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Propagation of vaccine strain of duck enteritis virus in a cell line of duck origin as an alternative production system to propagation in embryonated egg

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ABSTRACT

Duck virus enteritis (DVE) also known as duck plague, is a viral infection of ducks caused by duck enteritis virus (DEV). The control of the disease is mainly done by vaccination with a chicken embryo-adapted live virus that is known to be poorly immunogenic and affords partial protection. Further, the risk of harboring other infectious agents in the embryo particularly the deadly and zoonotic avian influenza virus is also high. In this paper, we report propagation of a chicken embryo-adapted vaccine strain of duck enteritis virus in duck embryo fibroblast (DEF) cell line. Thirty serial passages were done in DEF cell that made the vaccine virus further attenuated which was tested in ducks. The growth behaviors of the virus in DEF cells were studied and at 30th passage level the virus titre was found to be $10^{6.8}$ $TCID_{50}$/ml. Ducks were immunized with this virus and challenged after 21 days with high dose of virulent DEV. All the immunized ducks withstood challenge with no clinical symptoms in any of the ducks while all the control ducks died. DEF cell which is free from other infectious agents appears to be a good system for cultivation of duck enteritis virus vaccine strain.

1. Introduction

Duck virus enteritis (DVE) also known as duck plague, is a fatal viral infection of ducks, geese, swans and other species of the order Anseriformes [1]. The etiological agent, duck enteritis virus (DEV) or anatid herpes virus-1 (AHV-1) is a member of the Herpesviridae family [2]. DVE was first diagnosed in the western hemisphere in a commercial duck producing area in Suffolk County, New York [3] and since then DVE epizootics are reported in USA [4,5]. The disease has spread in many countries through out the world causing heavy mortality in domestic ducks and wild mallards. It had been frequently recorded in the Netherlands where it first appeared in 1923. The disease has also been reported from Belgium [6], Britain [7], Vietnam and other South East Asian countries [8].

In India, DVE was first diagnosed from a severe outbreak in the state of West Bengal during early nineteen sixties [9]. There after, the disease has spread in different parts of the country and frequent outbreaks have been recorded in the duck-rearing areas including Kerala [10], Tamil Nadu [11], West Bengal [12] and Assam [13]. DVE causes significant economic loss in duck production in many places of India and the socio-economic group most affected by the disease is the small and marginal farmers.

Control of DVE is mainly done by preventive vaccination. A chick embryo-adapted live DVE vaccine (Holland strain) is available commercially for use in the country. However, the production and supply of the vaccine is highly insufficient considering the large population of domestic and wild ducks. Moreover, this vaccine has some inherent disadvantages like, poor immunogenicity and partial protection in addition to the chances of harboring other infectious agents in the chicken embryo including the deadly and zoonotic avian influenza virus (H5N1). The continuing spread of this highly pathogenic influenza virus in wild waterfowls and domestic poultry in South East Asia and other parts of the world represents the most serious human pandemic influenza risk [14].

In this present situation, a safe, potent and cheap DVE vaccine is required which will be easy to produce and free from other adventitious infectious agents. The use of a certified cell line may, probably, be the best option to achieve this. DEV can be propagated in primary cell culture such as, chicken embryo fibroblast (CEF), duck embryo fibroblast, duck embryo liver cells [15] and also in a duck embryo fibroblast (DEF) cell line [16]. In this paper, we report propagation of duck enteritis virus vaccine strain (Holland strain) in DEF cell line and demonstrate protection of immunized ducks against virulent challenge.

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2. Materials and methods

2.1. Cells

The primary chicken embryo fibroblast (CEF) culture was made from 9 day-old embryonated hen's eggs and the vaccine virus was grown on this culture for first three passages. The duck embryo fibroblast (DEF) certified cell line (CCL-141) was procured from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and the vaccine virus was also adapted and propagated in this cell line.

2.2. Viruses

The DEV vaccine strain (Holland strain, freeze-dried chicken embryo tissue) was obtained from the Institute of Veterinary Biologicals and Animal Health (IVBAH) Ranipet, Tamil Nadu, India. This vaccine virus was originally imported from the Netherlands and maintained by passage in chicken embryo in the above Institute. The commercial vaccine produced from this strain is commonly known as Ranipet vaccine of duck plague in Southern India. In our laboratory, the virus designated as DEV Ranipet strain [17], was initially grown in primary chicken embryo fibroblast cells and then adapted to DEF cell line. The virulent DEV (Palode strain), isolated from a natural outbreak, was obtained from the IVBAH, Palode, Kerala. The virulent DEV was inoculated through the subcutaneous route to healthy ducks and upon their death, liver suspension (10% w/v in PBS, pH 7.2) was prepared and stored at −80 °C for challenge studies.

2.3. Passage of virus in primary CEF and DEF cell line

The primary CEF cell monolayers were grown in tissue culture flasks (25 cm^2) at 37 °C under 5% CO_2 in Glasgow modified Eagle's medium (GMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 0.3% tryptose phosphate broth and antibiotics (Penicillin G and Streptomycin sulfate, 100 unit and 100 μg per ml respectively). The cells were inoculated with 0.2 ml of the reconstituted chicken embryo-grown freeze-dried duck enteritis vaccine virus (Holland/Ranipet strain). Virus was adsorbed for 1 h and then the cell layer was washed and submerged in GMEM containing 2% FBS. Flasks were observed for cytopathic effects (CPE) and harvested after 72 h post-infection (p.i.) by three freeze–thaw cycles Thus, three serial passages were done in CEF after which, the virus was adapted and passaged in DEF cell line. The DEF cells were grown to confluence in tissue culture flask at 37 °C under 5% CO_2 in Eagle's minimum essential medium (EMEM, Sigma) supplemented with 10% FBS and antibiotics. The CEF-adapted virus (0.2 ml) at third passage was added to DEF cell layer (25 cm^2) and incubated at 37 °C for 1 h for virus to adsorb. The inoculum was removed and cells were maintained in EMEM containing 2% FBS at 37 °C. The infected cells were observed for CPE and virus was harvested between 48 and 72 h by freezing and thawing. Thus, several passages were done in DEF and the virus titre was determined. In subsequent passages (up to 30), DEF cells were infected at 0.1 multiplicity of infection (MOI) and virus was harvested between 36 and 48 h post-infection when satisfactory CPE was observed. Harvested virus was stored at −80 °C for further use.

2.4. Multiplication assay for infectious progeny virus

For studying the virus multiplication, DEF confluent monolayers (25 cm^2 flasks) were infected with vaccine virus (between 5 and 8 passage) at 0.1 MOI. After 1 h adsorption, the inoculum was removed and the monolayers were washed three times with PBS. The cell layers were then covered with culture medium containing 2% FBS and incubated for three days at 37 °C. The nature of CPE was recorded and the virus was harvested at 12, 24, 36, 48 and 72 h p.i. Virions were released from the cells by three cycles of freezing thawing and diluted ten folds. The diluted virus was assayed in DEF cells in the 96-well plates to determine the yield or titre following the method of Reed and Muench [18]. The virus titre was also determined at 30th passage level.

2.5. Detection of viral DNA in infected DEF cells by PCR

The DEV DNA at different passage level was detected in the infected DEF cells using a PCR described by Hansen et al. [19] with little modifications. The primers (5F: 5′-GGCTGGTATGCGTGACAT-3′ and (5R: 5′-GTATTGGT TTCTGAGTTGGC-3′) were designed for identification of DEV by the above workers on the basis of partial nucleotide sequence of DNA polymerase gene (GenBank Accession No. AF064639) of a DEV vaccine strain developed at USDA, Veterinary Services Diagnostic Laboratory in 1977. The expected length of the PCR product is 602 bp. The reaction was performed in 50 μl volume containing approximately 50 ng of DEV genomic DNA (isolated from infected cells), 0.2 μM each of forward (5F) and reverse primer (5R), 1.5 mM $MgCl_2$, 200 μM each dNTP, 10× PCR buffer and 2 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR was done in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) for 30 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The final cycle was at 72 °C for 7 min. PCR products were examined by electrophoresis in 1% agarose gel. The expected DNA band was gel purified and cloned in a TA cloning vector and three positive clones were sequenced for confirming the identity of the insert.

2.6. Experimental inoculation and challenge

The DEV vaccine strain (at 30th passage in DEF) was inoculated to 13 healthy Khaki Campbell ducks about 3–4 months of age. Each duck received 10^3 $TCID_{50}$ virus in 0.5 ml culture fluid through the subcutaneous route under the wings at several sites. Ten ducks of same age were kept as control and each of them received 0.5 ml culture medium through the same route. After inoculation, the vaccinated and control ducks were housed together in the same enclosure and they were monitored for clinical signs for a week. All the ducks were challenged on 21-day post-inoculation (or vaccination) with 1 ml (10% w/v, liver suspension) of virulent DEV (Palode strain) per duck through the subcutaneous route. Clinical signs and temperature of the challenged ducks were recorded for a week.

2.7. Virus neutralization test (VNT)

VNT was done in 96-well flat bottom tissue culture plates as per the method described by Thayer and Beard [20] with few modifications. Serial two-fold dilutions (from 1:2 to 1:128) of sera (heat inactivated at 56 °C) were prepared in 50 μl medium. Approximately 100 $TCID_{50}$ of DEV (vaccine strain) in 50 μl was added to each well and incubated at 37 °C for 1 h. One hundred micro liters DEF cell suspension (3×10^5 cells/ml) was added to each well, incubated at 37 °C under 5% CO_2 and observed daily for CPE. The titre of virus neutralization activity was expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there was no evidence of CPE and therefore, complete virus neutralization has occurred.