

DUCK VIRAL ENTERITIS IN EGYPT: ISOLATION AND DETECTION OF THE CIRCULATING VIRUS DURING OUTBREAKS FROM 2016-2018

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Abstract: Duck viral enteritis (DVE) is an acute and contagious herpes virus infection with a potential threat to domestic and wild waterfowl. In this study, 75% mortality rate with 40% drop in egg production was recorded among the examined flocks. Diagnosis of the disease was carried out based on the clinical signs, pathology and supported by laboratory confirmation. Microscopically, the presence of eosinophilic intranuclear inclusion bodies (IN/IB) in the hepatocytes revealed duck enteritis virus (DEV) infection. DEV was isolated in embryonated duck eggs; 42.6% of the samples showed evidence of infection. The nucleic acid of the DEV was detected by Polymerase Chain Reaction (PCR) in 19 out of 68 collected samples (27.9%) with positive amplification of the DNA polymerase gene. This investigation disclosed variation in the disease prevalence among the different ages, breeds, seasons, and immune status of ducks, as the disease was more prevalent among vaccinated flocks (34.8%) than in non-vaccinated ones (24.4%). Accordingly, control efforts can be done to reduce the further outbreaks of DVE in terms of adoption of good management practice, biosecurity measures and increasing awareness about the disease spread and application of proper vaccination regimens. Continuous studies of DEV at the molecular and immunological levels are important for the effectiveness of any control strategy of the disease including vaccination.

Key words: DVE; duck plaque; histopathology; PCR; DNA polymerase gene; Egypt

Introduction

Ducks are considered a relatively resistant species of domestic poultry. However, there are many viral and bacterial diseases seriously attacking ducks and cause serious losses (1). Being, one of the most contagious, highly infectious and lethal viral diseases among domestic (2) and wild ducks, swans, geese, and other waterfowl of different ages is the duck viral enteritis [DVE; synonym: duck plaque (DP)]. This disease results in significant economic losses due to morbidity and mortality varying from 5-100%, carcass condemnation and reduction of egg production and hatchability (3-7).

The etiological agent of DVE is Duck Enteritis Virus (DEV), a member of *Herpesviridae* family, *Alphaherpesvirinae* subfamily, *Mardivirus* genus as Anatid

Herpesvirus 1 denoted after the host family Anatidae (8). Like other herpesviruses, this virus has a linear double-stranded DNA genome of 119×10^6 Daltons and is comprised of approximately 158,091 base pairs (9). All strains of the virus are antigenically similar, but their virulence is greatly varied (10). Hence, the severity of DVE infection varies according to the strain of the virus, and the species, age, and sex of affected birds (11, 12).

Since the first report of DVE in the Netherlands in 1923 (13), the virus had been spread all over the world resulting in a dramatic impact on the commercial duck industry (14, 15). Several outbreaks have been detected in many countries including Egypt (7, 15-18), where the disease was first reported in Egypt in 1986 (19).

The disease is usually transmitted through direct or indirect contact with the diseased birds

and/or the contaminated environment. Migratory and domestic waterfowls have a crucial role in the spread of infection (6, 7, 20, 21). Several reports indicated that the significance of virus transmission through the eggs of infected birds remains unclear in the cycle of disease (22-24).

In most cases, the affected birds with DVE die without any detectable symptoms (25, 26). Nevertheless, clinical signs and mortalities are intelligible especially in adult ducks, and they are associated with eruptions in the digestive mucosal, degenerative lesions in parenchymatous and lymphoid organs, vascular damage, and consequent internal hemorrhages (15, 16, 27).

Similar to other herpes viruses, DEV can cause unapparent infection in the recovered birds; this state is referred to as latency (in the trigeminal ganglion). The reactivation of the latent virus has the ability to cause further disease outbreaks (19, 28, 29).

Prevention and control of the DVE outbreaks in duck farms are based on using attenuated DEV vaccines (30-33). In spite of using attenuated vaccines for disease eradication, the infection is not completely controlled (9, 34). Therefore, researches have been in a progress to study the epidemiological and pathological characteristics of the DEV, besides proper diagnosis and vaccine development (24). Since the DVE emerged in Egypt, few studies addressed the epidemiology and surveillance of the disease (35-37). Consequently, this study aimed to investigate the current situation of DVE among commercial and backyard duck flocks in Egypt during 2016-2018.

Material and methods

Clinical Samples

Clinical samples were collected from 68 duck flocks containing 197635 ducks; commercial (n=61) and backyard (n=7) duck flocks of different ages (30-398 days) and breeds including; Native (n = 12), Pekin (n =11), Muscovy (n =42) and Mallard (n =3) located in six provinces; Sharkia, Dakahlia, Gharbiya, Qalubiya, Damietta and Port-Saied, Egypt during 2016–2018 and suspected to be infected with DEV. The observations of clinical symptoms and autopsy examinations were carried out as a primary diagnostic step of the suspected duck flocks. Tissues from the in-

testinal tract, liver, spleen, and heart were collected from DVE suspected diseased/dead ducks during autopsies for histopathology and/or virus isolation.

Virus Isolation

Tissue samples from the liver, spleen and, intestine were collected from suspected ducks with clinical signs resembling DVE. The tissue homogenates (1:20 w/v) were prepared (38) and inoculated into 10-14 days old embryonated Muscovy duck eggs via chorioallantoic membrane (CAM) route (39). The inoculated EDEs (embryonated duck eggs) were incubated at 37°C for up to 7 days and monitored daily for embryonic death. The allantoic fluid (AF) and CAM were harvested separately. The collected AF was screened by a slide hemagglutination (HA) assay by using 10% (v/v) washed chicken red blood cells. The harvested CAM was subjected to histopathological examination and Polymerase Chain Reaction (PCR) for confirmatory detection of DEV. Before isolation, two to four serial blind passages of the homogenized CAM may be required.

Histopathological examination

Different tissue specimens including liver, spleen, heart and, intestine were collected from the necropsied birds and fixed in 10% neutral buffered formalin solution and processed in a normal way. Thin paraffin sections (4 microns thickness) were prepared and stained with haematoxylin and eosin. For histopathological diagnosis, the stained sections of tissue samples were observed using light microscopy (40).

DNA Extraction and PCR amplification

Viral DNA was extracted from the inoculated CAM using DNA extraction kit (viral GeneSpin™, Cat#17151, iNtRON Biotechnology, Inc), following the manufacturer's instructions. The primers used for DNA-directed DNA polymerase gene (UL30) amplification are Forward 5'-GAAGGCGGGTATGTA-ATGTA-3' and Reverse 5'-CAAGGCTCTA-TTCGGTAATG-3' to amplify the targeted DNA segments of DEV (39). The amplification reaction was carried out in a 50 µl reaction mixture using 2x PCR master mixture solution (*i*-Taq™) (Cat#25027, iNtRON Biotechnology, Inc). The cycling conditions were

2 min at 94°C (initial denaturation), followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 2 min at 72°C (extension), and the cycle of final extension at 72°C for 7 min. The amplified PCR products were separated by agarose gel electrophoresis and then visualized using ultra-violet trans-illuminator.

Results

Virus Isolation

The isolation of virus on embryonated Muscovy duck eggs revealed embryo deaths at 4-6 days post inoculations with specific lesions on both dead and survived embryos at the end of the observation period. Dead embryos (42.7%) showed congestion and hemorrhages all over the body especially in the head, neck, legs, abdomen and beak regions in 27.1% of the embryos (Fig. 1A). The lesions of the liver were included swelling, and white necrotic foci in few cases reached 2.2% (Fig. 1B). Thickening of the CAM with irregular white necrotic patches (pock lesions) were observed in several inoculated embryos with a percentage of 29.7% (Fig. 1C). Based on embryo lesions, 42.6% (n= 29/68) of samples showed evidence of DEV infection and their AF were found negative to slide HA. The characteristic eosinophilic intranuclear inclusion bodies in the epithelial lining were observed during microscopical examination of some harvested CAMs (Fig. 1D).

Molecular detection of DEV in the field samples

The DEV was detected in 19 out of 68 duck flocks (27.9%) using a specific primer for the DNA polymerase gene. The expected PCR amplicon appeared at 446-bp (Fig. 1E). Our targeted DNA polymerase gene usually encodes UL30 protein. Extraction of DNA from the commercial live attenuated vaccine (Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt) and the RNase-free water were used as a positive and negative control, respectively.

Pathological findings of positive DEV flocks

The main observed clinical manifestations among the examined duck flocks were depression, dehydration, and nasal and ocular discharge with sometimes corneal opacity. Nearly, positive

DEV flocks (n= 19) showed at least one or more lesions specific to the disease (Table 1) with a mortality rate ranged from 1-75%. Watery diarrhea was observed in all examined flocks, while the bloody diarrhea was detected in 42.1% (8/19) of flocks and greenish diarrhea in only 2 flocks. Also, in mature males, the prolapsed penis was observed (15.8%; 3/19). Among the breeder duck flocks, drop in egg production was recorded in 4 flocks and reached up to 40%.

Furthermore, the main lesions noted during the examination (Figure 2) were hemorrhages on the epicardium and at the esophago-proventricular sphincter and were detected in 47.4% and 15.8% of the examined flocks, respectively. The common intestinal lesions were intense annular congestion and enlargement (94.7%; 18/19), followed by mucosal hemorrhages (42.1%; 8/19), and then pseudo-membranous or diphtheritic mucosal eruption (15.8%; 3/19). Necrotic degenerative changes were evident in the parenchymatous organs including the liver and spleen. On the liver, irregularly distributed pinpoint hemorrhages and necrotic foci were seen (31.6%; 6/19 and 26.3%; 5/19, respectively).

In histopathological examination, the intestine revealed massive necrosis of the mucosa with lymphocytes and plasma cells infiltration in mucosa and submucosa, and the lumen contained a large number of necrotic cells with leucocytes.

Moreover, the intestinal capillaries all over the wall were hyperemic and the muscular coat showed congestion, hyaline degeneration, edema, and leucocytic infiltration (Fig. 3A). Partial destruction of intestinal crypts and intestinal glands were seen (Fig. 3B). Necrosis of the hepatic tissue was detected (Fig. 3C), and others showed severe congestion of hepatic sinusoids and focal hemorrhage. In addition, eosinophilic intranuclear inclusion bodies (IN/IB) were apparent in the hepatocyte (Fig. 3D). Focal coagulative necrosis of cardiomyocytes with partial replacement by inflammatory cells as well as interstitial edema with leucocytic infiltration was observed in the heart (Fig. 3E). Moreover, depletion and multifocal necrosis of the lymphoid tissue of the white pulp (Fig. 3F), congestion of splenic sinusoids and hemosiderosis were noticed.

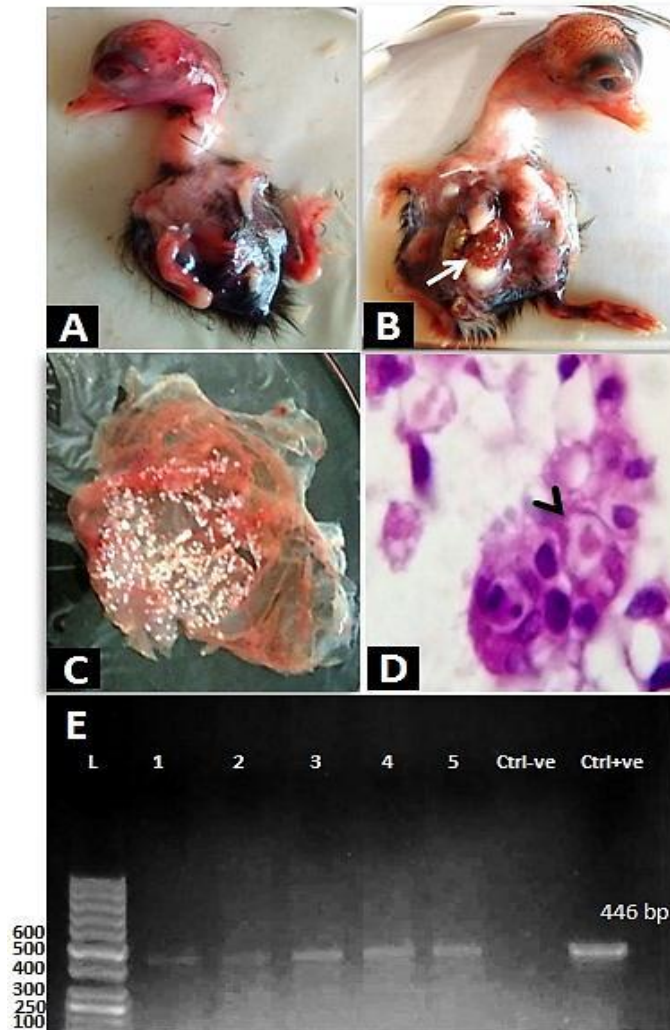


Figure 1: Detection of duck enteritis virus using embryonated Muscovy duck eggs and PCR

(A) Congestion and hemorrhages all over the body of embryo. (B) Swelling with white necrotic foci on liver of embryo. (C) Thickening of the CAMs with irregular white necrotic patches. (D) Microscopic examination of CAM showing characteristic eosinophilic intranuclear inclusion bodies in epithelial lining, located in the nucleus (arrow head). H&E X1000. (E) PCR amplification of DNA samples using DNA polymerase gene specific primer showing band of 446 bp. Lane "L": Molecular ladder; Lane 1-5: positive field samples; Ctrl +ve: (Control positive; vaccine strain) and Ctrl -ve: (Control negative; RNase-free water)

The situation of DEV in duck farms detection by PCR

The DEV infection among the positive duck flocks varies according to several factors including study area, year, season, flock category, breed, age, and immune status (Table 2).

According to the locality of sampling, the highest positive infection rate of DEV infection (100%) was detected in Port-Saied, followed by Sharkia (40%), and Gharbiya (30.8%) province. However, no virus was detected in Qalubiya and Damietta provinces. The trend of DEV isolation in the provinces of Egypt and the case number

analyzed (n/n) are shown in Figure (4). The detection rate of DEV was ranged from 26.7% - 50% during 2016- 2018. The infection rate was 11.8% in spring and 100% in the summer season. Regarding the flock categories, DEV incidence rates were 28.6% and 27.9% in the backyard and commercial duck flocks, respectively. Muscovy ducks were the most affected breeds with a percentage of 35.7%. Furthermore, the virus detection rate was higher in duckling (2-7 weeks) than adult age; 33.3% and 25.5% correspondingly. Vaccinated duck flocks showed a higher detection rate (34.8%) of DEV than that of non-vaccinated ones (24.4%).

Table 1: Mortalities and pathological findings in the positive DEV flocks in Egypt, during 2016-2018

No	Age / day	Mort. rates (%)	Drop in Egg prod. (%)	Clinical signs				Gross lesions				
				Diarrhea	Ophth. signs	Prol. penis	Hg's at the esophago-proventricular sphincter	Swelling and hemorrhagic intestinal annular bands	Hg's enteritis	diphtheritic mucosal lesions	Hg's on Heart	Lesion on Liver
1	40	10%	-	+	+	-	+	-	-	-	+	Hg's
2	45	75%	-	+	-	-	-	+	+	+	-	Hg's
3	250	10%	-	Greenish	-	-	-	+	-	-	-	Nec.
4	258	4%	20%	+	-	-	-	+	-	-	+	Hg's
5	61	24%	-	Bloody	-	-	-	+	+	-	+	-
6	270	6%	-	+	-	-	-	+	-	-	+	Hg's
7	240	21%	-	+	-	+	-	+	-	-	+	Nec.
8	120	30%	-	+	+	-	-	+	-	-	+	-
9	250	8%	-	-	-	+	-	+	-	-	-	-
10	260	2%	40%	Bloody	-	-	-	+	+	-	-	-
11	38	15%	-	Bloody	+	-	-	+	+	-	+	Hg's
12	30	20%	-	+	+	-	-	+	-	-	-	-
13	398	1%	20%	+	-	-	+	+	-	-	-	-
14	329	1%	20%	Greenish	-	+	+	+	-	-	-	Nec.
15	60	2%	-	Bloody	+	-	-	+	+	-	-	-
16	35	15%	-	Bloody	+	-	-	+	-	-	+	-
17	50	3%	-	Bloody	+	-	-	+	+	+	-	Nec.
18	65	10%	-	Bloody	+	-	-	+	+	-	-	Nec.
19	30	2.8%	-	Bloody	+	-	-	+	+	+	+	Hg's

Prod.: Production; Mort.: Mortality; Ophth.: Ophthalmic; Prol.: Prolapsed; Hg's: Haemorrhages; Nec.: Necrosis

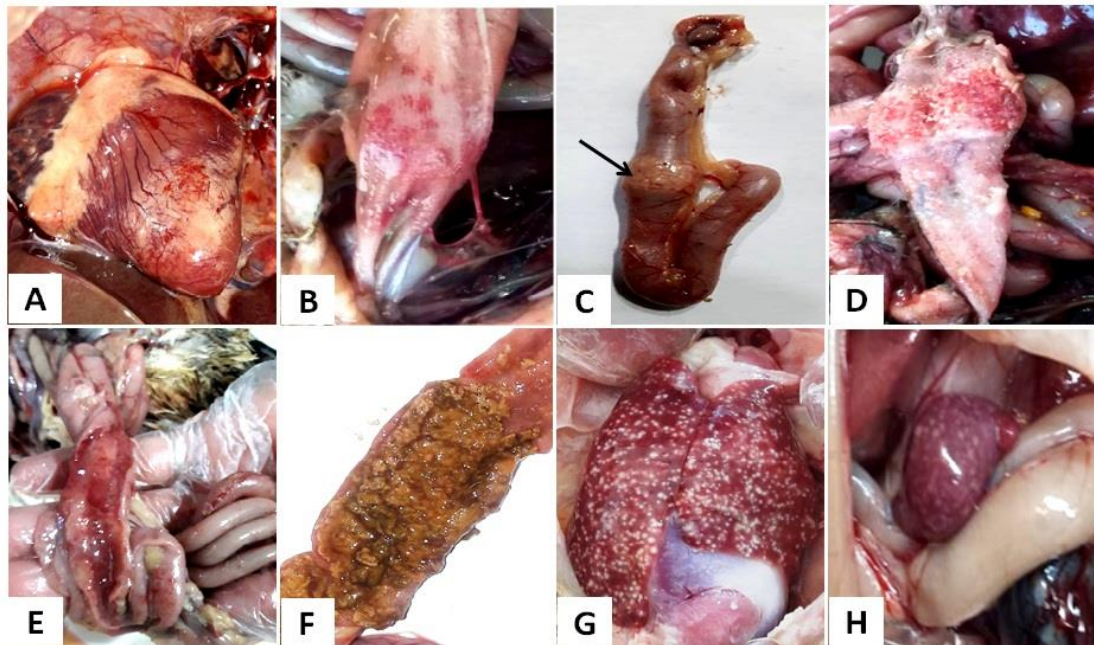


Figure 2: Ducks infected with duck enteritis virus

(A) Haemorrhages on epicardium. (B) Haemorrhages at the esophageal-proventriculus junction. (C) Distinct necrotic intestinal annular bands/ necrosis overlaying annular lymphoid bands. (D) Hemorrhagic intestinal annular bands. (E) Haemorrhages on intestinal mucosa. (F) Diphtheritic mucosal lesions in intestine. (G) White necrotic areas on surface of liver. (H) White necrotic foci on surface of spleen.

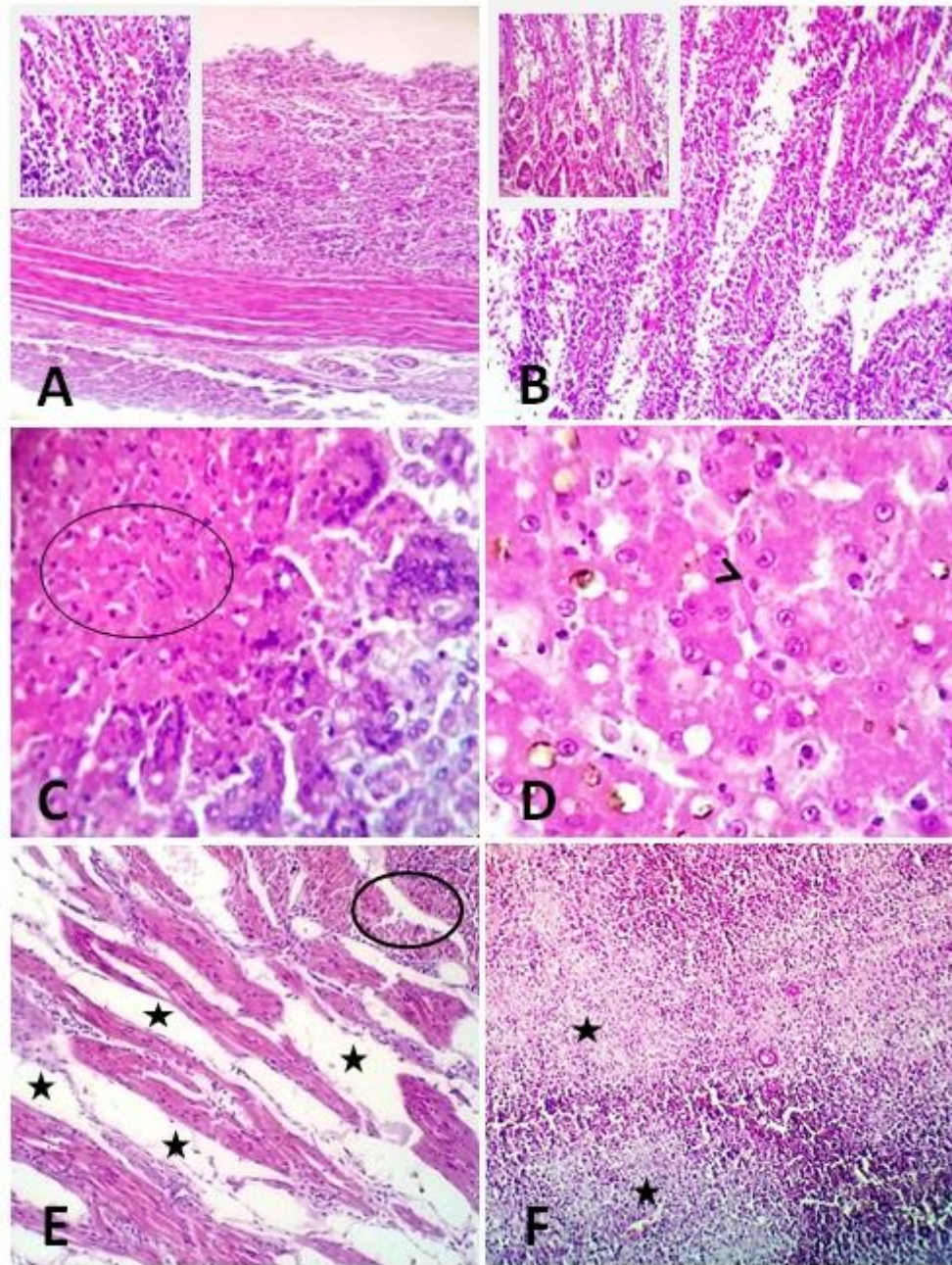


Figure 3: Microscopic lesions of infected ducks with duck enteritis virus

(A) Duck intestine showing massive necrosis of the intestinal mucosa including the villi and the crypts with the presence of some intact and partially distracted intestinal glands, the intestinal lumen containing large number of necrotic cells and leucocytes, the mucosa and submucosa are massively infiltrated with lymphocytes and plasma cells, the intestinal capillaries all over the intestinal wall are hyperemic, the muscular coat showed congestion, hyaline degeneration, edema and leucocytic infiltration, H&E X100; Inset: Higher magnification, H&E X400. (B) Duck intestine showing necrotic enteritis, H&E X100; with partial destruction of intestinal crypts and intestinal glands, Inset: Higher magnification, H&E X400. (C) Duck liver showing necrosis of the hepatic tissue (circle), H&E X400.. (D) Duck liver showing degenerative change mainly cloudy swelling, vacuolar degeneration in addition to eosinophilic IN/IB inclusion bodies (arrow head). H&E X400. (E) Duck heart showing focal coagulative necrosis of cardiomyocytes (circle) and interstitial edema (star). H&E X400. (F) Duck spleen showing multifocal necrosis of the lymphoid elements of the white pulp which partially replaced by histocytes (stars). H&E X100

Table 2: Molecular detection of DEV in duck farms in Egypt during 2016-2018

Criteria	Flocks Number	Number of Positive samples (%)	95% CI
Provinces			
Sharkia	15	6 (40)	16.3- 67.7
Dakahlia	36	8 (22.2)	10.1- 39.1
Gharbiya	13	4 (30.8)	9.1- 61.5
Qalubiya	2	0 (0)	0
Damietta	1	0 (0)	0
Port-Saied	1	1 (100)	2.5- 100
Year			
2016	6	2 (33.3)	43.3-77.7
2017	60	16 (26.7)	16.1-39.7
2018	2	1 (50)	1.3- 98.7
Season			
Winter	46	13 (28.3)	15.9- 43.5
Spring	17	2 (11.8)	1.5- 36.4
Summer	2	2 (100)	15.8- 1.0
Autumn	3	2 (66.7)	9.4- 99.2
Flock Category			
Backyard flocks	7	2 (28.6)	3.7- 70.9
Commercial flocks	61	17 (27.9)	17.1- 40.8
Breed			
Native	12	3 (25)	54.9- 57.2
Muscovy	42	15 (35.7)	21.5- 51.9
Mallard	3	0 (0)	0
Pekin	11	1 (9.1)	0.2- 41.3
Age (weeks)			
Ducks ≤ 7 weeks	21	7 (33.3)	14.6- 56.9
Ducks > 7 weeks	47	12 (25.5)	13.9- 40.2
Immunity status			
Vaccinated	23	8 (34.8)	16.4- 57.3
Non-vaccinated	45	11 (24.4)	12.9- 39.5

Note: the detection rate and 95% CI were computed using WinPepi software, version 11.65



Figure 4: The trend of duck enteritis virus isolation in different Egyptian provinces and the analyzed case number (n/n) during 2016-2018

Discussion

Duck enteritis virus is an important pathogen of ducks that has a serious impact on the duck industry worldwide as a result of mortality and low egg production rates (18). Controlling of DVE is considered as one of the largest challenges facing poultry industry (24, 41). Thus, it is important to investigate the current situation of the disease in Egypt. In this study, the diagnosis of DEV infection was confirmed by histopathological examinations, clinical and pathological features, isolation and molecular detection, and thorough analyses of epidemiological data.

The clinical signs and post mortem lesions findings confirmed the suspicion of DEV infection as previously mentioned (24, 42-45). Among the examined duck flocks, the recorded mortality rate was up to 75% and the drop in egg production was about 20-40%. These findings were consistent with Sandhu and Shawky (23) and Sarker (46) who recorded high mortality (60%–90%) and drop in egg production (25%–40%) in DEV infected flocks. The highest mortality rate of 75% was detected in young age (45-days-old) ducklings which are inconsistent with Campagnolo et al (15) who mentioned that high mortality rate of DVE was detected in adult ducks. As DEV has an immunosuppressive effect, so the secondary bacterial infections such as *Escherichia-coli*, *Riemerella anatipestifer* and *Pasteurella multocida* were identified in young ducklings (42). Moreover, other virus such as paramyxovirus 6 was found as another complicating factor that may induce high mortality with a percentage of 75% (47).

The most prominent detected pathological lesion was hemorrhages; this may be attributed to the affinity of DEV to replicate in the vascular endothelial cells of small blood vessels, capillaries and venules causing severe hemorrhages, eruptions, and degenerative alterations of internal organs (10). As well, most of the pathological lesions were detected in the intestinal tract and liver. Primarily, DEV replicates in the digestive tract mucosa and then spreads into other organs (29, 48, 49).

In the histopathological examination, eosinophilic intranuclear inclusion bodies were detected in the hepatocytes. From a hallmark feature of the infection with herpes virus including DEV is the presence of intra-nuclear inclusion bodies in the infected cells (15, 16, 44, 50, 51). The finding of

lymphoid tissue depletion and multifocal necrosis in the spleen was compatible with other studies which mentioned that the main targets of DEV infection are the immune organs resulting in severe immunosuppression (3, 52, 53).

The characteristic pathological findings are helpful in the preliminary diagnosis of DVE. However, these lesions should be distinguished from other duck diseases producing similar changes in Anseriformes (54-56). Confirmatory diagnosis in this work included isolation DEV in embryonated Muscovy duck eggs and identification using PCR. Twenty nine (42.6%) of the examined samples induced characteristic lesions in the inoculated embryos and on the CAM. Similar embryonic and CAMs findings were previously reported (46, 57, 58). Embryonic deaths were recorded after 4-6 days of inoculation with a percentage of 42.7% and this indicated that not all DEV strains are lethal for the embryos (59).

Herein, PCR was used for the detection of DEV in the inoculated CAM. By using a specific primer for the DNA polymerase gene, the results showed presence of DEV in 19 flocks (27.9%). Negative DEV detection was recorded in 10 samples, although pathological lesions were demonstrated in the inoculated eggs. This could be explained by presence of other duck diseases that produce similar pathological lesions in the inoculated egg embryos such as duck hepatitis virus, Reovirus and parvovirus (60-62). Moreover, there were reports indicated presence of some other diseases may be caused by herpes virus infection in waterfowl (63).

The infection rate of duck viral enteritis was 33.3% in duckling less than 7 weeks of age and 25.5% in ducks more than 7 weeks of age. Many authors recorded consistent results and found that all ages of ducks (from 7 days of age to adulthood) are susceptible to DVE (23, 24, 36, 44, 64). Due to the lack of vaccination programmes in duck flocks under 7 weeks of age, higher infection rate was common in young ducks than in adults.

Seasonal variation of DVE outbreaks was also recorded as 66.7% in autumn to 100% in summer. It has been shown that ducks reared under high temperature and humidity during the summer season; become stressed and highly susceptible to infectious diseases such as DVE (65). Besides, the autumn is considered as an important

seasons for migratory birds in Egypt, since, these migratory waterfowls play an important role in the spread of this disease (6, 7, 30, 66).

All members of the family Anatidae are likely susceptible to infection with DVE, but not all species are equally responding. The highest infection percentage (35.7%) rate was detected in Muscovy duck flocks as authorized by (16, 67). However, no virus was detected in Mallard breed flocks. Wobeser and Docherty (68) proved that the Mallard breed of ducks is more resistant to lethal effects and is thought to be a natural reservoir for DEV infection.

Regarding the results of the rearing system, the rate of infection with DEV was 28.6% in the backyard and 27.9% in the commercial flocks. In the backyard flocks, all types of poultry are kept at the same area with a lack of biosecurity measures or vaccinations. Additionally, this sector of rearing has several chances to contact with wild birds. Hoque et al. (26) and Khan et al., (65) considered that all of the above mentioned factors are significant for duck plague outbreaks and transmission.

Immunization of ducks is an efficacious way to prevent DEV infection and attenuated live vaccine that commonly used, can provide a good protection (41, 69). In the same line, our results revealed that the detection rate of DEV was 34.8% in vaccinated ducks, while it was 24.4% in non-vaccinated flocks. According to the history of flocks, there were improper immune procedures. Bordolai et al. (70), Wang et al. (7) and Dhama et al. (24) mentioned some causes of vaccination failure against DVE like vaccine dose inconsistencies, lack of the biosecurity practice in duck farms and the presence of other immune-suppressive diseases that increases the susceptibility of ducks to DEV infection after vaccination.

Duck viral enteritis is an important and prevalent disease in Egypt. The major thrust of activities to prevent and control of such infection are taking some necessary steps such as improving the diagnostic techniques, enhancing the appropriate biosecurity measures and using of effective vaccines. Consequently, we still need to determine the virulence of the circulating DVE viruses and their sequences as a step to develop an efficient vaccine.

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