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Bioinformatics and *in-silico* epitope prediction analysis of highly conserved pathogenic *Leptospira* genes

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Abstract

The aim of this study was to identify potential candidates suitable for the development of a multivalent DNA vaccine that can stimulate significant antibody production that will aid the control and prevention of leptospirosis. Antigenic B cell epitopes from highly conserved pathogenic leptospiral genes *lip*L32, *Lip*L41, *omp*L1, *loa*22 and *lig*A were predicted using bioinformatics tools as potential vaccine candidates. The vaccine constructs were composed of the lipopolysaccharide genes (*lip*L32, *lip*L41), the outer membrane protein and outer membrane-like protein (*omp*L1, *loa*22) and the immunoglobulin-like protein (*lig*A). Up to 250 sequences from different isolates with identities ranging from 54% to 100% across all sequences were obtained. The Bepipred software predicted 13 different overlapping and potentially immunogenic regions within the selected genes. This study was able to use a high throughput *in-silico* process in identifying potential vaccine candidates for use in the development of leptospira vaccine.

Keywords: BepiPred, DNA vaccine, Epitope, *In*-silico prediction, Leptospirosis

Introduction

Leptospirosis is recognized as an important public health problem due to the increasing incidence of the disease and its occurrence in epidemic proportions in both developing and developed countries. The disease is an infectious one caused by pathogenic spirochetes of the genus *Leptospira* that has about nine pathogenic species with over 200 antigenically distinct serovars (McBride *et al.,* 2005). Since its initial demonstration by Weil, sporadic outbreaks have occurred throughout the world with fatal outcomes (Adler, 2015). In the past century, several epidemics of leptospirosis have been reported worldwide (Garba *et al.*, 2017; Garba *et al.*, 2018a). Current killed vaccines against leptospirosis are directed against surface-exposed lipopolysaccharide coat of leptospires, which are characterized by small antigenic differences between various strains, thus limiting cross-protection (Bharti *et al.*, 2003). The identification of immunogenic proteins expressed during infection is important for the development of new immune-protective strategies (Guerreiro *et al.*, 2001; Bashiru & Bahaman, 2018). Information on mechanisms involved in immunity against leptospiral infection is still scanty, hence current emphasis is

placed on discovering cross-species protective antigens that can ensure long-lasting protection from multi-species *Leptospira* infection (Zhao *et al.*, 2012; Garba *et al.*, 2018b). Experimental epitope-based vaccines represent an alternative strategy for the development of an effective leptospiral vaccine with heterologous protection against a wide range of serovars. The potential advantages, however, are increasing safety level, ability to rationally engineer epitopes for increased potency as well as focusing immune response on conserved epitopes (Sette & Fikes, 2003; Garba, *et al.*, 2018b). Hence, the objective of this study was to determine the antigenicity of predicted B cell epitope from highly conserved leptospiral genes (*lip*L32, *lip*L41, *omp*L1, *loa*22 and *lig*A). This is because humoral-mediated immunity had been shown to be essential and capable of conferring protection against pathogenic infection in humans, dogs and pigs (Fraga *et al*., 2011).

Materials and Methods

Retrieval of the nucleotide sequence

The complete amino acid sequences of genes *lip*L32 (*L. interrogans* serovar Icterohaemorrhagiae), *lip*L41 and OmpL1 (*L. interrogans* serovar Lai), *loa*22 (*L. interrogans* serovar Grippotyphosa) and *lig*A (*Leptospira kirschneri* serovar Grippotyphosa) were retrieved from the UniProt knowledgebase (UniprotKB) NCBI data base using the following search parameters and filters;

- Data base: UniProtKB/Swiss-Prot- non-redundant protein sequences (nr)DBSOURCE: UniProtKB: locus Q72SM7_LEPIC, accession Q72SM7
- Max E-value: 1e-1

The search was conducted in all five (5) genes under the entry UniProtKB/TrEMBLNCBI and protein accession numbers for each was retrieved (Q72SM7; AAP04735; AAT48511; AAT48493; AGH20068). All the sequences were analyzed on BLAST using UniProtKB BLASTP, Matrix: Blossum 62 and threshold 10.

Multiple sequence alignment

The retrieved protein sequences were subjected to Multiple Sequence Alignment with Multiple Sequence Comparison by Log-Expectation (MUSCLE). Muscle uses two distance measures: a *k* mer distance for unaligned sequence pairs and a Kimura distance for aligned pairs (Edgar, 2004). Excess sequences were trimmed and consensus sequences for all five genes were generated from each data set after removing sequences with gaps or ambiguities.

Phylogenetic analysis

Neighbour-Joining trees were constructed with 2000 bootstrap value using MEGA6 software (Tamura *et al.,* 2013). The amino acid poissons correction model with complete deletion of gaps was used. The selected 2000 bootstrap replication is to estimate the reliability of the phylogenetic tree by giving an accurate representation of the historical branching order of the sequences. The purpose of the molecular phylogenetic tree was to estimate the relationships among the species represented by the sequences and to understand the relationships among the sequences themselves regardless of the host species.

Physicochemical parameters

The amino acid composition, molecular weight, instability index, aliphatic index and grand average of hydropathicity of the protein sequences were analyzed using the ProtParam tool (Gasteiger *et al.,* 2003). The Swiss-Prot/TrEMBL accession number for each of the protein sequence was inputed and the search was done for the entire sequence based on default settings.

Linear B cell epitope prediction

Computational analysis of the consensus sequences of the identified genes to map potential B-cell epitopes was done using the IEDB Bepipred 1.0 prediction server for the prediction of the location of linear B cell epitopes based on a combination of hidden Markov model and a propensity scale method (Larsen *et al.,* 2006). Based on the physicochemical properties of the sequences analyzed, the IEDB BepiPred software program (Jespersen *et al.,* 2017) was used to predict B-cell epitopes. VaxiJen v2.0, a server for the identification of immunogenic antigens for use in the development of subunit vaccines was used for the determination of highly antigenic proteins (Doytchinova & Flower, 2007).

Analysis of variability or conservation of epitopes

The IEDB conservancy analysis tool was used to scrutinize the selected epitopes. The tool is capable of analysing the conservancy or variability of epitopes within a given set of the protein sequence (Bui *et al.*, 2007). The analysis was done based on epitope linear sequence conservancy and the sequence identity threshold was set at default (≥ 100%).

Results

The retrieved protein sequences analyzed with BLASTP tool in the NCBI database yielded 250 similar searches after adjusting the algorithm parameter for

searches up to 250 isolates identities ranging from 54% to 100% across all sequences. The selected genes and their accession numbers are: *lip*L32- Q72SM7; *lip*L41- AAT48511; *omp*L1- AAT48493; *loa*22- AGH20068 and, *lig*A- AAP04735. From the BLAST results, nine isolates (sequences) associated with human and animal infections were selected. These were *Leptospira interrogans, L. borgpetersenii, L. kirshneri, L. kmety, L. noguchi, L. santorasai, L. weilii, L. alstoni and L. alexanderii*. The sequences were aligned and a phylogenetic tree was constructed. From the resultant aligned results, consensus amino acid sequence was obtained based on the accession number of the selected proteins with LipL32 having 272 aa, LipL41 (355 aa), OmpL1 (320 aa), Loa22 (195 aa) and LigA (1224 aa).

Multiple sequence alignment by MUSCLE revealed the presence of *indels* (insertions-deletions polymorphism) across all gene sequences aligned although with very high similarity (Figure 1). The average percent amino acid identity in pairwise comparison was 0.0305 corresponding to 90% identity for *lip*L32. In other words, it indicates that the sequences are accurately aligned and could produce a reliable phylogenetic tree (Thompson *et al.,* 1999). There were mutations in *lig*A occurring either as deletions or insertions when compared with sequences from eight pathogenic *Leptospira* species in the NCBI data-base such that percentage identity ranged from 53-100%. Average percent amino acid identity in pairwise comparison was 0.3533. Duplicate sequences were detected using MEGA6 pairwise distance. Average percent amino acid identity in pairwise comparison for OmpL1 and Loa22 which both belong to the OMP family with 87-100% similarity across all pathogenic species considered in this study was 0.5716 and 0.0511 corresponding to 80% and 92% respectively. Finally, LipL41 which is an outer membrane lipopolysaccharide had 0.0553 amino acid identity using pairwise comparison.

The phylogenetic analysis was conducted by using the *lip*L32 gene which is the most abundant gene among pathogenic *Leptospira* species and it is a conserved gene. The analysis on the selected sequences shows that they all belong to the pathogenic *Leptospira* group (*L. interrogans, weilii, borgpetersenii, santorasai, kmety, kirshneri* and *noguchii*). Evolutionary analyzes were conducted in MEGA6. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.09244552 is shown in Figure 2. The evolutionary distances were computed using the poisson correction method because the analysis was conducted using amino acid sequences and are in the units of the number of amino acid substitutions per site. The analysis involved 8 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 266 positions in the final data set. The ProtParam results represent the physicochemical properties of the proteins. LipL32 had 272 aa, LipL41 355 aa, OmpL1 320 aa, Loa22 195 aa and LigA 1224 aa as shown in Table 1. Since a protein with instability index lower than 40 is stable, only Loa22 was found to have instability index above 40. Similarly, Loa22 had a lower aliphatic index which represents the volume occupied by aliphatic side chains (alanine, valine, isoleucine and leucine) compared to the other genes. The result also shows that the proteins were highly immunogenic and stable.

The IEDB Bepipred predicted 13 different overlapping and potentially immunogenic regions

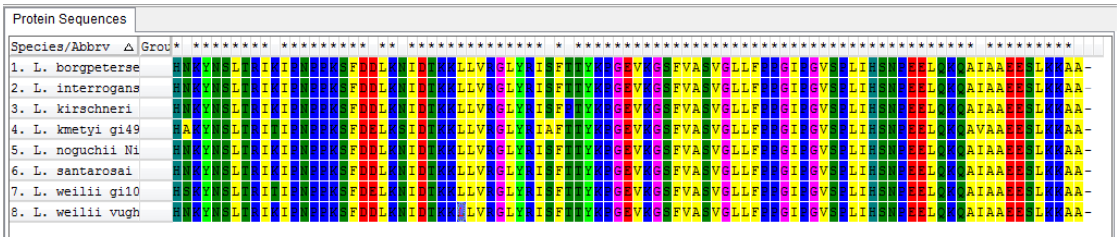

Figure 1: Multiple sequence alignment for N-terminal portion of LipL32 gene (haemolysis associated protein for *L. interrogans, L. borgpeeterseni, L. kirschneri, L. kmetyi, L. noguchi, L. santorasai and L. weilii*) showing high and low similarities between sequences aligned

within the LipL32, LipL41, OmpL1, Loa22 and LigA proteins respectively. BepiPred predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method. The residues with scores above the threshold (default 0.35-sensitivity and specificity are maximal in BepiPred) are predicted to be part of an epitope and coloured in yellow on the graph (where Y-axis depicts residue scores and X-axis residue positions in the sequence) as shown in Figures 3 and 4 for gene(s) *lip*L32 and *lip*L41 of pathogenic leptospira respectively. For each input sequence, the server returns a prediction score and annotation for every residue. The positions of the linear B-cell epitopes are predicted to be located at the residues with the highest scores. The predicted epitope was subjected to VaxiJen analysis which is an independent alignment approach for antigen prediction which is based on auto cross covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties. Predicted epitopes and their scores are given in Table 2.

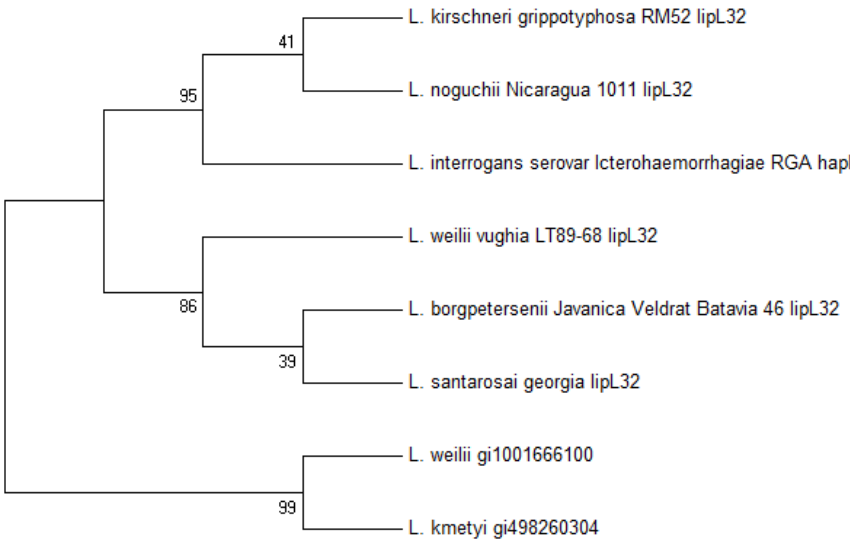

Figure 2: Phylogenetic tree for LipL32 gene using a neighbour joining method based on amino acid sequences. The figure shows all amino acid residues having 100% match with the nine commonly isolated leptospires

Table 1: Physicochemical properties of protein sequences based on the ProtParam results

Parameters	Genes				
	LipL32	LipL41	OmpL1	Loa22	LigA
Molecular weight	29612.9	38939.7	33461.0	20884.5	124947.3
Theoretical Pi	6.34	6.01	8.91	8.56	6.26
Instability Index	32.53	26.19	27.6	47.70	22.82
Aliphatic Index	85.74	87.15	89.09	78.21	88.46
Grand average of hydropathicity	-0.257	-0.244	0.099	-0.430	-0.109
Instability Index	32.53	26.19	27.6	47.70	22.82

Bepipred prediction result identified 5 epitopes as antigenic for *lipL32* (Table 2). VaxiJen 2.0 further analyzed these and the two highly antigenic epitopes selected were YVKPGQAPDGLVDGNK at position 62-77 and IAKAAKAKPVQKLDDDDDGDDTYKEERHNK at position 148-177 respectively. The two selected epitopes can be seen to have a larger score on the Y-axis (Figure 3) which is interpreted as the residues with the higher probability of being part of an epitope. Epitope conservancy analysis using the IEDB programme shows that YVKPGQAPDGLVDGNK (62-77) had the higher identity score (87%) and IAKAAKAKPVQKLDDDDDGDDTYKEERHNK (148-177) hadh 75%.

LipL41 epitopes predicted by Bepipred yielded five antigenic epitopes with scores ranging from 0.8006 to 0.9825 (Table 2). The threshold for the antigen to be immunogenic according to VaxiJen server is 0.4; hence selected epitope were analyzed further by IEDB epitope conservancy analysis tool. The epitope prediction graph also shows the interaction of the predicted epitopes with surface membrane, which indicates their binding properties with the antigens of the bacterial organism (Figure 4).

Furthermore, epitope conservancy analysis indicated that PVFPKDKEGR, ATGKDVNTGNEPVSKPTG, KPYTECSTENKID are 87.5%, 50% and 62.5% common in *lip*L41 gene of all the pathogenic *Leptospira* isolates analyzed in this study (Table 2).

In this study, three epitopes were selected after BepiPred prediction and VaxiJen analysis. They represent the C-terminal portion of the gene. Epitope conservancy analysis shows that NASDSHG, GGIQGSTDFK and ASGEEGRGKAIS had 60% and 100%

minimum and maximum matches across the eight pathogenic *Leptospira* species used in this study.

OmpL1 and Loa22 are both an outer membrane and outer membrane-like proteins of Leptospira and they are transmembrane proteins expressed during natural infection with pathogenic leptospires. The combined B cell epitopes predicted by BepiPred and VaxiJen is given in Table 2. Epitope conservancy analysis shows that SDGTDPVTTR had 33.33% sequence match while AVGKTQSVGGATNLSPFPA and WSLNGSNNIKG had 47.37% and 33.33% protein sequence matches for OmpL1 gene while Loa22 epitopes AEKKEESAAPEPSAQEQSAAANRNVDVNSPEAIADS, TDAIGPEQAEGAKK and GVGSSEPVSGLDAKDAKN had 22.22%, 55.56% and 11.11% equivalent to 55.56%, 93.33%, 88.89% minimum identity/protein sequence matches.

Discussion

Bioinformatics has given room for selecting potential epitopes without the risk involved in propagating the pathogen of interest (Bashiru & Bahaman, 2018). This technique represents a considerable advantage over conventional methods of vaccine production in addition to faster output and lower cost (Soria-Guerra *et al.*, 2015). The efficacy and safety of whole cell bacterins in preventing human and animal diseases has been reported in several countries (Martínez Sánchez *et al.*, 2000; McBride *et al.*, 2005; Chang *et al.*, 2007; Garba *et al.*, 2018b). Due to their inability to elicit long term immunity against different pathogenic serovars, efforts have shifted to search for subunit vaccine candidates that can provide heterologous protection (Zuerner *et al.*, 2000). Several outer membrane proteins and immunoglobulin-like proteins have been reported to provide protection against challenge with several *Leptospira* organisms (Lottersberger *et al.*, 2009). Many of the protections claimed are not clear cut, due to inappropriate statistical analysis, inadequate challenge dose and virulence of the challenge strain and number of animals used (Adler and Klaasen, 2015). These limitations observed by Adler were put into consideration during the design of this present study, which eventually led to the development of novel multi-epitope gene constructs.

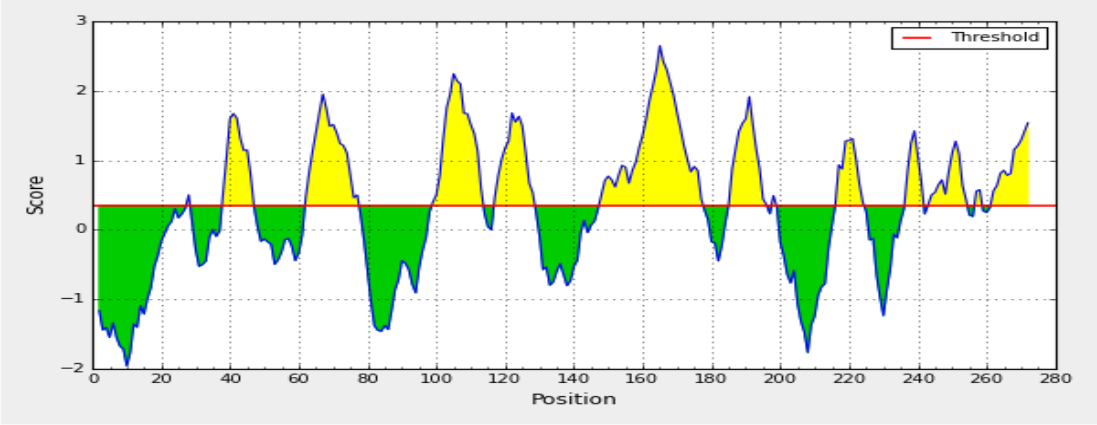

Figure 3: Predicted B cell epitopes for gene LipL32 of pathogenic leptospira showing potentially antigenic epitopes (yellow peaks) above the threshold (red line)

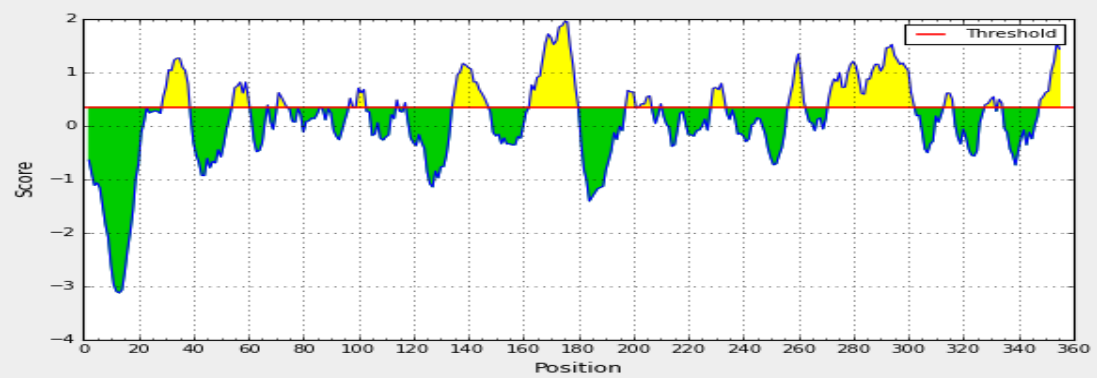

Figure 4: Predicted B cell epitopes for gene LipL41 of pathogenic leptospira showing potentially antigenic epitopes (yellow peaks) above the threshold (red line)

Table 2: BepiPred predicted epitopes from the selected conserved genes

Genes	Proteins/epitopes	Amino acid position	VaxiJen score
lipL32	YVKPGQAPDGLVDGNK	62-77	0.9096
	IAKAAKAKPVQKLDDDDDGDDTYKEERHNK	148-177	1.2556
lipL42	ATGKDVNTGNEPVSKPTG	163-180	0.8006
	VEAPEKS	54-60	0.9825
	PVFPKDKEGR	29-38	0.9697
ligA	NASDSHG	365-371	2.4576
	GGIQGSTDFK	388-397	1.5995
	ASGEEGRGKAIS	725-736	2.5603
Ompl1	WSLNGSNNIKG	206-216	1.5026
	SDGTDPVTTR	234-243	1.2271
	AVGKTQSVGGATNLSPFPA	284-302	0.9276
loa22	AEKKEESAAPEPSAQEQSAAANRNVDVNSPEAIADS	24-59	1.1122
	HTDAIGPEQAEGAKK	119-133	1.2149
	GVGSSEPVSGLDAKDAKN	164-181	1.4977

Sequences were retrieved from the UniProt knowledge base. Sequences with significant identity were aligned with MUSCLE in the MEGA6 software, trimmed and a consensus sequence for each gene was obtained. A reverse vaccinology approach using bioinformatics to predict highly conserved surface exposed immunogenic epitopes was employed. B cell epitopes are recognized by B cell receptors or antibodies in their native structure. Continuous B cell epitope prediction is based on the amino acid properties such as hydrophilicity, charge, exposed surface area and secondary structure (Soria-Guerra *et al.*, 2015). However, using a single scale amino acid propensity profile is not sufficient to predict epitope location reliably, hence the use of BepiPred which is a combination of hidden Markov model and propensity scale has been shown to improve prediction accuracy compared to single model (Blythe & Flower, 2005; Garba *et al.*, 2018b).

In this study, all the genes considered belong to the pathogenic *Leptospira* group and their antigenic epitopes are more likely to be accessible by antibodies because they are surface exposed (Haake *et al.*, 1999). To identify vaccine epitopes that can generate cross species and cross serovar protection against a diverse group of serovars, phylogenetic analysis and epitope conservancy analysis were conducted to identify epitopes that are conserved among nine pathogenic species involved in most of the infections (Hu *et al.*, 2014). All selected epitopes had 100% amino acid residue match with sequences from nine of the pathogenic *Leptospira* isolates. In an epitope-based vaccine strategy, the use of conserved epitopes would be expected to provide broader protection across multiple strains or even species, than epitopes derived from highly variable genome regions (Bui *et al.*, 2007).

Although the threshold for VaxiJen is 0.4, in this present study only epitopes with a score of 0.8 and above was considered for inclusion in the design of the vaccine. The selection of epitopes with high score is to overcome the limitation of *In-silico* techniques, which occasionally score non-epitopes as epitopes and vice versa (Soria-Guerra *et al.*, 2015). Prediction of epitope peptides is essential not only in diagnostics but also for the vaccine and these small segments of proteins are sufficient in eliciting a desired immune response (Ramasamy *et al*., 2014). The physicochemical analysis shows that the instability index for all the proteins were: 32.53, 26.19, 27.6, 22.82 for LipL32, LipL41, OmpL1 and Loa22 respectively, implying that the proteins are stable except for Loa22 which is unstable with 47.7. This indicates that the net protein charge is zero (the isoelectric point of the protein) and could lead to easy degradation of the protein. Another possible reason could be that because the protein is rich in Pro (P-6.85), Glu (E-8.1%), Ser (S-8.1%) and Thr (T-4.7) which are termed PEST, they are prone to degradation. However, fusing the protein with a tag or a fusion partner can change the N-terminal sequence of the protein and therefore increase the yield and stability of the protein (Singh *et al.*, 2013).

In conclusion, **o**ne of the most innovative strategies in vaccinology in recent time is DNA vaccine against infectious diseases including leptospirosis. In this

study, high throughput *in-silico* process in determining potential vaccine candidates against were predicted from five highly conserved, surface-exposed pathogenic *Leptospira* genes.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

Adler B (2015). Vaccines Against Leptospirosis. In: Leptospira and Leptospirosis (B Adler, editor). *Current Topics in Microbiology and Immunology*, doi.org/10.1007/978-3-662-45059-8_10.

Adler B & Klaasen E (2015). Recent advances in canine leptospirosis: Focus on vaccine development. *Veterinary Medicine: Research and Reports*, doi: 10.2147/VMRR.S59521

Bashiru G & Bahaman AR (2018). Advances and challenges in leptospiral vaccine development. *Indian Journal of Medical Research*, **147**(1): 15-22.

Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E & Vinetz JM (2003). Leptospirosis: A zoonotic disease of global importance. *The Lancet Infectious Diseases*, **3**(12): 757–771.

Blythe MJ & Flower DR (2005). Benchmarking B cell epitope prediction: Underperformance of existing methods. *Protein Science*, **14**(1): 246–248.

Bui HH, Sidney J, Li W, Fusseder N & Sette A (2007). Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. *BMC Bioinformatics*, **8**(1): 361.

Chang YF, Chen CS, Palaniappan RU, He H, McDonough SP, Barr SC, Yan W, Faisal SM, Pan MJ & Chang CF (2007). Immunogenicity of the recombinant leptospiral putative outer membrane *Leptospira* infection was performed. Thirteen potentially immunogenic epitopes as listed in table 3 proteins as vaccine candidates. *Vaccine*, **25**(48): 8190–8197.

Doytchinova IA & Flower DR (2007). VaxiJen: A server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics*, **5**(8): 4.

Edgar RC (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, **19**(5): 113.

Fraga TR, Barbosa AS & Isaac L (2011). Leptospirosis: Aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. *Scandinavian Journal of Immunology*. **73**(5): 408-419.

Garba B, Bahaman AR, Khairani-Bejo S, Zakaria Z & Mutalib AR (2017). Retrospective study of leptospirosis in Malaysia. *EcoHealth*, **14**(2): 389–398.

Garba B, Bahaman AR, Bejo SK, Zakaria Z, Mutalib AR & Bande F (2018a). Major epidemiological factors associated with leptospirosis in Malaysia. *Acta Tropica*, doi.org/10.1016/j.actatropica.2017.12.010.

Garba B, Bahaman AR, Zakaria, Bejo SK, Mutalib AR, Bande F & Suleiman N (2018b). Antigenic potential of a recombinant polyvalent DNA vaccine against pathogenic leptospiral infection. *Microbial Pathogenesis*, doi.org/10.1016/j.micpath.2018.08.028.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD & Bairoch A (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, **31**(13): 3784–3788.

Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvão Reis M, Levett PN, Ko AI & Haake DA (2001). Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. *Infection and Immunity*, **69**(8): 4958–4968.

Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J & Wagar EA (1999). Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infection and Immunity*, **67**(12): 6572–6582.

Hu W, Lin X & Yan J (2014). Leptospira and leptospirosis in China. *Current Opinion in*

Infectious Diseases, **27**(5): 432–436.

Jespersen MC, Peters B, Nielsen M & Marcatili P (2017). BepiPred-2.0: Improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Research*, **45**(1): 24–29.

Larsen JEP, Lund O & Nielsen M (2006). Improved method for predicting linear B-cell epitopes. *Immunome Research*, **2**(2): 1-7.

Lottersberger J, Guerrero SA, Tonarelli GG, Frank R, Tarabla H & Vanasco NB (2009). Epitope mapping of pathogenic Leptospira LipL32. *Letters in Applied Microbiology*, **49**(5): 641–645.

Martínez Sánchez R, Pérez Sierra A, Baró Suárez M, Alvarez AM, Menéndez Hernández J, Díaz González M, Cruz de la Paz R, de los Reyes G, Montoya Batista B, Sierra González G, Armesto del Río M, Saltarén Cobas A & Sabournin Ramos O (2000). Evaluation of the effectiveness of a new vaccine against human leptospirosis in groups at risk. *Revista Panamericana de Salud Pública; Pan American Journal of Public Health*, **8**(6): 385–392.

McBride AJA, Athanazio DA, Reis MG & Ko AI (2005). Leptospirosis. *Current Opinion in Infectious Diseases*, **18**(5): 376–386.

Ramasamy Victor AA, Sunil A, Manimaran NC, Madanan GM & Jebasingh T (2014). Phylogenetic characterization and threading based-epitope mapping of leptospiral outer membrane lipoprotein Lipl41. *Journal of Proteomics and Bioinformatics,* **07**(08). 222-231.

Sette A & Fikes J (2003). Epitope-based vaccines: An update on epitope identification, vaccine design and delivery. *Current Opinion in Immunology*, **15**(4): 461–470.

Singh H, Ansari HR & Raghava GPS (2013). Improved method for linear B-cell epitope prediction using antigen's primary sequence. *PloS One*, **8**(5): e62216.

Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO & Rosales-Mendoza S (2015). An overview of bioinformatics tools for epitope prediction: Implications on vaccine development. *Journal of Biomedical Informatics*. doi.org/10.1016/j.jbi.2014.11.003**.**

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30** (12): 2725–2729.

Thompson JD, Plewniak, F & Poch O (1999). A comprehensive comparison of multiple sequence alignment programs. *Nucleic Acids Research*, **27**(13): 2682–2690.

Weil A (1886). A strange acute infection with spleenomegaly, icterus and nephritis [Ueber eine eigenthümliche, mitMilztumor, Icterus und Nephrits einhergehende, akuteInfektionskrankheit]. *Deutsches Archiv für klinische Medizin*, **39**: 209–232.

Zhao C, Sun Y, Zhao Y, Wang S, Yu T, Du F, Yang XF & Luo E (2012). Immunogenicity of a multi-epitope DNA vaccine against hantavirus. *Human Vaccines and Immunotherapeutics*, **8**(2): 208–15.

Zuerner R, Haake D, Adler B & Segers R (2000). Technological advances in the molecular biology of Leptospira. *Journal of Molecular Microbiology and Biotechnology*, **2**(4): 455–462.