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Antigen-Specific Antibody Profiles in TB-Exposed and Infected Patients: A Cohort Study from Pakistan

Hina Farzana[1*], Natasha Anwar[2]

[1]Kauser Abdulla Malik School of Life Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan
[2]Aga Khan University Hospital Lahore Outreach, Department of Laboratory and Clinical Medicine, Lahore, Pakistan
Email: *hananrana@gmail.com

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Abstract

Background: Circulating antibodies against specific *M. tuberculosis* (*M. tb*) antigens offer promising diagnostic biomarkers and an appealing alternative due to their ease of application, cost-effectiveness, and relatively non-invasive nature. This study aimed to quantify and characterize the IgG antibody response against a panel of eight *Mycobacterium tuberculosis* (*M. tb*) antigens in TB-exposed and infected patients. The overarching hypothesis was that the IgG profiles and median fluorescence intensity (MFI) against each *M. tb* antigen could potentially differentiate between pulmonary, extrapulmonary, index contacts and healthy controls in a high TB burden setting. **Methods**: A multiplex microbead immunoassay (MMIA) was used to measure the serological response in 524 blood samples against eight *M. tb* antigens namely Rv-3881, Ag85-a, Ag85-b, Ag85-c, P-38, HspX, CFP-10 and MPT-32, with antibody concentrations determined using MFI and antigen's cut off was established to determine the percentage of patients with positive antibody response for each antigen. Diagnostic accuracy of panel antigens was determined through ROC (Receiver operating characteristic) curve analysis and AUC (area under the curve) with 95% CI by using SPSS version 22. **Results**: ROC analysis demonstrated that all eight TB antigens in the panel are suitable for diagnosing TB infection in endemic areas, with an AUC ranging from 0.996 to 0.999 ($p < 0.001$) and a specificity greater than 95%. Among patients with pulmonary TB (P-TB), 61% of smear-positive (SS+ve) individuals exhibited positive IgG antibody response for antigen Ag85-a, 50% for Ag85-b, and 49% for Rv-3881 and P-38 antigens. In smear-negative (SS-ve) patients, 43% showed positive MFI for Rv-3881, 40% for Ag85-a, and 32% for P-38. Groupwise comparisons revealed significantly differential and higher IgG antibody response profiles in P-TB group (both SS+ve and SS-ve) and index TB contacts against all panel

antigens with a $p < 0.001$ except for HspX in SS-ve patients compared to controls with lower median MFIs. The extrapulmonary TB (EP-TB) group exhibited significantly higher IgG response levels against Rv-3881 (Md = 85, $p = 0.003$), Ag85-a (Md = 6, $p = 0.001$), Ag85-b (Md = 35, $p < 0.001$), P-38 (Md = 9, $p = 0.002$), CFP-10 (Md = 14, $p = 0.006$), and MPT-32 (Md = 20, $p = 0.026$) compared to controls, who showed lower MFIs for these antigens. **Conclusions**: The *M. tb* antigen-specific IgG antibody profiles, with established cut-offs, could be incorporated into current immunological tests to enable rapid and accurate diagnosis and differentiation of TB infection types in TB endemic settings.

Keywords

M. tuberculosis (*M. tb*), MMIA, Antibody Response

1. Introduction

Tuberculosis (TB) is one of the major infectious diseases, as globally WHO has estimated in year 2022 about 10.6 million cases with 1.6 million deaths due to TB [1]. Pakistan, ranks fifth among high-burden countries worldwide with high prevalence, incidence and mortality rate per 100,000 population [1]-[5]. The WHO recommends controlling pulmonary TB (P-TB) and screening individuals for latent TB infection (LTBI) in endemic areas [1]. Absence of TB contact investigation leads to continuous transmission of TB/MDR strains in high burden settings [1]-[4]. In Pakistan, traditional TB detection procedures are based on 2 - 3 sequential sputum smears (microscopy), X-ray and *M. tb*. culture, are slow, inefficient and cumbersome. Though sputum-based microscopy and solid/liquid culture is the most common and traditional method of TB diagnosis, blood-based antibody assays can be an effective alternative to diagnose active TB and non-symptomatic TB (LTBI) patients who cannot expectorate/produce sputum for diagnosis in resource limited settings [6]-[13]. Interferon-gamma (IFN-γ) release assays (IGRAs) like T-SPOT assays have been widely used in clinical diagnosis to detect LTBI by using early secretory antigenic target 6 (ESAT-6) and culture filtrate protein-10 (CFP-10) but have limitations in diagnosing active infection in TB endemic areas because of low spot forming units (SFUs) threshold with higher false positive results rate [14]-[20].

B-cell responses after infection or vaccination are often measured as serum titers of antigen-specific antibodies. Blood based antibody immune assays could serve as cost effective yet efficient diagnosis tests but the challenge to look for improvement is, find a way to measure the differential response of patients against different antigens of TB. This is why, need of the time is to bring multiplex immunoassay approach like (MMIA) in practice [7] [8].

Circulating antibodies in the peripheral blood are the last but lasting host immune response against TB. Different studies have shown that well defined IgG

antibody profiles against several TB antigens acquired by using MMIA could serve as potential biomarkers for TB diagnosis [7] [8]. First MMIA validation studies for TB serodiagnosis was conducted on nonhuman primates and then field evaluation was conducted to screen P-TB in "Pakistani population" with a sensitivity and specificity of approximately 90% [9] [10]. Considering this all, current study was designed to determine, whether differentiating IgG response profiles and MFI values against multiple *M. tb* antigens in patients and index contacts could efficiently differentiate P-TB, EP-TB, TB patient contacts (HHCs) and healthy controls (HCs) in TB endemic settings of Pakistan or not. The logical reason behind this hypothesis was that higher MFIs with higher IgG response levels against different TB antigens, indicates ongoing TB infection while lower MFIs with reduced IgG antibody response levels reflects resolving/latent stage of infection.

2. Methods

2.1. Study Population

This study included a total of 524 subjects registered in Ghulab Devi and Mayo Hospital Lahore, Pakistan, divided into P-TB (Sputum Smear positive (SS+ve) = 167, Sputum Smear negative (SS-ve) = 175), extra pulmonary TB (EP-TB) = 90, household contacts (HHCs) = 40 and healthy controls (HCs) = 52. Tuberculin Skin Test (TST) and Interferon Gamma Release Assay (IGRA) T-Spot tests were conducted to screen index TB contacts. A Blood sample (10 ml) was drawn after informed and written consent of participants.

2.2. Ethical Approval

Research clinical approvals for this study were obtained from the Institutional Review Board, Forman Christian College, (A Chartered University) Lahore (IRB Ref: IRB-96/06-2018) and from Institutional Review Board, King Edward Medical University Lahore-Pakistan (No. 230/RC/KEMU).

2.3. Tuberculosis Skin Test (TST)

The TST was performed by injecting 0.1 ml of tuberculin Purified Protein Derivative (PPD) into the inner surface of the forearm and measured in millimeters of the induration across the forearm. An induration of 5 or 10 millimeters is considered positive in HIV-infected persons, recent immigrants from endemic countries, residents and employees of high-risk areas, and laboratory personnel. False-positive reactions may occur due to infection with non-tuberculosis mycobacteria and BCG vaccination, and people can be TST positive even though they are not infected/ exposed to *M. tb* [21] [22] [23].

2.4. MMIA

Multiplex Multibead assay is the advance form of ELISA, that can study more than two parameters of immune responses (Antibody/cytokine) at a time. This assay used antigen coated beads in a suspension microarray. 50 µl of bead mixture was

added along 50 µl of diluted plasma of patients and HCs to a final dilution of 1:200 in 96 well plate (Transparent bottom) and incubated in dark for (2 hours) at room temperature on a plate shaker. Plate was then fixed on magnetic washer for washing with 100 µl of PBS-T Secondary antibody, phycoerythrin conjugated antihuman IgG (Jackson Immunoresearch cat#109-116-098), was diluted and added to each well and then incubated in the dark for (1 Hour) on plate shaker. At the end of incubation final resuspension was done in 100 µl PBS-T and mixed on plate shaker for 1 - 2 minutes. The plate was then read on Luminex 100 [7] [8].

2.5. Statistical Analysis

Multiplex data was collected as median fluorescence intensity (MFI). Mann Whitney U test was used to compare response profiles of IgG antibody under non-parametric analysis (NPA) because obtained data was not normally distributed. Mann Whitney U test usually takes the median values and convert these scores into the ranks and then conclude which group is showcasing significant response against selected panel of antigens. In case of significant results, we need effect size (r) which can be calculated by z value by using following formula.

$$R = z/\sqrt{N}$$

Magnitude of effect size was decided according to Cohen's classification of effect sizes (1988, pp. 79-81) guidelines which are:

0.1 (small effect);

0.3 (moderate effect);

0.5 and above (large effect).

ROC (Receiver operating characteristic) curve analysis and AUC (area under the curve) with 95% CI analysis were used to calculate the diagnostics accuracy of each panel antigen by using SPSS version 22. SPSS was also used to calculate positive predictive value (PPV), negative predicted value (NPV), sensitivity (SN) and specificity (SP).

3. Results

Earlier validation studies of MMIA for serological diagnosis of P-TB in Pakistani population showed > 90% sensitivity with >80% specificity [9] [10]. In this study MMIA assay performance test was determined by calculating the assay baseline (cut-off) for each TB antigen of panel from MFI response values of HCs. Formula used to calculate the cut-off was

Cut-off value: Mean MFI of antigen + (3*Standard Deviation).

Patient samples having higher MFIs than calculated cut-off values were considered as positive for that particular antigen. Overall, a great number of P-TB patients showed positive IgG antibody response against all panel antigens compared to EP-TB and HHCs. In brief, individual antigen reactivity was determined by calculation of percent positivity for each group of patient category which showed that 61% SS+ve patients of P-TB group predominantly showed a positive IgG

antibody response against Ag85-a, 50% for Ag85-b and 49% for Rv-3881 and P-38 antigen, while SS-ve patients showed 43% positivity for Rv-3881 antigen followed by a positivity of 40% and 32% for antigen Ag85-a and P-38. EP-TB patients showed 33% positive IgG antibody response for antigen Ag85-a and 23% for P-38. Positive antibody response of 28% was seen against MPT-32 antigen in enrolled HHCs of active TB patient's despite of negative TST status as shown in Figure 1. One possible reason of this un-predicted higher response against MPT-32 antigen could be their recent exposure to TB (LTBI) means they are at the verge of developing active TB infection.

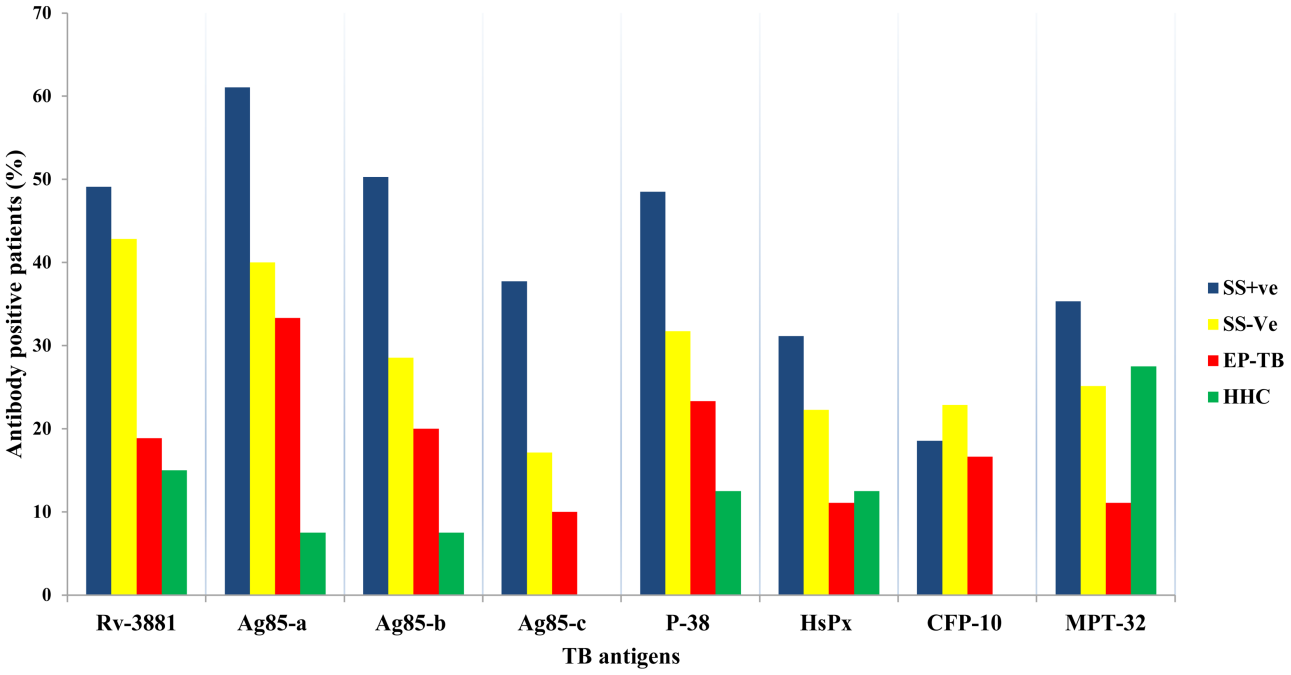

Figure 1. No. of TB patients with positive IgG antibody response of study groups against *M. tb* antigen panel. Blue bars represent SS+ve patients of P-TB group (n = 167), yellow bars represent SS-ve patients of P-TB group (n = 175), Red bars represents EP-TB patients (n = 90) and green bars represents Household Contacts (HHC, n = 40).

A cluster analysis of individual response MFIs of each group was obtained by using heatmap as shown in Figure 2. Heat map showed a separate response cluster for each study groups against TB antigens in ascending order with P-TB patients response cluster at the top and HCs response cluster at the bottom. It was observed that P-TB patient's plasma was predominately reactive to all TB antigens with higher antibody response while EP-TB group and HHCs contained lower antibody response. HCs plasma samples were found to be non-reactive against any antigen of the panel.

Quantitative response of IgG antibody ($\log_{10}$ MFI) was plotted as box and whisker plots for comparing baseline response profiles of HCs with P-TB, EP-TB and HHCs as shown in Figures 3(a)-(h).

These plots revealed that both SS+ve and SS-ve patients of P-TB group have higher MFI response values against all TB antigens with great data spread. EP-TB

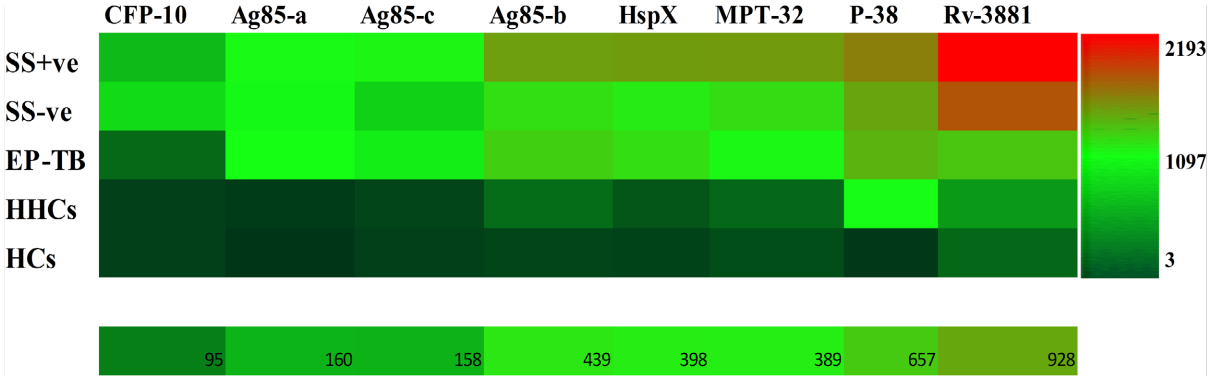

Figure 2. Clustered heat map based on avg MFI response of group samples against eight TB antigens. Signal intensity scale ranges from red as maximum to dark green as minimum antibody response.

group plasma showed maximum reactivity for Rv-3881 and Ag85-a antigen with great data spread and outliers as well. HHCs showed higher MFI against Rv-3881 and MPT-32 antigen as compared to HCs. Presence of outliers in box plots, represents presence of extreme low or higher MFI values in data.

The predominant and differential IgG response profiles of P-TB group against all *M. tb* antigens in comparison of EP-TB and HHCs could be that P-TB patients retain fast replicating bacteria, with higher antigen presentation which induce greater immune response and additionally may be B cell responses are stronger in lungs due to formation of granuloma and weaker in other parts of the body.

The MFIs and profiles of individual samples and groups suggests *M. tb* differentially modulate the host immune response during TB infection, we see a differential and positive IgG antibody profiles in all study groups in comparison of HCs. To determine, how significant this differential response was, NPA was conducted on calculated results. SS+ve patients of P-TB group showed significantly higher and differential IgG response distribution for all *M. tb* antigens except assay control total IgG with $p < 0.001$ and with moderate effect size $r = 0.4$, however SS-ve patients of P-TB group showed differential response distribution of IgG antibody against all panel antigens except HspX and assay control total IgG with $p < 0.001$, with small effect size $r = 0.2$ in comparison of HCs. EP-TB group showed significantly higher response against Rv-3881 (Md = 85, n = 142, U = 3033, z = 2.938, p = 0.003, with moderate effect size r = 0.25), Ag85-a antigen (Md = 6, n = 142, U = 3086, z = 3.399, p = 0.039, with small effect size r = 0.28), Ag85-b (Md = 35, n = 142, U = 3292, z = 4.034, $p < 0.001$, with moderate effect size r = 0.33), P-38 (Md = 9, n = 142, U = 3044, z = 3.160, p = 0.002, with small effect size r = 0.26), CFP-10 (Md = 14, n = 142, U = 2984, z = 2.750, p = 0.006, with small effect size r = 0.23) and MPT-32 (Md = 20, n = 142, U = 2863, z = 2.225, p = 0.026, with moderate effect size r = 0.19) compared to HCs with lower MFIs for Rv-3881 (Md = 40), Ag85-a (Md = .00), Ag85-b (Md = 13), P-38 (Md = 0.00), CFP-10 (Md = 5), MPT-32 (Md = 9). Plasma antibody IgG response levels of HHCs and HCs were found significantly different for all antigens except assay control total IgG with $p \leq 0.001$and moderate effect size $r \geq 0.3$.

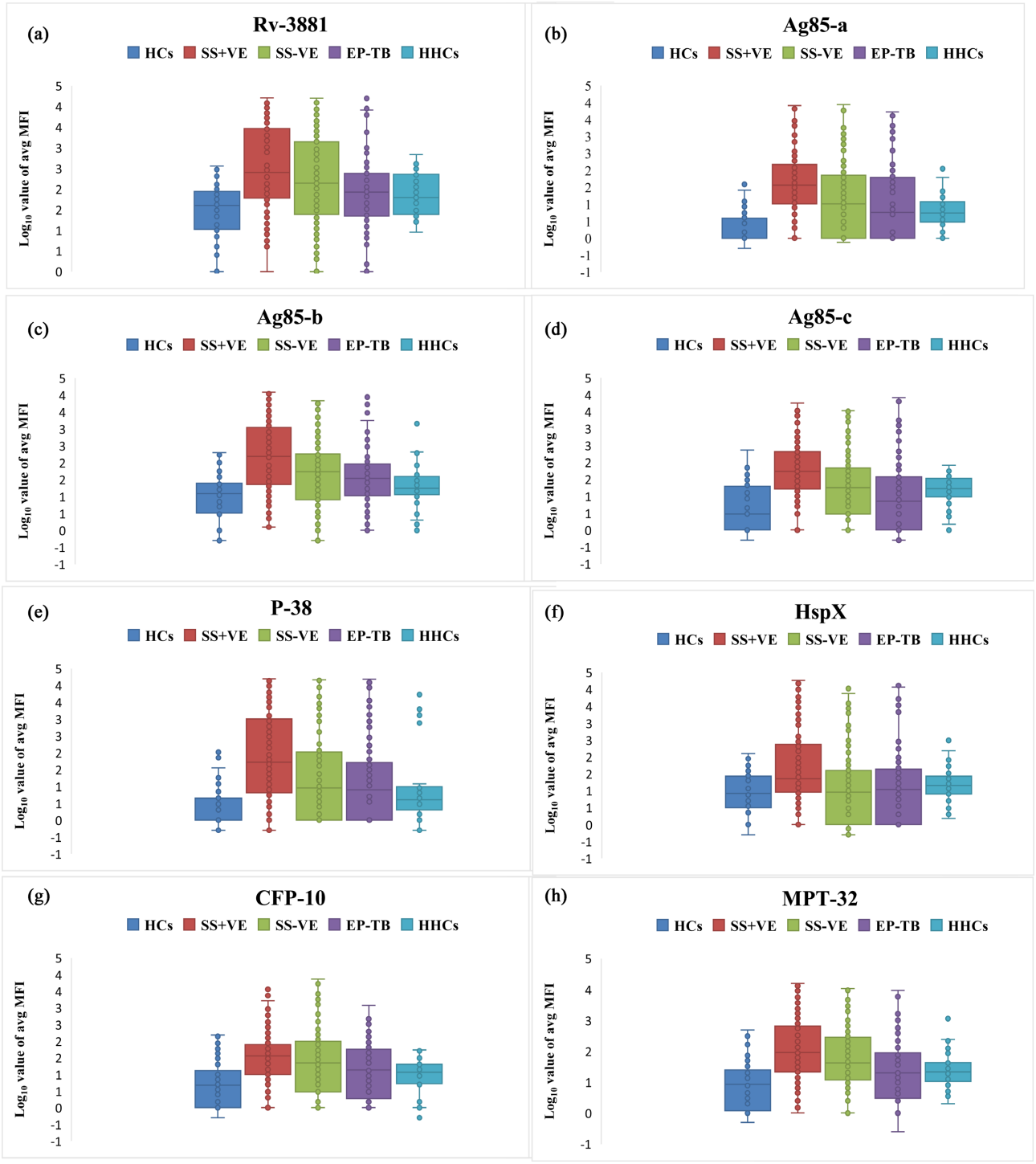

Figure 3. Box and whisker plots for average MFI response of IgG in all study groups and Healthy Controls against antigens (a) Rv-3881; (b) Ag85-a; (c) Ag85-b; (d) Ag85-c; (e) P-38; (f) HspX; (g) CFP-10; (h) Mpt-32.

Percent positive IgG antibody response profiles along p value revealed response profiles against Rv-3881, Ag85-a, Ag85-b, P-38, HspX, CFP-10 and MPT-32 could potentially differentiate P-TB (SS+ve, SS-ve), EP-TB from HHCs and baseline response profiles of HCs.

Consistent but differential profiles obtained against antigen Rv-3881, Ag85-a, Ag85-b, P-38, HspX and MPT-32 in this cohort of TB patients can also be explained by looking into the biological functions of these secretory antigenic proteins of *M. tb*. Antigen Rv-3881 is alanine and glycine rich protein which act as T cell stimuli in TB patients. Ag85-a and Ag85-b are important and newly explored diacylglycerol acyltransferases which are involved in lipid body formation of bacteria. P-38 protein is an important MAPK (Mitogen Activated Protein Kinase) signaling regulator, when expressed by the *M. tb*. inhibits several anti-microbial processes of host defense system. HspX is ubiquitous protein, produced under stressful hypoxic condition to regulate *M. tb* replication. This protein has the potential to differentiate latent TB infection from active infection and could also be promising target of new vaccine development. MPT-32 is alanine and proline rich antigenic protein involved in phagosomal capturing of bacteria [23]-[30]. Higher expression of these secretory antigens in this cohort of people reflects the strategy opted by this smart bacterium to escape the host immune system with the help of these proteins.

Diagnostic accuracy of panel antigens along SN, SP, PPV, NPV was obtained by considering three variables, first samples positive against one or more antigens, second positive for two or more antigens and last one three or more antigen positive samples. Study results revealed that one or more antigen positive variable showed a SN of 48% with 96% SP along 99% PPV and 18% NPV, two or more antigen positive variable showed a SN of 42% with 96%SP along 99% PPV and 17% NPV while a SN of 36% with 96% SP along 99% PPV and 15% NPV was seen in samples positive for three or more antigens. Diagnostic efficacy of individual antigens was determined using ROC curve analysis, summarized in **Table 1** along calculated SNs and SPs. This analysis showed that all TB antigens of panel were suitable for diagnosing TB infection in high TB burden areas like Pakistan with an AUC of antigens ranged between 0.996-0.999 with $p < 0.001$.

Table 1. Analysis of antigen panel SN, SP, PPV, NPV and ROC.

Study variables	SN* (%)	SP* (%)	PPV (%)	NPV (%)	AUC*	CI (95%)	p-value
One or more antigen positive samples	48	96	99	18	0.996	0.994 - 0.999	$p < 0.001$
Two or more antigen positive samples	42	96	99	17	0.999	0.999 - 1.000	$p < 0.001$
Three or more antigen positive samples	36	96	99	15	0.998	0.999 - 1.000	$p < 0.001$

AUC = area under the curve, PPV = positive predictive value, NPV = negative predicted value, SN = sensitivity, SP = specificity.

In this study we were able to differentiate TB infection from latent and other non-TB strains of *M. tb* based on different antigen positivity in just one day to avoid potential delay in anti-TB treatment in Pakistan which is TB endemic country.

4. Discussion

This study has shown that higher and differentiating *M. tb* antigen specific IgG

response profiles are present in TB patients of P-TB (SS+ve and SS-ve) and EP-TB group while lower response profiles are present in HHCs. Diagnostic efficacy of individual antigens, determined through ROC analysis showed that all 8 selected TB antigens for this study are capable of diagnosing TB infection with a specificity of 96% by ruling out the false positivity in TB endemic areas which is supported by the work done by Khaliq *et al.* in 2017 but with higher assay sensitivity. Heat map analysis showed heterogenous and differentiating response clusters for antigen Rv-3881, Ag85-b, P-38, HspX and MPT-32 in study groups with clear view of reactive and non-reactive response of plasma samples. Cut-off values, individual MFIs and IgG response profiles differed among HCs, P-TB, EP-TB and HHCs. Group wise comparison analysis reported significantly differential distribution pattern of IgG antibody against Rv-3881, Ag85-a, Ag85-b, P-38, HspX and MPT-32 antigens in P-TB, EP-TB and HHCs group, indicating the potential of these antigens as biomarkers for TB diagnosis and differentiation between P-TB and EP-TB, index TB patient contacts (HHCs) and HCs because higher IgG response profiles of antigens Rv-3881, Ag85-a, Ag85-b, MPT-32 are linked and indicates ongoing TB infection while P-38 and HspX are dormancy related antigenic proteins and their higher expression indicates the latent infection [23]-[30]. This study included the screening of TB contacts in TB endemic settings of Pakistan that will help in development of preventive therapies in terms of treatment to prevent transmission of TB/ MDR strains to others from viable TB contacts which in turn will help to decrease the incidence and mortality rate in Pakistan [4]. The results from this study confirm previous work done by Khaliq *et al.* in 2017 for the field evaluation of multiplex assay in P-TB patients in Pakistan with the exception that they had not included EP-TB and HHCs. In addition to this calculated sensitivity of antigen panel of 8 antigens in this study is almost half as compared to their calculated sensitivity. Probable reason of this lower sensitivity in our study could be shifting of MMIA from 11plex to 8plex TB antigen panel for this cohort. Most of the cited research proposed utility of T-SPOT in diagnosis and differentiation of active in PTB (P-TB) from LTBI but in low TB endemic settings [15]-[19]. In TB endemic countries like Pakistan, it can have higher false positive rates due to lower threshold of 6 SFUs [17] [18]. Antigen CFP-10, which is one of the T-Spot assay antigens was part of this study and data analysis showed that CFP-10 is a good antigen to distinguish P-TB, EP-TB infection from HHCs (with LTBI), because ROC and NPA showed that CFP-10 is 100% specific in diagnosing TB from non-TB infection and HCs with an AUC of 0.999 and $p < 0.001$. This additionally favors the idea of serological screening of antibody responses against multiple protein antigens rather than using single or two antigens as in IGRA assays. Multiplex analysis with stringent antigen cutoffs makes MMIA approach more suitable for accurate diagnosis and differentiation of TB infection and their contacts in comparison of IGRA tests in high burden areas. Major limitation of this cross-sectional study was small sample size of HCs and contacts of index TB patients (HHCs). Furthermore, longitudinal studies on

larger cohort of HCs, HHCs and follow-ups (FUPs) till the end of treatment could help in calculation of optimum cutoffs for TB antigens with higher assay sensitivity.

5. Conclusion

In low middle income and TB endemic country like Pakistan, traditional TB diagnosis methods and absence of TB contacts screening leads to potential delay in start of anti-TB treatment and continuous transmission of TB/MDR strains. This research findings suggest that following *M. tb* antigen specific IgG antibody response profiles with established cutoffs by using MMIA could be incorporated in current immunological tests for rapid and accurate diagnosis, differentiation of pulmonary, extrapulmonary, index TB patient contacts and healthy controls in just one day.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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