International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Development of Serodiagnostic Method with the Help of Combination of Immunodominant TB Antigens

Shalmali S. Pendse[1] Ajitha Rani R[2]

[1]Guru Nanak Khalsa College of Arts, Science and Commerce, Mumbai
Corresponding Author Email: *pendseshalmali[at]gmail.com*

[2]Guru Nanak Khalsa College of Arts, Science and Commerce, Mumbai
Email: *ajitha.rani[at]gmail.com*

Abstract: ***Introduction: Tuberculosis (TB) remains one of the world's oldest and most persistent infectious diseases, posing a significant global health challenge. The development of a more efficient and quicker diagnostic method is crucial to stopping the tuberculosis (TB) epidemic. TB infection is known to elicit a robust humoral immune response, making serological tests an attractive approach for diagnosis. In this study, we designed a combination of epitopic peptides derived from various immunodominant TB proteins for detection of antibodies directed against these primary invader sites. Method: Using ELISA, an attempt was made to assess the antibody response to peptides generated from proteins Rv1886c, Rv0934, Rv1926c, Rv1980c and Rv0538 of M. tb in TB patients and healthy individuals. Result: Antibody levels against M. tb peptides were evaluated in 80 TB patients and 150 healthy controls. TB patients showed a stronger immunological response in comparison to healthy individuals and there was a significant difference between the patients and healthy controls (P < 0.000*). Conclusion: Significant statistical differences were observed in the antibody levels against these peptides between PTB patients and healthy individuals in our investigation. These peptides may provide a novel candidate (or candidates) for the early detection of tuberculosis.***

Keywords: TB Diagnosis, Immunodominant proteins, ELISA, B cell epitopic peptides, humoral immunity

1. Introduction

Tuberculosis (TB) is a contagious bacterial disease that causes illness and is one of the top causes of death globally. Prior to the coronavirus (COVID-19) pandemic, tuberculosis (TB) was the most common infectious disease to cause death, surpassing HIV/AIDS. The COVID-19 pandemic still negatively affects both the burden of TB disease and access to TB diagnosis and treatment. Global TB objectives are not on pace, and progress made in the years leading up to 2019 has slowed, stopped, or even reversed [1].

Without treatment, the TB disease has a significant mortality rate (about 50%). Approximately 85% of patients can be treated with the currently suggested therapies (a 4-6 month course of anti-TB medications). To treat TB infection, there are regimens ranging from one to six months [1].

Eight of the 30 nations with significant TB burdens accounted for more than two thirds of the world's estimated incident cases, accounting for 87% of the total. India is a major contributor to the worldwide burden of TB, accounting for 28% of it [1].

Fast and accurate TB detection is a crucial component of global health initiatives to combat the illness. Acid-fast bacilli (AFB) cultures and sputum inspection have historically been used to diagnose tuberculosis (TB). However, in areas where tuberculosis is prevalent, mycobacterial culture facilities are typically rare, and even when they are, the results of the cultures are sometimes too late to have an impact on initial care. The sensitivity of the sputum smear for AFB is also quite low in these areas. The Mantoux Tuberculin Skin Test (TST), which employs a pure protein derivative of five tuberculin units, is a widely used method for identifying M. tuberculosis infection. The false-negative rate cannot be evaluated because TST is typically used to identify asymptomatic infection. A negative TST result does not exclude childhood TB. False-positive TST results are frequently attributed to asymptomatic environmental infection with non-tuberculous mycobacteria. TST is particularly beneficial for those who have a high risk of contracting TB infection or disease but is not recommended for those who do not. This is due to the relatively low sensitivity and specificity of the test. The QuantiFerron TB test was recently created to address some of the shortcomings of the TST, however its 12 hour processing time restriction for whole blood is a significant drawback for its use in reference lab settings. Recently, it has been discovered that not all clinical laboratories accurately execute diagnostic polymerase chain reaction (PCR), which uses specific primers as markers for M. tuberculosis and exhibits varied sensitivity and specificity. Numerous serological tests have been explored, but almost none have been able to outperform the tried-and-true sputum smear and culture method [2].

One of the methods most frequently used to identify infectious diseases is the detection of antibodies. Serological procedures are straightforward, frequently quick, and don't need isolating or cultivating pathogens. However, the sensitivity of antibody detection based on a single antigen in TB diagnosis is typically low. The antibody repertoire is quite variable in cases of intracellular infections like tuberculosis, with sera from various individuals responding with various antigens. The breadth of the antibody response necessitates a cocktail of numerous antigens for accurate

serodiagnosis of such infectious illnesses. The detection of antibodies against a group of immunogenic antigens of *M. tb* can increase the sensitivity and specificity of serodiagnosis. The whole genome sequencing of Mtb strain H37Rv, which provided sequence information and the location of open reading frames (ORF), opened the door to enormous discoveries and the recombinant manufacture of the antigens. The Mtb H37Rv genome has 4,411,529 base pairs (bp) and 4000 ORFs, according to sequencing details [3], [6].

Proteins are very bulky molecules and hence it is difficult to pool multiple such antigens in one assay. To resolve this issue we have done B cell epitope prediction of the immunodominant proteins and have selected most suitable B cell epitopic peptides as a representative of each protein.

In the current study we have worked on immunodominant antigens like Ag85B, pstS1, MPT63, MPT64 and Ptrp. The antibody responses against B cell epitopic peptides of these antigens have been identified in 150 healthy volunteers and 80 TB positive. The combinations of these antigens were also studied to evaluate the synergistic reaction of the combination of peptides.

2. Materials and methods

A. Serum samples

Sera was obtained from 150 healthy volunteers from Samruddhi medical systems path lab, these individuals were visiting the diagnostic lab for their routine blood testing and did not have any disease. Serum samples of 80 TB positive patients were obtained from Central India Institute of Medical Sciences (CIIMS), Nagpur, these individuals were tested positive for TB with the help of AFB staining and PCR TB detection test.

B. Antigens

B cell epitopic peptides of immunodominant proteins of TB such as Ag85B, pstS1, MPT63, MPT64 and Ptrp were used in this study as an antigen individually as well as in combination.

Table 1: The list of B cell epitopic peptides selected for the study

S. No.	Name of the Ag	RV No.	Peptide Sequence
1	Ag85B	Rv1886c	RSSNLKFQDAYNAAGGHNAV
2	38kDa	Rv0934	QYLSKQDPEGWGKSPGFGTT
3	MPT63	Rv1926c	AVSQFNARTADGINYRVLWQ
4	MPT64	Rv1980c	DQAYRKPITYDTLWQADTDP
5	Ptrp	Rv0538	PVPIIIPPFPGWQPGMPTIP

C. ELISA

Wash solution A: 20mM Tris, 150mM NaCl pH 8.0
Wash solution B: 20mM Tris, 150mM NaCl pH 8.0, 0.05% Tween 20
Blocking Solution: 2% skim milk in washing solution
Stop Solution: 2N HCL

Polystyrene 96 well microtiter plates (Nunc) were coated with 100µL of single/ combination of antigens (B cell epitopic peptides) at concentration of 5µg/mL in 50mM Tris pH 8.0 at 37°C for 1 hour. After coating the plates were washed with wash solution a twice and blocked with 400µL of blocking solution overnight at 4°C. The blocking solution was discarded after incubation and the plates were washed with wash solution A once and they were store in refrigerator at 4°C till further use as pre-coated plates. The pre-coated plates were removed from refrigerator prior use to bring them at room temperature. A 100µL of serum was added to each well with 1: 100 dilution in blocking solution and was incubated for 1 hour at room temperature. After incubation the plates were washed with wash solution A 3 times, wash solution B once and wash solution A again twice. After washing, the plates were incubated with HRP conjugated anti-human goat antibody, diluted 1:2500 in blocking solution for 45 minutes at 37°C. After incubation the plates were washed with wash solution A 3 times, wash solution B once and wash solution A again twice. The plates were tapped onto the blotting paper for drying and were incubated with TMB for 15 minutes in dark condition for assaying the enzyme activity. After the incubation the reaction was stopped by stop solution. The optical density was measured at 405nm by using automatic microtiter plate reader (Biorad).

These antigens were screened using 150 healthy volunteer's sera and 80 TB positive patients using above mentioned protocol.

D. Statistical analysis

The sensitivities and specificities of developed Indirect ELISA based test for diagnosis of TB was calculated. Receiver operating curve (ROC) was used to calculate the cut off value and comparison between TB patients and healthy volunteers.

3. Result

B cell epitopic peptides as representatives of immunodominant proteins of *M.tb* and their combinations were screened with healthy as well as TB positive patient's sera. Lot of person to person variation in the immune response was found against all the selected antigens, but there was a distinguishing threshold between the immune response of healthy population and the TB patients. Figure 1 shows the illustrative variation in the immune response of representative volunteers healthy and TB patients against the selected antigens.

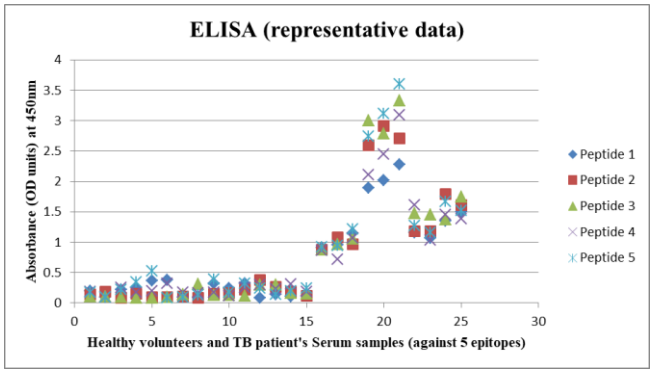

Figure 1: Graphical representation of healthy and TB patients immune response to antigens

Table 2 shows that even with numerous variations in the immune response the absorbance values of antibody response in ELISA against the antigens was statistically significant, with acceptable standard deviation. There was a statistically highly significant difference seen for the values between the TB positive and healthy volunteers group ($p<0.01$) for all the peptides and their 3 combinations.

Table 2: Inter group comparison of ELISA test result

Group		N	Mean	Std. Deviation	Std. Error Mean	Mann-Whitney U value	Z value	p value of Mann-Whitney U test
Peptide 1	1	80	1.22324	0.577893	0.06461	0	-12.484	0.000**
	2	150	0.19859	0.072956	0.005957			
Peptide 2	1	80	1.35356	0.637203	0.071241	4	-12.476	0.000**
	2	150	0.19093	0.083755	0.006839			
Peptide 3	1	80	1.39489	0.688582	0.076986	0	-12.484	0.000**
	2	150	0.14845	0.066122	0.005399			
Peptide 4	1	80	1.27756	0.687123	0.076823	7	-12.469	0.000**
	2	150	0.1884	0.089217	0.007285			
Peptide 5	1	80	1.36369	0.703261	0.078627	2	-12.48	0.000**
	2	150	0.19512	0.091665	0.007484			
Mix 1	1	80	1.53886	0.763567	0.085369	4	-12.476	0.000**
	2	150	0.13657	0.081008	0.006614			
Mix 2	1	80	1.60242	0.742502	0.083014	0	-12.484	0.000**
	2	150	0.19356	0.07747	0.006325			
Mix 3	1	80	1.5925	0.73693	0.082391	0	-12.484	0.000**
	2	150	0.13603	0.055185	0.004506			

Figure 2 show that the antibody response of the candidates was significant for all the selected antigens and their combinations. This reduces the chances of getting false positive and negative responses. The ROC curve and the table 3 shows that the assay is very specific and sensitive for the detection of TB.

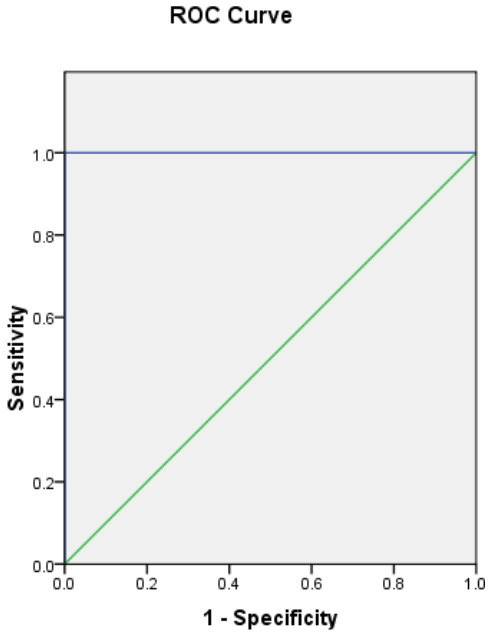

Figure 2: ROC curve for sensitivity and specificity of the ELISA

Table 3: Area under the curve- Test Result Variable(s)

	Area	Std. Error[a]	Asymptotic Sig.[b]	Cut off (Absorbance)	Sensitivity (%)	Specificity (%)
Peptide 1	1	0	0	0.4185	100	100
Peptide 2	1	0	0	0.4445	100	99.993
Peptide 3	1	0	0	0.4315	100	100
Peptide 4	0.999	0.001	0	0.363	100	99.973
Peptide 5	1	0	0	0.415	100	99.987
Combination 1	1	0	0	0.542	100	99.993
Combination 2	1	0	0	0.4995	100	100
Combination 3	1	0	0	0.458	100	100

The optimized protocol with low background from healthy volunteers was used for TB +VE patients to study their immune response against 5 B cell epitopic peptides and their 3 combinations. TB patients showed lot of person-to-person variation in their immune responses against B cell epitopic peptides and their combinations. The cut off for the peptides and combinations was found to be around 0.36-0.49 OD units where maximum healthy volunteers have shown response in the range of 0.09-0.3 OD units and the TB patients have shown the significant increase in the OD values in the range of 0.8 and nearing to 3.0 OD units.

4. Discussion

The World Health Organization reports state that tuberculosis is one of the most serious illnesses. Basic research in this field is essential since a lot of work is focused on the diagnosis of tuberculosis [1].

Given the rising prevalence of MDR-TB-XDR-TB, the gold standard for identifying active TB should continue to be cultured. Since immunoassays are easy to use, quick, affordable, and have the potential to identify instances that traditional sputum smear microscopy is unable to detect, the serological approach is an interesting advancement in the diagnosis of tuberculosis in vitro [4].

Numerous immunoassays with varying sensitivity and specificities have been developed to detect antigen or antibody in serum samples. But only a small number of diagnostic tests are available for tuberculosis (TB) despite much research in this area [2].

A complex cocktail of antigens known as pure protein derivative (PPD) from MTB has long been utilized as a skin test for tuberculosis diagnosis. Although the tuberculin skin test (TST) is straightforward technically, it is not very specific in diagnosing BCG-vaccinated persons. The polymerase chain reaction (PCR), which uses particular primers as markers for MTB, is another frequently used

diagnostic test. However, not all clinical laboratories perform this test correctly, and its sensitivity and specificity can vary [5].

Latent, reactivating, and active TB are the three primary stages of the *M. tb* life cycle. We can therefore ascertain the immunological response to antigens particular to each stage by examining the variations in *M. tb* gene expression that each stage represents. Secreted during the latency phase of mycobacterial growth, Hsp16.3 plays a crucial role in enabling MTB survival during latent human infection. It has been demonstrated that active TB has much stronger immune responses to the *M. tb* antigens Ag85B and ESAT6/CFP10 than LTBI. Evaluating the usefulness of the *M. tb*-secreted antigens in the serodiagnosis of active TB or LTBI was therefore considered reasonable [4].

In low-income and high-burden populations, serodiagnosis of tuberculosis (TB) may be a useful strategy if the sensitivity levels are raised to a manageable level. Previously fusion molecules from multiple clinically significatnt antigens of RD (region of difference) were used for improving the sensitivity of the serodiagnosis. The fusion protein showed AUC values of 0.947 which was higher than the individual proteins involved in the assay [6].

Narayana et al. assessed the antibody response to the PE protein families Rv1818c, Rv0978, and Rv1169c. IgG and IgM immune reactivity to Rv1818c was statistically significant across a range of clinical characteristics of tuberculosis patients. Rv0978c demonstrated an IgG response and Rv1169 shown B-cell reactivity to sera from adult patients who experienced a recurrent infection in the case of children with pulmonary illness. When compared to healthy controls, children with extrapulmonary infections showed a robust IgG response to Rv0978 and Rv1169c. In adult patients, Rv1818c demonstrated 93% specificity for IgG, 94% for Rv0978c for IgG, and 98% and 96% for IgM [7].

The serodiagnostic ability of eleven recombinant proteins (Rv0251c, Rv0934, Rv1973, Rv1984c, Rv2031c, Rv2185c, Rv2376c, Rv2537c, Rv2785c, Rv2878c, and Rv3873A) has been determined by Zhang et al. There were findings of 18%–60% sensitivity and 94%–100% specificity [8].
In the present study we have used B cell epitopic peptides of 5 immunodominant proteins of MTB and have screened them against healthy as well as TB positive patients using indirect ELISA. The combinations of these 5 peptides were also used to study the immune response of the individuals. The individual B cell epitopic peptides as well as the combination of them showed a very good sensitivity and specificity in the assay while showing lot of person to person variation in the absorbance. The high sensitivity and the specificity could be the result of selecting TB specific antigens, which are immunodominant and hence the infected individuals would have promising antibody titer in the sera. These immunodominant antigens are present only during the actively growing phase of the bacteria which reflected in negligible false positive results in the assay.

Previous studies have shown that the variation in the immune response against different antigens can be observed in differently located populations. The variation can also be found in different socio-economic backgrounds due to their lifestyle differences resulting in their corresponding differences in immunity.

These antigens should be further used for screening the extensive population of various regions of India, patients from different socio-economic background, from different cities to rural areas. Population with compromised immunity, co-infection, comorbidities, and different age groups should also be screened for evaluation of the antibody response against these study antigens.

These tests should also be used to screen the different clinical stages of the TB patients such as latent TB, pulmonary- extra pulmonary TB patients, reinfection cases. The test should be able to discriminate between the present and past infection. The patient undergoing treatment should also be able to screen to understand the status of the effectiveness of the treatment.

A plethora of novel proteins specific to the organism inhabiting this stubborn mycobacterium await to be explored. Screening of many more antigens, their respective sub molecular domains against wide spectrum of clinical TB patients should lead to a simple diagnostic tool in future.

References

[1] Global Tuberculosis Programme (GTB) TVAC. Global tuberculosis report 2022 [Internet]. 2022 [cited 2023 Dec 31]. Available from: https://www.who.int/publications/i/item/9789240061729
[2] Kashyap RS, Rajan AN, Ramteke SS, Agrawal VS, Kelkar SS, Purohit HJ, et al. Diagnosis of tuberculosis in an Indian population by an indirect ELISA protocol based on detection of Antigen 85 complex: a prospective cohort study. BMC Infect Dis [Internet]. 2007 Jul 10;7:74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17620147
[3] Lyashchenko KP, Singh M, Colangeli R, Gennaro ML. A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases. J Immunol Methods [Internet]. 2000 Aug 28;242(1–2):91–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10986392
[4] Zhang C, Song X, Zhao Y, Zhang H, Zhao S, Mao F, et al. Mycobacterium tuberculosis Secreted Proteins As Potential Biomarkers for the Diagnosis of Active Tuberculosis and Latent Tuberculosis Infection. J Clin Lab Anal [Internet]. 2015 Sep;29(5):375–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25131423
[5] F Song 1, X Sun, X Wang, Y Nai, Z Liu, Early diagnosis of tuberculous meningitis by an indirect ELISA protocol based on the detection of the antigen ESAT-6 in cerebrospinal fluid. Ir J Med Sci. 2014; 183(1): 85–88
[6] MohsinaAkhter, ShaistaArif, AasiaKhaliq, Zaib un Nisa, Imran H. Khan, Muhammad WaheedAkhtar, Designing fusion molecules from antigens of Mycobacterium tuberculosis for detection of multiple antibodies in plasma of TB patients, Tuberculosis,

Volume 124, 2020, 101981, ISSN 1472-9792, https://doi.org/10.1016/j.tube.2020.101981. (https://www.sciencedirect.com/science/article/pii/S1472979220301487)

[7] Narayana Y, Joshi B, Katoch VM, Mishra KC, Balaji KN Differential B-cell responses are induced by Mycobacterium tuberculosis PE antigens Rv1169c, Rv0978c, and Rv1818c. Clin Vaccine Immunol 2007;14:1334–41

[8] Zhang G, Zhang L, Zhang M, Pan L, Wang F, Huang J, et al. Screening and assessing 11 Mycobacterium tuberculosis proteins as potential serodiagnostical markers for discriminating TB patients from BCG vaccinees. Genomics Proteomics Bioinformatics 2009;7:107–15.

Author Profile

Shalmali Pendse received BSc degree in Biotechnology and MSc degree is Bioanalytical Sciences. Currently pursuing PhD in the Subject of Bioanalytical Sciences from Mumbai University.

Dr. Ajitha Rani R received BSc, MSc and PhD degree in Chemistry. Currently she is the HOD of department of Chemistry in Guru Nanak Khalsa College of Arts, Science and commerce.