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Expression of Segment a of Infectious Bursal Disease Virus in *Pichia pastoris*

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Recombinant plasmid containing segment A open reading frame 2 (ORF2) gene of infectious bursal disease virus (IBDV) of a very virulent subtype from local outbreak (strain 3529/92) was constructed. The gene encoding the IBDV structural polyprotein (N-VP2-VP3-VP4-C) was inserted into an expression vector, pPICZ prior to its transformation into *Pichia pastoris* by electroporation. After the induction of *P. pastoris* transformant with 0.5% methanol, the production of IBDV polyprotein was observed using Western blot. In P. pastoris, co- or post-translational processing of the large polyprotein occurred, generating a stable C-terminal product (VP3) of correct size, but without any detectable N-terminal product (VP2). The failure to observe the VP2 protein in Western blot analysis was probably due to the conformational epitope problem.

Key words: Segment A, IBDV Expression, *Pichia pastoris*, Vaccine.

Infectious bursal disease virus (IBDV) is the causative agent of "Gumboro disease" which is a highly immunosuppressive disease in juvenile chickens. The IBDV viral genome consists of two dsRNA molecules which are segment A and B. Segment A of IBDV consists of two open reading frames (ORFs), ORF 1 encodes for VP5 and ORF 2 encodes for a large polyprotein which is auto-proteolytically cleaved into pVP2, VP4 and VP3. pVP2 is then further cleaved to form mature VP2. VP2 and VP3 are the major structural proteins which form the outer and inner layer of the virus capsids. VP4 is the virus encoded-protease. In previous studies, it was demonstrated that expression of ORF2 gene in various host systems such as *Escherichia coli*[1], insect cells[2] and animal cells[3] produced proteolytically processed pVP2/VP2, VP3 and VP4 proteins. However, there were no reports on cloning of ORF2 gene in *Pichia pastoris* and confirmation of the auto-proteolysis in this organism. In this study, segment A IBDV ORF2 of local isolate (strain 3529/92) was cloned and expressed in *P. pastoris*. The whole ORF2 Segment A IBDV was cloned in order to study the expression of the polyprotein genes in *Pichia pastoris*. The expression of the polyprotein gene in *P. pastoris* is hoped to automatically form virus like particles (VLPs) that can later be developed into effective subunit vaccine.

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MATERIAL AND METHODS

Cloning of Segment A ORF2 Gene In pPiczA

A set of primer was synthesized in reference to Segment A ORF 2 IBDV strain 3529/92 [4] (Ghazali, *et al.,* 2012). The primer pPicza_Forward (5' – TCT GCA GAA TTC GCA ATG ACG AAC CTG CAA GAT- 3') and pPicza_Reverse (5'- GTT TTC GGT ACC TCA CTC AAG GTC CTC ATC- 3') was designed to have *EcoR1* and *Kpn1* to facilitate the insertion of the gene into expression vector, pPiczA(Invitrogen, USA). The amplified DNA fragment was expected to have 3039 bp in size. The PCR was done by using AmpOne PFU DNA Polymerase (Geneall, Korea). The PCR reaction was according to the following conditions; 95°C denaturation for 30 sec, 1 min 45 sec at 60°C for annealing, 72°C for extension for 30 cycles and final extension of 72°C for 10 min. The PCR product was then subjected to enzymatic digestion with *EcoR1-HF* (NEB, England) and *Kpn1-HF* (NEB, England) for 4 hours. The segment A ORF2 PCR products which had been subjected to enzymatic digestion was then separated by using 1% agarose gel electrophoresis. The DNA fragment was then purified by using Qiaquick gel extraction kit (Qiagen, USA). The same protocol was also applied to expression vector. The ligation was then conducted using T4 DNA ligase (Invitrogen, USA). Subsequently, the *E. coli* TOP10 cells were transformed with the ligation mixture. The transformed cells were streaked on low salt LB/ Zeocin plate for screening. The positive clones were selected, and their plasmids were extracted by alkaline lysis methods. The clones were first confirmed by PCR and double digestions with *EcoR1-HF* and *Kpn1-HF* before be sequenced to further confirmed the sequence and the orientation. The resulting plasmid, pPiczA-sA was linearized with *SacI* before transformed into *P. pastoris* strain X33 by electroporation. The positive clones were screened using Zeocin and confirmation of the integration of the gene in *P. pastoris* genome was carried out using PCR colonies. Three Mut^+ phenotypes which were able to grow at high Zeocin concentration (2000 µg/ml) were selected and proceeds for expression.

Expression of Segment A ORF2 protein of IBDV in the methylotrophic yeast P. pastoris

The expressions of the ORF2 protein were carried out according to the manufacturer's protocols (Easy select kit, Invitrogen). Briefly, the transformants, together with the negative controls (non-transformed *P. pastoris* and *P. pastoris*trans formed with expression vector pPiczA only) were growing in BMGY until OD_{600} reached 2-6. Yeast cells were harvested by centrifugations and the cells concentrations were adjusted to concentration of OD_{600} 1.0 in BMMY medium. To induce the expression of the gene, 0.5% of methanol was added to the recombinant culture for every 24 hours until the incubation time reached 96 hours. 2 ml of the expression culture was collected every 24 hours until 96 hours, the culture was harvested and the resulting pellet was stored in -80°C

Western blot analysis

The samples were prepared according to the manufacturers protocols using acids washed glass beads. The samples were added with equal amount of sample buffer, boiled for 10 minutes and separated by SDS-PAGE. Gels were then electro blotted onto PVDF membranes. The proteins were then detected with monoclonal antibodies against VP2, VP3 and polyclonal antibodies against IBDV.

RESULT AND DISCUSSION

IBDV Segment A ORF 2 gene was amplified by PCR. The PCR product was subjected to enzymatic digestion with *Ecor1* and *Kpn1* before purified with Qiaquick gel extraction kits (Qiagen, USA). Figure 1 shows the gel electrophoresis of Segment A ORF2 of IBDV 3529/92. As expected, a band about 3 kbps in size can be observed. The digested and purified PCR product was ligated to the pPiczA vector (Invitrogen,USA). Ligation mixture was transformed into *E. coli* Top10 (Invitrogen, USA). The transformed cells were spread onto low salt LB/Zeocin plate for selections. The positive colonies were then selected and grown in low salt LB/ Zeocin broth and plasmids were harvested and tested for the insert by digestion with Restriction enzymes and PCR.

Figure 2 shows the gel electrophoresis of positive colony digest with *EcoR1* and *Kpn1*, the digestion with the enzymes proved that the

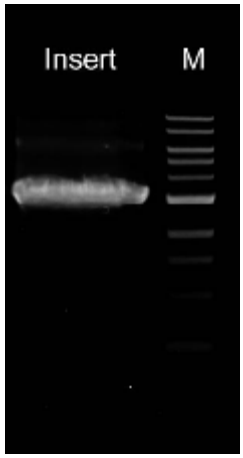

Fig. 1. Agarose gel electrophoresis of PCR amplified cDNA fragment of Segment A IBDV ORF2. Lane M 1 kb DNA ladder NEB; lane Insert the IBDV insert

insertion part were still available. When single digested with *EcoR1* or *Kpn1* as expected a single DNA fragment of 6.3 kb in size can be observed (insert 3039bp and pPiczA vector 3.3 kbp, that make total of 6.3 kbp in size when single digested). When digested simultaneously, it was expected to yield two bands but because of the size of the insert was almost the same pPiczA, only one band can be observed but with higher intensity. The positive clones were sent for sequencing; the inserted DNA sequence was checked and confirmed to have hundred percent similarities with the previously

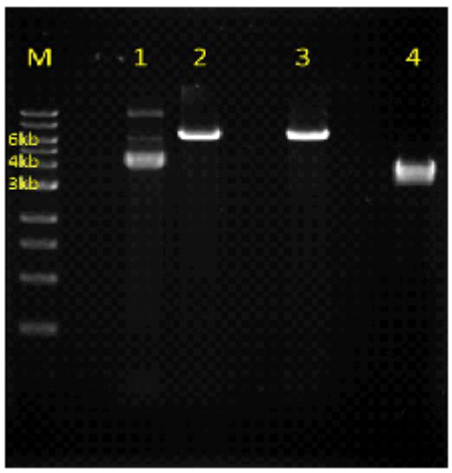

Fig. 2. Agarose gel electrophoresis of plasmids Segment A gene (pPICZA-sA) *EcoR1* and *Kpn1*. Lane M M= marker NEB , 1= uncut plasmid, 2= Plasmids digest with EcoR1, 3=plasmids digest with Kpn1, 4= Plasmids double digest with *EcoR1 and Kpn1*.

reported vvIBDV strain 3529/92[4]. The positive clone (pPICZA-sA) was used to transform *Pichia pastoris* strain X33. The transformation of the insert involved two steps in which involved the linearization of the insert by RE Sac1 and secondly transformation of *P. pastoris* by electroporation.

Figure 3 shows the gel electrophoresis of pPiczA-sA linearized by enzymes Sac1 and as expected only single band approximately of 6 kbps was observed. The uncut plasmids in the figure served as control to show the plasmids have been completely linearized by the enzyme Sac1. The electroporated P. *pastoris* was then spread on YPDS/Zeocin plates for screening. The integration of Segment A ORF 2 genes were confirmed by running PCR colony.

Figure 4 showed the gel electrophoresis of the PCR colony. As expected, the positive clones will have two bands when amplified with 5'AOX1

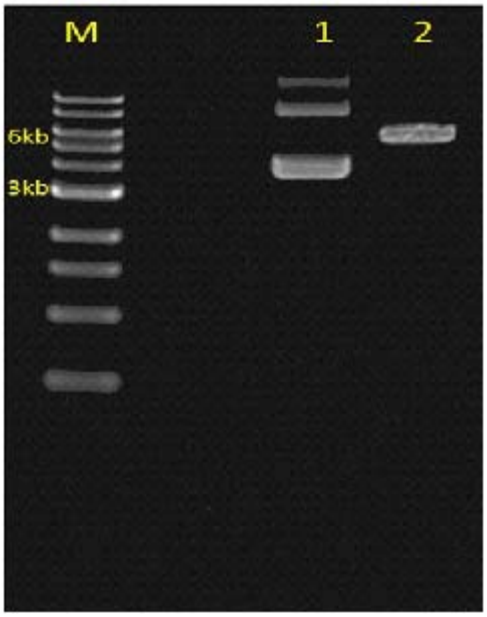

Fig. 3. Linearized pPicZA containing Segment A gene (pPICZA-sA) with SacI M= marker NEB, 1= uncut plasmid, 2= Plasmids digest with Sac1

and 3'AOX1 primer. The bands corresponded to the insert (about 3.3 kbp in size, insert 3 kbps plus 300 bp A0X1 sites of the vector, pPicZA) and other bands corresponded to the native AOX1 sites of the yeast (about 2.2 kbps).

3 multi copy integrantsmut$^+$ phenotypes selected from colonies which were able to grow on high concentrations of Zeocin (2000 µg/ml) were

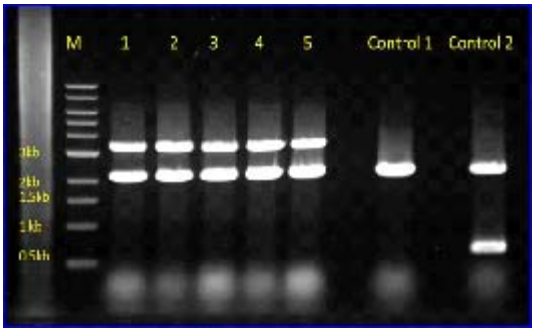

Fig. 4. PCR colonies of *Pichiapastoris*harboring insert. Lane M = 10 kb DNA Ladder (NEB); Lanes 1-5 = Colony 1-5 of *Pichiapastoris*harboring insert (3.6 kb) in its genome; lane Control 1= *Pichiapastoris strain x-33 only*; Lane Control 2 = *Pichiapastoris*

chosen to be expressed.

Expression of Segment A ORF 2

Segment A ORF2 genes as described earlier encoded for a large polyprotein which will later undergo autoproteolysis to form three proteins which is VP2, VP4 and VP3. As described earlier from the sequence study of vvIBDV 3529/92[5]the proteins pVP2 is estimated to have 54 kDa size and 48 kDa in matured formed, VP4 size is 26 KDa and VP3 is estimated to have 28 kDa size. The proteins were detected by the western blot using antibodies specifics against VP2 (Acris antibodies, German), anti-VP3 (Acris antibodies, German) and anti-IBDV polyclonal antibodies. comment: replace the long yellow text with the following: When *P. pastoris* harboring Segment A gene lysate was probe by anti-IBDV polyclonal antibodies (IBDV pABs), bands of 54 kDa and 28 kDa in size was detectable on the membrane (Figure 5, lanes 3-5). These two bands have the expected size of VP2 and VP3 proteins, respectively. However, the band with the size of 54 kDa was also observed in *P. pastoris* haboring pPicZ vector only (Figure 5, lane 2) indicating that the band in recombinant lysates was probably not VP2 protein. Many unspecific bands were observed on the membranes when probe by polyclonal antibodies (Figure 5, lanes 2-5) as the antibody is not specific and can cross reacts with host's proteins thus explain the detection of the 54kDa band in both *P. pastoris* with and without segment A gene. Unfortunately, probing the blot with anti-VP2 monoclonal antibody (mAb) failed to detect the band of 54 kDa in all samples (data not shown) and cannot further confirm the protein in *P. pastoris* harboring Segment A gene is the VP2 protein. We speculated that the anti-VP2 that we used probably can only bind to the conformational epitope of VP2 proteins which was destroyed during the harsh denaturing conditions of SDS-PAGE treatment. The VP2 protein is previously reported to be highly conformational[5] and the loss of the epitopes rendered the mAb failed to detect the proteins.

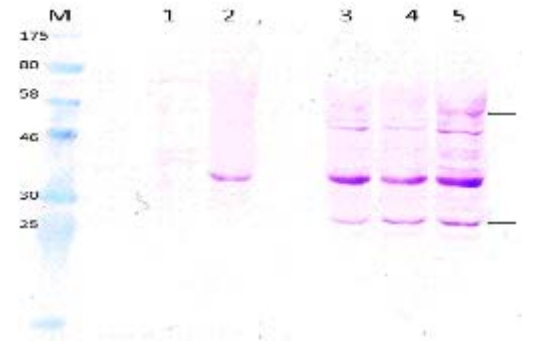

Fig. 5. Western blot results of *Pichia pastoris* harboring segment A gene. The blot was probbed by anti-IBDV polyclonal antibodies. The cells were collected at 24 hours post-inductions with 0.5% methanol. M= Marker (Broad range marker, NEB); 1= host only (*Pichia pastoris* strain X33); 2 = *P. Pastoris* harboringp PicZa only; 3-5 = Colony 1-3, *P. pastoris* harboring ORF2 Segment A IBDV insert

The VP3 protein can be clearly seen on membranes when detected by anti-VP3 (Figure 6, lanes 3-5) and polyclonal antibodies against IBDV (Figure 5, lanes 3-5). The VP4 protein is undetected on the membrane when probe with anti IBDV due to the fact that the protein was being lowly expressed.

CONCLUSION

In conclusion, IBDV polyprotein was produced by *P. pastoris* when it is transformed with pPICZA harboring Segment A (ORF 2) gene. In the organism, co- or post-translational processing of the large polyprotein occurred, generating a stable C-terminal product (VP3) of correct size, but without any detectable N-terminal product (VP2). The failure to detect the VP2 protein was probably due to the conformational epitope problem in Western blot analysis.

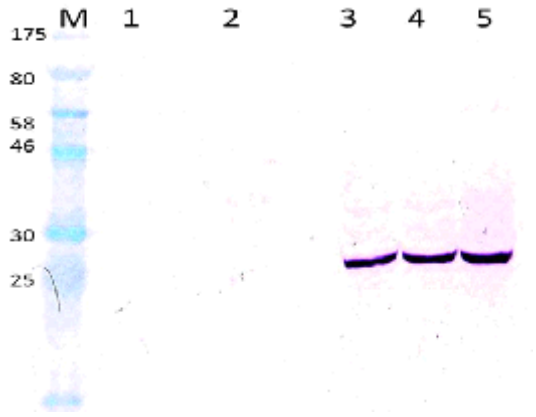

Fig. 6. Western blot results of *Pichiapastoris*harboring segment A gene. The blot was probbed by anti-VP3 monoclonal antibodies. The cells were collected at 24 hours post-inductions with 0.5% methanol. M= Marker (Broad range marker, NEB); 1= host only (*Pichiapastoris* strain X33); 2 = *P. Pastoris* harboringp PicZa only; 3-5 = Colony 1-3, *P. pastoris* harboring ORF2 Segment A IBDV insert

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