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Recombinant chicken anaemia virus viral protein 1 gene expression in insect cell system

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ABSTRACT

CAV specific PCR positive tissue samples of Namakkal region available in the Department of Animal Biotechnology, Madras Veterinary College, were used in this study. A multiple host expression vector pTriEx 1.1 Neo was selected to clone the CAV VP1 gene (1353 bp) and transformed into BL21 (DE3) strain of *E. coli* and confirmed. The CAV VP1 recombinant pTriEx vector was co- transfected to Bacvector triple cut virus DNA with Sf9 insect cells. The plaques formed by CAV VP1 recombinant baculovirus were purified and propagated for protein expression. The recombinant CAV VP1 protein expression was confirmed by indirect FAT and SDS-PAGE analysis followed by western blot analysis using specific serum.

Key words: Baculovirus, Chicken anaemia virus, Cloning, Insect cells, Protein expression, Viral protein 1

Chicken infectious anaemia caused by chicken anaemia virus (CAV) is an acute viral infection of chickens and is worldwide in existence. Chicken anaemia virus is classified as Circovirus (family Circoviridae) on the basis of morphology and circular genome characteristics (Lukert *et al*. 1995). CAV has a circular single-stranded 2.3 kb DNA genome contained within an icosahedral capsid, 25 nm in diameter. CAV genome is an un-spliced polycistronic mRNA of about 2100 nucleotides encoding 3 proteins of about 51.6 kDa (VP1) capsid protein; 24 kDa (VP2) scaffold protein and 13.6 kDa (VP3) apoptin which are synthesized in CAV infected cells. CAV can infect chickens of all ages but disease is only seen in young chickens and is characterized by depression, anorexia, anaemia, hemorrhage and a sudden rise in mortality due to immunosuppression. The virus was first isolated in Japan in 1979 and was given its name because of the serious anaemia it caused in young chicks (Yuasa *et al.* 1979). Many attempts were made to produce the CAV proteins in prokaryotic and eukaryotic expression systems. The VP1 gene was cloned and expressed in prokaryotic expression vector pRSET-B by Soliman *et al.* (2006) to have a recombinant protein containing immunogenic epitope. CAV VP1 gene was cloned and expressed in prokaryotic expression vector - pPro Ex Ht 'b' and the recombinant protein was confirmed by immunobloting (Chakravarthy 2007). Recombinant baculovirus co-expressing VP1 and VP2 is a potential production system for a subunit vaccine against CAV infection (Koch *et al.* 1995, Noteborn *et al*. 1998). An *in vitro* baculovirus cloning system was developed for direct cloning of foreign DNA into baculovirus genomes. Advantages of using the baculovirus expression system (BVES) includes — high expression levels, limitless size of the expressed protein, efficient cleavage of signal peptides and processing of the protein, post-translational modifications and simultaneous expression of multiple genes. In addition to these advantages, expressed proteins are correctly folded and biologically active. Having understood the diagnostic importance of VP1 protein in the CAV infection, this study was undertaken to clone and express the immunogenic gene of CAV with the strategy of using it in diagnostic assays.

MATERIALS AND METHODS

Samples: Tissue samples such as liver, spleen, bone marrow, thymus, and serum collected during 2006 from birds of different poultry farms in and around Namakkal, Tamil Nadu, for the diagnosis of chicken anaemia virus infection, available at the Department of Animal Biotechnology, Madras Veterinary College, were used in this study.

Cloning and expression

Polymerase chain reaction: The following primers were designed using the LASERGENE software to amplify the

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VP1 gene of chicken anaemia virus with built in restriction enzyme sites to enable directional cloning of the PCR product into the expression vector - pTriEx 1.1 Neo.

Forward primer

5'-GGG GAA$^{EcoR\ I}$ TTC GAT GGC AAG ACG AGC TCG CAG ACC-3'

Reverse primer

5'-GGG CTC$_{Xho\ I}$ GAA TCA GGG CTG CGT CCC CCA GTA CAT GGT-3'

Amplification of VP1 gene was carried out using the above primers with the isolated DNA from CAV positive tissues by using DNAzol reagent. PCR cycle conditions were, 94° C for 45 sec, 55° C for 1 min, 72° C for 2 min for 30 cycles and a final extension at 72°C for 5 min followed by holding at 4°C. Pure link quick gel extraction kit was used to purify the PCR product as per the kit protocol.

pTriEx 1.1 Neo was transfected in Nova Blue cells and the plasmid was extracted by using plasmid extraction kit. Both the purified plasmid and PCR product were digested with the restriction enzymes *EcoR I* and *Xho I*, ligated and transformation was done by heat shock method in *E. coli* BL21 (DE3) cells. The recombinant colonies were screened by colony PCR using gene specific primers and further confirmation was done by digesting the recombinant plasmid with restriction enzymes to release the inserted CAV VP1 gene.

CAV VP1 protein expression in insect cell system: Sf9 insect cell culture was maintained and the recombinant plasmid was transfected to the insect cells using DNA 1000 transfection kit. Briefly, the recombinant plasmid (500 ng in 5 µl), triple cut virus DNA in linearized form (100 ng in 2 µl) were added with insect cell medium to make up to 25 µl volume. In another tube, 20 µl of nuclease free water and 5 µl of insect transfection reagent were taken and added immediately to the above mixture, vortexed and incubated for 15 min at room temperature. Meantime, the Sf9 cells were prepared by washing with serum and antibiotic free insect cell medium. After the incubation, 450 µl of insect cell medium was added to the mixture and 1/10, 1/50, 1/250, 1/1250 dilutions were made and 100 µl was added to the medium drained cells and incubated for one hour at room temperature for direct plaquing. Bacplaque agarose (1%) overlay was done which was prepared with insect cell medium and foetal bovine serum. After 4 days of incubation, the sf9 cells with agar overlay was stained with Neutral red and the individual plaque was picked up for the recombinant baculovirus separation from the agar for further confirmation. The plaques were screened by indirect FAT and sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) followed by western blotting and immuno-detection using CAV positive reference serum.

RESULTS AND DISCUSSION

Total DNA isolated from the pooled tissue samples suspected for CAV was used to amplify CAV VP1 gene by PCR with designed primers to get the complete open reading frame. An expected amplicon of 1353 bp was confirmed by 1.2% agarose gel electrophoresis (Fig. 1). The PCR product and the cloning vector pTriEx 1.1 Neo were digested with *EcoR1* and *Xho1*. The digested PCR product and the pTriEx 1.1 Neo vector were ligated and transformation was done. Large numbers of colonies were seen in LB plates and transformation efficiency was 2×10^4 colonies/µg of DNA. VP1 gene specific primers were used to screen the recombinant pTriEx 1.1 Neo vector in the growing colonies. Four colonies were positive by colony PCR out of 5 colonies screened. PCR amplicon size of 1353 bp CAV VP1 gene was observed in 1.2 % agarose gel electrophoresis and in the

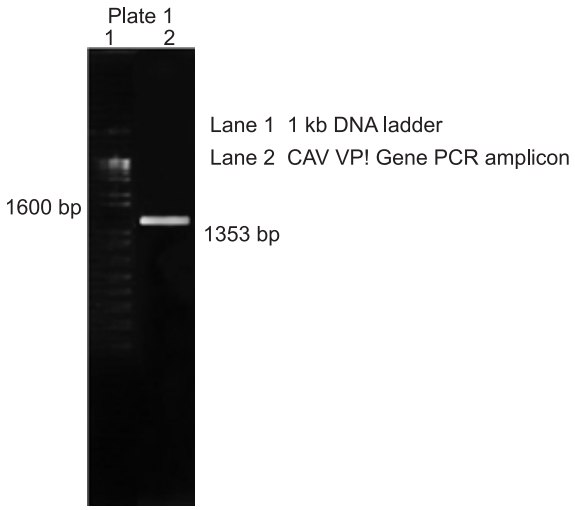
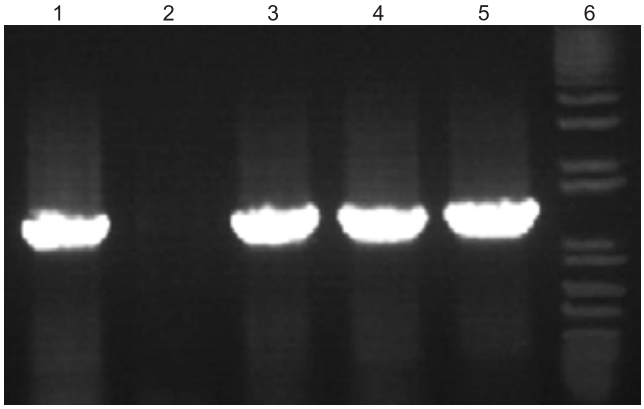
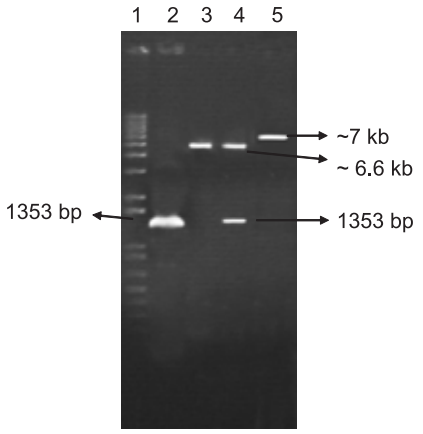


Fig. 1.CAV VP1 gene amplification from CAV infected tissue samples shown in 1.2 % agarose gel.



Lane 1, 3, 4, 5: Positive colonies with CAV VP1 insert
Lane 2: Negative control
Lane 6: 1kb ladder

Fig. 2. Colony PCR results showing CAV VP1 gene amplification from the recombinant colonies seen in 1.2 % agarose gel.



Lane 1- Trackit 1 kbp DNA Marker
Lane 2 - Amplified VP1 Colony PCR product
Lane 3 - pTriEx Vector
Lane 4 - Digested Recombinant Plasmid
Lane 5 -pTriEx Recombinant Vector

Fig. 3. 1.2 % agarose gel showing CAV VP1 gene insert released from the recombinant pTriEx vector.

restriction digestion of the recombinant plasmid (Figs 2, 3).

Co-transfection of Sf9 cells with purified transfer vector (500 ng) and BacVector triple cut virus DNA (100 ng) using insect gene juice transfection reagent was done at different dilutions as per the kit protocol. At 96 h post transfection the plates were processed for neutral red staining. The neutral red staining (0.33% w/v) of the Sf9 control (Fig. 4a) showed a confluent smooth monolayer. The transfected cells showed positive baculovirus plaques at 1/10, 1/50 and 1/250 dilution of the transfection mixtures. The plaques were identified as clear zone among the stained cells (Fig. 4b). The indirect fluorescent antibody technique showed cytoplasmic fluorescence in all the infected cells mostly seen as clumps or cell aggregation when compared to the uninfected control Sf9 cells (Fig. 5a, b).

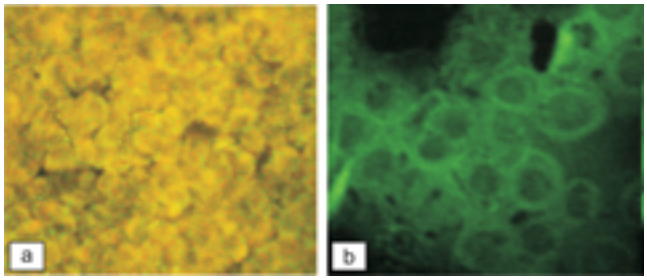


Fig. 4a. Neutral red stained uninfected control Sf9 cells (200×). b. Transfected Sf9 cells with recombinant pTriEx vector showing plaques. 100×.

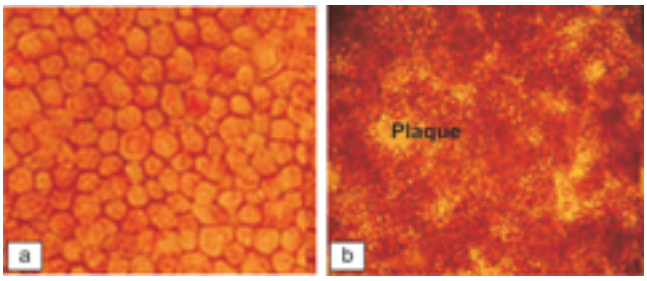
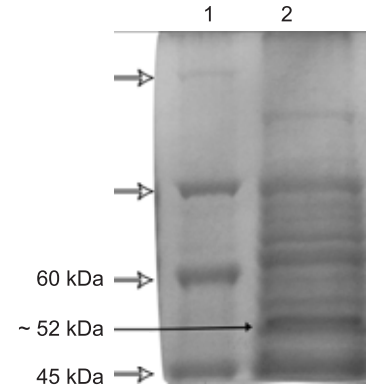


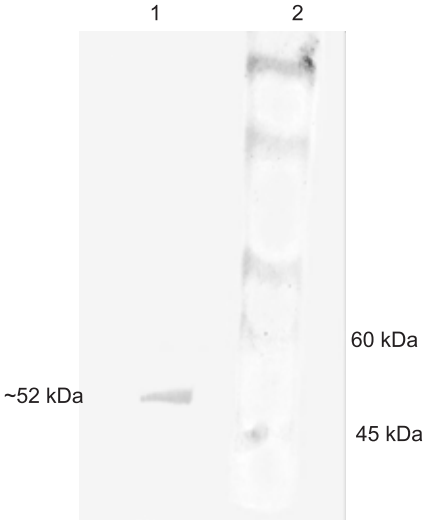
Fig. 5a. Control Sf9 cells stained, b: Transfected Sf9 cells showing intra-cytoplasmic fluorescence (200×).

The recombinant baculovirus infected Sf9 cell crude proteins were fractionated in polyacrylamide gel with wide range standard protein marker and stained with coomassie brilliant blue. The expressed recombinant CAV VP1 was discerned at ~ 52 kDa position when compared with protein standards along with other crude proteins (Fig. 6). The result of immuno-blotting of transferred proteins of recombinant of CAV VP1 along with the baculovirus infected Sf9 cells crude proteins are presented in Fig. 7. It was clearly demonstrated that CAV specific polyclonal serum, could react only with the capsid protein CAV VP1 at the position of ~52 kDa size.



Lane 1 Protein marker
Lane 2 Insect cell lysate with CAV VP1 protein-52 kDa

Fig. 6. SDS-PAGE analysis showing recombinant CAV VP1 protein.



Lane 1 CAV VP1 recombinant protein 52 kDa size
Lane 2 Protein marker

Fig. 7. Immunblotting of the recombinant CAV VP1 protein.

After the first report of CAV in commercially produced chickens in Japan (Yuasa *et al*. 1979), the virus was detected by isolation or serology or by molecular methods in most other countries in both laying and broiler chickens. Namakkal is one of the leading poultry production centres in India and sero-prevalence was reported earlier. In the present outbreak, the mortality recorded was only 4 % but the production loss due to stunted growth was high. But the recorded mortality rate so far varies between 10 and 60%. It is a sturdy virus which resists chloroform and heat treatment (70°C for 5 min) and is very difficult to eliminate from an infected poultry farm (Verma *et al.* 2005).

In this study, the main capsid protein of CAV VP1 was taken for cloning and expression in the baculovirus system. Previously many workers cloned and expressed the VP1 protein in both prokaryote and eukaryote systems. pGEM vector was used by Pallister *et al.* (1994) and pRSET-B vector by Soliman *et al*. (2006) for bacterial expression and Kato *et al*.(1995) also used *E. coli* cells for VP1 protein expression with ß-galactosidase as fusion protein. Koch *et al.* (1995) stated that co-expression of CAV VP1 and VP2 proteins in insect cell system, are required for inducing neutralizing antibodies and suggested to be used as sub unit vaccine and later it was done by Iwata *et al*. (1998). Noteborn *et al.* (1998) also used insect cell system for expressing CAV VP1 and VP2 proteins.

CAV DNA was isolated along with the total tissue DNA by DNAZol method and this method was more efficient than the conventional method of DNA isolation. Todd *et al.* (1992) extracted DNA by guanidine isothiocyanate method, wherein they used diatoms for nucleic acid purification which is a tedious method. In another study, Chakravarthy (2007) used Chargeswitch gDNA mini tissue extraction kit. Though it yielded highly purified DNA it took 4–5 h duration. The current method is quicker and the results can be obtained in 4 h duration. In the present study, only the open reading frame of CAV VP1 gene was targeted for cloning into pTriEx 1.1 Neo vector. Advantage of this vector is that a single cloning in this vector would be sufficient to go for expression in multiple system like prokaryotic, baculovirus and mammalian expression system depending upon the requirement. But others have used primers to amplify the gene open reading frame along with small fragments of non-coding regions at both upstream and downstream of the open reading frame (Koch *et al.* 1995, Noteborn *et al.* 1998). For diagnostic purpose, Anci (2007) analyzed several primer sets to amplify the 3 CAV genes separately for sensitive detection of the CAV infection.

Further confirmation of the recombinant pTriEx 1.1 Neo vector for CAV VP1 gene was done by sequencing, though the double enzyme digestion naturally helps in proper orientation of the inserted gene in cloning vector. The linearized recombinant pTriEx 1.1 Neo vector was used in the co- transfection with the Baculovector triple cut virus DNA in the Sf9 insect cells using insect gene juice transfection reagents. Agar overlay was done to plaque purify the recombinant baculovirus after 96 h post infection. The pTriEx 1.1 Neo vector as a p10 promoter which is a late activating promoter with the expression of the recombinant protein would be at the end of the baculovirus infection cycle of 96 h.

FAT was used as one of the confirmative tests in detecting CAV infection and was used by Hoop (1991) and Bhardwaj *et al*. (2003) for detection of CAV infection in tissue impression smears as well as MDCC- MSB1 infected cell cultures, and by Noteborn *et al*. (1991) in Sf9 infected cells.

Immunoblotted nitro cellulose membrane also confirmed the expression of the recombinant CAV VP1 when reacted with the CAV positive polyclonal serum. A similar immunoblot confirmation of the CAV VP1 recombinant protein expressed in prokaryotic system was done by Noteborn *et al.* (1992) and Todd *et al.* (1990), and it ranged between 49 and 54 kDa. The recombinant baculovirus expressed CAV VP1, VP2 and VP3 proteins was confirmed by radioimmunoprecipitation assay by Koch *et al*. (1995) and Noteborn *et al.* (1998), which showed expression of each proteins separately or in combinations.

The focus of this study was to develop a recombinant antigen for diagnosis of chicken anaemia virus infection. CAV VP1 gene cloning and expression in insect cell system was confirmed in this study. Further, the insect cells expressed recombinant CAV VP1 protein could be purified easily with the help of His tag fusion protein and could be used in various diagnostic assays for CAV antibody detection and also be used as subunit vaccine along with CAV VP2 protein.

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