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Adaptation of vaccine strain of duck plague virus in chicken embryo fibroblast cell culture

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ABSTRACT

The duck plague vaccine virus was adapted to grow in chicken fibroblast cell culture. Adaptation of the virus in chicken fibroblast cell culture was confirmed by detection of viral infection in infected cells by PCR and AGPT. In PCR, amplification of DNA dependent DNA polymerase gene of duck plague virus yielded 446 bp in vaccine virus against no amplification in negative template control. Total time required to complete the PCR including extraction of viral DNA is about 5 h. On the other hand in AGPT, faint precipitation lines were observed following 24 h and distinct bands were seen after 48 h of incubation at room temperature. The precipitate lines were obtained with acetate (pH 5.6) and phosphate (pH 7.2) buffer incorporating additional 8% NaCl. The cell adapted duck plague virus in the present study provides a scope to undertake further research on duck plague in India towards development of cell culture vaccine for use in the country.

Key words: AGPT, Cell culture adaptation, Chicken fibroblast cell culture, Duck plague virus, PCR

Ducks are considered relatively resistant birds compared to other members of domestic poultry but some viral and bacterial diseases seriously attack ducks and cause havoc. Duck plague (DP) is considered to be highly infectious and thereby hampering development of duck husbandry. In India, DP was first diagnosed from a severe outbreak in West Bengal during early 1960's (Mukerji *et al*.1963). Thereafter, the disease was reported from duck-rearing Kerala (Kulkarni *et al.*1995), Tamil Nadu (Chellapandian *et al*. 2005), West Bengal (Bhowmik and Ray 1987) and Asom (Sarmah *et al.* 1997). Duck plague also known as duck viral enteritis (DVE), a fatal viral infection (Leibovitz *et al.* 1991), is caused by Anatid Herpesvirus-1 (Gardner *et al*. 1993). It affects all ages and species in the family *Anatidae*. It is clinically characterized by vascular damage, tissue haemorrhages, eruption on the digestive mucosa, lesion on lymphoid organs, and degenerative changes in the parenchymatuos organs (Shawky *et al*. 2000). Depending on the virulence of virus and immunological status, morbidity and mortality of birds due to duck plague virus (DPV) infection ranging from 5 to 100% (Jansen 1961). It is established that the virulent strain of DPV can be adapted by several passages in suitable host system like in developing duck embryo and chicken embryo fibroblast cell culture. This method is not only important for diagnostic purposes but is equally important for molecular characterization and also for development of vaccine strain of the virus and hence the present study was undertaken.

MATERIALS AND METHODS

The freeze dried form of the DP vaccine virus was procured from A H & Veterinary Biological, Government of Kerala. The DP vaccine virus was propagated in the 9–11 day-old chicken embryo to multiply the virus. As per the manufacturer's guidelines freeze dried vaccine virus was reconstituted and 0.2 ml of the inoculums per embryo was inoculated into 9 to 11 day-old chicken embryos by CAM route. After 4 to 5 days of inoculation the CAM was harvested.

Embryonated chicken eggs (9 to 11 days-old) were used for the preparation of the chicken embryo fibroblast (CEF) cell culture in MEM growth medium containing 4% fetal calf serum (Rai 2005). A 10% suspension of DPV infected CAM was prepared in PBS (pH 7.4) and supernatant was collected by centrifugation at 5,000 rpm for 30 min. Virus

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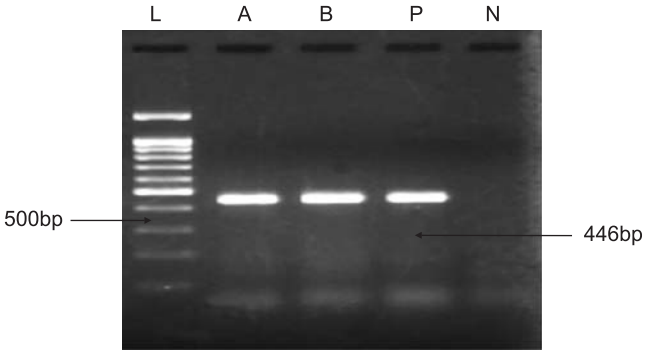

Fig. 1. Electrophoresis analysis of PCR product of DNA directed DNA polymerase gene of duck plague virus. L,100 bp ladder; A, known DP vaccine virus; P, B, CEF adapted DPV isolates; N, negative control.

containing supernatant was treated with penicillin G and streptomycin sulphate @ 100 unit and 100 µg/ml, respectively. After thorough washing of 70% completed cell monolayer, virus inoculum was added onto cell monolayer @ 0.2 ml/25 mm cell culture flask, allowed to adsorve and finally maintenance-medium containing 2% fetal calf serum was added. Virus infected monolayer was incubated at 37°C for 3–4 days. As control un-inoculated healthy monolayer was maintained. After completion of CPE, infected cell culture bottle along with control cell monolayer were stored at –20°C. The cell culture supernatant was harvested by repeated freezing and thawing for 3 to 4 times and the cell suspension was clarified by centrifugation at 5,000 rpm for 15 min at 4°C. The supernatant collected was stored at –20°C for confirmation of presence of virus.

For confirmation of DPV polymerase chain reaction (PCR) and agar gel precipitation test (AGPT) were used. PCR was done as per *OIE Terresterial Manual* (2008). The DPV DNA was extracted from infected *CEF* cells from first passage onwards as well as from uninfected CEF cells by DNA extraction kit. The primer sets forward (5'-GAAGGCGGGTATGTAATGTA-3') and reverse (5'-CAAGGCTCTATTCGGTAATG-3') were used to amplify 446bp of DNA-dependent DNA polymerase gene of DPV. The targeted gene of the DPV was amplified using viral DNA 5µl, Taq polymerase 0.5µl, forward primer (20pmol) 1ul, reverse primer (20pmol) 1µl, dNTP 1µl, $MgCl_2$ 3µl, 10× buffer 5µl and nuclease free water to a total reaction volume of 50µl. Amplification was performed in a thermocycler at 94°C for 2 min, 37°C for 1 min, 72°C for 3 min (1 cycle), 94°C for 1 min, 55°C for 1min, 72°C for 2 min (35 cycles) and 72°C for 7 min (1 cycle). After PCR, the amplified products were confirmed by agarose gel electrophoresis using 1.7% agarose gel containing ethidium bromide at 78V in 0.5× tris borate EDTA (TBE) buffer. At the end of electrophoresis, the gel was visualized on a UV translluminator. AGPT was done as per John *et al.* (1989).

RESULTS AND DISCUSSION

The virus inoculum did not induce any toxic effects on the cells and cytopathic effect (CPE) could be observed on first passage onwards. Propagation of DPV in primary CEF was confirmed by appearance of CPE comprising rounding and clumping of cells, marked cytoplasmic granulations, formation of large number of intracellular vacuoles and finally death. Characteristic CPE appeared by 12 h post infection, within 48 h more than 60% cells died and by 72 h cell monolayer detached from the surface of the culture vessels. Along with passage of virus CPE appeared earlier and complete CPE could be detected by 48 h. The virus was readily adapted to CEF within first 3 passages. Similar CPE was also observed for DPV at fifth passage. The chicken embryo-adapted DPV was readily adapted to grow in a CEF cell line probably being homologous. Similar observation was recorded by Mondal *et al*. (2010). Therefore, CEF can be an alternative source for propagation of vaccine virus in high titer.

Confirmation of DPV in CEF cells was done by PCR as well as by AGPT. PCR has become a routine technique in many diagnostic laboratories (Phuc *et al*. 2004). In the present study viral nucleic acid could be detected from first passage onwards. In India there seems to be only one report on use of PCR (Mondal *et al*. 2010) for detection of DPV that too in infected duck embryos fibroblast cell culture. For detection of DPV nucleic acid, DNA directed DNA polymerase gene was targeted in the present study. Various workers had targeted the gene because of highly conserved nature (Xufeng *et al*. 2008). This region is widely used for phylogenetic analysis between alpha, beta and gamma *Herpesvirinae* (Thureen and Keeler 2006). Extraction of viral DNA by DNA extraction kit showed the yield of viral DNA ranged between 80 and 120 ng/µl. In order to check the specificity of PCR using these primers, in the present study DNA was also isolated from normal CAM suspension of chicken embryo and used as negative control. Amplification of DNA dependent DNA polymerase gene of the DP virus yielded 446 bp in vaccine virus (Fig. 1) against no amplification in negative template control. Pritchard *et al.* (1999) used PCR technique for detection of DPV in infected tissue and from virus grown in cell culture. They found this molecular diagnostic technique to be of great benefit when applied for the identification of disease outbreaks because of its simplicity, reliability and speed. The AGPT was done as per John *et al.* (1989). AGPT is a simple, user friendly routine diagnostic test used for detection of DPV antigen. However, presence of viral antigen was detected from third passage onwards. A faint precipitation lines could be observed following 24 h of incubation and distinct bands were seen after 48 h of incubation at room temperature. No precipitation lines appeared between the negative control wells consisting of normal CAM following incubation at room temperature for 48 h. Comparing with antibody detection technique PCR

is robust and rapid for detection of DPV nucleic acid.

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REFERENCES

Chellapandian M, Piramamayagam S and Balachandram S. 2005. Incidence of duck virus enteritis in Tirunelveli district of Tamil Nadu. *Indian Veterinary Journal* **82**: 913.

Ferguson N M, Cummings D A, Cauchemez S, Fraser C, Riley S and Meeyai A. 2005. Strategies for containing an emerging influenza pandemic in South East Asia. *Nature* **437**: 209–14.

John K, Sarma K, Boro B R, Das S K and Mukit A. 1989. Attenuation and pathogenecity of locally isolated strain of Duck plague virus. *Indian Journal of Animal Sciences* **60**: 615–17.

John K. 1988. 'Adaptation and attenuation of locally isolated strain of duck plague virus in embryonated eggs.' M.V.Sc. Thesis, Assam Agricultural University, Guwahati, Asom.

Kulkarni D D, James P C and Sulochana S. 1995. Isolation of duck plague virus from ducks in Kerala state. *Indian Veterinary Journal* **72**: 446–50.

Leibovitz L. 1991. Duck virus enteritis. *Diseases of Poultry.* 9th edn, (Ed.) Calnek B W. Iowa State University Press, Ames, Iowa, USA.

Mondal B, Rasool T J, Ram H and Mallana S. 2010. Propagation of Vaccine strain of Duck virus enteritis in a cell line of duck origin as an alternative production system to Propagation in embryonated egg. *Bilogicals* **38:** 401–06.

Mukherjiee A, Das M S, Ghosh B B and Ganguly J L. 1963. Duck plague in West Bengal. Part 1. *Indian Veterinary Journal* **40**: 457–62.

OIE. 2008. Principle of validation of diagnostics assays for infectious disease. OIE *Terrestial Manual* 2008 Chapter 1.14. pp 190–217.

Phuc K V, Hung D, Tu T D, Trung N T, Morrissy C J and Prichard L L. 2004. Control of Newcastle disease and duck plague in village poultry. (Eds) Jonne Meere, Peter B, Spradbrow and Tran Din Tu. *ACIAR proceedings* **117**.

Pritchard L I, Morrissy C, Van P K, Daniels P W and Westerbury H A. 1999. Development of a polymerase chain reaction to detect Vietnamese isolates of duck virus enteritis. *Veterinary Microbiol*ogy **68**: 149–56.

Rai A. 2005. *Method in Veterinary Virology.* Pp. 86–95. Indian veterinary research Institute, India.

Sandhu T S and Shawky S A. 2003. Duck viral enteritis (duck plague). (Eds) Saif H M, H J Bernes, A M Fadly, J R Glisson, L R McDougald and D E Swayne. *Diseases of Poultry*. 11th edn. Iowa State University Press, Ames, IA, USA, pp: 354–63.

Sarmah R, Sarmah A K, Sarma P C and Sharma K. 1997. Physico-chemical characterization of a local isolate of duck plague virus. *Indian Journal of Virology* **13:** 119–20.

Sharma K. 1992. **'**Studies on antigenic characterization and immune response of locally isolated strain of Duck plague virus in embryonated eggs.' Ph.D. Thesis, Assam Agricultural University, Guwahati, Asom.

Shawky S, Sandhu T and Shivaprasad H L. 2000. Pathogenecity of low virulence duck virus enteritis isolate with apparent immunosupressive ability. *Avian Diseases* **44**: 590–99.

Thureen D R and Keeler C L. 2006. Psittacid herpesvirus 1 and infectious laryngotracheitis. *Journal of Virology* **80**(16):7863–72.

Xuefeng Q, Xiaoyan Y, Anchun C, Mingshu W, Dekang Z and Renyong J. 2008. The pathogesis of the duck virus enteritis in experimentally infected ducks: A quantitative time- course study using TaqMan Polymerase chain reaction. *Avian Pathology* **37**(3):307–10.