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Antigenic Characterization of *Mycoplasma agalactiae* by SDS-PAGE and Immunoblotting

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ABSTRACT

Contagious agalactia is an endemic disease in most of the parts of the world with the classical etiology of *M. agalactiae* which accounts for 90% outbreaks of contagious agalactia syndrome in goats. The disease is responsible for high economic losses due to loss in milk yield and kids/lambs because of abortions, neonatal deaths and loss of animals. It has been reported from various parts of the country including India. However, till date no specific diagnostic is available in India due to cross reactivity of mycoplasma antigens. The conventional methods of diagnosis are cumbersome and lack specificity and sensitivity. Keeping this in mind the present study was undertaken with the objective to conduct the antigenic analysis of Indian isolates of *M. agalactiae* by separation of proteins by SDS-PAGE and estimating their immunogenicity and diagnostic potential. SDS-PAGE revealed 24 polypeptides in Whole Cell Antigens (WCA) and Sonicated Supernatant Antigens (SSA) of both the isolates, respectively in the range of 20.89-181.97 kDa with the seven major proteins of 63.10, 60.25, 58.88, 47.86, 44.66, 33.88 and 28.84 kDa molecular weights. On immunoblotting with polyclonal rabbit serum raised against *M. agalactiae* RPNS 216. All the major proteins appeared immunogenic with 12-14 immunogenic polypeptides. Out of them proteins of 47.86, 44.66, 33.88 and 28.84 kDa might be of protective and diagnostic importance for *M. agalactiae* and could be the source for future diagnostics in India.

Key words: *Mycoplasma agalactiae*, SDS-PAGE, immunoblotting

INTRODUCTION

Contagious agalactia is an endemic disease in most of the parts of the world which severely affects the sheep and goats (Lambert, 1987; Sarris, 1996; Kumar *et al.*, 2011, 2012). The disease originally included mastitis, arthritis and kerato conjunctivitis with the classical etiology of *M. agalactiae* which accounts for 90% outbreaks of contagious agalactia syndrome in goats (Garrido *et al.*, 1987; Kumar *et al.*, 2012) and almost 100% in sheep (Lambert, 1987; Nicholas *et al.*, 2000). The disease is responsible for high economic losses due to loss in milk yield and kids/lambs because of abortions, neonatal deaths and loss of animals (MacOwan *et al.*, 1984; Lagakis, 1996; Kumar *et al.*, 2002). In India, Bawa (1944) first time reported the disease. Later on it has been reported from various parts of the country (Dhanda *et al.*, 1959; Banerjee *et al.*, 1979; Sikdar, 1979; Gupta and Verma, 1984; Kumar and Chandiramani, 1987;

Srivastava *et al.*, 1996). As India has large sheep and goat population, contributing significantly in Indian economy (FAO, 2008). Therefore it is important to have better understanding of mechanisms implicated in the pathogenesis as well as knowledge about the nature of the major antigenic substances of *M. agalactiae* for controlling contagious agalactia and to develop a diagnostic add for the early diagnostic of the infected and carrier animals (Kumar *et al.*, 2010b). It can be developed based on the immunogenic protein of *M. agalactiae* (Kumar *et al.*, 2010a; Kumar and Singh, 2011). Keeping this in mind the present study was undertaken with the objective to conduct the antigenic analysis of Indian isolates of *M. agalactiae* by separation of proteins by SDS PAGE and estimating their immunogenicity which can be used, in future, for the development of diagnostic as well as immuno prophylactic agents for the control of contagious agalactia in India.

MATERIALS AND METHODS

Mycoplasma strains: Two strains of *M. agalactiae* RPNS 216 and RPNS 200 isolated from pneumonic goats were obtained from National Referral Laboratory on Mycoplasma, Division of Bacteriology and Mycology, IVRI.

Culture media: The Modified Beef Horse Serum liquid (MBHS-L) medium was prepared according to the method of Srivastava (1982) for the propagation of *M. agalactiae* strains. The MBHS-solid medium was prepared by the addition of 1.2% bacto agar (Difco) in MBHS-L medium.

Whole cell antigens (WCA): WCA were prepared as per the method of Solsana *et al.* (1996) with slight modifications. Actively growing 2-5 mL of *M. agalactiae* culture was inoculated in 10 mL MBHS-liquid medium and incubated at 37°C for 48 h. The growth was confirmed by the change in pH (change of color red to yellowish orange). This growth was subsequently transferred to larger volume of media and incubated for 4-5 days to obtain sufficient growth. Simultaneously the growth was checked for purity on Robertson cooked meat, Sabouraud's dextrose agar and Blood agar media. The growth was centrifuged at 10,000 rpm for 25 min using a refrigerated centrifuge (Sorvell, RC-5C). The pellets washed thrice with PBS (pH 7.2) and finally resuspended in 10 mL of PBS. The protein concentrations of WCA were estimated by the method of Lowry *et al.* (1951).

Sonicated supernatant antigen (SSA): Sonicated antigens of both the isolates were prepared from the whole cell antigen by the method of Bhanuprakash and Srivastava (1996) and the protein concentration of SSA was estimated by the method of Lowry *et al.* (1951).

Raising of hyperimmune sera: The antiserum against *M. agalactiae* (RPNS 216) was raised in white New Zealand rabbits (obtained from LAR, IVRI, Izatnagar), according to the method of Jones (1989). These antisera were tested by slide agglutination test (Srivastava *et al.*, 1986) and titer was estimated by Indirect Haemagglutination (IHA) by the method of Krogsgaard-Jensen (1971) as modified by Srivastava *et al.* (1992). Hyperimmune sera raised in rabbits were finally filtered through 0.20 µm filter (Sartorius) and then stored at -20°C for further use.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE of both WCA and SSA under denaturing, reducing conditions was performed by the method of

Laemmli (1970) as modified by Solsana *et al.* (1996). The determination of molecular weights was based on the distance migrated by the polypeptides in the gels in comparison to the distance migrated by polypeptide markers of known molecular weights (Bioered) (Neville, 1971).

Immunoblotting: The WCA and SSA were separated on 12.5% (w/v) SDS-PAGE slabs (Laemmli, 1970) and transferred electrophoretically on nitrocellulose membrane papers (NCP) (Sartorius) by the method of Towbin *et al.* (1979).

RESULTS AND DISCUSSION

The sonicated supernatant and whole cell antigens of the isolates of *M. agalactiae* were separated under reducing and denaturing conditions by SDS-PAGE (Fig. 1). SDS-PAGE revealed 24 polypeptides in WCA and SSA of the isolates, respectively in the range of 20.89 to 181.97 kDa. The protein profiles of WCA and SSA of respective isolates were similar and identical. The polypeptide of 120.22 kDa was present only in *M. agalactiae* RPNS 216 whereas the polypeptide of 62.85 kDa was present only in the profile of *M. agalactiae* RPNS 200. These are of diagnostic importance and can be used for the differentiation of isolates. The major proteins appeared in the range of 20 to 80 kDa. As per the intensity of proteins the seven major proteins were of 63.10,

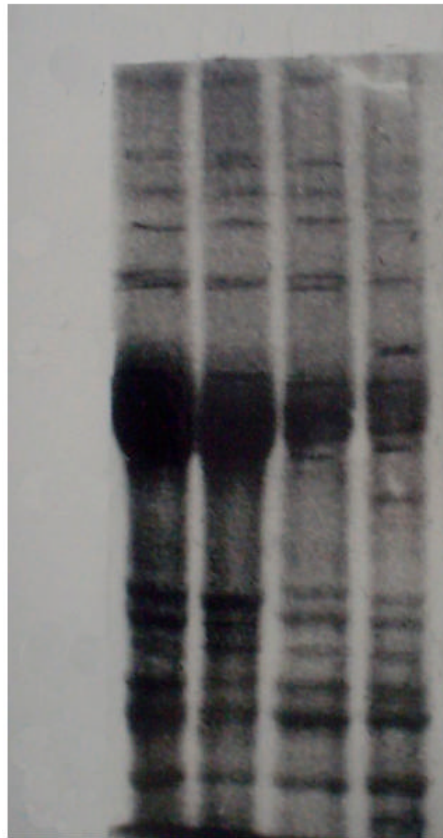


Fig. 1: SDS-PAGE profile of *M. agalactiae*, Lane A: WCA of *M. agalactiae* RPNS 216, Lane B: WCA of *M. agalactiae* RPNS 200, Lane C: SSA of *M. agalactiae* RPNS 216, Lane D: SSA of *M. agalactiae* RPNS 200

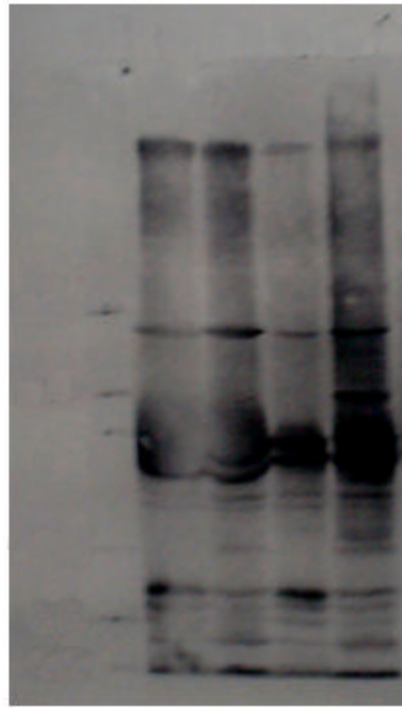


Fig. 2: Immunoblot of *M. agalactiae*, Lane A: WCA of *M. agalactiae* RPNS 216, Lane B: WCA of *M. agalactiae* RPNS 200, Lane C: SSA of *M. agalactiae* RPNS 216, Lane D: SSA of *M. agalactiae* RPNS 200

60.25, 58.88, 47.86, 44.66, 33.88 and 28.84 kDa molecular weight. The protein profile of the Indian isolate resemble to that of *M. agalactiae* isolates from other parts of the world (Boothby *et al.*, 1983; Bergonier *et al.*, 1996; Gummelt *et al.*, 1996) with the difference of number of polypeptides and minor differences in major proteins. Similar to present study, Bergonier *et al.* (1996) observed almost identical protein profile of 52 isolates of *M. agalactiae*, isolated from different geographical regions with 5-7 major polypeptides in the range of 20-90 kDa. Boothby *et al.* (1983) and Gummelt *et al.* (1996) also reported 5-8 major polypeptides and almost similar protein profiles of different isolates with minor differences in whole cell proteins. Moreover the naïve PAGE also confirmed the presence of 5 major proteins in *M. agalactiae* (Kumar *et al.*, 2010a) which appear to remain intact even after fractionation (Kumar and Singh, 2011). These proteins which persisted in fractions and are persistent in presence can be the antigens of choice for diagnostics and vaccine candidature (Kumar *et al.*, 2011). There are reports of using whole cell of *M. agalactiae* in vaccine preparation in different animal models (Sunder *et al.*, 2001; Kumar *et al.*, 2009). However, the use of whole organism is a burden upon the host immune system if a suitable protein is capable enough to protect against pathogen. Thus immunoblotting, a more sensitive and more specific confirmatory method to find out the immunogenic proteins when the results of common serological tests are ambiguous (Hung *et al.*, 1991) was applied to provide the information of immunogenic proteins responsible for protective immunity (Solsana *et al.*, 1996; Kumar *et al.*, 2011). The polypeptides bands were electrophoretically transferred on nitrocellulose membranes and blotted with polyclonal rabbit serum produced against *M. agalactiae* RPNS 216 (Fig. 2). All the major proteins appeared

immunogenic with 12-14 immunogenic polypeptides. Both the polypeptides (120.22 and 62.85 kDa) which were present in one isolates (*M. agalactiae* RPNS 216 and *M. agalactiae* RPNS 200, respectively) were found non immunogenic. The findings are in resemblance to the findings of Boothby *et al.* (1983) who also reported almost similar immunogenic protein profile with the major immunogenic proteins in the range of 20-80 kDa. In contrast to the findings of present study (Gummelt *et al.*, 1996) reported high variability in 19 strains of *M. agalactiae* with *M. agalactiae* serum however, the range and major proteins coincide to present study. Solsana *et al.* (1996) recommended 29 kDa proteins as the mean of diagnosis on the basis of immunoblot analysis of 31 strains of *M. agalactiae*. Similarly the present study also suggests that the major polypeptides may be of protective and diagnostic importance for *M. agalactiae*. Thus the presence of major immunogenic polypeptides suggested the candidate for diagnostics as well as vaccine development.

CONCLUSION

The presence of major immunogenic proteins particularly the proteins of 28.84 kDa suggested the potential diagnostic as well vaccine candidates for the prevention and control of the disease conditions produced by *M. agalactiae*. However, further challenge as well as exhaustive studies to assess the selectivity is required.

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