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ISOLATION AND MOLECULAR DETECTION OF FOWL POX AND PIGEON POX VIRUSES FROM RECENT OUTBREAK IN BANGLADESH

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ABSTRACT

Present study was undertaken for the isolation and molecular detection of recently circulating fowl pox (FP) and pigeon pox (PP) viruses in Bangladesh. Isolation of the viruses was done via CAM route of avian embryos inoculation. Out of 40 FP suspected field samples, 27 (67.5%) were found positive and 13 (32.5%) were negative. Similarly, out of 40 PP suspected field samples, 30 (75%) were positive and 10 (25%) were negative. Results of molecular detection of FP suspected field samples 32 (80%) were positive and 8 (20%) were negative. On the contrary, out of 40 PP suspected samples 35 (87.5%) were positive and 5 (12.5%) were negative by PCR. Rate of molecular detection of avi-pox viruses from recent outbreak found higher than that of virus isolation in avian embryos. Results of experimental infection showed re-establishment of FP and PP infection with similar pox lesions (nodule) as natural infection under the wing (100%) and nasal areas (100%) after day 6 and day10 of post inoculation in chicken and pigeon respectively. Results of experimental infection clearly indicated that both chicken and pigeon got infected with fowl pox viruses regardless route of infection whereas pigeon pox viruses were able to infect pigeon only in this study.

KEYWORDS : Fowl Pox Virus, Pigeon Pox Virus, Polymerase Chain Reaction

Poultry farming in Bangladesh is being considered as an important component under livestock sub-sector for a marked contribution in gross domestic product (GDP). It has a great potential in income generation and poverty alleviation as well as improving human dietary nutrition by supplying good quality protein source from meat and eggs. Rural poultry Occupies 75-80% of the total poultry population in Bangladesh (Amin and Siddik, 2003), but the advancement of poultry farming is being hampered seriously due to outbreak of some fatal infectious diseases, among which, Fowl pox (FP) and Pigeon pox are notable. Pox is a common viral disease of commercial poultry (chickens and turkeys) as well as of pet and wild birds (Tripathy and Reed, 2003) of the approximately 9000 bird's species, about 232 in 23 orders have been reported to have acquired a natural poxvirus infection (Bolte et al., 1999). The disease has been described in chickens, turkeys, pigeons, ostriches, quails, pheasants, and canaries other species of wild birds particularly of young birds up to 4 to 6 weeks of age (Back et al., 1995). The lesions may vary according to the stage of development; i.e. papules, vesicles, pustules or crusts formations. The lesions are usually observed in the region of the head, wing, vent, and nodular lesion with large necrotic and crustous changes on feather-free regions of the body, namely comb, around the beak, wattles, eyelids or even legs. In some cases, fibrio-necrotic lesions or diphtheritic membranes may develop in the mucous membrane of mouth, pharynx and esophagus (Tripathy and Cunningham, 1984). The disease is still a malady and an enzootic to the growing chicken of any ages, sexes and breeds either in organized or in backyard poultry firming system in Bangladesh (Siddique et al., 1997). This disease is economically important in commercial poultry farming (chicken and pigeons), as it may cause decline in egg production, mortality and lower growth rate (Isa et al., 2002; Ariyshi et al., 2003). For the control and prevention of fowl pox and pigeon pox viruses confirmatory diagnosis is prerequisite so the present experiment was aimed for the isolation and molecular detection of avi-pox virus from chicken and pigeon using avian embryos and PCR with P4b gene specific primer (of fowl pox virus strain HP444) and the degree of their host specificity.

MATERIALS AND METHODS

Sample Collection

A total of 40 fowl pox and 40 pigeon pox suspected field samples (nodular lesion) were collected from six different outbreak districts (Khulna, Jessor, Gazipur, Pabna, Mymensingh and Netrokona) of Bangladesh during

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October 2013 to March 2014. In this study, fresh, larger and younger nodular as well as small nodular and crust type samples were collected randomly from Avi-pox suspected live, sick and recovered chickens and pigeons (Fig. 1 a, b, c and d). The birds were sacrificed and then the nodular lesions were collected. Both FP and PP suspected birds samples (nodules and crust) were subjected to grinding for the preparation of inoculums (20% suspension) for further studies (virus isolation and molecular detection).

Isolation of Viruses

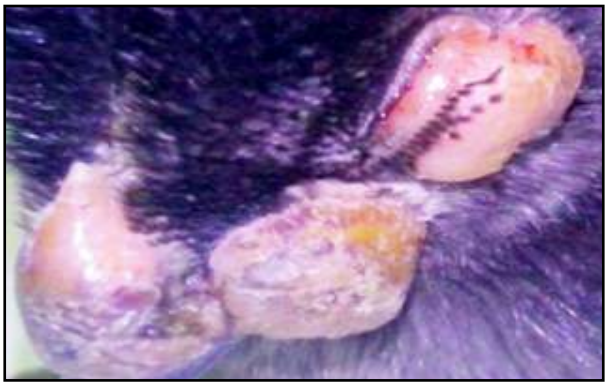
10-day-old embryonated hen eggs were selected for the isolation of avi-pox viruses. For this eggs were marked with a pencil at the centre of the air cell; an artificial air cell was created at the point of inoculation over the CAM by applying suction with the help of small rubber bulb at the hole on the air sac make artificial sac on the CAM. Using a sterilized 1 ml tuberculin syringe fitted with a 1/2 inch needle, 0.5 ml of sterile inoculums was inoculated onto the CAM. The inoculated eggs sealed with melted wax and were incubated for five to six days at 37°C in egg incubator. Alter 5 to 6 days of inoculation, the embryos which either died or remained alive chilled in refrigerator at 4°C to 8°C for 1-2 hour. The CAM which showed confluent growth of pocks and thickened was harvested as a source of avi-pox viruses. Several passages were given to increase the concentration of virus and the CAMs with pock lesions were collected and preserved for further study.

Molecular Detection (PCR)

Primer used

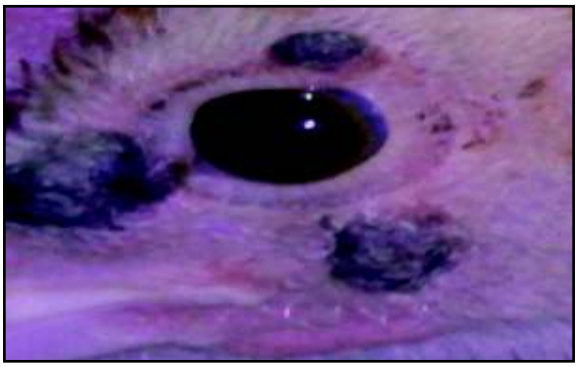
A set of the APV-specific primers, forward 5'-CAGCAGGTGCTAAACAACAA-3' (F) and reverse primer 5'-CGGTAGCTTAACGCCGAATA-3' (R) described by Huw Lee & Hwa Lee (1997) based on P4b sequence of fowl pox virus strain (HP444) were used for the amplification of 578 bp amplicons of pox viruses.

a. Fresh nodule



b. Medium Old Fresh Nodule

c. Recover or Crust Nodule



d. Almost Recover

Figure 1: Naturally Infected Avi-Pox Suspected (Nodular Lesion) Samples of Chicken and Pigeon

DNA Extraction

The viral DNA was extracted using 200 µl samples of AF and 20% w/v suspension of CAM using the QIAamp mini DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Then reaction mixture was prepared per sample as followed 10X LA buffer 2.5 µl, 50mM $Mgcl_2$ 1.0 µl, 10mM dNTPs 1.5 µl, LA taq 0.2 µl, specific primer of forward (F) 1.5µl, reverse (R) 1.5 µl, viral DNA 10 µl, dH_2O 6.8 µl and mixed properly with the help of the minispin. The tubes were then placed in a forty eight wells thermocycler (Mastercyclear, Eppendorf, Hamburg, Germany) and applied the thermal profile as 94°C for 5 minutes, initial denaturation, 35 cycles were continued at 94°C for 45 seconds, denaturation; 48°C for 1.5 minutes, annealing; 60°C for 2 minutes, extension; 60°C for 10 minutes, final extension. Each PCR product was detected by 2% NA Agarose gel electrophoresis.

Establishment of Experimental Infection

A total of 20 young pigeon (squabs) age of 1 month, 20 adult pigeon of 3 months, 20 adult chicken of 5 month and 20 local chicks of 1 month age groups were used. All these birds were inoculated with 20% w/v suspension of 0.5 ml FPV and PPV inoculums in three different routes wing web punctures, nasal and oral routes. Five birds of each group remained as control. All the birds were reared in the Laboratory animal shed of Bangladesh Agricultural University, Mymensingh maintaining strict biosecurity condition. All birds were checked daily for the appearance of any lesion on wing, comb, wattle, eyes, and legs for a total period of 4 weeks.

RESULTS AND DISCUSSION

Virus Isolation

Out of 40 FP suspected field samples, 27 (67.5%) were found positive for virus isolation in 10-day-old embryonated hen eggs. Virus could not be isolated from remaining 13 (32.5%) samples because 5 samples were smaller nodule of recently recovered and 8 samples were crust form of recovered nodules (Table 1). Findings of the present study partially agree with the findings of Masola et al. (2014) in their study they stated that out of 154 investigated samples 66 (42.86%) were found positive for FPV isolation in 10-day-old embryonated hen eggs. In the present study virus isolation rate was found higher than that of previous study done by Masola et al. (2014).

Of the 40 PP suspected field samples, 30 (75%) were found positive for virus isolation in 10-day-old embryonated hen eggs. Virus could not be isolated from remaining 10 (25%) samples because 5 samples were smaller nodule of recently recovered and 5 samples were crust form of recovered nodule (Table 1). Findings of the present study agree with the study of Prukner Radovcie et al. (2006) in their report they also performed isolation of PPV using same route and same age of hen embryos.

Molecular Detection

All of the field samples and isolated samples were subjected to molecular detection for the confirmation of FPV and PPV by PCR using the APV-specific primer pair described by Huw Lee & Hwa Lee (1997) based on P4b sequence of fowl pox virus strain (HP444). Both FPV and PPV showed specific bands on 578 bp on 2% NA Agarose gel electrophoresis (Fig. 2).

Out of 40 fields FP suspected samples, 32 (80%) were found positive and remaining 8 (20%) samples were negative by polymerase chain reaction (Table 1). Molecular detection rate of FPV in this study was 80%, whereas the genome detection rate of FPV by Roy et al. (2013) was almost 100% by PCR. Result of the molecular findings of the present study slightly differs with the findings of the previous study done by Roy et al. (2013). Failure of isolation and molecular detection of FPV from the remaining 8 (20%) samples may be due to faulty collection of samples at recovery stage of the diseases and also may be due to presence of very minimal concentration of viruses in the collected samples from avi-pox suspected chicken. This discrepancies in virus isolation and genome detection of the present study with the previous report of other study might be due to number of samples size, method of samples processing, samples treatments, age of the embryos and quality of samples of the field samples during the period of collection.

On the other hand, out of 40 PP suspected field samples, 35 (87.5%) were found positive and remaining 5 (12.5%) samples were negative by PCR (Table 1).

Table 1 : Summery of the Isolation, Molecular Detection (PCR) and Re-isolation of Avi-Pox Viruses

Types of Samples	Total no. of samples	Younger fresh and bigger nodule	Smaller nodule of recently recovered	Crust form of recovered nodule	Isolation positive	PCR positive	Re-isolation positive form experimental infection
Fowl pox	40	27	5	8	27 (67.5%)	32 (80%)	5* (100%)
Pigeon pox	40	30	5	5	30 (75%)	35 (87.5%)	5** (100%)

5*= Indicates Re-isolation of Fowl Pox Form Experimental Infection

5**= Indicates Re-isolation of Pigeon Pox Form Experimental Infection

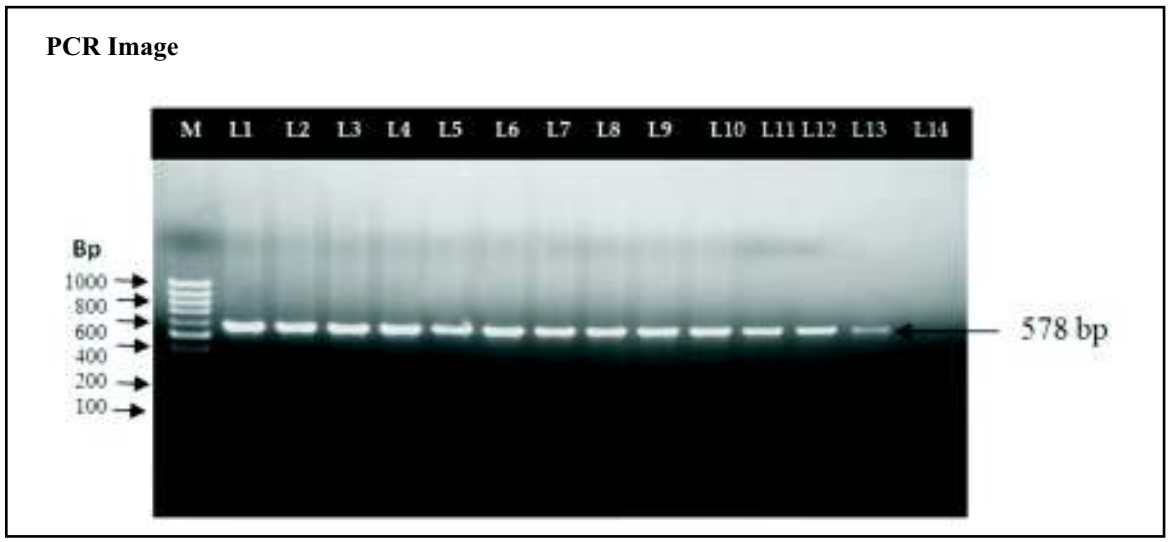


Figure 2 : Electrophoresis Results of PCR Products of Fowl Pox Virus Isolates Showing Specific Bands on 2% Agarose NA gel. M = 100 bp DNA Marker. L1 = Positive Control (Fowl Pox) L2 = Positive Control (Pigeon Pox), L3-L5 = Fowl Pox Field Virus Isolates, L6-L8 = Pigeon Pox Field Virus Isolates, L9-11= Re-Isolation of Fowl Pox Virus, L12-13= Re-Isolation of Pigeon Pox Virus and L-1 4 = Negative Control

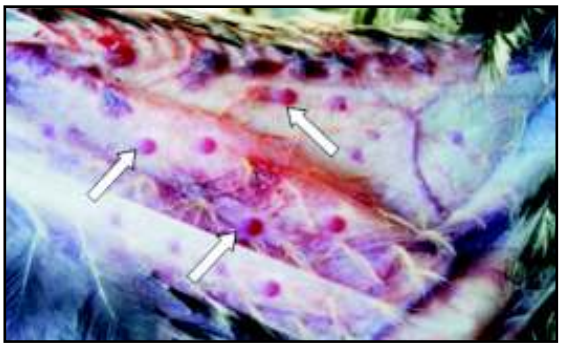
Molecular detection rate of PPV in this study was 87.5%, whereas the detection rate of PPV by Fahmy et al. (2009) was only 62.5% by PCR. Results of the molecular findings of the present study partially agree with the findings of Fahmy et al. (2009) in their study they reported that out of the 8 suspected field samples only 5 (62.5%) were positive. In the present study genome detection rate was higher than that of previous study done by Fahmy et al. (2009). Failure of molecular detection of PPV from the remaining 5 (12.5%) samples may be due to similar reason as mentioned earlier for the FPV in this study.

Experimental Infections

Experimentally established infection of FP and PP in pigeon and chicken using the isolation and PCR positive representative recent isolates of FP and PP viruses (Table 1). Out of three routes of infection, wing web and nasal routes of both the young and adult pigeons manifested pox lesions (nodule) under the wing (100%) and nasal area (100%) after 6 days of post inoculation with the exception of oral route of inoculation (Table 2). Failure of development of pox lesion in pigeon through oral route might be due to failure of multiplication of the viruses locally at the site of

Table 2 : Experimental Infection with FPV and PPV via Three Different Routes and Rate of Infection

Hosts	Virus isolates	No. of birds	Routes of inoculation	Lesions appeared in days	Lesions appearing site	Number of bird showed lesion	Percentage (%) of infection
pigeon	FPV	5	Wing web	6	Under the wing	5	5 (100%)
		5	Nasal	6	Nasal area	5	5 (100%)
		5	Oral	-	-	-	0 (0%)
	PPV	5	Wing web	10	Under the wing	5	5 (100%)
		5	Nasal	10	Nasal area	5	5 (100%)
		5	Oral	12	Mouth commissure	5	5 (100%)
chicken	FPV	5	Wing web	5	Under the wing	5	5 (100%)
		5	Nasal	-	-	-	0 (0%)
		5	Oral	5	Under the wing	5	5 (60%)
	PPV	5	Wing web	-	-	-	0 (0%)
		5	Nasal	-	-	-	0 (0%)
		5	Oral	-	-	-	0 (0%)



a. Wing Web Puncture Route In Chicken, Wet Nodular Lesion (White Arrow)

b. Wing Web Puncture Route In Pigeon, Large Nodular Lesion (White Arrow)

Figure 3 : Experimentally Infected Avi-Pox Suspected (Nodular Lesion) Samples of Chicken and Pigeon

inoculation. On the other hand, layer birds exhibited take reaction (crust form) in the wing web site inoculation except nasal and oral routes. Local chicks possess dispersed as well as local pox lesion in wing web (100%) and oral routes (66.66%) after 5 days of post inoculation with the exception of nasal route (Table 2). FPV indicated the wider host range means it can infect the chicken and pigeon regardless of age of birds. The clinical signs and lesions observed in these experimental birds were similar to those observed in cases of natural infection of pigeons and chickens with avi-pox virus (Fig. 3, a and b). Findings of the present study highly agree with finding reports of Khodir and Mikhail (2006). In their study they had reported that FPV is not extremely host specific and also produces the characteristics pox lesions in quails. The infections of FPV following wing web puncture route of inoculation was manifested more severe (100% in pigeon and 60% in chicken) compared to that of oral route (Table 2). In case of PPV, same routes and doses were used. Wing web puncture and nasal routes both of young and adult pigeons manifested pox lesions (large wet nodule) under the wing and the nasal area (100%) after 10 days of post inoculation (Table 2). Oral route exhibited the pox lesion (large nodule) both of young and adult pigeon on mouth commissure (100%) after 12 days of post inoculation (Table

2). On the contrary naturally infected PPV failed to produce any lesion in the experimentally infected chicken. None of the birds (chicken) exhibited any pox lesion irrespective of any inoculation routes indicated that the host specificity of the PPV. The results of the present study were in agreement with the findings of Siddique et al. (2011) showed that the field isolates of FPV manifested considerable host specificity to pigeons. The results of the present study were partially in agreement with the findings of Tripathy & Cunningham (1984) who described that FPV and PPV are host specific and produced disease in their respective hosts only. Incubation period of both the FPV and PPVs was experimentally established in this study. Regardless of doses and route of inoculation FPV incubation period was more or less 5 to 6 days where incubation period of PPV was more or less 10 to 12 days which indicated that FPV incubation period is shorter than that of PPV. Overall findings of experimental infection of both pigeon and chicken with pigeon pox and fowl viruses clearly indicated that fowl pox virus can infect both chicken and whereas pigeon pox virus infect pigeon only.

Overall findings of this study indicated that when samples were collected from 6 different districts (Khulna, Jessor, Gazipur, Pabna, Mymensingh and Netrokona) of Bangladesh. Pigeon pox samples were collected from exclusively in Khulna and Jessor but there was no outbreak of fowl pox in these two districts. Fowl pox samples were also collected from exclusively in Mymensingh and Netrokona but there was no outbreak of pigeon pox in these two districts. On other hand, there was a massive outbreak of fowl pox and pigeon pox in Gazipur and Pabna districts. Results of PCR and virus isolation from chicken and pigeon indicated that both the viruses are either same or different lineage of avi-pox virus those are circulating in Bangladesh. Without molecular characterization and phylogenetic analysis, we are not sure yet about the origin and clades of circulating avi-pox viruses in Bangladesh.

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