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Towards Improvement of Tuberculin Skin Test: Five Potential Antigenic Proteinselicited *in vitro* Specific Immune Reaction Against *Mycobacterium tuberculous* Species Using Sensitized Guinea Pig Models

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Abstract: Description of a new reagent of either single or multiple antigens to replace PPD remains challenging. Therefore, the current study attempted to fractionate culture filtrate of *Mycobacterium bovis* (*M. bovis*), using RF-HPLC. Obtained fractions were *in vitro* evaluated for their antigenicity by Lymphocytic Proliferation Assay (LPA) using PMBC from guinea pig models sensitized by heat killed *M. bovis*. Antigenic fractions were analyzed for its protein contents using SDS-PAGE. Multi-protein fractions were re-fractionated using shallower gradients of RF-HPLC. Obtained proteins were re-evaluated *in vitro* for their antigenic specificity by LPA and Gamma Interferon (γ-INF) using PMBC from sensitized guinea pigs by both *Mycobacterium tuberculous* (*M. tuberculosis and M. bovis*) and non-tuberculous Mycobacterium (*M. intercellularae*, *M. avium*, *M. kansasi* and *M. fortuitum*). The study revealed five proteins that elicited variable degrees of specific antigenicity only against tuberculous Mycobacterium sensitized PMBC. On SDS-PAGE analysis, selected proteins ranged between ~5 kDa up to ~25 KDa. Interestingly, negative skin reaction was revealed by all of the five selected proteins. Absence of pro-inflammatory factors, present in crude PPD from these pure proteinscould explain the *in vivo* failure of these *in vitro* provred antigenic proteins. The N-terminus sequencing of these proteins were carried out and the obtained sequences were searched for related Mycobacterium proteins using NCBI-protein blast. At this stage, the ORFs of the genes coding those proteins were characterized and currently, we are working on the cloning of these genes for mass production of corresponding proteins to be tried in different combinations with or without adjuvant.

Key words: Tuberculin PPD, SC-PPD, chromatographic fractionation, lymphocytic proliferation assay, gamma interferon

INTRODUCTION

Bovine tuberculosis is caused by Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex. All animal species and humans of all age groups are susceptible to the disease. Human tuberculosis is caused mainly by *Mycobacterium tuberculosis*. However, *M. bovis* can also infect human causing clinically undistinguishable disease from that of human origin which makes it an important zoonotic (O'Reilly and Daborn, 1995; Etchechoury *et al*., 2010). In spite of the progress in the diagnosis, treatment and control programs of tuberculosis during the past decades, this disease is still a threat to the public, especially in the developing and underdeveloped countries (WHO, 2013).

Eradication of this zoonotic disease remains an important goal in several countries. In animals, the strategy of test and slaughter has been used widely in an attempt to control dissemination of the disease and is based on the tuberculin skin test for diagnosis of bovine tuberculosis-infected animals. The tuberculin skin test has been in use for decades as an asset in the control of bovine tuberculosis. The test is based on the Delayed-Type Hypersensitivity (DTH) reaction elicited by *Mycobacterium tuberculosis* antigens (tuberculin). Intradermal injection of Purified Protein Derivative (PPD) of tuberculin results in a DTH response in infected animals that peaks 48-72 h after injection (Monaghan *et al*., 1994). However, the test specificity complicates its use as a tool for accurate diagnosis and

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control of active disease. Cross-reactions occur due to exposure to or infection with other non-tuberculous environmental mycobacteria. These cross-reactions are generally attributed to the fact that the PPD of tuberculin has many antigens shared by other Mycobacterium species as *Mycobacterium avium*, *M. interacellularae*, *M. scrofulaceum*, *M. paratuberculosis*, *M. kansasii* and *M. fortuitum* (Pollock and Andersen, 1997). Single Intradermal Comparative Tuberculin Test (SICTT) was proposed to overcome this problem. The SICTT compares skin responses to parallel injections of bovine PPD and avian PPD on the two sides of the animal neck. Animals are considered positive when the responses to bovine PPD is greater than parallel responses to avian PPD Nevertheless, although the results of the SICTT constitute a good indication of mycobacterial exposure, even this test does not always discriminate between cattle with tuberculosis and those exposed to nonpathogenic organisms (Whelan *et al*., 2003).

Alternatively and in order to enhance the success rate of infection detection, an assay system for IFN-γ has been developed to detect and quantify release of cytokine when whole blood is cultured with bovine tuberculin (Wood and Jones, 2001). Low-molecular-weight antigens such as ESAT-6 and CFP10 have been identified as having great potential as IFN-γ inducing antigens of tuberculous mycobacteria (Pollock and Andersen, 1997; Van Pinxteren *et al*., 2000). Although, such *in vitro* immunogenic assay has been shown to be a practical possibility for diagnosis of bovine tuberculosis, the test sensitivity is variable and is reported to be as low as 55% in some cases and as high as 97% in other cases (Monaghan *et al*., 1997). In addition, the test is far from being a simple field screening assay as it needs special laboratory skills and much more expensive as comparing to skin testing. It was shown that the combined use of the tuberculin test together with the IFN-γ assay could improve the early detection of *M. bovis* infected cattle (Neill *et al*., 1994). However, disadvantages of the combined assay include the possible influence of prior skin testing on the IFN-γ assay results. In addition, time factor plays an important role due to the requirement that the blood samples have to be processed for the assay within few hours of collection which might not always be feasible when testing large population with large geographic distances separate them from the diagnostic laboratory (Ryan *et al*., 2000; Gormley *et al*., 2004). The importance of PPD skin test as a significant asset for the control of tuberculosis cannot be denied. The test is extremely simple to perform and does not require extensive training for its application and subsequent scoring of the result (Monaghan *et al*., 1994). However,

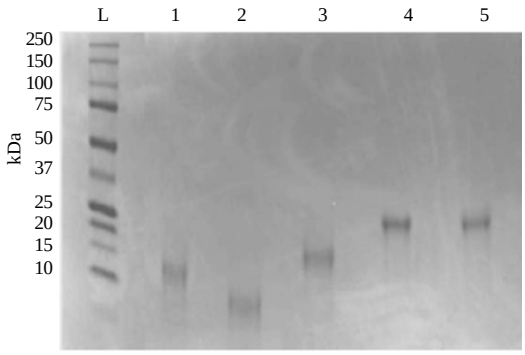

Fig. 1: The kDa range analysis due to L

better skin-test reagents for the diagnosis of tuberculosis are needed. A replacement antigen for PPD that improve skin test specificity has been a long-standing research goal.

Attempts to identify candidate antigens have led to the purification and characterization of many proteins from *M. tuberculosis* and *M. bovis*. Several suchproteins have been investigated as candidate skin test reagents in guinea pig models of tuberculosis. These include MPT64, MPT59, 38-kDa antigen, KatG, MPT32, MTC28, MPT51, MPT70, 19-kDa antigen, MPT63 and ESAT-6. *In vitro* evaluation of these proteins have given very promising results (Fig. 1). Recombinant ESAT-6 and MPT64 differentiated *M. tuberculosi* infected guinea pigs from animals given *M. bovis* BCG or *M. avium* (Lyashchenko *et al*., 1998; Wiker *et al*., 1998). However, efforts to replicate results obtained in guinea pigs for the diagnosis of natural tuberculosis infection have been disappointing and so far no purified antigens have been successfully *in vivo* tested as skin test reagents (Van Pinxteren *et al*., 2000; Pollock *et al*., 2000; Lightbody *et al*., 2008). In conclusion, the efforts for developing a more reliable diagnostic assay for the control of bovine tuberculosis should be directed towards the improvement of the currently used skin testing method in term of increasing its specificity without affecting its sensitivity. Therefore, we hypothesize that the failure of all previously described antigenic proteins to produce comparable skin testing results comparable to their *in vitro* proven specific antigenicity is related to the sensitivity of such individual proteins and the need for a cocktail of such specifically antigenic proteins to produce the required reaction upon skin testing in cattle. The current research aimed to characterize different specifically antigenic proteins from *M. bovis* PPD to be cloned and used as recombinant proteins in different combination. At this stage, we were able to molecular define five different antigenic proteins as candidates for further application.

MATERIALS AND METHODS

Bacterial strains: A total of 5 different reference mycobacterial species were used for the study (Microbiologics, MN, USA). These included *Mycobacterium tuberculosis* (ATCC 25177), *M. intercellularae* (ATCC 13950), *M. avium* ssp. *avium* (ATCC 25291), *M. kansasi* (ATCC 12478) and *M. fortuitum* ssp. *Fortuitum* (ATCC 6841). In addition, *M. bovis* AN5 reference strain was kindly provided by the Central Veterinary Laboratory, Weybridge, England. All reference mycobacterial species were received as lyophilized strains and were rehydrated in 1 mL of Middlebrook 7H9 liquid medium (Difco, Detroit, MI) and then were cultured on Middlebrook 7H11 and Lowenstein-Jensen solid medium (Difco, Detroit, MI) at 37°C for 1-4 weeks with or without 2% CO_2.

Culture filtrate and tuberculin PPD production: Culture Filtrate PPD (CF-PPD) and tuberculin PPD was produced as previously described (Rennie *et al*., 2010) with some modification. Briefly, *M. bovis* AN5 reference strain was grown aerobically at 37±2°C to log phase in Middlebrook 7H9 liquid medium supplemented with Middlebrook ADC enrichment then was grown in modified Sauton medium without Tween-80 on an orbital shaker at 37°C for additional 7 days in 2 different culture flask. Culture supernatant from the first flask was inactivated by autoclaving at 121°C and 110 kPa for 45 min. Tuberculin produced from this culture supernatant was referred to as Heat Killed PPD tuberculin (HK-PPD) and was used for skin testing of sensitized guinea pigs. The CF from the second flask was sterile filtered through a 0.22 μm Millipore membrane (Millipore Corporation, Bedford, MA, USA) to remove remaining mycobacteria. Tuberculin produced from this culture supernatant was referred to as Sterile Filtrate PPD tuberculin (SF-PPD) and was used for analysis and fractionation by SDS-PAGE and RF-HPLC, respectively. Proteins of both culture filtrates were then precipitated with ammonium sulfate (55%) for 18 h at 4°C and the resulting precipitate was dissolved and dialyzed against 1 mM sodium phosphate buffer (pH 6.8) for 18 h at 4°C. The protein concentration of the CF was adjusted to 2 mg mL^{-1} as measured spectrophotometrically at A280 (Denovix DS-11+Spectrophotometer, Wilmington, DE, USA). The antigen preparations were stored at -20°C till further uses.

Fractionation of culture filtrate: The SF-PPD was first evaluated for protein content by SDS-PAGE analysis. Then, it was subjected to fractionation by RF-HPLC **(**PerkinElmer series 200 HPLC binary solvent delivery system, San Diego, Canada**)** on a Vydac C18 column using different run conditions included double linear gradient (0.1 and 0.5 mL min^{-1}) of buffer B (60-80% acetonitrile/0.1, 0.2 and 0.5% of Trifloroacetic (TFA) acid). The temperature has been maintained constant at 25°C inside a column oven. Eluted protein fractions were collected manually based on the retention time of each fraction. Obtained protein fractions were evaluated for protein content by SDS-PAGE. Multiple protein-containing fractions were refractionated on shallower gradients and higher concentrations of TFA, obtained fraction were re-evaluated for its protein content again using SDS-PAGE. Protein concentrations od single protein-fractions were measured spectrophotometrically at A280 (Denovix DS-11+Spectrophotometer, Wilmington, DE, USA).

Sensitization of guinea pigs: For preparation of Mycobacterium sensitized guinea pig models, six different groups, 10 animals each of healthy guinea pigs weighting 300-400 g (Animal House facility, Collage of Medicine, King Saud University, KSA) were injected intramuscularly (I/M) with 0.5 mL of 2 mg mL^{-1} heat killed*M. tuberculosis*, *M. bovis*, *M. avium*, *M. intercellularae*, *M. kansasi* and *M. fortuitum*, respectively (Worsaae *et al*., 1987). An additional group of healthy non-sensitized guinea pigs were included as a negative control. All animals were skin tested 4-weeks post infection using HK-PPD to ensure sensitization. All animal experimental research has been carried out in accordance with the code of ethics of EU Directive 2010/63/EU for animal experiments.

***In vitro* evaluation of obtained protein fractions:** For evaluation of the antigenicity of obtained fractions, Peripheral Blood Mononuclear Cells (PBMC) from sensitized guinea pigs with *Mycobacterium tuberculous* species (*M. tuberculosis* and *M. bovis*) was used while for evaluation of specificity of antigenic proteins, PMBC from sensitized guinea pigs with both tuberculous and non-tuberculous Mycobacterium species were used. Blood was collected from all sensitized as well as healthy non-sensitized guinea pig groups three months post injection. PMBC were separated by centrifugation over lymphocytic separation media as instructed by the manufacturer (Lonza, B-4800 Verviers, Belgium). Viability of the PBMC preparation was determined by Trypan blue exclusion. Microcultures were prepared with 10^6 cells mL^{-1} in RPMI 1640, supplemented with 10% fetal

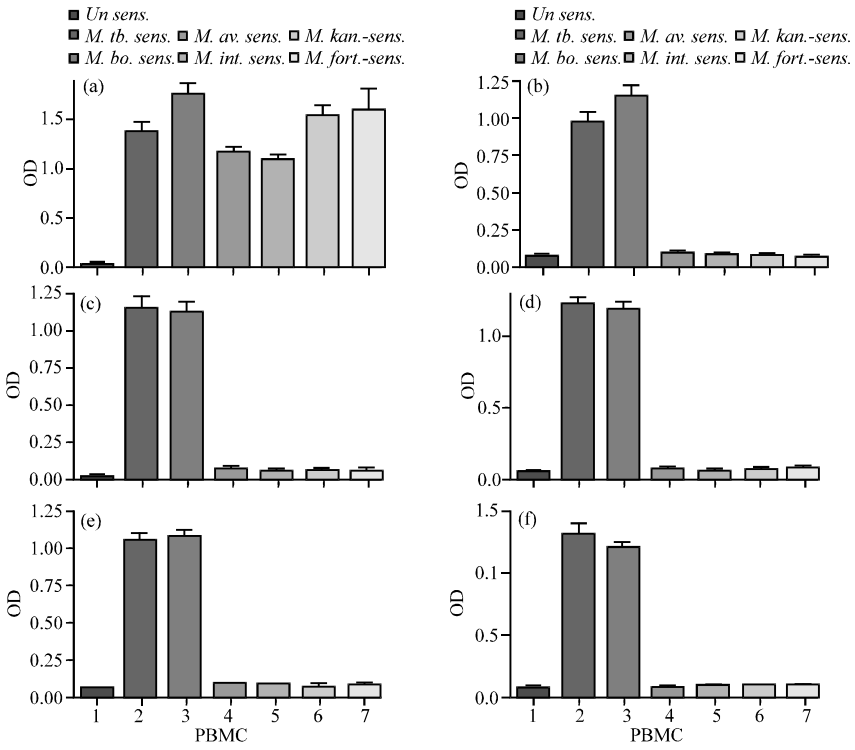

Fig. 2: Lymphocytic proliferation assay analysis through PPD, F1-7

bovine serum, 2 mM L-glutamine and 25 μg mL^{-1} gentamicin sulfate. Lymphocytic Proliferation (LPA) Assay was used for evaluation of the antigenicity of obtained fractions while both LPA and Gamma Interferon (INF-γ) assay were used for the evaluation of the specificity of the selected antigenic fractions.

Lymphocytic proliferation assay: Lymphocytic proliferation assay were conducted using colorimetric cell proliferation ELISA, BrdU (Roche Applied Science, Penzberg, Germany) according to the manufacture protocol. Briefly, selected protein fractions were added to PMBC cultures (in triplicate) at a standard protein concentration of 1 μg/well in a 96 well MP in a final volume of 100 μL/well (Fig. 2). PPD was used as a positive antigen control and Phytohemagglutinin was used to measure the viability of separated PBMC. PBMC cultures were incubated in a humidified atmosphere at 37°C for 3 days. For each well, 10 μL of BrdU labeling solution were added and cells were re-incubated for additional 24 h at 37°C. Plates were then centrifuged at 300 g for 10 min with the subsequent removal of the labeling media by flicking off and cells were dried at 60°C for 1 h. About 200 uL/well of FixDenat (fixation and DNA denaturation) were added and cells were re-incubated for further 30 min at 25°C, then FixDenat were thoroughly removed by flicking off and tapping. About 100 μL/well antiBrdU-POD (antibody conjugate) working solution were then added to the cells and followed by incubation for 90 min at 25°C. The antibody conjugate were then removed by flicking off and rinsing wells 3 times with 200 μL/well washing solution which was then removed by tapping. Substrate solution (100 μL/well) was then added and cells were incubated for 10 min at 25°C until color development was sufficient for photometric detection. The absorption ODs of the samples were then measured in an ELISA reader (HumReader HS, Human, Wiesbaden, Germany) at 370 nm (reference wave length ~492).

Gamma interferon production assay: For measuring of INF-γ production levels, guinea pig interferon gamma ELISA kit were used as constructed by manufacturer (CUSABIO BIOTECH CO., Ltd.). Briefly, after incubation of selected protein fractions with PMBC cultures as previously mentioned, supernatants were collected and used as samples for the measuring assay. A total of 100 μL of tested samples in triplicate were added per well and the plates were covered and incubated for 2 h at 37°C. Then the liquid was removed from the wells and 100 μL of Biotin-antibody was added to each well,

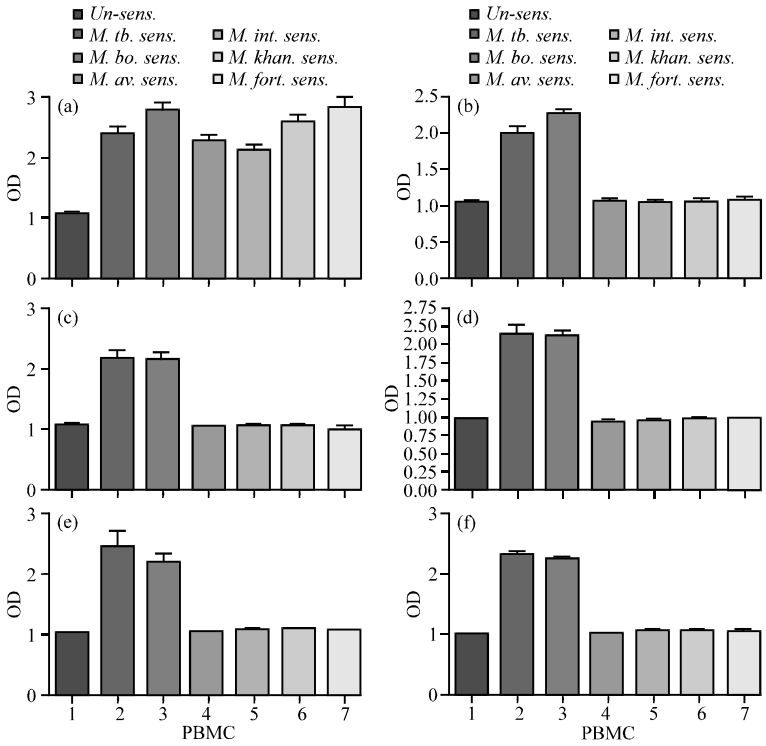

Fig. 3: Gamma interferon production assay analysis through PPD, F1-7

covered and incubated for 1 h at 37°C. The plated were then washed 3 times with 200 μL washing buffer, 100 μL of HRP-avidin was added to each well and the plates were covered and incubated for 1 h at 37°C. The plates were then washed 5 times as previously mentioned and 90 μL of TMB substrate was added to each well and the plates were incubated for 30 min at 37°C. About 50 μL of stop solution were added to each well and the OD of the plates was measured within 5 min using ELISA reader (HumReader HS, Human, Wiesbaden, Germany) at 450 nm wavelength (Fig. 3).

***In vivo* evaluation of selected proteins:** Selected protein fractions were evaluated by skin testing in *Mycobacterium bovis* sensitized guinea pigs to determine the sensitivity of the antigen in comparison with the HK-PPD tuberculin. *Mycobacterium bovis* sensitized guinea pig groups were prepared as mentioned earlier. For the skin tests, guinea pigs were anesthetized, side shaved and injected intradermally with 0.1 mm of physiological PBS (pH 7.4) containing 1 μg of each of the tested selected proteins and tuberculin PPD protein and reactions were read at 48 and 72 h.

Molecular characterization of selected proteins: Selected purified proteins were subjected to SDS-PAGE analysis to ensure purity and to estimate their molecular weights. Selected proteins were subjected to N-terminus sequencing by Edman degradation using automated Gas Phase Sequencing (GPS) as previously described (Matsudaira, 1987). Obtained sequences of the N-terminus of selected proteins were used for search analysis for corresponding Mycobacterium proteins using NCBI-protein blast.

Statistical analysis: Analysis of the mean of OD values that reflect either the proliferation degree or the level of induced INF-γ of sensitized lymphocytes against selected protein fractions including whole PPD protein were done using the program Statistical Package for Social Sciences (SPSS) version 16.0. One way Anova was used to compare data from different groups of sensitized guinea pigs with control group while student t-test was used to compare between data of the 2 groups of *M. bovis* sensitized guinea pigs and control group. Data were presented as the mean OD±SE. The $p<0.05$ were considered statistically significant.

RESULTS

Fractionation of culture filtrate: SDS-PAGE analysis of SF-PPD revealed a protein complex structure (Fig. 4).

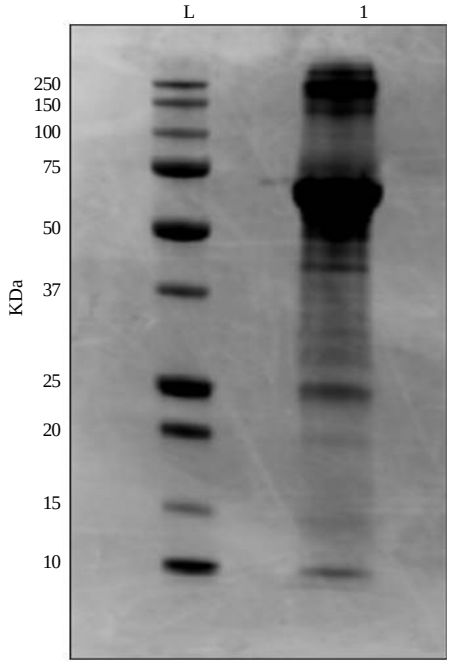

Fig. 4: Electrophoresis analysis of culture filtrate using 12% SDS-PAGE. L, Precision plus protein standard (BIO-RAD); 1, SC-PPD preparation from *M. bovis*

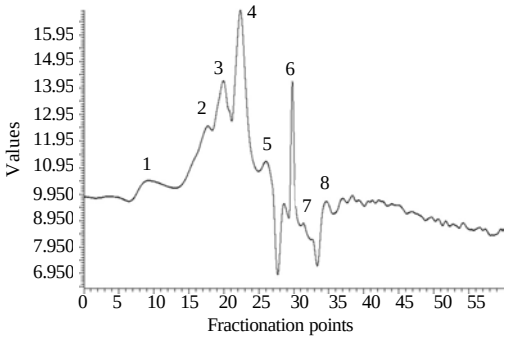

Fig. 5: Chromotographic fractionation of SC-PPD of *M.bovis* using RF-HPLC with variable run conditions with linear gradient (0.1 and 0.5 mL min^{-1}) of buffer B (60 and 80% acetonitrile/0.1% of 0.2 and 0.5% trifloroacetic acid). Initial fractionation (A-C) produced 16 different fractions

Fractionation of SF-PPD from *M. bovis* using RF-HPLC under different run conditions resulted in the production of a total of 21 different fractions. Initially, 16 different fractions were obtained at 3 different run conditions (Fig. 2-5). Three fractions (F6, F10 and F12) out of the original 16 fractions showed multiple protein content on SDS-PAGE (Fig. 6).

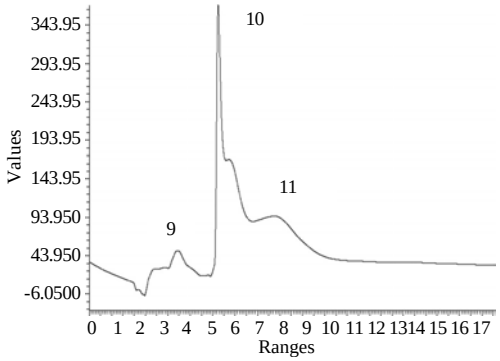

Fig. 6: SDS-PAGE analysis of obtained fractions revelead 3 fractions (6, 10 and 12) with multiple protein contents: 1) protein content of fraction 6 (3 proteins); 2, protein content of fraction 10 (2 proteins); 3, protein content of fraction 12 (3 proteins)

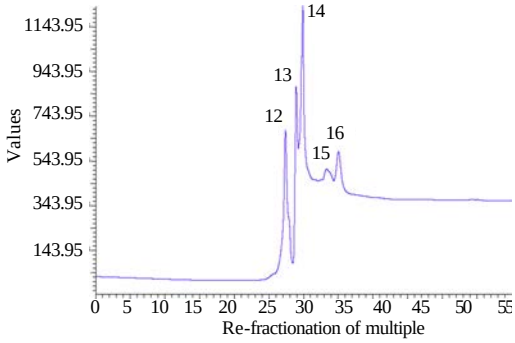

Fig. 7: Re-fractionation of multiple protein-containing fractions at shallower gradient and higher concentration of TFA. A, fraction 6 produced 3 sub-fractions; B, fraction 10 produced 2 sub-fractions; C, fraction 12 produced 3 sub-fractions

re-fractionation, fraction 6 produced 3 sub-fractions (Fig. 7a), fraction 10 produced 2 sub-fractions (Fig. 7b) and fraction 12 produced 3 sub-fractions (Fig. 7c).

***In vitro* evaluation of the antigenicity and specificity of obtained fractions:** The results of the antigenicity evaluation study of the obtained fractions using LPA revealed 12 out of the 21 total obtained fractions induced significant proliferation of sensitized PBMC as compared to un-sensitized ones (Table 1). Specificity evaluation of the selected antigenic fractions by both LPA and INF-γ production assay revealed consistent results for 5 fractions that produced significant

Table 1: Evaluation of antigenicity of obtained fractions vs tuberculin PPD using Lymphocytic proliferation assay of PBMC from *M. bovis* sensitized and un-sensitized guinea pigs

	Mean OD±SE of PBMC of sensitized and un-sensitized guinea pigs			
Protein	Un-sensitized PBMC	*Mbovis*-sensitized PBMC	p-value	Sig.
PPD	0.03±0.010	1.37±0.10	p<0.01	S
F 1	0.075±0.02	0.98±0.06	p<0.01	S
F 2	0.07±0.020	0.06±0.00	p?0.05	NS
F 3	0.036±0.01	1.10±0.11	p<0.01	S
F 4	0.063±0.01	0.07±0.01	p>0.05	NS
F 5	0.066±0.02	1.12±0.08	p<0.01	S
F 6	0.053±0.01	1.14±0.10	p<0.01	S
F 7	0.078±0.01	0.08±0.01	p>0.05	NS
F 8	0.073±0.01	1.01±0.06	p<0.01	S
F 9	0.068±0.02	1.21±0.05	p<0.01	S
F 10	0.093±0.01	0.071±0.0	p>0.05	NS
F 11	0.077±0.01	0.06±0.01	p>0.05	NS
F 12	0.096±0.01	1.07±0.04	p<0.01	S
F 13	0.031±0.01	1.22±0.03	p<0.01	S
F 14	0.065±0.01	0.07±0.01	p>0.05	NS
F 15	0.074±0.01	1.31±0.09	p<0.01	S
F 16	0.047±0.01	1.10±0.11	p<0.01	S
F 17	0.080±0.01	1.14±0.08	p<0.01	S
F 18	0.073±0.01	0.07±0.02	p>0.05	NS
F 19	0.051±0.02	1.03±0.04	p<0.01	S
F 20	0.069±0.00	0.07±0.01	p>0.05	NS
F 21	0.065±0.01	0.07±0.02	p>0.05	NS

S = Significant; NS = Non-Significant; Fraction: F

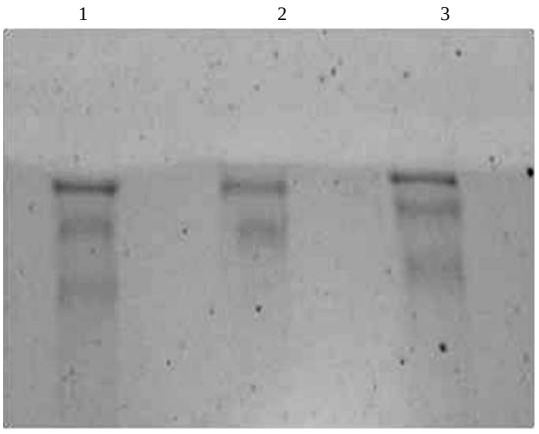

Fig. 8: LPA responses to conventional PPD and selected specific antigenic fractions (1, 3, 4, 6 and 7). PPD crude protein induced significant LPA reaction in PBMC from all tuberculous and non-tuberculous Mycobacterium sensitized guinea pigs as compared to un-sensitized PBMC while selected specific fractions produced significant proliferation in PBMC only from *Mycobacterium tuberculous* sensitized guinea pigs

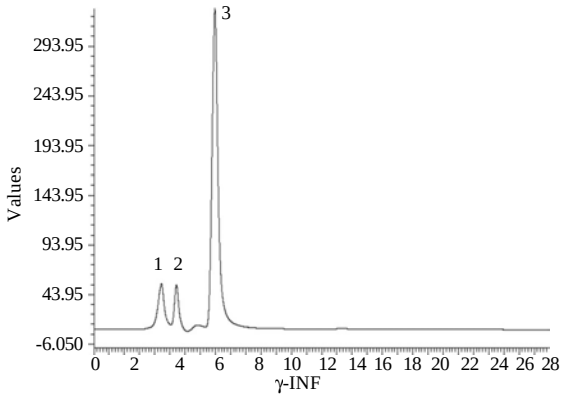

Fig. 9: The γ-INF responses to conventional PPD and selected specific antigenic fractions (1, 3, 4, 6 and 7). PPD crude protein induced significant γ-INF responses in PBMC from all tuberculous and non-tuberculous Mycobacterium sensitized guinea pigs as compared to un-sensitized PBMC while selected specific fractions produced significant γ-INF responses in PBMC only from *Mycobacterium tuberculous* sensitized guinea pigs

proliferation (Table 2, Fig. 8) and significant INF-γ production (Table 3, Fig. 9) only in tuberculous *Mycobacterium*-sensitized PBMC and not in non-tuberculous *Mycobacterium*-sensitized cells.

***In vivo* evaluation of selected proteins:** Skin testing of *Mycobacterium tuberculous* sensitized guinea pigs with each of the selected 5 proteins revealed very low sensitivity of all selected proteins as compared with HK-PPD.

Table 2: Evaluation of the specificity of selected antigenic fractions vs tuberculin PPD using Lymphocytic proliferation assay of PBMC from *Mycobacterium* spp. sensitized and un-sensitized guinea pigs

	Mean OD±SE of PBMC of sensitized and un-sensitized guinea pigs						
Protein	Un-sensitized	*M. Tb* sensitized	*M. bovis* sensitized	*M. avium* senitized	*M. intercell.*-sensitized	*M. kansasi* sensitized	*M. fortuitum* sensitized
PPD	0.03±0.00	1.38±0.10	1.77±0.11	1.17±0.04	1.10±0.06	1.55±0.10	1.60±0.21
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S
F 1	0.08±0.02	0.98±0.06	1.14±0.09	0.10±0.01	0.08±0.01	0.07±0.01	0.06±0.02
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 2	0.07±0.02	0.97±0.06	0.96±0.03	0.96±0.08	1.04±0.09	0.95±0.15	0.95±0.03
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S
F 3	0.04±0.01	1.15±0.10	1.10±0.11	0.09±0.01	0.07±0.01	0.07±0.02	0.07±0.02
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 4	0.06±0.01	1.22±0.05	1.19±0.06	0.09±0.00	0.05±0.01	0.09±0.00	0.09±0.01
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 5	0.07±0.02	1.13±0.08	1.03±0.04	0.98±0.03	1.08±0.05	0.98±0.09	0.13±0.02
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05
F 6	0.05±0.01	1.08±0.04	1.10±0.05	0.09±0.01	0.09±0.01	0.09±0.01	0.09±0.01
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 7	0.08±0.01	1.32±0.09	1.23±0.03	0.09±0.01	0.09±0.00	0.09±0.00	0.09±0.01
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 8	0.07±0.01	1.14±0.08	1.03±0.05	0.99±0.02	1.08±0.05	0.18±0.02	1.01±0.04
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p<0.01^S
F 9	0.07±0.02	1.03±0.04	1.20±0.05	0.87±0.03	0.94±0.03	0.95±0.03	0.24±0.03
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.05^S
F 10	0.09±0.01	1.16±0.09	1.01±0.06	0.96±0.03	1.09±0.05	1.00±0.06	1.00±0.01
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S
F 11	0.08±0.01	0.98±0.05	0.93±0.03	0.97±0.10	0.94±0.05	0.18±0.06	0.94±0.06
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p<0.01^S
F 12	0.10±0.01	1.20±0.05	1.03±0.04	0.97±0.03	0.18±0.03	1.00±0.07	0.11±0.01
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p<0.01^S	p>0.05

Table 3: Evaluation of the specificity of selected antigenic fractions vs tuberculin PPD using gamma interferon production assay from PBMC of *Mycobacteriu* ssp. sensitized and un-sensitized guinea pigs

	Mean OD±SE of PBMC of sensitized and un-sensitized guinea pigs						
Protein	Un-sensitized	*M. Tb* sensitized	*M. bovis* sensitized	*M. avium* senitized	*M. intercell.* sensitized	*M. kansasi* sensitized	*M. fortuitum* sensitized
PPD	1.06±0.02	2.39±0.11	2.77±0.11	2.27±0.06	2.13±0.05	2.58±0.11	2.81±0.14
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S
F 1	1.05±0.02	1.99±0.11	2.25±0.05	1.07±0.03	1.06±0.01	1.07±0.02	1.07±0.06
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 2	1.07±0.01	1.85±0.10	1.97±0.02	1.98±0.09	2.06±0.09	1.87±0.05	2.65±0.20
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S
F 3	1.07±0.01	2.18±0.12	2.15±0.11	1.05±0.01	1.07±0.02	1.07±0.02	1.01±0.05
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 4	1.07±0.01	2.38±0.13	2.33±0.08	1.04±0.01	1.07±0.02	1.09±0.01	1.08±0.02
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 5	1.09±0.01	2.20±0.10	2.02±0.05	2.00±0.01	2.12±0.08	1.98±0.09	1.07±0.00
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05
F 6	1.04±0.01	2.44±0.28	2.19±0.11	1.06±0.02	1.09±0.01	1.09±0.01	1.07±0.01
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 7	1.03±0.00	2.34±0.06	2.27±0.02	1.05±0.01	1.07±0.02	1.08±0.00	1.06±0.02
p-value		p<0.01^S	p<0.01^S	p>0.05	p>0.05	p>0.05	p>0.05
F 8	1.06±0.00	2.18±0.11	2.06±0.09	1.97±0.04	2.09±0.05	1.07±0.01	1.27±0.15
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p>0.05
F 9	1.05±0.01	2.04±0.06	2.74±0.31	1.94±0.02	1.96±0.03	1.93±0.02	1.08±0.01
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05
F 10	1.05±0.00	2.34±0.07	2.06±0.08	2.00±0.06	2.13±0.07	1.10±0.06	2.90±0.20
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p<0.01^S
F 11	1.04±0.01	2.15±0.08	1.94±0.02	2.02±0.08	1.17±0.05	1.07±0.01	2.33±0.28
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p>0.05	p<0.01^S
F 12	1.04±0.01	2.23±0.04	2.01±0.07	2.09±0.05	1.07±0.01	2.28±0.27	1.06±0.01
p-value		p<0.01^S	p<0.01^S	p<0.01^S	p>0.05	p<0.01^S	p>0.05

^{S}significant; Fraction: F

Molecular characterization of selected proteins: Protein content analysis of the selected 5 fractions by SDS-PAGE revealed that all fractions are single protein-fractions with MW range of ~5 to ~25 kDa (Fig. 10 and 11).

NCBI-Protein Blast search analysis of N-terminus sequences of selected proteins against Mycobacterium proteins revealed 5 known proteins. Those proteins are CFP10 (NP_218391.1), ESAT6 (YP_178023.1), MPB64

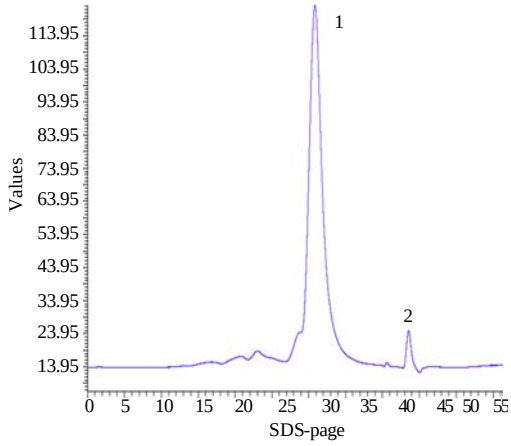

Fig. 10: The SDS-PAGE (12%) analysis of selected antigenic protein fractions showing the purity and the single protein content of all selected fractions. L, precision plus protein standard (BIO-RAD); 1, fraction 1 showing~10 kDa protein; 2, fraction 3 showing~5 kDa protein; 3, fraction 4 showing~15 kDa protein; 4, fraction 6 showing~20-25 kDa protein and 5, fraction 7 showing~20-25 kDa protein

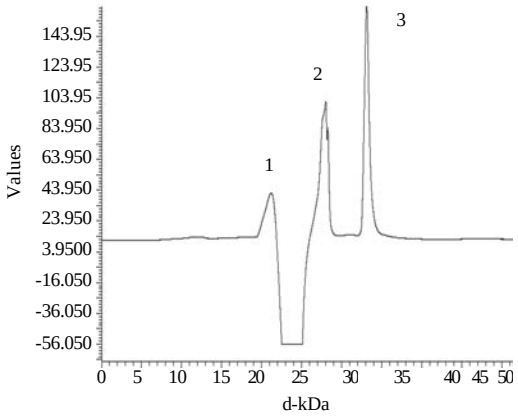

Fig. 11: The analysis of different parameters of kDa

(WP_003409954.1), MPB83 (WP_003414630.1) and DPPD (WP_003908298.1). The ORFs of the genes coding those proteins were characterized and currently, we are working on the cloning of these genes for mass production of corresponding proteins.

DISCUSSION

The Tuberculin Skin Test (TST) has been a useful diagnostic and epidemiological tool for control of bovine tuberculosis for several decades (Monaghan *et al*., 1994). However, suboptimal specificity of TST has frequently been reported in somebovine tuberculosis eradication programs (O'Reilly and Dabron, 1992). This suboptimal specificity could be attributed, in part, to the nature of the poorly characterized antigens used which are mycobacterial extracts containing components that are not species specific with the subsequent failure of the present test to discriminate between individuals infected with tuberculosis and those sensitized by vaccination or exposure to environmental mycobacteria (Pollock and Andersen, 1997). Description of a new reagent of either single or multiple antigens to replace PPD remains a research challenge. The current Study Fractionated locally produced bovine tuberculin PPD (SF-PPD) with subsequent evaluation of the obtained fractions both *in vitro* and *in vivo* in a trial to characterize mycobacterial proteins that can elicittuberculous Mycobacterium specific DTH reaction as a potential candidate for reliable and specific skin testing.

The study revealed 5 different single-protein fractions that elicited specific *in vitro* DTH using both LPA and INF-γ production of PBMC from guinea pig models sensitized with both tuberculous and non *Mycobacterium tuberculous* strains. The N-terminus sequences of selected proteins and NCBI protein blast search analysis of obtained sequences against mycobacterial proteins revealed 5 known proteins CFP10, ESAT6, MPB64, MPB83 and DPPD. ESAT-6 and CFP-10 are specific tuberculous mycobacterial proteins typically consisting of about 100 amino acids. They belong to a large family of mycobacterial proteins whose members have been identified as potent T-cell antigens (Lightbody *et al*., 2008). MPB 64, 70 and 83 are relatively larger antigens that are more abundantly expressed by *M. bovis* than *M. tuberculosis*. MPT83 (a homologue in *M. tuberculosis*) is a cell wall associated lipoglycoprotein whose function is still unknown. The MPB 64, MPB70 and MPB83 are proposed to play a role in promoting the initiation of DTH reaction which can help in production of a more potent DTH response to other dominant antigens (Wiker *et al*., 1998; Hewinson *et al*., 1996; Whelan *et al*., 2010). The DPPD is a major component of the PPD protein mixture complex. It is a small protein composed of 84 amino that induces strong and specific DTH in *M. tuberculosis*-infected guinea pig models (Coler *et al*., 2000; Whipple *et al*., 2001). Further studies of DPPD on TB patients suggest that DPPD is a potential alternative for PPD (Campos-Neto *et al*., 2001; Liu *et al*., 2004). Over the past 20 year, a wide range of antigenic proteins had been identified and validated as candidate skin test reagents in guinea pig models of tuberculosis (Reed and Campos-Neto, 2003), however,

efforts to replicate results obtained in guinea pigs for the diagnosis of natural tuberculosis infection have been disappointing and so far no purified antigens have been successfully tested as skin test reagents in cattle (Lyashchenko *et al*., 1998; Jones *et al*., 2013). In the current study, despite the *in vitro* promising results of those antigenic fractions as potential candidates for better skin testing, *in vivo* evaluation of those proteins revealed their failure to stimulate measurable DTH comparable to that of conventional PPD in *M. bovis* sensitized guinea pigs. This may be due to the small size of the proposed protein (~5-25 kDa) as compared to tuberculin PPD which may result in rapid diffusion away from the injection site. One obvious difference between tuberculin PPD and the proposed proteins is physical composition where PPD is a complex of protein and non-protein components while the proposed antigenic proteinsare single proteins in a more purified state. In addition, the crude nature of tuberculin PPD and the presence of pro-inflammatory factors which are missing from the more pure fractionated proteins is proposed to play a possible stimulatory influence of cytokine and chemokine networks that promote the DTH reaction elicited in response to main antigenic proteins (Pollock *et al*., 2003).

It has long been recognized that poor humoral and cell-mediated immune responses usually occur antigenic proteins injected without suitable adjuvant. It has been proposed that adjuvant stimulates the release of TNF-α and IL-1 (pro-inflammatory cytokines) which in turn stimulate migration of Dentritic Cells (DC) to the antigen injection site. After digestion of the antigen, it reaches draining lymph nodes. The presentation and recognition of the antigen by memory T cells stimulate an influx of monocytes, macrophages and T cells to the antigen injection site (Pollock *et al*., 2003). Absence of adjuvant with the subsequent lack of pro-inflammatory conditions with the low level of DC migration that occurs under non-inflammatory conditions usually result in insufficient presentation of the antigen (Jenkins *et al*., 2001). Therefore, the poor performance of our selected proteins as a skin test reagent in guinea pig model could be attributed in part to the absence of a suitable proinflammatory stimulus. Bacterial lipoproteins and lipopeptides from a diverse range of bacteria including *E. coli*, *Borrelia burgdorferi*, *Treponema pallidum*, *Mycoplasma fermentans* and *M. tuberculosis* have been shown to induce pro-inflammatory cytokine production from monocytes and macrophages through triggering of the innate pattern recognition signal Toll-like receptor 2 (Brightbill *et al*., 1999). Inclusion of these lipoproteins in any proposed mixture of antigenic proteins could enhance their DTH reactions and produce better skin testing results.

CONCLUSION

In conclusion, the current study characterized 5 different antigenic proteins that elicited specific *in vitro* DTH in *Mycobacterium tuberculous* sensitized guinea pig models but not in non-tuberculous Mycobacterium sensitized ones. However, none of these proteins succeeded to produce any DTH reaction comparable to that of conventional PPD when used for skin testing on *Mycobacterium tuberculous* sensitized guinea pigs. Given the complex nature of the tuberculin could have support the notion that these proteins could have represent potential candidates for skin testing not individually but in a cocktail. Inclusion of some synthetic bacterial lipoproteins as an adjuvant in the proposed specific antigenic protein cocktails could provide the assumed required pro-inflammatory stimulus and enhance the DTH reaction elicited by proposed proteins. Therefore, currently we are working on the cloning and production of these antigens as recombinant proteins to be used in different combinations with or without adjuvants for specific skin testing against tuberculous infections.

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