ANALYZING 16S rRNA SEQUENCES FROM VIETNAMESE PATHOGENIC LEPTOSPIRA STRAINS AND IN-SILICO PREDICTION OF POTENTIAL ANTIGENIC EPITOPES ON LIPL21, LIPL32 OUTER MEMBRANE LIPOPROTEINS

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SUMMARY

Leptospirosis, a zoonosis caused by \textit{Leptospira}, is recognized as an emergent infectious disease. In currently, the lack of adequate diagnostic tools, vaccines are an attractive intervention strategy. In this experiment, a 550 bp fragment of large ribosomal RNA gene (16S rRNA) was sequenced and constructed phylogenetic tree from a panel of six Vietnamese pathogenic strains of \textit{Leptospira} spirochetes (e.g., Pomona, Canicola, Mitis, Icterohaemorrhagiae, Bataviae, and Grippotyphosa). The results showed a close relationship of \textit{L.Pomona VN} and \textit{L.Hardjo} (bootstrap: 99%). \textit{L.Canicola VN} and \textit{L.Icterohaemorrhagiae VN} appeared to be weak related to the classic \textit{L.Canicola}, \textit{L.Grippotyphosa}, these assemblage have a bootstrap support of 62%. The other strains (\textit{L.Mitis VN} and \textit{L.Grippotyphosa VN}) were appeared monophyletic, while their sister group (\textit{L.Bataviae VN}) relationship found only weak support (bootstrap: 62%). We also selected six genes [e.g. the immunoglobulin like proteins A and B (LigA and LigB genes), outer membrane protein (OmpL1 gene), and lipopolysaccharide (Lipl32, Lipl41, and Lipl21 genes)] and checked gene expression in these \textit{Leptospira} strains by polymerase chain reaction (PCR) method. There were three genes (e.g., Lipl32, Lipl21, and LigA genes) expressed in all strains, OmpL1 gene occurred in 4 strains (L.Bataviae VN, L.Canicola VN, L.Grippotyphosa VN and L.Mitis VN), whereas Lipl41 and LigB genes did not appear in any \textit{Leptospira} strains. A multi-antigenic epitope potential of two gene (Lipl21 and Lipl32) was predicted by bioinformatic tools for designing a recombinant vaccine against leptospirosis. There were 3 multi-epitope regions (1 region and 95 antigenic epitope for B and T cells of Lipl21 peptide; 2 regions and 124 antigenic epitope for both B and T cells of Lipl32 peptide). It should be more of the deeply molecular biology studies to confirm the level agglutinating, antigen cleavage, peptide specificity matrices as well as neutralizing antibodies in the immune responses of DNA vaccine of these genes.

Keywords: Antigenic genes, Leptospiraceae, Leptospirosis recombinant vaccine, 16S rRNA gene sequencing

INTRODUCTION

Leptospirosis constitutes an important public health problem in developing countries particularly those that are impoverished. The disease is responsible for economic losses in animal production as well as exerts a burden on human health. The World Organisation for Animal Health (OIE) manages leptospirosis in 2\textsuperscript{nd} group of the most dangerous diseases (OIE, 2008) and currently it adds to the group of occupational diseases in Vietnam (TLDLVN, 1991). Leptospirosis commons everywhere in the world, mostly in Africa, South America, and Asia regions, causing considerable damage to livestock and human health in many countries, especially the tropics (Vanasco \textit{et al.}, 2008). According to the World Health Organization (WHO) estimates that there are annually from 7 to 10 million people in the worldwide infected with the \textit{Leptospira interrogans} (NHS, 2014). In developing countries, the disease is mainly farmers and poor people in the city, especially, people often working outdoors in warm and humid places have the risk of infection. The disease transmits to humans through mucus, skin, and eyes, which exposure to infected animal urine (Slack, 2010). Currently, pathogenic
leptospirae are classified into nine pathogenic and four intermediate species, containing more than 260 serovars, and six saprophytic species, including over 60 serovars (Adler, de la Pena Moctezuma, 2010; Levett, 2001). The worldwide leptospirosis disease burden estimates are hampered by the lack of scientifically data from countries with probable high endemicity and limited diagnostic capacities. In Sri Lanka (2008) 404 possible cases were defined, in which 155 were confirmed to have leptospirosis with serovars Pyrogenes, Hardjo, Javanica, and Hebdomadis (Agampodi et al., 2011). Leptospirosis has been prevalent sporadically in China in recent years, and Leptospira interrogans Icterohaemorrhagiae serovar Lai is the most common pathogen in Chinese leptospirosis patients and Apodemus agrarius is its major animal host (Hu et al., 2014). Nowadays, there are several highlight reviews in the leptospirosis, e.g. susceptible population, disease transmission and epidemiology, treatment, trends and advances in diagnosis, vaccines and vaccination strategies in humans and animals. Many molecular tools like PCR-RFLP, real-time PCR, multiplex PCR, qPCR and immunocapture PCR have been found useful for rapid and confirmatory detection and differentiation of pathogenic and non-pathogenic leptospires to against all emerging pathogens with success stories. Available vaccines can only provide limited protection, which is short lived. The most commonly used leptospiral vaccines are based on inactivated whole-cell bacterins or leptospiral cell membrane components. These preparations although effective, are associated with several side-effects like pain, irritation and discomfort in addition to limited protection. In addition, the vast majority of the vaccines are for animal use, albeit for few countries that have licensed bacterins for human use (Silveira et al., 2017). As a result, the last few decades have seen research efforts shifting focus to the classical identification of antigens for the development of recombinant vaccines against leptospirosis. While significant progress have been recorded, the development of a broad-range leptospiral vaccine still remains elusive (Dellagostin et al., 2017).

The advanced techniques like recombinant DNA technology, reverse genetics, DNA vaccination, molecular genetics and proteomics approaches are being explored for search of novel antigens, proteins and genes as potential candidates to discover safer, efficient and better vaccines for leptospirosis (Verma et al., 2012). The recent advancements of recombinant outer membrane protein (OMP) vaccines, lipopolysaccharide (LPS) vaccines and DNA vaccines against leptospirosis are also reviewed (Dellagostin et al., 2011). Moreover, a vaccine ontology database was built for the scientists working on the leptospirosis vaccines as a starting tool (Wang et al., 2007). DNA vaccines containing multi-epitope encoded gene have been reported to confer protection against many infectious diseases (Ding et al., 2012; Sela-Culang et al., 2015; Sette, Fikes, 2003). DNA vaccine stimulates antibody production in a similar way as foreign antigens are processed and presented during natural infection. Similarly, multi-epitope DNA vaccines were reported to induce more potent immunoreactions than whole protein vaccines (Zhao et al., 2012). Such vaccines are also able to induce powerful cross-reactive immunological response due to the fact they are derived from multiple antigens packaged into a relatively small chimeric molecule. Moreover, immunity induced by multi-epitope DNA vaccine includes both the humoral and cellular immune component. Hence, they are considered suitable for protection against a wide range of serovars, especially in endemic regions. The construction of such highly complex synthetic vaccine may potentially have higher efficacy than assembly of naturally occurring sequences (Yu et al., 2015). Chemically synthesized genes could produce a significant impact on the immuno-reactivity against diverse leptospiral outer membrane proteins (Rollauer et al., 2015; Wang et al., 2002). Overall, recognition of special clinical symptoms of leptospirosis and determination exactly the target immune factors are important not only to have rapid diagnosis and treating in time but also to reduce risks of fatality by vaccination.

MATERIALS AND METHODS

Leptospira strains, cultivation, and serogroup identification

Among twelve epidemiologically related Leptospira strains collected from human or rats in Leptospira outbreaks in Vietnam, we selected six Leptospira strains that were used to make the inactivated vaccine currently in Vietnam, including L.Pomona_VN, L.Canicola_VN, L.Mitis_VN, L.Icterohaemorrhagiae_VN, L.Bataviae_VN, and L.Grippotyphosa_VN. Serogroup identification of these leptospiral
strains was carried out by Microscopic agglutination test (MAT) with 12 international standard serogroup-specific rabbit antisera from the National Center for Veterinary Diagnosis (NCDV), Vietnam. All of the 6 strains were maintained by the NCDV. Leptospires were stored long-term at −70°C and have been passaged every six months. When needed, they were subcultured in 10ml Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium at 30°C for 7-10 days to stationary phase and checked for the presence of contaminating aerobic bacteria by overnight culture on 8% (w/v) horse-blood agar at 30 and 37°C (Ellinghausen, McCullough, 1965).

Total genomic DNA extraction

Genomic DNA was extracted using Phenol:Chloroform:Isoamyl (25:24:1) (Sambrook, Russell, 2001) and was diluted to a final concentration of 20 ng/ml. All total DNA samples were quality and quantity checks by electrophoresis of 3 µl of each reaction on 1% agarose gel for 30 min at 100V and Nanodrop.

Sequence assembly and alignment using 16S rDNA gene sequencing

16S rRNA PCR products (Table 1) were purified with mini spin columns provided in GeneJET™ PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. The PCR products were sequenced according to dideoxynucleotide chain termination method (Sanger et al., 1977). Sequencing PCR reactions were performed on each template on the automated ABI PRISM 3500 DNA Sequencer (Applied Biosystems) at Institute of Genome Research, Vietnam Academy of Science and Technology, Vietnam. Amplified fragments were set up in 10 µl reaction volumes (2 µl BigDye Terminator v3.1, 1 µl BigDye Sequencing buffer, 1 µl primer (10 µM), 2 µl DNA, and 4 µl water). Sequencing was carried out according to the following procedure: denaturation at 96°C for 1 min, and 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, elongation at 60°C for 4 min. Then the samples were purified and incubated with 20 µl Hi-Di, and denatured at 98°C for 2 min. Finally, these samples transferred to plate and sequenced with the sequencer using POP6 and a 36 cm capillary array. The run module conditions were as follows: 22 s for injection time, 1 kV for injection voltage, 15 kV for run voltage, 10 Amps for run current, and 55°C for run temperature.

| Gene name | Sequences F | Sequences R | Size (bp) | Tm (°C) |
|-----------|-------------|-------------|----------|---------|
| 16S rRNA  | F GCCTACGGGAGGCAGCAG | R CCCTCACTCTCCACGAG | 550 | 50°C for 30s |
| OmpL1     | F TTGATTTGACTACTGATAGTTTTA | R AAGGAAGGCTATGATCCTAAGT | 960 | 56°C for 50s |
| LipL32    | F TTACCGCTGAGGTGTCCTTATTCCAGC | R TTGTAACCCGGGTGTAATGTCCTCAGA | 782 | 50°C for 30s |
| LipL41    | F AGAGAAGATAATTTTCTCCCGATGGAACCA | R GAAAGCAACGCGCGATGGTAATCGC | 1065 | 50°C for 50s |
| LipL21    | F ATGATCGATAGCTTTAGTGA | R TTATGTGGGACACGCTTGA | 560 | 50°C for 60s |
| LigA      | F CGGCCGCTATCAGATCCAGCA | R CGCGGCGCGTTATGTTTAGA | 991 | 50°C for 50s |
| LigB      | F CGCGCGCTATCGCTGTTTATGAAAGAA | R ACTCTCGAGCGATATTAGGAAT | 620 | 50°C for 50s |

Sequencing analysis

To explore the genetic diversity and evolutionary relationship between the isolates in Vietnam and other countries, twenty-four accessible *Leptospira* species reference sequences obtained from GenBank database were added into our analysis (Table 2). The sequences of all the *Leptospira* strains in this study and the twenty-four representative sequences from GenBank were compared using CLUSTALW multiple alignments and Phylogenetic analysis was conducted with MEGA 6.0. The
Maximum Likelihood method based on Kimura 2-parameter model was constructed using bootstrapping at 1,000 bootstrap replications (Kim et al., 2002).

**Table 2.** Other *Leptospira* species references from GenBank database.

| Strains             | Serovar          | Accession number (NCBI GenBank) |
|---------------------|------------------|---------------------------------|
| *Leptospira interrogans* | Hardjo-prajitno  | JQ765630.1                      |
| *Leptospira interrogans* | Hardjo-prajitno  | CP013147.1                      |
| *Leptospira interrogans* | Hardjo           | CP012603.1                      |
| *Leptospira interrogans* | Kennewicki       | FJ154571.1                      |
| *Leptospira interrogans* | Pyrogenes DB60   | JQ988842.1                      |
| *Leptospira interrogans* | Pomona DB37      | JQ988858.1                      |
| *Leptospira interrogans* | Pomona           | KR091971.1                      |
| *Leptospira interrogans* | Copenhageni      | NR 074524.1                     |
| *Leptospira interrogans* | Copenhageni      | KR091970.1                      |
| *Leptospira interrogans* | Linhai           | CP006723.1                      |
| *Leptospira interrogans* | Grippotyphosa    | JQ906628.1                      |
| *Leptospira interrogans* | Grippotyphosa    | JQ906625.1                      |
| *Leptospira interrogans* | Saxkoebing       | KR107202.1                      |
| *Leptospira interrogans* | Manilae          | CP011931.1                      |
| *Leptospira interrogans* | Canicola         | KU053945.1                      |
| *Leptospira interrogans* | Canicola         | KR080516.1                      |
| *Leptospira interrogans* | Bratislava       | CP011410.1                      |
| *Leptospira interrogans* | Bratislava-DB38  | JQ988859.1                      |
| *Leptospira interrogans* | Lai              | NR 074481.1                     |
| *Leptospira interrogans* | Australis-DB42   | JQ988863.1                      |
| *Leptospira interrogans* | Icterohaemorrhagiae -DB69 | JQ988845.1 |
| *Leptospira interrogans* | Icterohaemorrhagiae | FJ154563.1 |
| *Leptospira interrogans* | Bataviae-DB59    | JQ988841.1                      |
| *Leptospira interrogans* | Muenchen         | FJ154565.1                      |

**Gene expression**

Gene expressions were performed based on six genes including the immunoglobulin like proteins A and B (*LigA* and *LigB* genes), outer membrane protein (*OmpL1* gene), and lipopolysaccharide (*LipL32, LipL41, and LipL21* genes). The genes were amplified in PCR fragments generated with specific primer pairs (Table 1). PCR was conducted using the following parameters: an initial denature step at 94°C for 3 min, followed by 30 cycles of specific primer pairs (Table 1). PCR was conducted using the following parameters: an initial denature step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 - 50 s, 50°C - 56°C for 30 - 50 s, 72°C for 40 - 60 s, then 72°C for 7 - 10 min. The PCR products were visualized on 1% TBE agarose gel electrophoresis and ethidium bromide stained to check the gene expressions.

**In-silico prediction and selection of vaccine epitopes**

This study used DNA were sequencing from six strains of *Leptospira* by 454 sequencing system. ExPASy tool ([https://web.expasy.org/translate/](https://web.expasy.org/translate/)) was used to translate DNA sequence to protein sequence. Protein sequences suitable to predict epitope were selected by aligning protein sequences on BioEdit software (version 7.2.5) (Hall et al., 2011). Multiple sequence alignment was done for all retrieved sequences using Bioedit software to determine the conserved region so as to predict the only conserved epitopes that might act as a peptide vaccine. In addition, to avoid the epitopes located in the signal peptide region, SignalP 3.0 Server ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) was used to predict the signal peptides. The detection of T and B cell epitopes were predicted based on the amino acid sequences of LipL21 and LipL32.
3D structure prediction

I-TASSER Server was used to predict protein 3D structures (Zhang, 2008). The best results were analyzed by ElliPro with default threshold values. ElliPro predicts linear and discontinuous antibody epitopes based on a protein antigen's 3D structure (Ponomarenko et al., 2008).

B-cell epitope prediction

B-cell epitopes were predicted base on sequence and structure of LipL21 and LipL32 protein. Protein sequence were analyzed by some B-cell prediction methods from IEDB including Bepipred Linear Epitope Prediction 1.0 & 2.0 (Jespersen et al., 2017; Larsen et al., 2006), Chou and Fasman beta turn prediction (Chou, Fasman, 2009), Emini Surface Accessibility Prediction (Emini et al., 1985), Karplus & Schulz Flexibility Prediction (Karplus, Schulz, 1985), Kolaskar & Tongaonkar Antigenicity (Kolaskar, Tongaonkar, 1990) and Parker Hydrophilicity Prediction (Parker et al., 1986) with default window and threshold values.

T-cell epitope prediction

To predict T-cell epitope linked to mayor histocompatibility complex (MHC) I or MHC II, the IEDB prediction tools were used. This study used MHC I Binding tool with all alleles of human, cow, mouse and pig (http://tools.iedb.org/mhci), other options set default. For MHC II Binding tool, all alleles of human and mouse were selected (http://tools.iedb.org/mhcii/), other options set default.

Analyzing distribution of epitopes

Predicted epitopes were aligned with protein sequence by Clustal Omega (Sievers et al., 2011). Microsoft Excel base on position of epitopes on protein sequences to analyze distribution of epitopes.

RESULTS AND DISCUSSION

Phylogenetic relationship between Vietnamese Leptospira strains and other Leptospira strains using 16S rRNA gene sequencing

Phylogenetic tree was constructed for the six leptospiral isolates in this study and the twenty-four international pathogenic L. interrogans representative strains obtained from GenBank database (Figure 1). Sequences 16S rRNA gene of Leptospira spp. strains were used for phylogenetic analysis.

16S rRNA sequencing used as a tool for phylogenetic analysis has led to a better understanding of evolution of Leptospira. To investigate the genetic diversity of leptospirosis, a total of six Vietnamese strains and twenty-four international reference strains belonging to serogroup L. interrogans were analyzed using 16S rRNA gene sequencing. The Maximum Likelihood data revealed the phylogenetic relationship between these different pathogenic species in this study and other international pathogenic species. The findings from the phylogenetic tree reveal the relationship and genetic diversity of various serovars of Leptospira interrogans, with their nearest phylogenetic relatives. The results showed that two pathogenic species of L.Pomona_VN and L.Hardjo seem to be more closely (bootstrap: 99%). A weak close genetic relationship of L. Canicola_VN or L. Ictero haemohagiae_VN and the classic L.Canicola, L.Grippotyphosa was also confirmed by using 16S rRNA sequencing in this study (bootstrap: 62%). From the phylogenetic analysis, L.Mitis VN and L.Grippotyphosa VN were clustered together and L.Bataviae_VN strain, their sister group, appeared a weak phylogenetic relationship (bootstrap: 62%). The phylogenetic analysis revealed that the genetically diverse strains of serogroup L. interrogans isolates from Vietnam were generally different with those isolated in other countries. The bootstrap percentages quoted are the percentage times a taxon at that node occurred, the percentages are ranging from 62% to 99%, which may indicate that Leptospira may evolve according to different locations and the epidemiology of leptospirosis in Vietnam relative independent from other countries. The genetic relationship of Leptospira species were also confirmed by several previous studies. Rettinger (2012) compared phylogenetic trees through 16S rRNA gene sequences of twenty-eight leptospiral strains, including pathogenic, non-pathogenic and intermediate strains. Statistical analysis of three pathogenic genospecies revealed peak differences at the species level in this study (Rettinger et al., 2012). Zhang (2015) used the 16S rRNA sequencing and MLST genotyping methods to investigate the genetic diversity of pathogenic Leptospira and understand the changing epidemiological and evolutionary trends of this serogroup in Mainland China. Their data revealed that the major Leptospira species from different countries were distinct and
had great genetic diversity in geographic epidemiology (Wang et al., 2006). Bourhy (2014) was inferred from sequence analysis of the 16S rRNA gene and analyzed the phylogenetic of the genus Leptospira in Mayotte (Indian Ocean). These data were phylogenetically consistent and reflected genetic relatedness among species of these genus

Leptospira (Bourhy et al., 2014). Our present study also indicated that 16S rRNA gene sequencing is a useful technique to explore the genetic diversity and molecular epidemiology of leptospirosis on a global and/or historical scale. Moreover, these results provide a blueprint for further phylogenetic research in pathogenic Leptospira strains.

Checking for some antigenic genes in Vietnamese pathogenic Leptospira serovars

In an effort to find the promising antigenic genes for launching a kind of recombinant leptospirosis vaccines in Vietnam, we selected six genes (e.g., LipL32, LipL21, and LigA genes) were expressed in all strains and OmpL1 gene occured in four strains (L. Bataviae_VN, L. Canicola_VN, L. Gryporthyposa_VN and L. Mitis_VN), whereas LipL41 and LigB genes did not appear in any Leptospira strains (data not shown) (Figure 2).

It has long been expected to find an effective vaccine to prevent leptospirosis through

Figure 1. Phylogenetic analysis based on based on 16S rRNA gene for the thirty pathogenic Leptospira strains (twenty-four sequences obtained from the NCBI database). Phylogenetic trees were constructed by Maximum likelihood and Neighbour-joining method. The bootstrap percentages quoted are the percentage times a taxa at that node occurred. The scale bar represents the number of base pairs differences. The arrows (→) indicate Leptospira strains in Vietnam.
immunization of high risk humans or animals. Although some leptospirosis vaccines have been obtained, the vaccination is relatively unsuccessful and millions of dollars spent (Wang et al., 2007). In Vietnam, mainly using inactivated vaccine in preventing and controlling leptospirosis. However, vaccination with inactivated whole-cell preparations (bacterins) has limited efficacy due to the wide antigenic variation of the pathogen. A intensive efforts towards developing improved recombinant vaccines are ongoing. During the last decade, many reports on the evaluation of recombinant vaccines have been published. Partial success has been obtained with some surface exposed protein antigens. Raja (2013) was surveyed the different types of OMPs of Leptospira and combines all the novel features of OMPs and put forth some views for future research (Raja, Natarajaseenivasan, 2015). Forster (2015) evaluated the N-terminal region of the leptospiral immunoglobulin-like B protein (LigBrep) as a candidate antigen for an effective vaccine against leptospirosis and emphasised the use of the DNA prime protein boost as an important strategy for vaccine development (Forster et al., 2015). Hu (2014) reported several outer membrane protein antigens exist in all the L. interrogans prevailing in China and suggested that predominant T- and B-cell combined epitopes in the outer membrane protein antigens can be used for developing novel universal leptospirosis vaccines (Hu et al., 2014). The research results of Dezhbord (2014) in cloning gene for expression and recombinant OmpL1 as an efficient and conserved antigen were suggested that OmpL1 gene may be a useful vaccine candidate against leptospirosis in Iran region (Dezhbord et al., 2014). Several researchers suggested the purified recombinant LigA protein is the most promising subunit vaccine candidate against leptospirosis reported to date, however, as purified proteins are weak immunogens the use of a potent adjuvant is essential for the success of LigA as a subunit vaccine (Bacelo et al., 2014; Kanagavel et al., 2014). In 2014, Maneewatch and Adisakwattana studied two LipL32-specific mouse monoclonal antibodies (mAbLPF1 and mAbLPF2) and suggested LipL32 recombinant protein as diagnostic and vaccine targets for leptospirosis (Maneewatch et al., 2014). In addition, Ye (2014) was tested and developed four recombinant proteins of Leptospira interrogans, namely, rLipL21, rLoa22, rLipL32, and rLigACon4-8 as antigens for the diagnosis of equine leptospirosis (Ye et al., 2014). Although were not commercialization, the first vaccines were put foundation for efficacy of new generations, which have outstanding developments to coincide the present needs. However, a crucial work on effective recombinant vaccine development and engineered antibodies will hopefully meet to solve the therapeutic challenges (Vedhagiri et al., 2009).

Figure 2. Gene expression products of Leptospira strains. Each species labeled as follow: Ba: L. Bataviae_VN; Ca: L. Canicola_VN; Ic: L. Icterohaemagglutinates_VN; Gr: L. Grippotyphosa_VN; Mitis: L. Mitis_VN; Pm: L. Pomona_VN; M: Marker DNA 1 Kb (10,000 bp; Thermo) or Marker DNA 100 bp (1,000 bp, Thermo).
Epitope candidates

The potential B cell and T cell epitopes were found to be distributed throughout the whole sequence, numerous fragments could be selected as candidates for the design of a vaccine. Nevertheless, it is necessary to define both B cell and T cell epitopes as candidates, to thereby generate a vaccine inducing both humoral and cellular response. Therefore, a more exhaustive analysis was performed to identify regions comprising both types of epitopes.

The combined T and B cell epitopes were predicted based on the amino acid sequences of LipL21 and LipL32. The LipL21 and LipL32 translated sequences had 163 and 215 amino acids (aa), respectively. Six sequences for each gene were aligned by multiple sequence alignment using BioEdit software, to obtain the conserved regions. The only one different amino acid at amino acid 114 of LipL32 sequences was detected. Thus one conserved regions for the LipL21 and two conserved regions for 1-113 aa residues (LipL32_{1-113}) and 115-215 aa residues (LipL32_{115-215}) of LipL32 were chosen to predict protein 3D structure and potential epitopes (Figure 3).

Figure 3. 3D structure of the conservative regions in outer membrane lipoproteins LipL21 and LipL32. Using seven different algorithms for B cell and two algorithms for T cell, 95 epitopes from LipL21 peptide, 46 epitopes from LipL32_{1-113} peptide, and 78 epitopes from LipL32_{115-215} peptide were predicted (Table 3). Candidate peptides were mapped to reference to determine the epitope density per base of each peptide.

The results of mapping showed that, all of three conserved peptides had two high levels of mapped epitopes regions. Because, these regions had many candidate epitopes positions, they may show the higher antigenicity than other regions. They were LipL21 residues 2-70 (LipL21_{2-70}), LipL21_{71-119}, LipL32_{41-123}, LipL32_{115-156}, and LipL32_{164-210}. The six peptide has length in range 45 - 69 aa (Figure 4).

Previous studies recommended that the application of only one protein as a subunit recombinant vaccine could not successfully prevent leptospirosis since it is not antigenic enough to stimulate the immune system. While, the applying multi-epitope vaccines, which use epitopes of several proteins, were the solution to vaccine antigenicity (Branger et al., 2001; Haake et al., 1999). Several researchers had used in silico approach for identifying and designing of vaccine candidates (Branger et al., 2001; Hasan et al., 2015; Khatoon et al., 2017) and some of them achieved promising clinical trial results (Groot, Rappuoli, 2004). Multi-epitope peptide DNA vaccines are effective against some viruses and they have recently been shown to have potential efficacy against some bacterial diseases including leptospires. Similarly, the fact that the DNA was expressed efficiently invitro is indicative of the fact that the DNA is correspondingly expressed after immunization as observed from the protection rendered. However, incorporating T-cell epitopes may likely improve the potency of the vaccine as Th-1 type immune response has previously been demonstrated in cattle vaccinated with killed L. Hardjo vaccine (Naiman et al., 2001). Overall, the present novel immunogenic multi-epitope DNA vaccine developed by chemical gene synthesis and delivered as a plasmid DNA vaccine may serve as a new candidate target for leptospiral vaccine development.

In the present study, we also used bioinformatic
tools to predict candidate epitopes. To stimulate antigen specific B cell and T cell immune response, epitopes were predicted for both B-cell antibody and T-cell MHC (I and II class) (Forouharmehr, Nassiry, 2015; Yousefi et al., 2015). The six peptide LipL21\(_{2-70}\), LipL21\(_{71-119}\), LipL32\(_{4-61}\), LipL32\(_{81-133}\), LipL32\(_{115-158}\), and LipL32\(_{164-210}\), did not only have the high antigenicity but also short enough for recombination. Therefore, with further test, these peptides can be candidates for a new recombinant vaccine for designing a recombinant multi-epitope vaccine against leptospirosis in Vietnam.

**Figure 4.** Antigenic epitope prediction showing potential B and T cell epitopes for LipL21 (A), LipL32\(_{1-113}\) (B), and LipL32\(_{115-215}\) (C).
Table 3. Number of antigenic epitopes in the conservative regions of outer membrane lipoproteins LipL21 and LipL32.

| LipL21 | LipL32 | LipL32 |
|--------|--------|--------|
| Tc cell (Cytotoxic T cell) | 53 | 18 | 45 |
| Th cell (T helper cells) | 19 | 10 | 19 |
| B cell | 23 | 14 | 14 |

CONCLUSION

From the initial research results, we suggested that the six Vietnamese *L. interrogans* strains were differentiated effectively in genetical distance by phylogenetic analysis. Moreover, three genes (LipL32, LipL21, and LigA genes) were expressed in six Vietnamese pathogenic strains of *Leptospira*. This work identified combined T and B cell immunodominant epitopes in LipL32 and LipL21 of *L. interrogans*. The identification of these immune dominant epitopes may greatly facilitate the development of novel leptospiral vaccines which may provide protections across different serogroups or serovars. The findings could also contribute to the development of effective cross-protective vaccine strategies for againsting leptospirosis in Vietnam.

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PHÂN TÍCH TRÌNH TỪ GEN 16S rRNA TỪ CÁC CHUNG LEPTOSPIRA GÂY BỆNH CỦA VIỆT NAM VÀ DỰ DOAN IN-SILICO CÁC EPITOPE TIỆM NĂNG TRÊN HAI LIPOPROTEIN MÀNG NGOÀI LIPL21, LIPL32

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Tóm tắt

Bệnh xơ xoan khuẩn (Leptospirosis) được xác định là một bệnh truyền nhiễm mới nổi, có khả năng gây bệnh trên động vật và lây lan sang người. Bệnh do Leptospira gây ra. Mặc dù đã có một số loại vaccine phòng bệnh do Leptospira nhưng đáp ứng miễn dịch không cao, thời gian bảo hộ không dài. Trong thí nghiệm này, một đoạn 550 bp của gen RNA ribosome (16S rRNA) của 6 chủng Leptospira gây bệnh xơ xoan và dẳng được sử dụng làm chủng vaccine ở Việt Nam (bao gồm: Pomona, Canicola, Mitis, ietero haemohagiae, Bataviae, và Grippotyphosa) đã được giải trình tự. Kết quả cho thấy một mô quan quan hệ di truyền gắn giữa các chủng này, mọc ở các chủng có điểm L.Canicula, L.Grippotyphosa (bootstrap: 62%). Các chủng khác như L.Mitis, VN, và L.Grippotyphosa, VN xuất hiện cũng bình thường, nhưng lại có mô quan hệ di truyền yếu với nhóm chi em của chúng là chủng L.Bataviae VN (bootstrap: 62%). Chủng tối cứng lứa chỉ có 6 gen, gồm: các globulin miễn dịch như protein A và B (gen Lig4 và LigB), protein mãng ngoài (gen OmpL1), và lipopolysaccharide (gen LipL32, LipL41 và LipL21) để kiểm tra sự xuất hiện của các gen này ở các chủng Leptospira trên bằng phương pháp phân ứng chuỗi polymerase (PCR). Có 3 gen (gen LipL32, LipL21 và LigA) xuất hiện trong tất cả các chủng, riêng gen OmpL1 chỉ có ở 4 chủng (Bataviae, Canicula, Griplphthysa và Mitis), trong khi đó gen LipL41 và LigB đã không xuất hiện trong bất kỳ chủng Leptospira nào. Một tập hợp các epitope tiềm năng trong cả tế bào lympho T và B của protein LipL21 và LipL32 đã được tìm kiếm bằng các công cụ tin sinh. Kết quả cho ra, có 3 vùng da epitope (1 vùng và 97 epitope kháng nguyên cho cả hai tế bào B và T của LipL32, 2 vùng và 124 epitope kháng nguyên cho cả hai tế bào B và T của LipL21). Các nghiên cứu sâu hơn về sinh học phân tử để tạo ra được một loại vaccine ti thể holopATTER ngưng trong phòng và điều trị bệnh leptospirosis cho动 vật và người ở Việt Nam.

Từ khóa: Gen quyết định kháng nguyên, Loài Leptospira, Vaccin tái tạo hợp leptospirosis, Trình tự gen 16S rRNA

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