Immuno-reactivity pattern of secretory proteins of *Mycobacterium avium* subspecies *paratuberculosis* vaccine strain ‘S 5’ with potential for diagnosis of Johne’s disease in early infection

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Diagnosis of Johne's disease (JD) is hampered by the lack of specific immune-reactive antigens. Therefore, studies should be focused on the search of new candidate antigenic epitopes as 'novel biomarkers' for early diagnosis of JD. Secretory proteins profile of novel biotype ('S 5') of 'Indian Bison Type' and their immuno-reactivity was studied in early growth period (4 & 6 wk). Analysis of harvested CF (culture filtrate) proteins was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting showed strong reactivity of 4 CF proteins (19, 36-38 and 65 kDa). Additional 48 kDa protein was recognized at 6 wk using MAP infected caprine serum. Diagnostic potential of early phase secretory proteins was evaluated using Indirect ELISA test. Results showed slightly lower sensitivity and 100.0% specificity with respect to whole cell sonicated semi-purified protoplasmic antigen (sPPA). Our earlier studies clearly exhibited that there cannot be universally effective diagnostic kits in case of chronic insidious diseases like Johne's disease. Therefore efforts should be to use antigen candidates from locally prevalent strains. By using commercially available universal kits we are unknowingly grossly under reporting the disease prevalence/incidence.

Keywords: Immunoblotting, Indirect ELISA, *Mycobacterium avium* subspecies *paratuberculosis*, secretory proteins, SDS-PAGE

Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of Johne’s disease (JD), is endemic in domestic livestock population and world-wide also1,2. This is mainly due to the difficulties in diagnosing sub-clinically infected animals shedding MAP in their feces silently. MAP has widest host range. Besides domestic and wild ruminants, it is prevalent in other animals species including primates and human beings1,3,4. JD has major impact on the US dairy industry and in US alone 250 million USD worth economic losses have been reported5. In the absence of control programmes at National level and preventive measures at local herd/flock level, prevalence of JD continues to increase1. Once MAP infection and disease enters a herd, it becomes firmly established. The economic losses are due to reduced milk productivity (10-25% below potential). In India, total losses due to outbreak of JD in the dairy farm were reported to be Rs. 5,67,176.0 including losses due to reduction in milk production 23.1% (Rs. 1,31,376.0) in 305 d of lactation6. Control of paratuberculosis has been hindered due to absence of sensitive, specific locally relevant diagnostic tests. Cross-reactivity of the antigens and lowered specificity of the commercially available diagnostic tests is a problem due to the sharing of antigens/epitopes by MAP with other species of *Mycobacterium*, *Corynabacterium* and *Nocardia*7,8. Sensitivity of commercially available ELISA kits prepared using purified protoplasmic antigen from different MAP strains (not local to the country using) have been found to be comparatively low (13.6-33.3%)9. Therefore, the present search should be focused on the new 'candidate antigenic epitopes' for the early diagnosis of paratuberculosis infection.

Recent studies have focused on the development of improved sero-diagnostics using species-specific multiple protein antigens from locally available strains10,11. Micro-fluidics, Lab-on-Chip, PARA-LP-01 and PARASAFE are some of the current technologies that can help in development of laboratory-free diagnostic for Mycobacterial infections12-14. Previous studies have identified several antigens inducing
strong humoral responses, which are highly conserved within Mycobacterial species and hence cross-react with other mycobacterial pathogens. Earlier reports identified different types of immuno-dominant MAP specific proteins/genes, which has been characterized, cloned, expressed and evaluated for their diagnostic potential. However, type of MAP strain may also affect the sensitivity and specificity of the tests. The purpose of the present study was to investigate profile of secretory protein present in the native strain of (‘S 5’) of MAP, a novel biotype of ‘Indian bison type’ and used as ‘vaccine strain’ and antigen source in indigenous ELISA test, with reference to determine the antigenicity/reactivity of these proteins to develop more precise diagnostics (using cocktail of secretory proteins) and evaluated the sensitivity and specificity of the test.

Materials and Methods

Mycobacterial Strain

*M. avium* subspecies *paratuberculosis* (MAP) ‘S 5’ strain was procured from the Mycobacterial Repository in the Animal Health Division of Central Institute for Research on Goats (CIRG), Makhdoom. The strain was maintained on modified Herrold’s egg yolk medium with mycobactin J (HEYM) as per Singh et al. and was sub-cultured in Middlebrook 7H9 medium (as per the manufacturer, Becton Dickinson, BD) supplemented with ADC (10% or 100 mL/L), PANTA antibiotics (Himedia) and mycobactin J (2 mg/L, Allied Monitor, USA). To avoid clump formation the cultures were constantly shaken at 100 rpm during incubation.

Secretory Protein(s)

Secretory protein(s) were harvested as per the method of Gupta et al. Briefly, secretory protein(s) were obtained by centrifugation of the bacteria in growth (4000× g, 20 min, 4°C) at different times points (4 & 6 wk of incubation), followed by filtration (using 0.22-µm pore size syringe filter, Millipore) and were concentrated with 10 kDa Amicon Ultra 2 mL centrifugal filters (Sigma-Aldrich, India). Concentration of proteins was quantified by Bradford protein assay kit (Genei) and was stored at –20°C.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Culture filtrate (CF) protein profile was analyzed by 12% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue (R-250) as per method given by Laemmli.

Immunoblotting

The electrophoresed secretory proteins were transferred on to PVDF-plus membrane (pore size 0.45 µm, Millipore) using Mini Trans-blot Cell (Bio-Rad) for 1.5 h at 60 V, 100 mA as per Towbin with some modifications. The membrane was blocked for 1 h at room temperature in 5% skim milk (Himedia) containing TBS with 0.05% Tween-20 (TBST) and secretory proteins were probed with polyclonal primary antibody (goat serum) diluted 1:100 in 1× TBST for 2 h at room temperature. After incubation, the membrane was washed 3 times with 1× TBST. Reactivity was seen by incubating the PVDF-plus membrane with peroxidase-conjugated anti-goat IgG (Sigma-Aldrich, USA) in 1:2000 dilution for 1 h at room temperature, followed by washing 5 times (5 min each) with TBST. Visualization of immuno-reactive protein bands was done by 3,3-Diaminobenzidine (DAB) (Sigma-Aldrich).

Semi-purified Protoplasmic Antigen of Goat Origin

Semi-purified protoplasmic antigen (sPPA) prepared from native strain S 5 of ‘Indian Bison Type’ MAP, isolated from a terminal case of JD in a goat, was initially developed as ‘Indigenous ELISA kit’.

Indigenous ELISA Kit (i-ELISA)

The i-ELISA developed by Singh and co-workers was used. Briefly, native sPPA was standardized at 0.1 µg per well of the micro-titer plate. Serum samples and goat anti-horseradish peroxidase conjugate (Sigma Aldrich, USA) were used in 1:50 and 1:5000 dilutions. Serum samples from culture positive and negative goats were used as positive and negative controls, respectively. Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per the method of Collins.

Analysis of OD (Absorbance) Values

S/P ratio value = [(Sample OD–Negative OD)/(Positive OD–Negative OD)]

Values of S/P Ratios and Corresponding Status of Johne’s Disease

The corresponding status of Johne’s disease with the calculated S/P ratio is shown in Table 1. The sensitivity and specificity of i-ELISA kit was 83.3 and 90.0%, respectively.

Secretory proteins based ELISA test (sp-ELISA)

Secretory proteins (as a pool) harvested at 4 and 6 wk of growth of MAP culture were used as per Singh et al. at 0.1 µg per well of the microtiter
plate (sp-ELISA) and rest steps were like i-ELISA kit, including interpretation of ELISA test results.

**Statistical Analysis**

Fisher’s exact test and kappa agreement was applied to analyze the statistical significance between results of two tests using GraphPad software, USA.

**Results**

**Immunoblot of MAP Secreted Proteins**

Polyclonal caprine sera naturally infected with MAP (confirmed by fecal microscopy, IS900 blood PCR and ‘Indigenous serum ELISA kit’) were tested for reactivity pattern to secretory proteins. The initial antigenic profiles were detected at 4 and 6 wk of growth of MAP culture in 7H9 medium. Analysis of harvested secretory proteins by sodium SDS-PAGE showed that greater part of the secretory proteins had molecular masses <70 kDa (Fig. 1). The antigenic secretory proteins of ‘S 5’ strain of MAP commonly recognized with MAP infected goat serum at 4 and 6 wk (early infection) were approx 19, 36, 38 and 65 kDa and showed wide variation in recognition pattern (Figs 2 & 3). However, an additional 48 kDa protein was

<table>
<thead>
<tr>
<th>No.</th>
<th>Calculated value of S/P ratio</th>
<th>Johne’s disease status in animal*</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00-0.09</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>0.10-0.24</td>
<td>Suspected or Borderline</td>
</tr>
<tr>
<td>3</td>
<td>0.25-0.39</td>
<td>Low Positive</td>
</tr>
<tr>
<td>4</td>
<td>0.40-0.99</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>1.0-10.0</td>
<td>Strong Positive</td>
</tr>
</tbody>
</table>

*Serum samples in positive (P) and strong positive (SP) categories were taken as positive.

![Fig. 1—SDS-PAGE profile of secretory proteins harvested at 4 and 6 wk incubation periods of MAP. (Lane M: Pre-stained protein marker (Fermentas, #SM0671); Lane 1 & 2: Secretory proteins harvested at 4 and 6 wk, respectively)](image)

![Fig. 2—Immunoblots with secretory proteins of MAP in naturally infected caprine 1 serum. (Lane M: Pre-stained protein marker; Lane 1 & 2: Secretory proteins harvested at 4 and 6 wk, respectively)](image)

![Fig. 3—Immunoblots with secretory proteins of MAP in naturally infected caprine 2 serum. (Lane M: Pre-stained protein marker; Lane 1 & 2: Secretory proteins harvested at 4 and 6 wk, respectively)](image)
recognized at 6 wk (Fig. 2). In the immuno-blotting studies, it was accrued that secretory proteins were highly specific. Therefore, immunogenicity of these reactive secretory proteins was evaluated in the ELISA test in order to identify the biomarker proteins

**Sensitivity and Specificity of Secretory Protein Pool Based Assay (sp-ELISA) vs sPPA Based Assay (i-ELISA)**

These biomarker protein pools were used in sp-ELISA test so as to assess the sensitivity and specificity of the assay as compared to i-ELISA (Indigenous ELISA). Results of sp-ELISA test using above (early) protein pools (4 & 6 wk) as antigen showed improved specificity (100.0%) as compared with the whole cell sonicated semi-purified protoplasmic antigen (sPPA) as exhibited in the ROC (Receiver Operating Characteristic) analysis (MedCalc, software). Sensitivity of sp-ELISA at 4 and 6 wk protein pools with respect to i-ELISA was 82.7 and 93.1%, respectively (Tables 2 & 3). Kappa agreement calculated between sp-ELISA$_{4w}$ vs i-ELISA and sp-ELISA$_{6w}$ vs i-ELISA were 0.843±0.047 and 0.938±0.030, respectively (Table 3). Comparison of two tests showed that none of the sample was false positive in sp-ELISA (Tables 4 & 5; combination no. 3). Strength of agreement was found very good (kappa value: 0.81-1.0) and statistically significant (P-value <0.0001) between the ELISA based on two types of native antigens (sPPA & secretory proteins).

<table>
<thead>
<tr>
<th>No.</th>
<th>Status*</th>
<th>i-ELISA based on s-PPA</th>
<th>sp-ELISA based on early secretory proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4W (36.3)</td>
<td>55 (41.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6W (35.6)</td>
<td>47 (35.6)</td>
</tr>
</tbody>
</table>

*Animals in positive and strong positive categories were considered as positive in the ELISA tests

4W & 6W: Secretory proteins harvested at 4 and 6 wk of growth of MAP culture in liquid medium

<table>
<thead>
<tr>
<th>Serum</th>
<th>Tests</th>
<th>*Two tailed P value</th>
<th>Kappa±SE</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goats</td>
<td>i-ELISA vs sp-ELISA$_{4w}$</td>
<td>&lt;0.0001</td>
<td>0.843±0.047</td>
<td>82.76</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>i-ELISA vs sp-ELISA$_{6w}$</td>
<td>&lt;0.0001</td>
<td>0.938±0.030</td>
<td>93.10</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Strength of agreement was very good

Table 4—Comparative evaluation of sp-ELISA$_{4w}$ with i-ELISA

<table>
<thead>
<tr>
<th>Tests</th>
<th>Combinations</th>
<th>Cumulative total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
<td></td>
</tr>
<tr>
<td>sp-ELISA$_{4w}$*</td>
<td>48 (36.3) 74 (56.1) 0 (0.0) 10 (7.5)</td>
<td></td>
</tr>
<tr>
<td>i-ELISA</td>
<td>54 (36.3)</td>
<td></td>
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</tbody>
</table>

*4W: Secretory proteins harvested at 4 wk of growth of MAP culture in liquid medium

Table 5—Comparative evaluation of sp-ELISA$_{6w}$ with i-ELISA

<table>
<thead>
<tr>
<th>Tests</th>
<th>Combinations</th>
<th>Cumulative total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4</td>
<td></td>
</tr>
<tr>
<td>sp-ELISA$_{6w}$*</td>
<td>54 (40.9) 74 (56.1) 0 (0.0) 04 (3.0)</td>
<td></td>
</tr>
<tr>
<td>i-ELISA</td>
<td>58 (43.9)</td>
<td></td>
</tr>
</tbody>
</table>

*6W: Secretory proteins harvested at 6 wk of growth of MAP culture in liquid medium
Discussion

Johnne’s disease (JD) is chronic enteritis of both domestic and wild ruminants caused by MAP. It is responsible for extensive economic losses to dairy industry worldwide. Use of recently developed immuno-diagnostics for JD is hampered by the lack of specific antigens. Diagnosis of the disease or identifying the causative agent quickly and accurately is critical to combat this disease and lack of it halts the progression of sub-clinical shedders into clinical shedders or epidemics like situation\textsuperscript{21}. Cross-reactivity of the MAP antigens, and poor sensitivity and specificity of the current diagnostics, is the main problem in correctly identifying infected animals. This is primarily due to extensive sharing of antigenic epitopes within Mycobacterium, Corynebacterium and Nocardial group of bacteria\textsuperscript{7,8}. Test results from the sub-clinically, MAP infected animals are challenge to interpret, because clinical signs are not present to assist the interpretation. Transmission of infection usually takes place prior the tests becoming positive and before clinical signs developed. Routinely used test for diagnosis of MAP infection include bacterial culture, IS\textsubscript{900} PCR amplification, interferon-\(\gamma\) assay and serum antibody detection using an ELISA platform. Wadhwa and co-workers used complement fixation (CFT), agar gel immuno-diffusion (AGID), and ELISA to determine the antibody response in JD\textsuperscript{12}. The CFT and AGID tests both suffer poor sensitivity\textsuperscript{22,23}. However, other studies reported that immunological assays based on ELISA platform are the best methods as compared to CFT and AGID for calculating MAP infection in dairy and beef herds\textsuperscript{24}.

Earlier studies have reported the use of different antigens as sPPA\textsuperscript{9,25}, lipoarabinomannan (LAM)\textsuperscript{26}, culture filtrate of MAP\textsuperscript{27}, and MAP recombinant proteins (MBP-fusion protein, Mod D, Map1152, Map1156, Map2609, Map2942c & Map0210c)\textsuperscript{11,15,16,28} for testing antibodies against MAP. Though LAM is one of the cell wall constituent, but it is shared with other mycobacterial species and thus has limitations in correctly identifying infected animals due to cross-reactivity\textsuperscript{25}. However, using recombinant proteins as the solid-phase antigen, low specificity (loss or decrease in antigenicity) was reported, which might be attributed to the conformational changes in the proteins and/or glycosylation during the cloning process\textsuperscript{15,30}. Some studies also suggested that use of secreted proteins may be better as solid-phase ELISA antigens, resulting in increased sensitivity by 25% for low shedding animals\textsuperscript{27,31,32}. On the other hand, Sung and Collins\textsuperscript{33} have reported that appearance of MAP secretory protein depends on the type and culture conditions. Mycobacterial infections, such as, tuberculosis (TB), bovine tuberculosis (bTB) and JD, are major infectious diseases of zoonotic concern\textsuperscript{12}. New technologies, such as, ‘microfluidics’ and ‘Lab-on-Chip’, are the technologies that can emphasize development of laboratory-free diagnostic devices for these mycobacterial infections\textsuperscript{12,13}. Further, Wadhwa et al\textsuperscript{34} have developed EVELISA, a sensitive ELISA assay using ethanol extract of MAP, especially for JD.

The present study shows that nearly all early-secreted proteins were of low mol wt ranging 14-70 kDa. Similar findings regarding secretory protein(s) of MAP have been reported with other field strains elsewhere\textsuperscript{11,35}. Further, immunoblotting studies indicate that the secretory proteins were quite specific, showed high intensity of bands and variability among naturally infected caprine sera in protein binding patterns observed. Cho and Collins\textsuperscript{31} also reported similar results.

In the immunoblotting studies, the reactive secretory proteins were evaluated as biomarkers to develop more sensitive and specific sp-ELISA assay. Combined application of these secretory proteins (pooled) as antigen showed slightly lower sensitivity and 100.0% specificity as compared with sPPA (containing a large number of antigens expressing early and late). Lowered sensitivity might be due to the antigens used in these assays are early secretory proteins harvested at 4 and 6 wk of culture growth. It is difficult to increase sensitivity of ELISA test because of poor Th 2 response during latent period/sub-clinical phase of the disease and may be large population of animals with prolonged latent/sub-clinical disease in the flocks and herds at any given time\textsuperscript{28}. Therefore, to enhance ELISA performance, secreted antigens that are expressed during early growth of MAP need to be identified. Infected goats showed slight difference in OD value at A\textsubscript{450} as compared antigen used in sPPA (Indigenous ELISA). Earlier study also reported that, during short incubation period, MAP may secrete immunoreactive proteins, which are not dominant in long term cultures\textsuperscript{36}. Low level antibody reactivity was also observed with caprine sera free of MAP infection. Strength of agreement (kappa value) between the ELISA based on two types of native antigens (sPPA & secretory proteins) was found perfect.
and statistically significant. Previous studies on ‘Indigenous ELISA’ (sPPA) developed from ‘Indian Bison type’ strain showed improved sensitivity and specificity as compared to commercial ELISA kits. Conceptually, the use of specific purified proteins as the solid phase antigen in ELISA assay has suffered from low diagnostic sensitivity. JD being spectral in nature, its immune response to MAP in infected animals was variable. Cocktail or pool of immunoreactive secretory proteins of the native ‘Indian Bison type’ strain provided significant advantage and improvement in the diagnosis of disease in animals with sub-clinical stages of infection. Other workers also reported existence of early immune (serological and/or CMI) response against secretory proteins of mycobacteria. Present study showed that immunoreactive secretory proteins can be utilized as ‘marker proteins’ for the diagnosis of JD in domestic livestock during sub-clinical and early stages of infection. These secretory proteins can also help to differentiate between infected and vaccinated animals (DIVA), since these proteins are not secreted by inactivated ‘vaccine strain’.

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Conflict of Interest
No potential conflict of interest to declare.

References