Production of Fused Protein Consisting of Vp2 From Infectious Bursal Disease Virus and Hn from Newcastle Disease Virus in Escherichia coli

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Abstract. Viral protein 2 (VP2) of infectious bursal disease virus (IBDV) and hemagglutinin-neuraminidase (HN) of Newcastle disease virus (NDV) are viral surface proteins which contain epitopes that are able to induce neutralizing antibodies for protection against Newcastle disease and infectious bursal disease. In the present study, recombinant fused protein consisting of VP2 and partial HN protein was produced. The fused protein was made by fusing VP2 gene to full length (fHN) and partial HN (pHN) gene, separately and cloned it into pRSETB expression vector. The recombinant construct is then transformed into E. coli BL21 (DE3) for production of the fused protein. The fused VP2-pHN protein was successfully produced in E. coli BL21 (DE3) strain with the size of 75 kDa detected by anti-His monoclonal antibody via Western blot analysis.

Keywords: Newcastle disease, Infectious bursal disease, Hemagglutinin-neuraminidase, Viral protein 2, Escherichia coli, Fused protein.

INTRODUCTION

Newcastle disease (ND) is one of the major poultry diseases that have been causing constant economic losses to the poultry industry worldwide. The causative agent of ND is Newcastle disease virus (NDV), also known as avian paramyxovirus serotype 1 (APM1), which is an enveloped virus consisting of negative-sense, single stranded RNA genome. Strains of NDV are classified into three types depending on the degree of virulence, which are non-virulent (lentogenic) strains, intermediate virulent (mesogenic) strains, and highly virulent (velogenic) strains. The strains can cause clinical signs such as respiratory and nervous signs that vary from inapparent infections to 100% mortality depending on the different types of strains (Alexander, 2003).

Besides ND, another major poultry disease is the infectious bursal disease (IBD) that is caused by infectiousbursaldiseasivirus(IBDV)whichisanenveloped virus that infects the actively dividing and differentiating lymphocytes of the B-cells in the bursa of Fabricius which retards the production of antibody and thus leads to susceptibility to other diseases (Burkhardt & Muller, 1987). From the two serotypes of IBDV recognized, only serotype 1 strains are the pathogenic IBDV strains. Serotype 1 IBDV is further classified into three main strains which are variant strains of IBDV (vIBDV), standard or classical virulent strains of IBDV (cIBDV) and very virulent strains of IBDV (vvIBDV). The vIBDV could infect and cause disease in chickens even though it has been vaccinated with the cIBDV (Ismail et al., 1990). The vvIBDV strains could cause infections of up to 50% mortality in chickens (Lin et al., 1993). IBDV belongs to the genus Avibirnavirus in the family Birnaviridae that contains genome which is composed of two segments of double-stranded RNA (dsRNA) (Dobos et al., 1979).

Despite the continuous efforts in the vaccination programme, outbreaks of Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) in vaccinated chickens of poultry farms are still occurring worldwide in various years as reported by previous studies. Several ND outbreaks have been reported in Malaysia (Tan et al., 1990). 

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MATERIALS AND METHODS

Virus: Chorioallantoic membrane (CAM) and allantoic fluid infected by IBDV strain 3529/92 and NDV strain 7979, respectively, were provided by Veterinary Research Institute (VRI) of Malaysia and used for plasmid construction. IBDV strain 3529/92 (very virulent strain) and NDV strain 7979 (velogenic strain) were previously isolated from infected broiler chickens in local poultry farm located at Perak and Selangor, respectively, and propagated in specific pathogen free (SPF) eggs by VRI.

Bacterial strains and vectors. E. coli TOP10 strain (Invitrogen, USA) was used for cloning and maintenance of plasmids while E. coli BL21 (DE3) strain (Novagen, Germany) was utilized as expression host. pCR2.1TOPO (Invitrogen, USA) and pRSETB (Invitrogen, USA) vectors were used as cloning vector and expression vector, respectively.

Amplification of Genes via RT-PCR. Total RNA was extracted from IBDV strain 3529/92 infected CAM and NDV strain 7979 infected allantoic fluid using Trizol LS reagent (Invitrogen, USA) following the modified manufacturer's instruction. Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized using Superscript III One Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA) to isolate VP2 and HN gene from the total RNA extracted previously based on the manufacturer's protocol. Primers (Table 1) for amplifying VP2 gene were designed based on the 5' and 3' ends of VP2 sequence of IBDV 3529/92 available in GenBank database with accession number of KC189836.1. For amplifying HN gene, primers synthesized were based on primers used in previously reported studies on HN gene (Kattenbelt et al., 2006; Maminiaina et al., 2010). A reaction mixture containing 12.5 µl of 2X reaction mix, 0.5 µl of 20 µM forward primer, 0.5 µl of 20 µM reverse primer, 0.5 µl of 5.0 U/µl platinum Taq DNA polymerase, 2.0 µl of RNA as template and sterile nuclease-free H2O was set to a volume of 25 µl. Three replications were prepared to obtain high amount of PCR product. Reactions were conducted according to the following protocol: 48 °C for 30 min and 94 °C for 5 min, followed by 40 cycles (94 °C for 1 min, 56 °C for 1 min, 68 °C for 2 min) and final elongation step of 68 °C for 5 min in a PCR machine. The amplified DNA fragments were separated on 1 % agarose gel and the appropriate bands were excised and purified.

Cloning of VP2 and HN genes into pCR2.1TOPO vector: The amplified VP2 and HN genes were cloned separately into a cloning vector pCR2.1TOPO, transformed into E. coli TOP10 strain and plated on LB agar containing X-gal and ampicillin (50 µg/ml) for blue/white screening. Recombinant plasmids (pCR2.1TOPO-VP2 and pCR2.1TOPO-HN) were extracted from white colonies and digested with restriction enzymes to confirm the presence of inserts. The complete nucleotide sequences of VP2 and HN genes were identified via sequencing (First BASE, Serdang) followed by multiple sequence alignment and Basic Local Alignment Search Tool (BLAST).

Production of fused VP2-HN protein in E. coli

Construction of fused gene in pRSETB expression vector

Specific primers with addition of restriction enzyme sites at the 5’ and 3’ ends were designed based on the complete sequence of VP2 and HN genes to facilitate the construction of fused gene in pRSETB vector (Table 1). Full length of VP2 gene was amplified from pCR2.1TOPO-VP2 plasmid using FVP2_XhoIF and FVP2_KpnIR primers via PCR before digested with restriction enzymes and ligated pRSETB expression vector. The resulting pRSETB-VP2 was extracted, digested with restriction enzymes, and subsequently sent for sequencing to confirm the presence of insert. Full length of HN gene (flHN) and partial HN (pHN) gene were amplified from pCR2.1TOPO-HN plasmid using specific primers via PCR before digested with restriction enzymes and ligated to pRSETB-VP2 to generate pRSETB-VP2-HN and pRSETB-VP2-pHN. After confirming the presence of inserts via restriction enzyme analysis and sequencing, the resulting recombinant plasmids were transformed into E. coli BL21 (DE3) strain to express the fused genes.

Production of Fused Protein in E. coli BL21 (DE3) Strain

The recombinant plasmid of pRSETB-VP2-HN was transformed into E. coli BL21 (DE3) strain via heat-shock transformation and plated on LB agar containing 50 µg/ml ampicillin for selection of positive transformant. The positive colony was grown in 5 ml LB broth supplemented with 50 µg/ml ampicillin at 37 °C with vigorous shaking for overnight. After approximately 15 h, 1 ml of the overnight culture was transferred to a 250 ml shake flask containing 40 ml LB broth and was grown at 37 °C until the cells reached OD600 of 0.6-1.0. At this point, IPTG was added to the culture to a final concentration of 1 mM to induce the expression of the fused gene to produce VP2-HN protein. Incubation was continued for another 4 hours at 30 °C prior to collection of the cells by centrifugation at 8000 rpm for 10 min at room temperature. The same procedure was performed on pRSETB-VP2-pHN to produce the fused protein.

Table 1: List of primers used in the present study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Fragment</th>
<th>Length (bp)</th>
<th>Restriction sites (underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2_forward</td>
<td>5’ ATGACGAACCTGCAAGATCAAACC CAA 3’</td>
<td>VP2</td>
<td>1356</td>
<td>-</td>
</tr>
<tr>
<td>VP2_reverse</td>
<td>5’ CCTTAGGGCCCGGAATTATGTCTTT GAA 3’</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>NDV_5724F</td>
<td>5’ AGATGACAACATGTAGATG 3’</td>
<td>Fragment HN</td>
<td>2670</td>
<td>-</td>
</tr>
<tr>
<td>NDV_8390R</td>
<td>5’ TCTGCCCTTTCAGGACCGGA 3’</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>FVP2_XhoIF</td>
<td>5’ GGTATTCTCGAGTAGACGAACCTGCAAAGA 3’</td>
<td>VP2</td>
<td>1356</td>
<td>XhoI – 5’ end</td>
</tr>
<tr>
<td>FVP2_KpnIR</td>
<td>5’ GCATTCCGTTACCCCTTAGGGCCCGGAT-TAT 3’</td>
<td></td>
<td></td>
<td>KpnI – 3’ end</td>
</tr>
<tr>
<td>pHN_KpnIF</td>
<td>5’ GGGTTTGGTACCCAATACCATGAG AAG GAC 3’</td>
<td>pHN</td>
<td>640</td>
<td>KpnI – 5’ end</td>
</tr>
<tr>
<td>pHN_EcoRIR</td>
<td>5’ ATCAAGGAATTCTTTAGAAGACCCCTCG-TAG 3’</td>
<td></td>
<td></td>
<td>EcoRI – 3’ end</td>
</tr>
<tr>
<td>flHN_KpnIF</td>
<td>5’ ATTATAGGTACCAGGACCGCGG GTT 3’</td>
<td>HN</td>
<td>1716</td>
<td>KpnI – 5’ end</td>
</tr>
<tr>
<td>flHN_EcoRIR</td>
<td>5’ AAGTCTGAATTCTAAAATCTATC ATCCGT 3’</td>
<td></td>
<td></td>
<td>EcoRI – 3’ end</td>
</tr>
</tbody>
</table>
**SDS-PAGE.** Production of the fused protein by *E. coli* BL21 DE3 was confirmed by extracting the total protein from the bacterial cells before subjecting to SDS-PAGE to separate the proteins based on molecular weight. The proteins were extracted using BugBuster Master mix (Novagen) and subjected to Bradford protein assay to measure the total protein concentration. Equal amount of total protein for each sample and negative control lysates were loaded into 12% SDS-PAGE gel. The protein bands were visualized by staining the gel with Coomassie blue dye.

**Western Blot Analysis** After separating the proteins in SDS-PAGE, the proteins were transferred to nitrocellulose membrane using semi dry transfer cell apparatus (BioRad, USA) according to manufacturer’s instructions. Prior to adding the specific antibodies, the membrane was incubated in blocking buffer (5% skimmed milk powder in TBST) at room temperature for 1 h with gentle shaking followed by washing with TBST for three times. Anti-his monoclonal antibody (1:1000; Merck Millipore, Germany) was added to the membrane and incubated for 1 h with gentle shaking. After washing, goat anti-mouse IgG AP conjugated (H+L) (Novagen, USA) was added to the membrane with 1:5000 dilution and incubation was continued for another 1 h at room temperature with gentle shaking. Western blue stabilized substrate for alkaline phosphatase (Promega, USA) was added to yield protein bands which were compared with the precision plus protein kaleidoscope standards (Bio-Rad, USA).

**RESULTS**

**Cloning of VP2 and HN genes into pCR2.1TOPO.** A VP2 gene of 1356 bp was amplified from IBDV 3529/92 isolate RNA genome (Figure 1) and cloned into pCR2.1TOPO vector. A fragment containing HN gene (Fragment HN) was amplified from NDV 7979 isolate RNA genome and cloned into pCR2.1TOPO vector. The primers amplified a region in the genome that contained HN gene with addition of few bp at the upstream and downstream of HN gene with the size of 2670 bp (Figure 1).

![Figure 1](image_url): PCR product of VP2 and FragHN genes. The VP2 and FragHN genes were amplified from RNA genome of IBDV and NDV respectively via RT-PCR using specific primers. 10 µl of the PCR mixture was loaded into 1% agarose gel and subjected to electrophoresis at 80 V for 40 min. Lane L: 1 kb DNA ladder; Lane 1: PCR product of FragHN gene (2.67 kb); Lane 2: PCR product of VP2 gene (1.356 kb).
Construction of Recombinant Plasmid Containing Fused Genes. The VP2 gene of IBDV 3529/92 isolate was cloned into pRSETB vector at the XhoI and KpnI restriction enzyme sites. The presence of VP2 gene in the vector was confirmed by restriction enzyme analysis (Figure 2) and sequencing.

Full length HN gene of 1716 bp and partial HN (pHN) gene of 640 bp genes were amplified from pCR2.1TOPO-FragHN (Figure 3). Subsequently, the resulting pRSETB-VP2 was fused with HN and pHN genes of NDV at KpnI and EcoRI restriction sites and generated recombinant plasmid pRSETB-VP2-HN and pRSETB-VP2-pHN respectively. The presence of the fused VP2-HN and VP2-pHN genes were confirmed by restriction enzyme analysis as shown in Figure 4 and Figure 5 respectively.

Production of Fused Protein in E. coli. Fused proteins were produced by E. coli BL21 (DE3)/pRSETB-VP2-HN and E. coli BL21 (DE3)/pRSETB-VP2-pHN following induction with IPTG and extracted from expressed cells using a protein extraction reagent. The proteins were separated by SDS-PAGE and analyzed by Western blot. Based on Western blot analysis (Figure 6), an expected protein band of FVP2-pHN protein with approximate size of 75 kDa was visualized on the membrane upon detection with anti-histidine monoclonal antibodies. However, a protein band lower than the expected size of VP2-HN protein was observed with approximate size of 60 kDa.

DISCUSSION

The ongoing reports on ND and IBD outbreaks worldwide have urged researchers to develop recombinant subunit vaccine as alternative for viral vaccine. Subunit vaccines are vaccines that are formulated with purified proteins that contain neutralizing epitopes capable of inducing antibodies against the virus. The basic principle for producing a recombinant subunit vaccine is to isolate and transfer the gene of interest into an expression host and followed by purification of the recombinant protein (or vaccine) produced by the host. The advantages of recombinant subunit vaccine are that the pathogen can be entirely excluded from the vaccine as well as eliminating the risks of reversion from non-virulent to virulent strains or incomplete inactivation of the virus (Liljeqvist & Stahl, 1999).

Figure 2: Restriction enzyme analysis of pRSETB-VP2. pRSETB-VP2 plasmids were extracted from the ampicillin resistant transformants of E. coli TOP10 strain and digested using XhoI and KpnI enzymes to confirm the presence of VP2 gene. The digestion products were loaded into 1% agarose gel and subjected to electrophoresis at 80 V for 40 min. Lane 1: Digestion product consisting of pRSETB (2.9 kb) and VP2 gene (1.356 kb); Lane 2: Digestion product consisting pRSETB (2.9 kb) as negative control; Lane L: 1 kb DNA ladder.

Figure 3: PCR product of HN and pHN genes. The HN and pHN genes were amplified from pCR2.1TOPO-FragHN using specific primers with addition of KpnI and EcoRI restriction enzyme sites for cloning into pRSETB-VP2 plasmid. 10 µl of the PCR mixture was loaded into 1% agarose gel and subjected to electrophoresis at 80 V for 40 min. Lane L: 1 kb DNA ladder; Lane 1: PCR product of HN gene (1.716 kb); Lane 2: PCR product of pHN gene (0.640 kb).
Previous studies have shown that the VP2 protein of IBDV contains conformational epitopes between amino acids 206 and 350 that can induce neutralizing antibodies (Azad et al., 1987). HN protein which is located at the surface of NDV has also been identified to contain neutralizing epitopes at amino acid residues of 346 to 353 (Chambers et al., 1988). To our knowledge, fused protein consisting of VP2 and HN proteins from IBDV and NDV have not been produced by any expression system. In the present study, two different fused proteins were produced whereby the first contained full length of VP2 protein fused with full length of HN protein while the second was fused with partial length of HN protein consisting of amino acid residues 280 to 488. The partial length of HN protein was selected to reduce the molecular weight of the fused protein while maintaining it antigenic site (346 to 353 amino acid residues).

Due to the advantages of E. coli as heterologous gene expression system, the production of fused VP2-HN and VP2-pHN proteins were attempted in E. coli. Many studies have been reported on the successful production of viral proteins in E. coli. E. coli expression system is the first choice among the bacterial expression systems for recombinant protein production due to its easiness to culture, rapid growth, high products yield, cost-effective, and rapid expression (Demain & Vaishnav, 2009). For example, hemagglutinin protein from avian influenza virus had been highly expressed in E. coli BL21 (DE3) strain (Behzadi et al., 2014). In other studies, nucleocapsid proteins from vesicular stomatitis virus and also from Nipah virus were expressed by BL21 (DE3) expression system under the T7 RNA polymerase promoter (Das & Banerjee, 1993; Tan et al., 2004). A study in Malaysia had demonstrated the successful expression of fused protein in E. coli such as the expression of fused gene consisting of viral protein 2 (VP2) from IBDV.

Figure 4: Restriction enzyme analysis of pRSETB-VP2-HN. The recombinant plasmid of pRSETB-VP2-HN was digested with XhoI and EcoRI restriction enzymes and confirmed the presence of VP2-HN fused gene by yielding two bands corresponding to pRSETB vector and VP2-HN fused gene. Lane L: 1 kb DNA ladder; Lane 1: Digestion products consisting of pRSETB vector (2.9 kb) and VP2-HN gene (3.0 kb); Lane 2: Digestion products consisting of pRSETB vector (2.9 kb), negative control.

Figure 5: Restriction enzyme analysis of pRSETB-VP2-pHN. The recombinant plasmid of pRSETB-VP2-PHN was digested with XhoI and EcoRI restriction enzymes and confirmed the presence of VP2-PHN fused gene by yielding two bands corresponding to pRSETB vector and VP2-PHN fused gene. Lane L: 1 kb DNA ladder; Lane 1: Digestion products consisting of pRSETB vector (2.9 kb) and VP2-PHN gene (2.0 kb).
Production of fused VP2-HN protein in E. coli and nucleocapsid (NP) from NDV in BL21 (DE3) (Rafidah Saadun et al., 2008). The results from Western blot showed that the fused protein retained its antigenicity by being able to bind with anti-IBDV and anti-NDV polyclonal antibody. According to Western blot analysis, a specific 75 kDa protein band corresponding to the expected molecular weight of VP2-pHN fused protein was visualized on the nitrocellulose membrane. There was no similar band appeared in the negative controls. However, a protein band of approximately 60 kDa was observed where the VP2-HN protein was loaded. This was different from the predicted molecular weight of VP2-HN protein which is 115 kDa. The production of truncated (shorter) version of the VP2-HN protein may have occurred due to some possible reasons. The expression of smaller fused protein of VP2-HN could have been due to proteolytic cleavage at a specific site on the HN protein that generated exactly a single protein size. This is believed because VP2 protein produced by pRSET vector in E. coli BL21 (DE3) has the size of 50 kDa as reported by (Omar et al., 2006). Even though protease inhibitor was used in the preparation of the protein sample, the protein may have been cleaved due to presence of numerous protease in the E. coli host (Gottesman, 1996).

Another possible reason for the expression of fused protein smaller than the expected size is due to a nonsense mutation occurred in the recombinant VP2-HN gene whereby a premature stop codon was introduced in the gene that may have terminated the translation process earlier (Glass, 1982). As reported by the author, the gene fragment is easily susceptible to many types of mutations. It is possible that the mutation could have occurred during the storage of the recombinant plasmids.

Despite the unsuccessful attempts to produce the intact VP2-HN fused protein, the fused VP2-pHN protein consisting of VP2 protein and partial HN protein carrying the major antigenic sites was successfully produced by pRSETB vector in E. coli BL21 (DE3) strain. This preliminary data could be used as a platform in developing a subunit vaccine against IBDV and NDV. Immunization studies could be performed on the fused VP2-pHN protein to analyse its antigenicity and immunogenicity by administering the fused protein into chickens. The first step would be to prepare the subunit vaccine of the recombinant fused protein. An example of the preparation of subunit vaccine is explained by Rong et al. (2005) in which the preparation involved depositing of the fused protein with ammonium sulphate, dialysing with PBS followed by addition of oil emulsion to make a water-in-oil emulsion vaccine. Once the recombinant subunit vaccine is prepared, the vaccine could be administered into the chickens. If the subunit vaccine comprises of the shorter fused protein (VP2-pHN) could give full protection against NDV and IBDV, there is no need for cloning and expression of the full fused protein (VP2-HN). This could ease the process of cloning, expression as well as the detection of the fused protein since shorter gene is easier to be cloned while lower molecular weight protein is easier to be detected by the Western blot. Furthermore, the cost of cloning and expression could be minimised. Therefore, the result presented in this study is of great importance and further studies using the resulting fused protein may be carried out in the future.

CONCLUSIONS

The successful construction of the recombinant vector containing VP2-pHN fused gene and the production of VP2-pHN fused protein by E. coli opens the pathway for the investigation of the fusion protein’s biological function in order to explore its potential as a bivalent subunit vaccine candidate against IBDV and NDV upon single immunization.
ACKNOWLEDGEMENT

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