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 memerianangkakan dalam merangsang imuniti perumah secara berkesan. Kajian ini berjaya 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 dikeluarkan dari 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 diamplifikasi dan dikeluarkan ke dalam vektor pRSET B. Koloni bakteria yang membawa pRSET B-NS1 boleh digunakan untuk menyiapkan vaksin influenza berasaskan NS1 pada masa akan datang, 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 pRSET 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/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’-AGCTCGAGAGATTTCAACACTGTTCA-3 and NS1-R: 5’-GAATTCACTTTGACATTTGCTGCT-3’. The underlined sequences are XhoI and EcoRI 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 the 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 plasmid 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 Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, 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 A₂₆₀ and A₂₈₀ using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, United States). The NS1 gene was liberated from pGEM-T-NS1 using EcoRI and XhoI at 37°C for 4 h. The E. coli expression plasmid prSET B was linearized with the same enzymes. The digested NS1 and prSET 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 PRSET B PLASMID**

Ligation of the purified NS1 gene with the linearized pRSET 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 positive colonies was carried out using blue-white and colony PCR screening. The

![FIGURE 1](image_url) The schematic diagram of the amplified NS1 gene (687 bp). The amplicon harbours XhoI and EcoRI cutting sites at its 5’- and 3’-ends, respectively.
The recombinant pRSET 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 revivied 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 pRSET B-NS1 plasmid were screened using PCR.

SEQUENCING OF PRSET B-NS1

pRSET 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 processes 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 PGM-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 positive 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](image-url)
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 PGM-T-NS1 AND PRSET B PLASMID

The primer pair used to amplify the NS1 gene was designed to harbour EcoRI and XhoI restriction sites at the 3'-end and 5'-end of the PCR product, respectively. The pGEM-T-NS1 gene and pRSET B plasmid were digested by those two restriction enzymes prior to ligation. This generated compatible cloning sites on the NS1 gene and pRSET 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 pRSET B). Upon incubation, the RE were heat inactivated at 65°C 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 PRSET B PLASMID

pRSET 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). pRSET 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, pRSET 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 PRSET 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 EcoRI and XhoI 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 pRSET B (Pb) was about 3000 bp in size. Lane L: 1kb DNA Ladder

FIGURE 5. pRSET 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
The NS1 gene of influenza A/Chicken/Malaysia/5858/2004 (H5N1) virus was successfully amplified and cloned into a pRSET B expression vector. The recombinant plasmid, pRSET 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.

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