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PURIFICATION OF 38 kDa ANTIGEN FOR DIAGNOSIS OF TUBERCULOSIS IN BUFFALOES

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SUMMARY

The 38 kDa antigen of Mycobacterium tuberculosis strain H37/Rv was prepared and purified from the unheated culture filtrate by alcohol fractionation after being precipitated with ammonium sulphate. The prepared antigen was evaluated in comparison with bovine tuberculin by skin sensitivity test and ELISA in guinea pigs experimentally infected with typical and atypical mycobacteria. Results indicated the ability of 38 kDa antigen to differentiate between guinea pigs infected with both types of mycobacteria. Also, the antigen was evaluated in comparison with bovine PPD using ELISA for serum samples taken from tuberculin positive and negative buffaloes. The antigen was able to distinguish infection of pathogenic potential forms from cross-reaction sensitization by environmental mycobacteria at serum dilution of 1/80. Specificity of the 38 kDa antigen was 88.9% versus to 66.7% for bovine PPD. Therefore, 38 kDa antigen is strongly suggested for serological diagnosis of bovine tuberculosis.

INTRODUCTION

Bovine tuberculosis, a zoonotic disease caused by infection with *Mycobacterium bovis*, remains a serious economic problem in several countries including Egypt. Many national eradication programs depended on intradermal tuberculin testing to identify diseased cattle, a procedure which is known to lack both sensitivity and specificity (Partchard, 1988).

Species specific antigens of *M. tuberculosis* complex are theoretically attractive immunodiagnostic reagents that are potentially able to distinguish infection of pathogenic potential forms from cross reaction sensitization by environmental mycobacteria (Wilkinson et al., 1997).

The 38 kDa antigen is one of the glycoprotein antigens. Glycoproteins represent large group of conjugated proteins of wide distribution and considerable biological significance. Glycoproteins of some pathogens are immunodominant antigens (Dalton and Strand, 1987), which make these molecules potentially suitable for immunodiagnosis on the same pattern of Pottumorthy et al. (2000) who used four serological tests utilizing the 38 kDa antigen for diagnosis of tuberculosis in sera of patients.

Consequently the aim of the present study was to prepare and purify the 38 kDa antigen from unheated culture filtrate of *M. tuberculosis* strain H37/Rv and its application in ELISA test for the serodiagnosis of bovine tuberculosis in buffaloes.

MATERIAL AND METHODS

I. Antigens:

A. PPD antigen: Bovine PPD was obtained from Dept. of Bacterial Diagnostic Products, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

B. 38 kDa antigen: The method used for preparation and purification of 38kDa antigen was followed in principle according to Espitia et al. (1989).

1. Preparation of the culture filtrate:

It was prepared from cultivated *M. tuberculosis* strain H37/Rv on Middle brook 7H9 media and incubated for 6 weeks at 37oC. Cells were removed and the culture filtrate was sterilized by Seitz filter.

2. Purification of the 38 kDa antigen:

The antigen was purified from the culture filtrate in 2 steps: primarily through precipitation with 45% pure ammonium sulphate $(NH_4)_2SO_4$ then via alcohol fractionation as shown in Fig. (1).

3. Precipitation with 45% (NH4)2SO4:

It was added and stirred to a final concentration of 45% for 2 hours at 4°C then centrifuged at 6000 rpm/20 minutes. The precipitate was discarded and the supernatant was harvested.

4. Alcohol fractionation: (Seibert, 1949)

It was performed by addition of $(NH_4)_2SO_4$ to the supernatant. The precipitate was dialyzed against 0.01 M PBS pH 7.4 at 4°C overnight and the pH of the supernatant was adjusted to 7.0 then sufficient alcohol was added to give 30% alcohol concentration. The supernatant was brought to pH 4.0 and centrifuged. Finally, alcohol was added to the obtained supernatant to give 70% alcohol concentration.

The various precipitates and the last supernatant were subjected to lyophilization to concentrate the antigen and to estimate protein concentration by the method of Ohnishi and Bar (1978).

5. Antigen characterization:

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) by using Biorad protein II gel apparatus. Gels were stained with Commassie blue dye and the molecular weight of purified protein was identified in relation to standard molecular weight marker.

6. Dot-Blot:

A sheet of supported nitrocellulose membrane was placed over 3 layers of 3µm filter paper in the bio-spot (Bio-Rad) apparatus and subjected to vacuum. Ten microlitre of each of protein fraction were loaded in separate wells and allowed for 10 minutes under vacuum till complete dryness. The membrane was then washed in TBS-T/5 minutes. The membrane was then blocked by 3% gelatin in TBS for one hour and primary antibody was diluted 1/1000 in PBS-T in which the membrane was left overnight at 4°C. The membrane was then washed 3 times by TBS-T to which 0.05% Triton X100 was added in each time. The membrane was left in the solution for 10 minutes over a rotating shaker. The secondary conjugated antibody protein A alkaline phosphate conjugate was diluted 1/3000 in TBS-T. The membrane was then washed as before and stained in solution of BCIP/NPT till clear visible blots were developed, then the reaction was stopped by washing the membrane in a running distilled water.

II. Buffaloe samples:

A total number of twenty blood samples were taken from 20 animals. Fifteen samples of tuberculin positive buffaloes; out of which 10 showed tuberculous like lesions (7 localized lesion and 3 generalized lesion) and the remaining 5 animals showed no visible lesion by proper inspection of their carcasses at the abattoir that confirmed by bacteriological examination. In addition to 5 blood samples from tuberculin negative buffaloes and from herd known to be free of tuberculosis. Sera were collected, centrifuged and stored at -20°C.

III. Experimental infection of Guinea pigs:

Thirty-five negative adult albino Guinea pigs (weighing from 250-300gm) were divided into seven equal groups. The first and second groups were infected by intramuscular injection with 5 mg (wet weight) of *M. bovis* and *M. tuberculosis*, respectively according to Mikhail et al. (1997).

The remaining 4 groups were infected with 20 mg (wet weight) of each of the following *M. kansasii*, *M. intracellulare*, *M. fortuitum* and *M. avium*, respectively according to Hass et al. (1968). The last 7th group was kept uninfected as a negative control.

A. Skin reactivity test:

Intradermal skin tests to estimate skin reactivity

were performed on the shaved flanks of all guinea pigs 4 weeks post infection using 38 kDa (1µg/ml) and bovine PPD tuberculin (1 µg/ml) antigens in a dose of 0.1 ml each (Worsaae et al., 1987). Reading of the reactions took place 24 hours post injection by two neutral readers who had no knowledge of the sequence of antigen injection sites in each animal (Payne and Daniel, 1980). Obtained results were statistically analyzed using Student's T-test (Aviva Petrie, 1987).

B. Enzyme-Linked Immunosorbent Assay (ELISA):

Blood samples of guinea pigs were collected from all groups 1 day before the application of tuberculin test. Sera were harvested and stored at -20°C until used, while, the blood samples of buffaloes were taken before tuberculin test and also stored at -20°C until used.

Specific humoral antibody was monitored using the modified ELISA test according to Thoen et al. (1983) and Espitia et al. (1989).

The 38 kDa and bovine PPD were the antigens used in the test. Protein "G" labeled with horseradish peroxidase as conjugate and orthophenylene diamine (OPD) as substrate were applied with both antigens. Optical density (OD) was measured at wave length 492 nm. A serum dilution was considered as positive ELISA reaction if it gave an OD value equal to or greater than the mean OD of the negative sera plus two standard deviations (Nassau et al., 1976). Serum dilutions of > 1:80 were considered as positive ELISA test (Dimitri et al., 1990).

Statistical analysis:

The evaluation of ELISA test result was carried out by determination of its sensitivity and specificity according to Daniel and Debanne (1987).

RESULTS AND DISCUSSION

The need for purified and well-standardized antigens of Mycobacteria is essential, not only for accurate diagnosis of tuberculosis infection, but also mycobacterial antigens serve as standards in the assessment of cell-mediated (T-lymphocyte) immunological functions for a variety of clinical and fundamental biological situations. Truly purified and standardized highly specific mycobacterial antigens should contribute broadly to our knowledge of major biochemical problems (Daniel and Janick, 1978).

In the current study, the purification of the 38 kDa antigen was done according to Espitia et al. (1989), with the combination of alcohol fractionation and variable pH degrees which resulted in purification of the 38 kDa antigen in a relatively high quantities. The 1st most of the contaminant

proteins were precipitated by 45% ammonium sulphate $(NH_4)_2SO_4$. The supernatant remained was subjected to the alcohol fractionation and pH adjustment as in Fig. (1). From the SDS-PAGE analysis single band migration at the 38 kDa was found in the 1st and 2nd precipitate and the last supernatant but with different concentrations (Photo 1). The most purified fractions was the last supernatant which contained only the 38 kDa antigen while other fractions were contaminated with other antigenic components. These results were confirmed from the previous studies which declared that the 1st precipitate contained the protein C (Seibert, 1949). This precipitate was identified by Coates et al. (1981) as shown in Table 1 by immunoblotting and they found that this fraction contained a complex protein of antigens 2, 6, 7 and 2 unidentified anodal antigens plus the 35 kDa antigen which they defined as antigen 5 (Lane V Photo 1). In the mean time, the second precipitate was found to contain a high MW glucan antigen plus antigen 3 according to US-Japan reference. These antigens constitute the polysaccharide II (Seibert. 1949). The third precipitate was shown to contain a MW mass ranging from 35 to 42 kDa (corresponding to antigen 5) plus antigens 1, 2, 6 and probably antigen 4 (all constituents of this fraction were referred to protein A) (Seibert, 1949), the last supernatant contained small amount of antigen 1, 2 and one anodal antigen. They were called by Seibert (1949) the polysaccharide I, while, the 38 kDa antigen was found to be in high concentration in this fraction.

From the SDS-PAGE analysis all the precipitates and the last supernatant were shown to give variable amounts of the 38 kDa antigen but the most purified fraction was the 2nd precipitate and the last supernatant. This may be due to these fractions have polysaccharide antigens which does not stained by Commassie blue but stained by periodic acid chieff.

Dot blot analysis (Photo 2) showed the reactivity of each fraction with the serum from tuberculous buffaloes. All fractions except P1 fraction gave positive signals, which vary in the intensity due to variation in both concentration of the 38 kDa antigen and the concentration of the other antigenic constituents of each fraction. Positive control was used (CF of *M. tuberculosis* strain H37/Rv) to test the validity of the serum used. The same results were obtained by Daniel and Affronti (1973) who used immunoelectrophoretic analysis to identify antigen fractions purified by Seibert fractionation method of Mycobacterial CF.

Evaluation of the prepared 38 kDa antigen was performed by different methods. At first, the skin sensitivity test of the experimentally infected and non-infected guinea pigs with different Mycobacterial strains, and 2nd by ELISA.

Skin sensitivity results depicted in Table (2) revealed a significant difference ($p > 0.05$) between every infected and non-infected groups of guinea pigs when tested by each of the two antigens (38 kDa and bovine PPD tuberculin) using student "F" test. Thus it was proved that the 38 kDa antigen could stimulate Delayed-type hypersensitivity (DTH) response as bovine PPD tuberculin, these findings comply with those reached by Wilkinson et al. (1997) who found that the 38 kDa antigen induced a significant skin reaction. A significant difference was also observed between typical mycobacterial infected groups with those infected with atypical mycobacteria as the genetic analysis of various atypical strains showed the absence of the 38 kDa antigen in such strains. It is of value to note that although *M. intracellulare* infected group was significantly different from those infected with *M. tuberculosis*, still the mean OD of the skin reaction is high (0.85± 0.04). This may be explained on the basis of Haslov et al. (1990) findings who reported that there was 30% homology between the 38 kDa antigen purified from *M. intracellulare* with that of *M. tuberculosis*.

Although the 38 kDa antigen was purified from the CF of *M. tuberculosis* strain H37/Rv yet, it gave a positive result with *M. bovis* infected guinea pigs. These findings may be explained on account of the study of Young et al. (1986) who found that the TB72 epitope was the main immunodominant epitope of the 38 kDa antigen. The T-cell proliferative in vitro assay was stimulated by the 38 kDa antigen in about equal proportions (60%) of the tuberculous patients and healthy BCG vaccinated controls. Furthermore, DTH skin reaction elicited by the 38 kDa antigen was not significantly different when guinea pigs were infected with *M. tuberculosis* or *M. bovis*.

The detection of anti-mycobacterial antibodies in the sera of the infected and non-infected guinea pigs with different mycobacterial strains was evaluated by the ELISA, using the 38 kDa antigen and bovine PPD. The obtained results are displayed in Table (3) which revealed that the 38 kDa antigen is able to differentiate between the serum of guinea pigs infected with typical mycobacteria from those infected with atypical mycobacteria at serum dilution 1/80 expect *M. intracellulare* infected group that became negative at serum dilution 1/320 where M. intracellulare was genetically approved to have 30% homology with the 38 kDa antigen of *M. tuberculosis*. These findings also were presented by De Smet (1996) who found that the genetic analysis of various *M. intracellulare* and *M. avium* strains showed the presence of the 38 kDa antigen homology in *M. intracellulare* but not in *M. avium* which correlated with the skin test results.

Approximate findings were mentioned by Haslov

et al. (1990) reported that the 38 kDa antigen was highly sensitive and specific when used for the diagnosis of tuberculosis by the ELISA technique.

It is of importance to assure that *M. kansasii* has a large number of cross reacting antigens with the members of family Mycobacteriaceae (Chaparas and Maloney, 1978). Yet, the *M. kansasii* infected guinea pigs gave a negative ELISA test when the 38 kDa antigen was adopted as an antigen; a finding which come in a accordance with Haslov et al. (1990) who discovered the ability of the 38 kDa antigen to distinguish between *M. tuberculosis* complex and *M. kansasii*. Bovine PPD failed to differentiate between sera of infected guinea pigs with typical mycobacteria from those infected with atypical mycobacteria at serum dilution of 1/80 except with *M. fortuitum*, which gave negative results at 1/80 serum dilution. This may be attributed to the fact that bovine PPD has many individual and common antigens (Stavri et al., 1982). Moreover, *M. kansasii* has the PQS with *M. tuberculosis* as high as 53 % causing interference in ELISA reactions (Chaparas and Maloney, 1978). According to the ELISA test results, the 38 kDa antigen failed to distinguish between groups of infected guinea pigs with *M. tuberculosis* and *M. bovis* at serum dilution up to 1/320. These findings may be explained according to Wiker et al. (1988) who reported that the 38 kDa antigen was found in the CF of *M. bovis* as in *M. tuberculosis* but to lesser extent.

As a field trial for the first time, the purified 38 kDa antigen was evaluated as an ELISA reagent for the serodiagnosis of bovine tuberculosis on sera from buffaloes (Table 4). Attention should be paid to the 2 tuberculin positive buffaloes which revealed MOTT on bacteriological examination and were negative in necropsy findings; these buffaloes gave positive ELISA results to bovine PPD while they were negative to the 38 kDa antigen at a serum dilution of 1/80.

In further extent, Stavri et al. (1982) found that PPD has many common antigens that may cross react with other MOTT. Moreover Daniel and Janicki (1978), pointed out that denaturation to the PPD protein due to the heating process leads to severe decrease of its specificity and sensitivity. On the other hand, the 38 kDa antigen was considered a highly purified antigen which avoids those non-specific reactions due to the absence of the other sharing antigen impurities (Haslov et al., 1990). It is important to notice from ELISA results by using a serum dilution of 1/80 gave variance between true tuberculin positive buffaloes which recovered *M. bovis* and negative ones from the false tuberculin positive buffaloes which yield MOTT at the bacteriological examination does not affect the sensitivity or the specificity of the 38 kDa antigen, while it reduces these properties

in the bovine PPD. This may be aligned to the 38 kDa antigen being highly sensitive and specific on using it for the diagnosis of tuberculosis by ELISA (Haslov et al., 1990)

Regarding the 4 false tuberculin positive buffaloes (2 yielded MOTT during the bacteriological examination and 2 were bacteriologically negative), the ELISA results using 38 kDa antigen as an antigen gave positive reaction at serum dilution of 1/40 only and was considered as negative ELISA test at serum dilution 1/80 except for one bacteriologically negative, while on using bovine PPD gave positive reaction at serum dilution of 1/80 and 1/160 expect one from those who gave negative culture which become negative at serum dilution of 1/80 and then positive at 1/160 serum dilution and was considered positive ELISA.

The high specificity of 38 kDa antigen over bovine PPD is deduced from the fact that its nature as a secreted protein antigen (Rhodes et al., 2000) which is found to be phosphate binding protein (Chang et al., 1994), thus it is likely to be recognized by the host immune system with subsequent of antibodies. From another angle, the 38 kDa antigen of M. tuberculosis displayed a significant heterology in about 6 immunodominant epitopes to that purified from MOTT (M. kansasii) (Andersen and Hansen, 1989).

In addition, bovine PPD contain highly complex mixture of antigens (Fifis et al., 1989) from these findings 38 kDa antigen could exclude MOTT infected buffaloes but not bovine PPD. Table (5) shows the difference between the sensitivity and the specificity of the 38 kDa antigen and those of bovine PPD. These findings agree with the results

Table (1): Different fractions of the unheated culture filtrate of *M. tuberculosis* strain H37/Rv condition of precipitation, antigenic constituents* and their protein content.

Fraction	Condition of precipitation	Antigenic constituents	Protein content (µg/ml)
The first precipitate (P1)	0.5 gm/ml $(NH_4)_2SO_4$ then dialysis against PBS. pH 7.4 and pH of precipitate was 4	2, 6, 7 antigens and two unidentified anodal antigens 100,000 MW glucan and antigen 3	400
The second precipitate (P2)	30% alcohol concentration and pH_7	35-42,000 MW protein and	150
The third precipitate (P3	70% alcohol concentration and pH was 4	1, 2, 5, 6 probably 4 antigens Antigen 2 and smaller	250
The last supernatant (S)	70% alcohol concentration and pH was 4	amount of 1 antigen and unidentified anodal components	250

*Antigenic constituent determined by Coates et al. (1981).

Table (2): Results of the skin test reactivity of infected and non-infected (control) guinea pigs using the 38 kDa antigen and bovine PPD tuberculin

Antigen	Infected guinea pigs						Non-infected guinea pigs (Control)
	Typical Mycobacteria			Atypical Mycobacteria			
	M. Tuberculosis	*M. bovis*	*M. Kansasii*	*M. intracellulare*	*M. fortuitum*	*M. avium*	
38 kDa antigen 0.1µg/0.1 ml	1.31±0.021 *	1.42±0.91	0.20±0.02 N.S.	0.85±0.04 *	0.45±0.057 N.S.	0.15±0.07 N.S.	0.092±0.001
Bovine PPD tuberculin 0.1µg/0.1 ml	2.06±0.005 *	1.9±0.035	1.25±0.003 *	0.60±0.001	0.50±0.28 N.S.	0.25±0.0 N.S	0.040±0.003

Values are expressed as the mean diameter of the induration + standard error.
N.B. Results were statistically evaluated using Student's (t) and (F) tests.
* Value is significant at P (0.05
N.S. Value is not significant at P ≤ 0.05

Table (3) :ELISA results of infected and non-infected (control) guinea pigs using 38 kDa and bovine PPD.

Serum dilution	38 kDa antigen							Bovine PPD						
	H	*B*	*K*	*I*	*F*	*A*	*C*	*H*	*B*	*K*	*I*	*F*	*A*	*C*
1/40	+	+	+	+	-	-	-	+	+	+	+	+	+	-
1/80	+	+	+	+	-	-	-	+	+	+	+	-	+	-
1/160	+	+	-	+	-	-	-	+	+	+	-	-	-	-
1/320	+	+	-	-	-	-	-	+	+	-	-	-	-	-
1/640	+	-	-	-	-	-	-	+	+	-	-	-	-	-

H. M. tuberculosis infected group.
B.M. bovis infected group.
K: M. kansasii infected group.
I: M. intracellulare infected group.
F. M. fortuitum infected group.
A: M. avium infected group.
C: Non infected control group.

Table (4) :ELISA results on the sera of previously examined tuberculin positive and negative buffaloes using 38 kDa and bovine PPD antigens

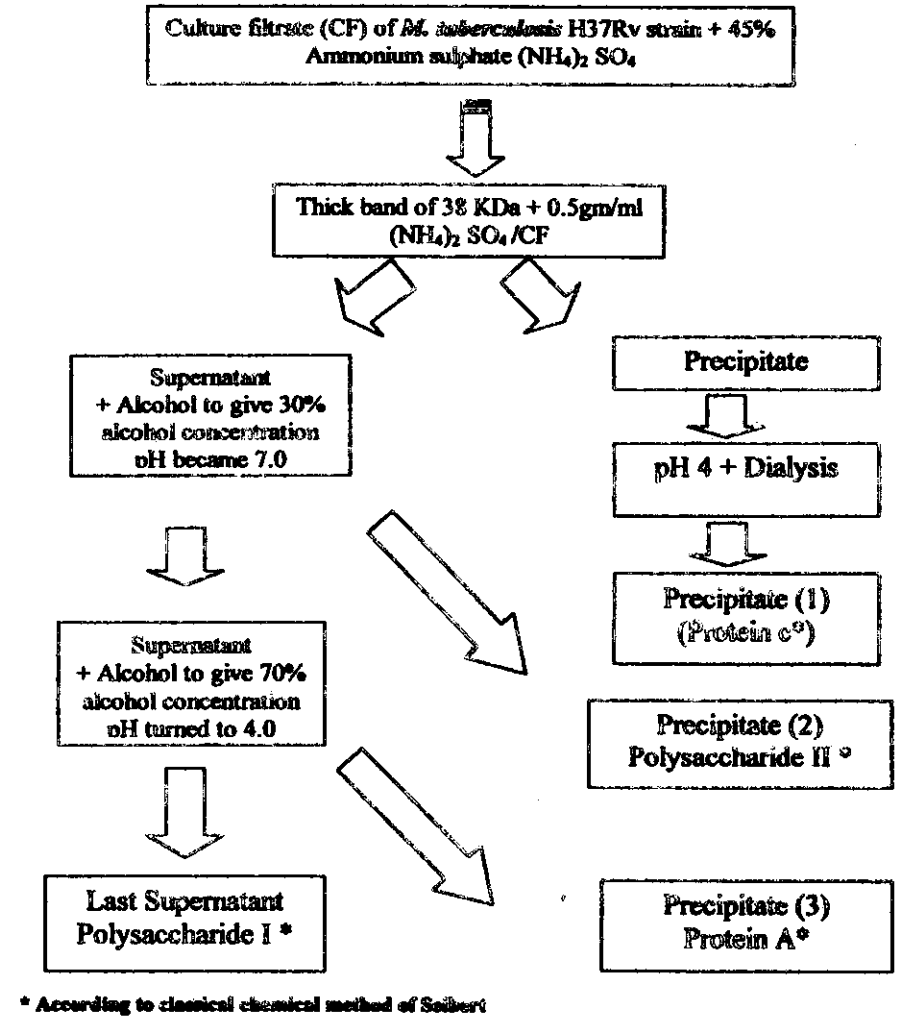
Serum dilution	Tuberculin test results	PM examination	Bacteriological Findings	ELISA reaction results using									
				38 kDa antigen					Bovine PPD				
				1/40	1/80	1/160	1/320	1/640	1/40	1/80	1/160	1/320	1/640
1	+ ve	Loc. Resp.	M. bovis	+	+	+	-	-	+	+	+	+	-
2	+ ve	Loc. Resp.	M. bovis	+	-	-	-	-	+	-	+	+	-
3	+ ve	Loc. Resp.	M. bovis	+	+	+	-	-	+	+	+	+	+
4	+ ve	Loc. Dig.	M. bovis	+	+	-	-	-	+	+	-	+	-
5	+ ve	Loc. Dig.	M. bovis	+	-	-	-	-	+	+	+	+	-
6	+ ve	Loc. Dig.	M. bovis	+	+	+	-	-	+	-	+	+	+
7	+ ve	Loc. Rwsp. + Dig	M. bovis	+	+	+	+	+	+	+	+	+	+
8	+ ve	Congested L. N.	M. bovis	+	-	-	-	-	+	+	+	-	-
9	+ ve	Severe Pneumo.	-ve culture	+	+	-	-	-	+	+	+	-	-
10	+ ve	Congested L. N.	-ve culture	+	-	-	-	-	+	-	+	-	-
11	+ ve	Calci. Mesen. LN	MOTT	+	-	-	-	-	+	+	+	-	-
12	+ ve	Fasciola	MOTT	+	-	-	-	-	+	+	+	-	-
13	+ ve	Generalized	M. bovis	+	+	+	+	+	+	+	+	+	+
14	+ ve	Generalized	M. bovis	+	+	+	+	+	+	+	+	+	+
15	+ ve	Generalized	M. bovis	+	+	+	+	+	+	+	+	+	+
16	- ve	NVL	-ve	-	-	-	-	-	-	-	-	-	-
17	- ve	NVL	-ve	-	-	-	-	-	-	-	-	-	-
18	- ve	NVL	-ve	-	-	-	-	-	-	-	-	-	-
19	- ve	NVL	-ve	-	-	-	-	-	-	-	-	-	-
20	- ve	NVL	-ve	-	-	-	-	-	-	-	-	-	-

Loc: Localized lesion. Dig.: Digestive. NVL: Non Visible Lesions.
L.N. : Lymph Nodes MOTT: Mycobacteria Other Than Tuberculosis.

Table (5): Statistical evaluation of ELISA results on serum of the naturally infected buffaloes

Antigen	Sensitivity	Specificity
38 kDa antigen	72.7 %	88.9 %
Bovine PPD	81.8 %	66.7 %

Figure (1) summarizing the purification steps of the 38 kDa antigen



* According to classical chemical method of Seibert

adopted by Espitia et al. (1989) who found that the sensitivity and specificity of the 38 kDa antigen were 68% and 96% respectively versus to 72.7% and 88.9% in this work, respectively.

Conclusively, the 38 kDa antigen is highly specific and sensitive purified glycoprotein being a potent skin test antigen in infected guinea pigs. It has the capacity to differentiate between typical and atypical mycobacteria infected guinea pigs on the basis of cellular and humoral immune response. Adding to that it can discriminate between tuberculin positive from the false tuberculin positive animals by ELISA test at serum dilution of 1/80.

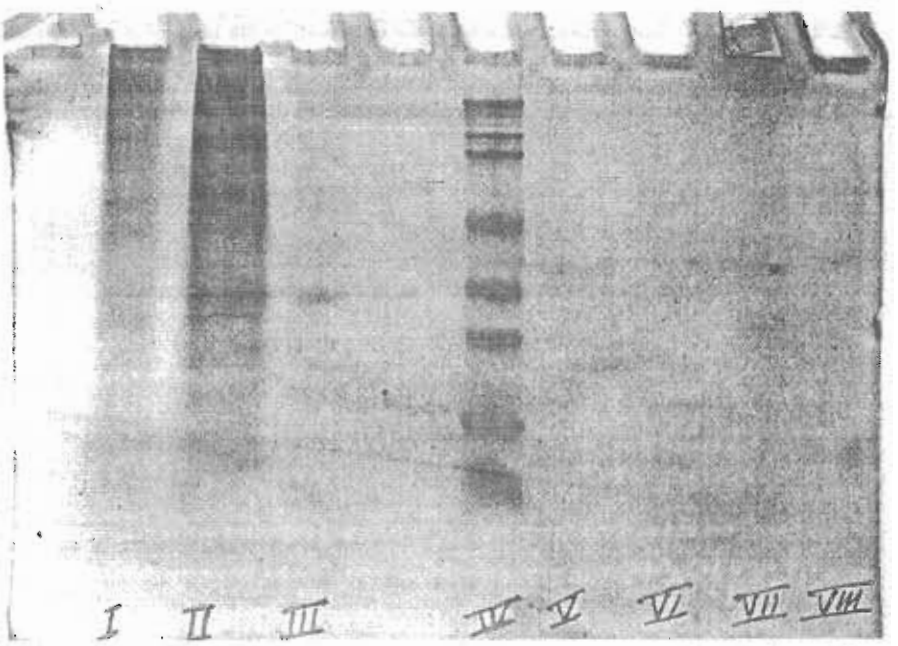


Photo (1): Isolation of the 38 kDa from the supernatants of the unheated culture filtrate precipitated with 45% $(NH_4)_2 SO_4$:

Lane (I, II): The complex pattern of the unheated culture filtrate.
Lane (III): P3 fraction had band of 32 kDa M.W.
Lane (IV): Protein marker (205-119-98-52.3-36.8-30.1-22-7.6 kDa).
Lane (V, VI, VII and VIII): P1, P2, P3 and S fractions respectively have visible band of the 38 kDa antigen with another band at 32 kDa MW in Lane VII.

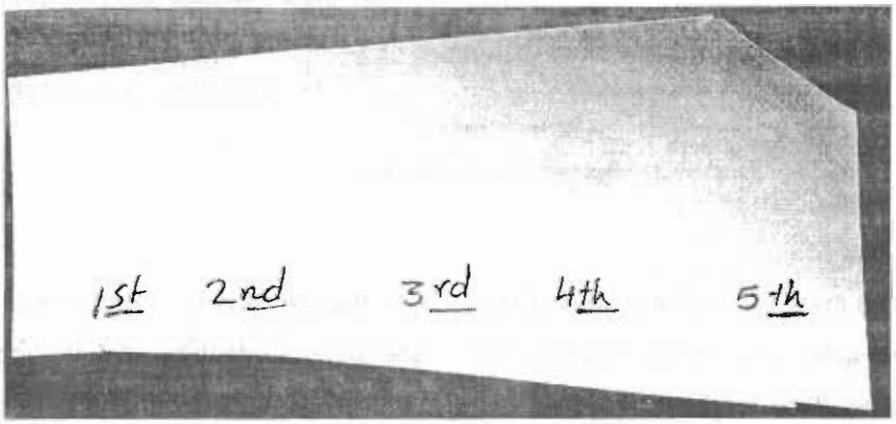


Photo (2): Dot-blot analysis of the alcohol fractionated 38 kDa antigen
1st dot is for CF of *M. tuberculosis* H37Rv strain (positive control)
- 2nd dot, P1 (Protein C*) No signals were detected.
- 3rd dot, P2 (Polysaccharide II*).
- 4th dot, P3 (Protein A*).
- 5th dot, Last supernatant (Polysaccharide I*).

* According to Seibert (1949)

For the construction of preparation containing a blend of defined mycobacterial protein to replace tuberculin PPD as a diagnostic reagent for *M. tuberculosis* complex infection the 38 kDa protein antigen is a prime candidate.

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