7. SUMMARY AND CONCLUSION

7.1. Graphical Summary
7.2. Overall Summary

The major obstacle in leptospirosis is lack of proper diagnostic assays and vaccines for complete protection. This is due to lack of potential antigen, which have the ability to overcome the disease. By keeping these facts in mind for the present study we have identified novel \textit{in vivo} genes from virulent \textit{Leptospira interrogans} serovar Autumnalis strain N2 and applied for the early diagnosis as well as for vaccine development. Overall the outcome was found to be appreciable and herewith we are summarizing the obtained results.

- A total of 274 sera samples and 37 urine samples were taken which includes MAT positive (n=118 sera and n=17 urine), other febrile illness (n=98 sera and n=10 urine), healthy controls (n= 58 sera and n=10 urine) and processed.
- Rabbit polyclonal sera (HIS) were raised for leptospiral heat extracted proteins of Autumnalis N2 (lab strain) and the titre was found to be >1,00,000.
- Genomic library screening identified 18 expressive clones and among them 3 were \textit{in vivo} expressed immunodominant clones (N2 + λ 4-3 III, N2 + λ 16-1 III and N2 + λ 17-3 III).
- Evaluation of these clones against patient’s sera using phage immunoscreening test shows good sensitivity (93.3 - 97.7%) and specificity (65.3 - 94.6%).
- Sequence analysis of 3 \textit{in vivo} immunogenic clones revealed 9 genes: \textit{argC} (1017bp), \textit{recA} (1101bp), \textit{glpF} (717bp), \textit{flaD} (1920bp), \textit{trmD} (696bp), \textit{rplS} (417bp), \textit{rnhB} (696bp), \textit{lp28.6} (744bp) and \textit{lrr44.9} (1185bp).
- The identified genes were cloned and over expressed in prokaryotic expression vectors pET15b / pRSETA as recombinant proteins.
- Recombinant proteins were expressed with a 6×His tag at the N-terminus and purified by metal-chelating chromatography.
- The purity of complete His6 tag fusion proteins expressed in either \textit{E. coli} BL21 (DE3) or \textit{E. coli} BL21 (DE3) pLysS was confirmed by SDS-PAGE using coomassie brilliant blue staining.
- Virulence strain (N2-MACS) (Mouse Adapted Challenge Strain) was selected from its parent strain N2 (laboratory strain) by passaging the isolate in BALB/c mice (cyclophosphamid treated) for ~15 times and its virulence was further evidenced by hematoxylin and eosin (H&E) staining.
Identified ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 were as in vivo expressed proteins and only transpires during natural infection.

Differential temperature regulation assay proved the identified ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 proteins are not as temperature regulated proteins further confirmed their in vivo expression.

Evaluated ArgC, RecA, GlpF, FliD, TrmD, RplS, RnhB, Lp28.6, Lrr44.9 recombinant proteins by IgM ELISA for the diagnosis of leptospirosis where except GlpF and RplS all proteins shows significant sensitivity (>74.5%) and specificity (>78.5%).

Combo ELISA sensitivity was 5 to 20% greater than that of the ELISA using proteins separately as antigen.

Comparison between conventional and qPCR based detection of leptospiral DNA in human urine was assessed for recA and fliD gene and qPCR results showed 100% efficacy whereas conventional PCR shows only 29.4% positivity.

Different vaccine formulations like heat killed vaccine, subunit vaccine, DNA vaccine and prime boost vaccine were prepared for immunization experiments

Heat killed vaccine was prepared from L. interrogans serovar Autumnalis strain N2 and the sterility checking was done to confirm the safety of developed vaccine.

For subunit vaccine, the RecA and FliD proteins were overexpressed as recombinant proteins, purified, dialysed against PBS and used for the immunization along with Freunds adjuvant.

DNA vaccine were constructed by cloning recA and fliD gene in multiple cloning site of pEGFP-N3 vector and purified by endotoxin free plasmid purification kit.

Immunizations were performed with 1 primary (Day 1) and 2 booster (Day 14 and 28) injections of different vaccine formulations followed by cyclophosphamide (Cy) treatment and challenge.

Expressions of recA and fliD DNA vaccine construct in tissues from visceral organs of mice were confirmed by immunoblotting and immunohistochemistry (IHC).

Humoral immune response was found to be induced by different vaccine formulations and showed significant O.D values from 0.6 to 2.5 at 490nm by protein specific IgG-ELISA. Subunit vaccination shows higher levels of antibody followed by Heat killed vaccine > Prime boost > DNA vaccination immunized groups.
• Cell mediated immunity was assessed by qPCR and the results clearly show that our vaccines could induce significant levels of both Th1 and Th2 cytokines (p<0.05). In particular prime boost vaccine shows highly statistical level (p<0.05) of mRNA expression levels for all cytokine analysed (TNF-α, IL-10, IL-4, IL-12p40 and IFN-γ).

• Biochemical parameters (creatinine, alkaline phosphatase and alanine aminotransferase) were analysed during immunization and post challenge days and the vaccine formulations was found to be safe by in vivo.

• Pathological changes were recorded through hematoxylin and eosin (H & E) staining and there is reduction of lesion and necrosis scores (p<0.05) in vaccinated mice in comparison with the unvaccinated controls. High levels of reduction were observed in prime boost vaccine and heat killed vaccine group followed by DNA vaccine.

• Survival of mice was observed among immunized groups heat killed leptospires (100%), rRecA (50%), rFliD (33%), recA/pEGFPN3 (83.3%) or fliD/pEGFPN3 (66.6%) or recA/pEGFPN3 + rRecA (100%) or fliD/pEGFPN3 + rFliD (83.3%).

• Interestingly in our study, along with bacterin vaccine, RecA prime boost vaccine induced 100% sterilizing immunity.

• Shedding of leptospires through urine after challenge were analysed using qPCR and all the vaccinated animals showed appreciable level of reduction and the prime boost vaccination was reported as highly statistically significant (p<0.05).

7.3. Conclusion

Identification of *Leptospira* virulence factors and understanding their properties is crucial for revealing the disease mechanisms. This study underlines the potential importance of the *in vivo* genes in leptospiral infections. The reported data from this study provide new information, which may lead to a better understanding of the disease. Over all our results concludes that *recA* and *fliD* gene were characterized as potential *in vivo* expressed genes, well conserved among pathogenic leptospiral serovars, and as a expressed protein they offer efficient diagnostic sensitivity and specificity, ability to induce humoral and cell mediated immune response and finally provide better protection against challenge with virulent *Leptospira* as prime boost vaccine.