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Characterization of *p30* membrane protein gene of *Mycoplasma agalactiae* isolates by polymerase chain reaction and restriction endonuclease enzyme assay

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Seven hundred and eight (708) samples collected from the goats of Gujarat were screened for *Mycoplasma agalactiae* by culture and PCR using 16S rRNA based genus specific and species-specific primers. Amplification of the *p30* membrane protein gene was carried out using specific primers and the resultant amplicons were subjected to restriction enzyme analysis. The isolates yielded 715 bp and 360 bp products with genus-specific and species-specific primers, respectively and were identified as *M. agalactiae*. Amplification of the *p30* membrane protein gene yielded a 730 bp product. Restriction enzyme analysis of the 730 bp amplicon of *p30* gene with *Rsa*I and *MboII* yielded 2 fragments (654 bp and 74 bp) and 3 fragments (111 bp, 375 bp and 244 bp), respectively. Digestion with *Sau3A*I yielded two fragments (461 bp and 269 bp) while digestion with *Alu*I resulted in 3 fragments (342 bp, 328 bp and 60 bp). The results of the present study revealed the presence of polymorphism at the respective positions of *p30* membrane protein gene of *M. agalactiae* isolates examined by the restriction digestion. These polymorphisms can result into changes in pathogenesis and persistence inside the host and require further investigation of immunological outcome of these polymorphisms.

Key words: *Mycoplasma agalactiae*, polymerase chain reaction (PCR), restriction enzyme, *p30*.

INTRODUCTION

In small ruminants, *Mycoplasma agalactiae* is responsible for a syndrome known as contagious agalactiae of small ruminants (CASR) (Solsona et al., 1996), which is generally characterized by mastitis, arthritis and keratoconjunctivitis. It causes reduction and suppression of milk production (De Garnica et al., 2013) and occasionally results into abortion and death (Madanat et al., 2001). For pathogenesis and clinical manifestations of mycoplasmal infections, adhesion to host cells and immune evasion are the major prerequisite for colonization. Different strategies used for the successful persistence of *M. agalactiae* inside host include a

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constantly changing surface structure and the capacity of some lipoproteins to induce the expression of up- and down modulating cytokines (Razin et al., 1998). Few constantly expressed surface proteins have also been described in *M. agalactiae* which includes *p30*, *p48* and *p80* as immunogens and other proteins belonging to the variable surface membrane proteins family (*Vpma*) (Cacciotto et al., 2010). *Vpma* phase variation has been reported to have importance for survival and persistent infection of *M*. *agalactiae* in host (Chopra-Dewasthaly et al., 2017).

The *M*. *agalactiae* variable gene (*avg*) system is a cluster of four genes that encode a family of surface lipoproteins characterized by high-frequency phase and size variations (Flitman-Tene et al., 2000). Stability and variation of these membrane proteins have implications for immunogenicity and pathogenicity. Among the immunodominant membrane proteins, expression of *p30* was detected consistently from several strains of *M. agalactiae* revealing its importance for serological analysis (Fleury et al., 2001). Membrane proteins p80 (Kashoo et al., 2011) and *p40* (Fleury et al., 2002) were also reported to have serological importance in the *M. agalactiae* infections and their importance as candidate proteins for diagnosis.

Several studies have targeted these membrane protein genes for identification of the organisms (Macun et al., 2010). Among the various molecular techniques, restriction analysis is an important technique of genetic characterization and to study the variation among the strains of *Mycoplasma* circulating in the field. In the present study, *p30* membrane protein gene was targeted to study the polymorphism among the isolates obtained from the samples collected from goats of Gujarat, which has significant number of goat and sheep population including several well-known milch and dual purpose breeds of India.

MATERIALS AND METHODS

Collection and processing of samples for cultural isolation

Ear (169), nasal (158) and ocular (28) swabs as well as lung tissues (94) and milk (259) samples were collected aseptically from healthy as well as sick animals and processed for isolation of *Mycoplasma*. After collection, each sample was placed directly in 2 ml of MBHS-L broth (Modified Balanced Hank's Salt Solution Liquid Media) and kept at 37°C for 1 h. After incubation for 1 h, 200 µl of each MBHS-L broth containing sample was transferred to 2 ml of fresh MBHS-L broth after filtration (with 0.45 µm filter). The fresh MBHS-L broths containing filtered inoculum were incubated at 37°C for 10-15 days. The broths were examined daily for sign of growth (floccular material) and positive cultures were further purified. The samples showing growth in broth were inoculated on solid media (Modified Balanced Hank's Salt Solution Agar Media, MBHS-A) and incubated anaerobically for 10 days at 37ºC under humid conditions in 5-10% carbon dioxide tension to obtain optimum growth (Carmichael et al., 1972).

Examination of the isolated colonies and biochemical characterization of isolates

The suspected colonies of *Mycoplasma* were examined morphologically under microscope (4X) after staining them with different stains viz. Dienes', Giemsa and Acridine orange stain. Biochemical tests as described by Erno and Stipkovits (1973) such as catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion, digitonin sensitivity test (Freundt, 1973) and film and spot formation were carried out to determine the biochemical activity of the suspected *Mycoplasma* isolates.

Confirmation of *Mycoplasma* isolates by polymerase chain reaction (PCR)

For preparation of the template DNA, 2 ml of broth culture of each isolate was centrifuged at 12000 rpm in a micro-centifuge at 4°C for 25 min. The pellets were washed in 500 µl of PBS twice; pellets were resuspended in 100 µl of nuclease-free water and boiled for 10 min. After boiling, the suspension was snap-chilled at -20°C for 5 min. After chilling, cell debris was removed by centrifugation and 3 µl of the supernatant was used as a DNA template in PCR after quantitation and quality assessment of DNA using Nano-drop spectrophotometer. For identification of genus and species of *Mycoplasma*, the PCR was carried out in a final reaction volume of 25 µl using 200 µl capacity PCR tube containing 3 µl of DNA template, 1 µl of each primer (10 pmole/µl), 12.5 µl of 2X PCR Master-mix (Fermentas) and 7.5 µl of DNase-RNase free water.

The isolates were confirmed as *Mycoplasma* sp. using genus specific forward, GPO-1 (5'-ACTCCTACGGGAGGCAGCAGTA-3') and reverse, MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') primers amplifying 715 bp fragment of 16S rRNA of *Mycoplasma* sp. (Kuppeveld et al., 1992) after PCR. After initial denaturation at 94°C for 2 min, amplification was carried out for 30 cycles (consisting of denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 1 min) with final extension at 72°C for 5 min.

For confirmation of species as *M. agalactiae*, PCR was performed using *M. agalactia*e specific forward, Maga (5'-CCTTTTAGATTGGGATAGCGGATG-3') and reverse Maga (5'-CCGTCAAGGTAGCGTCATTTCCTAC-3') primers for an expected amplification product of 360 bp fragment of the 16S rRNA gene (Chávez-González et al., 1995). After initial denaturation at 95°C for 5 min, amplification was carried out for 40 cycles (consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 68°C for 1 min) with final extension at 70°C for 10 min.

To detect the targeted amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading buffer and electrophoresed on 1.5% agarose gel at a constant 80V for 30 min in 0.5X TBE buffer along with 100 bp DNA Ladder (GeneRuler-Fermentas). It was then stained with ethidium bromide (1% solution at the rate of 5 µl/100 ml), the size of the product was visualized under UV light and documented by the gel documentation system (SynGene, Gene genius bioImaging System, UK).

Amplification of *p30* gene and restriction enzyme analysis

The membrane protein gene *p30* of four representative isolates of *M. agalactiae* was amplified using the specific forward, P30(F) (5'-CAGGGGGATGAACATTTATG-3') and reverse, P30(R) (5'-TTACCTCCATCTTTTTCAAC-3') primers (Fleury et al., 2001) in a final reaction volume of 25 µl (containing 3 µl of DNA template, 1 µl of each primer (10 pmole/µl), 12.5 µl of 2X PCR master-mix

Figure 1. Agarose gel electrophoresis of PCR product of *p30* gene of *M. agalactiae*. Lanes 1 - 4: Positive representative samples. L: 100 bp DNA molecular weight marker

(Fermentas) and 7.5 µl of DNase-RNase free water) for an expected amplicon of 730 bp. After initial denaturation for 2 min at 94°C, thermal cycling was carried out for 35 cycles (denaturation at 94°C for 30 s, annealing at 52°C for 45 s and extension for 1 min at 68°C) with final extension for 5 min at 72°C.

The amplified PCR products of membrane protein gene were further processed and characterized by RE analysis. Four different REs, *viz. Rsa*I, *Mbo*II, *Sau3A*I and *Alu*I were selected from restriction map created using the sequences of *p30* membrane protein gene available in GenBank at *http://www.ncbi.nlm.nih.gov/Genbank/index.html* and NEBcutter V2.0 software available online at *http://tools.neb.com/NEBcutter2/index.php.* A (30 µl) reaction mixture (containing 10 µl of PCR product, 1 µl of RE (10 U/µl), 2 µl of 10X restriction buffer and 17 µl of nuclease free water) was prepared and incubated in a water bath overnight according to the conditions specified by the manufacturer (Fermentas). After restriction digestion, an aliquot (10 µl) of each digested PCR product was mixed with 2 µl of gel loading buffer and electrophoresed along with 100bp DNA molecular weight marker on 2% agarose gel containing ethidium bromide (1% @ 5 µl/100 ml) by submarine gel electrophoresis apparatus at constant voltage of 60V for 45 min in 0.5X TBE buffer. After completion of electrophoresis, the gel was examined on UV transilluminator to observe the various fragments and photographed by gel documentation system (SynGene, Gene Genius BioImaging System,UK).

RESULTS

All the 13 isolates were identified on the basis of colony morphology and biochemical characters. In broth, floccular deposits were observed whereas on Modified Balanced Hank's Salt Solution Agar **(**MBHS-A) medium, the typical fried egg appearance of colonies of Mycoplasmas were observed after staining. Biochemically, the isolates were sensitive to digitonin and positive for tetrazolium reduction, phosphatase production and film and spot test. All the isolates were

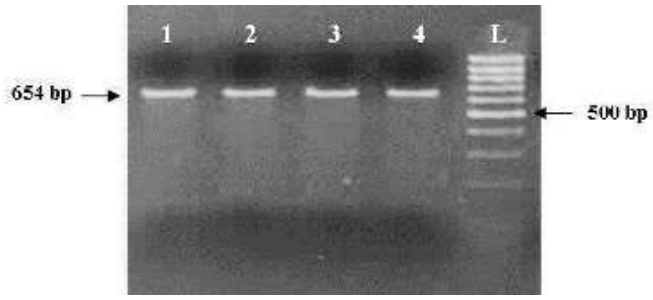

Figure 2. *Rsa*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing larger restriction fragment (654 bp) and unresolved smaller fragment (76 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

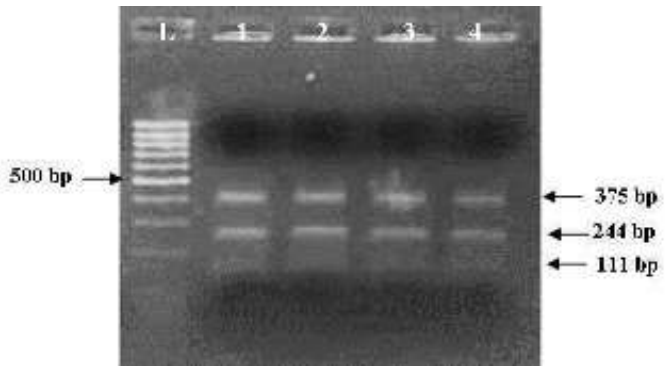

Figure 3. *Mbo*II generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 3 restriction fragments (375 bp, 244 bp and 111 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker

negative for glucose metabolism, arginine hydrolysis and serum liquefaction. The isolates were further confirmed as *M. agalactiae* by PCR using genus and species specific primers which yielded specific amplification product of 715 bp and 360 bp respectively. The *p30* gene with amplicon size of 730 bp was detected in all the representative *M. agalactiae* isolates analyzed (Figure 1).

Digestion of the 730 bp product of the *p30* gene with *Rsa*I yielded a single large fragment of 654 bp along with the smaller 76 bp fragment which could not be resolved because of its smaller size (Figure 2). Analysis of the products with *Mbo*II revealed the presence of three fragments of 111 bp, 375 bp and 244 bp in size (Figure 3). Digestion of the amplified products with the *Sau3A*I yielded two fragments of 461 bp and 269 bp (Figure 4). Digestion of the amplified product with *Alu*I yielded two fragments resolved into bands of 342 bp and 328 bp, whereas one smaller fragment of 60 bp could not be resolved (Figure 5).

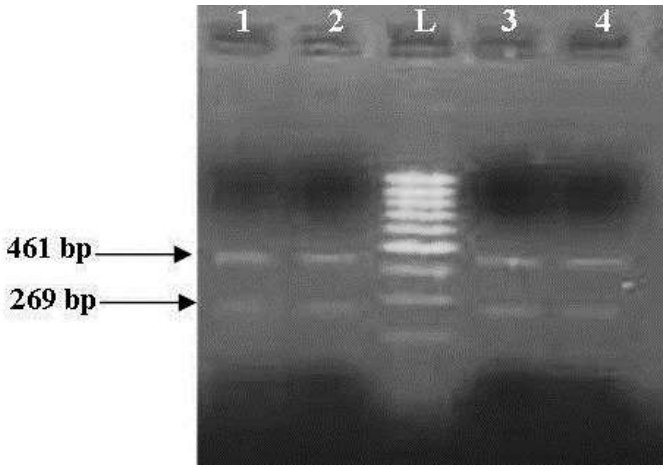

Figure 4. *Sau3A*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 2 restriction fragments (461 bp and 269 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

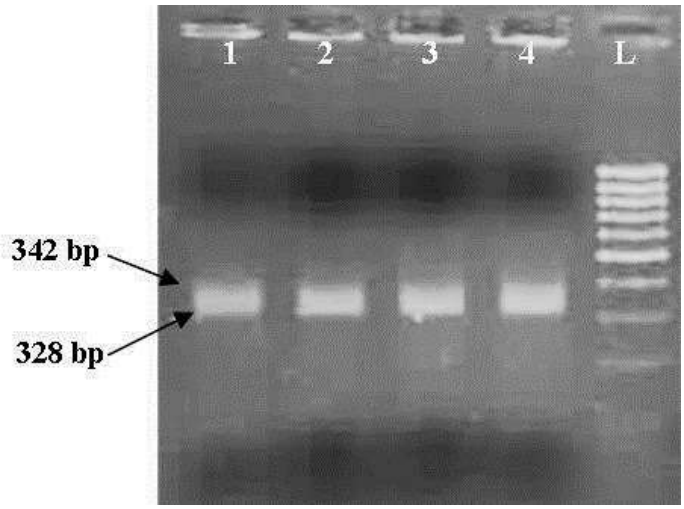

Figure 5. *Alu*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 2 restriction fragments (342 bp and 328 bp, unseparated) with unresolved smaller fragment (60 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

DISCUSSION

Restriction patterns produced by *RsaI* and *MboII* were in accordance with the expected restriction of *M. agalactiae p30* gene revealing the presence of one and two restriction sites respectively. Variations in the expected pattern were observed in case of digestion with *Sau3A*I and *Alu*I. Digestion with *Sau3A*I yielded only two fragments of 461 bp and 269 bp in contrast to the three expected fragments of 461 bp, 105 bp and 164 bp according to the restriction map showing the absence of one restriction site. Digestion of *p30* amplicons with *Alu*I revealed the absence of one restriction site resulting into a larger fragment of 328 bp in contrast to the expected two fragments of 282 bp and 46 bp along with other fragments of 60 bp and 342 bp fragments. Thus, the samples revealed the presence of only two restriction sites in contrast to the expected three restriction sites as per the expected restriction map with *Alu*I.

Several studies have been undertaken on the genetic characterization of the isolates by restriction digestion of various genomic segments of *M. agalactiae* including membrane protein gene. Glew et al. (2002) studied the polymorphism of the *vpma* locus of *M. agalactiae* by RE analysis using *Ase*I, *Alw*I and *Hind*III. An identical banding pattern was observed for both *Ase*I and *Alw*I whereas different patterns were observed with *Hind*III. In a similar study, Sung et al. (2006) analyzed the nested PCR products of various mycoplasmal strains by restriction enzyme digestion with *Sau*3AI enzyme to further identify and differentiate between the *Mycoplasma* species including *M. agalactiae*. Macun et al. (2010) targeted the 81 kDa membrane protein gene for the detection of *M. agalactiae* from cases of contagious agalactiae by PCR. In agreement with the present study, Kashoo et al. (2011) carried out the RE analysis of PCR products of *p80* gene of *M. agalactiae* using *Rsa*I and *Xho*I restriction enzymes to confirm the identity of the amplified products which produced the expected fragments of 146 and 868 bp with *Rsa*I enzyme, while 176 and 838 bp fragments were obtained with *Xho*I enzyme on 2.5% agarose gel electrophoresis.

Conclusion

The present study reveals the presence of polymorphism using PCR-RE analysis among the strains of *M. agalactiae* prevailing in the field in Gujarat region of India. This western province has significant population of sheep and goat which are affected by several disease condition and many disease outbreaks have been reported in them. Screening of those flocks is important to understand the cause of morbidity and mortality. Molecular tools are very important for rapid screening of those flocks as well as for characterization of the associated pathogens. The present study also shows the importance of molecular tools in the study of genomic variation among the field strains of these microorganisms causing covert and overt manifestations utilizing various mechanisms to evade host immune response. Many studies globally have shown the importance of RE analysis in the study of polymorphism in various genomic segments of field strains of *M. agalactiae* and development of detection system for different species.

These findings further underline the need to study the immunological implications of these variations occurring in the membrane proteins of these mycoplasmal pathogens which can ultimately affect their pathogenesis and persistence in the host.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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