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## MOLECULAR CHARACTERIZATION OF DUCK PLAGUE VIRUS FOR DETERMINATION OF TCID<sub>50</sub>

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### ABSTRACT

Duck plague is an enveloped DNA virus that belongs to the Anatid Herpes Virus; the Herpesviridae family is an acute and highly infectious duck, geese, and swan disease that causes tremendous economic losses of duck rearing in Bangladesh and other duck rearing countries. Therefore, we decided to isolate duck plague virus from recent fields' outbreaks area and performed molecular detection and phylogenetic analysis to find out the similarities between our findings and other isolates around the world. Visceral organs of 13 suspected ducks from recent outbreaks area were collected by post-mortem examination for inoculum preparation. Several passages were performed to harvest into 9-11 old embryonated eggs Chorioallantoic membrane (CAM) route and duck embryo fibroblast (DEF) primary cell culture. DNA polymerase (446bp) and DNA polymerase (UL, 602bp) genes were used for molecular detection by Polymerase chain reaction (PCR). Pathogenicity was done with duckling and TCID<sub>50</sub> on DEF. Molecular characterization was performed from extracted DNA of duckling and 2 Positive PCR products were partially sequenced for phylogenetic analysis of their origin and nucleotide variations. Sequenced data was analyzed to reveal genetic relationships among constructed phylogenetic tree for understanding potential transmission with origin of virus and data was then submitted to gene bank and got accession number for DPV-BR1-MN937272 and DPV-BR-2-MN937273. Among 13 samples, 4(30.77%) were found positive by PCR using DNA polymerase at 446 bp and UL at 602 bp gene. Chorioallantoic membrane (CAM) was observed hemorrhagic after 72 days and duck embryo fibroblast (DEF) become round as showed cytopathic characteristics after 48h of infection. Duckling showed that isolated virus was highly pathogenic as characteristics signs of post-mortem examination. Therefore, this has found that recent isolates have similarity with Bangladesh, India and China isolates. Moreover, TCID<sub>50</sub> has confirmed the isolates have accepted titer to be a vaccine strain.

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## INTRODUCTION

Household poultry rearing is an important part of Bangladesh's poor rural communities where duck production occupies the second position next to chicken approximately 54.016 million in Bangladesh and 3rd largest duck population in East and South Asia for the production of table eggs and meat in Bangladesh (Khan et al., 2018, Ahamed et al., 2015b, Hoque et al., 2011). The popularity of duck production is increasing in Bangladesh because ducks are relatively resistant as compared to chickens by Considering infectious diseases (Ahamed et al., 2015b, Hossain et al., 2005b, Islam et al., 2005). Duck plague virus (DPV), a member of the subfamily Alphaherpesvirinae of the Herpesviridae family, is the causative agent of duck plague, also known as duck enteritis virus (DEV), known as Anatidalphaherpesvirus 1 genus Mardivirus (Liu et al., 2017, Li et al., 2009). In 1923 the first known occurrence of DVE was recorded in the Netherlands (Mondal et al., 2010, Baudet, 1923). The first DP Virus (DPV) isolation and identification study was published in Bangladesh in 1982, subsequently evaluating the immunogenicity of the DPV vaccine from local isolates (Wu et al., 2012a, Ahamed et al., 2015a, Hossain et al., 2005a, Islam et al., 2005). The morbidity and mortality rates of suspected birds vary between 5% and 100%, depending on the virulence of bird infection and immunological status (Neher et al., 2019) that causes acute hemorrhagic, septic, infectious, and fatal diseases among waterfowls such as ducks, geese, and swans, (Li et al., 2009, Liu et al., 2017, You et al., 2018, El-Tholoth et al., 2019, Wu et al., 2012a), which caused major economic losses in the commercial waterfowl industry around the world as a result of sudden deaths, condemnations, and reduced production of eggs (Cai et al., 2010, Liu et al., 2017, Lian et al., 2010). Natural infections of waterfowls are susceptible at all ages and there reported that in young ducks from 7 days of age (El-Samadony et al., 2013, El-Tholoth et al., 2019) and infected with direct or indirect contact and developed clinical manifestation within 3 to 7 days after infection (Wang et al., 2013). After recovering from virus infection, ducks become the carriers of the virus and shed the virus repeatedly and spread the virus through feces (Li et al., 2009). According to Clinical signs and pathology including tremor, sudden death, paresis, partial to complete closure of eyelid, photophobia, polydipsia, loss of appetite, Thirst, nasal discharge, ataxia, ruffled feather, greenish and watery diarrhea, soiled vent (Dhama et al., 2017, El-Samadony et al., 2013, Huang et al., 2014, Li et al., 2009), grossly, lesions of vascular damage, blood in the body cavities, penile prolapse in the penis in drakes, pseudomembrane in the mucosal secretions, cyanosis of the bill in the young, pinpoint haemorrhage and white foci in liver were observed in the susceptible ducks (Pazhanivel et al., 2019, Li et al., 2016, Sandhu and Shawky, 2003). The DEV is an enveloped virus size ranging from 160–380 nm, and their nucleocapsids are about 75–100 nm in diameter (Li et al., 2009, El-Tholoth et al., 2019) and a linear double-stranded DNA is approximately 158-162kb size (Hu et al., 2017, Wu et al., 2012b, Liu et al., 2017, Wu et al., 2012a) containing 64.3 % G+C (Lian et al., 2010, Cai et al., 2010, Zhao et al., 2019, Wu et al., 2012a, El-Tholoth et al., 2019). Becoming the enveloped virus, DEV has the sensitivity to heat, several chemicals as ether and chloroform, disinfectants, and rapidly gets inactivated at pH 3–11 as well (Li et al., 2009, El-Tholoth et al., 2019). The key sites of DPV replication are the lining of these organs' epithelial cells, macrophages and lymphocytes, from where the virus spreads through Fabricius's liver, thymus, spleen, and bursa, respectively, leading to pathological lesions in several different organs (Dhama et al., 2017, Li et al., 2016, Lian et al., 2010, Cai et al., 2010, Wu et al., 2012b).

Different Confirmatory diagnosis methods can isolate DPV which comprise commercial ELISA and dot-enzyme-linked immunosorbent assay (Wu et al., 2011, Kataria et al., 2005, Neher et al., 2019), propagation of 9-11 days of embryonized duck eggs into the chorioallantoic membrane (CAM), Chicken embryo fibroblast (CEF) virus isolation or duck embryo fibroblast isolation (DEF) (Wu et al., 2011, Sandhu and Shawky, 2003, Gao et al., 2014, You et al., 2018, Li et al., 2016, JING et al., 2017), passive hemagglutination (PHA) (Ahamed et al., 2015a), targeting DEV-specific gene segment by PCR or real time PCR (Lian et al., 2010, Mandal et al., 2017, Cai et al., 2010), serum neutralization tests (SNT) (Wu et al., 2011, Manual, 2012), restriction fragment length polymorphism (RFLP) (Aravind et al., 2015), nucleotide sequencing (Wu et al., 2012b) and Recent research has been performed to detect LAMP duck plague virus DNA (Ji et al., 2009), respectively.

In the present study, the DPV isolated from the recent field outbreaks using duck embryo and DEF cell culture systems for biological and molecular characterization of isolated DPV to develop a vaccine from the local strains.

## MATERIALS AND METHOD

### Study of outbreaks area

The research work was conducted the study from June to September 2018 in two unfamiliar areas of the Mohanganj Upazila of Netrokona (24°34.5'N90°23.5'E), and Trishal Upazila of Mymensingh districts (24°34.5'N90°23.5'E). Where active affected areas have been evaluated by regular observation and contact with veterinary professionals working in veterinary clinics of the district government. Besides this field we conducted investigations based on information on previous vaccination history and clinical signs where an active outbreak of DPV was reported. During we made sample collection visual inspection to observe typical clinical signs of DPV and it did a detailed physical examination on affected ducks.

### Methods of sample collection

After taking the permission from the owners, we collected 13 dead and live ducks from the commercial farms and households. It targeted Ducks showing typical clinical signs of DP including the in contact Healthy ducks and the dead birds suspected to be dead of DPV infection to collect different clinical and postmortem samples for the study. Post Mortem (PM) examination was conducted according to (Neher et al., 2019, El-Tholoth et al., 2019) for dead and live infected birds and it scrutinized all internal organs for presence of typical DP lesions. During the PM review, visceral organs like the heart, esophagus, proventriculus, spleen, liver and intestine were collected. The post mortem examination was done aseptically and placed the collected organs in sterile falcon tubes with proper labeling. Then the samples were transported to the Virology Laboratory at the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), immediately after collection by maintaining a proper cold chain. The samples were then stored at -20°C till used for molecular identification and molecular characterization of DEV.

### Preparation of inoculum and sterility test

Each sample was crushed under sterile conditions prior to the analysis of collected samples according to a 10 % suspension using phosphate buffer solution for preparation (PBS) (Manual, 2012). Then the suspension was centrifuged at 6000 rpm for 10 minutes and it collected supernatant in a sterile falcon tube treated with 100 µg/ml antibiotics (Gentamicin). The sterile suspension was examined for sterility in fresh nutrient and blood agar as described at 37°C for 24 hours. Then stored the prepared sterile solution at -20°C for until use (Ahamed et al., 2015a).

### Propagation of DPV in embryonated duck eggs

It propagated the antibiotic-treated suspension containing viruses in the allantoic cavities of 10-day-old embryonated duck eggs through CAM route (Liu et al., 2011, Manual, 2012, Ahamed et al., 2015a, El-Samadony et al., 2013). Inoculated eggs were incubated at 37°C with proper humidified condition and observed twice daily for mortality of the embryo. The embryos that died within 72 hours were discarded as nonspecific deaths (Manual, 2012). After 6-10 days of inoculation, it chilled embryonated eggs at 4°C for overnight and allantoic fluid (AF) and CAM were collected.

### Isolation of Duck Plague Virus in duck Embryo Fibroblast (DEF) cell culture

Duck embryo fibroblasts (DEFs) cells were prepared freshly as described (Shi et al., 2019, Ager-Wick et al., 2018, Mondal et al., 2010, Zhao et al., 2019) from 11-day-old day-old embryonated duck eggs for isolation of wild strain DPV from the prepared solution. The duck embryo fibroblast (DEF) monolayer cells were grown in 25 cm<sup>2</sup> tissue culture flasks incubated at 37°C with 5% CO<sub>2</sub> humidified incubator (Liu et al., 2017) with Eagle's minimal essential medium (EMEM, Sigma, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin. For infection, it inoculated 1 ml of tissue suspension into 70% confluent cell culture flask with EMEM containing 3% FBS. The inoculated flasks were observed under Inverted microscope (Carl Zeiss, Germany) after 48-72 h postinfection (hpi) when the cytopathic effect (CPE) was over 75% (Lian et al., 2010, You et al., 2018, JING et al., 2017) and harvesting by freeze thawing cycle and stored at -80° C for further use.

### Molecular detection of DPV by PCR

The viral DNA from cell suspension was then extracted using a DNA extraction kit (Promega®, USA), according to the manufacturer instructions. For confirmation of the target gene, it used two pairs of primers (DNA polymerase gene, UL gene) as described by (Wu et al., 2011, Zou et al., 2010) (Table 1) and it performed PCR for amplification. For

amplification of DNA polymerase gene and UL gene, total 25 µl PCR mixture was prepared containing 12.5 µl master mix (Promega-Madison, WI, USA), Nuclease free water 1.5 µl, Primer Forward 2 µl, Primer Reverse 2 µl and Template (Extracted DNA) 7 µl. Thermal condition used for DNA polymerase gene amplification was: initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec followed by a final extension at 72°C for 7 min. Further, the PCR condition for UL gene amplification was: initial denaturation at 94°C for 5 min; followed by 30 cycles of reaction denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension 72°C for 30 sec, and a final extension at 70°C for 7 min and stored the amplified PCR products at -20°C until confirmed by gel electrophoresis.

**Table 1.**Primer used for DPV isolation

Name of Gene	Primers	Sequence 5'-3'	Amplicon size	Reference
DNA Polymerase	Forward primer	5'-GAA GGC GGG TAT GTA ATG TA-3'	446 bp	(El-Tholoth et al., 2019)
	Reverse primer	5'-CAA GGC TCT ATT CGG TAA TG-3'		
UL	Forward primer	5'-GGC TGG TAT GCG TGA CAT-3'	602 bp	(Mondal et al., 2010)
	Reverse primer	5'-GTA TTG GTT TCT GAG TTG GC-3'		

#### Determination of DPV from PCR products by agarose gel electrophoresis

The amplified PCR products were examined by using agarose gel electrophoresis. For UL gene confirmation 1% (Mondal et al., 2010)(Mondal, B., et al.2010) and DNA polymerase (UL30) gene confirmation 1.5% (El-Tholoth et al., 2019)(El-Tholoth, Mohamed, et al.2019) agarose gel were used, respectively. An amount of 7 µL amplified PCR products was mixed well with 1 µL 6X loading dye (Promega, USA), and loaded to the agarose gel well. It stained the DNA with ethidium bromide after electrophoresis and using UV transilluminator (Biometra, Germany) for visualizing stained DNA.

#### Determination of TCID<sub>50</sub>

The determination of virus titers by infection of duck embryo fibroblasts cell to be a  $10^{6.166}$ /mL median tissue culture infective dose (TCID<sub>50</sub>)/mL. For this purpose, there used 48 well cell culture plate seeding with 100 µl of DEF cells and 500 µl of cell growth media to each well. After 24 hours, the plate observed under the inverted microscope (Carl Zeiss, Germany) for confluent growth and added 100 µl of virus suspension in each wells dilution with  $10^{-1}$  to  $10^{-8}$  (You et al., 2018, Li et al., 2016, Mondal et al., 2010, Zhao et al., 2019). Then the plates were incubated at 37°C for 48 hours and calculated the TCID<sub>50</sub>/mL titer followed by the Reed & Muench (1938) method.

#### Pathogenicity test in adult duckling

A small group of 4-week-old adult ducks was used for these studies. The ducks were inoculated with 1 mL 106.166 TCID<sub>50</sub>/mL serum-free minimum essential medium diluted virus through the subcutaneous and intramuscular route under the wings at several sites (Liu et al., 2011). Few adult ducks were kept separately for control and each of them inoculated with 1 ml culture medium through the same route. After inoculation, ducks also observed two weeks for clinical signs of disease, and it happened postmortem to re-isolate DPV, which was confirmed by PCR.

#### Sequencing data analysis and construction of phylogenetic tree

For the further experiment, two positive PCR products of UL gene (602 bp) were sent to Invent Technology Ltd. for partially sequencing. The nucleotide sequence generated in this study has been deposited in the GenBank to obtain accession number. Data analysis and multiple alignments were performed using Codon Code analyzer and MEGA X software. The sequence was compared with the GenBank database using the BLASTn. The evolutionary history was inferred with the Phylogenetic tree using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the p-distance

method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 132 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Nei and Kumar, 2000).

## RESULTS

### Isolation of DPV

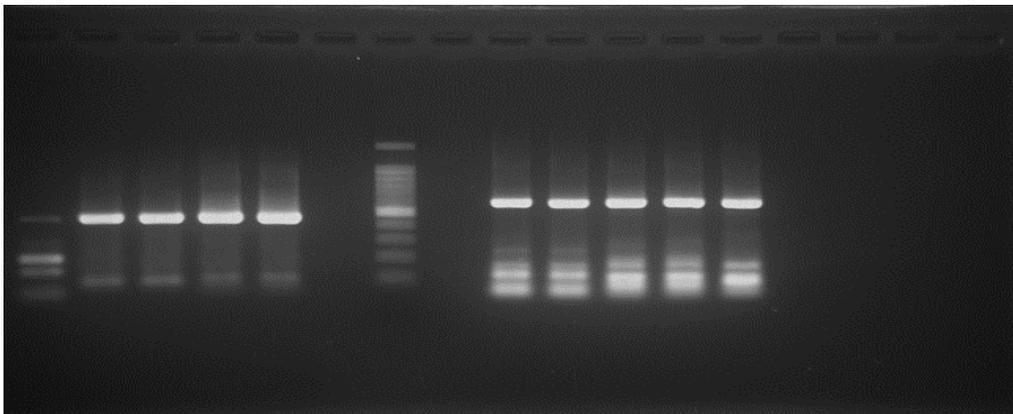
Among the 13 DPV suspected cases 4 ducks (30.77%) were found positive for duck plague which propagated and subsequently confirmed by PCR using specific primer. Three ducks of Mymensingh and 1 duck of Netrokona were confirmed as positive for duck plague virus by PCR (Table 3). Overall prevalence rate of duck plague virus was 30.77% where 33.33% was in Netrokona, and 30% in Mymensingh district (Figure 4).

**Table 2.** Prevalence rate of Duck plague virus after growth in duck embryo

District	Number of samples was collected	Number of positive samples	Prevalence rate as Percentage (%)
Netrokona	3	1	33.33
Mymensingh	10	3	30
<b>Overall</b>	<b>13</b>	<b>4</b>	<b>30.77</b>

### Molecular detection of viral DNA by PCR

The desired band positive at specific 446 bp and 602 bp were observed for DNA polymerase primer (446bp) and UL (602bp) gene primer, respectively which was the indication of duck plague virus.



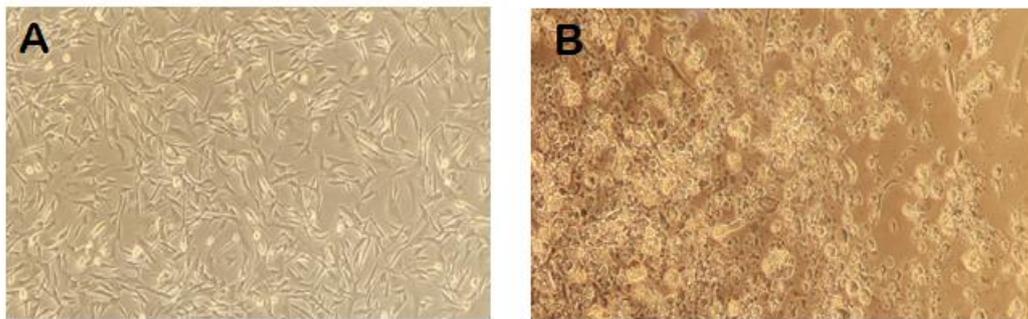
**Figure 1.** PCR amplicons of duck plague virus with DNA polymerase (446bp) (Left) and UL (602bp) (Right) gene specific primer

### Propagation of DPV in embryonated duck eggs

The inoculated embryonated eggs death began after 3-5 days of inoculation. The embryos showed dwarfism, subcutaneous hemorrhage in legs, head, neck and abdomen. The CAM was found with hemorrhagic lesions throughout the membrane and irregular congestion resulting in thickening. Besides, positive control of uninfected eggs remains alive without no hemorrhagic lesions on CAM and PCR showed positive results.

**Isolation of Duck Plague Virus in duck Embryo Fibroblast (DEF) cell culture**

DEF adapted dpv was observed after 72h of infection under inverted microscope that appeared 90% of cell died and detached from the cell culture plate surface. On the other hand, positive control plates remain confined to the cell culture plate surface. The cells changed their morphology by clumping and rounding from their normal spindle like shapes. Therefore, it formed large intracellular vacuoles and cytoplasmic granulations. Molecular detection by PCR of adapted DPV showed positive.



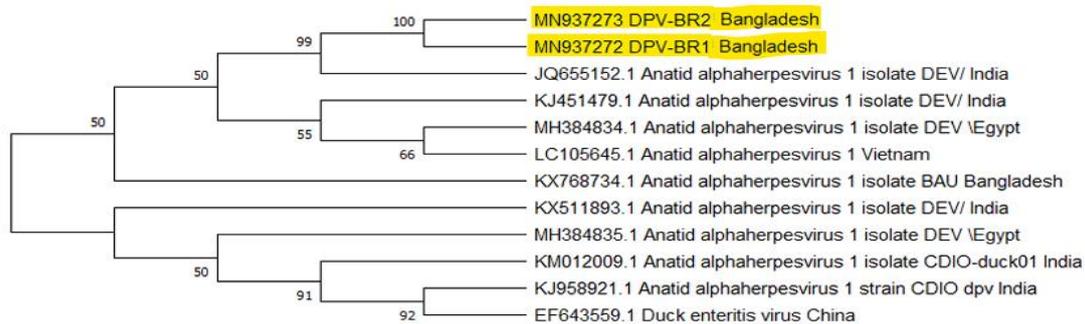
**Figure 2.** (A) Normal duck embryo fibroblast cells showing confluent growth of spindle shaped fibroblast cells. (B) Observation of DPV infected DEF cell after 24 hours of infection.

**Determination of TCID<sub>50</sub>**

After 48 observations of inoculated wells were found the DEF cell was infected with the DPV and showed cytopathic effect under the inverted microscope. The result was evaluated followed by Reed & Muench (1938) (Table 2) and found the 50% endpoint 0.166 at 10<sup>-5</sup> dilution. Therefore, the titer (TCID<sub>50</sub> /mL) of the virus suspension found 10<sup>6.166</sup>/mL.

**Pathogenicity test in adult duckling**

The infected group ducks revealed typical clinical signs and symptoms after 10 days of post-inoculation, which comprised paralysis of legs, dropping wing, back arched position, greenish diarrhea, soiled vent, pasty eyes, depression, loss of appetite, swelling of the head and neck. During postmortem examination, body cavity was found filled with blood and it observed hemorrhage on the esophagus. The liver was enlarged with blood spots and gray-white areas on the surface. Moreover, hemorrhage in the bursa of Fabricius, annulus trachealis and intestine with annular band was observed. Besides, the positive control remains healthy and found no postmortem lesion. It showed the confirmation of DPV by PCR on targeted gene location.



**Figure 3.** Phylogenetic tree of DPV

**Study of sequence and phylogenetic analysis**

The obtained two partial nucleotide sequence data of UL gene were submitted to GenBank and got accession number MN937272 for DPV-BR1 and MN937273 for DPV-BR-2. After submission of nucleotide sequence data, it confirmed that our isolated viruses are DPV. Analysis of acquired data and phylogenetic tree in comparison with other sequences of

NCBI BLAST showed that our isolated DPV has close genetic relationship and from the constructed phylogenetic tree we concluded that our isolates have 100% similarities with the accession number of LC105645 in Vietnam, KX768734 in Bangladesh, KX511893 in India, KJ958921 in India, EF643559 in China and JQ655152 in India, MH384835 in Egypt, KM012009 in India has 99% similarities, furthermore, KJ451479 in India, MH384834 in Egypt has 98% similarities.

## DISCUSSION

DPV is a highly contagious, and fatal disease among the waterfowls that has relatively high mortality and broad host range (Li et al., 2016, Ager-Wick et al., 2018). Due to high mortality, morbidity rates DPV became one of the most widespread and devastating diseases that causes enormous economic losses in commercial waterfowl-based industry throughout the world (Sandhu and Shawky, 2003, El-Samadony et al., 2013, Li et al., 2016). In Bangladesh rural farming has become an integral part but DPV has caused tremendous losses every year due to lack of vaccination and poor immune responses through the several imported vaccines. Thus, in the duck's industry DPV Control became one of the serious challenges, therefore, using quality vaccines can prevent this deadly duck disease. This recent work was carried out to isolate and molecularly characterize duck plague (DP) virus by partially sequencing local strain DP virus vaccine seeds from recent field outbreaks in Bangladesh for the production of vaccine seeds.

During this investigation, 13 suspected dead ducks were collected from DPV affected farms of Netrokona, and Mymensingh for virus isolation. Among the DPV suspected cases overall prevalence rate was 30.77%, which was similar to (Ahamed et al., 2015a, El-Samadony et al., 2013, Wang et al., 2013, Shawky and Schat, 2002). We found hemorrhage on several visceral organs of suspected ducks as described by (Li et al., 2016, Mandal et al., 2017, El-Samadony et al., 2013). and liver, spleen, esophagus, proventriculus and intestine were collected from dead and live ducks by post-mortem examination as described by (Shawky and Schat, 2002) Shawky et al. (2002), (Wang et al., 2013) Wang et al. (2013) and (Ahamed et al., 2015a) Ahamed et al. (2015). The collected visceral organs were prepared for making 10% inoculum (Mandal et al., 2017, El-Tholoth et al., 2019, El-Samadony et al., 2013). For molecular detection as described by (Mondal et al., 2010, Ahamed et al., 2015a). PCR were done with UL gene (602 bp) (Mandal et al., 2017) and DNA polymerase (446 bp) gene (El-Tholoth et al., 2019, Ahamed et al., 2015a) specific primers were used in this study where it was found positive band at specific gene location after agarose gel electrophoresis. For virus propagation, blindly 5-6 passages were performed into 9-12 days old duck embryonated eggs from the positive DPV samples and (El-Samadony et al., 2013, Ahamed et al., 2015a, Mandal et al., 2017, Li et al., 2009, Zhao et al., 2019) the inoculated eggs were found dead within 3-5 days of post inoculation. After the postmortem of inoculated eggs showed hemorrhagic lesion on dead embryo, CAMs and thickened CAMs (Liu et al., 2011, Manual, 2012, Ahamed et al., 2015a, El-Samadony et al., 2013), further, 10% inoculum also prepared from infected CAMs and confirmed by PCR. DPV has broad tissue tropism to infect ducks and replicate rapidly in a diversity of tissues, especially the bursa of Fabricius and spleen by causing serious pathological lesions (Li et al., 2016, Huang et al., 2014). Another report has demonstrated that the infection levels of DPV in visceral organs and tissue had a close relation for the disease progression. Moreover, DEF cells were prepared according to the study (Shi et al., 2019, Ager-Wick et al., 2018, Mondal et al., 2010) for isolation of virus and confluent cell culture plates were infected with 10% suspension. The infected cells were found rounding and clumping similar to (Lian et al., 2010, You et al., 2018, JING et al., 2017, Zhao et al., 2019) by changing their normal spindle like morphology after 72h of post infection, which was then confirmed by PCR for molecular detection. Before determination of DPV pathogenicity in duckling, DPV titer (TCID<sub>50</sub>/mL) according to (You et al., 2018, Li et al., 2016, Mondal et al., 2010, Zhao et al., 2019) was measured in 48 well cell culture plates and the result found 106.166/mL with 50% endpoint 0.166 at 10<sup>-5</sup> dilution (Reed & Muench, et al., 1938;). Experimentally infected ducklings with 1ml 106.166/ mL TCID<sub>50</sub>/ml were showed clinical signs and symptoms including paralysis of legs, dropping wing, greenish diarrhea, pasty eyes, depression, loss of appetite, swelling of the head and neck similar to previous study (Li et al., 2016, El-Samadony et al., 2013, Huang et al., 2014).

Postmortem examination found hemorrhagic intestine and esophagus, body cavities full of blood, enlarged blood spotted liver as found in previous research work (Li et al., 2016, El-Samadony et al., 2013, Huang et al., 2014). Ducklings were also confirmed by PCR. In this research molecularly detected viruses with UL gene were partially sequenced and obtained GenBank accession number MN937272 for DPV-BR1 and MN937273 for DPV-BR-2 respectively. After analyzing sequenced data and constructed phylogenetic trees showed that our isolated virus was DPV and which is genetically 100% similar to LC105645 in Vietnam, KX768734 in Bangladesh, KX511893 in India and EF643559 in China and other has 98-99% genetically relationship.

## CONCLUSION

Our present research reveals the recent affected areas were conducted with DPV, which was confirmed by molecular characterization. The isolation of DP was carried out for development of vaccines with local strains. DPVs showed the adaptation of embryonated duck eggs and DEFs cell culture with definite characteristics. Moreover, DPVs showed the higher pathogenicity in duckling with specific clinical characteristics while infected with titer of TCID<sub>50</sub>/mL at 10<sup>-5</sup> dilution. Our sequenced data and phylogenetic tree analysis reveal that isolated DPV has higher similarity with China, India, Vietnam and Bangladesh isolates. Further research is needed for the whole genome sequence to reveal proteins sequence for development of attenuated vaccine of local strain.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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