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Expression of the VP2 gene of classical D78 infectious bursal disease virus in the methylotrophic yeast *Pichia pastoris* as a secretory protein

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ABSTRACT

Infectious bursal disease virus (IBDV) is the causative agent of Gumboro disease, an infectious disease of global economic importance in poultry. The expression of heterologous proteins in *P.pastoris* is fast, simple and inexpensive. In this study, VP2 encoding gene of classical D78 IBDV was amplified using reverse transcription (RT) polymerase chain reaction (PCR) and cloned into pPICZαA vector. Recombinant plasmid DNA was integrated into the chromosome of the transformed *Pichia pastoris* by electroporation and expressed protein identified by SDS- PAGE and western blotting. A recombinant protein was secreted into the supernatant from the yeast when induced with methanol. The expressed target protein in supernatant was bound with chicken anti IBDV Polyclonal antibodies. Western blotting with antibodies against D78 IBDV indicated that the recombinant VP2 protein retained its antigenicity. The concentration of secreted VP2 protein was 0.67mg/l.The production of recombinant VP2 protein indicated that *P. pastoris* was an efficient secreted expression system for D78 IBDV.

Keywords: IBDV, Secreted expression, VP2, *Pichia pastoris*

INTRODUCTION

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens (kibenge *et al* 1988). The etiological agent, IBD virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage (Burkhardte *et al* 1987). The chickens are susceptible to clinical disease at 3-6 weeks of age. The virus belongs to the family *Birnaviridae* of the genus Avibirnavirus (Murphy *et al* 1995). Members of the family contain a double stranded RNA genome consisting of 2 segments, designated A and B, within a non enveloped single–shelled icosahedral capsid of 60nm diameter. The IBDV genome segment A (3254bp) contains 2 open reading frames (ORF); small ORF Preceding and Partially

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overlapping the larger ORF encodes VP5 (Kibenge *et al* 1991). The larger ORF encodes a 109 KDa precursor polyprotein (N-VPx-VP4-VP3-c) which is processed into 2 structural proteins VP2(40-45KDa) and VP3 (32-34KDa) and the putative viral protease VP4 (28-30.5KDa) (Kibenge *et al* 1991). VP2 contains the major antigenic site responsible for eliciting neutralizing antibodies and VP3, the group specific site responsible for eliciting neutralizing antibodies specific antigens (Becht *et al* 1988) and a minor neutralize site (Jagadish and Azad 1991). In the last two decades many studies have been conducted in order to develop vaccines based on genetic engineering methods (Dertzbaugh 1998). VP2 expressed in *Escherichia coli* failed to promote neutralizing antibodies (Azad *et al* 1991, Omar *et al* 2006). On the other hand, immunization with VP2 expressed in yeast led to the formation of a high level of neutralizing antibodies that, when injected into specific-pathogen free (SPF) birds, conferred protection against wild IBDV challenge (Azad *et al* 1991).

The first commercial subunit vaccine was developed against hepatitis B virus and was expressed in *Saccharomyces cervisiae* (Valenzuela *et al* 1982). Another yeast expression system is the facultative yeast *Pichia pastoris* which utilizes methanol. The methanol metabolic pathway of *P. pastoris*, as in other methylotrophic yeasts, involves a unique set of enzymes. In the first step of this pathway, methanol is oxidised to generate formaldehyde and hydrogen peroxide which is then decomposed to water and molecular oxygen by catalase. The oxidation is carried out by two alcohol oxidase genes Aox1 and Aox2 (Cregg *et al* 1985). Aox1 is more active alcohol oxidase and may reach as much as 30% of the total Protein in the cell when cultured under growth limiting rates of methanol. This gene's promotor is utilized for expression of heterologous genes. The heterologous proteins in *P. pastoris* is fast, simple and inexpensive (Pitcovski *et al* 2003). Strong aerobic growth allows culturing at high cell densities. High levels of foreign protein expression have been shown for this vector and eukaryotic protein processing, modifications and folding can be performed. The objective of this work was the VP2 gene of D_{78} strain cloned, analyzed and expressed in a secreted yeast expression system.

MATERIALS AND METHODS

Virus and viral RNA Purification. The classical IBDV D_{78} strain (sequence in Genbank as IBDV D_{78}, the accession number AF499929) was grown in primary chicken embryo fibroblast (CEF). The fibroblast cells IBDV strain were derived from 10-day–old embryonated eggs and purified using methods described by Tsukamoto (Tsukamoto *et al* 1990). Total RNA from purified virus was extracted by using kit reagents, (Roche, Germany) according to the manufacture protocol. RNA samples were dissolved in nuclease free water.

Reverse transcription Polymerase chain reaction amplification. The VP2 of D78 was amplified with the forward and reverse primers of VP2 (Table 1) by RT- PCR Kit (One step-Titan, Roche) using a mixture of enzymes (superscript RT-PCR and expand high fidelity) in the following conditions 45 ºC for 45 min and 94 ºC for 2 min followed by 35 cycles of 94 ºC for 30 sec, 55 ºC for 1 min and 68 ºC for 2 min and finally 72 ºC for 10 min.

Table 1 . List of PCR Primers

Primer	DNA sequence
VP_2F	5´-GCCGGAATTCATGACAAACCTGCAAGAT-3´
VP_2R	5´-GCCGTCTAGAAACCTTATGGCCCGGAT-3´
5-Aox_1	5´-GACTGGTTCCAATTGACAAGC-3´
3-Aox_1	5´-GCAAATGGCATTCTGACATCC-3´
SD_1F	5´-TCAGGATTTGGGATCGC-3´
SD_1R	5´-CTCACCCCAGCGACCGATAACGACG-3´

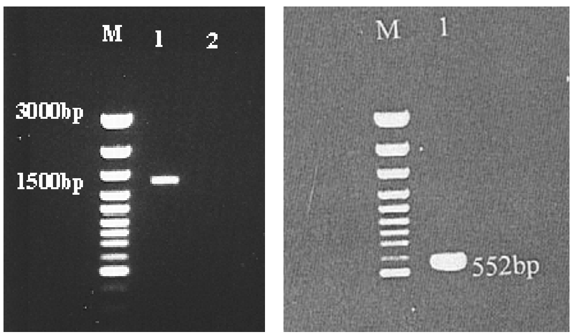
Cloning of VP2 gene into pPICZαA vector. VP2 PCR products were separated in a 1% low melting agarose gel and purified by high pure PCR product purification kit (Roche). The specificity of the PCR fragment was verified by nested PCR that reactions were 50 µl and contained 2 mM Mgcl2, 10 mM dNTPs, 10 Pmol of each primer SDF & R (Table 1), 2 µl RT-PCR product, 4 µl of PCR buffer and 1 unit of Taq DNA polymerase. The PCR program was as follows: 3 min at 94 ºC, (30 sec at 94 ºC, 60 sec at 55 ºC, 2 min at 72 ºC)×30 cycles and 10 min at 72 ºC. The pPICZαA Vector (Easy Select *Pichia* Expression Kit, Invitrogen) and the resulting PCR fragments of 1355bp were purified from agarose gel were subsequently digested with *EcorI* and *XbaI*. The 1355bp insert (VP2) was ligated to digested pPICZαA. *E. coli* TOPl0F´ cells were transformed with the resulting ligation products and plated on low salt LB/Zeocin medium containing 1% trypton, 0.5 % yeast extract, 0.5 % Nacl, pH 7.5 and 25 mg/ml zeocin (Leber *et al* 1999). The resulting transformants were tested by restriction analysis, and the positive clones were amplified to make larger amounts of DNA. The final DNA construct was linearized with *MSSI* restriction enzyme, the construct was transformed into the yeast *P. pastoris* KM71H by electroporation according to manufactures protocol (Easy Select Kit, Invitrogen).The resulting electroporated cells were Plated on YPDS/ zeocin medium containing 1% yeast extract, 2% glucose, 1M sorbitol, and 100 Mg/ml zeocin, following the manufacture's instructions. Transformants bearing the chromosomally integrated copies of VP2 gene in cassettes were then detected by genomic PCR using the primers VP2 or 3´ and 5´Aox1 primers (Table 1). Four positive clones of each transformants were used to inoculate 100 ml of buffered minimal glycerol medium (containing 100 mM potassium phosphate, pH 6, 13.4 g/liter of yeast nitrogen base without amino acids, 400 ng/lit biotin, 40 mg/liter L-histidine, and 1% glycerol) overnight at 30 ºC. The cells were then harvested and resuspended in 20 ml of buffered minimal methanol medium and incubated for 6 days at 30 ºC. To induce expression, the culture were supplemented every 24 hrs with methanol (100%) to a final concentration of 1% (v/v). 1 ml aliquots were withdrawn for cell viability determination and expression analysis.

SDS–PAGE and immunoblot analysis. Electrophoresis of the protein was performed as described by Laemmli (Laemmli, 1970), using 12% acryl amide gels followed by staining with coomassie blue or immunoblotting. For western blot analysis pellet and supernatant of samples were subjected to 7% sodium dodecyl sulphate–Polyacrylamide gel electrophoresis (SDS–PAGE) and transferred from the gels on to nitrocellulose membranes using buffer consisting of 25m MTris, 192 mM glycine,and 20% methanol. The membranes were blocked for one hour at room temperature in MPBST buffer (0.137 NaCl, 2.7 mM KCl, 8mM Na_2PO_4, 8 mM KH_2Po_4 0.1%, tween20, and 5%Skimmed milk Powder). Subsequently, the membranes were incubated for one hour at room temperature with polyclonal chicken serum against IBDV (KPL, USA) were used at 1:200 dilution. Rabbit anti–chicken IgG labeled with HRP (Sigma, Germany) were used at 1:500 dilution was added to the membrane and incubated for 1h at room temperature. the membranes were washed 3x10 min with phosphate buffer saline (PBS) containing 0.01% tween20 and 2x10 min with PBS the immunoblot was visualized with DAB (3,3-diaminobenzidine substrate) (Ausbel *et al*, 1999).

RESULTS

The VP2 was amplified by RT-PCR followed by PCR (Figure1A). The 1355bp fragment was obtained after amplification and by nested-PCR fragment in a 552bp, indicating the specificity of the PCR fragment (Figure 1B).

Cloning of VP2 in *E. Coli*. The 1355bp inserts were ligated into the multiple cloning site region downstream of the *P. pastoris* Aox1 promotor and the α-factor signal sequence of pPICZαA vector using the *EcorI / XbaI* restriction sites. The resulting plasmids were transformed into the *E. coli* strain TOP10F´. The pPICZαA plasmid contained the zeocin resistance gene for selection in *E. coli*. The construct digested with *EcorI* and *XbaI* (Figure 2).



A **B**

Figure 1. A: PCR product of amplified VP2. *Lane 1*: amplified VP2 (1355bp); *Lane 2*: negative control, *Lane M*: molecular size markers (100bp). **B:** Nested PCR product of VP2. *Lane 1*: amplified SDI (552bp); *Lane M*: molecular size marker (100 bp).

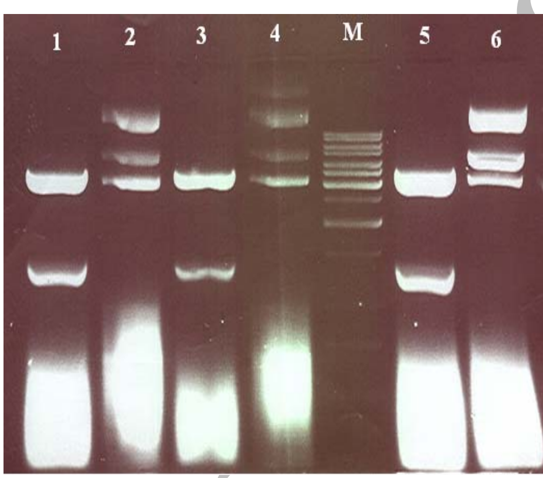


Figure 2. Agarose gel electrophoresis of construct (VP2 + pPICZαA) digested by *EcorI* and *XbaI*. *Lane 1,3 and 5:* digested with *EcorI* and *XbaI* (construct); *Lane 2, 4 and 6*: not construct; *Lane M*: molecular size marker (1kb).

Expression of construct in yeast. The construct linearized with *MSSI* enzyme and following amplification of the plasmid transfected into yeast cells (KM71H strain).

Yeast transformants were detected by a genomic PCR assays using the Primers VP2 and the 3´ and 5´Aox1 primers (Figure 3).

A

B

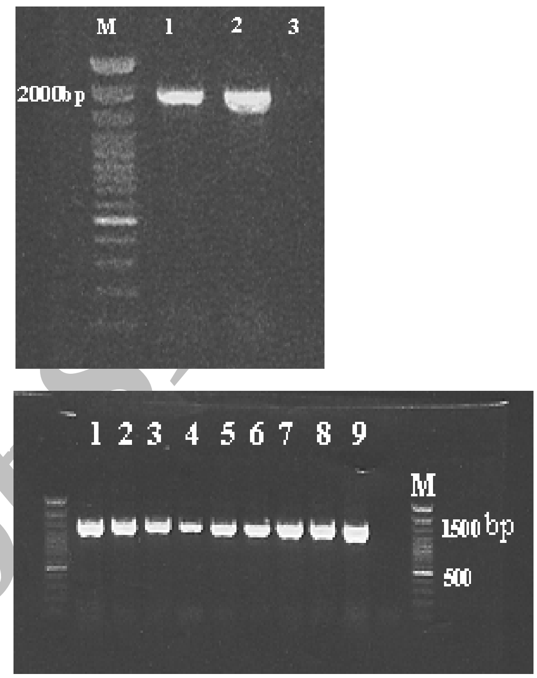


Figure 3. Typical genomic PCR assay of *P. pastoris* transformants on 1% agarose gel. **A:** genomic PCR analysis using 3´and 5´Aox1 primers. *Lanes 1 and 2*: positive clones. **B:** genomic PCR analysis using VP2 F and VP2 R primers. *Lanes 1-9*: positive clones (1355 bp); *Lane M:* molecular weight marker (1kb).

The VP2 protein was found in the supernatant and analyzed by SDS-PAGE (Figure 4A) and western blot. A band of the VP2 appeared at the same size as viral VP2 (Figure 4B). The concentration of secreted VP2 protein was 0.67 mg/l of the original fermentation volume.

DISCUSSION

As a major capsid protein of IBDV with multiple roles in IBDV evolution, VP2 has been the main target for many research groups around the world.VP2 is the major host- protective immunogen of IBDV.

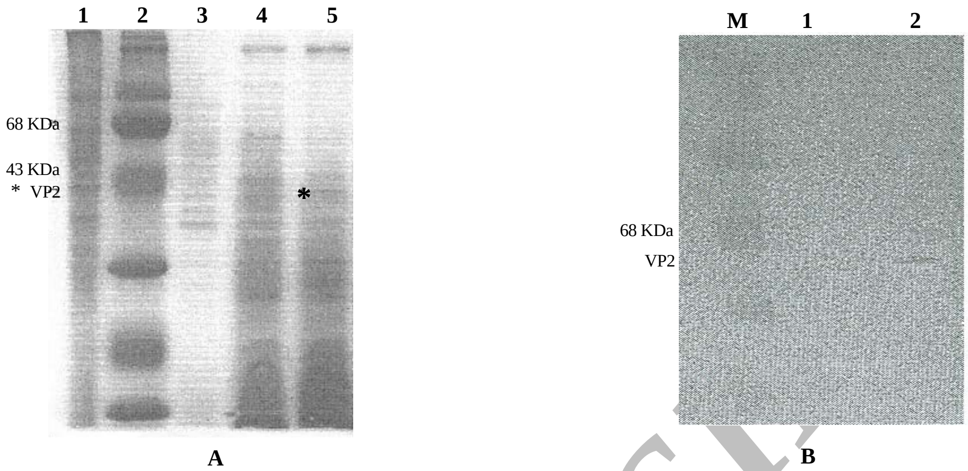


Figure 4. A: Yeast transformation proteins in SDS-PAGE analysis shown in a representative coomasie blue-stained 12% polyacrylamide gel. *Lane1:* whole D78 IBDV; *Lane 2:* molecular mass standards; *Lane 3*: negative control; *Lane 4*: KM71H strain that transformed by pPICZαA; *Lane 5:* KM71H strain that transformed by pPICZαA+VP2 grown at minimized culture. **B:** Western blot analysis of recombinant VP2. *Lane1:* negative control, yeast transformed with pPICZαA. *Lane 2:* one clone of yeast VP_2. *Lane M:* molecular weight size markers.

At least three neutralizing epitopes have been identified on the VP2 protein. VP2 gene has been expressed in different expression systems. The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins. Generally, *P. pastoris* produces higher amount of heterologous proteins than other expression system and is particularly advantageous for smaller proteins (Cereghino & Cregg 2000). Protein expression in *P. pastoris* is based on the of the alcohol oxidase, gene Aox1.Transcription of the gene is regulated by the Aox1 coding sequence is replaced by a gene of interest. The *P. pastoris* expression system is a eukaryotic system. That is efficient and less expensive than expression in other eukaryotic system (e.g. mamalian, insect).This system has been used for the production of various recombinant proteins (Sreekrishna *et al* 1988). Furthermore it was shown that *P. pastoris* yeast is not pathogenic to chicken even administered live at a very high concentration (Pitcovski *et al* 2003). Recently, the use of yeast *P. pastoris* has resulted in recombinant VP2 (rVP2) production level of up to 1 mg/l (Wu *et al* 2005).

In this study, we employed the *P. pastoris* expression secretional vector system to produce of rVP2 that concentration was 0.67mg/l. However, high levels of production necessitate some degree of optimization. As the first step to study the function of VP2 and develop a VP2 subunit vaccine for IBDV, we have expressed the VP2 gene of D78 strain in *P. pastoris*. Our yield definitively pointed out that the secreted yeast expression would be the choice for production of the VP2 protein. We are currently modifying cell growth conditions to optimize and obtain higher expression levels.

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