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Rapid latex agglutination test for serodiagnosis of fowl adenovirus serotype 4 using recombinant antigen

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ABSTRACT

Hydropericardium hepatitis syndrome (HPS) is a newly emerging disease of poultry, which is caused by fowl adenovirus serotype 4. The virus was propagated in a primary chicken embryo liver culture. The cytopathic effect was observed from the third passage onwards. DNA was isolated from the infected culture and used as a template for amplification of a partial hexon gene (700 bp) using hexon gene specific primers. The amplified product was cloned into pProEX HT b vector and the ligated product was transformed into DH5α cells. The recombinant clones were analyzed by colony PCR and plasmid isolation, followed by restriction digestion to check the insert release. The positive clones were induced by IPTG. The induced culture fractions were checked at different hours and the induction was high at the 4th hour onwards. The expressed proteins were purified and confirmed by using hyperimmune serum against FAV4 by western blot analysis and the protein size of 50kda was obtained. The purified recombinant FAV4 protein was used as a serodiagnostic agent using enzyme linked immunosorbent assay and latex agglutination test.

Key words: fowl adenovirus, hexon gene, cloning, expression in *E. coli,* ELISA, latex agglutination test

Introduction

Hydropericardium hepatitis syndrome (HPS) is a newly emerging disease of poultry and it occurs due to immunosuppressive adenovirus serotype 4. The disease was first observed in a broiler flock 3-4 weeks of age in Pakistan during 1987. GOWDA and SATYANARAYANA (1994) reported the incidence of HPS outbreaks in different states of India. Pathogenic lesions were characterized by the accumulation of amber colour jelly-like fluid in the pericardial sac. Diagnosis was done mainly by post-mortem examination and laboratory confirmation was done by serological tests.

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More recently molecular techniques such as polymerase chain reaction, restriction fragment polymorphism, sequencing, hybridization technique, SDS-PAGE have also been developed for sub-clinical infection. The hexon gene was used for both differentiation of fowl adenovirus serotypes and diagnosis. The development of a recombinant antigen against the hexon gene of fowl adenovirus serotype 4 (FAV4) will avoid cross reaction with other serotypes of fowl adenovirus. Hence in the present study, a recombinant protein for hexon gene was developed and used for serodiagnosis.

Materials and methods

Virus isolation. The local field sample available in our department was passaged through chicken liver cells, the infected culture was observed for cytopathic effect and the uninfected culture was used as control. Titration of virus was done in the chicken liver cells using a 96 well plate and $TCID_{50}$ was calculated according to REED and MUENCH's (1938) formula.

Hyperimmune serum. Hyperimmune serum raised against FAV4 in chicken obtained from the Vaccine Research Centre, Madhavaram, Chennai, Tamil Nadu India was used for this study.

DNA isolation was carried out on the basis of the method described by GANESH et al. (2002) using the proteinase K method.

Polymerase chain reaction. The Hexon gene primers designed by GANESH et al. (2002) were used in this study. The PCR mixture consisted of 2X red dye master mix (Bangalore GeNei, India), 50 picomoles of both forward and reverse primers, 100 ng of template DNA and the volume made up to 25 µL with DNase free water. The temperature profile included initial denaturation at 94 °C for 5min, followed by 30 cycles of denaturation at 94 °C for 1 min; annealing at 57 °C for 1 min and extension at 72 °C for 2 min with final extension of 72 °C for 5 min. The amplified product was run in 1.5% agarose gel electrophoresis. The PCR product was purified and sequenced. DNA extracted from Marek's disease virus was used as a control to check the cross reactivity of primers.

Cloning of the hexon gen. The PCR amplicon and the expression vector (pPROExHT b, Invitrogen, USA) were digested with *Pst*I and *Bam*HI restriction enzymes. The digested product was ligated with expression vector in a ratio of 3:1, using T_4 DNA ligase and incubated at 16 °C overnight. The mixture was stored at -20 °C until further use. Then 3 µl of the ligation mixture was transformed into DH5α cells. The transformants were spread onto LB ampicillin plates for selection of transformed colonies.

Analyzing transformants. Colony PCR. The white colonies formed by the recombinant clones were picked up from the ampicillin containing Luria Bertani (LB) agar (Himedia, India) plate and streaked onto another LB ampicillin plate and grown overnight at 37 °C.

Next day, a few cells from the LB ampicillin plate were mixed with the PCR components and colony PCR was carried out.

Plasmid isolation and insert release. The colonies positive by colony PCR were further grown in LB broth containing 100 µg of ampicillin. Plasmid isolation was done using Qiagen plasmid extraction kit and digested with *Pst*I and *Bam*HI enzymes (Promega, USA). The insert release was checked in 1.5% agarose gel.

In vitro expression of recombinant proteins in E. coli DH5α cells. Fifty mL of LB media with ampicillin (100 µg/mL) was inoculated with 1/100th volume of overnight culture of positive clones. The cultures were incubated in a shaking incubator at 37 °C. When the optical density at 600 nm reached 0.4 to 0.6, 1mM IPTG (Isopropyl β- D thiogalactoside) (Promega, USA) was used for induction. The cultures were incubated in a shaking incubator at 37 °C for an additional 6 hours and the cultures were collected at 1 hour intervals to check the induction level. Another 50 mL of cultures in LB media kept in a shaking incubator without IPTG was used as uninduced controls.

SDS PAGE for analyzing the induced proteins. The induced and uninduced cells were lysed by heat denaturation and resolved in 12% SDS-PAGE along with the protein molecular weight marker (Bangalore Ge Nei, India).

Purification of recombinant hexon protein. The culture pellet was lysed using guanidium lysis buffer and sonicated. The cell lysate was used for purification. The recombinant hexon fusion protein was purified by affinity chromatography using a Ni^{2+}-NTA affinity column. The histidine molecule present in the fusion protein bound to the immobilized nickel (Ni^{2+} -NTA) column (Invitrogen, USA) containing anti-histidine molecule and the recombinant fusion protein was eluted maximally with 3M imidazole (pH 6.0) and analyzed in 12% SDS-PAGE.

Western blot analysis. The 6th hour induced lysed cells were transferred to a nitrocellulose membrane (NCM) (Amersham International PLC, UK) from PAGE. The NCM was treated with anti-chicken polyclonal serum raised against fowl adenovirus serotype 4 to check the specificity of the expressed protein along with pre stained protein marker (Bangalore GeNei, India). The blotting was done on the basis of the method followed by BALAMURUGAN et al. (2002).

Enzyme linked immunosorbent assay (ELISA). The recombinant hexon protein was used as the coating antigen in ELISA. The uninfected chicken serum samples were used as negative controls where as the hyperimmune FAV4 serum was used as the positive control. The ELISA test for FAV4 was performed on the basis of the method described by MOCKETT and COOK (1983).

Latex agglutination test. The carboxylate modified polysterne latex beads (size 0.8 µm, Sigma Aldrich, USA) were used. The recombinant hexon protein was coated on the

latex beads and used to screen the sera samples. The test was done as per the method described by JIANFENG (2007).

Results

The cytopathic effect, cell rounding, clumping and grouping of cells, was observed after 48 hrs of post infection from the 3rd passage onwards. The $TCID_{50}$ was calculated using Reed and Muench's formula and the value was found to be $10^{6.89}$/100 µL. The crude Fowl adenoviral DNA was extracted by the proteinaseK method and its concentration was measured by a UV spectrophotometer at A260 / A280. The ratio was found to be between 1.7 and 1.8, which indicates the purity of the DNA.

The extracted plasmid DNA of 4.7 kbp was observed in 0.8% agarose gel along with 1kb DNA ladder. Five µL aliquot of the amplified product was electrophoresed and the expected amplicon size of 700 bp was observed in 1.5% agarose gel, along with 100 bp DNA ladder (Fig. 1). BLAST analysis revealed that the hexon gene nucleotide sequence showed 98% homology with other fowl adenovirus serotype 4 available in the GenBank. Phylogenetic analysis revealed the close relationship with other fowl adenoviruses serotype 4.

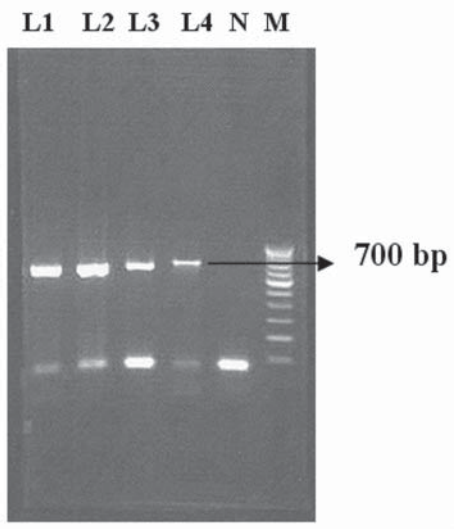

Fig. 1. Agarose gel electrophoresis of PCR amplified product from FAV4 infected cell culture fluid. Lane L1, L2, L3- Hexon gene amplicon (700 bp); Lane L4-Positive control; Lane N-Negative control; Lane M-100 bp DNA Ladder

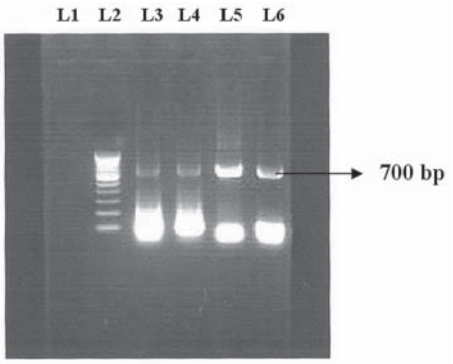

Fig. 2. Colony PCR for the presence of hexon gene (700bp) in pProEX HT'b'. Lane L2-100 bp marker; Lane L3, L4, L5, L6 - Positive colonies.

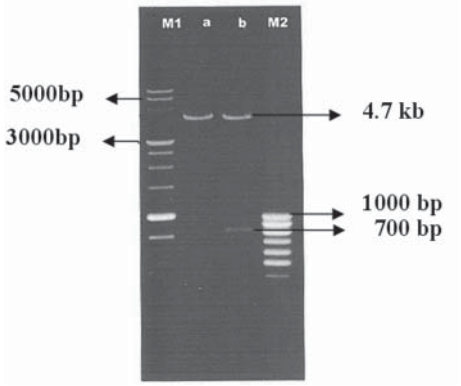

Fig. 3. Digestion of recombinant plasmid with restriction enzymes. Lane M1-1000 bp marker; Lane M2-100 bp marker; Lane a & b - restriction enzyme digested plasmid with insert release.

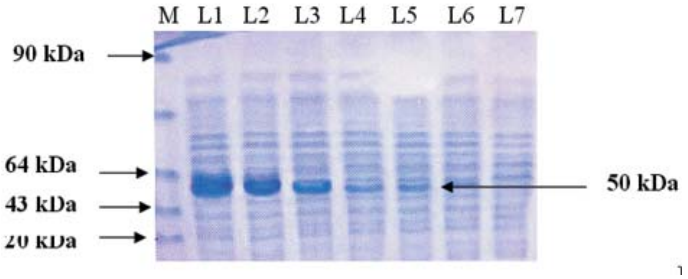

Fig. 4. SDS PAGE analysis of induced hexon protein of FAV4. Lane M-protein molecular marker (Medium range); Lane L1-L7 sixth, fifth, fourth, third, second, first and zero hour induced recombinant proteins.

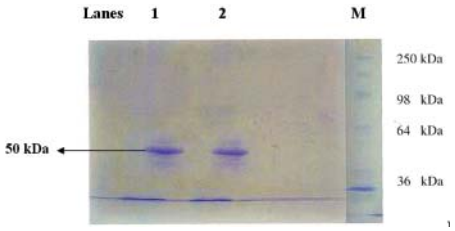

Fig. 5. Purified recombinant protein using nickel column. Lane 1 and 2 - purified recombinant protein; Lane M - protein molecular marker (High range).

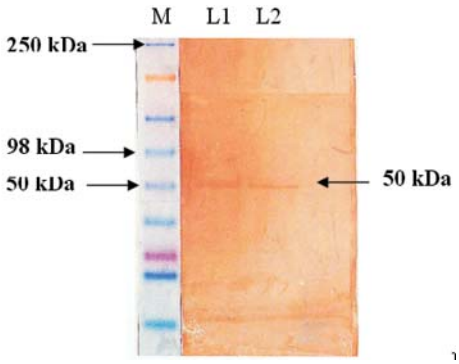

Fig. 6. Western Blot analysis of recombinant protein. Lane M - prestained protein molecular marker (High range); Lane L1 and L2 - recombinant purified hexon protein band.

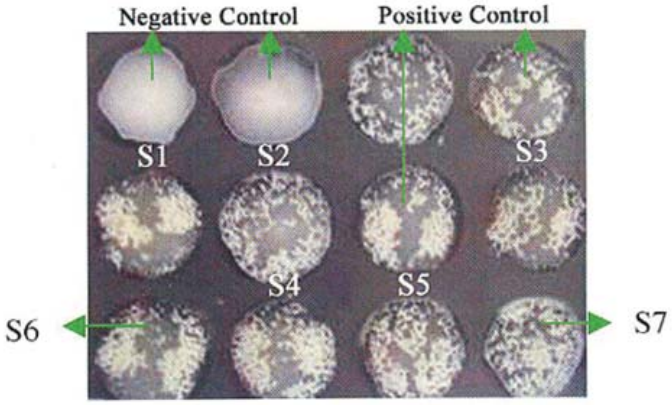

Fig. 7. Latex agglutination test. NC - negative control; PC - positive control, S1, S2, S3, S4, S5, S6, S7 - Field samples

The ligated product was transformed into *E. coli* DH5α cells (Promega, USA). The transformants were spread onto LB ampicillin plates for selection of transformed colonies. The growth of the white colonies were indicative of recombinant clones. The recombinant clones were selected by colony PCR, using forward and reverse hexon gene specific primers (Fig. 2). It was further confirmed by plasmid isolation followed by restriction enzyme digestion using *Pst*I and *BamH*I enzymes (Fig. 3).

The IPTG induced and uninduced cells were lysed by heat denaturation and resolved in 12% SDS-PAGE. The expression of hexon protein was noticed at the second, third, fourth, fifth and sixth hours of post induction as a band of 50 kDa. No band was observed in uninduced controls of equivalent molecular weight (Fig. 4). Nickel (Ni^{2+} -NTA) affinity column purified protein was checked in 12% SDS-PAGE which resulted in a band observed with molecular weight of approximately 50 kDa (Fig. 5). The sixth hour induced lysed cells were transferred to Nitrocellulose membrane (NCM) from Polyacrylamide gels. The NCM was treated with anti-chicken polyclonal serum raised against fowl adenovirus to check the specificity of the expressed protein. The antiserum reacted with the protein and gave a single band with molecular weight of approximately 50 kDa (Fig. 6).

The recombinant hexon gene was used for screening field sera samples. From the total of 61 sera samples tested in ELISA, 29 samples were positive. The OD value doubled so the negative value was taken as positive. On the other hand, out of 61 same sera samples tested using the latex agglutination test, 42 samples were positive. Agglutination was observed against the positive control and no agglutination was observed in the negative control (Fig. 7).

Discussion

Hydropericardium hepatitis syndrome is a newly emerging disease of poultry which has shown a higher percentage of mortality in broilers than layers. FAV4 isolate was propagated in a chicken embryo liver culture and the infected culture showed the characteristic cytopathic effect after 48 hrs of post infection, as reported by JADHAO et al. (1997). DNA was isolated from the infected culture using the proteinaseK method, because proteinaseK was found to be effective in the lysis of the viral coat and releasing the nucleic acid (GANESH et al., 2001). In PCR analysis, the hexon gene has been used for differentiation of 12 fowl adenovirus (MEULEMANS et al., 2004). Hence in the present study the hexon gene was used for PCR analysis and subsequently for cloning and expression study to produce a recombinant hexon protein which could specifically react with FAV4, as reported by BARUA and RAI (2005). The primers are highly specific to FAV and do not react with other poultry virus such as Marek's disease virus, as reported by PARTHIBAN et al. (2004). In the present study the pPROEXHT b prokaryotic vector

was used for cloning the FAV hexon gene containing restriction sites *Pst*I and *Bam*HI, as previously described by ERNY et al. (1995).

SAMBROOK et al. (1989) reported that the temperature and buffer condition affect the ligation efficiency. Hence the optimum temperature of 16 °C was used and ligation efficiency was good. Transformation was done with calcium chloride, which was found to be a simple method, as reported by SAMBROOK et al. (1989). In the present study, transformation efficiency was found to be 85%, similar to the findings of BARUA and RAI (2003). The recombinant plasmid colonies were selected on the basis of an ampicillin resistant gene. The recombinant plasmid was screened by colony PCR and restriction enzyme digestion, to release the insert. The sequencing of the recombinant clone showed 95% homology with other Indian isolates on blast analysis. Similar results were found by PARTHIBAN et al. (2005). The recombinant clones showed a high level of expression under the *trc* promoter of the vector when induced with IPTG after 4 hrs of induction and SDS-PAGE analysis revealed a high level of expression of the fusion protein of 50KDa. Here, the recombinant protein was expressed as the fusion protein tagged with polyhistidine to the 5' end. This peptide tag was exploited for purification of the recombinant hexon protein using a Nickel-Chelate acetic acid (NI-NYA) column, as reported by HAAKE et al. (1999).

In this study, unimmunized chicken sera were used as the negative control and hyperimmune serum was used as the positive control. The expressed recombinant FAV4 hexon antigen was used for serodiagnosis. Out of the 61 serum samples, 29 sera samples were found positive by ELISA, whereas in the case of LAT, 42 samples were positive. More samples were found to be positive by the latex agglutination test than ELISA due to the increased specificity of the assay. The latex agglutination test was found to be more simple and easier to perform when compared to ELISA, as reported by JIANFENG (2007). In the case of ELISA, a non-specific reaction occurred due to the unpurified virus used as a coating antigen and the purified antigen reduced the prevalence of the non specific reaction (MARQUARDT et al., 1980). Hence, in the present study the purified recombinant antigen was used as the coating antigen, which increased the specificity of the assay.

In conclusion the recombinant hexon protein can be used as a serodiagnostic agent. It can be coated in latex beads and used as a field based diagnostic test.

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SAŽETAK

Sindrom hidroperikarda i hepatitisa nova je bolest peradi uzrokovana serotipom 4 ptičjeg adenovirusa. Virus je bio umnožen u primarnoj staničnoj kulturi podrijetlom od jetrenoga tkiva pilećega zametka. Citopatski učinak javio se nakon treće pasaže. DNK je bila izdvojena iz zaražene kulture i rabljena kao kalup za umnožavanje dijela gena heksona (700 bp) uz upotrebu specifičnih početnica. Umnoženi odsječak bio je kloniran u vektoru pProEX HT b, a proizašli proizvod prebačen u DH5α stanice. Rekombinantni klonovi bili su analizirani lančanom reakcijom polimerazom i izdvajanjem plazmida nakon čega je pomoću restrikcijske digestije provjerena uspješnost postupka. Pozitivni klonovi bili su inducirani pomoću IPTG-a. Frakcije induciranih stanica bile su provjeravane svakog sata, a indukcija je bila velika nakon četiri sata. Proizvedene bjelančevine bile su pročišćene i identificirane uporabom hiperimunoga seruma za FAV4 Western blot analizom te se pokazalo da je proizvedena bjelančevina veličine 50 kda. Pročišćena rekombinantna bjelančevina FAV4 bila je rabljena kao antigen u imunoenzimnom testu i lateks aglutinacijskom testu.

Ključne riječi: ptičji adenovirus, gen hexona, kloniranje, *E. coli*, imunoenzimni test, lateks aglutinacijski test