Review Article

Advances & challenges in leptospiral vaccine development

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Considerable progress has been made in the field of leptospiral vaccines development since its first use as a killed vaccine in guinea pigs. Despite the fact that the immunity conferred is restricted to serovars with closely related lipopolysaccharide antigen, certain vaccines have remained useful, especially in endemic regions, for the protection of high-risk individuals. Other conventional vaccines such as the live-attenuated vaccine and lipopolysaccharide (LPS) vaccine have not gained popularity due to the reactive response that follows their administration and the lack of understanding of the pathogenesis of leptospirosis. With the recent breakthrough and availability of complete genome sequences of *Leptospira,* development of novel vaccine including recombinant protein vaccine using reverse vaccinology approaches has yielded encouraging results. However, factors hindering the development of effective leptospiral vaccines include variation in serovar distribution from region to region, establishment of renal carrier status following vaccination and determination of the dose and endpoint titres acceptable as definitive indicators of protective immunity. In this review, advancements and progress made in LPS-based vaccines, killed- and live-attenuated vaccines, recombinant peptide vaccines and DNA vaccines against leptospirosis are highlighted.

Key words DNA - immunity - *Leptospira* - leptospirosis - recombinant technology - subunit vaccines

Leptospirosis a zoonotic disease is caused by spirochetes of the genus *Leptospira* and it occurs in diverse epidemiological settings affecting vulnerable populations[1]. The disease remains endemic in most tropical and sub-tropical regions of the world including Latin America, Middle East, Africa and Asia[2,3]. Leptospirosis can be acquired by humans when they come in direct contact with urine and other body fluids from infected animals, but in endemic regions, people and other susceptible animal species can acquire the disease by indirect contact with water or soil contaminated with viable leptospires[4,5].

Leptospirosis is perceived as an important public health problem as a result of epidemic cases recorded in both developed and developing countries[6]. Since its initial demonstration by Weil[7] (also known as Weil's disease), sporadic outbreaks have occurred throughout the world .with fatal outcomes[8]. In the past century, several epidemics have been reported worldwide including India, where the disease has been endemic since the 20th century[9]. Epidemiological investigations indicate that the infection is commonly associated with certain occupational groups such as farmers, sewage workers, veterinarians, and animal handlers[10-12] and most of the circulating serovars reside in rodents and

other domestic animal reservoirs such as dogs, pigs and cattle[13-15]. Hence, these domestic animals may be the source of both human and animal infection.

Development of an effective vaccine against leptospirosis remains a challenge[16]. The pathogen has evolved mechanisms to evade the protective function of the complement system, multiply in blood, adhere to host cells and penetrate into organs and tissues at a faster rate[17]. This ability to rapidly colonize multiple organs poses high threat to the host and is the main reason for the need to develop a safe and efficient leptospirosis vaccine. Non-living vaccines, which confer protection primarily through the immunity elicited by surface-exposed lipopolysaccharide (LPS), generally provide short-term homologous protection against serovars included in the vaccine preparation[18]. In contrast, live-attenuated vaccines can mobilize both the cellular and humoral immune responses and help develop long-term immunity. While the process of attenuation can adversely lower the antigenicity of live vaccines, it can however, be more challenging when multiple serovars are targeted[9].

Leptospirosis vaccines

Live vaccine

These vaccines are known to elicit a strong cellular and humoral immune response and confer lifelong immunity with a few doses. These vaccines are produced by attenuating a pathogenic organism but retaining its ability to replicate and induce protective immune response without being able to cause disease. Live leptospiral vaccines have not gained prominence due to a lack of detailed knowledge about the pathogenicity, diversity in serovar distribution and the need for genetic tools that allow for manipulation of pathogenic *Leptospira* species[19,20]. Experimental live attenuated Pomona vaccine has been shown to be effective in protecting pigs against leptospiral challenge and also prevented establishment of renal reservoirs of the organism[21,22]. Serovar Pomona attenuated by laboratory passage was also used as a live vaccine in the cattle. The leptospires were attenuated by passage through egg and the vaccine was used in an aborting cattle herd. The vaccine was shown to elicit protective immune response and reduced the abortion rate[23]. The application of these vaccines in leptospirosis was limited due to their instability, undefined genetic basis for attenuation and lack of strict quality control measures necessary for their production[24]. Efforts by Srikram *et al*[24] in developing a transposon mutant with LPS-associated mutations of *Leptospira interrogans* serovar Manilae conferred a significant cross-protective immunity in hamsters against homologous as well as heterologous challenges. Although the live-attenuated vaccines were able to induce higher antibody titres compared to bacterins, their acceptance and use among veterinary biologic manufacturers were hampered by the difficulty of maintaining their viability during storage as well as the potential for reversion to virulent state in the host animal[25]. Thus, most commercially available *Leptospira* vaccines are bacterins[26].

Bacterin vaccines

Killed whole-cell bacterins against leptospirosis have been in use for over five decades. This follows successful immunization of guinea pigs with phenol inactivated leptospires[9]. However, side effects such as reactivity due to components of the growth media, local and systemic reactions and restriction of protection to closely related serovars have hindered its application[18]. Furthermore, local variability of endemic pathogenic leptospiral strains from region to region as well as virulence and lack of cross-reactivity have greatly hampered its application in the prevention of leptospirosis. Others factors include lack of consistency of production or repeatability, high production costs, lack of immunological memory and short duration of immunity requiring several booster doses[18].

Efforts to grow leptospires in protein-free medium have yielded mixed results with some reporting no significant difference in the side effects produced between leptospires grown in bovine serum albumin-supplemented medium and protein-free medium[9]. Due to lack of cross-reactivity among different serovars, a polyvalent vaccine containing multiple serovars has been used largely in dogs, swine and cattle and yielded a satisfactory response[27]. Despite reports indicating lack of heterologous protection, they are able to confer protection against serovars included in the vaccine as well as antigenically related serovars[9]. The serovar-specific LPS component of *Leptospira* is responsible for inducing antibody response, although the response lacks immunological memory except for *Leptospira interrogans* serovar Hardjo infection in cattle which is characterized by T-helper I and interferon gamma (INFγ) T-cell response[9,28]. The duration of immunity is also said to range from six months to three years depending on the choice of adjuvants[17,18]. Recent reports in the United States indicate up to 30 per cent of vaccinated dogs do not respond to vaccinations and immunizations are also associated with adverse effects[10]. This is attributed to change in the epidemiology of

the disease and ingress of new serovars in endemic areas. Not only is there failure to provide protective immunity by certain commercial bacterin vaccines, but also claims of vaccines (Nobivac L4) attributed death or transmission of infection among vaccinated dogs[29]. These controversies further highlight the importance of leptospirosis as important zoonosis and emphasized the need for safe and effective vaccine for both humans and animals. This has led to development of new strategies such as the use of sub-unit vaccines (Duramune, by Fort Dodge, USA) composed only of the immunogenic component of the *Leptospira* organism instead of the entire organism[30].

As observed in animals, use of bacterins in humans also has limited protection against serovars contained in the vaccine. The bacterins for human use are composed of monovalent or polyvalent cellular suspension, for example, French SPIROLET (IMAXIO) from Pasteur Merieux against *Leptospira icterohaemorrhagiae* serovar. Although this vaccine is specific for icterohaemorrhagiae infection, it is capable of protecting against antigenically related serovars[31]. In addition to the SPIROLET vaccine, there is a bivalent vaccine from Shanghai Institute of Biological Products and a trivalent vaccine (Vax-Spiral) from Cuba, both of which have successfully undergone phase four clinical trial as reported by Martinez *et al*[31]. The vaccine was registered in 1998 and has shown improved safety with low reactivity[31,32]. However, these vaccines are only recommended for workers with high risk of contracting leptospirosis infection. Other benefits of bacterins include anti-shedding effect which helps reduce transmission, stability during storage, unlikely to cause disease due to residual disease-causing characteristics, induction of strong antigen-specific proliferative response by peripheral blood mononuclear cells and production of cross-protective immunity against closely related serovars[17,33,34].

Leptospiral lipopolysaccharide antigen vaccine

Leptospiral lipopolysaccharide (L-LPS) constitutes the major component of the outer membrane which greatly influences the virulence of pathogenic *Leptospira*[19]. It is a complex structure with approximately 100 genes all encoded in a single double-stranded DNA molecule within its biosynthetic loci[35]. However, there is a lack of adequate knowledge on its structure and the role of individual proteins involved in its synthesis[12,19]. During infectious process, L-LPS plays a significant role in the stimulation and activation of innate immune response by eliciting a differential recognition via toll-like receptors (TLRs 2 and 4)[36,37]. L-LPS is a protective immunogen as demonstrated by protections rendered by monoclonal antibodies against lethal infection in hamsters[38]; however, there are conflicting views on the ability of LPS-based *Leptospira* vaccine to protect against heterologous challenge[17,39]. Hence, there is a need to carry out further studies to determine whether LPS vaccines are serovar-specific or heterologous. The disadvantage of the strong biological activity of LPS is its contribution to vaccine reactivity[40]. As mentioned earlier, there is lack of knowledge on the complex structure of L-LPS and the genes involved in its biosynthesis; thus, modifications of the LPS structure that would have allowed triggering of immune response needed in a vaccine and the lowering of its toxicity would make LPS vaccines more potent and efficient.

Recombinant vaccines

Efforts to develop effective leptospirosis vaccine with ability to protect against multiple serovar infection have led to the adoption of recombinant technology. This technology has witnessed success in protection against many infectious diseases, for example, human hepatitis B vaccine[41]. This technique involves inserting the foreign DNA molecule encoding an antigen such as outer membrane protein that stimulates an immune response into bacterial or mammalian cell, expressing the antigen in these cells and then purifying them for use as vaccines. Recombinant vaccines based on highly immunogenic genes conserved among pathogenic leptospires such as LipL32, LipL41, OmpL1 and LigA and virulence factor gene *Loa22* have all shown promise in different animal models[9,11,14,42,43]. Immunoprotection of leptospiral recombinant protein has been demonstrated based on OmpL1 and LipL41 genes. The genes were amplified, cloned and expressed in BL21 *Escherichia coli* cells. The expressed proteins were used to immunize dogs and later challenged and the protection conferred was 83 per cent[44]. Similarly, rising antibody titre against conserved region of recombinant LigA has been reported in hamster model of leptospirosis[45]. In this experiment, the gene was expressed as a fusion protein with glutathione-*S*-transferase in *E. coli* cells. The recombinant protein was able to protect hamsters against lethal challenge with pathogenic *Leptospira interrogans* serovar Pomona. In a related study, the efficacy of a synthetic consensus DNA vaccine

developed against lipoprotein LipL45 was tested[46]. Intramuscular immunization of mice with the synthetic LipL45 DNA vaccine via *in vivo* electroporation induces a significant Th1 cellular immune response as well as development of specific anti-LipL45 antibodies. Cloning of synthetic DNA encoding immunogenic protein into a plasmid vector, propagating the plasmid carrying the foreign DNA in bacteria such as *E. coli* and then isolation and purification for immunization have shown promise as important strategy for vaccine development[46]. Genetic engineering has been applied for many different purposes in research and medicine. The technology enables creation of multiple copies of genes or insertion of foreign genetic materials into an organism with the aim of acquiring desirable features such as resistance or multivalency. Mammalian expression system can also be used when the protein antigen requires post-translational modification such as glycosylation.

Recombinant subunit protein vaccine

Live-attenuated vaccine mimics natural infection, and as a result, antibody production is achieved for a prolonged period of time. However, the use of live-attenuated vaccines against leptospirosis poses a risk due to its potential ability to revert to virulence, cause infection, continuous shedding in the urine and accidental infection resulting from handling of live strains.

An ideal vaccine for human and animal use should be effective, avirulent and produce long-lasting neutralizing immunity[47]. Subunit vaccines such as recombinant protein vaccines have been reported to be promising vaccine candidates because these are avirulent, less bio-hazardous, non-infectious, non-viable and well-defined[47,48].

Recombinant proteins vaccines

Selection of an optimal antigen is paramount for designing an effective vaccine. These antigenic proteins, depending on the response desired, should contain appropriate epitopes to B-cell receptors and can be recognized by the T-cell receptor in a complex with major histocompatibility complex molecule[31].

Several recombinant protein vaccines such as the outer membrane protein (OMPs), lipoproteins and virulence factor vaccines have been developed using various biotechnological methods[5,32].

The first successful recombinant leptospirosis vaccine was reported in 1999[5]. Immunization with recombinant OmpL1, a transmembrane OMP that functions as a porin in leptospiral outer membrane and lipoprotein LipL41 conferred significant protection against intraperitoneal challenge with virulent *Leptospira kirschneri*[49]. The leptospiral outer membrane contains both transmembrane proteins exposed on the leptospiral surface and may play a potential role in the pathogenesis of the disease. The location of OmpL1 on the leptospiral surface has also been demonstrated by immune-electron microscopy and is presumed to have surface-exposed epitopes[5]. Evaluation of the immunoprotective ability of OmpL1 and the outer membrane lipoprotein LipL41 in the Golden-Syrian hamster revealed a synergistic effect[49]. The two membrane-associated proteins OmpL1 and LipL41 were expressed in *E. coli* using specialized expression plasmids to enhance their expression and reduce toxicity to the cells. Active immunization of hamsters with the expressed recombinant proteins provided significant protection against challenge with *Leptospira kirschneri* serovar Grippotyphosa[49]. The survival rate was 75 per cent compared with 25 per cent in the control group, although immunization with OmpL1 alone did not produce significant level of protection at 28 days after challenge. Analysis of time course of survival of hamsters after immunization with OmpL41 alone indicated significant protection at nine days after challenge[5]. Surface-exposed proteins are potential candidates for inducing immune responses following infection and are also said to act as adhesins that mediate the initial process of pathogen attachment to host cells. Furthermore, well-conserved outer membrane proteins and leptospiral lipoprotein LipL41 are promising vaccine candidates with potentials to induce cross-protective immunity[49].

LipL32 [haemolysis-associated protein 1 (Hap1)], is the most abundant outer membrane protein and it is highly expressed both during infection and by *in vitro* culture[50]. The nucleotide sequence coding for LipL32 is conserved among pathogenic *Leptospira* species and absent in non-pathogenic leptospires[51]. Branger *et al*[11] have reported that adenovirus-mediated Hap1 vaccinations induce significant immune response against heterologous challenge with virulent *Leptospira interrogans* serovar Canicola in gerbils, whereas a similar OmpL1 construct failed to protect the animals[52]. The 31- to 34-kDa protein fraction of *L. interrogans* serovar Autumnalis was obtained by cloning of the gene in pET-29b expression vector using PCR fragments digested and ligated into the

*Not*I and *Xho*I site of the vector. The adenovirus construct was generated by ligation into the pET-29b vector carrying the desired gene. The 6-12 wk old Mongolian gerbils were immunized with 26-31 kDa for Hap1 and 31-34 kDa for OmpL1 intramuscularly via the quadriceps. The result of this experiment showed that the cross-protective effect exhibited by pathogenic *Leptospira* was shared by Hap1 protein mediated by an adenovirus vector[11]. In another study, immunization of hamsters with recombinant BCG expressing LipL32 were found to render protection against lethal challenge with *L. interrogans* serovar Copenhageni[32]. For the purpose of this experiment, four *E. coli*-mycobacteria shuttle vectors were constructed by cloning of the gene into the expression vectors and introduced into *Mycobacterium bovis* BCG Pasteur strain by electroporation. Hamsters immunized with recombinant BCG expressing LipL32 were protected against lethal challenge with *L. interrogans* serovar Copenhageni. Histopathological examination did not indicate any clinical sign indicative of leptospirosis during the 28-day period after challenge. Importantly, recombinant rBCG-*LipL32* was able to induce sterilizing immunity in animals that survived the lethal challenge as indicated by the absence of clinical and histopathological signs of the disease as well as negative result from lung and kidney culture[32]. These results show that immunogenicity increases with the expression of multiple genes and is enhanced by the use of suitable adjuvant.

Recombinant LipL32 purified from an *E. coli* expression system was assessed for protective immunity in a group of five hamsters challenged with virulent *L. interrogans* serovar Canicola strain Kito. However, no significant survival was observed as compared with the unvaccinated control group[53]. Subsequent histological analysis revealed reduced amount of *L. interrogans* in the kidneys of vaccinated hamsters[53]. Furthermore, due to conflicting reports on the inability of LipL32 subunit vaccine to stimulate protective immune response, the protein was co-administered with the B subunit of *E. coli* heat-labile enterotoxin in a hamster model of leptospirosis to enhance its immune stimulating capability. The highly conserved *lipL32* coding sequence was amplified by PCR after obtaining the DNA material from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and then cloned into the corresponding enzyme sites on pAE/*ltb* vector to form pAE/*ltb-lipL32*. The construct was used to transform competent BL21 *E. coli* cells

and the expressed protein was purified using affinity chromatography. The 4-5 wk old hamsters were injected in the quadriceps muscle with the vaccines (one group received LipL32 coupled with heat labile enterotoxin while another group received LipL32 co-administered with heat labile enterotoxin) and control groups received the recombinant heat-labile enterotoxin alone. After challenge with 5×50 per cent lethal dose of *L. interrogans,* hamsters vaccinated with LipL32 coupled with heat-labile *E. coli* enterotoxin had significantly higher survival rates than animals in the control group[54]. Based on this finding, it was claimed that this was the first reported protective immune response afforded by a subunit vaccine using LipL32 and could serve as an important contribution to the development of improved leptospirosis vaccine[54]. This may be a valid argument considering a number of subunit proteins have been evaluated as potential vaccine candidates[55].

DNA vaccines

DNA vaccines are a novel approach for inducing an immune response. These are the simplest embodiment of vaccines that consist of the antigen itself, and provide genes encoding the antigen[56]. In this approach, purified plasmid DNA containing the coding sequences of an immunogenic gene and the essential regulatory elements to transcribe and translate them is introduced into the tissue intramuscularly. This is preceded by tissue expression and induction of potent, long-lasting heterologous immune response. The efficiency of leptospiral DNA vaccines has been demonstrated in animal models[48,57]. This approach is seen as a positive development in the efforts to prevent leptospirosis for which the conventional vaccines have failed. In addition, DNA vaccines also allow for inclusion of multiple genes so as to improve the coverage and ability to protect against infection due to a wide range of serovars.

DNA vaccines provide prolonged antigen expression and amplification of the immune response, while simultaneously offering several advantages over other vaccine preparations such as ease of construction, low cost of mass production, high-level temperature stability and the ability to elicit both humoral and cell-mediated immune response[58,59].

A DNA construct encoding *L. interrogans* Hap1 was designed to enhance direct gene transfer of the protein into gerbils followed by challenge with virulent strain of serovar Canicola. The genomic DNA from *L. interrogans*

Autumnalis strain 32 was amplified by PCR and cloned in PCRII.1 vector. The amplified fragment was later digested and sub-cloned into pUC 19 expression vector using the corresponding restriction enzyme site. The DNA construct was used to immunize hamster, and after the second immunization, the hamsters were challenged with virulent *L. interrogans* Canicola. The result revealed significant protection against the Canicola challenge[4], and the survival rate of the gerbils vaccinated with the vaccine was significantly higher than the control[4]. These findings indicated the potentials of DNA vaccine and it was able to protect gerbils against the pathogenic challenge similar to the protection conferred by Hap1 expressed by adenovirus vector.

In a related study[60], OmpL1 plasmid DNA vaccine rescued some vaccinated animals from lethal challenge with heterologous *L. interrogans* serovar Pomona at one week after vaccination. The vaccine also delayed death time and reduced morbidity and the number of *Leptospira* in the tissue of vaccinated animals. In this study, the OmpL1 plasmid DNA vaccine was generated by cloning PCR amplified fragment of the gene into expression vector pcDNA3.1 and propagated by transforming competent Top10 *E. coli* cells. Outer membrane proteins although occur in small amounts, play a significant role as porins or receptors for soluble molecules[60]. However, further efforts are required to optimize the dose and formulation to maximize its efficacy.

Immunization with LigA DNA vaccine provided significant protection against virulent *L. interrogans* serovar Pomona challenge in hamsters[42]. This protection was achieved by both humoral and cell-mediated immunity as revealed by increase in antibody titres after administration of a booster, significant proliferation of lymphocytes from vaccinated animals and the enhancement of both Th1 and Th2 cytokines[42]. The vaccine was constructed in two truncated forms with the conserved and variable portion of the *LigA* gene. The conserved and variable regions of the gene were cloned in a eukaryotic expression vector and used to immunize hamsters. The high-level stimulation of cell-mediated immunity was indicated by substantial lymphocytes proliferation among the LigA-vaccinated animals. However, there was greater response in the conserved LigA group compared to the variable region of LigA[42].

Although the ability of plasmid DNA vaccine to induce strong and specific immune response has been established, there are still concerns regarding its safety specifically the potential to induce deleterious autoimmune disease and development of tolerance in response to the persistent exposure to a foreign antigen[58]. However, questions on how to maximize immune responses by optimization of the route of application and delivery methods of the plasmid are being researched. Some bacterial and viral vectors been shown to be a good source of plasmid DNA due to their ability to transfer plasmids across phylogenetic borders to mammalian host cells[61].

In the last few years, immense progress has been made in the field of DNA vaccine and it has been proven to be a successful approach using animal models. Despite these achievements using animal models, the same cannot be said about human clinical trials due to the low immunogenicity observed. Currently, efforts are under way to enhance the immunogenicity of plasmid DNA to allow for its application in humans.

Conclusion

The prospect of developing a universal leptospiral vaccine capable of providing protection against heterologous challenge is unlikely in the foreseeable future. However, while earlier vaccines were developed using empirically approach including laboratory egg passage and LPS-based vaccines, recent technological advances in molecular biology and genetic engineering have fuelled rapid progress in vaccine technology, leading to the production of new products. Newly introduced vaccine delivery systems using nano-particles or adjuvants and highly efficient protein expression systems have led to the discovery of potentials for new vaccine approaches that may provide solutions for vaccines against leptospirosis. In addition, technological advancement is creating new opportunities for design of effective immunogens. This progress is demonstrated by the ability to predict or identify highly immunogenic surface proteins from circulating pathogenic strains and cloning into plasmids and then co-expression with a set of genes responsible for stimulating immune response. It also allows deletion or editing of undesirable traits in the DNA to further enhance safety, combination of genes from multiple pathogenic serovars as well as isolation of human monoclonal antibodies. Consequently, this will provide new strategic approaches for selecting vaccine antigens and formulations. Further studies are required to elucidate the molecular mechanisms of pathogenesis and immunity that will contribute to the development of a novel vaccine for the treatment of this important zoonotic disease among human and animal populations.

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