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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 *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 *Leptospira* spirochetes (e.g., Pomona, Canicola, Mitis, Ictero haemohagiae, Bataviae, and Grippotyphosa). The results showed a close relationship of *L.*Pomona_VN and *L.*Hardjo (bootstrap: 99%). *L.*Canicola_VN and *L.*Ictero haemohagiae_VN appeared to be weak related to the classic *L.*Canicola, *L.* Grippotyphosa, these assemblage have a bootstrap support of 62%. The other strains (*L.*Mitis_VN and *L.*Grippotyphosa_VN) were appeared monophyletic, while their sister group (*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 *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 occured 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 *Leptospira* strains. A multi-antigenic epitope potential of two gene (*Lip L21* and Lip *L32*) 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, Leptospiraceac, 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 2nd group of the most dangerous diseases (OIE, 2008) and currently it adds to the group of occupational diseases in Vietnam (TLĐLĐVN, 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 *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 *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 mucous, skin, and eyes, which exposure to infected animal urine (Slack, 2010). Currently, pathogenic

leptospires 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). Nowaday, 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 leptospira. 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.*Ictero haemohagiae_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 DNA extraction

Genomic DNA was extracted using Phenol:Chlorofrom: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 3µl of each reaction on 1% agarose gel for 30 min at 100V and Nanodrop.

Sequence assembly and alignment using *16S rRNA* 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, 1µl 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.

Table 1. Primer pairs used for successful amplification of *16S rRNA* sequencing and gene expressions.

Gene name	Sequences		Size (bp)	Tm
16S rRNA	F	GCCTACGGGAGGCAGCAG	550	50°C for 30s
	R	CCGTCAATTCMTTTGAGTTT		
OmpL1	F	TTGATTGAATTCTTAGAGTTCGTGTTTATA	960	56°C for 50s
	R	AAGGAGAAGCTTATGATCCGTAACATAAGT		
LipL32	F	TTACCGCTCGAGGTGCTTTCGGTGGTCTGC	782	50°C for 30s
	R	TGTTAACCCGGGTTACTTAGTCGCGTCAGA		
LipL41	F	AGAGAAGATAATTTTCTCCCCATGGGTAACA	1065	50°C for 50s
	R	GAAAGCAACGCAAAGTAACTCGAGTCCTTT		
LipL21	F	ATGATCAATAGACTTATAGCTC	560	50°C for 60s
	R	TTATTGTTTGGAAACCTCTTGA		
LigA	F	CGGTCGACTATCGTTACTCCAGCA	991	50°C for 50s
	R	CGGCGGCCGCAATATCCGTATTAGA		
LigB	F	CCGCTCGAGGTGTTTATGAAGAAA	620	50°C for 50s
	R	ACTCTCGAGCGTATTAGAGGAAT		

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 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/) 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/) 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 taxa 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 genomospecies 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.

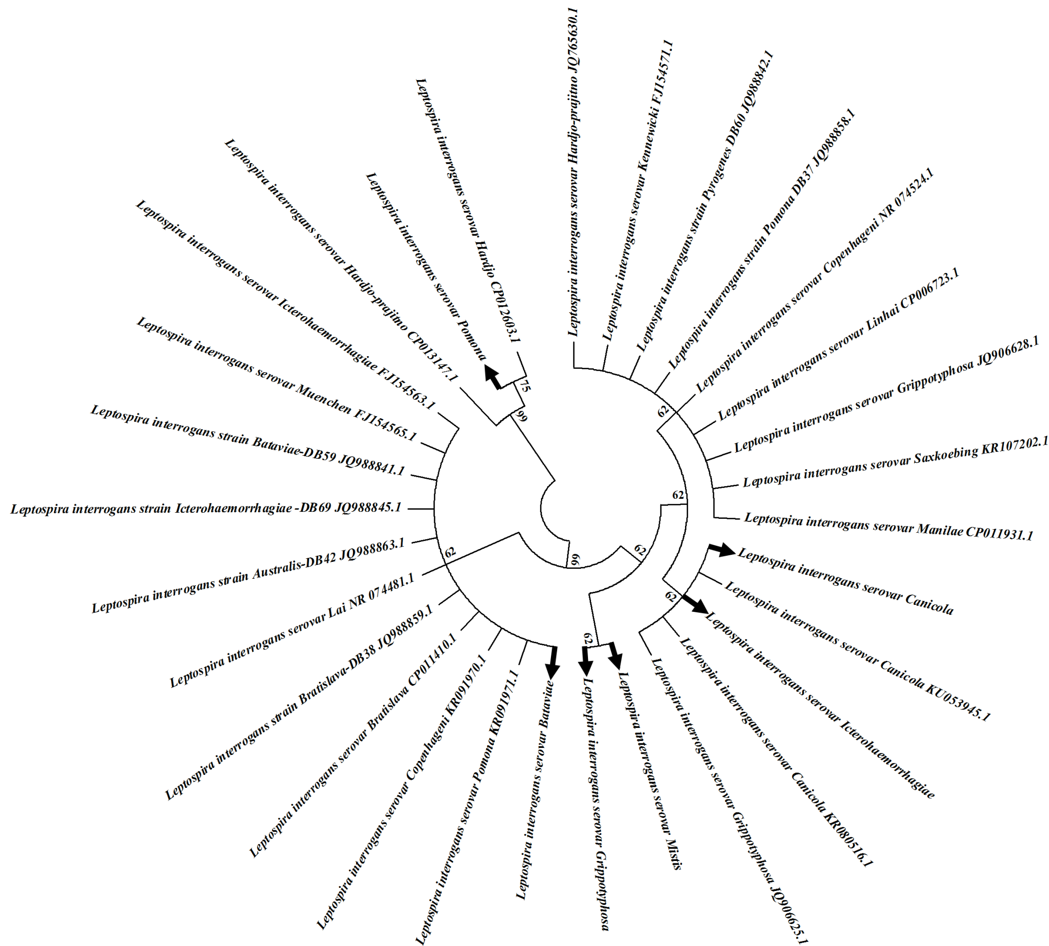

Figure 1. Phylogenetic analysis based on based on 16S rRNA gene for the thirty pathogenic *Leptospira* strains (twenty-four sequences obtained from the NCBI database). Phylogenetic trees were constructed by Maximum likelihood and Neighbour-joining method. The bootstrap percentages quoted are the percentage times a taxa at that node occurred. The scale bar represents the number of base pairs differences. The arrows (→) indicate *Leptospira* strains in Vietnam.

Checking for some antigenic genes in Vietnamese pathogenic *Leptospira* serovars

In an effort to find the promising antigenic genes for launching a kind of recombination leptospirosis vaccines in Vietnam, we selected six genes (e.g. *LigA*, *LigB*, *OmpL1*, *LipL32*, *LipL41* and *LipL21* genes) and checked gene expression in these *Leptospira* strains by PCR method. Only three 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.*Grypothyphosa_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

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).

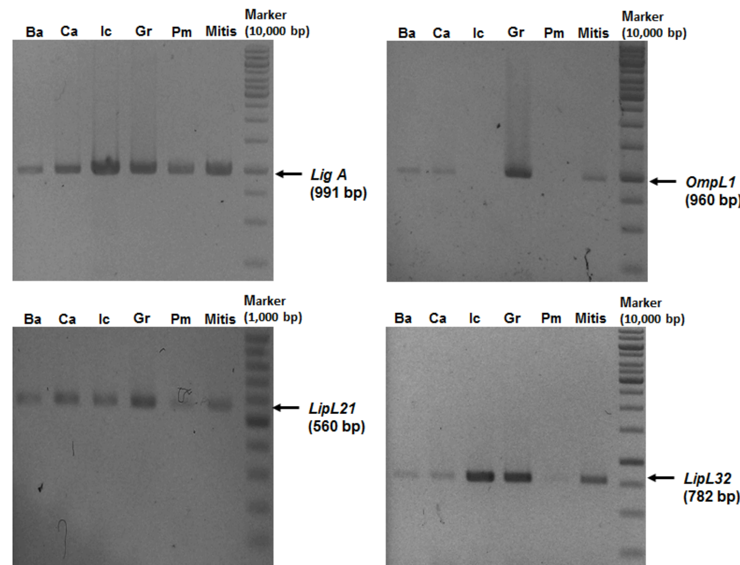

Figure 2. Gene expression products of *Leptospira* strains. Each species labeled as follow: Ba: *L.*Bataviae_VN; Ca: *L.* Canicola_VN; Ic: *L.*Ictero haemohagiae_VN; Gr*:* *L.*Grippotyphosa_VN; Mitis: *L.*Mitis_VN; Pm: *L.*Pomona_VN; M*:* Marker DNA 1 Kb (10,000 bp; Thermo) or Marker DNA 100 bp (1,000 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 114th 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).

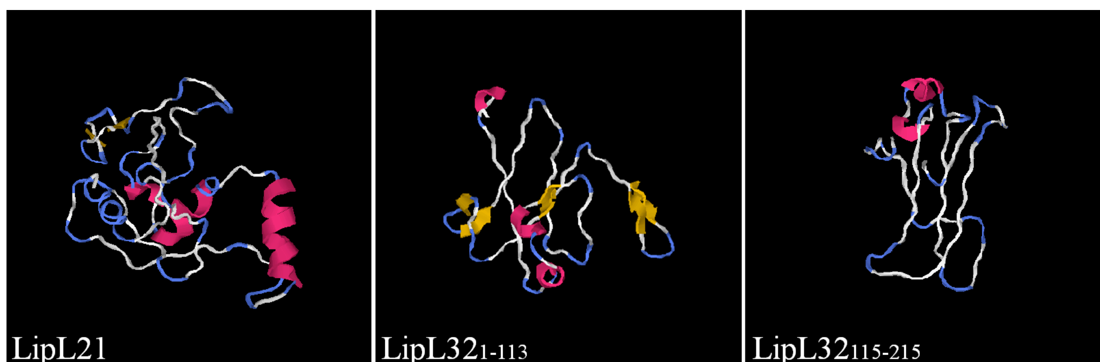

Figure 3. 3D structure of the conservative regions in outer membrane lipoproteins LipL21 and LipL32. Using seven different algorithms for B cell and two algorithms for T cell, 95 epitopes from LipL21 peptide, 46 epitopes from $LipL32_{1-113}$ peptide, and 78 epitopes from $LipL32_{115-215}$ peptide were 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 ($LipL21_{2-70}$), $LipL21_{71-119}$, $LipL32_{4-61}$, $LipL32_{81-133}$, $LipL32_{115-158}$, 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.

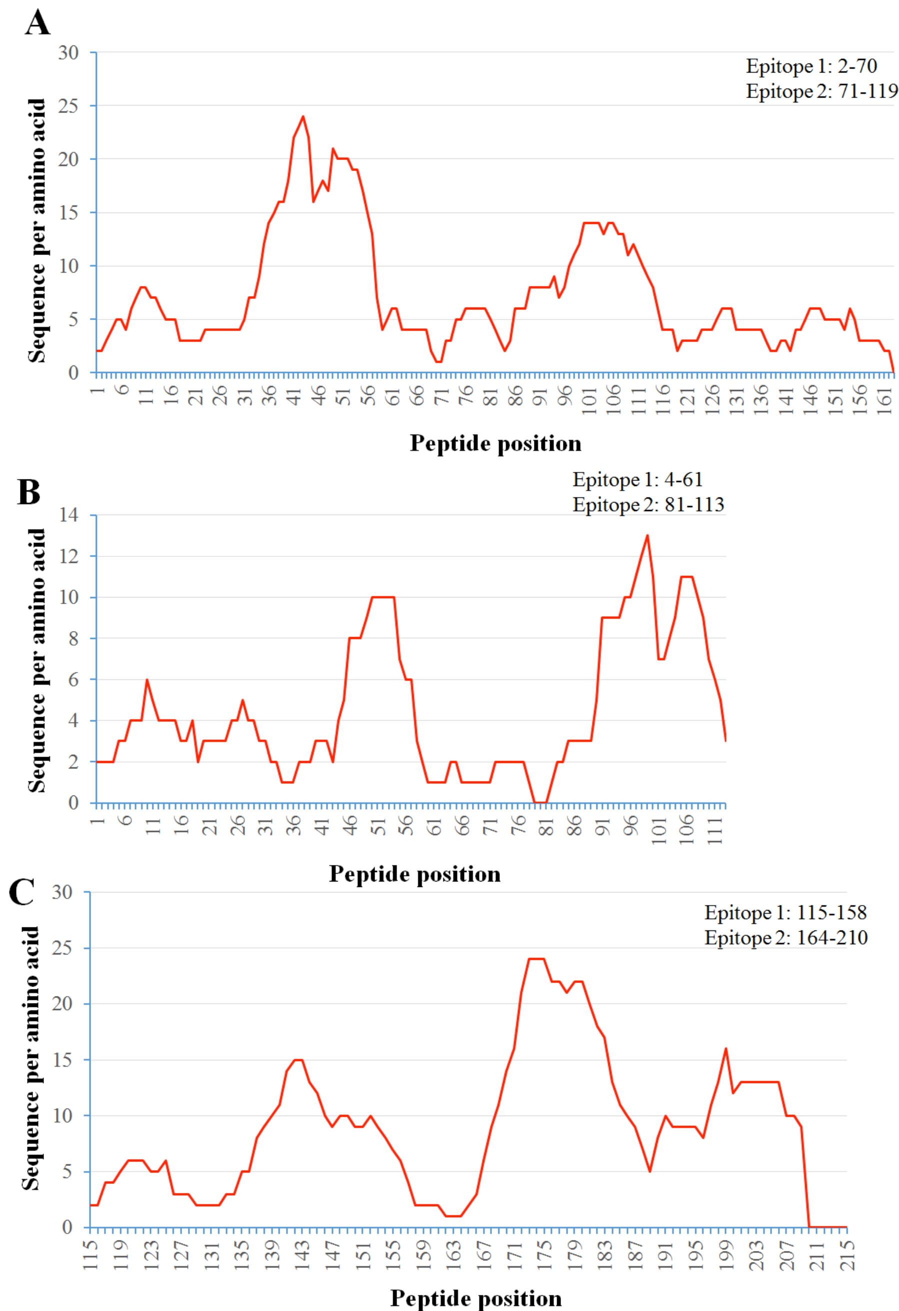

Figure 4. Antigenic epitope prediction showing potential B and T cell epitopes for LipL21 (A), $LipL32_{1-113}$ (B), and $LipL32_{115-215}$ (C).

Table 3. Number of antigenic epitopes in the conservative regions of outer membrane lipoproteins LipL21 and LipL32.

	Number of epitope		
	LipL21	**$LipL32_{1-113}$**	**$LipL32_{115-215}$**
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. interrogan* 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 TỰ GEN *16S rRNA* TỪ CÁC CHỦNG *LEPTOSPIRA* GÂY BỆNH CỦA VIỆT NAM VÀ DỰ ĐOÁN *IN-SILICO* CÁC EPITOPE TIỀM NĂNG TRÊN HAI LIPOPROTEIN MÀNG NGOÀI LIPL21, LIPL32

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TÓM TẮT

Bệnh xoắn 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 vacxin 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 xoắn khuẩn và đang được sử dụng làm chủng chế vacxin vô hoạt ở Việt Nam (bao gồm: Pomona, Canicola, Mitis, Ictero haemohagiae, Bataviae, và Grippotyphosa) đã được giải trình tự. Kết quả cho thấy một mối quan hệ di truyền gần gũi của chủng *L.*Pomona_VN với *L.*Hardjo (bootstrap: 99%). Ngược lại, *L.*Canicola_VN và *L.*Ictero haemohagiae_VN dường như có mối quan hệ di truyền yếu với các chủng cổ điển *L.*Canicola, *L.* Grippotyphosa (bootstrap: 62%). Các chủng khác như *L.*Mitis_VN và *L.*Grippotyphosa_VN xuất hiện cùng nhánh, nhưng lại có mối quan hệ di truyền yếu với nhóm chị em của chúng là chủng *L.*Bataviae_VN (bootstrap: 62%). Chúng tôi cũng lựa chọn 6 gen, gồm: các globulin miễn dịch như protein A và B (gen *LigA* và *LigB*), protein màng 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 phản 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, Canicola, Grypothyphosa 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 chỉ ra, có 3 vùng đa epitope (1 vùng và 95 epitope kháng nguyên cho cả hai tế bào B và T của LipL21; 2 vùng và 124 epitope kháng nguyên cho cả hai tế bào B và T của LipL32). Cần nghiên cứu sâu hơn về sinh học phân tử để tạo ra được một loại vac xin tái tổ hợp mới ứng dụng trong phòng và điều trị bệnh leptospirosis cho động vật và người ở Việt Nam.

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