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Molecular characterization of p80 gene of Indian *Mycoplasma agalactiae* isolates

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ABSTRACT

Serological tests are considered to be the most suitable for screening the goat pens for contagious agalactia, mainly caused by *Mycoplasma agalactiae*, but these have certain limitations due to the cross-reactive antigens among different mycoplasma species. The present study was undertaken to characterize the membrane protein p80 of *M. agalactiae* for specific diagnosis of disease as p80 protein is expressed in all field strains as well as throughout the course of infection. Five Indian isolates of *M. agalactiae* and one isolate of *Mycoplasma bovis* were used in the study after checking the purity by cultural examination, various biochemical tests and group specific and *M. agalactiae* specific PCR assays. Using group specific primers GPO-1 and MGSO, an amplified fragment ~715 bp was observed whereas, *M. agalactiae* specific PCR primers *Mbo*F2 and *Mbo*R2 produced an amplicon of size ~734 bp in all the 5 isolates. The cell lysates of all *M. agalactiae* isolates were subjected to SDS-PAGE western blot analysis, which resulted in a prominant band of ~80 kDa. The gene specific primers were designed and custom-synthesized for amplification of p80 which amplified the amplicon of 1014 bp from all strains. Our findings indicated that the membrane protein P80 is conserved antigen in the Indian isolates of *M. agalactiae* and can be used for specific diagnosis of contagious agalactia.

Key words: Contagious agalactia, p80 gene, Polymerase chain reaction

Mycoplasma agalactiae, the first identified mycoplasma in small ruminants, causes reduction of milk and leads to increased mortality in young animals (Mondal *et al.* 2004, Zendulkova *et al.* 2007). The disease affects many pens without manifesting clear and characteristic symptoms particularly when these animals are under any of the stress conditions. The situation is even more worrying in pens subjected to genetic improvement (Corrales *et al.* 2007). It has been reported in a number of countries including India but prevalence of infection is largely under estimated as very few laboratories in our country routinely monitor the infections. Its isolation is also hampered when extensive antibiotic treatment is used (Nicholas and Ayling 2003). Therefore, serological detection is considered the method of choice for screening the pens. These tests are used to detect and discriminate the antibody responses elicited in both vaccinated and infected sheep (Tola *et al*. 1997, Fusco *et al*. 2007).

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Several workers have reported the different types of serological tests for the diagnosis of contagious agalactia using whole cell bacterial lysate or membrane antigens, recombinant based antigens for epidemiological and diagnostic studies (Kittelberger *et al*. 2006, Fusco *et al*. 2007, Cheema 2009). It was found that a surface membrane protein with an apparent molecular mass of 80 kDa was expressed in all the strains and it also induced the antibody response in early phase of infection and maintained throughout the course of infection (Tola *et al.* 1997, 2001). The conserved nature of this major outer membrane protein among *M. agalactiae* strains makes it an extremely interesting candidate for the development of an effective diagnostic assay for diagnosis of contagious agalactia (Fusco *et al*. 2007). However, no information is available with regard to P80 in Indian isolates of *M. agalactiae*. Therefore, the present study was carried out to characterize the p80 surface antigen in available Indian isolates of *M. agalatiae*.

MATERIALS AND METHODS

Indian isolates (VP20-L15/02, VP12/05, RL 14/95–96 and RL 17/95–96) and 1 standard strain of *M. agalactia*e (UK) along with 1 standard strain of *M. bovis* were obtained from National Referral Laboratory on Mycoplasma, Division of Bacteriology and Mycology, Indian Veterinary Research

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Institute, Izatnagar (Uttar Pradesh). The liquid mycoplasma medium (MBHS-L) was prepared for propagation of strains according to Carmichael *et al.* (1972) as adopted by Singh (1990).

Characterization of the M. agalactiae *strains*

Giemsa staining: The culture smears were stained as per Singh (1990). The air-dried smears of the cultures were fixed in methanol, stained by Giemsa stain and observed under oil-immersion.

Biochemical characterization: Various biochemical tests, viz. glucose fermentation, phosphatase activity, arginine hydrolysis, liquification of coagulated serum, tetrazolium reduction and film and spot formation were performed (Aloutto *et al*. 1970).

Digitonin sensitivity test: The sensitivity of the organism to digitonin was tested according to Ernø and Stipkovits (1973). A zone of inhibition of more than 2 mm around the disc was considered to be sensitive for a culture.

Growth inhibition (GI) test: Growth inhibition test using hyperimmune serum discs against *Mycoplasma agalactiae* standard strain was carried out by the method of Clyde (1964) as adopted by Singh (1990).

Molecular characterization

Preparation of cell lysate: The cultures of mycoplasma were processed for direct analysis by PCR as per Johansson *et al*. (1996).

Group specific PCR: PCR was performed employing the primers based on 16S rRNA gene, viz. GPO-1 (5'-ACT CCT ACG GGA GGC AGC ACT A-3') and MGSO (5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3'), specific for *Mycoplasma*, *Acholeplasma*, *Spiroplasma* and *Ureaplasma* species (Kuppeveld *et al*. 1992). The reaction mixture consisted of 5µl of 10X PCR buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl, 1.5 mM $MgCl_2$, 0.08% Nonidat P-40), 200µM dNTP mix, 20 pmol of each of the primers, 1 unit of Taq polymerase and 6 µl of culture lysate. PCR program consisted of an initial denaturation at 94˚C for 2 min followed by 30 cycles of denaturation (94˚C for 45 sec), annealing (55˚C for 1 min) and extension (72˚C for 1 min) followed by final extension at 72˚C for 5 min.

M. agalactiae *specific PCR:* PCR assay was performed using the primers MboF2 (5'- GAA GAA AAA GT AGC ATA GGA AAT GAT-3') and MboR2 (5'-CGT CGT CCC CAC CTT CCT CCC G-3'), which amplify a segment of 734 bp between the variable region V6 and semi-conserved region S5b of 16S rRNA gene of *M. bovis* and *M. agalactiae*. The reaction mixtures as well as cyclic conditions were same as that of group-specific PCR except that the annealing temperature was set for both strains at 55°C and 65°C. At 65˚C, only *M. bovis* strain gets amplified while at 55°C, both *M. bovis* and *M. agalactiae* strains gets amplified (Johansson *et al*. 1996, Das 2001).

Characterization of p80 surface lipoprotein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The whole cell antigens (WCA) of all the 5 strains of *M. agalactiae* were prepared according to Solsona *et al*. (1996) for confirmation of P80 in all strains in native form. Protein concentration of WCA was estimated by the method of Lowry *et al*. (1951).

The SDS-PAGE of whole cell antigens was performed with the method of Laemmli (1970) as adopted by Solsona *et al*. (1996).

Western blotting: Sonicated antigen of standard strain of *M. agalactiae* was prepared according to Bhanuprakash and Srivastava (1996) for raising the antiserum. The whole cell antigens separated on 12.5% (w/v) SDS-PAGE slabs (Laemmli 1970) was transferred on nitrocellulose membrane (NCM) using the method of Towbin *et al*. (1979). The gel and the filter papers were soaked in the transfer buffer for 10 to 20 min. A transfer sandwich was prepared by placing the gel between NCM and filter papers. The whole cassette was assembled and transferred to the tank. The blotting was carried out at 1 mA/cm^2 of the gel for 1 h. After the completion of transfer, the cassette was unloaded from the blotter and then the gel was stained with coomassive brilliant blue to check the efficacy of transfer.

The NCM was blocked by 3 to 5% skimmed milk in TBS (Tris buffered saline) for 1 h at room temperature or overnight at 4˚C with occasional shaking. Then, the blocking agent was removed and NCM was washed thrice for 5 min each with TBS-T (Tween-20)) and once with TBS by continuous agitations. Subsequently, NCM was treated with primary antibodies as hyperimmune serum (1:4000 in TBS) at room temperature for 1.5 hr with occasional shaking.

The NCM was again washed thrice with PBS-T and then incubated with goat antirabbit HRPO conjugate in 1:2000 dilution in 1% BSA for 1 h at room temperature. After washing, immunoblot was developed with substrate solution (di aminobenzidine and peroxide) in dark at room temperature for 2–3 min till colour developed. The reaction was stopped by washing with distilled water, air dried and then it visualized.

Isolation of genomic DNA for PCR amplification of P80 from all strains

The genomic DNA of *M. agalactiae* isolates was extracted as per Sambrook and Russel (2001).

PCR amplification of p80*gene:* For amplification of *p80*gene of *M. agalactiae*, the primers P80F (5'- GGA TCC ATG GAA AAT GGT AAG GGC -3') and P80R (5'- TCT AGA AGC TTC ATT TAC CTG GCA CAG G -3') were self designed using GENE tool and DNA Star softwares based on published sequence (Tola *et al*. 2001) and custom synthesized.

The PCR reaction mixture consisted of 10 ng of the genomic DNA, 2.5 µl of 10X PCR buffer (10 mM Tris-HCl,

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pH 8.8; 50 mM KCl; 0.08% Nonidet P40), 2.5 µl of $MgCl_2$ (25 mM), 200 µM of dNTP mix, 20–30 pmol of each of the primers and 1 unit of *pfu* DNA polymerase. The cycling conditions consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min.

The amplified product was analyzed by submarine gel electrophoresis on 1% agarose gel containing 0.5µg/ml of ethidium bromide. Finally, the gel was visualized and photographed under UV gel documentation system.

Restriction endonuclease analysis of PCR products: The PCR products were further confirmed by restriction enzyme analysis using *Rsa*I and *Xho*I restriction enzymes. A 15µl of PCR product was digested separately using 10 IU of *Rsa*I or *Xho*I enzyme at 37°C for 4 h and the digested products were analysed on 2% agarose gel electrophoresis.

RESULTS AND DICUSSION

Characterization of cultures

Morphology and cultural characters: All the strains of *M. agalactiae* produced a change of pH from alkaline (red) to acidic (yellow) in liquid medium after 2–5 days of incubation at 37°C but, none of the strains showed any turbidity. On solid mycoplasma medium, typical fried egg colonies were observed in all the strains of mycoplasma. However, no growth was observed on blood agar and Robertson's cooked meat medium indicating the purity of the strains. Giemsa stained smears revealed typical pleomorphic purplish pink coloured organisms under oil immersion.

Biochemical tests: All the 6 strains (5 *M. agalactiae* + 1 *M. bovis*) used in the study produced film and spot reaction and were found positive for phosphatase test and also reduced the tetrazolium. But, these were not able to utilize glucose, hydrolyse arginine and digest the serum.

Digitonin sensitivity test: The strains were sensitive for digitonin as indicated by a zone of inhibition of more than 4 mm in diameter.

Disc growth inhibition test: All the strains of *M. agalactiae* were inhibited with homologous antiserum showing more than 2 mm of inhibition.

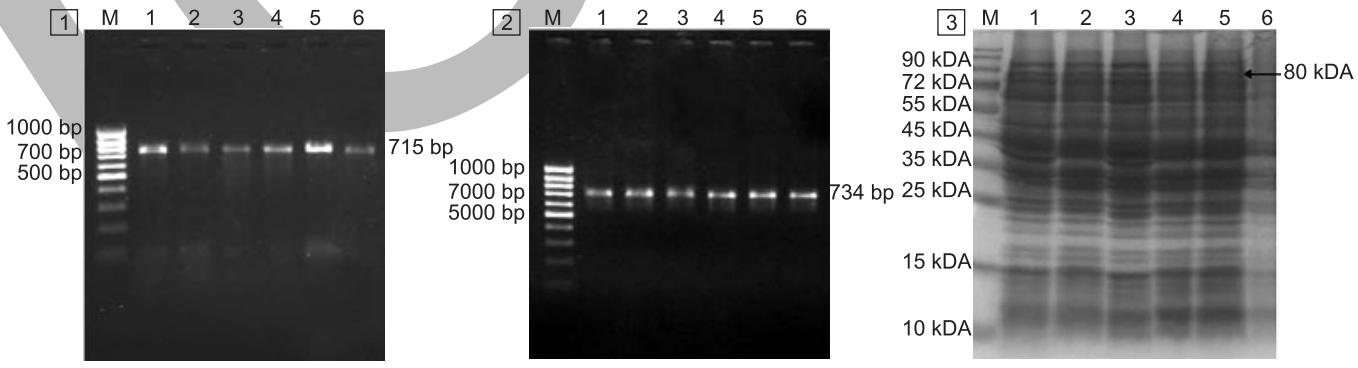
Molecular characterization

Polymerase chain reaction: The DNA samples from the different strains of *M. agalactiae* and *M. bovis* were found intact and without smearing in gel electrophoresis. The purity of DNA was between 1.7–1.8 which indicated the free of protein or RNA contamination. The concentration of DNA was about 1.41 to 4.61 µg/µl in different strains.

Using mycoplasma group specific primers GPO-1 and MGSO: The group specific primers, viz. GPO-1 and MGSO with mycoplasma genomic DNA was amplified product size of 715 bp in all the strains of *M. agalactiae* and *M. bovis* on 1.25% agarose gel electrophoresis (Fig. 1).

Using M. bovis specific primers MboF2 and MboR2: All the Indian strains of *M. agalactiae* and *M. bovis* had exhibited amplification of approximately 734 bp fragment on using *Mbo*F2 and *Mbo*R2 primers at the annealing temperature of 55°C, while at 65°C, only *M. bovis* strain showed amplification (Fig. 2).

All the 6 Indian isolates of *M. agalactiae* and *M. bovis* used in the study were pure as indicated by various tests like colony morphology, biochemical tests, digitonin sensitivity, growth inhibition and the absence of growth on blood agar, Robertson's cooked meat medium and Sabouraud's dextrose agar. Similar observations were also made by others using different species of mycoplasma (Das 2001, Kathiresan 2007, Cheema 2009, Zahid 2010). The genomic analysis, which is considered as highly specific, by using group specific and



Figs 1–3. **1.** Group specific PCR using primers MGSO and 16srRNA gene, viz. GPO-1.Lane M, 100 bp plus DNA ladder; lane 1, *M. agalactiae* standard; lane 2, *M. agalactiae* VP20L15/02; lane 3, *M. agalactiae* VP12/05; lane 4, *M. agalactiae* RL14/95–96; lane 5, *M. agalactiae* RL17/95–96; lane 6, *M. bovis* 17K. **2.** *M. agalactiae* specific PCR using primers *Mbo*F2 and *Mbo*R2. Lane M, 100 bp plus DNA ladder; lane 1, *M. agalactiae* standard; lane 2, *M. agalactiae* VP20L15/02; lane 3, *M. agalactiae* VP12/05; lane 4, *M. agalactiae* RL14/95–96; lane 5, *M. agalactiae* RL17/95–96; lane 6, *M. bovis* 17K. **3.** SDS-PAGE analysis of whole cell antigens of *M. agalactiae.* Lane M, Protein Marker; lane 1, *M. agalactiae* standard; lane 2, *M. agalactiae* VP20L15/02; lane 3, *M. agalactiae* VP12/05; lane 4, *M. agalactiae* RL14/95–96; lane 5, *M. agalactiae* RL17/95–96; lane 6, *M. bovis* 17K.

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M. agalactiae specific PCR also confirmed the *M. agalactiae* based on the results of Kuppeveld *et al*. (1992), Johansson *et al*. (1996), Kathiresan (2007) and Cheema (2009).

Characterization of native P80 lipoprotein

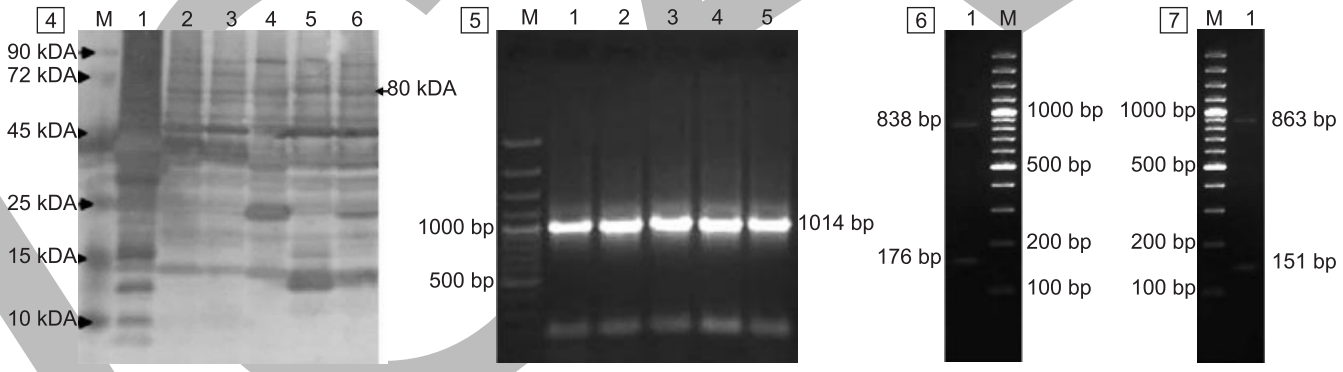
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The whole cell antigen was prepared from all the strains of *M. agalactiae.* The protein concentration varied from 8.15 to 12.42 mg/ml. The SDS-PAGE analysis revealed that a prominent polypeptide of about 80 kDa was present in all the Indian strains of *M. agalactiae*. Apart from the protein of 80 kDa, other protein bands of different molecular weight, viz. 95, 63, 48, 33, 32, 29, 25, 15 kDa were also found in all the strains (Fig. 3). Recently, number of immunodominant surface proteins P30, P48, P55 and P80 of *M. agalactiae* were targeted for the development of a specific serodiagnostic tests (Rosati *et al*. 2000, Fleury *et al*. 2001, 2002, Fusco *et al*. 2007, Cheema 2009). Amongst them, P80, a stable lipoprotein was targeted as a diagnostic marker for *M. agalactiae* infection as it is detected in maximum number of isolated strains (Tola *et al.* 1997, 2001). The conservation of this major outer membrane protein among *M. agalactiae* strains makes it an effective candidate for diagnostic purpose (Fusco *et al*. 2007). Furthermore, this protein was recognised in infected serum collected during early phase of infection and remained higher throughout of infection (Tola *et al.* 1997, Fusco *et al*. 2007).

Western blotting: Western blot analysis using hyper immune serum against *M. agalactiae* showed that an immunodominant protein of molecular weight about 80kDa was present in all the strains of *M. agalactiae*. It had also cross reacted with other immunodominant proteins of *M. bovis* (Fig. 4). However, species specific nature of these 2 organisms *(M. agalactiae* infect only small ruminants and *M. bovis* specific of large ruminants), the p80 candidate can be used as a marker antigen for CA diagnosis (Zahid 2010).

Amplification of p80 gene: PCR amplification of p80 gene in all the strains of *M. agalactiae* was carried out using the specific primers, viz. p80F and p80R at annealing temperature of 54.5°C and obtained 1014 bp amplified product in all the strains (Fig. 5). As per the result of amplification in the agarose gel electrophoresis, the expression of p80 was found higher and primer annealed specifically.

Restriction endonuclease analysis: The eluted amplicon was subjected to restriction endonuclease analysis with *Rsa*I and *Xho*I enzymes, which produced the bands of 146 bp and 868 bp with *Rsa*I enzyme while, 176 bp and 838 bp fragments were obtained with *XhoI* enzyme on 2.5% agarose gel electrophoresis (Figs 6,7). The results were in accordance with Tola *et al*. (2001), who studied the p80 gene from the Italian isolates. Also, the result of restriction enzyme patterns match with the predicted published p80 gene sequences analysis by bioinformstic tools such as GENE TOOL and DNA STAR.

Our findings indicated that the membrane protein p80 is an immunodominant antigen conserved in all the examined Indian isolates of *M. agalactiae.* The p80 antigen based ELISA might be specific, sensitive and reliable assay for the future specific detection of serum antibodies against *M. agalactiae.*



Figs 4–7. **4.** Western Blot analysis with polyclonal antiserum against *M. agalactiae.* Lane M, Protein marker; lane 1, *M. agalactiae* standard; lane 2, *M. agalactiae* VP20L15/02; lane 3, *M. agalactiae* VP12/05; lane 4, *M. agalactiae* RL14/95–96; lane 5, *M. agalactiae* RL17/95–96; lane 6, *M. bovis* 17K. **5.** PCR Amplification of p80 gene. Lane M, 100 bp plus DNA ladder; lane 1, *M. agalactiae* standard; lane 2, *M. agalactiae* VP20L15/02; lane 3, *M. agalactiae* VP12/05; lane 4, *M. agalactiae* RL14/95–96; lane 5, *M. agalactiae* RL17/95–96. **6.** Restriction endonuclease analysis: Lane M,100 bp plus DNA ladder; lane 1, *M. agalactiae* VP20-L15/02. **7.** Restriction endonuclease analysis of p80 of p80 digested with *Xho*I. product digested with *Rsa*I: Lane M,100 bp plus DNA ladder; lane 1, *M. agalactiae* VP20-L15/02.

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