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Review

Adjuvanted leptospiral vaccines: Challenges and future development of new leptospirosis vaccines

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A B S T R A C T

Leptospirosis is a neglected infectious disease of global importance. Vaccination is the most viable strategy for the control of leptospirosis, but in spite of efforts for the development of an effective vaccine against the disease, few advances have been made, and to date, bacterin is the only option for prevention of leptospirosis. Bacterins are formulations based on inactivated leptospires that present a series of drawbacks, such as serovar-dependence and short-term immunity. Therefore, bacterins are not widely used in humans, and only Cuba, France and China have these vaccines licensed for at-risk populations. The development of recombinant DNA technology emerges as an alternative to solve the problem. Recombinant protein-based vaccines or DNA vaccines seem to be an attractive strategy, but the use of adjuvants is critical for achievement of a protective immune response. Adjuvants are capable of enhancing and/or modulating immune responses by exposing antigens to antigen-presenting cells. In the last years, several components have been tested as adjuvants, such as aluminum salts, oil based-emulsion adjuvants, bacteria-derived components and liposomes. This review highlights the use of adjuvants in the multiple vaccine approaches that have been used for leptospirosis and their most important immunological aspects. Immune response data generated by these strategies can contribute to the understanding of the immune mechanisms involved in protection against leptospirosis, and consequently, the development of effective vaccines against this disease. This is the first review on leptospiral vaccines focusing on adjuvant aspects.

Contents

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1. Introduction

Vaccination is the most effective and cost-efficient tool for preventing a variety of infectious diseases. According to the World Health Organization (WHO) (2018) [1], vaccination prevents an estimated of 2–3 million deaths every year. Despite the substantial success of vaccines and development of many vaccine strategies, only three different types of vaccine are currently used in humans: live-attenuated vaccines, inactivated vaccines and subunit vaccines [2,3]. Although effective stimulation of the immune system can be achieved using attenuated or inactivated pathogens, several safety issues are associated with these formulations [4]. Subunit vaccines are designed to be safer than whole cell-based vaccines, and are generally less toxic. However, the absence of key molecules in attenuated or inactivated pathogens results in lowered immunogenicity levels [5,6]. Thus, the use of adjuvants is absolutely necessary to increase the immunogenicity of these vaccines [7].

Adjuvants are defined as agents that are incorporated into vaccine formulations to enhance antigen immunogenicity and induce protection against infection [4]. The fact that immune response to antigens can be improved by the addition of certain compounds was first demonstrated by Glenny in 1926, when aluminum salts were introduced into vaccine formulations against diphtheria. Since then, it is believed that vaccine formulations containing appropriate adjuvants is a worthwhile strategy towards eliciting protective and long-lasting immunity. Nevertheless, only a limited number of adjuvants are currently licensed for human vaccination, and aluminum salts remain the most widely used adjuvant in human vaccines [8–13]. The understanding of the mechanism of action of new adjuvants could provide guidance for the development of novel vaccines for emerging diseases that are still not completely under control, such as leptospirosis.

Leptospirosis is a bacterial disease that occurs worldwide and has a significant impact on humans and animals. The disease is considered a major public health problem with approximately one million cases every year [14]. Additionally, due to the lack of appropriate sanitation, rodent infestation, and extreme poverty, leptospirosis incidence is increasing in urban slums of developing countries [15,16]. On the other hand, in developed countries, leptospirosis incidence has been associated with adventure races and triathlons involving fresh water events [17,18]. Infection by pathogenic *Leptospira* species occurs through direct contact with an infected animal's urine or indirectly through contaminated water or soil. Symptoms range from a mild influenza-like illness, which may be misdiagnosed as other diseases, to a severe and potentially fatal infection that is characterized by kidney damage, liver failure and respiratory distress [19–21].

Vaccination is the most viable strategy for the control of leptospirosis. However, there is currently no available vaccine against leptospirosis for human use worldwide. Only a few countries such as France, Cuba and Japan have approved whole cell-inactivated leptospiral vaccines for risk populations [22–24]. Most available vaccines on the market are composed of inactivated leptospires and are routinely used for the immunization of livestock and companion animals. Although these vaccines are highly effective, they elicit an immune response mainly against leptospiral lipopolysaccharide (LPS), a T-independent antigen [19,25–27]. As expected, a short-term immunity is produced, and there is a lack of cross-protection against serovars not included in the preparations. As the goal of vaccination is to induce a pathogen-specific immune response and long-lasting protection against infection, the identification of protein targets for use in vaccine subunits has been proposed. Thus, this review outlines the progress in the use of adjuvants in leptospirosis vaccines and the most important immunological aspects of the multiple strategies that are being explored to develop more effective vaccines.

2. Aluminum salt-based adjuvants

Alum is the oldest and most commonly tested adjuvant in leptospirosis vaccines. Despite being extensively used as an adjuvant, its mechanism of action remains elusive. Several hypothesis such as depot formation, antigen targeting and inflammation induction have been reported [28–31]. It is interesting that in the local of cells accumulation, alum particles bound to neutrophil extracellular traps (NETs) are observed, suggesting that host DNA could increase the adjuvant activity of alum [32,33]. Moreover, it has been suggested that the cytokine IL-1β is responsible of alum's adjuvant action, since it activates NALP3 inflammasome [30]. However, others have shown an activation of inflamassome independent manner [34]. Independently of the mechanism, alum has been considered a safe adjuvant and acknowledged to induce a Th2-type immune response [35–37].

The protective potential of leptospirosis vaccines that use alum as an adjuvant is diverse and various literature data, including survival, sterility and immunogenic activities of protein antigens, are listed in Table 1. Only some vaccine formulations containing LigA and LigB protein fragments have been able to confer 100% protection after challenge with virulent leptospires [38–40]. LigA and LigB are surface exposed proteins that contain bacterial immunoglobulin-like (Big) domains similar to the proteins intimin and invasin from enteropathogenic *E. coli* and *Yersinia* spp., respectively [41–44]. These proteins have a highly conserved N-terminal region and a less conserved C-terminal region, and interestingly, both regions are recognized during acute host infection and are considered to date as promising vaccine antigens [45–47]. However, *ligA* gene is not common to all leptospiral species, which could limit its use in a broad-spectrum vaccine. Since LigB protein is found in all pathogenic *Leptospira* spp., it tends to be a more promising antigen. Several studies that evaluated vaccine formulations containing the LigB protein showed that the conserved region is responsible for a higher level of protection, regardless of the vaccine approach used, such as, recombinant protein, DNA vaccine or prime-boost [48–50]. Despite the fact that several studies have been performed with the LigA and LigB proteins, none of these candidates was able to promote complete sterilizing immunity [38–40,48,49,51].

Interestingly, LipL32, the major outer membrane protein of pathogenic *Leptospira* spp., was not able to promote protection in hamsters after challenge with virulent leptospires [52,53]. Although the immune response generated by these vaccine formulations has not been evaluated, LipL32 is a highly immunogenic protein, recognized by over 95% of confirmed human leptospirosis serum samples [54]. LipL41 is the third most abundant leptospiral outer membrane protein [55] and was the first recombinant candidate tested in an animal model. Immunization with the membrane-associated forms of leptospiral proteins OmpL1-M and LipL41-M were shown to elicit significant protection against homologous bacterial challenges. These protective effects were synergistic because protection was not observed in animals immunized with either OmpL1-M or LipL41-M alone. Contrasting with these data, animals inoculated with the recombinant forms of LipL41 and OmpL1 were not protected, suggesting that membrane fractions may act as an adjuvant, playing a role in immune protection [56].

OmpL1 is a transmembrane porin, and like most porins, it has the potential to serve as a target for a protective immune response due to its surface exposure [57]. Despite these facts, OmpL1 alone has been ineffective in promoting immune protection [56]. A topological model of OmpL1 suggested the presence of 5 surface-exposed loops, which could contain antigenic determinants [58]. Thus, some combined B and T cell epitopes were defined [59],

Table 1
Vacines containing aluminium adjuvants.

	Surviving (%)	Sterility (%)	Via	Immune response	Reference
OmpL1	11	ND	Subcutaneous	Total IgG	[56]
LipL41	22				
OmpL1 + LipL41	55				
Control	22				
rLigA	100	ND	Subcutaneous	Total IgG	[38]
Control	75				
Lp0607/Lp1118/ Lp1454	37.5	*	Subcutaneous		[135]
Control	0				
LigAvar	50	33.34	Subcutaneous	Total IgG/IL-4/IL-10	[136]
Control	0	8.4			
rLigBcon-Alum	71	*	Subcutaneous	IL-4/IL-10/IL-12p40/ IFN-γ	[48]
rVarB1-Alum	54			IL-4/IL-10/IL-12p40/ IFN- γ	
rVarB2-Alum	33				
rLigBcon-Alum/ rVarB1-Alum/ rVarB2-Alum	83				
Control	16				
rLIC10494	40	20	Subcutaneous	Total IgG	[62]
rLIC12730	44	20			
rLIC12922	30	0			
Control	10	0			
rLIC11859	16.7	ND	Intramuscular	–	[63]
rLIC12253	16.7				
rLIC10561	16.7				
rLIC10508	16.7				
rLIC10091	12.5				
rLIC13059	33.3				
rLIC10054	16.7				
rLIC11567	25				
rLIC20172	12.5				
rLIC10191	0				
rLIC12099	0				
rLIC11947	0				
rLIC10011	16.7				
rLIC12730	0				
rLIC12538	16.7				
rLIC10501	16.7				
rLIC13306	16.7				
rLIC13006	0				
rLIC11184	16.7				
rLIC10645	16.7				
rLIC10021	0				
rLIC10325	33.3				
rLIC12555	0				
rLIC11087	0				
rLIC12632	0				
rLIC10009	0				
rLIC13305	0				
Control					
Soluble LipL32	0	ND	–	–	[52]
LipL32 _155–200	0				
LigANI	0				
LipL32_155-200_ LigANI	20				
Control	20				
Lsa21	25	16.67	Subcutaneous	total IgG	[146]
Lsa66	25	41.67			
rLIC11030	30	67			
rLIC10821	20	25			
Lsa25	25	25			
rLIC10672	20	0			
Control	10	ND			
pTARGET/LigAni	0	ND	Intramuscular	Total IgG	[49]
pTARGET/LigBni	0	ND			
pTARGET/LigBrep	62.5	80			
pTARGET/LigBct1	0	ND		–	
pTARGET/LigBct2	0	ND			
Control	0	ND			
rLemA	50	50	–	Total IgG	[88]
rLigANI-Al	66.7	0	Subcutaneous	Total IgG	[121]

(*continued on next page*)

Table 1 (*continued*)

	Surviving (%)	Sterility (%)	Via	Immune response	Reference
Control	0	ND			
Mannosylated LigANI	83	0	Intramuscular	Total IgG	[147]
Control	0	ND			
LipL32	20	ND	Subcutaneous	–	[53]
Control	0				
pTARGET/ ligBrep + rLigBrep	83	ND	Intramuscular	Total IgG	[50]
rLigBrep	0				
pTARGET/ligBrep	40				
Control	0				
Lp11 + Lp21 + Lp22+ Lp25 + Lsa30 + Lp35	50	0	Subcutaneous	Total IgG	[39]
Lp11 + Lp21 + Lp22+ Lp25 + Lsa30 + Lp35 + LigAC	87	0			
LigAC	100	0			
Control	10	0			
rOmpL37	0	ND	Intramuscular	Total IgG/IFN-γ/ IL-1α	[64]
Control	8.3	100			
r4R protein [#]	80	*	Subcutaneous	IgG1/IgG2a/IFN-γ	[60]
Control	0				
rLigANI	67	ND	Subcutaneous	IgG1/IgG2/3 / IgG3	[51]
Control	0				
rLigB(131–645)	80–100	77.8–100	Intramuscular	IgM/ IgG1/ IgG2/3	[40]
Control	0–30	ND			
rChi	41.5	66.66	Subcutaneous	IgG2/3	[61]
Control	10	0			
Lsa46	44	15	Subcutaneous	IgG1/ IgG2/3	[65]
Control	13	0			
Lsa77	50	56		IgG1/IgG2/3	
Control	39	72			
Lsa46 + Lsa77	90	55		IgG1/IgG2/3	
Control	58	29			
recA- pEGFPN3 + rRecA	100	100	Intramuscular	Total IgG/TNF-α/ IL-10/IL-4/ IL-12p40/IFN-γ	[66]
fiD- pEGFPN3 + rFliD	83.3	83.4			
recA-pEGFPN3	91.6	91.7			
fiD-pEGFPN3	75	75			
rRecA	58.3	58.4			
rFliD	41.6	41.7			
WCL	100	100		–	
pEGFPN3	0	0		–	
PBS	0	0		–	
recA- pEGFPN3 + rRecA	100	100	Intramuscular		[66]
fiD- pEGFPN3 + rFliD	83.3	83.4			
recA-pEGFPN3	91.6	83.4			
fiD-pEGFPN3	83.3	66.7			
rRecA	66.7	66.7			
rFliD	58.3	58.4			
WCL	100	100			
pEGFPN3	0	0			
PBS	0	0			

ND: not determined.
* Data not shown.
\# Guinea pigs was used as animal model.

and OmpL1 was tested in a chimeric vaccine containing other leptospiral protein fragments. The first chimeric vaccine designed was based on immunodominant epitopes of OmpL1, LipL32 and LipL21 proteins [60]. This chimeric protein, designated r4R, contained four of the six T and B cell combined epitope repeats and was able to promote an increasing production of IgG2 antibodies in immunized guinea pigs. Moreover, r4R stimulated lymphocytes to produce high levels of IFN-γ cytokine, pointing to a Th1-polarized immune response. This pattern of immune response resulted in 80% protection and bacterial clearance from kidneys after challenge with *L. interrogans* serovar Lai [60]. The second chimeric protein was designed on the basis of amino acid sequences of OmpL1, LigA, LipL41, Mce and Lsa45, resulting in a recombinant protein called rChi [61]. Hamsters immunized with rChi and alum showed high titers of IgG2 antibodies and the presence de IgG1 was observed after booster with rChi vaccine. Even with high levels of IgG2, only 40% protection was achieved [61].

Other proteins tested with alum adjuvant showed variable efficacy. Atzingen et al. [62] reported that immunization with rLIC12730, rLIC10494 and rLIC12922 proteins conferred only low protection levels in hamsters, ranging from 30 to 40%, and sterilizing immunity was not reached. Immunization with the recombinant proteins rLIC11859, rLIC12253, rLIC10561, rLIC10508, rLIC10091, rLIC13059, rLIC10054, rLIC11567, rLIC20172, rLIC10561 and rLIC10508 resulted in more survivors than in the negative control groups, but the differences were not significant [63]. OmpL37 is a surface-exposed protein that fulfils several requirements for a potential vaccine candidate. However, hamsters immunized with OmpL37 recombinant protein plus alum adjuvant were not protected after challenge with virulent leptospires [64].

Recently, it was shown that animals subcutaneously immunized with Lsa46 or Lsa77 protein or a combination of both in alum adjuvant induced a strong IgG response. Th2- and Th1-biased immune responses were observed when Lsa46 or Lsa77 was individually administered, while a combination of both proteins induced a Th1-biased immune response. Despite the immunogenic activity of these proteins, a complete immune protection was not obtained [65]. On the other hand, when the leptospiral recombinase A (RecA) and flagellar-hook associated (FliD) proteins were tested as DNA vaccine and prime boost scheme in combination with alum adjuvant, significant protection was provided against homologous and heterologous challenge. Moreover, RecA prime-protein boost vaccine showed 100% sterilizing immunity, with heterologous protection [66]. Given that one of the major goals for a vaccine against leptospirosis is to provide heterologous protection, it is anticipated that this formulation will be a potential vaccine candidate worth further investigation.

3. Emulsion-based adjuvants

Emulsion-based adjuvants have been used safely and successfully in influenza vaccines [67–70]. MF59 and ASO3 are the most widely used emulsion adjuvants approved for human use [13]. Nonetheless, these adjuvants have still not been tested in leptospiral vaccines, and only Freund's adjuvant, Adda Vax and EMULSIGEN-D have been evaluated (Table 2) [71–77].

Complete Freund's adjuvant (CFA) consists of heat-killed *Mycobacterium tuberculosis* emulsified in paraffin oil, incomplete Freund's adjuvant (IFA) lacks the bacteria. CFA induces a strong immune response, but it is highly toxic. Thus, CFA is usually used for the initial immunization and booster doses are performed with IFA to minimize the adverse effects [78,79]. Leptospirosis vaccines containing Freund's adjuvant were tested only with LipL32, LigA and LigB proteins. The protective effect elicited by LipL32 recombinant protein immunization was not achieved [72]. In contrast, vaccine formulations containing LigA and LigB proteins showed promising results [71,73–75]. Coutinho and coworkers conducted a thorough study showing which LigA10-13 domains were involved in immune protection after lethal infection with virulent *Leptospira* [74]. Interestingly, Koizumi and Watanabe [71] demonstrated that mice immunized with LigB protein exhibited 90% survival after challenge, while Silva et al observed that immunization with the LigBrep and LigBNI fragments did not induce protection [73]. It is possible that differences in the animal models and bacterial strains used in challenging experiments may have influenced these results. Nonetheless, CFA shows reactogenic properties, and it is reported to cause pain and distress in animals. Thus, many recommendations and regulatory issues exist related to the use of CFA, and the use of alternative adjuvants is recommended [80,81]. Furthermore, due to these limitations, its approval for humans is unlikely. On the other hand, IFA has been widely used, and recently, Garba and colleagues (2018) [82] developed a chimeric leptospiral DNA vaccine, encoding the immunogenic epitopes of LipL32 and LipL41 proteins in a formulation with this adjuvant. Their results, after challenge with a sublethal dose of virulent leptospires, showed a high level of survival with significant decrease in renal colonization.

AddaVax is a squalene-based oil-in water adjuvant, which has a similar formulation as MF59. Squalene-based adjuvants such as MF59 promote a more Th1/Th2 directed immune response. Although their mechanism of action is not completely understood, they are believed to act through recruitment and activation of dendritic cells and stimulation of chemokine secretion by macrophages and monocytes [83–85]. The efficacy of subunit vaccines containing rLemA and rErp Y-like mixed with AddaVax adjuvant has been promising. Hamsters vaccinated with rLemA showed 87.5% protection, while immunization with rErp_Y-like produced 62.5% protection [77]. Interestingly, humoral immune response produced by Erp Y-like was lower than that in the LemA group, but kidney cell cultures were negative for both groups [77]. LemA protein is a promising antigen, conserved in different pathogenic serovars and differentially expressed by *L. interrogans* cultivated within dialysis membrane chambers implanted in the rat peritoneum [86]. Despite its unknown role in pathogenesis, LemA possesses a M3 epitope similar to that of *Listeria*, which could facilitate antigen presentation [87]. Since LemA induces high levels of IgG2, it is possible that other immune response mechanisms are involved in the greater protection observed. When, Hartwig et al. [88] tested a DNA vaccine containing *lemA*, they observed that even with high survival rates, no measurable IgG levels were detected, suggesting a cellular immune response. Thus, the vaccine potential of this protein deserves to be explored.

EMULSIGEN-D adjuvant is an oil-in-water emulsion free of ingredients of animal origin. EMULSIGEN-D is manufactured with micro size oil droplets, which increase the surface area available to antigens, and reduce the quantity of oil required in the final formulation, diminishing the side effects observed in the water-in-oil adjuvants. Its mode of action seems to be similar to other oil-in-water adjuvants. It can act forming a depot effect of antigen, targeting the immune cells. Moreover, since dimethyldioctadecylammonium bromide (DDA) is a known T-cell immune stimulator, this compound is present in the EMULSIGEN-D fomulation as an additional immunostimulant, resulting in good levels of immune response (MVP laboratories, Inc., Omaha, NE, USA). The adjuvant properties of EMULSIGEN-D were evaluated with 6 putative outer membrane proteins that possess an OmpA domain at the C-terminus [76]. Usually, proteins with an OmpA domain play an important role in bacterial pathogenesis and are considered potential vaccine candidates [89]. The OmpA-like proteins Lp4337, Lp3685 and Lp0222 of *L. interrogans* exhibited a notable immunoprotective activity, associated with an increase in antibody response, lymphocyte proliferation and upregulation of Th1 and Th2 cytokines [76]. A greater level of protection was associated with a higher lymphoproliferative response, suggesting the importance of the cellular immune response for leptospirosis control.

Cao and colleagues (2011) [90] used fused fragments of the extracellular matrix (ECM)-binding domain of the proteins LigB and LipL32 in various combinations in vaccine formulations containing EMULSIGEN-D as adjuvant, and evaluated the protective efficacy of these vaccines in the hamster model of leptospirosis. The fusion products induced significant protection against challenge with *L. interrogans* serovar Pomona, correlating with the level of antibody immune response. It was suggested that protection might be due to antibody blockade of the ECM-binding site of the bacteria required for adhesion to the host.

4. Vaccines formulated with bacteria-derived adjuvants

Bacteria-derived components exhibit an adjuvant potential, since they are recognized by cell receptors, and consequently

Table 2
Vaccines containing emulsion-based adjuvants.

Protein + Freund's	Surviving (%)	Sterility (%)	Via	Immune response	Reference
LigA-m_Nc [#]	90	ND	Subcutaneous		[71]
LigB-m_Nd	90				
LigB-m_N + LigB-m_N	100				
Control	40				
r-Hap1 [&]	0	ND	Subcutaneous	Total IgG	[72]
control	13.3				
LigANI	80.76	ND	Subcutaneous	Total IgG	[73]
LigBNI	10.71				
Control	0				
LipDNA-01-pBudCE4.1.	100	*	Intramuscular	ND	[82]
Control	100				
LigA7′-13	100	0	Subcutaneous	Total IgG	[74]
LigA7′-11	50	50			
LigA7′-9	0	0			
LigA10-13	100	0			
LigA10-12	100	0			
LigA11-13	100	0			
LigA11-12	25	25			
LigA12-13	50	100			
Control	0	0			
LigA7′-13	100	0	Subcutaneous	Total IgG	[75]
LigB0-7	37.5	12.5			
LigA7′-13 + LigB0-7	100	0			
Control	0	0			
AddaVax					
rErp Y-like	62.5	100	Intramuscular	IgG2/3	[77]
rLemA	87.5	100		IgG1/IgG2/3/IgG3	
Control	0	0			
EMULSIGEN-D					
Lp0222	38	ND	Subcutaneous	Total IgG/IL-4/IL-10/IFN-γ	[76]
Lp3685	55				
Lp4337	72				
Lp0056	0				
Lp3615	16.7				
Lbp328	16.7				
Control	16.7				
LigBcon4-7.5-LigBcen2	50	ND	Subcutaneous	Total IgG	[90]
LigBcon4-LigBcen2	50				
LigBcon4-7.5-LigBcen2-LipL32-C-Terminus	50				
LigBcon4-LigBcen2-LipL32-C-Terminus	50				
LigBcon	34				
LipL32	0				
Control	0				

ND: not determined.
[*] Data not shown.
[#] C3H/HeJ mice used as animal model.
[&] Mongolian gerbils.

initiate signaling events that activate the host immune system [78,79]. Potent adjuvants derived from the cell wall of Gram-negative bacteria are the lipopolysaccharides (LPS) [91,92]. The lipid A moiety is responsible for its adjuvant effect, but it is too toxic for humans [93]. The development of a less toxic compound, which retains the adjuvant activity, is called monophosphoryl lipid A (MPLA) [94]. MPLA derived from *Salmonella minnesota* R595 formulated with alum has been approved for human use in Europe [95], and *Bordetella pertussis* MPLA has been obtained after treatment of pertussis whole-cell vaccine [96]. MPLA of *Salmonella minesota* has been described to enhance T-cell response by having an effect on dendritic cells (DC) and T-cells. It was demonstrated that DC treated with MPLA increased the costimulatory molecules expression and IL-12 production [97–99].

The adjuvant potential of *Bordetella pertussis* MPLA against leptospirosis was evaluated in combination with chimeric protein rChi [61]. Immunization with rChi plus MPLA produced a high level of IgG2 and resulted in 55% protection of animals after virulent leptospiral challenges. However, controversial results were obtained in relation to sterilizing immunity, since in the first experiment

100% of surviving hamsters were free from leptospires in their kidney, while in the second experiment only 16% sterilization was achieved [61].

Flagellin is a TLR5 agonist protein extracted from the flagella of both Gram-positive and Gram-negative bacteria. The signaling pathway activated by flagellin results in an inflammatory pattern with direct effects on the adaptive immune response [100]. After the flagellin-TLR5 binding, genes MyD88-dependent or-independent pathways can be activated, resulting in the production of cytokines and costimulatory molecule expression, such as TNF-α, IL-10, IL8, IL6 and a monocyte activation marker [101]. Flagellin adjuvant properties have already been investigated in several vaccine formulations [102–105]. Monaris and collaborators evaluated the immunoprotective activity induced by seven *L. interrogans* outer membrane proteins combined with *Salmonella enterica* serovar Typhimurium flagellin. Although survival rate was similar as with alum adjuvant, the incorporation of the flagellin adjuvant reduced renal colonization of infecting leptospires in challenged hamsters, suggesting their potential in conferring sterilizing immunity [39].

Another bacteria-derived component that exhibits a strong adjuvant activity is the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) [106]. LTB has the capacity to induce a potent immune response, possibly because it induces the expression of activation markers MHC class II, B7, CD40, CD25 and ICAM-1 on B cells and enhances B7-2 expression on antigen-presenting cells leading to the costimulation of $CD4^{+}$ T cells [107–109]. LTB adjuvant efficacy has been demonstrated for antigens of *Helicobacter pylori* [106], *Mycoplasma hyopneumoniae* [110], *Pseudomonas aeruginosa* [111] and influenza virus [112]. For leptospirosis, LTB coupled or coadministered with recombinant protein LipL32 was highly immunogenic, protecting approximately 80% of hamsters after lethal challenge [113].

Synthetic oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs mimic bacterial DNA and activate the innate immune system by interacting with TLR-9 [114,115]. Thus, CpG ODN activation results in a marked increase in antibody and T-cell responses to a variety of protein antigens [116–120]. CpG DNA has also been tested as a vaccine adjuvant for leptospirosis. Although this adjuvant boosts the generation of vaccine-specific humoral and cellular immune responses, the use of CpG ODN as an adjuvant in a subunit vaccine containing the LigA protein of *Leptospira* did not produce significant protection with less than 20% survival after lethal challenge [51,121]. In addition, the data from Oliveira's group showed that animals immunized with CpG ODN and LigA did not develop an IgG response while in the case of Bacelo's group, IgG levels were only detectable after the booster. The results of these groups suggest that improving the induction of humoral and cellular immune responses of this adjuvant depend on the protein antigen used.

A polysaccharide derived from *Xanthomonas* spp., the causative agent of many plant diseases, has been suggested as an adjuvant in a vaccine formulation against virulent *Leptospira* [121]. Xanthan gum is commonly used as a food additive, since it can improve texture, consistency and flavor and stabilize food [122]. Its adjuvant potential was investigated in the 1980s [123] and more recently,

Table 3
Bacteria-derived adjuvants.

B. pertussis MPLA	Surviving (%)	Sterility (%)	Via	Immune response	Reference
rChi	55	60	Subcutaneous	IgG2/3	[61]
Control	10	0			
Salmonella flagellin					
Lp11 + Lp21 + Lp22+ Lp25 + Lsa30 + Lp35	3	0	Subcutaneous	Total IgG	[39]
Lp11 + Lp21 + Lp22+ Lp25 + Lsa30 + Lp35 +LigAC	86	70			
LigAC	93	0			
Control	10	0			
LTB adjuvant					
rLTB::LipL32	80	ND	Intramuscular	Total IgG	[113]
rLTB _ rLipL32	87				
rLTB	40				
Control	27				
Xanthan Gum					
rLigANI-Xa	100	0	Subcutaneous	Total IgG	[121]
Control	16.7	0			
CPG					
rLigANI-CpG	16.7	0	Subcutaneous	Total IgG	[121]
Control	0	ND			
rLigANI-CpG	17	ND		IgG2/3	[51]
Control					
Xanthan Gum + CPG					
rLigANI-CpG-Xa	100	0	Subcutaneous	Total IgG	[121]
Control	0	ND			
E. coli expressing					
LigA7-13- i.p route	37.5	ND	Oral	Total IgG	[126]
LigA7-13- i.d route	62.5				
BCG adjuvant					
rBCG (pUS977/lipL32)	32.35	100	Intraperitoneal	Total IgG	[127]
rBCG (pUS2000/lipL32)	55.88	100			
Control	11.76				

ND: not determined.

Table 4
Liposome as adjuvant.

Smegmosome	Surviving (%)	Sterility (%)	Via	Immune response	Reference
Lp0607/Lp1118/Lp1454	75	*	Subcutaneous	Total IgG/IL-4/IL10/IFN-γ	[134]
Control	0				
Leptosome					
Lp0607/Lp1118/Lp1454	75	*	Subcutaneous	Total IgG/IL-4/IL-10/IFN-γ	[135]
Control	0				
Liposomes					
LigAvar-Lipo	87.5	83.34	Subcutaneous	Total IgG/ IL-4/ IL-10/ IL-12/ IFN-γ	[136]
Control	0				
PLGA microspheres					
LigAvar-MS	75	66.67	Subcutaneous	Total IgG/ IL-4/ IL-10/ IL-12/ IFN-γ	[136]
Control	0				

ND: not determined.
* Data not shown.

Table 5
Other adjuvants used in leptospiral vacines.

Carboxyl MWCNTs	Surviving (%)	Sterility (%)	Via	Immune response	Reference
rLigANI-COOH-MWCNTs	0	ND	Subcutaneous	IgG1/IgG2/3	[51]
Control	0				
rLipL32-COOH-MWCNTs	0	ND	–	Total IgG	[142]
Control	0				
rLigANI-CpG-COOH-MWCNTs	17	ND	Subcutaneous	IgG1/IgG2/3	[51]
Control	0				
HNTs					
rLipL32-HNTs	0	ND	–	Total IgG	[142]
Control	0				
Alum + QS21					
r-Hap1 &	50	ND	Subcutaneous	Total IgG	[72]
control	60				
Adenovirus					
Ad-hap1 &	87	ND	Intramuscular	Total IgG	[144]
Ad-ompL1	36.6				
Ad-hap1+Ad-ompL1	75.8				
Ad-null	51				

ND: not determined.
& Mongolian gerbils used as animal model.

as bioadhesive liposomes for an avian influenza virus vaccine [124]. The combination of xanthan gum, alone or mixed with CpG, with leptospiral rLigANI protein elicited 100% immune protection and a strong IgG response. However, surviving animals harbored leptospires in their kidneys, indicating that this vaccine formulation was ineffective in *Leptospira* clearance [121].

Live attenuated bacteria can act as an antigen delivery system by expressing foreign proteins. Recombinant bacteria as carriers are advantageous due to the effective humoral and cellular immune responses at systemic and mucosal levels [125]. *E. coli* expressing a lipidated form of LigA (domains 7 to 13) was evaluated as an oral vaccine in hamsters. The results obtained showed 37.5% survivals in intraperitoneal challenge experiments and 62.5% survival with intradermal challenge [126]. In the aforementioned study, although there was better survival with intradermal challenge, renal cultures of all animals were positive for leptospires. Another promising candidate for delivery of foreign antigens tested for leptospirosis is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). A recombinant BCG expressing LipL32 protein elicited protective and sterilizing immunity in 55.8% of animals [127]. Although only MPLA from *Salmonella minnesota* R595 has been approved for human use, some vaccine formulations have shown promising results and therefore deserve further in-depth assessment. Table 3 shows a compilation of data for vaccine formulations with bacteria-derived adjuvants tested for leptospirosis.

5. Liposome adjuvants

Concentric spheres consisting of phospholipid bilayers separated by aqueous compartments were reported as an adjuvant by Gregoriadis and Allison in 1974 [128]. Since then, their use as vaccine adjuvants has been investigated. Liposome-based adjuvants may be used for either delivery systems of subunit antigens or

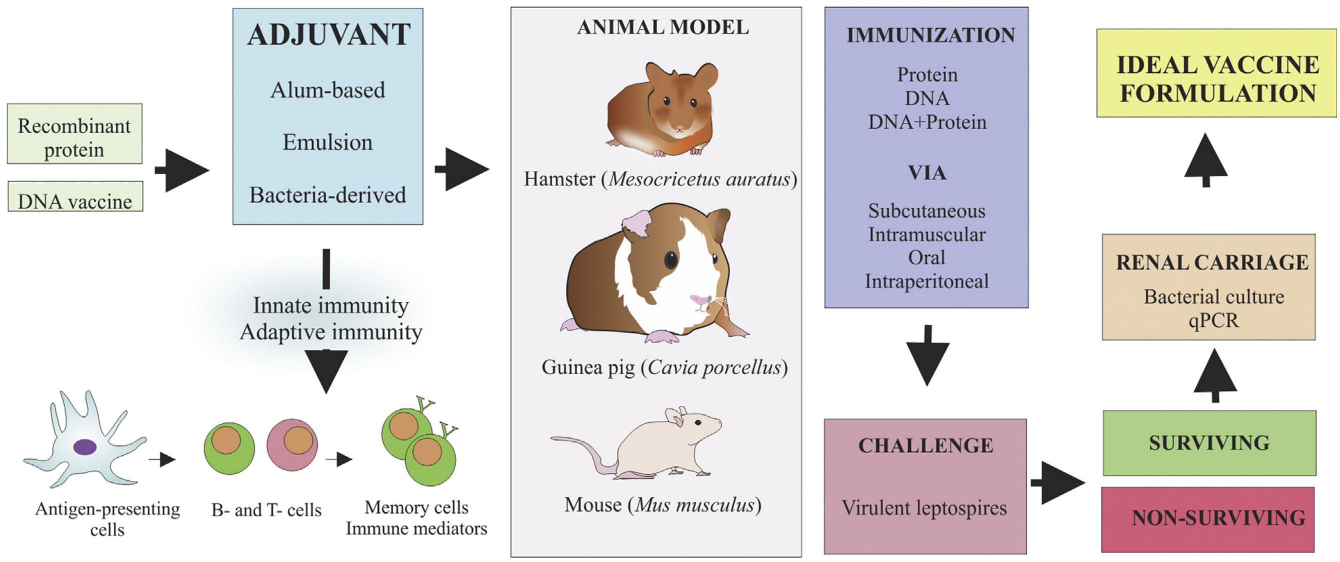

Fig. 1. Overview of approaches used in animal model for leptospirosis vaccine development.

immunopotentiators. The effectiveness of this adjuvant depends on electrical charge, method of preparation, number of lipid layers and composition. Thus, they are considered versatile molecules, since their composition and preparation can be chosen to achieve desired features [129]. Liposomes are able to interact with antigen-presenting cells and enhance the exposure time of antigen to these cells, which results in an increase in humoral and cellular responses [130]. It seems that the size of the particle can also influence the type of immune response obtained [131].

Liposomes of attenuated mycobacterium strain BCG vaccines have been described as potent immune stimulators [132,133]. The adjuvant potential of liposomes composed of lipids of non-pathogenic *Mycobacterium smegmatis*, having a similar lipid profile as BCG, was evaluated with three leptospiral proteins (Lp0607, Lp1118 and Lp1454). This vaccine formulation called smegmosome, induced a mixed Th1/Th2 immune response able to protect 75% of animals. However, 50% of surviving animals were positive for *L. interrogans*, showing that despite the high rate of protection, this formulation was not sterilizing [134]. When liposomes composed of lipids from non-pathogenic *E. coli* or from *L. biflexa* serovar Patoc were constructed, the same level of protection was achieved. Although the immune response pattern was similar, leptosome-based formulation conferred approximately 80% bacterial clearance [135]. The variable region of LigA protein incorporated into conventional liposomes, prepared with egg phosphatidylcholine and cholesterol, were constructed and evaluated for their immunoprotective potential. It is interesting to note that this vaccine formulation induced a polarized Th2 response but produced nearly 90% protection and also elimination of bacteria from the kidney [136]. Along with the high levels of IL-4 and IL-10 induced by this formulation, a significant level of IFN-γ was found, which could contribute to the elimination of leptospires. The data on a liposome-based vaccine approach against leptospirosis are summarized in Table 4.

6. Other adjuvants

Other uncommon vaccine adjuvants have also been tested for leptospirosis (Table 5). Nanomaterials are versatile because of their unique physicochemical properties. Among the variety of nanomaterials, carbon nanotubes (CNTs) and halloysite clay nanotubes (HNTs) possess interesting characteristics for vaccine development, such as large-scale production and biocompatibility, which allows functionalization with different biomaterials [137–139]. Moreover, nanotubes can be internalized by a wide variety of cell types, allowing their use in intracellular drug-delivery strategies [140,141]. The potential of nanotube as adjuvants was evaluated in immunized female golden Syrian hamsters with the LipL32 recombinant protein and functionalized multi-walled carbon nanotubes (COOH-MWCNTs) or HNTs. Although both vaccine preparations increased IgG levels, no protection was achieved and all the immunized hamsters died 9–12 days after the challenge [142]. Similar results were obtained when vaccine formulations containing the LigANI protein and nanotubes were tested [51].

Defective adenovirus has been developed as a vaccination vector because of its inability to replicate in cells, and its ability to induce the synthesis of high levels of a foreign protein [143]. Branger et al. [144] studied the potential of adenovirus expressing the Hap1 and OmpL1 proteins of *L. interrogans*. Immunized gerbils showed 87 and 36.6% survival with Hap1 and OmpL1, respectively. After administration of both vectors, there was a decrease in survival rate, suggesting that adenoviruses expressing OmpL1 may have reduced the protective effect.

QS21, a purified plant extract, was shown to act as an adjuvant, inducing high antibody responses with the production of both IgG1 and IgG2a [145]. QS21 was used in combination with aluminum hydroxide as adjuvant for a vaccine against leptospirosis. However, no protection was observed and gerbils immunized with recombinant protein Hap1 plus adjuvants showed the same mortality rates as the control group, including delay in the onset of death [72].

Fig. 1 summarizes the approaches used in protective immunity against lethal challenge in animal model of leptospirosis.

7. Conclusion

Although there have been many efforts to develop effective vaccines against leptospirosis, several questions still remain. Despite the large number of published studies on immune protection activity, there has been little progress in finding a good vaccine candidate against leptospirosis. The data on vaccine efficacy presented in the literature are conflicting, independently of the protein/adjuvant used. One possibility that may contribute to the contradictions observed is the animal model used. Most studies have used hamsters as the model for acute leptospirosis, because they are considered particularly sensitive to infection with virulent leptospires. However, unexpected results arise from the non-immunized control group, in which, for unclear reasons, the animals survive after a challenge test. A possible alternative to this situation is to use endpoint criteria, such a rapid or progressive weight loss, lethargy or persistent recumbency, or any other condition that interferes with the animal's daily activities and that may define signs of illness. Overall, this would contribute to more consistent survival data. However, the great limitation of assessing immune response would continue due to the lack of specific antibodies. The use of mice as an animal model would solve the problem of immunological analyses, but they are generally unsuitable hosts for acute lethal leptospirosis because they develop severe signs of disease only within a short window of time after birth. Regardless of the animal model used, it is clear that the use of different adjuvants specifically amplifies the immune response, to leptospiral antigens and, in some cases, is able to exert an immunomodulating effect. Interestingly, the combination of antigen and adjuvant seems to be essential to achieve this goal, since the same protein used with different adjuvants produces a different protection profile. Moreover, we note that high antibody titers are not necessarily related to protection and also that cytokine profile analysis does not reveal the immune mechanisms involved in protection against disease. Unfortunately, the lack of a good correlation between adjuvants to immune response, and animal protection has limited our understanding of the immune protection mechanisms against lethal leptospiral challenge. It is anticipated that the study of new vaccine strategies, such as combination of antigens, mapping of the protective epitopes and new delivery systems, is needed and shall contribute to advancing the development of new vaccines against leptospirosis.

8. Declarations

Ethics approval and consent to participate. Not applicable.
Consent for publication. Not applicable.
Availability of date and material. Not applicable.

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10. Authors' contributions

All authors participated in the literature revision, discussion and preparation of manuscript, including table and the figure.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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