Chapter 6: Summary and Conclusions
Historically, mycobacterial infections have been a major cause of death and suffering since ancient times. Among the major infectious diseases, mycobacterial infections including JD, leprosy and TB have alone proven as one of the major global public health problem. These infections are worldwide in distribution but preferably more prevalent among the Asian and African people. Tuberculosis, caused by \textit{Mtb}, is responsible for more than 1.4 million deaths and nearly 8.7 million new cases each year (Phelan et al., 2016). The global registered prevalence of leprosy, whose causative agent is \textit{M. leprae}, at the end of the first quarter of 2014 stands at 180,464 cases. While JD infections caused by MAP has steadily dispersed around the world and has now been reported on every continent with the global trade in animals (Nacy and Buckley, 2008). Exact prevalence estimates in domestic species are not available due to limited testing, but nearly all ruminant animal industries have listed JD as a common problem. The reported prevalence of the infected patients is at least partially a reflection of the diligence with which researchers and clinicians look for the mycobacterial diseases.

Eradicating JD, leprosy and TB would mean complete sanitization of these mycobacterial infections across the world. Over the past decades, a number of mycobacterial infection control programs have been implemented in order to completely eradicate and eliminate these diseases. Introduction of regime of antibiotics and existing BCG vaccines, together with the global effort of the WHO to eliminate TB and leprosy as a public health problem, has not met the demand of complete control and eradication of these mycobacterial infections resulting in a global crisis. This may be attributed to lack of early diagnosis and effective medical treatment resulting in increase in
mycobacterial infections prevalence worldwide. The advancement towards the accurate diagnosis and treatment of the long-lasting disease has met with a complete failure as presently there are no reliable diagnostic assays available to detect the sub-clinical infection. Acknowledging these clinical realities, there is an inevitable need of novel intervention strategies in order to better comprehend the control and eradication of JD, leprosy and TB from the world. Improved strategies may include application of various tools to design and develop assays for early detection of the disease as well as vaccines intended for pre-exposure and post-exposure prophylaxis of JD, leprosy and TB.

The SASPs in bacteria such as lipoproteins, OMPs and secretory proteins have been well reported in the literature as the most promising antigenic targets acting as vaccine leads (Miao et al., 2015). Among these, the bacterial lipoproteins are membrane proteins and play significant roles during infection and hence have been commonly targeted for the generation of antibacterial agents (Kovacs-Simon et al., 2011). Similarly, many of the bacterial secretory proteins released into the host have also been targeted and reported to play very important roles in potent vaccines (Miao et al., 2015; Rana et al., 2014, 2015a; Rana and Akhter, 2016). OMPs in eubacteria have several important roles, which ranges from membrane transport to the host-pathogen interactions. These are directly involved in pathogen attachment, entry and activation of several pathogen-induced signaling cascades in the cell. At this juncture, the OMPs may be considered as the most promising antigenic targets acting as vaccine leads since these represent the most exposed proteins on the surface of bacteria and hence vulnerable targets that can be recognized by immune defense system of the host (Smith, 2003).
The challenge of working with these extremely slow-growing mycobacterial species, has markedly narrowed down the experimental knowledge about the pathophysiology of such infections. Therefore, the *in silico* approaches only remain in hand for most of the unveiled target identification studies against MAP, *M. leprae* and *Mtbc*.

In recent years, expediting growth of computational biology and its applications together with the significant amount of clinically relevant data has given rise to a new field called immunoinformatics (Patronov and Doytchinova, 2013). It has been used to identify and characterize potential immunogenic epitopes in pathogens to design and develop novel vaccines in an approach called reverse vaccinology (Vani et al., 2006; Guerfali et al., 2009). Such a combinatorial approach has the potential to be an effective method of epitope discovery against various antigenic microbes.

This present thesis addresses a much more advanced approach to identify and study the novel SASPs including lipoproteins, secretory proteins and OMPs. OMP genes from the completed MAP, *M. leprae* and *Mtbc* genomes by using standardized bioinformatics tools/resources. These tools have been exploited to identify proteins with unique features like the existence of specialized peptide signatures indicating their cellular location and secretion across the cell membrane. The proteins of interest have been prioritized based on their immunologic values having potential T-cell or B-cell antigenic epitopes. Finally, the selected protein epitopes have been further utilized to design novel thermodynamically stable multi-epitope vaccine against the MAP infections. Accordingly, new effective vaccines for leprosy
and TB may be designed which may be proven effective for such mycobacterial infections control by vaccination.

The work started with exploiting the cardinal sequence and structural features of SASPs (lipoproteins, secretory proteins and OMPs). In the case of OMPs, it includes the presence of β-barrel, signal peptide and the absence of the transmembrane helix for the proteome-wide identification of OMPs of ruminant pathogen, MAP. The complete proteome of MAP was analyzed using a pipeline of algorithms, which screens the amino acid sequences and structural features shared by OMPs in other bacteria. Secondary structure of these proteins was also analyzed and scores were calculated for amphiphilic β-strands. From the set of 588 exported proteins, the work identified 264 proteins to be inner membrane proteins while 83 proteins were identified as potential OMPs in MAP. Finally, the study identified 57 proteins as top candidates, on the basis of computed Isoelectric points, as the core set of OMPs. Significantly, the resulting data on OMPs from the present analysis is not only useful in designing novel vaccines but may also open avenues for the development of early serodiagnostic tools for MAP.

In the next part of the study, the challenge to identify the MAP OMPs, which may be immunologically recognized by the host immune system, was addressed. The identified fifty-seven MAP OMPs were evaluated for the epitope selection and analysis employing a computational approach. Thirty-five MAP OMPs were reported with nine-mer peptides showing high binding affinity to MHC Class I molecules and twenty-eight MAP OMPs with fifteen-mer peptides of high binding affinity for MHC Class II molecules. The presence of MHC binding epitopes indicates the potential cell mediated
immune response inducing capacity of these MAP OMPs in infected host. To further investigate the humoral response inducing properties of OMPs of MAP, potential B cell epitopes based on the sequences of peptide antigens and their molecular structures were also identified. In the present analysis, ten proteins having epitopes for both B and T cells representing potential candidates which may invoke both humoral and cellular immune responses in the host were also identified. The findings from the present work will greatly accelerate and expedite the formulation of effective and cost-efficient vaccines and diagnostic tests against MAP infection.

Further, protein structure based strategies were implemented to design an efficient multi-epitope subunit vaccine against MAP. The earlier identified immunodominant peptide epitope sequences from MAP1611 protein were conjugated together with a stretch of conserved amino acid residues of Heparin-Binding Hemagglutinin, reported as a TLR4 agonist was employed as an adjuvant to polarize the cellular responses towards host protective Th1 responses. These three types of component peptides were combined with the help of relevant linkers for efficient separation to improve and intensify the antigen processing and presentation. The primary structures of these multi peptides were three-dimensional homology modeled to yield the final chimeric vaccine. Further, its conformational correctness and stability enhancement was assessed using MD simulations. Finally, disulfide engineering in the most flexible regions of the molecule yielded three potential mutants, Y593C-E610C, Q631C-A634C and a double mutant Q631C-A634C/Y593C-E610C. The double mutant represents thermodynamically most stable version among them. It is potentially highly antigenic, soluble and non-allergen molecule.
interacting with the TLR receptor expressed on the immune cells. This vaccine contains both T-cell and several B-cell epitopes and an adjuvant which potentially possess protective cellular and humoral immune responses triggering properties. The presented vaccine strategy will be proven a promising pathogen specific candidate with wide therapeutic application against MAP which have been be extended to other prevalent infections in future.

For centuries *M. leprae*, etiological agent of leprosy, has been afflicting mankind regardless of the extensive use of recommended live attenuated vaccines and assorted antibiotics. The enhanced global strategies implemented by World Health Organization aims to curtail the rate of new incidences which presents us with a new demand of novel drugs and vaccines vital to restrain the worldwide epidemic of leprosy. The proteins secreted and surface anchored in the outer membrane are considered as attractive therapeutic targets for the treatment of numerous bacterial infections in humans. To investigate suitable antigenic targets in *M. leprae* and *Mtb*, a proteome wide multi-step bioinformatics strategy was employed by integrating the biological knowledge with the computational approaches for the identification of novel and understudied SASPs. Further, computational evaluation was carried out for identifying the immunodominant epitopes as coordinates of prospective antigenic candidates in one of the important classes of SASPs, the OMPs of *M. leprae*. Exploiting the known sequential and structural characteristics shared by the SASPs from bacteria, 17 lipoproteins, 11 secretory and 19 novel OMPs (including 4 essential proteins) were identified in *M. leprae* and 47 lipoproteins, 49 secretory and 36 OMPs in *Mtb*. 

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As OMPs represent the most frequently exposed protein antigens on the cell-surface, the immunoinformatic analysis of 19 *M. leprae* OMPs showed that these harbor T-cell MHC Class I epitopes and Class II epitopes against HLA-DR alleles (fifty-four), while 15 OMPs present potential T-cell Class II epitopes against HLA-DQ alleles (six) and 7 OMPs contain potential T-cell Class II epitopes against HLA-DP alleles (five) of humans. Additionally, 11 *M. leprae* OMPs were found to contain B-cell epitopes while Penicillin binding protein was identified with epitopes which may elicit both antibody- and cell-mediated immune responses. The findings in the present work strongly points to the unique systems present in *M. leprae* and *Mtb* which may be considered as prime candidates for the development of new medical interventions against leprosy and TB.

Information in the present thesis could be targeted in future, to design and develop next generation subunit vaccines against JD, leprosy and TB. Also, the identified antigenic peptides may also be used to express in BCG and other attenuated vaccine strains and the generated recombinant bacteria can be exploited to elicit more robust immune responses against these bacilli as shown earlier (Yang et al., 2016). Such recombinant BCG vaccine together with multi-epitope subunit vaccine option may provide vast opportunity to improve the existing vaccine efficacy against prevalent mycobacterial infections. The specific epitopes discovered in the present study may be targeted for the development of artificial antigens and may be proven as better serodiagnostic markers against the mycobacterial infections. The antigenic molecules identified in the present thesis warrant immediate attention of the
scientific and medical workers for carrying out *in vitro/in vivo* testing and further trials in clinical settings.