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Effect of colibacillosis on the immune response to a rabbit viral haemorrhagic disease vaccine

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ABSTRACT

Viral haemorrhagic disease (VHD) and colibacillosis are common diseases in rabbits that cause economic losses worldwide. The effect of colibacillosis on the immune response of vaccinated rabbits against rabbit haemorrhagic disease virus (RHDV) was studied. Four groups (G1-G4) were included. G1 was the negative control group; G2 was the RHDV vaccine group; G3 was the *E. coli*-infected group; and G4 was the *E. coli*-infected + RHDV vaccine group. The *E. coli* infection and RHDV vaccination were simultaneously performed, with another previous infection, 3 days before vaccination. At 28 days post-vaccination (PV), the rabbits (G2-G4) were challenged intramuscularly with 0.5 ml of RHDV at a dose of 10^3 50% median lethal dose (LD_{50})/rabbit. The rabbits were observed for clinical signs, body weight gain and mortality rates. Tissue, blood, serum, and faecal samples and rectal swabs were collected at 3, 5, 7, 14, 21 and 28 days PV. Significant clinical signs and mortality and a decrease in BW were observed in the infected + RHDV vaccine group. On the 3rd day post-infection (PI), compared with all the other groups, the vaccinated group (G2) had significantly upregulated hepatic tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels; however, the infected + RHDV vaccine group had significantly higher intestinal levels of TNF-α and IL-6 than the other groups. Furthermore, *E. coli* infection in vaccinated rabbits led to immunosuppression, as shown by significant decreases ($P < 0.05$) in heterophil phagocytic activity, the CD4+/CD8+ ratio, and HI antibody responses to RHDV and a significant increase in the heterophil to lymphocyte (H/L) ratio. In conclusion, colibacillosis leads to immunosuppression involving a shift in the equilibrium of cytokines and reduced weight gain and mortality in vaccinated rabbits and could be a contributing factor in RHDV vaccination failure in rabbit farming.

1. Introduction

Colibacillosis is a major infectious disease endangering the rabbit industry, and rabbit haemorrhagic disease virus (RHDV) is a common disease in rabbits that causes economic losses worldwide (Wang, 2000). Enteropathogenic *Escherichia coli* (EPEC), Gram-negative bacteria, is the most important cause of enteritis and mortality in rabbits (Thouless et al., 1996). Clinically apparent *E. coli* infection is generally indicative of immunosuppression (Mc Gruder and Moore, 1998).

Rabbit haemorrhagic disease virus (RHDV) is a contagious and fatal viral pathogen capable of infecting rabbits, and it is an RNA virus in the family *Caliciviridae* (Ohlinger et al., 1990). RHDV is extremely lethal with high mortality rates in adult animals (60–90%), whereas infected rabbits younger than the age of 2 months usually survive (Xu and Chen, 1989). The clinical signs post-challenge with RHDV were studied by Elkady et al. (2016), who observed respiratory signs, bloody nasal discharge, diarrhoea and death; in addition, postmortem lesions showed punctate haemorrhages in the respiratory tract, liver, spleen, cardiac muscle and kidneys. Vaccines against RHDV have been developed for production; however, in recent years, failure to produce

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Table 1
Grouping and experimental design.

Group	No.	*E. coli* O55: H7 strain Infection (1.0 ml X 10^8 CFU, Orally)				RHDV Vaccination (0.5 ml $X2^{10}$ HAU, S/C)		Challenge (n = 6) 28 days PV (0.5 ml $X10^3$ LD_{50}, I/M)	
		Infection 3 days before vaccination	Infection during vaccination	Test PI	+/-	Test PV	+/-	Test PC	
G1 Negative control group	5X3 replicates	-ve	-ve	**-Colonization and shedding tests** (n = 6) at 3, 5, 7, 14, 21, and 28 DPI. **-BW** (n = 6) at 0 and 28 DPI and **BWG** (n = 6) at 28 DPI. **-Haematological and biochemical assays** (n = 6) at 0, 7, 14, 21 and 28 DPI **-Gene expression (n = 3 per group)** at 3, 5 and 7 DPI. **-CD4+ and CD8+ (n = 3)** at 3, 5 and 7 DPI. **-Histopathology** (n = 3 per group) at 7 DPI.	-ve	**- HI test** (n = 6 per group) at 0, 7, 14, 21 and 28 days PV.	-ve	**- Clinical signs, mortality rate and post-mortem lesions** observed daily for 10 days PC **-RT-PCR** to confirm the cause of death	
G2 Vaccinated group	5X3 replicates	-ve	-ve		+ ve		+ ve		
G3 Infected group	5X3 replicates	+ ve	+ ve		-ve		+ ve		
G4 Infected+ vaccinated group	5X3 replicates	+ ve	+ ve		+ ve		+ ve		

PI, post-infection; PV, post-vaccination; PC, post-challenge; DPI, days post-infection.

protective immunity has often appeared. Vaccination of rabbits against RHDV is the only way to control this endemic disease (Wang, 2000; Day, 2006).

Leukogram, the heterophil to lymphocyte (H/L) ratio and phagocytic activity measured by the nitroblue tetrazolium (NBT) heterophil slide adhesion test have been used in rabbits infected with *E. coli* (Panda et al., 2010). Tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine that causes cell damage, and Shiga toxins of *E. coli* can induce a TNF-α-based immune response (Lee et al., 2015).

There are no published articles on the effects of colibacillosis on the immune response to RHDV vaccination. Consequently, the present work was mainly aimed at evaluating the potential effects of *E. coli* infection on the cellular and humoral immune responses of vaccinated rabbits against RHDV. Therefore, this study was planned to (i) describe the effects of *E. coli* infection on rabbit health using clinical signs, gross lesions, histopathology, and faecal shedding; (ii) detect the effects of *E. coli* infection on humoral and cellular immune responses in RHDV-vaccinated rabbits (haemagglutination inhibition (HI) test, H/L ratio determination, heterophil glass adhesion assay, and CD4+/CD8+ ratio determination); (iii) identify the effects of *E. coli* on serum marker levels and gene expression (TNF-α, IL-6, and IFNγ); and (iv) evaluate the efficacy of the vaccine using a challenge with an isolated and identified RHDV field strain.

2. Materials and methods

2.1. Experimental rabbits

Sixty domestic male rabbits (*Oryctolagus cuniculus*) of the Baladi breed (8 weeks old, 1.3–1.5 kg) were used. The rabbits were purchased from a private rabbitry without a previous history of RHDV outbreaks or vaccination against RHDV. The rabbits were acclimatized for 2 weeks prior to the experiment. After the acclimatization period, rectal swabs were taken to confirm the absence of *E. coli* infection. The animals were reared under highly hygienic measures with daily observation until the end of the experiment. They were provided with water and feed *ad libitum*. The pelleted feed was free of any medicines. The animals were housed in stainless steel cages (n = 5 per cage) at 26–29 °C with a 14-h light/day cycle. All rabbits were tested for anti-RHDV antibodies before the experiment started using HI test (OIE Manual, 2008).

2.2. E. coli strain inoculation, RHDV vaccination and challenge strain

- The *E. coli* strain was a field EPEC serotype O55: H7 strain that was isolated, classified and serotyped in the Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Egypt. Bacterial colonies were grown overnight in Trypticase soy broth (Oxoid) at 37 °C on a shaker for bacterial enrichment. Cultures were washed twice with phosphate-buffered saline (PBS) by centrifugation at 500 × *g* for 10 min, and the bacteria were suspended in a 10% sodium bicarbonate (First Logistic Egypt Company, Egypt) solution at a chosen density using a spectrophotometer. The infection with *E. coli* was performed orally with 1.0 ml $X10^8$ CFU/rabbit using a nasogastric tube (Pai et al., 1986).
- The inactivated RHDV vaccine (Batch no. 17041) was purchased from the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt (containing 2^{10} haemagglutination unit (HAU)/ml). This commonly used vaccinal strain in Egypt is RHDV-Giza 2006; JQ995154.1 clustered in genogroup 6 RHDVa (Awad and Kotb, 2018). The infection with *E. coli* and vaccination (subcutaneously with 0.5 ml of RHDV vaccine) were simultaneously carried out, but there was a previous dose of *E. coli* infection, 3 days before vaccination.
- The RHDV challenge strain was titrated in groups of six seronegative adult rabbits (4-month-old) with ten-fold serial dilution of the stock

material (OIE Manual, 2008; Read and Kirkland, 2017) and titrations were calculated as described by Reed and Muench (1938). The stock material was then diluted in phosphate-buffered saline to give a dose of 10^3 LD_{50}/rabbit.

2.3. Grouping and Experimental design (Table 1)

2.3.1. Design description

Animals (n = 60) were randomly allocated into 4 groups, with fifteen animals in each group (5 animals × 3 replicates). The first group (G1), 2nd (G2), 3rd (G3) and 4th (G4) were the negative control group, vaccinated group, infected group and infected + vaccinated group, respectively. The negative control group was uninfected, unvaccinated and unchallenged with RHDV.

2.3.2. Rabbits examination

Bacterial colonization and shedding (n = 6) were examined at 3, 5, 7, 14, 21 and 28 days post-infection (PI) with faecal samples and rectal swabs. Clinical signs and mortality associated with *E. coli* infection were recorded. The average body weight (BW) of the rabbits (n = 6) was calculated at the beginning of the experiment (at 0 day PI) and at the end of the experiment (at 28 days PI). Body weight gain (BWG) was also calculated at 28 days PI. Clinical signs, postmortem lesions and mortality were observed post-challenge with RHDV. After euthanasia (n = 3 each group), organs were collected (the liver and intestine) for gene expression analysis of TNF-α and IL-6 at 3, 5 and 7 days PI with *E. coli*. Haematological and biochemical assays (n = 6) were performed at 0, 7, 14, 21 and 28 days PI, while CD4+ and CD8 + T lymphocytes (n = 3) were assessed at 3, 5 and 7 days PI with *E. coli*. At 7 days PI with *E. coli*, other liver and intestine tissue samples (n = 3 each group) were taken for histopathological examination.

2.3.3. Rabbits handling during experiment

All rabbits were handled and managed according to the appropriate biosecurity guidelines, and animal experiments were performed in accordance with all regulations and recommendations of the "Guide for the Care and Use of Laboratory Animals", were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University and were conducted at the Educational Veterinary Hospital, Faculty of Veterinary Medicine, Mansoura University (Mansoura, Egypt).

2.4. Bacterial analysis

Ten-fold dilutions of samples were plated on eosin methylene blue (EMB) agar medium **(Oxoid)** (peptone, lactose, dipotassium hydrogen phosphate, eosin Y, methylene blue and agar) and incubated overnight at 37 °C for faecal content enumeration of *E. coli* cells. After the incubation, colonies were picked and serotyped as described previously (Kok et al., 1996).

2.5. Challenge test

RHDV was a local field isolate of naturally infected rabbits used as a challenge strain. The naturally infected rabbits with RHDV outbreak were collected from a local farm in Kafrelsheikh Governorate, Egypt during March 2018 with suspected clinical signs and lesions of RHDV. The infection was confirmed by RT-PCR from liver tissue. Liver fragments were homogenized in 10–20% (W/V) phosphate buffered saline at pH 7.2, filtered through cheesecloth and clarified by centrifugation at 5000*g* for 10 min as described by OIE Manual (2008). The prepared liver homogenates were titrated against rabbit hyperimmune serum obtained from rabbits experimentally vaccinated with an inactivated RHDV vaccine (RHDV-Giza 2006; JQ995154.1; genogroup 6 RHDVa) purchased from the VSVRI (Abbasia, Cairo, Egypt) (Salman, 1999; Awad and Kotb, 2018). In this study, six rabbits per group (G2-G4) were challenged intramuscularly with 0.5 ml of virus at a dose of 10^3 LD_{50} /rabbit at 28 days PV. The rabbits were observed daily for clinical signs, and the mortality rate was determined at 10 days post-challenge.

2.6. Molecular identification of RHDV

2.6.1. RT-PCR analysis

Liver samples from rabbits (naturally infected or challenged) were tested using RT-PCR. The naturally infected rabbits were used for isolation of the challenge strain and the challenged rabbits used in this study. RNA extraction from samples was performed using a QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH). Briefly, 140 µl of sample suspension was incubated with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA at room temperature for 10 min. After the incubation, 100% ethanol (560 µl) was added to the lysate. The sample was then washed and centrifuged as described by the manufacturer's instructions. Nucleic acids were eluted with 60 µl of elution buffer provided in the kit. Primers supplied from Metabion (Germany) were used. Primer sequences, target genes, and amplicon sizes are indicated. The primer sequences (5′-3′) for the target gene VP60 were P33: CCACCACCAAC ACTTCAGGT and P34: CAGGTTGAACACGAGTGTGC. The length of the amplified product was 538 bp (Fahmy et al., 2010). Primers were utilized in a 25-µl reaction containing 12.5 µl of Quantitect probe RT-PCR buffer (Qiagen, GmbH), 1 µl of each primer at a concentration of 20 pmol, 0.25 µl of RT-enzyme (enzyme mix of the QuantiTect probe RT-PCR kit; Omniscript Reverse Transcriptase and Sensiscript Reverse transcriptase), 4.25 µl of water, and 6 µl of template. The reaction was performed with a Biometra thermal cycler. Reverse transcription was performed at 50 °C for 30 min, and a primary denaturation step was performed at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 40 s and 72 °C for 45 s. A final extension step was performed at 72 °C for 10 min. PCR products were separated by electrophoresis using a 1.5% agarose gel (Applichem, GmbH, Germany) in 1x TBE buffer, and 15 µl of products was loaded in each gel slot for gel analysis. A 100-bp DNA ladder (Qiagen, GmbH, Germany) was used to detect the fragment size. A gel documentation system (Alpha Innotech, Biometra) was used for gel imaging, and then data analysis was performed using computer software.

2.6.2. Sequencing and phylogenetic analysis of challenge strain

PCR products were purified using QIAquick PCR Product extraction kit (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (Hitachi, Japan), a BLAST® analysis was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Lasergene DNAStar and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

2.6.3. GenBank accession number

The VP60 gene nucleotide and the amino acid sequences from our challenge strain were deposited in the GenBank. The accession number of this strain was presented as MN397253.1.

2.7. Quantitative real-time PCR for immune gene expression analysis

RNA extraction from tissue samples (liver and intestine) was performed using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH); 30 mg of tissue sample was homogenized and processed as described by the manufacturer's instructions. Amplification and quantification of immune gene expression were conducted using Real-time PCR SYBR green (Thermo Fisher Scientific). The primer sequences (Metabion, Germany) used for real-time PCR to quantify immune gene expression are listed in Table 2. The primers were utilized in a 25-µl reaction containing 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix

Table 2
Target genes, primers sequences, amplicon size of immune genes.

Target gene	Primers sequences (5′-3′)	Amplicon size (bp)	References
GAPDH	F: TGACGACATCAAGAAGGTGGTG R: GAAGGTGGAGGAGTGGGTGTC	120	Schnupf and Sansonetti (2012)
IL6	F: CTACCGCTTTCCCCACTTCAG R: TCCTCAGCTCCTTGATGGTCTC	135	
TNF-α	F: GTCTTCCTCTCTCACGCACC R: TGGGCTAGAGGCTTGTCACT	335	Godornes et al. (2007)

(Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL), 0.5 µl of each primer at a concentration of 20 pmol, 8.25 µl of water, and 3 µl of RNA template. The cycling conditions for SYBR green RT-PCR were as follows: reverse transcription (50 °C, 30 min), primary denaturation (94 °C, 15 min), and then amplification (40 cycles) with secondary denaturation (94 °C, 15 s), annealing (60 °C, 30 s) and extension (72 °C, 30 s); a dissociation curve was produced under the following conditions: 1 min at 94 °C, 1 min at 60 °C and 1 min at 94 °C. The results of the SYBR green RT-PCR were analysed. Stratagene MX3005 P software was used to calculate amplification curves and CT values. The expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization of fold expression. The CT value of each sample was compared with that of the positive control group as described by the "ΔΔCT" method (Yuan et al., 2006).

2.8. Haematological, biochemical and immunological assays

Blood samples were collected with and without heparin from the rabbit ear vein on 0, 7, 14, 21 and 28 days PI. The heparinized samples were collected for estimation of the total leukocyte count (TLC) and differential white blood cell (WBC) count as well as for use in a heterophil slide adhesion test. Serum samples were frozen until subsequent analysis by an HI test. Haematological parameters of the leukocyte (WBC) response were estimated (Coles, 1986) using an improved Neubauer haemocytometer and Turk's diluents (Bio- diagnostic, Egypt) for WBCs. The NBT slide adhesion test (Gifford and Malawista, 1972) is a test for assessing the ability of heterophils to phagocytose and reduce yellow NBT (Sigma-Aldrich) to insoluble blue formazan and is quantified by comparing with heterophils without particles. Preserved serum samples were also used for the assessment of the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) (Randox Co., UK), creatinine (Human, Germany), total protein, albumin (Stanbio, USA), and globulin according to the manufacturer's instructions. IFNγ and TNFα were assessed with ELISA kits (Quantikine Company, USA). Peripheral blood samples (n = 3, each group) were collected in tubes with heparin on the 3rd, 5th and 7th days PI for evaluation of CD4 + and CD8 + T lymphocytes *via* flow cytometry by isolating mononuclear cells using Ficoll-Paque medium (Nieto et al., 2012). Flow cytometry was performed at Mansoura Children Hospital using reagent protocols from Becton Dickinson (Sunnyvale, CA, USA).

2.9. HI test

HI test were carried out according to OIE Manual (2008) in a round-bottom microtiter plate (U shaped) at 0, 7, 14, 21 and 28 days PV. The Haemagglutination (HA) test was carried out using type-O human erythrocytes (0.5%) to determine the HA titer of RHDV before performing the HI test. The antigen of HI test (a local isolate of RHDV-Giza 2006; JQ995154.1; genogroup 6 RHDVa) with a titer of 2^{10} HAU identified against reference immune serum was kindly supplied by VSVRI, Abbassia, Cairo (Salman, 1999; Awad and Kotb, 2018). HI test was used for detection of RHDV antibodies before the experiment and to assess the humoral immune response post-vaccination (n = 6 per group).

2.10. Histopathology

Tissue samples were processed for paraffin embedding. Then, the paraffin sections were cut and prepared. Deparaffinization, dehydration and staining with haematoxylin and eosin were performed as described in the protocol published by Bancroft and Gamble (2007).

2.11. Statistical analysis

The experimental data were statistically analyzed using SAS program (version 9.2) with GLM (Generalized linear model) procedure and Duncan's multiple range tests ($P < 0.05$).

3. Results

3.1. RHDV isolated and identified challenge strain

RHDV was a local field isolate of naturally infected rabbits used as a challenge strain. The clinical signs and lesions of naturally infected rabbits were respiratory signs, high mortality and hemorrhagic liver, kidneys and lung. The infection was confirmed by RT-PCR from liver tissue. The GenBank accession number was MN397253.1. This strain was closely related to recent Egyptian RHDV strains with 95.7–99.8% identity and showed 95.9% identity with the strain FN552800.1 France, which represents genogroup G5 of the classic RHDV (Fig. S1 and Table S2).

3.2. Growth performance, clinical signs and mortality after E. coli infection

The BW and BWG of the vaccinated group (G2) at the end of the experiment were significantly increased ($P = 0.017$ and $P = 0.028$, respectively) when compared to the infected vaccinated group (G4) (Table 3). Mild diarrhoea was recorded in the *E. coli*-infected groups (G3 and G4) during the first week. Most rabbits were asymptomatic and no mortality due to *E. coli* was observed. Moreover, no clinical signs were observed in the control group (G1) and the vaccinated group (G2) till the time of challenge with RHDV.

Table 3
Body weight (BW), Body weight gain (BWG), HI, mortality rates post-challenge with Rabbit haemorrhagic disease virus (RHDV) at 28 days post-vaccination (PV) and infection with *E. coli*.

Group	BW (gram) 0 day PV	BW (gram) 28 days PV	BWG (gram)	HI (Log2) Days PV					Mortality % post-challenge with RHDV
				0	7	14	21	28	
G1 C	1475 ± 42.5^{a}	2063 ± 91.7^{a}	588 ± 32.8^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{a}	**0/6 (0)^{c}**
G2 V	1420 ± 32.7^{a}	1942 ± 33.5^{b}	522 ± 82.1^{b}	0.0 ± 0.0^{a}	2.4 ± 1.5^{a}	4.1 ± 2.1^{a}	6.3 ± 1.2^{a}	7.2 ± 1.3^{a}	**0/6 (0)^{c}**
G3 E	1382 ± 81.6^{a}	1823 ± 82.1^{c}	441 ± 63.3^{c}	0.0 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{a}	**6/6 (100)^{a}**
G4 E + V	1513 ± 73.6^{a}	1794 ± 82.3^{d}	281 ± 37.2^{d}	0.0 ± 0.0^{a}	1.6 ± 1.5^{b}	3.6 ± 2.4^{b}	4.1 ± 1.2^{b}	5.3 ± 1.3^{a}	**2/6 (33.3)^{b}**

C, V, E and V + E were negative control, vaccinated, infected and infected + vaccinated groups respectively.
The different letters within the same column were significantly different at $P < 0.05$.

3.3. Mortality rate and clinical examination post-challenge with RHDV

No clinical signs or mortality were observed in the rabbits in the G1 negative control (unvaccinated-unchallenged) group. Moreover, vaccinated group (G2) did not show any clinical signs or mortality post-challenge with RHDV. However, the mortality percentage of G3 group was 100% (6/6) and that of the G4 group was 33.3% (2/6) with a significant difference ($P = 0.013$) (Table 3); the deaths occurred between 2 and 3 days PC. The obtained protection rates correlated with the HI antibody titres. The clinical signs in the challenged rabbits included bloody nasal discharge, diarrhoea, respiratory manifestations and death. Postmortem lesions included scattered haemorrhages in the trachea, lungs, liver, heart, spleen and kidneys. The livers from all dead rabbits in the G3 and G4 groups were evaluated by RT-PCR and shown to be positive for RHDV. Meanwhile, no virus was detected in the liver of surviving rabbits.

3.4. Recovery of E. coli O55

Faecal samples and rectal swabs from all rabbits inoculated with 10^8 CFU were cultured and positive for the respective inoculum strain until 28 days PI. Infection was followed by a significant increase in faecal *E. coli* levels in both the G3 and G4 groups on the 3rd day PI. In G3 & G4, the number of *E. coli* decreased from 10^8 to 10^1 CFU/g between 3 and 21 days PI. The *E. coli* colonization in G4 was relatively higher than that in G3 from 3 days PI onwards. G1 and G2 were negative for *E. coli* faecal shedding throughout the course of this study (Table 4).

3.5. Analysis of cytokine expression

Hepatic and intestinal TNF-α and IL-6 exhibited highly significant differences between the group means corrected for the time (Type III *F* test $P < 0.0001$ of each) (Fig. 1). A higher significant elevation in the hepatic TNF-α of G2 than G3 was detected at 3 and 5 dpi ($P = 0.0001$ and $P = 0.024$, respectively). Meanwhile, the hepatic IL-6 in G2 was significantly higher than those of G3 and G4 at 3 dpi ($P = 0.0001$ and $P = 0.001$ respectively). Conversely, the intestinal TNF-α level in G2 was significantly lower than those of G3 and G4 on 3rd dpi ($P = 0.01$ and $P = 0.004$, respectively). The intestinal IL-6 level was significantly up-regulated in G4 than those of G2 and G3 on 3rd ($P = 0.004$ and $P = 0.035$ respectively) and 5th dpi ($P = 0.0001$ and $P = 0.002$, respectively) (Fig. 1).

3.6. Haematological and biochemical changes

3.6.1. Haematological changes

Fig. 2(A–E) demonstrates the TLC, lymphocytes, heterophils, monocytes and the H/L ratio. The vaccinated group (G2) showed a significant lymphocytic ($P = 0.001$) leukocytosis ($P = 0.019$) at 7 dpi, then lymphocytosis at 14 dpi ($P = 0.002$) and at 28 dpi ($P = 0.017$) compared to G3. The G4 showed a significant leukocytosis ($P = 0.002$, $P = 0.034$ and $P = 0.036$, respectively) at 7, 21 and 28 dpi compared to G1. In G3, a significant increased H/L ratio ($P = 0.001$, $P = 0.001$ and $P = 0.002$ respectively) was detected at 7, 14 and 21 dpi when compared to G2. At 28 dpi, H/L ratio was significantly ($P = 0.004$) increased. The cellular NBT ratio of G2 was significantly elevated at 7 dpi ($P = 0.0001$, $P = 0.34$ and $P = 0.01$, respectively) and 14th dpi ($P < 0.001$ of each) when compared to G1, G2 and G3. Meanwhile, G4 had a significant decrease ($P = 0.001$, $P = 0.008$ and $P = 0.002$, respectively) of the NBT ratio compared to those of other groups at 21 dpi (Fig. 2F).

3.6.2. Biochemical changes

The serum TNF-α was elevated significantly in G1, G2 and G3 at 7 dpi ($P = 0.003$, $P = 0.002$ and $P = 0.001$ respectively) and 14 dpi ($P = 0.002$, $P = 0.007$ and $P = 0.003$, respectively) compared to control group (G1) (Fig. 3A). G3 and G4 continued its elevation at 21 dpi ($P = 0.014$ and $P = 0.005$ respectively) and 28 dpi ($P = 0.016$ and $P = 0.005$, respectively) compared to G1, as well as G2 at 21 dpi ($P = 0.037$ and $P = 0.012$ respectively) and 28 dpi ($P = 0.003$ and $P = 0.002$, respectively). Serum IFNγ of G2 achieved a higher elevation than G3 and G4 at 7 dpi ($P = 0.001$ and $P = 0.002$, respectively), 14 dpi ($P = 0.004$ and $P = 0.045$ respectively), 21 dpi ($P = 0.003$ and $P = 0.001$, respectively) and 28 dpi ($P = 0.006$ and $P = 0.005$, respectively) (Fig. 3B). Serum ALT was significantly elevated in G3 ($P = 0.043$) at 14 dpi and in G4 ($P = 0.045$ and ($P = 0.002$ respectively) at 21 and 28 dpi compared to G1 (Table S3). At 7 dpi, G3 had significantly higher AST but lower TP and globulin levels than those of G2. In addition, a higher significant elevation of creatinine level was detected in G3 than G2 group at all time-points (Table S3).

3.7. Assessment of CD4+ and CD8 + T lymphocytes

The CD4+ and CD4+/CD8+ ratio were significantly increased in G2 group compared to G1 group at 3, 5 and 7 dpi ($P = 0.028$, $P = 0.037$ and $P = 0.018$) and at 5 and 7 dpi ($P = 0.022$ and $P = 0.031$, respectively) (Fig. 4). Conversely, these parameters (CD4+ and CD4+/CD8+ ratio) were significantly suppressed in the infected groups (G3 and G4) at 5 and 7 dpi. CD8+ subsets showed non-significant changes in between groups, times and groups with time effect (Type III *F* test $P = 0.309$, $P = 0.876$ and $P = 0.941$, respectively).

3.8. HI antibody titers

No maternal HI antibody titers of RHDV were detected in the experimental rabbits on day 0 PV (8 weeks old) immediately before vaccination. The vaccinated group **(G2)** showed significantly higher ($P = 0.024$, $P = 0.019$, $P = 0.014$ and $P = 0.016$) HI antibody titers against RHDV than the infected vaccinated group (G4) at 7, 14, 21, and 28 days PV. No HI antibody titers were found in the non-vaccinated groups; G1 control (uninfected, unchallenged) and G3 at the tested time-points (Table 3).

3.9. Histopathology

The liver of G3 showed acute cell swelling, congested blood vessels and a few extravasated erythrocytes (Fig. 5B). In G4, focal necrotic hepatic parenchyma and recent thrombi consisting of fibrin-containing

Table 4
Recovery of the inoculum *E. coli* strain from rabbits (log^{10} CFU/g).

Group		Diarrhea in 1st week	Colonization of *E. coli* in faecal samples						Shedding of *E. coli* in rectal swabs					
			3d	5d	7d	14d	21d	28d	3d	5d	7d	14d	21d	28d
G1	C	–	–	–	–	–	–	–	–	–	–	–	–	–
G2	V	–	–	–	–	–	–	–	–	–	–	–	–	–
G3	E	+	7.49	3.18	2.08	1.30	0.60	0	+	+	+	+	+	–
G4	E + V	+	8.39	4.63	3.49	2.38	0.69	0	+	+	+	+	+	–

C, V, E and V + E were negative control, vaccinated, infected and infected + vaccinated groups respectively.

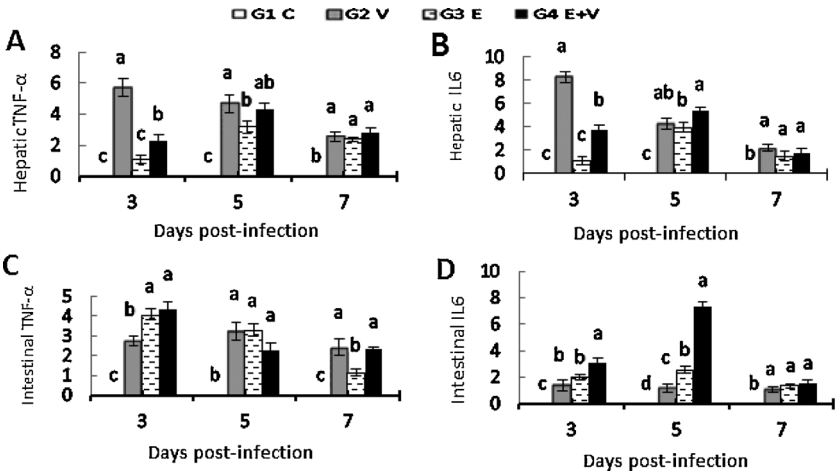

Fig. 1. Fold change expression of mRNA cytokines genes in the liver (A, B) and intestine (C, D) relative to C (control) group of experimental rabbits after normalization with the housekeeping gene (GAPDH). V, vaccinated with rabbit viral hemorrhagic disease vaccine; E, *E. coli* infected; E + V, infected and vaccinated with *E. coli*. Different letters (a–c) on the bars (mean ± SE) expressed significance level at $P < 0.05$.

inflammatory cells were seen (Fig. 5C). The G3 showed partial loss of villus tips with mucosal and submucosal oedema containing round cells in the intestine (Fig. 6B). In **G4**, necrotic enteritis characterized by destruction of the superficial mucosa with luminal exudate and intense oedema with inflammatory cells in the mucosa was observed (Fig. 6D).

4. Discussion

Vaccination is still one of the most important methods to control diseases and avoid economic losses in the rabbit industry. In particular, the efficacy of vaccination against RHDV in rabbits should be evaluated. This study aimed to assess the effects of colibacillosis on the immune response to a RHDV vaccine. The commonly used vaccinal strain in Egypt is RHDV-Giza 2006 clustered in genogroup G6 RHDVa (Awad and Kotb, 2018). The challenge strain in this study is more closely related to recent RHDV strains from Alexandria, Egypt with 95.7–99.8% identity and showed 95.9% identity with the strain with accession number FN552800.1 France, which represents genogroup G5 of the classic RHDV (Marchandeau et al., 2005). The RHDV G6 and G3-G5 genotypes are currently circulating in different Egyptian governorates as available sequence data (Awad and Kotb, 2018).

In the present study, mild diarrhoea was recorded in the *E. coli*-infected groups during the first week. In a previous work, Gallois et al. (2007) observed that the clinical signs in rabbits with *E. coli*infection are prostration, weight loss and diarrhoea. Moreover, the response to experimental infection with an *E. coli* strain was previously studied in rabbits (Panda et al., 2010). In this study, there were significant decreases in BW and BWG in the *E. coli*-infected groups. Similarly, Zhao et al. (2017) found that BW and BWG decreased significantly in rabbits with colibacillosis.

In our work, faecal colonization by *E. coli* was significantly higher in the infected vaccinated group than in the infected without vaccination group. The results concurred with those of previous studies and showed that faecal shedding of *E. coli* was significantly higher during the first dpi than during later days, particularly in infected vaccinated rabbits (Panda et al., 2010). Moreover, the RHDV vaccine significantly increased faecal colonization by *E. coli* in the infected vaccinated group (Arafat et al., 2017). The severity of the IBV variant was shown to be increased by the presence of *E. coli* O78 (Galal et al., 2018). Furthermore, EPEC O55 was detectable for long periods of time in the infected groups in this study. Earlier studies have shown that *E. coli* can persist (at a 10^8 challenge dose) for days to weeks in the rabbit intestinal tract

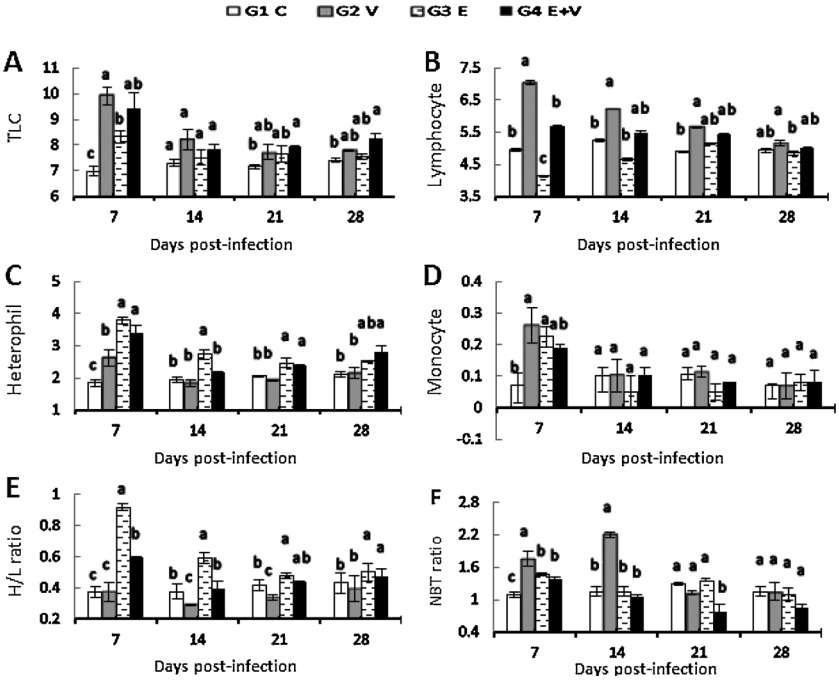

Fig. 2. Leukogram (10^3/μL); (A) total leukocyt count (TLC); (B) lymphocyte, (C) heterophil; (D) monocyte; (E) H/L; heterophil/lymphocyte ratio and (F), NBT; nitroblue tetrazolium ratio of the experimental rabbits; C, V, E and V + E were G1 negative control, G2 vaccinated, G3 infected and G4 infected + vaccinated groups respectively. Different letters (a–c) on the bars (mean ± SE) expressed significance level at $P < 0.05$.

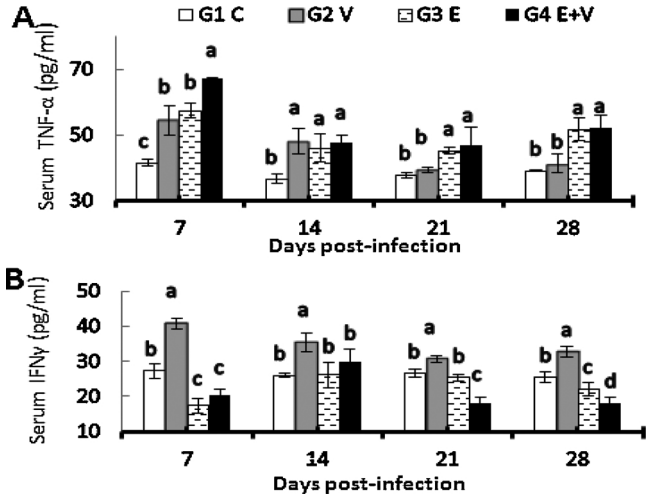

Fig. 3. (A) Serum TNFα and (B) IFNγ of the experimental rabbits. C, V, E and V + E were control negative group, vaccinated group, infected group and infected + vaccinated group respectively. Different letters (a–d) on the bars (mean ± SE) expressed significance level at $P < 0.05$.

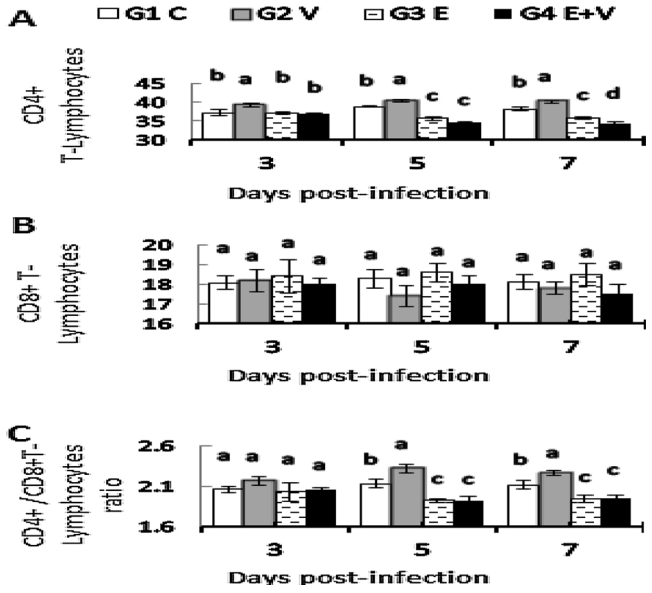

Fig. 4. The CD4 + (A) and CD8 T-lymphocyte (B) subsets and CD4 + and CD8 + ratio (C) of the peripheral blood samples of experimental rabbits. C, control group; V, vaccinated with rabbit viral hemorrhagic disease vaccine; E, *E. coli* infected; E + V, infected and vaccinated. Different letters (a–d) on the bars (mean ± SE) expressed significance level at $P < 0.05$.

before clearance. This evidence supports the principle that *E. coli* strains can cause immunosuppression (Guo et al., 2017).

In the present study, no clinical signs or mortality was observed in vaccinated rabbits post-challenge with RHDV. However, the mortality rate of the infected vaccinated group was 33.3% (2/6), and the deaths occurred between 2 and 3 days post-challenge. Similar to the vaccinated only group in our study, Yang et al. (2015) showed that 8-week-old rabbits inoculated with one vaccine dose of inactivated RHDV and challenged with lethal RHDV had not any mortality with 100% survival rates. Read and Kirkland (2017) indicated that vaccinated rabbits are protected against RHD, showing that a commercial vaccine is effective against different strains of RHDV due to the production of detectable antibodies before virus exposure. They also revealed that unvaccinated rabbits that received virus died between 48 and 72 h after inoculation. Therefore, the interaction between *E. coli* infection and RHDV vaccination can be considered as a cause of vaccination failure rather than simply assuming that RHDV antigenic changes were the main problem as previously suggested by Ismail et al. (2017).

In this study, the gene expression results clarified the link between stress represented by *E. coli* infection, RHDV vaccination and the statuses of proinflammatory cytokines (TNF-α and IL-6) in rabbits. The results revealed overexpression of the IL-6 and TNF-α genes and serum TNF-α. IL-6 and TNF-α have potential roles in the immune response as their levels increase with RHDV infection in rabbits (Trzeciak-Ryczek et al., 2017) and with *E. coli* infection in the liver at 3 dpi (Guo et al., 2017). The present study showed a general elevation in serum IFNγ level in the vaccinated groups compared to the control group, except at 7 dpi, which showed a decline. This decline may be because IFNγ was exhausted in the serum for a temporary period and then increased again. Exhaustion of the T cells had associated with persistent viral infection. This exhaustion may affect some function as specific cytokine production as IFNγ as well as some chemokines. However, regenerative immunity returned after the demise of the cause (Wherry and Kurachi, 2015). Meanwhile, the IFNγ of the vaccinated rabbits had significantly elevated. The cytotoxic lymphocytes produced the IFNγ which enhanced their motility and function leading to the antiviral action of CD8 T cells (Bhat et al., 2017). IFNγ is produced by natural killer cells and lymphocytes after antigen-specific immunity has developed, and IFNγ prevents viral replication (Schoenborn and Wilson, 2007). IFN-γ expression is upregulated with haemorrhagic disease virus infection in rabbits (Trzeciak-Ryczek et al., 2017).

The leukogram of the infected group rabbits in this study indicated heterophilic leukocytosis, in addition to an increased H/L ratio in the infected group compared to the control group. The results for the leukogram, H/L ratio and phagocytic activity measured by the NBT test were in agreement with the findings of a study of rabbits infected with *E. coli* (Panda et al., 2010). In rabbits, H/L ratio showed a higher elevation with bacterial infections than the elevation of the TLC (Panda et al., 2010). At 7th dpi, the TLC of G2 and G3 had a higher significant elevation than the control group and then it changed to normal till the end of the experiment. This may be attributed to the elevation in the level of the proinflammatory cytokine TNF-α (the cytokine responsible for the regulation of immune cells), which promoted the migration of leukocytes into the inflamed tissue (Hodgson, 2006). However, vaccination in addition to infection with *E. coli* changed the balance of cytokines away from increasing inflammation (Belkaid and Hand, 2014). The phagocytic heterophil levels detected in the NBT test were elevated in the infected group at 7 dpi, which may be due to fragments and lactoferrin from the bacteria encouraging the phagocytic process (Hashimoto et al., 1998). In addition, the reduced phagocytic activity of the infected group may be related to stress of infection (Ali et al., 2013; Eladl et al., 2019). The creatinine level was increased in the infected vaccinated group compared to control group. This difference may be attributed to the release of epinephrine due to the stress of infection that can decrease renal perfusion (Melillo, 2007).

CD4 + and CD8 + T lymphocytes are mirrors of the humoral and cellular immune responses, respectively, and the CD4 + /CD8 + T lymphocyte ratio reflects T lymphocyte activities (Seyidoglu et al., 2017). The serum IFNγ level was increased on subsequent days. CD4 signalling stimulates T helper cells, consequently leading to the secretion of IFNγ (Seyidoglu et al., 2017). In the current study, the CD4 + T lymphocyte level was significantly elevated in the vaccinated group, but the CD8 + T lymphocyte level did not change. Similarly, administration of a myxomatosis vaccine to rabbits elevated blood CD4 + T lymphocyte numbers without changing CD8 + T cell numbers (Ghodratizadeh et al., 2014). In this study, the infected vaccinated group showed a significantly lower CD4 + level and CD4 + /CD8 + ratio than the other groups. These outcomes may be attributed to the stress of infection and vaccination (Eladl et al., 2014; Arafat et al., 2018).

In this work, compared to the infected vaccinated group, the uninfected vaccinated group showed significantly elevated HI antibody

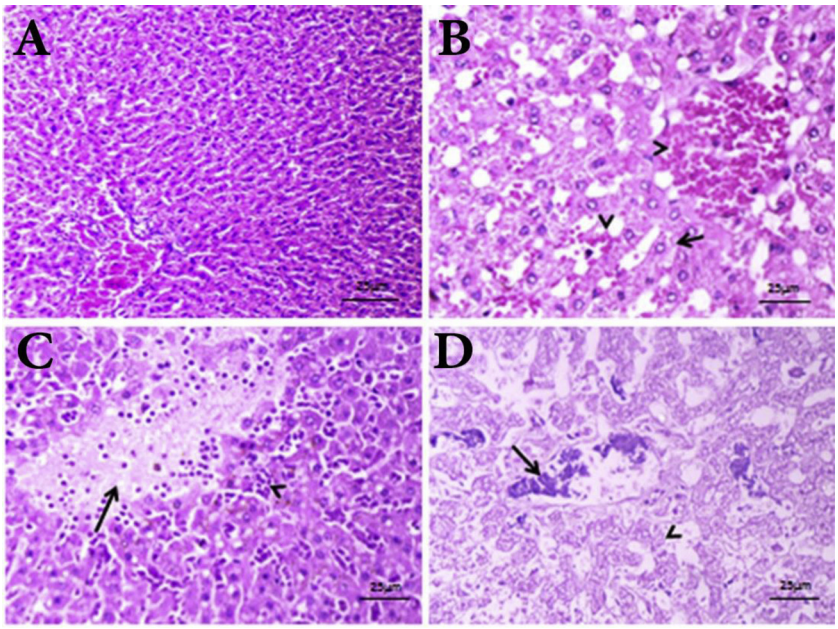

Fig. 5. Photomicrograph of sections from liver of vaccinated rabbits at 7 days post-infection with *E. coli* stained with H&E. (A) histological picture of liver show normal picture in G1 (Control group) & G2 (V group); (B) acute cell swelling of the hepatic cells (arrow) congested blood vessels and a few extravsated erythrocytes (arrow head) in G3 (E group); (C) recent thrombus consists of fibrin containing lymphocytes and heterophils (arrow) beside clusters of inflammatory cells in the adjacent hepatic sinusoids (arrow head) in G3 (E group); (D) disseminated basophilic bacterial emboli in hepatic vasculature (arrow) and necrotic hepatic parenchyma (arrow head) in G4 (E + V group).

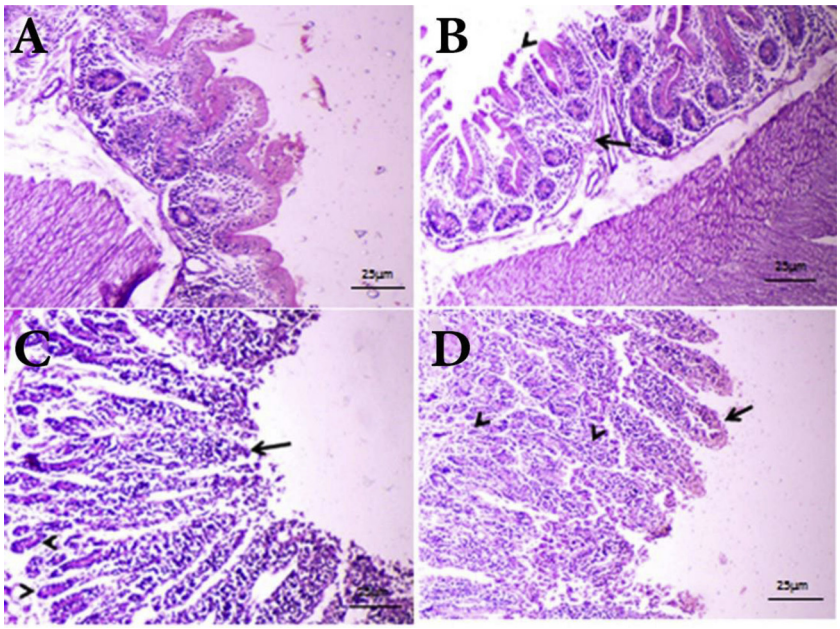

Fig. 6. Photomicrograph of sections from intestine of vaccinated rabbits at 7 days post-infection with E. coli stained with H&E. (A) histological picture of intestine show normal picture in G1 (Control group) & G2 (V group); (B) mild mucosal and sub mucosal edema and inflammatory cells (arrow) and partial loss of villus tips (arrow head) in G3 (E group); (C) partial loss of villous enterocytes (arrow) and proliferative intestinal crypts (arrow head) in G3 (E group); (D) destructed superficial mucosa (arrow) with intense edema and inflammatory cells in mucosa and sub mucosa (arrowhead) in G4 (E + V group).

titers. The HI test is used to detect RHDV antibodies and the RHD prevention is still dependent on vaccination (Day, 2006). Recent work in Australia for instance, indicated that myxomatosis suppresses the rabbit's immune system increasing mortality among those subsequently infected with RHDV (Barnett et al., 2018). However, no experimental work has been carried out to confirm this. The experiment described in this study is a step in that direction and suggests that such interactions between pathogens might be far more widespread than many people imagine.

In this study, histopathological examination displayed milder lesions in the liver and intestine samples from the infected unvaccinated group than in those from the infected vaccinated group. These findings may be due to the immunosuppression and stress induced by infection and vaccination (Galal et al., 2018). Our results were in agreement with those of a study by Lateef et al. (2018), who showed that the intestine of rabbits experimentally infected with *E. coli* exhibited proliferation in Peyer's patches and mononuclear cell infiltration. The liver of infected rabbits displayed coagulative necrosis and loss of cells due to karryolysis of hepatocyte nuclei. These results also agree with those of Lateef et al. (2018), who reported that systemic syndromes are induced by the action of bacterial toxins.

5. Conclusion

This paper reports the first evidence of the effect of colibacillosis on the immune status of rabbits vaccinated against RHDV, specifically that colibacillosis leads to immunosuppression, poor WG and mortality. Colibacillosis with vaccination shifted the equilibrium of cytokines away from excessive inflammation. RHDV vaccination at 8 weeks old is effective in rabbits, as displayed by HI titers, while *E. coli* infection had a depressive effect on the immune response. Colibacillosis might be a contributing factor in RHDV vaccination failure in rabbit farming. The application of good hygienic measures is highly recommended, and frequent examination for bacterial diseases such as colibacillosis is

required. While it is true that hygienic measures are extremely important, RHDV vaccination still has an important role to play in the control of this disease.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108429.

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