The *Leptospira interrogans* vaccines currently available are serovar specific and require regular booster immunizations to maintain protection of the host. In addition, a hamster challenge batch potency test is necessary to evaluate these vaccines prior to market release, requiring the use of a large number of animals, which is ethically and financially undesirable. Our previous work showed that the N terminus of the outer membrane protein LipL32 was altered in *Leptospira interrogans* serovar Canicola vaccines that fail the hamster challenge test, suggesting that it may be involved in the protective immune response. The aim of this study was to determine if vaccination with LipL32 protein alone could provide a protective response against challenge with *L. interrogans* serovar Canicola to hamsters. Recombinant LipL32, purified from an *Escherichia coli* expression system, was assessed for protective immunity in five groups of hamsters (*n = 5*) following a challenge with the virulent *L. interrogans* serovar Canicola strain Kito as a challenge strain. However, no significant survival against the *L. interrogans* serovar Canicola challenge was observed compared to that of unvaccinated negative controls. Subsequent histological analysis revealed reduced amounts of collagen type IV and plasma fibronectin, in a calcium-independent manner, than 230 pathogenic serovars of *Leptospira* have been identified to date (3), and higher prevalences of some serovars in particular species, such as *Leptospira interrogans* serovar Canicola in dogs and *Leptospira interrogans* serovar Hardjo in sheep and cattle have been observed (4). The initial presentation of symptoms can include (5) fever, headache, myalgia, nausea, coughing, diarrhea, vomiting, and tubulointerstitial nephritis (1); left untreated, infections can result in hepatic or renal failure and death (6). Other symptoms, such as cessation of milk production and fetal loss, have also been observed in cattle (7), making leptospirosis particularly economically damaging to the farming community. Vaccination is used to protect against infection; however, the vaccines currently available are serovar specific (8), and regular booster immunizations are required to maintain immunity (9).

The proteins LipL32 (Hap-1), LipL41, LipL45, and OmpL1 have been previously identified as possible vaccine candidates (10–12) and, in some cases, have undergone *in vivo* trials (13, 14). LipL32 has also been shown to bind to the extracellular membrane proteins, collagen type IV and plasma fibronectin, in a calcium-independent manner (15). A LipL32 DNA vaccine was shown to confer protective immunity against *L. interrogans* serovar Canicola in the gerbil model (16); however, the protective effect of LipL32 against *L. interrogans* serovar Canicola in the hamster model has yet to be determined. The exact component(s) of the *L. interrogans* serovar Canicola vaccines that provide protective immunity are currently defined. However, in our previous study (17), we demonstrated that the concentration of an N-terminal LipL32 region was reduced in failed batches of *L. interrogans* serovar Canicola vaccines, suggesting that it may be involved in the protective immune response. The aim of the present study was to determine if LipL32 could confer protective immunity against *L. interrogans* serovar Canicola in hamsters. This would be an important step in establishing whether the N-terminal alteration of LipL32, observed in failed vaccine batches (17), was responsible for the inability of the failed vaccines to confer immunological protection following challenge with *L. interrogans* serovar Canicola.

MATERIALS AND METHODS

Ethical approval. All animal procedures in this study were covered under the Animals (Scientific Procedures) Act 1986 by Home Office Project License No. PPL 70/7249 and were approved by the Animal Ethics Committee at the Animal Health and Veterinary Laboratories Agency (AHVLA) where all of this work was performed.
LipL32-pRSET C construct was generated using SECentral (Sci-Ed, USA). This method is used to insert the LipL32 gene into the plasmid. The restriction sites KpnI and EcoRI, which are associated with the primers, were used to confirm the insertion of the LipL32 gene. Promoter (T7), the polyhistidine tag (6×His), and the primers used are also shown. The plasmid diagram of the LipL32-pRSET C construct was generated using SECentral (Sci-Ed, USA).

Vaccine and bacteria selection and growth conditions. A randomly chosen bivalent vaccine, giving protection against L. interrogans serovars Canicola and Icterohaemorrhagiae, which had passed the in vivo vaccine potency test and had been released for commercial sale, was purchased from the manufacturer for analysis. This was designated vaccine A and used to vaccinate the positive-control group. The commercial sensitivity relating to this product did not allow precise formulations to be determined for vaccine A to be 1% (wt/vol) ethylene-maleic anhydride (EMA) and 3% (vol/vol) NeoCryl A-640.

L. interrogans serovar Canicola (strain Kito), for use as the challenge strain, was prepared by inoculation of 20 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton, Dickinson, USA) with 1 ml of pure culture (1 × 10⁸ cell/ml) and incubated for 7 days at 30°C with orbital agitation at 50 rpm.

Expression and purification of recombinant LipL32 protein. Cloning of the lipL32 gene into an expression vector, downstream from a polyhistidine tag, was performed using a method modified from that of Haake et al. (18). In this process, genomic DNA from Leptospira kirschneri was substituted with genomic DNA from L. interrogans serovar Canicola strain Kito, which also necessitated that the restriction enzymes XhoI and SmaI be substituted with KpnI and EcoRI (and associated primers changed accordingly); in addition, the expression strain BLR(DE3)/pLysS was substituted for BL21(DE3)/pLysS as it was more readily available. PCR was used to amplify the portion of the LipL32 gene encoding the mature protein beginning with the first residue after the amino-terminal cysteine.

The PCR amplicon product size was assessed using gel electrophoresis, and the product was cleaned using a QIAquick PCR purification kit (Qiagen, USA) as per the manufacturer’s instructions, and the DNA concentration was estimated using a NanoDrop ND-1000 instrument (Thermo Scientific, United Kingdom) at 260 nm. The ligation mixture was then transformed into chemically competent Escherichia coli BL21(DE3) pLysS cells (Promega, United Kingdom) as per the manufacturer’s instructions, and transformants were selected on Luria-Bertani (LB) agar plates (35 μg/ml chloramphenicol and 50 μg/ml ampicillin) at 37°C overnight. The plasmid diagram of the LipL32-pRSET C construct (Fig. 1) was generated using SECentral (Sci-Ed, USA).

LipL32 protein was expressed from the recombinant E. coli strain (500 ml) as per the manufacturer’s instruction (Invitrogen, United Kingdom), using a final concentration of 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce lipL32 expression. Cells were centrifuged (6000 × g) for 15 min at 4°C and resuspended in 10 ml lysis buffer (100 mM sodium phosphate, 6 M guanidine hydrochloride [pH 8]). Cells were lysed by 6-s pulses of probe sonication (amplitude 60) using a Vibra-Cell ultrasonic processor (Sonics and Materials, USA) for 6 min on ice and centrifuged (4000 × g) for 10 min at 4°C to remove cellular debris. The polyhistidine-tagged LipL32 protein was then purified from the retained supernatant using PureProteome nickel magnetic beads (Millipore, United Kingdom), a wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole [pH 8]), and an elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole [pH 8]) according to the manufacturer’s instructions. The eluted protein was washed once with 2 ml of 1 M phosphate-buffered saline (PBS) (pH 7.2) using a 5-kDa MWCO filter (Sartorius Stedim, France) and resuspended to a final volume of 200 μl in PBS. Total protein, lipopolysaccharide (LPS), and LipL32 concentrations of the purified recombinant LipL32 protein, and vaccine A were determined (Table 1) using the Bradford, Limulus amebocyte lysate (LAL), and multiple reaction monitoring (MRM) assays as previously described (17). The purified LipL32 protein (10 μg) was assessed on a 4 to 12% NuPAGE gel stained with Coomassie blue (Fig. 2) to evaluate protein elution from the nickel magnetic beads, which showed one band at the predicted size of mature length LipL32 (27.8 kDa). An enzyme-linked immunosorbent assay (ELISA) using a

<table>
<thead>
<tr>
<th>Substance</th>
<th>Protein (mg/ml)</th>
<th>LPS (µg/ml)</th>
<th>LipL32 (fmol/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine A</td>
<td>9.69 ± 0.40</td>
<td>0.01 ± 0.00</td>
<td>1.07 ± 0.22</td>
</tr>
<tr>
<td>LipL32</td>
<td>21.75 ± 0.49</td>
<td>0.47 ± 0.01</td>
<td>3,096.17 ± 1,449.91</td>
</tr>
</tbody>
</table>

FIG 1  Plasmid schematic diagram of pRSET C following insertion of the LipL32 gene. Promoter (T7), the polyhistidine tag (6×His), and primers used to confirm insertion are shown in blue. The KpnI and EcoRI restriction sites used to insert the LipL32 gene are also shown. The plasmid diagram of the LipL32-pRSET C construct was generated using SECentral (Sci-Ed, USA).

FIG 2 Purified LipL32 protein (lane 2; 10 μg) run on a 4 to 12% NuPAGE gel stained with Coomassie blue to detect protein. Lanes 1 and 3 contain a 3.5- to 260-kDa protein ladder.

[Table 1](#) Concentrations of LipL32 (N and C termini using MRM), total protein, and LPS in recombinant LipL32 protein and vaccine A

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LipL32-specific antibody was used to confirm the immunogenicity of the recombinant LipL32 protein prior to use in the hamster model (data not shown).

**Immunization of hamsters with test products.** Four groups of five female hamsters (120 g; Charles River, Germany) were vaccinated subcutaneously with either purified LipL32 protein (868 pmol N-terminal LipL32) (group 1), purified LipL32 protein (868 pmol N-terminal LipL32) with Imject alum adjuvant (0.25 ml; Thermo Scientific, USA) (group 2), vaccine A (diluted 1/40) (group 3), or 0.9% (wt/vol) physiological saline (group 4). All test products were prepared in 0.9% (wt/vol) physiological saline to a final volume of 0.5 ml. Two additional control groups of hamsters were also used to confirm that the challenge strain was appropriately virulent (group 5; n = 5) and to provide an unchallenged control (group 6; n = 3) for histological comparison.

Fifteen days following vaccination, groups 1 to 5 were challenged by intraperitoneal inoculation with 1 ml of virulent *Leptospira interrogans* serovar Canicola (1 × 10^8 cells/ml) strain Kito. The hamsters were routinely monitored, and their conditions were assessed using a clinical score sheet developed at the AHVLA (Table S1 in the supplemental material). Hamsters with a score of 3 or higher were judged to be in distress, likely to end in death, and were therefore humanely euthanized using halothane; all surviving hamsters were humanely euthanized on day 24 (which was 14 days after the fourth hamster in the negative control [group 5] succumbed to infection). All euthanized hamsters were observed for 5 min after halothane was administered to confirm cessation of life prior to performing any procedures.

Kidneys were excised from all hamsters at postmortem examination and dissected for assessment of infection. Half were retained for histological processing, and half were disrupted with a 10-ml syringe and cultured in EMJH medium. The presence of *Leptospira* was assessed in 10 fields of view using a dark-field microscope (×400 magnification).

**Histology.** Samples from liver, spleen, and kidney were collected from all hamsters at postmortem examination and fixed in 10% (vol/vol) buffered formalin. The tissue samples were routinely processed and embedded in paraffin wax using a Hypercenter XP tissue processor (Thermo Shandon, United Kingdom). Consecutive 4-μm-thick sections were cut using a Leica RM2025 (Leica, Germany) rotary microtome. Sections were stained with hematoxylin and eosin (H&E) for microscopic examination and with Warthin-Starry silver impregnation for the visualization of leptospires in the tissues (19). Renal lesions indicative of infection, if present, were graded according to their severity (0 as normal [0% coverage], 1 as minimal [≤10% coverage], 2 as mild [11 to 25% coverage], 3 as moderate [26 to 50% coverage], and 4 as severe [≥50% coverage]) (Fig. 3) using a semiquantitative scoring system modified from that of Palaniappan et al. (20); one slide, containing approximately 100 nephrons, was assessed per animal. The number of leptospires present on the various tissues were also

![FIG 3 Microphotographs of kidneys representative of the scoring system used to assess the extension and severity of histopathological changes in hamsters following infection with *L. interrogans* serovar Canicola. Infected animals displayed variable amounts of tubules containing eosinophilic protein casts (arrows) with different degrees of distension and attenuation, intratubular inflammatory infiltration (white arrows), and eosinophilic material in the urinary spaces of Bowman’s capsules (*`). (a) Control, normal structure of the cortex with the presence of a glomerulus (G) among the tubules, (b) score 1; (c) score 2; (d) score 3; (e) score 4. Hematoxylin and eosin (H&E). Scale bars, 100 μm and 50 μm (insets).](http://cvi.asm.org/figure/FIG_3)

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graded with 0 as absent, 1 as rare, 2 as few, 3 as numerous, and 4 as profuse. Slides were examined in a Leica DM4000B microscope (Leica, Germany). Pictures were taken using a Leica DFC480 digital camera and Leica Application Suite software. Adobe Photoshop Elements 4.0 (Adobe, USA) was used to adapt images for publication.

**Statistical analysis.** Where appropriate, the data are presented as means and standard deviations of the means. Comparisons of the severity of the renal lesions and the invasion of *Leptospira* in different tissues between hamster groups 1 to 6 were performed using Student’s t test; a *P* value of ≤0.05 was taken to be statistically significant. Kidney (~100 nephrons), liver (2 cm²), and spleen (1 cm²) tissues from each animal were examined. Comparisons of the survivals of hamster groups 1 to 6 were performed using Fisher’s exact test; a *P* value of ≤0.05 was taken to be statistically significant.

**RESULTS**

Assessment of the protective effect of recombinant LipL32 in the hamster vaccine batch potency test model. Following challenge with virulent *L. interrogans* serovar Canicola, groups 1 and 2 failed the vaccine potency test on days 10 and 12, respectively, with 4/5 hamsters either succumbing to infection or having to be euthanized. One hamster from each group survived until the end of the test (day 24); however, this increased survival was not statistically significant (Table 2). Group 3 (vaccine A) passed the vaccine potency test (day 24); however, this increased survival was not statistically significant. One hamster from each group survived until the end of the vaccine potency test on days 10 and 12, respectively, with 4/5 hamsters free of disease (*nd*).

Histopathological analysis of hamsters immunized with LipL32. Variable degrees of diffuse tubulointerstitial nephritis, consistent with *Leptospira* infection, were observed in the hamsters that succumbed to infection (or had to be euthanized) following challenge with *L. interrogans* serovar Canicola. The microscopic changes consisted of minimal infiltration of the interstitial spaces with lymphocytic cells and the frequent presence of strongly eosinophilic hyaline casts in the lumen of tubules, often associated with degeneration, necrosis, and attenuation of tubular epithelial cells. Occasional tubules also displayed a mixture of sloughed cells and leukocytes in their lumen. Lower scores for renal lesion severity were observed in groups 1, 3, and 6 (Table 3) than in groups 2, 4, and 5 (Table 3); treatment group 1, which comprised LipL32 without adjuvant, had a significantly lower score (*P* ≤ 0.01) than the negative control (group 4). Only one survivor from group 3 (euthanized on day 24) showed evidence of renal pathology as a minute focal lesion; no lesions were observed in group 6 (Table 3).

Leptospires, when present in the kidneys, could be observed in the interstitial spaces and tubular lumina in the renal cortex and occasionally in the lumen of blood vessels of the cortex or medulla, with no preference for any particular vascular structure. A significantly lower (*P* ≤ 0.01) score for *Leptospira* kidney invasion was observed (Table 3) in group 1 than in the negative control (group 4); no leptospires were observed in group 3 or 6.

In addition to microscopic analysis of hamster kidneys at postmortem examination, culturing was also performed to determine if *Leptospira* were still viable after infection. Leptospires were not observed in the kidney cultures of the animals euthanized at day 24 (groups 1 to 3 and 6), which is in agreement with the histological findings. No leptospires were observed by histological staining in the kidneys of a hamster from group 3 that died at day 17 (see Fig S2 in the supplemental material); however, confirmatory data could not be obtained for this animal using kidney culturing due to the detection of bacterial contamination during processing.

All hamsters that died (or had to be euthanized) following challenge with *Leptospira* displayed liver plate disarray, with loss of the normal hepatic sinusoid architecture, increased volumes of hepatocyte cytoplasm with eccentric nuclei and frequent multinucleation (Fig. 4), and multifocal infiltrations by lymphohistioctytic cells. A few random multifocal areas of necrotic hepatocytes were observed in two animals from group 4; no hepatic lesions were observed in the hamster from group 3 that died at

**TABLE 2** Treatments protocols applied to hamster groups 1 to 6 (*n* = 5) and numbers of survivors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of survivors/no. immunized</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LipL32 (no adjuvنت) + challenge</td>
<td>1/5</td>
</tr>
<tr>
<td>2</td>
<td>LipL32 + adjuvant + challenge</td>
<td>1/5</td>
</tr>
<tr>
<td>3</td>
<td>Vaccine A + challenge</td>
<td>4/5</td>
</tr>
<tr>
<td>4</td>
<td>Saline + challenge</td>
<td>0/5</td>
</tr>
<tr>
<td>5</td>
<td>No treatment + challenge</td>
<td>0/5</td>
</tr>
<tr>
<td>6</td>
<td>No treatment + no challenge</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*a* Group 6 comprised 3 hamsters. *P* values are 2-sided and were obtained through comparisons with the negative control (group 4) using Fisher’s exact test. *b* ND, not determined.

**TABLE 3** Severity of renal lesions and invasion of *Leptospira* in hamster tissues determined through staining with hematoxylin and eosin and Warthin-Starry stains, respectively

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Severity of renal lesion score Mean ± 1 SD</th>
<th><em>P</em> value</th>
<th>Invasion of <em>Leptospira</em> score in:</th>
<th>Kidney</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± 1 SD</td>
<td><em>P</em> value</td>
<td>Mean ± 1 SD</td>
<td><em>P</em> value</td>
<td>Mean ± 1 SD</td>
<td><em>P</em> value</td>
</tr>
<tr>
<td>1</td>
<td>1.8 ± 1.1</td>
<td>0.004</td>
<td>1.2 ± 0.8</td>
<td>0.009</td>
<td>2.4 ± 1.3</td>
<td>0.070</td>
</tr>
<tr>
<td>2</td>
<td>2.6 ± 1.5</td>
<td>0.374</td>
<td>2.0 ± 1.4</td>
<td>0.189</td>
<td>1.6 ± 1.8</td>
<td>0.034</td>
</tr>
<tr>
<td>3</td>
<td>0.6 ± 0.9</td>
<td>0.001</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>3.0 ± 0.7</td>
<td>ND</td>
<td>3.0 ± 0.0</td>
<td>ND</td>
<td>3.6 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>2.6 ± 0.5</td>
<td>0.178</td>
<td>2.8 ± 0.4</td>
<td>0.374</td>
<td>2.8 ± 1.6</td>
<td>0.242</td>
</tr>
<tr>
<td>6</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
<td>0.0 ± 0.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* Scores were obtained using a semiquantitative scoring system modified from Palaniappan et al. (20). Means and standard deviations of the means for the observed scores are shown. *P* values were obtained through comparison with the negative control (group 4) using a Student’s t test. *b* ND, not determined.
day 17. Groups 1 and 2 showed reduced liver invasion scores (2.4 ± 1.3 and 1.6 ± 1.8, respectively) (Table 3) compared to those for group 4 (3.6 ± 0.5); however, only group 2 showed a significant (P ≤ 0.05) difference. An example of the histopathological effects of *Leptospira* on hamster livers is shown in Fig. 4, where loss of the normal hepatic sinusoid architecture, an increased volume of hepatocyte cytoplasm with eccentric nuclei, and frequent multinucleation can be observed. The spleens of animals that succumbed to infection (or had to be euthanized) following challenge with *Leptospira* showed marked hypertrophy and hyperplasia of macrophages of splenic cords in red pulp in animals dying of the disease (Fig. 5). Very few leptospires could be observed in the red pulp of hamsters showing splenic pathology, and no significant difference in splenic invasion was observed between groups (Table 3).

**DISCUSSION**

The recombinant LipL32 generated in this study did not result in a statistically increased survival against challenge with *L. interrogans* serovar Canicola (Table 2). However, a decreased score of kidney invasion (P ≤ 0.01) was observed in the group treated with recombinant LipL32 (group 1) prior to challenge with *L. interrogans* serovar Canicola (Table 3), which corresponded to decreased scores of kidney lesions (P ≤ 0.01), indicating that while recombinant LipL32 was unable to provide complete protection, it was able to reduce the severity of infection in the hamsters.

Interestingly, group 2, which received aluminum hydroxide adjuvant in conjunction with LipL32, did not show significantly decreased kidney invasion or lesions compared with those for the negative control (group 4). Further work is required to elucidate the mechanism behind this finding; however, it may be suggested that aluminum hydroxide is either not suitable for presentation of a single protein against *L. interrogans* serovar Canicola or requires an alternate dosage to elicit an effective response. As the N-terminal concentration of LipL32 in group 1 was in excess (868 pmol) of that used in group 3 (641 fmol), the results may suggest that vaccine A either contains additional components required to initiate protective immunity or possesses an increased immunostimulatory effect.
LipL32 Reduces Kidney Invasion of L. interrogans Serovar Canicola

(either through the use of an adjuvant or other naturally occurring bacterial components). A recent study (21) demonstrated that LipL32 could provide protective immunity against L. interrogans serovar Copenhageni in hamsters when coadministered with the B subunit of E. coli heat-labile enterotoxin (LTB) as an adjuvant. It is conceivable, therefore, that the immunogenic effect of LipL32 against L. interrogans serovar Canicola will also be increased through use of LTB as an adjuvant. The initial results reported here support the need for larger studies using a range of LipL32 concentrations, in conjunction with a range of adjuvants, to fully elucidate the role of LipL32 in the vaccines. Further, it has been observed previously that adsorption with aluminum hydroxide can reduce antigen immunogenicity (22), which could explain the results seen in group 2; however, no known mechanism of action for the aluminum hydroxide interference is currently known.

It should also be noted that although aluminum hydroxide was assessed as a proxy for the adjuvant used in vaccine A, subsequent release of commercially sensitive data relating to the precise formulation of vaccine A confirmed that the adjuvants are EMA and NeoCryl. Clearly, in retrospect, an analysis of the precise formulation of vaccine A confirmed that the adjuvants are EMA and NeoCryl. Clearly, in retrospect, an analysis of the precise formulation of vaccine A confirmed that the adjuvants are EMA and NeoCryl. Clearly, in retrospect, an analysis of the precise formulation of vaccine A confirmed that the adjuvants are EMA and NeoCryl.

ACKNOWLEDGMENTS

We thank Mathieu Picardeau at the Pasteur Institute (Paris, France) for the donation of L. interrogans serovar Canicola strain Kito and Jarlath Nally at the University College of Dublin, Ireland, for the donation of an anti-LipL32 antibody. This work was supported by a grant (G0700633) from The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NCR3).

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