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Some Microbial Causes of Mortality in Rabbit in Northwest of Delta, Egypt

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Abstract

This study aimed to investigate pathogenic microbial causes of mortalities in twenty rabbit flocks located in 4 governorates in Northwest of Delta Egypt during a period from 2019 to 2022.The mortality rate was varied from 10 to 90 % in either sex of different breeds and ages (15 day-18 month). Ten flocks (50%) were positive for Rabbit hemorrhagic disease virus (RHDV) by HA and RT-PCR. Phylogenetic analysis of the *Vp60* gene indicated one sample (MW455128) was classic RHDV and eight strains (MW455120 - MW455127) were variant RHDV2.Enteropathogenic *Escherichia coli* (EPEC) was the predominant isolated bacteria (60%), belonging to various serotypes (poly 1, O26 K60, poly 2, O55 K59, poly 2, O126 K71, poly 1, O111, and poly 3, O114), followed by *K. pneumoniae* (35%), which was determined to be virulent by PCR detection of the *Uge* gene (80%) and *rmpA* (40%), while the *kfu* gene was absent. *Staph. aureus* and *Pasteurella multocida* represented (30%) for each, while *pseudomonas aeruginosa* (20%) and *Salmonella* was negative. Pathologically, apoptosis was the most prominent lesion observed in liver, kidney, intestine, spleen, lung and heart, also there were severe enteritis, abscess in lung, liver and kidney and suppurative bronchopneumonia. By immunohistochemistry the RHDV antigen was detected in hepatic, splenic, renal, pulmonary, and cardiac tissues. The findings of this investigation highpoints on the significance of RHDVs (classic G3-G5 and variant RHDV2 which become more predominant), EPEC, *K. pneumonia*, *S. aureus*, *P. multocida* and *P. aeruginosa* as rabbit pathogens causing mortalities in studied Egyptian provinces. So that, these results are important in any effort to control rabbit pathogens in Egypt.

KEYWORDS
Escherichia coli, *K. pneumoniae*, Pathological finding, Rabbit hemorrhagic disease virus (RHDV)

INTRODUCTION

Bacterial, viral, parasitic, and mycotic infections are the main reasons behind rabbit deaths (Eid and Ibraheem, 2006). Rabbit hemorrhagic disease virus (RHVD) is among the most common viral causes of mortality in rabbits, its morbidity reached 100% and mortality 70–90%, and characterized by sudden deaths, fever, dyspnea, abdominal respiration, and frothy bloody nasal discharges (Guittre *et al*., 1995; OIE, 2000).

The causative agent is RHDVs. It is a lago viruses within the Family Caliciviridae, the disease was initially discovered in 1984 in China and quickly spread to other countries of world. Phylogenetic analysis of pathogenic RHDV strains shows three main groups: ''classical'' RHDV with the genogroups G1–G5, the antigenic variant RHDV a/G6 (Le Gall *et al*., 2003), and the new type RHDV2/RHDVb (Le Gall *et al*., 2013).This new type known as RHDV2 or RHDVb, generate a disease very identical to that induced by RHDV strains and it became also known as Lagovirus europaeus GI.2 (Carvalho *et al*., 2017). RHDV2 emerged in France (Le Gall *et al*., 2011) then spread rapidly across Europe (Almeida *et al*., 2015) and was detected in Australia in 2015 (Hall *et al*., 2015) and in Canada in 2016 (World Organization of Animal Health, 2016). RHDV2 is now regarded as endemic in Europe (Mahar *et al*., 2018). The first case of RHDV in Egypt was noted in Sharkia governorate in 1991, then spread to other Egyptian governorates (Ghanem and Ismail, 1992). Recently, natural recombinant RHDV2 strains were discovered to have the structural proteins of RHDV2 (*Vp60* and minor protein-encoding genes). The genomic structures of RHDV and RHDV2 have similar arrangement, and both include two open reading frames (ORFs). ORF1 encodes the nonstructural proteins (RNA-dependent RNA polymerase and the major capsid protein *Vp60*), while ORF2 encodes a minor structural protein called vp10 (Dalton *et al*., 2015). So the *Vp60* gene was then chosen for detection of caliciviruses by RT-PCR (Le Gall *et al*., 2017), and detection of lagoviruses by developing the universal RT-PCR method using primers that span a highly conserved region (Strive *et al*., 2009).

Numerous clinical disorders caused by the bacterial infection result in rabbit deaths particularly enteric pathogens, due to various reasons as undeveloped intestinal microbiota, poor digestive development, and changes in gut pH (Pakandl, 2009). *E. coli* represented the most common species resulting in enteritis and mortality in rabbits (Hamed *et al*., 2013; Mohammed *et al*., 2013). EPEC is a subacute mucoid enteropathy affected rabbits at 5–7

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weeks age causing watery diarrhoea with mucus, dehydration, and high rates of mortality that varied from 16.7% to 35.2% (Garcia *et al*., 2010). Also *K. pneumoniae* causes high rates of morbidity and mortality by inducing pyogenic liver abscess, pneumonia, renal failure and septicemia (Paczosa and Mecsas, 2016). *K. pneumoniae* incorporated many virulence genes, one of them is the *Uge* gene which is essential for capsule synthesis and its absence render *K. pneumoniae* unable to cause urinary tract infections, pneumonia and sepsis (Regué *et al*., 2004). While *rmpA* highly correlated with hypervirulent *K. pneumoniae* and purulent tissue infections like liver abscesses (Li *et al*.,2019), It responsible for colony mucoviscosity on media which may aid in the early diagnosis of putative virulence isolates of *K. pneumoniae* (Kawai, 2006). *S. aureus* associated with high mortality by causing necrotizing pneumonia, abscesses formation and severe respiratory failure (Tsai and Ku, 2012) and *P. multocida* participated as an important cause of rabbit mortality through various lesions as abscesses, pneumonia, rhinitis, sepsis, mastitis, and rhinitis (Coudert *et al*., 2006). Deaths, septicemia, respiratory symptoms, and diarrhoea in rabbits also were all linked to *P. aeruginosa* infection (Gong *et al*., 2018).

So the aim of this investigation was to determine certain pathogenic viral and bacterial causes of rabbit mortality in order to control these infections and prevent its spread.

MATERIALS AND METHODS

Ethical approval

There were no animal experiments conducted as part of this study. Infected rabbits were euthanized then samples were collected in accordance with the regulations of Animal Health Research Institute and the General Organization for Veterinary Services.

History of investigated rabbit flocks

During a period of May 2019 to February 2022, a total number of twenty rabbit flocks suffered from high mortality rates (10 to 90 %) with different symptoms and post mortem lesions were submitted by the owners to the reference Laboratory for veterinary Quality control on Poultry production (RLQP), Damanhour branch and kafrelsheikh provenical laboratory for detection of possible causes. These flocks ranged in age from young (15-70 days) to adult (5-18 months) and included different sexes and breeds (8 New Zealand, 3 High Plus, 5 V-Line, 1 Dutch and 3 Alexandria). These rabbits were localized in various regions of Northwest of Delta (El Boheira, Alexandria , Kafr-El Sheikh and El -Gharbia governorates) and flock capacity ranged from 250 to 3000 rabbits of different sexes and ages. Three flocks had been vaccinated twice per year by local vaccine (SVR1), sixteen flocks had been vaccinated one shot yearly by Cunipravac (Hipra) and one flock unvaccinated against RHDV and all vaccinated against *P. multocida*.

Viral investigation

Samples

Liver, spleen, and lungs were collected from freshly dead rabbits for PCR analysis and gene sequencing of RHDV. Collected tissues were ground with a mortar and pestle in PBS with addition of antimycotic and antibiotic mixture (5 µg amphotericin B + 10000 I.U. penicillin/ml + 5 mg streptomycin sulphate /ml) to prepare a 10% tissue homogenate. Freezing and thawing were applied three times then the homogenates were centrif*Uge*d at 3000 rpm for 20 minutes. The collected supernatant was stored at –80 °C until further use for nucleic acid isolation.

Molecular detection of RHDV

Nucleic acid extraction of RHDV

QIAamp viral RNA Mini kit (Qiagen, Gmbh, Germany) was used for RNA extraction from the prepared tissue homogenate, according to the manufacturer's instructions. Briefly, 560 µl pf AVL Lysis buffer and 5.6 µl of carrier RNA was incubated with 140 µl of the sample supernatant at room temperature for 10 min. After incubation, 560 µl of 100% ethanol was added. The sample was then washed and centrif*Uge*d twice, a 60 µl of elution buffer was used to elute RNA.

Polymerase Chain Reaction (PCR) for amplification of RHDV

Supplied oligonucleotide primers from Metabion, (Germany) were utilized to amplify 538 bp of the *Vp60* gene listed in (Table 1) were used in a 25 µl reaction tube containing 1 µl of each primer at a concentration of 20 pmol, 12.5 µl of Quantitect probe RT-PCR buffer (Qiagen, Gmbh, Germany), 0.25 µl of reverse transcriptase enzyme, 7.25 µl of pcr water, and 3 µl of RNA template. The reaction was performed in a Biometra thermal cycler. Reverse transcription condition are presented in Table 1.

Analysis of the PCR products for RHDV

15 µl of the amplified *Vp60* gene PCR products were evaluated by gel electrophoresis using ultrapure 1.5% agarose (Invitrogen, Thermo Fisher Scientific, Germany) in 1×Tris-borate-EDTA (TBE) buffer at room temperature. Fragment sizes was determined by Gelpilot 100 bp DNA ladder (Qiagen, Gmbh, Germany). Gel documentation system (Alpha Innotech, Biometra) was used to photograph amplified pcr products. Data were analyzed using Automatic Image Capture Software (Protein Simple, formerly Cell Biosciences, San Jose, CA, USA).

Gene sequencing and phylogenetic analysis for RHDV

QIAquick PCR Product extraction kit (Qiagen, Gmbh, Germany) was used to purify PCR products. Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used to perform sequence reaction, and Centri-Sep spin columns (Thermo Fisher, Germany) was used for purification. *Vp60* gene sequences were obtained using a 3130 genetic analyzer (Applied Bio-systems, Life technologies, Thermo Fisher, Germany). Basic Local Alignment Search Tool BLAST® (Altschul *et al*., 1990). alignment was performed for establishing sequence similarities to the sequences deposited in the GenBank database. To determine phylogenetic distances among the analyzed strains, the Meg Align module of Lasergene DNA-Star version 12.1 was used (Thompson *et al*., 1994) and MEGA6 was used to create a phylogenetic tree using maximum composite likelihood with 1000 bootstrap replications, neighbor-joining, and maximum parsimony (Tamura *et al*., 2013).

Slide and microplate hemagglutination (HA) tests

According to previously published methods (Chasey *et al*., 1995), positive RT-PCR samples were tested for hemagglutination by slide and microplate HA tests. For slide HA, 100 µl of

each isolate was mixed with 100 µl of 0.75% washed human red blood cells (RBCs) type O for one minute. For microplate HA, in brief, 50 µl of phosphate buffered saline (PBS, pH 7.2) was added to all wells including positive and negative controls. Then 50 µl of prepared isolates was added to the first well only followed by twofold serial dilution. Finally, 50 µl of 0.75% washed human Red Blood Cells (RBCs) type O was added to all wells followed by incubation at 4°C for 1 hour.

Bacterial investigation

Bacterial isolation and identification

Tissue samples from liver, heart, lung, kidney, spleen, uterus, blood and suppurative lesions were collected. The samples were initially incubated in buffer peptone water, after that were cultivated on different specific media.

For E coli isolation, the samples were cultivated on both MacConkey and Eosin methylene blue agar and confirmed by (IMVIC) (Quinn *et al*., 2002). For *K. pneumoniae*, the samples were inoculated on MacConkey and Blood agar and identified based on Gram's staining, and biochemical tests (Arya *et al*., 2020) then viscosity of colony was examined by a string test (Podschun and Ullmann, 1998) to determine hyper virulent *K. pneumoniae* (hvKP). The collected samples for *P. multocida* were inoculated in brain heart infusion (BHI) broth and incubated at 37°C for 24 hours before being plated on 7% sheep's blood agar media then colonies were identified by Gram's staining and biochemical reactions (Glisson *et al*., 2008). *P. aeruginosa* was isolated on MacConkey agar and Pseudomonas agar base medium and identified up on colonial morphology, Gram staining, pigment production, detection of fruity smell, and biochemical tests (Quinn *et al*., 2002). Detection of *Salmonella* was done according to ISO 6579 2002. Isolation of *Staph. aureus* was performed on Baird parker agar and mannitol salt agar then identified by biochemical tests according to Quinn *et al*. (2002).

Serotyping of E. coli strains

Serotyping of E coli was done according to Edwards and Ewing (1986) in Animal Health Research Institute, Dokki, Egypt.

Surveying of K. pneumoniae virulence genes by PCR

Hypervirulent *K. pneumoniae* isolates were surveyed for the presence of (Uridine diphosphate galacturonate 4-epimerase) *Uge* gene, (regulator of mucoid phenotype A) *rmpA* gene and (Klebsiella ferric iron uptake) *kfu* gene by PCR.

DNA extraction of bacterial isolates

QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used for DNA extraction from samples with modifications from the manufacturer's recommendations. Briefly, 200 µl of lysis buffer and 200 µl of the sample suspension was incubated with 10 µl of proteinase K at 56°C for 10 min. then, 200 µl of 100% ethanol was added to the lysate. The sample was washed and centrif*Uge*d following the manufacturer's recommendations. DNA was eluted with 100 µl of elution buffer provided in the kit.

PCR amplification

Used oligonucleotide primers that were supplied from Metabion (Germany) were listed in Table 1. Primers were utilized in a 25 µl reaction tube containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer with concentration of 20 pmol, 5.5 µl of PCR water, and 5 µl of DNA template. Applied biosystem 2720 thermal cycler was used for performing of the reaction. Reverse transcription condition illustrated in Table 1.

Analysis of the PCR products

PCR products were separated by gel electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. fragment sizes were determined by Generuler 100 bp ladder (Fermentas, Germany). Gel documentation system (Alpha Innotech, Biometra) was used to photograph the amplified PCR products and the data was analyzed through computer software.

Histopathological examination

Pieces of lung, spleen, liver, kidney , heart and intestine were collected from sacrificed diseased rabbits in the 20 farms, immediately placed in 10% neutral buffered formalin, sectioned, stained with hematoxylin and eosin (H&E) then evaluated for histological lesions (Bancroft and Gamble, 2008). Immuno peroxidase technique was done on paraffin sections for detection of RHDV antigen according to Suvarna *et al.* (2013).

Table 1. Oligonucleotide Primers sequences, target genes for *K. pneumonia* and RHDV

Target gene	Primers sequences	Amplified segment (bp)	reverse transcription	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
(1) *rmpA*	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535	-	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Yeh et al. (2007)
(2) *Uge*	TCTTCACGCCTTCCTTCACT GATCATCCGGTCTCCCTGTA	534	-	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Osman et al. (2014)
(3) *kfu*	GAAGTGACGCTGTTTCTGGC TTTCGTGTGGCCAGTGACTC	797	-	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Osman et al. (2014)
(4) *Vp60*	CCACCACCAACACTTCAGGT CAGGTTGAACACGAGTGTGC	538	45 °C 30 min.	95°C 15 min.	94˚C 30 sec.	56°C 40 s.	72˚C 45 sec.	72˚C 10 min.	Fahmy et al. (2010)

1-2-3: Virulence genes of *K. pneumonia*; 4: Detection gene of RHDV

RESULTS AND DISCUSSION

Epidemiological data

The collected data of the investigated rabbit flocks (Tables 2 and 3) indicated variation in mortality rates from 10 to 90%, which may be attributed to the age, breed and immune status of infected rabbit flocks, type of RHDVs and/or co-infection with pathogenic bacteria. The reported mortality rate of 90% in rabbit flock No. 11 was attributed to classic RHDV, and mortality rate varied from 20 in young to 70% in adult of flocks No.1-4 ,6 and 7 were attributed to RHDV-2. Meanwhile, mortality rate varied from 29 % in young to 70%, in adult of flocks No.5 and 10 was attributed to mixed infection by RHDV-2 and EPEC. This finding agreed with that previously mentioned by Rocchi and Dagleish (2018); Bonvehí *et al.* (2019) and Harcourt *et al.* (2020) and indicated that naturally the virus is very contagious to all breeds and ages of rabbits (from 15 days till 18 month).

Clinical signs and gross lesions

Only a few of the affected rabbits showed signs of sudden death with frothy bloody discharge from the nostrils. Whereas other rabbits displayed signs of lethargy, dullness, tremors, fever with cyanosis of lips and nostrils, and dyspnea associated with abdominal respiration. In some cases, there were blood in urine and vaginal discharge. On other hand, abortion of doe at late pregnancy stage and abscesses were detected in other rabbit flocks. Watery diarrhea, distended, bloated, and/or painful abdomen, grinding of teeth, anorexia, weight loss, dehydration, restlessness, poor coat quality and hypothermia were detected in almost young, affected rabbits. The major postmortem of investigated affected rabbit flocks included liver in some affected rabbits was congested and enlarged (Fig.1A) while, in others, liver showed excessive area of necrosis and suppuration (Fig.1D). Congested and enlarged spleen in almost affected rabbits. Flabby and congested heart with engorged blood vessels ((Fig.1A). In almost affected rabbits, the lungs were congested (Fig.1A) and the trachea was often hyperemic and contains frothy, blood-stained mucus. While in others, lung showed focal and massive abscess (Fig.1C). Congested stomach and mesenteric blood vessels were engorged with blood (Fig.1B). Kidneys were congested in some affected rabbits and showed focal abscess in others (Fig.1.B). Uterus was congested and filled with dead fetus in some affected females. (Fig.1E). Here, clinical signs and gross lesions in rabbit flock No 11 was attributed to classic RHDV and in flocks No.1-7 and 10 were attributed to RHDV-2. This finding agreed with that previously mentioned by Rocchi and Dagleish (2018); Bonvehí, *et al.* (2019) and Harcourt-Brown *et al.* (2020).

Virological findings

Ten PCR positive samples were also positive for RHDV by HA test, and HA titer varied from 26 to 211. These results agree with those reported by other authors (Erfan and Shalaby, 2020; Hemida *et al*., 2020) and indicated that RHDV powerfully agglutinates human type O RBCs and endorsed using HA as a screening tool for detection of RHDVs in infected samples.

Table 2. Epidemiological data of investigated rabbit flocks infected with identified RHDV and/or EPEC.

Farm No.	Accession No	Locality & date	Vaccine	Age	Mortality	Breed	Identified agents
1	MW455120 RHDV GH-19	GH -May 2019	Hipra	Adult young	40% 20%	N	V-. RHDV2
2	MW455121 RHDV BH-R-19	BH –R-May 2019	Hipra	Adult young	65% 20%	V-Line	V- RHDV2
3	MW455122 RHDV KFS-1-19	KFS -June 2019	Hipra	Adult young	70% 22%	N	V- RHDV2
4	MW455123 RHDV KFS-2-19,	KFS -July 2019	Hipra	Adult young	50% 25%	Alexandria	V- RHDV2
5	MW455124 RHDV BH-K-19	BH- K-August 2019	Hipra	Adult young	60% 29%	N	V- RHDV2+ EPEC,
6	MW455125 RHD-VAlex 19	Alex-August 2019	SVR1	Adult young	50% 22%	V-Line	V- RHDV2
7	MW455126 RHDV BH-D-19	BH-D- September 2019	SVR1	Adult young	55% 25%	N	V- RHDV2
10	MW455127 RHDV KFS-3-19	KFS -December 2019	Hipra	Adult young	70% 32%	N	V-RHDV2+ EPEC,
11	MW455128 RHDV BH-D-20	BH-D- April 2020	Unvac.	Adult	90%	N	C- G3-G5 RHDV

KFS: Kafr-ELSheikh; GH: El Gharbia; BH: El-Beheira; Alex: Alexandria; N: New Zealand; V: Variant; C: Classic; K: Kom Hamadah; D: Damanhour; R: Rashid; Unvac: Unvaccinated

Table 3. Mortality percent in related to identified causes:

Serial No.	Identified agents	No. of farms	Mortality
1	+ve Classic G3-5 RHDV1or variant RHDV2	8 (40%)	20-90%
2	+ve variant RHDV2, EPEC.	2 (10%)	29-70%
3	+ve *S. aureus, EPEC*, *P. multocida*	3 (15%)	15 – 32 %
4	+ve *EPEC*, *K. pneumonia, P. aeruginosa*	4 (20%)	10 -25%
5	+ve *EPEC*, *S. aureus, K. pneumonia, P. multocida*	3 (15%)	13– 35%

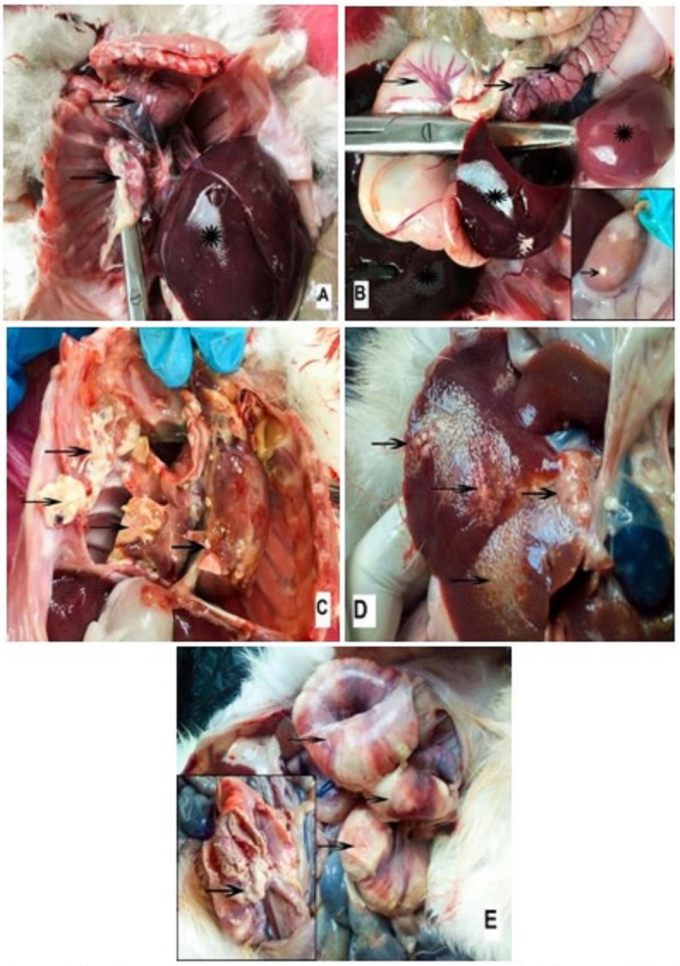

Fig. 1. (A) liver is congested and enlarged (asterisk), heart is congested with engorged blood vessels and the lung is congested (arrow). (B) congested and enlarged liver, stomach and mesenteric blood vessels are congested and engorged with blood (arrow), congested kidney (asterisk) with focal abscess (arrow).(C) massive abscess in lung (D) liver showed excessive area of necrosis and suppuration. (E) uterus is congested and filled with puss and dead fetus.

RT-PCR was done for detection of RHDV-specific nucleic acid (Fahmy *et al*., 2010). Ten out of twenty samples (50%) were positive for RHDV by *Vp60* gene based RT-PCR assay. This result suggests a high incidence of this disease among rabbit populations, and this finding is compatible with those reported in earlier studies (Magouzi *et al*., 2017; Hemida *et al*., 2020) in Egypt.

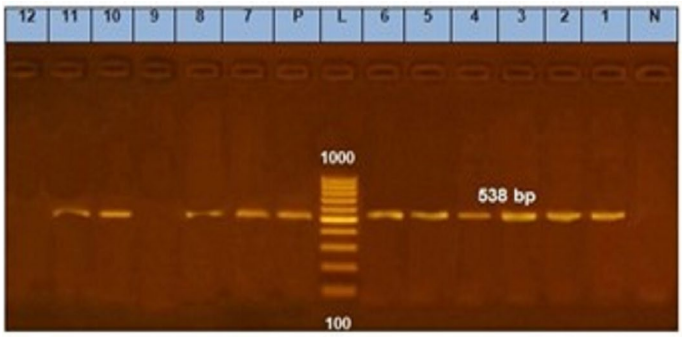

Fig. 2. RT-PCR of RHDV-*Vp60* gene. Lane L: 100bp DNA ladder, Lane P : positive control, Lane N: negative control, Lane 1, 2, 3, 4, 5, 6, 7, 8, 10 and11 samples positive.

Nucleic acid sequencing of the *Vp60* gene amplicons were done for nine representative RHDV strains that were detected in this study and revealed a range of 79.8-100% nucleotide sequence identity for each other. Moreover, the selected isolates showed 78.2-89.5% nucleotide sequence identity with the commonly used vaccine strain (RHDV-GIZA 2006, accession number JQ995154.1). Where they showed 79.8-98.8% nucleotide sequence identity with RHDV representative strain Qena AZ10 (GenBank accession number MN295023) and Assiut AZ12 (GenBank accession number MN295025) respectively as shown in (Table 4). The new sequences were assigned GenBank accession numbers (MW455120 RHDV GH-19, MW455121 RHDV BH-R-19, MW455122 RHDV KFS-1-19, MW455123 RHDV KFS-2-19, MW455124 RHDV BH-K-19, MW455125 RHDVAlex 19, MW455126 RHDV BH-D-19, MW455127 RHDV KFS-3-19 and MW455128 RHDV BH-D-20).

Phylogenetic tree of the RHDVs based on partial nucleotide sequences of the *Vp60* gene using maximum likelihood, neighbour joining and maximum parsimony in MEGA6. The isolates of the present study are indicated by a circle and solid triangle Reveled that isolated strains divided into two major clusters, the classic G3-G5 RHDV group and the variant-type RHDV2. It was shown

Table 4. Results of nucleotide sequence identity of the present RHDV isolates with selected references and vaccine strains on GenBank.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
1		91.3	92.1	90.3	98.8	79.0	90.5	90.7	78.8	90.7	78.6	78.4	78.4	78.2	78.6	78.6	78.8	78.2	89.5	78.4	79.0	77.8	77.8	77.8	78.0	1	JQ995154 RHDV GIZA-2006
2	9.4		96.2	89.9	91.1	79.2	96.4	96.6	79.2	96.6	79.6	79.4	79.4	79.2	79.2	80.0	79.4	79.2	95.8	79.4	80.0	79.2	79.6	79.2	79.4	2	DQ189077 RHDV Bahrain
3	8.5	4.0		90.9	91.9	79.6	95.6	95.8	79.6	95.8	79.4	79.2	79.2	79.0	79.0	79.8	79.2	79.0	94.5	79.2	79.8	79.0	79.4	79.2	79.4	3	EF558575 RHDV Ascot
4	10.6	11.0	9.8		90.3	78.8	89.5	89.7	78.8	89.7	79.0	78.8	78.8	78.6	79.0	78.6	79.0	78.6	88.7	78.8	79.4	78.2	78.6	78.2	78.4	4	KY498582 RHDV Zar2008_1
5	1.2	9.6	8.6	10.5		79.0	90.3	90.5	78.8	90.5	78.6	78.4	78.4	78.2	78.6	78.6	78.8	78.2	89.3	78.4	79.0	77.8	77.8	77.8	78.0	5	KX133721 RHDV Menofia/2012
6	25.6	25.2	24.5	25.7	25.5		78.8	79.0	99.8	79.0	93.3	93.1	93.1	92.9	93.3	93.3	93.1	92.9	78.6	93.1	93.1	93.9	93.9	96.0	95.6	6	MN295018 RHDV Sharkia AZ5
7	10.4	3.8	4.6	11.5	10.6	25.9		99.8	78.8	99.8	80.0	79.8	79.8	80.0	79.6	80.0	79.8	79.6	98.6	79.8	80.4	79.2	79.6	79.2	79.4	7	MN295019 RHDV Menia AZ6
8	10.1	3.5	4.4	11.3	10.3	25.6	0.2		79.0	100.0	80.2	80.0	80.0	79.8	79.8	80.2	80.0	79.8	98.8	80.0	80.6	79.4	79.8	79.4	79.6	8	MN295023 RHDV Qena AZ10
9	25.9	25.1	24.5	25.7	25.8	0.2	25.8	25.5		79.0	93.1	92.9	92.9	92.7	93.1	93.1	92.9	92.7	78.6	92.9	92.9	93.7	93.7	95.8	95.4	9	MN295024 RHDV Dakahlia AZ11
10	10.1	3.5	4.4	11.3	10.3	25.6	0.2	0.0	25.5		80.2	80.0	80.0	79.8	79.8	80.2	80.0	79.8	98.8	80.0	80.6	79.4	79.8	79.4	79.6	10	MN295025 RHDV Assiut AZ12
11	26.2	24.5	24.8	25.4	26.1	7.1	24.0	23.7	7.3	23.7		99.8	99.8	99.6	99.6	99.2	99.4	99.6	80.2	99.8	99.0	98.6	98.6	96.8	96.6	11	MW455120 RHDV GH-19
12	26.5	24.8	25.1	25.7	26.4	7.3	24.3	24.0	7.6	24.0	0.2		100.0	99.4	99.4	99.0	99.2	99.4	80.0	99.6	98.8	98.4	98.4	96.6	96.4	12	MW455121 RHDV BH-R-19
13	26.5	24.8	25.1	25.7	26.4	7.3	24.3	24.0	7.6	24.0	0.2	0.0		99.4	99.4	99.0	99.2	99.4	80.0	99.6	98.8	98.4	98.4	96.6	96.4	13	MW455122 RHDV KFS-1-19
14	26.8	25.1	25.4	26.0	26.7	7.6	24.0	24.3	7.8	24.3	0.4	0.6	0.6		99.2	98.8	99.0	99.6	79.8	99.4	98.6	98.2	98.2	96.4	96.2	14	MW455123 RHDV KFS-2-19
15	26.2	25.1	25.4	25.4	26.1	7.1	24.6	24.3	7.3	24.3	0.4	0.6	0.6	0.8		98.8	99.4	99.2	79.8	99.4	98.6	98.2	98.2	96.4	96.2	15	MW455124 RHDV BH-K-19
16	26.2	23.9	24.2	26.0	26.1	7.1	24.0	23.7	7.3	23.7	0.8	1.0	1.0	1.2	1.2		98.6	98.8	80.2	99.0	98.2	98.2	98.2	96.4	96.2	16	MW455125 RHDV Alex 19
17	25.9	24.8	25.1	25.4	25.8	7.3	24.3	24.0	7.6	24.0	0.6	0.8	0.8	1.0	0.6	1.4		99.0	80.0	99.2	98.4	98.0	98.0	96.2	96.0	17	MW455126 RHDV BH-D-19
18	26.8	25.1	25.4	26.0	26.7	7.6	24.6	24.3	7.8	24.3	0.4	0.6	0.6	0.4	0.8	1.2	1.0		79.8	99.4	98.6	98.2	98.2	96.4	96.2	18	MW455127 RHDV KFS-3-19
19	11.6	4.4	5.7	12.5	11.8	26.2	1.4	1.2	26.1	1.2	23.7	24.0	24.0	24.3	24.3	23.7	24.0	24.3		80.0	80.6	79.4	79.8	79.4	79.6	19	MW455128 RHDV BH-D-20
20	26.5	24.8	25.1	25.7	26.4	7.3	24.3	24.0	7.6	24.0	0.2	0.4	0.4	0.6	0.6	1.0	0.8	0.6	24.0		98.8	98.4	98.4	96.6	96.4	20	MN276176 RHDVVet-Abotaleb
21	25.5	23.9	24.2	24.7	25.4	7.3	23.3	23.0	7.5	23.0	1.0	1.2	1.2	1.4	1.4	1.8	1.6	1.4	23.0	1.2		97.6	97.6	96.2	96.0	21	MN276177 RHDVVet-Alex-B18
22	27.4	25.1	25.4	26.6	27.3	6.4	25.2	24.8	6.7	24.8	1.4	1.6	1.6	1.8	1.8	1.8	2.1	1.8	24.8	1.6	2.5		99.6	97.4	97.6	22	MH341508 RHDV 1990
23	27.4	24.5	24.8	25.9	27.3	6.4	24.5	24.2	6.7	24.2	1.4	1.6	1.6	1.8	1.8	1.8	2.1	1.8	24.2	1.6	2.5	0.4		97.4	97.6	23	MH341504 RHDV 1178
24	27.5	25.1	25.1	26.6	27.4	4.2	25.2	24.9	4.4	24.9	3.3	3.5	3.5	3.8	3.8	3.8	4.0	3.8	24.9	3.5	4.0	2.7	2.7		99.2	24	MG763938 RHDV SOS129
25	27.2	24.8	24.8	26.3	27.1	4.6	24.9	24.6	4.8	24.6	3.5	3.8	3.8	4.0	4.0	4.0	4.2	4.0	24.6	3.8	4.2	2.5	2.5	0.8		25	MG763947 RHDV SOS158
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		

from the phylogenetic tree (Fig. 3) that eight of the sequenced samples including strains with GenBank accession numbers (MW455120- MW455127) were related to the variant RHDV-2 group. While, sample MW455128 only was clustered with classic RHDV (G3-G5. The result revealed that the classic RHDV (G3-G5) and the variant RHDV2 were still circulated in Egyptian rabbit populations and considered as a main cause of mortality in rabbit flocks. This agrees with Hemida *et al*. (2020). Regarding variant, RHDV2 is considered the most dominant circulating strain in rabbits, which is suppoted by the finding of Erfan and Shalaby (2020). The multiple alignment of deduced amino acid between the commonly vaccinal strain (GIZA 2006) and other reference strains on GenBank in comparison to RHDV2 isolates showed four constant amino acid substitutions at position T 92 C, A 264 G, C 300 T and A 462 G, while on comparing to the classic isolate many differences or substitutions are shown (Fig. 4), including substitution at position C 476 A which identified positive selected codons (PSCS) in the F region. These substitutions may be characterized as differential amino acid substitutions between them. The present study revealed that there was amino acid substitution in C 476 A region in the classic isolate (MW455128 RHDV BH-D-20). This change may cause changes with respect to polarity according to Esteves *et al.* (2008), who identified positive selected codons (PSCs) in F region (amino acid number 476). They mentioned that amino acid substitutions at the PSCs resulted in changes in the polarity or charge of the protein that are import-

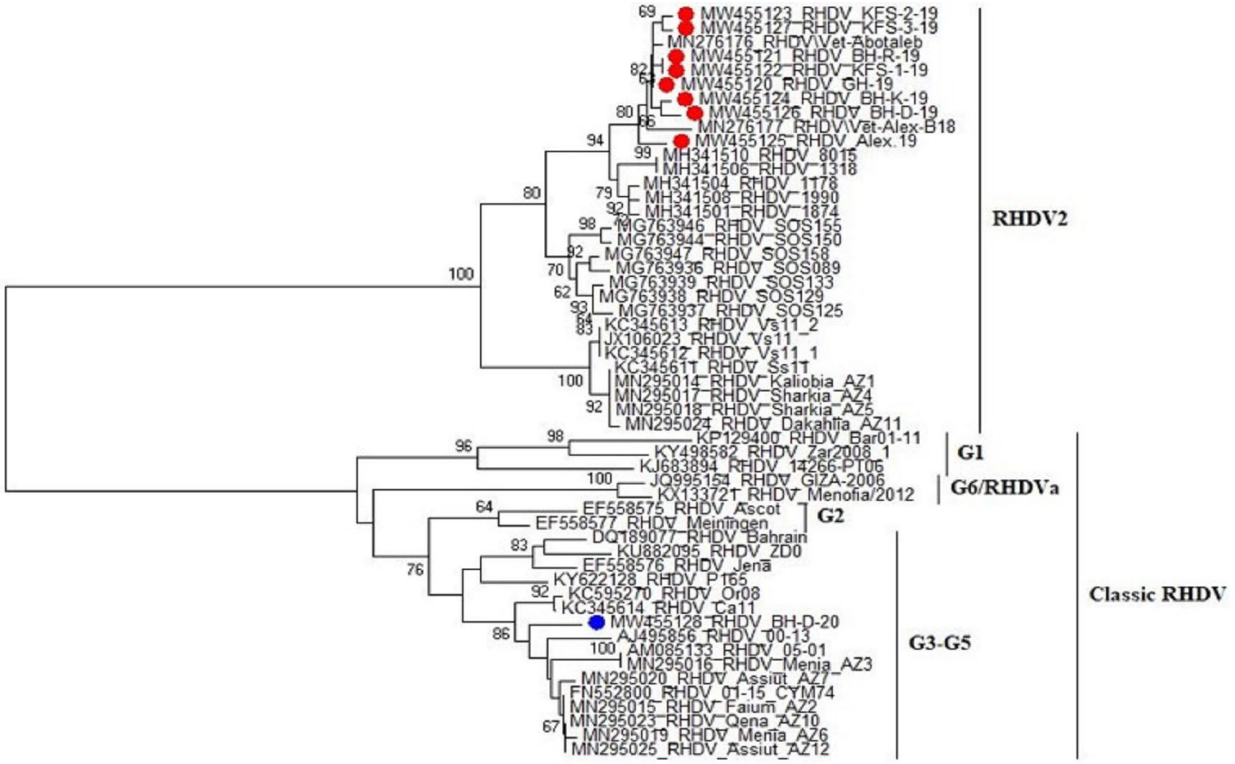

Fig. 3. Phylogenetic tree of the RHDV based on partial nucleotide sequences of the *Vp60* gene using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. The isolates of the present study are indicated by a circle.

Table 5. The severity of pathological lesions in different organs according to virological and bacteriological investigation

Organ	Lesion	1	2	3	4	5
liver	Fatty, vacuolar degeneration and necrosis	+++	+++	++	++	++
	Chronic hepatic abscesses	-	-	++	++	+++
	Apoptotic hepatocytes	+++	+++	-	-	-
Spleen	Lymphoid depletion	+++	+++	-	++	-
	Apoptosis of splenic lymphocytes	+++	+++	-	-	-
Lung	Desquamation of bronchial epithelium, intra alveolar edema and hemorrhage, congested blood vessels	++	++	+++	+++	+++
	abscesses	-	-	++	++	+++
	Apoptosis	+++	+++	-	-	-
Intestine	Erosion of intestinal villi	++	+++	++	++	++
	Apoptosis	+++	+++	-	-	-
Kidney	Abscesses	-	-	++	++	+++
	Degeneration and necrosis of renal tubules	+++	+++	++	++	++
	Hyaline thrombi and haemorrhages in the renal corpuscles	++	++	-	-	-
	Apoptosis	+++	+++	-	-	-
Heart	Myocardial necrosis	+++	+++	-	-	-
	Apoptosis in myocardium and inside blood vessels	+++	+++	-	-	-

+++: Severe; ++: Moderate; +: Mild; -: No positive cells

1 (+ve RHDV), 2 (+ve RHDV, EPEC) , 3 (+ve *S. aureus*, EPEC, *P. multocida*), 4 (+ve EPEC, *K. pneumonia*, *P. aeruginosa*), 5 (+ve EPEC, *S. aureus*, *K. pneumonia*, *P. multocida*).

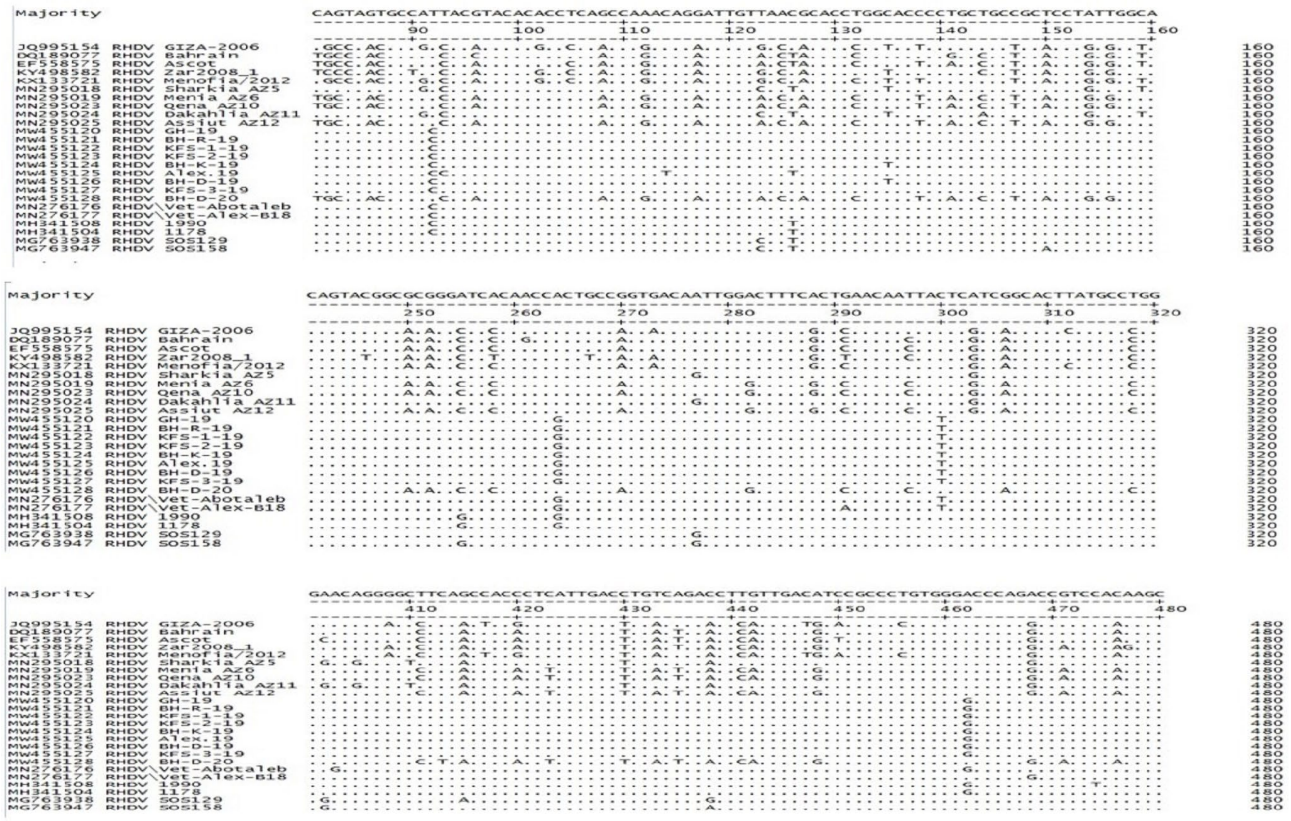

Fig. 4. nucleotide alignment report: Amino acid sequences of RHDV isolates, vaccine strain (Giza 2006) and reference Strains on the gene bank. Dots denote identical Amino acid sequence while other letters denote the mutated amino acids.

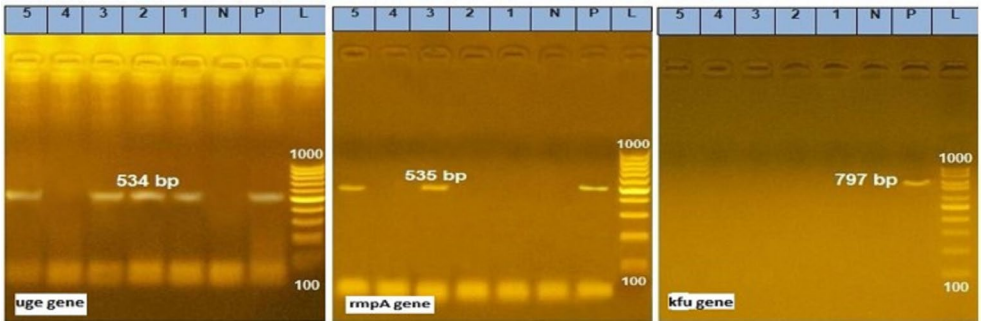

Fig. 5. Electrophoretic pattern of *K. pneumonae.*
Uge gene): Lane (L): DNA ladder (1000 bp), Lane (P) positive control, lane (N): Negative control, Lane (1, 2, 3 & 5) positive sample (534 bp) Lane (4) negative.
rmpA gene): Lane (L): DNA ladder (1000 bp), Lane (P) positive control, lane (N): negative control, Lane (3 & 5) positive sample (535bp) Lane (1, 2 & 4) negative sample.
kfu gene): Lane (L): DNA ladder (1000 bp), Lane (P) positive control, lane (N): negative control, Lane (3 & 5) positive sample (535 bp) Lane (1, 2 & 4) negative sample

ant for the protein structure and protein–protein interaction. The nucleotide divergence among the variant RHDV2 isolates was 0.0-1.4%, the nucleotide divergence within classical strain and the vaccinal strain (RHDV-GIZA 2006) was 11.6% while nucleotide divergence within the variant RHDV2 isolates and the vaccinal strain ranged from 25.9-26.8%, (Table 4) this agree with Lopes *et al.* (2015) who mentioned that continuous distribution of original strains, appearance and gradual replacement of RHDV2 in Egypt could be due to vaccine mismatch.

Bacteriological findings

Several pathogenic bacteria were isolated from rabbits in the investigated farms represented in EPEC, *K. pneumoniae*, *P. multocida S. aureus* and *P. aeruginosa*, with mortalities as 60%, 35%, 30%, 30% and 20% respectively. The obtained results declared that E coli isolates represented the majority of detected bacteria and most of them found combined with other infection (Table 3) which may contribute to enhancing its virulence to cause diarrheal and extra intestinal diseases in an immune-suppressed host (Croxen *et al.*, 2013), resulting in septicemia, organ failure and death (Licois *et al.*, 2005). Several studies associated mortality in rabbits with E coli infection EL-Sayed Hatab and Moustafa (2007) 17.71 %, Elsayed *et al.* (2014) 26.7%, Saeed *et al.* (2020) 100% of pre-weaning mortality and Fatma *et al.* (2019) 56.6% of weaning rabbit mortality. Serotyping of pathogenic E coli declared several serotypes represented in four poly 1, O26 K60 which recorded as highly pathogenic strain causing 80% mortality by Abd El Gwad (1988), three poly 2 ,O55 K59, two poly 2 ,O126K71 , two poly 1, O111 and one isolate belong to poly 3, O114. All of them were EPEC (Sansonetti 1985; Frits and Ida 1992), the same observation was recorded also by (Swennes *et al.*, 2012). A doe can spread EPEC to her kits Through faecal exposure (Okerman, 1994) resulting in acute intestinal infection distinguished by inflammatory lesions (Licois 2004) and consequently results in death.

The second isolated bacteria was *K. pneumoniae* (35%) compared with previous studies as a cause of rabbits mortality in 14.06% and 20% by EL-Sayed Hatab and Moustafa (2007) and Sumitha and Sukumar (2014) respectively. Regarding the post-mortem lesions of *K. pneumoniae* on internal organs of affected rabbit (lung and liver abscesses ,pus in uterus) surveying of some virulence genes *Uge* gene, *rmpA* gene and *kfu* gene in five isolates was conducted by PCR which revealed presence of *Uge* gene in most isolates (4/5 ,80%) and *rmpA* (2/5,40%) while we did

not detect any isolate that was positive for *kfu* gene (Fig.5). These virulence genes contribute in pathogenesis of *K. pneumoniae* on host organs as both *rmpA* and *Uge* genes play a significant role in the manufacture of capsules, phagocytosis resistance, liver abscess, and blood contamination.

The most staph aureus isolated in this study (30%) was from pus content of uterus of does and abscesses of internal organ and this indicate that *S. aureus* was one of the main factors of pyogenic infection as recorded by (Segura *et al*., 2007) who isolated *S. aureus* from15.9% of does pyometra, and José Espinosa *et al*. (2020) who isolated it with 14.28% from condition of septicemia characterized by widespread abscesses in internal organs mostly in adults rabbits. Also, it was considered as a cause of mortality reaching 16.67% as stated by EL-Sayed Hatab and Moustafa (2007) and Heba *et al*. (2021) (16.9%).

P. multocida prevalence rates represented 30%, the bulk of them from lung samples, when compared to earlier investigations (Mahrous *et al*., 2022) 22%, (Fatma *et al*., 2020) 62.5%, (Jinxiang Wang *et al*., 2019) 21.21 to 53.06% and (Heba *et al*., 2021) 13.2%. It was identified as one of the main infections causing respiratory disease in rabbit farms in Egypt (Gergis *et al*., 1992).

P. aeruginosa isolated from (20%) examined samples compared with previous studies as a cause of mortality in 6% of rabbit by Abd-El-Gwad (1997) and 9.38 % by EL-Sayed Hatab and Moustafa (2007). The positive recovery was from lung and pus content of uterus which revealed that it may be incriminated as a cause of pyometra as mentioned by von Degerfeld *et al*. (2020) who initially reported pyometra in a pet rabbit attributed to *P. aeruginosa* or engaged in it as a secondary infection because it is an opportunistic pathogen. Finally, the variability of the isolated microorganisms explains the multiple clinical forms associated with rabbit mortality.

Histopathological findings

The intestine in rabbits was infected by EPEC and RHDV, showed severe enteritis, sloughing of the intestinal villi and infiltration of lymphocytes, bacterial and cellular debris also there were edema and hemorrhage in the submucosa (Fig. 6A), same findings were reported by Rodrı´guez *et al*. (2008) and Alton *et al*. (2012) due to EPEC infection and due to RHDV infection (Kevin *et al*., 2012; Fábio *et al*., 2021). The pathogenesis of this class of *E. coli* depends on a particular chromosomal region known as the locus of enterocyte effacement in order to create attaching and effacing lesions of bacteria at the surface of the intestinal mucosa (McDaniel *et al*., 1995). Firstly, the bacteria make close attachment to enterocytes, then effacement of surface-absorptive microvilli which causes it to be destroyed and diffusely shortened. These lesions severely decrease the ability of absorption in this part of intestine, increasing the luminal contents osmotic load, and promoting water loss and diarrhea (Goosney *et al*., 2000).

Rabbits infected by *S. aureus* and *K. pneumoniae* showed suppurative inflammation in different internal organs as uterus, lung, liver and kidney leading to small and large abscess appeared as focal structure less eosinophilic and basophilic substance infiltrated with leukocytes especially neutrophil and dead cells, surrounded by fibrous connective tissue capsule with degenerative changes (Fig. 6 B, C & D), the same findings were noted by Zainab *et al*. (2018) and El-Mashad *et al*. (2019) for *S. aureus*, and by Yingchun *et al*. (2014) and Muna *et al*. (2018) for *K. pneumonia* infection. The pathogenesis of abscess formation in case of *S. aureus* infection may result from numerous factors as coagulase and von Willebrand factor-binding protein which clot plasma as it activate prothrombin and convert fibrinogen to fibrin. Also clumping factor, A which binds fibrinogen and aggregates platelet leading to fibrin clot which is critical to abscess formation (Kobayashi *et al*., 2015). Then it grows in communities at the center of the abscess and are enclosed by pseudo-capsules or may be phagocytized and killed, activating inflammatory reactions and immunological defenses that attract neutrophils, macrophages and other phagocytic cells (Lowy, 1998), While hyper virulent *K. pneumoniae* produces its effect by utilizing a variety of virulence factors, particularly lipo- and capsule-polysaccharides, this thick capsule protects it from phagocytosis by macrophages and neutrophils causing its survival and immune evasion during infection (Pan *et al*., 2011). Even If neutrophils phagocytized it can steadily escape and spread to other organs like the liver, leading to an abscess (Wu *et al*., 2008).

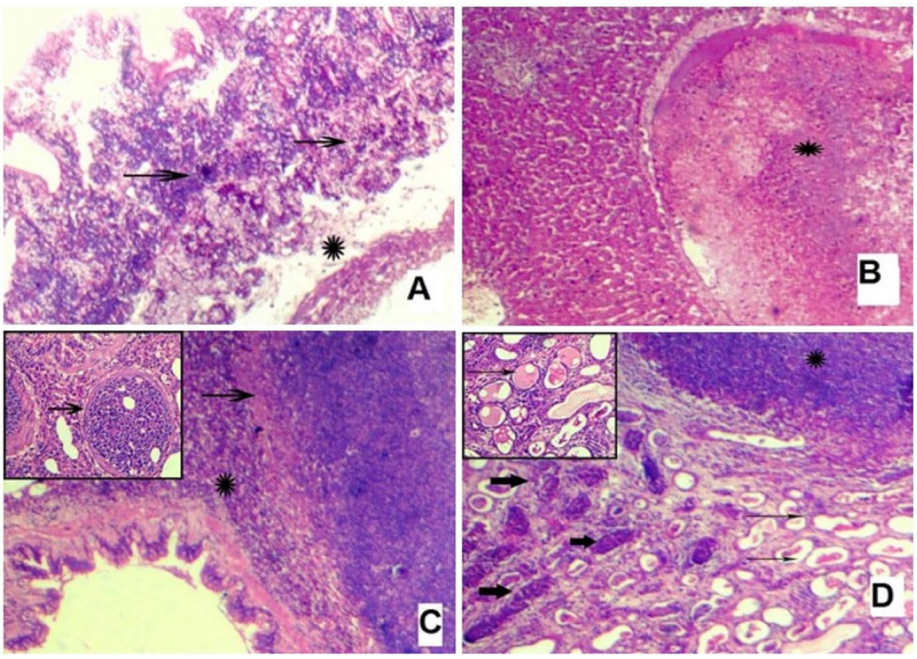

Fig. 6. (A) Intestine, necrosis of intestinal villi (arrow) , edema and hemorrhage in the submucosa (asterisk) .(B) Liver, chronic hepatic abscesses (C) Lung, large abscesses (arrow) surrounded by heavy leukocytic cellular infiltration (asterisk) (D) Kidney: excessive large surface abscesses (asterisk) and numerus small ones (thick arrow), hyaline thrombi and hemorrhage within blood vessels (thin arrow) .

Also the lung of rabbits infected by *K. pneumoniae* and *P. multocida* suffer from suppurative bronchopneumonia which appeared as marked desquamation of bronchial epithelium filled with inflammatory cells and some debris, surrounded by thick layer of fibrin, the alveolar walls were thickened by fibrinous exudates and heterophilic infiltration, with intra alveolar haemorrhages and perivascular edema (Fig. 7 A), similar findings were reported by Muna, *et al*. (2018) for *K. pneumoniae* and by Patel *et al*. (2016) and Moshira *et al*. (2017) for *P. multocida*

While liver, heart and kidney in rabbits infected by RHD undergo degeneration, necrosis and infiltration of heterophils in hepatocytes, myocardium and renal tubules (Fig. 7 B,C). In addition to apoptosis (programed cell death) which was the most prominent lesion observed in different organs including the liver, kidney, intestine, spleen, lung, heart and circulating inside small blood vessels (Fig. 8), these results agree with Harcourt *et al*. (2020).

Apoptosis is a common feature in RHD (Elmore, *et al*., 2016). This virus targets hepa¬tocytes and phagocytic cells such as Kupffer cells, circulating monocytes, alveolar macrophages and

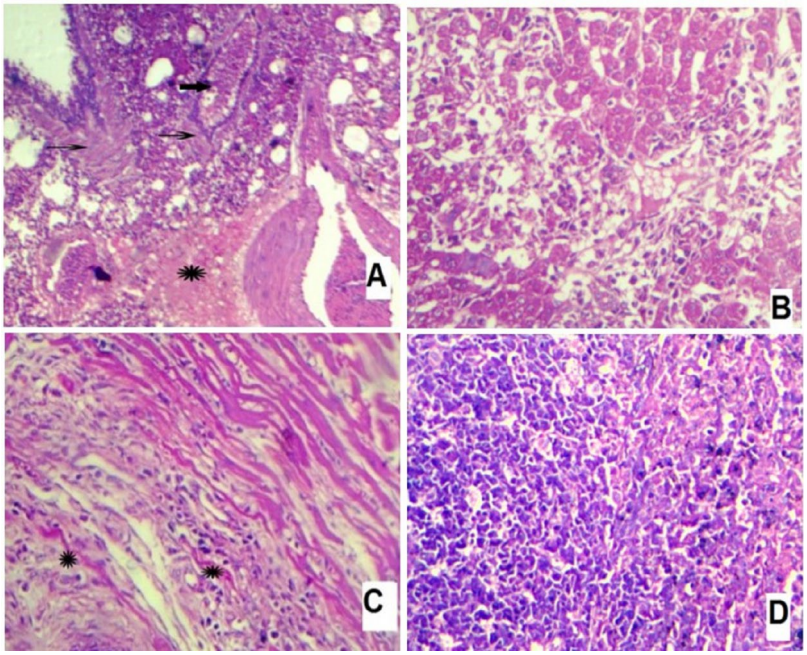

Fig. 7. (A) Lung, desquamation of bronchial epithelium, intrabronchiolar inflammatory cells and debris (thick arrow), peribronchiolar infiltration of inflammatory cells and fibrin (thin arrow), edema, hemorrhage, and congestion of blood vessels (asterisk). (B) Liver, vacuolar degeneration and necrosis with lymphocytic infiltration. (C) Heart, focal area of myocardial necrosis and infiltration of mononuclear cell (asterisk). (D) Spleen, diffuse lymphoid depletion giving a starry sky appearance. (H&EX100).

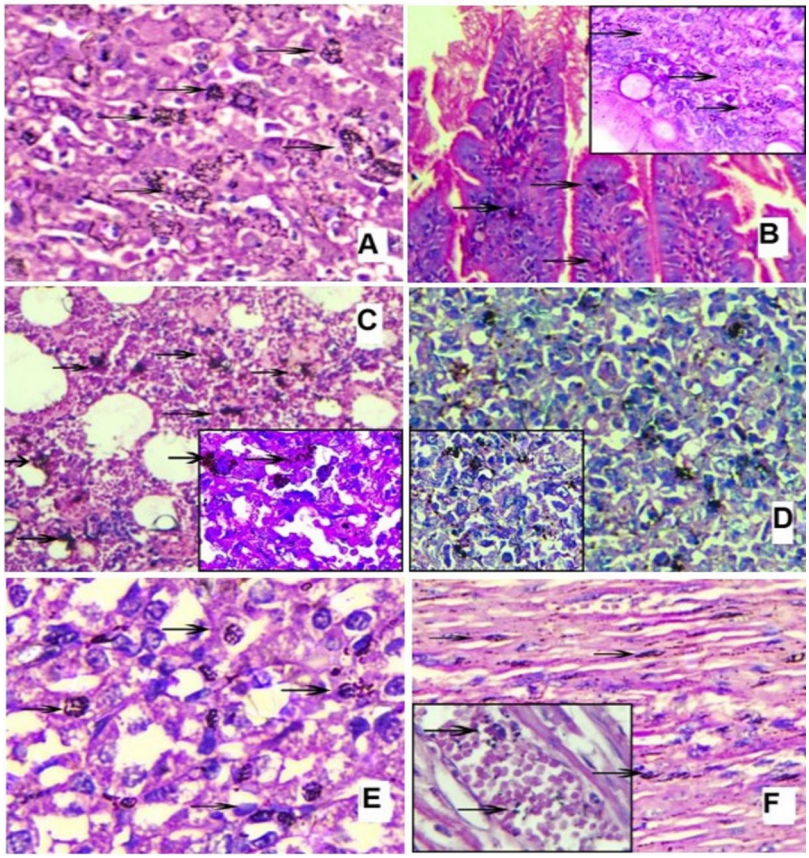

Fig. 8. Marked apoptosis and excessive apoptotic bodies in (A) hepatocytes, (B) intestinal villi, (C) interstitial tissues and lining alveoli , (D) splenic lymphocytes, (E) renal tubules and in the interstitial tissues of kidney, (F) degenerated myocardium and circulating inside small blood vessels of heart (H&EX200- 400).

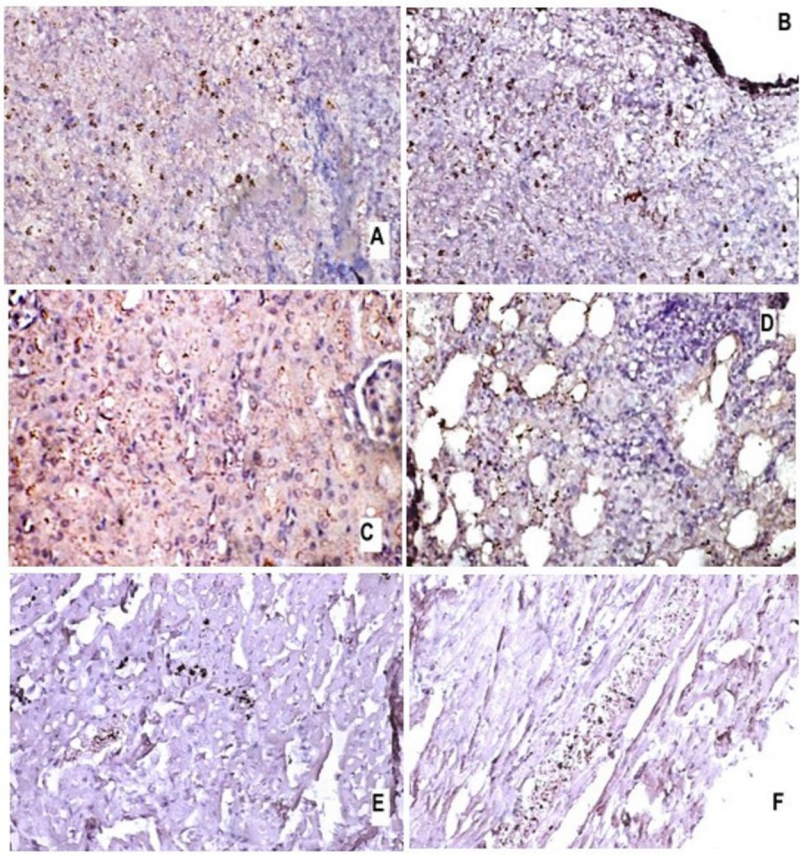

Fig. 9. Immunopositive reaction were found in (A) Liver: in intracytoplasmic or intrasinuosoidal in hepatic tissues (Immunopperoxidase Counter Stain Myer's Heamatoxylene X200). (B) Spleen: in splenic tissues (X200) . (C) Kidneys: in the basement membrane of renal tubules and lining the lumen of others and in the interstitial tissues (X400). (D) Lung: in the interstitial tissues and lining air alveoli (X400). Heart (E) in the degenerated muscular fibers (X600) and (F) in the blood vessels (X400).

endothelial cells (Neimanis *et al*., 2018). Liver failure or diffuse intravascular coagulopathy were reported to cause death in rabbits (Ueda *et al*., 1992). Disseminated intravascular coagulopathy is caused by the apoptosis of intravascular monocytes and endothelial cells (Ramiro-Ibáñez *et al*., 1999), also hepatocyte apoptosis produces extensive parenchymal destruction, resulting in an acute and lethal hepatitis that develop the pathogenesis of this disease (Elie *et al*., 2010). Spleen in cases of RHD and *P. aeruginosa* was affected by diffuse lymphoid depletion and necrosis giving a starry sky appearance (Fig. 7D), these findings agree with Shijun *et al*. (2020) for RHD and with Kiyoyasu *et al*. (2008) for *P. aeruginosa* .

Using immunohistochemistry for detection of RHDV antigen, viral antigen was detected intracytoplasmic or intrasinuosoidal in hepatic tissues, splenic tissue, basement membrane and interstitial tissues of renal tubules, pulmonary interstitial tissues, and lining air alveoli, degenerated cardiac muscular fibers and inside the blood vessels (Fig. 9), these results agree with Jie *et al*. (2017) and Aleksija *et al*. (2018).

CONCLUSION

In this study, we successfully isolated and identified some pathogenic microbial causes associated with mortality in rabbits in North West Of Delta of Egypt which summarized in RHDV (either classic RHDV (G3-G5) and the variant RHDV2), *E. coli*, *K. pneumoniae*, *S. aureus*, *P. multocida* and *P. aeruginosa* in addition to their pathological effect on different organs. Consequently, effective disease prevention strategies and a development plan for the rabbit industry should be implemented.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this work.

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