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MDR-TB Antibody Response (Western Blot) to Fractions of Isoniazid and Rifampicin Resistant Antigens of *Mycobacterium tuberculosis*

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Abstract Drug-resistant TB poses a major threat to control of TB worldwide. Despite progress in the detection of Multidrug-resistant TB (MDR-TB) cases, a major diagnostic gap remains: 55 % of reported TB patients estimated to have MDR-TB were not detected in 2013. MDR-TB antigens were conjugated to CNBr-activated Sepharose 4B. Specific polyclonal antibodies against MDR-TB Ags were prepared in rabbits using two boosted injections of the MDR-TB antigen. The antibodies were purified and treated with susceptible TB to remove any non-specific and cross-reactive antibodies. In the present study, comparative analysis of electrophoretic pattern of different antigens of INH/RIF-resistant TB were studied for identifying protein profiles. A RIF-resistant TB antigen was shown here to have different protein profiles from INH-resistant TB isolate. The results of Western blotting analysis showed that in the RIF- and INH-resistant antigenic fractions some bands of 14.4 and 45 kDa as immunogenic were common. Moreover, four bands of RIF-resistant TB antigen fractions (16, 19, 21, and 45 KDa) and one band of INH-resistant TB (about 26 KDa) were detected as diagnostic antigens. This study suggests that the Western blot is an accurate test to survey INH- and RIF-resistant TB antigens of *M. tuberculosis* infection. These findings indicate that MDR-TB diagnosis (based on Ag detection) could be useful in the identification of disease stages that precede symptomatic and microbiologically positive TB, such as subclinical and incipient TB.

Keywords MDR-TB · INH- and RIF-resistant TB · MDR-TB antibody response · DR-TB detection

Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360,000 of whom were HIV-positive [1].

Despite this enormous global burden, case detection rates are low, posing major hurdles for TB control. Conventional TB diagnosis continues to rely on smear microscopy, culture, and chest radiography. These tests have known limitations [2]. The main obstacles to controlling TB worldwide are multidrug resistance, the absence of concise diagnostic methods, and variations in the protective effects of the BCG vaccination [3]. As part of the post-2015 global TB strategy's, the early diagnosis of TB is emphasized, and inclusion of drug susceptibility testing (DST) is now targeted as a universal standard for patient care, including for both new and previously treated patients. Detection of TB without investigation for drug resistance can lead to ineffective treatment, further development, and spread of drug-resistant strains and additional suffering and costs for patients [1]. Conventional tests for detection of drug resistance are slow, tedious, and difficult to perform in field conditions [2]. On the other hand, the most TB serological tests rely on detection of the humoral antibody immune response to *M. tuberculosis*, while WHO recently announced its first negative policy in TB, against

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the use of current TB serological tests based on antibody detection [4]. For this reason, this study defined the electrophoretic pattern of INH/RIF-resistant TB antigens using SDS-PAGE technique and consequently, using Western blotting method to determine the specific antigens of these resistance patterns. Actually, the determination of antigenic fractions in resistant TB (INH+RIF/MDR-TB) can be an important and valuable tool for rapid diagnosis of MDR-TB isolates.

Methods

Settings and Definitions

This study was conducted in the Protein Chemistry Laboratory of Tuberculosis Department, Pasteur Institute, Tehran. Multidrug-resistant TB (MDR-TB), Susceptible TB, Isoniazid-resistant TB (IR-TB) and Rifampicin-resistant TB (RR-TB) strains were obtained from the Iranian Mycobacterial Collection, Pasteur Institute-Tehran, Iran. MDR-TB was defined as TB caused by strains of *Mycobacterium tuberculosis* that are resistant to at least isoniazid and rifampicin [5]. A Rifampicin-resistant TB (Rifampicin mono-resistance) strain was defined as a strain resistant to rifampicin and susceptible to isoniazid, on the basis of first-line DST analysis on solid or liquid media [6]. Isoniazid-resistant TB also was defined as a strain resistant to Isoniazid and susceptible to rifampicin [7].

Culture and Drug Susceptibility Testing (DST)

Cultures were done on 7H9 broth with ADC enrichment. DST against isoniazid, rifampicin, ethambutol, and streptomycin was performed by the proportional method on Lowenstein–Jensen (LJ) media at a concentration of 0.2, 40, 2.0, and 4.0 μg/ml, respectively. Drugs were procured from Sigma (USA), and for each batch of DST, a sensitive strain of H37Rv was used as a control [8]. Both proportional and molecular methods, to confirm the resistance to INH and RIF, were done on each identified strains.

Mycobacterium tuberculosis Antigen Preparations

The antigen was obtained from *M. tuberculosis* strains (MDR-TB, Susceptible TB, INH and RIF-resistant TB) grown in 7H9 broth medium. Bacteria were separated from the culture medium by centrifugation at 5000 rpm and then washed with PBS, pH 7.4, containing PMSF 1 mM, EDTA 20 mM, Sodium azide 0.02 %, Triton X114 0.5 %, Glycerol 10 %, Sucrose 12.5 mM, DNAse 1 μg/ml, and DTT 10 mM. The bacilli were subjected to sonication for 1 h at 50 Hz, using a cell sonicator (Bandelin, GM-220, Germany) in ice, and subsequently centrifuged at 5000 rpm for 45 min at −4 °C. Proteins were precipitated by adding refrigerated ethanol (−20 °C) to the supernatant. After centrifugation at 13,000 rpm for 45 min at −15 °C, pellets were resuspended in PBS and then dialyzed for 24 h against saline [9]. The protein content was measured by Bradford's method [10].

SDS-PAGE of *Mycobacterium tuberculosis* Antigens

SDS-PAGE was performed with the Bio-rad system for vertical electrophoresis in a Mini-Protean Tetra Cell unit (Bio-Rad Laboratories, Hercules, California, US) by the method of Laemmli [11]. Samples were prepared in SDS-mercaptoethanol buffer by boiling for 5 min. Fifty microgram per lane of crude mycobacterium extract was loaded on 12 % vertical polyacrylamide slab gels. The gel was run at a constant current of 10 mA until the tracking dye entered the resolving gel; the current was then increased to 15 mA. Proteins were visualized by staining with Coomassie blue staining. Molecular weight marker in the range 25.0–116.0 kDa was used (Fermentas, Ontario, Canada). β-galactosidase (116.0 KDa), Bovine serum albumin (66.2 KDa), Ovalbumin (45.0 KDa), Lactate dehydrogenase (35.0 KDa), REase Bsp981 (25.0 KDa).

MDR-TB Antibody Purification Procedures

Rabbits

Animals were supplied by Animal Lab Facility, Pasteur Institute of Iran, Karaj, Iran. The purified antigen (MDR-TB antigen) was injected subcutaneously. The rabbits were re-immunized (boosted) at 21-day intervals until peak antibody titers were reached. Freund's incomplete adjuvant was used, at 1:1 ratio with antigen [12, 13].

Antibody Purification

Affinity chromatography was used to purify specific polyclonal antibodies (rabbit's sera) against MDR-TB antigen. This step was carried out on an affinity column based on the use of MDR-TB antigen covalently coupled to CNBr-activated Sepharose 4B (CNBr-activated Sepharose 4B, Pharmacia, Uppsala, Sweden) according to affinity chromatography technique as described previously [14].

Preabsorption of MDR-TB Antibody

The antibodies were treated with susceptible TB antigens to remove any non-specificity and cross-reactivity. Cross-reactive immunoglobulin was removed by incubation of antibodies for 2 h at room temperature with sensitive-TB antigen extracts. This step was repeated at least three times [15].

Western Blot

The fractionated proteins, Isoniazid- and Rifampicin-resistant TB proteins were electrophoretically transferred onto nitrocellulose membranes (PROTRAN^{R} Schleicher & Schuell Bioscience GmbH, Germany) in a Mini Trans-Blot Cell unit (BioRad, CA, USA). Protein staining of transfers was performed using ponceau S solution (sigma). Membranes were blocked with 3 % BSA, bovine serum albumin (Sigma), and then incubated overnight at four. After extensive washing with TTBS buffer (Tween 20+TBS), MDR-TB antibody was diluted 1:10 and allowed vortex at room temperature for 2 h. The blots were washed again followed by the addition of anti rabbit-IgG conjugated to peroxidase (Sigma). Antibody binding was revealed at room temperature using DAB 3, 3-Diaminobenzidine tetra hydrochloride (Sigma-Aldrich). The reaction was stopped by adding water [16].

Results

Mycobacterium tuberculosis isoniazid- and rifampicin-resistant protein extracts were subjected to gel electrophoresis and Western blot analysis with MDR-TB polyclonal antibodies.

Electrophoresis

The coomassie blue stain gave very good results with sharp and clear protein bands. The silver staining did not increase the number of bands detected (gels not shown). Only the gels stained with coomassie blue were used for further analysis. Protein banding patterns for susceptible TB, and INH- and RIF-resistant TB are presented in Fig. 1. Several prominent protein bands were present in all isolates. Electrophoretic pattern of susceptible TB is shown in the lanes 6 and 7. There were at least four sharp bands ranging from 35 to about 85, 45 kDa band was well noticeable. Generally, the sensitive banding patterns could be recognized on the gel. However, the banding pattern of INH-resistant TB isolate was distinguishable from that formed by RIF-resistant TB strain. As shown in Fig. 1, there were 16 protein bands with the molecular weights ranging from 25 to more than 116 kDa in INH-resistant TB. Protein bands weighing less than 116KDa were sharp. There were 15 protein bands from 25 to less than 116 kDa in RIF-resistant TB. There were 3 sharp bands 45 to 66.2 kDa common in the INH and RIF-resistant-TB, lanes 1–4.

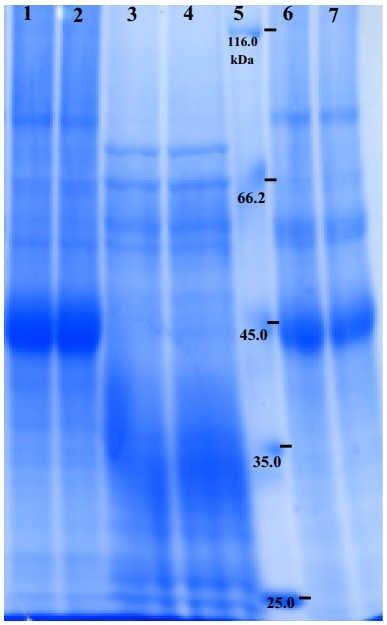

Fig. 1 Protein banding patterns of susceptible TB, and isoniazid- and rifampicin-resistant TB after SDS-polyacrylamide gel (10 %) electrophoresis and staining with coomassie blue. *Lanes 1* and *2* isoniazid (INH)-resistant TB isolates, *Lanes 3* and *4* rifampicin (RIF)-resistant TB isolates, *Lane 5* Marker, *Lanes 6* and *7* sensitive MTB isolates

Western Blotting

In order to determine the specific antigens, antigenic fractions were analyzed by Western blotting technique. Results from the Western blotting of antigens obtained from the INH- and RIF-resistant TB versus MDR-TB antibodies are shown in Fig. 2 lanes 1–4, respectively. There were three blurred bands in the RIF-resistant TB patterns ranging from 25 to 45 kDa. Band of about 23 kDa is common for the two antigens of INH- and RIF-resistant TB, but in the RIF-resistant TB patterns, four obvious bands (lanes 1, 2, bands A, C, D, and E, respectively) with molecular weights of about 14.4–45 kDa were observed. As well as recognizing the bands mentioned above, antibodies from sonicated MDR-TB identified a low molecular weight band of about 16 kDa (Lane 2, Rif E), which was not apparent on the profiles of the INH-resistant TB strain (Fig. 2 lane 3, INH). The results of Western blotting analysis showed that in the RIF- and INH-resistant antigenic fractions some bands of 14.4 and 45 kDa as immunogenic were common. Moreover, four bands of RIF-resistant TB antigen fractions (16, 19, 21, and 45 KDa) and one band of INH-resistant TB (about 26 KDa) were detected as diagnostic antigens. Antibodies from sonicated MDR-TB produced a stronger reaction with INH-resistant TB antigens than that was observed with RIF-resistant TB

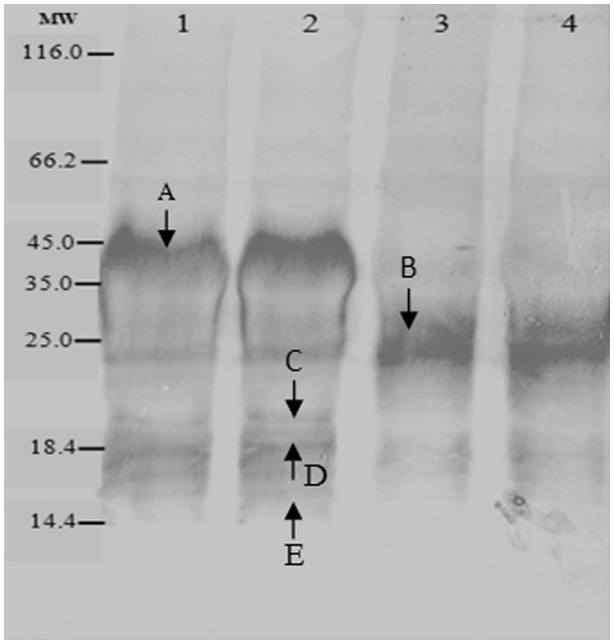

Fig. 2 Western blotting of different TB resistant antigens versus MDR-TB antibody absorbed with susceptible TB isolates. *Lanes 1, 2* rifampicin-resistant TB, *Lanes 3, 4* isoniazid-resistant TB

isolates particularly with about 24 and 28 kDa bands (Fig. 2). These antibodies also identified a band at 45 kDa, unique to the RIF-resistant TB strain, (Fig. 2 lane 1, a) while only an undefined region of staining between 35 and 45 kDa was observed with the MDR-TB antibodies against INH-resistant TB antigens. As well as recognizing the bands mentioned above, antibodies from sonicated MDR-TB identified a low molecular weight band of about 16 kDa (Fig. 2 lane 2, e), which was not apparent on the profiles of the INH-resistant TB strain (Fig. 2 lane 3, INH). The Western blot findings are summarized in Table 1.

Discussion

We studied four *Mycobacterium tuberculosis* strains, MDR and susceptible TB, Isoniazid- and Rifampicin-resistant TB strains, using protein electrophoresis and Western blot analysis. We concluded that major TB resistance proteins, Isoniazid and rifampicin protein contents were characteristic of strains, and protein banding patterns could be used in the identification of MDR-TB isolates. A very high of similarity was detected between INH- and RIF-resistant TB. Similarities in the SDS-PAGE protein profiles were evident between the extracts of INH- and RIF-resistant TB, and these were markedly different to the profile obtained with susceptible TB strains (Fig. 1). The results suggest that the 16 and 45 kDa bands (Fig. 2a, e) and 26 kDa band (Lane 3, INH) are immunogenic for diagnostic of MDR-TB isolates. The existence of four bands of about 14.4 to 45 kDa related to the RIF-resistant TB and especially one band in INH-resistant TB can be regarded as diagnostic antigen for MDR-TB. Isoniazid- and rifampin-resistant TB protein profiles as revealed by Coomassie Brilliant Blue R were possible to identify specific bands that could act on diagnostic marker for MDR-TB isolates, although they differed between the 2 strains. The polyclonal antibody (MDR-TB Abs) showed a great specificity with INH- and RIF-resistant TB antigens. The high reactivity of MDR-TB antibodies with both INH- and RIF-resistant TB isolates, tested here, suggests that there are significant amounts of these antigens in the protein components of MDR-TB strains. In immunoblotting of INH-resistant-TB antigen with MDR-TB antibodies, one band (Fig. 2 lane 3, b) of about 25 to 35 kDa was observed, which shows this band can be considered as specific diagnostic patterns for INH-resistant TB isolates.

Jiang et al. [17] have reported that the differentially expressed proteins from INH-resistant strains might be used as potential immunodiagnosis antigens and candidate novel drug targets against drug-resistant tuberculosis. In another study, Garbe et al. [18], showed the major upregulated 32 KDa protein band in *M. tuberculosis* cultures exposed to isoniazid inducing expression of the Ag85 complex. They also detected a 27-KDa protein that was probably identical to the 27 KDa polypeptide observed in the experiments with isoniazid.

These results are approximately similar to our findings. A possible explanation for the discrepancy and differences in molecular weight of these bands may be due to alterations in its structural confirmation during bacterial growth. The 26 kDa band was clearly identified on INH-resistant TB strains by MDR-TB antibody. This band was also identified in humoral response to low molecular weight antigens of *Mycobacterium tuberculosis* by beak et al. [19]. In addition, the 45 kDa band was identified on RIF-resistant TB strains and this is consistent with the results of Gamboa-Suasnavart et al. [20]. Thus, the lack of any reaction with the other bands suggests that these bands have been shown to be immunogenic and these findings have serious implications for the development of diagnostic marker for MDR-TB isolates.

Table 1 Patterns of Western blot analysis of isoniazid (INH)- and rifampin (RIF)-resistant *Mycobacterium tuberculosis* sonicate antigens

Patterns	Antibody	Sonicate Ag (INH)	Sonicate Ag (RIF)
A	MDR-TB		45 KDa
B	MDR-TB	26 KDa	
C	MDR-TB		21 KDa
D	MDR-TB		19 KDa
E	MDR-TB		16 KDa

The results of Western blot analysis suggest that INH-resistant TB is closely related or possibly not distinct from RIF-resistant TB. Additional verified isolates need to be compared. Our results also suggest that Western blot and SDS-PAGE represent distinct MDR-TB isolates.

The SDS-PAGE results did demonstrate that INH and RIF-resistant TB have high degree of microbiological similarity. In addition, MDR-TB antibody displays multiple distinct patterns on Western blotting that appears to identify proteins not previously recognized. This antibody proved an effective diagnostic tool for identifying MDR-TB strains/isolates, particularly by Western blot methods and compares favorably with conventional method. It would be interesting to use this antibody for immunochromatographic assay based on antigen detection test to compare reactivity of the other TB antigens involved in Rapid-TB test. Moreover, specific anti-mycobacterial antibodies, such as MDR-TB antibody, could be essential tools in the identification of species-restricted antigens like INH and RIF's Drug-resistant TB (DR-TB) antigens and finally the present MDR-TB antibodies may prove of value in the identification of Mycobacterial antigens during the course of infection.

In conclusion, the clinical use of this assay would also be greatly enhanced if a simple inexpensive point of care version of this assay were developed. However, well-designed, prospective cohort studies are needed to evaluate the prognostic value of panel of such antigens. The detection of early and often asymptomatic disease stages would be extremely valuable, especially in HIV-infected persons in whom treatment of subclinical TB suggests an association with reduced mortality [21].

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