

Original Article

Isolation and Purification of Low Molecular Weight Proteins from Culture Filtrate of *Mycobacterium Tuberculosis* Strain C

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Abstract

In the last couple of years, a number of new and rapid tests for the diagnosis of Tuberculosis (TB) have been developed based on the low molecular weight antigens from *Mycobacterium tuberculosis* (*Mtb*) culture supernatant. This study aimed to isolate and purify low molecular weight antigens secreted by *Mtb* strain C for diagnostic purpose. The secretory proteins from culture filtrate of *Mtb* were extracted using ammonium sulphate precipitations and sephadex-G50 gel chromatography. The obtained antigen fractions were analyzed for their protein concentrations and approximate molecular weight using Lowry method and SDS-PAGE (12.5%), respectively. DOT-ELISA and Western blot assay was performed to confirm the presence of purified low molecular weight proteins isolated from *Mtb* using sera from pulmonary tuberculosis patients (polyclonal antibodies). During chromatography, low molecular weight proteins were separated, that was approximately 0.7 mg/ml of the total proteins (1.662 mg/ml). The purified protein fractions in molecular weight range of 14 kDa-41kDa appeared during SDS-PAGE analysis. The chromatographic band fraction in the weight range of 30-41 kDa was identified in the TB patients' sera using Western blotting. The low molecular weight proteins in the culture filtrate of *Mtb* strain C were purified using ammonium sulphate and chromatography. These fractions were confirmed using Western blotting. The obtained results might support the hypothesis that the *Mtb* culture filtrate antigens could be used as a rapid and sensitive assay for the detection of patients with pulmonary TB.

Keywords: Chromatography, Dot enzyme-linked immunosorbent assay, *Mycobacterium tuberculosis*, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis, Western-blot

Isolement et Purification des Protéines de Faible Poids Moléculaire à Partir du Filtrat de Culture de la Souche C de *Mycobacterium Tuberculosis*

Résumé: Au cours des deux dernières années, un certain nombre de tests nouveaux et rapides pour le diagnostic de la Tuberculose (TB) ont été développés sur la base des antigènes de faible poids moléculaire du surnageant de culture de *Mycobacterium tuberculosis* (*Mtb*). Cette étude visait à isoler et purifier les antigènes de faible poids moléculaire sécrétés par la souche C de *Mtb* à des fins de diagnostic. Les protéines sécrétoires du filtrat de culture de *Mtb* ont été extraites en utilisant des précipitations au sulfate d'ammonium et une chromatographie sur gel Sephadex-G50. Les fractions d'antigène obtenues ont été analysées pour leurs concentrations en protéines et leur poids moléculaire approximatif en utilisant la méthode de Lowry et SDS-PAGE (12,5%), respectivement. Le test DOT-ELISA et Western blot (transfert de protéines) a été réalisé pour confirmer la présence de protéines purifiées de faible poids moléculaire isolées de *Mtb* en utilisant des sérums de patients atteints de tuberculose pulmonaire (anticorps polyclonaux). Au cours de la chromatographie, les protéines de faible poids moléculaire ont été séparées, soit environ 0.7 mg/ml des protéines totales (1.662 mg/ml). Les fractions de protéines purifiées

dans la gamme de poids moléculaire de 14 kDa-41 kDa sont apparues pendant l'analyse de SDS-PAGE. La fraction de bande chromatographique dans la gamme de poids de 30 à 41 kDa a été identifiée dans les sérums des patients tuberculeux en utilisant le Western blot (transfert de protéines). Les protéines de faible poids moléculaire dans le filtrat de culture de la souche *Mtb* C ont été purifiées en utilisant du sulfate d'ammonium et une chromatographie. Ces fractions ont été confirmées par Western blot. Les résultats obtenus pourraient étayer l'hypothèse selon laquelle les antigènes de filtrat de culture *Mtb* pourraient être utilisés comme test rapide et sensible pour la détection des patients atteints de tuberculose pulmonaire.

Mots-clés: Chromatographie, test d'immunosorbant lié aux enzymes Dot, *Mycobacterium tuberculosis*, électrophorèse sur gel de sulfate de sodium et de polyacrylamide, Western-blot

1. Introduction

Tuberculosis (TB) is a major health problem with a high mortality rate worldwide. The standard diagnosis methods of TB include clinical examination, direct sputum microscopy, and bacterial culture. Limitations of these methods include low sensitivity of acid-fast smears (only 40%-50% of patients with pulmonary TB are smear-positive using Ziehl-Neelsen direct microscopy), a lengthy period of bacterial cultivation (approximately eight weeks) with undetectable growth in 10% to 20% of cases (Chakravorty et al., 2005; El-Masry et al., 2008). However, rapid testing methods, such as PCR, though sensitive, are expensive and scarce in most diagnostic or health care centers (Beck et al., 2005a; Chakravorty et al., 2005).

The tuberculin skin test (TST) using purified protein derivative (PPD) has limitations in differentiating between active disease and latent TB infection due to cross-reactions with *Bacillus Calmette-Guérin* (BCG) vaccine substrain or infection with environmental *nontuberculous mycobacteria* (NTM) (Beck et al., 2005b). Moreover, 10%-25% of TB patients have a negative TST result, and this percentage increases up to 50% in patients with advanced disease or people infected with human immunodeficiency virus (HIV) (Beck et al., 2005a; Getahun et al., 2010). Therefore, the development of an easy, rapid, and inexpensive diagnostic tool for TB is an important goal, particularly in view of a global increase in the number of cases of active TB.

In recent years, much effort has been devoted to identify immunologically important antigens of *Mtb* that could react specifically with antibodies from TB patients' sera. Antigens secreted into the extracellular environment by *Mtb* are the usual target of immune response in the infected host (Arend et al., 2000). Many of these antigens have been studied, purified, and characterized.

For a number of years, the components of culture filtrate have been investigated using narrow-molecular-mass fractions as a guide to identifying immunologically active single molecules. Low molecular proteins demonstrated to be strongly recognized by T cells isolated from human TB patients and those of experimentally infected mice and cattle.

A large number of mycobacterial antigens have been identified and purified which include Ag85A, Ag85B, MTB81 (malate synthase), MTB48, MPT 51, MPT 32, crystalline, 38-kDa antigen, CFP-29 (29 kDa culture filtrate protein), MTC28 (28 kDa *Mtb* complex), CFP-10 (10 kDa culture filtrate protein), ESAT-6 (6 kDa early secretory antigenic target), thiol peroxidase, ICD I (Isocitrate dehydrogenase 1) and ICD II (Young et al., 1992; Beck et al., 2005b; Wu et al., 2010). The 38-kDa protein is an immunodominant antigen isolated by gel chromatography and specific only for the *Mtb* complex. This antigen has the potential to be used in Enzyme-Linked Immunosorbent Assay (ELISA) and detects up to 85% of smear-positive cases (Young et al., 1986). The 30/32-kDa complex of major secretory proteins is among the abundant proteins produced by *Mtb* growing

either extracellularly in broth culture or in human mononuclear phagocytes. G. Harth et al. (1996) purified the 30/32-kDa proteins from the culture filtrate of *Mtb* by gel filtration chromatography. Their immunocytochemical analyses conclusively demonstrated that the 30/32-kDa proteins were abundantly expressed by intracellular bacteria within infected human monocytes.

CFP-29 is present in both the culture filtrate and the membrane fraction from *Mtb*, suggesting that this antigen is released from the envelope to culture filtrate during the growth (Young et al., 1992; Weldingh et al., 2005; Sonawane et al., 2012).

The ESAT-6 and CFP-10 are present in form of a complex in the cytosol and the culture filtrate of tubercle bacilli, before and after secretion, respectively (De Jonge MI et al., 2007). A combination of these two molecules provides specific and sensitive targets for the detection of *MTC* infection and has been used for vaccines, skin tests, and the gamma interferon release assay (IGRA) (Shen et al., 2011).

In this study, low molecular weight antigens were purified from the culture filtrate of *Mtb* strain C (ATCC 35808 TMC 116) by ammonium sulphate precipitations and gel chromatography and later identified the antigens using Dot-ELIZA and Western Blotting assay and sera from pulmonary tuberculosis patients.

2. Material and Methods

2.1. Mycobacterial Cultures and Growth Conditions

Mtb standard strain C (ATCC 35808 TMC 116), provided by the Tuberculin and Mallein Production and Research Department in Razi Vaccine and Serum Research Institute, Alborz, Karaj, Iran, were grown on Lowenstein-Jensen medium with glycerol at 37°C for eight weeks. Bacteria grown on the Lowenstein-Jensen medium were inoculated in a biphasic medium of Potato-Dorset-Henley.

After incubation at 37°C for 6-8 weeks in biphasic medium, the bacterial suspension was transferred to a 1-liter flask containing 250 ml of Dorset-Henly liquid

medium and incubated at 37°C for up to 6 weeks, without shaking.

2.2. Inactivation of Bacteria and Filtration

Six-week-old culture filtrates from *Mtb* were inactivated at 68 °C for 1 h. After the completion of inactivation, the bacterial mass was removed from the culture filtrate by filtration under pressure on filter membrane type 0.5 µm and 0.22 µm, first passing the cultures through 0.5µm filter, followed by 0.22 µm filter and culture supernatant was harvested.

2.3. Protein Precipitation by Ammonium Sulphate

For the isolation of low molecular weight proteins, the obtained culture supernatant was twice subjected to ammonium sulphate precipitation (20% saturation) at 4°C overnight. Subsequently, the precipitated proteins were sediment by centrifugation at 3000 rpm for 20 min. The collected precipitates were dissolved in 200 µl phosphate-buffered saline (PBS, 0.01 M), and stored at 4°C until further use. The proteins were dialyzed using Dialysis Cassettes with a molecular weight cut off (MWCO) of 6,000 Da to remove the salt. The protein solution was dialyzed against 0.01 M PBS and left with stirring at 4 °C for 72 hours. The PBS was changed thrice at regular intervals.

2.4. Purifications of Culture Filtrate Protein (CFP) by Gel Chromatography

The protein solution was purified by gel filtrations on Sephadex G-50 columns (with the height and diameter of 150 cm and 2 cm, respectively). The final volume of concentrated protein solution after dialysis was 5 ml with 1.6 mg/ml density. The chromatography was monitored at 280 nm and antigen fractions were separated. Additionally, the protein concentrations were determined using the Lowry method at 750 nm.

2.5. Protein Estimation and Molecular Weight Determination

Protein concentrations were determined using the Lowry method and bovine serum albumin as the standard. Calibration curves were prepared for the absorptions measurements and the protein content of the samples (unknown model) was calculated using this

curve. Purified protein fractions obtained during the gel filtration were separated using 12.5% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) according to Laemmli SDS PAGE procedure (1970). Prestained protein markers, ranging from 10kDa to 250kDa (Sinaclo) were used in this study. The SDS-PAGE was conducted at 80 V for 2 h. Afterward, the components in the fractions were visualized by silver staining assay.

2.6. Dot Blot Immunoassay

The qualitative dot-blot immunoassay was carried out to detect the low molecular weight antigens fractions of *Mtb*. Samples were applied as a spot to strips of pre-treatment Polyvinylidene fluoride (PVDF) membranes and were allowed to dry. Pre-treatment involved submersion in 100% methanol for five min or until the membrane was translucent, followed by equilibration in the transfer buffer (3.03 gr of Tris base, 14.25 gr Glycine, 200 ml Methanol, and 5 ml SDS 20 %) until the membrane no longer floated on the surface. After 1 h, the PVDF membrane was blocked with 3% Bovine Serum Albumin (BSA) in 1X Tris Buffered Saline (TBS) with 10% Tween 20 (TBST) pH 7.6. The membrane strips were washed thrice with 1X TBST, and incubated with serum samples from *Mtb* infected patients and healthy individuals (diluted 1/50 in TBST containing 3% BSA) or 1 h at 4°C as a primary antibody. Following another wash cycle, the strips were immersed in diluted 1/5000 horseradish peroxidase (HRP) goat anti-human (Abcam 102420), as a secondary antibody at room temperature with shaking for 1 h. The membrane strips were washed again thrice with 1X TBST and the blots were developed by addition of the 0.005 gr diaminobenzidine (DAB: Sigma D8001) as substrate, in 10 ml PBS and 500 µl H₂O₂ (30%) and incubated for 5-15 min in the dark. The reaction was stopped by washing the blot in distilled water. All incubations were carried out under constant shaking.

2.7. Western Blot Immunoassay

Western blotting of *Mtb* culture filtrate antigen was performed with serum samples from both *Mtb* patients and healthy individual human. In brief, 20 µl of the

protein samples were transferred electrophoretically onto PVDF membranes (400 mA for 2 h). The purified chromatographic fractions were separated on SDS-poly-acrylamide 12.5% gels and transferred onto PVDF membrane. Transfer efficiency was monitored by checking for the presence of prestained low molecular weight marker bands on the membrane.

Sinaclo prestained protein marker ranging from 10 kDa to 250 kDa was used in this study. After transfer, the detection method was carried out the same as dot blot assay detection. Primary and secondary antibody dilutions were prepared from the dot-blot assay.

3. Results

3.1. Protein Estimation by Lowry Method

After precipitating the proteins in the culture filtrate medium of *Mtb*, protein concentration in the precipitated solution was approximately 1.05 mg/ml (estimated by Lowry assay). However, this concentration was slightly increased after dialysis and was shown to be about 1.662 mg/ml.

3.2. Gel Chromatography

The precipitated protein solution was loaded on a Sephadex G-50 column for gel chromatography. On Sephadex G-50, the proteins were eluted with phosphate buffer saline 0.5 molar (pH: 7.4). Elution was followed by recording the absorbance at 280 nm.

At this stage, three fractions related to the obtained peaks were collected. Figure 1 shows the elution profile after the chromatography of the protein on the Sephadex G-50 column.

The protein concentrations in the collected fractions F1, F2, and F3 were approximately 0.121, 0.6128, and 0.063 mg/ml, respectively.

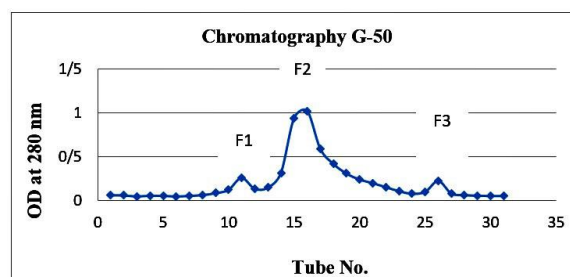


Figure 1. Chromatograph of exit of proteins in the culture filtrate from Sephadex-G-50 column

3.3. Molecular Weight Determination

Figure 2 shows the SDS-PAGE of the fractions obtained after chromatography. Based on these results, a series of proteins in the approximate range of 14-41 kDa were observed in F2 fraction by SDS-PAGE analysis. However, F1 and F3 showed no significant protein bands and were omitted from the study.



Figure 2. SDS-PAGE analyses of F2 fraction obtained after chromatography on Sephadex G50 columns. Lane1, prestained protein marker, ranging from 10kDa to 250kDa (Sinaclone); lane 2, purified protein fraction.

3.4. Dot Blot Analysis

Defined antigens present in the filtrate were identified by immunoblot analysis using polyclonal antibodies. Results of dot blot analysis are shown in Figure 3. The proteins in the F2 fraction were recognized by the used TB patients' polyclonal sera and confirmed the presence of the antigenic proteins in this fraction (A). No spots were detected on membranes treated with healthy patients' sera which indicated the specificity of the test. The tuberculin-negative control did not respond significantly to any of the fractions (B).

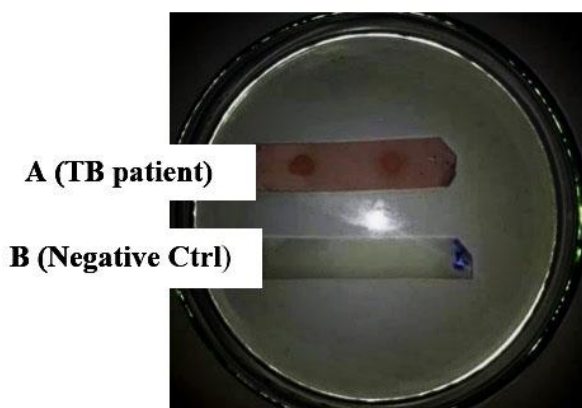


Figure 3. Dot blotting with HRP Goat Anti-Human (diluted 1/5000) and human serum samples (diluted 1/50) after SDS-PAGE of culture fluids from *Mtb*. The strips were incubated with *Mtb* patients' serum samples (A) and healthy individual human (B) as the polyclonal antibody.

3.5. Western Blot Analysis

Figures 4 and 5 show the Western blotting results of F2 fraction proteins with the polyclonal antibodies in the serum samples collected from both *Mtb* patients and healthy individuals. The TB patients' serum predominantly recognized antigen fractions with a molecular weight between 30kDa and 41kDa on PVDF membrane (Figure 4), while, serum from healthy individuals had negative results.

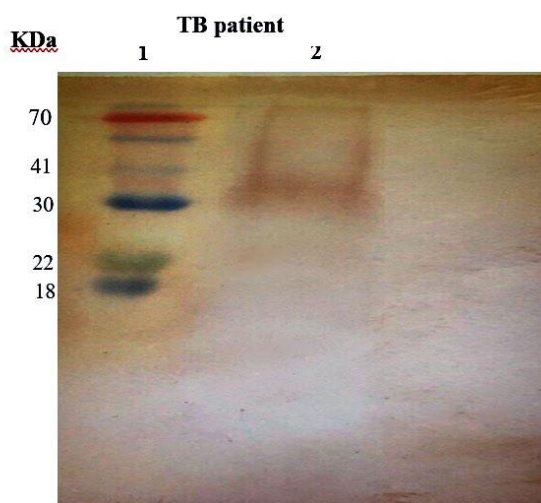


Figure 4. Western blot of *Mtb* culture filtrate with TB patient sera. Lane 1, prestained protein ladder, ranging from 10-kDa to 250-kDa (Sinaclone); lane 2, Tuberculous patient sera identified bands at approximate range of 30 to 41 kDa.

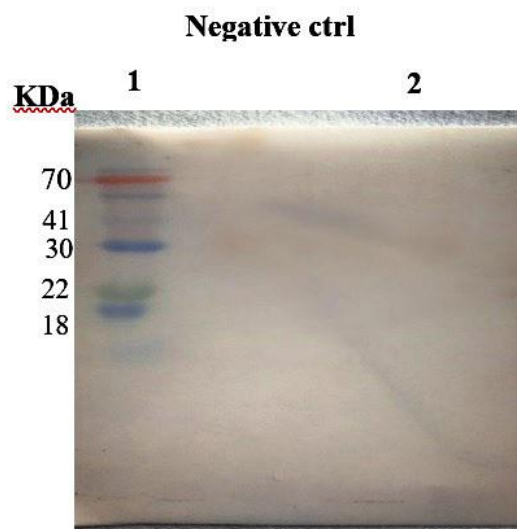


Figure 5. Western blot of *Mtb* culture filtrate with negative control sera. Lane 1, prestained protein ladder (10 to 250 kDa, Sinaclone); lane 2, healthy individual human serum

4. Discussion

This study aimed to isolate and purify low molecular weight antigens secreted from *Mtb* and use them for diagnostic purposes. The results demonstrated that these purified proteins showed significant protein bands approximately in the range of 14kDa -41 kDa on SDS-PAGE (12.5 %) and in Western blotting assay using TB patients' sera as a primary antibody. Bands in the range of 30-41 kDa were identified.

In the present study, the immunoblotting assay was used to detect the purified low molecular weight antigen fractions of *Mtb* in TB patients' sera. Obtained results showed that positive patients' sera identified bands at the approximate range of 30 to 41kDa. Therefore, the results support this hypothesis that the low molecular weight secreted antigens in culture filtrates of *Mtb* could make the protein complex at 30-41 kDa regions and that it is responsible for immune responses in tuberculosis patients. The 30-kDa protein, the major secretory protein of *Mtb* accounted for almost a quarter of the total extracellular protein in the culture filtrate (Harth et al., 1996; Horwitz et al., 1996). Moreover, some studies have shown that 38-kDa *Mtb* protein is one of the major secretory proteins of *mycobacteria* (Abebe et al., 2018).

Applied methods were employed in this study to purify the natural proteins from the Dorset-Henley liquid medium and decrease the purification stages. Accordingly, in this study, the bacteria were cultured in a high volume of Dorset-Henley liquid medium, and as described in the Methods section *Mtb* standard strain C were grown on dedicated culture media.

The secreted low molecular weight antigens from *Mtb* were precipitated by ammonium sulfate with 20% saturation and were purified further by Gel filtration (GF) chromatography on Sephadex-G50.

There are many different methods for protein precipitation. Protein samples should have an appropriate concentration after precipitation to be suitable for electrophoresis (Fountoulakis and Takcs, 2002). Horwitz et al. (1996), reported that the antigenic

proteins from the culture supernatant could be harvested by filtration and saturation with 10-60 % ammonium sulfate. However, during gradient analysis, maximum precipitation of the proteins was achieved using 20 % ammonium sulphate.

The GF chromatography separates proteins solely on the basis of molecular size and proteins are eluted from the GF column in descending size order (Babaie M., et al., 2013). In this study, three protein fractions were obtained using Sephadex-G50 gel chromatography.

Both HPLC (High-performance liquid chromatography) and GF chromatography methods are used to isolate proteins. The HPLC method is recommended for the separation of proteins due to the higher quality of the proteins separated by HPLC, compared to those separated by GF chromatography and regarding higher sensitivity of this method (Fong and Lam, 1991).

In this study, the Lowry method was used for the estimation of protein fraction concentrations, and molecular weight was determined by SDS-PAGE. Based on the results, protein concentration in the collected fraction F2 was at maximum (approximately 0.7 mg/ml). The purified protein fraction F2 appeared at regions with macular weight range of 14-41 kDa on silver staining SDS-PAGE, and immunoblotting assay showed that these regions contained antigens recognized strongly by TB patient sera.

Several physical and chemical methods, such as sonication and high temperatures have been employed for the inactivation of bacterial mass; however, these methods could destroy the existing proteins.

Normally, PPD is produced by inactivation of the bacterial mass by subjecting the bacterial culture to high temperatures for 3 h. The early work demonstrated that subsection of the mycobacterial culture filtrates to high temperatures led to a significant loss by denaturation of the antigenic protein; therefore, the PPD test had a low specificity for making a distinction between *Mtb* and other non-tuberculous mycobacteria (Yang et al., 2012). Therefore, in this study, the protein

from the bacterium was extracted using moderate heat shock, i.e., subjection of live bacterial *Mtb* cultures to a temperature of 68 °C for 1 h.

Based on the results, TB patient sera recognized antigen fractions with a molecular size between 30 kDa and 41 kDa on PVDF membrane. Various parameters are effective on the quality of immunoblotting after the transfer of antigen to the membrane, which includes optimal blocking conditions, antibody dilution, and washing buffers. It should be noted that not only is the affinity of the primary antibody for the antigen important, but primary and secondary antibody concentrations also have a profound effect on signal intensity. Accordingly, too much HRP on the blot can be caused by either primary and secondary antibody concentrations or both. The minimal primary antibody is advantageous since it promotes target-specific binding and low background (Alegría-Schaffer et al., 2009). Therefore, the Dot-ELISA assay was used to optimize the primary and secondary antibody dilutions for use in Western blotting.

In the present study, TB patients' sera were used as the primary polyclonal antibody. Although the monoclonal antibody has a high specificity to a single epitope, the polyclonal antibody is both inexpensive and has the high ability to capture the target protein (it is recommended as the capture antibody in a sandwich ELISA).

The results obtained in the present study were compatible with those obtained in the previous studies. Until now many low molecular weights *Mtb* antigens have been identified, including 71, 65, 38, 23, 19, 16, 14, and 12-kDa proteins (El-Masry et al., 2008). The 38-kDa protein is an immunodominant antigen isolated by affinity chromatography and is specific only to the *Mtb complex* (Beck et al., 2005b). The study conducted by E. Rovatti et al., in 1996 showed that a semi-quantitative western blot test against the 38, 28, 24, and 19 kDa protein fractions can identify individuals with mycobacterial infection (Rovatti et al., 1996).

The 22- to 32-kDa antigen has been reported to be the major component of culture filtrate when bacteria are grown in cultures without shaking. C. Espitia et al. (1989), purified culture filtrate antigens in the weight range of 122-14 kDa from *MTB*. Immunoblot analysis of the antibody response in patients with pulmonary TB and in healthy individuals with these antigens showed that 32-31 kDa band was found reacting with 90% of TB sera. Another antigen band with similar reactivity was a 60 kDa antigen which was recognized by 82% of pathologic sera.

The 16-kDa antigen is an immunodominant antigen, frequently called 14 kDa, related to the family of low molecular weight heat-shock proteins. This antigen contains B-cell epitopes specific to the *Mtb complex*. In another study conducted by Senol et al. (2007), Western blot analysis showed that 30 (58%), 26 (50%), and 31 (60%) of the patients produced specific IgG antibodies against antigens in the 6 kDa, 16 kDa, and 38 kDa ranges, respectively. The important point is the low expression of these proteins under *in vitro* conditions. Therefore, cloning methods and gene expression should be employed to obtain these proteins. However, the recombinant proteins do not have natural protein abilities (Berthet et al., 1998).

During TB infection, several antigenic proteins are produced by *Mtb*. Many of these proteins have been studied, purified, and characterized for their role in serological and cellular immune responses. New serological tests are used to detect the host antibody response to low molecular weight mycobacterial antigens (Min et al., 2015). The key to success in controlling the wide spread of TB is the development of novel and more effective methods to detect TB. Improved detection of the latent bacilli will lead to early intervention strategies, and will likely reduce disease morbidity and break the cycle of disease transmission. Taken together, the results of this study indicated that the protein antigens detected in the range of 30-41 kDa could discriminate NTM and *MTBC*

infections. However, further *in vivo* studies are required to confirm these results.

Authors' Contribution

Study concept and design: N. M.

Acquisition of data: N. M.

Analysis and interpretation of data: N. M.

Drafting of the manuscript: N. M.

Critical revision of the manuscript for important intellectual content: N. M.

Statistical analysis: N. M.

Administrative, technical, and material support: N. M.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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