The Leptospiral General Secretory Protein D (GspD), a secretin, elicits complement-independent bactericidal antibody against diverse *Leptospira* species and serovars

EJA. Schuler, RT. Marconi *

Department of Microbiology and Immunology, Virginia Commonwealth University Medical Center, 1112 E Clay St., Richmond, VA 23298, USA

**A R T I C L E   I N F O**

Article history:
Received 29 October 2020
Received in revised form 18 February 2021
Accepted 20 February 2021
Available online 23 February 2021

Keywords:
Canine
GspD
Leptospirosis
Secretin
Spirochetes
Type 2 secretion

**A B S T R A C T**

Leptospirosis, the most common zoonotic infection worldwide, is a multi-system disorder affecting the kidney, liver, and lungs. Infections can be asymptomatic, self-limiting or progress to multi-organ system failure and pulmonary hemorrhage. The incidence of canine and human leptospirosis is steadily increasing worldwide. At least sixty-four *Leptospira* species and several hundred lipopolysaccharide-based serovars have been defined. Preventive vaccines are available for use in veterinary medicine and limited use in humans in some countries. All commercially available vaccines are bacterin formulations that consist of a combination of laboratory cultivated strains of different lipopolysaccharide serotypes. The development of a broadly protective subunit vaccine would represent a significant step forward in efforts to combat leptospirosis in humans, livestock, and companion animals worldwide. Here we investigate the potential of General secretory protein D (GspD; LIC11570), a secretin, to serve as a possible antigen in a multi-valent vaccine formulation. GspD is conserved, expressed in vitro, antigenic during infection and elicits antibody with complement independent bactericidal activity. Importantly, antibody to GspD is bactericidal against diverse *Leptospira* species of the P1 subclade. Epitope mapping localized the bactericidal epitopes to the N-terminal N0 domain of GspD. The data within support further exploration of GspD as a candidate for inclusion in a next generation multi-protein subunit vaccine.

© 2021 Virginia Commonwealth University. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Leptospirosis is a potentially life-threatening multi-systemic zoonotic infection of veterinary and human concern that occurs worldwide [1,2]. The incidence of leptospirosis is highest in tropical and subtropical regions where socioeconomic conditions are poor. The genus *Leptospira* consists of at least 64 pathogenic and saprophytic species that form two pathogenic and saprophytic clades (P and S, respectively). These clades can be further divided into subclades (P1, P2, S1 and S2) [3]. While the virulence potential of many *Leptospira* species remains to be determined, most well characterized pathogenic species belong to the P1 subclade [3]. *Leptospira* have also been classified based on the antigenic properties of their lipopolysaccharide (LPS) resulting in the designation of hundreds of serovars.

*Leptospira* are maintained in nature by a wide array of reservoir hosts [3]. As a consequence of the ability of pathogenic species to establish chronic colonization of the kidneys, leptospires are continually shed in urine. Contact with animals (livestock, wildlife and companion), contaminated soil, and water serve as common sources of infection [4]. The severity of leptospirosis can range from asymptomatic colonization to mild or serious infection with life-threatening clinical manifestations including compromised kidney and liver function and pulmonary hemorrhage [2,5]. Over 1,000,000 cases of human leptospirosis are documented annually with over 60,000 deaths worldwide [6]. While the incidence of leptospirosis is on the rise in companion animals, an accurate count of cases per annum is elusive [7]. In dogs, cases are most common in unvaccinated dogs <6 months of age and <14 lbs in weight with approximately 25% of cases being fatal (American Kennel Club; https://www.akcchf.org/educational-resources/library/an-update-on-canine.html; accessed on 01/07/2021).

In veterinary medicine (small and large animal) vaccination serves as the primary preventative strategy for leptospirosis [8]. *Leptospira* bacterin vaccines consist of inactivated cell lysates of multiple strains. The antibody response to *Leptospira* bacterin vaccines is directed largely at LPS. As a result, the protection afforded by bacterins is limited primarily to the serovars represented in the vaccine formulations [9,10]. The dominant circulating serovars in...
the United States are Icterohaemorrhagiae, Canicola, Grippotyphosa, Pomona, Bratislava, and Autumnalis. In Europe, Icterohaemorrhagiae, Grippotyphosa, Australis, Sejroe and Canicola are dominant [11]. In addition to limited cross-protection [12], concerns have also been raised about the recombinogenicity of Leptospira bacterin vaccines, particularly those used in canines [13,14]. Specific protein antigens that convey broad protection remain to be identified [reviewed in [9]]. The development of recombinant protein based subunit vaccines represents an attractive alternative to bacterin based vaccines.

In this study we assessed the vaccine potential of the L. interrogans serovar Copenhageni strain Fiocruz L1-130 general secretory protein D (GspD) [15]. GspD is a 67 kDa protein that is predicted to function as a secretin for a leptospiral type 2 secretion (T2S) system [16,17]. Here we demonstrate that GspD is an outer membrane protein antigen produced in vitro cultivation and during natural infection in canines. Immunization of rats with recombinant GspD (GspD) elicited potent bactericidal antibody that effectively targeted multiple Leptospira species, strains and serovars of the P1 subclade. Antibody mediated killing was demonstrated to occur through a complement independent mechanism. Epitope mapping revealed that the bactericidal epitopes of GspD reside within its N-terminal ‘N0’ domain [18,19]. The data presented within support the potential use of GspD in a subunit-based vaccine formulation.

2. Materials and methods

2.1. Bacterial strains and cultivation

Leptospira isolates were cultured at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with Probufin vaccine grade BSA (Millipore) as previously described [20]. Growth was monitored by wet-mounts using dark-field microscopy. All isolates assessed in vitro or in silico are described in Table 1. Note that strain virulence was not assessed as part of this study.

2.2. Phylogenetic analyses

GspD amino acid sequences from diverse Leptospira isolates were aligned using Mega X [21]. GspD homologs from Leptotena illini, Turneriella parva, and Escherichia coli K-12 served as outliers. A phylogenetic tree was constructed in Mega X using the neighbor-joining tree algorithm with 500 bootstraps, a poisson model of evolution, and pairwise deletion of gaps [21]. Mega X was also used to calculate percent amino acid identity and similarity values. GspD accession numbers are listed in Table 1.

2.3. Cloning, expression and purification of recombinant proteins

PCR and cloning were performed using standard conditions as previously described [22]. LipL32, gspD, qlp42 and gspD gene fragments were PCR amplified from L. interrogans serovar Copenhageni strain Fiocruz L1-130 genomic DNA using Phusion polymerase (Thermo Scientific) and primers that harbor restriction sites for cloning (Table 2). Signal sequences predicted by SignalP-5.0 [23] were excluded from the amplified lipL32 and qlp42 sequences. The amplicons were purified using QIAquick PCR Purification kits (Qiagen), digested with the appropriate restriction enzymes (New England Biolabs), ligated with linearized pET-45b(+) transformed into E. coli BL21(DE3) cells, and protein production induced with 1.0 mM Isopropyl β-D-1-thiogalactopyranoside [24]. All recombinant proteins were produced with an N-terminal hexa-histidine tag. Gene sequences were verified on a fee for service basis (Gene-wiz). Recombinant proteins that separated with the soluble fraction (Qlp42 and LipL32) were purified by nickel affinity chromatography using an AKTA purification platform as previously described [25]. Recombinant Treponema denticola Factor H binding protein B (FhbB), generated as part of an earlier study, served as control in several experiments [26]. Recombinant proteins that partitioned in the insoluble fraction (GspD and GspD subfragments F1 through F5) were purified using urea-denaturing conditions and then dialyzed stepwise into 1X PBS [24]. Protein concentrations were determined by bicinchoninic acid assay (BCA; Pierce).

2.4. Generation of antisera

Antisera against recombinant GspD, Qlp42 and LipL32 were generated as previously described [27]. In brief, Sprague Dawley rats were immunized (intraperitoneally) with 50 µg of recombinant protein in Freund’s complete adjuvant (Sigma-Aldrich) with

| Table 1 | Bacterial isolates included in this study. |
|---------|-----------------------------------------|
| Isolate | Source | GspD Amino Acid Access No. a |
| L. interrogans sv. Copenhageni str. Fiocruz L1-130 | Human, Brazil | AAS70166.1 |
| L. interrogans sv. Lai str. 56601 | Human, China | AAN49574.2 |
| L. interrogans sv. Canicola str. LT1962 | Human, Taiwan | EMF70412.1 |
| L. interrogans sv. Canicola str. Mex1 | Unknown | Not determined |
| L. interrogans sv. Canicola str. Kito | Canine, Brazil | EMK18369.1 |
| L. interrogans sv. Manilae str. 1495 | Human, Philippines | EJO79568.1 |
| L. interrogans sv. Autumnallis str. Bonito | Brazilian | EMI64742.1 |
| L. interrogans sv. Autumnalis str. ZUN142 | Human, Peru | EMO42106.1 |
| L. interrogans sv. Autumnalis str. Schwine | Swine, USA | EJO70340.1 |
| L. interrogans sv. Autumnalis str. Bonito | Freshwater, USA | Not determined |
| L. interrogans sv. Autumnalis str. LT 821 | Human, Taiwan | WP_004460673.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Cattle, Thailand | WP_002751252.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Mouse, Puerto Rico | Not determined |
| L. interrogans sv. Autumnalis str. LT 821 | Human, Thailand | WP_004503249.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Human, Peru | EPG65857.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Unknown, Canada | EKJ85129.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Freshwater, Brazil | EOQ90642.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Freshwater, Italy | ABF47508.1 |
| L. interrogans sv. Autumnalis str. LT 821 | Cattle, USA | EHQ5871.1 |
| L. interrogans sv. Autumnalis str. LT 821 | N/A | Not determined |
| L. interrogans sv. Autumnalis str. LT 821 | N/A | AFM14754.1 |
| L. interrogans sv. Autumnalis str. LT 821 | N/A | NP_417784.4 |

a All isolates were provided by Drs. James Matsunaga, David Hatke (UCLA), Elsio Schuler and RT. Marconi.
2 boosts of 25 μg recombinant protein in Freund’s incomplete adjuvant (Sigma-Aldrich) delivered 2 weeks apart. One week after the final boost, rats were sacrificed, blood collected via cardiac puncture and the sera harvested using Grenier Bio-one Vaccuette Z Serum Sep Clot Activator tubes (Grenier). All animal use protocols were reviewed and approved by the VCU IACUC and followed the Guide for the Care and Use of Laboratory Animals (Eighth edition).

2.5. SDS-PAGE, immunoblot, and dot-blot analyses

Cell lysates (0.3 OD₅₀₀ units per lane) were separated using AnykD Criterion Precast Gels (Bio-Rad) and transferred to PVDF membranes using the Transblot Turbo System per the manufacturer’s protocol (Bio-Rad). Dot-blot arrays were generated by spot application of 500 ng of GspD, LipL32, and FhbB (negative control) on to membranes using the Transblot Turbo System per the manufacturer’s protocol (Bio-Rad). Gels (AnykD Criterion Precast Gels (Bio-Rad) and transferred to PVDF (Sigma-Aldrich) delivered 2 weeks apart. One week after the final boost, rats were sacrificed, blood collected via cardiac puncture and the sera harvested using Grenier Bio-one Vaccuette Z Serum Sep Clot Activator tubes (Grenier). All animal use protocols were reviewed and approved by the VCU IACUC and followed the Guide for the Care and Use of Laboratory Animals (Eighth edition).

2.5. SDS-PAGE, immunoblot, and dot-blot analyses

Cell lysates (0.3 OD₅₀₀ units per lane) were separated using AnykD Criterion Precast Gels (Bio-Rad) and transferred to PVDF membranes using the Transblot Turbo System per the manufacturer’s protocol (Bio-Rad). Dot-blot arrays were generated by spot application of 500 ng of GspD, LipL32, and FhbB (negative control) onto nitrocellulose membranes (22 μM pore size; BioRad) followed by air drying. Immuno blots and dot-blot arrays were incubated with blocking buffer (PBS with 5% nonfat dried milk; 0.2% Tween 20; 2 h). Microscopic agglutination test (MAT) positive canine sera (1:200 dilution; Novus Biologicals) was added. IgG binding was detected using ECL substrate (Bio-Rad) and images were captured using a ChemiDoc Touch Imaging System (Bio-Rad). Note that the immunoblot and dot-blot images were cropped to generate the figures.

2.6. Triton X-114 extraction and phase partitioning

Triton X-114 extraction and phase partitioning were performed as previously described [28] with some modifications. Approximately 10¹¹ mid-log phase L. interrogans serovar Copenhageni strain Fiocruz L1-130 cells were recovered by centrifugation, washed twice (1X PBS, 5 mM MgCl₂, 1% protease inhibitor cocktail (PIC; Sigma-Aldrich). Lysate was diluted to OD₅₀₀ = 3.0 and pelleted by centrifugation (3000 g, 15 min, 4 °C). The pellet was resuspended in 1 mL extraction buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1% Triton X-114, 1% PIC), incubated on ice (2 h) and centrifuged (17,000 g, 10 min, 4 °C) to separate the detergent soluble and insoluble fractions. The supernatant was pipetted into a separate tube and 20 mM CaCl₂ was added, followed by incubation (37 °C, 15 min) and centrifugation (2000g, 10 min). The aqueous supernatant was decanted into a separate tube and the pelleted detergent phase was washed twice with PBS and resuspended. Aqueous and detergent phases were acetone precipitated with 10X volumes of ice-cold acetone. Precipitate was collected by centrifugation (12,600g, 30 min, 4 °C) and resuspended in PBS.

2.7. Bactericidal assays

Bactericidal activity of anti-GspD antisera was assessed as previously described [27,29] with some modifications. In brief, 4 μL of mid-log phase Leptospira cultures (density of ~100 cells per field of view under 400× magnification) were combined with 8 μL of EMJH media. Four μL of heat-inactivated (HI; 56 °C, 30 min) rat anti-GspD antiserum, 4 μL of complement certified normal human serum (NHS; Innovative) or 4 μL of HI-NHS was added. All assays were performed in triplicate. Cells incubated in EMJH, NHS, and rat pre-immune (PI) serum served as negative controls. The samples were incubated at 30 °C for 2 h, and the average number of live cells per 5 fields of view determined by visual counting under dark-field microscopy. Percent killing was calculated by determining the decrease in the number of live cells in the treatment groups versus the NHS + rat pre-immune serum negative control. Statistical analyses were conducted using Student’s t test.

2.8. Epitope localization

Recombinant proteins (500 ng) were coated onto 96-well plates (Corning) in triplicate and screened with serum (1:100) from nine client-owned, MAT positive canines [30]. Secondary antibody (rabbit anti-canine IgG HRP-conjugated; Novus Biological; 1:15000) was added and absorbance measured at 405 nm using a microtiter plate reader (BioTek). To localize the bactericidal epitopes, anti-GspD antiserum was adsorbed (overnight; 30 °C) with 250 ng μL⁻¹ of full-length (FL) GspD, GspD fragment (F) 1, GspD F2, GspD F3, GspD F4, GspD F5 (Table 2), or FhbB [26]. Bactericidal activity was assessed as detailed above. Cells incubated with rat PI serum alone and non-adsorbed anti-GspD antiserum alone served as negative and positive controls, respectively. The Treponema denticola FhbB protein served as an additional negative control [31].
3. Results and discussion

3.1. Analysis of GspD conservation amongst Leptospira species and strains

Evolutionary distances and phylogenetic relationships among GspD sequences were determined through pairwise comparisons. A phylogenetic tree is presented in Fig. 1A. The Leptospira GspD sequences assessed divide into three distinct phyletic clusters that are well-supported by bootstrap analyses. Amino acid identity and similarity values are presented in Fig. 1B. Identity values among Leptospira P1 subclade isolates ranged from 90.1 to 100%. GspD sequences derived from isolates of subclades P2 and S1 display approximately 75 and 60% identity with P1 subclade isolates, respectively. Hence, while GspD is conserved within a subclade, divergence between subclades is significant.

3.2. In vitro expression and subcellular localization of GspD

In vitro expression of GspD by diverse P1 strains was demonstrated by screening immunoblots of Leptospira whole cell lysates with anti-GspD antiserum. A single protein of approximately 67 kDa was detected in all strains tested with the exception of the saprophyte, L. biflexa strain Patoc I, which belongs to subclade.
S1 (Fig. 2A). While it is possible that \textit{L. biflexa} does not produce GspD in vitro, an alternative possibility is that its GspD protein, which shares only 60% amino acid identity with the \textit{L. interrogans} serovar Copenhageni strain Fiocruz L1-130 GspD protein used to generate the sera, is antigenically distinct. In light of the conservation of GspD at the intra-clade level, the difference in signal intensity among P1 subclade strains observed in the immunoblot most likely suggests variable levels of expression in vitro (Fig. 2A).

The subcellular localization of GspD was determined through Triton X-114 extraction and phase partitioning of \textit{L. interrogans} serovar Copenhageni strain Fiocruz L1-130. The resulting fractions were immunoblotted and screened with anti-GspD, anti-LipL32 and anti-Qlp42 antisera (Fig. 2B). GspD was detected in the whole cell lysate, the detergent insoluble phase (protoplasmic cylinder), and the detergent soluble phase (outer membrane). Based on earlier studies that demonstrated that GspD homologs of other bacterial species interact with both the inner and outer membrane \[18,19,32\], the detection of GspD in both the detergent insoluble and soluble phases was expected and is consistent with its putative function as a secretin in type 2 secretion\[17\]. As controls, identical immunoblots were screened with anti-LipL32 and anti-Qlp42 antisera. LipL32 and the Qlp42 have been demonstrated to partition with the outer membrane and aqueous phases, respectively \[28,33–36\]. Both proteins partitioned as expected.

3.3. GspD is expressed during natural infection in client-owned canines

Serum from forty-one MAT positive and nine MAT negative client-owned dogs were screened for IgG to GspD using a dot-blot format. Recombinant LipL32 and FhbB (\textit{T. denticola} FhbB [26]) served as positive and negative control detection antigens, respectively. Of the forty-one MAT positive dogs, 92.7% were IgG positive for antibody to GspD and all were immunoreactive with LipL32, a known surface exposed in vivo antigen \[3\] [28,33]. MAT negative sera were IgG negative for antibody to all proteins tested and all sera were antibody negative for FhbB. The detection of antibodies to GspD in canines is consistent with the detection of anti-GspD antibodies in the sera of confirmed human leptospirosis patients \[37\]. It can be concluded that GspD is actively produced during \textit{Leptospira} infections in canines.

3.4. Anti-GspD antiserum is bactericidal and kills through a complement independent mechanism

Bactericidal assays revealed that anti-GspD antibody can kill diverse species and strains of \textit{Leptospira} of the P1 subclade (Fig. 4). Antibody dependent killing (60–74%) occurred independent of complement (Fig. 4) with no significant difference in killing in the presence of active NHS vs HI-NHS (\(P = .41\)). The mechanism of killing by anti-GspD antibody remains to be determined but could be linked to the critical functions mediated by secretin proteins and T2SS in general. The binding of antibody to GspD may interfere with the passage of effector molecules from the cell into the extracellular milieu \[38\]. Importantly, anti-GspD antibody effectively killed all strains tested including those of differing serotypes suggesting that GspD or domains derived from it, may be good candidates for inclusion in a multi-protein subunit based vaccine.

3.5. Localization of the bactericidal epitopes of GspD

To localize the immunodominant epitopes of GspD that are presented during natural infection, GspD fragments harboring 20 amino acid overlaps were generated (Table 2) and screened with MAT positive canine sera by ELISA (Fig. 5A). The sera reacted most strongly with full length GspD and the F1 fragment (residues 1–135). IgG binding to other fragments was low and variable among individual dogs. The difference in the level of antibody binding to F1 versus all other fragments was significant (\(P \leq 0.0001\)). The results demonstrate that the immunodominant epitopes of GspD reside within a segment of the N-terminal N0 domain within amino acids 1 through 135.

To verify that the epitopes of GspD that elicit bactericidal antibody reside within the F1 fragment, bactericidal assays were performed with anti-GspD antiserum absorbed with full length GspD, GspD sub-fragments or FhbB prior to conducting the assay (Fig. 5B). Bactericidal activity was significantly reduced by both full length GspD or the F1 fragment (\(P < 0.01\)), but not by adsorption with fragments F2, F3, F4, F5 or the irrelevant FhbB antigen. Hence, it can be concluded the bactericidal epitopes of GspD reside within the first 135 amino acids which comprise a majority of the N0 domain. The N0 domain extends from the outer membrane across the periplasm where it interacts with substrates and inner membrane components \[19\]. Antibody binding to the N0 domain may disrupt T2S and thus explain the basis for the complement independent bactericidal activity of anti-GspD antibody. A BLASTP
Fig. 3. GspD is antigenic during natural infection in client-owned canines. Dot-blot of full-length recombinant proteins (indicated to the left) were screened with sera from MAT positive and MAT negative client-owned dogs (1:200 dilution) as indicated below each panel. For each serum sample, the serovar(s) that yielded the highest MAT titers are indicated above each dot-blot. Abbreviations were as follows: Pomona (P), Icterohaemorrhagiae (I), Canicola (C), Grippotyphosa (G), Hardjo-bovis (H), Bratislava (B), Autumnalis (A), and Sejroe (S). For sera that had equal titers to two serovars, both serovar abbreviations are provided. LipL32 was included as a positive control and the T. denticola FhbB protein served as a negative control. Molecular weight standards are indicated on the left.

Fig. 4. Anti-GspD antisera is bactericidal and kills diverse strains through a complement-independent mechanism. The bactericidal activity of rat anti-GspD antisera against diverse Leptospira strains (as indicated in the figure key) was assessed as described in the methods. Abbreviations are as follows: NHS (complement-certified normal human serum), HI-NHS (heat-inactivated normal human sera), and Rat PI (rat pre-immune serum). Equivalent numbers of cells were incubated with NHS, HI-NHS, rat PI, or anti-GspD antisera as indicated below the figure. Percent killing was determined by visual examination of cell numbers per field of view using dark field microscopy as detailed in the text. Differences in cell counts per field of view in the negative control (NHS + rat PI) versus in the treatment groups (NHS + Anti-GspD and HI-NHS + Anti-GspD) were assessed using the two-tailed student’s t-test (* indicates P ≤ .05).

search using GspD amino acid residues 1 through 135 as the query revealed that anti-GspD antibodies are bactericidal and kill through a complement-independent mechanism. The epitopes that elicit bactericidal antibodies were localized within the within the N0 domain of GspD that spans the N-terminal 135 residues. Anti-GspD antibody displayed significant killing activity against antigenically diverse subclade P1 strains including L. interrogans serovars Canicola, Copenhageni and Pomona, L. kirschneri serovar Grippotyphosa, L. noguchii serogroup Autumnalis, and L. borgpetersenii serogroup Ballum. While GspD is conserved amongst subclade P1 isolates, the GspD sequences of other subclades show significant divergence. Hence, a GspD vaccine formulation may need to be multivalent, including the F1 fragment of at least one P1 and P2 subclade isolate. While this study was in review, it was demonstrated that L. interrogans serovar Canicola (LOCas46) (subclade P1) full length GspD conveyed partial protection to hamsters against lethal challenge with the homologous strain of Leptospira [39]. The data presented here further support the potential utility of GspD as a component of a subunit vaccine for leptospirosis.

A few studies have sought to develop recombinant chimeric antigens that can elicit antibody against multiple Leptospira antigens [40–44]. An OmpL1-LipL32-LipL21 chimeric conferred significant protection against lethal homologous challenge with L. interrogans sv. Lai in hamsters [45] and a LigAc-LenA-LcpA-Lsa23 significantly reduced leptospiral renal burden [46]. However, the potential for these chimeric protein vaccinogens to elicit protection against heterologous strains has not yet been assessed. Recently, a recombinant chimeric epitope based vaccine antigen, referred to as a chimeritope, was successfully developed as a vaccine antigen for canine Lyme disease. The sequences encoding the immunodominant and variable L5 and H5 epitopes from several Borrelia burgdorferi outer surface protein C variants were linked to form a single contiguous gene sequence. Protein expressed from this chimeritope encoding sequence, designated as Ch14, was demonstrated to elicit broadly cross reactive and protective antibodies [27,47]. The Ch14 chimeritope is one of two recombinant antigens that comprise the commercially successful canine Lyme disease vaccine, VANGUARD®-crLyme (Zoetis) [47,48]. The remarkable diversity of the Leptospira suggests that a chimeritope approach may offer a path to a broadly protective vaccine antigen for both human and veterinary leptospirosis. Future work will seek to

4. Conclusions

In this study we have demonstrated that the Leptospira secretin protein, GspD, is expressed in vitro and antigenic during natural infection in canines. Recombinant GspD delivered to rats elicited a high titer IgG antibody response. In vitro assays demonstrated that anti-GspD antibodies are bactericidal and kill through a complement-independent mechanism. The epitopes that elicit bactericidal antibodies were localized within the N0 domain of GspD that spans the N-terminal 135 residues. Anti-GspD antibody displayed significant killing activity against antigenically diverse subclade P1 strains including L. interrogans serovars Canicola, Copenhageni and Pomona, L. kirschneri serovar Grippotyphosa, L. noguchii serogroup Autumnalis, and L. borgpetersenii serogroup Ballum. While GspD is conserved amongst subclade P1 isolates, the GspD sequences of other subclades show significant divergence. Hence, a GspD vaccine formulation may need to be multivalent, including the F1 fragment of at least one P1 and P2 subclade isolate. While this study was in review, it was demonstrated that L. interrogans serovar Canicola (LOCas46) (subclade P1) full length GspD conveyed partial protection to hamsters against lethal challenge with the homologous strain of Leptospira [39]. The data presented here further support the potential utility of GspD as a component of a subunit vaccine for leptospirosis.

A few studies have sought to develop recombinant chimeric antigens that can elicit antibody against multiple Leptospira antigens [40–44]. An OmpL1-LipL32-LipL21 chimeric conferred significant protection against lethal homologous challenge with L. interrogans sv. Lai in hamsters [45] and a LigAc-LenA-LcpA-Lsa23 significantly reduced leptospiral renal burden [46]. However, the potential for these chimeric protein vaccinogens to elicit protection against heterologous strains has not yet been assessed. Recently, a recombinant chimeric epitope based vaccine antigen, referred to as a chimeritope, was successfully developed as a vaccine antigen for canine Lyme disease. The sequences encoding the immunodominant and variable L5 and H5 epitopes from several Borrelia burgdorferi outer surface protein C variants were linked to form a single contiguous gene sequence. Protein expressed from this chimeritope encoding sequence, designated as Ch14, was demonstrated to elicit broadly cross reactive and protective antibodies [27,47]. The Ch14 chimeritope is one of two recombinant antigens that comprise the commercially successful canine Lyme disease vaccine, VANGUARD®-crLyme (Zoetis) [47,48]. The remarkable diversity of the Leptospira suggests that a chimeritope approach may offer a path to a broadly protective vaccine antigen for both human and veterinary leptospirosis. Future work will seek to
incorporate epitopes several leptospiral antigens into a single recombinant chimeritope protein.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Leptospira isolates were generously provided by Drs. Haake, Matsunaga (University of California Los Angeles), Ko and Wunder (Yale University). We thank Dr. Wunder for his helpful advice and members of the Marconi lab for their review of this manuscript.

References

[1] Divers TJ, Chang YF, Irby NL, Smith JL, Carter CN. Leptospirosis: An important infectious disease in North American horses. Equine Vet J 2019;51:287–92.
[2] Bulach D, Adler B. Leptospiral Genomics and Pathogenesis. Curr Top Microbiol Immunol 2020;387:251–72.
[3] Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis 2015;9:e0003898.
[4] Adler B, de la Pena Montezuma A. Leptospira and leptospirosis. Vet Microbiol 2010;140:287–96.
[5] Cilia G, Bertelloni F, Fratini F. Leptospirosis Infections in Domestic and Wild Animals. Pathogens 2020;9.
[6] Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis 2015;9:e0003898.
[7] White AM, Zambrana-Torrello C, Allen T, Rostal MK, Wright AK, Ball EC, et al. Hotspots of canine leptospirosis in the United States of America. Vet J 2017;222:29–35.
[8] Bashiru G, Bahamn AR. Advances & challenges in leptospirosis vaccine development. Indian J Med Res 2018;147:15–22.
[9] Adler B. Vaccines against leptospirosis. Curr Top Microbiol Immunol 2015;387:251–72.
[10] Bouvet J, Lemaître L, Caruso C, Scotto M, Blain C, Oberli F, et al. A canine vaccine against Leptospira serovars Icterohaemorrhagiae, Canicola and Grippotyphosa provides cross protection against Leptospira serovar Copenhageni. Vet Immunol Immunopathol 2020;219:109855.
[11] Ellis WA. Control of canine leptospirosis in Europe: time for a change?. Vet Rec 2010;167:602–5.
[12] Spati AM, Rodriguez-Campos S, Matos JM, Glaus TM, Riond B, Reusch CE, et al. Clinical, serological and echocardiographic examination of healthy field dogs before and after vaccination with a commercial tetravalent leptospirosis vaccine. BMC Vet Res 2017;13:138.
[13] Robbins H. Adverse events in dogs given Leptospirosis vaccine. Vet Rec 2017;180:257.
[14] Yao P, Stephenon N, Foley JE, Toussieng CR, Farver TB, Sykes JE, et al. Incidence rates and risk factors for owner-reported adverse events following vaccination of dogs that did or did not receive a Leptospirosis vaccine. J Am Vet Med Assoc 2015;247:1139–45.
[15] Reyes EA, Cullen P, Paladino T, Dr Adler B, Haake D, de la Peña Montezuma A. Expression in Escherichia coli of the gspdA gene of the type II secretion system in Leptospira borgpetersenii serovariety hardjo. Rev Cubana Med Trop 2005;57:45–6.
[16] Haake DA, Matsunaga J. Leptospira: a spirochaete with a hybrid outer membrane. Mol Microbiol 2010;77:805–14.
[17] Zeng L, Zhang Y, Zhu Y, Yin H, Zhuang X, Zhu W, et al. Extracellular proteome analysis of Leptospira interrogans serovar Lai. OMICS 2013;17:527–35.
[18] Korotkov KV, Pardon E, Steyaert J, Hol WG. Crystal structure of the N-terminal domain of the secretin GspD from ETEC determined with the assistance of a nanobody. Structure 2009;17:255–65.
[19] Yan Z, Yin Y, Xu D, Li X. Structural insights into the secretin translocation channel in the type II secretion system. Nat Struct Mol Biol 2017;24:177–83.
[20] Matsunaga J, Sanchez Y, Xu X, Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LtgA and LtgB and the extracellular release of LtgA. Infect Immun 2005;73:70–8.
[21] Kumar S, Stecher G, Li M, Knysz C, Tamura K, MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 2018;35:1547–9.
[22] O’Brien NS, Patel DT, Oliver JR, MD, Miller DP, Marconi RT. Development of an FhBb based chimeric vaccinogen that elicits antibodies that block Factor H binding and cleavage by the perisoprogen Treponton denticola. Mol Oral Microbiol 2020.
[23] Almagro Armenteros JJ, Tsirigos S, Sandherby CK, Petersen TN, Winther O, Brunnak S, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019;37:420–3.
[24] Izac JR, Oliver Jr LD, Earnhart GC, Marconi RT. Identification of a linear epitope in the OspA protein of the Lyme disease spirochete that elicits bactericidal antibody responses: Implications for vaccine development. Vaccine 2017;35:3178–85.
[25] Mallerov K, Miller DP, Oliver Jr LD, Freedman JC, Kostick-Dunn JL, Carlyon JA, et al. Cyclic-di-GMP binding induces structural rearrangements in the PtzA and PtzC proteins of the Lyme disease and relapsing fever spirochetes: a possible switch mechanism for c-di-GMP-mediated effector functions. Pathog Dis 2016;74.
[26] Miller DP, Oliver Jr LD, Teglos BK, Reed LA, O’Brien NS, Kurniyati K, et al. The Treponton denticola FhB Protein Is a Dominant Early Antigen That Elicits FhBb Variant-Specific Antibodies That Block Factor H Binding and Cleavage by Dentilisin. Infect Immun 2016;84:2051–8.
[27] Izac JR, O’Brien NS, Oliver Jr LD, Camire AC, Earnhart CG, LeBlanc Rhodes DV, et al. Development and optimization of OspC chimerite vaccinogens for Lyme disease. Vaccine 2020;38:1915–24.
[28] Haake DA, Chao C, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein Lpl32 is a lipoprotein expressed during mammalian infection. Infect Immun 2000;68:2276–85.
[29] Hsieh CL, Ptak CP, Tseng A, Suguura IMS, McDonough SP, Sriratul T, et al. Extended low-resolution structure of a Leptospira antigen offers high bactericidal antibody accessibility amenable to vaccine design. Elife 2017;6.
Izac JR, Camire AC, Earnhart CG, Embers ME, Funk RA, Breitschwerdt EB, et al. Analysis of the antigenic determinants of the OspC protein of the Lyme disease spirochetes: Evidence that the C10 motif is not immunodominant or required to elicit bactericidal antibody responses. Vaccine 2019;37:2401–7.

McDowell JV, Huang B, Fenno JC, Marconi RT. Analysis of a unique interaction between the complement regulatory protein factor H and the periodontal pathogen Treponema denticola. Infect Immun 2009;77:1417–25.

Cianciotto NP, White RC. Expanding Role of Type II Secretion in Bacterial Pathogenesis and Beyond. Infect Immun 2017;85.

Pinne M, Haake DA. Lipl32 Is a Subsurface Lipoprotein of Leptospira interrogans: presentation of new data and reevaluation of previous studies. PLoS ONE 2013;8:e51025.

Verma A, Artiushin S, Matsunaga J, Haake DA, Timoney JF. LruA and LruB, novel lipoproteins of pathogenic Leptospira interrogans associated with equine recurrent uveitis. Infect Immun 2005;73:7259–66.

Matsunaga J, Young TA, Barnett JK, Barnett D, Bolin CA, Haake DA. Novel 45-kilodalton leptospiral protein that is processed to a 31-kilodalton growth-phase-regulated peripheral membrane protein. Infect Immun 2002;70:323–34.

Pinne M, Haake DA. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of Leptospira interrogans. PLoS ONE 2009;4:e6071.

Lesia-Aquino C, Lindow JC, Randall A, Wunder E, Pablo J, Nakajima R, et al. Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis. PloS Negl Trop Dis 2017;11:e0005349.

Silva YRdO, Contreras-Martel C, Macheboeuf P, Dessen A. Bacterial secretins: Mechanisms of assembly and membrane targeting. Protein Sci 2020;29:893–904.

Llanos Salinas SP, Castillo Sánchez LO, Castañeda Miranda G, Rodríguez Reyes EA, Oritoñez López I, Mena Baluñuelos R, et al. GspD, The Type II Secretion System Secretin of Leptospira, Protects Hamsters against Lethal Infection with a Virulent L. interrogans Isolate. Vaccines (Basel) 2020;8:759.

Validi M, Karkhah A, Prajapati VK, Nouri HR. Immuno-informatics based approaches to design a novel multi epitope-based vaccine for immune response reinforcement against Leptospirosis. Mol Immunol 2018;104:128–38.

Fernandes LCV, Teixeira AF, Filho AFS, Souza GO, Vasconcellos SA, Heinemann MB, et al. Immune response and protective profile elicited by a multi-epitope chimeric protein derived from Leptospira interrogans. Int J Infect Dis 2017;57:61–5.

Garba B, Baharman AR, Zakaria Z, Bejo SK, Mutalib AR, Rande F, et al. Antigenic potential of a recombinant polyvalent DNA vaccine against pathogenic leptospiral infection. Microb Pathog 2018;124:136–44.

Oliveira TL, Ruzzi C, da Cunha CEP, Dorneles J, Seixas Neto ACP, Amaral MG, et al. Recombinant BCG strains expressing chimeric proteins derived from Leptospira protect hamsters against leptospirosis. Vaccine 2019;37:776–82.

da Cunha CEP, Bettin EB, Bakry A, Seixas Neto ACP, Amaral MG, Dellagostin OA. Evaluation of different strategies to promote a protective immune response against leptospirosis using a recombinant LigA and LigB chimera. Vaccine 2019;37:1844–52.

Lin X, Chen Y, Yan J. Recombinant multiepitope protein for diagnosis of leptospirosis. Clin Vaccine Immunol 2008;15:1711–4.

Techawiwattanaboon T, Barnier-Quer C, Palaga T, Jacquet A, Collin N, Sangjun N, et al. Reduced Renal Colonization and Enhanced Protection by Leptospirot Factor H Binding Proteins as a Multisubunit Vaccine Against Leptospirosis in Hamsters. Vaccines (Basel) 2019;7.

Marconi RT, Garcia-Tapia D, Hoovers J, Honsberger N, King VL, Ritter D, et al. VANGUARD® crLyme: A next generation Lyme disease vaccine that prevents B. burgdorferi infection in dogs. Vaccine X 2020.

Marconi RT, Honsberger N, Teresa Winkler M, Sobell N, King VL, Wappel S, et al. Field safety study of VANGUARD® crLyme: A vaccine for the prevention of Lyme disease in dogs. Vaccine X 2020;6.