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CHARACTERIZATION AND PATHOGENICITY OF DUCK PLAGUE VIRUS ISOLATED FROM NATURAL OUTBREAKS IN DUCKS OF BANGLADESH

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ABSTRACT

Characterization and pathogenicity studies on Duck plague virus isolated from natural outbreaks of 170 ducks belonged to the districts of Mymensingh (n = 50), Netrokona (n = 70), and Kishoregonj (n = 50) were carried out during the period from January to June 2002. The collected samples were processed virologically for isolation and identification of Duck plague virus (DPV). Characterization and pathogenicity of the isolated DPV were studied in developing duck and chicken embryo, confluent monolayer of DEF, and experimentally induced infection in ducks. The DPV was identified by using neutralization (NT) and passive haemagglutination (PHA) tests. No remarkable differences were observed in the clinical characteristics, course of the disease and macroscopic lesions between naturally and experimentally infected ducks. The results NT of DPV showed that all the local virus isolates (DPV-Mymensingh, DPV-Netrokona, and DPV-Kishoregonj) were neutralized by the antisera of duck plague vaccine virus (DLS, Mohakhali) which indicate that the virus isolates are homogenous and specific to the antisera to DPV. The PHA test was also found sensitive for the identification of DPV. Therefore, both the NT and PHA tests could be used for the identification of DPV. In addition, the local isolate of DPV-Netrokona was found to be more antigenic compared to other isolates which could be selected for vaccine preparation to control duck plague under field condition.

Key words: Duck plague virus, duck plague vaccine, neutralization test, passive haemagglutination test

INTRODUCTION

Duck virus enteritis (DVE), also known as Duck plague (DP), is an acute contagious viral disease of wild and domestic ducks, geese and swans, caused by a herpesvirus. The disease was first observed in the Netherlands in 1923 (Baudet, 1923), and initially it was mistaken for avian influenza, but in 1942 it was clearly differentiated from avian influenza and was termed 'duck plague' (Bos, 1942). Subsequently, the disease has been reported in many countries of the world (Calnek *et al.*, 1997). Although the DPV is a single antigenic type but the antigenicity of the different isolates may vary. Therefore, it is necessary to compare the antigenicity and pathogenicity between the vaccines and preventing field isolates of DPV. This paper describes the isolates of DPV from the field outbreaks and its experimental pathogenicity in developing embryos and ducks.

MATERIALS AND METHODS

Blood, liver, spleen, colon and cloacal swabs were collected from 170 sick and/or dead ducks from the different outbreaks areas of the districts of Mymensingh, Netrokona, and Kishoregonj during the period from January to June 2002. These samples were collected and processed with a view to isolate the duck plague virus (DPV) using developing duck embryos and susceptible ducks.

Duck and chicken eggs were obtained from the apparently healthy birds of the backyard poultry rearing units of the Backyard Poultry Development Project of Bangladesh Agricultural University (BAU), Mymensingh. These eggs were inoculated for 10-14 days at 37^0C for the development of embryos and the eggs containing well developed and active embryos were used for inoculation of virus.

Experimental ducks of either sex of day-old to four months old were purchased from the BAU Poultry Farm, and also from the local market. These birds were kept separately in two age groups in duck houses and observed for at least 10 days before inoculation with virus.

Tissue suspension from outbreaks of disease were inoculated into 10-12 days old embryonated duck eggs and chicken eggs by chorioallantoic membrane (CAM) route @ 0.2 ml / embryo using standard techniques of embryo inoculation. Each of the inoculated embryos was monitored for embryopathy daily for six days. The allantoic fluid (AF) and CAM were harvested separately from embryos that died during the period of observation. The harvested CAM, livers of duck and chicken embryos were tested separately with 2% suspension of chicken erythrocytes for haemagglutinating activity (HA) by using slide haemagglutination test. The ducks of both the age groups i.e., 2-week-old and semi-mature (4 months old) were injected intramuscularly with tissue suspension from different outbreaks of disease as well as CAM suspension of duck embryos @ 1.0 ml and 2.0 ml / duck respectively. Each group of birds was housed separately were observed daily for any signs of illness up to 21 days of post-infection. Necropsy was conducted on the sick or dead birds. The tissue samples of liver and spleen from the sick and/or dead birds were harvested for virus isolation.

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Inoculation of virus in cell culture

Confluent monolayer of duck embryo fibroblast (DEF) grown in 24 wells of tissue culture plates were used for the isolation of virus. Within 48 hours after seeding when the cells were fully confluent, the growth medium was removed from the wells with a pipette and 0.5 ml of each of the filed sample was inoculated on the cell sheet in the well. Two to three wells were used for each sample and at least two wells in each plate were left as uninfected controls. The plates were incubated at 37^{0}C in a humidified incubator for one hour to allow the virus to absorb. After that, 1 ml of maintenance medium was added to each well and the plates were taken back to the incubator. The cells were examined daily under an inverted microscope for the appearance of any cytopathic effect (CPE). On day 5 of post-infection, the cells in the plates were frozen at –20^{0}C irrespective of the appearance of CPE. Two more blind passages were conducted before the samples were considered to be negative for virus isolation in cell culture. For this purpose, the cells were disrupted by vigorous pipetting with sterile Pasteur pipettes. Then the tissue culture fluids from two wells, inoculated with the same samples, were pooled and then reinoculated to fresh cell monolayers as before.

Neutralization test (NT)

20% suspension was prepared in nutrient broth with the CAM and liver of embryos infected with virus isolates / vaccine virus (DLS, Mohakhali) and was used as antigen. Antiserum prepared against the virus isolates and the vaccine virus (DLS, Mohakhali) separately in ducks were used as the sole source antibody. The duck eggs of 12 days old were used for the test by CAM route of inoculation. Five duck eggs were inoculated for each dilution of virus and serum virus mixture separately. Each of the embryonated duck eggs was inoculated with 0.1 ml of virus dilution and 0.2 ml of serum virus mixture separately through CAM. After inoculation, the embryonated eggs were incubated at 37^{0}C for 6 days and candled twice daily to observe embryopathy. Death of the embryos was recorded daily. For the determination of neutralizing index (NI) of each of the serum, the same technique was used for all the virus isolates and the antisera to duck plague vaccine virus (DLS, Mohakhali) and each isolate of DPV as described by Reed and Muench (1938).

Passive haemagglutination (PHA) test

20% suspension of the CAM and liver tissues of duck eggs infected with virus isolates (DPV-Mymensingh, DPV-Netrokona and DPV-Kishoregonj) separately were used as antigen and 2.5% tanned sheep RBC was used for coating each of the antigens. Antisera prepared in rabbits against the DPV isolates and duck plague vaccine virus (DLS, Mohakhali) were used as the test antibodies.

The results were recorded by deposition of a different thin layer of clumping of RBC at the bottom of the tube. The end point was determined by observing the highest dilution at which cells agglutinated. Agglutination was indicated by a flat or deposition of a diffuse thin layer of clumping RBC at the bottom of the well, i.e., folded of cells with irregular margins covering the bottom of the well. A compact mass of cells forming a smooth edge button with clear zone was considered as evidence of a negative. The results of the test that calculated on the basis of the highest dilution of serum causing agglutination of sensitized sheep RBC (sRBC) was considered as titre of the serum. Triplicate tests were conducted to make final observation for the test following the method of Zyambo *et al.* (1973).

RESULTS AND DISCUSSION

All the duck embryos inoculated separately with blood, liver, colon and spleen suspension from suspected cases died within 4-6 days of inoculation exhibiting characteristic pathological lesions of duck plague in embryonic tissue. Ranjan *et al.* (1980) reported death of inoculated embryos within 3-5 days while Sarker (1982) recorded death of embryos within 4-6 days of inoculation of duck plague virus. The virus concentration of different samples of infected duck embryos with different virus isolates were determined in relation to $DELD_{50}$ and is presented in Table 2.

Clinical observations

The clinical manifestations in the affected ducks were anorexia, drowsiness with ruffled feathers, watery nasal and lacrimal discharges which became profuse with advancement of the disease causing the external nares and eyelids sticked together with dirty, purulent exudates. Eyes were swollen and wet with congested and opaque membrana nictitans and half closed eyes showing evidence of photophobia. The birds were lethargic with swollen face and almost all of the affected birds were unable to stand due to incoordination of movements and partial or complete lameness.

Body temperature was as high as 44^{0}C at the onset of disease but gradually falling with the abundant of profuse greenish watery diarrhoea. The vent, eyelids and nostrils were soiled rendering the bird ugly looking. In some cases there was evidence of dehydration especially in ducklings showing excessive thirst and rapid loss of weight but alertness was evident in all cases. In some cases the adult ducks were frightened with harsh quacks and breathed rapidly and a few affected ducks showed tremors of head and neck. In extreme of the cases the birds became unwilling to move or be moved. Similar clinical observations were recorded by Ranjan *et al.* (1980) in natural outbreak of duck plague. Death occurred within 3 to 8 days of showing clinical signs of duck plague. The morbidity and mortality ranged up to 100%.

Gross pathological lesions recorded in the study were slight enlargement of liver with discoloration, necrotic and haemorrhagic changes. Splenomegaly with ecchymotic or petechial haemorrhagic spots were present. Showing haemorrhagic signs in mouth cavity all parts of the intestine revealed haemorrhagic and catarrhal inflammation. The intestinal annular bands in ducklings were intensely haemorrhagic and revealed as haemorrhagic bands. Diphtheritic pseudomembrane or caseous plaque formation with hemorrhages in the underlying surface was seen in esophagus and cloaca. Heart revealed hemorrhages in pericardium. Brain, lungs, crop and pharynx showed congestion of varying degrees. Pancreas and kidneys revealed petechiation. Bursa of Fabricius was haemorrhagic with caseous mass in the cavity. Skeletal muscles were free from gross lesion. Ovarian follicles showed degeneration, rupture and haemorrhage.

Experimental observations

The clinical signs recorded in experimentally infected birds were almost similar to those of the naturally infected cases. The incubation period ranged from 2-8 days. However, slight variations were observed in different age groups. This finding is in agreement with the findings of Calnek *et al.* (1997) and Sarker (1982) who stated this period to be 3-7 days and 4-6 days, respectively. The 10 days old duckling did not exhibit appreciable clinical signs except slight lameness, mild exudation from nasal and lacrimal passages and death in 1-2 days. The experimentally infected adult duck showed signs as described in natural infection. The death rate of duck embryos, day-old ducklings and chicken embryos indicating the presence of virus in the selected field samples (Table 1).

Table 1. Death rate of duck and chicken embryos and ducklings indicating the presence of virus in the selected field samples tested

Type of samples	Doses and routes		Duck embryos		Ducklings		Chicken embryos	
	Per embryos (CAM route)	Per duckling (IM route)	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died
Blood	0.2 ml	1.0 ml	5	5	5	5	5	0
Liver	0.2 ml	1.0 ml	5	5	5	5	5	0
Colon	0.2 ml	1.0 ml	5	5	5	5	5	0
Tissue of upper palate	0.2 ml	1.0 ml	5	5	5	5	5	0

Although characteristics gross pathological lesions were not observed in 10 days old duckling, all the changes described for natural infection were recorded in other experimentally infected ducks and ducklings. The gross pathological lesions observed in this study are similar to those of reported by Bos (1942), Snyder *et al.* (1973) and Gough (1984).

Irregular patches of congestion and petechial haemorrhage throughout the body particularly in the head, neck, legs, abdomen and beak region of the embryo inoculated with field isolates of duck plague virus obtained from naturally and experimentally infected ducks. The lesions of the chorioallantoic membrane included irregular patches of congestion. These findings are in agreement with Ranjan *et al.* (1980). Petechial ecchymotic haemorrhage, small areas of edema and small irregular white necrotic patches resulting in the thickening of the CAM (pock lesion). The lesions were irregularly distributed throughout the membrane.

Table 2. Determination of virus concentration of different samples of infected duck embryos in relation to $DELD_{50}$

Samples	Dilution of the infected suspension								$Log_{10}DELD_{50}$ titre of virus
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
CAM of DPV-Mymensingh isolate	$5^n/5^d$	5/5	5/5	4/5	2/5	0/5	0/5	–	4.25
Embryo of DPV-Mymensingh isolate	$5^n/5^d$	5/5	5/5	5/5	3/5	1/5	0/5	–	5.80
AF of DPV-Mymensingh isolate	$5^n/5^d$	5/5	5/5	1/5	0/5	0/5	0/5	–	3.80
CAM of DPV-Netrokona isolate	$5^n/5^d$	5/5	5/5	3/5	1/5	0/5	0/5	–	4.80
Embryo of DPV-Netrokona isolate	$5^n/5^d$	5/5	5/5	5/5	2/5	0/5	0/5	–	5.50
AF of DPV-Netrokona isolate	$5^n/5^d$	5/5	5/5	2/5	0/5	0/5	0/5	–	3.50
CAM of DPV-Kishoregonj isolate	$5^n/5^d$	5/5	5/5	4/5	1/5	0/5	0/5	–	4.57
Embryo of DPV-Kishoregonj isolate	$5^n/5^d$	5/5	5/5	5/5	3/5	1/5	0/5	–	5.80
AF of DPV-Kishoregonj isolate	$5^n/5^d$	5/5	5/5	1/5	0/5	0/5	0/5	–	3.80

n = Numerator represents number of embryos died, d = Denominator represents number of embryos inoculated, – = No embryo died.

Morphology of duck embryo cultured cells

Following attachment the cells spread on the substrate and multiplied to from islands of cells. The cells further multiplied to form a confluent monolayer. In the first few hours of growth many cells had multipolar appearance but as the growth continued the cells took bipolar elongated shape and formed parallel arrays and occasionally whorls was present. The findings are in conformity with the findings of Kunst (1967) and Docherty and Solta (1988).

Neutralization test (NT)

The neutralization test was carried out in duck eggs using antiserum to duck plague virus vaccine (DLS, Mohakhali) and the virus isolates (DPV-Mymensingh, DPV-Netrokona and DPV-Kishoregonj) as test antiserum (antibody) and 20% suspension of CAM of duck embryos infected with the virus isolates separately as antigen. Neutralization index (NI) in relation to $DELD_{50}$ was expressed as antilogarithm of the deference between the virus titre and the titre of the serum-virus mixture. Virus titre, titre of serum-virus mixture and the NI of each serum were calculated and expressed as $DELD_{50}$ per 0.1 ml. The results of NT have been presented in Table 3.

Table 3. Determination of the neutralizing indexes of the anti-DPV serum in relation to $DELD_{50}$

Test components	Dilution of the infected CAM suspension								Log_{10} $DELD_{50}$ titre of virus	Log_{10} $DELD_{50}$ titre of serum-irus	NI of diluted serum in relation to $DELD_{50}$
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
DPV-DLS	$5^n/5^d$	5/5	4/5	3/5	0/5	0/5	–	–	4.83	–	–
DPV-Myn	$5^n/5^d$	5/5	5/5	4/5	2/5	0/5	–	–	4.25	–	–
DPV-Net	$5^n/5^d$	5/5	5/5	3/5	1/5	0/5	–	–	4.80	–	–
DPV-Kis	$5^n/5^d$	5/5	5/5	4/5	1/5	0/5	–	–	4.57	–	–
DPV-DLS + ∝-DPVV-DLS	$5^n/5^d$	1/5	0/5	–	–	–	–	–	4.83	1.44	3.39
DPV-Myn + ∝-DPVV-DLS	$5^n/5^d$	3/5	0/5	–	–	–	–	–	4.25	2.83	1.42
DPV-Net + ∝-DPVV-DLS	$5^n/5^d$	3/5	0/5	–	–	–	–	–	4.80	2.83	1.97
DPV-Kis + ∝-DPVV-DLS	$5^n/5^d$	3/5	0/5	–	–	–	–	–	4.57	2.83	1.74

DPV = Duck plague virus, DPVV = Duck plague virus vaccine, Myn = Mymensingh, Net = Netrokona, Kis = Kishoregonj, n = Numerator represents number of embryos died, d = Denominator represents number of embryos inoculated, – = No embryo died.

The NT of antisera to duck plague virus (DLS, Mohakhali), DPV-Mymensingh, DPV-Netrokona and DPV-Kishoregonj against the duck plague virus vaccine (DLS, Mohakhali) were $10^{3.39}$, $10^{1.42}$, $10^{1.97}$,and $10^{1.74}$, $DELD_{50}$ respectively. It appears that the antisera of duck plague virus (DLS, Mhakhali) and the virus isolates possess specific neutralizing antibodies against the isolated virus isolates and duck plague virus (DLS, Mohakhali) respectively as was evident by the neutralization of the virus isolates and by antisera to duck plague virus vaccine (DLS, Mohakhali) and the neutralization of duck plague virus vaccine (DLS, Mohakhali) by antisera to duck plague virus (DLS, Mohakhali) as presented in Table 3, showed that the duck plague virus vaccine (DLS, Mohakhali) induced more antibody responses possessing higher neutralizing titre the DPV-Kishoregonj possessing high neutralizing titre than of other virus isolates.

The results indicate that the antisera to duck plague virus vaccine (DLS, Mohakhali) and the virus isolates neutralized the virus isolates and duck plague virus (DLS, Mohakhali) respectively which demonstrated that all the virus isolates are related to duck plague vaccine virus (DLS, Mohakhali).

The NI in relation to $\log_{10}$ $DELD_{50}$ with duck plague virus (DLS, Mohakhali), DPV-Mymensingh, DPV-Netrokona and DPV-Kishoregonj antisera were 3.39, 1.42, 1.97 and 1.74 respectively. Dardiri and Hess (1967) reported the virus NI of 1.75 $\log_{10}$ $DELD_{50}$ as positive evidence of the presence of specific DPV antiserum. The differences in the NI of the antisera to virus isolates and duck plague virus (DLS, Mohakhali) were probably due to the differences in the antigenic capacity of the virus isolates and the vaccine virus.

Passive haemagglutination (PHA) test

Passive haemagglutination test with the virus isolates (DPV - Mymensingh, DPV - Netrokona, DPV - Kishoregonj) and antiserum prepared in rabbits against the duck plague virus vaccine (DLS, Mohakhali) was carried out to study the suitability of the test for rapid and accurate diagnosis of duck plague. Twenty percent suspension of CAM and liver of duck embryos infected with the virus isolates separately was used as antigen and antiserum prepared in the rabbits against the virus isolate (DLS, Mohakhali) was used as antibody. The results of passive haemagglutnation test are presented in Table 4.

Table 4. Results of passive haemagglutination test for DP virus isolates

Virus isolates	Dilution								NRS control	SsRBC	Ag control	TsRBC
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256				
DPV – DLS	+	+	+	+	+	–	–	–	–	–	–	–
DPV - Myn	+	+	+	+	+	–	–	–	–	–	–	–
DPV – Net	+	+	+	+	+	–	–	–	–	–	–	–
DPV – Kis	+	+	+	+	+	–	–	–	–	–	–	–

DPV = Duck plague virus, Myn = Mymensingh, Net = Netrokona, Kis = Kishoregonj, NRS = Normal rabbit serum, SsRBC = Sensitized sheep RBC, AG = Antigen, TsRBC = Tanned sheep RBC, + = Agglutination of RBC, and – = Non agglutination of RBC.

The clumping of the sheep RBC sensitized with the virus isolates separately was found to occur in presence of antiserum to DP-192. The haemagglutination reaction was found positive for all the virus isolates up to a serum dilution of 1:32. The serum dilution of 1:64 and higher produced no haem agglutination of sheep RBC sensitized with the virus isolates separately. These indicate that the antiserum and the virus isolates used in this test are specific resulting haemagglutination of sensitized sheep RBC and the test can safely be used for their detection. The results of this study correlate with those of Ming *et al.* (1984) who demonstrated reverse passive haemagglutination of duck plague virus.

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