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(54) Title: A METHOD OF DIAGNOSING TUBERCULOSIS

(57) Abstract: The present invention relates to a method of diagnosing tuberculosis in a subject, said method comprising steps of: detecting anti-Mycobacterium tuberculosis (M.tb.) isocitrate dehydrogenase (ICDs) antibody in the subject, and diagnosing tuberculosis in the subject.

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A METHOD OF DIAGNOSING TUBERCULOSIS

FIELD OF THE INVENTION

INTRODUCTION

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains a major threat to human population, roughly responsible for 2 – 3 millions deaths every year worldwide (1 - 3). The secret of the pathogen's success is its ability to escape the host immune system and remain undetected in lungs for decades. Only in 10% of the infected people, the number being higher in immuno-compromised patients, TB erupts as a full-blown disease (4). Delay in diagnosis and treatment impedes the downstream management and control of the disease. With the increasing emergence of multi drug resistant strains and co-infection with HIV the problem is getting further compounded (5 - 7). Early diagnosis, therefore, is a matter of utmost concern not just for TB disease management but also for epidemiological investigations (8). Current diagnostic tools for tuberculosis often lack sensitivity and can be time consuming. TB diagnosis in developing countries largely banks upon tuberculin skin test and staining and culture methods. The epidemiological relevance of tuberculin test with purified protein derivative (PPD) is questionable in areas where BCG vaccination is compulsory because PPD is not sensitive enough to distinguish between vaccinated and infected individual (9). Microscopic determination of the bacilli in the sputum samples is a direct way of examining pulmonary tuberculosis (5). This however requires high titers of bacilli (5000 – 10000 / ml) in sputum – a condition seen only in full blown tuberculosis patients. Culture techniques can detect very low titers but are time consuming taking approximately 3 – 6 weeks (10).

The importance of the major extracellular proteins of the pathogen as candidate components of a subunit vaccine has been reported earlier (11). Current discovery of the RD1 locus in the *Mtb* genome, encoding mainly the proteins actively secreted by mycobacteria into the culture medium, such as CFP-10 and ESAT-6, have further encouraged immunological tests as an adjunct to conventional diagnosis (12-15). Proteins that are released from *Mycobacterium tuberculosis* during late

logarithmic growth phase, such as, superoxide dismutase and isocitrate dehydrogenase are employed as autolysis markers (16). The use of isocitrate dehydrogenase as a potential antigen for serodiagnosis along with malate dehydrogenase has been suggested (17, 18). The *Mycobacterium tuberculosis* genome carries two isoforms of isocitrate dehydrogenase, *M.tb* ICD-1 and *M.tb* ICD-2. Multiple sequence alignment revealed a closer similarity of *M.tb* ICD-1 to eukaryotic $NADP^+$ dependent ICDs, while *M.tb* ICD-2 groups with bacterial ICDs (manuscript in preparation). We have evaluated the utility of ICDs as immunogenic markers for tuberculosis through detection of anti-*M.tb* ICD antibody in sera of different well characterized categories of TB patient through enzyme linked immunosorbent assays. We describe the sensitivity and specificity of ICDs to distinguish TB patients from those vaccinated with BCG, and also from those patients infected with non-tuberculous mycobacteria or other pathogens *vis-à-vis* the conventional antigen – HSP 60 (19) and purified protein derivative (PPD).

MATERIALS AND METHODS

Cloning, expression and purification of *M.tb* ICD-1 and *M.tb* ICD-2: The ORFs, corresponding to *M.tb* ICD-1 (Rv3339c, 1.230 kb) and *M.tb* ICD-2 (Rv0066c, 2.238 kb) were PCR amplified from the genomic DNA of H37Rv. *Bam*HI and *Hin*dIII restriction sites were incorporated in the 5' end of forward and reverse primers respectively for both *M.tb* ICD-1 and *M.tb* ICD-2. The primers and parameters for thermal cycle amplification have been tabulated in table 1. The amplicons carrying the full length *M.tb* ICD-1 and *M.tb* ICD-2 were cloned at the *Bam*HI and *Hin*dIII sites of the expression vector pRSET-A (Invitrogen, USA) with six histidine sequence tag at N-terminal. The generated constructs 'setAicd1' and 'setAicd2' were further transformed into the BL21 (DE3) strain of *E.coli*. The clones were confirmed by sequencing using the T7 promoter primer, on an ABI prism 377 DNA sequencer (PE Biosystems, USA).

The genes were over-expressed in the pRSET-A/ *E.coli* BL-21 (DE3) expression system. The over-expressed his-tagged recombinant protein was purified by Ni^{2+}-nitrilotriacetate affinity chromatography. The cells transformed with the constructs were grown in Terrific Broth (TB) containing ampicillin (100µg /ml) to an OD_{600} of 0.4 to 0.5 at 37°C, cooled to 27°C, induced with 0.1mM isopropyl β-D-thiogalactoside and grown overnight at 27°C. The cells were lysed by sonication, followed by centrifuging at 13000 rpm for 30 minutes at 4°C. The clear lysate was loaded onto Ni^{2+}-NTA column, which was then washed with 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8. The protein was eluted in the same buffer supplemented with 200mM imidazole. The proteins were 90-95% pure as seen on 10% SDS-PAGE followed by Commasie Blue staining (Figure 1). The purified recombinant proteins were dialyzed against 20mM TrisCl, pH7.5 with 100mM NaCl and 3% glycerol and quantified using Bradford Reagent (20).

Human Sera: The study population (n= 215) comprised of the *M.tb* infected human sample population reporting to Mahavir Hospital and Research Centre, Hyderabad and Central JALMA Institute for Leprosy, Agra. These were categorized into three groups, namely group 1 (n=42 patients), group 2 (n=32 patients) and group 3 (n=35 patients). In addition to the above 44 clinically healthy donors, 30 NTM cases and 32 non – TB patients who were proven culture negative for acid fast bacteria were also included as controls in this study. Group 1 comprised of patients with fresh infection with no history of TB treatment. Group 2 comprised of patients with relapsed cases, i.e. those who were treated earlier for tuberculosis but the symptoms re-emerged after the completion of the treatment. Group 3 included patients with extra-pulmonary tuberculosis. Group1 and group 2 patients were diagnosed by sputum examination (acid-fast bacillus smear positive and negative) while the extra-pulmonary cases were confirmed by tissue biopsy. Clinically healthy donors were *M. bovis* BCG vaccinated and had no

symptoms of TB at the time of sera collection. Randomly picked individuals from the population of healthy controls were subjected to PCR test for TB and were found to be PCR negative. Mycobacteria other than *M. leprae* that are not included in the *M. tuberculosis* complex are referred to as nontuberculous mycobacteria or NTM (21). However, the group referred to as NTMs in this study included sera collected from patients infected with non-tuberculous mycobacterial species (n= 14), such as, *M. avium*, *M. xenopi*, and *M. fortuitum* as well as sera from patients with *M. leprae* infection (n= 16). The non - TB patient category included infected individuals who were tested negative for acid fast bacteria by staining and culture based techniques. These patients were also negative for HIV and HBV. These randomly picked patients were suffering from either pneumonia, lower respiratory infections, septicemia, urinary tract infections, gastrointestinal infections, cirrhosis or fever of unknown origin. The study population had no sex or age bias. This study was approved by the Institutional Ethics Committee.

Immunosorbent assays: Enzyme linked immunosorbent assays (ELISA) were performed to check the B cell immune response in human to the *M.tb* ICD-1 and ICD-2 proteins and control antigen HSP 60 and PPD. HSP 60 used was *M. tb* HSP65/GroEL. In brief, the 96 well microtitre plates (Corning, Costar, USA) were coated with ~500ng of either control antigens or recombinant *M.tb* ICD-1 and *M.tb* ICD-2. The plates were incubated overnight at 4°C, washed thrice with phosphate buffer saline (PBS) and blocked with 100 µl of blocking buffer (2% BSA in PBS) for 2 hour at 37°C. The plates were then washed thrice with wash buffer PBST (0.05% Tween 20 in 1 X PBS). The *M. tuberculosis* infected human sera belonging to different clinical groups were diluted 200 times in blocking buffer (1% BSA in PBS). 50 µl of sera were added to antigen coated wells followed by incubation for 1hr at 37°C. The plates were thoroughly washed with PBST and further incubated with anti-human IgG-horseradish peroxidase (HRP)

(Sigma, USA) at 37°C for 1hr. HRP activity was detected using a chromogenic substance *o*-phenylenediamine tetrahydrochloride (Sigma, USA) in citrate-phosphate buffer (pH 5.4) and H_2O_2 (Qualigens, India) as 1 µl/ml. The reactions were terminated using 1N H_2SO_4, and the absorbance values were measured at 492 nm in an ELISA reader (BioRad, USA). Each ELISA was repeated at least twice with some randomly picked sera samples tested thrice for confirmation, with and without replicates for each sample within individual ELISA.

Data analysis: t-test was performed to compare the means of two variable groups, healthy and infected classes, using the online scientific calculator of GraphPad (http://www.graphpad.com/quickcalcs/ttest1.cfm) to calculate means, standard error of means (SEM) and p values.

RESULTS

Expression and purification of *M.tb* ICD-1 and *M.tb* ICD-2

The over-expressed N-terminal His-tagged *M.tb* ICD-1 was purified to 95% homogeneity on a Nickel affinity column (Figure 1). The molecular size of the recombinant ICD-1 was determined to be 49.2 kDa. The purification was carried out under native conditions from soluble fraction with an yield of 3.25 mg protein per 500 ml of start culture. Similarly *M.tb* ICD-2, a 83 kDa protein, was purified to 90-95% homogeneity (Figure 1) with an yield of about 20.4 mg per 1000 ml of start culture.

***M.tb* ICD-1 and *M.tb* ICD-2 show high reactivity to patient sera as opposed to BCG-vaccinated healthy controls**

Humoral immune responses directed against the *M.tb* ICD-1 and *M.tb* ICD-2 were compared between patients with tuberculosis and BCG-vaccinated healthy controls (Figure 2A and 2B). The recombinant proteins were used to screen the infected and the healthy sera by ELISA using anti-human IgG-HRP as conjugates. The sera were also tested

against *M.tb* HSO 60 and the purified protein derivative (PPD) (Figure 2C and 2D). The immunoreactivity of ICD-1, ICD-2, HSP 60 and PPD were statistically analysed and compared with respect to both infected and healthy sera. These data demonstrate that sera of all the infected patients mounted a statistically significant ($p < 0.0001$) antibody response against recombinant *M.tb* ICD-1 and *M.tb* ICD-2 proteins as compared to that of the healthy controls. PPD, on the other hand, reacted against both healthy as well as TB infected sera. It is interesting to note that as compared to ICD-1 and ICD-2 ($p < 0.0001$) the difference in the reactivity of PPD to total infected and healthy sera was negligible and statistically insignificant ($p = 0.2301$). Since PPD, a mixture of proteins, showed statistically insignificant discrimination between healthy and different categories of infected population (Figure 2D), the reactivities of the recombinant proteins were compared with the B-cell response to *M.tb* HSP 60 (Figure 2C) in different categories of patients. The difference between reactivity to HSP 60 between TB patients and healthy controls was statistically not quite significant ($p= 0.0645$).

A correlation between reactivity against *M.tb* ICD-1 and *M.tb* ICD-2 in patient sera with the state of disease, fresh or relapse, was attempted by comparing the antibody responses to *M.tb* ICD-1 and *M.tb* ICD-2 between various clinical categories (Figure 2A and 2B respectively). *M.tb* ICD-1 failed to discriminate between fresh, relapsed and extra-pulmonary TB cases as no significant differences in immunoreactivity in different patient groups were observed (Figure 2A). Yet as compared to BCG-vaccinated healthy controls, each category yielded p values less than 0.0001 indicating that *M.tb* ICD-1 can differentiate substantially between BCG-vaccinated healthy population and any category of *M.tb* infected patients, pulmonary or non-pulmonary. *M.tb* ICD-2, on the other hand, could also discriminate relapsed cases from both fresh infections ($p< 0.0001$) and extrapulmonary infections ($p= 0.0003$). Like *M.tb* ICD-1, *M.tb* ICD-2 could also distinguish substantially between BCG-vaccinated

healthy population and any category of *M.tb* infected patients. Surprisingly, HSP 60, even though,. could discriminate Group1 and Group 2 from healthy controls (p= 0.0011 and 0.0036 respectively), failed to distinguish the extrapulmonary infections from BCG vaccinated healthy controls (p= 0.2177). These results demonstrate that (i) recombinant *M.tb* ICD-1 and ICD-2 proteins could differentiate sera from TB infected patients vis-à-vis healthy BCG vaccinated controls, (ii) the extrapulmonary infections that could not be distinguished from healthy controls by HSP 60, could be significantly identified and categorized by *M.tb* ICDs and (iii) *M.tb* ICD-2 mounted a stronger antibody response in relapsed cases and could significantly discriminate them from Group 1 and Group 3 categories. These proteins, which have an apparently important metabolic role, are thus able to elicit a strong B-cell response as a function of the TB infection.

Immunodominace of ICDs over HSP 60

We compared the immunogenicity of ICDs over HSP 60. Humoral response to HSP 60 in all the three categories of TB patients was tested and compared with those to ICDs (Figure 3). The data clearly indicate that the mean reactivity (represented by the horizontal bands in Figure 3) of HSP 60 in all the classes of patient sera was much lower than either ICD-1 or ICD-2 (Figure 3). Thus ICDs are immunodominant and serologically more sensitive than HSP 60. The mean values for ICD-1 in the Groups 1, 2 and 3 were 0.481, 0.565 and 0.457 respectively, while those for ICD-2 were 0.165, 0.362 and 0.188 respectively. It is therefore apparent that ICD-1 elicited a stronger response in all the three categories of patients tested than ICD-2. The data also confirm the discriminatory power of ICD-2 for relapsed case as compared to other categories.

Immunospecificity of *M.tb* ICDs

The potential of *M.tb* ICDs to specifically distinguish between TB, NTMs and non-TB patient sera (those essentially culture negative for acid fast bacteria but harboring other pathogens) was tested by examining the cross-reactivity of the recombinant proteins with NTMs and non-TB patient sera. Thirty NTMs and thirty two non-TB patient sera were tested for their immunogenic response against *M.tb* ICD-1, *M.tb* ICD-2 and HSP 60. The data were statistically analyzed to check if ICDs could significantly distinguish between TB infected patients and NTMs or non-TB patients. Figure 4A and 4B show that ICDs could significantly distinguish TB-infected sera from NTMs ($p < 0.0001$) and non-TB ($p < 0.0001$ for ICD-1 and $p = 0.0008$ for ICD-2), thus ruling out any cross reactivity with non-tuberculous mycobacteria and other bacterial pathogen tested. HSP 60 appeared to react more broadly to the population under study and could not differentiate between TB infections and NTMs ($p = 0.4461$) or non-TB ($p = 0.3464$) significantly. This was apparent by calculating the average reactivity for each group; infected, NTMs and non-TB; where reactivity to HSP 60 remain almost the same (Figure 4A and 4B). This is in contrast to mean humoral response to ICD-1 and ICD-2, where a distinctly higher response was seen in TB infected sera as compared to NTMs or non-TB cases.

DISCUSSION

The main objective of our study was to evaluate *M.tb* ICD-1 and *M.tb* ICD-2 in terms of their immune features as compared to the control antigens HSP 60 and PPD that are ferquently used for diagnosis of tuberculosis. ICDs serve as marker of autolysis (16, 17) and are amongst the secretory proteins released during late logrithmic phase. While earlier efforts have pointed to the antigenic potential of *M.tb* ICDs (17), the present study is the first systematic investigation of their potential as an immune marker.

The cases of tuberculosis were identified and enrolled based on their history of treatment as fresh infections, relapsed cases and

extrapulmonary infections. The categorization of patients largely depended upon the treatment history dictated by the patients or their family members. As evident from Figure 2, there is little doubt about the ability of either *M.tb* ICD-1 or *M.tb* ICD-2 to elicit a strong B-cell response, irrespective of patient category. When compared to *M.tb* ICD-2, *M.tb* ICD-1 was more antigenic (Figure 2 and 3). It would be interesting to explore this disparity. A comparative analysis of ELISA reactivities amongst different categories of patients for *M.tb* ICDs (Figure 2A and 2B) revealed higher reactivity in the Group 2 as compared to fresh (Group 1) and extrapulmonary (Group 3) infections. More specifically, the antigenic response in this category of patients to ICD 2 was significantly higher than that in Group 1 and Group 3. Since, these patients had undergone treatment earlier, high number of autolysed infected macrophages and autolysed pathogens could possibly explain the comparative high antibody response against *M.tb* ICDs in this category. Drugs, like isoniazid, are known to affect the cell envelope architecture of mycobacteria and hence the increase in the production of the secreted proteins (22).

Comparative immunoreactivity of *M.tb* ICD-1, *M.tb* ICD-2 and HSP 60 clearly indicates that the antigenic distinction between healthy and tuberculosis patients is statistically significant for both *M.tb* ICD-1 and *M.tb* ICD-2 ($p < 0.0001$), but not quite so for Hsp60 ($p = 0.0645$). Earlier reports have shown cross-reactive epitopes between microbial HSP60/65 and human HSP60, which often serve as autoimmune targets in conditions like atherosclerosis (19). This probably could explain the broader reactivity of HSP 60 to healthy and infected sera. Since negligible antibody responses were obtained in the BCG-vaccinated healthy control group as compared to TB infected patients and a statistically significant difference in the immunoreactivity between infected and healthy sera was observed, it can be argued that *M.tb* ICD-1 and *M.tb* ICD-2 can be used for diagnosis of *M. tuberculosis* infection even in areas where BCG vaccination is routinely followed. The poor performance of PPD can be

attributed to its non-specific immune reaction in BCG-vaccinated healthy controls. Interestingly, the extrapulmonary TB cases (Group 3) in the study population did not show any significant humoral response to HSP 60 to distinguish them from healthy controls. On the other hand, Group 3 patients mounted a very significant B-cell response to ICD-1 and ICD-2, separating them from BCG vaccinated healthy controls.

Immunodominance is a parameter that we defined to compare the antibody titers against the tested proteins, i.e, ICD-1, ICD-2 and HSP 60, in the patient sera. Our data clearly showed that ICD-1 was most antigenic and mounted a very strong B-cell response in all the patient categories, followed by ICD-2 and HSP 60 (Figure 3). Having shown that *M.tb* ICDs elicit a B-cell response much higher than HSP 60, we checked for immune specificity of these proteins. Cross-reaction with sera of NTMs and non-TB patient is one of the critical parameters that needed to be checked before establishing any antigenic marker for possible serological studies in *M.tb. Mycobacterium tuberculosis* complex, including *M. bovis* and *M. africanum*, is responsible for more illness worldwide than any other bacteria. However, there are more than 82 recognized species of mycobacteria that occasionaly infect mammalian hosts. These are referred to as nontuberculous mycobacteria (NTM). NTMs are omnipresent in the environment and most species are either non-pathogenic for humans or are rarely associated with disease, except a few like *M. avium*, that are opportunistic pathogens, more frequently associated with immunocompromised patients (23, 24). The clinical significance of many NTM remains unclear, however it is important to check the crossreactivity of *M.tb* antigens with this group of mycobacteria. Our experiments could establish that *M.tb* ICDs do not cross-react with either NTMs or non-TB patient sera (Figure 4A and 4B).

The existing diagnostic tests for tuberculosis, even to this day, largely depends on tuberculin skin test and staining and culture techniques. These methods are slow, cumbersome and lack sensitivity and specificity

in BCG vaccinated cases. As more and more recombinant antigens are being tested (25 - 31) serological methods are likely to be favoured over others. ELISA *per se* is unlikely to replace the current tuberculosis diagnosis, however in combination or parallel with other rapid PCR based diagnostic techniques, ELISA can largely improve the accuracy and rapidity of tuberculosis diagnosis for an effective disease management. Our data, for the first time, reveal the antigenic potential of recombinant *M.tb* ICD-1 and also present a systematic study on immunogenicity of recombinant *M.tb* ICD-2. *M.tb* ICD-1 and *M.tb* ICD-2 can be further analyzed for their pathogen specific antigenic epitopes. Given their important role in the energy cycle, we are currently evaluating these two enzymes of *M.tb* as possible drug targets. That such important enzymes can also have strong antigenic attributes which enable them to significantly discriminate between BCG-vaccinated healthy controls and TB patients and at the same time TB from other pathogenic infections is a very exciting and novel proposition possibly pointing to their immunomodulatory function.

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LEGENDS

Table1: PCR primers and thermal cycle parameters for amplification of *M.tb* ICD-1 and ICD-2

Figure 1: Affinity purification of *M.tb* ICD-1 and *M.tb* ICD-2. Histidine-tagged recombinant protein was purified by nickel column chromatography under native condition and stained with Coomassie Blue following electrophoresis on 10% SDS polyacrylamide gels. The different lanes are: lanes 1 and 2: *M.tb* ICD-2; lane M: protein molecular size markers (200 kDa, 116 kDa, 97 kDa, 66 kDa, 45 kDa, 31 kDa and 21.5 kDa); lanes 3 and 4: *M.tb* ICD-1.

Figure 2: *M.tb* ICD-1 and ICD-2 show high B-cell reactivity to sera from TB infected patients from different groups as opposed to BCG vaccinated healthy controls. The humoral immune responses directed against the recombinant proteins, *M.tb* ICD-1 (2A) and *M.tb* ICD-2 (2B) and control antigens HSP 60 (2C) and PPD (2D) were compared between different categories of patients and healthy controls. Group 1: fresh infections, Group 2: relapsed infection and Group 3: extrapulmonary TB. The respective sample numbers and p values are shown.

Figure 3: *M.tb* ICDs are more immunogenic than HSP 60. The ELISA reactivity to *M.tb* ICD-1, *M.tb* ICD-2 and control antigen HSP 60 was compared in different patient groups. Horizontal bands represent the mean reactivity or average levels of humoral response in each category.

Figure 4: *M.tb* ICDs could significantly distinguish TB-infected sera from NTMs and non-TB patient sera. Recombinant *M.tb* ICD-1 and *M.tb* ICD-2 as well as HSP 60 were tested against sera of NTM (4A) and non-tuberculosis patients (4B). The respective humoral responses were compared to TB – infected sera, the p values for which are given in the figures. HSP 60 could not distinguish TB-infected patient from either NTM or non-TB significantly. Horizontal bands represent the mean reactivity in each category.

Table 1: PCR primers and thermal cycle parameters for amplification of *M.tb* ICD-1 and ICD-2

Primers	Sequence	PCR Parameters	Amplicon Size
*M.tb icd-1*FP	ggatccATGTCCAACGCACCCAAGATA	94°C for 2'	~1.2 Kb
*M.tb icd-1*RP	aagcttCTAATTGGCCAGCTCCTTTTC	(35 Cycles)	
		94°C for 30"	
		50°C for 1'	
		72°C for 3'	
		72°C for 7'	
M.tb icd-2 FP	AGCTTggatccATGAGCGCCGAACAGCC	94°C for 2'	~2.23Kb
M.tb icd-2 RP	CATGGaagcttTCAGCCTTGGACAGCCT	(10 Cycles)	
		94°C for 30"	
		50°C for 30"	
		72°C for 3.30'	
		(25 cycles)	
		94°C for 30"	
		58°C for 30"	
		72°C for 3.30'	
		72°C for 7'	

Claims

1. A method of diagnosing tuberculosis in a subject, said method comprising steps of:

    a. detecting anti-*Mycobacterium tuberculosis* (*M.tb.*) isocitrate dehydrogenase (ICDs) antibody in the subject, and

    b. diagnosing tuberculosis in the subject.

2. A method as claimed in claim 1, wherein the subject is mammal including human.

3. A method as claimed in claim 1, wherein the antibodies are detected using enzyme linked immunosorbent assays (ELISA).

4. A method as claimed in claim 1, wherein the ICDs are antigenic in two isoforms ICD-1 and ICD-2.

5. A method as claimed in claim 1, wherein the method distinguishes subject with tuberculosis from healthy BCG-vaccinated subject.

6. A method of developing drug against tuberculosis, said method comprising step of:

    a. targeting the proposed drug towards enzyme isocitrate dehydrogenase (ICD), and

    b. developing the drug against tuberculosis.

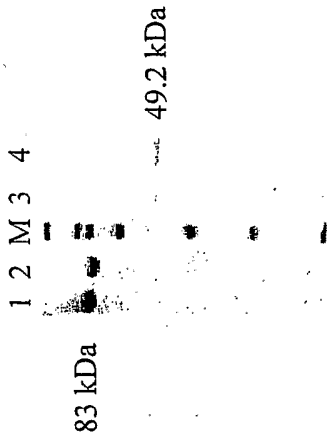

Figure 1

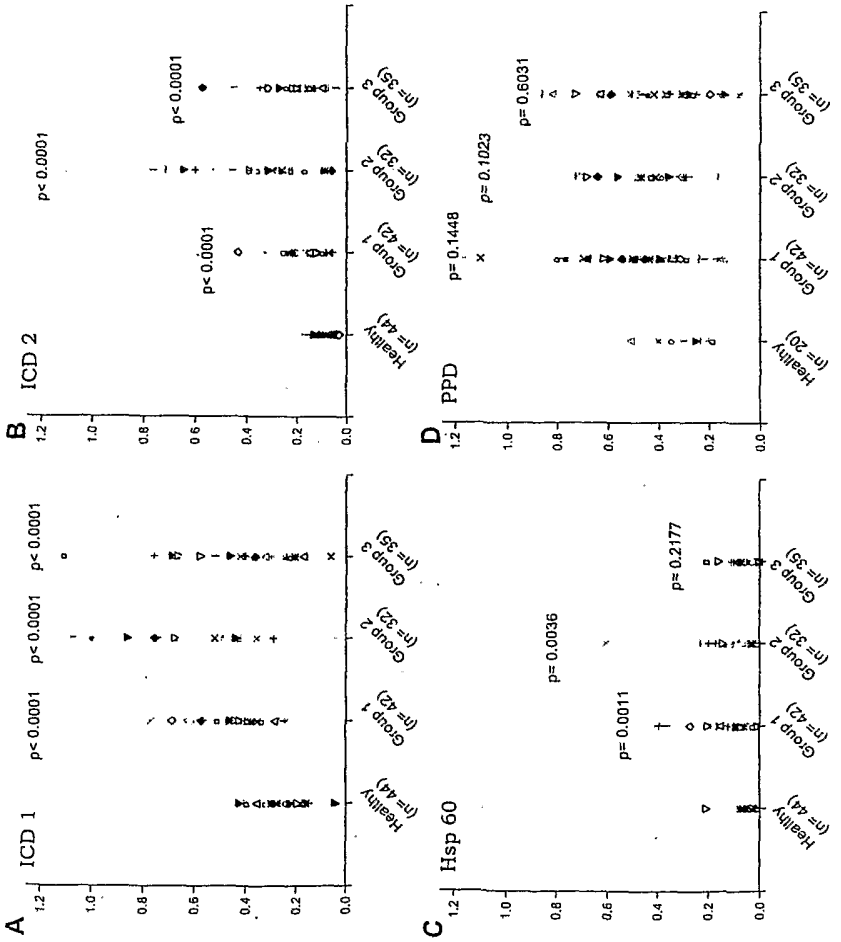
A
ICD 1
p< 0.0001
p< 0.0001
p< 0.0001
B
ICD 2
p< 0.0001
p< 0.0001
C
Hsp 60
p= 0.0011
p= 0.0036
p= 0.2177
D
PPD
p= 0.1448
p= 0.1023
p= 0.6031
Healthy (n= 44)
Group 1 (n= 42)
Group 2 (n= 32)
Group 3 (n= 35)
Healthy (n= 20)
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Figure 2

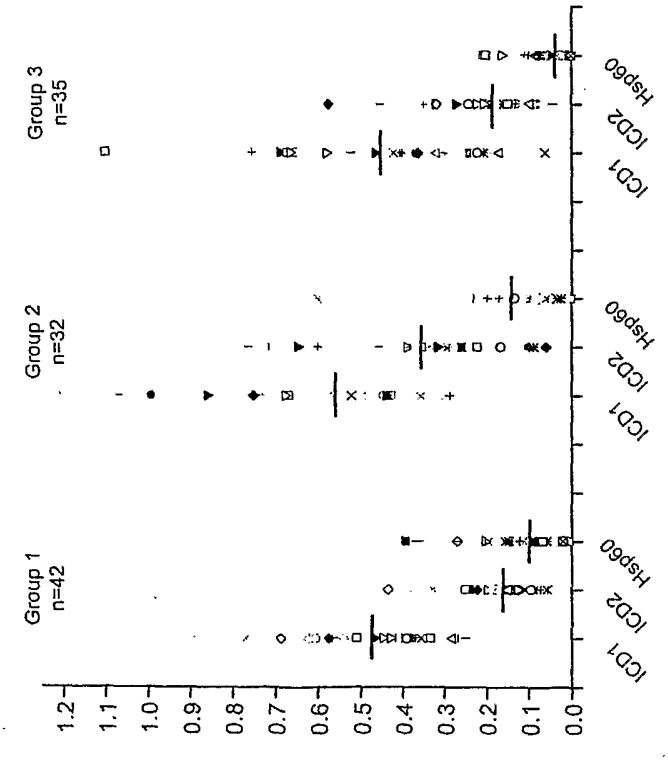
Group 1
n=42
Group 2
n=32
Group 3
n=35
ICD1
ICD2
Hsp60
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Figure 3

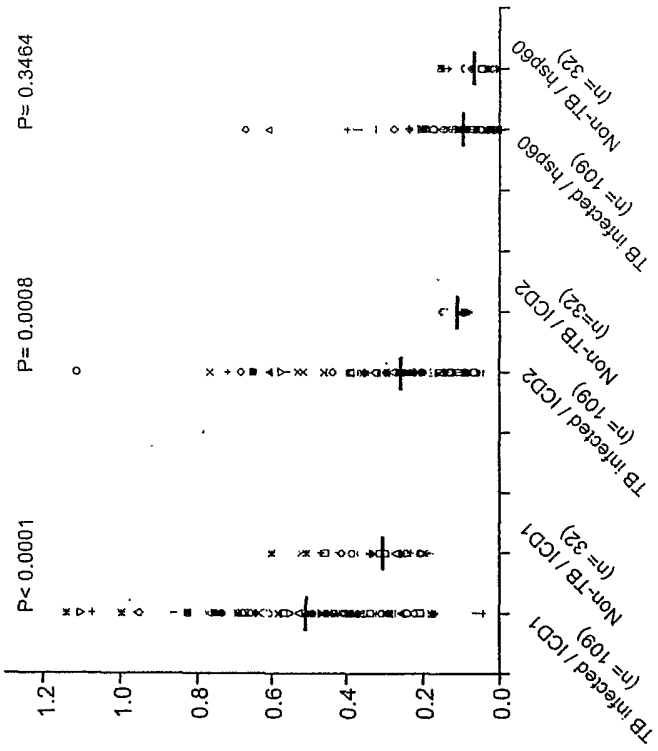

Figure 4B

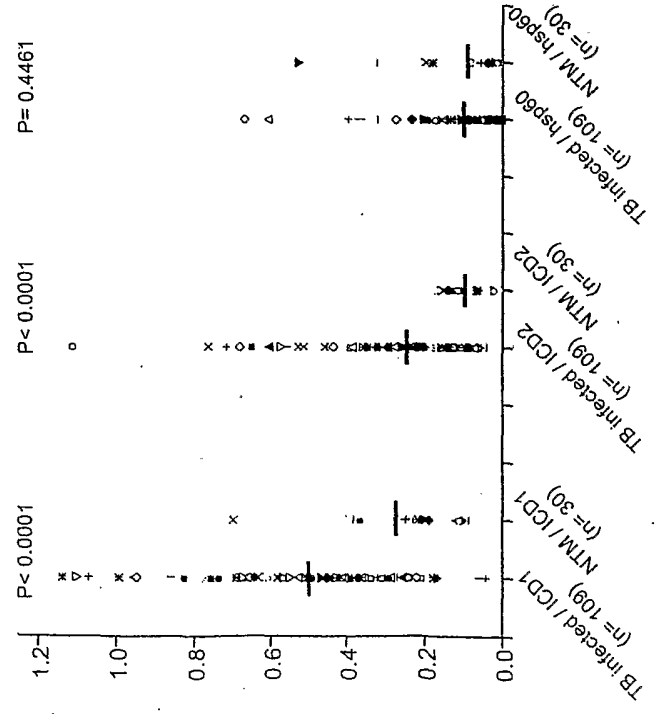

Figure 4A