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Isolation and Molecular Detection of Duck Viral Hepatitis

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Abstract: A total of nine liver samples were collected from different duck breeds (average age 2-21 days) had clinical suspicion of infection with duck hepatitis virus (DHV) from six different governorates in Egypt at period started from September 2014 to February 2016. The liver samples were processed for isolation of virus in specific pathogen free embryonated chicken eggs (SPF ECEs). Histopathological examination for embryonic livers of inoculated samples showed pronounced hyperemic blood vessels, dilated sinusoids with haemolysed RBCs, vacuolar degeneration, hepatocellular necrosis, perivascular infiltration with granulocytes and perivascular amyloidosis. Liver impression smears collected from the field cases were examined with indirect immune fluorescence antibody test (IIFAT) by using rabbit anti-duck hepatitis virus serum. The seven out of nine samples showed positive results appeared as green apple fluorescent coloration of DHAV antigen in hepatic cells. DNA fragment of 467 bp was amplified 3D gene by one-step RT-PCR using RNA extracted from livers of the collected samples. Seven positive lanes were obtained at 467 bp. One step real-time RT-PCR with the use of TaqMan probe whereas the primers were designed to detect the conserved region in the 3D gene that encodes the RNA dependent RNA polymerase. One step real-time RT-PCR (one-step rRT-PCR) revealed five positive samples. This paper confirmed that the one step methods (one-step RT-PCR and one-step rRT-PCR) were able to selectively recognize ducks infected with DHAV. So, the use of two tests will be a powerful tool for detection of suspected cases of DHAV and molecular epidemiological screening. This paper demonstrated that the two PCR methods were reliable and accurate tools for detection of field exposure with DHAV.

Key words: DHAV · SPF · Histopathology of embryonic liver · IIFAT · One step RT-PCR and one step real-time RT-PCR

INTRODUCTION

Duck hepatitis virus (DHV) was described firstly in Long Island, New York in 1949 [1]. The disease is an acute, contagious, rapidly spreading and highly fatal for young ducklings. The disease causes significant economic losses in duck raising farms as a result of high mortality and condemnations [2]. The disease is caused by three heterologous serotypes of duck hepatitis virus (DHV- 1-3) but no antigenic relationships are found among these serotypes [3-6]. DHV-1 is the most virulent and most common serotype that spread worldwide while DHV-2 and DHV-3 are reported from the UK and in the USA, respectively. Both DHV-2 and DHV-3 were identified later as astrovirus, known as duck astrovirus type 1 (DAstV-1) and duck astrovirus type 2 (DAstV-2) respectively [7]. DHV-1 is a small, non-enveloped virus with a positive-sense, single-stranded RNA genome of approximately 7.8 kb [8-11]. Currently, DHV-1 is renamed duck hepatitis A virus (DHAV) and is assigned to the novel genus Avihepatovirus within the family Picornaviridae [12]. DHAV is divided genetically into

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three different genotypes found in south Asia: the original genotype DHAV-1, a new genotype DHAV-2 isolated in Taiwan and a new genotype DHAV-3 isolated in South Korea and China [13, 14].

The disease is characterized by sudden onset and the affected ducklings fall on their sides, causes high losses close to 100 % within 3-4 days in a flock of ducklings up to 3 weeks old, kick spasmodically with both legs and die with heads in opisthotonos position. Pale swollen liver and hemorrhages are typical gross lesions of disease in affected ducklings in addition to swollen kidney and spleen [5].

The typical clinical signs may appear or sudden death may occur within 24 hr after infection. So, both prevention and rapid diagnosis are the logical and most effective strategies for control of the disease. DHAV is a positive, single stranded RNA virus containing a single, long open reading frame that encoding a polyprotein of more than 2000 aa. This long open reading frame is preceded by a 5' untranslated region, followed by a short 3' untranslated region and a poly (A) tail. This polyprotein is processed into a leader protein (L), four structural proteins, (1A, 1B, 1C and 1D) and seven non-structural proteins, (2A, 2B, 2C, 3A, 3B, 3C and 3D) [8, 9, 11, 15].

Experimentally, DHAV can be transmitted by parental or oral routes, but egg transmission has not been occurred yet. The virus is rapidly propagated in chick and duck embryos [5]. Recently, DHAV was only associated with causing disease in Mallard and Pekin ducklings but now, it is reported to cause pancreatitis and encephalitis in Muscovy ducks [16]. The diagnosis of DHAV is usually based on epidemiological information, clinical signs, pathological changes and viral isolation. Conventional detection methods include direct fluorescence-antibody technique on livers obtained from naturally occurring cases or on duck embryos inoculated with DHAV [17, 18] and the methods for detection of antibody against DHAV in duck sera include neutralization tests [19, 20] indirect hemagglutination tests [21] gel diffusion assays [22] and ELISA [23]. These assays are time-consuming and don't provide the needed sensitivity to detect low-level of virus [24].

Nucleic acid-based assays such as reverse transcriptase-polymerase chain reaction (RT-PCR) [25, 26] and multiplex RT-PCR [27] can detect DHAV RNA either directly from samples or from viral isolates without standardized serological reagents. But, none of them show a reliable evaluation on accumulation of virus in infected tissues. Real-time RT-PCR assays (rRT-PCR) [28, 29] provide high sensitive, reproducible and specific results in addition to quantifying the viral genomic copies number.

In the late 1970, the virus appeared in Egypt [30] but the disease situation is little known [31]. Recently, epidemiology and molecular characterization of DHAV from different duck breeds in Egypt was studied by Erfan *et al*. [32] and Bayoumie and Abd EL-Samie [33]. DHAV usually affects ducklings under 6 weeks of age. The morbidity is 100% and the mortality may reach 95-100 % in the first week of age [34].

This study was aimed to confirm virus exposure in ducks breed in Egypt so we isolated DHAV from ducklings with a history of high mortality from several provinces using specific pathogen free embryonated chicken eggs (SPF ECEs) then identified by IIFAT and confirmed by RT-PCR and real-time RT-PCR. For detection of suspected cases of DHAV, different diagnostic methods could be reliable in monitoring DHAV outbreaks.

MATERIALS AND METHODS

Collection of Samples: Nine samples were collected from different duck breeds in 9 commercial farms in Egypt, from six governorates as shown in Table 1. These commercial duck farms had a history of nervous signs and high mortality rate during the first 3 weeks of life.

Preparation of Tissue Sample Suspension: Liver tissue have been collected from freshly dead or ducks showed clinical sings, homogenized in saline containing 2000 iu/ml Penicillin and 200 mcg/ml Streptomycin. These organs were ground making homogenized (20% W/V), then centrifuged at 3000 rpm for 15 minutes. After centrifugation the clear supernatant fluid put at -20°C until used [35].

Table 1: Collected samples from commercial duck farms in Egypt from September 2014 to February 2016

Sample No.	Governorate	Age	Breed	Time period
1	Sharkia	3 days	Mallard ducks	September 2014
2	Gharbia	7 days	Pekin ducks	November 2014
3	Gharbia	11 days	Pekin ducks	November 2014
4	Gharbia	14 days	Pekin ducks	November 2014
5	Gharbia	10 days	Mallard ducks	June 2015
6	Menoufiya	21 days	Muscovy ducks	October 2015
7	Assiut	5 days	Pekin ducks	January 2016
8	Qaliubiya	2 days	Mallard ducks	January 2016
9	Beni Suef	15 days	Hurcara ducks	February 2016

Egg Inoculation: According to OIE [31] 20 % liver suspension was inoculated into allantoic sac of SPF embryonated chicken eggs (aged 8-10 days); 0.2ml /embryo using standard techniques of embryo inoculation. Each of inoculated embryos was monitored for embryopath daily for 5-8 days.

Vaccine Strain: Local strain of duck virus hepatitis obtained from Veterinary Serum &Vaccine Research Institute- Abbassia- Cairo- Egypt.

Production of Duck Virus Hepatitis Antiserum: Anti-duck hepatitis antiserum was produced in rabbits. The vaccinal strain 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 hepatitis antibody using serum neutralization test (SNT). Serum was harvested when titer was high. This hyper immune serum was used in IIFAT [36]. The secondary antibody used: KPL (Fluorescein-Labeled Antibody to rabbit IgG (H+L) produced in goat. Catalog No.172-1506.

Histopathological Examination: Livers from embryonating chicken eggs of inoculated 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 (H&E) [37].

Staining of Impression Smears: Impression smears were prepared from livers taken from field samples. Stain using indirect immune fluorescence antibody test (IIFAT). Smears were stained using rabbit anti-duck hepatitis virus polyclonal serum and an anti-rabbit FITC conjugate according to Hanaa *et al*. [38].

Detection of RNA of Avihepatovirus causing Duck Hepatitis Virus

RNA Extraction Methods: Liver suspensions supernatants were treated with the Patho Gene-spin™ DNA / RNA Extraction Kit following the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea). The nucleic acids are used for one step RT-PCR and one-step real-time RT-PCR. After measuring RNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE), the samples were stored at –20°C [31].

Primers Design: The one-step RT-PCR is conducted using the specific primer (DHV-1 ComF and DHV-1 ComR) DHV-1 ComF (5'-AAG-AAG-GAG-AAA-ATY-[C or T]-AAG-GAA-GG-3') DHV-1 ComR (5'-TTG-ATG-TCA-TAG-CCC-AAS- [C or G]-ACA-GC-3') Flanked by a 467 bp DNA sequence in the 3D gene [31].

In one-step rRT-PCR, the primers P3, P4 and probe (FP) were designed for amplification of the conserved region of 3D gene for DHAV isolate Sichuan using the primer express 3.0 software (Applied Biosystems, USA). The rRT-PCR product of 3D gene produced by P3 and P4 was 87 bp, covering the nucleotides 65-151 of DHAV isolate Sichuan.

P3: 5'-TGATGAGATATGGCAGGTAGAAGGA-3'
P4: 5'-CACGCAAGCTGATTCACAATAGA-3'. The Taq Man™ probe (FP) was labeled with the 5' terminal reporter dye 6-carboxyfluorescein (FAM) and the 3' quencher dye 6- carboxytetramethyl-rhodamine (TAMRA), FP: 5' FAM-TGTGTTCAGGATCCCCATGTACTACCGTG-TAMRA-3' [28].

One-Step RT-PCR: The one-step RT-PCR is conducted using the 20µl reaction mixtures. DHV-1 ComF and DHV-1 ComR, a T-gradient thermal cycler (Biometra, Gottingen, Germany) was used for one-step RT-PCR. Reverse transcription is performed at 45°C for 30 minutes, after which the enzyme is inactivated at 94°C for 5 minutes. PCR amplification is conducted using an initial denaturation for 20 seconds at 94°C; followed by 40 cycles of annealing for 30 seconds at 52°C, extension for 30 seconds at 72°C, denaturation for 20 seconds at 94°C; final extension for 5 minutes at 72°C, reactions were stored at 4°C [31].

One-Step Real-Time RT-PCR: One step real-time RT-PCR was performed in a 20 µl reaction mixture. The reactions were carried out in a AB Applied Biosystems (step one real-time RT-PCR System). One-step real-time RT-PCR conditions consisted of 10 sec at 45°C, 2 min at 95°C followed by 45 cycles of 5 sec at 95°C, 20 sec at 54°C and 20 sec at 60°C [28]. The one step real-time RT-PCR data acquisition and analysis were performed using Analysis computer system, V 2.2.2 software (AB Applied Biosystems).

RESULTS

Inoculated Eggs: Chicken embryos were more variable and erratic in their response and usually take from 5 to 8 days to die. There are 7 out of 9 inoculated samples (No. 1, 2, 3, 4, 6, 7 and 8) showed gross pathological changes as stunting embryos with subcutaneous hemorrhages covering the body and oedema in the hind limb and

abdominal regions. The embryonic livers were swollen, yellow or red in color, also showed some necrotic foci. In case of embryos that take longer to die, the allantoic fluid color changed to green, but both stunting and liver lesions become more evident.

Histopathological Findings: The liver of 7 out of 9 samples of inoculated embryos showed pronounced hyperemic blood vessels in addition to dilated sinusoids with hemolysed RBCs. Hepatocellular necrosis in addition to vacuolar degeneration was observed associated with perivascular infiltration with granulocytes. Also, perivascular amyloidosis was appeared as shown in Fig. (1-4). The positive samples are No. 1,2,3,4,6,7 and 8.

Immunofluorescence Findings: The Indirect Immunofluorescence of impression smears of hepatic cells showing a green apple fluorescent coloration of DHAV antigen in hepatic cells as shown in Fig. (6 and 7). This green apple fluorescent coloration was seen in 7 out of 9 examined samples. The positive samples No. are 1, 2, 3, 4, 6, 7 and 8.

One-Step RT-PCR: A DNA fragment of 467 bp was amplified 3D gene by one-step RT-PCR using RNA extracted from the livers of collected samples. The detection limit of this part for general detection of common DHAV, as shown in Fig. (8), positive lanes are 1, 2, 3, 4, 6, 7 and 8 at 467 bp.

One-Step Real-Time RT-PCR: The primers P3, P4 and the TaqMan™ probe (FP) were designed based on the conserved region of 3D gene for DHAV isolate Sichuan. The positive control cut threshold at cycle 26. The positive samples No. are 3, 6, 1, 4 and 8 which cut threshold at 6, 7.5, 9, 36, 37 cycles on respective as shown in Fig. (9).

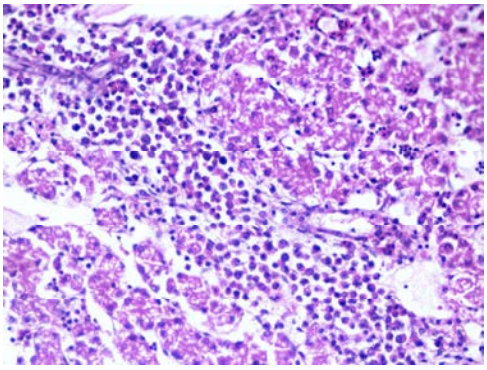

Fig. 1: Liver of embryo showed heterophils infiltration (H&E, X400)

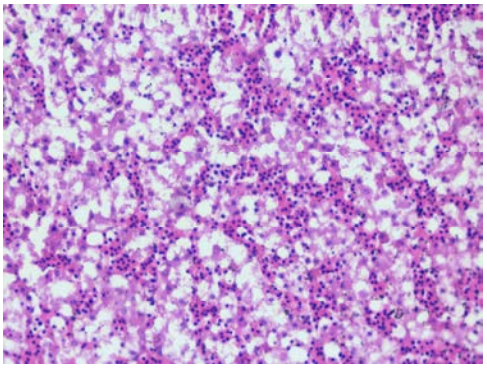

Fig. 2: Liver of embryo showed congested blood vessels and vacuolar degeneration (H&E, X400)

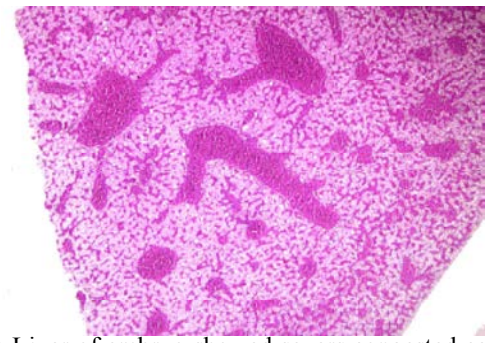

Fig. 3: Liver of embryo showed severs congested central vein sinusoids (H&E, X50)

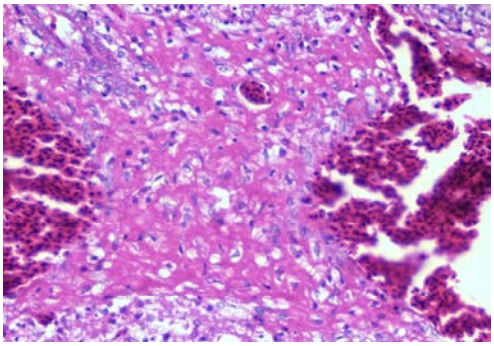

Fig. 4: Liver of embryo showed amyloidosis (H&E, X400)

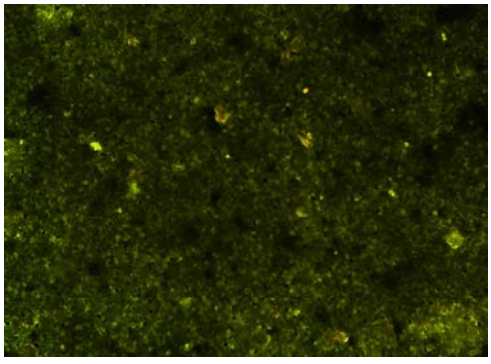

Fig. 5: Immunofluorescence of liver of negative control group showing no signal (-ve)

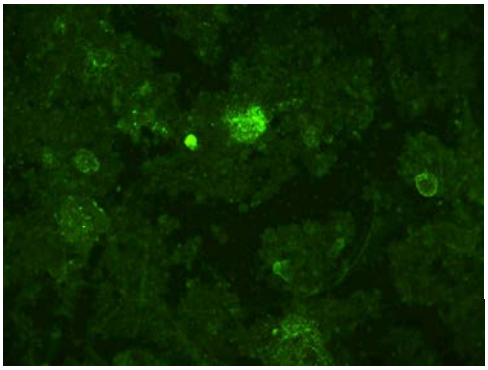

Fig. 6: Impression smears of liver sample No. 7 showing green apple signal in the hepatic cells

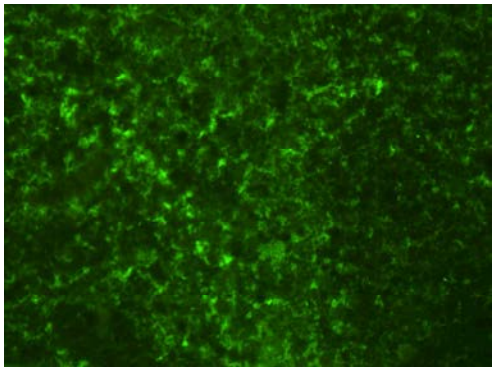

Fig. 7: Impression smears of liver sample No. 8 showing green apple in the hepatic cells

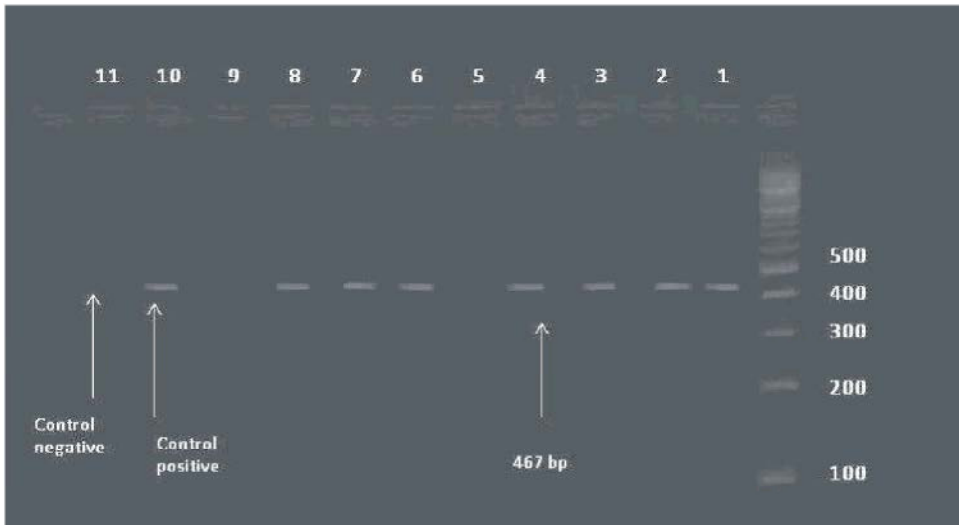

Fig. 8: PCR picture of 9 field samples, showed positive lanes 1, 2, 3, 4, 6, 7 and 8 at 467 bp

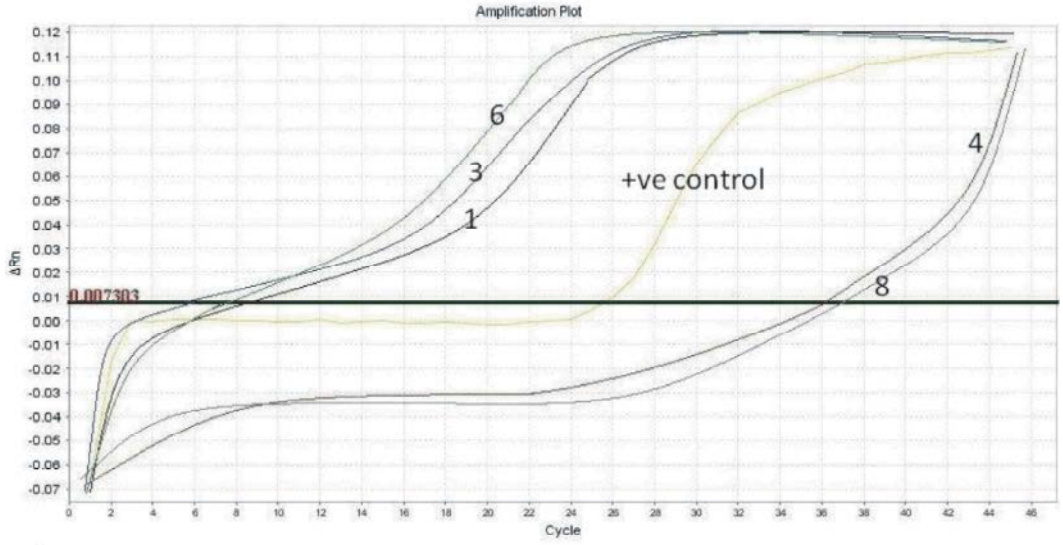

Fig. 9: Amplification curve and corresponding cycle threshold (CT) values of extracted RNA from examined livers sample

DISCUSSION

Traditionally, classification of DHV strains has been divided into three serotypes: DHV-1, DHV-2 and DHV-3. The viruses DHV-2 and DHV-3 are members of Astrovirus [7, 9]. DHV-1 causes acute hepatitis with high mortality in ducklings. This disease was first reported at 1949 in Long Island, but later, it had a worldwide outbreak. DHV-1 is an important serotype in the duck- producing areas worldwide as it causes high mortality (More than 80%) in ducklings less than 3 wk old [39]. Originally, DHV-1 was classified as an enterovirus and recently it is reclassified as a member of family Picornaviridae, genus Avihepatovirus. DHV-1 is renamed DHAV according to International Committee on Taxonomy of Viruses. Three variants of DHAV are found in south Asia based on phylogenetic analyses and neutralization tests: DHAV-1 (the classical serotype 1), DHAV-2 (isolated in Taiwan) and DHAV-3 (isolated in China and South Korea) [10, 14, 40].

In Egypt, the cause of ducks infection with DHAV is not well known. It may be due to antigenic variation in field virus, thus the used vaccine cannot produce good protection or due to low maternal antibody titer. So, studies on virus antigenic characterization is important to assess good protection. Nucleotide homology of the Egyptian strains with the 3D of the local strain of DHAV (vaccine strain) was 95-95.9% and also 98% identity to the closest Asian strains [32]. The GxxCSGxxTxxNS motif, highly conserved region in the 3D of all picornaviruses, is present in the Egyptian strains also like other DHAVs [41].

Diagnosis of DHAV may be confirmed by high mortality and rapid onset of clinical signs in susceptible ducklings, inoculation of tissues homogenate into the allantoic sacs of embryonated duck eggs (EDEs) and embryonated chicken eggs (ECEs) or infection of cell culture [31]. In contrast to the laborious and time-consuming viral isolation and serological tests [20, 23] rapid and specific detection of the DHAV RNA using different RT-PCR assays were developed [10, 13, 25, 26, 31].

In this study, we collected nine liver samples from duck farms with different locality (7 samples from 4 central delta governorates and 2 samples from 2 south delta governorates), different breeds as Pekin, Mallard, Muscovy and Hurcara, also with different ages (from 2 days and up to 21 days) with clinical sings of duck hepatitis virus infection (sudden death within 24 hr after infection with lethargy, weakness, lateral recumbency and opisthotonos), at period started from September 2014 to February 2016. So, we found that DHAV infections were detected all over the year in different localities of Egypt, under 3 weeks age and varied duck breeds and these agreed with that reported by Haider and Calnek [4], OIE [31] and Jin *et al*. [39]. Our results found that gross lesions were restricted to the liver; which was swollen with multiple punctuate hemorrhages. The liver samples were processed for virus isolation in specific pathogen free embryonated chicken eggs (SPF ECEs). Histopathological examination of emberyonic livers of inoculated samples showed pronounced hyperemic blood vessels, dilated sinusoids with hemolysed RBCs, vacuolar degeneration, hepatocellular necrosis, perivascular infiltration with granulocytes and perivascular amyloidosis. These results were parallel to those reported by Fitzgerald *et al*. [42] and Sheng *et al*. [43].

In our study diagnosis using indirect immunofluorescence antibody test (IIFAT) on hepatic cell impression smears stained with rabbit anti-duck hepatitis polyclonal serum and an anti-rabbit FITC conjugate; we found that seven out of nine samples showed positive result as green apple fluorescent coloration for duck hepatitis virus antigens [18, 33, 38].

The advantage of one step RT-PCR assay is that it can be used for DHAV RNA detection during outbreak before the immune response development. Also, the result of RT-PCR appears within 3-5 hours although other conventional laboratory detection methods as serological tests or virus isolation can take several days [27]. In this study, seven out of nine samples were positive at 467 bp, lanes No. 1, 2, 3, 4, 6, 7 and 8. The amplified 3D gene fragment using RNA extracted from the livers of collected samples and the detection limit of this part is for general detection of common DHAV.

One step real-time RT-PCR systems were improved by the development of fluorogenic labeled probes which use the 5′ nuclease activity of TaqMan DNA polymerase. The fluorogenic probes availability enabled the real-time method for detecting specific products after amplification. Thus, one step real-time RT- PCR with high sensitivity and specificity for detection of DHAV was applied according to designing a specific probe and a pair of primers based on the highly conserved region of the DHAV 3D gene that encodes RNA-dependant-RNA polymerase. The conditions of the real-time RT-PCR were designed for obtaining highly specific fluorescent signals [28, 29].

In this study, one-step real-time RT-PCR that applied using primers P3, P4 and probe FP revealed five positives out of nine tested samples. The positive control cut threshold at cycle 26, whereas the positive sample No. are 3, 6, 1, 4 and 8 cut threshold at 6, 7.5, 9, 36, 37 cycles on respective.

This difference of results between one-step RT-PCR and one-step rRT-PCR may be due to genetic diversity between isolates so missing of (primer or probe) target homology in one-step rRT-PCR leading to false negative results [44] as the used primers (P3 and P4) and TaqMan TM probe (FP) in this study for rRT-PCR were designed according to the result of sequencing DHV-1 isolate Sichuan that obtained by the Avian Disease Research Center of Sichuan Agricultural University (GenBank accession No. EF064886). Bayoumie and Abd EL-Samie [33] reported that the resemblance % ranging between (63.1-63.8%) in sequence and phylogenic analysis of 3D gene of DVH/EG.Bayoumieh-Sharkia-2015 isolate compared to the Egyptian isolates of Erfan *et al*. [32]. So, the cause of these obtained results requires further study to be determined through sequence analysis.

In conclusion, although impression smear of IIFAT is economic, sensitive, specific and able to detect DHAV rapidly than virus isolation, but also RT-PCR and rRT-PCR provide a potentially powerful technique in microbiological diagnostics, rapid, specific and sensitive tool for detection of suspected cases of DHAV in addition to molecular epidemiological screening.

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