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Immunological Studies on Cattle Naturally Infected with FMD Compared with the Vaccinated Cattle in Sharkia Governorate, Egypt

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Abstract

Foot and mouth disease (FMD) is a highly contagious viral disease. Despite annual mass vaccination programs in Egypt, the incidence and economic losses are high. The Samples were collected after clinical examination of animals in 2022 and divided into three groups; Group 1(G1): Fifteen samples were collected from apparently healthy cattle (control group). Twenty-five samples were collected from cattle with suspected symptoms of FMD and divided into 2 groups 15 non-vaccinated Group 2(G2) and 10 vaccinated cattle Group 3(G3). Isolation of FMDV using BHK-21 cells was done. Molecular identification, sequencing, and phylogenetic analysis of new FMDV serotypes A and O circulating variants from G2 and G3 regarding targeting the VP1 gene. Results demonstrated that genotype O was related to East Africa-3 (EA-3) topotype. Identification of FMDV using PCR for field isolates, revealed that genotype A was related to genotype IV of the African topotype. The isolated virus on BHK-21 showed characteristic cytopathic effects (CPE). Hematological analysis showed a significant decrease in most hematological parameters with neutrophilia and lymphopenia. Serum biochemical constituents revealed a significant increase in AST, ALT, urea, creatinine, and cortisol, while a significant decrease in the total protein, albumin, and globulins in (G2). The vaccinated group showed a significant increase in serum AST and cortisol. The highest values of IL10, CRP, Troponin 1, Haptoglobin, and Complement 3 in the infected group (G2). Pathological examination revealed ulcerative dermatitis on the tongues and the coronary bands of the hoof. The heart revealed severe lymphocytic myocarditis with depletion of lymph nodes and spleen. While vaccinated (G3) revealed a significant decrease in RBC count and Hb concentration also lymphocytosis, neutropenia, and leukocytosis were founded. while the lowest values of IL10, and Haptoglobin were reported in the vaccinated one (G3). The recorded level of IgG showed a severe decrease in G2 and a high increase in G3. The pathological lesions recorded in G3 were milder than in G2. It could be concluded that the isolates in this study from both groups non-vaccinated and vaccinated were matching each other while not matching with the local vaccinal strain. The vaccinated cattle in 2022 had mild to moderate immunity against the disease.

KEYWORDS
FMDV vaccine, BHK, RT-PCR, Sequence, Hematological, Biochemical parameters, Histopathology.

INTRODUCTION

FMD is an acute disease in cloven-footed animals as well as more than 70 species of wild animals (Knowles and Samuel, 2003). Egypt has long been regarded as one of the continent's FMDV-endemic nations with frequent outbreaks (Lin *et al*., 2010).

Fever, anorexia, and excessive stinging or frothy salivation with vesicles or blisters on the tongue are the disease's hallmarks. Although it often does not cause death in adult livestock, it does increase the likelihood of abortion in pregnant animals and mortality in young livestock (Saravanan *et al*., 2020).

FMDV has a non-enveloped, 8.5 kb in length, positive-sense, single-stranded, non-segmented RNA genome that is encased in an icosahedral capsid that contains roughly 60 copies of four structural viral proteins (VP1, VP2, VP3, and VP4). The prototypical member of the genus Aphthovirus, FMDV is a member of the Picornaviridae family. FMDV has a great potential for genetic and antigenic diversity due to the immunological variations between serotypes, which has resulted in the classification of 7 FMDV serotypes (A, O, C, Asia1, SAT-1, SAT-2, and SAT-3) discovered among the circulating viruses (Faruk *et al*., 2021).

It seems improbable that one serotype causes protection to another based on its capacity to generate cross-protection in animals (Botton *et al*., 2006). The significant amount of genetic variability between virus serotypes, which share approximately 86% homology, is caused by a high level of replication mistakes that are susceptible to occur during the FMD viral RNA replication process (Belsham, 2020). The main immunogenic epitopes are included in the extremely changeable protein known as VP1. In the global molecular epidemiology study of FMDV, this variable protein is crucial (Lloyd-Jones *et al*., 2017). According to Gab-Al-

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lah *et al*. (2018), the lesions included tongue ulcerative dermatitis and vesicle development. Emphysema, pulmonary cornifications, and thickening of the alveolar septa were also noted. Focused myocardial degenerations and necrotic degenerations were observed by Salim *et al*.,2019.

A tried-and-true method for preventing FMD is immunizing susceptible animals against it. Although vaccination is used as the primary form of control, several instances of immunization failure have been documented. Because the inactivated viruses cannot reproduce in animals that have received vaccinations, it is crucial to utilize inactivated virus vaccines. The risk of return to virulence makes the use of live virus vaccines unacceptable (Sarah *et al*., 2002; OIE 2012).

This study was taken to isolate and identify the emerging FMDV circulated in the naturally infected cattle in Egypt in 2022. Moreover, a comparison between the naturally infected cattle with or without local vaccines was done in terms of immunological, biochemical, and histopathological approaches.

MATERIALS AND METHODS

Animals

We investigated an FMDV outbreak in cattle in Sharkia Governorate, Egypt during the natural outbreaks of FMD in 2022. We approached this outbreak and examined 90 (ninety) farm animals (foreign and native cattle) of different ages and sexes. Cattle and calves that seemed to be infected with the characteristic clinical signs (epithelial, hoof lesions) and cardiac and respiratory distress were observed by using a stethoscope. The rectal temperature of infected cattle was determined using a thermometer. Clinical signs and postmortem examinations among diseased and emergency slaughtered animals were recorded.

Animals were divided into 3 groups as follows: Group 1 (G1), 15 healthy animals from the same localities (control group). Group 2 (G2), 15 diseased non-vaccinated animals showing characteristic clinical signs of FMD. Group 3 (G3), 10 diseased and vaccinated animals from the same localities and reared on the same pastures.

All experimentation, transportation, and care of the animals in this study were performed in compliance with the formal approval of the Agriculture Research Center, and Animal Health Research Institute's policy on animal use and ethics. IACUC protocol Number is ARC-AH-22-11.

Sampling

The samples were collected randomly as follows:

Samples for Virology

Twenty-five samples in all were taken from cattle that had clinical symptoms of FMD. From the tongue or buccal mucosa, epithelial tissue was removed from vesicles that had not yet ruptured or had ruptured recently, and it was then placed in a viral transport medium with antibiotics added (OIE, 2012). Samples were given to the Virology section and the Animal Health Research Institute for laboratory diagnosis while taking biosecurity precautions (OIE, 2021). Samples from clinically diagnosed cattle were taken in the year 2022. Groups of immunized (n = 10) and unvaccinated animals (n = 15) were separated. Moreover, samples from apparently healthy animals were also collected and served as a control (n = 15)

Blood samples

Blood samples were collected from the jugular vein of cattle under aseptic precautions. The first sample was 2 ml of blood collected on EDTA for hematological examination. The second blood sample was 5 ml of blood taken without anticoagulant for biochemical analysis (n=10).

Organs were obtained from slaughtered cattle of G1 and Cattle of G2 and G3 that died due to FMD and were investigated for necropsy (n= 2/each group).

Virus Isolation and identification

A baby hamster kidney cell line (BHK-21) was inoculated with prepared samples (Ferris *et al*., 2009). The cultures were examined microscopically for the development of a cytopathic effect (CPE) through three successive passages (Barry *et al*., 2009). It was applied in the Virology department, Animal Health Research Institute. BHK-21 cells were received from the Virology department, Animal Health Research Institute, Dokki, Egypt.

A reference FMD virus was obtained from the Virology department, Animal Health Research Institute, Egypt, and used as a positive control for PCR.

Polymerase chain reaction

It was applied in the Biotechnology unit, Reference lab. for veterinary quality control, Animal Health Research Institute, Egypt according to previous methods (OIE 2012; Kandeil *et al*., 2013; Othman 2018).

Nucleic acid extraction

Whole nucleic acid extraction from samples was performed using the QIA ampminielute virus spin kit (Qiagen, Germany, GmbH). Oligonucleotide primers were supplied from (Metabion Germany) and listed in Table 1.

PCR amplification

FMD screening PCR: 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, 4.25 µl of DDW, and 6 µl of the template was used to test the primers. A thermal cycler applied biosystem 2720 was used to carry out the reaction. The reverse transcription process was carried out at 50 °C for 30 min, followed by 35 cycles of 94 °C for 30 sec., 55 °C for 30 sec., and 72 °C for 30 sec. A final extension step was performed for 7 min at 72 °C.

FMD typing PCR

Reverse transcription was performed at 50 °C for 30 min, followed by a primary denaturation stage at 95 °C for 5 min, 35 cycles of 94 °C for 30 sec (FMD A, O: annealing at 55 °C for 40 sec; for SAT-2: annealing was performed at 60 °C for 40 sec), and 72 °C for 1.2 min. A final extension step was carried out for 12 min at 72 °C.

Analysis of the PCR Products

The PCR products were separated using 5V/cm gradient electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. Each gel slot had 15 µl of

the PCR product inserted for the gel analysis. The fragment sizes were calculated using DNA ladders from Generuler 100bp (Fermentas, Thermo Scientific, Germany) and gel pilot 100 bp plus (Qiagen, GmBh, Germany). A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel, and computer software was used to analyze the information.

Hematological studies

Red blood cells (RBCs), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total, and differential leukocytic counts were determined by the standard hematological procedures as described by Feldman *et al*. (2000).

Biochemical studies

All biochemical parameters were carried out using commercial kits, and the used protocol for each parameter was done as recommended by the manufacturer manual. The liver transferases (alanine aminotransferase, ALT and aspartate aminotransferase, AST) activities were estimated according to Murray (1984). Serum urea was determined according to Kaplan (1984) and the serum creatinine was estimated according to Henry (1974). The serum total protein was tested according to Tietz (1995). The serum albumin was estimated according to Doumas (1971). Serum globulins level was calculated. Serum levels of cortisol were detected using the ELISA technique and test kits supplied by CUSABIO, Catalog Number, CSB-E05112r. Determination of IgG by sandwich ELISA was carried out according to Kim *et al*. (1989). Interleukein10 (IL-10) was measured by using Quantikine ELISA, Catalog Number, R1000. Serum cardiac troponin I (cTn-1) was estimated according to Collinson *et al*., (2001). C-Reactive Protein (CRP) was measured by using BDTM ELISA. Haptoglobin was detected using the ELISA technique and test kits supplied by CUSABIO, Catalog Number, CSB-E08587r. A sandwich enzyme-linked immunosorbent assay (ELISA) kit from CAT was used to measure the serum complement 3 levels in the meantime (Life Span Biosciences, Inc., Seattle, WA, USA). LS-F9287), following Lie *et al*., (1986).

Histopathological identification

Tissue specimens from different organs (tongue, coronary band of the hoof, heart, sublingual lymph nodes, and spleen) from different groups were fixed on neutral buffered formalin then they were processed by routine methods (Suvarna *et al*., 2013). and examined microscopically.

Statistical analysis

According to Tamhane and Dunlop (2000), the statistical analysis of the data gathered from this study was performed using the MSTAT-C computer program.

RESULTS

Clinical findings

The animals showed FMD signs which included fever, dullness, anorexia, salivation, and lameness with the development of vesicular eruptions on the buccal mucosa, and the coronary band in all infected animals of G2. Also vaccinated group (G3) showed the same signs with a milder degree.

Postmortem Examination

The heart showed streaks (stripping) mainly in the wall of the left ventricle besides some petechiae and thickness on the pericardium. Hyperemia and minute ulcers in the forestomach, glandular stomach, and intestine were recorded in the G2 and G3. The spleen and lymph nodes were atrophied with multi-focal irregular whitish raised streaks on the surface of G2.

Viral identification

Tissue culture

Propagation of the suspected samples on the BHK-21 cell line resulted in the characteristic CPE for three successive passages 24-48 h post-inoculation. The cytopathic effects were in the form of flattening of the cells, and formation of multinucleated giant cells, the breaking of the intracellular bridges, and cytotoxicity. Characteristic CPE appeared after 12 h post-infection, within 48 h, more than 60% of cells died, and by 72 h cell monolayer detached from the surface of the culture vessels (Fig. 1).

Molecular characterization and Typing of FMDV by PCR

The successful isolation of 10 FMDV samples tested for the presence of VP3-2B (target gene), followed by typing of the 10 FMDV against serotypes O, A, and SAT- 2 at expected size: 1301 bp, 863 bp, and 1279 bp, respectively. This revealed detection of serotypes A & O. Typing of FMD revealed 5 positive serotype A (4 vaccinated and 1 nonvaccinated), 5 positive serotype O (1 vaccinated and 4 nonvaccinated) and negative serotype SAT 2 (Fig. 2).

Table 1. Primers sequences, target genes and amplicon sizes.

Target agent	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
FMD	5'UTR	GCCTGGTCTTTCCAG GTCT CCAGTCCCCTTCTCAGATC	326	OIE (2012)
FMD A	VP3-2B	TACCAAATTACACACGGGAA GACATGTCCTCCTGCATCTG	863-866	Kandeil *et al*. (2013)
FMD O		ACCAACCTCCTTGATGTGGCT GACATGTCCTCCTGCATCTG	1301	
FMD SAT-2		TGAACTACCACTTCATGTACACAG ACAGCGGCCATGCACGACAG	1279	Othman (2018)

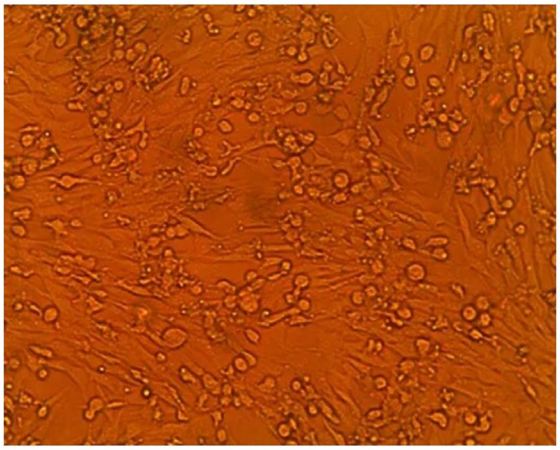

Fig. 1. 3rd passage Characteristic CPE of FMDV post inoculation on BHK 21 cells.

Nucleotide sequencing of serotype O

As shown in Fig. 3, the successful isolation of FMDV reported in this paper and two samples representative of FMDV serotype O were sequenced and submitted to Genbank with accession number: Banklt2580845 seq 1 ON455107 Egypt 3SD: from the nonvaccinated animal. Banklt2580845 seq 1 ON455108 Egypt 5SD: from the vaccinated animal.

Phylogenetic analysis of serotype O

Phylogenetic trees were constructed based on the nucleotide sequence alignment of the VP1 resulting in two strains of FMDV serotype O showing that they are belonging to topotype EA-3 which was detected in Egypt recently (Figs. 4 & 5).

Nucleotide sequencing serotypes A

The successful isolation of FMDV reported in this paper and two samples representative of FMDV serotypes A were sequenced and submitted on Genbank with accession number:
Banklt2577688 seq1 ON380439 FMDV/A/Egy/Egypt 1SD/2022.
Banklt2577688 seq2 ON380440 FMDV/A/Egy/Egypt 2SD/2022.

Phylogenetic analysis serotypes A

A phylogenetic tree was constructed based on the nucleotide sequence alignment of the VP1 resulting in two strains of FMDV serotype A showing that they are belonging to the G-IV African topotype which was detected in Egypt recently acid identity (Figs. 6 - 8).

Hematological analysis

Table (2) illustrated a significant decrease in most hematological parameters (RBCs, Hb, PCV, MCV, MCH, and TLCs) with neu-

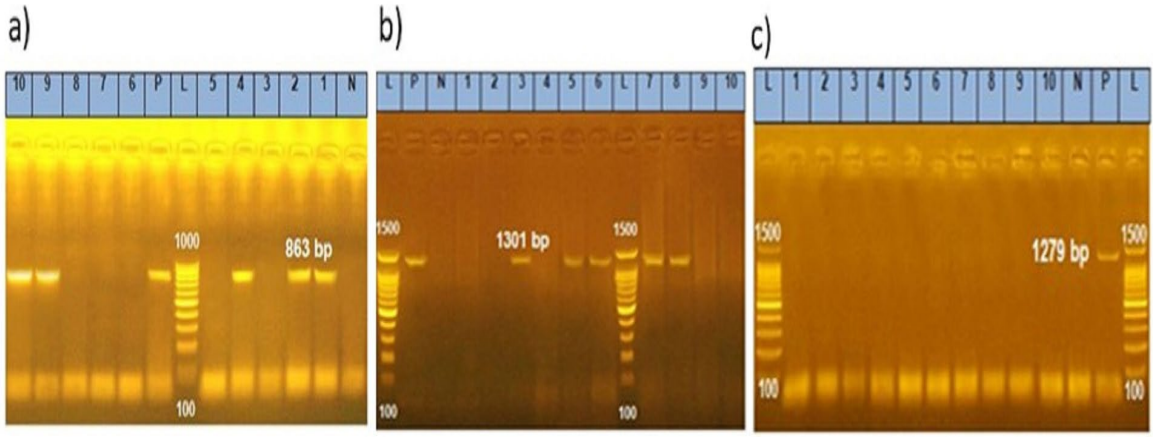

Fig. 2. Agarose gel electrophoresis of amplified PCR products of serotypes a) A, b) O, and c) SAT2 with product size of 1301 bp, 863 bp and 1279 bp, respectively. compared to positive control sample. L: Molecular weight marker (ladder).

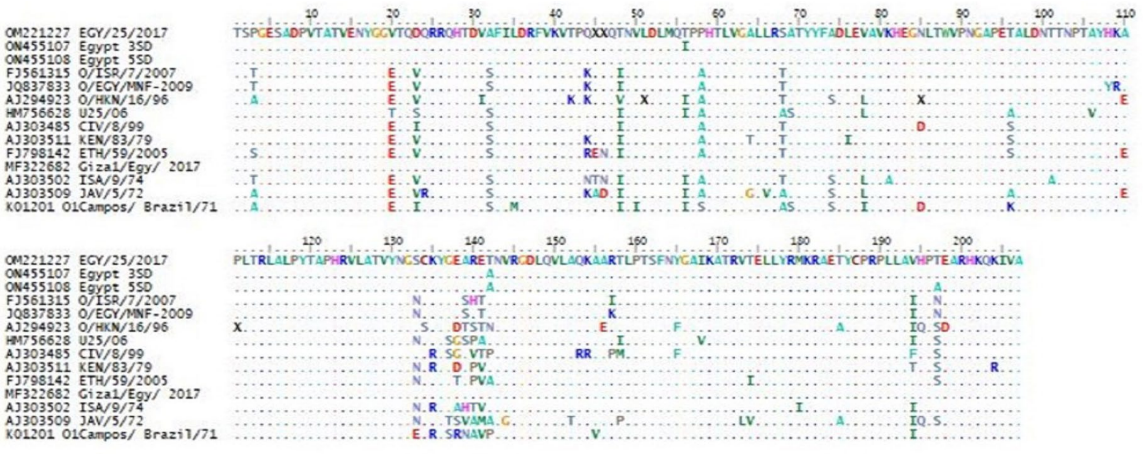

Fig. 3. Amino acid alignment of FMD virus serotype O isolated from Egypt.

trophilia and lymphopenia in FMD infected group (G2) compared with the control one. While the vaccinated group (G3) revealed a significant decrease in RBC count and Hb concentration also lymphocytosis, neutropenia and leukocytosis were founded. The lowest values were recorded in the infected group.

Biochemical analysis

The tested biochemical parameters are presented in Table (3). The serum activities of AST and ALT showed a significant increase in G2 (infected one) when compared with G1 (control group). G3 (vaccinated) showed a significant increase in AST level compared with G1. Serum urea and creatinine showed a significant increase in G2 (infected one) compared with the control group. The vaccinated G3 showed non-significant changes in serum urea and creatinine compared with the control group. Serum total protein, albumin, and globulin showed a significant decrease in G2 compared with the control one, while G3 vaccinated group showed a non-significant increase in total protein. G2 infected group showed a significant increase in serum cortisol concentration compared with the control group. G3 vaccinated showed a non-significant increase in serum cortisol concentration compared with G1. The immunological parameters are shown in Table (4), the highest values of IL10, CRP, C, Trop1, haptoglobin, and complement 3 in the infected group (G2) while the lowest values of IL10, haptoglobin were founded in vaccinated one (G3). IgG recorded a high increase in the vaccinated group and severely decreased in the infected one compared with the control group (G1).

Histopathological finding

Histopathological examination of the tongue was presented in Fig. 9. Tongue of G1 was normal. The histopathological alternation of the infected tongue (G2) showed distortion of papillae and vesicle formation which coalesce with each other forming bullae of different sizes and leukocyte infiltration. Ballooning of epithelial cells and eosinophilic inclusion bodies in the dermal layer were seen. In advanced cases, ulceration of the epidermal layer represented by cellular debris and round cells mainly neutrophils and lymphocyte aggregations forming large, circumscribed depression (pustule formation) were noticed. The stratum muscularis showed severe hyalinization, interstitial hemorrhages, and Zenker's necrosis. The tongue of the infected vaccinated (G3)

Table 2. hematological parameters in control (G1), FMD-infected cattle (G2) and vaccinated one (G3).

Parameters	G1 (Control)	G2 (Infected)	G3 (Vaccinated)
RBCs (X 10^6 /µl)	5.46±0.17a	4.55±0.19c	5.12±0.16b
Hb(g/dl)	8.41±0.25a	6.35±0.24c	7.96±0.26b
PCV%	38.18±0.47a	28.44±0.34b	34.78±0.40a
MCV (fl)	69.93±0.82a	62.51±1.44b	67.92±1.35a
MCH (pg)	15.40±1.30a	13.96±1.03b	15.55±1.20a
TLC X 10^3 /µl	9.08±1.40b	7.10±1.30c	10.36±0.91a
Neutrophils X 10^3 /µl	3.1±1.60b	4.6±1.90a	2.8±0.85c
Lymphocytes X 10^3 /µl	5.8±2.06b	2.2±1.80c	7.3±1.29a
Monocytes X 10^3 /µl	1.4±0.40b	2.1±0.70a	1.6±0.70b

Data are presented as mean ± SE; Different letters at the same column means that there was a significant change at $p<0.05$

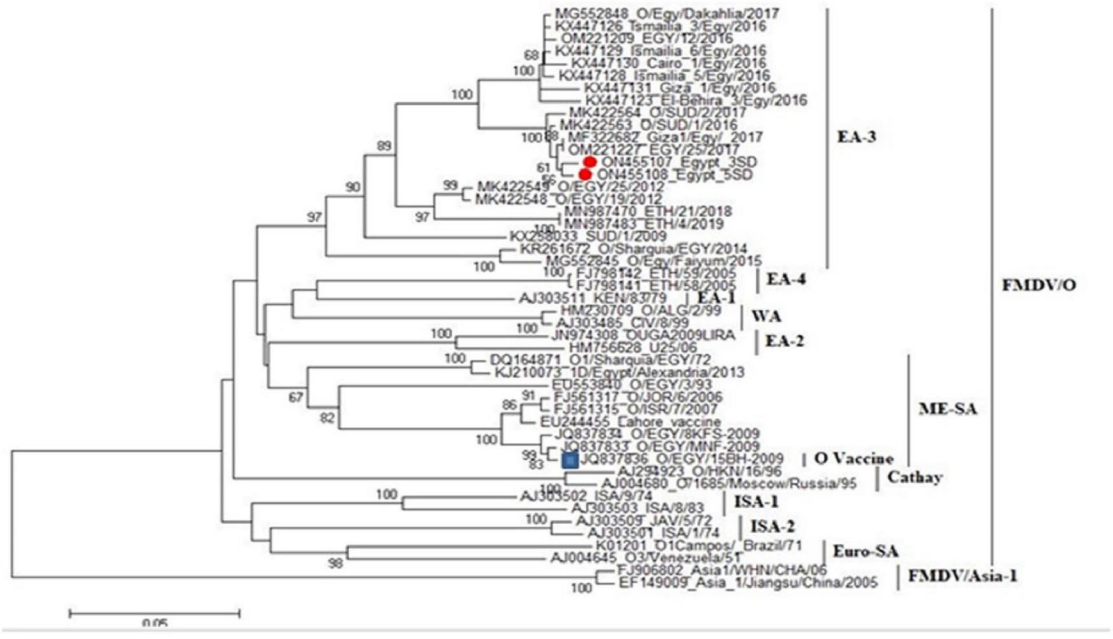

Fig. 4. Phylogenetic tree of the VP1 sequence of FMDV serotype O isolated from Egypt. The FMDV serotype O strains of this study are in bold font labeled with red circles and the Vaccine Strain is in bold font labeled with blue square.

showed papillae distortion, ballooning degeneration of the stratum spinosum, and intense inflammatory cells mainly lymphocytes and neutrophils. The hoof of G1 was histologically normal. The coronary bands of the hoof of G2 showed focal hemorrhages, intermuscular edema, and partial necrosis of collagen fibers were reported in the affected hoofs (Fig. 10). The later, hyalinized and necrotic collagen fibers of the subdermal layer, infiltrated with neutrophils (pus cells) and lymphocytes (abscess formation) could be seen. The Haversian system of the hoof showed osteomalacia and degenerative changes in the severely affected animals. The coronary bands of G3 showed focal leucocytic cell infiltration (mainly neutrophils) among the dermal layer, destruction of the cornfield epithelial cells or vesicle formation, and interstitial edema. The heart of G1 showed normal histomorphology structures with minor cardiac interstitial edema (Fig.20). Heart of G2 showed multifocal lymphohistiocytic infiltration, few neutrophils, diffuse edema, and necrosis of cardiac muscles fibers (Fig. 11). Serofibrenous pericarditis and perivascular edema represented by serofibrinous exudate and numerous inflammatory cells mainly lymphocytes, neutrophils, and macrophages. The heart of G3 showed endothelial hyperplasia with perivascular edema, the pyknotic nucleus of the myocardial cells with disarrangement of the myofibrils, and intermuscular edema. The lymph node and spleen of G1 were of normal histological structure and immunologically active. Submandibular lymph nodes of both G2 and G3 showed lymphoid depletion, hemosiderosis, and reduction of lymphocytic aggregations in the lymphoid follicles of both cortex and medulla, and proliferation in histiocytes in the necrotic lymphoid follicles could be seen (Fig, 12).

Table 3. Serum biochemical profile in control (G1), FMD-infected cattle (G2) and vaccinated one (G3).

Parameters	G1 (Control)	G2 (Infected)	G3 (Vaccinated)
AST(U/L)	50.90±1.8c	109.80±6.3a	73.83±3.1b
ALT(U/L)	41.6±4.4b	70.4±5.6a	30.7±1.1b
Urea (mg/dl)	40.7±6.17b	55.8±2.28a	37.7±1.50b
Creatinine (mg/dl)	1.3±0.05b	1.8±0.08a	1.2±0.05b
T. protein (g/dl)	6.57±0.o8a	5.35±0.09b	6.69±0.09a
Albumin (g/dl)	3.25±0.13a	2.65±0.09b	3.48±0.14a
Globulin (g/dl)	3.32±0.04a	2.7±0.08b	3.20±0.23ab
Cortisol (µg/dl)	62.50±4.3c	164.8±3.1a	88.3±11b

Data are presented as mean ± SE; Different letters at the same column means that there was a significant change at p<0.05

Table 4. Immunological and inflammatory markers profile in control (G1), FMD-infected cattle (G2) and vaccinated one G3.

Parameters	G1 (Control)	G2 (Infected)	G3 (Vaccinated)
Igg (ng/ml)	369.6±32b	65.1±9c	515.2±61a
IL10 (pg/ml)	90.3±11.8c	376.7±19.2a	160.10±5.8b
CRP (ng/ml)	61.3±8.2b	237.7±15.1a	58.5±7.1b
C.Trop1 (pg/ml)	114.7±14.2b	382.03±31.4a	89.8±4.8b
Haptoglobin (ug/ml)	0.41±0.08b	2.03±0.12a	0.72±0.11b
C3 (mg/dl)	37.90±5.4b	111.19±7.2a	34.99±3.01b

Data are presented as mean ± SE; Different letters at the same column means that there was a significant change at p<0.05

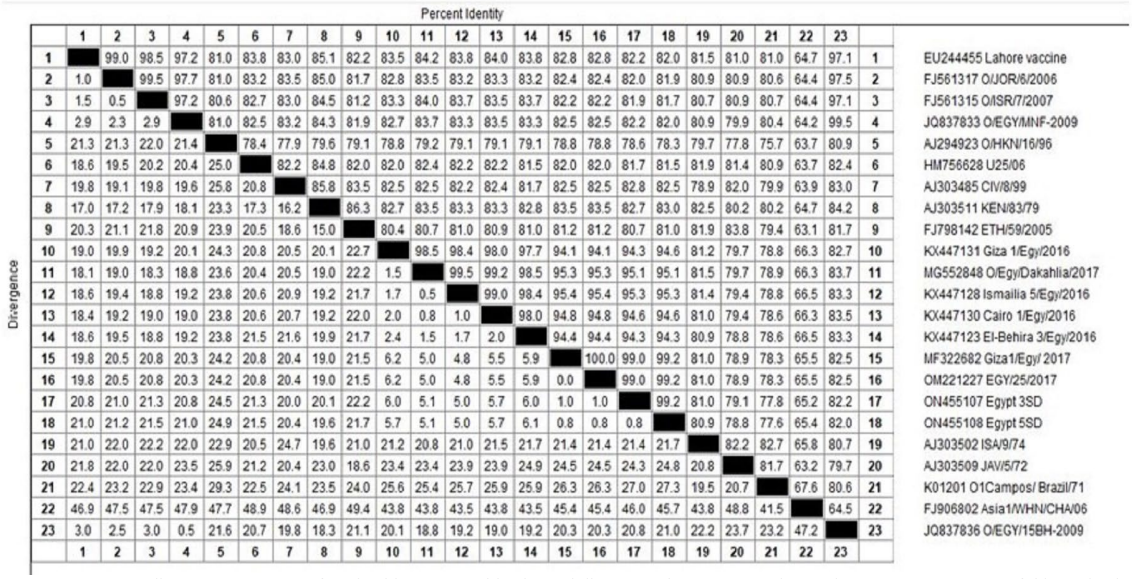

Fig. 5. FMD O serotype sequence distance. Percentage of nucleotide sequence identity and divergence between Egyptian strains FMDV serotype O of this study, the vaccine strains and other serotype O reference strains.

DISCUSSION

The detection of FMDV in Egypt during the outbreak of 2022 was conducted as a surveillance study. The outbreak affected both non-vaccinated and vaccinated farm animals with milder symptoms and lesions. This study showed that diseased cattle were suffering from anorexia, fever, and the development of dermatitis in the mouth and coronary bands associated with lameness. The same signs were described by Abd-Alhameed and Rhaymah (2010) and Constable *et al.* (2017). The same clinical signs appeared on the vaccinated group to a mild degree. This might be due to the development of immune responses of vaccinated animals (Hemida, *et al*., 2018).

Egypt has implemented a stringent FMD control program that involves immunizing animals with a locally produced inactivated polyvalent vaccine made up of serotypes O pan Asian II (EGY/2010), A Iran 05 (A/EGY/1/2012), SAT-2 (EGY/Gharbia/2012), and SAT-2 (LIB/2018). Despite this, FMD is still an endemic disease in the country. The immunization effort was effective in lowering the prevalence of FMD; nonetheless, FMDV strains continue to circulate as a result of unchecked animal migration. There have been at least eight different FMDV serotype strains over the previous ten years (A, O, and SAT-2). These strains included (A-Iran 05, A-African genotype IV, A-African genotype VII, O-Pan Asia II, O East Africa, SAT-2 Ghb-12, SAT-2 Lib-12, and SAT-2 Alx-12) with alternate displacement and winter peak of 2 to 3 circulating strains and an annual dominant circulating strain. (El-mayet *et al*., 2020). Due to the tremendous mutation rate of the FMDV RNA genome, the virus may be able to emerge in a variety of genotypes within each serotype with little to no cross-protection between serotypes or even genotypes. Failure to immunize against a variety of strains that are distinct from field strains is possible (Santos *et al*., 2018). Serotype O of the sequenced FMDV isolates in the current investigation is associated with the EA 3 topotype, whereas serotype A is related to the African topotype's genotype IV. The nucleotide sequence alignment of the VP1 resulted in two strains of FMDV serotype O from non-vaccinated and vaccine-exposed animals, respectively (ON455107 Egypt 3SD and ON455108 Egypt 5SD). Phylogenetic trees were constructed based on these alignments, showing that it is a member of topotype EA-3, which was recently discovered in Egypt. With 99.2% amino acid identity, the two strains from vaccinated and nonvaccinated animals are closely related to one another. The scale bar shows the number of changes per nucleotide when compared

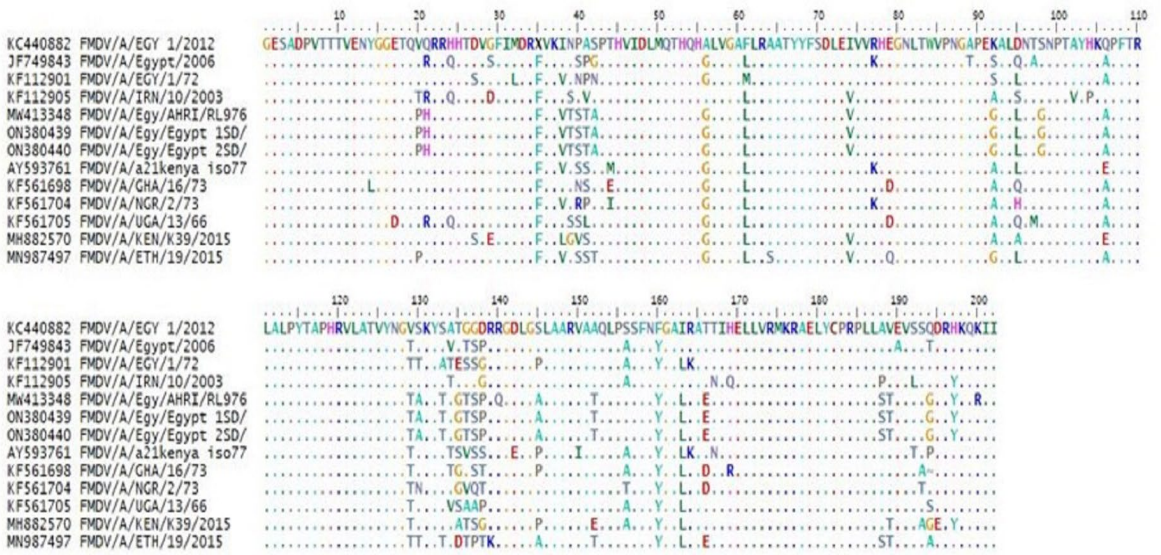

Fig. 6. Amino acid alignment of FMD virus serotype A isolated from Egypt.

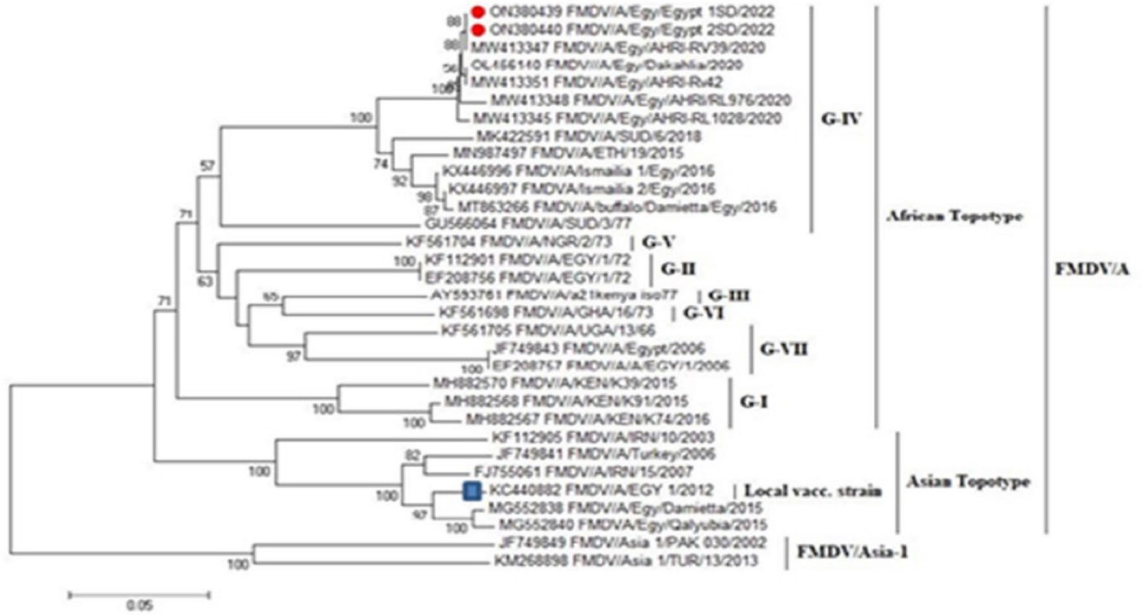

Fig. 7. Phylogenetic tree of the VP1 sequence of FMDV serotype A isolated from Egypt. The FMDV serotype A strains of this study are in bold font labeled with red circles and the Vaccine Strain is in bold font labeled with blue square.

to other reference strains in the Genbank database. It shares 99% amino acid similarity with MF322682 Giza1/Egy/2017 and OM221227 EGY/25/2017. With 95.4% amino acid identity, it is closely related to KX447128 Ismailia 5 /EGY/ 2016. It is closely related to MG552848 O /EGY/Dakahlia/2017 with 95.3% amino acid identity. It is closely related to KX447130 Cairo 1/EGY/ 2016 with 94.8% amino acid identity. It is closely related to KX447123El-Behira 3 /EGY/ 2016 with 94.4% amino acid identity. It is closely related to KX447131Giza 1 /EGY/ 2016 with 94.1% amino acid identity. The characterized strains differ from the Egyptian vaccine strain (JQ837836 O/EGY/15BH-2009) with 82.5 amino acid identity. Phylogenetic trees were constructed based on the nucleotide sequence alignment of the VP1 resulting in two strains of FMDV serotype A showing that it is belonging to IV of the African topotype which was detected in Egypt recently.

Both two isolates of FMDV serotype A from vaccinated and nonvaccinated animals are closely related to each other with 100% amino acid identity. The generated FMDV sequences in the present study also showed similarity to previously reported sequences from Ethiopia (MN987497FMDV/A/ETH/19/2015) and Sudan (MK422591FMDV/A/SUD/6/2016) and with 94.1 and 92.9% amino acid identity respectively. Compared with other reference strains in the Genbank database, the scale bar represents the number of substitutions per nucleotide, and it is closely related to OL456140FMDV/A/Egy/Dakahlia/2020 with 99.7% amino acid identity. It is closely related to MW413345FMDV/A/Egy/AHRI-RV42 with 99.7% amino acid identity. It is closely related to MV413345FMDV/A/Egy/AHRI-RL1028/2020 with 99.3% amino acid identity. It is also closely related to MW413348FMDV/A/Egy/AHRI/RL976/2020 with 98.8% amino acid identity. It is also closely related to KX446997 FMDVA /Ismailia2/ Egy/ AHRI/RL 976/2016 with 94.2% amino acid identity. With 75.7% amino acid identity, the characterized strains diverge from the Egyptian vaccine strain (KC440882 FMD/A/EGY 1/2012). When compared to the vaccinal strains used in the regional FMD vaccines in Egypt, further analysis of the key antigenic regions of VP1 revealed the presence of numerous amino acid changes in the newly circulating strains. These findings indicated a potential for the direct

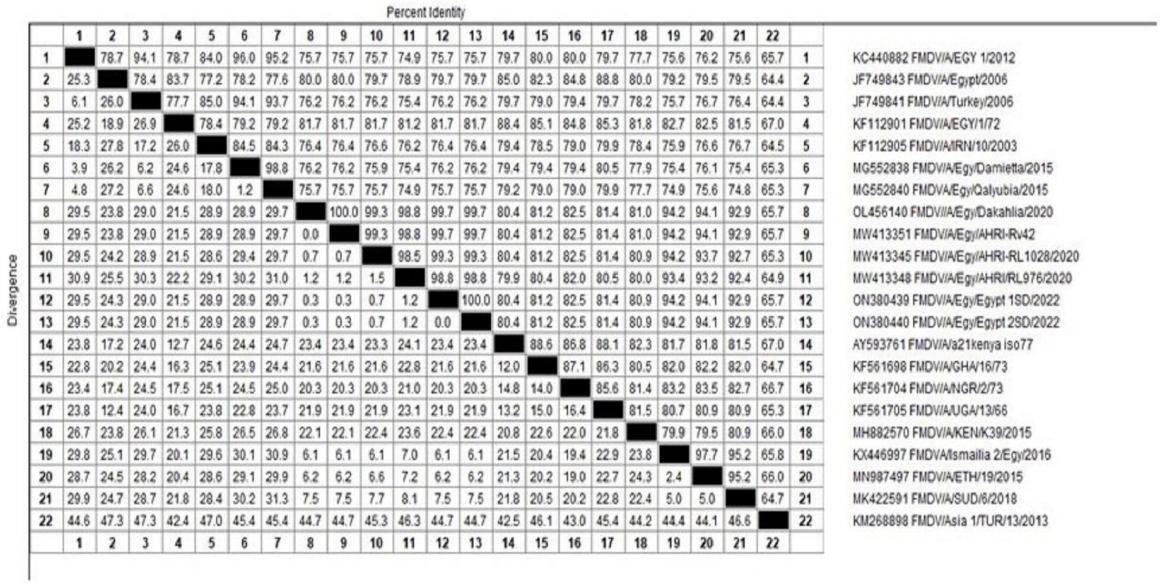

Fig. 8. FMD A sequence distance. Percentage of nucleotide sequence identity and divergence between Egyptian strains FMDV serotype A of this study, the vaccine strains and other serotype A reference strains.

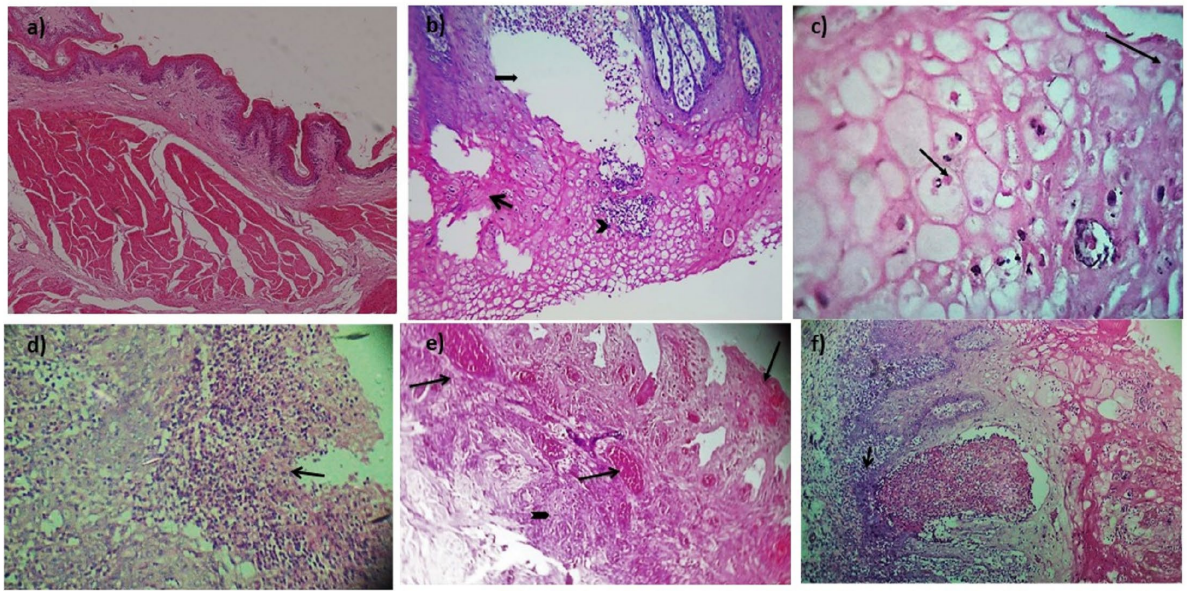

Fig. 9. Histopathological changes of tongue. Tongue of G1 showing normal architecture of fungiform papillae and stratum spinosum (H&E X100), b) Tongue of G2 showing vacuolar and hydropic degeneration in the lining epithelium, vesicles formation in the tongue mucosa which coalesce each other forming bullae of different size (arrows) and leukocytes infiltration (arrowhead) (H&E X200), c) Tongue of G2 showing ballooning of epithelial cells and eosinophilic inclusion bodies (arrows) (H&E X400), d) Tongue of G2 showing pustular formation represented by circumscribed depression filled with mixture of necrotic epithelium and leucocytes aggregation (arrow) (H&E X200), e) The tongue of G2 showing interstitial hemorrhages in the stratum muscularis (arrows) and Zener's necrosis in the submucosal layer (arrowhead) (H&E X100), f) Tongue of G3 showing ballooning degeneration of the stratum spinosum (arrow) with the intense inflammatory cells mainly lymphocytes, neutrophils (black arrow) (H&E X200).

introduction of genotype-IV from Ethiopia or Sudan into Egyptian territory (Duchatel *et al*., 2019). The genomes of the FMDV serotype A isolates that are now in circulation differ from the previously reported Egyptian sequences of the African genotype. Therefore, the goal of this study was to describe the molecular and genetic characterization of the FMDV serotypes that were recently circulating in the Sharkia governorate in 2022 and were responsible for an outbreak. This was done to hasten diagnosis, choose the best emergency vaccine, and assist in tracing the origin and spread of an outbreak. The isolates used in this investigation did not match the locally generated vaccine strain. The challenges of FMDV vaccination include non-vaccination, disrupted herd immunity, animal health, FMDV carrier status, lack of vaccine matching with field isolates, large populations, and species interface. In addition, animal markets are creating an ideal environment for FMDV spread because animals are brought in from different areas and kept in one location without proper vaccine history (Sobrino and Domingo, 2017).

RBCs, Hb, PCV, MCV, and MCH levels were significantly lower in the group (G2) based on hematological analysis. This may have been caused by mouth lesions that reduced feed intake and enteritis-related malabsorption. According to El-Mandrawy and Farag (2017); Ghanem and Abdel-Hamid (2010) and Gokce *et al.* (2004), endocrinopathy that develops as a result of FMD viral infection could be the cause. However, the large drop in TLC with lymphocytopenia may have been brought on by T and B cells being infected with the FMD virus shortly after an infection, at the height of viremia, which temporarily suppressed the immune system (Roussel *et al*., 1997).

This agreed with Burtis and Ashweed (1994); Reid *et al.* (2000), and Grunwaldt *et al.* (2005) who reported leukopenia in cattle during the virus infection taking into account the positive association between virus load and leukopenia during acute viral infections. El-Mandrawy and Farag (2017) disagreed, recording leukocytosis in FMD-infected cattle; this may be due to the difference in age, species, or the time of In G2, neutrophilia and monocytosis may be attributed to crucial phagocytic cell responses that take part in innate immunity (Grunwaldt *et al*., 2005; Nasr., 2013). Neutrophilia may also result from tissue degradation. Moreover, cows vaccinated G3 showed no significant changes in the arthrogram and mild leukocytopenia and lymphopenia which may be attributed to viral infection of the vaccinated group, this was confirmed by PCR results which recorded isolation of two serotypes of FMD virus. These results were disagreeing with Red-

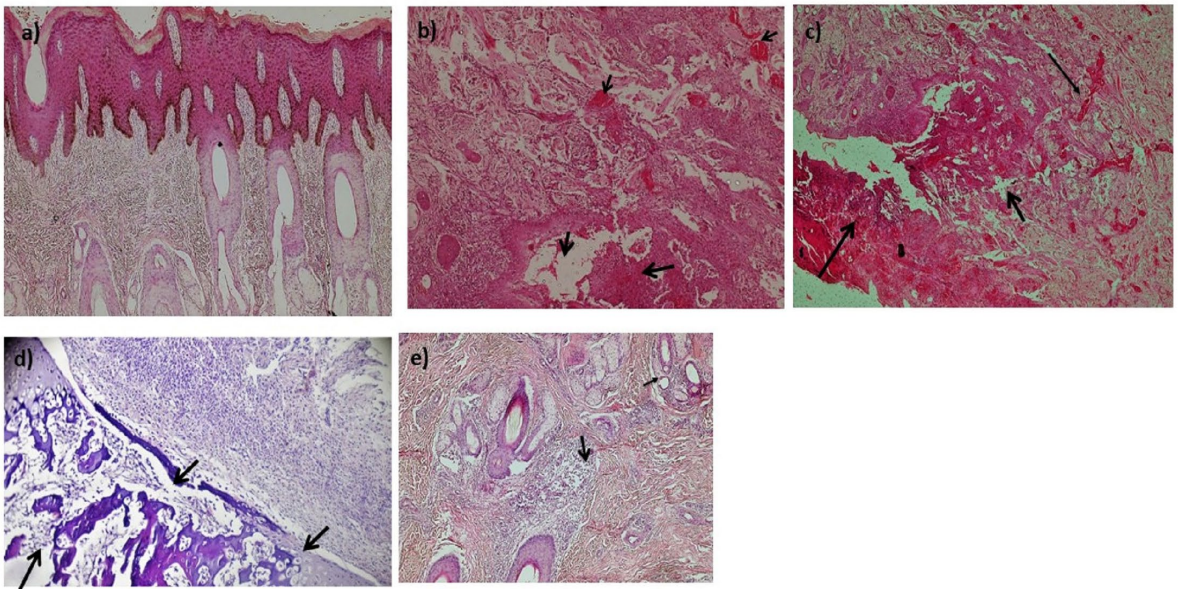

Fig. 10. Histopathological changes of Coronary band of Feet. a) Coronary band of Feet of G1 showing normal histological picture of coronary band (H&E X 100), b) Coronary band of feet of G2 showing hemorrhages, edema and necrosis of collagen fibers (arrows) (H&E X 200), c) Coronary band of feet of G2 showing hyalinized, sloughing of the epidermal layer, necrotic collagen fibers and interstitial hemorrhages (H&E X 200), d) Hoof of G2 showing osteomalacia and degenerative changes of the Haversian system morphology (arrows) (H&E X 200), e) Coronary band of feet of G3 showing focal leucocytic cell aggregation among the dermal layer and multiple microvesicles with in the epidermis (arrows) (H&E X 200).

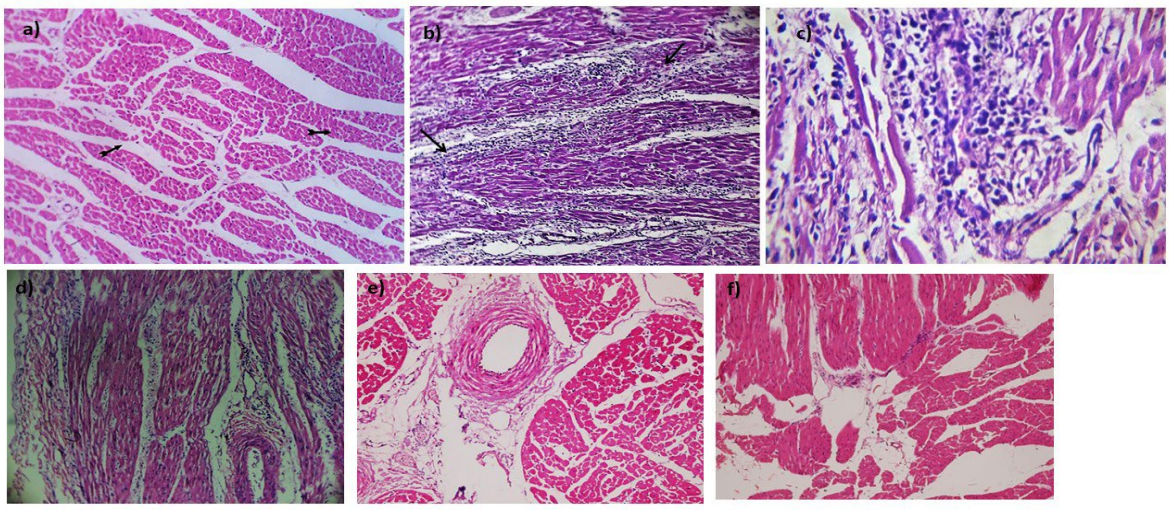

Fig. 11. Histopathological changes of heart. a) Heart of G1 showing nor mal histomorphological structures with minor cardiac, b) Heart of G2 showing myocardial necrosis with focal lympho- histiocytic infiltration and few mononuclear cells (H&E X100), c) High power of the previous figure showing focal lympho- histiocytic aggregation, and coagulative necrosis of myocardial cells with loss of striation(arrows) (H&E X400), d) Heart of G2 showing serofibrenous pericarditis and perivascular edema represented by serofibrinous exudate and numerous inflammatory cells (H&E X200), e) Heart of G3 showing endothelial hyperplasia with perivascular edema (H&E X100), f) Heart of G3 showing myocardial cells were pyknotic nuclei with disarrangement of the myofibrils and intermuscular edema(H&Ex100).

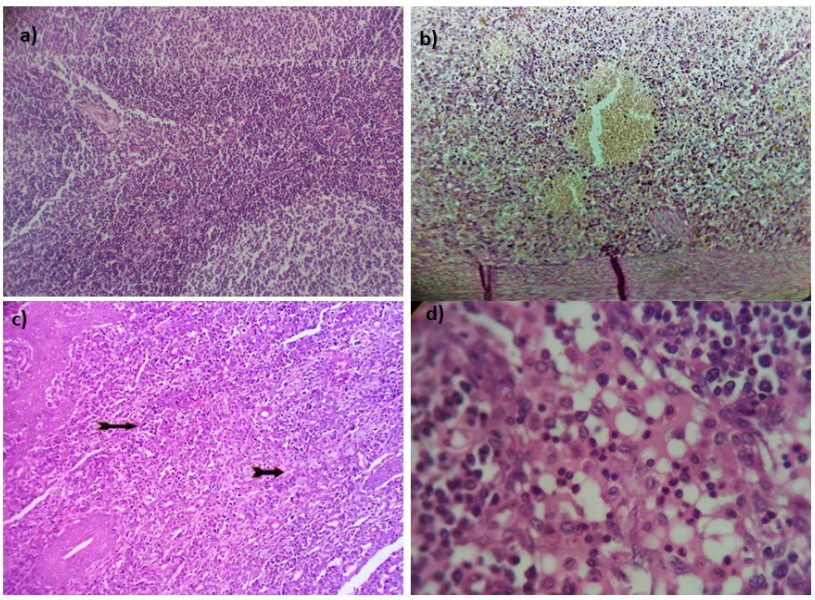

Fig. 12. Histopathological changes of spleen and lymph nodes. a) Spleen of G1 showing normal histological struc ture and immunologically active (H&E X100), b) Lymph nodes of G2 showing depletion of the lymphoid follicles and hemosiderosis (H&E X100), c) Spleen of G3 showing lymphoid depletion (H&E X100), d) necrotic lymphocytes and histocyte infiltration (H&E X400).

dy and Sivajothi (2020) who recorded elevations in the levels of total leukocyte count, neutrophil, lymphocyte, and eosinophil count this may be attributed to the differences in species, ages, or FMD viral infection. The significant increase in leukocytes and lymphocytes compared with infected ones may be a result of the enhancement of the B cell function for antibody production.

The data of biochemical parameters revealed a significant increase in AST and ALT in infected cattle. Aktas *et al*. (2015) investigated that AST elevation contributed to muscle, heart, and liver diseases. Salim *et al*. (2019) suggested that the degenerative alterations of the damaging effects of the FMD virus on the liver and heart and/or hepatocellular damage could be responsible for the increase in AST and ALT in FMDV-infected calves. The same results were obtained by Nath *et al*. (2014) and Nasr El-Deen *et al*. (2017). Also, Hashem *et al*. (2018) added that the level of AST and GGT were highly significant increase in infected cattle with FMDV of all ages.

The increase in AST levels in vaccinated group G3 may be due to some hepatic dysfunction Shawky *et al*. (2016) found that there was a significant increase in AST activity in vaccinated cattle with the trivalent-hexavalent vaccine. Kaneko *et al*. (1997) reported that AST increases combined with increased cortisol levels. A significant increase in serum urea and creatinine was observed by Hashem *et al*. (2018) in infected cattle with FMDV of different ages. Also added that this increase might be due to decreasing in renal blood flow and renal damage. The same results were found by Mansour *et al*. (2016) who investigated that guinea pigs infected with FMDV showed a significant increase in serum creatinine levels. On the other hand, non-significant changes in serum levels of creatinine and blood urea nitrogen in infected buffaloes with FMDV were detected by Nasr El-Deen (2013).

A reduction in total protein and albumin in the infected group may be associated with starvation, and hepatic and renal damage (Gokce *et al*., 2004). Hashem *et al*. (2018) reported that there was a significant decrease in the mentioned parameters in infected cattle with FMDV and a significant decrease in total protein and albumin concentration was observed in FMD-infected cattle at the advanced stage (Faruk *et al*., 2021). Reduced feed intake disrupted liver metabolism, maldigestion, malabsorption caused by enteritis, and plasma exudation from ulcers on the lips, tongue, and between claws may all contribute to hypoalbuminemia in infected cattle (Mousa and Galal, 2013). Hypoglobulinemia in FMD-infected cattle may be attributed to increased interleukin- 10 levels which act as anti-inflammatory causing inhibition of immunity (Hashem *et al*., 2018).

Cortisol is known as the stress hormone because it is secreted in increased amounts during the body's 'fight or flight response to stress and during the body's response to a challenge, and it is responsible for many stress-related changes in the body (Dhabhar, 2009; Martin, 2009). G2 infected group showed a significant increase in serum cortisol concentration Similar results were detected by Saleh (2019). No significant increase in serum cortisol was observed in G3 vaccinated group. Shawky *et al*. (2016) demonstrated increases in serum cortisol in vaccinated cattle. Kim *et al*. (2011) added that stress like vaccination, weaning, mixing and transportation lead to an increase in serum cortisol concentration.

Our results illustrated that the highest values of IgG were recorded in G3, and the lowest values were in G2 the increase in G3 may be due to immune response to the vaccine which is accompanied by an increase in lymphocytes which is responsible for immunoglobulins production. These results were in agreement with Mulcahy *et al*. (1990), Capozzo *et al*. (1997) and Xiao *et al*. (2007), who mentioned that the changes in serum IgG levels increased significantly after the animals were immunized with FMDV antigens. Also, they found a significant decrease, 1 day after infection, in the FMDV- IgG. They illustrated that in some of the animals, circulating antibodies might have found virulent virus administered.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that inhibits the activity of Th1 cells, NK cells, and macrophages, which are required for optimal pathogen clearance. Serum IL10 revealed a significant increase in infected and vaccinated cattle in comparison with the control group this may be attributed to the effect of FMDV which was confirmed by PCR results. Hashem *et al*. (2018) added that the increase in IL-10 in infected cattle is due to inflammation in the buccal cavity and other parts of the body.

C-reactive protein is a major acute phase protein. Acute phase proteins are blood proteins primarily synthesized by hepatocytes as part of acute phase responses (Cray *et al*., 2009). Our results recorded a significant increase in c-reactive protein in G2 compared with G1. This agreed with Lee *et al*. (2019) who mentioned that C-reactive protein, a major acute phase protein gradually increased in non-immunized infected animals compared with the immunized group.

Determination of specific cardiac biomarkers is very important for the diagnosis of myocarditis in animals as serum cardiac troponin is measured in various animal species including cattle

as indicators of myocardial injury (Constable *et al*., 2017). Serum cardiac troponin showed a significant increase in the infected group compared with the control one this may be attributed to the effect of virus FMD on cardiac tissue which is confirmed by PCR results by the recorded presence of 2serotyps of FMD virus and this is agreed with Tunca *et al*. (2008); Aktas *et al*. (2015); Dawood and Alsaad (2018) and Salim *et al*. (2019). Serum cardiac troponins are the earliest appearing biochemical markers during myocardial damage (Boccara *et al*., 2000). Haptoglobin, a marker of acute inflammation and tissue damage in ruminants, is a glycoprotein synthesized in the liver by pro-inflammatory cytokines (Pradeep, 2014). In the present study haptoglobin concentrations were defined as mild prognosis in G3 to poor prognosis in G2 (Skinner *et al*., 1991).

Complements are a collection of circulating and membrane-associated proteins, they have an important role in defense against microbes, and most of them are proteolytic enzymes. Complement3 is the major proteolytic fragment, it acts by coating the microbes and promotes the binding of these microbes to phagocytes under receptors forc3 as mentioned by (Abbas *et al*., 2022). The present study illustrated a significant increase in C3 in the group (G2) compared with the control one this may be due to FMD viral infection and increasing phagocytes (neutrophilia and monocytosis).

Histological changes represented by Vesicular dermatitis developed with erosion and ulceration on the epithelial of the tongue and coronary bands. Our results revealed hemorrhages, edema, and necrosis of collagen fibers. Hemida *et al*. (2018) and Ranjan *et al*. (2016) reported forming of small vesicles then progressed to rupture and lead to erosions, ulcerations on the buccal cavity and coronary bands, and partial sloughing of the hoof. This finding osteomalacia of the hoof and degenerative changes of the Haversian system morphology. The heart showed multifocal lympho- histiocytic myocarditis and perivascular sero-fibrinous exudate (Sheire 2016; Hemida *et al*., 2018). Some theories suggested that the heart muscles could not be able to send impulses for the contracting process. These lesions agree with those previously reported by Gulbahar *et al*. (2007) and Sheire (2016). To our knowledge, these lesions in the heart revealed by hyaline degeneration make it capable to pump blood to the body developing large vasculature clots (currant jelly clots) than heart failure and death (Oem *et al*., 2008). While the vaccinated infected group showed moderate cardiac lesions represented by edema and necrosis with disarrangement of the myofibrils. We suggested that this is due to the higher level of immunological IgG combat with spreading necrosis of heart muscle and decreasing the dramatic evidence of heart failure (Oem *et al*., 2008; Hemida *et al*., 2018). The lymph nodes and spleen of both G2 and G3 showed lymphoid depletion, hemosiderosis, and reduction of lymphocytic aggregations in the lymphoid follicles and proliferation in histiocytes in the necrotic lymphoid follicles. These results were in the same direction as Arzt *et al*. (2011) who reported transient lymphopenia during the viral early infection and viremia coinciding with depletion of the T- cells in the lymph node and spleen. Also, Gab-Allah *et al*. (2018) and Juleff *et al*. (2008) discussed the congested and hemorrhagic spleen (acute cases) while depletion of lymphocytes in the spleen (chronic cases).

CONCLUSION

We believe in the importance of the periodical vaccine with the recent circulating serotype to achieve maximum protection for the farm animals. The occurrence of infection in the vaccinated cattle may be attributed to the type of vaccine with the strain currently circulating in the field strain. Requiring continuous monitoring of the FMD molecular level to ensure the preparation of the effective vaccine.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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