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Expression studies of fowl adenovirus penton base

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Abstract
The fowl adenovirus infections are responsible for high morbidity and high mortality in poultry flock throughout the world. There is no effective vaccine against these viruses. The present study was conducted to express penton base gene which is one of the major immunogenic proteins of virion. The penton base gene was amplified and cloned into plenti6/V5-D-TOPO vector system which is HIV-1 based vector. After transfection, the pseudotyped lentivirus particles were synsthesized which were found to be able to transform as into vero cells. The expression of penton base protein in vero cells was observed by immunofluorescence studies.

Keywords: Fowl adenovirus, hydropericardium-hepatitis syndrome, penton base, lentivirus expression vectors

Introduction
The Fowl Adenoviruses, classified under family *Adenoviridae*, are involved in many pathological conditions of poultry, like Hydropericardium-hepatitis syndrome, gizzard erosions and some minor respiratory and enteric conditions [1-3]. Domestic poultry is primarily affected however they have wide host range [4, 5]. Transmission occurs by horizontal route (Oro-feacal route) [2, 6, 7]. Significant morbidity and mortality has been reported by various workers [1, 2]. The virion is non-enveloped isometric particle, composed of 252 capsomeres including 240 hexons and 12 pentons bases along with fibers are arranged in icosahedral symmetry. Hexons and pentons are major capsid proteins which are exposed to host immune responses and play critical role in the pathogenesis of virus [1, 2, 6].
The present study was designed to express penton base gene in eukaryotic cells. The expression of penton base protein was done using ViraPower lentiviral expression system. This expression system has a HIV-1 based vector; plenti6/V5-D-TOPO vector. The HIV based vector systems have many advantages over other viral and non viral vectors. These vectors effectively transduce both dividing and non-dividing cells, whereas Moloney leukemia virus based retroviral systems cannot transducer non dividing cells [8]. This vector system is able to provide stable long term expression of a target gene while adenoviral systems can only provide transient expressions [8, 9]. The viral particles produced in this system have envelope of vesicular stomatitis virus (VSV) (derived from VSV-G gene). The VSV glycoprotein has high lipid content. This virus can fuse with lipid bilayer of any cell [10]. Thus this system produces pseudotyped virus particles with a broadened host range. The gene is expressed under the effect of cytomegalovirus promoter (pCMV).

Materials and Methods
The virus isolate, designated as HPS-K used in this study was previously characterized by us by cloning into pGEMT vector and sequencing along with eight other adenovirus isolates [11] and stored at -20 ^{0} C in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, G B Pant University of Agriculture and Technology, Pantnagar.
The expression of penton base protein was attempted in Vero cell line using Vira Power lentiviral directional TOPO expression kit (Invitrogen, USA).

PCR amplification of penton base gene
The *E.coli* DHFα cells, containing penton base gene of Fowl adenovirus cloned in pGEMT-easy vector, were revived in Luria Bertani (LB) agar at 37 ^{0} C temperature. The recombinant plasmids were isolated from bacteria by alkaline lysis method [12] and used as template for PCR.

For this amplification, nested PCR approach was used. The sequences of primers used for first round of amplification were as follows:
PFC9 (Forward): 5' CGC TTT TAC TTA CGA GAG CTA GAT ACC 3'
PRA10 (Reverse): 5' TGG ACA TGT TCA GTC CTA CTG CAA 3'.
The amplification was carried out in 50µl reaction mixture containing 30 ng of plasmid DNA, 100 picomoles of each primer, 10 mM DNTPs each and 1 Unit of Taq polymerase (Fermantas, Germany). The amplification conditions used were standardized at; initial denaturation 94 ^{0} C for 4 minutes followed by 35 cycles of denaturation (at 94 ^{0} C for 1 minute), annealing (at 55 ^{0} C for 1 minute) and extension (at 68 ^{0} C for 1.5 minutes) [11].
Second round of amplification was done using touch done strategy [13]. To obtain high fidelity sequences. These primers were designed using the open reading frame of penton base of HPS-K sequence using DNASTAR software. The sequence of primers is as follows:
PVK-F (Forward): CAC CAT GGG GGG GTT GCA GCC GC
PVK-R (Reverse): CTA CTG CAA GGT CGC GGA ACT CAG
The amplification was carried out in 50µl reaction mixture containing 30 ng of DNA, 100 picomoles of each primer, 10 mM DNTPs each and 45 µl of *pfx* supermix (Invitrogen, USA). The amplification conditions used were standardized at; initial denaturation 95 ^{0} C for 4 minutes followed by 25 cycles of denaturation (at 94^{o}C for 1 minute), annealing (at 60 ^{0} C -0.5 ^{0} C for 1 minute) and extension (at 68 ^{0} C for 1.5 minutes); followed by 25 cycles of denaturation (at 94 ^{0} C for 1 minute), annealing (at 57 ^{0} C for 1 minute) and extension (at 68 ^{0} C for 1.5 minutes).
Positive control DNA fragment was amplified using primers supplied with the kit using conditions as per recommendations. PCR products were confirmed by running on 1% agarose gel along with 1 kb marker.
The PCR products were further purified using gel extraction kit (PureLink gel extraction kit, Invitrogen, USA).

Cloning of Penton base gene into expression vector
The expression vector used in this study was pLenti6/V5-D-TOPO expression vector supplied with the kit.
Three reactions; Standard, Positive control and Blank control, were set up for ligation into expression vector and transformation of competent *E. coli* DHF α cells. The Standard reactions contained penton base gene, positive reaction used positive control insert whereas no insert was used in blank reaction. All ligation procedures and transformation was carried out as per manufacturer`s recommendation.
The transformed *E. coli* cells were incubated on LB agar plates containing ampicillin at 30 ^{0} C. Six bacterial colonies were randomly picked and screened for positive clones by colony PCR. The recombinant plasmids were isolated using QIAprep Spin Miniprep kit (Qiagen, Germany). Integrity of plasmids was checked by digestion with *BamH1* enzyme.

Checking expression of cloned gene
The plasmids with correct orientation were used to transform *E.coli DHF* α cells, grown in LB broth and bulk plasmid isolation was done using QIA filter plasmid midi kit (Qiagen, Germany).
Expression of the cloned gene was checked by transfection of HeLa cells with purified plasmid. The cells were grown on cover slips in 6-well plates using lipofectamine and optiMEM media supplied with the expression kit. Negative control was also used.
After 72 hrs. of incubation the cells were checked for expression by immunofluorescence staining. Briefly, cover slips were fixed with chilled acetone (for 30 min.), washed with wash buffer (Triton X-100 0.01% in PBS) for 5 minutes, blocking with 10 % Fetal calf serum, followed by addition of Fowl adenovirus -4 specific antiserum for 1hr. then rabbit anti-chicken FITC conjugate (Sigma) was added. Then cover slips were examined under fluorescent microscope.

Lentivirus production in Cos-7 cell line
The purified expression plasmid was further used for transfection of Cos-7 cell line to produce pseudotyped ectropic lentiviral particles expressing penton base gene. The cells were transformed using lipofectamine, Virapower packaging mix (supplied in kit) and recombinant plasmid as per instructions. The cells were grown in 6-well plate. Three wells were kept for lentivirus production, two as transformation control and one as negative control. Cell culture supernatant was removed, clarified after 48 hrs and 72 hrs. and stored at -20 ^{0} C.

Transient expression in Vero cell line
The cell culture supernatant was further used to inoculate vero cells in 6-well plates. After inoculation, the transformation of cells for expression of penton base was confirmed by immunofluorescence staining.

Results and Discussion
PCR amplification of penton base gene
Nested PCR approach was applied for amplification. First round amplification a product of 1675 bp size was obtained. After, second round amplification product size was 1580 bp (Fig.1). Second round of amplification without touchdown procedure produced multiple bands, therefore, touchdown PCR approach was applied. The touchdown PCR utilizes the exponential nature of PCR, which starts at higher temperature than the expected annealing temperature of the primer [14].
Size of positive control fragment was 750 bp.

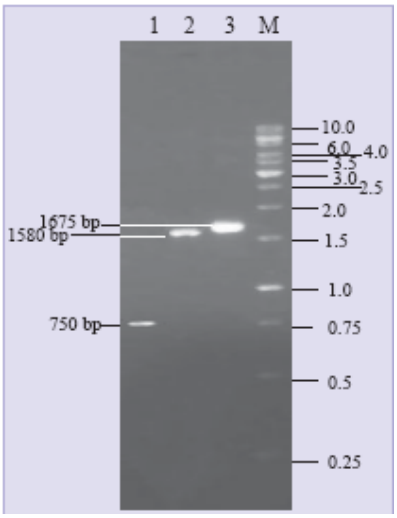

Fig 1: lane 1. PCR product of positive control, lane 2. PCR amplification with second round of amplification of Nested PCR 3. PCR product of first round of amplification, Lane M 1 Kb mol. size marker.

Cloning of Penton base gene into expression vector

The PCR products were further cloned into pLenti6/Vs-D-TOPO vector. *E. coli* DHFα was used for transformation. The HIV based vectors have two long terminal repeats (5' LTRs and 3' LTRs of) derived from HIV-1. These LTRs are responsible for stable integration of vector with the genome of eukaryotic cells. But presence of theses LTRs makes these vectors susceptible to recombinase enzymes present in *E. coli.* cells. The DHFα strain is a *rec*+ strain. To suppress the activity of recombinase, the transformed bacterial cells were grown at 30°C. Numerous small uniformly sized and a few large colonies were obtained on LB agar plate. Randomly six colonies were tested by colony PCR (Fig.2).

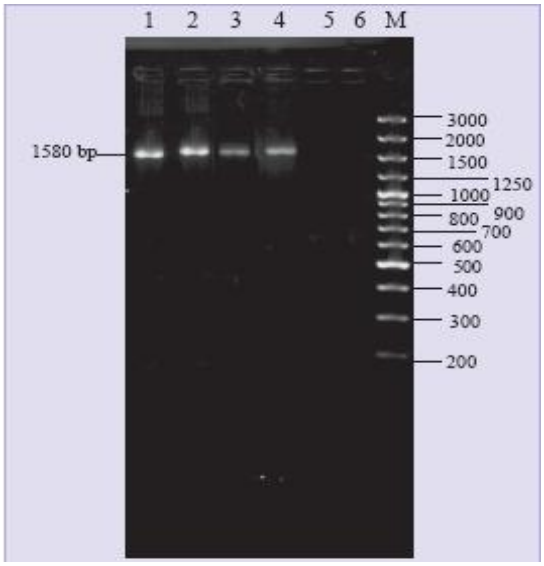

Fig 2: Lane 1. colony no. 1., Lane 2. colony no. 2., Lane 3. colony no. 3., Lane 4. colony no. 4., Lane 5. colony no. 5., Lane 6. colony no. 6., Lane M. 100 bp mol. size marker.

Plasmids were isolated from positive colonies and subjected to RE digestion by *Bam*H1 enzyme. The vector has only one site for *Bam*HI whereas penton base sequence does not contain any site for this enzyme. (Fig. 3).

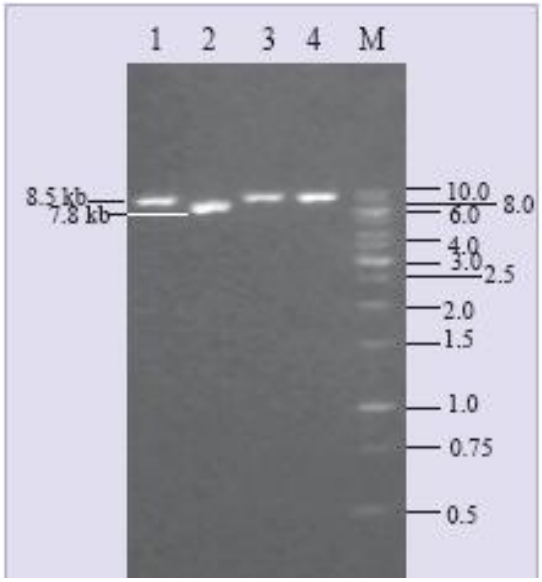

Fig 3: Lane 1. Digested product of colony 6 plasmid., Lane 2. Digested product of colony 5 plasmid. Lane 3. Digested product of colony 4 plasmid, Lane 4. Digested product of colony 3 plasmid Lane M. 1 Kb mol. size marker

Plasmid with insert was further grown in bulk by retransformation in DHFα. Once the correct clone was confirmed, further study was done to check the expression of penton gene.

Expression of Recombinant Plasmids in HeLa cells

After 48 hrs. of transfection, the expression was studied by Immunofluorescence staining. A greenish yellow intracytoplasmic fluorescence was observed (Fig. 5, 6). It confirmed the correct orientation of insert in vector and the gene of interest was being expressed inside cells detected by polyclonal sera of Fowl adenovirus-4.

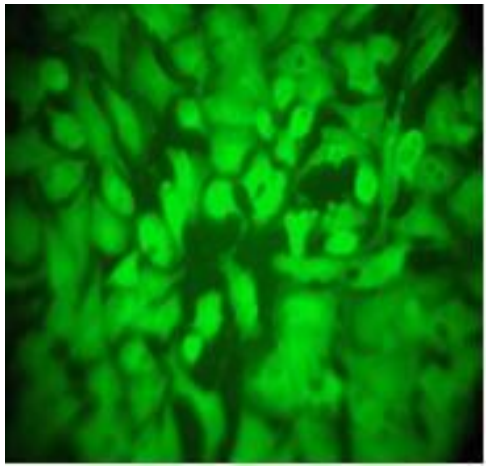

Fig 4: HeLa cells showing intracytoplasmic fluorescence

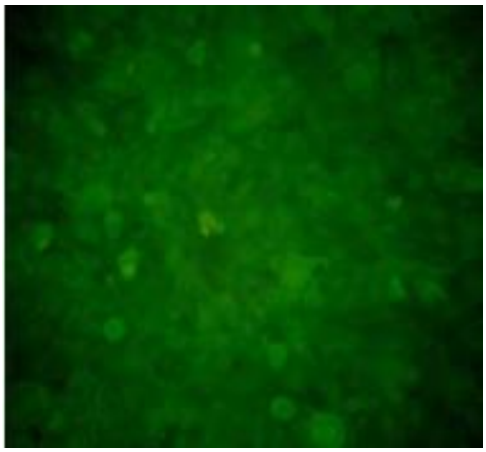

Fig 5: Negative control HeLa cells.

Lentivirus production in COS-7 cell line and transient expression of Vero cell line

The pLenti6/V5 vector contains SV 40 early promoter and origin derived from Simian virus 40 genome. It allows episomal replication of vector in cells expressing SV 40 large T antigen [15]. Cos-7 is such cell line in which SV 40 genome is integrated stably and it expresses SV 40 large T-antigen under normal culture conditions. This cell line was chosen for production of lentiviral particles. The ViraPower lentiviral expression system contains ViraPower lentiviral packaging mix which is a mixture of three plasmids, pLP1, pLP2 and pLP/VSVG. These three plasmids contain genes for structural proteins of lentivirus. pLP1 has *gag* and *pol* genes, pLP2 has *rev* gene and pLP2 contain *VSVG* gene. The recombinant plasmid containing penton base gene and Virapower packaging mix were cotransfected into Cos-7 cell line by using lipofectamine.

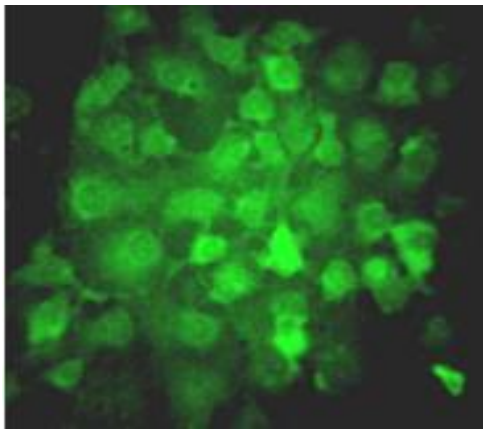

Fig 7: Intracytoplasmic fluorescence in Vero cells.

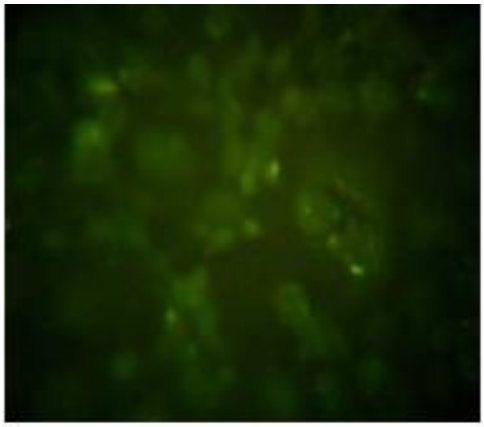

Fig 8: Negative control

The supernatant from transfected cell line was collected at regular intervals and checked for presence of virus by infecting Vero cells and detecting expression of penton base gene. Immunofluorescence staining produced intense intracytoplasmic fluorescence (Fig. 7, 8).

The studies for expression of penton base are lacking. Few attempts have been made to express other structural proteins of Fowl adenovirus.

Sheppard and Werner [16] constructed expression library of FAV 10 in *E. coli* using pGEX expression vector and identified approximately 600 clones. The penton base gene [17] and hexon [18] were mapped in a *Sau*3a genomic fragment of FAV 10.

Gutter *et al*. 2008 [19] cloned knob and part of the shaft domain of the fiber protein into pQE30 expression vector and transformed in *E. coli*. Bacterial cells were lysed and knob-s protein (25 kDa) was detected. They used this knob-s protein as a sub-unit vaccine against EDS and found high haemagglutination inhibition antibody titres.

Conclusion

The Lentivirus particles so generated have the capacity to transduce cell lines, which can be stably selected for production of penton base protein by antibody selection. The penton base protein can be used in diagnostic kits or it can be checked for its potential for use as subunit vaccine against HHS. The virus particle can also be used for *in vivo* expression of penton base protein.

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