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Leptospirosis-persistence of a Dilemma: An Overview with Particular Emphasis on Trends and Recent Advances in Vaccines and Vaccination Strategies

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Abstract: Leptospirosis, caused by pathogenic spirochetes of the genus *Leptospira*, affects both humans and animals and is among the most common but neglected direct zoonotic disease in the world, particularly in untreated or undiagnosed animals as well as humans. Now, it has been considered as a re-emerging disease causing global health problem due to its increasing incidences in developing as well as developed nations. It is a multisystemic disease leading to death. Diagnostic tests of importance are Latex Agglutination Test (LAT), lateral flow and immunoglobulin M (IgM) based ELISA, PCR based assays, Multiple-microscopic Agglutination Test (MAT), Loop-mediated Isothermal Amplification (LAMP) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Molecular tools like PCR-RFLP, real-time PCR, multiplex PCR, qPCR and immunocapture PCR have all been found useful for rapid and confirmatory detection and differentiation of pathogenic and non-pathogenic leptospires. Inactivated/killed and attenuated vaccines are always attempted, since the beginning of vaccine and vaccination story, against all emerging pathogens with mixed success stories. The advanced tools and techniques like recombinant DNA technology, reverse genetics, DNA vaccination, molecular genetics and proteomics approaches are being explored for search of novel antigens, proteins and genes as potential candidates to discover safer, efficient and better vaccines for leptospirosis. The present review highlights the leptospirosis, susceptible population, disease transmission and epidemiology, treatment, trends and advances in diagnosis, vaccines and vaccination strategies in humans and animals with a view to combat this organism having public health significance.

Key words: Leptospirosis, *Leptospira*, vaccine, killed vaccine, serovar, protein antigen, recombinant vaccine, DNA vaccine, reverse genetics, proteomics

INTRODUCTION

The major problems, which the human beings are facing, are tremendous growth of population and risk of diseases to human and animals (Mahima *et al*., 2012) due to increased incidence of wide variety of bacterial zoonotic diseases among which leptospirosis is a prominent one (Silva *et al*., 2011; Deb *et al*., 2012) etc. in human as well as animals. Timely diagnosis, regular surveillance and adapting suitable control measures for these emerging bacterial pathogens is essential in order to prevent both animal and human deaths and zoonotic impacts. Leptospirosis, an endemic disease, otherwise known as Weil's disease/syndrome, caused by pathogenic spirochetes of the genus *Leptospira,* affects both humans and animals, causes significant morbidity and mortality. It is among the most common but neglected direct zoonotic disease in the world, due to dearth in diagnosis and treatment (Atzingen *et al*., 2012; Murray *et al*., 2012). Now, it has been considered as a re-emerging disease with resurgent zoonosis (Hartskeerl *et al*., 2011; Lim, 2011) causing global health problem (Murray *et al*., 2012). The disease emerged as an occupational zoonosis and first reported in 1907 and

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subsequently reported in rats in 1916. Since then the disease has been reported from almost all the parts of the world and is endemic particularly in tropical and subtropical climatic countries (Gaynor *et al*., 2007; Pradhan *et al*., 2012). It is a multi-systemic disorder, has an incubation period of 2-30 days, having wide variety of clinical manifestations and mortality may reach upto 40% (Gulati and Gulati, 2012; Pradhan *et al*., 2012; Puliyath and Singh, 2012). A sound managemental approach including rapid serological and molecular detection, appropriate vaccination regimen, effective antibiotic treatment and amending of some environmental factors are altogether helpful in minimising threats (Lim, 2011). Adhesins (LenA) help the pathogen to escape innate defence, allowing them to translocate through cell monolayers with higher rate, thereby helping them to spread through blood stream and disseminate to various organs, creating challenges for scientists to develop safe and effective vaccine against this bacteria (Gordon, 2002).

The present review highlights leptospirosis, it's etiological agent, epidemiology, treatment and trends in diagnosis, vaccines and vaccination strategy in humans as well as animals with a view to combat this organism having public health significance.

ETIOLOGY AND EPIDEMIOLOGY

Leptospires are genetically diverse, corkscrew-shaped, finely coiled, thin, motile, obligate, slow-growing anaerobes, classified in the family leptospiraceae of order Spirochaetales and the genus was originally thought to comprise only 2 species: *Leptospira interrogans* (pathogenic) and *L. biflexa* (saprophytic) (WHO-ILS, 2003). Recent studies have classified Leptosipres into at least 12 pathogenic, 4 distinct saprophytic, 17 named species and more than 250 serovars. Some of the serovars are *L. pomona* and *L. interrogans* in cattle and pigs; *L. grippotyphosa* in cattle, sheep, goats and voles; *L. ballum* and *L. icterohaemorrhagiae* in rats and mice and *L. canicola* is dogs and so also *L. autumnalis*, *L. hebdomidis* and *L. australis*. Moreover, there are 4 unnamed (1, 3, 4 and 5) species viz. *Leptospira alstonii* sp. nov., *Leptospira vanthielii* sp. nov., *Leptospira terpstrae* sp. nov., *Leptospira yanagawae* sp. nov., respectively (Smythe *et al*., 2012; Arent *et al*., 2012; Lau *et al*., 2012).

Leptospira affects at least 160 mammalian species and has been recovered from rats, swine, dogs, cats, raccoons, cattle, horse, dogs (even vaccinated) (Gamage *et al*., 2012; Koizumi and Yasutomi, 2012, Hamond *et al*., 2013), rats (most common), domestic and feral animals, bats, California seals and squirrels being the reservoirs (Lim, 2011; Dzupova *et al*., 2012; Koma *et al*., 2012; Muhldorfer, 2012). In humans, majority of leptospirosis occur as occupational hazards (Hartskeerl *et al*., 2011; Nafeev *et al*., 2012), prominently being encountered in tropical regions. The organism enters the body via mucous membrane via splitted milk, contaminated moist soil and vegetation, ingestion and inhalation of food and droplet aerosol of contaminated, leading to subsequent infection through conjunctivae or abraded skin while swimming or immersion in contaminated water and even can penetrate broken down skin (Wang *et al*., 2007; Dellagostin *et al*., 2011; Dzupova *et al*., 2012). Globally, rising incidence rates with few deaths and several outbreaks have been observed in all the continents (Abela-Ridder *et al*., 2010). In India, monsoon season is favourable for the disease to occur. Waterborne and post flood outbreaks along with outbreaks in athletes and travellers participating in white water rafting have also been reported (Amilasan *et al*., 2012; Dechet *et al*., 2012; Smith *et al*., 2012).

DIAGNOSIS AND TREATMENT

Diagnosis helps in elimination of reservoirs and to adopt suitable prevention and control measure. Initial diagnosis is based on Latex Agglutination Test (LAT), lateral flow, IgM based ELISA (Tanganuchitcharnchai *et al*., 2012; Signorini *et al*., 2013); this is followed by confirmatory and definitive diagnosis by isolation, PCR based confirmation, PCR-RFLP, real-time PCR, multiplex PCR, quantitative PCR, immunocapture PCR, or Microscopic Agglutination Test (MAT), Loop-mediated Isothermal Amplification Method (LAMP) and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (WHO-ILS, 2003; Balassiano *et al*., 2012; Koizumi *et al*., 2012; Djelouadji *et al*., 2012; Suwancharoen *et al*., 2012; Villumsen *et al*., 2012; Stoddard, 2013). Serotyping method based on PCR would be highly useful, allowing detection as well as characterisation of *Leptospira* field isolates (Tulsiani *et al*., 2011). For studying genetic diversity, molecular typing with Variable Number of Tandem Repeats (VNTR) and Multilocus Sequence Typing (MLST) are useful (Caimi *et al*., 2012). Treatment requires to be started well in advance. However, antimicrobial therapy with tetracyclines, amino and benzyl penicllin, quinolones, as well as third generation antibiotics are recommended in severe form (Brett-Major and Coldren, 2012; WHO-ILS, 2003; Brett-Major and Coldren, 2012).

PREVENTION AND CONTROL

Vaccines and vaccination strategies: Inactivated/killed and attenuated vaccines are always attempted, since the

beginning of vaccine and vaccination story, against all emerging pathogens with a mixed success story but in case of leptospirosis, there is lack of adequate vaccine. The conventional vaccines have been used in animals like cattle and dog. Currently available vaccines are the killed whole cell bacterins, being used widely in animals (but less so in humans), for generating mainly humoral immunity by generating antibody against Lipo-polysacchride (LPS). Vaccines for successful prevention of leptospirosis in humans are not available. Leptospiral antigens (vaccine candidates), conserved among pathogenic leptospires induce cross-protection to a variety of serovars. Newer approaches for vaccines utilizing defined protective and recombinant protein antigens, along with DNA vaccines and reverse genetics vaccinology show promise for the development of effective and safer leptospiral vaccines (Koizumi and Watanabe, 2005; Murray *et al*., 2012).

Vaccines for humans: Long back, heat killed whole cell vaccine was used but their specificity is limited to serovar specificity. Certainly there are reports available with a limited period of cross protection but majority of studies are documented with serovar-specific protection. There is a need to develop more effective and wider spectrum vaccines against *Leptospira* sero diversity. Thus immunization and selection of vaccine for human leptospirosis is a challenge. Under such scenario it is always appropriate to have safe and effective vaccine particularly in endemic areas of world. Vaccines are offered to high-risk workers in some European, South American and Asian countries (eg, rice workers in Italy and Cuba) however, vaccines are not used in the United States. That is due to the painful swelling, especially after revaccination from present available vaccines (Philip and Tennent, 1966).

In human, inactivated leptospirosis vaccines were also tested (Chapman *et al*., 1990) but the only leptospirosis vaccine licensed for humans is being produced in Cuba since 2006. The issue with inactivated vaccines is short period of immunity, safety, adverse reaction and need of booster (McBride *et al*., 2005; Srivastava, 2006) and can be overcome by proper selection of adjuvant. In general, it has been observed that bacterin-derived vaccines against leptospirosis do not even provide protective immunity (Dellagostin *et al*., 2011) for a year.

Human vaccines are available in a few countries, including Cuba and China (McBride *et al*., 2005) but not in US. A killed vaccine is being used in China, Japan and Vietnam to prevent leptospirosis in humans (McBride *et al*., 2005). Different types of vaccines, even though are having pros and cons, still are discussed in detail.

Vaccines for animals: Prevention and control strategies for leptospirosis in animals would help reduce the incidences in humans, as it is a zoonotic disease (Lim, 2011). Vaccines are available for domestic livestock but their effectiveness is under question. Likewise in humans, all the available animal vaccines are specific for one or few serovars that are present in the vaccine, thereby it becomes crucial that vaccine given should contain the serovars known to be prevalent in the area (Koizumi and Watanabe, 2005). Subunit canine vaccine contains only the immunogenic component of the *Leptospira* with reduced potential side effects and annual protection from disease (Grassmann *et al*., 2012). Animal vaccines with few strains like in dog vaccines are reported to be effective for at least one year, whereas multiple vaccination is required in case of calves (Wallace, 2005). Induction of a type 1 immune response has been found to be consistent with protective immunity to serovar Hardjo infections (Brown *et al*., 2003). Double doses of monovalent leptospiral vaccine and pentavalent vaccine containing *L. interrogans* serovar hardjo type hardjoprajitno prevents renal colonization and urinary shedding, when challenged with *L. borgpetersenii* serovar hardjo strain 203 four months after vaccination (Zuerner *et al*., 2011; Rinehart *et al*., 2012). This provides evidence of cross-protection during *Leptospira* vaccination but the efficacy of such vaccines are a matter of concern.

Advances in *Leptospira* vaccine developments: Advanced tools and techniques like of recombinant DNA technology, reverse genetics, DNA vaccination strategy and others, are being explored employing novel antigens, proteins and genes as potential candidates to get safer, efficient and better vaccines for leptospirosis (Koizumi and Watanabe, 2005; Lutticken *et al*., 2007; Adler and de la Pena Moctezuma, 2010; Murray *et al*., 2012). Virulence of *Leptospira* is attributed to multiple virulence factors which play vital role in pathogenesis. These virulence factors include hemolysins, outer membrane proteins (czcA and its subunit peptides) (Umamaheswari *et al*., 2012) and lipoproteins and appeared to be a potent candidate for vaccine development. *Leptospira* cell components like Hsp58, hemolysin SphH, ChpK, FlaA, FlaB, outer membrane proteins OmpL1 (Gebriel *et al*., 2006; Vedhagiri *et al*., 2009), omp like proteins Lag42, Loa22, Lk73.5 (Koizumi and Watanabe, 2003a, b; Artiushin *et al*., 2004)

hemolysis-associated protein 1 (Hap1) (Branger *et al*., 2001); immunoglobulin-like (Lig) protein LigA and LigB (Palaniappan *et al*., 2006; Silva *et al*., 2007; Coutinho *et al*., 2011), Lipoproteins eg. LipL21, LipL32, LipL41, LipL45 (Matsunaga *et al*., 2002; Cullen *et al*., 2003; Seixas *et al*., 2007; Feng *et al*., 2009; Luo *et al*., 2009; Vedhagiri *et al*., 2009; Grassmann *et al*., 2012; Murray, 2012), lipoprotein-like complex Glycolipoproteins (GLPs) (Diament *et al*., 2002) are major virulence factors and potential candidates for recombinant vaccine. Among them a pore forming protein 'Hsp58' and a factor encoded for genes responsible for cytotoxicity 'ChpK' have been targeted as vaccine agents (Picardeau *et al*., 2003).

Probably, development of recombinant vaccines against the outer membrane proteins (omp) of *Leptospira* might allow concurrent protection against multiple serovars (Nascimento *et al*., 2004). Moreover, variation in the expression of omp in different serovar also suggests these omps viz., Lsa21, Lsa66 and rLIC11030 either combined or used in a mixture with novel adjuvant have the potential candidate for vaccine development (Umamaheswari *et al*., 2012; Atzingen *et al*., 2012). Studies have proved that recombination of protective antigen genes viz., lipL32, lipL41 and ompL1 and a DNA-protein gives better immune responses than single-component, LipL32, or single DNA or protein immunization against *Leptospira* (Feng *et al*., 2009). Thus the recombinant vaccine with these potential candidates provides a window to develop a safe and effective vaccine against human leptospirosis. Although all the trials are in preliminary stages and a long path is to be travelled to get effective and safe recombinant vaccine. In a recent study, conducted to evaluate the immune protective potential of leptospiral recombinant antigens, out of 27 antigens 13 were found to induce protective immune responses in the hamster model and only two recombinant proteins LIC10325 and LIC13059 induced significant protection against mortality (Felix *et al*., 2011). These results have important implications for advancing the development of a successful recombinant subunit vaccine against leptospirosis.

Being Gram negative bacteria *Leptospira* also express lipopolysaccharides (LPS) and these could be another potential candidate for the development of good and safer vaccine. Initially LPS was reported to have dose and administration dependent serovar-independent potential with some contrasting findings (Matsuo *et al*., 2000) keeping it open for further investigation. During this journey identification of LPS fractions and LPS like substances (LSSs) with antigenic potential and capability of protection were also attempted and *Leptospira*-derived LPS has revealed a promising immune potential. Moreover, cross-protection against multiple serovars due to mutation of LPS is suggested (Srikram *et al*., 2011). Scientific community is exploring LPS for more than last two decades with diverse results and success of LPS and LPS factor based vaccines but it is still under question (Humphryes *et al*., 2012).

The concept of "reverse vaccinology" using the desired proteins and characteristics with the help of computer data based are the newer opportunity for the development of bacterial vaccines (Serruto *et al*., 2004; Murray *et al*., 2012). The known full genome sequence of *Leptospira* strains (Nascimento *et al*., 2004) have been used for the identification of potential antigen (Gamberini *et al*., 2005). On the basis reverse vaccinology, 226 genes encoding conserved and potentially surface-exposed proteins in the bacterium have been identified as vaccine candidates in the genome of *L. interrogans and* might also be helpful for diagnosis of leptospirosis (Yang *et al*., 2006). However, the immune status with these candidates is yet to be established.

Similar to other bacterial diseases scientists are also trying for DNA vaccines against *Leptospirosis* (Branger *et al*., 2005), following the concept that these might be produced at low cost and be easily administered with safety (Wang *et al*., 2004; Meykadeh *et al*., 2005). However, the trial for *Leptospira* DNA vaccines are still in early stages and only laboratory trials with limited success are reported with genes encoded for hemolysis-associated protein 1 (Hap1), endoflagellin gene flaB2 (Branger *et al*., 2005), ompL1 DNA vaccine in hamster (Maneewatch *et al*., 2007) and lipL21 DNA vaccine in guinea pig (He *et al*., 2008). The CpG motifs within the flaB2 gene could give the DNA vaccine an additional immunostimulatory property, without the use of adjuvants (Dai *et al*., 2003). Research has proved that the recombination of antigen genes like lipL32, lipL41 and ompL1 and a DNA-protein may provide better results in comparison to that of single components (Feng *et al*., 2009). No doubt DNA vaccine in bacterial diseases revealed encouraging results but success for *Leptospira* vaccine is still questionable due to sero-variations and need more extensive investigations (Wang *et al*., 2007).

Novel and efficacious vaccines developed for emerging infections like of *Leptospira* in animals can safeguard animal health and also prevent transmission of zoonotic pathogens to humans. Also vaccination of reservoir hosts provides additional advantages to combat zoonotic diseases having public health implications. Options for changes in the *Leptospira* components of dog vaccines have been suggested for inclusion of serovars Bratislava and Grippotyphosa in Europe. But continued inclusion of serovars Icterohaemorrhagiae and

Canicola, with other serovars, such as Pomona, to be considered in the future only after necessary research to support their inclusion (Ellis, 2010). Emergence of *Leptospira* serovars in particular countries like after the recent report in Greece regarding the presence of serogroup Pomona and serovar Bratislava, emphasizes a need for considering these serovars to be included in future *Leptospira* vaccines for dogs (Arent *et al*., 2012). Recently, Kulpa-Eddy (2012) reported successful development and validation of an *in vitro* replacement assay (ELISA based) for *Leptospira* vaccine potency tests.

A better understanding and more insights are required about *Leptospira* spp. biology, pathogenesis, immune mechanisms, identifying leptospiral immunogens, genetic makeup and molecular genetics, which would altogether have important implications for the future prevention of leptospirosis and pave the development of novel intervention strategies to curb this neglected disease. The recent availability of complete genome sequences for *Leptospira* spp. is a great success in this direction. Recent proteomic studies applied to leptospirosis, characterizing outer membrane proteins and identifying potential leptospiral immunogens, may lead towards the development of rapid and more accurate diagnostic tests and vaccine and vaccine development (Thongboonkerd, 2008). The published full-length genome sequences of *Leptospira* strains need to be screened for identifying potent leptospiral vaccine candidates utilizing advances in biotechnological and molecular tools and techniques like that of recombinant DNA technology, genetic tools for their transformation, high-throughput cloning and expression, in-silico analysis, comparative genomic hybridization (CGH) analysis, transcriptional analysis, reverse vaccinology, bioinformatics and others (Yang *et al*., 2006; Ko *et al*., 2009). More and more genetic information pertaining to *Leptospira* need to be exploited and explored to combat the disease.

LIMITATIONS OF VACCINES

Presently available vaccines against leptospirosis are of low efficacy, possess undesirable side-effects, induces only short-term protection, does not provide cross-protection against different pathogenic serovars of *Leptospira* (Koizumi and Watanabe, 2005; Yang *et al*., 2006). There is a study of development of auto immune disease such as uveitis due to whole cell *Leptospira* vaccine. In addition, there can be painful swelling after revaccinations (WHO-ILS, 2003) as reported in Cuba when vaccine against leptospirosis was studied to evaluate efficacy and safety, it was found safe with minor side effects (Martinez *et al*., 2004). Moreover, vaccines are not always recommended due to lack of effectiveness and also due to adverse events (Levett and Haake, 2012). In addition, vaccines produce only agglutinating antibody to specific serovars thus individual once vaccinated will always produce hindrance in diagnosis as these agglutinating antibodies cannot be differentiated from antibodies produced by natural infections. However, the generation of agglutinating antibodies does not always reflects the protection (Levett, 2003) and developments of antibodies against those serovars which were not included in vaccine, this also further create hindrance in diagnosis (Goldstein *et al*., 2006). It is further aggravated as most of the presently available leptospirosis vaccines for humans are serovar-specific and require repeated yearly vaccination i.e. yearly boosters (Koizumi and Watanabe, 2005; Levett and Haake, 2012). Post vaccination renal infection and persistent leptospiruria has been observed in immunized dogs along with transmission to human from asymptomatic immunized dogs due to shedding of leptospires in their urine. Moreover, the development of carrier state in animals is of major concern (Green-McKenzie and Shoff, 2010).

In view of the above limitations, the current major focus in search for an efficacious and safer leptospirosis vaccine is to discover conserved protective antigens eliciting longer-term protection against a wide range of *Leptospira*, providing cross-protection against different serovars of pathogenic leptospires.

Key points for prevention and control:

- All the precautionary steps should be taken to avoid the exposure of infected excreta of domestic and wild carrier animals
- Proper personal, protective measures like wearing gumboots, goggles and rubber gloves should be used in high risk areas
- Strict hygienic measures should be practiced in farms, kennels and animal sheds to avoid transmission of infection through contaminated feed and water by urine
- Some disinfectants like calcium chloride, sodium hydroxide or sodium hypochlorite can be used
- Isolation of the infected persons or animals from the infected one
- Recovered animals should be kept separated for at least two months
- Measures to control rodents, an important reservoirs
- Pre-exposure antibiotic prophylaxis by doxycyclin can be used in endemic areas

CONCLUSION AND FUTURE PERSPECTIVES

Since vaccines are used in apparently healthy humans as well as animals to make them immune to diseases, so it should be safe enough to be administered. Presently existing two types of *Leptospira* vaccines, attenuated and inactivated, are being used in animals but still have safety concern. Inactivated vaccines are comparatively safer than attenuated vaccines but the level and duration of immune response is to be compromised. The combination of attenuated and inactivated vaccines are also reported with better immune response, however attempts for the inactivation of virulence factors without compromising immune response might be another option. Still, a long way to go to get a safe, effective, single vaccine to get protection against all the serovars of *Leptospira* as the selection of type of vaccine is not easy. Recent study on leptospirosis is being focused mainly on identifying leptospiral immunogens and protective antigens to be used for diagnostics and vaccine developments. Use and development of recombinant and DNA vaccines with potent immunogenic moiety and genes encoded for the expression of potent immunogens, respectively show a spark of hope. Regular and continuous epidemiological and environmental surveillance actions are suggested, utilizing advanced diagnostic tools, to know the genetic diversity and emergence of *Leptospira* serovars/strains, which would be much helpful in designing appropriate prevention and control strategies for this important pathogen affecting animals and in humans. It would also help formulating newer multivalent vaccines with most prevalent leptospires and also developing novel and safer vaccines employing advanced research in the field of vaccinology. However, till then application of presently existing vaccines with different adjuvant and routes could be attempted to enhance the immune response with more safe administration.

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