6. DISCUSSION

Leptospirosis is the worldwide zoonosis caused by the spirochete bacteria named *Leptospira* spp. This infectious disease is an important public health problem in both developing and industrialized countries. Humans acquire the disease by direct exposure to *Leptospira* or contact with soil or water contaminated with this organism. Leptospirosis patients used to present with a wide spectrum of symptoms ranging from asymptomatic or a flu-like syndrome to severe disease complications with multi-organ failure. Weil’s disease, the severe form of leptospirosis is manifested by jaundice and renal failure. Leptospirosis has been identified as a re-emerging infectious disease, particularly in Nicaragua, Brazil, India and Malaysia, and in other tropical and subtropical regions (Costa et al., 2015). About 5–10% of leptospirosis patients exhibit severe manifestations and the overall mortality rate is 1–5% (Dolhnikoff et al., 2007; Gouveia et al., 2008).

The major obstacle in overcoming the leptospirosis is still we are in search for the potential antigen for the early diagnosis and vaccine development. Even though a number of *Leptospira* virulent proteins have been identified, molecular mechanisms underlying pathologies observed in leptospirosis are not well understood. Leptospirosis patients with different degrees of disease severity may acquire different numbers of organisms. In addition, patients with severe disease may be infected with high-virulence *Leptospira*. Host genetic background and immune response have also been proposed to involve in different degrees of disease severity. Little is known about the pathogenesis of leptospirosis and the expression pattern of virulent proteins inside the host. Identification of antigenic proteins that triggers the innate immune response in host will help to diagnose the disease in early stage (Natarajaseenivasan et al., 2004). The survival of leptospires upon transition into host conditions and leptospirosis pathogenesis include the modulation of bacterial gene expression. Recent studies evidenced that shifts from environmental to mammalian host simulating conditions (temperature or osmolarity) lead to major transcriptional modifications in *L. interrogans* (Matsunaga et al., 2007), especially for genes involved in pathogenesis. Exposure to guinea pig serum or mammalian macrophages also influenced genome-wide *Leptospira* gene expression (Xue et al., 2010). It was notable that expression of several outer membrane proteins (OMPs) was downregulated. These results emphasized the transcriptomic regulation of pathogenic *Leptospira* upon a shift to host-simulating physiological conditions. However, the regulation of the leptospiral transcriptome has been evaluated *in vitro* only with
host-simulating parameters that poorly reflected real in vivo conditions. Thus, its regulation during infection that is during real in vivo conditions remains to be analyzed. So, we identified the genes, which are turns to be expressed in vivo. Over the past few years, novel methods such as in vivo expression technology, signature tag mutagenesis, and differential fluorescence induction have been used to identify host-induced antigens. The lack of a methodology for genetic manipulation of the Leptospira genome limits application of these approaches to study the immunogenic proteins of pathogenic Leptospira spp.

Our approach for the identification of leptospiral host-inducible antigens is to use a modified in vivo-induced antigen technology (Handfield et al., 2000) in which a gene library of Leptospira interrogans serovar Autumnalis strain N2 is screened with a pooled patient sera. Followed by that a second screen with antiserum to bacterin of in vitro-cultured Leptospira to identify clones positive for genes expressed only in vivo. Using this approach, we have identified three novel, in vivo-induced immunogenic clones namely N2 + λ, 4-3 III, N2 + λ 16-1 III and N2 + λ 17-3 III. Previous studies identify several leptospiral genes like lipL36, lipL45, lsfA, hsp15, lag42, loa22, dnaK, qlp42, lr4A, lr4B, lr4C, lr4D, lK73.5 by genomic library screening (Haake et al., 1998; Nally et al., 2001; Palaniappan et al., 2002; Koizumi and Watanabe, 2003; Artiushin et al., 2004; Verma et al., 2005, 2006, 2012). However none of them can able to provide complete protection from leptospirosis. This drawback may be due to screening of chromosomal DNA libraries with custom made antibodies raised against in vitro-grown high passaged Leptospira. In our study we find out three immunoreactive clones, which found to express with convalescent-phase patient sera but not with bacterin-specific antibody. To further check the diagnostic sensitivity and specificity of the identified clones we have performed the phage immunoscreening test using these immunodominant clones. The outcome of the results showed that antibodies specific to all the three clones are found in the patients sera and can be detected by phage immunoscreening test. Sequence analysis of the identified clones showed nine genes, which are found to be responsible for the expression of novel immunoreactive proteins. The genes were characterized as argC, recA, glpF, fliD, trmD, rplS, rnhB, lp28.6 and lrr44.9. Characterization of these proteins in Leptospira is first of its kind and not reported so far globally. Molecular cloning and expression of the identified immunogenic genes were done by designing gene specific primers, amplified and cloned in prokaryotic (pRSETA/pET15b) expression vector. Gene conservation analysis was done by PCR and protein BLAST analysis.
and the results reveals all the genes were found to be present among pathogenic *Leptospira* species and they were showing less or no homology among non-pathogenic *L. biflexa* species and other bacterial species. Structural prediction and annotation was done for the identified proteins using Modeller 9v7 and Ramachandran plot 2.0 and the constructed model has a good quality. To go for immunoblotting and diagnostic assay, proteins were overexpressed as recombinant proteins using BL21 (DE3)/BL21 (DE3) PlysS and purified by IMAC Ni2+ resin.

A detailed study in leptospiral genes expression profile confirms there was major difference between *in vitro* and *in vivo* condition (Matsui et al., 2011). Till now tons of immunogenic leptospiral proteins were reported but none found to be up to the mark and also found to be downregulated during *in vivo* condition. These results emphasized the need for identification of genes, which up regulated during leptospiral infection. So we identified the genes, which up regulates during *in vivo* condition that is during infection. Even though we have identified three immunoreactive clones from genomic library screening as *in vivo* clones the sequence analysis shows each clone has more than one or two genes, this urge us to further confirm among nine genes identified which one is express under *in vivo* condition. So we performed immunoblots for our purified recombinant proteins against pooled patients’ sera or rabbit HIS (high passaged laboratory culture N2) and the blots of the purified proteins developed with rabbit HIS did not reveal reactivity of the identified proteins except for RplS, RnhB and Lp28.6. This made us to hypothesize that the newly identified and characterized proteins ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 could possibly be expressed during natural infection of the host and not *in vitro*. To further prove that these proteins to be expressed *in vivo* we designed an immunoblotting experiment using virulence strain N2-MACS and non-virulent strain N2 (laboratory strain). For these we used a highly sensitive 1-Step Ultra TMB Blotting Solution (Thermo Scientific, Rockford, IL) for detecting protein even at low concentrations. As a first step raised rabbit polyclonal sera for recombinant proteins like ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 and the titre of specific antibody was found to be >1:100000 for all the recombinant proteins. Then analyzed the raised recombinant proteins specific rabbit HIS against heat-extracted proteins of virulent N2-MACS. Interestingly the expression was found only in virulent N2-MACS for ArgC, RecA, GlpF, FliD, TrmD, and Lrr44.9 proteins and found to be absent in laboratory grown high passaged N2 strain. Absence of differential LipL32 expression confirms the sensitivity of
assay performed. Still to convince strongly that our identified proteins are purely in vivo and not temperature regulated proteins, further performed immunoblotting against heat extracted proteins of L. interrogans serovar Autumnalis strain N2 grown at 30, 37 and 42°C and the results revealed it doesn’t have any detectable level of these proteins and show positive detection only for leptospiral LipL32. Overall the results strongly confirm that the proteins namely ArgC, RecA, GlpF, Flid, TrmD, and Lrr44.9 were found to be expressed only during natural infection of the host and not in vitro.

The search for new tools for the diagnosis and treatment of leptospirosis, especially an early sensitive and reliable diagnostic test, would improve patients’ quality of life (Lin et al., 2010), as pathogenic Leptospira can rapidly disseminate to multiple organs and cause multi-organ system complications, including jaundice, meningitis, pulmonary hemorrhage, hepatic and renal dysfunction, and cardiovascular collapse. Due to the fastidious and slow-growing nature of Leptospira and difficulty in observing the organism in body fluid, diagnosis of leptospirosis often relies on serology, which can be difficult to interpret due to confusing with other bacterial illness (Natarajaseenivasan et al., 2008). Currently, the standard reference method for serologic diagnosis of leptospirosis is the microscopic agglutination test (MAT), in which sera are reacted with live antigen suspensions of various Leptospira serovars (Levett, 2001). However, MAT can be a challenging assay to implement because it requires considerable expertise to perform and interpret and necessitates the continual maintenance of a panel of live strains representing all serogroups in addition to locally isolated strains, which is technically demanding and biohazardous. Therefore, MAT is usually restricted to reference laboratories (Woodward et al., 1997). The current interpretive criteria indicative of active infection for the Leptospira MAT require a 4-fold rise in titer between the acute and convalescent-phase sera (Levett, 2001). Although it is well recognized that seroconversion or increasing antibody titers in paired serum specimens provide strong evidence for true infection, the samples need to be taken 2 to 3 weeks apart in order to see changes in titer, which is not practical in clinical settings. The complexities associated with MAT highlights the need to develop a simple and rapid screening test to detect Leptospira antibodies. Commercial enzyme-linked immunosorbent assay (ELISA) kits using antigens derived from a nonpathogenic Leptospira biflexa serovar Patoc have generally been found to have lower sensitivity than that of MAT, because the ELISA antigens do not detect all infecting serovars (Fairbrother et al., 1984). Moreover, the antigenic preparations are generally crude in nature.
and from a single serovar, with lipopolysaccharide as the major antigenic component. Since lipopolysaccharide is serovar specific, the antigens may not detect antibodies produced against serovars other than that used for antigen preparation, thus limiting the widespread use of the assay (Natarajaseenivasan et al., 2011b). Other than these several diagnostic tests like whole cell-killed leptospiral antigen-based tests like macroscopic slide agglutination test, microcapsule agglutination test, lepto dipstick, lepto dridot and polymerase chain reaction (PCR). All these techniques were found to have low sensitivity during the acute stage of the disease and laborious to be performed in all laboratories. Therefore, researchers are focusing on development of recombinant protein based assays that would be more sensitive during the early stage of the disease (Raja and Natarajaseenivasan, 2015). Previously, several studies have attempted to utilize different recombinant proteins from *Leptospira* spp. as antigens in an indirect enzyme-linked immunosorbent assay (ELISA) as a serodiagnostic test (Bomfim et al., 2005; Flannery et al., 2001; Croda et al., 2007; Natarajaseenivasan et al., 2008; Oliveira et al., 2008; Chalayon et al., 2011; Natarajaseenivasan et al., 2011b; Vedhagiri et al., 2013; Anita et al., 2016). However still we need a novel antigen with a potential diagnostic technology for efficient diagnosis of leptospirosis disease without false positives and false negatives.

There is an urgent need to develop a rapid, sensitive and appropriate diagnostic test that could be used in a routine diagnostic laboratory to detect antibodies against leptospires. Immune responses include antibodies to numerous leptospiral proteins constitutively expressed or upregulated during infection (Lessa-Aquino et al., 2013). Antibodies of the IgM isotype appear about 4 days after onset of fever and headache and are responsible for clearance of leptospires from the bloodstream. Enzyme-linked immunosorbent assay (ELISA) offers reasonable sensitivity and the possibility of handling many samples at a time. Recombinant protein-based ELISA is a suitable and safe procedure for the examination of a large number of sera as it involves an immunodominant antigen and lacks the non-specific moieties present in whole-cell preparations (Flannery et al., 2001). Therefore, it is essential to identify novel candidate antigens to improve diagnostic methods to assist in early treatment. For the evaluation of recombinant ELISA, we have applied our identified immunogenic proteins as antigen, which is also identified as conserved among pathogenic serovars, and also systematically characterized as potential in vivo antigens. Even though we have screened only six genes as potential producers of in vivo out of nine genes identified, evaluation of
recombinant IgM ELISA was done for all of our identified proteins. Among all the recombinant proteins analyzed except GlpF and RplS, remaining proteins had the highest sensitivity (>74.5%) and specificity (>77.9%) for the diagnosis of leptospirosis. The use of purified recombinants in combination proved to be an effective antigen to provide conclusive diagnosis of leptospirosis. Percentage sensitivity and specificity were >90% based on comparisons with sera of healthy individuals and of patients with other febrile infectious diseases.

Humoral immune responses to pathogenic bacteria are mainly directed at surface-exposed and secreted antigens accessible to antibody in plasma and mucosal secretions. Thus, a screen of an expression gene library with immune serum identifies a disproportionately large number of proteins with sequences predictive of surface exposure (Zysk et al., 2000; Etz et al., 2002; Timoney et al., 2007). A consequence of this approach has been failure to consider cytoplasmic proteins. The proteins identified as in vivo in our study are cytoplasmic or periplasmic proteins of L. interrogans that participate in host immune responses. Neither of these proteins was identified in a recent high-density protein microarray of serovar Copenhageni for which the selection criteria included potential biological importance and antigenic features (Lessa-Aquino et al., 2013). The majority of studies of putative virulence factors important in microbial pathogenesis have focused on non-metabolic gene products. This is due, in part, to the belief that housekeeping and other genes common to pathogens and non-pathogens are unimportant for pathogenesis. In fact, during the crucial early phase of microbial proliferation in vivo, the ability of the pathogen to synthesize or acquire scarce/unavailable nutrients becomes critical. For example, iron acquisition factors are well accepted as important for leptospiral pathogenesis (Asuthkar et al., 2007; Velineni et al., 2008). Acquisition or synthesis of other nutritional factors is less well appreciated but supported by a variety of reports (Leung et al., 1991; Mahan et al., 1993; McAdam et al., 1995). The frequent identification of biosynthesis genes following library screening of random gene fusions or randomly generated transposon mutants argues for their importance in pathogenesis (Fields et al., 1986).

Before begin the vaccination experiments a potential gene has to be taken from the previous results. Since all the six in vivo genes identified shows good diagnostic sensitivity and specificity, we went for a literature survey in related species and found that recA and fliD
have been reported as potential virulent genes by mutational studies. RecA is a ubiquitous bacterial recombination protein essential both for DNA transformation (Vosman et al., 1991; Quivey et al., 1992) and for mediation of bacterial SOS responses (Walker et al., 1984). Colonization and infection of host organs are potentially stressful for invading pathogens because they include the effects of antibacterial factors such as pH, elevated temperature, osmolarity, and factors released by other microorganisms on the mucosal surface. Some stresses result in DNA damage, the repair of which involves RecA, an enzyme critical for pairing of single-strand DNA with complementary regions of double-stranded DNA (Kameni et al., 2002). Single-strand DNA - RecA produced following DNA injury activates self-catalysis of LexA protein responsible for blocking expression of SOS genes for proteins that repair DNA. Thus, RecA also functions as a LexA coprotease. A significant decrease in intracellular pH has been shown to result both in DNA damage via depurination in Salmonella enterica serovar Typhimurium and in induction of the DNA repair protein PolA (Foster et al., 1994). RecA may enhance resistance to oxidative killing by phagocytes based on evidence that a ΔrecA mutant of Salmonella Typhimurium is sensitive to the killing effect of the oxidative burst of macrophages (Buchmeier et al., 1993). Furthermore, recA mutation diminishes adherence to and colonization of rabbit intestine by Vibrio cholerae (Kumar et al., 1994). Infection by serovar Autumnalis and other Leptospira serovar is clearly associated with consistent expression of this immunogenic protein in early stages of invasion/bacteremia, raising the possibility that it has a role in pathogenesis in addition to that of DNA repair.

Pathogen attachment is a crucial early step in causing infections. This step is mediated by important virulence factors, such as flagellar proteins. In particular, a known mucin adhesin, the flagellar cap protein, Flid, appears to engage in a highly effective, specific interaction with mucin (Arora et al., 1998). The flagellar cap protein could play a role in adherence by mediating initial binding of the flagellar tip to mucin during the first stage of pathogenesis. The expression of flagella correlates with different aspects of bacterial pathogenicity, ranging from adherence to host cells to activation of inflammatory responses by the innate immune system. Flid protein because of his low inter strain variability and their high immunogenicity, could be interesting test antigens for active immunization (Péchiné et al., 2005). Moreover, flagellin has already been used as a vaccine antigen in other infections, such as salmonellosis. Oral or nasal immunization of mice with flagellin allowed a lower
degree of infection in the immunized group than in the control group (Strindelius et al., 2004). Recently it is found that binding of the enteropathogenic *Escherichia coli* (aEPEC) strain to enterocytes was significantly impaired in strains deleted of *fliD* genes and it could not form flagella on the bacterial surface (Sampaio et al., 2016). Further the results revealed that the role of flagella in the adherence of aEPEC to human enterocytes was mediated by FliD in the adhesion process (Sampaio et al., 2016). The identified *in vivo*-expressed proteins RecA and FliD would have been involved in infection and stress responses of *L. interrogans* and thus are upregulated during infection and elicited specific IgM responses in patients during the acute phase. So based on these results these two *in vivo* expressed genes were considered for vaccination experiments. However, still before going to vaccination experiments a preliminary examination of testing these two genes, recA and fliD in patient’s urine for the antigen based detection will further support the potential of these genes in early diagnosis. Because only few genes like *lipL32*, *ligA* and *ligB* were evaluated in urine samples of diseased humans and animals and also only these genes show good immunoprotection in vaccine studies.

The need for rapid diagnostics at the time of admission for patients with suspected leptospirosis has led over the last two decades to the development of numerous assays to detect antigen in a range of samples using the polymerase chain reaction (PCR). Conventional and real-time PCR have been described for the detection of *Leptospira* in urine samples taken from humans (Natarajaseenivasan et al., 2012; Villumsen et al., 2012). This reduces time to diagnosis and can be performed outside of the reference laboratory. In diagnostic laboratories, real-time PCRs are increasingly being used instead of conventional PCR. These techniques are faster, require less laboratory personnel and are performed in a closed system, thereby reducing the risk of DNA cross-contamination. Potentially, they further allow quantification of the targeted organisms as a quantitative real-time PCR (qPCR). Assays fall into two categories based on the detection of genes that are universally present in bacteria (for example, gyrB (Slack et al., 2006), *rrs* (16S rRNA gene) (Merien et al., 1992; Natarajaseenivasan et al., 2012) and secY (Ahmed et al., 2009), or detection of genes that are restricted to pathogenic *Leptospira* spp. (e.g. *lipL32* (Levett et al., 2005; Stoddard et al., 2009), *ligA* and *ligB* (Palaniappan et al., 2005). Here, we compare the conventional and real-time PCR assays targeting *recA* or *fliD* where the primer sequences were designed which can detect only the pathogenic *Leptospira*. Our findings suggest that all
relevant *Leptospira* spp. are detected by our *recA* or *fliD* real-time PCR with high sensitivity. Absence of amplification in non-pathogenic *Leptospira* and other bacterial species shows the specificity of the developed qPCR assay. We then validated the developed *Leptospira* specific real-time PCR targeting *recA* or *fliD* gene for application on urine samples from humans. Our qPCR assay proves 100 % efficacy by detecting *Leptospira* in all the submitted urine samples. This confirms the potential of our identified genes as diagnostic antigen equal to currently using *lipl32* or *lig* genes. While the clinical sensitivity of 16S real-time PCR was very high, it is reported that unacceptable high rate of false-positive reactions were observed when the 16S real-time PCR was used for either with blood cultures or urine samples. Most PCR assays used in diagnostic laboratories have a clinical specificity very close to 100%. These observations (Stoddard et al., 2009; Ganoza et al., 2010), suggest that the 16S real-time PCR can no longer be recommended for primary diagnostic use. So use of our *in vivo* genes will be an alternative positive strategy in antigen based detection methods for leptospirosis.

Different vaccine formulations have been tested against leptospirosis in animal models like whole cell based killed, attenuated leptospiral vaccines, recombinant protein vaccines or DNA vaccines (Raja and Natarajaseenivasan, 2015). Killed whole-cell leptospiral vaccines for prevention of human leptospirosis are available in some countries, including France, Cuba, and Japan (Martínez et al., 1998; Koizumi and Watanabe, 2005). However whole cell vaccines were staggered by several limitations like short-term immunity, LPS reactogenicity, possess undesirable side effects, requirement of annual boosters and lack of cross protection against the various leptospiral serovars. Several studies have evaluated different formulations of recombinant vaccine candidates with the intention of improving leptospirosis vaccines. To date, the most promising results were obtained using the Lig (Leptospiral immunoglobulin-like) proteins. Mice immunized with LigA or LigB survived lethal challenge, showing 90–100% of protection (Koizumi and Watanabe, 2004). In a vaccine efficacy study using the C-terminal portion of the LigA protein, induced protection ranging from 63% to 100% in hamsters (Silva et al., 2007). Of note, sterilizing immunity has not yet been achieved (Dellagostin et al., 2011) highlighting the need for new conserved antigens for vaccine development.

In case of DNA vaccines it has more advantages, which include ease of manipulation, low production costs, stability, and lack of a cold-chain requirement that can induce both humoral responses as well as cellular responses. Even though several recombinant protein
vaccines and DNA vaccines were tested in animal models these candidate vaccines have
down variable level of immunogenicity and protection in animal models and majority of the
vaccines tested to date do not induce significant protection against mortality (Ko et al., 2009;
Raja and Natarajaseenivasan, 2015). These drawbacks highlight the need to develop
alternative vaccines strategies, such as prime boost based immunization against leptospirosis.
Traditionally the same vaccines are given multiple times as homologous boosts. New
findings suggested that prime-boost could be done with different types of vaccines containing
the same antigens. In many cases such heterologous prime-boost can be more immunogenic
than homologous prime-boost. Heterologous prime-boost represents a new way of
immunization and will stimulate better understanding on the immunological basis of vaccines
(Lu et al., 2009). Guinea pigs and hamsters were reported to be the most suitable animal
model for reproducing acute lethal infection for leptospirosis. Due to availability,
maintenance and handling of these animals we planned to design a vaccine experiment using
BALB/c mice as animal model. But laboratory strains of mice are in general not susceptible
to virulent leptospires. To overcome this and to provide a better animal model for vaccine
experiment alternative use of animal should be considered for more vaccination studies. In
supporting this statement, earlier reports are their using cyclophosphamide (Cy) treated
BALB/c mice in experimental infection of *Leptospira* in place of hamsters or guinea pigs
(Adler and Faine, 1976, 1977; Masuzawa et al., 1991). In the present study we used BALB/c
mice treated with Cy.

This study evaluated, for the first time, the recombinase A (*recA*) and flagellar cap
protein (*fliD*) from *L. interrogans* Autumnalis strain N2 as a vaccine antigen against
leptospirosis. In addition to the *in vivo* properties of *recA* and *fliD* gene, orthologues of these
genes were present in different serovars, suggesting it is a potential vaccine candidate.
Independent experiments using subunit, DNA vaccine, and prime boost immunization
strategies were performed using RecA and FliD. One limitation that hinders the development
of vaccines against leptospirosis is the lack of correlates of protection, immune markers that
have contributed to many vaccinology studies (Pizza et al., 2000; Etz et al., 2002). To date,
no markers have been identified for leptospirosis and the animal model of lethal leptospirosis
remains the preferred method of assessing vaccine efficacy (Haake et al., 2006). Both
humoral and cellular immune responses have been reported to play roles in the response
against leptospirosis (Fraga et al., 2011) as such, this study included a protein-boost strategy
that aimed to improve the immunogenicity of the recA or fliD DNA vaccine, as previously demonstrated for other antigens (Feng et al., 2009; Hartwig et al., 2013).

The subunit vaccine preparation included Freund's complete and incomplete as an adjuvant. Animals immunized with the subunit vaccine demonstrated the highest IgG response; however, only 33.3 - 50.0% of animals survived the challenge. Several studies have shown the effectiveness of DNA vaccines and reported that protection was associated with a strong antibody response (Branger et al., 2005; Faisal et al., 2008; He et al., 2008). However, in the present study the levels of antibody for DNA vaccine even though lower when compare to subunit vaccine the protection was found to be greater (66.6 – 83.3%). In supporting this statement earlier report states that the outer membrane protein LipL32 of Leptospira was able to impart protection when used as a DNA vaccine but failed to impart a significant level of protection when used as recombinant protein (Branger et al., 2001; 2005). A recent study also suggested that protection by LigA was not due solely to the humoral immune response induced by the protein or its fractions (Coutinho et al., 2011). This shows that both humoral and CMI is needed for better protection. Remarkably, high level of protection was conferred by prime boost vaccine in our study (83.3 % for FliD and 100% for RecA).

Still we can’t able to reach a conclusion whether both arms of immunity is needed because the positive control used, bacterin vaccine shows 100% protection from challenge. It is probable that Leptospira could have an intracellular phase, which protects them from antibiotics and the host immune system, as their presence in monocytes/macrophages and/or Vero cells have been demonstrated (Merien et al., 1997). Moreover invasion of Madin-Darby canine kidney (MDCK) cells only by pathogenic leptospires and not by saprophytic leptospires further validates this hypothesis (Barocchi et al., 2002). This invasive ability of virulent leptospires may help them to persist in areas protected from the immune response, such as meninges or eyes (de Souza et al., 2006; Parma et al., 1997). Since immunity to leptospirosis is mediated mainly by humoral mechanisms, the presence of virulent L. interrogans in renal tubular cells, where they are protected from specific and nonspecific defense mechanisms, could explain this persistence in host animals (Faine et al., 1999). This bacterial persistence is also well established for other spirochetes, such as Treponema pallidum in syphilis and Borrelia spp. in Lyme disease (Appel et al., 1993; Chang et al.,
Thus, it seems likely that antibody itself is not sufficient to eliminate these spirochetes from the host. Hence, an effective vaccine against *Leptospira* would be one that should not only prevent invasion of spirochete but should also eliminate those that somehow escaped the immune response and have formed successful niche in various organs. By keeping these facts in mind, we evaluated the CMI response in immunized mice spleen using qRT-PCR. The results show that our vaccines can able to induce both Th1 and Th2 cytokines. Even though down regulation of IL-10, IL-12p40 and IFN-γ was observed for subunit vaccines. Upregulation of TNF-α, IL-10, IL-4, IL-12p40 and IFN-γ were observed for both DNA vaccine and prime boost vaccine. This discloses the level of protection provided by DNA vaccine and prime boost vaccine in comparison with the subunit vaccine. The key success of vaccine development is achieving sterilizing immunity. In a previous report, sterilizing immunity was observed in hamsters immunized with a recombinant *Mycobacterium bovis* BCG strain expressing LipL32 (Seixas et al., 2007). However majority of vaccine candidates against leptospirosis have so far failed to induce sterilizing immunity (Raja and Natarajaseenivasan, 2015). Bacterin vaccines have been shown to induce sterilizing immunity in previous studies (Schreiber et al., 2005). Interestingly in our study, along with bacterin vaccine, RecA prime boost vaccine induced 100% sterilizing immunity. The burden of infection and its effects on vaccinated animals, qPCR and a histopathology scoring system were included as quantitative outcome measures. To our knowledge, this is the first vaccine study to use qPCR to quantitate leptospiral burden in animal urine samples after challenge. The application of qPCR to leptosporal vaccine studies allows for the accurate determination of the leptospiral burden, especially in the urine samples. We found that the prime boost vaccine confer good reduction in leptospiral load followed by DNA vaccine and subunit vaccine.

This study reports on the successful evaluation of a novel vaccine candidate that offers significant protection against mortality in the susceptible mice model of leptospirosis. Furthermore, due to the considerable level of conservation of *recA* and *fliD* among *Leptospira* spp., it is a potential candidate for the development of a vaccine that could generate cross-protection against a wide range of *Leptospira* serovars; however, this needs to be better evaluated in trials of heterologous challenge. Our results further demonstrate that prime boost vaccine using *recA* or *fliD* could impart a significant level of protection as revealed by enhanced survival and reduced histopathological lesions in vital organs of
immunized animals. Keeping in mind the limitations associated with the currently available vaccines against leptospirosis, there is urgent need for development of more efficacious and better-defined vaccines. Finally, we conclude that our DNA prime protein boost vaccine can activate a strong humoral and CMI thus seems to be an ideal vaccine candidate for leptospirosis.