Prokaryotic expression of chicken infectious anemia apoptin protein and characterization of its polyclonal antibodies

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In the present study recombinant VP3 (rVP3) was expressed in E.coli BL21 (DE3) (pLysS) and its polyclonal antibodies were characterized. SDS–PAGE analysis revealed that the expression of recombinant protein was maximum when induced with 1.5 mM IPTG for 6 h at 37°C. The 6×His-tagged fusion protein was purified on Ni-NTA and confirmed by Western blot using CAV specific antiserum. Rabbits were immunized with purified rVP3 to raise anti-VP3 polyclonal antibodies. Polyclonal serum was tested for specificity and used for confirming expression of VP3 in HeLa cells transfected with pcDNA.cav.vp3 by indirect fluorescent antibody test (IFAT), flow cytometry and Western blot. Available purified rVP3 and polyclonal antibodies against VP3 may be useful to understand its functions which may lead to application of VP3 in cancer therapeutics.

Keywords: Chicken infectious anemia, Polyclonal sera, Prokaryotic expression, VP3 gene

Chicken anemia virus (CAV), genus Gyrovirus and family Circoviridae, is the causative agent of chicken infectious anemia. It is primarily an immunosuppressive disease of young chickens\(^1\) and appears to be ubiquitous in all major chicken producing countries of the world\(^2\).

CAV is one of the smallest avian viruses; it is 23-25 nm in size, icosahedral in shape and non-enveloped, having a 2.3 kb, circular, negative sense single-stranded DNA genome\(^3,4\). The ssDNA genome has 3 open reading frames (ORFs), which partially overlap and code for VP1, VP2 and VP3 proteins\(^5,7\). VP1 is a 51 kDa protein, which is the major component responsible for the assembly of viral capsid\(^8\), VP2 is a 28 kDa non-structural protein with phosphatase activity of dual-specificities and is hypothesized to be a scaffolding non-structural protein required for correct folding of VP1\(^9,11\) and VP3 is a 13 kDa protein, called apoptin that can induce apoptosis in chicken or tumor cell lines\(^12,14\).

Among the viral proteins, VP1 and VP2 are the protective proteins that induce neutralizing antibodies\(^15\). VP2 induces apoptosis but, its apoptotic activity is much weaker than VP3. VP2 induces apoptosis both in normal and tumorigenic cells and apoptin induces p53-independent apoptosis in a tumor-specific way\(^16\). In tumor cells, apoptin is phosphorylated and mainly nuclear, whereas in normal cells it is un-phosphorylated, cytoplasmic and becomes readily neutralized. It acts as a multimeric complex and forms superstructures upon binding to DNA. Interestingly, apoptin phosphorylation, nuclear translocation, and apoptosis can transiently be induced in normal cells by co-transfecting SV40 large T oncogene, indicating that apoptin recognizes early stages of oncogenic transformation\(^16\).

Properties of, selective apoptotic activity against transformed and cancer cells, sensing early stages of oncogenic transformation and inducing p53 (most commonly mutated tumor suppressor gene in cancer) independent apoptosis make apoptin a potent candidate for viral oncotherapy. In our lab to establish the oncolytic potential of VP3 in HeLa cells the gene was cloned in pcDNA\(^17\) (pcDNA.cav.vp3). To confirm the expression of the VP3 in pcDNA.cav.vp3 transfected HeLa cells there was a need to raise polyclonal serum against CAV-VP3. Therefore, this study was carried out to express and purify the recombinant VP3 (rVP3), and characterize the polyclonal serum.
Materials and Methods

Amplification of apoptin gene and cloning in pET32a+—VP3 was amplified and cloned in pcDNA3\(^{15}\) and pcDNA.cav.vp3 was used as a template to amplify VP3 gene. Primer sequences, 5'-ATGAACGCTTCAAGAAG-3' (Forward); 5'-CTTACAGTCCTACACCTT-3' (Reverse); with specific restriction sites (EcoRI and BamHI) for directional cloning into pET32a+, were used to amplify a 367bp amplicon covering the complete apoptin open reading frame. Amplification was carried out in 50 \(\mu\)l final volume containing 1.5 mM MgCl\(_2\), 50 mM Tris-HCl (pH 9.0 at 25 °C), 15 mM (NH\(_4\))\(_2\)SO\(_4\) and 0.1% Triton-X; 0.4 \(\mu\)M of primers; 200 \(\mu\)M of each dNTP's and 1 unit of Taq polymerase. After an initial denaturation at 94 °C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 94 °C for 45 sec, annealing at 54 °C for 45 sec and extension at 72 °C for 30 sec. A final extension step of 10 min at 72 °C ended the reaction. PCR product was resolved in 2% agarose gel. PCR product was recovered from low melting point agarose and purified (QIAex II gel-extraction kit, Qiagen). Amplified VP3 and expression vector pET32a+ were digested with restriction enzymes EcoRI and BamHI, ligated and transformed into E.coli BL21 (DE3) (pLysS) competent cells. The desired recombinant plasmid pET32a+, VP3 was confirmed by PCR and restriction enzyme digestion with NcoI/XhoI.

Expression of recombinant protein—E. coli strain BL21 (DE3) (pLysS) (Stratagene, La Jolla, CA) was used as a host for expression of VP3 gene. Induction procedure for gene expression was as follows: 5 mL of Luria–Bertani (LB) broth containing chloramphenicol (34 \(\mu\)g/mL) and ampicillin (100 \(\mu\)g/mL) was inoculated with a bacterial colony and incubated overnight at 200 rpm at 37 °C. The overnight culture was transferred into a flask containing 500 mL of LB medium with the same antibiotics and agitated at 200 rpm at 37 °C until the culture density reached an OD\(_{600}\) of 0.7-0.8. IPTG was added to final concentration 1.5 mM with subsequent incubation at 200 rpm at 37 °C for 6 h (A time and dose course of expression to determine the optimal induction conditions for maximum expression of protein was measured by taking aliquots of cells at 2, 4 and 6 h after induction with IPTG at final concentrations of 1, 1.5, 2 and 2.5 mM). After incubation, the bacterial cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C and frozen at -80 °C till further use. A non-induced culture was used as a negative control. Expression was analyzed by 12% SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250.

Protein Extraction and purification—Bacterial pellet was suspended in lysis buffer (100 mM sodium di hydrogen ortho phosphate and 50 mM di sodium hydrogen ortho phosphate with 8M urea, pH 8.0, (10 mL/50ml bacterial culture)) and kept on a rocker platform for 3 h for complete lysis. The cleared supernatant was collected by centrifugation at 12000 rpm for 30 min. 1 ml of Ni-NTA (Qiagen) was added to supernatant and gently mixed on a rock for 1 h and then loaded into a column to collect the unbound fraction of the protein. The column was washed with 8 mL wash buffer, \(pH\) 6.3 (100 mM Na\(_2\)HPO\(_4\) and 50 mM Na\(_2\)HPO\(_4\) and 8 M urea) before collecting 0.5 mL fractions with 5 mL of elution buffer \(pH\) 4.5 (100 mM Na\(_2\)HPO\(_4\) and 50 mM Na\(_2\)HPO\(_4\) and 8 M urea). All the fractions were then loaded on SDS-PAGE to identify the fraction with purified protein. Purified recombinant protein was confirmed by Western blot using CAV infected antiserum (available in the lab) and anti-His monoclonal antibody conjugated to HRP. The concentration of the protein was determined by Bradford method.

Before injecting the recombinant VP3 into rabbit to raise antiserum, the protein fraction containing the recombinant VP3 was dialysed to remove urea and other impurities.

Specificity of Polyclonal antibodies against VP3—Purified recombinant VP3 (rVP3) was used to prepare antibodies in albino rabbit. Briefly, rabbit was immunized subcutaneously with rVP3 (200 \(\mu\)g) in complete Freund’s adjuvant. Two booster injections at a weekly interval were given in incomplete Freund’s adjuvant. Serum was collected 7 days after the 3rd immunization to determine the VP3 antibody specificity. The last immunization was performed one week later, and the antiserum was collected through heart puncture after 7 days. Polyclonal serum was tested for specificity by indirect ELISA and Western blot using cell lysate infected with CAV.

Indirect ELISA—To detect the presence of anti VP3 antibody in rabbit antiserum indirect ELISA was done. Briefly, positive reference antigen (infected cell lysate) was diluted 1:200 in 1x PBS (coating buffer). Diluted antigen (50 \(\mu\)L) was added to each well of a micro-ELISA plate in quadruplicates and incubated
overnight at 4 °C. Four wells added with 50 µL PBS and not coated with antigen, were taken as negative control. After incubation the antigen solution was discarded and 50 µL of blocking solution (1% BSA in coating buffer - w/v) was added to each well and incubated for 1 h at 37 °C. After blocking, the plates were washed three times in phosphate buffered saline pH 7.2 containing Tween 20 (PBS-T). Test sera at the same dilution were run in quadruplicates. The plates were incubated for 2 h at 37 °C and then washed three times in PBS-T. Goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:2000 in PBS-T, was then added and a further incubation for 1 h carried out at 37 °C. The plates were again washed three times, a substrate-chromogen solution of H₂O₂ containing O-phenylenediamine was added, and after ten minutes, the reaction was stopped by adding 100 µL of 2 M sulfuric acid. Intensity of colour development was determined by measuring absorbance using a micro-ELISA reader equipped with a 420 nm filter. The same was repeated with infected lysate 6 times.

**Western Blot**—Western blot analysis was done using the total proteins of cell lysate infected with CAV and uninfected cells as control. The protein samples were separated on 12% SDS-PAGE and electroblotted onto nitrocellulose membrane. After blocking overnight in 2% BSA the membrane was incubated with serum at a dilution of 1:100 for 2 h. The membrane was then washed 3 times with Tris buffered saline containing Tween 20 (TBST) and then incubated with goat anti-rabbit FITC conjugated secondary antibody (KOMA BIOTECH) at 1:100 dilution in PBS containing 1 % BSA at 37 °C for 1 h. The membrane was then washed thrice with TBST for 10 min each and bands were visualized following incubation with diaminobenzidine tetrahydrochloride (DAB system, GeNei).

**Expression of VP3 in HeLa transfected with pcDNA.cav.vp3**—The expression of VP3 in HeLa transfected with pcDNA.cav.vp3 was confirmed by flowcytometry, IFAT, and Western blot analyses using HeLa cells transfected with pcDNA.cav.vp3 construct. Untransfected Hela cells were used as control.

**Flowcytometry and IFAT**—HeLa cells showing 60-70% confluency in a six-well culture plate (Nunc) were transfected with pcDNA.cav.vp3 using Lipofectamine reagent (Invitrogen). 4 µg of the plasmid (pcDNA.cav.vp3) was mixed in 250 µL OptiMEM medium (Invitrogen) and were allowed to stand at room temperature for 5 min. Simultaneously, 10 µL Lipofectamine was mixed with 250 µL OptiMEM medium in another tube and also kept at room temperature for 5 min. The contents of both the tubes were then mixed together and kept at 37 ºC for 30 min. The monolayer of cells in each well was washed with OptiMEM three times. After 30 min, 1 mL of OptiMEM medium was added drop-wise to the Lipofectamine/DNA mixture, mixed well, added to the cell monolayer in each well drop-wise and incubated at 37 ºC with 5 % CO₂. After 4 h incubation, the medium was replaced with growth medium containing 5% fetal bovine serum. At the end of 48 h, medium was removed completely and cells were washed with PBS (pH 7.5). Cells in a few wells were fixed for IFAT and rest were harvested for FACS.

For IFAT, transfected cells were rinsed once with ice-cold PBS and fixed in 4 % paraformaldehyde (PFA) for 20 min at RT. Cells were then washed twice with ice-cold PBS for 5 min each, permeabilized by treating with 0.2 % Triton X 100 in PBS for 5 min, washed again twice with PBS for 5 min each and subsequently blocked in 2 % bovine serum albumin (BSA) dissolved in PBS for 1 h at 37 ºC. After rinsing thrice with PBS the cells are incubated overnight at 4 °C with antisera at 1:100 dilution in PBS containing 1 % BSA. Next day, the cells were washed thrice with PBS for 5 min and incubated with goat anti-rabbit FITC conjugated secondary antibody (KOMA BIOTECH) at 1:1000 dilution for 1 h at 37 °C. The cells were washed thrice with PBS for 5 min each and were observed for green fluorescence using the fluorescence microscope.

For FACS, transfected cells were trypsinized and cells were pelleted at 6000 rpm for 5 min. The cells were then fixed, permeabilized and blocked as described above. After rinsing thrice with PBS the cells are incubated overnight with antisera at 1:100 dilution in PBS containing 1 % BSA at 37 ºC for 1 h. The cells were washed thrice with PBS and incubated with goat anti-rabbit FITC conjugated secondary antibody at 1:2000 dilution for 30 min at 37 °C. After incubation cell pellet was washed again thrice with PBS and resuspended in 400 µL of 1X wash buffer and analyzed by flow cytometer using FL1 filter. 

**Western Blot**—Western Blot analysis was done using the total proteins of HeLa cells transfected with pcDNA.cav.vp3 construct and the total proteins of untransfected HeLa cells as control. For western blot, transfection was carried on monolayer of HeLa cells...
grown in a 25 cm² tissue culture flask. The rest of procedure was as described above.

Statistical analysis—Indirect ELISA data was analysed for sensitivity and specificity with MedCalc software. Flow cytometry data was analysed using Kruskal-Wallis non parametric test in JMP 8.0 (SAS institute 2009). Difference was considered significant at \( P \leq 0.05 \).

**Results and Discussion**

*Expression and purification of rVP3—*PCR amplification with specific primers yielded a 367bp amplicon covering complete CDS of VP3 gene (Fig. 1a). Amplified PCR product was gel purified and cloned into pET32a+ prokaryotic expression vector. Recombinant plasmids with VP3 gene insert were confirmed by PCR and restriction digestion (Fig. 1b). The pET32a+ vector was used in the present study for getting high-level expression of recombinant VP3 (rVP3) protein containing cleavable 6×His-tag sequence for efficient detection and purification.

The most useful host organism for overproducing recombinant proteins is *Escherichia coli* (*E. coli*), though the approach has a number of weaknesses such as the organism’s codon usage, the production of cytoplasm inclusion bodies and a lack of posttranslational modification\(^{19-21}\). Currently, many eukaryotic and prokaryotic functional proteins such as enzymes, cellular receptors, hormones and many viral proteins are expressed in *E. coli*\(^{22-25}\). The rVP3 was expressed in *E. coli* BL-21(DE3) (pLysS). Initially, *E. coli* cells containing recombinant plasmid pET32a+.VP3 were cultured in 5 mL of LB culture tubes and a time and dose dependent study was carried out by inducing cultures with different concentrations of IPTG and collecting samples at regular intervals after induction, respectively. SDS-PAGE analysis revealed maximum expression in *E.coli* induced with 1.5mM IPTG for 6 h at 37 ºC. The expressed protein was approximately 34 kDa in size (Fig. 2a) which included 13.4 kDa VP3 and 20.4 kDa fusion peptide having the 6xHis-tag. The protein was finally expressed from 500 mL induced *E.coli* culture, extracted and purified. The purified protein was confirmed by Western blot using anti-His monoclonal antibody conjugated to HRP and CAV

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Fig. 1—Amplification and cloning of CAV-VP3 (a)-PCR amplification of pET32a+.VP3 , M - 100bp DNA ladder; Lanes 1 & 2 – amplified VP3; Lane 3 – negative control (b)-Confirmation of recombinant plasmid pET32a+.VP3, Lane 1 – PCR amplicon ; Lane M - 100 bp DNA ladder and Lanes 2 & 3- release of the insert on digestion with Neol / XhoI (The release is of approximately 418bp because of the nucleotides (51bp) added from the plasmid to the insert (367bp) after digestion).
infected antiserum (Fig. 2b & 2c). The purified recombinant VP3 (rVP3) after dialysis, was used to raise polyclonal antibodies in albino rabbit. The serum collected was tested for its VP3 antibody specificity.

Specificity of Polyclonal antibodies against VP3—Polyclonal serum raised against CAV-VP3 was tested for specificity by Western blot and indirect ELISA using cell lysate infected with CAV. Antisera against rVP3 reacted with CAV infected cell lysate as shown.
by western blot (Fig. 2d) and indirect ELISA (Fig. 2e). The area under curve (AUC) indicated that the anti rVP3-ELISA test was on an average 97.2% accurate. The 95% confidence interval of the AUC for this ELISA ranged from 85.5% to 99.9%. The ROC analysis indicated that the optimal cut-off point was 0.141; this resulted in a sensitivity of 100% and specificity of 83.3%.

Expression of VP3 in HeLa transfected with pcDNA.cav.vp3—Construct pcDNA.cav.vp3 was confirmed as to harbor the VP3 gene by sequencing. Expression of VP3 in HeLa transfected with pcDNA.cav.vp3 was confirmed by flowcytometry, IFAT, and western blot analyses. IFAT in cells transfected with pcDNA.cav.vp3 using the polyclonal sera showed specific binding of antibody to the expressed VP3 protein when compared to the control untransfected cells (Fig. 3a). The specificity was confirmed by Flow cytometry analysis. The transfected cells showed significantly ($P \leq 0.05$) increased fluorescence as compared to control (Fig. 3b and c). This increase was due to the binding of VP3 specific antibody to VP3 protein expressed in HeLa cells. Further, western blot analysis of the cell lysate reaffirmed the specificity of the polyclonal serum to CAV-VP3. The Western blot showed a specific band of 13.4 kDa specific for CAV-VP3. Expression of VP3 in HeLa cells by the gene construct - pcDNA.cav.vp3 is now established and the gene construct is being used for evaluating the oncolytic potential of VP3 in HeLa cells.

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References


17 Singh P K, Elucidation of molecular mechanism involved in apoptin induced oncosynthesis in tumour cell line, MVSc, thesis, Indian Veterinary Research Institute, Bareilly, India, 2010.


