A prime-boost strategy using the novel vaccine candidate, LemA, protected hamsters against leptospirosis.

Running title: LemA recombinant vaccines against leptospirosis.

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ABSTRACT
Towards developing an effective vaccine capable of conferring heterologous protection the putative lipoprotein LemA (present an epitope M3 similar to Listeria) was evaluated as a vaccine candidate in the hamster model of leptospirosis. LemA is conserved (> 70% pairwise identity) among the pathogenic Leptospira spp., indicating its potential in stimulating a cross-protective immune response. Using different vaccination strategies, including prime-boost, DNA vaccine and a subunit preparation, recombinant LemA conferred different levels of protection in hamsters. Significant protection against mortality was observed for the prime-boost and the DNA vaccine strategies, 87.5% ($P < 0.01$) and 62.5% ($P < 0.05$) efficacy, respectively. Although the subunit vaccine preparation protected 50.0% of immunized hamsters it was not significant. None of the hamsters in the control groups survived challenge with a virulent strain of Leptospira interrogans serogroup Icterohaemorrhagiae. Characterization of the immune response found that the strongest antibody response was stimulated by the subunit vaccine preparation, followed by the prime-boost strategy. The DNA vaccine failed to elicit an antibody response among immunized hamsters.

KEYWORDS: Leptospirosis; Vaccine; Leptospira spp.; LemA; Protection; Hamster challenge model.
INTRODUCTION

Leptospirosis is a widespread zoonosis caused by pathogenic *Leptospira* spp. that can infect almost all mammals (1-3) and it is an emerging public health problem, with an estimated 500,000 cases per year (4). Humans are generally infected through direct or indirect contact with the urine of infected animals, primarily rats in urban leptospirosis (1,5). Symptoms range from a mild influenza-like illness to a severe infection, as Weil’s disease or leptospirosis-associated pulmonary haemorrhage syndrome (LPHS) with a case-fatality rate of > 50% (6,7).

Currently available vaccines are based on inactivated whole bacteria or membrane preparations from pathogenic leptospires that require annual booster immunizations and do not confer cross-protective immunity (8). There are more than 270 pathogenic serovars of *Leptospira* spp. and this antigenic diversity has been attributed to the distribution and composition of the lipopolysaccharides (LPS) in the outer membrane (OM) (9), thereby precluding its use in multivalent vaccine preparations.

Several leptospiral vaccine candidates have been evaluated in animal models, reviewed in (8). However, the majority of the subunit vaccines tested to date do not induce significant protection against mortality (10). Many of these antigens are localized in the OM, e.g. OmpL1, LipL41, LipL32 and the Lig proteins, are highly conserved among the pathogenic species (11,12) and are important for the development of vaccines that can offer heterologous protection (10,13). The OM contains a diverse range of proteins, including lipoproteins, predicted by genome sequencing projects (14-17), and some are unique to the pathogenic serovars (14). Spirochaete lipoproteins represent the most abundant proteins in the OM (18). Furthermore, several lipoproteins as LipL41, LipL32, LIC10325 and LIC13059 have been shown to stimulate a protective immune response against leptospirosis, indicating their potential as vaccine candidates.
(19-21). The hypothetical lipoprotein LemA (LIC11058), that presents an epitope M3 similar to *Listeria*, contains a transmembrane helix with an extracellular amino terminus and its subsequent modification releases an epitope that is presented on the surface of phagocytes (22), yet the biological function of this protein remains unknown.

DNA vaccines stimulate both humoral and cell-mediated immunity in different animal models and this may be a promising strategy in the development of new vaccines against leptospirosis. DNA-type vaccine candidates have been evaluated in several leptospirosis vaccine studies (23-25), including prime-boost strategies (26). In a previous study, we reported that the LemA subunit vaccine preparation induced a strong humoral immune response in immunized mice and that it was recognized by antibodies present in sera of patients with severe leptospirosis (27). The aim of the present study was to determine whether LemA could induce a protective immune response in hamsters against challenge with a virulent strain of *L. interrogans* using prime-boost, DNA and protein based immunizations. The current paper demonstrates that LemA is a good candidate to compose a vaccine for leptospirosis control.

**MATERIAL AND METHODS**

**Bacterial strains and culture conditions**

The *L. interrogans* strains were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco, USA) supplemented with *Leptospira* Enrichment EMJH (Difco, USA) at 30 °C (32). The strains used in this study included *L. interrogans* (serovars Autumnalis, Bataviae, Bratislava, Canicola, Djasiman, Hebdomadis, Icterohaemorrhagiae, Muenchen and Pomona), *L. borgpetersenii* (serovars Ballum, Castellonis, Javanica, Mini, Poi and Sejroe), *L. kirschneri* (serovars Cynopteri and Grippotyphosa) and *L. santarosai* (serovar Pomona). The *E. coli* strains TOP10 and...
TOP10F’ (Invitrogen, Brazil) were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37 °C with the addition of the ampicillin to 100 µg mL⁻¹.

**Presence and conservation of lemA in Leptospira spp.**

The presence of lemA among Leptospira spp. was confirmed by PCR amplification using genomic DNA from 17 serovars, including *L. borgpetersenii*, *L. interrogans*, *L. kirschneri* and *L. santarosai*. The PCR product was amplified with the primers used to clone the LemA coding region, as described previously (27). The 16S gene was amplified using the fD1_F and rP2_R primer pair (28) to confirm the identity of the DNA template. The coding sequences were aligned using Geneious (Biomatters Ltd.), based on the ClustalW algorithm, at the default settings (29). Protein sequences were generated by translation of the CDS and were aligned using the default settings.

**Amplification, expression and purification of recombinant LemA**

The lemA coding sequence was amplified using oligonucleotides based on the lemA gene sequence from the *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genome. The cloning into the vector pQE30 and the expression and purification of recombinant LemA (rLemA) protein was performed as previously described (27). The protein concentration was determined by BCA Protein Assay Kit (Pierce, USA).

For construction of the DNA vaccine, lemA was amplified using the primer pair lemApTARGET_F: 5'-atgATTCAAGAAGAAGATGA and lemApTARGET_R: 5'–gggAAGCTTAATTGTAACGTTGTA and cloned into the mammalian expression vector pTARGET (Promega, USA). The identity and orientation of the cloned inserts was confirmed by DNA sequencing. *E. coli* TOP10 competent cells were transformed
with the pTARGET-lemA construct and plasmid DNA was purified using the Perfectprep Plasmid Maxi kit (Eppendorf, Germany). The plasmid DNA concentration was determined using the Qubit Fluorometer (Invitrogen, Brazil).

To confirm the expression of rLemA in the pTARGET-lemA construct, Vero cells were transfected with either the lemA construct or pTARGET (control), using the transfecting reagent lipofectamine (Invitrogen). Briefly, Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 10% (v/v) heat inactivated foetal bovine serum. When 60-75% confluence was reached, the cells were transfected with 15 µg of plasmid DNA pre-condensed with lipofectamine in serum-free DMEM. Forty-eight hours after transfection the cells were removed from the flasks, washed with phosphate buffered saline (PBS) and suspended in DMEM. The transfected cells were suspended in lysis buffer (PBS containing 4% (v/v) NP-40), and incubated on ice for 30 min. The expression of rLemA by cell lysates was analysed by Western blotting (WB) assay using mouse anti-LemA sera (1:100 in PBS) (27). The reactions were developed with ECL WB Detection Reagents (GE Healthcare, Brazil).

Immunization and challenge of hamsters

The vaccines preparations used in this study were rLemA-alhydrogel, pTARGET-lemA, pTARGET, PBS-alhydrogel and heat-killed whole-leptospires (bacterin) and for protection experiments, 6 or 8 female hamsters aged 4-6 weeks were used (Table 1). The various doses for the vaccination strategies were as follows: subunit: rLemA (2× 100 µg); prime-boost: pTARGET-lemA (100 µg) + rLemA (100 µg); DNA vaccine: pTARGET-lemA (2× 100 µg) and bacterin: 10⁹ heat-killed whole-leptospires, the second dose of each strategy was administered 21 days later. Blood samples were
collected from the retro-orbital venous plexus before each immunization and challenge
and the sera were stored at -20 ºC. Forty-two days after the first immunization the
hamsters were challenged intraperitoneally with a dose of 10 leptospires, equivalent to
5× LD_{50} (19; 30) of the *L. interrogans* serogroup Icterohaemorrhagiae, strain Spool
(31). Hamsters were monitored daily and were euthanized upon the appearance of
clinical symptoms of leptospirosis. Animal experiments described in this study were
carried out in strict accordance with the recommendations approved by the Ethics
Committee in Animal Experimentation, Federal University of Pelotas, Brazil (Permit
Number: 2348). All surgery was performed under sodium pentobarbital anesthesia, and
all efforts were made to minimize suffering.

**Evaluation of the humoral immune response in hamsters**

The antibody responses were monitored by indirect ELISA using rLemA as
previously described (27). Briefly, each well was coated with 50 ng of rLemA diluted in
carbonate-bicarbonate buffer, pH 9.6. The ELISA plates were washed three times with
PBST (PBS with 0.05% (v/v) Tween 20), blocked and hamster sera, diluted 1:50, was
added for 1 h at 37 ºC, and then washed 3× with PBST. Anti-Golden Syrian Hamster
IgG Antibody Peroxidase Conjugated (Rockland), 1:8,000 dilution, was added,
incubated at 37 ºC for 1 h, washed 5× with PBST and the reaction was visualized with
*α*-phenylenediamine dihydrochloride (Sigma-Aldrich) and hydrogen peroxide. The
reaction was stopped by the addition of 0.1 M sulphuric acid and absorbance was
determined at 492 nm using a Multiskan MCC/340 ELISA plate reader (Titertek
Instruments, USA).

**Culture, histopathology and imprint detection of leptospires**
The presence of leptospires in the kidney and lung tissue samples was determined by culture, histopathology and imprint in survivors. For histopathological studies, kidney and lung tissues samples were fixed in 10% formalin (pH 7.0) and embedded in paraffin. Six sections, 5-6 µm, were stained with haematoxylin and eosin and examined by a qualified pathologist for evidence of interstitial nephritis or pulmonary haemorrhage (19). Kidney samples were used to confirm sterilizing immunity by culture as described (32). The direct detection of leptospires in the kidneys of hamsters was evaluated by the imprint method as previously described (33,34).

Statistical analysis

The Fisher exact test and the Wilcoxon log-rank test were used to determine significant differences for protection against mortality and survival, respectively, using Epi Info 6 (Centres for Disease Control, USA) and Prism 5 (Graphpad, USA) software packages. Variance analysis was used to determine significant differences between the assay results. The Student’s t-test was used to determine significant differences in the serological assays. Differences were considered significant at a P-value of < 0.05.

RESULTS

Distribution and conservation of lemA among Leptospira spp.

The PCR assay found that the gene was present in *L. interrogans* (nine serovars), *L. borgpetersenii* (six serovars), *L. kirschneri* (two serovars) and *L. santarosai* (one serovar). Only the *L. interrogans* serovar Hebdomadis strain was negative in the PCR assay. Furthermore, the genome sequences available in GenBank indicated that *lemA* is present in all *Leptospira* spp. sequenced to date. The overall pairwise identity of the DNA sequences was 70.8%, compared to 68.0% for the amino acid sequences. The *L.
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interrogans lemA sequences were highly conserved: 99.9 and 100% pairwise identity for the DNA and protein sequences, respectively and, comparing between species, together with the L. santarosai sequences, the pairwise identity was 93.4% and 93.5%, respectively. The L. noguchii sequences reduced the mean pairwise identity to 90.6 and 96.0% for the DNA and amino acid sequences, respectively. Similarly, including the L. weilii sequences resulted in a pairwise identity of 89.2 and 94.5%, respectively. Of note, the L. biflexa genome contains a lemA ortholog, although the pairwise identity of the DNA and amino acid sequences with the pathogenic Leptospira spp. was 61.5 and 56.3%, respectively.

Subunit and DNA vaccine preparation

Even though rLemA was expressed as a truncated fragment (17 kDa) rather than the full length of the native protein (22.6 kDa) and was engineered to avoid potential hydrophobic regions (27), the recombinant protein was insoluble when expressed in E. coli TOP10F’. The rLemA protein was recovered from the E. coli pellet, solubilised in 8 M urea, purified by IMAC and the yield of rLemA was > 40 mg L⁻¹ (Fig. 1A). The expression of LemA in the pTARGET construct was confirmed by detection of rLemA in transfected Vero cells with the expected molecular mass of 17 kDa (Fig. 1B).

Humoral immune response in rLemA immunized hamsters

To quantify the humoral antibody response, an ELISA was performed with the sera collected on days 0, 21 and 42 post-immunization (pi). Significant levels of circulating anti-rLemA antibodies were detected ($P < 0.05$) (Fig. 2). Twenty one and 42 days pi there was a significant induction of IgG in hamsters immunized with the rLemA subunit preparation compared to the negative control group (PBS-alhydrogel), $P < 0.05$. 

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The IgG response in hamsters immunized using the prime-boost strategy significantly differed from the negative control group on day 42 pi ($P < 0.05$). In the group immunized with the DNA vaccine there were no detectable levels of IgG (Fig. 2).

**Efficacy of the LemA vaccine preparations**

The protective efficacy of the LemA vaccine preparations was evaluated and the prime-boost vaccination strategy significantly protected 87.5% of the hamsters against lethal challenge ($P < 0.01$) and 62.5% of hamsters immunized with the DNA vaccine survived ($P < 0.05$) (Fig. 3, Table 1). The rLemA subunit vaccine preparation protected 50.0% of vaccinated animals ($P = 0.08$). Of note, among the hamsters immunized with the rLemA subunit preparation that died following challenge, death occurred significantly later than observed for animals in the control group ($P < 0.05$, Fig. 3).

Histopathological analysis of the organs collected from hamsters 34 days post-infection revealed evidence for acute leptospirosis with pulmonary and kidney lesions (Table 1). Surviving animals immunized using the DNA (100%), prime-boost (71.4%) or subunit (50.0%) vaccine strategy were culture-positive, indicating a lack of sterilizing immunity (Table 1) and the imprint evaluation confirmed these results (data not shown). In contrast, none of the hamsters immunized with bacterin vaccine showed evidence of the presence of leptospires (culture or imprint).

**DISCUSSION**

The identification of leptospiral OMPs represents an important step in the discovery and development of novel vaccine candidates for leptospirosis. The sequencing and annotation of the bacterial genomes of pathogenic *L. interrogans* serovars Lai (16), Copenhageni (15) and *L. borgpetersenii* (17) resulted in the
identification of many putative OMPs. Further bioinformatics analysis of the *L. interrogans* serovar Copenhageni genome identified more than 200 proteins containing motifs suggesting localization to the OM (27). A major limitation in the field of leptospirosis has been the identification of membrane associated proteins through conventional laboratory methods. However, the post-genomic age has made it possible to characterize new targets for vaccine candidates and diagnostic tests for leptospirosis.

Recently, our group characterized the immunogenic and antigenic potential of eight putative OMPs from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (27). Bioinformatics was used to identify genes predicted to encode OMPs, the recombinant proteins from seven of these genes induced significant IgG responses in mice and they were recognised by sera collected from patients with severe leptospirosis. In the present study we evaluated the immune-protective potential of the LemA recombinant protein in the hamster model against lethal challenge.

Using the *lemA* sequences available in GenBank, sequence analysis demonstrated that *lemA* was conserved at the DNA and protein level (>70% overall pairwise identity) in *L. interrogans*, *L. noguchii*, *L. santarosai* and *L. weilii* strains. An ortholog of *lemA* was also identified in the *L. biflexa* genome, albeit with less homology to the gene found in the pathogenic serovars. The absence of *lemA* in the Hebdomadis serovar is difficult to explain as it is conserved in all tested serovars to date. It is possible that the PCR failed, possibly due to significant differences in the level of identity of *lemA* in the genome. The genome of *L. interrogans* serovar Hebdomadis is currently being sequenced at the J. Craig Venter Institute and once available this issue will be resolved.

In leptospirosis a major effort has been to find a potent and effective immunotherapy. However, the available vaccines do not confer cross-protection against heterologous *Leptospira* serovars. The presence of the *lemA* gene in different serovars
indicates it has a potential role as a vaccine candidate. In *Leptospira* spp., LemA is a putative lipoprotein and has similarities with orthologous proteins described in other bacteria. This protein belongs to a family that is predicted to contain a transmembrane helix with an extracellular amino-terminal region. However, the biological function of LemA and its involvement in the pathogenesis of leptospirosis is unknown. At the time of writing, only one leptospiral virulence factor, Loa22, has fulfilled Koch’s molecular postulates (35). Experimentally, only a few proteins have been shown to be present on the leptospiral surface (36), therefore our knowledge of the superficial layer of leptospires remains limited.

Immune-strategies using LemA (subunit, DNA vaccine and prime-boost) were evaluated using the hamster model of lethal leptospirosis. The subunit vaccine preparation included alhydrogel as an adjuvant. This is regularly used in commercial animal vaccines and is approved for use in human vaccines (37). Animals immunized with the subunit vaccine demonstrated the highest IgG response however, only 50.0% of hamsters survived the challenge. The subunit vaccine had a significant impact on survival time (Fig. 3). There were no significant differences observed between the surviving and dead hamsters except on day 21 pi, when the IgG response was found to be higher among the susceptible animals. Even though the hamsters immunized using the prime-boost strategy produced significantly lower amounts of IgG, this significantly protected the hamsters (87.5%). Of note, the susceptibility of the only animal that did not survive the challenge dose could not have been predicted from the IgG response observed. Furthermore, while there was no significant IgG response in the hamsters immunized using the DNA vaccine, a significant number were protected against mortality (62.5%). Several studies have shown the effectiveness of DNA vaccines and reported that protection was associated with a strong antibody response (23,24,25).
is contrary to the results presented in the current study that suggest the involvement of a cellular immune response in protection, although this is mere speculation as cytokine levels were not determined. However, there are reports in the literature that support the involvement of a cellular immune response in protection against leptospirosis (38-41).

Bacterin vaccines have been shown to induce sterilizing immunity in previous studies, e.g. (42). In our study none of the rLemA vaccine strategies, except the bacterin, induced 100% sterilizing immunity as there was evidence of lesions in the lungs and kidneys among all of the surviving hamsters. In hamsters immunized using the prime-boost or subunit vaccine strategy 28.5 and 50.0% of the survivors were culture negative, respectively (Table 1). In a previous report, sterilizing immunity was observed in hamsters immunized with a recombinant *M. bovis* BCG strain expressing LipL32 (19). However, the majority of vaccine candidates against leptospirosis have so far failed to induce sterilizing immunity, reviewed in (8).

This study reports on the successful evaluation of a novel vaccine candidate that offers significant protection against mortality in the hamster model of leptospirosis. Furthermore, due to the considerable level of conservation of LemA among *Leptospira* spp. it is a potential candidate for the development of a vaccine that could generate cross-protection against a wide range of *Leptospira* serovars, however this needs to be better evaluated in trials of heterologous challenge.

**COMPETING INTERESTS**

DDH, AJAM and OAD are inventors on a patent submission entitled: *The Leptospira spp. LemA protein for immunobiological use*. The other authors declare no competing interests.
ACKNOWLEDGMENTS

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REFERENCES


Figure 1. rLemA vaccines preparation. (A) SDS-PAGE of the rLemA protein expressed in *E. coli* and purified by IMAC using Ni²⁺ Sepharose HisTrap columns. Lanes M: molecular mass protein ladder (kDa). Lanes 1 - 6: purified rLemA eluted with imidazole 250 mM (17 kDa). (B) Expression of LemA in Vero cells visualized in a Western blotting assay using anti-LemA mouse sera. Lane 1: pellet of cells transfected with pTARGET and incubated with anti-LemA sera. Lane 2: pellet of cells transfected with pTARGET/lemA and incubated with anti-LemA sera.

Figure 2. Systemic antibody levels in hamsters inoculated with rLemA vaccines and controls. Recombinant rLemA expressed by *E. coli* was used as antigen in the ELISA. Mean values were calculated from sera samples assayed in triplicate. Results are expressed as the mean absorbance and *significance a P value < 0.05 in comparison to the control group.

Figure 3. Survival of hamsters challenged with virulent *L. interrogans* after immunization with the various rLemA vaccines preparations. Post-challenge survival is shown for a representative vaccine evaluation (Experiment 1, Table 1). The Fisher exact test and the Wilcoxon log-rank test were used to determine significant differences for mortality and survival between the immunized and control groups, respectively.
Table 1. Protection conferred by immunization with different LemA vaccination strategies against lethal challenge in the hamster model

<table>
<thead>
<tr>
<th>Vaccine preparation</th>
<th>% Protection (No. survivors/total)</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Culture positive&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haemorrhage and necrosis</td>
<td>Lymphocyte infiltration</td>
</tr>
<tr>
<td>rLemA</td>
<td>50 (4/8)</td>
<td>0.08</td>
<td>50 (2/4)</td>
<td>100 (4/4)</td>
<td>100 (4/4)</td>
</tr>
<tr>
<td>pTARGET-lemA</td>
<td>62.5 (5/8)</td>
<td>0.03</td>
<td>100 (5/5)</td>
<td>100 (5/5)</td>
<td>100 (5/5)</td>
</tr>
<tr>
<td>pTARGET-lemA + rLemA</td>
<td>87.5 (7/8)</td>
<td>0.005</td>
<td>71.4 (5/7)</td>
<td>100 (7/7)</td>
<td>100 (7/7)</td>
</tr>
<tr>
<td>Bacterin</td>
<td>100 (6/6)</td>
<td>&lt; 0.001</td>
<td>0 (0/6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alhydrogel</td>
<td>0 (0/6)</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pTARGET</td>
<td>0 (0/6)</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not determined

<sup>a</sup> Compared to the relevant control, Fisher exact test (two-tailed P-value).

<sup>b</sup> In immunized hamsters that were protected against lethal challenge.

<sup>c</sup> The percentage of animals positive with evidence of a particular lesion.