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Production and Evaluation of Polyclonal Antibodies Against Surface Protein of *Mycobacterium tuberculosis*: Diagnostic and Vaccine Implications

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ABSTRACT

Mycobacterium tuberculosis (MTB), which causes tuberculosis (TB), is a significant health threat in the world. The diagnosis of early active and latent TB is still a problem. Serological methods have potential benefits of offering a fast and cost-effective diagnosis, although they need to be further refined to become clinically reliable. **Objectives:** To generate and test polyclonal antibodies (pAbs) of surface proteins of MTB as a potentially useful immunodiagnostic application. **Methods:** Extraction of surface proteins of MTB was done with the use of PBS and Tween-20, and rabbits were immunized with antigen-adjuvant mixtures. The serum was gathered, and titers of antibodies were determined by an indirect ELISA. **Results:** Rabbits vaccinated against the surface proteins of MTB generated high-titer antibodies, and the median absorbance of the 1:1600 dilution was 1.42 0.15, which was significantly higher ($p<0.001$) when compared to their controls that were not immunized. The endpoint titer, which was 1:12,800, was geometric, indicating that immunization and antibody were successful. **Conclusions:** The paper shows that it is possible to produce polyclonal antibodies against the proteins of the surface of *Mycobacterium tuberculosis*, which is going to serve as a basis for the future development of serological diagnostic instruments.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that is transmitted by *Mycobacterium tuberculosis* (MTB) bacterium which is one of the most efficient pathogens in the human population and is mostly associated with the lungs [1-3]. It is a pathogenic bacterium that is a part of the Mycobacterium genus, which is characterized by its capacity to survive and reproduce in the human body (particularly the lungs). MTB is an aerosol infection whose primary mode of transmission is through inhaling of coughs or sneezes of an infected individual. The cell wall of MTB is complex and rich in lipids that render it resistant to most widely used antibiotics, and among other factors, it causes its survival in the host immune system [4]. Nevertheless, TB may also disseminate into other body parts such as the lymph node, kidney, and brain, among many others, in a condition referred to as extrapulmonary TB. As mentioned in the WHO Global TB Report 2021, 25% of the world's population has immunologic evidence of previous infection with Mtb in surveillance testing, and 10 million people developed ATB in 2020. TB is one of the most lethal

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infections globally and, together with malaria and HIV/AIDS, has affected human beings socially and economically most severely [5]. According to the latest mortality statistics, 1.4 million people died of TB in 2020, which makes it the second leading infectious cause of death after COVID-19. Despite being discovered over a century ago, an effective vaccine capable of preventing pulmonary TB in adults remains unavailable. Vaccination with Bacillus Calmette–Guérin (BCG), an attenuated *M. bovis* strain, protects children against disseminated forms of TB but offers variable protection against adult pulmonary infection. Although BCG remains the only licensed TB vaccine, its limited efficacy in high-burden regions underscores the urgent need for more effective immunization strategies [6, 7]. Recent data indicate a possible underestimated role in TB protection by humoral immunity as well as cell-mediated immunity. The development of antibodies against *M. tuberculosis* proteins has been studied by many researchers, mostly in the field of diagnostic and immunological uses [8, 9]. In the first stages of infection, the immune system is mainly focused on surface and secreted proteins of MTB that are the main virulence factors and host-pathogen interactions [10]. The study made use of surface proteins of *M. tuberculosis* to produce polyclonal antibodies in an animal model. Surface proteins play an important role in virulence, adhesion, and signaling [11]. Host immune modulation has been linked to surface proteins of MTB, including pili, antigen85 complex, hyperpolarized antigenic glycoprotein of 19 kDa size, and Proline-Glutamic acid proteins [12]. The relevance of these surface-exposed proteins in the formation of antibodies can be used in the development of better diagnostic reagents and next-generation vaccines. The suggestion that protective antibodies might be used to block the progression to persistence induced by the primary infection has revived interest in the study of the humoral component of TB immunity [13].

Tuberculosis remains a major global health burden, and despite advances in molecular diagnostics, early, rapid, and cost-effective detection particularly for latent and early active disease continues to be challenging. Current serological assays lack sufficient sensitivity and specificity, largely due to suboptimal antigen selection and incomplete understanding of humoral immune responses against Mycobacterium tuberculosis. Although surface-exposed MTB proteins play a crucial role in host–pathogen interactions and represent promising immunological targets, their application in reliable immunodiagnostic platforms remains underexplored. In this context, the present study aimed to address this gap by generating and evaluating polyclonal antibodies against MTB surface proteins and assessing their immunoreactivity using an indirect ELISA, thereby providing foundational evidence for the potential development of serology-based diagnostic tools.

METHODS

The experiment was carried out at the Institute of Molecular Biology and Biotechnology, the University of Lahore, Pakistan, from May 2024 and October 2024. A modified buffer extraction technique was used to isolate surface proteins of *Mycobacterium tuberculosis* H37Rv. In a few words, bacterial pellets were washed with the phosphate-buffered saline (PBS; 10 mM, pH 7.4) twice and resuspended in the PBS with 0.05% (v/v) Tween-20 inclusion (Sigma-Aldrich, USA). The suspension was left rotating at 4°C and 1 hour after incubation, centrifuged at 10000 rpm and 10 minutes at the same temperature. The solubilized surface proteins in the supernatant were then collected, filtered with the use of a 0.22 µm syringe filter, and the concentration of protein was measured through Bradford assay (Bio-Rad, USA). The purified antigen was aliquoted and kept at -20°C until its use. Complete Adjuvant (FCA) and Incomplete Adjuvant (FIA) were procured from Sigma-Aldrich (Cat. No. F5881 and F5506). The choice of these particular adjuvants was based on their established immune-stimulatory activity in the production of polyclonal antibodies in rabbits. To achieve a total of 1 mL in each rabbit, a purified antigen was emulsified with an equal amount of adjuvant (1:1 v/v) in every immunization. The emulsion was made through the gentle mixing with the help of a glass syringe till a stable water-in-oil suspension was obtained. This experiment involved six healthy adults of New Zealand White rabbits (2.0- 2.5 kg, female). The immunized group had three animals, and the controls, or non-immunized, had three animals. All animal experimentations were carried out in accordance with the international animal welfare regulations. Ethical approval was secured at the University of Lahore. according to the ARRIVE and OECD animal care requirements. The immunization mixture was administered subcutaneously (s.c.) at multiple dorsal sites using a sterile 1 mL syringe. The immunization protocol consisted of five injections over two months: Day 0: 100 µg antigen + FCA (primary immunization), Days 14, 28, 42, and 56: 100 µg antigen + FIA (booster doses), and Control rabbits received PBS mixed with the same volume of adjuvant. This schedule was designed to allow sustained antigen release and effective antibody production while minimizing tissue stress. Two weeks after the final booster, blood samples (approximately 5 mL per animal) were collected via cardiac puncture under light ketamine anesthesia (35 mg/kg). Blood was allowed to clot at room temperature for 30 minutes, followed by centrifugation at 5000 rpm for 10

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minutes at 4°C. The resulting serum was collected, aliquoted (1.5 mL micro-tubes), and stored at -20°C until further analysis. An indirect ELISA was conducted to establish the levels of antibodies, using the procedures of Grange *et al.* [14] with some adjustments that were confirmed in the study under the antigen. Overnight incubation of polystyrene 96-well micro-titer plates (Nunc MaxiSorp 3.0, Thermo Fisher) was done with 0.5 0g/well of the purified *M. tuberculosis* surface protein in PBS (pH 7.4). Plates were blocked with 2.5% (w/v) PBS containing 0.05% Tween-20 (PBST) for an hour at room temperature. Rabbit sera were added in serial two-fold dilution (1:100 to 1:51,200) and incubated at room temperature (90 min). Following washing, plates were incubated with goat anti-rabbit IgG-HRP conjugate (1:5000; Sigma-Aldrich, Cat. No. A6154) for 1 hour, and ABTS substrate (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; Sigma, Cat. No. A1888) was added. Measurement of absorbance was done at 450 nm, using a microplate reader (Bio-Rad iMark™). Validation: On each plate, positive and negative control sera were placed. Three independent assays were conducted to ensure the reproducibility of the assay. Triplicate analysis of serum samples of immunized rabbits (n=3) and control rabbits (n=3) was done. The outcomes were provided as mean + standard deviation (SD). The absorbance surpassing the mean + (3 x SD) of pre-immune sera became the endpoint titer. An unpaired, two-tailed Student t-test was used in Graph-Pad Prism v9.0 to determine statistically significant differences between the groups; $p<0.05$ was used as a statistically significant value. Sample size justification: The sample size of three animals per group was selected due to the previous literature, which showed that the author has credible immunogenicity data towards polyclonal antibody production with n = 3 animals per treatment group.

RESULTS

The triplicating of the analysis (n=3) was conducted on immunized and pre-immune serum in order to replicate the results. Rabbit anti-*M. tuberculosis* surface protein assays with serum (n=3). The immunogenicity of a fraction of the *M. tuberculosis* surface protein was detected strongly and dilution-dependently in the serum of rabbits immunized with the protein, whereas in the serum of the same rabbits, no binding activity was observed in pre-immune serum (n=3). The median absorbance (SD) of the immunized group at 1:1600 dilution was 1.42 (SD 0.15), which was much greater ($p=0.001$, unpaired t-test) than that of the pre-immune control (0.08, SD 0.02). Strong antibody formation was evident as a statistically significant increase ($p<0.001$) was realized with all the dilutions tested. The endpoint of the antibody titer was identified as 1:12,800. The above findings are a quantitative confirmation of the successful production of polyclonal antibodies that are highly reactive to the *M. tuberculosis* surface protein. Indirect ELISA was done on the serum of immunized rabbits (blue) and controls (red) (serum of pre-immune rabbits). Data points are the standard deviation and mean absorbance of triplicate measurements at 450 nm. The dashed line is used to indicate the cutoff value (mean + 3SD of the pre-immune control). Statistical significance was calculated where an unpaired t-test was used, whereby the statistically significant results were compared to a control group at the same dilution, **, $p<0.001$. The evident distinction between groups in the ELISA absorbance indicates specific antibody production of high titer (Figure 1).

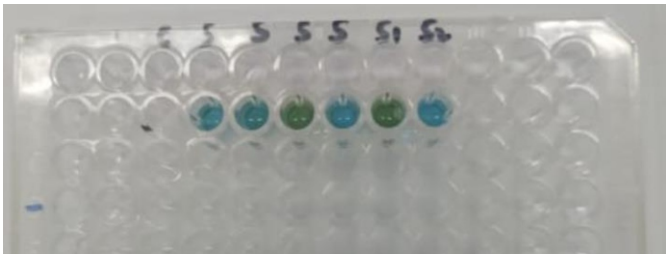

Figure 1: Quantitative Analysis of Polyclonal Antibody Response Against *Mycobacterium tuberculosis* Surface Protein

DISCUSSION

The production and characterization of pABs against surface proteins of *Mycobacterium tuberculosis* (MTB) is an important approach in tuberculosis (TB) research, offering potential applications in diagnostics, vaccine development, and therapeutic interventions [15, 16]. MTB proteins are important virulence factors involved in immune evasion, adhesion, and nutrient acquisition; therefore, they are suitable for the production of antibodies [17]. Any diagnostic test is a keystone of the antigen on the basis of immune reactions that mediate its sensitivity and specificity. In tests involving ELISA, it is important that the antigens used are small enough, uniform, and can attach to the surface of the solid phase of the ELISA plate. Various antigens are typically prepared using high temperature, ultrasound, and chemical methods, but in the process, they alter the structure of the antigen. Thus, milder forms of processing, such as the extraction of surface cellular structures with salt solutions of various ionic strengths with mild detergents, have become more popular. The initial targets of immune response are surface antigens of the pathogens, both of pathogenic mycobacteria and those excreted by the pathogens. Currently, convergent diagnostic kits using serological detection methods have not been established, allowing separation of the humoral response, depending on antibody recognition of M. TB, *M. bovis*, BCG vaccine, and other mycobacteria, though this is underway. The *M. tuberculosis* antigens in the serum of infected patients are

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not dependent on the intact host immune response, and therefore, they are less variable in patients compared to the TB-related antibodies. Moreover, antigen manifestation following *M. tuberculosis* infection and its removal following the cure are manifested earlier than antibodies. The properties render serologic testing of *M. tuberculosis* antigens a more appropriate method of diagnosis of TB. The production of polyclonal antibodies against surface proteins of M. tuberculosis provides valuable tools for studying tuberculosis. In this work, MTB surface proteins key to bacterial survival and host infection were purified and used to immunize rabbits. Antibodies were subsequently isolated from the serum and tested for specificity. In this study, an indirect ELISA demonstrated the successful production of polyclonal antibodies in rabbits immunized with a surface protein fraction of MTB, showing a significant serological response compared to pre-immune controls. ELISA quantitative assays with a mean absorbance of 1.42 /0.15 at a 1:1600 dilution and a geometric mean endpoint titer of 1:12,800 validated the presence of strong immunogenicity of the surface protein extract. These results can be compared to the earlier outcomes of Anderloni [18, 19], who also reported similar antibody titers in rabbits vaccinated with mycobacterial antigens, which proves the applicability of the given method. Although such immunogenicity is encouraging, it has a number of limitations that should be considered. First, a crude antigen fraction was used instead of the purified protein; hence, the antibody response is to a mixture of proteins. This restricts the possibility of ascribing the immune-reactivity to a particular antigen and enhances the possibility of cross-reactivity. The same limitations were observed in previous trials on mycobacterial surface antigens, and it is advised to refine the antigens, followed by diagnostic use. Second, the pilot character of this research, as well as the small size of the sample (n=3), implies that the findings confirm a demonstration of the concept but have to be proved in a larger cohort to ensure that the findings are consistent and strong. Conversely, larger-scale antibody generation biomarkers have demonstrated that they can be recapitulated through batch-to-batch reproducible experiments and that they produce species-specific responses, which underscores the need to scale the number of experimental replicates to apply the biomarkers in a translational context [20]. The results indicate that the preparation of antigens is immunogenic and capable of inducing an antibody response that can be measured. Initial research will be required in the future in the purification of the involved antigen(s), rigorous specificity testing against other mycobacterial species, and the analysis of the antibodies using clinically relevant assays. In general, this paper is a step in the right direction as it confirms the generation of reactive pAbs, yet it emphasizes the importance of additional characterization before any type of application would be a realistic endeavor.

This study has several limitations that should be acknowledged. First, the use of a crude surface protein extract may have resulted in antibody responses against multiple antigens, increasing the risk of cross-reactivity and limiting antigen-specific interpretation. Second, the small sample size (n = 3 per group) restricts the generalizability of the findings and reflects the pilot nature of the work. Additionally, antibody specificity was not evaluated against other mycobacterial species or clinical samples, which is essential for diagnostic validation. Future studies should focus on the purification and characterization of individual immunodominant surface proteins, assess antibody cross-reactivity, and validate performance in larger animal cohorts and human clinical samples. Such investigations will be critical to translating these preliminary findings into clinically reliable diagnostic or vaccine-related applications

CONCLUSIONS

This study produced polyclonal antibodies to the surface protein of Mycobacterium tuberculosis (MTB) by immunizing rabbits. The resulting antibodies were very reactive in indirect ELISA, which demonstrated specific antibodies to antigens. These findings are evidence of the concept of the immunogenicity of MTB surface proteins. Nevertheless, additional research needs to be done to evaluate the specificity of antibodies, cross-reactivity, and their performance with purified antigens and different strains of Mycobacterium. On the whole, the given research is a first step towards creating a set of reliable immunological tools in the sphere of tuberculosis research and paves the further diagnostic and characterization research.

Authors' Contribution

Conceptualization: MUR
Methodology: AB, SAK, MAB
Formal analysis: MFG
Writing review and drafting: MUR, BAG
Review and Editing: MUR, AB, SAK, MAB, MFG, BAG

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

The authors declare no conflict of interest.

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