

Subunit Approach to Evaluation of the Immune Protective Potential of Leptospiral Antigens[∇]

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Leptospirosis is the most widespread zoonosis in the world. Current vaccines are based on whole-cell preparations that cause severe side effects and do not induce satisfactory immunity. In light of the leptospiral genome sequences recently made available, several studies aimed at identification of protective recombinant immunogens have been performed; however, few such immunogens have been identified. The aim of this study was to evaluate 27 recombinant antigens to determine their potential to induce an immune response protective against leptospirosis in the hamster model. Experiments were conducted with groups of female hamsters immunized with individual antigen preparations. Hamsters were then challenged with a lethal dose of *Leptospira interrogans*. Thirteen antigens induced protective immune responses; however, only recombinant proteins LIC10325 and LIC13059 induced significant protection against mortality. These results have important implications for the development of an efficacious recombinant subunit vaccine against leptospirosis.

Leptospirosis is a disease caused by pathogenic spirochetes of the *Leptospira* genus (1). Transmission occurs through direct or indirect exposure to urine of mammalian reservoirs, especially during floods, occupational exposure, and the practice of water sports (3). The infection usually manifests as asymptomatic or as a self-resolving febrile illness. However, up to 15% of all human infections progress to severe leptospirosis, with complications such as kidney failure and pulmonary hemorrhage and fatality rates of up to 50% (11, 21). Mortality remains high because of delays in diagnosis due to lack of infrastructure and adequate clinical suspicion and to other poorly understood reasons, especially in underdeveloped and developing countries (3).

Although vaccination is the recommended method of prevention in at-risk settings (17), the immune response generated by most of the currently available vaccines (bacterins) is attributable to the outer membrane lipopolysaccharide (LPS) component (29). As over 250 leptospiral serovars have been identified thus far (7), with the main antigenic differences attributed to the LPS, the efficacy of such vaccines has been found to be limited to short-term, serovar-specific immunity. Bacterin-type vaccines have been approved for use in humans in Cuba, China, Japan, and France. However, bacterins induce adverse reactions and side effects and, in general, their use has been restricted to animals (21), especially dogs, cattle, and pigs (1). Therefore, considerable effort is being made to identify novel leptospiral vaccine candidates with

fewer side effects that can induce a cross-protective immune response to the pathogenic serovars.

In recent years, many potential vaccine candidates have been tested in animal models, and several different approaches, including those employing subunit, DNA, adenovirus, and *Mycobacterium bovis* BCG constructs, have been used (2, 5, 6, 12, 13, 15, 19, 24, 26, 27). Most of these studies identified their protein targets by screening for antigenicity through the use of sera from patients with leptospirosis (14) and/or proteins with predicted surface exposure (10). However, a recent review highlighted the difficulties of evaluating the reports of efficacy for these vaccine candidates due to the different animal models and statistical methods used. The authors of the review reported that when the same method of statistical analysis of protection against mortality was used, very few candidates were found to offer significant immunoprotection (1). In the majority of reports, furthermore, protection did not induce sterilizing immunity.

Recently, our group used an approach based on reverse vaccinology to identify eight putative lipoproteins in the *L. interrogans* genome and to subsequently characterize those lipoproteins in terms of immunogenicity and antigenicity (16). The eight putative lipoproteins and an additional 19 proteins, predicted to be surface exposed and recognized by sera from convalescent patients with leptospirosis (14), were evaluated using a hamster model of lethal leptospirosis. The aim of the study was to identify potential vaccine candidates that could protect hamsters against lethal challenge; the endpoints used in the present study included survival and protection against mortality.

MATERIALS AND METHODS

Leptospira strains. *L. interrogans* strain Fiocruz L1-130 (serogroup Icterohaemorrhagiae, serovar Copenhageni) was used in this study. Leptospire were

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TABLE 1. Primer and protein information

Antigen	Primer	Molecular mass (kDa)	Vector
rLIC10191	F CGGGATCCTCAACGCAAGAGCA R GGGGTACCTTGTGTGGTGC	22.9	pAE
rLIC12099	F TTGGTACCGCTCAAACGGCAAG R GGGAAGCTTTTTATATTTGACGATGA	52.7	pAE
rLIC11947	F CCGGATCCCCTGTGAAAGAAA R GGGAAGCTTTTTTCTGGAGGAA	17.9	pAE
rLIC10011	F TAGGTACCACGGATGGACTTTTGAA R CGGAATTCTTATTGTTTGAAACCTC	19.8	pAE
rLIC12730	F GCGGATCCATTTAGTCTTTACCTC R CCCAAGCTTGATCAATTCGGTTC	57.8	pAE
rLIC10561	F GCGGATCCTTAATATTTCTGGTCTTTC R CCCAAGCTTGATCAATTCGGTTC	30.22	pAE
rLIC10508	F CCGGATCCAATTCAATAACTATG R GGGAAGCTTACAACCAGGACCTT	22.99	pAE
rLIC12538	F GGGATCCGCAGACGAAAAGGAAA R CCAAGCTTTCAGCTAGTCAGAGTAAAA	24.9	pAE
rLIC10501	F CACCGATAACAAAGAGAAGGGAGG R CTACTCCACACATTCGGGACTATTG	48.8	pET100-D/TOPO
rLIC13006	F GACTCGAGAAGCTCTGCTTAAAGTGGCTTAA R GGCCATGGTTATTGTTCTACACAACTAAA	47	pAE
rLIC13306 ^a	F CACCTCCAAGAGAAAATGTTTATTC R TCATTTCCGAACCGGATGACCGT	17.7	pET100-D/TOPO
rLIC12253	F TTCTCGAGGAGAAACCGGACGATACTACTT R CCCCATGGTTAGGGAAGACTTCTAACAAC	23.4	pAE
rLIC11184	F CACCTGTGAAGATGAAAAAAGGA R TTAGTAACCACTCACTCGCAGC	18.8	pET100-D/TOPO
rLIC10645	F CACCAAAAAAGATAAGGACGATACCTT R TTAACGAACTAGTACAGTCGGTAAATG	42.3	pAE
rLIC10021	F GACTCGAGAATTGTTCTGTCAAGCCC R CCAAGCTTTCATAAATCCACGGAAAGT	63.8	pAE
rLIC11859 ^a	F CACCGAATTTATGAAGGTCACG R TTAAAAAGCACTTAAGGCAGCC	30	pET100-D/TOPO
rLIC10325 ^a	F CACCATTCAAGACGAAGATTCCAAAC R TCAATCCAATTTTCGGTTTCTAG	40.6	pET100-D/TOPO
rLIC12555	F CATCTCGAGAGCCCAGTACAAATGAAAGT R TCCATGGTTAAAGATTGTAAACGAGATTCC	43	pAE
rLIC11087	F CACCGTTGGAGATTCCAGAAAGGAA R TAAAAATAAATTACAACCAGTCTGATATAA	29.9	pET100-D/TOPO
rLIC12632	F GTCTCGAGTGTAACCTGGCAAACAAAATT R GGCCATGGCTAATGATGATAGATTAAATCT	63.8	pAE
rLIC10054	F TTTTTTGGATCCGAGTCTAAACGAAG R TTTTTTAAGCTTCACCGTATTCTGTGC	32.1	pQE30
rLIC20172	F CCGGATCCGATACGGACAAGGACGGG R CCCAAGCTTTTCGGAATCCTCGTCCGG	28.1	pQE30
rLIC13059	F CCGGATCCGAATCCATGGTATATTAT R CCCAAGCTTACTTTGACGAATCAATGC	14.4	pQE30
rLIC11567	F CCGGATCCAACAGATTGATTCGTAAA R CCCAAGCTTCTTTTTGTATTCCACAAG	13.9	pQE30
rLIC10091	F CCGGATCCAACACTATTTTAGCTCCTTTG R CCCAAGCTTGATTTCAAAGAAGTATG	14.6	pQE30
rLIC10009	F TTTTTTTGATCACAAGAAGCGCAGATCT R CCCAAGCTTGAATCATCTGTTTA	24.8	pQE30
rLIC13305	F TTTTTTTGATCAAACACTATGATCGTGAC R TTTAAGCTTGATATCACCACCCAAA	20.3	pQE30

^a Expressed as soluble protein and did not require the use of urea for purification.

cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories) at 28°C. Growth was monitored by counting leptospire in a Petroff-Hausser chamber (Fisher) and by dark-field microscopy as described previously (11). *Escherichia coli* strains TOP10 (Invitrogen) and BL21(DE3) STAR (Novagen) were used in this study. They were grown in Luria-Bertani (LB) or Terrific broth (TB) media at 37°C.

Plasmid construction and expression and purification of recombinant proteins. All proteins used in this study were identified in previous studies (14, 16, 23). The genomic DNA of *L. interrogans* was used as a template for amplification of the target sequences. PCR products were cloned into pAE (25), pQE30 (Qiagen), or pET100-D/TOPO (Invitrogen) plasmid vector. Those vectors con-

tain a 6×His tag that is expressed fused to the recombinant protein. In general, primers were designed to include most of the target genes but not their highly hydrophobic signal sequences. They also included a restriction enzyme site to allow direct cloning of the PCR product. Full primer information is presented in Table 1. Recombinant plasmids were used to transform *E. coli* strains by electroporation, and *E. coli* organisms containing the constructs were then cultured at 37°C. Expression was induced by the use of IPTG (isopropyl-β-D-thiogalactopyranoside) at a 1 mM final concentration. Cells were harvested by centrifugation, resuspended in column buffer containing 8 M urea (no urea was used for soluble protein; see Table 1), and disrupted by sonication. His-tagged proteins were purified by affinity chromatography in a nickel (Ni⁺²)-charged Sepharose

TABLE 2. Survival timeline of vaccinated animals

Antigen	Name or feature	No. of days to death for each animal	No. (%) of surviving animals/total no. of animals
rLIC10191	OmpA-like	10, 10, 10, 10, 11, 13	0/6 (0)
rLIC12099	Hypothetical protein	10, 13, 13, 13, 13, 14	0/6 (0)
rLIC11947	Putative lipoprotein	12, 12, 14, 14, 16, 17	0/6 (0) ^b
rLIC10011	LipL21	11, 12, 13, 13, 14	1/6 (16.7) ^b
rLIC12730	Hypothetical protein	11, 11, 12, 13, 14, 14	0/6 (0)
rLIC10561	Hypothetical protein	11, 11, 11, 11, 13	1/6 (16.7)
rLIC10508	Putative lipoprotein	11, 13, 13, 13, 18	1/6 (16.7)
rLIC12538	SecD	11, 11, 13, 13, 14	1/6 (16.7) ^b
rLIC10501	Putative lipoprotein	10, 11, 11, 11, 13	1/6 (16.7) ^b
rLIC13306	Hypothetical protein	12, 12, 13, 14, 17	1/6 (16.7)
rLIC13006	Putative lipoprotein	10, 11, 11, 12, 12, 13	0/6 (0)
rLIC12253	Putative lipoprotein	12, 12, 12, 12, 12	1/6 (16.7)
rLIC11184	Putative lipoprotein	12, 12, 12, 12, 13	1/6 (16.7) ^b
rLIC10645	Putative lipoprotein	10, 11, 12, 12, 14	1/6 (16.7) ^b
rLIC10021	Putative lipoprotein	11, 11, 11, 13, 14, 20	0/6 (0) ^b
rLIC11859	Hypothetical protein	10, 11, 11, 12, 14	1/6 (16.7)
rLIC10325	Hemolysin	11, 12, 13, 13	2/6 (33.3) ^c
rLIC12555	Hypothetical protein	11, 12, 12, 12, 12, 15	0/6 (0)
rLIC11087	Putative lipoprotein	11, 11, 12, 13, 13, 14	0/6 (0)
rLIC12632	Hemolysin	11, 11, 12, 13, 14, 14	0/6 (0) ^b
rLIC10054	Putative lipoprotein	9, 10, 10, 10, 13	1/6 (16.7)
rLIC20172	Lipoprotein	10, 13, 13, 13, 13, 15, 16	1/8 (12.5) ^a
rLIC13059	Putative lipoprotein	8, 9, 10, 10	2/6 (33.3) ^c
rLIC11567	Putative lipoprotein	9, 10, 10, 10, 13, 13	2/8 (25) ^a
rLIC10091	Putative lipoprotein	10, 10, 10, 11, 11, 11, 11	1/8 (12.5) ^a
rLIC10009	Putative lipoprotein	9, 10, 10, 10, 10, 13, 14, 15	0/8 (0) ^a
rLIC13305	Putative lipoprotein	10, 10, 10, 11, 11, 11, 11	1/8 (12.5) ^a

^a The experiment was conducted with eight hamsters per group.

^b One animal from the PBS control group survived in the corresponding experiment.

^c The rate of survival of vaccinated animals was significantly different from the rate seen with the PBS control group (log rank sum test).

column. Columns containing bound protein were washed with 10 volumes of wash buffer containing 10 mM imidazole. His-tagged proteins were eluted from the column with elution buffer containing 250 mM imidazole. A dialysis procedure was used to remove urea and imidazole and to promote refolding of the recombinant proteins. Proteins in the final preparation were quantified by the Bradford (4) and bicinchoninic acid (BCA) (Pierce) methods (Table 1 contains further information regarding solubility, vectors, and protein size).

Protein gel electrophoresis. All proteins were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to verify molecular weight and sample purity. These gels were stained with Coomassie blue solution to reveal the proteins.

Hamster immune protection studies. Female golden Syrian hamsters (4 to 5 weeks of age) were obtained from the animal house facility at the Federal University of Pelotas. The animals were immunized twice in the quadriceps muscle with the recombinant protein in an aluminum hydroxide solution (15%) on day 0 (zero) and day 14. All vaccine doses contained 60 µg of purified recombinant protein, with administration of a standard volume of 200 µl at a single injection site. Negative-control groups in all experiments were inoculated with a 200 µl of phosphate-buffered saline (PBS)-aluminum hydroxide (15%). Positive-control groups were immunized with 200 µl of a bacterin inoculum containing approximately 10⁸ leptospire per dose. All hamsters were challenged on day 28 (at age 8 to 9 weeks) with an intraperitoneal inoculum of 100 leptospire (~2 × the 50% lethal dose [LD₅₀]) of strain Fio Cruz L1-130 (28) 14 days after the last immunization. The inoculum was produced from log-phase cultures and consisted of a 1-ml dose. Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared.

Twenty-seven recombinant proteins were tested in 11 individual experiments; each group consisted of six hamsters except where otherwise noted (Table 2). All experiments included negative-control (PBS) and positive-control (bacterin) groups. All animal studies were approved by the Ethics Committee for the Use of Experimental Animals of the Universidade Federal de Pelotas.

Statistical analysis. The log-rank test was used to determine significant differences in survival among the vaccinated and the negative-control groups. All *P* values were calculated using two-sided tests, and a *P* value of <0.05 was considered to indicate statistical significance. Prism 4 software (GraphPad Software) was used to perform the statistical analysis.

RESULTS

Production of recombinant proteins and vaccine preparation. All PCR products were successfully cloned and expressed in *E. coli* as 6×His tag N-terminus fusion proteins, which allowed purification of the proteins by affinity chromatography. The recombinant proteins required urea-promoted denaturing conditions for purification (except where noted otherwise) followed by prolonged dialysis to obtain soluble protein preparations. The integrity and purity of the recombinant proteins used in this study were verified by SDS-PAGE analysis (Fig. 1). When necessary, proteins were concentrated prior to dialysis, permitting the standardization of vaccine doses at 200 µl. All vaccine doses were prepared at least 1 day prior to vaccination, and proteins were allowed to adsorb onto the aluminum hydroxide overnight.

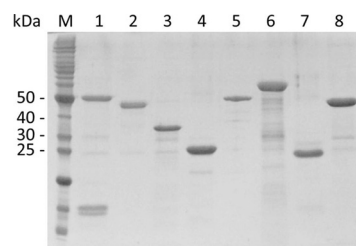


FIG. 1. SDS-PAGE (12%) of purified proteins. Lanes: M, molecular mass marker; 1, LIC10501; 2, LIC12555; 3, LIC11087; 4, LIC12253; 5, LIC10645; 6, LIC10021; 7, LIC11184; 8, LIC13006.

Protection of hamsters immunized with recombinant proteins against lethal challenge with *L. interrogans*. Immunization with the majority of the proteins did not prevent death among the challenged hamsters. Although immunization with the recombinant LIC11859 (rLIC11859), rLIC12253, rLIC10561, rLIC10508, rLIC10091, rLIC13059, rLIC10054, rLIC11567, rLIC20172, rLIC10561, and rLIC10508 proteins did result in more survivors than were seen with the negative-control groups, the differences were not significant. rLIC10325 and rLIC13059 significantly ($P < 0.05$) increased survival among the vaccinated hamsters (log-rank test). Table 2 shows the timeline of days to death for all the experiments. One animal survived in the negative-control group in each experiment, whereas animals in the positive-control group were fully protected in all experiments.

DISCUSSION

Several studies have employed the recombinant subunit vaccine approach to try to develop a vaccine against leptospirosis; however, the results have been variable and difficult to interpret (1). Although LigA seems to be the most promising antigen (24, 27), immune protection resulting from the use of that antigen in an adjuvant approved for human use has not been shown. Some authors reported protection induced by LipL32, LigB, and other outer membrane proteins (2, 6, 15, 19, 26); however, those proteins have yet to demonstrate practical applicability. In an effort to identify novel vaccine candidates, we evaluated 27 recombinant leptospiral proteins. In our assays, a total of 15 recombinant proteins were incapable of inducing a protective immune response to challenge with *L. interrogans*. A total of 12 recombinant proteins did improve survival compared to the negative-control group results. However, only two of those recombinant proteins (rLIC10325 and rLIC13059) induced survival at a rate that reached significance ($P < 0.05$). Those two proteins may constitute potential vaccine candidates.

The majority of the proteins evaluated in this study were identified as putative lipoproteins or hypothetical proteins (see Table 2). Of the protective antigens, LIC10325 was annotated as a hemolysin whereas LIC13059 was identified as a putative lipoprotein (23). A BLAST analysis revealed that the corresponding genes are present in all pathogenic leptospiral genomes described to date and are highly conserved among the pathogenic *Leptospira* spp. These are important features for antigens intended for use as vaccines aimed at affording cross-protection against different *Leptospira* spp. and serovars.

Previous reports have shown that vaccine preparations that include two or more proteins can be more effective than the individual components (8, 15). Several of the leptospiral virulence factors and outer membrane proteins have exhibited redundancy; this was demonstrated by the fact that knockout of some of these genes did not reduce virulence (9, 20, 22). Therefore, immunity responses directed toward one of these proteins may not be effective. Thus, further investigation of the protective proteins (fused or coadministered with each other and/or with proteins described elsewhere) identified in this study may increase the effectiveness of our preparations.

Although characterization of the immunogenicity and antigenicity of immunogens is an important step in identifying

surface-exposed proteins, there is no correlation between the amplitude of the immune response and protection against leptospirosis. Highly immunogenic and antigenic surface proteins such as LipL32 do not induce protective immunity (18). For this reason, we used a challenge assay to screen for protective antigens. This approach produces relatively fast and practical results. Antigens that fail to induce a protective immune response need not be further assessed.

In this study, we identified two potentially protective antigens. These, in combination with other leptospiral antigens already described, may result in the formulation of an effective and cross-protective vaccine against human and animal leptospirosis. Studies are being conducted to test not only different immunization protocols and antigen combinations but also different adjuvants and forms of antigen presentation.

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REFERENCES

- Adler, B., and A. de la Peña-Moctezuma. 2010. *Leptospira* and leptospirosis. *Vet. Microbiol.* **140**:287–296.
- Atzingen, M. V., et al. 2010. Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with *Leptospira interrogans*. *J. Med. Microbiol.* **59**:1005–1015.
- Bharti, A. R., et al. 2003. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* **3**:757–771.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- 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.
- Branger, C., et al. 2001. Identification of the hemolysin-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. *Infect. Immun.* **69**:6831–6838.
- Cerqueira, G. M., and M. Picardeau. 2009. A century of *Leptospira* strain typing. *Infect. Genet. Evol.* **9**:760–768.
- Chang, Y. F., et al. 2007. Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. *Vaccine* **25**:8190–8197.
- Croda, J., et al. 2008. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. *Infect. Immun.* **76**:5826–5833.
- Cullen, P. A., D. A. Haake, and B. Adler. 2004. Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol. Rev.* **28**:291–318.
- Faine, S. B., B. Adler, C. Bolin, and P. Perolat. 1999. *Leptospira* and leptospirosis, 2nd ed. MedSci, Melbourne, Australia.
- Faisal, S. M., et al. 2008. Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine* **26**:277–287.
- Felix, S. R., et al. 2009. Leptospirosis vaccine: search for subunit candidates. *Procedia Vaccinol.* **1**:110–114.
- Gamberini, M., et al. 2005. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. *FEMS Microbiol. Lett.* **244**:305–313.
- Haake, D. A., et al. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect. Immun.* **67**:6572–6582.
- Hartwig, D. D., F. K. Seixas, G. M. Cerqueira, A. J. McBride, and O. A. Dellagostin. 2011. Characterization of the immunogenic and antigenic potential of putative lipoproteins from *Leptospira interrogans*. *Curr. Microbiol.* **62**:1337–1341.
- Hotez, P. J., and M. T. Ferris. 2006. The antipoverty vaccines. *Vaccine* **24**:5787–5799.
- Ko, A. I., C. Goarant, and M. Picardeau. 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* **7**:736–747.
- Koizumi, N., and H. Watanabe. 2004. Leptospiral immunoglobulin-like proteins elicit protective immunity. *Vaccine* **22**:1545–1552.
- Lin, Y. P., and Y. F. Chang. 2007. A domain of the *Leptospira* LigB contributes to high affinity binding of fibronectin. *Biochem. Biophys. Res. Commun.* **362**:443–448.

21. **McBride, A. J., D. A. Athanazio, M. G. Reis, and A. I. Ko.** 2005. Leptospirosis. *Curr. Opin. Infect. Dis.* **18**:376–386.
22. **Murray, G. L., et al.** 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. *Infect. Immun.* **77**:810–816.
23. **Nascimento, A. L., et al.** 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* **186**:2164–2172.
24. **Palaniappan, R. U., et al.** 2006. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. *Infect. Immun.* **74**:1745–1750.
25. **Ramos, C. R., P. A. Abreu, A. L. Nascimento, and P. L. Ho.** 2004. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz. J. Med. Biol. Res.* **37**:1103–1109.
26. **Seixas, F. K., et al.** 2007. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine* **26**:88–95.
27. **Silva, E. F., et al.** 2007. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine* **25**:6277–6286.
28. **Silva, E. F., et al.** 2008. Characterization of virulence of *Leptospira* isolates in a hamster model. *Vaccine* **26**:3892–3896.
29. **Wang, Z., L. Jin, and A. Wegrzyn.** 2007. Leptospirosis vaccines. *Microb. Cell Fact.* **6**:39.