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Molecular Characterization and Pathogenicity of an Indian Isolate of Duck Enteritis Virus Recovered From a Natural Outbreak

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ABSTRACT

Background: Duck plague is a highly contagious viral disease reported in our country very often with significant economic loss. There are some bottlenecks with the currently used 'Holland strain' vaccine that involves cumbersome process of vaccine production in embryonated chicken eggs. With the future goal of development of an indigenous cell culture vaccine for duck plague, the present study is aimed at isolation of an Indian strain of DEV from a natural outbreak and its characterization for the seed virus purpose.

Methods: Liver samples were collected from the suspected ducks died during a natural outbreak in Kerala and subjected to polymerase chain reaction (PCR) to confirm presence of viral DNA. The duck enteritis virus (DEV) was isolated by inoculation of PCR positive samples in embryonated duck eggs/ducklings and its pathogenicity was studied. Further, the DEV recovered from the infected duck embryo and duckling liver was confirmed by PCR amplification of the viral DNA polymerase gene and its sequence analysis.

Result: Out of 12 liver samples tested eight (8) were found to be positive for duck plague by PCR. The DEV infected duck embryos and ducklings died showing typical signs and characteristic gross and microscopic lesions. PCR amplification of viral DNA targeting the DNA polymerase gene yielded amplicon of expected size of 446bp. The amplicon sequence showed 99-100% homology with other DEV isolates, thus confirming the new isolate as DEV, named as DEV/India/IVRI-2016 and the gene sequence has NCBI acc. no. KX511893.

Key words: Duck enteritis virus, Ducklings, Molecular characterization, Pathogenicity, Phylogeny.

INTRODUCTION

Although, ducks are relatively resistant to many infectious diseases compared to chickens, they suffer from few diseases including duck plague. Duck plague (DP), which is also known as duck viral enteritis (DVE) is primarily a disease of waterfowls like duck, geese and swans. It is the major infectious viral disease of ducks reported in our country very often with significant economic loss due to high mortality and decreased egg production. DP is an acute or sometimes chronic contagious viral disease affecting the birds of the order *Anseriformes*. It is caused by duck enteritis virus (DEV), which is *anatid alphaherpesvirus*-1 belonging to the genus *Mardivirus,* subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Fauquet *et al.* 2005). It is a double stranded DNA virus, enveloped, non-hemagglutinating having diameter of 120 to 130 nm with globular shape and 158 kb genome. Mortality ranges from 5 to 100% and almost all the age groups are susceptible (Sandhu and Shawky, 2003).

Duck plague is presently controlled in India by using a chicken embryo adapted live attenuated vaccine prepared from the "Holland strain", which was developed by Jansen *et al.* (1963) and imported to India during 1970s. This currently used DEV vaccine has been reported to be poorly immunogenic, affords only partial protection (Kulkarni *et al.* 1998) and disease outbreaks have also been reported despite vaccination (Rani *et al.* 2015), indicating the need of an improved vaccine. Further, large scale production of vaccine by propagation in chicken embryos is a cumbersome process and virus titres fluctuate from batch to batch. Therefore, it is envisaged that there is a need for development of an indigenous cell culture attenuated vaccine using an Indian strain of DEV, which may be more efficacious against the prevailing strains in India. The present study describes isolation of the DEV from a natural outbreak of duck plague in the Kuttanad region of Kerala, India and its molecular characterization including phylogeny, antigenic

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characterization and pathogenicity in the duck embryos and ducklings to confirm its virulence or ability to reproduce the disease in the natural host. This was a pre-requisite study with the goal of using this isolate subsequently for development of an indigenous cell culture attenuated vaccine for duck plague.

MATERIALS AND METHODS

Screening of field samples for DEV by PCR

Twelve liver samples were collected from the suspected ducks died during a natural outbreak in the Kuttanad regions of Kerala during November - December, 2015 and the PCR confirmed liver samples were received from the Avian Disease Diagnostic Laboratory, Thiruvalla, Kerala. The liver samples were screened for presence of viral DNA by PCR amplification of the *DNA polymerase* gene of DEV using the OIE recommended primers: 5′-GAA-GGC-GGG-TAT-GTA-ATG-TA-3′ (forward) and 5′-CAA-GGC-TCT-ATT-CGG-TAA-TG-3′ (reverse) with annealing temperature of 55°C. Briefly, the DNA was extracted according to the manufacturer's recommendation by using DNeasy Blood and Tissue Kit (Qiagen, Germany). The reaction was carried out in 0.2 ml PCR tubes containing 2X Master mix (12.5µl) (Thermo Fisher scientific, USA), 10pmol of each forward and reverse primer (1.25 µl), DNA template (0.1 µg/ 3µl) and nuclease free water (7.0 µl) in 25 µl volume of reaction. The PCR reaction was set up with the following cycling conditions: one cycle of 94°C for 2 min, 37°C for 1 min, 72°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and finally one cycle of 72°C for 7 min and then 4°C. Amplified products were resolved by agarose gel electrophoresis (1.5%, w/v) with 0.5 µg/ml ethidium bromide and viewed under UV transilluminator (Bio Rad, Gel Doc XR+ imaging system).

Isolation of DEV and its pathogenicity in duck embryos

From the PCR positive liver samples, inoculum was prepared as per the standard procedures (OIE, 2018). Briefly, 10% liver suspension was prepared by sonication of the liver tissue in PBS containing antibiotics (GIBCO, USA), followed by centrifugation at 3000 rpm for 15 min (swing-out rotor, REMI, R-8C), supernatant was collected and filtered through 0.45 µm syringe filter. For isolation of virus, 10-day old embryonated duck eggs were inoculated with 0.1 ml of the inoculum through chorio-allantoic membrane (CAM) route with slight modification of the procedure described by Burnet and Galloway, (1934).

The inoculated duck embryos were incubated at 37°C and examined daily by candling for any embryopathy or death of the embryos. The gross lesions in the dead embryos were recorded and histopathological examination of the embryo liver and CAM were also conducted. The CAM was collected from the dead embryos and 10% suspension was prepared in Hank's balanced salt solution (HBSS, pH 7.2) containing antibiotics and was used for subsequent passages. Presence of viral DNA in the CAM and liver suspension was detected by PCR amplification of the *DNA polymerase* gene of DEV as described above.

Pathogenicity of the DEV field isolate in ducklings

A 10% suspension of PCR positive field liver tissue sample was prepared in sterile HBSS, centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was collected and filtered through 0.45 µm syringe filter. The filtrate was inoculated into 3 ducklings of around four-week-old @ 0.5 ml each, i.m. Two ducklings were injected with liver suspension from healthy ducklings and kept as control and housed separately. Inoculated ducklings were observed daily for the clinical signs and death up to 7 days post infection (d.p.i.). After death, post-mortem examination was conducted and gross lesions were recorded.

Detection of viral DNA in the liver samples of infected ducklings

The liver samples from the dead experimental ducklings were collected and the viral DNA was detected by PCR amplification using a set of primers targeting the *DNA polymerase* gene as described earlier.

Sequencing of amplicons and phylogenetic analysis

The PCR product (partial DNA polymerase gene) was gel purified by using gel extraction kit (Invitrogen, USA) and got sequenced commercially and the nucleotide sequence was compared by BLAST analysis with the sequences of *DNA polymerase* gene of other DEV isolates, available in the NCBI GenBank (Table 1) for confirmation of our DEV isolate and phylogenetic analysis was also done. Clustal *W* was used for alignment of obtained sequence data and the output files were used for phylogenetic analysis by Neighbour Joining (N J) method with 1000 repeats bootstrap value in MEGA X (Ver. 10.1) software.

Antigenic detection of DEV in liver tissue by dot-ELISA

The viral antigens were detected in the liver suspension of experimentally infected ducklings by dot-ELISA using the hyper immune serum raised against DEV following the method described by Lu *et al*. (2010) with slight modifications. The anti-DEV hyperimmune serum at the dilution of 1: 40 was used as primary antibody and the anti-duck IgY-HRPO conjugate (KPL, Sera care, USA) at 1:2000 dilution was used as secondary antibody in this test.

Ethical statement

The experimental procedures in the ducklings were carried out according to the recommendations and approval of the Institute Animal Ethics Committee (IAEC) as per the guidelines of the CPCSEA.

RESULTS AND DISCUSSION

Screening of field samples for DEV by PCR

Eight (8) out of 12 samples tested, were found to be positive for presence of the viral DNA by PCR amplification of *DNA polymerase* gene of DEV, as the amplicons of expected

molecular size (446bp) were obtained. PCR has been widely used for detection of DEV *DNA polymerase* gene (El-Samadony *et al*. 2013; Ahmed *et al*. 2015; El- Tholoth *et al*. 2019) and is also recommended by the OIE as DNA polymerse gene is highly conserved among all herpes viruses (OIE, 2018; VanDevanter *et al*., 1996). Further, PCR is a highly sensitive technique, even moribund tissues also can be utilized to detect viral DNA. Although, at the field level the disease is presumptively diagnosed by history, symptoms and pathological lesions, confirmatory diagnosis can be made by PCR and sequencing of the amplicon or by virus isolation. So, PCR is an indispensable tool in diagnosis of duck plague.

Isolation of DEV and its pathogenicity in duck embryos

The infected duck embryos died within 5 to 7 d.p.i., showing the gross lesions like hemorrhages in head, legs and dorsal surface of the body (Fig 1), enlarged hemorrhagic liver with focal necrosis and the CAM appeared to be congested, hemorrhagic and thickened (Fig 2). On histopathological examination, liver showed congestion of blood vessel, degenerative hepatocellular necrosis, eosinophilic intranuclear inclusion bodies and vacuolar degeneration and the CAM showed congestion and hemorrhages (Fig 3). Characteristic intracytoplasmic and intranuclear inclusion bodies in the liver tissue of infected duckling/duck embryo have also been reported by other workers (Jana *et al.* 2014). Further the presence of DEV viral DNA in the CAM and liver of the infected duck embryo was confirmed by PCR which yielded the expected amplicon size of 446 bp in agarose gel electrophoresis. Usually, the isolation of virus from field samples is best achieved by duckling inoculation compared to other methods like propagation in cell cultures and in duck embryos etc. However, if the susceptible ducklings are not available or if there is any ethical issue for use of natural host, the virus can alternatively be isolated in 10-12 day old embryonated duck eggs, through CAM route inoculation (OIE, 2018). We observed that for inoculum preparation, sonication of the infected tissue is better than manually triturating it as sonication helps to release the virus from the cells, being the virions are cell associated in nature.

Pathogenicity of the DEV field isolate in ducklings

Experimentally infected ducklings on 5th- 6th d.p.i. showed depression, soiled vent, greenish white watery diarrhoea, nasal discharge, less feed intake, ataxia or uncoordinated movement as both the legs showed paralysis, typical posture of breast touching on the ground and were unable to walk. Finally, all the three ducklings died within 5-7 d.p.i. Gross lesions like enlarged pale copper coloured liver (Fig 4) with irregularly distributed pinpoint hemorrhages and white foci of necrosis giving speckled appearance were found during postmortem examination. Spleen was also highly enlarged (Fig 4) and congested, in the intestine petechial hemorrhage was visible and diphtheritic lesions with bran like crusted plaque deposits on the oesophageal mucosa was observed. All the above clinical signs and lesions were in accordance

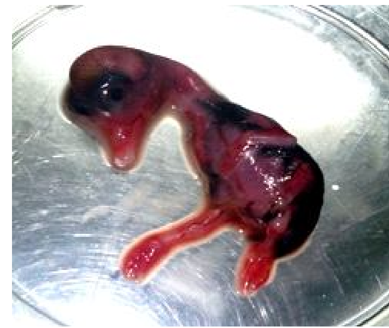

Fig 1: DEV infected duck embryo died on the 5th day post infection, showing haemorrhages in head, legs and dorsal surface of the body.

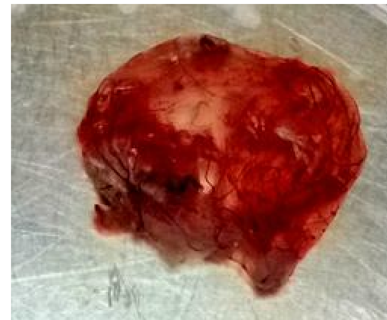

Fig 2: Gross lesions in the chorioallantoic membrane (CAM) of DEV infected duck embryos, showing haemorrhages and thickening.

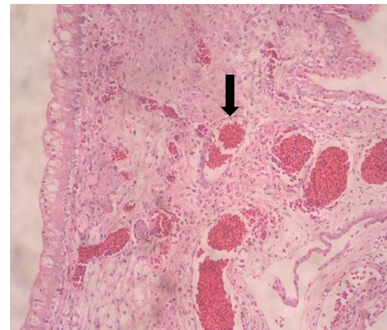

Fig 3: Microscopic lesions in the chorioallantoic membrane (CAM) of DEV infected duck embryos: arrow mark showing blood vessel congestion.

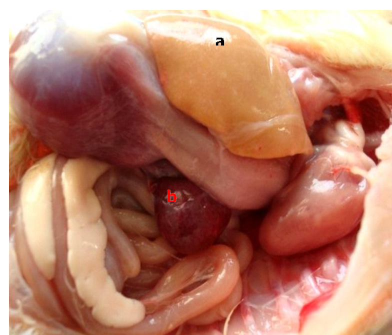

Fig 4: Gross postmortem lesions in the visceral organs of ducklings died after inoculation with DEV isolate, showing enlarged and copper coloured liver (a) and enlargement of spleen (b).

with the findings of the earlier workers (Akter *et al.* 2004; Hanaa *et al.* 2013; Rani *et al.* 2015; El Tholoth *et al.* 2019). In DP the gross lesions are usually haemorrhage throughout the body and degenerative changes in the GI mucosa, parenchymatous organ and petechial haemorrhage in various visceral organs (Konch *et al.* 2009). Most successful and efficient DEV isolation from the field samples are achieved by duckling inoculation as the young ducklings are highly susceptible to this virus and being the natural host.

Detection of viral DNA in the liver samples of infected ducklings

The viral DNA was detected in the liver samples of all the three experimentally infected ducklings by PCR amplification of the *DNA polymerase* gene and the amplicons showed sharp and single band with expected molecular size of 446 bp in agarose gel electrophoresis (Fig 5). Although, the virus is pantropic in nature, it prefers to grow with high titer in liver and spleen and these are the preferred tissue samples for virus isolation.

Sequencing of amplicons and phylogenetic analysis

The DNA polymerase gene amplicon was sequenced and BLAST analysis of the nucleotide sequence showed about 99 to 100% homology with the already published sequences available in the NCBI GenBank, thus confirming our isolate as DEV, which was named as DEV/India/IVRI-2016 and the gene sequence has NCBI acc. no. KX511893.

For phylogenetic analysis, the sequence of *DNA polymerase* gene of our DEV isolate (DEV/India/IVRI-2016) was compared with the sequences of other 23 isolates of DEV available in the NCBI GenBank (Table 1), which showed that our isolate has close genetic relationship especially with Chinese isolates, having 100% similarity (Fig 6). It seems that most of the Indian isolates including this one seems to be acquired from West Bengal, where DEV outbreak was first reported in India. However, earlier workers have reported that there is no much strain variation in this virus, as the whole genome sequencing of the DEV from different countries did not show much variation in nucleotide sequence (Wu *et al.* 2012).

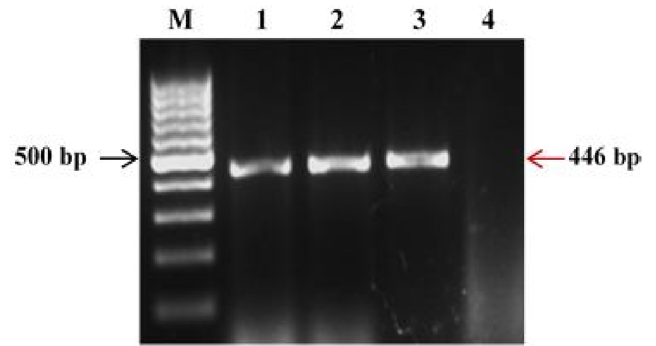

Fig 5: PCR amplified *DNA polymerase* gene of duck enteritis virus showing amplicon size of 446 bp in agarose gel electrophoresis. Lane M: Molecular size marker (100 bp ladder), Lane 1, 2, 3: Liver samples from infected ducklings, Lane 4: Negative control (Normal duck liver suspension).

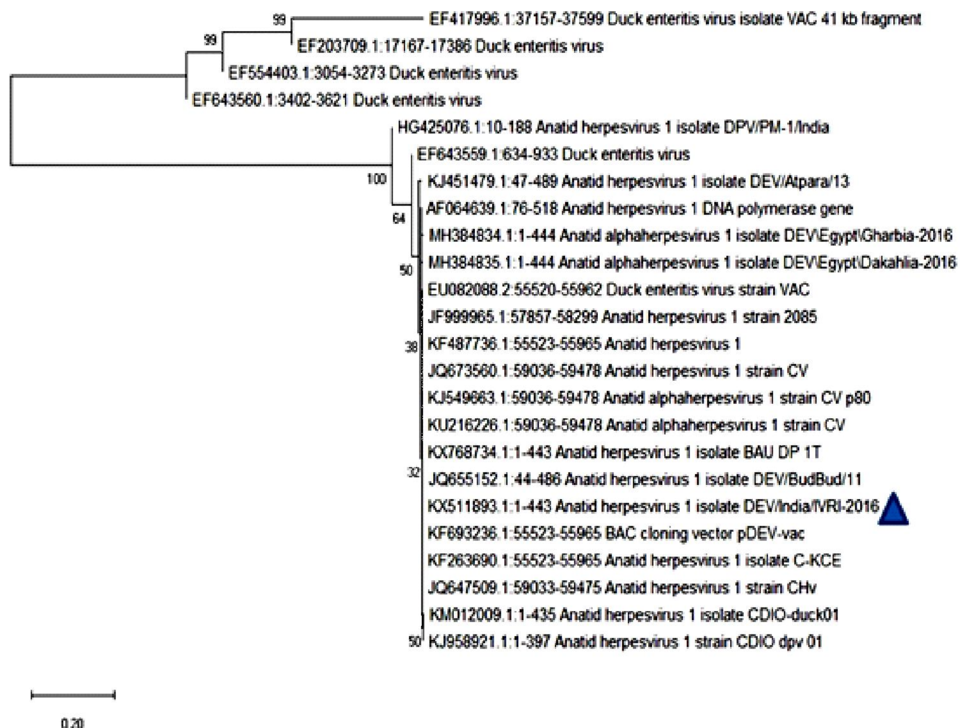

Fig 6: Phylogenetic tree on comparison of *DNA polymerase gene* sequences of our DEV isolate, (KX511893.1, marked with a triangle) with other DEV isolates taken from the NCBI GenBank.

Table 1: Nucleotide sequence homology of DNA polymerase gene of our DEV isolate (KX511893) with other isolates available in NCBI GenBank.

Accession No	Country	Year reported	(%) homology
KX511893(Our isolate)	India, Kerala	2016	100%
KJ958921	India, Kerala	2014	100%
KM012009	India, Kerala	2014	99%
HG425076	India, West Bengal	2013	97%
KJ451479	India, West Bengal	2014	98%
JQ655152	India, West Bengal	2012	99%
KX768734	Bangladesh	2016	100%
KU216226	China	2015	100%
KJ549663	China	2014	100%
JQ673560	China	2012	100%
KF487736	China	2013	100%
KF693236	China	2013	100%
KF263690	China	2013	100%
JQ647509	China	2012	100%
EF643559	China	2007	100%
EU082088	China	2009	100%
EF643560	China	2007	100%
EF417996	China	2007	100%
EF554403	China	2007	100%
EF203709	China	2006	100%
MH384834	Egypt	2016	99%
MH384835	Egypt	2016	99%
JF999965	Germany	2005	100%
AF064639	USA	1973	99%

Fig 7: DEV viral antigens detected by dot-ELISA in the liver suspension of infected duckling, showing brown coloured dots. 1: Liver suspension of infected duckling, 2: Negative control (normal duck liver suspension), 3: Positive control (purifed virus DEV).

Antigenic detection of DEV in liver tissue by dot-ELISA

The infected liver suspension showed brown coloured dots similar to the positive control indicating its reactivity with anti-DEV serum; however, the negative control did not show any dot (Fig 7). Earlier we have reported detection of DEV antigens by immuno-histochemical techniques in the liver tissues of the experimentally infected ducklings (Kumar *et al.* 2018) indicating immunoreactivity of the viral antigens in the tissues with the anti-DEV antibody. Thus, detection of DEV antigens by using specific serum antibodies is one easier method, but its sensitivity and specificity are not yet established.

CONCLUSION

This study describes isolation of duck enteritis virus (DEV) from a natural outbreak of duck plague, its pathogenicity in duck embryos/ducklings, antigenic detection in liver samples and its molecular characterization including PCR amplification of *DNA polymerase gene,* sequencing of the amplicon, BLAST and phylogenetic analysis. Thus, the virus isolate was confirmed to be DEV based on molecular and patho-biological properties, which were the prerequisites for its ultimate use in the vaccine development. Further, adaptation and serial propagation of this virus has been done in the duck embryo fibroblast (DEF) and chicken embryo fibroblast (CEF) primary cell cultures (data not shown) for developing an indigenous cell culture attenuated vaccine candidate of DEV, which is presently not available in our country.

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