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Expression Study of *Mycobacterial* Hsp70 Protein in Serum Samples of Pulmonary Tuberculosis Patients

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Abstract

Clinically dreaded diseases are those that are difficult to diagnose. One among such heavy toll wipers is pulmonary tuberculosis. The problem with the disease is its clinical detection that usually occurs late in the course of the disease. Clinical researchers have been facing the challenge of rapid and exact diagnosis of TB since long. Their efforts access new branches of studies that include diverse classes of proteins for their possible candidature as detection markers. One of such classes is the family of heat shock proteins (Hsps). The current study deals with detection of *Mycobacterial* Hsp70 protein in pretested serum samples. From a total of 240 serum samples 87 were confirmed TB positive using the routine 30kD test (patented by Central India Institute of Medical Sciences, Nagpur), and the rest (153) were found to be negative. With these samples a standardized protocol for ELISA detection of *Mycobacterial* Hsp70 was carried out, which showed remarkable results. Out of 153 non-TB samples 77 (i.e. 50.33%) were found to be positive for *Mycobacterial* Hsp70 AND a total of 31 (i.e. 35.63%) clinically tested TB positive samples were found to show lack of *Mycobacterial* Hsp70.

The results were suggestive of the probable cross reactivity shown by the antibody that was used to detect *Mycobacterial* Hsp70. This compelled us to perform sequence alignment and structure comparison in between Hsp70 proteins from both the species (i.e. *Mycobacterium tuberculosis* and *Homo sapiens*). Similarity searches were also performed to find out similar motifs and domains lying between the two proteins.

Keywords: Mycobacterium tuberculosis, Hsp70, ELISA, structure comparison.

Introduction

Infectious diseases present not only the challenge of heavy spread but also the problem of accurate diagnosis. Pulmonary tuberculosis is one of the toughest to diagnose and defeat due to its latency period and quick spread [25]. Clinical researchers are always in search of ideal detection markers, which provide early, cheap yet an exact diagnosis. They have explored many different classes of proteins for this purpose. Proteins from the 'Heat shock protein (Hsp) - family' [15] are being clinically tested for their possible candidature to serve as ideal markers [2]. Heat shock proteins represent a family of proteins having similar functions but differing in their molecular weights. They are expressed at high levels when exposed to heat or other stress. One of the members of the family is Hsp70 protein [4] [6].

The current study deals with the observations made on detection of *Mycobacterial* Hsp70 protein in pre-tested serum samples of pulmonary tuberculosis patients [9] [10] [11]. To our surprise, we got unexpected outcomes from an ELISA protocol standardized for detecting *Mycobacterial* Hsp70 [10]. A considerable number of clinically suspected TB samples did not show the presence of *Mycobacterial* Hsp70 protein and another striking number of samples from the non-TB group were found to be positive for *Mycobacterial* Hsp70 protein. These results compelled us to carry out bio-informatic analysis of the question. A sequence alignment study of Hsp70 from *Mycobacterium tuberculosis* and *Homo sapiens* was done to find out the similar patterns lying between the two proteins [13]. It was found and confirmed that the structures of the two proteins were not available in PDB. This added a new dimension to the current work and the structures of the two proteins were predicted and aligned [18]. This opened more options for comparing the two structures and understand the homology better [12].

Materials and Methods

***Subjects*:** The subjects admitted to Central India Institute of Medical Sciences, Nagpur, India were selected for the study. A total of 240 serum samples were tested by using routine 30 kD test patented by CIIMS, Nagpur [9].

***Specimens*:** Venous blood was collected from all the patients as well as the control subjects. Blood was allowed to clot and centrifuged at 2000 rpm for 2 minutes to collect serum. All the samples were stored at -20^{o} C until use[9].

***Primary antibody*:** The benefactor of the primary antibody against *Mycobacterial* Hsp70 is Colorado State University, Colorado.

A routine diagnosis of TB was performed using 30kD test patented by CIIMS, Nagpur [9] [11]. This determined if the samples were positive for TB or not. All the samples were stored at -20^{o}C until use. To determine the most accurate concentration of antibody to be used to detect *Mycobacterial* Hsp70, a series of ELISA and Western Blot experiments was carried out exploiting different dilutions of the antibody [9] [10] [11]. Based upon observations from these experiments a dilution 1:1000 of the

antibody was selected *Fig 1*. This helped us to standardize a protocol for ELISA detection of *Mycobacterial* Hsp70 in all the samples.

From the 240 samples, 87 were clinically suspected TB (i.e. TB positive) samples and 153 were non-TB (i.e. TB negative) samples. Out of 87 clinically tested TB positive samples a total of 31 *(i.e. 35.63%)* were found to show lack of *Mycobacterial* Hsp70 while from 153 non-TB samples 77 *(i.e. 50.33%)* were found to be positive for Hsp70 mycobacterium.

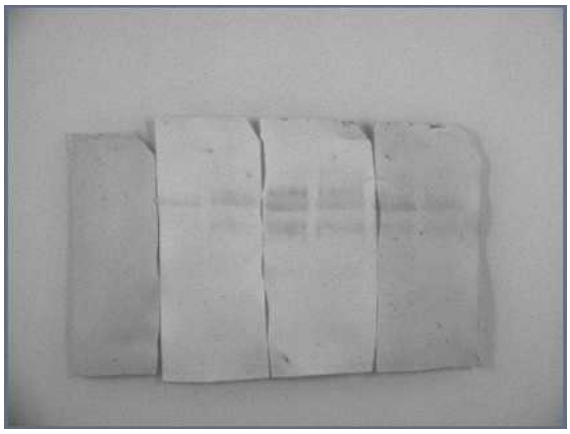

Figure 1: Shows 4 pieces of nitrocellulose membrane. Iry Antibody dilutions tested. From left 1:4000, 1:2000, 1:1000, 1:500.Most prominent results can be observed in dilution 1:1000.

Groups	70kDa positive	70kDa negative
TB patients (Total 87)	56 (64.37%)	31 (35.63%)
NTB patients (Total 153)	77 (50.33%)	76 (49.67)

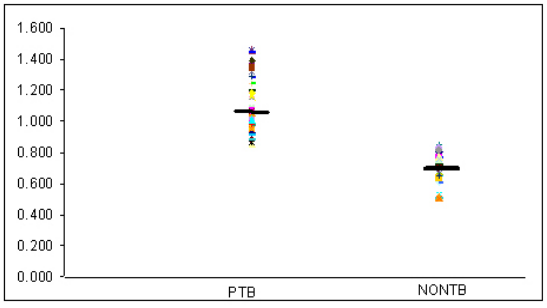

Figure 2: A. Signifies indirect ELISA results for detection of 70kDa Hsp in TB and NTB patients. B. Hsp 70 levels in TB and non-TB individuals (A plot of absorbance)

The sample types, non-TB but Hsp70 positive (NTB: Hsp70+) and TB positive but Hsp70 negative (TB: Hsp70-) were presenting unexpected outcomes. Hence it was hypothesized that the antibody used for detection of *Mycobacterial* Hsp70 would have

shown some extent of cross reactivity. To ensure this we compared in between Hsp70 proteins from both the organisms i.e. *Mycobacterium tuberculosis* and *Homo sapiens*.

The amino acid sequences of the Hsp70, from *Mycobacterium tuberculosis* and *Homo sapiens* were obtained from the sequence database of NCBI (Accession No. ACE79189, AAA02807 and GeneID: 190576826, 292160) respectively. BLAST [1] and ClustalX [13] were used to perform global sequence alignment to find similar patterns. It was assured that the three dimensional structures of both the proteins were not available in Protein Data Bank. This generated a need to predict and design 3D models of these two proteins. The 3D models were predicted and generated. The predicted models of both the proteins were then compared to study structural similarity and homology [12]. The two proteins were found out to be exactly 92.8% similar. The steps followed for structure prediction are dealt below.

Template Searching

Attempts were made to find suitable template proteins for the modeling of the target proteins. The template proteins were searched through Geno3D server [5], (an online tool for searching similar sequences, based on sequence and structure-wise similarity). From the homology searching 'NMR-RDC / X-RAY structure of E. coli HSP70 (DNAK) chaperone (1-605) complexed with ADP and substrate [2KHO: A] Chain A' was selected as template protein for *Mycobacterium tuberculosis* Hsp70. From the homology searching 'Structure of the Hsp110:Hsc70 Nucleotide Exchange Complex from *Bos taurus* [3C7N: A] Chain A' was selected as template protein for *Homo sapiens* Hsp70.

Sequence Alignment

Amino acid sequence alignment of target and template proteins was derived using the Swiss-PdbViewer package. Default parameters were applied and the aligned sequences were inspected and adjusted manually to minimize the number of gaps and insertions.

Rough Model

Rough 3-D models (20 models) were generated from the sequence alignment between Hsp70 proteins from *Mycobacterium tuberculosis* and *Homo sapiens* and the template proteins using MODELLER 9v7 [24].

Evaluation of Models

The predicted models were subjected to a series of tests for testing their internal consistency and reliability [20]. Backbone conformation was evaluated by the inspection of the Psi/Phi Ramachandran plot [20] obtained from PROCHECK [14] analysis. Packing quality of the structures was investigated by the WHATIF.

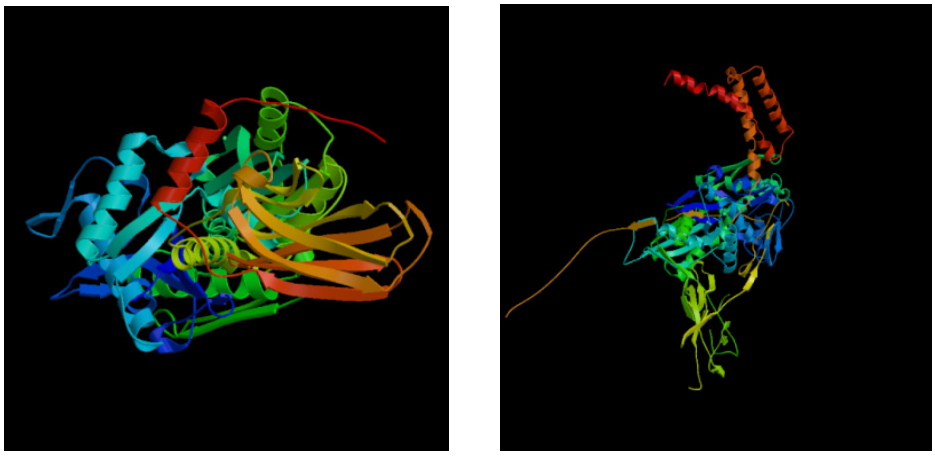

Figure 3: Predicted 3D models of A. Hsp70 [Mycobacterium tuberculosis] B. Hsp70 [Homo sapiens]

Structure comparison

Structural comparison was performed using R Console Programming Script and other different structure comparison tools like MAMMOTH [18]. The two proteins were found out to be exactly 92.8% similar.

Results and Discussion

Surprisingly, we found out that 35.63% of TB positive samples showed no significant concentrations of *Mycobacterial* Hsp70, where as 50.33% of TB negative samples showed significant levels of *Mycobacterial* Hsp70 protein. The results were suggestive of probable cross reactivity shown by the antibody used to detect *Mycobacterial* Hsp70. The basis of this cross reactivity can be understood better by attempting comparison of the sequences and structures of the Hsp70 proteins from both the sources. The first and foremost requirement for comparing any two structures is the availability of both the structures. Since this requirement was not met in this case, we had to exercise the steps for structure prediction of both the proteins. Based on the best template selected for predicting structures, 3-D models for both the proteins were generated. A comparison between the two predicted structures revealed that the similarity between the two proteins was remarkable 92.8%. This made us understand the basis of the cross reactivity shown by the antibody used to detect *Mycobacterial* Hsp70.

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