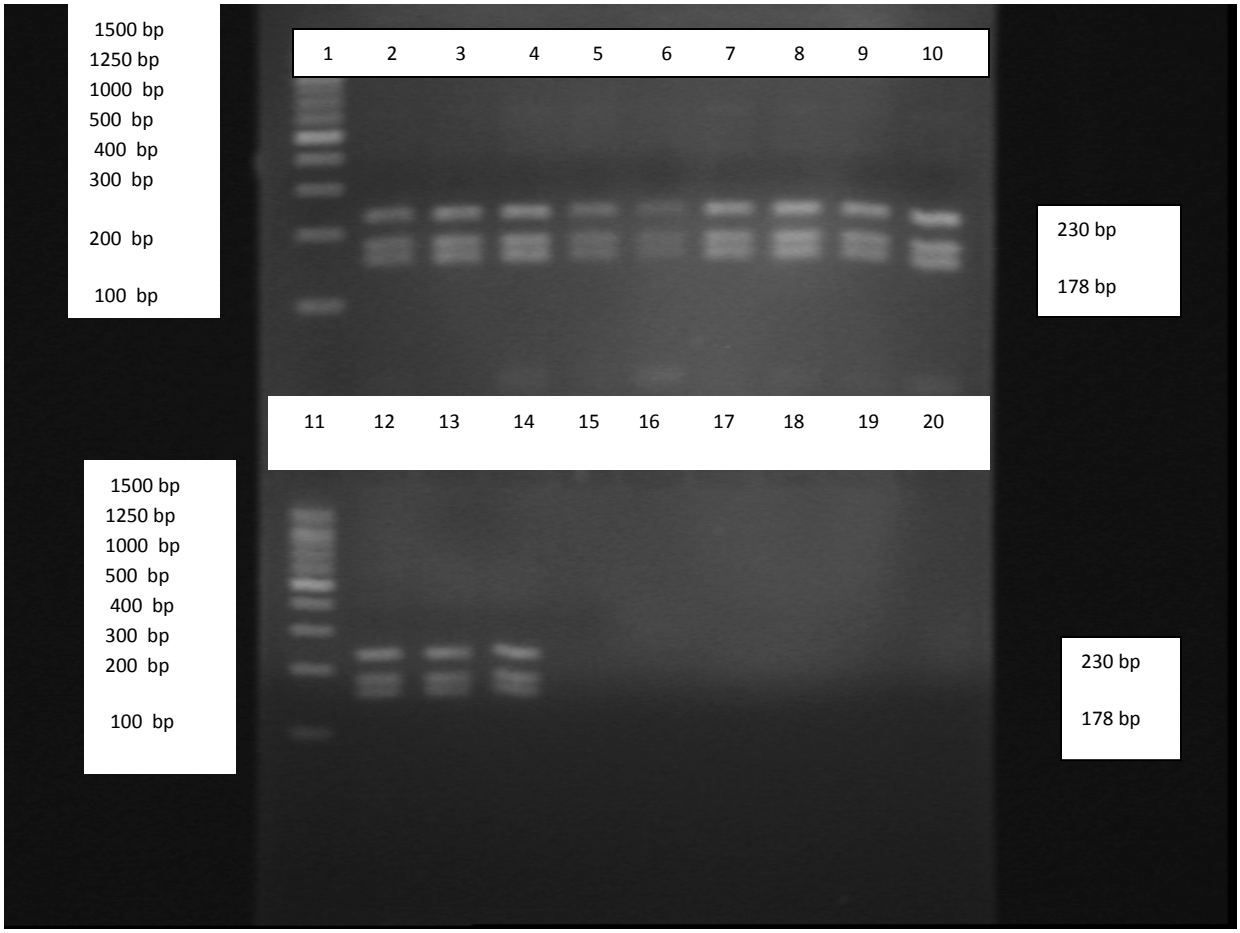

Fig 3 Showing the RFLP profile of Mycoplasma mycoides subcluster (574 bp) PCR product. Lane 1 and 11 is ladder. Lane 10 is a positive control of Mmc showing three bands 230,178 and 153 sizes. 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 are positive samples for Mmc. Non of sample run in lane 15,16,17,18. Lane 20 is negative control.

Similarly *M. bovirhinis* and *M. arginini*, were frequently isolated from cattle and from all ruminants, respectively, but their presence in a sample is of no diagnostic significance since their pathogenicity has never been established (Gourlay et al., 1979). Similarly *M. canis* and *M. alkalescens*, suspected to cause emerging epizootic diseases in UK cattle (Ayling et al., 2004 :Lawes et al., 2006).

The isolation of *Mycoplasmamycoides subsp.capri(Mmc)* in bovine lungs was significant. PCR based identification of the *Mycoplasma* species is a rapid, specific, and less laborious as compared to the traditional biochemical tests. It is also indicated that there is possibility of strains to cross the species barriers.

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