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Evaluation of 238 antigens of *Leptospira borgpetersenii* serovar Hardjo for protection against kidney colonisation

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ABSTRACT

Leptospirosis is a zoonotic disease affecting animals and humans worldwide. Leptospiral infection in cattle can cause reproductive failure and reduced weight gain, and importantly, infection represents a significant disease risk for farmers. Current bacterin vaccines offer protection that is short-lived and restricted at best to related serovars. The development of protective vaccines that stimulate immunity across multiple leptospiral serovars would therefore be advantageous. This study used a reverse vaccinology approach to evaluate a set of *Leptospira borgpetersenii* proteins in the hamster infection model. The *L. borgpetersenii* serovar Hardjo strain L550 genome sequence was analysed and genes encoding 262 predicted outer membrane or secreted proteins were selected. From this list, 238 proteins or protein fragments were successfully expressed and purified; 28 proteins (12%) were soluble, while the remaining 210 proteins (88%) were insoluble and purified under denaturing conditions. Proteins were mixed into 48 pools of up to five each and tested for protection against infection as assessed by renal colonisation in the hamster model of infection. None of the pools of antigens protected the hamsters against infection, despite a detectable antibody response being mounted against the majority of proteins (71%). This study is the first large scale evaluation of individual leptospiral proteins for ability to induce a protective immune response in the hamster infection model. It thus constitutes an important reference of protein immunogenicity and non-protective antigens that should be consulted before embarking on any future subunit vaccine experiments.

1. Introduction

Leptospirosis in animals and humans is caused by spirochaetes of the genus *Leptospira*. Remarkably, leptospirosis is the most widespread zoonosis, yet remains grossly under-recognised. It is estimated that there are more than half a million cases of severe human leptospirosis annually with 100,000 deaths [1]. Pathogenic leptospires are maintained in reservoirs of wild and domestic carrier hosts such as rats, cattle, pigs, and dogs, where they colonise the kidneys and are dispersed into the environment *via* urine. Once outside the host, leptospires may persist in soil and water. Transmission to a new host can occur through contact with environmental sources of the bacteria or direct contact with animals shedding the organism [2].

In humans, leptospirosis can range from an asymptomatic infection to severe disease resulting in multiple organ failure and death. Fatality rates in untreated cases may reach 15% [3], and in the most severe form (pulmonary haemorrhagic syndrome) fatality rates are greater than 50% [4]. Leptospirosis is also a major cause of economic loss in the production of livestock. Disease in cattle is caused mainly by serovar Hardjo where it may account for more than 40% of MAT positive sera [5], but is also caused by other leptospiral serovars [6]. Moreover, even in the absence of disease, the ability of domestic livestock to act as reservoirs for pathogenic *Leptospira* represents a significant health risk to a wide range of workers in the industry, in particular farmers, veterinarians and abattoir workers [2].

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Currently only bacterin (killed whole-cell) vaccines are available for leptospirosis. Bacterins have a number of shortfalls, including possible failure to prevent disease transmission [7,8], reactogenicity, and conferral of only short term immunity [2]. Moreover, bacterins elicit only a narrow range of serovar protection. There are more than 250 serovars of pathogenic *Leptospira*, necessitating knowledge of local serovar prevalence for vaccine formulation protocols. Hence there is potential for the development of improved vaccines with broader specificity.

Subunit vaccines have the potential to overcome some of the deficiencies of bacterins, including the stimulation of cross-protection. There is compelling evidence that protective protein antigens exist, making a subunit vaccine feasible; in some documented cases animals that survived infection with one serovar of *Leptospira* were protected against re-infection with leptospires from a different serogroup [9,10]. More recently, cross-protection against acute infection was observed after vaccinating hamsters with an attenuated, live vaccine [11]; in the absence of a response to the heterologous LPS, protection mediated by proteins was hypothesised. There has been progress towards the identification of antigens that might be useful in a subunit leptospiral vaccine. Outer membrane proteins LipL32, LipL41 combined with OmpL1, and LigA have shown potential as protective antigens [12–14]. However, not all experimental animals were protected in the reported studies [2], and LigA is absent in some pathogenic serovars of *Leptospira interrogans* [15,16]. A recent study assessed 27 recombinant leptospiral proteins as vaccine candidates against *L. interrogans*; none was significantly protective [17]. While it is clear that cross-protective antigens exist, there is still considerable work required for the development of effective subunit vaccines for *Leptospira*.

The recent publication of multiple leptospiral genome sequences [15,16,18,19] has opened the way for a systematic approach to the identification of protein antigens that elicit protection and are therefore potential subunit vaccine candidates. While it is not feasible to express each of the 3412 predicted proteins of serovar Hardjo to apply a strict reverse vaccinology approach, we have used a bioinformatics-based approach to establish a shortlist of candidate genes for analysis in this reverse vaccinology study. The analysis included comparative genomics, inference of cellular localisations and similarity to known vaccine antigens. In total, 238 predicted leptospiral surface antigens or secreted proteins were expressed and evaluated as potential vaccine antigens.

2. Methods

2.1. Bacterial strains and culture conditions

L. borgpetersenii serovar Hardjo strain L550 was isolated in Australia from a human patient with leptospirosis contracted from exposure to infected cattle, while strain L664 was provided by Pfizer Animal Health, Melbourne, Australia. Bacteria were cultured at 30 °C in EMJH medium (Difco).

2.2. Genome sequence analysis

The predicted protein coding regions annotated on the genome of *L. borgpetersenii* serovar Hardjo L550 (GenBank Accession NC_008509.1) [16] were evaluated for a series of features to infer sub-cellular localisation. Preference was given to proteins that were predicted to be secreted, lipoproteins or have an outer membrane location. Signal peptidase I cleavage sequence was predicted using SignalP 3.0, with a score of >0.5 considered significant [20]. The presence of α-helical transmembrane domains was predicted using TMHMM [21] and proteins with less than two predicted transmembrane domains were prioritised. Proteins predicted to be lipoproteins by the LipoP programme [22] or SpLip programme [23] and those with high β-sheet structure predicted using WHAT [24] were considered likely to be found in the outer membrane.

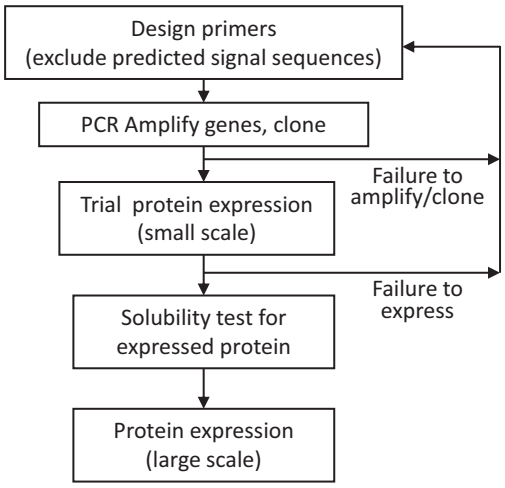

Fig. 1. Workflow for the expression of protein antigens.

Predicted proteins encoded in the *L. borgpetersenii* serovar Hardjo L550 genome were compared with those of the *L. interrogans* serovar Lai [15] and Copenhageni [18], and *L. biflexa* [19] genome sequences to identify orthologues by analysis in the computer program Wasabi [16]. Priority was given to proteins with orthologues in other pathogens but not in *L. biflexa*.

2.3. Cloning and expression of candidate antigens

The sequences of primers used to amplify genes are shown in Table S1. The process for cloning genes and expression of proteins is summarised in Fig. 1. Genes were amplified by PCR from L550 chromosomal DNA, omitting sections of the coding regions encoding predicted signal sequences. Amplicons were cloned by two different methods, either directly into pET151 (containing N-terminal hexa-histidine and V5 tags), or alternatively into pENTR using the Gateway™ high throughput cloning system (Invitrogen) followed by transfer to the expression vector pDEST41BA or pDEST17 (containing an N-terminal hexa-histidine tag).

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2.4. Protein expression and purification

Proteins were expressed in *Escherichia coli* BL21 Codon Plus (Agilent Technologies). Proteins were initially expressed on a small scale using Overnight Express™ Autoinduction System (Merck) at 28 °C according to the manufacturer's instructions. For instances where protein expression was not detected, the cloning and expression process was repeated with the design and synthesis of new oligonucleotide primers.

Where small-scale expression was successful, larger scale protein expression and purification was performed by the Monash University Protein Production Unit. For the expression of recombinant proteins, 200 ml cultures (Overnight ExpressTM, Merck) containing ampicillin (100 μg/ml) were grown overnight at 28 °C, with constant shaking at 250 rpm. The cells were collected by centrifugation at 3500 × *g* for 10 min and resuspended in nickel affinity buffer (100 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 10 mM imidazole). The cells were then lysed

by sonication on ice for six rounds of 30 s with a 10 mm sonication probe, interspersed with 30 s rest intervals. After sonication the soluble and insoluble fractions were separated by centrifugation at 7500 × *g* for 20 min. For the soluble proteins, the soluble fraction prepared above was filtered through a 0.22 μm filter and loaded on to a HisTrap FF nickel affinity column (GE Healthcare – Life Sciences) at a flow rate of 1 ml/min. After washing the column with the nickel affinity buffer, the recombinant proteins were eluted from the column with 100 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.5 M imidazole. The eluted proteins were then loaded on to a HiLoad 16/60 Superdex 200 or Superdex 75 size exclusion chromatography column (GE Healthcare – Life Sciences). The fractions containing the protein of interest were collected in 100 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl.

For the insoluble proteins, the insoluble fraction was washed twice with 100 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl and 1 mM 2-mercaptoethanol, 1% (v/v) Triton X-100 (centrifugation at 7500 × *g* for 20 min). The third wash was with 100 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl and 1 mM 2-mercaptoethanol and the insoluble fraction again pelleted by centrifugation at 7500 × *g* for 20 min. The pellet was resuspended in 100 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl, 10 mM 2-mercaptoethanol and 8 M urea and mixed for one hour at room temperature. Any proteins that remained insoluble were removed by centrifugation at 27,000 × *g* for 20 min, and the urea-solubilized material was loaded on to a HisTrap FF column at 1 ml/min. After washing this column with 100 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl, 1 mM 2-mercaptoethanol and 8 M urea, the recombinant proteins were eluted with the same buffer containing 0.5 M imidazole. The eluted proteins were loaded on to a Hiload 16/60 desalting column (GE Healthcare – Life Sciences). The fractions containing the protein of interest were collected in 100 mM sodium phosphate buffer, pH 7.4, with 150 mM NaCl, 1 mM 2-mercaptoethanol, and 8 M urea. Analysis by Coomassie Blue stained SDS-PAGE gels and western immunoblotting with antibodies directed against the appropriate tag was used to analyse the purified recombinant proteins [25]. In the event that migration of the purified protein did not correlate with the predicted protein size, identity was verified by mass spectrometry as described previously [25].

2.5. Evaluation of hamster protection

Golden Syrian hamsters of either sex (4 weeks old, groups of 10) were vaccinated subcutaneously with pools of proteins (containing 25 μg of each protein) in Alhydrogel™ adjuvant (Invitrogen) and then boosted with the same dose 14 days later (Fig. 2). Two weeks later a serum sample was taken by retro-orbital bleed under anaesthesia and animals were challenged intraperitoneally with 0.1 ml containing 2×10^6 *L. borgpetersenii* serovar Hardjo strain L664. Strain L664 was chosen to challenge hamsters because strain L550 was not infectious in the hamster model. Clinical signs were observed twice daily and two weeks post challenge animals were euthanized and kidneys cultured in EMJH medium as described previously [26]. Cultures were monitored by microscopy until positive cultures were observed or for up to 6 weeks where cultures were negative. A positive control of Spirovac™ (100 μl of commercial preparation, Pfizer) was included in each experiment and negative controls of PBS. Where appropriate, a positive control of Spirovac™ in 8 M urea and a negative control of 8 M urea in PBS were also included. Animal experiments were approved by Khon Kaen University Animal Ethics Committee. Statistical significance was determined using Fisher's exact test.

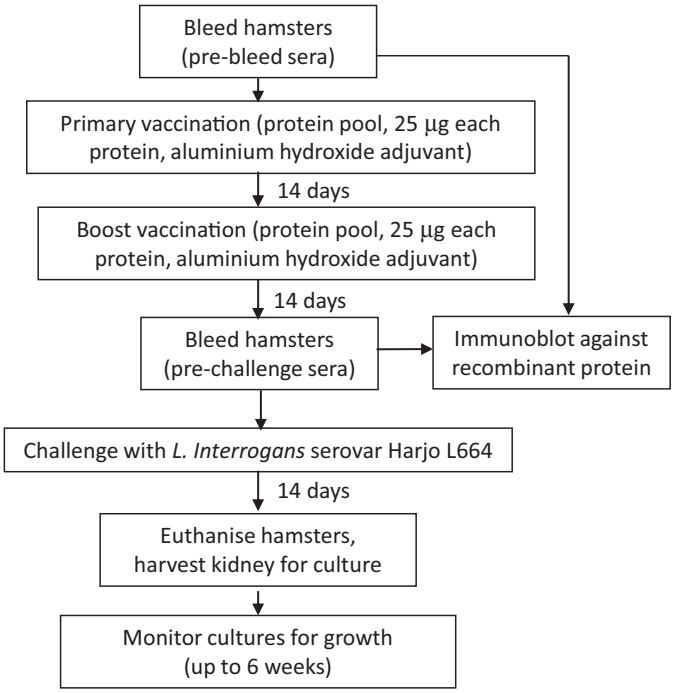

Fig. 2. Overview of the vaccination protocol.

2.6. Analysis of hamster sera

Immunoblotting was conducted against purified recombinant antigen, or whole cell lysates of induced *E. coli* expression strains using hamster sera pooled from each of the antigen evaluation groups. Sera were also analysed against whole cell lysates of strain L550 by immunoblotting. However, a cross-reaction to leptospiral serovar Hardjo LPS was identified in all hamster groups, including naive animals, preventing meaningful interpretation of immunoblotting against whole cell lysates of *Leptospira*; sera from these hamsters did not agglutinate serovar Hardjo suggesting that an unexposed LPS epitope was recognised. This cross-reaction with LPS did not influence the results of antigen evaluation in the hamsters, as 100% of hamsters in all unvaccinated control groups were colonised by serovar Hardjo.

3. Results

3.1. Genome analysis for identification of antigens

Proteins encoded by the predicted coding regions for the *L. borgpetersenii* serovar Hardjo L550 genome were individually evaluated for predicted cellular location. Proteins predicted to be secreted, located in the outer membrane or lipoproteins were selected for further evaluation (expressed proteins are detailed in Table S2). The list was further refined by evaluating the distribution of orthologues in the genus *Leptospira* for each of the candidate proteins. Proteins with orthologues in *L. interrogans* serovars Lai or Copenhageni [15,18] were prioritised. This analysis was performed in conjunction with a literature survey to identify any characterised leptospiral proteins that were either surface exposed or implicated in immunity. No putative autotransporters were identified. Proteins related to characterised virulence factors from other genera were added to the list of proteins. Of the expressed proteins (Table S2), 99% had orthologues in the second *L. borgpetersenii* serovar Hardjo genome (strain JB197) [16], while 89% had orthologues in the genomes of *L. interrogans* serovar Lai or Copenhageni [15,18]. Fourteen percent of expressed proteins had an ortholog in *L. biflexa*

(>69% amino acid sequence identity). The selected genes accounted for 7.7% of coding sequences of the *L. borgpetersenii* serovar Hardjo genome.

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3.2. Cloning and expression of antigens

A total of 238 proteins was expressed, accounting for 223 unique proteins; where expression of full-length proteins was unsuccessful, one or more gene fragments were cloned and expressed (Table S2). Soluble proteins were purified under non-denaturing conditions. The majority of proteins (210/238, 88%) was insoluble and were purified under denaturing conditions using buffers containing 8 M urea.

3.3. Evaluation of recombinant antigens

Recombinant antigens were evaluated using the hamster kidney colonisation model. Antigens were tested in pooled sets of up to five recombinant proteins per group of hamsters, constituting seven pools of soluble proteins (S1–S7) and 41 pools of insoluble proteins (U1–U41); the antigens tested are tabulated in Table S2. Some strains of *L. borgpetersenii* serovar Hardjo are not hamster-lethal, but establish a chronic renal infection with similarity to bovine infection [27]. This was found to be the case with our challenge strain *L. borgpetersenii* serovar Hardjo strain L664. Infection with 2×10^6 L664 resulted in 100% renal colonisation of hamsters by day 14 post infection; hence, kidney colonisation was used as an indicator of infection.

Each group of hamsters was primed and boosted with a set of protein antigens in Alhydrogel™ adjuvant, challenged with *L. borgpetersenii* serovar Hardjo strain L664 and assessed for kidney colonisation by culture. Control animals vaccinated with Spirovac™ or Spirovac™ in 8 M urea were never colonised. Hamsters that were vaccinated with PBS or 8 M urea in PBS were not protected against colonisation. However, none of the vaccine pools elicited significant protection against infection.

3.4. Analysis of the antibody response in vaccinated hamsters

Sera taken from vaccinated hamsters prior to challenge were analysed for reaction by immunoblotting against the corresponding recombinant protein. A total of 71% (169/238) proteins reacted with respective vaccine group sera, while the remaining 29% were negative (Table S2). Hamsters responded to a total of 75% (21/28) of soluble proteins, compared to 70% (148/210) of insoluble proteins, indicating no significant difference in immunogenicity resulting from preparation and administration of denatured proteins in urea.

4. Discussion

This study describes the large-scale production of recombinant proteins from *L. borgpetersenii* and the screening of these antigens for their ability to induce protective immunity in the hamster kidney colonisation model of leptospirosis. It thus supersedes the previous *ad hoc* testing of small numbers of proteins as subunit vaccines.

A total of 263 proteins with predicted surface exposure or role in virulence was selected for this study. This group included antigens that have shown promise as subunit vaccines against leptospirosis, including LigB, LipL32 and LipL41. Previously, vaccination with LigB protected a significant proportion of animals in two out of three experiments [28]. However, these animals were not protected against colonisation. In contrast, LipL41/OmpL1 in combination protected a majority of animals against death (71%), and surviving animals were not colonised by *Leptospira* [12]; however, 25% of control hamsters also survived challenge. Use of LipL32 as a vaccine antigen has yielded variable results, with the majority of studies showing no protection [2]. However, in one study significant protection was conferred by LipL32 in 56% of vaccinated hamsters; in surviving animals sterilising immunity was achieved [13]. Despite these previous encouraging results, LigB, LipL32 and LipL41 did not elicit protection against renal colonisation in this study. OmpL1 was not tested in this study because it expressed very poorly and purification was unsuccessful.

In this study, 223 unique proteins (excluding protein fragments) were expressed and purified from the 263 selected genes, yielding an 85% success rate. Previous reverse vaccinology studies have had success rates for protein expression in *E. coli* varying from 53% up to 89% [29–32]. Our solubility rate of 12% compares favourably with rates reported in other studies; for example, only 8% solubility for proteins from *Porphyromonas gingivalis* was reported by Ross et al. [31]. Our rate of solubility was not unexpected, due to the high proportion of selected genes encoding predicted outer membrane proteins. Previous studies evaluated candidate antigens by screening for reactivity against sera from convalescent humans or animals [31,33,34]. This step was not included in the current study because serological reactivity does not correlate with protection, as clearly demonstrated by reported non-protective responses to leptospiral proteins LipL32 and LigA [11,35]. Conversely, lack of antibody reactivity in sera does not exclude the possibility that the antigen is protective [36].

Previous reverse vaccinology studies have benefited from *in vitro* correlates of protection, such as opsonophagocytosis or complement-fixing bactericidal antibody [29,34]. For *Leptospira*, no such correlate is available, so all antigens were tested in an animal challenge model. While statistical significance has not been enforced as a measure for evaluating the outcome of many previous leptospiral vaccine studies [2], we have taken measures to avoid this problem. In this study, groups of ten animals were used, providing a sufficient statistical buffer for failure in a small number of control animals. The majority of proteins stimulated a detectable antibody response in hamsters and for the insoluble proteins the presence of urea had no impact on the rate of seroconversion. Despite most proteins stimulating an antibody response, none protected animals against infection.

Previous large scale reverse vaccinology studies have identified multiple protective vaccine antigens, including two against *P. gingivalis* (from 120 expressed antigens) [31], four against group B *Streptococcus* (from 312 expressed antigens) [30], up to five against *Neisseria meningitidis* group B (from 350 expressed antigens) [29,37], and six against *Streptococcus pneumoniae* (from 108 expressed antigens) [32]. Some of these protective antigens have been developed into vaccines in various stages of clinical trials for group B *N. meningitidis*, *S. pneumoniae*, and group B *Streptococcus* [38].

As found in other reverse vaccinology studies, we could not express all selected proteins. Consequently, some potentially protective antigens may not have been tested. Pathogenic leptospires are known to post-translationally modify proteins through lipidation, methylation and acetylation [39] and proteins expressed in *E. coli* may lack immunologically relevant post-translational modifications. Lipidation may contribute to the immunogencity of recombinant lipoproteins, such as OspA of *Borrelia burgdorferi* [40]. It is possible that in the current study non-lipidated recombinant lipoproteins may have altered immunogenic properties. In future studies predicted lipoproteins could be expressed in plasmids designed to lipidate expressed proteins in the *E. coli* host [41], and this may contribute to a protective immune response.

5. Conclusions

While this study did not identify protective antigens against infection with *L. borgpetersenii* serovar Hardjo, a total of 238 protein antigens was successfully expressed, purified and tested. The results of this study will thus provide a valuable reference for immunogenic and non-protective antigens that will inform a more targeted approach to future subunit vaccine research.

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