Analysis of Multiple Leptospira interrogans Serovar Canicola Vaccine Proteomes and Identification of LipL32 as a Biomarker for Potency

P. C. Humphries,¹ M. E. Weeks,¹ A. Gielbert,¹ G. Thomson,¹ and N. G. Coldham¹
Animal Health and Veterinary Laboratories Agency, Addlestone, New Haw, Surrey,¹ and UCL Institute of Child Health, London,¹ United Kingdom

The current batch potency test for Leptospira interrogans serovar Canicola vaccines requires the use of a large number of hamsters and has severe effects (i.e., hepatic and renal failure resulting in death); while this vaccine is effective, a safer, cheaper, more ethical replacement is desired. The aim of this study was to analyze vaccine proteomes and identify target molecules common to all L. interrogans serovar Canicola vaccines which could be used to design an in vitro potency test. Initial analysis of L. interrogans serovar Canicola vaccines (A to E) from different manufacturers, using the Limulus amebocyte lysate assay and silver-stained sodium dodecyl sulfate polyacrylamide gels, indicated that lipopolysaccharide was not present in all vaccines, preventing it from being a suitable target molecule. The protein contents of vaccines A to E were therefore determined by two-dimensional liquid chromatography mass spectrometry ([2D-LC/MS] 221 ± 31, 9 ± 8, 34 ± 4, 21 ± 5, and 34 ± 17 proteins [mean ± 1 standard deviation] found, respectively). The outer membrane protein LipL32 was established to be common to all and to be present at a significantly higher (P < 0.05) relative spectral abundance in a batch of vaccine which passed the in vivo potency test than in one which had failed. Further analysis using multiple reaction monitoring revealed that the concentration of the N terminus of LipL32 was significantly lower (P < 0.001) in failed batches (n = 2) of vaccine than in passed batches (n = 2); the concentration of the C terminus between the two batches was approximately the same. An in vitro Leptospira vaccine potency test, based on N-terminal amino acid quantification of LipL32, was subsequently developed.

Prevalent worldwide, Leptospira interrogans is a waterborne zoonotic pathogen capable of infecting virtually any mammal it comes into contact with (1). Primarily contracted through exposure to infected urine, infection causes symptoms that can be diverse, ranging from fever to hepatic failure, and that often result in death if left untreated (22). Other symptoms, such as cessation of milk production and miscarriage, are frequently observed in cattle (8), making leptospirosis particularly economically damaging to the farming community. Vaccination is widely used to protect against infection; however the vaccines currently available are serovar specific (21), and regular boosters are required to maintain immunity (20). Large volumes of vaccine are therefore produced every year to protect the domestic animal population. The majority of Leptospira vaccines available on the market are either bi- or multivalent and are derived from heat-inactivated cultures of the bacteria which have been grown (19) in medium containing a high level of Tween 80 and albumin (typically 1%, wt/vol).

Potency testing of Leptospira vaccines is required prior to new batches being released onto the market. The current test, as laid out by the European monograph status review (25), requires that for each serovar the vaccine provides protection against, 10 hamsters be challenged with a virulent strain of that serovar. Half of these receive the vaccine prior to challenge while the control group does not; the endpoint of this test is death, and euthanasia is not typically employed. Additionally, Leptospira has a predisposition to lose virulence during in vitro culture (39) and liquid nitrogen storage (28), due in part to alterations in the expression of lipopolysaccharide (LPS) and outer membrane proteins (15, 17, 26); it is therefore routine practice to maintain the virulence of strains by passage through hamsters (34).

An in vitro replacement for the hamster potency test has been sought for some time as the use of such a large number of animals, accrued through regular usage of the test and in vivo challenge strain maintenance, is ethically and financially undesirable. Enzyme-linked immunosorbent assay (ELISA)-based vaccine batch potency tests for some Leptospira serovars, such as Leptospira borgpetersenii serovar Hardjo, have been successfully developed and implemented (12); however similar attempts at the development of an in vitro potency test (10) for Leptospira interrogans serovar Canicola have had mixed success. In the United States ELISA potency tests for certain specific L. interrogans serovacanica vaccines are in usage (30); however, they require that adjuvants be removed prior to testing, which is impractical for widespread implementation due to the wide variety of adjuvants used by different manufacturers. It has been clearly established (13, 29), however, that both protein and LPS derived from Leptospira are capable of eliciting an immune response.

Prior studies (24, 31) have successfully described the proteome of L. interrogans. The proteins LipL32 (Hap-1), LipL41, LipL45, and Ompl1 have been identified as possible vaccine candidates (7, 9, 38) and, in some cases, used in in vivo trials (16, 33). A LipL32 DNA vaccine has been shown to confer protective immunity against L. interrogans serovar Canicola in the gerbil model (3); however, the protective effect of LipL32 against L. interrogans serovar Canicola in the hamster model has yet to be determined. The exact mechanism by which Leptospira vaccines provide protective immunity remains poorly understood, and to date no reliable in vitro method exists for their analysis or quantitation. The aim of

Received 16 November 2011 Returned for modification 17 January 2012 Accepted 1 February 2012 Published ahead of print 8 February 2012 Address correspondence to N. G. Coldham, nick.coldham@ahlva.gsi.gov.uk. Supplemental material for this article may be found at http://cvi.asm.org/.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/CVI.05622-11
the present study was to identify biomarkers present in *L. interrogans* serovar Canicola vaccines that could be used as the basis for the development of an *in vivo* vaccine batch potency test. The protein and LPS content of *L. interrogans* serovar Canicola vaccines were chosen for analysis due to their known immunogenic effects.

**MATERIALS AND METHODS**

**Vaccine selection and preparation.** Bivalent vaccines (giving protection against *L. interrogans* serovars Canicola and Icterohaemorrhagiae) which had passed the *in vivo* vaccine batch potency test and been released for commercial sale were purchased from five different manufacturers for analysis. Vaccine C was of subunit manufacture comprising outer membrane proteins, whereas vaccines A, B, D, and E were derived from heat-inactivated bacteria. Test batches of vaccine C (*n* = 4) which had not been subjected to the vaccine potency test were also obtained from the manufacturer; test batches were available only for vaccine C.

The vaccines (1 ml) were concentrated using 5-kDa molecular-weight-cutoff concentrators (Sartorius Stedim, France), washed once with 0.5 ml of 2.5 mM ammonium bicarbonate (pH 8.0), and concentrated again to a final volume of 0.5 ml (the protein content of vaccine B was too low to process in this manner, so it was therefore analyzed without washing). The protein concentration in the washed vaccines was then determined using the Bradford method (Sigma-Aldrich, United Kingdom); five control hamsters were kept unvaccinated. Fifteen days following 0.5 ml of virulent saline); five control hamsters were kept unvaccinated. Fifteen days following 1 ml of vaccine (diluted 1/40 with 0.9% [wt/vol] physiological saline); five control hamsters were kept unvaccinated. Fifteen days following 1 ml of vaccine (diluted 1/40 with 0.9% [wt/vol] physiological saline); five control hamsters were kept unvaccinated. Fifteen days following 1 ml of vaccine (diluted 1/40 with 0.9% [wt/vol] physiological saline); five control hamsters were kept unvaccinated.

**Vaccine batch potency test.** The test batches (*n* = 4) of vaccine C were assessed for potency according to the guidelines laid out in the most recent European monograph (25). For each batch analyzed, five female hamsters (weight, ≤120 g) (Charles River, Germany) were inoculated subcutaneously with 0.5 ml of vaccine (diluted 1/40 with 9.9% [wt/vol] physiological saline); five control hamsters were kept unvaccinated. Fifteen days following vaccination all the hamsters were challenged by intraperitoneal inoculation with a 1 ml of virulent *L. interrogans* serovar Canicola (−1 × 10⁸ cells/ml); for the test to be valid at least four of the five unvaccinated controls had to die within 14 days of infection. Vaccine batches were deemed potent if at least four of the five vaccinated hamsters survived for 14 days longer than the unvaccinated controls. All animal procedures used in this study were covered under the Animals (Scientific Procedures) Act 1986 by Home Office Project License number PPL 70/7249 and were approved by the Animal Ethics Committee at the Animal Health and Veterinary Laboratories Agency (AHVLA) where this work was performed.

**Proteome analysis by two-dimensional liquid chromatography mass spectrometry (2D-LC/MS).** Three replicates of each vaccine, normalized by dilution in 2.5 mM ammonium bicarbonate (pH 8.0) to 100 μg of protein, were heat denatured at 95°C for 5 min and then digested overnight with 2 μg of sequencing grade trypsin (Promega) (5). Digestion was terminated by the addition of 1 μl of 25.2 M formic acid (Fluka). Due to the low concentration of protein found in vaccine B (see Fig. 2), only 6 μg was used for each replicate of that vaccine.

Tryptic digestes were centrifuged (5,000 × *g* for 1 min) to remove particulates, and 50 μl of supernatant was taken for strong cation exchange (SCX) fractionation by high-pressure liquid chromatography (HPLC). Tryptic peptides were chromatographed using a Hewlett-Packard 1100 system on a Biobasic SCX HPLC (2.1 by 100 mm) column (Thermo Scientific). Peptide sequence Terminus Molecular mass (Da) Precursor ion for MRM (m/z) Product ion for MRM (m/z) Retention time (min)

| Peptide sequence | Terminus | Molecular mass (Da) | Precursor ion for MRM (m/z) | Product ion for MRM (m/z) | Retention time (min) |
|------------------|----------|---------------------|----------------------------|--------------------------|----------------------|
| SSFVLSEDTIPGNETVK | N        | 1924.07             | 962.50                     | 845.40                   | 13.16                |
| ISFTTKPKGVEK      | C        | 1369.56             | 457.30                     | 628.80                   | 10.80                |

The full amino acid sequence of LipL32 and the locations of the tryptic peptides used for quantitation (underlined) are as follows: MKKLSILAISVALFASITACGAFGGLPSLK

**TABLE 1** Tryptic peptides and product ions selected for detection and quantification of LipL32 using LC-MRM

The search engine Spectrum Mill (Agilent, United Kingdom) was chosen for analysis due to its known immunogenic effects. **TABLE 1** Tryptic peptides and product ions selected for detection and quantification of LipL32 using LC-MRM

The search engine Spectrum Mill (Agilent, United Kingdom) was chosen for analysis due to its known immunogenic effects.

**Protein identification.** The search engine Spectrum Mill (Agilent, United Kingdom) was used to extract MS/MS data from MassHunter acquisition files, and proteins were subsequently identified by comparison of tryptic peptide product ion mass spectra against those generated *in silico* from a protein database. Search parameters included selection of trypsin as the proteolytic enzyme with up to two missed cleavage sites and a variable modification for oxidation of methionine residues; precursor and product mass tolerances were set to 20 and 50 ppm, respectively. Identified protein lists (and associated information) with a Spectrum Mill protein score higher than 11 were exported as tab-separated files for bioinformatics analysis; proteins identified had at least two distinct tryptic peptides and were present in all three technical replicates. To date the com...
complete genome of *L. interrogans* serovar Canicola has not been sequenced; therefore, a custom-made database derived from chromosomes I and II of *L. interrogans* serovar Copenhageni (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/Leptospira_interrogans_serovar_Copenhageni; accessed on 6 December 2010) was used for database interrogation.

The relative abundance of proteins present in all three technical repeats was determined through spectral counting (40). The normalized spectrum abundance factor (NSAF), calculated as described by Zybailov et al. (41), was used to account for differences in peptide length, allowing for comparison of protein abundance among individual vaccines.

**Quantitation of LipL32 by LC-MS/MS.** Synthetic peptide analogues, corresponding to N- and C-terminal tryptic peptides found in LipL32, were obtained at a purity of 98% (Peptides Synthetics, United Kingdom) for detection and quantification using multiple reaction monitoring (MRM) (Table 1 and Fig. 1). Passed (*n* = 2) and failed (*n* = 2) batches of vaccine C (PH1 to PH4) (Table 2) were prepared, quantitated, and digested as described above. SCX separation was also performed as described above; however, the 15 fractions of each replicate (containing 50 μg of total protein equivalents) were recombined prior to being taken to dryness, dissolved in 0.1% (vol/vol) formic acid (50 μl), and analyzed on an Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies) with an HPLC chip cube source. The chromatography chip consisted of a 160-nl enrichment column (Zorbax 300 SB C18; 5-μm particle size) and a 75-μm by 150-mm analytical column (Zorbax 300 SB C18; 5-μm particle size) driven by the Agilent Technologies 1200 series nano/capillary liquid chromatography system. Both systems were controlled by MassHunter Workstation Data Acquisition for Triple Quadrupole (version B.02.01; Agilent Technologies). Tryptic peptides (1 μl) were loaded onto the enrichment column of the chip and washed with eight column volumes of 0.1% TFA. Peptides were then separated on the analytical column using an acetonitrile gradient (4.5% to 90% [vol/vol]) over 25 min and eluted directly into the mass spectrometer. The mass spectrometer was run in positive ion mode, with the electrospray voltage set to 1900 V and gas temperature and pressure set at 300°C and 4 liters/min, respectively. Optimal transitions and conditions for the peptides of interest (Table 1 and Fig. 1) were obtained using the MS and MS/MS data from previous Q-TOF analysis of vaccine C. The acquired data were quantified using the Agilent MassHunter Quantitative Analysis software (version B.03.01; Agilent Technologies).

**Analysis of total LPS content in vaccines by PAGE.** The vaccine(s) LPS content was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the Xcell Surelock system (Invitrogen) using a 4 to 12% gradient NuPAGE gel run at 150 W for 80 min. Gels contained a 3.5-kDa protein ladder (Invitrogen), two concentrations of LPS (10 μg and 500 ng, *Escherichia coli* derived; Sigma, United Kingdom) and aliquots (20 μg) of untreated vaccine (A, C, D, and E); vaccine B was loaded in a smaller amount (2.5 μg) due to its lower concentration of protein (Fig. 2). LPS was detected in the gel using the silver staining method described by Tsai et al. (37).

**Statistical analysis.** All results are presented as means of the standard deviations of the mean (except proteomics data). Comparison of the concentration and relative abundance of proteins between vaccines was performed using a Student’s *t* test; a *P* value of *<*0.05 was taken to be statistically significant.

### RESULTS

**Evaluation of vaccine LPS content.** The LPS content of the five vaccines (A to E) was evaluated by PAGE using an LPS-specific silver stain (Fig. 3). This staining method works through interaction with the polysaccharide part of the molecule (11) and showed that vaccine C did not contain any detectable LPS. For confirmation, the LAL assay (Fig. 2), which functions by interaction with the lipid A (36) and core region (6) of LPS, was also performed on vaccines A to E. The LAL assay did not detect LPS in vaccine C,
suggesting that it is either absent or present at levels below the detection limits of these assays.

**Vaccine proteome characterization.** Analysis of vaccines A to E revealed that the total number of proteins in each varied considerably (see Table S1 in the supplemental material). However, two proteins, LipL32 and flagellin, were found to be common to the five vaccines analyzed (Table 3); due to the low number of proteins identified in vaccine B and their correspondingly low coverage, a separate comparison of vaccines A and C to E was also performed, which identified two additional conserved proteins (Table 3, shaded area). Two batches of vaccine C were available to establish whether LipL32 or flagellin protein could be used as a biomarker for vaccine potency; one had passed the in vivo potency test, batch PH1 (Table 2), while the other, batch PH3, had failed (Table 2). Both were subsequently analyzed by 2D-LC/MS and quantitated using the NSAF (Table 4; see also Table S2 in the supplemental material).

The average number of proteins detected in the three replicates of the failed batch was lower than that found in the passed batch (Table 4); however, the total numbers of conserved proteins (i.e., proteins detected in all three replicates) found in the passed and failed batches (see Table S2) were approximately the same (22 and 18 proteins, respectively). Based on the NSAF, LipL32 and histidine sensor kinase protein were shown to be

![FIG 3](image3.png) Vaccines A to E (lanes 2, 4, 5, and 6 contain 20 μg; lane 3 contains 2.5 μg) were run on a 4 to 12% NuPAGE gel stained with silver to detect LPS. Lanes 1 and 9 contain a 3.5-kDa protein ladder, and lanes 7 and 8 contain 10 μg and 500 ng, respectively, of *Escherichia coli* LPS as a positive control.

![FIG 2](image2.png) Concentrations of protein and LPS in vaccines A to E (as sold) as determined by the Bradford and LAL assays, respectively. Two replicates of each vaccine were analyzed for each assay; mean and standard deviation of the mean are shown.

**TABLE 3** Conserved proteins present in commercially available vaccines from five different manufacturers

| Accession no. | Protein identification | Protein abundance by vaccine<sup>a</sup> | % Coverage | No. of peptides | NSAF | % Coverage | No. of peptides | NSAF | % Coverage | No. of peptides | NSAF | % Coverage | No. of peptides | NSAF |
|---------------|-----------------------|-------------------------------------------|------------|----------------|------|------------|----------------|------|------------|----------------|------|------------|----------------|------|
| 450577230     | LipL32                | 4.11                                      | 27.00      | 12.00          | 6.00 | 20.00      | 4.00           | 3.33 | ND         | ND             | 2.33 | ND         | ND             | 2.33 |
| 450575053     | Flagellin             | 3.41                                      | 31.00      | 25.00          | 5.00 | 30.00      | 5.00           | 2.33 | ND         | ND             | 2.00 | ND         | ND             | 2.00 |
| 963593991     | LipL41                | 3.35                                      | 33.33      | 33.33          | ND   | ND         | ND             | 2.33 | ND         | ND             | 2.00 | ND         | ND             | 2.00 |
| 15960111      | LIC10411              | 3.25                                      | 35.00      | 35.00          | ND   | ND         | ND             | 2.00 | ND         | ND             | 1.00 | ND         | ND             | 1.00 |

<sup>a</sup> Values are means. NSAF, ln relative abundance (higher values indicate greater abundance); ND, protein not detected. The total numbers of proteins per vaccine group (mean ± standard deviation) are as follows: A, 221 ± 31; B, 98 ± 17; C, 344 ± 4; D, 215 ± 5; E, 341 ± 17. Shaded areas represent additional conserved proteins identified when vaccine B is excluded from comparison.

<sup>b</sup> Hypothetical protein.
present at a higher abundance ($P \leq 0.05$) in the batch of vaccine which had passed the in vivo potency test than in the failed batch; since LipL32 was also known to be highly conserved in vaccines A to E, it was chosen for further analysis.

Spectrum Mill was subsequently used to identify LipL32 tryptic peptides which were present in each batch (Table 5). The number of LipL32 vaccine peptides identified was significantly lower than that predicted by in silico digestion; this can be attributed to a variety of factors including alterations to the protein during vaccine manufacture, peptide loss during chromatographic fractionation (which is consistent with 2D-LC/MS experiments), and the fact that not all tryptic peptides can be detected using a mass spectrometer (32). However, both batches were analyzed in parallel, and so any losses would be consistent between the two.

**Quantitation of LipL32 in passed and failed batches of vaccine.** To enable accurate determination of the concentration of LipL32 in the vaccines, two tryptic peptides from Table 5, corresponding to the N and C termini of the protein, were selected for quantitation using multiple reaction monitoring (MRM). The limits of detection (LOD) of the N and C terminus peptides were determined (2) as 0.025 and 0.018 fmol/µg, respectively, using the empirical method. Whereas the limits of quantification (LOQ; defined as the lowest concentration of the standard with a coefficient of variation lower than 20% [2]), were 0.02 and 0.05 fmol/µg for the N and C terminus peptides. Calibration curves for both peptides were constructed using MassHunter software (Agilent, United Kingdom) over the tested concentration range (0.01 to 100 fmol/µg) allowing determination of sample concentration (Table 6) by interpolation. The concentration of the N terminus region was significantly lower ($P \leq 0.01$) in failed batches (Table 2, PH3 and PH4) of vaccine (Table 6) than in passed batches (Table 2, PH1 and PH2); no statistical difference was observed in the concentration of the C terminus regions. The C-terminal peptide ISFTTYKPGEVK was not detected in the passed batch (PH1) of vaccine C during Q-TOF analysis (Table 5); however, subsequent targeted MRM analysis revealed it to be present (Table 6) as this method is more sensitive than Q-TOF analysis.

**DISCUSSION**

*Leptospira*-derived LPS has been shown to be highly immunogenic (29), and it is has been suggested that mutation of LPS can elicit cross-protection against multiple serovars (35). However, it was not detected in vaccine C, suggesting that at least in this vaccine it may not be necessary to induce protective immunity against *Leptospira*; further work is required to elucidate its function in the other vaccines analyzed. An alternative biomarker candidate, which is conserved across all the vaccines, was sought by comparison of the proteomes of the five vaccines under investigation. Protein content was assessed initially by the Bradford assay, which revealed that the concentration of protein in vaccine B was much lower than that found in the other vaccines. A smaller quantity of vaccine B was therefore analyzed using 2D-LC/MS which could account for the lower number of proteins identified (Table 3).

| Accession no. | Protein identification                     | NSAF | % coverage | No. of peptides | NSAF | % coverage | No. of peptides | P value |
|---------------|-------------------------------------------|------|------------|----------------|------|------------|----------------|--------|
| 45657309      | Histidine kinase sensor                    | –4.92| 1.00       | 2.00           | –4.57| 1.00       | 2.00           | 9.23E−03|
| 45657230      | LipL32                                    | –2.83| 21.33      | 9.00           | –3.07| 16.33      | 5.00           | 3.20E−02|
| 45658246      | Isocellul-rrNA synthetase                  | –4.46| 2.00       | 6.00           | –3.89| 2.00       | 7.67           | 8.58E−02|
| 45658988      | Hypothetical protein LIC13166             | –3.54| 15.33      | 5.00           | –3.70| 10.00      | 3.00           | 9.39E−02|
| 45656611      | Putative lipoprotein                      | –3.94| 9.33       | 5.00           | –3.63| 9.00       | 4.67           | 1.05E−01|
| 45657748      | Putative lipoprotein                      | –3.78| 16.00      | 5.67           | –4.27| 5.00       | 2.33           | 1.70E−01|
| 45658793      | LipL41                                    | –2.65| 32.33      | 14.33          | –2.44| 31.33      | 12.33          | 1.90E−01|
| 45656945      | Cell division protein                     | –5.68| 0.33       | 2.00           | –5.24| 0.00       | 2.00           | 2.04E−01|
| 45657213      | Chaperonin GroEL                          | –3.22| 22.00      | 12.33          | –3.35| 15.33      | 7.67           | 3.63E−01|
| 45655648      | Acyl carrier protein                      | –2.71| 16.33      | 3.00           | –2.36| 20.00      | 3.00           | 3.65E−01|
| 45657869      | ATP-dependent protease                    | –4.58| 1.00       | 5.00           | –4.73| 1.00       | 3.00           | 3.83E−01|
| 45657753      | Flagelin                                  | –3.79| 9.33       | 3.67           | –4.12| 6.67       | 2.00           | 4.66E−01|
| 45656175      | Cell wall hydrolase                       | –1.97| 3.00       | 17.33          | –1.88| 3.00       | 13.67          | 5.42E−01|
| 45657078      | Putative citrate lyase                    | –3.95| 18.67      | 3.67           | –3.91| 15.33      | 2.67           | 8.55E−01|

*Values are means. NSAF, in relative abundance (higher values indicate greater abundance). The mean total numbers of proteins (± 1 standard deviation) were 54 ± 8 in passed batches and 42 ± 4 in failed batches.*

April 2012 Volume 19 Number 4 cvl.asm.org 591

Downloaded from http://cvi.asm.org on July 20, 2018 by guest
TABLE 5 Comparison of tryptic peptides detected in two batches of vaccine C against an in silico digest of LipL32

| In silico digest | Presence of peptide in: | Passed vaccine | Failed vaccine |
|------------------|-------------------------|----------------|----------------|
| (K)AYLYLVWPAVIAEMGVR(M) | - | - |
| (K)IPNPPK(S) | - | - |
| (K)LDGDGDDDTYK(E) | - | - |
| (K)SMHPWFDTWIR(V) | + | + |
| (K)SSFVLSEDITPGNETV(T) | + | - |
| (R)IKIPNPPK(S) | - | - |
| (R)ISFTTTYKPGVEK(G) | - | - |
| (R)ISPTGEIGEPGDGLVSDAFK(A) | - | - |
| (K)MSAIMPDQIAK(A) | + | + |
| (K)AAKAPVQK(L) | - | - |
| (K)ATPEEKSMHPWFDTWIR(V) | - | - |
| (K)KPVQKLDDGDDDTYK(E) | - | - |
| (K)EEHHNK(Y) | - | - |
| (K)GFVASVGLFPFPGVSPLIHSNPEELK(Q) | - | - |
| (K)IPNPPKSFDDLK(N) | - | - |
| (K)KAYLYLVWPAVIAEMGVR(M) | - | - |
| (K)KLSIALVAFAITACGEFGLPSLK(S) | - | - |
| (K)LDGDGDDDTYK(E) | - | - |
| (K)LLVRGLYR(I) | - | - |
| (K)KLSIALVAFAITACGEFGLPSLK(S) | - | - |
| (K)QIAAASELKK(K) | - | - |
| (K)QIAAASELKK(K) | - | - |
| (K)SFDDLKNDIKTR(K) | - | - |
| (K)SMHPWFDTWIRVER(M) | - | - |
| (K)TLLPGVISYGGYVKPQAPDGDLGNK(K) | - | - |
| (K)TLLPGVISYGGYVKPQAPDGDLGNK(K) | - | - |
| (K)YNSTRIK(I) | - | - |
| (R)GRISFTTTYKPGVEK(G) | - | - |
| (R)HKYNSTRIK(K) | - | - |
| (R)ISPTGEIGEPGDGLVSDAFKAAKK(E) | - | - |
| (R)MSAIMPDQIAK(A) | - | - |
| (R)VERMSAIMPDQIAK(A) | - | - |

In silico digest | Passed vaccine | Failed vaccine |
|----------------|----------------|----------------|

Mean % coverage 21 16

*The presence (+) or absence (−) of the tryptic peptide is indicated.

TABLE 6 Concentrations of the N- and C-terminal tryptic peptides of LipL32 in passed and failed vaccines

| Region of protein | Passed (n = 2) | Failed (n = 2) | Fold difference | P value |
|-------------------|---------------|---------------|----------------|---------|
| N terminus        | 1.28 ± 0.68   | 0.08 ± 0.03   | 15.87          | 0.007   |
| C terminus        | 0.88 ± 0.29   | 0.79 ± 0.23   | 1.11           | 0.586   |

* Three replicates of each vaccine were analyzed.

In conclusion, this study demonstrates 2D-LC/MS to be an effective tool for vaccine analysis which could be utilized in the development of in vitro potency tests for a number of different bacterial vaccines (tests for other serovars of Leptospira in particular); 2D-LC/MS-based techniques could also be developed for the quality assurance stage of vaccine manufacture. This study has also shown that MRM-based N-terminal amino acid quantitation of LipL32 has the potential to act as a novel assay target for the determination of potency in vitro. Further validation will be required, with access to a larger sample cohort of passed and failed batches from multiple manufacturers, to determine if it can replace the hamster challenge batch potency test.

ACKNOWLEDGMENTS

This work was supported by a grant from The National Centre for the Replacement, Refinement and Reduction of Animals in Research.

We thank William Newell for his assistance with the NSAF calculations.

REFERENCES

1. Adler B, de la Pena Moctezuma A. 2010. Leptospira and leptospirosis. Vet. Microbiol. 140:287–296.
2. Armbruster DA, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29(Suppl 1):S49–S52.
3. Branger C, et al. 2005. Protection against Leptospira interrogans sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect. Immun. 73:4062–4069.
4. Branger C, et al. 2001. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of Leptospira interrogans by adenovirus-mediated vaccination. Infect. Immun. 69:6831–6838.
5. Coldham NG, Woodward MJ. 2004. Characterization of the Salmonella typhimurium proteome by semi-automated two dimensional HPLC-mass spectrometry: detection of proteins implicated in multiple antibiotic resistance. J. Proteome Res. 3:595–603.
6. Conrad ML, Pardy RL, Armstrong PB. 2001. Response of the blood cell of the American horseshoe crab, Limulus polyphemus, to a lipopolysaccharide-like molecule from the green alga Chlorella. Biol. Bull. 201:246–247.
7. Cullen PA, et al. 2005. Surfaceome of Leptospira spp. Infect. Immun. 73:4853–4863.
8. Department for Environment, Food, and Rural Affairs. 2008. Zoonoses report: United Kingdom 2008. Department for Environment, Food, and Rural Affairs, London, United Kingdom. http://archive.defra.gov.uk.
9. Dong H, et al. 2008. Characterization of the ompL1 gene of pathogenic Leptospira species in China and cross-immunogenicity of the OmpL1 protein. BMC Microbiol. 8:223.

in passed vaccines than in failed vaccines, indicating that it could make a good target molecule candidate for an in vitro potency test. To investigate this further, multiple reaction monitoring (MRM) was used to accurately quantitate (27) the concentration of LipL32 in passed (PH1 and PH2) and failed (PH3 and PH4) batches (Table 6) of vaccine C. MRM quantitates proteins based on specific peptide sequences; to ensure good coverage of LipL32, two peptide sequences, corresponding to the start (N terminus) and end (C terminus) of the protein, were selected for quantitation. The concentration of the C terminus peptides was approximately the same across both conditions (0.88 and 0.79 fmol/μg in the passed and failed batches, respectively) (Table 6); however the concentration of the N terminus peptide was substantially (P ≤ 0.01) lower in failed batches. This suggests that LipL32 is somehow altered in failed batches of vaccine, which would result in fewer spectra being detected during Q-TOF analysis, accounting for the lower abundance observed through spectral counting. It is proposed that this N-terminal region of LipL32 may be used as an effective biomarker for quantifying Leptospira vaccine potency in vitro. Its effectiveness as an epitope for protective efficacy has not been determined; however, it is plausible that this truncation to the primary full-length structure of LipL32 in failed batches could alter its conformational structure, which could conceivably be the cause of its inability to convey protective immunity; to determine this, further work using X-ray crystallography of the LipL32 found in passed and failed batches will be required.
10. Ebert E. 1999. Guinea pig serology as an alternative to the hamster challenge test for potency testing of *Leptospira* hardjo vaccines, p 102–109. In Proceedings of the 1999 International Conference on Alternatives to Animal Challenge Tests in the Batch Control of *Leptospira* Vaccines for Veterinary Use. European Directorate for the Quality of Medicines and Healthcare, Strasbourg, France.

11. Fomsgaard A, Freudenberg MA, Galanos C. 1990. Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. J. Clin. Microbiol. 28:2627–2631.

12. Goddard RD, Hopkins IG, Thornton DH. 1986. The development of a potency test for *Leptospira* hardjo vaccines: a comparison of protection in calves and serology in guinea-pigs. J. Biol. Stand. 14:337–344.

13. Guerreiro H, et al. 2001. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect. Immun. 69:4958–4968.

14. Haake DA, et al. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68:2276–2285.

15. Haake DA, et al. 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect. Immun. 66:1579–1587.

16. Haake DA, et al. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. 67:6572–6582.

17. Haake DA, et al. 1991. Changes in the surface of *Leptospira interrogans* serovar grippotyphosa during in vitro cultivation. Infect. Immun. 59:1131–1140.

18. Hauk P, et al. 2008. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. Infect. Immun. 76:2642–2650.

19. Johnson RC, Harris VG. 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. 94:27–31.

20. Klaasen HL, Molkenboer MJ, Vrijenhoek MP, Kaashoek MJ. 2003. Duration of immunity in dogs vaccinated against leptospirosis with a bivalent inactivated vaccine. Vet. Microbiol. 95:121–132.

21. Koizumi N, Watanabe H. 2005. Leptospirosis vaccines: past, present, and future. J. Postgrad. Med. 51:210–214.

22. Levet PN. 2001. Leptospirosis. Clin. Microbiol. Rev. 14:296–326.

23. Lucas DS, et al. 2011. Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. Vaccine 29:3413–3418.

24. Malmstrom J, et al. 2009. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature 460:762–765.

25. Marbehant P. 1999. Leptospiral vaccines batch potency testing. European monograph status review, p 11–16. In Proceedings of the 1999 International Conference on Alternatives to Animal Challenge Tests in the Batch Control of *Leptospira* Vaccines for Veterinary Use. European Directorate for the Quality of Medicines and Healthcare, Strasbourg, France.

26. Matsunaga J, et al. 2002. Novel 45-kilodalton leptospiral protein that is processed to a 31-kilodalton growth-phase-regulated peripheral membrane protein. Infect. Immun. 70:323–334.

27. Ong SE, Mann M. 2005. Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1:252–262.

28. Reed NE, Varney WC, Goddard RD, Wyeth PJ. 2000. The maintenance of challenge strains used in the potency test for canine leptospirosis vaccines. Biologicals 28:25–28.

29. Ruby KW. 1999. Development of in vitro assays for measuring relative potencies of leptospirosis bacteria containing serovars Pomona, Canicola, Grippotyphosa and Icterohaemorrhagiae. p 35–45. In Proceedings of the 1999 International Conference on Alternatives to Animal Challenge Tests in the Batch Control of *Leptospira* Vaccines for Veterinary Use. European Directorate for the Quality of Medicines and Healthcare, Strasbourg, France.

30. Ruby KW, Walden DM, Wannemuehler MJ. 1996. Development of an in vitro assay for measuring the relative potency of leptospirosis bacteria containing serovar Canicola and its correlation to the hamster potency assay. Dev. Biol. Stand. 86:341.

31. Sakolvaree Y, et al. 2007. Proteome and immunome of pathogenic *Leptospira* spp. revealed by 2DE and 2DE-immunoblotting with immune serum. Asian Pac. J. Allergy Immunol. 25:53–73.

32. Sanders WS, Bridges SM, McCarthy FM, Nanduri B, Burgess SC. 2007. Prediction of peptides observable by mass spectrometry applied at the experimental set level. BMC Bioinformatics (Suppl 7):S23.

33. Seixas FK, et al. 2007. Evaluation of different ways of presenting LipL32 to the immune system with the aim of developing a recombinant vaccine against leptospirosis. Can. J. Microbiol. 53:472–479.

34. Silva EF, et al. 2008. Characterization of virulence of *Leptospira* isolates in a hamster model. Vaccine 26:3892–3896.

35. Srikram A, et al. 2011. Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. J. Infect. Dis. 203:870–879.

36. Takayama K, et al. 1984. Influence of fine structure of lipid A on *Limulus* amebocyte lysate clotting and toxic activities. Infect. Immun. 45:350–355.

37. Tsai CM, Frasch CE. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119:115–119.

38. Wang Z, Jin L, Wegrzyn A. 2007. Leptospirosis vaccines. Microb. Cell Fact. 6:39.

39. Woodward MJ. 2001. *Leptospira*, p 2137–2158. In Sussman M (ed), Molecular medical microbiology. Academic Press, New York, NY.

40. Zybaivol B, Coleman MK, Flores L, Washburn MP. 2005. Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal. Chem. 77:6218–6224.

41. Zybaivol B, et al. 2006. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. J. Proteome Res. 5:2339–2347.