

Molecular Characterization of Circulating Duck Viral Enteritis in Egypt During 2012-2013

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Abstract: Isolation trials were made on Duck Viral Enteritis (DVE) collected from natural outbreak in 2012 and 2013 from different breeds, age and location. The collected samples were processed for isolation and identification of DVE. Characterizations and pathogenesis of the isolates were studied by inoculation in specific pathogen free; embryonated Muscovy eggs and one-day old Muscovy ducklings, then keep for observation for clinical signs and mortality. Liver, esophagus, small intestine and kidney collected from field samples, inoculated embryos and ducklings are examined histopathologically. Supernatant from tissue suspension of original samples was examined with PCR. Impression smears from organs of both field cases and inoculated ducklings examined with Indirect Fluorescent Antibody Test (IFAT). Many basophilic and eosinophilic intranuclear inclusion bodies were observed histopathologically in 2 samples of 4, in livers of SPF, embryonated Muscovy eggs, ducklings one-day old. Fluorescent green light colors were seen in the same samples and in Muscovy ducklings' liver and esophagus. PCR results were agreed with isolation on SPF, embryonated Muscovy eggs, one-day old Muscovy ducklings and Indirect Fluorescent Antibody Test (IFAT).

Key words: DVE • Molecular Diagnosis • Embryonated Muscovy duck eggs • Muscovy duckling one • Day old • IFAT • Histopathology.

INTRODUCTION

Duck viral enteritis (DVE) is an acute, highly contagious disease of ducks, geese and swans of all ages, characterized by sudden death, high mortality (particularly among older ducks) and hemorrhages and necrosis in internal organs. It has been reported in domestic and wild waterfowl in Europe, Asia, North America and Africa, resulting in limited to serious economic losses on domestic duck farms and sporadic, limited to massive die-offs in wild waterfowl. In the USA, considerable losses due to DVE have been reported in the concentrated duck-producing areas located in Long Island, New York. There are differences in susceptibility to the virus, with Muscovy ducks being the most susceptible. However, naturally occurring infections have been reported in a variety of domestic ducks such as Pekin, khaki Campbell, Indian runners and mixed breeds. The age at infection ranges from 7 days to mature ducks [1].

DVE reported in domestic ducks and ducklings in birds ranging from 7 days of age to mature breeders in susceptible flocks the first signs are often sudden high and persistent mortality with a significant drop in egg production in laying flocks. In domestic ducks the incubation period ranges from 3-7 days. Mortality usually occurs 1-5 days after the onset of clinical signs and is often more severe in susceptible adult breeder ducks. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be latently infected carriers may shed the virus in the feces or on the surface of eggs over a period of years [2-5].

In breeder ducks the range of signs include 'sudden deaths', photophobia associated with partially closed, pasted eye-lids, polydipsia, loss of appetite, ataxia and nasal discharge. Birds often have ruffled feathers, watery diarrhea and soiled vents. Sick birds may maintain an upright stance by using their wings for support, but their overall appearance is one of weakness and depression. In ducklings 2-7 weeks of age, losses may be lower than

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in older birds and the signs associated with DVE include dehydration, loss of Weight, conjunctivitis and serous ocular discharge, a blue coloration of the beaks and blood-stained vents. Prolapsed of the penis may occur in addition to haemorrhages in ovarian follicles [6]. The gross lesions are characterized by vascular damage, with tissue haemorrhages, free blood in the body cavities and intestinal lumen and a range of lesions affecting the digestive tract mucosa. These latter lesions progress with the course of the disease and include initial mucosal haemorrhages and eruptions and intense annular congestion, leading to pseudo-membranous or diphtheritic mucosal lesions. Necrotic degenerative changes are evident in the lymphoid and parenchymatous organs. In the liver showed irregularly distributed pin point haemorrhages and white foci giving a speckled appearance. In ducklings lesions of the lymphoid tissues tend to be more prominent than visceral haemorrhages. Microscopic lesions are eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present. Birds that recover from natural infection are suggested to be immune to re-infection, but latency (in the trigeminal ganglion) and reactivation of virus is recognized [7].

In Egypt, the Duck Plague was reported for the first time in large flock of white Pekin ducks in Bahem Provinces, where the disease caused high morbidity and mortality [8]. Duck plague caused great economic losses with mortality rate ranging 1-16% in breeders and 1-40% in broilers and drop in egg production ranged from 0.5 - 99.5 % [9, 10]. Sequence analysis of the glycoprotein envelope gene of DP and they found a great similarity was found between the UL35 gene amplified from either local or imported vaccinal strain but the antigenicity profile alone blot matrix revealed that the UL 35 proteins antigen (antigen VP26) from local isolate is more antigenic and thus the genome of the local strain would be a suitable templates for the gene [11]. The present study was aimed to confirm virus exposure in ducks breed in Egypt by deferent diagnostic methods and determine if these methods could be reliable in monitoring duck plague outbreaks.

MATERIELS AND METHODS

Collection of Sample: Four samples were collected, the 1st sample from Beni Suef, Pekin ducks, 270 days age, in February 2012. The 2nd sample from Al-Dakahlia, Pekin

ducks, 310 days, in April 2013. The 3rd sample from Giza, Mallard ducks, 24 day, in May 2013 while the 4th sample was from Alexandria, Muscovy ducks, 66 days, May 2013.

Preparation of Tissue Sample Suspension: Liver, spleen, esophagus, small intestine and kidney tissue have been collected from ducks (had clinical signs resembling Duck Plague signs) homogenized in saline containing 2000 iu/ml Penicillin and 200mcg/ml Streptomycin. These organs were made pulled then ground in a Tenbroeck tissues grinder (20% W/V), then centrifuged at 3000 rpm for 15 minutes. After centrifugation the clear supernatant fluid from sample was extracted and preservative at -20°C until used [12].

Egg Inoculation: Twenty % tissue sample suspension (Liver, esophagus, small intestine and kidney) was inoculated into chorioallantoic membrane (CAM) of 10 to 13 day-old Muscovy duck embryonating eggs and SPF. About 0.2ml /embryo were inoculated using standard techniques of embryo inoculation. Each of inoculated embryos was monitored for embryopath daily for 6 days. The allantoic fluid (AF) and CAM were harvested separately from embryos that died during period of observation. The harvested CAM, livers of embryonated Muscovy ducks and SPF were ground then examined against standard serum for Agar Gill Precipitation test (AGPt) [13].

Duckling: Tissue suspension of sample No. 1 experimentally inject 1ml intramuscularly in 10 one day-old Muscovy duckling and keep 2 weeks for observing clinical signs and death [1].

Histopathologically Examination: Liver, esophagus from different samples were fixed in 10% buffered formalin solution, processed in normal way, paraffin sections (5 microns thickness) were prepared and stained with haematoxylin and eosin [14] and Phloxine tartrazin stain [15].

Production of Duck Plague Antiserum: Anti-duck plague antiserum was produced in rabbits. The vicinal strain (local strain gets from VAC Sera Abbasia) was used to inoculate rabbit. There were 2 inoculations using adjuvant (Montnide ASA50 1:1) with virus and third with virus alone. The animals were monitored for duck plague antibody using serum neutralization test (SNT). Serum was harvested when titer were high. This hyper immune serum was used in IFAT [16].

Staining of Impression Smears: Impression smears were prepared from tissues taken from field samples and experimentally infected Muscovy duckling one-day old. Smears were stained using rabbit anti-duck plague polyclonal serum and an anti-rabbit FITC conjugate [17].

Detection of DNA Polymerase Gene of Herpes Virus Causing DVE

DNA Extraction Method: The extraction was performed on 4 field samples of duck viral enteritis outbreak using DNA easy blood and tissue Qiagen kit [18].

PCR for Duck Viral Enteritis: The reaction was performed in a volume of 50 µl of green taq DNA polymerase (Promega) in the Promega PCR buffer containing 400nM of each primer and 1.5 mM MgCl₂.

B5f: 5'-CCA GAT GCT CTC AAC CTA TAT AAG-3'
B6r:5'- GAC TGT GTG CTC TAA TGA GAC GA-3'

The PCR product was analyzed in 1.5% agarose in TAE buffer gel contain 0.5 µl/ml ethidium bromide.

RESULTS

Inoculated Eggs: Samples No. 1 and 4, when inoculated in embryonated Muscovy eggs and SPF, death began after 4-6 days of inoculation and embryo showed irregular patches of congestion and petechial haemorrhage throughout the body particularly in the head, neck, legs, abdomen and beak region of the embryos. The lesions of the CAM included irregular patches of congestion. 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.

Agar Gel Precipitation Test: Collected CAM and Livers from inoculated embryonated Muscovy eggs and SPF examined against refrains' serum gave precipitating line with samples No.1 and 4.

Duckling: 10 Ducklings die within 3-12 days with clinical signs of Duck Viral Enteritis. After 3 days of inoculation nervous signs began to appear as tremors of head, neck and body. Duckling was unable to stand; they maintain a posture with drooping outstretched wings and head down suggesting weakness, depression, off food, ataxia, diarrhea and death. The gross lesions are characterized by

vascular damage, with tissue haemorrhages, free blood in the body cavities and intestinal lumen and range of lesions affecting the digestive tract mucosa. In the liver this manifests as irregularly distributed pinpoint haemorrhages and white foci giving a speckled appearance. Lesions of the lymphoid tissues tend to be more prominent than visceral haemorrhages.

Histopathologically Lesions: The entail lesion occurs in the wall of blood vessels. Smaller blood vessels, venules and capillaries, instead of larger blood vessels, are more markedly involved. The endothelial lining is disrupted and a connective tissue of the wall becomes less compact, with visible separations at points where extravasations of the blood pass from the lumen through the thin ruptured wall into surrounding tissues. Hemorrhages are especially pronounced in certain locations: interlobular venules of the proventriculus, hepatic and portal venules at the margins of liver lobules.

The liver of both SPF and embryonated Muscovy eggs inoculated by sample No. 1 showed congestion of blood vessels, thrombus, degenerative hepatocellular necrosis and eosinophilic intranuclear inclusion bodies.

Duckling liver showed congested blood vessels and proliferation of bile ductules, thrombus formation and per vascular hemorrhage, red intranuclear inclusion bodies on yellowish background. Also vascular degeneration was seen in duckling.

Liver of inoculated SPF and embryonated Muscovy eggs from sample No.4 showed red intranuclear inclusion bodies. Also liver from SPF and embryonated Muscovy eggs inoculated by field sample No.4 showed intranuclear inclusion bodies.

Impression Smears: Stained immersion smears with rabbit anti-duck plague polyclonal serum and an anti-rabbit FITC conjugate from esophagus, liver, spleen and kidney from field samples or duckling showing positive reaction in samples No.1, 4 and ducklings inoculated by emulsion of sample 1. The organs that were most frequently and strongly affected were the esophagus, kidney, liver. Specific fluorescence (green light colors) in the nucleus of organs

PCR: A product 209 bp in size was amplified from emulsion of 4 field samples known to be obtained from farms with clinical signs of duck viral enteritis. As shown in photo [Fig 13]. Positive sample No.1 and 4.

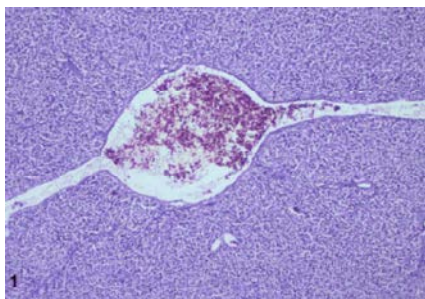


Fig. 1: Liver of SPF showed thrombus formation (HandE X100).

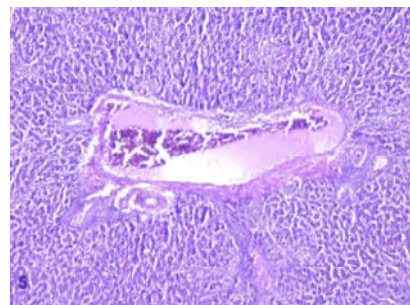


Fig. 5: Liver of Muscovy duckling showed congested blood vessels and proliferation of bile ductules (HandE X200).

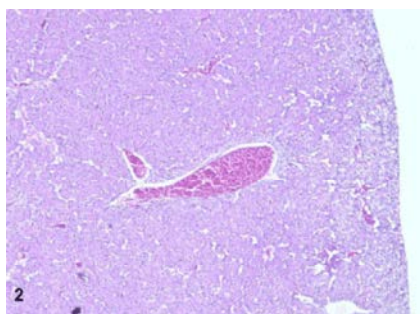


Fig. 2: Liver of embryonated Muscovy eggs showed vacuolar degeneration (HandE X400).

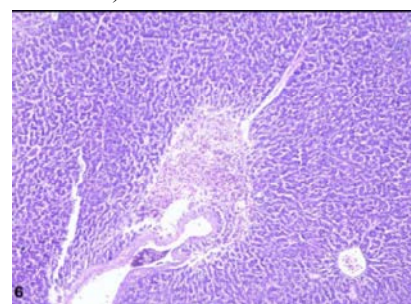


Fig. 6: Liver of Muscovy duckling showed per vascular hemorrhage (HandE X100).

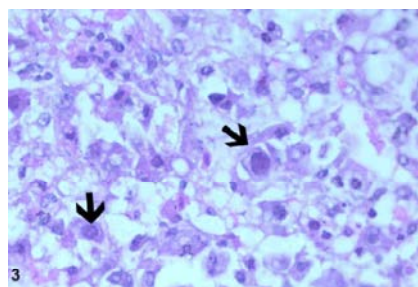


Fig. 3: Liver embryonated Muscovy eggs showed vacuolar degeneration, hepatocellular necrosis and eosinophilic intranuclear inclusion bodies (arrow) (HandE X1000).

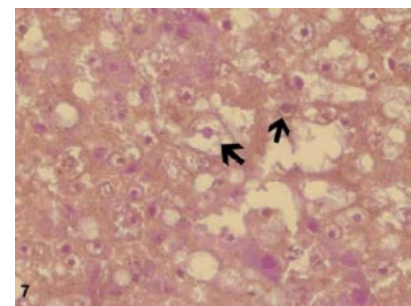


Fig. 7: Liver of Muscovy duckling showed red intranuclear inclusion bodies on yellowish background (arrow) (Phloxine Tartrazine stains X 1000).

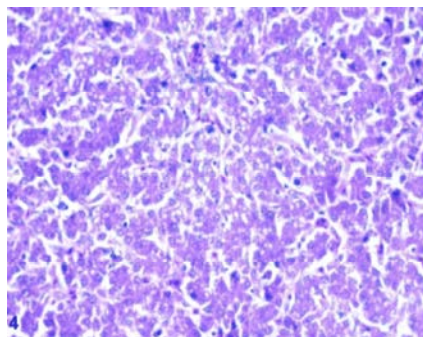


Fig. 4: Liver of embryonated Muscovy eggs showed vacuolar degeneration (HandE)

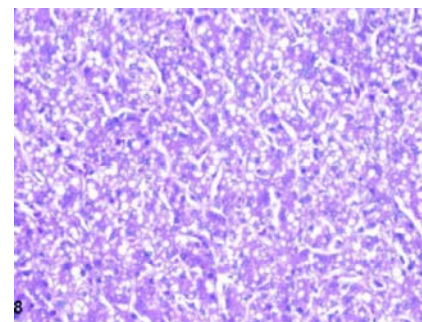


Fig. 8: Liver of Muscovy duckling showed vacuolar degeneration (HandE X400).

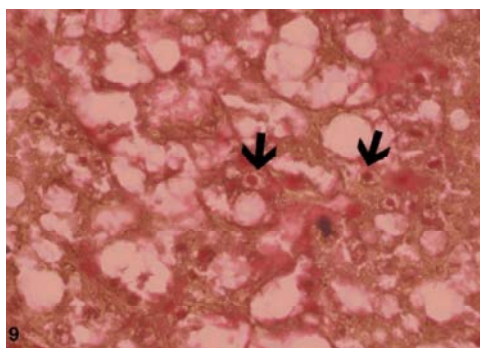


Fig. 9: Liver embryonated Muscovy eggs showed red intranuclear inclusion bodies on yellowish background (arrow) (Phloxine Tartrazine stain X 1000).

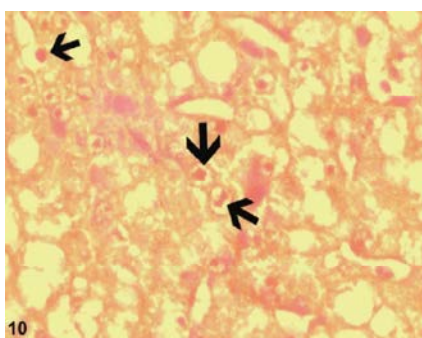


Fig. 10: Liver of embryonated Muscovy eggs showed red intranuclear inclusion bodies on yellowish background (arrow) (Phloxine Tartrazine stain X 1000).

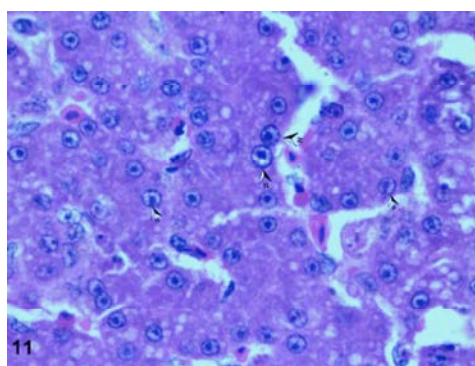


Fig. 11: Liver of duck infected with field strain showed intranuclear inclusion bodies (arrow) (HandE X1000).

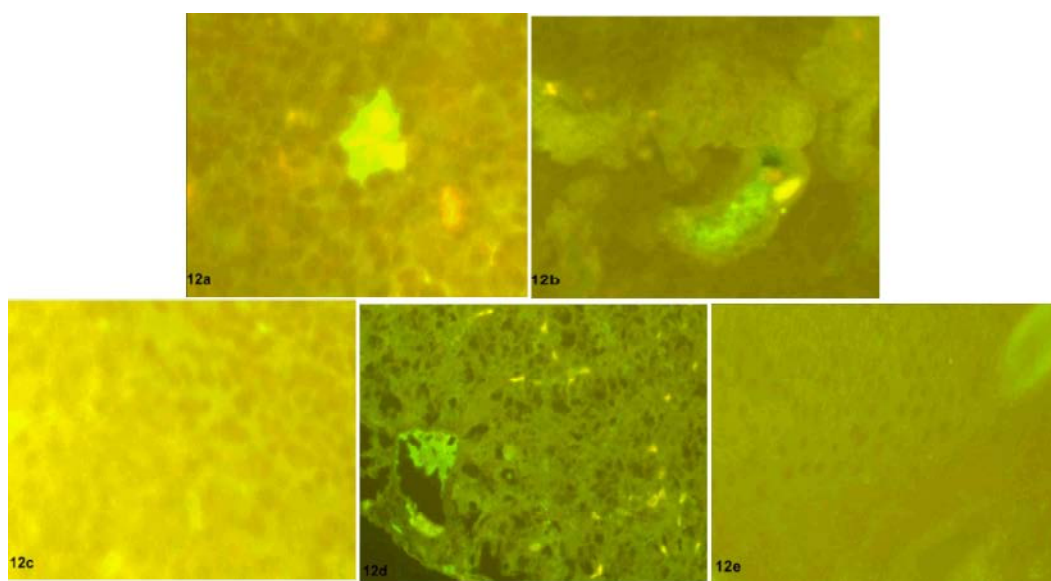


Fig. 12: DVE-specific fluorescence in the nucleus of the infected cells. DVE field samples obtained from ducks suspected of having DVE. Tissue from liver stained with antirabbit monoclonal antibody. a: liver sample 2, b: liver of sample 3, c: liver, control negative. X 400, d: esophagus of sample 3, e: esophagus, control negative.

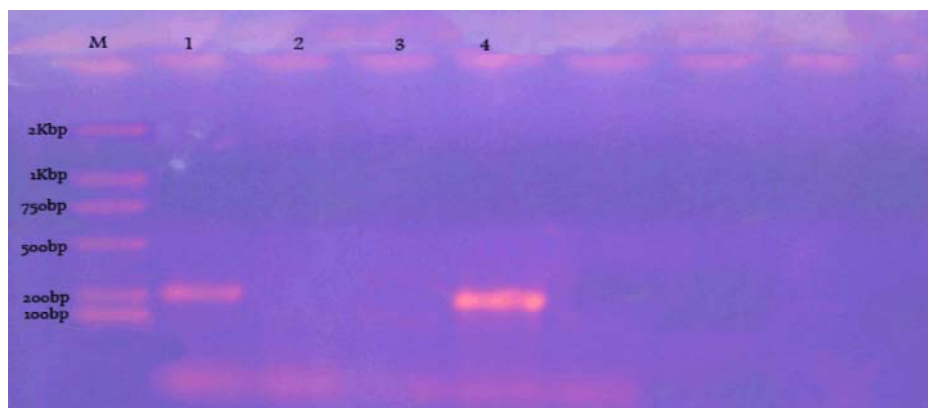


Fig. 13: PCR picture of 4 field samples, showed positive lanes 1 and 4 at 209 bp.

DISCUSSION

Duck plague (DP) or Duck Viral Enteritis (DVE) is an acute contagious disease of waterfowl, which occurs throughout Asia, Europe, Africa, America and spread by natural migration and international trade. In this study the samples collected from deferent age 270,310, 24 and 66 days also deferent breeds Pekin, Mallard and Muscovy and location Beni Suef, Dakahlia, Giza and Alexandria on respectively. The sample 1 and 4 of age 270,66 days witch from Beni Suef, Alexandria (2 deferent location and age) gave lesion of duck plague in Muscovy embryos and SPF [6]. We find that duck plague affected young and old age, Pekin and Muscovy ducks in hot and cold weather. AGP test also gave precipitating line with CAM and livers of samples No. 1, 4. [6, 7, 13, 18, 20].

The incubation period of duck plague in infected duckling one-day old ranged from 2-3days. Also clinical sings began to appear after 3 days of inoculated duckling with emulsion of sample No.1. After 3 days of inoculation nervous sings began to appear as tremors of head, neck and body. Duckling was unable to stand; they maintain a posture with drooping outstretched wings and head down suggesting weakness, depression, off food, ataxia, diarrhea and death [20, 21]. Also, in this study, the experience of infection occurring in Muscovy ducklings, has proved more sensitive than cell culture method. [6,9,12,20]. The presence of DVE is often indicated by the presence of Cowdry type inclusion bodies in the nucleus [3].The same results were observed in this study in the nuclei of hepatocytes, no inclusions were observed histopathologically in the esophagus of examined samples. This is may be as a result of replication of virus primarily in the mucosa of digestive tract especially in the esophagus and then spread to bursa of Fabricius, thymus, spleen and liver [2].In this research we found that severity

of lesions in liver of SPF inoculated with field strain were less than the lesions of duckling 4 days old infected with the same field strain, embryonated Muscovy eggs and the liver of embryo inoculated with vaccinal strain [1,16,20]. The impression smears of fresh organs (field samples and Muscovy duckling), the ability of detecting the virus could be arranged as follows, eosophagus, liver, spleen, small and large intestine [22]. The impression smears was strong particularly in epithelial tissues, lymphoid tissues and some small blood vessels. The organs that were most frequently and strongly affected were the eosophagus, kidney and liver [16]. Indirect Immunofluorescence (IFAT) staining techniques allow detection of duck plague virus in impression smears for fresh tissue sample and in duckling used for virus isolation. Duck plague primers were shown to be specific to DVE and did not react with other avian herpes virus and used for the detection of duck plague viral DNA in tissue samples, the assay was also able to differentiate duck plague from other avian virus as Infectious Laryngotracheitis virus and Marker's. The PCR test was able to detect field isolates of duck plague virus [23].

The evaluation of the various diagnostic methods for detecting DVE infection in ducks, in this study has demonstrated that impression smear of IFA offered advantages among the other diagnostic method. It was sensitive, specific and economic and it was able to detect DVE rabidly than isolation in duckling one-day old or embryonated Muscovy eggs. Impression smear detect virus in fresh organs and results can be available in few hours. Allowing the convenience of a single sample submission and ability to perform retrospective study on tissue smears finally, in our opinion it could provide rabid and definitive diagnostic and may be useful for eradication programs.

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