Jurnal Sains Kesihatan Malaysia **18** (2) 2020: 73 - 81
DOI: https://doi.org/10.17576/jskm-2020-1802-08

Kertas Asli/*Original Article*s

Construction of an ***Escherichia coli*** expression vector for the non-structural (NS)-1 protein of avian influenza virus H5N1

(Pembangunan vektor pengekspresan ***Escherichia coli*** untuk protein non-struktural (NS)-1 virus influenza unggas H5N1)

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ABSTRACT

In the search for universal vaccine candidates for the prevention of avian influenza, the non-structural (NS)-1 protein of avian influenza virus (AIV) H5N1 has shown promising potential for its ability to effectively stimulate the host immunity. This study was aimed to produce a bacterial expression plasmid using pRSET B vector to harbour the NS1 gene of AIV H5N1 (A/Chicken/Malaysia/5858/2004 (H5N1)) for protein expression in Escherichia coli (E. coli). The NS1 gene (687 bp) was initially amplified by polymerase chain reaction (PCR) and then cloned into a pGEM-T Easy TA vector. The NS1 gene was released from pGEM-T-NS1 using EcoRI and XhoI restriction enzymes (RE). The pRSET B vector was also linearized using the same RE. The digested NS1 gene and linearized pRSET B were ligated using T4 DNA ligase to form the expression plasmid, pRSET B-NS1. The NS1 gene sequence in pRSET B-NS1 was confirmed by DNA sequencing. To prepare recombinant bacterial cells for protein expression in the future, pRSET B-NS1 was transformed into E. coli strain BL21 (DE3) by heat-shock. Colonies bearing the recombinant plasmid were screened using PCR. The DNA sequencing analysis revealed that the NS1 gene sequence was 97% homologous to that of AIV H5N1 A/Chicken/Malaysia/5858/2004 (H5N1). These results indicated that the NS1 gene of influenza A/Chicken/Malaysia/5858/2004 (H5N1) was successfully amplified and cloned into a pRSET B vector. Bacterial colonies carrying pRSET B-NS1 can be used for the synthesis of NS1-based influenza vaccine in the future and thereby aid in the prevention of avian influenza.

Keywords: NS1; avian influenza; H5N1; vaccine; protein expression

ABSTRAK

Dalam usaha menemui calon vaksin universal untuk pencegahan influenza unggas, protein bukan struktur (NS)-1 virus flu burung (AIV) H5N1 telah menunjukkan potensi yang memberangsangkan dalam merangsang imuniti perumah secara berkesan. Kajian ini bertujuan untuk menghasilkan plasmid pengekspresan bakteria dengan menggunakan vektor pRSET B bagi membawa gen NS1 virus AIV H5N1 (A/Chicken/ Malaysia/5858/2004 (H5N1)) untuk tujuan pengekspresan protein dalam Escherichia coli (E. coli). Gen NS1(687 bp) diperbanyak melalui reaksi berantai polimerase (PCR) dan kemudian diklonkan ke dalam vektor TA pGEM-T Easy. Seterusnya, gen NS1 dibebaskan dari pGEM-T-NS1 dengan enzim restriksi EcoRI dan XhoI. Vektor pRSET B juga dilinearkan dengan enzim restriksi yang sama. Gen NS1 dan pRSET B kemudian digabungkan dengan menggunakan enzim T4 DNA ligase untuk membentuk plasmid pengekspresan, pRSET B-NS1. Jujukan gen NS1 dalam pRSET B-NS1 disahkan melalui kaedah penjujukan DNA. Untuk menyediakan sel bakteria rekombinan bagi pengekspresan protein kelak, pRSET B-NS1 dimasukkan ke dalam E. coli strain BL21 (DE3) melalui kaedah kejutan panas. Koloni yang membawa plasmid rekombinan disaring dengan menggunakan PCR. Keputusan penjujukan DNA menunjukkan bahawa jujukan gen NS1 adalah 97% homologus dengan gen NS1 AIV H5N1 A/Chicken/Malaysia/5858/2004 (H5N1). Hasil ini menunjukkan bahawa gen NS1 influenza A/Chicken/Malaysia/5858/2004(H5N1) telah berjaya diamplifikasikan dan diklonkan ke dalam vektor pRSET B. Koloni bakteria yang membawa pRSET B-NS1 boleh digunakan untuk mensintesis vaksin influenza berasaskan NS1 pada masa akan dating, dengan itu, membantu dalam pencegahan influenza unggas.

Kata kunci: NS1, flu unggas, H5N1, vaksin, pengekspresan protein

INTRODUCTION

Avian influenza virus (AIV) strain H5N1 is a highly pathogenic influenza A virus that causes severe respiratory illnesses in birds. Close contact with infected poultry increases the opportunity of AIV H5N1 to spill over and cause infections in human (Alexander et al. 2018). The first reported H5N1 infection in human occurred in Hong Kong in 1997 (Claas et al. 1998), followed by re-emergence in Asia in 2003 and 2006 (Peiris et al. 2004; WHO 2009). According to World Health Organization (WHO), AIV H5N1 has caused 861 cases with 455 death in 17 countries since 2003 (WHO 2020). The ability of AIV H5N1 to breach the birds-to-human species barrier is driven by 2 major mutational changes: (1) point mutations that alter the affinity of the viral hemagglutinin (HA) protein to sialic acid receptors on host cells (Crusat et al. 2013), and (2) gene reassortment with human-adapted influenza A subtypes (Schrauwen et al. 2013). This gives rise to the emergence of new AIV strains which possibly cause global influenza pandemics with high morbidity and mortality (Webster & Govorkova 2014).

Vaccination is by far one of the most effective preventive measures against influenza (Lee & Shah 2012). The commercially available influenza vaccines are made based on virus surface antigens: hemagglutinin (HA) and neuraminidase (NA) of recently circulating strains (Wong & Webby 2013). In general, production of influenza vaccines involves embryonated chicken eggs and therefore requires long production time (Lu et al. 2017). Furthermore, the vaccines are only effective against homologous influenza subtypes and may potentiate allergic reactions against egg proteins in certain individuals (Soema et al. 2015). Due to the rapid antigenic change among the circulating influenza strains, it requires annual reformulation of flu vaccines. In addition, since the conventional influenza vaccines only target the circulating influenza subtypes, they do not protect humans from a novel, potential pandemic strain. Therefore, a universal vaccine candidate that can induce cross-protection against the majority of influenza subtypes is needed (Sautto et al. 2018).

The influenza non-structural (NS)-1 protein is a relatively conserved protein among influenza A subtypes. It is a multifunctional protein involved in the viral replication and inhibition of host immune responses (Tynell et al. 2014). Although the NS1 protein has been shown to inhibit the host immunity especially the type-1 interferon (IFN) responses when it is produced abundantly on infected cells (Kuo et al. 2016; Li et al. 2010), a number of studies demonstrated that influenza viruses carrying mutated NS1 genes or small quantities of NS1 protein on virus particles could induce protective responses against the virus infections and hence attenuation of the disease symptoms in animal models (Richt & Garcia-Sastre 2009; Steel et al. 2009). Surprisingly, this protective response were not only limited to the homologous influenza strains but also effective against heterologous strains, thus indicating a broad, cross-neutralizing response induced by the recombinant influenza viruses (Steel et al. 2009). Given its conserved amino acid sequences among influenza strains and ability to induce protective immune responses, this suggests the potential of influenza NS1 protein as a universal vaccine candidate.

Owing to newer advances in the field of recombinant DNA technology, several types of pathogen-free vaccines such as recombinant subunit vaccines have been developed and proven to be effective in preventing contagious infections, for instance, hepatitis B (Michel & Tiollais 2010) and human papillomavirus (Schiller et al. 2012). The use of recombinant DNA technology in vaccine development is less tedious and labourious and relatively faster compared to the conventional methods used to produce seasonal influenza vaccines (Harding & Heaton 2018). Moreover, the availability of various bacterial expression systems has made the vaccine production even more easier, quicker and less complicated (Legastelois et al. 2017, Mahalik et al. 2014). In this study, an expression plasmid anchoring the *NS1* gene of AIV H5N1 was generated by recombinant DNA techniques and subsequently transfected into *Escherichia coli* (*E coli*) BL21 strain (DE3). The recombinant *E. coli* can be employed to produce a NS1-based universal influenza vaccine in the future.

MATERIALS AND METHODS

BACTERIAL STRAIN, PLASMID AND CHEMICALS

The viral RNA of AIV H5N1 A/Chicken/Malaysia/5858/2004 (H5N1) was provided by Associate Professor Dr. Sharifah Syed Hassan from the Monash University Sunway. Bacterial hosts used in this study were *E. coli* strain Top10 for plasmid maintenance and *E. coli* BL21 strain (DE3) (Invitrogen, United States) for protein expression. Plasmid vectors used in this study were pGEM-T Easy (Promega, United States) for TA-cloning and pR*SET* B (Invitrogen, United States) for protein expression.

AMPLIFICATION OF *NS1* GENE

The viral RNA was converted to first-strand cDNA with oligo-dT primers (Promega, Madison, USA) and AMV

reverse transcriptase (Promega, Madison, USA). The *NS1* gene of AIV A/Chicken/Malaysia/5858/2004 (H5N1) was amplified from the first-strand cDNA using the Accura High-Fidelity DNA polymerase (Lucigen Corporation, USA). The PCR reaction contained: Accura High-Fidelity DNA polymerase (1U), Accura HF reaction buffer (1X), dNTPs (200 µM), forward and reverse primer (1 µM), DNA template (55.5 ng) and nuclease-free water. The following primers were employed, NS1-F: 5'-AGCTCGAGAGATTCCAACACTGTGTCA-3 and NS1-R: 5'- GAATTCAACTTCTGACTCAATTGTGCT- 3'. The underlined sequences are *Xho*I and *Eco*RI digestion sites, respectively. The thermal cycling conditions were as follows: 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 60 s, and a final extension step at 72°C for 10 min. The PCR product (Figure 1) was mixed with the 6X Purple Gel DNA loading dye (NEB, United Kingdom), analyzed on a 1% (w/v) agarose gel at 80 V for 1 h and visualized under an UV transilluminator. The resulting PCR product was then purified using the Wizard® SV Gel and PCR Clean-Up system (Promega, United States) prior to TA-cloning in pGEM-T Easy plasmid. Before ligation, A-tailing of PCR product was performed to add an adenine residue to the 3'-end using the GoTaq Flexi DNA polymerase. The reaction mixture contained GoTaq Flexi DNA Polymerase (2.5 U), GoTaq Flexi Colorless Buffer (1X), dATP (200 µM), magnesium chloride (4 mM), the purified *NS1* gene as a template and nuclease-free water.

CLONING OF NS1 GENE INTO PGEM-T EASY TA VECTOR

Cloning of the purified *NS1* gene into pGEM-T Easy TA vector was carried out using T4 DNA ligase (NEB, United Kingdom) at 1:3 (vector:insert) ratio. The ligation reaction was incubated overnight at 4°C and transformed into competent *E. coli* strain Top10 at 42°C for 90 s. The bacterial cells were then revived in 200 µl of LB broth at 37°C and 150 rpm for 1.5 h. The culture was spread on LB agar containing IPTG (0.1 mM), X-gal (40 µg/mL) and ampicillin (100 µg/mL) for 18 h.

BLUE-WHITE AND COLONY PCR SCREENING

Positive colonies carrying the pGEM-T-*NS1* plasmid were identified by blue-white screening in the presence of X-gal and IPTG. Bacterial colonies containing the PGEM-T-*NS1* plamid appeared as white colonies while blue colonies indicated negative colonies. The white colonies were further validated using the colony PCR screening. In this study, five white colonies were picked and mixed with Phosphate Buffer Saline (PBS: 4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCL, 1.4 mM KH_2PO_4, pH 7.6). The cell suspension was then added to the EconoTaq Plus 2x Mastermix containing the NS1 primer pair for PCR screening. The PCR product was analyzed on a 1% (w/v) agarose gel as described in 2.2.

DIGESTION OF PGEM-T- *NS1* AND PRSET B PLASMID

The pGEM-T-*NS1* was isolated using the HiYield Plasmid Mini Kit 2.0 (Yeastern Biotech, Taiwan). The purity and yield of the plasmid was measured at A_{260} and A_{280} using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, United States). The *NS1* gene was liberated from pGEM-T-*NS1* using *Eco*RI and *Xho*I at 37°C for 4 h. The *E. coli* expression plasmid pR*SET* B was linearized with the same enzymes. The digested *NS1* and pR*SET* B were mixed with 6X Purple Gel DNA loading dye and analyzed using agarose gel electrophoresis as described earlier. The digested products were then excised and purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up system (Promega, USA).

CLONING OF *NS1* GENE INTO PR*SET* B PLASMID

Ligation of the purified *NS1* gene with the linearized pR*SET* B plasmid was carried out overnight at 16°C using T4 DNA ligase. The ligation product was first transfected into competent *E. coli* strain Top10 cells using heat shock at 42°C for 90 s. Selection of postitive colonies was carried out using blue-white and colony PCR screening. The

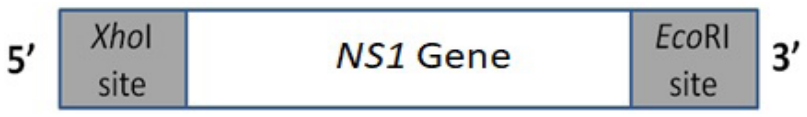

FIGURE 1. The schematic diagram of the amplified *NS1* gene (687 bp). The amplicon harbours *Xho*I and *Eco*RI cutting sites at its 5'- and 3'-ends, respectively

recombinant pR*SET* B-*NS1* plasmid was then isolated and transfected into competent *E. coli* BL21 strain (DE3) cells using heat shock at 42°C for 45 s. The bacterial cells were revived with 200 µl of LB broth at 37°C, 150 rpm. The bacterial suspension was spread on LB agar containing 100 µg/ml ampicillin. The bacterial colonies carrying the pR*SET* B-*NS1* plasmid were screened using PCR.

SEQUENCING OF PR*SET* B-*NS1*

pR*SET* B-*NS1* was isolated and purified as previously described in section 2.4. The plasmid was then sequenced to determine the *NS1* gene sequence in the plasmid. The nucleotide sequences of the *NS1* gene was compared with that of AIV A/Chicken/Malaysia/5858/2004 (H5N1) using the Basic Local Alignment Search Tool (BLAST).

RESULTS AND DISCUSSION

AMPLIFICATION OF *NS1* GENE

The *NS1* gene (687 bp) of influenza A strain H5N1 (A/chicken/Malaysia/5858/2004) was successfully amplified as observed in Figure 2. Although the amount of template used in the PCR reaction was relatively low (55.5 ng), due to the high performance of Accura High-Fidelity DNA polymerase, the amount of amplicon was sufficiently produced for the A-tailing and TA cloning experiments (Ishino & Ishino 2014). The Accura High-Fidelity *Pfu* DNA polymerase possesses higher ability to prevent the occurrence of gene mutations in the amplification processess than *Taq* DNA polymerases (McInerney, Adams & Hadi 2014). However, *Pfu* DNA polymerase produces blunt-end PCR products, therefore, additional adenosine residues are added to the 3'-end of PCR products using *Taq* DNA polymerase (Yu et al. 2014) in order to facilitate the subsequent TA cloning process in pGEM-T Easy. The PCR product was later purified to remove unwanted traces of enzyme, primers and salts that will interfere with the subsequent DNA ligation (Carson et al. 2019).

CLONING OF *NS1* GENE INTO PGEM-T EASY TA VECTOR

The 687-bp amplicon was cloned into a pGEM-T Easy vector in order to improve restriction digestion efficiency (Sambrook & Russel 2001). The pGEM-T Easy vector is a TA-cloning vector which employs complementarity between 3'-T overhang in the vector and 3'-A overhang in the PCR fragment (Aranishi & Okimoto 2004). Transformation of pGEM-T-*NS1* into *E. coli* strain Top 10 cells was made possible by using divalent or multivalent cations such as calcium and magnesium that enable foreign DNA molecules to cross the bacterial membrane (Tan et al. 2017). The *ampicillin-resistant* gene in the pGEM-T vector confered the antibiotic resistance to the positive transformants. This allows selection of postive colonies on LB agar supplemented with ampicillin (Tan, Syed Hassan & Yap 2017). Besides, the insertion of *NS1* gene into the pGEM-T Easy vector inactivates β–galactosidase enzyme that is responsible to oxidize lactose medium into a blue product under the influence of *lac/tac* promoter (Sambrook & Russel 2001). The activation of *lac/tac* promoter is usually induced by the addition of IPTG (Yao, Hart & An 2016). Therefore bacterial colonies carrying the pGEM-T-

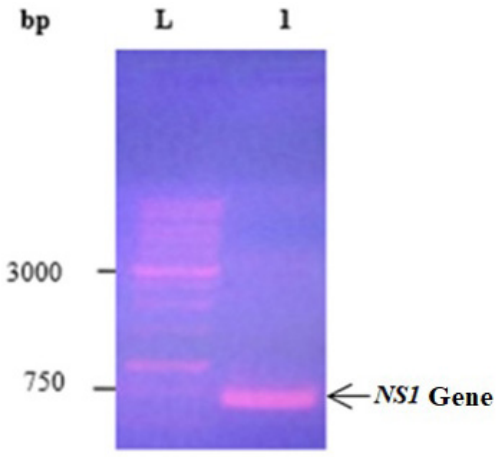

FIGURE 2. The PCR product of AIV H5N1 *NS1* gene. Lanes L: 1 kb DNA ladder; 1: amplified *NS1* gene. A bright band below 750 bp was observed and it correlated well with the molecular size of the *NS1* gene

NS1 appeared as white colonies whereas the negative transformants turned out as blue colonies (results not shown). In order to better validate the presence of pGEM-T-*NS1* in those white colonies, the colony PCR (Tan, Syed Hassan & Yap 2017) was performed and the amplicon was then visualized using agarose gel electrophoresis. Among the five selected colonies, four of them showed a band below the 750-bp marker (Figure 3). This is in parallel to the expected size of the *NS1* gene (687 bp).

DIGESTION OF PGEM-T-*NS1* AND PR*SET* B PLASMID

The primer pair used to amplify the *NS1* gene was designed to harbour *Eco*RI and *Xho*I restriction sites at the 3'-end and 5'-end of the PCR product, respectively. The pGEM-T-*NS1* gene and pR*SET* B plasmid were digested by those two restriction enzymes prior to ligation. This generated compatible cloning sites on the *NS1* gene and pR*SET* B vector. The RE digestion was carried out for 4 h in this study in order to ensure high digestion efficiency (Ihle & Michaelsen 2000). Figure 4 shows the restriction products, P (pGEM-T Easy and *NS1*) and Pb (linearized pR*SET* B). Upon incubation, the RE were heat inactivated at 65ºC for 10 min to avoid unspecific cutting (Fischer, Mgboji & Liu 2018). Before proceeding to ligation, the digested products were purified from unwanted materials that may interfere with the ligation (Lessard 2013).

CLONING OF *NS1* GENE INTO PR*SET* B PLASMID

pR*SET* B-*NS1* (Figure 5) was multiplied in *E. coli* Top10 in order to produce sufficient amount of plasmid for transformation into *E. coli* BL21 (DE3) (Casali & Preston 2003). pR*SET* vectors are designed to ensure high-level protein expression of cloned genes in *E. coli* carrying the *lambda (λ) phage DE3* gene that tightly regulates the activities of RNA polymerase (Horiuchi 2018; Rosano & Ceccarelli 2014). In addition, pR*SET* vectors contain a polyhistidine tag that is useful for metal binding in affinity chromatography protein purification (Terpe 2006) and an enterokinase cleavage recognition sequence to allow subsequent removal of N-terminal fusion peptides from the purified recombinant proteins in order to prevent structural and functional disruption (Ramos et al. 2004).

SEQUENCING OF *NS1* GENE IN PR*SET* B-*NS1*

The DNA sequencing analysis showed that the *NS1* gene was located downstream of the N-terminal fusion peptides and in frame with the cloning sites in the vector (Figure 5). The nucelotide sequence was also aligned with that of influenza A/Chicken/Malaysia/5858/2004 (H5N1). The BLAST findings indicated that the amplified *NS1* gene was 97% similar to that of the A/Chicken/Malaysia/5858/2004 (H5N1). Some point mutations were introduced in the *NS1* gene and this is mainly due to the low fidelity of the viral RNA polymerase that renders a high mutational rate to the viral genome meanwhile lacks of proofreading ability when the viral genome is replicated in host cells (Kautz & Forrester 2018). When the *NS1* gene sequence was translated to amino acid sequence, the similarity was retained as high as 96%. There were nine amino acid changes in the NS1 sequence: G47S, K75E, A81T, K95R, A106V, V122N, T190S, F196Y and P210L (Figure 6). However, this alteration did not affect the antigenic sites of NS1 protein: NTVSSFQV at position 4-11 (Ishizuka et

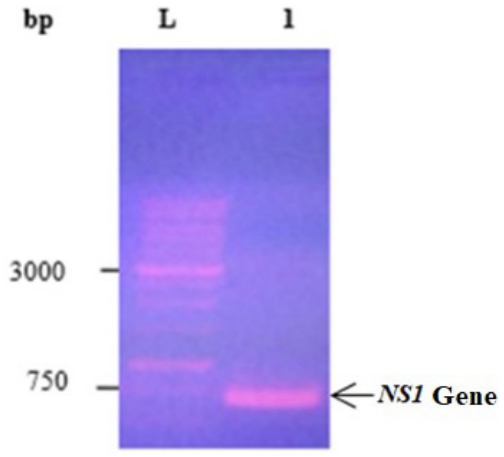

FIGURE 3. Colony PCR screening of 5 white colonies formed on the LB agar plate. Lanes 1-5: white colonies; L: 1-kb DNA ladder. The arrow indicates the amplified *NS1* gene

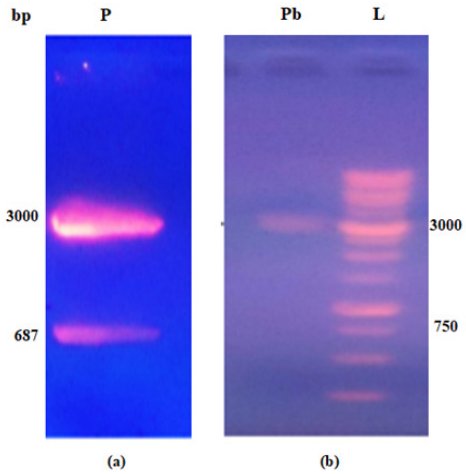

FIGURE 4. Restriction digestion using *Eco*RI and *Xho*I enzymes. In (a), two distinct fragments, around 3000 bp and 687 bp, were observed on the gel after pGEM-T-*NS1* (P) was digested with the RE. In (b), the linearized pR*SET* B (Pb) was about 3000 bp in size. Lane L: 1kb DNA Ladder

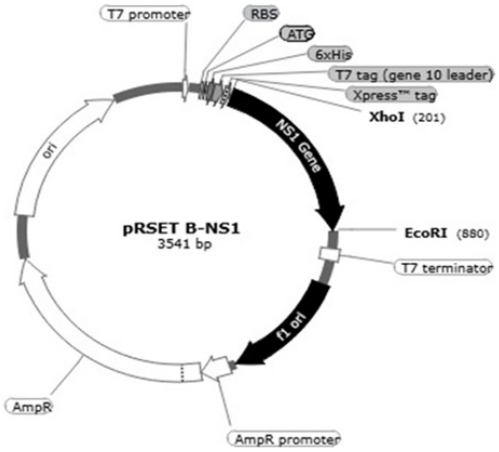

FIGURE 5. pR*SET* B-*NS1* expression vector. The *NS1* gene is located downstream of the N-terminal fusion peptides

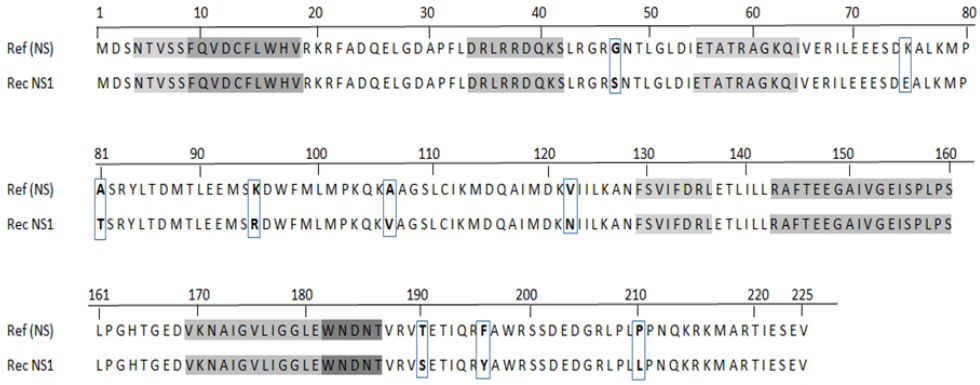

FIGURE 6. The mutated amino acid in the recombinant NS1 protein. [Ref (NS)] denotes the NS1 protein of AIV H5N1 A/Chicken/Malaysia/5858/2004 (H5N1); [Rec (NS1)] is the NS1 protein expressed from pRSET B-NS1. The bold sequences are antigenic sites and mutated amino acids are bold and boxed. The mutations are not located in the antigenic sites

al. 2009), FQVDCFLWHV at position 9-18 (Alexander et al. 2010), DRLRRDQKS at position 34-42 (Jameson et al. 1999), ETATRAGKQI at position 55-64 (Sequence Feature Details (Influenza A_NS1_experimentally-determined-epitope_55(10), accessed on 29.02.2020), FSVIFDRL at position 129-136 (Zhong et al. 2003), VKNAIGVLIGGLEWNDN within residues 169-185 (Richards et al. 2018), RAFTEEGAIVGEISPLPS at position 143-160 (Richards et al. 2011) and WNDNT at position 182-186 (Wen et al. 2015). It is therefore believed that the NS1 protein expressed from the amplified *NS1* gene will retain its antigenicity. However this needs to be autheticated using immunoassays such as Western blotting and ELISA using anti-NS1 antibody or antisera.

CONCLUSION

The *NS1* gene of influenza A/Chicken/Malaysia/5858/2004 (H5N1) virus was successfully amplified and cloned into a pR*SET* B expression vector. The recombinant plasmid, pR*SET* B-*NS1* was successfully transformed into *E. coli* BL21 strain (DE3). The DNA sequencing analysis showed that the *NS1* gene sequence was 97% similar to that of the parental H5N1 virus. This recombinant NS1 can be further tested with immunoassays for its antigenicity and immunogenicity, and potential as a universal influenza vaccine candidate.

AKNOWLEDGEMENTS

The project was supported by the Fundamental Research Grant Scheme (FRGS/1/2017/STG05/UKM/02/9) granted by the Malaysia Ministry of Education. The authors would like to thank Associate Professor Dr. Sharifah Syed Hassan fom Monash University Sunway for her generosity in providing the viral RNA.

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