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Lip L protein antibodies: A new promising diagnostic marker for tuberculosis

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***Abstract*---**Background: Tuberculosis (TB) is as yet quite possibly the most over the top dreadful sickness, primarily in Southeast Asia, with the highest incidence in five countries i.e. India, Indonesia followed by China, Philippines, and Pakistan (in that order). In order to combat the rising tuberculosis (TB) load, new diagnostic tests are urgently needed. Because immunoassays are fast, quite simple, and cheap and may suggest the prospect of identifying cases overlooked by routine sputum smear microscopy, an efficient in vitro diagnostic test of tuberculosis based on serological approaches would be considered as an appealing advancement. Lipolytic enzymes, like lipases and esterases, are more important for mycobacterial survival. Lipolytic enzymes, including as lipases and phospholipases, have been found to help *M. tuberculosis* access host lipids. The main purpose of the current study was to compare the level of antibodies against membrane bound lipolytic enzymes in Tuberculosis patients and

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control subjects. Methods: To detect serum IgG antibodies against lipolytic enzymes, recombinant proteins were created and employed in an ELISA test. LipL antibody assay were done in serum samples of patients of tuberculosis (PTB, EPTB & MDR TB) and healthy controls. The immunological response to mycobaterial LipL protein was compared in patients and healthy controls to evaluate its utility as a diagnostic immunogenic marker for tuberculosis. Results: Patients with active tuberculosis had a substantial humoral response, whereas antibodies to the lipolytic enzyme were rarely found in control group or in people with latent infection. Conclusion: These findings show that lipolytic enzymes are immunogenic and can be used as strong biomarkers as they showed high sensitivity and high specificity for active TB diagnosis.

***Keywords*---**Lipolytic enzymes, Tuberculosis (TB), IgG antibodies.

Introduction

Tuberculosis (TB) continues to be the most horrible illness that humanity has ever faced. TB affects over one-third of the world, making it the infectious illness with the highest number of deaths worldwide (1). In 2016, more than 10.4 million cases of TB were accounted globally (range: 8.8 million to 12.2 million), or to 140 cases per 100,000 people (2). PTB has a prevalence of 320 per 100,000 people in India (2018) (3), and EPTB has a prevalence of 15 to 20% in India (4).

M. tuberculosis is a slow-growing intracellular disease that can survive in a dormant condition for decades in an infected host (5). M. tuberculosis devises methods of evading both adaptive and innate immune responses. To get through the host's defences, it manipulates macrophage death pathways (6). Due to the extended sub-clinical latent phase, TB in humans appears in a variety of clinical symptoms that are phenotypically varied (7). In the form of TB-HIV co-infection and the advent of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria, tuberculosis remains a significant general medical condition not just in underdeveloped nations but also in industrialised ones (8).

Early detection of the infection and prompt administration of appropriate medication can assist to improve the disease's prognosis. Currently, tuberculosis is diagnosed in many countries on the basis of clinical signs and laboratory tests, such as the identifying acid-fast bacilli in sputum (9). The gold standard test for diagnosing tuberculosis, microbiological culture, is extremely time-consuming. (10). In most poor countries, the tuberculin skin test on the basis of purified protein derivative (PPD) is currently the only immune-based analytic test accessible for clinical use. However, the lack of specificity of this century-old test is because of prior BCG vaccination and cross-reaction with other mycobacterial species (11). For this reason, there is an urgent need to develop new diagnostic tools that can detect tuberculosis early.

These days' significant attempts have been undertaken to build up quick tuberculosis diagnostic tests based on the identification of antibodies against *M.*

tuberculosis antigens as a substitute to TB diagnosis, particularly in developing nations (12).

As a result, finding acceptable M. tuberculosis antigens for immunodiagnosis that are easy to detect, have high specificity and sensitivity, and allow active disease patients to be distinguished from latently infected and BCG-vaccinated persons is extremely enviable (13,14)

Identification of novel antigens, such as lipolytic enzymes, is one of the possible options for developing new TB diagnostic assays. Lipids are so specific to mycobacteria that they could be utilised as biomarkers to track the progression of tuberculosis (15). Lipolytic enzymes such as lipases and phospholipases, which operate as significant virulence factors in M. tuberculosis, acquire host lipids, making lipids an important factor for the pathogenicity and survival of *M. Tuberculosis* (16) (17). Lipases hydrolyze lipid membranes of host cells which lead to liberate fatty acids (FA) and these FA are supplied to *M. tuberculosis* during infection (18).

Based on the motif 'GXSXG', a distinctive property of the members of the / hydrolase-fold family, in silico assay of the *M. tuberculosis* genome suggested the occurrence of roughly 24 potential lipase/esterase genes which belongs to the family of “lip” gene (lipC to lipZ) (19). Lip L protein has been shown in previous research to include a conserved pentapeptide –GXSXG-, which is feature of lipolytic enzymes, at the C-terminus, as well as a consensus -lactamase motif 'SXXK'. The lipL gene was cloned, produced in E.coli, and purified to homogeneity on a Ni-NTA column for further characterisation of the protein. Both esterase and -lactamase activity were found in the Lip L protein. It appears to be tightly linked to the cell surface of Mycobacteria, and the production of a robust specific humoral response in patients with TB supports its ease of access to the immune system of host (15).

Thus, the goal of this study was to look into the humoral response of a mycobacterial lipolytic protein called LipL in patients with different types of tuberculosis, such as PTB, EPTB, and MDR TB, in order to assess its diagnostic usefulness.

Materials and Methods

The current study was a cross-sectional and observational study conducted between March 2017 to October 2018. Blood samples were withdrawn from patients diagnosed with TB attending the Outpatient clinics of Department of Pulmonary Medicine, Government Medical College & Hospital (GMCH) Chandigarh. Written Patient consent form was obtained in written form taken from the subjects at the time of enrolment. The study was granted by the Institutional ethics committee (IEC).

Study population

A total randomly selected 150 patients were categorised into three groups:

Group 1 (n = 54): Patients diagnosed with pulmonary TB for the very first time and had no history of TB treatment.
Group 2 (n =50): Patients diagnosed with extra pulmonary TB for the very first time and had no history of TB treatment.
Group 3 (n =46): Patients diagnosed with Multidrug resistant TB.
Apparently healthy volunteers (n = 41) were enrolled as Controls.

Bacterial strains and culture condition
E. coli DH5α and *E. coli* M15 strains were the bacterial stains which were used in the study.

Gene amplification and expression plasmids construction

LipL protein, a membrane bound mycobacterial lipase, was chosen using bioinformatics reports. The SVMTrip tool (sysbio.unl.edu/ SVMTrip/) was used to identify antigenic epitopes. For the gene cloning inpQE30 vector, M. tuberculosis H37Rv chromosomal DNA was employed. Primers were created utilising 18 to 24 nucleotides from the gene's terminal region.

Cloning, expression, and purification of LipL

For cloning of *lipL*, gene sequence was amplified by designated set of primers by using genomic DNA of *M. tuberculosis* H37Rv as template. The chromosomal DNA of *M. tuberculosis* H37Rv strain, used for amplification of *lipL* gene, was a kind present from Dr. U. D. Gupta (JALMA, India). *lipL* gene of *M. tuberculosis* H37Rv, was PCR amplified at 65˚C resulting in an amplified product of 1.3 kb corresponding to the size of *M. tuberculosis* H37Rv *lipL* on 1.2% agarose gel**.** PCR product *(lipL)* and the pQE30 vector were broken down with BamHI and HindIII at restriction sites to facilitate cloning into the pQE30 vector. Digested products were analyzed on 0.8% agarose gel. Intact bands for plasmid and gene were observed. Double digested fragment of pQE30 vector and amplified gene were ligated and used to convert freshly prepared ultra-competent *E. coli* DH5α cells through heat shock method. Recombinant clone was identified through sequencing (Agri Genom, Banglore).

Gene expression and protein purification:

E.coli M 15 strains were transformed using the positive recombinant clone. Insoluble fraction was used to produce LipL protein. For purification, 1% of an overnight developed culture of E. coli M15 cells harbouring lipL was inoculated into 250 ml of Luria broth medium which is supplemented with kanamycin (30 g/ml) and incubated at 37 °C and 180 rpm until the OD600 reached 0.6. The culture was next stimulated for 3 hours at 37 °C and 180 rpm with 0.5 mM IPTG. Centrifugation at 9000 rpm for 15 minutes at 4°C harvested the induced cells. 35 mL cold lysis buffer was used to suspend the culture pellet. Ultrasonication (21 percent amplitude, 10 sec on and 10 sec off pulse) was used to disturb cells for 10 minutes with occasional cooling. Centrifugation of cell lysates was done for 20 mins at 7000 rpm. The sonicated pellet was dissolved in 8 M urea before being placed onto a Ni-NTA column that had been pre-equilibrated using equilibration buffer. The columns were washed in 10 volumes of wash buffer, and elution of

bound protein was performed in 1 ml fractions with elution buffer. The absorbance at A280 was used to determine the protein concentration in each fraction. The fractions with a high protein concentration were combined and dialyzed. On a 12 percent SDS-PAGE, the integrity and purity of the protein were examined.

Protein estimation

A commercially available BCA (bicinchoninic acid) assay was used to assess the protein concentration (Banglore-Genei, India). The standard was bovine serum albumin. At 562 nm, the absorbance was measured. Mix Reagent A (bicinchoninic acid solution) and Reagent B (Copper (II) Sulfatepentahydrate 4 percent solution) in the ratio of 50:1 included in the kit to make the BCA working reagent. A 1.0 mg/ml BSA stock solution was diluted to produce solutions with concentrations of 50, 100, 150, 200, 250, and 500 g/ml, respectively. 100 l BSA/test protein sample was added to 1 ml BCA working reagent. For 30 minutes, reaction mixtures were incubated at 37 °C temperature. The absorbance A562 was determined, and a standard curve was created by graphing BSA protein content vs absorbance (562 nm). The standard curve was used to determine the concentration of test samples.

Enzyme activity

The activity of enzyme esterase of the recombinant protein was also demonstrated by tributyrin plate assay method. One percent tributyrin emulsion was prepared in 50mM phosphate buffer (pH 8.0). Autoclave the emulsion after addition of 1.5% agar and pour the plates when the mixture was cooled to room temperature. Add 100 µl of enzyme and control sample (50 mM phosphate buffer, pH 8.0) in wells created in the plate. Incubate the plate at 37 °C for overnight and the occurrence of zone of clearance was observed after 24 h.

Immunological Assay

In 96-well plates, enzyme-linked immunosorbent assays (ELISAs) were done (Genetix, Biotech Asia Pvt. Limited). Overnight at 4°C, the ELISA plates were coated with 100 l pure LipL protein (50 g/ml) diluted in coating buffer (PBS buffer). As a negative control, BSA protein was used. For all samples, ELISA was done in triplicate. The plates were cleaned thrice, twice with PBST solution and once with PBS buffer, before being blocked in PBST with 5% skimmed milk. The plates were cleaned after bloackage and incubated at 37°C with human sera (1:10 dilution in PBS) overnight. After three washes, two with PBST and one with PBS buffer, the plates were incubated with goat anti-human IgG antibody conjugated to alkaline phosphatase at a 1:5,000 dilution (Anti–Human IgG whole molecule–Alkaline Phosphatase antibody made in goat, Sigma Aldrich). After washing, 50ul of BCIP NBT (Genei) substrate was applied to each well. An ELISA Absorbance Microplate Reader (Synergy – H1 microplate reader) was used to measure the absorbance values at 405 nm (Biotek). Each experiment was carried out three times in a row.

Statistical analysis

Statistical analysis was performed by GraphPad PRISM software. The mean±SD were computed using the student's t-test. ANOVA with Tukey's Numerous Comparison Test was employed to analyse multiple samples statistically. Statistical significance was described as a p-value <0.05.

Results

Prediction of antigenic epitopes

lipL gene was found to be annotated as a possible lipase from tuberculist database, from *M. tuberculosis* H37Rv. Antigenic epitopes of *lipL* were predicted using the SVMTriP program. *lipL* is predicted to be localized in the membrane fraction of bacterium. *lipL* has more than 9 probable antigenic epitopes each of 16 amino acids in length predicted by the program but three have significantly high.

Rank	Location	Epitope	Score	Recommend*
1	149 - 168	QDLLDHVVMEERLAAAVPGR	1.000	
2	272 - 291	APLLDAEIPAANGVATARAL	0.856	
3	379 - 398	RLLSPLVMTDHAGFVGIYHL	0.851	
4	114 - 133	AEYWPAFGANGKATLTVRDV	0.742	
5	3 - 22	VDTGVDHRAVSSHDGPDAGR	0.729	
6	172 - 191	KSAYHALTFGWLMSGLARAV	0.722	
7	219 - 238	PADAPTRVAEIIMPQDIAAN	0.717	
8	331 - 350	LLVPLNFHLGYHGMPIGNVM	0.647	
9	69 - 88	WADRAGWVPWSADSAPMVFS	0.635	

Immunogenicity of LipL

An indirect-ELISA was used to examine the reactivity of the sera of TB patients with recombinant protein LipL to investigate the involvement of LipL protein in the host humoral immune system.

The results of the indirect ELISA demonstrated that the sera of all three groups of TB patients had considerably stronger antibody reactions against LipL than healthy controls. The IgG antibody against LipL protein was found to be 2.5 fold higher in patients of PTB and EPTB (Figure 8 a, b) (Figure 9 a ,b) as compared to controls, while the increase in patients with MDRTB was found to be 3 fold. (Figure 10 a, b)($p<0.005$)**.**The results strongly indicate that a LipL-specific humoral immune response is induced in all three groups of TB patients.

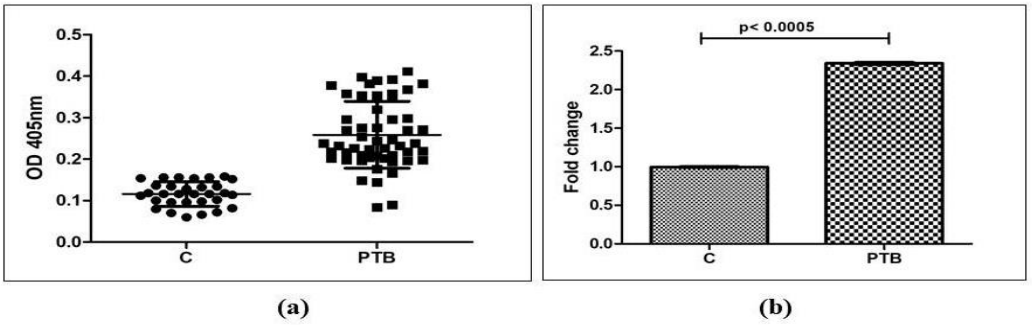

Figure 8(a): The humoral responses induced by the recombinant LipL protein inPTB (b) Fold change in PTB patients with healthy control.Each dot represents the mean OD of independent experiment for respective sample.

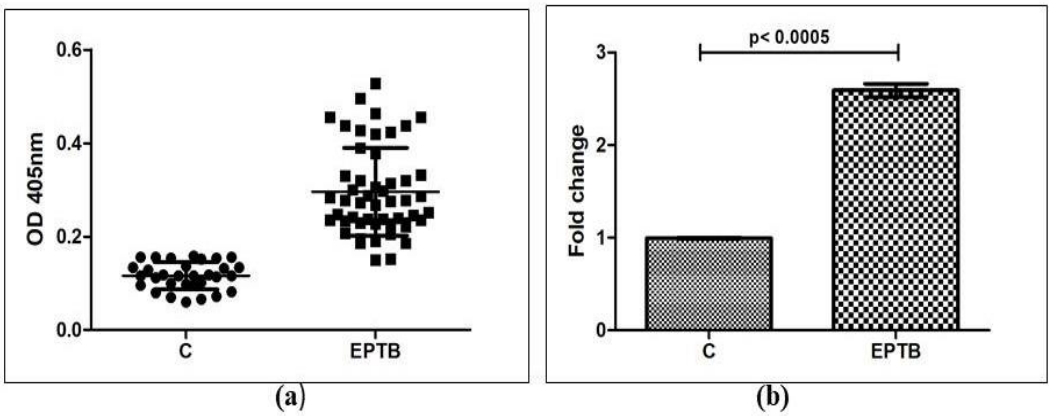

Figure 9(a): The humoral responses induced by the recombinant LipL protein in EPTB(b) Fold change in EPTB patients with healthy control. Each dot denotes the mean OD of independent experiment for respective sample.

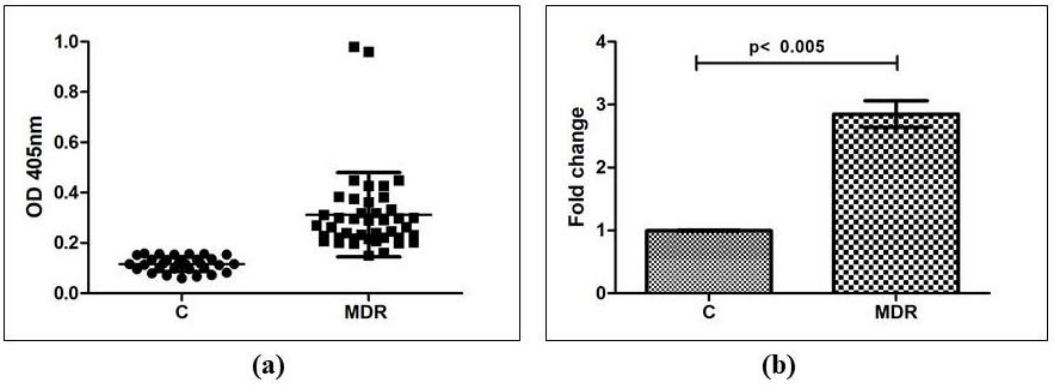

Figure 10(a): The humoral responses induced by the recombinant LipL protein in MDR(b) Fold change in MDR patients with healthy control. Each dot denotes the mean OD of independent experiment for respective sample.

Discussion

TB is one of the most severe infectious diseases which is caused by intracellular bacteria, and the T-cell response is the primary source of protective immunity (20). A large number of research on circulating antibodies in tuberculosis patients have been conducted in recent years with the purpose of evaluating them as indicators of active illness (21). It is necessary to identify genes that are crucial for the bacterium's survival and pathogenicity in order to gain a better knowledge of its physiology (22). *M. tuberculosis* laboratory strain H37Rv was successfully sequenced in 1998 which has nearly 4,000 protein-coding gene (23). Out of these genes, 40% of the genes encode for hypothetical proteins (24). Since then attempts are being made to characterize these hypothetical proteins. Recently several genes with lipase and esterase activity have been reported in *M. tuberculosis* genome.

It has been reported that various extracellular esterases and lipases and produce an immune response *in vivo* (25). Looking at the burden of the tubercular diseases, a need was felt to investigate more mycobacterial lipases, which if found antigenic could aid in early diagnosis of tuberculosis. The studies so far had revealed that LipL protein of the *M. tuberculosis* H37Rv had esterase activity and the protein contained conserved pentapeptide –GXSXG-, at C- terminus instead of N-terminus location which is characteristic feature of lipolytic enzymes (19).

With this baseline knowledge about LipL, bioinformatic analysis was done in the present study using using SVM Trip program which showed several antigenic epitopes. The CD spectroscopy was done which verified α/β hydrolase nature of LipL protein as predicted by bioinformatics analysis. Subsequently *lipL* gene was cloned from H37rv genome, amplified and its purified protein product was used for detection of IgG antibodies by Indirect ELISA in the sera of TB patients.150 patients of TB were enrolled in the present study and grouped into three groups PTB (n=54), EPTB (n=50) and MDRTB (n=46).

The results revealed that increased reactivity against LipL protein was found in the serum of all the 3 groups of TB patient's i.e PTB ($p<0.0005$), EPTB ($p<0.0005$) and MDR TB ($p< 0.005$) in comparison to the healthy controls. The results indicate that LipL, an extracellular esterase of *M. tuberculosis* could interrelate with the host immune response, and thus, could be used as a diagnostic immunogenic marker.

Conclusions

lipL gene of *M. tuberculosis* was selected for cloning and characterization as it was present on the cell wall and also on cell membrane, which is likely to evade the host immune system by activating humoral immunity in the patients suffering from various types of TB. Cloning, expression of *lipL* gene, purification, and characterization of LipL protein of *M. tuberculosis* was done and utilized to evaluate immunogenic response in sera of patients of PTB, EPTB, MDR TB.

LipL protein was found to be highly immunogenic in all the three groups of TB: PTB, EPTB & MDR TB than healthy controls. ($p < 0.0005$ & $p < 0.005$

respectively) and is thus, a promising new immunodiagnostic marker for detection of TB. The predictive, diagnostic and prognostic abilities of mycobacterial LipL protein are required to be evaluated in future with large group studies.

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Conflict Of Interest
None

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