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Assessment of Non-Sputum Biomarkers: An Early Diagnosis in Tuberculosis

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The detection of Mycobacterium tuberculosis (Mtb) specific human antibodies has been an important diagnostic aid in the diagnosis of tuberculosis (TB) cases with smear-negative sputum samples, especially for the screening of high-risk population. This study focused on the analysis and comparison of the two potential Mtb-secreted proteins, Early Secreted Antigenic Target 6 (ESAT6) and Ag85B as new markers in the serodiagnosis between active TB and latent TB infection (LTBI).The results showed that ESAT6 and Ag85B were the best-associated antigens for serology diagnosis of the active TB and LTBI individuals because of their specificity, sensitivity. Early diagnosis and treatment of TB patients is crucial for the control of TB spreading (Pollock et al.,1997). In many countries, especially in developing countries, because of resource constraint the diagnosis of TB largely relies on the detection of acid-fast bacilli in sputum in conjunction with assessment of clinical symptoms and X-ray radiographic evidence.However, these evaluations are time-consuming and unable to distinguish between active TB and latent infection.Thus, a combined test of multiple Mtb-secreted proteins Ag85B and ESAT6 may be the ascendant preliminary screening antigens for active TB or LTBI patients.

Keywords: Early Secreted Antigenic Target 6 (ESAT6), Ag85B, active TB, latent TB infection (LTBI), Acid-Fast bacilli, M. tuberculosis (Mtb),Non-Sputum Biomarkers

1. Introduction

Tuberculosis (TB) is a communicable infectious disease, spread almost exclusively by coughed aerosols carrying pathogens from the Mycobacterium tuberculosis (Mtb) complex. TB is characterized pathologically by necrotizing granulomatous inflammation usually in the lung, although almost any extra-pulmonary site can be involved. TB remains one of the most significant infectious causes of mortality and morbidity worldwide. As reported by the World Health Organization (Delia Golettiet al.,2016) it causes disease among 9.6 million people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide.

INTERNATIONAL STATUS

TB is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS. In 2016, there were an estimated 1.3 million TB deaths among HIV-negative people (down from 1.7 million in 2000) and an additional 374 000 deaths among HIV-positive people (WHO-2017 REPORT). An estimated 10.4 million people fell ill with TB in 2016: among that, 90% were adults, 65% were male, 10% were people living with HIV (74% in Africa) and 56% were in five countries: India, Indonesia, China, the Philippines and Pakistan (GLOBAL TUBERCULOSIS REPORT 2017). Drug-resistant TB is a continuing threat word wide, in 2016; there were 600000 new cases with resistance to rifampicin (RRTB), the most effective first-line drug, of which 490000 had multidrug-resistant TB (MDR-TB) (https://www.tbfacts.org/mdr-tb/). Almost half (47%) of these cases were in India, China and the Russian Federation (GLOBAL TUBERCULOSIS REPORT 2017). Globally, the TB mortality rate is falling at about 3% per year. In 2016, 6.3 million new cases of TB were reported (up from 6.1 million in 2015), equivalent to 61% of the estimated incidence of 10.4 million; TB disease burden Most of the estimated number of incident cases in 2016 occurred in the WHO South-East Asia Region (45%), the WHO African Region (25%) and the WHO Western Pacific Region (17%); smaller proportions of cases occurred in the WHO Eastern Mediterranean Region (7%), the WHO European Region (3%) and the WHO Region of the Americas (3%). The purpose of WHO's Global Tuberculosis Report is to provide a comprehensive and up-to-date assessment of the TB epidemic and of progress in care and prevention at global, regional and country levels (WHO) For the period 2016–2035, these are WHO's End TB Strategy and the UnitedNations' (UN) Sustainable Development Goals (SDGs), which share a common aim: to end the global TB epidemic.

NATIONAL STATUS

India is the country with the highest burden of TB. The World Health Organization (WHO) TB statistics for India - 2016 gives an estimated incidence figure 28 lakh cases occurred and 4.5 lakh people died. 5.05% prevalence of TB is notified in epidemiology of tuberculosis: status of India (2004) (Chakraborty AKet al., 2004).

We were focused mainly on host and pathogen side TB diagnosis. There are different types of biomarkers identification in various levels of research including Hepatocytic Nuclear Factor, CD27-MF1 ratio, Serum metabolic profile, Transthyritin, Neoptirin, ESAT-6, Ag85b-mycolic acid-chronic diseases, IP10, Matrix Metallo protinase-9, CCR5, CCR3, Cancer related TB-MDSC, MCP1, fract alkine, T-cell activation marker in children RFP, CFF Stage specific

immune markers-IP10, Th17, 19kd protein, IFR, TGF-ALPHA, PBMC, markers of TLR4 family, SCD40L, remote infection related biomarkers like RU2628, RU3407, Alpha crystaline, IFGAMMA, 19KD-HLA, Homohemoxiginase-I. From this, we were focused observation of ESAT-6 and Ag85b biomarkers in levels of infection in study groups.

In this study, we tested the recombinant proteins ESAT6 and Ag85B by ELISA to evaluate the usefulness of different combinations of these antibodies in diagnosing active TB patients and latent TB infected individuals.

AIM AND OBJECTIVE

1. To isolate the specific biomarkers of TB in study population.
2. Interpretation of the results.

2. PLAN OF WORK, METHODS AND TECHNIQUES:

STUDY DESIGN: Prospective analytical study

STUDY SETTING: Study subjects are patients infected with TB and without TB. Study subjects are divided in to 3 groups.

1. Active TB group (Usually person has a skin test or blood test result indicating TB infection, May have an abnormal chest x-ray, or positive sputum smear or culture, Has active TB bacteria in his/her body, Usually feels sick and may have symptoms such as coughing, fever, and weight loss, May spread TB bacteria to others and Needs treatment to treat TB disease)[CDC https://www.cdc.gov/tb/publications/factsheets/general/ltbiandactivetb.htm].

2. Latent TB group : Usually has a skin test or blood test result indicating TB infection, Has a normal chest x-ray and a negative sputum test, Has TB bacteria in his/her body that are alive, but inactive, Does not feel sick, Cannot spread TB bacteria to others, Needs treatment for latent TB infection to prevent TB disease; however, if exposed and infected by a person with multidrug-resistant TB (MDR TB) or extensively drug-resistant TB (XDR TB), preventive treatment may not be an option. [CDC-https://www.cdc.gov/tb/publications/factsheets/ general/ltbiandactivetb.htm]. In high TB burden countries the populations that are most strongly recommended for the treatment of latent TB infection are health practitioners have been prioritizing testing and treatment of people living with HIV and children under the age of 5 who have been in contact with people who have TBhttps://to-prevent-latent-tuberculosis-maximize-access-to-testing-and-treatment-whos-updated-guidelines/

3. Uninfected controls (Those who are not fit to above criteria).

The study was conducted in department of Microbiology, Sri Venkateswara Medical College, Tirupati, Andhrapradesh, India.

METHOD:

Study samples (5ml of blood) was collected from TB ward and TB OP of SVRRGG Hospital, Tirupati, Andhrapradesh, India.The serum of blood was separated with centrifugation and stored in -20^{o}c until the analysis. ELISA was performed to check the TB biomarkers of ESAT-

6 (6Kda/ESAT 6 kit), and Ag85B (Human anti-Mtb Ag85b-IgG ELISA kit). These markers are robust enough to withstand the heterogeneity associated with host and pathogen derived sources of variation. As per the literature and current reviews, we were focused to check the biomarkers related to host as well as pathogen in samples. All patients and TBUninfected controls in this study were negative for HIV antibodies.

TECHNIQUES WERE USED: ELISA

INCLUSION CRITERIA: The patients with ATB, LTB and UC that to those are willing to give consent only. 20-60 years age groups of both genders are included.

EXCLUSION CRITERIA: Patients associated with other morbidities and age group below 20 and above 60 years are excluded.

ETHICAL CLEARANCE: ByInstitutional Ethics Committee, Sri Venkateswara Medical College, Tirupati, Andhrapradesh, India.

STATISTICAL ANALYSIS:

Allthestudy data wereenterdintothecomputerdatabaseusing standard format, checkedforerrors and verified. Data maintained in thecomputersheetswereorganisedby SPSS version 20 software for Windows. Data will be peresented in aprropriateTablesbycaliculatingthepercentage andrate etc.

3. RESULTS:

Table 1. ClinicalCharacteristics of theSerumSamples

Tuberculosisgroups	Total Number	Smear Positive	Culture positive	Skin test-Positive
Active TB	40	40	40	70
LTBI	30	0	0	30
TB- UC	30	0	0	0

LTBI-latent TB infectedindividuals; TB−UC: TBUninfected controls;

The 30 LTBI patientswereSkin test-Positive and negativebyculture.

A total of 100 serum samples, 40 were active TB patientswhowerehavingtreatmentfor TB, 30 werelatent TB infectedindividuals, whowere skin-positive, culture negativeand 30 were TBUninfected controlsfrom TB ward and TB OP of SVRRGG Hospital, Tirupati, Andhrapradesh, India, wereselected to analysis (Table 1).

AntibodydetectionbyELISA in a total of 100 serumsamples

To detectthespecificIgGantibodiesby ELISA, wefollowedthemanufacturersprotocol and the response of specificantibodieswasmeasured at a wavelength of A490 nm in Bio-Rad 680.

Table 2. ROC analysis of theantibody response againsttwoantigens in TB patients, in LTBI and TB Uninfectedcontrols.

Antigens	Tuberculosisgroups(number)	SD	AUC	95%CI	Pvalues
ESAT6	Active TB (40)	0.7434	0.94	0.91-0.95	<0.0001
	LTBI (30)	0.7668	0.74	0.68-0.86	<0.0001
	TB- UC (30)	0.4640			
Ag85B	Active TB (40)	1.126	0.76	0.69-0.84	<0.0001
	LTBI (30)	1.324	0.74	0.64-0.86	<0.0001
	TB- UC (30)	0.4340			

SD, standard deviation; AUC, areaunderthe ROC curves; CI, confidenceinterval.

Table 3. ROC analysis of theantibody response againsttwoantigens in active TB patients, LTBI and TB Uninfectedcontrols.

Antigens	Tuberculosisgroups(number)	Cutoffvalues	Specificity (%)	Sensitivity (%)	YI values
ESAT6	Active TB (40)	2.200	95.64	86.44	0.820
	LTBI (30)	2.000	84.68	60.00	0.438
Ag85B	Active TB (40)	2.155	80.38	63.38	0.438
	LTBI (30)	2.688	97.78	54.33	0.508

YI, Youdenindex.

IgGAntibody Response AgainstthetwoAntigens in Active TB Patients, LTBI and TB- UC

Theantibody responses to theantigenswereanalyzedby ELISA. The AUC of eachantibodyresponse isprovided in Table 2. The AUC for ESAT6, and Ag85B of Active TB and LTBIwere0.94 (0.91-0.95) and 0.74 (0.68-0.86) respectively.Theoptimalcombination of specificity (95.64%) and sensitivity (86.44%) (Table 4) weregeneratedfromcutofflevelsbasedonthe AUC values. Thelevels of antibodiesagainsteachantigen in TB patientsweresignificantlyhigherthanthose in TBUninfected controls (P <0.0001, Table 2).

Evaluation of theDiagnosticValue of thetwoProteinsBetween Active TB and LTBI.

The AUC values and 95% CI of TB and LTBI groupsare calculated in Table 2. TheoptimalcutoffvalueswerechosenwhentheYoudenindex(YI) valuewasmaximum (Table 3).

ThespecificityfordetectingActive TB antibody responses to antigens Ag85B and ESAT6was 80.38%, and 95.64%, respectively, and thesensitivitywas 63.38% and 86.44%, respectively. Furthermore, the YI valueswere0.438 and 0.820, respectively.

Theserological responsesagainstantigens Ag85B andESAT6,whendetectingLTBI individualsshowedthatthespecificitywas 97.78% and 84.68 %, respectively, and thesensitivitywas 54.33% and 60.00%, respectively. Furthermore, the YI valueswere 0.508 and 0.438, respectively (Table 3). Ithad no statisticalsignificancebetweentheantibody response

results of detecting LTBI patientsagainstthetwoantigens.

4. DISCUSSION

Targetedtestingisanessential TB prevention and control strategythatisused to identify, evaluate, and treatpersonswho are at highriskforlatent tuberculosis infection (LTBI) or at highriskfordeveloping TB disease once infectedwith M. tuberculosis. Identifyingpersonswith LTBI isimportant to thegoal of TB control and eliminationbecausetreatment of LTBI can preventinfectedpersonsfromdeveloping TB disease and stop thefurther spread of TB.Centers for Disease Control and Prevention (CDC) Division of Tuberculosis Eliminationhttp://www.cdc.gov/tb/.

Althoughthegolden standard fordiagnosing active TBshould be culture andshouldremain so especially in the light of theincreasingburden of MDR-TB and XDR-TB. Theserologicalmethodisaneffectivediagnostic of TB in vitro, whichwould be anattractiveprogress as immunoassaysare simple, rapid, inexpensive, and mayofferthepossibility to detect cases missedby standard sputumsmearmicroscopy (Steingart KR et al.,2007). Selection-specificantibodyagainstMtbproteinsisveryimportantforserologicaldiagnosis of TB. TheMtblifecycle can be separatedintothreemainstages: latent, reactivating, and active TB. Eachstagerepresentsdifferences in Mtbgene expressionand thereforewe can determine theimmune response to stage-specificantigens (Sutherland JS et al.,2013).Immune responses to MtbantigensESAT6/CFP10 and Ag85B havebeenshown to be significantlyhigher in active TB and LTBI (Caccamo N et al.,2010). Thus, itwasrational to evaluatethevalue of thoseMtb-secretedantigens in serodiagnosis of active TB orLTBI.

In thisstudy, tworecombinantMtbproteins—Ag85B and ESAT6—weresuccessfullyobtained andevaluatedfortheirdiagnosticpotential in detectingserumantibodiesby ELISA in active TB patients, latent TB infectedindividuals, and TB uninfectedcontrols of studygroup. Wefoundthatboth active TB and LTBI individualshadhigherlevels of antibodiesagainstthesetwo individual antigens, comparedwiththatin healthycontrols ($P < 0.05$, Table 2). Althoughthesensitivity and specificitywerelowerthanWu X' study(Wu X, Yang Y et al.,2010), thedifferencemay be attributed to differences in thepopulations andoptimization of ELISA (Brust Bet al.,2011).The YI valueof ESAT6 (0.820)indicatingthat ESAT6 wastheoptimalantigenforthedetection of active TB fromtheresults in Table 2 (Caiqin Zhang et al.,.2015).

Theresultspresented in Table 2demonstratedthattheantibody response against ESAT6 wastheoptimal to detect LTBI individualsaccording to thevalues of SD,AUC, and 95%CI. However, the YI value of Ag85B washigherthanthat of ESAT6 antigen (Table 3), and thepositive rate of ESAT6had no differencefromthat of Ag85B in latent TB infectedpatients ($P > 0.5$). Thedetectionof antibodiesdirectedagainstmultipleantigenscouldprovideanimprovement in sensitivitycomparedto single antigen (Achkar JM et al.,2006).

ELISA is a simple, rapid, and inexpensive test of TB diagnosis,this can be prime screeningmethodforTB, especiallyfor LTBI. The HIV positive patients, immune-

suppressedindividuals, infants, and severelyillpatients—alsosolidorgandonors can behighlyconsideredforMtbscreening. Peoplewith positive resultsshould be consideredforhighrisk of TB, furtherdifferentialclinical diagnosis should be performed, whichissignificantlyimportantforcontrollingTB (Scott Vet al.,2012).

In conclusion,theresults of thisstudyshowedMtbsecretedproteins ESAT6 and Ag85B, whichshould be thepreliminaryscreeningantigensof active TB patients and LTBI individuals and as anadditionaltest aftersmearmicroscopy. This test mayalso be usedforearly diagnosis of the TB infectionorscreeningthehigh-riskpopulation (Steingart KR 2011 and Small PM 2010).

Furtherclinicalstudiesonvariousconditionsmaybettercharacterizetheseproteins. Single use of thesemarkersortheircombineduse withotherpromisingbiomarkersmay be a usefultool to aidthedevelopment of new effectivetherapies and vaccines.

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