

Isolation and expression of recombinant viral protein (VP2) from Iranian isolates of Infectious Pancreatic Necrosis Virus (IPNV) in *Escherichia coli*

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Abstract

Infectious Pancreatic Necrosis Virus (IPNV) is a member of the family Birnaviridae that has been linked to high mortalities in salmonids. Bacterial based systems as live vectors for the delivery of heterologous antigens offer a number of advantages as vaccination strategies. VP2 is a structural viral protein of IPNV with immunogenicity effects. In this study IPNV was isolated from diseased fry of rainbow trout *Oncorhynchus mykiss* (Walbaum) using CHSE-214. Then an expression vector was constructed for expression of viral protein VP2. The designed vector was constructed based upon pET-26b (+) with T7 promoter. A fragment containing the full length of the VP2 gene of Iranian Sp strain was amplified by PCR using genomic RNA of IPNV as template and cloned in pET-26b(+) plasmid. Recombinant structural viral protein VP2 was expressed as a soluble, N-terminal PelB fusion protein and secreted into the periplasmic space of *Escherichia coli* BL21(DE3) and Rosetta (DE3). The glucose, Isopropyl- β -D- thiogalactopyranoside (IPTG) was used as a chemical inducer for rVP2 production in 37° C. The rVP2 was extracted from the periplasm by osmotic shock treatment. The presence of gene in bacterial system of *E. coli* was confirmed by gel electrophoresis technique. The constructed vector could efficiently express the rVP2 into the periplasmic space of *E. coli*. The successful cloning and expression of the structural viral protein gene into *E. coli* can be used for developing a useful and safe vaccine to control IPNV infection in Iranian fish industry.

Keywords: VP2, Infectious Pancreatic Necrosis Virus, Recombinant viral protein, Periplasmic space, *Escherichia coli*

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Introduction

The IPNV is a small, non-enveloped virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus* (Dobos, 1995) and has been isolated from marine and fresh water fish, molluscs and crustaceans (Heppell *et al.*, 1995). The genome of virus has two segments of double-stranded RNA that are surrounded with a single shelled, icosahedral capsid with 60 nm in diameter (Dobos *et al.*, 1979; Macdonald and Gower, 1981). Segment A is 3097 bp long and encodes four viral proteins, namely structural proteins VP2 and VP3, and nonstructural proteins VP4 and VP5 (Dobos, 1995). Segment A contains a large open reading frame (ORF) that encodes a 106-KDa polyprotein (2916 bases) which is co-translationally cleaved by VP4 to produce pre-VP2 (pVP2) and VP3 (Dobos and Rowe, 1977; Duncan *et al.*, 1987; Galloux *et al.*, 2004; Dadar *et al.*, 2013). The protease activity dependable for this cleavage has been associated with the viral protein VP4 (Duncan *et al.*, 1987; Magyar and Dobos, 1994). Pre- Viral protein 2 is further progressed by host cell proteases to form the mature outer capsid protein VP2 (Magyar and Dobos, 1994) which builds about 60% of virion (Dobos, 1995) and contains the antigenic region depended on induction of neutralizing antibodies in the host (Heppell *et al.*, 1995).

Viral Protein 2 and VP3 are the major structural and immunogenic polypeptides of the virus (Dobos and Roberts, 1983). There is a small ORF which overlaps with the amino terminal end of the large ORF and generates a 15-KDa (VP5) nonstructural polypeptide (Dadar *et al.*, 2013). Also the structural proteins of many

viruses through self-assembly make specific aggregates in a kind of different expression systems (Noad and Roy, 2003; Garcea and Gissmann, 2004; Chackerian, 2007; Roy and Noad, 2008). These virally derived particles generally resemble the native viruses in size and morphology that refer as virus like particles (VLPs) (Ludwig and Wagner, 2007) or these particles do not mimic the virus capsid in size and they have been ascribed to as subviral particles (SVPs) (Dhar *et al.*, 2010).

Due to structural similarities, VLPs and SVPs show effective immune responses and serve as excellent candidates for the production of vaccines (Noad and Roy, 2003; Ramqvist *et al.*, 2007; Dhar *et al.*, 2010; Roldão *et al.*, 2010). They have developed vaccines through attractive platform for the display of foreign epitopes or targeting molecules such as antigens, drugs and plasmids (Samuel, 2001; Garcea and Gissmann, 2004; Georgens *et al.*, 2005; Patient *et al.*, 2009; Brun *et al.*, 2011). Virus like particles and SVPs have been produced from a wild range of viruses and have very advantages regarding safety and immunogenicity over previous approaches (Moon *et al.*, 2004; Roy and Noad, 2008). Economic losses due to IPNV in salmonid farms were estimated to be very high (Akhlaghi and Hosseini, 2007; Ahmadi *et al.*, 2013; Munang'andu *et al.*, 2013) and these losses can be reduced as vaccines for salmonids become available based on killed virus or recombinantly produced viral protein (Mikalsen *et al.*, 2004; Allnutt *et al.*, 2007; Fridholm *et al.*, 2007; Munang'andu *et al.*, 2013). Among the five proteins of IPNV, VP2 has been demonstrated to possess neutralizing

epitopes (Wolf *et al.*, 1960; Dobos and Roberts, 1983; Heppell *et al.*, 1995; Moon *et al.*, 2004). Recently it is demonstrated that VP2 gene of IPNV expressed in a yeast expression system forms SVPs that is antigenic and is capable of providing immunity against the native IPNV (Allnut *et al.*, 2007). Also findings of another researchers showed that the VP2 is important for immunogenicity of IPNV vaccine (Fridholm *et al.*, 2007; Dhar *et al.*, 2010; Munang'andu *et al.*, 2013). Based on these reports, recombinant VP2 can be used as vaccine against IPNV. In this study a VP2 sequence was isolated from Iranian strain of IPNV (Dadar *et al.*, 2013). Then the recombinant protein (rVP2) of Iranian IPNV expressed in periplasmic space of *E. coli* and the recombinant proteins characterized by SDS-PAGE. The aim of this study was to produce a recombinant structural protein of VP2 of Iranian IPNV in a prokaryotic system for possibly developing a native vaccine.

Materials and methods

Cell line and virus isolation

Iranian isolate of Infection pancreatic necrosis virus (IRIPNV strain) was isolated from diseased fry of rainbow trout using CHSE-214. Moribund rainbow trout fry were collected during an outbreak of IPNV from 2010 to 2012 in different fish farms in North and West provinces of Mazandaran, Chaharmahal and Bakhtiari, and Kohgiluyeh and Boyer Ahmad of Iran. The farms were run on a flow-through system of fresh water with a temperature range of 12 to 15°C. From each farm, 30 moribund fish were selected and transferred to laboratory. Virus isolation from fry samples with

disease clinical signs, such as darkening of the skin, abdominal swelling, cast-like pseudofaeces and loss of appetite was performed, according to the procedure described by the OIE with minor modification (Noad and Roy, 2003; Garcea and Gissmann, 2004; Chackerian, 2007; Roy and Noad, 2008). Each pool contained ten fry fishes (Dadar *et al.*, 2013). Briefly, 2 g of specimen were homogenized in 2ml of minimum essential medium (MEM, Sigma, St. Louis, Missouri, USA) and centrifuged at 3000x g for 10 min. The supernatant was used directly for cell culture inoculation. Chinook salmon embryo (CHSE-214) cells were cultured in MEM containing Earle's salts, L-glutamine, 25 mM HEPES, 10 % fetal bovine serum (FBS), 100 ng/ml of streptomycin sulphate, 100 IU penicillin G., and were incubated at 20 °C, up to 70-90 % confluence in 24-well plates before inoculation. Then they were inoculated with 200 microliters of 1:10 and 1:100 dilution of each prepared sample in parallel wells containing CHSE and incubated at 15°C. At 7 days post inoculation (dpi), the cultures were observed for cytopathic effect (CPE) and if they were negative, the cultures were used for the second passage. After subjecting to 1 freeze/thaw cycle, the cell culture lysates from 2 dilutions of each sample were pooled and centrifuged at 2000 x g for 5 min. Then fresh CHSE cells were inoculated with the pooled first passage supernatant. Plates were incubated at 15°C and monitored daily up to 21 dpi, for development of viral CPE. If no CPE was observed after a period of 21 dpi, the sample was recorded as negative for IPNV. When CPE was observed, IPNV was

confirmed by using an IPNV antigen (Ag) ELISA kit (BIO-X, Jemelle, Belgium), and the culture medium was removed and stored in -80 °C.

VP2 isolation

Genomic RNA was extracted from IRIPNV strain (Roche, Mannheim, Germany). Since there was no data about Iranian IPNV isolates; the IPNV sequences was took from NCBI (<http://www.ncbi.nlm.nih.gov/>) and were aligned with Mega5 (Tamura, *et al.*, 2007) and NCBI (Mutoloki & Evensen, 2011). The primers were including FVP2 (5' ATGAACACAAACAAGGCAAC 3') and RVP2 (5' GAC TAT GTC TCT CCA GCC CCA TGC 3') for VP2. Then the specific primers were designed according to VP2 conserved region. The genomic RNA was amplified using a one-step RT-PCR procedure regarding manufacturer's protocol (Qiagen). Then the VP2 gene of Iranian Sp strain was amplified by one-step RT-PCR method (Qiagen, Germany). The PCR products were purified and sequenced. The sequence was registered in NCBI.

Bacterial Strain

E. coli strain BL21 F- *ompT hsdS_B* (rB- m_B⁻) *gal dcm* λ (DE3) and *E. coli* strain rosetta F- *ompT hsdS_B* (rB- m_B⁻) *gal dcm* λ (DE3) was used as a host for expression of rVP2 of IPNV.

Construction of -pET-26b (+) plasmid

The PET-26b (+) vector carrying the viral protein gene from IPNV was constructed in this work. Briefly, the designed plasmid consist of the strong inducible T7 promoter under the control of the lac- operator sequences, pel B leader, the ORF protein of

interest, followed by the 6His- Tag and T7 transcription terminator, an origin of replication, the β- lactamase gene under its own promoter to confer resistance to Kanamycin with the strong transcription terminator of phage λ. The viral protein gene was amplified in a 50 μl reaction mixture by using a Mastercycler with one step RT-PCR (Qiagen, Germany). The viral protein gene was amplified using DNA KAPA polymerase with primers incorporating specific recognition site for two restriction enzymes (RE) *NcoI* and *BamHI* (Fermentas, Lithuania). The REs were selected in order to achieve a directional cloning of the genes of interest into the expression vector *pET-26b (+)* at its multiple cloning sites (MCS). The amplified 1347 bps fragment was digested by *NcoI* and *BamHI* restriction enzymes and VP2 was inserted into the *NcoI* and *BamHI* cloning sites and followed by ligation into the corresponding site on the *pET-26b (+)* plasmid by T4 DNA Ligase (Fermentas, Lithuania) (Sambrook *et al.*, 2001).

Transformation of recombinant plasmid into host cells

The constructed plasmid was transformed using CaCl₂ method into *E. coli* cells (Dagert and Ehrlich, 1979; Inoue *et al.*, 1990) and transforming clones were selected on LB (Luria- Bertani) medium (Merck, Germany) with 50 μg/ml Kanamycin. Plasmids were prepared by the alkaline lysis method (Bimboim and Doly, 1979; Kado *et al.*, 1981) and purified using plasmid kits (Fermentas, Lithuania) and followed by restriction digestion analyses.

The insertion was sequenced to confirm its integrity (Bioneer).

Expression studies and protein gel electrophoresis

A well grown colony of recombinant *E. coli* cells (carrying the VP2 gene of IPNV) inoculated in 5ml of LB broth containing 50 µg/ml of Kanamycin and followed by incubation at 37°C overnight. It was grown in shaking incubator at 37°C and after reaching $OD_{600nm}=0.7-1.0$. The cells induced with 0.1 M IPTG for 3 hours (Fermentas, Lithuania). A part of the culture was used as negative control without adding IPTG (pre- induction). After 3 hours of additional growth at 37°C; cells were harvested by centrifugation at 5000 g for 10 min at 4°C. Electrophoresis was performed in the presence of SDS according to the Laemmli method (Laemmli, 1970). The discontinuous gel consist of a 4% stacking and a 10% separating gel run on a vertical electrophoresis unit. The pellet of bacteria was resuspended in 200 µl of 2X sample buffer (5 ml of dH₂O, 1 ml of 1 M Tris-HCl, pH=6.8, 1 ml glycerol, 2 ml of 10% w/v SDS, 5 mg of bromophenol blue, 1 ml of β-mercaptoethanol) prior to boiling at 100°C for 5 min. After boiling, the samples were centrifuged at 11000 g for 2 min and supernatant was used as sample for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R250. The total protein pattern of recombinant bacteria was visualized on Coomassie brilliant blue stained gel.

Results

Virus isolation

The Iranian IPNV isolate were separated from samples after the second passage on CHSE-214 cells. Moribund rainbow trout fries were collected during outbreaks of IPNV from 2010 to 2012 in different fish farms in North and West provinces of Mazandaran, Chaharmahal and Bakhtiari, and Kohgiluyeh and Boyer Ahmad of Iran. The farms were run on a flow-through system of fresh water with a temperature range of 12 to 15°C. From each farm, 30 moribund fish were selected and transferred to laboratory. The virus CPE were spindle-shaped cells and pyknosis of nuclei that typically appear in 5-10 days after inoculation (Fig. 1). Isolation of the virus in cell culture was confirmed by IPNV antigen ELISA Kit.

VP2 Gene Isolation

The specific primer pair amplified the full length of VP2 gene from extracted viral RNA successfully. The reactions amplified the fragment of 1347 bp as expected. The amplified fragment were sequenced and deposited in the NCBI database with accession number KC489465.

Over expression of VP2

The plasmid *pET-26b (+)* was used for targeting VP2 to the *E. coli*. The viral VP2 gene was amplified using DNA KAPA polymerase with specific primers incorporating specific recognition site for two restriction enzymes (RE) *NcoI* and *BamHI* (Fig. 2). PCR amplification showed a band of 1347 bp which was in agreement with observations by other researchers. The schematic map of the construct carrying the

VP2 gene has shown in Fig. 3. Ten recombinant colonies were selected and sub-cultured in medium supplemented with 50 mg/l Kanamycin. The extracted plasmids, pET-26b (+) rVP2, were analyzed by RE digestion and showed successful cloning of VP2 gene into *E. coli*. Cloning was further verified by DNA sequencing. For recombinant protein production, the constructed vector encoding rVP2 was transformed into *E. coli* BL21 (DE3) and Rosetta (DE3). The expression of the recombinant protein was

induced using IPTG at final concentrations of 0.2 mM at 37°C. Following the induction, the total protein of non-induction and induction cells were on a SDS-PAGE after 3 hours (Fig. 4). The presence of corresponding protein bands with a weight of 54 kDa, confirmed the expression of rVP2. Expression of rVP2 could be detected in both strains of *E. coli* (BL21 and Rosetta).

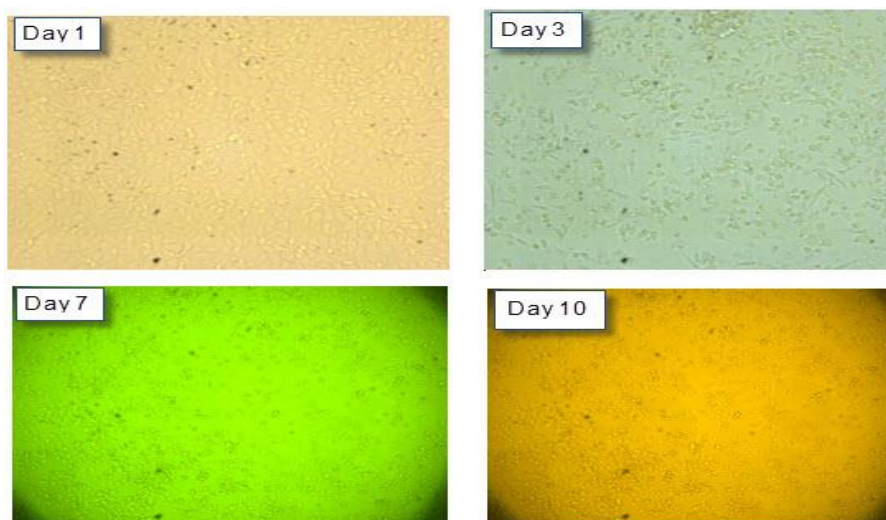


Figure 1: Phase contrast micrographs of CHSE-214 cells infected with IPNV.

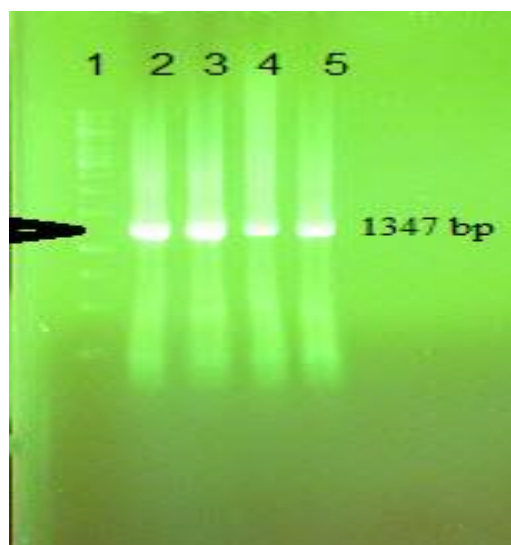


Figure 2: PCR amplification of the structural viral protein gene of Iranian Infectious pancreatic necrosis virus isolated from cell culture. Lane 1: Gene Ruler 1 kb DNA Ladder #SM0311 Fermentas.TM, Lanes 2, 3, 4 and 5: PCR products of the VP2 gene of Iranian Infectious pancreatic necrosis virus which is isolated from the cell culture.

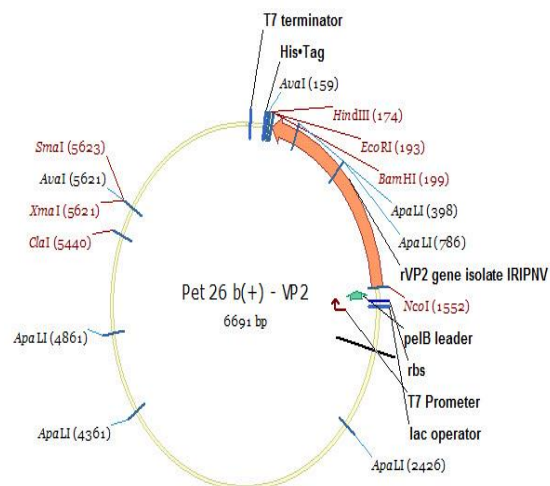


Figure 3: Schematic map of the recombinant *pET-26b (+) VP2* plasmid. The unique restriction sites inserted into the designed plasmid are *NcoI* and *BamHI*.

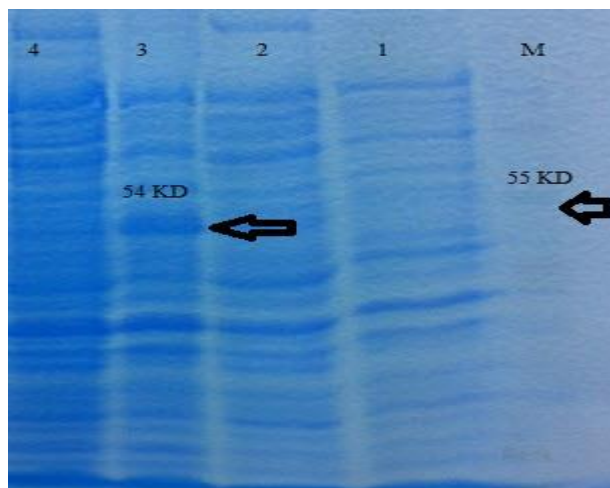


Figure 4: SDS-PAGE analysis of rVP2 periplasmic expression in *E. coli* BL21 and Rosetta. M: Protein marker, Page Ruler prestained protein ladder (#SM0671 Fermentas). Lane1: Control before IPTG addition. Lane2: Control 3h after IPTG addition; total soluble proteins extracted from untransformed *E. coli* BL21 and Rosetta strains having the pET-26b (+) plasmid. Lane 3: Total soluble proteins extracted from transformed *E. coli* strains containing the recombinant pET-26b (+) rVP2 plasmid and 3 h after induction by 0.2 mM IPTG, Lane 4, Total soluble proteins extracted from transformed *E. coli* strains containing the recombinant pET-26b (+) rVP2 plasmid, without induction.

Discussion

Viral diseases are usually regarded as the major factors of mortality and economic losses in aquaculture. Despite the importance of these diseases, there are few antiviral drugs for treatment of such cases (Kibenge *et al.*, 2012). Vaccination is a very effective way of protecting animals against infectious disease which can significantly reduce mortality (Tarrab *et al.*, 1995; Munang'andu *et al.*, 2013).

Since antiviral drugs have not yet found their way into aquaculture, vaccination against viral diseases can be considered as an approach for reducing mortality. An IPNV vaccine from company Norvax® Protect (NP) has been developed on the basis of rVP2 (Frost and Ness, 1997). Infection pancreatic necrosis vaccines have been available for a long time but their efficacy has been variable. The presence of a structural resembling neutralization epitope on recombinant VP2 (rVP2) for controlling Sp serotype strain has been demonstrated (Frost and Ness, 1997).

Most of neutralizing monoclonal antibodies which react with epitopes of IPNV, are located in VP2 region (Caswell-Reno *et al.*, 1989; Tarrab *et al.*, 1995; Frost and Ness, 1997; Labus *et al.*, 2001). Two variables and one conserved neutralization epitopes are located in the central third part of VP2 (Frost and Ness, 1997). Different genetic constructs have been made to express the capsid proteins for the expression and self-assembly of these proteins into VLPs and SVPs in different hosts including bacteria (Brun *et al.*, 2011), insects (Shivappa *et al.*, 2004), yeast (Allnutt *et al.*, 2007), plant (Santi *et al.*, 2008) and mammalian cells (Labus *et al.*,

2001). The reason for the varying immune response to these vaccines has not been well defined and studies on the importance of using vaccine trains homologous to the virulent field strain has been conclusive (Munang'andu *et al.*, 2013).

In this study the VP2 gene from IRIPNV isolate were amplified and sequenced using specific primers by RT-PCR. The gene of interest was cloned into pET-26b (+). Cloning verification was carried out by restriction enzyme digestion and DNA sequencing. The band of 54 kDa were detected in SDS-PAGE. Also the results showed that the recombinant VP2 can be expressed into both of strains of *E. coli* BL21 (DE3) and Rosetta (DE3). To our knowledge; this is the first report of isolation and expression of VP2 in Iranian IPNV isolates. The VP2 gene from IPNV was previously cloned and shown to form SVPs in yeast (Allnutt *et al.*, 2007; Dhar *et al.*, 2010), bacteria (Tarrab *et al.*, 1995; Moon *et al.*, 2004) expression systems. The SVPs and VLPs containing VP2 induce an immune response in salmon and rainbow trout (Galloux *et al.*, 2004). Moreover, recent studies showed that the residues of the VP2 capsid play a key role for immunogenicity of IPNV vaccines (Munang'andu *et al.*, 2013). Furthermore, several homologous and heterologous genes are successfully expressed in *E. coli* (Inoue *et al.*, 1990) and they were extensively engineered for the production of heterologous therapeutic proteins. The periplasmic space of *E. coli* is known for functional production of a variety of recombinant proteins, specially those which show toxic effects on host cells, or need oxidizing environment of periplasm

for appropriate formation of structural disulfide bonds (Hannig and Makrides, 1998; Harvey *et al.*, 2004; Memari *et al.*, 2010; Gholipour *et al.*, 2010; Morowvat *et al.*, 2014). SDS-PAGE analysis of rVP2 showed the expression of the viral protein in a same molecular weight that was in agreement with the reported and expected size. The results of our study can be used in development of recombinant vaccines for IPNV. While vaccination in aquaculture is yet in initial steps (Evensen, 2009), one of the most promising and urgent applications of rVP2 is to investigate their use in introducing a recombinant vaccine (Dhar *et al.*, 2014). The recombinant VP2 can be used as part of a commercial injectable polyvalent or monovalent vaccines as well as development of new diagnostic kits.

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