Mycobacterium Tuberculosis Heat Shock Protein 16 as a Potential Marker for Latent TB: A Preliminary Findings

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Abstract

Identifying latent Tuberculosis (TB) is a key component of global efforts to eliminate TB. Heat shock proteins (Hsps) of mycobacteria are up regulated in response to various environmental stresses during infection. The present study was undertaken to evaluate the diagnostic efficacy of *Mycobacterium tuberculosis* (MTB) Hsp16, MTB Hsp65 and MTB Hsp71 in latent and active TB in a high TB prevalence area by ELISA method. The mean optical density for Hsp16 as compared to Hsp65 and Hsp71 in latent TB subjects was significantly higher than the active subjects with significant P value of 0.0004. A preliminary finding indicates that the Hsp16 is more specific to latency and may be useful as a diagnostic marker for latent TB infection.

Keywords: Latent TB; Heat shock proteins; Chaperones; ELISA

Background

Tuberculosis (TB) remains one of the world's top ten leading causes of death. More than 2.0 million deaths were attributed to TB in 2000 [1]. One third of the world's population infected with *Mycobacterium tuberculosis* (MTB) contains the infection in a latent form without any clinical symptoms [2,3]. It is assumed that protective immunity maintains MTB infection in a latent state [4]. Following primary infection, the lifetime cumulative risk for active TB is estimated to be 10-20%. Certain factors (HIV infection, malnutrition, low age, immunosuppressive drugs etc) increases the risk of reactivation when first infected [5,6].

Identifying latent TB is a key component of global efforts to eliminate TB. Due to unavailability of gold standard methods to identify the latent TB cases are increasing exponentially [7]. For the last many years, tuberculin skin test (TST) have been used worldwide, however this test was not able to differentiate MTB infected individuals from those vaccinated with Bacillus Calmette-Guerin (BCG) [8]. Recently, *QuantiFERON-TB Gold* test is developed alternative to TST for diagnosis of latent TB [8,9]. However, in developing and underdeveloped countries this is under trial and not currently used in many laboratories due to its high cost and time with reference to availability of results. In absence of simple and reliable diagnosis, this latent infection becomes relatively resistant to current treatment regimen and is converted into active disease at favorable condition. This situation renders the global eradication of TB even more difficult to achieve than is the already ambitious goal embraced by the World Health Organization (WHO) of treating all active cases of TB [10].This stress the urgency of identification of biomarkers which can be used in differential diagnosis of latent TB from active infection.

Heat shock proteins (Hsp) of MTB have received a great deal of attention recently because Hsp appears to be one of the major immunologically active mycobacterial antigens following infection and is expressed at high levels by bacterial pathogens during adaptation for intracellular survival [11,12]. Earlier from our laboratory and various other workers have reported the diagnostic applications of Hsps in pulmonary and extra-pulmonary TB [13-16], however the role of Hsp is not known in patients who are in contact with TB patients. To the best of our knowledge, not much work is done on MTB specific Hsp in latent or exposed TB population despite of lot of experimental *in vitro* work is done to study latent TB. It will be interesting to see whether Hsps can be useful for differential diagnosis of active from latent TB.

In the present study, we have evaluated the diagnostic efficacy of Hsp16, Hsp65 and Hsp71 in cases of latent and active TB population in high TB prevalence area of Nagpur district, Maharashtra, India.

Materials and Methods

Study subjects

We prospectively selected serum samples from 22 active TB (04 male, 18 female; age 6 to 50 years) and 24 latent TB subjects (11male, 13 female; age3 to 75 years) from Macca Masjid, Teka Naka , Nagpur, India having high prevalence of TB and also from Central India Institute of Medical Sciences (CIIMS), Nagpur, India . All the latent TB patients, having normal chest radiographs and no signs of clinical impairment. Serum samples were obtained from active TB patients before initiation of Anti Koch Treatment (AKT) and were stored at -20°C until they were tested. In India, BCG vaccination is given within one week of the birth of child; all subjects included in the study had been vaccinated with BCG. Samples were collected from all study groups for which patient's consent was obtained. The CIIMS Ethical Committee, Nagpur, India approved the study and all the analyses were performed double blinded. All patients were grouped as follows:

Active TB (n=22): To diagnose active TB, sputum microscopy was done on two serial sputum samples by staining with Ziehl Neelson Stain as per the guidelines of Revised National TB Control Programme (RNTCP, India).

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TB was confirmed if acid fast bacilli (AFB) and/or culture of sputum specimens were positive for MTB. When both tests were negative, the patients were diagnosed by clinical symptoms. Clinical suspicion of TB was based on minimum of 3 symptoms of the following a) Chronic cough with or without expectoration/hemoptysis/ chest pain of more than 2–3 weeks or past history of TB b) Fever more than 2–3 weeks c) Progressive unexplained weight loss d) loss of appetite e) night sweats. Radiographic features supporting the clinical diagnosis considered were lung parenchymal infiltration mainly involving apical and/ or mid zone, miliary shadows and pleural effusion. Along with the above mentioned clinical features, any one radiological feature was considered sufficient as supportive evidence. Ultrasound examination of chest was done in suspected cases of pleural effusion, which was also utilized for diagnostic pleural tap. Sputum samples of 22 patients were collected. Among that twelve were positive to culture/AFB staining. All patients received AKT (not covered under DOT) in absence of any other therapy. Daily dosages were given as isoniazid 300 mg, rifampicin 450 mg or 600 mg, pyrazinamide 1.5 g or 2.0 g and ethambutol 25 mg/kg for the first 2 months. In the next 2 months rifampicin 450 mg or 600 mg, isoniazid 300 mg and ethambutol were given followed by ethambutol, rifampicin 450 mg or 600 mg and isoniazid 300 mg for the next 4 months.

Latent TB (n=24): All the subjects who have exposure of TB or having family history of TB and TST and /or *QuantiFERON®-TB Gold* test were positive with no clinical, bacteriological features of TB with normal radiographic evidences were considered as latent TB subjects [1].

Control Group (n=16): All the disease control cases included in the group are *QuantiFERON®-TB Gold* and tuberculin skin test negative with no clinical, bacteriological features of TB. Normal chest radiographs and no history of AKT treatment confirm the cases as Non-TB.

Specimen: Sputum specimens for ordinary examination by AFB and cultivation were obtained over three consecutive days. The sputum sample was digested and decontaminated with 2% sodium hydroxide and then processed for further investigation. Ziehl-Neelson acid fast staining was used to confirm the presence of AFB. Venous blood was collected from all the patients and control subjects. Blood was allowed to clot, and after centrifugation ($1000 \times g$, 10 min) the serum was separated and stored at -20°C until it was used.

Antibodies: Monoclonal antibody against Hsp16 (alpha-crystallin-like-Rv2031c, hspX), Hsp65 (Rv0440, cpn60.2, GroEL) and Hsp71 (Rv0350, DnaK), were obtained from Colorado State University, USA under the TB Research Materials and Vaccine Testing Contract (NO1-AI-75320) derived from MTB, strain H37Rv, The secondary antibody (rabbit anti rat) obtained from Bangalore Genei, Bangalore, India.

ELISA: Prior to sampling, the assay was standardized using different concentration of Hsp16 (1–1000ng/ml) in PBS (P^H 7.4). After standardization, wells of flat-bottom microtitre plates were coated with 100 µl of serum samples (1:400 dilution in PBS) of selected groups and incubated for 90 min at 37°C. The wells were then washed with PBS and blocked with 100 µl of 0.5% BSA in PBS at 37°C for 45 min. After blocking, monoclonal antibodies generated against Hsp16 or Hsp65 or Hsp71 were added to all the wells (1:5,000 dilution in PBS) and incubated at 37°C for 45 min. The wells were washed with the PBS followed by addition of 100 µl of affinity purified anti-rat IgG conjugated to horseradish peroxidase (Genei, Bangalore, India) with 1:10,000 dilution in PBS, and incubated at 37°C for 45 min. After incubation, the wells were washed extensively with PBS followed by addition of 100 µl of TMB/H_2O_2 substrate and incubated at room temperature for 10 min. The reaction was stopped with addition of 100 µl of 2.5 N H_2SO_4. The absorbance of each well was read at 450 nm OD. Each sample was tested in triplicate.

Statistical analysis

The statistical analysis was performed in SPSS V10.0 and MedCalc® Software using T test for continuous variables and Mann-Whitney for the non-parametric data analysis. Results are expressed as means of OD and 95%Cl. The yield of test, the selection of cut-off points and the comparison of three Hsps (Hsp16, 65 and 71) were performed with a Receiver Operating Characteristic Curve (ROC) analysis.

Results

The sample populations included 22 TB patients and 24 latent TB patients. There were 04 males and 18 females, with a mean age of 27.86 years (range 6 to 50 years) in TB group. In the latent TB group, there were 11 males and 13 females, with a mean age of 32.02 years (range 3 to 75 years). None of the patients were positive for anti-HIV antibodies, and none were receiving immunosuppressive drugs. Among 22 TB patients, only 2 patients had initial positive results for AFB in sputum samples. Final cultures for MTB on Lowenstein-Jensen medium obtained after 6 weeks were positive.

The results of heat shock proteins detection in serum were analyzed for all cases, expressed as OD are shown in Table 1. The mean OD for Hsp16 was 1.101 [0.495-1.904]) for latent TB significantly higher than the active group 0.892 [95%Cl: 0.437-1.203] (P = 0.0004) along with control group having mean OD of 0.755 [0.623-0.943]. The area under the ROC curve for latent TB by Hsp16 detection was 0.755 [95%Cl 0.606-0.869] with 77.27% sensitivity and 75% specificity for a cut-off value of > 0.636 (Table 2). Scatter plots of the Hsp16 activity in sera from TB and latent TB patients with mean value are shown in Figure 1.

Hsp test in serum	Latent TB	Active TB	Control Group	P-value**	P-value **	P-value **
	Mean OD with range	Mean OD with range	Mean OD with range	**Latent vs Control group**	Latent vs Active TB	**Control vs Active TB**
Hsp16, mean OD (95% CI)*	1.101 [0.495-1.904]	0.892 [0.437-1.203]	0.705 [0.623-0.943]	**P= 0.0001**	P = 0.0004	**P = 0.01**
Hsp65, mean OD (95% CI)*	0.856 [0.528-1.401]	0.871 [0.585-1.105]	0.777 [0.527-1.371]	**P= 0.03**	P = 0.5582	**P = 0.04**
Hsp71, mean OD (95% CI)*	0.637 [0.431-1.013]	0.694 [0.435-1.218]	0.682 [0.428-1.208]	**P > 0.05**	P = 0.5886	**P > 0.05**
*95% CI: 95% confidence intervals; **Mann-Whitney test; OD = Optical Density						

Table 1: Distribution of Hsps across each study group.

Table 1 shows the occurrence of Hsp65 antigen in sera from the TB and latent TB groups as determined by the ELISA method along with mean absorbance. The mean OD was almost equal (0.871 [0.585-1.105]) for active TB as compared to latent cases 0.856 [0.528-1.401], P = 0.5582. The mean OD for control group was 0.777 [0.527-1.371]. Scatter plots of the Hsp65 activity in sera from TB and latent TB patients with mean value are shown in Figure 2. The area under the ROC curve for latent TB by Hsp65 detection was 0.55 [95%Cl 0.397-0.697] with 59.09% of sensitivity and 62.5% of specificity for a cut-off value of > 0.825 (Table 2).

Serum from both the TB and latent TB groups were also probed with antibodies against the Hsp71 antigen, using the ELISA method along with mean absorbance (Table 1). The results for Hsp71 are corresponding with the Hsp65 as no significant difference was noted in the two study groups. The mean absorbance for active TB is 0.694 [0.435-1.218] and 0.637 [0.431-1.013] for latent patients with P value of 0.5886. The mean OD for control group is 0.682 [0.428-1.208]. The area under the ROC curve for latent TB by Hsp71 detection was 0.546 [95%Cl 0.393-0.694] with 31.82% sensitivity and 83.33% specificity. For a cut-off value of > 0.75 (Table 2). Scatter plots of the Hsp71 activity in sera from TB and latent TB patients with mean value are shown in Figure 3.

Discussion

In absence of specific test for latent TB, majority of patients remain undiagnosed and serve as a pool from which active cases of TB are drawn annually. Secondly 5–10% of recently exposed individuals

Figure 2: Expression of Hsp65 across latent (n=24) and active (n=22) TB groups. The average OD value is shown with a bar along the group.

Figure 3: Expression of Hsp71 across latent (n=24) and active (n=22) TB groups. The average OD value is shown with a bar along the group.

	Hsp16	Hsp65	Hsp71
Area under the ROC curve (AUC)	0.755 [95% CI 0.606-0.869]*	0.55 [95% CI 0.397-0.697]*	0.546 [95% CI 0.393-0.694]*
	(cut-off>0.636)	(cut-off>0.825)	(cut-off>0.75)
Sensitivity	77.27% [95% CI 54.6-92.1]*	59.09% [95% CI 36.4-79.3]*	31.82% [95% CI 13.9-54.9]*
	(cut-off>0.636)	(cut-off>0.825)	(cut-off>0.75)
Specificity	75.00% [95% CI 53.3-90.2]*	62.5% [95% CI 40.6-81.2]*	83.33% [95% CI 62.6-95.2]*
	(cut-off>0.636)	(cut-off>0.825)	(cut-off>0.75)
*95% CI: 95% confidence intervals, ROC: receiver operating characteristic			

Table 2: Diagnostic accuracies for ELISA of each heat shock protein.
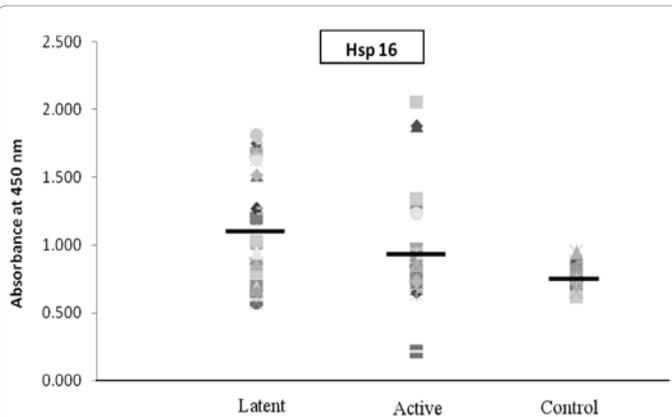

Figure 1: Expression of Hsp16 across latent (n=24) and active (n=22) TB and control (n=16) groups. The average OD value is shown with a bar along the group.
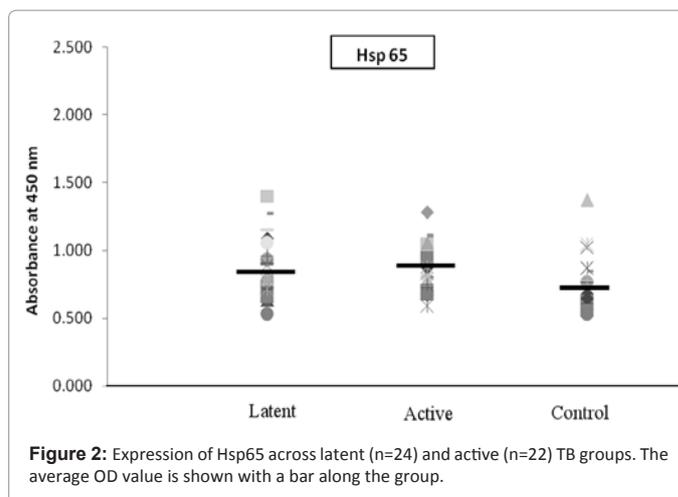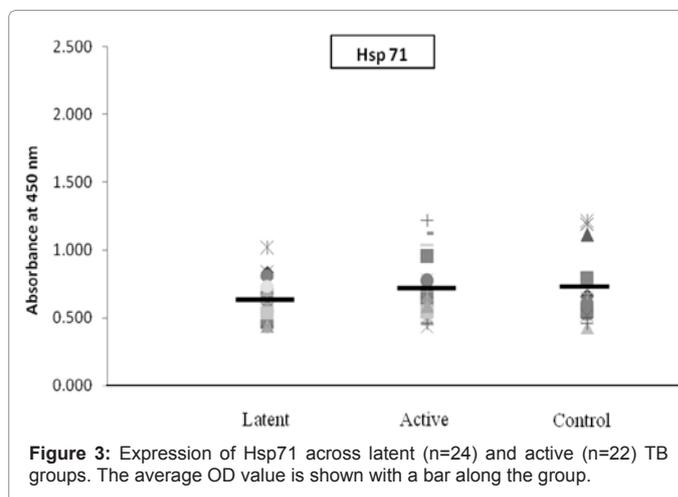
develop clinically active TB in the first two years after exposure, together with the often casual nature of exposure; makes diagnosis of latent TB extremely difficult among recently exposed and potentially infected individuals [14]. In our laboratory we have identified few cases of latent TB infections which developed into active TB within one and half year of tenure (personal observations). This stresses not only the development of diagnostic tool for latent TB and but also to perform differential diagnosis with active infections.

To the best of our knowledge, this is the first published study, where we are evaluating Hsp16, Hsp65 and Hsp71 as a biomarker for latent TB in adult population.

Hsp are believed to play a role in the pathogenesis of TB. Earlier various workers have reported the diagnostic value of Hsp in serum samples of pulmonary TB [11,18,19]. Similarly, elevated levels of Hsp have also been noted in patients with cerebral TB [15]. It is not surprising that due to their wide distribution and their homology among different species, Hsp represent target antigens of the immune response [20,21]. In the present study, we have assessed the role of Hsp in Latent TB infection patients.

We have shown among all the Hsps which were used in the present study, expression of Hsp16 is elevated latent TB cases as compared to active TB and control population, demonstrating its potential as a

biomarker for latent TB in adult population. In earlier studies, using *in vivo* models various scientists have demonstrated that it is the most abundant protein found in latent state of MTB Infection [16,22]. Similarly Yanmin et al. [23], have proposed that Hsp16 plays an active role in slowing the growth of MTB *in vivo* immediately following infection. Demissie et al. [24], have shown that bacteria which are Hsp16 deficient showed reduced ability to grow inside the macrophages while reactivation revert its expression back to the low level. There are also many studies which suggest that maximum expression of Hsp16 is found in stationary phase of MTB growth [22]. These all evidences make this protein a strong candidate in the list of markers of latent infection.

Similarly the Hsp65 and 71 were also evaluated in the same set of samples active and latent infection. The expression of Hsp65 was elevated in both latent and active cases in comparison to control population. On the other hand Hsp71 level was not raised in either latent or TB cases. Overall, this finding of our study suggests that Hsp71 is not helpful in diagnosis of latent TB while Hsp65 can be used as potential markers for latent TB diagnosis but useful in differential diagnosis of latent from active TB. However, Hsp16 is expressed significantly high in latent TB groups as compared to active TB and control population. With this results it seems that Hsp16 can be used for differential diagnosis of latent TB with active TB However, we are not able to make solid conclusion unless we will evaluate this in high number of samples.

Earlier several others markers have been identified and evaluated for the latent TB diagnosis. Whittaker et al, have shown elevated level of IP-10 following MTB antigen specific stimulation of whole blood in both active and latent TB cases [25]. Similarly Ravn et al., have suggested monocyte derived chemokines inducible protein (MCP-10) as biomarkers for latent TB [26]. All the above mentioned biomarkers have diagnostic potential however like IFN-gamma and Hsp65, these markers does not distinguish between active and latent TB.

Conclusion

In conclusion, we have evaluated Hsp16, Hsp65 and Hsp71 for Latent TB. Among these**,** Hsp16 appears the most promising marker for latent TB as its expression in very high in latent TB subjects as compared to active TB and control population. Additional studies with high number of Latent TB subjects recommended determining the sensitivity and specificity of this potential protein as latent TB specific markers.

Acknowledgement

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