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Article

Isolation and molecular detection of duck plague virus for the development of vaccine seed

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Abstract: The present study was carried out for the isolation and molecular detection of duck plague virus (DPV) for the development of inactivated vaccine seed from the local outbreaks. A total of 12 suspected dead duck samples were collected from commercial farms and local market at Sunamganj, Netrokona and Mymensingh districts. Then, the samples were processed and prepared inocula were inoculated into 9-12 days old duck embryonated eggs. In duck embryonated eggs, several passages (3-4) were performed before infection into DEF cell culture. Presence of viral DNA was confirmed by PCR using the primer for *DNA polymerase* gene. After PCR confirmation, virus cultured in DEF cell was used for the preparation of formalin (0.12%) inactivated and oil based adjuvanted vaccine and was experimentally injected to 18 ducklings and 5 were kept as control. $TCID_{50}$ of the selected virus for vaccine preparation was $10^{8.70}$/ml. The mean passive haemagglutination assay (PHA) titre of sera of samples at 0 days, 7 days and 14 days post vaccination were 4.0±0, 14.22±1.78 and 44.44±4.4, respectively, which indicated significant ($p<0.01$) increase of antibody titre. Embryonated duck eggs and DEF cell culture are effective for virus isolation and on the basis of PHA test, it could also be suggested that the experimentally developed DP vaccine can be used successfully for the prevention of DP in Bangladesh.

Keywords: duck plague virus; killed vaccine; PCR; cell culture

1. Introduction

Bangladesh is an agricultural country with various resources. Among these, poultry farming is considered to have potential effect on both for poverty remission and food production. After chicken, duck rearing is very usual in our country especially in villages. In Bangladesh, the total number of ducks is 522.40 lakhs (DLS, 2015-16).

Ducks are relatively resistant to infectious diseases compared to the chicken (Hoque *et al.*, 2011). But the most serious disease of duck is duck plague causing devastating lose to flocks. Duck viral enteritis (DVE) or duck plague (DP) is an acute, infectious and often lethal disease (Kaleta *et al.*, 2007). The causative agent of DVE is duck enteritis virus (DEV) also known as duck plague virus (DPV), which falls into *Herpesviridae* family, *Alphaherpesvirinae* subfamily, *Mardivirus* genus as Anatid alphaherpesvirus 1 denoted after the host family *Anatidae* (ICTV, 2017). Though this disease was first reported in Netherlands in 1923 (Baudet, 1923), it has been reported in many countries (Leibovitz and Hwang, 1968; Wobeser and Docherty, 1987; Wu *et al.*, 2012; El-Samadony *et al.*, 2013; Wozniakowski & Samorek-Salamonowicz, 2014). It is also a great concern in our country nowadays in the duck farming industry. In Bangladesh, DPV was first reported and confirmed by Sarker (1980, 1982). DPV was also isolated and characterized by Akter *et al.* (2004), Islam and Khan (1989) and Ahamed *et al.* (2015).

Most of the affected birds die without abundant clinical manifestations and even sometimes the carcasses are found floating on the water surface (Montali *et al.*, 1976). However, when clinical symptoms are evident, high mortalities especially in older ducks, vascular damage and subsequent internal hemorrhages, lesions in lymphoid organs, digestive mucosal eruptions, severe diarrhea and degenerative lesions in parenchymatous organs, following fatal outcomes (Montali *et al.*, 1976; Davison *et al.*, 1993; Shawky *et al.*, 2000; Campagnolo *et al.*, 2001) are noticed. Partially closed eyelids with photophobia, extreme thirst, loss of appetite, ataxia, nasal discharge, tremors, a drooped-wing appearance, watery diarrhea, prolapse of penis in male are also observed.
Susceptible host of this virus are domestic and wild ducks, geese and swans of all ages. This infection may show chronicity and latency (Richter and Horzinek, 1993). Duck enteritis virus (DEV) exhibits latent infection in trigeminal ganglia (TG) after establishing primary infection. Re-activation of the virus can occur that results in disease outburst (Shawky and Schat, 2002). Depending upon the virulence of infection and immunological status of birds, morbidity and mortality of birds range from 5 to 100% (Jansen, 1961).
Duck plague virion encoded about 78 genes or ORFs. Viral envelope glycoproteins are involved in interaction of DEV with host cells (Li *et al.*, 2009). Duck plague virus can be propagated in adult duck, avian embryo, duckling, avian fibroblast cell, kidney cell, liver cell etc. (Calnek *et al.*, 1997). This virus can be diagnosed by passive haemagglutination (PHA) test, virus neutralization test, or by inoculation into 9-11 days old embryonated duck eggs through CAM route, by propagation in duck embryo fibroblast cell, by inoculation in ducklings or in ducks and molecular detection by PCR with specific primer (Ahamed *et al.*, 2015).
In avian medicine, the control of DP is considered as one of the biggest challenges. Vaccination is an efficient way to prevent DPV. In Bangladesh, DPV vaccine produced at Livestock Research Institute (LRI), Mohakhali is reported to provide a good immunity in protection of DP infection. However, in many circumstances, the mentioned DPV vaccine has been shown ineffective to protect ducks despite regular vaccination, and the main reason behind this failure may be not using local strain in vaccine seed preparation. Considering above points, this study was conducted to isolate, molecular detection and development of duck plague vaccine seed from the local duck plague virus isolates.

2. **Materials and Methods**

2.1. **Study area and period**

A total of 12 duck plague suspected dead ducks were collected from farms of the Mohanganj upazilla of Netrokona, Dharmapasha upazilla of Sunamganj and Mymensingh Sadar Upazilla of Mymensingh districts during the period of July 2016 to July 2017. Among these, 7 ducks were collected from Netrokona, 3 ducks from Sunamganj and rest 2 ducks from Mymensingh.

2.2. **Method of sampling**

The samples were collected and then transported to the Laboratory at the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh by maintaining proper cool chain. Postmortem examination of the dead ducks was performed in the laboratory and visceral organs including esophagus, proventriculus, liver, spleen and intestine were collected. Samples were collected aseptically from dead ducks for the detection DPV and were kept separately in sterile falcon tube after labeling. Collected samples were stored at -20^0 C until used**.**

2.3. **Preparation of inocula**

Samples were cut into small pieces by a sterile scissors and forceps and grinded by sterile mortar and pestle with sterile sea sands. Then sufficient amount of PBS was added to make 10% suspension. Then sample was centrifuged at 4000 rpm for 10-20 minutes (OIE, 2012). The supernatant fluid was collected and treated with antibiotic-antimycotic (Gentaren, Renata) solution.

2.4. **Sterility test**

Antibiotic treated inocula were tested for sterility in fresh blood agar media at 37°C for 24 hours (Rana *et al.*, 2010). Bacteriologically sterile suspension was used as inoculum and was stored at -20^0C for further use.

2.5. **DNA extraction and PCR for confirmation**

DNA was extracted by chemical method using kits. DNA extraction kit (Promega®, USA) was used to extract DNA of the virus according to the manufacturer's instruction. Primers used to amplify the target segmented gene of DPV were described by Wu *et al.* (2011) (Table 1). A 25 µl of PCR mixture was prepared by mixing PCR master mixture (Promega®, USA) (12.5 µl), forward primer (1 µl), reverse primer (1 µl), nuclease free

water (6.5 µl) and template DNA (4 µl). Thermal profile used for the amplification of DNA polymerase gene was: initial denaturation at 94°C for 2 min; followed by 35 cycles of reaction consisting of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, along with final extension at 72°C for 7 min (Ahamed *et al.*, 2015). The PCR products were analyzed using 1.5% agarose gel and stained with ethidium bromide for visualization in gel documentation system (UV-Solo, Biometra, Germany).

2.6. **Propagation of virus in embryonated duck eggs and cell culture**

PCR positive inoculums were injected into 9-12 days old embryonated duck eggs (EDE) through CAM route (Ahmed, 2015; OIE, 2012). PCR positive inoculums were also injected into duck embry fibroblast (DEF) cell culture. The culture flask with confluent growth was used for virus inoculation. Firstly the flask was washed smoothly two times with PBS. Then 500 µl virus suspension was inoculated into each of 25 cm^2 flask. It was tilted for about one hour, kept in the incubator at 37^0C to observe the cyotopathic effect (CPE) after 24h of post infection.

2.7. Pathogenicity tests of DPV

At first, the $TCID_{50}$ was determined and then ducklings were inoculated with 0.5 mL ($TCID_{50}$ $10^{8.7}$/mL) intramuscularly, and adult ducks were inoculated with 1 mL ($TCID_{50}$ $10^{8.7}$/mL) of the infectious cell culture fluid in same route.

2.7.1. **$TCID_{50}$ determination**

48-well plates were prepared by seeding each well with 100 µl of DEF cells and 500 µl of cell growth media. After confluent growth after 24 hours it was used for infection. From 100 µl of CAM virus suspension, a 10-fold serial dilution was made (10^{-1} to 10^{-12}) and 6 wells were inoculated with 100 µL of (10^{-4} to 10^{-9}) diluted virus. The plates were placed at 37ºC for 48 hours and then monitoring of CPE using the inverted microscope (Carl Zeiss, Germany). The number of positive and negative wells was recorded (Table 3). Result of the $TCID_{50}$ titer was calculated by the Reed & Muench (1938) method.

2.7.2. Pathogenicity test in day old duckling

About 0.25 ml virus (10^{-8} dilution with $TCID_{50}$ $10^{8.7}$) was inoculated intramuscularly into 2 ducklings and 2 were kept as control. Then, the ducklings were observed for 8 days. Virus inoculated ducklings were affected and controls were remain apparently healthy. Virus was reisolated from sample of dead ducklings and was confirmed by performing PCR.

2.8. **Vaccine preparation**

2.8.1. **Formaldehyde inactivation**

The infectious DEF suspension of DPV having $TCID_{50}$ $10^{8.7}$/mL was inactivated with 0.12% formaldehyde. The virus suspension was mixed with measured amount of formalin and incubated for 24 hours at 37^0C in shaker incubator.

2.8.2. **Sterility test**

Bacteriological and fungal sterility of the inactivated virus suspension and the experimental vaccine suspension was tested on blood agar.

2.8.3. **Safety test**

The inactivated virus suspension was then used to infect EDE, DEF cell and duckling for safety test.

2.8.4. **Emulsification and oil based vaccine preparation**

Oil based DPV vaccine was prepared as described by Ahmed *et al.* (2015). One part of the virus suspension having $TCID_{50}$ $10^{8.70}$ /ml was mixed with four parts of oil base. The oil base will contain oil emulsifier (Arlacil), Tween-80 and liquid paraffin.

2.9. **Vaccination of the duckling**

For experimental purposes, 25 Khaki campbel ducklings were purchased from local markets with history of no Duck plague vaccination. Experimentally prepared inactivated DP vaccine was administered at the dose rate of 0.5 ml through subcutaneous route in each duckling (n=18).

2.10. **Passive haemagglutination (PHA) test**

To determine the serum anti DPV antibody titer of vaccinated ducks (n=18), PHA test was performed as described by Tripathy *et al.* (1970), Islam *et al.* (2005), Das *et al.* (2009), Khan *et al.* (1990), Ahamed *et al.* (2015).

3. Results and Discussion

3.1. Isolation of duck plague virus

The prevalence rate of duck plague virus in Netrokona, Sunamganj and Mymensingh was 71.42% (n=5/7), 66.67% (n=2/3), 50% (n=1/2), respectively. Overall prevalence was 66.67% (n=8/12). The results were confirmed by PCR (Table 2). These findings were almost similar to the reports of Hansen *et al.* (2000), Campagnolo *et al.* (2001) and Ahamed *et al.* (2015).

3.2. Propagation in embryonated duck eggs and cell culture

The embryo mortality started from 5 days of Post infection (PI). Hemorrhages were observed on the dead embryos subcutaneous region. Hemorrhage and thickening were also found in CAM (Figure 1). Similar findings were also reported by Marius-Jestin *et al.* (1987), Akter *et al.* (2004), Hanaa *et al.* (2013) and Ahamed *et al.* (2015). In DEF, After inoculation of viruses infection increased with passage of time and complete CPE were observed 72 hours of post inoculation. CPE observed as enlareged, rounded, clumped, degenarated and necrosed of fibroblast cells which agree with Akter *et al.* (2004), OIE, (2012) and Doley *et al.* (2013).

3.3. Detection of DPV by PCR

The expected PCR amplicon was settled on agarose gel at 446-bp (Figure 2) for *DNA polymerases*. Our targeted *DNA polymerase* gene usually encodes UL31 protein according to the reports of Pritchard *et al.* (1999), Hansen *et al.* (2000), Zou *et al.* (2010), and Wu *et al.* (2011, 2012).

Table 1. Primer used for PCR.

Target gene	Primers	Sequence 5′--3′	Amplicon size	Reference
DNA polymerase	Forward Reverse	GAAGGCGGGTATGTAATGTA CAAGGCTCTATTCGGTAATG	446 bp	Wu *et al.* (2011)

Table 2. Confirmation of DPV by PCR using *DNA polymerase* gene specific primer.

Sampling area	Number of samples	Confirmation by PCR		Overall isolation rate
		Positive sample	% of positive sample	
Netrokona	7	5	71.42%	
Sunamganj	3	2	66.67%	66.67%
Mymensingh	2	1	50%	

Table 3. Determination of $TCID_{50}$.

Dilution	Infected	Non infected	Accumulated value			Percentage of infection (A/A+B×100)
			Infected (A)	Non infected (B)	Total (A+B)	
10^{-4}	6	0	22	0	22	100
10^{-5}	6	0	16	0	16	100
10^{-6}	5	1	11	1	12	91.66
10^{-7}	3	3	8	4	12	66.66
10^{-8}	2	4	6	8	14	42.85
10^{-9}	0	6	6	14	14	0

Calculation of Proportionate Distance (PD) between the two dilutions above and below 50% end point:

$$PD = \frac{\text{(\% of CPE at dilution next above 50\%)} - 50\%}{\text{(\% CPE at dilution next above 50\%)} - \text{(\% CPE at dilution next below 50\%)}}$$

= (66.66% - 50%) / (66.66% - 42.85%) = 16.66 / 23.81= 0.699

Table 4. PHA titers of sera of duckling pre-vaccinated and post-vaccinated with DP vaccine determined by *t*- test.

Route of vaccination	Serum collection interval	PHA titer (Mean±SE)	P- value
SC	Day 0	4±0.00	
SC	Day 7	14.22±1.78	<0.01
SC	Day 14	44.44±4.4	

SC= Subcutaneous

Figure 1. Image showing subcutaneous hemorrhages over embryo's body (Left)) in DP infected embryo and hemorrhagic thickened CAM (Right).

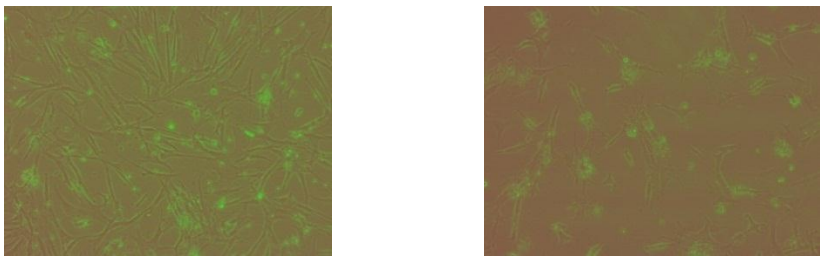

Figure 2. Normal duck embryo fibroblast cells after 24 hours at 10X magnification showing confluent growth of spindle shaped fibroblast cells (Left). Observation of DP infected DEF cell after 24 hours of infection (Right).

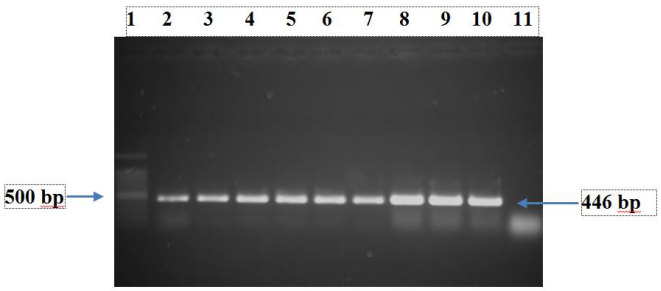

Figure **2. PCR amplicons of duck plague virus with DNA polymerase gene specific primer. Lane 1: 100 bp ladder; Lane 2-9: positive samples of duck plague virus; Lane 10: positive control; and Lane 11: negative control.**

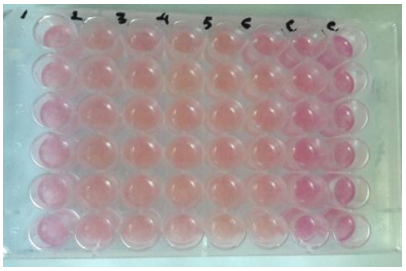

Figure 3. 48 well cell culture plate containing DEF cells infected with DP virus. Last two columns were control. From 1 to 6 columns contain 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} dilutions of virus.

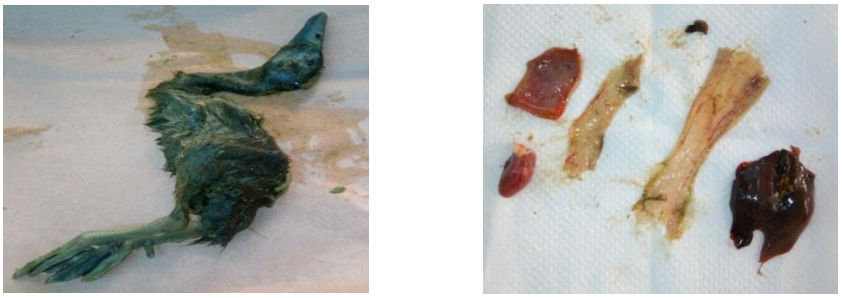

Figure 4. DP infected dead duckling (Left). Visceral organs after postmortem of the duckling (Right).

3.4. Results of pathogenicity tests

3.4.1. **$TCID_{50}$**

The 50% end point was calculated using the formula (mentioned above) to the dilution that killed the cells at rate immediately above 50%=$10^{-7.7.}$ This dilution of the virus suspension contained one $TCID_{50}$ unit of virus in 0.1 mL. So, 1 mL of the virus suspension will contain ten times the reciprocal of the calculated dilution. Therefore, infectivity titer ($TCID_{50}$) of virus suspension/mL = 10 x$10^{7.7}$=$10^{8.8}$ (Table 3) (Figure 3).

3.4.2. Pathogenicity test in day old duckling

The inoculated ducklings were observed. All the inoculated ducklings were affected within 7 days post infection with the appearance of clinical signs. Nervous sings began to appear as tremors of head, neck and body. Ducklings were unable to stand and they maintain a posture with drooping wings outstretched and head down suggesting weakness, depression, off food, ataxia, diarrhoea. On postmortem examination pinpoint hemorrhages white necrotic foci in pale liver, deep mottled spleen, epicardial haemorrhages were found (Figure 4). These findings were similar as described by Hanaa *et al*. (2013) and Ahamed *et al*. (2015). Virus was re-isolated from visceral organs and reconfirmed with PCR.

3.5. PHA test Result for determination of antibody titer

Antibody titer of post vaccinated vaccination was determined by PHA test using statistical analysis. The mean and p-value of antibody titre was calculated by one sample t-test using SPSS software. The lowest serum antibody titer after was 7days of vaccination. The highest serum antibody titer was found in 14 days post vaccination serum. The whole data was described in (Table 4). These findings were similar with the reports from Shawky & Sandhu (1997), Samia & Sandhu (1997) and Farouk *et al.* (2012).

4. Conclusions

A total of 8 (71.42%) DPV isolates were obtained from 12 suspected samples. All the isolates were confirmed by PCR. The pathogenicity tests revealed that the isolates were highly pathogenic for ducklings and ducks. All DPV isolates were successfully propagated in embryonated duck eggs and duck embryo fibroblast cell culture. Inactivated vaccine induced significant antibody production in ducklings after 14 days post vaccination. Duck embryo cell culture technique can be an important method to obtain high concentration of virus in a shorter period of time than conventional old techniques for vaccine propagation using chicken embryo inoculation.

Conflict of interest

None to declare.

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