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Designing a Recombinant Multi-epitope DNA Vaccine as Candidate for Protection against Pathogenic *Leptospira* Infection in Animals

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ABSTRACT

Leptospirosis can cause severe disease and probable death in humans. Antigenic epitopes from pathogenic strains of the bacteria have shown potential for serving as vaccine candidates and play a key role in the sensitivity and specificity of immunodiagnostic tests. This *in-vitro* analysis was undertaken to develop a prototype recombinant DNA vaccine using *in-silico* epitope prediction method. Epitope prediction software programs predicted the most antigenic linear B-cell epitopes of *OmpL1*, *LipL32*, *LipL41*, *Loa22*, and *LigA*. Thirteen epitopes were predicted, connected by the Gly-Ser linker, and synthesized. The purity of the concentrated recombinant multi-epitope protein was assessed by restriction enzyme digestion and gel electrophoresis. *In-vitro* expression on mammalian Chinese Hamster Ovary cell line indicated strong cytoplasmic fluorescence produced based on an indirect immunofluorescence antibody test. The green color of the cytoplasm indicates successful transcribed and translated DNA as against the blue-stained nucleus observed in the un-transfected control group based on the indirect immunofluorescence antibody test. The findings of the current study showed high antibody binding potentials of the vaccine constructs, which could be used for diagnostic applications or as polyvalent vaccine candidates.

Keywords: B-cell epitopes, Indirect immunofluorescence antibody test, Leptospirosis, Multi-epitope vaccine, Recombinant vaccine

INTRODUCTION

Leptospirosis is a zoonosis affecting a wide range of mammals, including humans, with significant public health implications (Garba et al., 2018a; Wang et al., 2022). The disease has a worldwide distribution, with varying severity depending on the infecting serovar, economic status, and prevailing environmental conditions (Azhari et al., 2018; Neela et al., 2019; Chacko et al., 2021). 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 (Hasoun et al., 2017; Garba and Moussa, 2021). Like many tropical diseases, most cases of the illness are characterized by mild self-limited disease episodes (Al-Rasheed et al., 2018; Abdulhaleem et al., 2019; Jesse et al., 2020; Zakaria et al., 2020). However, in severe cases, degenerative changes can be seen in the liver and kidney dysfunction (Garba and Moussa, 2021).

Currently available vaccines for both medical and veterinary applications, which are predominantly whole-cell killed bacterins, can not protect against serovars not included in the vaccine preparations (Bashiru and Bahaman, 2018). Also, experimental evaluations have shown that Leptospiral vaccines can effectively prevent disease and reduce shedding after challenges with the serovar included in the vaccine (Lin et al., 2016; Garba et al., 2018b). However, there is a lack of cross-reactivity among antigenically distinct serovars, which further limits the ability of available vaccines to provide heterologous protection (Branger et al., 2005; Garba et al., 2018b). DNA vaccines are a novel approach for inducing an immune response. They are the simplest embodiment of vaccines that, rather than consisting of the antigen itself, provides genes encoding the antigen (Donnelly et al., 2005). In this approach, purified plasmid DNA containing the coding sequences of an immunogenic gene and the essential regulatory elements to transcribe and translate them is introduced into the tissue parenterally. This is followed by tissue expression and induction of potent, long-lasting heterologous immune response. The efficiency of Leptospiral DNA vaccines has been demonstrated in animal models (Branger et al., 2005; Maneewatch et al., 2007; Garba et al., 2018b). This is seen as a positive development in the efforts

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to prevent leptospirosis for which conventional vaccines have failed. In addition, DNA vaccines also allow for the inclusion of multiple genes to improve the coverage and the ability to protect against a wide range of serovar infections.

The *Leptospira* outer membrane proteins *OmpL1*, *LipL32*, *LipL41*, *Loa22*, as well as the immunoglobulin-like proteins; *LigA*, LigB, and LigC are among the major markers of pathogenicity among *Leptospira* specie (Koizumi and Watanabe, 2005). These proteins are conserved among the pathogenic *Leptospira* specie and can confer immunogenic protection, as reported in some animal models (Wang et al., 2007; Alia et al., 2019).

This research aimed to develop a synthetic B-cell epitope gene from conserved pathogenic *Leptospira* genes (*OmpL1*, *LipL32*, *LipL41*, *Loa22*, and *LigA*) using an *in-silico* bioinformatics approach to identify highly antigenic B-cell epitopes for construction of the multi-epitope chimeric gene. The study will also examine the *in-vitro* expression and reactivity using the indirect immunofluorescence antibody test.

MATERIALS AND METHODS

Ethical approval

All the experimental procedure was approved by the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia (UPM/IACUC/AUP-R0012/ 2016).

Selection of the epitopes

The criteria for selection of B-cell epitopes for inclusion in the multivalent vaccine include the ability for spontaneous clearance of *Leptospira* from animals with infection and prevention of renal colonization, conservation of the sequence among the most isolated *Leptospira* serovars and potential for the epitopes to induce an immune response (Vijayachari et al., 2015; Dellagostin et al., 2017). The complete amino acid sequences of genes *LipL32* (*L. interrogans* serovar Icterohaemorrhagiae), *LipL41* and *OmpL1* (*L. interrogans* serovar Lai), *Loa22* (*L. interrogans* serovar Grippotyphosa) and *LigA* (*Leptospira kirschneri* serovar Grippotyphosa) were retrieved from the UniProt knowledgebase (UniprotKB) NCBI database using the following search parameters and filters.

- Database: UniProtKB/Swiss-Prot- non-redundant protein sequences (nr) DBSOURCE: UniProtKB: locus Q72SM7_LEPIC, accession Q72SM; Max E-value: 1e-1. The search was conducted on all five genes under the entry UniProtKB/TrEMBLNCBI, and protein accession numbers for each were retrieved (Q72SM7, AAP04735, AAT48511, AAT48493, AGH20068). All the sequences were analyzed on BLAST using UniProtKB BLASTP, Matrix: Blossum 62, and threshold 10 (Boutet et al., 2007).

Construction of multi-epitope containing plasmid

The selected epitopes were engineered together and linked using the glycine-rich linker (Trinh et al., 2004). These chimeric genes encoding multiple *Leptospiral* epitopes were codon optimized according to preferred codon usage for hamsters to enhance their expression (Chung and Lee, 2012). To enhance their immune-stimulatory capabilities and improve expression, a CpG motif (Class C) was added at both the N and C terminus, and a Kozak sequence was incorporated at the initiator terminal, respectively. The epitopes were combined and assembled in the following manner: epitopes from *LipL32* and *LipL41* together to give LipDNA01; epitopes from *OmpL1* and *Loa22* combined and designated OmpDNA02; epitopes from *LigA* were named LigDNA03, and the final construct is CompDNA04 which is the overall combination of all the epitopes. All the multi-epitope DNA constructs were designed with suitable restriction enzyme sites at the 5' and 3' ends, respectively (*LipDNA01- NotI* and *XhoI*; *OmpDNA02- SalI* and *BamHI*; *LigDNA03- KpnI* and *XhoI*; *CompDNA04- HindIII* and *Xbal*). The DNA molecule was synthesized by GeneScript (860 Centennial Ave. Piscataway, NJ 08854, USA) and was supplied cloned in pUC57 standard vector transformed in *E. coli* Top10 cells.

Cloning of multi-epitope gene(s) in pBudCE4.1 mammalian expression vector

The mammalian expression vector pBudCE4.1 is a 4.6 kb vector designed with the ability for simultaneous expression of two genes independently. It contains a human cytomegalovirus (CMV) immediate-early promoter and a human elongation factor 1α-subunit (EF-1α) promoter for high-level, constitutive, independent expression of two recombinant proteins. In addition to the CMV and human elongation factor 1α sub-unit, it contains a C-terminal peptide encoding the *myc* (*c-myc*) epitope, the V5 epitope, and a polyhistidine (6xHis) metal-binding tag that allows the detection and purification of expressed proteins. The vector also contains a Zeocin™ resistance gene for selection in *E. coli* and the creation of stable mammalian cell lines (Ly et al., 2005). Although the pBudCE4.1 plasmid is a bicistronic vector, only one synthesized gene fragment was cloned to each plasmid (monocistronic), resulting in the construction of four different plasmids designated LipDNA-pBudCE01 (520bp), OmpDNA-pBudCE02 (609bp), LigDNA-pBudCE03 (236) and CompDNA-pBudCE04 (1299bp). Furthermore, LipDNA-pBudCE01 and LigDNA-pBudCE03 were cloned upstream of the V5 epitope tag at the 5' end, while OmpDNA-pBudCE02 and CompDNA-pBudCE04 were fused upstream of the cmyc epitope tag at the 5' end as well.

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Transformation and sub-cloning of multi-epitope genes in pBudCE4.1 expression vector

The standard $CaCl_2$ chemical transformation protocol (Sambrook et al., 1989) was used with essential modifications. Then, 1.25µl of pBudCE4.1 plasmid from the frozen stock was mixed with 25µl competent *E. coli* cells in a 1.5ml centrifuge tube. The tubes were swirled and flicked gently to suspend the cells and then incubated on ice for 30 minutes, followed by exposure to heat shock using a water bath at 42°C for 40-45 seconds. The tubes were placed on ice for 2-5 minutes, and 900µl of SOC medium was added. The suspended bacteria were then incubated at 37°C with gentle shaking (200-250*g*) for 1 hour. Cells were centrifuged (Thermo Scientific™, USA) at 4000 *g* for 2 minutes, the supernatant was discarded, and then the cells were re-suspended in 1 mL of phosphate-buffered saline (PBS), and 200µl was plated on LB agar plate containing 25 µg mL^{-1} Zeocin. The plates were incubated at 37°C for 12-16 hours, and the resulting colonies were selected using the blue-white screening (Green and Sambrook, 2019).

Similarly, 1µg of purified pBudCE4.1 plasmid was digested using a combination of *NotI/XhoI*, *BamHI/SalI*, *KpnI/XhoI*, and *HindIII/XbaI* for genes 1, 2, 3, and 4, respectively. The compatibility of the restriction enzymes and their presence within the multiple cloning site of the plasmid vector was ascertained with the help of the New England Biolab NEB double digest finder. The optimized protocol for 40µl restriction enzyme digestion reaction comprises 5µl DNA (1µg), 2µl 10X NEB buffer, 0.5µl Bovine Serum Albumin (BSA), 1 µl each of restriction enzyme RE I and II, and nuclease-free water 30.5 µl.

The reaction mixtures were mixed thoroughly by finger tapping the tubes and then shortly spun down before incubating at 37°C for 1hr. After incubation, the reaction was deactivated by heating at 65°C for 15 minutes on a heating block. The digested products were separated in 0.8% agarose, stained with Gel red stain (Biotium, USA), and visualized using an Alpha imager™ gel documentation system (Alpha Innotech, Germany). Gel fragments were excised and purified using the QIAquick Gel extraction kit according to the manufacturer's instructions (QIAquick®, Germany).

The *NotI-XhoI*, *BamHI-SalI*, *kpnI-XhoI*, and *HindIII-XbaI* fragments for genes LipDNA-01, OmpDNA-02, LigDNA-03, and CompDNA-04 earlier prepared from pUC57 cloning vector were ligated into the corresponding sites of the digested pBudCE4.1 expression vector, generating LipDNA01-pBudCE.4.1, OmpDNA02-pBudCE4.1, LigDNA03-pBudCE4.1 and CompDNA04-pBudCE4.1 respectively. The ligation of the insert DNA fragments to the expression vector was achieved using the NEB T4 DNA ligase quick ligation kit (New England Biolabs®, England) according to the manufacturer's instructions. Briefly, a 20µl reaction containing 10µl of insert DNA, 5µl of the expression vector, 2µl of 10X buffer, 2µl of T4 DNA ligase, and 1µl of nuclease-free water was incubated at room temperature for 5 minutes. The ligation reaction was chilled on ice before using to transform chemically competent Top10 *E. coli* cells.

Confirmation of insert DNA in pBudCE4.1 eukaryotic expression vector

Colony PCR

Colony PCR to determine the presence of insert DNA in pBudCE.4.1 plasmid constructs were conducted. Individually transformed bacterial colonies were added to the PCR reaction and lysed during the initial heating step. This initial heating step was extended to 5 minutes to have complete lysis and release of the plasmid DNA from the cell, thereby serving as a template for the amplification reaction (Walch et al., 2016). The reaction condition used according to the NEB OneTaq® Quick-Load® 2X Master Mix Kit involved initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes.

The NEB OneTaq® (Quick-Load®, England) 2X master mix was used as the source of polymerase, dNTP, $MgCl_2$, buffer components, stabilizers, and tracking dyes. The manufacturer's instructions and reaction protocol are shown in Table 1.

Table 1. Gene-specific primers designed using Primer3 software (v. 0.4.0) for colony PCR confirmation

Gene	Forward primer	Reverse primer	Size
LipDNA01-pBudCE	GCG GCC GCA CCA TGG GCT CCA GCT T	CTC GAG GCA GCG CGT CTA AAG	520bp
OmpDNA02pBudCE	GTC GAC ACC ATG GGC AGC TCC	GGA TCC GGC GCG CCG GAA GGA GCT A	609bp
LigDNA03-pBudCE	GGT ACC ACC ATG GGC AGC	CTC GAG GCG GCG CGC CGA AAG	236bp
CompDNA04pBudCE	AAG CTT ACC ATG GGC AGC TCC TTC	TCT AGA GGC GCG CCG AAA AGA GGA GC	1299bp

Source of gene sequence: NCBI genebank (Q72SM).

Restriction enzyme digestion

Restriction enzyme digest was used to determine the presence of the insert DNA in the pBudCE4.1 plasmid vector (Ly et al., 2005). The restriction enzymes used were based on the REs inserted during the design and synthesis of the genes. The reaction mixes and incubation were based on NEB protocol, and the product was analyzed using gel electrophoresis in 0.8% agarose to determine both the insert and vector fragments.

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Sequence analysis of cloned genes

Sanger sequencing technique involves using a DNA polymerase, a primer, unlabelled deoxynucleotide triphosphates (dNTPs), and fluorescently labeled dideoxynucleotide triphosphates (ddNTPs) was used to determine the presence and orientation of the insert DNAs. The sequencing was done by Next gene Scientific, SDN. BHD, Malaysia.

Transfection of the mammalian cell line with plasmid DNA-carrying gene insert

The lipid-based Lipofectamin® LTX transfection method was used to transfect CHO cells grown on cover slips in a 6 well cell culture plate. Six well plates were seeded with 10^6 cells per well re-suspended in F12-K1 medium containing 10% FBS and incubated for 24 hours until they became 70-90% confluent. pBudCE4.1 plasmid containing a multi-epitope gene and a control plasmid were used to transfect the confluent cells using the Lipofectamin® LTX transfection reagent. According to the protocol, 200 µl of the Lipofectamin® LTX reagent (Thermo Fisher, USA) was diluted with 200 µl of serum-free opti-MEM medium (Thermo Fisher, USA) in a 1.5ml Eppendorf tube and 3 µg of plasmid DNA was mixed with 2.5 µl PLUS™ reagent containing 200 µl opti-MEM medium in another 1.5ml Eppendorf tube. The diluted DNA was then added to the diluted Lipofectamin® LTX reagent in a ratio of 1:1 and the mixture was incubated for 5 minutes at room temperature. The DNA-lipid complex was then finally dispensed into the respective wells of LipDNA01-pBudCE.4.1, OmpDNA02-pBudCE4.1, LigDNA03-pBudCE4.1 and CompDNA04-pBudCE4.1 as well as an additional well containing un-transfected cell as control. The plates were incubated in a humidified 5% CO_2 incubator for 5 minutes while agitating it occasionally to prevent drying of the cells. After 4 hours of incubation, the transfection medium was replaced with F12-K1 medium containing 5% FBS. Finally, 24, 48, and 72 hours after transfection, the plates were withdrawn, and the functionality and expression of DNA plasmids were evaluated.

Analysis of expression of plasmid by indirect immunofluorescence test IIFT

The c-myc Tag and V5 Tag monoclonal antibodies (ThermoScientific, USA) were used as the primary antibodies to detect the LipDNA01-pBudCE.4.1, and LigDNA03-pBudCE4.1 fused to the V5 epitope tag region of the pBudCE 4.1 plasmid vector and the CompDNA04-pBudCE4.1 and OmpDNA02-pBudCE4.1 equally cloned at the c-Myc epitope tag. However, the secondary antibody used was the Goat anti-mouse IgG conjugated to fluorescein dye Alexa Fluor 488 (ThermoFisher Scientific, USA)**.**

The instructions of the manufacturer in each case were followed. Working concentrations for all the listed antibodies were reconstituted as recommended by the manufacturers. Briefly, spent media from transfected cells withdrawn after 24- and 48-hours incubation were discarded, and the cells were washed twice with an interval of 5 minutes between washes with sterile PBS; the cells were then fixed with 4% formaldehyde and incubated for 15 minutes at room temperature (Sambrook et al., 1989). The cells were rinsed with PBS 3-4 times after fixation and then permeabilized with 0.2% buffered Triton X100 for 5 minutes. The cells were further rinsed with PBS and then blocked using 1% Bovine Serum Albumin (BSA) diluted in PBST (sterile PBS in Tween 20), followed by 1-hour incubation with primary antibody at 37^{o}C. Unbound primary antibodies were removed by rinsing 3-4 times with PBS before incubation with secondary antibody for 1 hour at 37^{o}C. The primary and secondary antibody incubation were performed under humidified and dark conditions. After the incubation period with the secondary antibody, the cells were rinsed once again with PBS, but this time it was done with PBS containing Tween 20 (0.05%) and then counter-stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) and incubated at room temperature for 5 minutes. The coverslips, upon which the cells were grown, were carefully lifted and placed on a clean microscope slide containing a drop of fluorescein mounting media with the cells facing downward. The mounted slides were kept drying in the dark before viewing with a confocal microscope.

RESULTS

Transformation of Top10 *E. coli* cells

The synthesized genes used in this study were cloned in pUC57 cloning vector as glycerol stock of Top10 *E. coli* cells. The plasmid was purified and sequenced using the M13 forward and reverse primers. The result indicated 100% similarity with the sequence submitted for synthesis. Restriction digestion of the pUC57 plasmid yielded two fragments with plasmid size 2.7kb while the sizes of the genes were LipDNA01-520bp, OmpDNA02-609bp, LigDNA03-236bp, and CompDNA04-1299bp, respectively.

The genes were then cloned in pBudCE4.1 expression vector to serve as the carrier of the vaccine gene. The genes were inserted upstream of the *myc* (OmpDNA02 & CompDNA04) and V5 (LipDNA01 & LigDNA03) epitope tags under the regulation of CMV and EF1-α promoter (Figure 1).

Each of the digested genes was ligated to its corresponding pBudCE4.1 vector using T4 DNA ligase (New England Biolabs). The ligated product was used to transform chemically competent *E. coli* cells. After overnight incubation at 37oC, successfully transformed cells were selected and further analyzed by colony PCR, restriction enzyme digestion, and sequencing (Swords, 2003).

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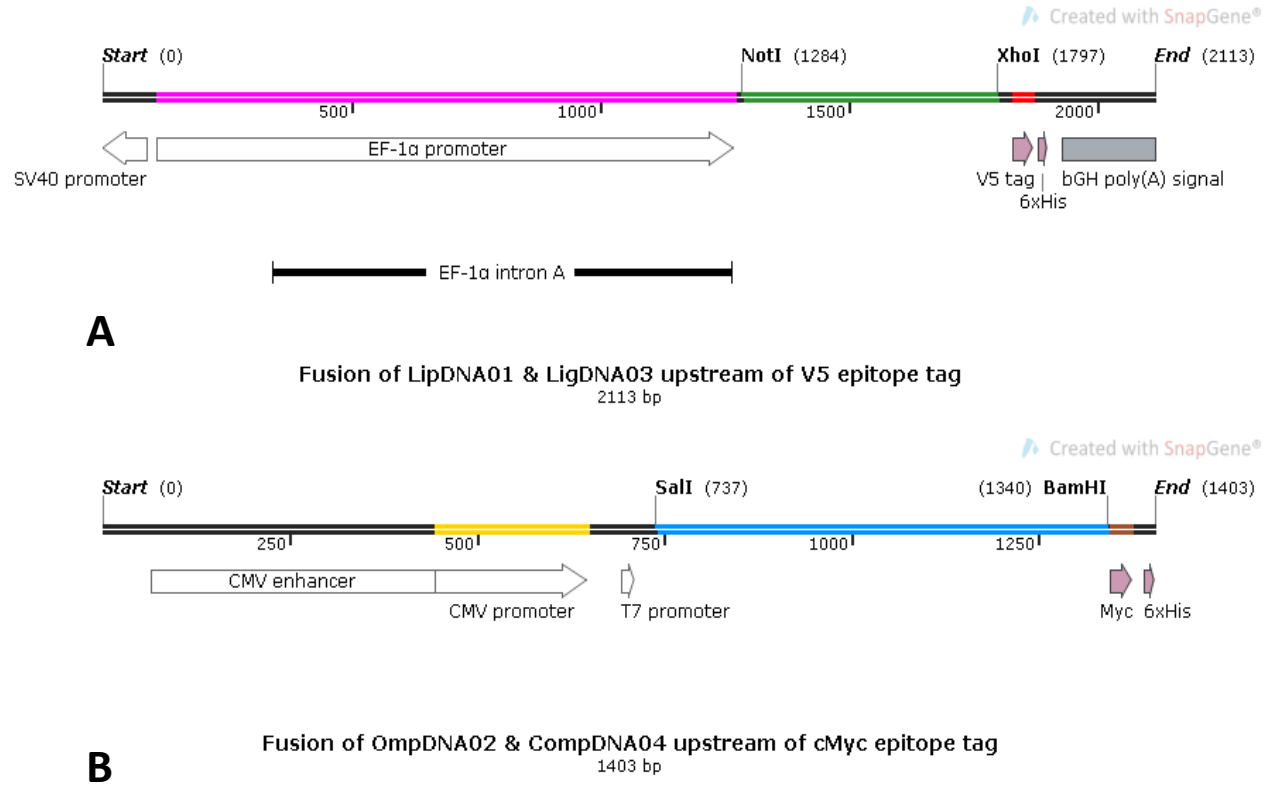

Figure 1. Schematic representation linear map of the segment of the plasmid vector showing V5 and cMyc epitope tags region. [A] fusion of LipDNA01 and LigDNA03 and [B] fusion of OmpDNA03 and CompDNA04 genes under the control of EF1α and CMV promoters, respectively (SnapGene® software).

Confirmation of positive clones by colony PCR

Using colony PCR, five bacterial colonies from each of the four plates with transformed Top10 E. coli cells were selected and screened for the presence of plasmid-carrying insert. The result (Figure 2) showed all colonies to be positive, indicating the incorporation of the inserted gene within the plasmid.

Confirmation of insert DNA by restriction enzyme digestion

Double digestion of purified plasmid DNA from bacterial colonies revealed that three colonies were positive for LipDNA01-520bp while OmpDNA02-609bp and LigDNA03-236bp had all clones carrying insert and for CompDNA04-1299bp, only four colonies were positive (Figure 3).

Figure 2. Showing bands for the screening of successfully cloned genes by colony PCR with positive bands against the 1kb DNA marker. Lane 1 DNA ladder; lane 2-4 LipDNA01 (520bp); lane 6 and 7 OmpDNA02 (609bp); lane 9 CompDNA04 (1299bp), lane 15-19 LigDNA03 (236bp), Lane 00 (empty)

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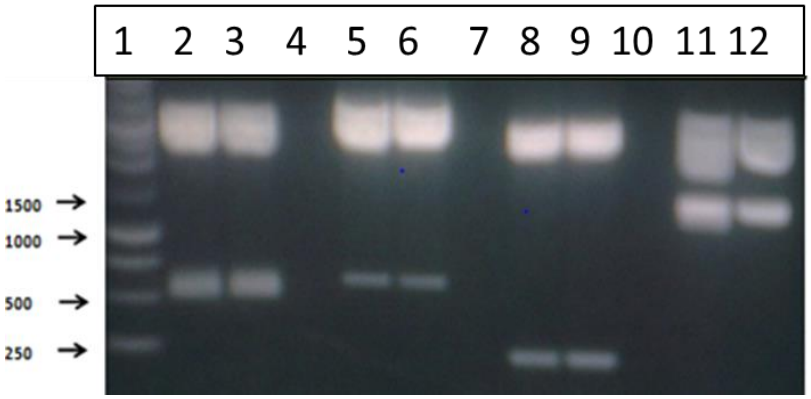

Figure 3. Gel electrophoresis of double-digested genes. Confirmation of bands from digested genes after subcloning in BudCE4.1 vector. Lane 1 is 1kb ladder, lane 2 and 3 is LiPDNA01 size 520bp, lane 5 and 6 is OmpDNA02 size 630bp, lane 8 and 9 is LigDNA03 size 236bp and lane 11 and 12 is CompDNA04 size 1299bp

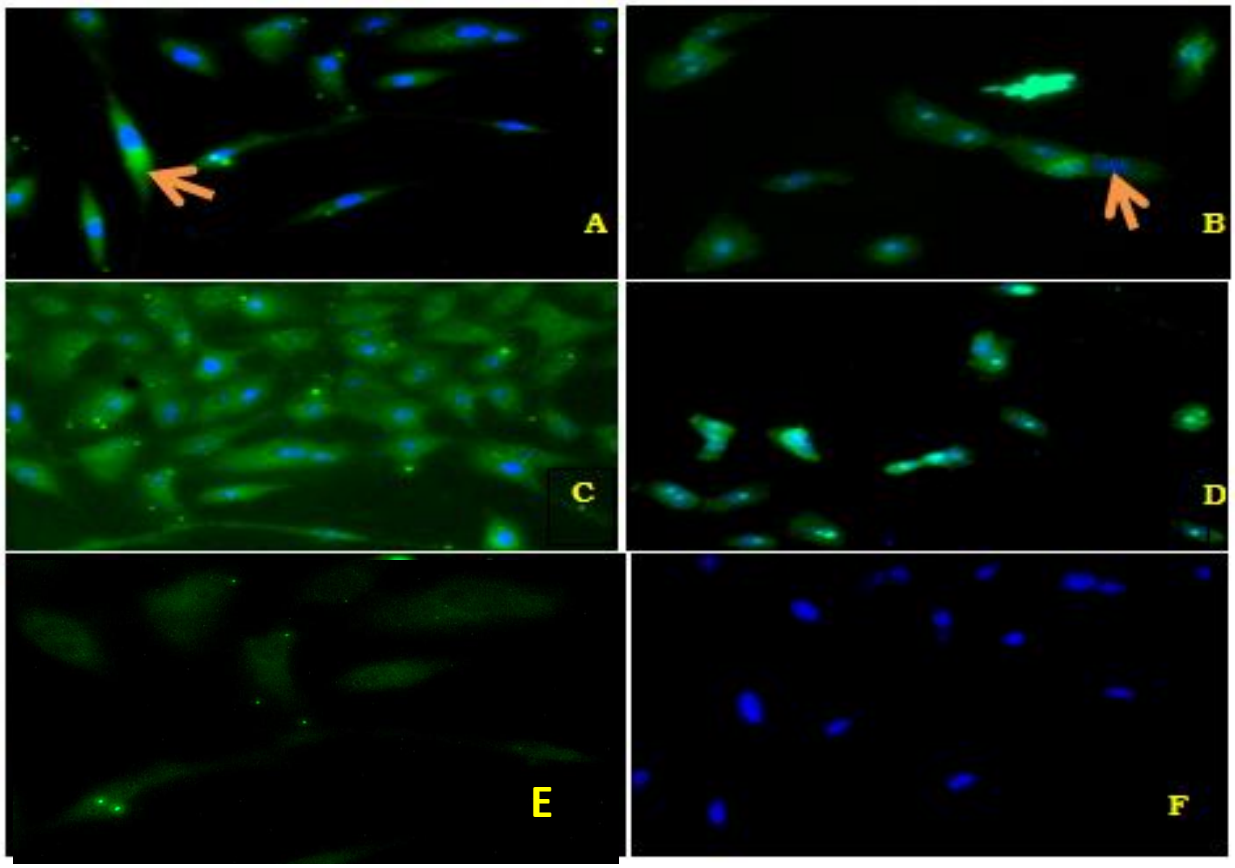

Figure 4. Demonstration of expression of plasmid DNA constructs in CHO-K1 cells at 48 hours' incubation after transfection with vaccine constructs as well as un-transfected control plasmid after counter-staining with DAPI. (A) LipDNA01-pBudCE.4.1 with an arrow showing expressed proteins in the cytoplasm indicated by green fluorescence emission of FITC (arrowhead-slide A). (B) OmpDNA02-pBudCE4.1; arrow showing the blue stained nucleus of the cells after picking the DAPI counter stain (arrowhead-slide B). (C) LigDNA03-pBudCE4.1 also well expressed due to the small size of the gene. (D) CompDNA04-pBudCE4.1 showing the green-coloured cytoplasm and the DAPI-stained nucleus. (E) Demonstration of expression of positive control GFP plasmid. (F) Negative control un-transfected CHO-K1 cells counter-stained with DAPI nucleic acid stain.

Confirmation of insert DNA by sequence analysis

Sequence analysis for pBudCE4.1 plasmid carrying each gene indicated a 100% homology with the reference sequence synthesized by Genescript (USA). The only exception was CompDNA04 with size 1299bp. Although, sequencing the gene in fragments would have sufficed. However, it should be noted that Sanger sequencing can only sequence DNA up to 1kb at a time. The result also revealed that the genes were inserted in the correct orientation as indicated by restriction enzyme sites inserted upstream and downstream of the insert DNA and the presence of nucleotide sequences of the plasmid flanking the N and C terminal portion of the insert.

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***In-vitro* expression of vaccine constructs**

The plasmid DNA constructs incubated for 48 hours gave the best result, with expression indicated by the strong cytoplasmic fluorescence produced (Figure 4). The green color of the cytoplasm indicates successful transcribed and translated DNA as against the blue-stained nucleus observed in the un-transfected control group. The fluorescence emission of FITC (Fluorescein isothiocyanate) conjugates produced green fluorescence, while DAPI, a nucleic acid stain that binds to A-T rich regions of DNA stains the nucleus blue. The positive control represented by GFP protein also produces solid green color, while the negative control represented by un-transfected cells only shows the blue stained nucleus material.

DISCUSSION

This study was undertaken to determine the potential of recombinant DNA vaccine construct based on *LipL32*, *LipL41*, *OmpL1*, *LigA*, and *Loa22* gene to serve as a vaccine candidate that can render protection against pathogenic *Leptospira* infection. The genes used are antigenically conserved among pathogenic *Leptospira* species, and their potential as vaccine candidates is enhanced by their expression during infection of mammalian host (Haake et al., 1999; Wang et al., 2007) This technology is mooted to provide better protection compared the routinely used inactivated vaccines due to the wide antigenic variation common among pathogenic *Leptospira* as reported by Dellagostin et al. (2011).

The synthesized gene was cloned into a plasmid in this study to understand their immune responses against leptospirosis. The fusion of the genes to the *myc* epitope and the V5 epitope permits co-expression of the insert genes as fused proteins to the epitopes and gives room for the detection and monitoring of the expression level of the genes. This approach is in congruence with earlier studies where *myc* and V5 epitope tags were used for protein expression due to their versatility and the ability for detection by immune cells (Traenkle et al., 2020). Similarly, the genes were expressed with the help of an expression vector using cell culture. The *in-vitro* expression of foreign genes is influenced by the promoter strength, the ribosomal binding site, which regulates the frequency of translation of the messenger RNA, the stability of the cloned protein, and the codons utilized in the foreign gene. In this study, the synthetic gene was codon optimized according to preferred codon usage for hamsters which is the chosen animal model and the origin of the cell line (Chung and Lee, 2012). It has been reported that optimal codons help achieve higher accuracy translation rates. However, the scientific basis of mammalian codon optimization indicates that codon usage is rate-limiting for protein expression (Lanza et al., 2014; Mauro and Chappell, 2014). Furthermore, the addition of flexible spacer sequences, as done in this study, ensures the stability of the insert DNA in the plasmid, allowing independent interaction of different segments without having any adverse effect on the outcome (Trinh et al., 2004).

Following the digestion of both genes and their corresponding plasmid vector, both were ligated using the T4 DNA ligase. T4 DNA Ligase catalyzes the joining of two DNA strands between the 5´-phosphate end of the insert DNA and the 3´-hydroxyl end of the digested plasmid nucleotides in a cohesive-ended configuration (Dickson et al., 2000).

The chemical competence conferred on Top10 *E. coli* cells was to ease the passage of the plasmid-carrying gene construct through the hydrophobic cell membrane, as earlier reported during vaccine development using the outer-membrane protein *LipL32* (Khodaverdi Darian et al., 2013; Humphryes et al., 2014). Although the Top10 *E. coli* cells' transformation efficiency was not determined, LigDNA03-pBudCE4.1 with 236bp had the highest success rate, while CompDNA04-pBudCE4.1 with 1299bp had the least. This observation may be due to the large size of the CompDNA04-pBudCE4.1. This is like studies that report that transformation efficiency decreases with an increase in DNA size (Ohse et al., 1995; Kung et al., 2013).

PCR and restriction enzyme digestion confirmed successful insertion of the genes into the plasmid. This was further supported by the sequencing results, which indicate the insertion of genes in the correct orientation by virtue of the restriction enzyme sequence and nucleotide sequences from the plasmid upstream and downstream of our insert gene. The sequencing result also shows 100% similarity with the synthesized sequence, indicating that no mutation occurred, particularly after exposure to UV light during the excision of bands from the gel. This is important as UV light is known to have mutagenic tendencies on DNA, which could have a detrimental effect on the integrity of the gene (Pfeifer et al., 2005; Rastogi et al., 2010). Although the quality of sequence result for CompDNA04-pBudCE4.1 was poor, especially at the beginning and towards the end of the sequence, this is one of the challenges of Sanger sequencing, and its due to poor quality in the first 15-40 bases of the sequence because of primer binding and deteriorating quality of sequencing traces after 700-900 bases (Ledergerber and Dessimoz, 2011). Sanger method is a popular sequencing technology for sequencing short pieces of DNA. However, the quality of a Sanger sequence is often not very good in the first 15 to 40 bases because that is where the primer binds (Tomotoch-Serra et al., 2017).

During pathogenic *Leptospira* infection, protection is dependent on the stimulation of an appropriate antibody response; highly potent neutralizing antibodies can intercept pathogenic *Leptospira's* before it attaches to its target cell. This ability is based on the antibodies' specific recognition of antigen epitopes (Gershoni et al., 2007). Thus, it is essential to evaluate the immunogenicity of B-cell combined epitopes for the development of novel vaccines. In this

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study, B-cell combined epitopes in the outer membrane proteins *LipL32*, *LipL41*, *OmpL1*, *LigA*, and *Loa22* from pathogenic *Leptospira*, which can induce the immune response against *Leptospiral* infection, were identified and characterized, and these will be used to develop vaccines or therapeutic strategies.

The production of recombinant proteins is a very important and powerful technique with multiple applications in life sciences (Pollet et al., 2021). Recombinant proteins have been used in industrial processes, diagnosis of infectious diseases, and production of subunit vaccines. Depending on the requirement of the cells, proteins are expressed and regulated to meet these purposes. The machinery for protein synthesis is stored in DNA and decoded by highly regulated transcriptional processes to create messenger RNA (mRNA). The mRNA is then translated into a protein (Pollet et al., 2021). Expressing the target protein is essential in producing the antigen required to initiate an immune stimulatory reaction. The expressed proteins can stimulate a specific response against invading microorganisms, eventually resulting in the clearance and development of immunological memory (Fraga et al., 2011). Hence, *in-vitro* gene expression has been used to explore the potential of DNA-induced immune response (Moreno et al., 2004).

In the current study, the expression of multi-epitope plasmid DNA for the purpose of producing a multivalent leptospirosis vaccine was successfully demonstrated. The use of a mammalian expression system in this study is to permit post-translational modifications, which ensures the regulation of the activity of the polypeptides, their location, and the completeness of their structure which enhances their biological activity (Duan and Walther, 2015). The efficient expression of a gene generally depends on how well it is transcribed. Transcription usually occurs when the RNA polymerase complex interacts with the promoter moving in the 5' to 3' direction leading to the production of mRNA transcript that dissociates from the gene at the transcription signal for subsequent translation. The gene expression in eukaryotic cells requires a suitable cell line and appropriate vectors that will act as a vehicle to transport the gene into the required cell lines. The pBudCE4.1 plasmid used in this study is designed for the independent expression of two genes in a single plasmid. It also possesses the CMV promoter for high-level transcription of genes with c-myc and V5 epitope tags for rapid detection of expressed proteins as well as 6xHis sequence for easy purification. In addition, it also has the human EF-1α promoter for high-level expression of genes. The lack of cross-reactivity among pathogenic serovars of *Leptispira* causing human and animal leptospirosis has greatly hampered the successful clinical application of many experimental vaccines developed (Barazzone et al., 2022). The results of the present study show promise that this lack of cross-protective immunity can be overcome using recombinant polyvalent epitope-based vaccines, which have been previously reported by other authors (Lauretti-Ferreira et al., 2020; Teixeira et al., 2020).

CHO-K1 cells are among the most common cell line utilized for protein expression. Similarly, CHO-K1 cells have been used to express the *Leptospiral OmpL37* gene (Oliveira et al., 2015). From the immunofluorescence images, it is obvious that LigDNA03-pBudCE4.1, which is the smallest with 236bp was expressed better compared to CompDNA04-pBudCE4.1 which is the longest with 1.3kb size. However, the expression level was not quantified. Although the actual expression levels were not determined, the judgment made in this research was based on subjective visualization and the number of cells per microscope field. Proximity to neighboring genes and size have all been reported to play significant roles in gene expression, with smaller genes being highly expressed, compared to longer ones (Chiaromonte et al., 2003).

CONCLUSION

In conclusion, this study was able to demonstrate the *in-vitro* expression of the synthetic genes and their reaction with antibodies against the *myc* and V5 epitope tags contained in the expression plasmid. This indicates the potential for these synthetic genes to serve as vaccine candidates for protection against multiple *Leptospira* infections in animals. It is, however, very important that further in-vivo evaluation be conducted using a suitable animal model to determine the efficacy and safety of the vaccine.

DECLARATION

Authors' contribution

Bashiru Garba conceptualized the idea and conducted the laboratory work. Both authors (Bashiru Garba and Najib Isse Dirie) contributed to the drafting, editing, and production of the final draft. All authors confirmed and consented to the final submission.

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Competing interests

The authors declare that they have no competing interests.

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Ethical considerations

The authors ensure that all ethical issues concerning plagiarism, approval to publish, errors in fabrication, double publication, and submission are adhered to.

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