

ORIGINAL ARTICLE

Investigation of Protein Profiles in Two Strains of *Mycobacterium bovis* and *Mycobacterium Tuberculosis* Cultivated in the Middlebrook 7H9 Medium

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ABSTRACT

Mycobacterium bovis and *mycobacterium tuberculosis* as the causes of bovine tuberculosis involves human in case by case basis and are considered as worldwide health complications. The current research aims to separate and compare the protein profiles of two strains in order to achieve effective biomarkers in identification and immunization in coming studies. *N*-acetyl-L-cysteine and Sodium hydroxide method was used to cultivate clinical samples in Lowenstein - Jensen Medium and biochemical and antibiotic susceptibility tests were used for strain differentiation. Colonies were cultivated in Middle brook 7H9 growth medium, then sonication, deposition by ammonium sulfate and alcohol precipitation were used to extract secretory proteins and Bradford method was applied to determine protein concentration. Finally, it was compared with the one-dimensional electrophoresis method. The 45 and 60 KDa bands in addition to 14-45 KDa bands of secretory proteins were the major dissimilarity between *M. tuberculosis* and *M. bovis* strains. It appears that the difference between protein bands of the *M. bovis* and *M. tuberculosis* can be used as marker protein and even effective biomarker in diagnosis of these strains. Furthermore, resemblances even can be used for immunization.

Key words: SDS-page, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, protein profile

Received 10/09/2015 Accepted 14/12/2015

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How to cite this article:

Sarafi, H, Bahremand, A, Masoomi, M. Investigation of Protein Profiles in Two Strains of *Mycobacterium bovis* and *Mycobacterium Tuberculosis* Cultivated in the Middlebrook 7H9 Medium. Adv. Biores., Vol 7 [1] January 2016: 139-143. DOI: 10.15515/abr.0976-4585.7.1.139143

INTRODUCTION

Tuberculosis as a primeval illness has always been prevalent in different communities and its agent was detached from the Stone Age human skeleton and ancient Egyptians' mummified bones [1]. 8.8 million Persons were infected with active tuberculosis and 1.1 million individuals were died consequently, in 2010 [2,3]. *Mycobacterium bovis* is one of the oldest and main etiologies of zoonotic tuberculosis and is considered as a widespread health problem. The rate of death due to *M. bovis* is more than *M. tuberculosis*. Although *Mycobacterium tuberculosis* complex (MTB) including *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. dassie bacillus* and *M. canettii* doesn't show unlike phenotypic features in biochemical tests, they have high homology genetically [4]. Distinction of tuberculosis components is essential to promote successful treatment especially in regions that the disease becomes epidemic or the interaction of human and animal is greater [5]. Classical methods of differentiation of *M. tuberculosis* and *M. bovis* and drug- susceptibility testing are based on Nitrate reduction, activity of Pyrazinamide enzyme, Niacin accumulation and growth in the Theophany 2carboxylic acid hydrazine [6]. Considering that the existing distinction approaches despite being valuable don't meet the needs for quick diagnostic tests and since the only way to keep healthy people in the community is immunization and rapid diagnosis and well-timed and complete treatment of patients. Trying to achieve and improve the identification, inhibition and treatment tools for Tuberculosis turn out to be a world necessity due to emergence of antibiotic resistant strains of *M.*

tuberculosis and lack of efficiency of BCG vaccine in adults [7]. One of the applicable strategies in this field is to explore protein profiles of *M. tuberculosis* and *M. bovis* strains.

Surface and secretory proteins play great role in stimulation of effective cellular immunity and TB diagnosis. Cultivation filters of the *M. tuberculosis* include different antigens. Many of them are defined and studied using monoclonal antibodies. Some are secretory proteins available in walls of bacteria or released proteins from dead bacteria which are able to generate immune response in the primary stages of bacterial infections [8]. Although detailed information of genomic and proteomic about *M. tuberculosis* have acquired till now, unfortunately suitable vaccine candidate and explanatory protein profile pattern have still not been introduced. Ying Xiong, *et al* [2] (05) surveyed the membrane proteins of *M. tuberculosis* H37Rv by one-dimensional Gel electrophoresis and mass spectrometry and reported 349 membrane proteins and 42 membrane proteins were discussed for the first time [9, 10].

This study objective is to investigate the protein patterns using SDS-page. Then the particular proteins of bacteria can be presented using proper techniques and new diagnostic, preventive and treating approaches can be attained through their comparison.

MATERIAL AND METHODS

This study was carried out from April to October, 2013. 100 samples were evaluated among submitted samples to mycobacteriology section of Pasteur Institute. Samples of 2011 and 2012 also were used to study *M. bovis*.

Received samples were cultivated by N-acetyl-L-cysteine in Lowenstein - Jensen Medium and thiophene 2-carboxylic acid hydrazide medium were used for differentiation of *M. bovis* and *M. tuberculosis*. After 8 weeks, Niacin, Catalase and Nitrate reduction [11] and antibiotic susceptibility tests were carried out to identify the strains [12]. Then, bacteria were moved from solid medium to liquid Middlebrook 7H9 medium for net growth of mycobacterium and 4 weeks were putting in incubator shakers. With the passage of time, the method of Ziehl - Neelsen staining were used to approve the growth of cultivates. Next, cultivations were centrifuged at 3000 rpm at 3°C for an hour and then were filtered with 0.2µm filter to check secretory proteins. In order to purify the protein, the method of deposition with alcohol was applied. Purification buffer were added to the deposition to extract secretory proteins. Finally, bacteria were decomposed by liquid Nitrogen, warm baths and sonication. The resulting solution was centrifuged at 3000 rpm at -3 °C for an hour. Composition with saturated solution of ammonium sulfate 70% was used to distinct available proteins in the resulted supernatant. Samples were centrifuged at -3°C at 14000 rpm for an hour and obtained deposits were solved in 1×PBS.

Dialysis in 1x PBS was used to remove existing salts in the protein solutions [13]. In order to prove the existence of protein in the solution, its absorption spectrum was taken by UV-visible spectrophotometer. Amount of protein in deposited solutions was obtained by Bradford protein analysis. Ultimately, deposited proteins were investigated by SDS-PAGE, Blue Silver Staining and Coomassie Blue R-250. SDS-PAGE for membrane and secretory proteins was performed with 10% and 12.5 % Gel.

RESULTS

From 100 random samples cultivated in Lowenstein - Jensen solid Medium, 5 samples of susceptible *M. tuberculosis* were selected by biochemical and antibiotic susceptibility testing methods along with 5 *M. bovis* strains cultivated in Middlebrook 7H9 medium. The concentration of proteins was defined by Bradford method after they were extracted and standard curve was drawn with bovin serum albumin 1 mg/ml as standard protein. The concentration of membrane and secretory proteins were reported 40-70 and 1-5, respectively.

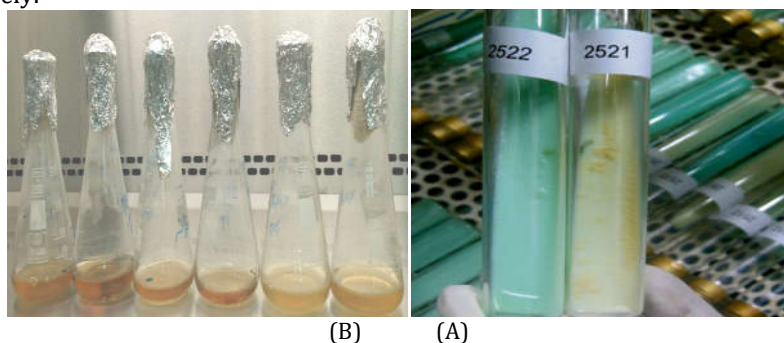


Figure 1- the growth of bacteria on two different mediums: A) Lowenstein - Jensen solid Medium b) specific liquid Middlebrook 7H9 medium.

SDS-PAGE of membrane proteins by 10% Gel was carried out with Connassi Blue R-250 and Blue Silver Staining and resolution of the blue silver staining was much more. It was resulted that all strains of *M. bovis* in 5 different patients have same protein patterns and all susceptible strains of *M. tuberculosis* in 5 different patients also have same protein pattern. 15-85KDa and 15-120KDa bands were observed in strains of *M. bovis* and *M. tuberculosis*, respectively.

Differences of candidate strains of the susceptible *M. tuberculosis* and *M. bovis* are revealed in picture (2) with arrow and it can be seen that major differences of these two strains is related to the weighted ranges of 45 and 60 KDa bands.

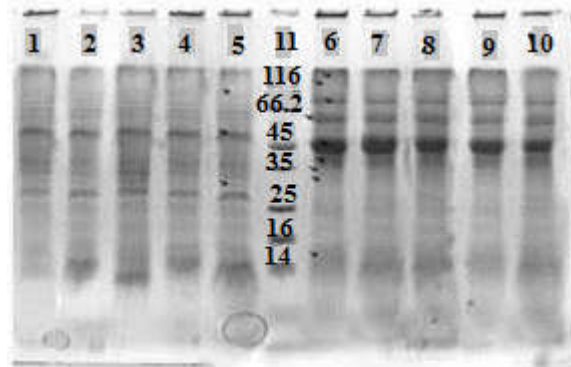


Figure 2- Gel 10%. Columns 1-5, membrane proteins of *M. bovis* strains- columns 6-10, membrane proteins of susceptible *M. tuberculosis* strains- column 11 is the marker. Protein extraction using Ammonium sulfate, Blue Silver Staining

SDS-PAGE of secretory proteins were done using 12.5% Gel with Blue Silver Staining and it was perceived that all strains of *M. bovis* in 5 patients are with same protein pattern and all strains of susceptible *M. tuberculosis* in 5 different patients also are with same pattern.

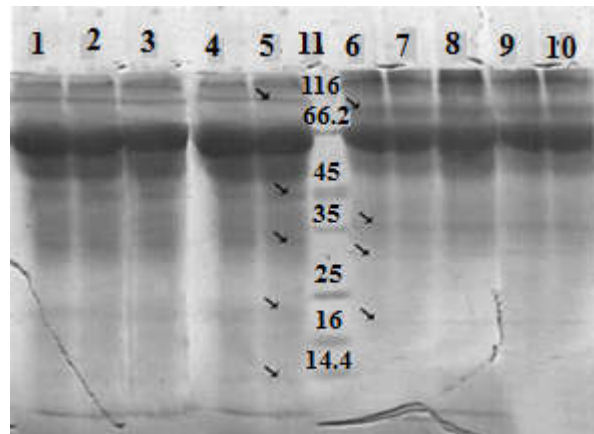


Figure 3- 12.5% Gel, columns 1-5 , secretory proteins of *M. bovis* strains - 6-11 columns, secretory proteins of susceptible *M. tuberculosis*- column 11, the marker. Protein extraction with alcohol, Blue Silver Staining

15-115 KDa and 18-114 KDa bands were observed in strains of *M. bovis* and *M. tuberculosis*. Differences of candidate strains are displayed by arrow in figure 3. Weighted range of 14- 45 bands is the most significant differences of these two strains.

DISCUSSION AND CONCLUSION

Considering that *M. tuberculosis* diagnostic methods including susceptible and resistant to drug susceptible mycobacterium and even *M. bovis* are based on microbiologic methods such as direct smear, cultivation and PCR methods. But cultivation always is considered as gold standard method. The necessity of rapid treatment and easy diagnosis are inevitable considering the Low susceptibility of some of these methods such as direct LAM or long response time to suspected patients to tuberculosis including cultivation in specific mycobacterium medium. In this study we tried to compare the weighted patterns of

membrane and secretory proteins between candidate strains of *M. tuberculosis* and *M. bovis* using SDS-PAGE method.

The liquid Middlebrook 7H9 medium was used in the current study and makes bacteria able to synchronize in addition to making possible the study of secretory proteins. Having mineral salts, enrichment materials (such as Albumin), Catalase, dextrose, Sodium Chloride, Glycerol and Polysorbate 80 are among its other advantages [14].

Bradford method was used to determine the amount of protein since it is capable of measuring small quantities in the range of μg and ng and even lesser.

In 1998, Cole *et al*, determined the complete genome sequence of *M. tuberculosis* H37Rv and defined the sequences of 3924 Gene. With the help of these genetic information, proteome analysis was done through combination of two-dimensional electrophoresis and mass spectrometry [15]. Almost 800 coding secretory proteins have been recognized by *M. tuberculosis* genome.

In 2003, Jens Mattow *et al*, analyzed supernatant proteins of mycobacterium tuberculosis cultivation using combination of two-dimensional gel electrophoresis and mass spectrometry and determined the N-terminal sequence. About 1250 protein pieces of *M. tuberculosis* H37Rv were identified. This study showed 137 different proteins from which 42 proteins were explained as secretory proteins. Comparison of *M. tuberculosis* H37Rv and weakened *M. bovis* BCG Copenhagen showed 39 specific protein pieces for *M. tuberculosis* which had 27 different proteins and can be as a candidate of antigens in order to produce new vaccine [16].

Xing Xiong *et al* [9] investigated the membrane proteins of *M. tuberculosis* H37Rv using one-dimensional electrophoresis and mass spectrometry and reported 349 integral fully membrane proteins and 42 membrane proteins was discussed for the first time.

Malen *et al* compared membrane proteins of *M. tuberculosis* H37Rv and H37Ra strains and examined the properties of more than 1700 proteins of both strains. Almost all identified proteins were too much similar, although strains were different in 5 or more proteins in 29 membrane or membrane associated proteins. 19 proteins and lipoproteins were the most frequent in H37Rv, while 10 proteins had the most frequency in H37Ra. 66 lipoproteins were the same in both strains, although 7 and 3 lipoproteins were just observed in H37Rv and H37Ra, respectively. Standard strains of ATCC and solid medium (7H10) were used in this study. Singhal *et al* [18] examined the intra cellular proteins of *M. tuberculosis* clinical isolates. Susceptible to drug *M. tuberculosis* (susceptible to at least 5 first-line drugs of ST-11 EA13-IND family) and resistant to drug *M. tuberculosis* (resistant to isoniazid rifampin and streptomycin from st288-CAS2 family) were selected from pulmonary disease center of JALMA (India). The liquid Sautons medium was used and bacteria were isolated in the late exponential phase (third week). Some protein were upregulate in comparison of 2DE and MS. 4 proteins were common in both groups AND 3 proteins belonged to metabolism and bacterial respiratory chain specifically. Results indicated that most proteins of upregulate /expressed were related to cellular metabolism and bacterial respiratory. Macrophage cell culture (THP-1) and cell infection with mycobacterium are also used. Overall, identified proteins contribute to bacteria compatibility with the environment and understanding the protein action is consistent with macrophage condition. Induction of their expression in *in vitro* may result in the interpretation of *M. tuberculosis* strategy in creation of infection and increase of TB cell survival. Accurate identification of the proteins allows us to involve them in structural operation of TB and growth of mycobacterium in the environment [18]. When comparing the bands of susceptible *M. tuberculosis* in the current study with membrane proteins of *M. tuberculosis* H37Rv in the Xiong study it can be state that clinical sample and standard strain of H37Rv are the same in terms of protein expression. Among main differences of the current study and the Xiong study, protein bands of 45 and 60 KDa can be noted.

It seems that the expression differences of protein bands of the two strains probably can be used as marker protein and even effective biomarker in differentiation of susceptible *M. tuberculosis* and *M. bovis* from each other in the case of more comprehensive purification and using a suitable method because differentiation of strains is important in cases that their distinguishing is not completely clear. Treatment of patients with *M. bovis* and *M. tuberculosis* is usually similar. Thus, difference of protein profile between these strains result in the early diagnosis and separation and consequently reducing health care costs. Selection of specific proteins able to show the differences of the strains can cause determination of these proteins to be as appropriate diagnostic biomarker. So that observation of differences in protein bands in the current study can be the beginning of different profile selection to determine the suitable candidate protein.

In this study, complementary studies including two-dimensional electrophoresis and mass spectrometry for protein difference in the observed weighted ranges will lead to more efficient purification of these

proteins in the future. Moreover, the protein difference can be useful to identify effective isotopes in immune responses of the host.

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