

Research Article

Immunoreactivity to Culture Filtrate Proteins of *Mycobacterium avium* Subspecies *paratuberculosis* in Naturally Infected Goat and Sheep Sera

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Abstract | *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of granulomatous chronic enteritis in ruminants (Johne's disease) is under reported due to difficulties in diagnosing pre-clinical cases. Compromised specificity is a problem due to extensive sharing of antigens /epitopes among MAP and other mycobacterial strains. Culture filtrate (CF) proteins profile of native 'Indian Bison Type' strain of MAP was studied at different harvesting times (2-8 weeks of growth) in Middlebrook 7H9 medium supplemented with ADC, PANTA antibiotics and mycobactin J. Analysis of harvested CF proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the greater part of CF proteins had molecular masses (<70 kDa) as 14, 19, 26, 34-41, 52-55, and 70 kDa. Immunoblotting showed reactivity of CF proteins commonly recognised (28, 34-36, 38-42, 45, and 56 kDa) with all four MAP infected goat and sheep sera at 2-8 weeks of growth. Collectively, these immunoreactive MAP CF proteins could be the potential targets for developing diagnostics against Johne's disease with improved sensitivity and high specificity instead of whole cell sonicated crude protoplasmic extracts (PPA).

Keywords | Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis*, Culture filtrate, SDS-PAGE, Immunoblotting

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INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP), a cause of Johne's disease (Paratuberculosis) is the most wide-spread and highly prevalent due to difficulties in diagnosing pre-clinical cases. MAP has wide host range including domestic and wild ruminants, free grazing animals and also the human beings (Singh et al., 2010; Singh et al., 2014a; Singh et al., 2014b). Paratuberculosis has high economic impact on dairy industry and > USD 250 million economic losses has been reported in US alone (Ott et al., 1999). Though disease is endemic in India and economic losses in dairy farm was estimated from reproductive disorders (Rs. 23400.0/cow/year), forced removal (Rs. 41,750.0/cow/year), reduced milk yield (Rs.

5,712.0/cow/ year) and increased mortality (Rs. 11,666.0/cow/year) (Rawat et al., 2014). Control of paratuberculosis has been hindered due to lack of efficient and accurate diagnostic tests. Lower specificity of tests can also be a problem due to the sharing of antigens or epitopes among MAP and other mycobacteria (Collins et al., 1991). Sensitivity of commercially available ELISA kits prepared using protoplasmic antigen has been reported comparatively low, 13.6-33.3% (Singh et al., 2007).

Recent studies have focused on developing improved serodiagnosics using species-specific multiple protein antigens (Singh et al., 2014c). Microfluidics and Lab-on-Chip are some of the recent technologies that can predicate development of laboratory-free diagnostic devices for

mycobacterial infections (Wadhwa et al., 2012a; Li et al., 2011). Earlier reports have identified several antigens inducing strong antibody responses but most of them have been found unsuitable for serodiagnosis because they are highly conserved within mycobacteria and hence cross-react with other mycobacterial pathogens. Recently, various MAP specific proteins/genes has been characterized, cloned, expressed and the protein was evaluated for their diagnostic potential (Cho et al., 2007). However, strain of MAP may also tune the sensitivity and specificity of test. Present study investigated CF protein profile of native Indian bison type ('S 5') MAP strain for the first time with reference to determine the antigenicity / reactivity of the CF proteins by immunoblotting with polyclonal antisera of goat and sheep infected with MAP and to develop diagnostic tests of higher sensitivity and specificity.

MATERIALS AND METHODS

MYCOBACTERIAL STRAIN

Mycobacterium avium subspecies *paratuberculosis* ('S 5') strain was procured from the mycobacterial repository, 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. (1996) and was sub-cultured in Middlebrook 7H9 medium (as per the manufacturer, Becton Dickinson, BD) supplemented with ADC (10% or 100ml/l), PANTA antibiotics and mycobactin J (2mg/l).

GROWTH PATTERN

Growth of MAP in middlebrook 7H9 medium was monitored by taking absorbance (OD) of the culture at third day, then every week at 600nm for a period of 8 weeks (Figure 1). Growth curves were obtained by plotting the absorbance (OD) values versus incubation time in weeks. Turbidity in the medium was measured by Mcfarland standards (0.5, 1, 2, 3, 4, 5 and 6) to check the growth of the culture. To avoid clump formation the cultures were constantly shaken at 100 rpm during incubation.

CULTURE FILTRATE (CF)

CF were obtained by harvesting (4000×g, 20 min, 4°C) bacterial growth at different times points (2, 4, 6 and 8 weeks of incubation) and was filtered (using 0.22-µm pore size syringe filter, Millipore). CF were precipitated with saturated ammonium sulfate (Rankem) due to the high amount of albumin in the broth followed by extensive dialysis overnight against 10 mM phosphate-buffered saline (pH 7.4) until free of ammonium ions at 4°C. Dialysed CF were concentrated 10-fold using vacuum concentrator (SPD Speedvac, Thermo Savant). Concentration of CF proteins was quantified by Bradford protein assay kit (Genie) and was stored at -20°C.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

CF protein profile was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie brilliant blue as per method given by Laemmli (1970).

IMMUNOBLOTTING

The electrophoresed CF proteins were transferred on to PVDF-plus membrane (pore size 0.45 µm, Millipore) using Mini Trans-blot cell (Bio-Rad) for 1.5 hrs at 60 V, 100 mA as per Towbin et al. (1979) with some modifications. Membrane was blocked for 1 hr at room temperature in 5% skim milk (Himedia) containing TBS with 0.05% Tween-20 (TBST) and were probed with polyclonal primary antibody (goat serum) diluted 1:100 in 1X TBST for 2 hrs at room temperature. After incubation membrane was washed 3 times with 1X TBST. Reactivity was seen by incubating the PVDF-plus membrane with peroxidase-conjugated anti-goat IgG (Sigma, USA) in 1:2000 dilution for 1 hr at room temperature followed by washing 5 times (5 min each) with TBST. Visualization of immuno-reactive protein bands was done by diaminobenzidine (DAB) (Sigma).

STATISTICAL ANALYSIS

The correlation (R^2) and Standard deviation of OD600 with time interval of growth measurements were analyzed using Graph Pad InStat.

RESULTS

GROWTH PATTERN

Mycobacterium avium subspecies *paratuberculosis* ('S 5') strain developed a granular growth with visible clumps suspended in the 7H9 medium up to 6 weeks, afterwards, clusters of floating cells were observed (Figure 1). The growth curve of MAP 'S 5' strain in 7H9 medium showed sharp bacterial growth phases delineation; however, after 3 weeks of incubation the culture was in log phase (early exponential) upto 8 weeks (mid to late exponential) in 7H9 medium (Figure 1). Total bacterial mass was with a maximum absorbance of 1.43 at 8 weeks. OD600 and time interval of growth measurements were highly correlated ($R^2 = 0.997$, Figure 1). We determined in Indian Bison type 'S 5' strain of MAP that 1 McFarland unit was nearly equivalent to 0.26 OD600.

IMMUNOBLOT OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS SECRETED PROTEINS

The initial antigenic profiles were detected at 2, 4, 6 and 8 weeks of incubation in 7H9 medium. Analysis of harvested CF proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the greater part of CF proteins had molecular masses (<70 kDa) as 14, 19,

Table 1: History of goats and sheep whose serum were used in the immunoblotting

Sn	Animal type/ Breed	Age/ Sex	Physical condi- tion	Diarrhea	Fecal Shedding (Microscopy)	IS900 blood PCR	Serology ('Indige- nous ELISA')
1	Sheep (Muzzaffarnagri)	Adult female	Extremely weak and emaciated	Yes	Yes	Positive	Positive
2	Goat 1 (Barbari)	Adult female	Weak and ema- ciated	Yes	Yes	Positive	Positive
3	Goat 2 (Jamunapari)	Adult female	Weak and ema- ciated	No	Yes	Positive	Positive
4	Goat 3 (Jakhrana)	Adult male	Weak	Yes	Yes	Negative	Positive

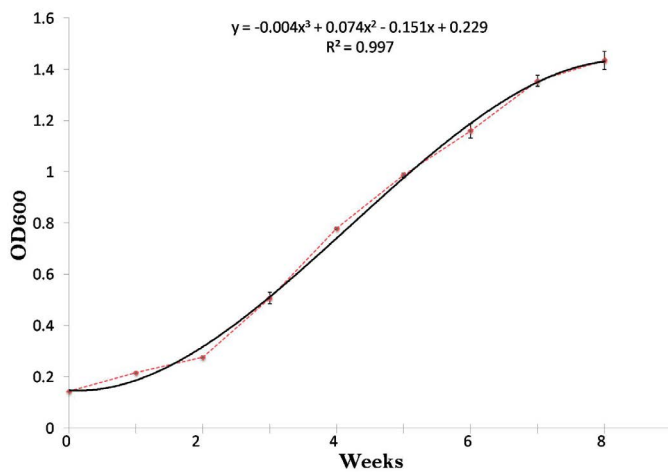


Figure 1: Growth curve of *Mycobacterium avium* subspecies *paratuberculosis* ('S 5') strain upto 8 weeks by taking OD at 600nm. Each point correlates to the mean of three determinations ± standard deviation

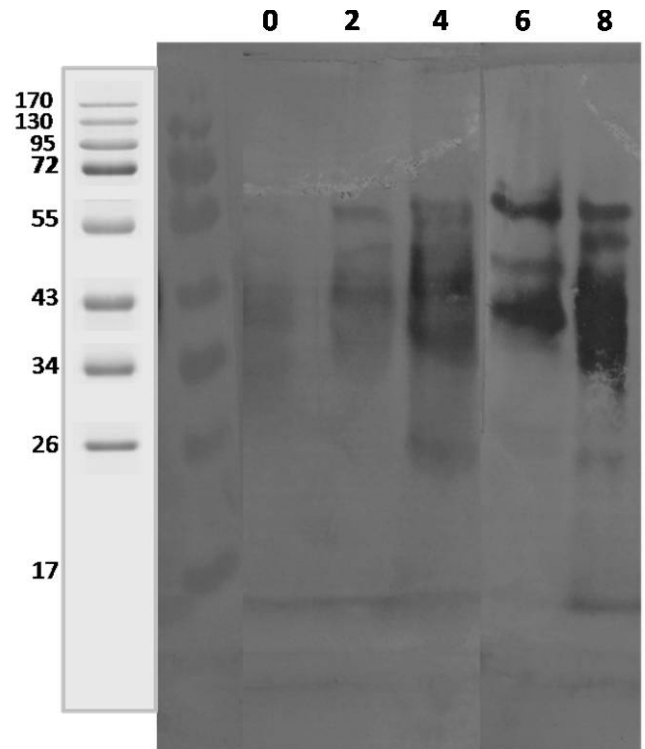


Figure 3: Immunoblots with CF proteins of MAP in naturally infected sheep serum
Lane 1: 0 Day; lane 2: 2 weeks; lane 3: 4 weeks; lane 4: 6 weeks; lane 5: 8 weeks.

26, 34-41, 52-55, 68-70 and 90-92 kDa (Figure 2). The antibody recognition patterns of secreted proteins using 4 positive sera of goats and sheep (Table 1) showed a high degree of variability among MAP infected animals (heavy shedders). Multiple proteins were bound by all the four positive sera, but the specific proteins bound by each serum sample were variable. However, the antigenic CF proteins of MAP 'S 5' strain which showed wide variation in recognition pattern with sera from clinical cases were ranged from 19 to 62 kDa at 2 to 8 weeks of growth (Figure 3, 4 and 5).

ANTIBODY RECOGNITION PATTERN TO SECRETED PROTEINS USING SERA FROM 4 CLINICAL CASES

Polyclonal sera from 3 goats and 1 sheep naturally infected with *Mycobacterium avium* subspecies *paratuberculosis* (confirmed by fecal microscopy, IS900 blood PCR and

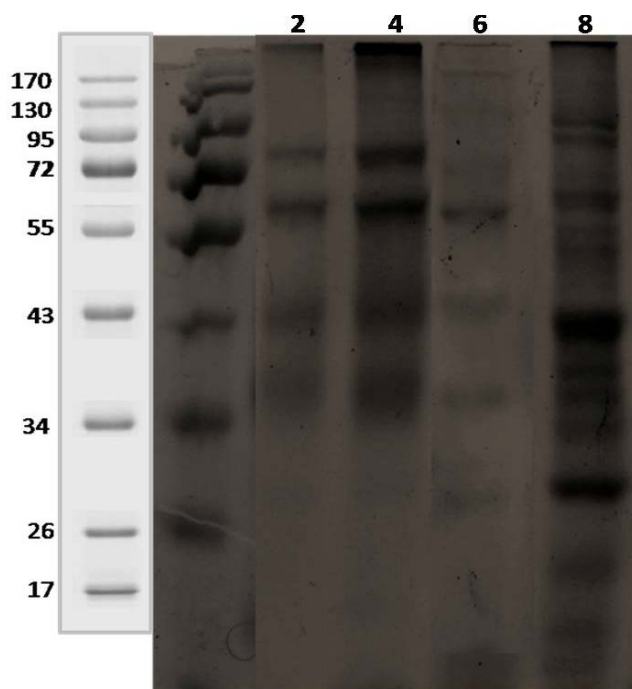


Figure 2: SDS-PAGE of CF proteins harvested at different incubation periods of MAP
Lane 1: 2 weeks; lane 2: 4 weeks; lane 3: 6 weeks; lane 4: 8 weeks.

‘Indigenous serum ELISA kit’) were tested for reactivity pattern to CF proteins. The CF proteins commonly recognised with all four MAP infected goats and sheep sera at 2 weeks (early growth) were approximately, 38-42, 45, and 56 kDa and at 8 weeks growth were approximately, 28, 34-36, 38-42, 45, and 56 kDa. Sera from goat 1, 2 and 3 reacted with 28, 38, 45 and 65 kDa secreted proteins at 2-8 weeks of growth (Figure 4 and 5). However, 38-42, 45 and 56 kDa CF proteins reacted with sheep serum (Figure 3).

poor sensitivity (Sherman et al., 1990; Pahangchopi et al., 2014), series of studies reported that immunological tests like ELISA is the best method as compared to complement fixation and AGID for controlling JD in dairy and beef herds (Wynne et al., 2011). Recent studies have focused to develop improved serodiagnostics using species-specific multiple protein antigens. Detection of MAP using ELISA have been reported in many previous studies using different antigens as protoplasmic antigen (PPA) (Collins et al., 2005; Singh et al., 2007; Sharma et al., 2008), lipoarabinomannan (LAM) (Sweeney et al., 1994), culture filtrate of MAP (Shin et al., 2008), and MAP proteins (Map1152, Map1156, Map2609, Map2942c and Map0210c) (Bannantine et al., 2011; Willemsen et al., 2006) for testing antibodies against MAP. Although PPA contains proteins very similar to proteins commonly found in closely related mycobacteria species. LAM is one of the constituent of the cell wall of mycobacteria species and its core structure is shared among mycobacterial species (Mishra et al., 2011). Some studies also suggested that secreted proteins may be better as solid-phase ELISA antigens resulting in a more sensitive assay (Cho and Collins, 2006; Willemsen et al., 2006). On the other hand, Sung and Collins (2003) have reported that expression of MAP CF protein depends on the type and culture conditions.

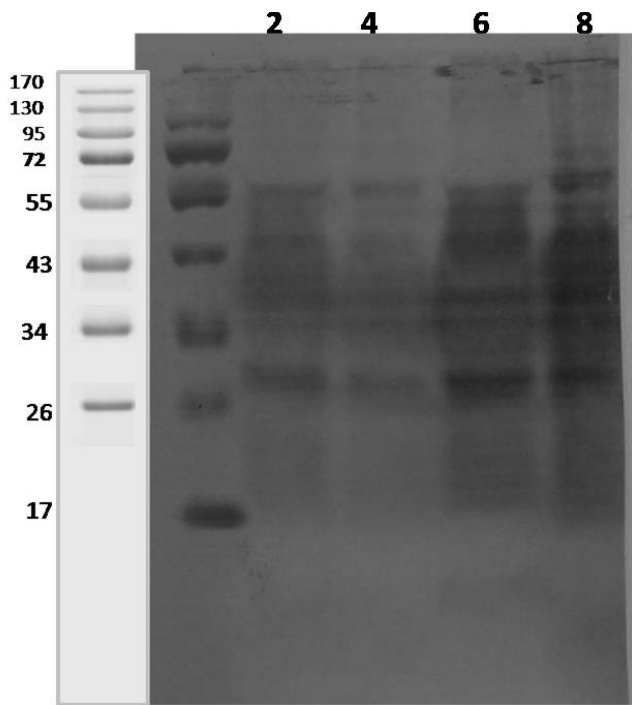


Figure 4: Immunoblots with CF proteins of MAP in naturally infected goat 1 serum
Lane 1: 2 weeks; lane 2: 4 weeks; lane 3: 6 weeks; lane 4: 8 weeks.

DISCUSSION

Current immuno-diagnostics for Johne’s disease, a chronic enteritis in ruminants, that is responsible for extensive economic losses to farmers and dairy industry worldwide caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) are hampered by the lack of specific antigens. The capability of rapidly diagnosing the disease and identifying its causative agent is critical to combat diseases and halt epidemics (Li et al., 2014). 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. Most frequently used test for diagnosis of MAP infection include bacterial culture, IS900 PCR amplification, interferon- γ assay and serum antibody detection using an ELISA platform. Wadhwa et al. (2012) used complement fixation, agar gel immunodiffusion (AGID), and ELISA to determine the antibody response in JD. The complement fixation and agar gel immunodiffusion tests both suffer

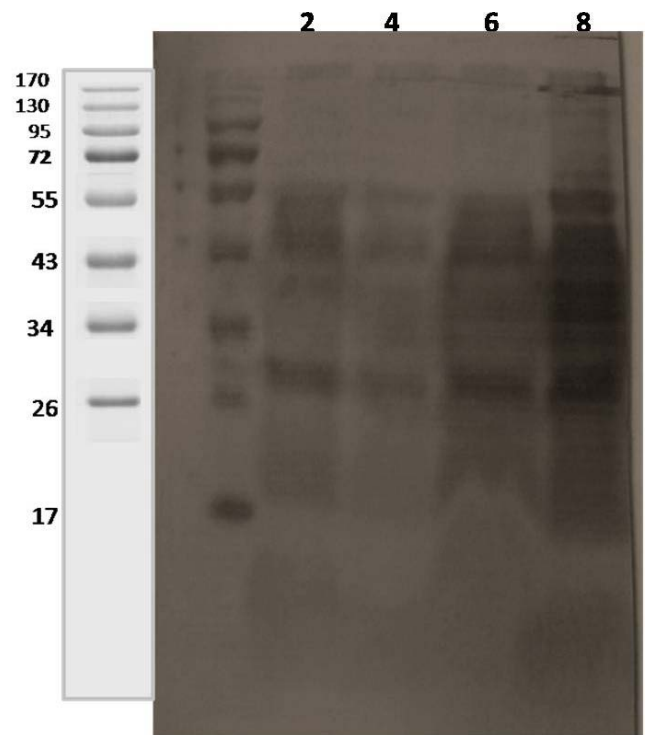


Figure 5: Immunoblots with CF proteins of MAP in naturally infected goat 2 serum
Lane 1: 2 weeks; lane 2: 4 weeks; lane 3: 6 weeks; lane 4: 8 weeks.

Recent technological developments have led to the proliferation of new, rapid diagnostic tests that hold promise for the improved management and control of infectious diseases. Mycobacterial infections such as tuberculosis

(TB), bovine tuberculosis (bTB), and Johne's disease (JD) are major infectious diseases of both human and animals (Wadhwa et al., 2012b). For tuberculosis and bovine TB, there has been recent progress in developing laboratory-free diagnostic methods. New technologies such as 'microfluidics' (Wadhwa et al., 2012a) and 'Lab-on-Chip' (Li et al., 2011) are examples of promising new technologies that can underpin development of laboratory-free diagnostic devices for these mycobacterial infections. Although there have been developed a sensitive serum ELISA test, ethanol vortex enzyme-linked immunosorbent assay (EV-ELISA), using ethanol extract of MAP especially for Johne's disease (Wadhwa et al., 2012c). Another study was conducted to assess the performance of EVELISA optimized to diagnose bovine TB using serum samples from various groups of red deer and cattle including animals experimentally infected with MAP and suggests that EV-ELISA can form a basis for development of a sensitive and specific test for bovine TB (Wadhwa et al., 2013; Wadhwa et al., 2014). Furthermore, EVELISA-based control measures increase the annual per capita revenue of US dairy farms when compared to no JD control and ELISA based JD control, respectively (Massaro et al., 2013). An AC electrokinetic impedance sensing can be used for rapid and sensitive detection of specific antibodies in serum samples and this could be a basis for development of a point of care diagnostic device for human and bovine tuberculosis (Cui et al., 2013). Garg et al. (2015) recently reported that the combination of milk ELISA and milk PCR may be adopted as a model strategy for the screening and diagnosis of JD in lactating cattle herds in Indian conditions.

Our results demonstrate the correlation between turbidity and OD600 measurements with time period of growth. We determined in Indian Bison type 'S 5' strain of MAP that 1 McFarland unit was nearly equivalent to 0.26 OD600. These measurements were not quite as precise, since *Mycobacterium avium* subsp. *paratuberculosis* tends to form clumps. Strain to strain variations were reported in growth curves and growth rate of MAP (Elguezabal et al., 2011). The present study also showed that 7H9 medium allowed to obtaining more and earlier CF proteins and nearly all these CF proteins were of low molecular weight ranging between 14 and 70 kDa; similar findings regarding CF proteins of MAP have been reported with other field strains elsewhere (Olsen et al., 2000; Cho and Collins, 2006). When immunoblotting test using CF proteins of MAP were performed, a high variability among naturally infected animals in protein binding was observed, similar results were reported by Cho and Collins (2006). Waters et al. (2004) have shown variable antibody binding patterns in assays with serum from white-tailed deer experimentally infected with *M. bovis*. High correlation has been shown between the level of shedding of MAP in feces and concentration of serum antibodies (Nielsen and Toft, 2006). However,

in agreement, our results showed high intensity and more number of bands were recognized with naturally infected goats and sheep sera. Several other factors may also affect serum antibody levels (Hendrick et al., 2006), including individual genetic variation (Sudgen et al., 1997). Valentin-Weigand and Moriarty (1992) also reported that during short incubation period MAP may secrete immunoreactive proteins which are not dominant in long term cultures.

There are studies evaluating sensitivity and specificity of commercial ELISA kits in domestic and wild ruminants derived from bovine strains of MAP (Whittington et al., 2003) however, 'Indigenous ELISA' developed from 'Indian Bison type' strain showed improved sensitivity and specificity as compared to commercial ELISA kits (Singh et al., 2007). Conceptually, the use of specific purified proteins as the solid phase antigen in ELISA assay have suffered from low diagnostic sensitivity (Huntley et al., 2005). Recombinant antigens can also alter antigenicity due to conformational changes or lack of post-translational modifications (Cho et al., 2007; Li et al., 2007). The use of MAP CF proteins as antigen in ELISA assay increased sensitivity by 25% over commercial ELISA kits for low shedding animals (Shin et al., 2008). Also the variability in responses among different animals to recognize single MAP-specific antigen in early and sub-clinical stages, a cocktail of immuno-reactive native proteins of 'Indian Bison type' provide significant advantages for improved serodiagnosis of MAP infection. Moreover, the immunoblotting findings concluded that the CF proteins were quite specific and the immunogenicity of these reactive CF proteins would be further evaluated as biomarkers to develop more sensitive and specific assays.

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CONFLICT OF INTEREST

No potential conflict of interest to declare.

REFERENCES

- Bannantine JP, Paulson AL, Chacon O, Fenton RJ, Zinniel DK, McVey DS, Smith DR, Czuprynski CJ, Barletta RG (2011). Immunogenicity and reactivity of novel *Mycobacterium avium* subsp. *paratuberculosis* PPE MAP1152 and conserved MAP1156 proteins with sera from experimentally and naturally infected animals. Clin. Vacc. Immunol. 18(1): 105-112. <http://dx.doi.org/10.1128/CVI.00297-10>
- Cho D, Collins MT (2006). Comparison of the proteomes

- and antigenicity of secreted proteins and cellular proteins produced by *Mycobacterium paratuberculosis*. Clin. Vacc. Immunol. 13: 1155-1161. <http://dx.doi.org/10.1128/CVI.00058-06>
- Cho D, Shin SJ, Tallat AM, Collins MT (2007). Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. Protein Expr. Purif. 53: 411-420. <http://dx.doi.org/10.1016/j.pep.2007.01.012>
 - Collins MT, Wells SJ, Petrini KR, Collins JE, Schultz RD, Whitlock RH (2005). Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin Diag. Lab. Immunol. 12(6): 685-692. <http://dx.doi.org/10.1128/CDLI.12.6.685-692.2005>
 - Cui H, Li S, Yuan Q, Wadhwa A, Eda S, Chambers M, Ashford R, Jiang H, Wu J (2013). An AC electrokinetic impedance immunosensor for rapid detection of tuberculosis. Analyst. 138: 7188-7196. <http://dx.doi.org/10.1039/c3an01112g>
 - Elguezabal N, Felix Bastida F, Sevilla IA, Gonzalez N, Molina E, Garrido JM, Juste RA (2011). Estimation of *Mycobacterium avium* subsp. *paratuberculosis* growth parameters: strain characterization and comparison of methods. Appl. Environ. Microbiol. 77: 8615-8624. <http://dx.doi.org/10.1128/AEM.05818-11>
 - Garg R, Patil PK, Singh SV, Sharma S, Gandham RK, Singh AV, Filia G, Singh PK, Jayaraman S, Gupta S, Chaubey KK, Tiwari R, Saminathan M, Dhama K, Sohal JS (2015). Comparative evaluation of different test combinations for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infecting dairy herds in India. BioMed Res. Intern. Article ID 983978 (In Press). <http://dx.doi.org/10.1155/2015/983978>
 - Hendrick SH, Kelton DF, Leslie KE, Lissemore KD, Archambault M, Bagg R, Dick P, Duffield TF (2006). Efficacy of monensin sodium for the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle. Prev. Vet. Med. 75: 206-220. <http://dx.doi.org/10.1016/j.prevetmed.2006.03.001>
 - Huntley JF, Stabel JR, Bannantine JP (2005). Immunoreactivity of the *Mycobacterium avium* subsp. *paratuberculosis* 19-kDa lipoprotein. BMC Microbiol. 5: 3. <http://dx.doi.org/10.1186/1471-2180-5-3>
 - Laemmli UK (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 227: 680-685. <http://dx.doi.org/10.1038/227680a0>
 - Li S, Cui H, Yuan Q, Wu J, Wadhwa A, Eda S, Jiang H (2014). AC electrokinetics enhanced capacitive immunosensor for point-of-care serodiagnosis of infectious diseases. Biosens. Bioelectron. 51: 437-443. <http://dx.doi.org/10.1016/j.bios.2013.08.016>
 - Li LL, Munir S, Bannantine JP, Sreevatsan S, Kanjilal S, Kapur V (2007). Rapid expression of *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins for antigen discovery. Clin. Vacc. Immunol. 14: 102-105. <http://dx.doi.org/10.1128/CVI.00138-06>
 - Liu X, Yang K, Wadhwa A, Eda S, Li S, Wu J (2011). Development of an AC electrokinetics based immunoassay system for on-site serodiagnosis of infectious diseases. Sens Actuators A Phys. 171: 406-413. <http://dx.doi.org/10.1016/j.sna.2011.08.007>
 - Massaro T, Lenhart S, Spence M, drakes C, Yang G, Agosto F, Johnson R, Whitlock B, Wadhwa A, Eda S (2013). Modeling for cost analysis of john's disease control based on EVELISA testing. J. Biol. Syst. 21(4): 1340010. <http://dx.doi.org/10.1142/S021833901340010X>
 - Mishra AK, Driessen NN, Appelmelk BJ, Besra GS (2011). Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. FEMS Microbiol. Rev. 35(6): 1126-1157. <http://dx.doi.org/10.1111/j.1574-6976.2011.00276.x>
 - Nielsen SS, Toft N (2006). Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. J. Dairy Sci. 89: 569-579. [http://dx.doi.org/10.3168/jds.S0022-0302\(06\)72120-8](http://dx.doi.org/10.3168/jds.S0022-0302(06)72120-8)
 - Olsen I, Reitan LJ, Holstad G, Wiker HG (2000). Alkyl hydroperoxide reductases C and D are major antigens constitutively expressed by *Mycobacterium avium* subsp. *paratuberculosis*. Infect. Immun. 68: 801-808. <http://dx.doi.org/10.1128/IAI.68.2.801-808.2000>
 - Ott SL, Wells SJ, Wagner BA (1999). Herd-level economic losses associated with John's disease on US dairy operations. Prev. Vet. Med. 40: 179-192. [http://dx.doi.org/10.1016/S0167-5877\(99\)00037-9](http://dx.doi.org/10.1016/S0167-5877(99)00037-9)
 - Pahangchopi D, Singh RV, Singh SV, Das P, Sharma D, Sardana T, Kumar N, Chaubey KK, Gupta S (2014). Evaluation of 'cattle' and 'Indian Bison' type antigens of *Mycobacterium avium* subspecies *paratuberculosis* for diagnosis of Bovine John's Disease using 'Indigenous ELISA' and AGPT. Indian J. Exp. Biol. 52: 1182.
 - Rawat KD, Chaudhary S, Gupta S, Chaubey KK, Jayaraman S, Kumar N, Sohal JS, Sachan TK, Dhama K, Singh SV (2014). Potential of 'goat based vaccine' using 'India bison biotype' of *Mycobacterium avium* subspecies *paratuberculosis* in salvaging a dairy farm consisting of high yielding Holstein Friesian cows from devastation and closure due to outbreak of bovine John's disease in Northern India. Adv. Anim. Vet. Sci. 2(12): 638-646. <http://dx.doi.org/10.14737/journal.aavs/2014/2.12.638.646>
 - Sharma G, Singh SV, Sevilla I, Singh AV, Whittington RJ, Juste RA, Kumar S, Gupta VK, Singh PK, Sohal JS, Vihan VS (2008). Evaluation of indigenous milk ELISA with m-culture and m-PCR for the diagnosis of Bovine John's disease (BJD) in lactating Indian dairy cattle. Res. Vet. Sci. 84(1): 30-37. <http://dx.doi.org/10.1016/j.rvsc.2007.03.014>
 - Sherman DM, Gay JM, Bouley DS, and Nelson GH (1990). Comparison of the complement-fixation and agar gel immunodiffusion tests for diagnosis of subclinical bovine paratuberculosis. Am. J. Vet. Res. 51(3): 461-465.
 - Shin SJ, Cho D, Collins MT (2008). Diagnosis of bovine paratuberculosis by a novel enzyme-linked immunosorbent assay based on early secreted antigens of *Mycobacterium avium* subsp. *paratuberculosis*. Clin. Vacc. Immunol. 15:1277-1281. <http://dx.doi.org/10.1128/CVI.00105-08>
 - Singh AV, Singh SV, Singh PK, Sohal JS (2010). Genotype diversity in Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* recovered from domestic and wild ruminants from different agro-climatic regions. Comp. Immunol. Microbiol. Infect. Dis. 33: e127-131. <http://dx.doi.org/10.1016/j.cimid.2010.08.001>
 - Singh N, Singh SV, Gupta VK, Sharma VD, Sharma RK, Katoch VM (1996). Isolation and identification of *Mycobacterium paratuberculosis* from naturally infected goat herds in India. Indian J. Vet. Path. 20: 104-108.
 - Singh SV, Kumar N, Sohal JS, Singh AV, Singh PK, Agrawal ND, Gupta S, Chaubey KK, Kumar A, Rawat KD, Deb R and Dhama K (2014a). First Mass Screening of the Human

- Population to Estimate the Bio-load of *Mycobacterium avium* subspecies *paratuberculosis* in North India. J. Biol. Sci. 14: 237-247. <http://dx.doi.org/10.3923/jbs.2014.237.247>
- Singh SV, Singh AV, Singh PK, Sohal JS, Singh NP (2007). Evaluation of an indigenous ELISA for diagnosis of Johne's disease and its comparison with commercial kits. Indian J. Microbiol. 47: 251-258. <http://dx.doi.org/10.1007/s12088-007-0046-2>
 - Singh SV, Singh PK, Singh, AV, Sohal JS, Kumar N, Chaubey KK, Gupta S, Rawat KD, Kumar A, Bhatia AK, Srivastav AK, Dhama K (2014b). Bio-load' and bio-type profiles of *Mycobacterium avium* subspecies *paratuberculosis* infection in the domestic livestock population endemic for Johne's disease: a survey of 28 years (1985-2013) in India. Transbound. Emerg. Dis. 61 (Suppl 1): 43-55. <http://dx.doi.org/10.1111/tbed.12216>
 - Singh SV, Sohal JS, Kumar N, Gupta S, Chaubey KK, Rawat KD, Chakraborty S, Tiwari R, Dhama K (2014c). Recent approaches in diagnosis and control of mycobacterial infections with special reference to *Mycobacterium avium* subspecies *paratuberculosis*. Adv. Anim. Vet. Sci. 2:1-11. <http://dx.doi.org/10.14737/journal.aavs/2014/2.1s.1.12>
 - Sudgen EA, Stilwell K, Michaelides A (1997). A comparison of lipoarabinomannan with other antigens used in absorbed enzyme immunoassays for the serological detection of cattle infected with *Mycobacterium paratuberculosis*. J. Vet. Diagn. Investig. 9: 413-417. <http://dx.doi.org/10.1177/104063879700900413>
 - Sung N, Collins MT (2003). Variation in resistance of *Mycobacterium paratuberculosis* to acid environments as a function of culture medium. Appl. Environ. Microbiol. 69: 6833-6840. <http://dx.doi.org/10.1128/AEM.69.11.6833-6840.2003>
 - Sweeney RW, Whitlock RH, Buckley CL, Spencer P, Rosenberger AE and Hutchinson LJ (1994). Diagnosis of paratuberculosis in dairy cattle, using enzyme-linked immunosorbent assay for detection of antibodies against *Mycobacterium paratuberculosis* in milk. Am. J. Vet. Res. 55(7): 905-909.
 - Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76: 4350-4354. <http://dx.doi.org/10.1073/pnas.76.9.4350>
 - Wadhwa A, Hickling GJ, and Shigetoshi Eda S (2012a). Diagnostics: Opportunities for Improved serodiagnosis of Human Tuberculosis, Bovine Tuberculosis, and Paratuberculosis. Vet. Med. Intern. Article ID 674238. <http://dx.doi.org/10.1155/2012/674238>
 - Wadhwa A, Hickling GJ, Eda S (2012b). Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis, and paratuberculosis. Vet. Med. Intern. 1-13. <http://dx.doi.org/10.1155/2012/674238>
 - Wadhwa A, Bannantine JP, Byrem TM, Stein TL, Saxton AM, Speer CA, Eda S (2012c). Optimization of serum EVELISA for milk testing of Johne's disease. Foodborne Pathog. Dis. 9: 749-754. <http://dx.doi.org/10.1089/fpd.2011.1115>
 - Wadhwa A, Johnson RE, Mackintosh CG, Griffin JF, Waters WR, Bannantine JP, Eda S (2013). Use of ethanol extract of *Mycobacterium bovis* for detection of specific antibodies in sera of farmed red deer (*Cervus elaphus*) with bovine tuberculosis. BMC Vet. Res. 9: 256. <http://dx.doi.org/10.1186/1746-6148-9-256>
 - Wadhwa A, Johnson RE, Eda K, Waters WR, Palmer MV, Bannantine JP, Eda S (2014). Evaluation of ethanol vortex ELISA for detection of bovine tuberculosis in cattle and deer. BMC Vet. Res. 10: 147. <http://dx.doi.org/10.1186/1746-6148-10-147>
 - Waters WR, Palmer MV, Bannantine JP, Whipple DL, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP (2004). Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. Clin. Diagn. Lab. Immunol. 11(5): 849-855. <http://dx.doi.org/10.1128/CDLI.11.5.849-855.2004>
 - Whittington RJ, Eamens GJ, Coussens DV (2003). Specificity of absorbed ELISA and agar gel immunodiffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats. Aust. Vet. J. 81: 71-75. <http://dx.doi.org/10.1111/j.1751-0813.2003.tb11437.x>
 - Willemsen PTJ, Westerveen J, Dinkla A, Bakker D, van Zijderveld FG, Thole JER (2006). Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. Vet. Microbiol. 114: 337-344. <http://dx.doi.org/10.1016/j.vetmic.2005.12.005>
 - Wynne JW, Bull TJ, Seemann T, Bulach DM, Wagner J, Kirkwood CD, Michalski WP (2011). Exploring the zoonotic potential of *Mycobacterium avium* subspecies *paratuberculosis* through comparative genomics. PLoS One, 6(7), Article ID e22171. <http://dx.doi.org/10.1371/journal.pone.0022171>
 - Valentin-Weigand P, Moriarty KM (1992). Protein antigens secreted by *Mycobacterium paratuberculosis*. Zentbl Veterinarmed. 39: 762-766.