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Epidemiological and molecular study on bovine malignant catarrhal fever in Assiut Governorate, Egypt

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ABSTRACT

Many domestic and wild animals are susceptible to malignant catarrhal fever (MCF), which is a lymphoproliferative, multisystemic, and fatal disease. The present study was conducted on 47 cattle. The clinical examination findings revealed a persistent high fever, enlargement of superficial lymph nodes, corneal opacity, respiratory symptoms, oral lesions, and ulcerative skin lesions. Buffy coat samples were collected for laboratory analysis. Semi-nested polymerase chain reaction (PCR) assay has been used. Ovine herpesvirus-2 (OvHV-2) Deoxyribonucleic acid (DNA) was detected in the buffy coat of 21 cattle. DNA sequencing and phylogenetic analysis of OvHV-2 were performed in our study. The phylogenetic analysis of the PCR product of the Egyptian strain of OvHV-2 (Assiut) showed close similarity with OvHV-2 strains of different governorates (Beni-suef, Giza and Fayoum) of Egypt, India, Turkey, Iraq, Italy, Brazil and United Kingdom. There was no significant variation ($P<0.05$) between the percentages of MCF infection and the sex, age, and breed (native and mixed breed) of molecularly tested cattle. According to the climatologic conditions of Assiut governorate, there is no discernible difference ($P<0.05$) between the MCF infection rate and the cold and hot months. To date, this is the first report of OvHV-2 in Assiut Governorate, so it is advisable to separate susceptible cattle from sheep, especially during lambing, and to euthanize any animals that are clinically infected with MCF.

Introduction

For more than a century, MCF was confined to Europe under the name "Kopfkrankheit," which referred to a sporadic, non-contagious illness to be distinguished from Rinderpest (Zaki *et al*., 2016). MCF is a very dangerous and fatal systemic lymphoproliferative infectious disease that affects numerous *Artiodactyla* species, including cattle, water buffalo, bison, deer, and pigs (Khudhair *et al*., 2019; Kumar *et al*., 2021). MCF disease belongs to the genus *Macavirus* in the family *Herpesviridae* and the subfamily *Gammaherpesvirinae* (Kumar *et al*., 2021). OvHV-2 is one of the most significant of these and is well-adapted to its natural host (sheep). As a result, it can infect sheep without causing any symptoms, which act as reservoir hosts, but it causes disease in susceptible hosts like cattle and buffalo, which is called sheep-associated MCF (SA-MCF) (Mananguit *et al*., 2021). MCF has a slightly higher prevalence in temperate areas during lambing season, but this increase is unrelated to lambing (Bastawecy and Abd El-Samee, 2012). The amount of virus shed by ewes during lambing does not increase, and newborn lambs are unaffected and do not shed the virus (Abd El Rahman *et al*., 2020). Clinically susceptible cattle contract the virus through inhalation, ingestion of virus-laden secretions, or possibly through contaminated feed and water, as it is shed in nasal secretions by their reservoir hosts (Coradduzza *et al*., 2022). OvHV-2 is not naturally transmitted from one clinically susceptible host to another, and animals with MCF do not endanger their mates (Abd El Rahman *et al*., 2020). MCF is a serious and frequently fatal condition that is characterised by lymphoproliferation and inflammation, primarily in blood vessels (arteritis) and mucosal surfaces (Kumar *et al*., 2021). After an incubation period of 2 weeks to 10 months, clinical signs typically start with depression and a high fever and are then followed by oculonasal discharge from keratoconjunctivitis and rhinitis (Zaki *et al*., 2016; Zakharova *et al*., 2020). Clinical forms of MCF are given descriptive names, such as "skin form" for cutaneous lesions, "nervous form" for nervous signs, "alimentary form" for gastrointestinal illness, "eye and head form" for eye and head affection, and "mild form" for mild clinical symptoms. Multiple mixed forms, however, could appear in one or more animals in the same herd (Zaki *et al*., 2016). The clinical picture allows for a presumptive diagnosis of MCF, but other diseases that also have the same symptoms (fever and oral lesions), such as mucosal disease (sequela of persistent infection with bovine viral diarrhoea virus), foot and mouth disease, and vesicular stomatitis, must also be taken into consideration and, if possible, ruled out through laboratory testing (Orono *et al*., 2019). Clinically affected susceptible hosts are regarded as dead end hosts because they do not discharge infectious viruses and do not transmit the virus to susceptible animals (Riaz *et al*., 2021). Histopathological analysis can provide evidence for MCF, but viral DNA detection in blood is the preferred technique for making the diagnosis (Orono *et al*., 2019; Abd El Rahman *et al*., 2020). Antibodies against one MCF virus that are virus-neutralizing cross-react with other members of the genus (Taus *et al*., 2015). Additionally, the SA-MCF causative agent has not yet been reliably propagated in vitro. As a result, it has been difficult to characterise the causative agent, and viral isolation has not been proven a useful technique for disease diagnosis. In addition, there is no vaccination for this disease that is readily available for prevention and control (Mananguit *et al*., 2021). The development of the PCR assay with targeted primers coupled with sequencing has enabled the detection and classification of organisms such as viruses that were previously impossible or difficult to identify using conventional virological approaches

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(Jacobsen *et al*., 2007; Khudhair *et al*., 2019; Orono *et al*., 2019). OvHV-2 has already been implicated in outbreaks of MCF in Egyptian cattle and buffalo in 2010 (Azzam *et al*., 2016) and 2012/2013 (Zaki *et al*., 2016). Still, there is no data available on studying MCF in Assiut Governorate so the current study's objectives were to molecularly identify OvHV-2 based on semi-nested PCR of the tegument gene and nucleotide sequencing of the PCR product with phylogenetic analysis, detect the clinical manifestations and study some epidemiological features such as sex, age, breed of cattle, and seasonal variation, with the infection rate of MCF in Assiut Governorate.

Materials and methods

Animals and Ethical approval

During the period of investigation, from August 2021 to September 2022, a total of 47 cattle of various sexes, ages, and breeds that belonged to different villages in Assiut Governorate were admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University. All examined cattle suffered from fever, oral lesions (erosions and ulcers), enlarged superficial lymph nodes, bloody diarrhea, respiratory signs (purulent nasal discharge and cough), corneal opacity and ulcerative skin lesions. All investigated cattle in this study were handled in accordance with ethical considerations. The study was approved by the Ethical Committee in the Faculty of Veterinary Medicine at Assiut University, Assiut, Egypt; the approval number was 06/2022/0005.

Clinical examination

Clinical examination of the investigated diseased cattle was done according to Jackson and Cockcroft (2002).

Sampling

Two millilitres of whole blood samples were collected into sterile ethylene diamine tetraacetic acid (EDTA) vacutainer tubes through the jugular vein while the cattle were properly restrained. Blood samples were centrifuged at 3000 r/min for 15 minutes, and the buffy coat layer was delicately removed (Riaz *et al*., 2021) and stored in separate tubes at -20°C for subsequent DNA extraction.

Molecular diagnosis

DNA extraction

The viral DNA was extracted from 47 frozen buffy coat samples using the EasyPure® blood genomic DNA extraction kit (TransGen Biotech, China), according to the instructions of the manufacturer. Extracted DNA was kept at -20ºC until further use.

Primers

The specificities of the chosen primers (Metabion International AG, Germany) used in the present study for the OvHV-2 tegument gene have been evaluated previously (Baxter *et al*., 1993). Sequences of primers and their positions in the viral genome are shown in Table 1.

Detection of OvHV-2 tegument gene of MCF by semi-nested PCR

Possibility of a semi-nested PCR assay specific for amplifying the highly variable region of the OvHV-2 tegument gene. For the first step, primer sets 556 forward and 755 reverse were used to amplify DNA fragments with a 442 bp length; for the second step, 2.5 µl of the first PCR product template was used with the 555 internal reverse and the same forward primer (Hristov *et al*., 2016; Khudhair *et al*., 2019). In the current study, the ABT red master mix (2X) (Applied Biotechnology, Egypt) was employed as a source for DNTPs and polymerase enzyme. PCR was carried out in two steps in a PCR thermocycler (Cole-Parmer, United States), with the following reagents used in the first step: A final volume of 25 µl containing 12.5 µl ABT red master mix (2X), 0.5 µl of each primer 556 and 755 (5 pmol), 5 µl DNA template, and 6.5 µl PCR molecular grade water. In second step: 12.5 µl ABT red master mix (2X), 0.5 µl of each primer 556 and 555 (5 pmol), 2.5 µl of the first PCR product template, and 9 µl PCR molecular grade water in a final volume of 25 µl. In a nutshell, thermal cycling conditions in two steps were carried out with initial denaturation at 95°C for 5 min, (40 cycles of denaturation 94°C for 1 min, 60°C for 1 min. for annealing step, and 72°C for 2 min. for extension), followed by 72°C for 10 min. for final elongation.

Analysis and detection of PCR products

For reaction visualization, 5 µl of amplified products and 3 µl of gel loading buffer were loaded. The amplicons were analysed by gel electrophoresis for 75 min at 90 V and 155 mA in 2% agarose gel stained with ethidium bromide (10 mg/ml) and their size was estimated with size marker DNA of 100 bp before being seen by a gel UV transilluminator (Syngene, United Kingdom).

Sequencing of PCR product of OvHV-2 tegument gene

To validate the semi-nested PCR result, one PCR product (422 bp amplicon) from the first amplification was submitted for DNA sequencing. The sequencing process utilized the same primers (primer 556 and primer 755) which were employed in the first PCR amplification step. The sequencing of the PCR product was performed in Macrogen (South Korea).

Phylogenetic Analysis

The obtained nucleotide sequence data was analyzed by using Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequence data used in this study had been registered in National Center for Biotechnology Information (NCBI) GenBank database under the following accession number: OR819791. The nucleotide sequence was submitted to BLAST analysis to find for sequence similarity against GenBank database. A phylogenetic analysis was conducted using reference sequences that were downloaded from GenBank. Fasta format was used to extract all query and reference sequences. The query and reference sequences were imputed into MEGA-X (version

Table 1. Nucleotide sequence of used primers, positions in the OvHV-2 tegument gene of MCF and size of the products obtained after PCR.

Primer	Nucleotide sequences	Position 5' -3'	Product size bp
556	5'-AGT CTG GGT ATA TGA ATC CAG ATG GCT CTC-3'	38-68	422
755	5'-AAG ATA AGC ACC AGT TAT GCA TCT GAT AAA-3'	460-431	
556	5'-AGT CTG GGT ATA TGA ATC CAG ATG GCT CTC-3'	38-68	238
555	5'- TTC TGG GGT AGT GGC GAG CGA AGG CTT C-3'	275-247	

10.2.4) software. With MEGA-X, multiple alignments were carried out using the ClustalW program. In MEGA-X, genetic distances were computed using the Kimura-2 parameter model. Using 1000 bootstrap replicates of sequence alignment datasets, the confidence values of internal nodes in phylogenetic trees were determined, and phylograms were created with MEGA-X using the Neighbor-Joining method.

Statistical analysis

The statistical package for the social sciences (SPSS) version 16 software was used to enrol and analyse the collected clinical findings and epidemiology data using the Chi-square of independence (2007).

Results

Field diagnosis of clinical cases associated with MCF

Cattle with MCF were diagnosed in the field based on clinical examination, identification of typical clinical symptoms, and interpretation of the disease's characteristic case history. It is possible that MCF was infected with OvHV-2 based on a history of sheep living on the same property as the clinically affected cattle. Cattle used in this investigation displayed the typical clinical symptoms of MCF, such as fever, oral lesions, enlargement of superficial lymph nodes, bloody diarrhea, respiratory signs, corneal opacity and skin lesions but sheep in contact with them had no clinical symptoms found. The presence of persistent high fever (40-41°C) and oral lesions such as erosions and ulcers were a distinguishing and the most significant features in 21 (100%) clinical cases of cattle associated with MCF (Table 2). Enlargement of superficial lymph nodes, particularly the prescapular and the prefemoral lymph nodes, was noted in 15 (71.43%) of the investigated diseased cattle (Table 2). Bloody diarrhea and respiratory signs like a cough and purulent nasal discharge were detected in 12 (57.14%) of examined diseased cattle (Table 2). Bilateral corneal opacity was observed in 7 (33.33%) of studied diseased cattle (Table 2). Two (9.52%) diseased cattle had skin lesions that resembled ulcerative dermatitis in the head, neck, back, and trunk areas (Table 2).

Semi-nested PCR assay for the diagnosis of SA-MCF

DNA samples were tested during the first round of this semi-nested PCR assay to produce the necessary bands at 422 bp (Fig. 1A), and products yielded from the first PCR reaction were tested throughout the second round to generate the specific diagnostic DNA band at 238 bp (Fig.1B). Twenty-one (44.68%) of the 47 buffy coat samples tested molecularly positive using semi-nested PCR.

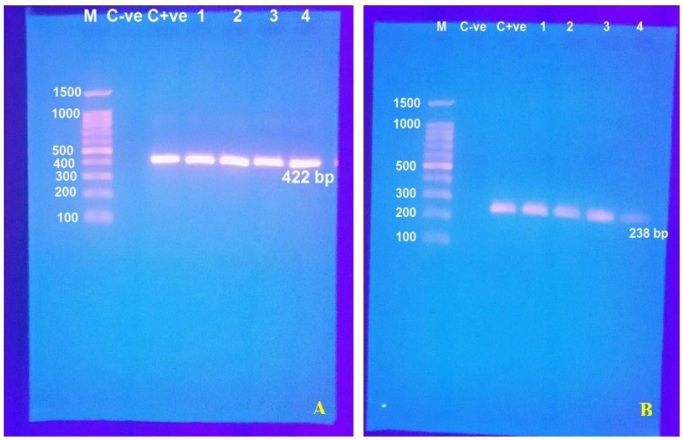

Fig. 1. Agarose gel electrophoresis of PCR after amplification of OvHV-2 tegument gene of MCF like disease infected cattle. Line M: DNA ladder 100 bp, line C-ve: Control negative distilled water, line C+ve: Control positive sample, and lines 1,2,3, and 4: positive DNA samples with primers specific for OvHV-2. (A): first step PCR of OvHV-2 DNA of tegument gene with amplified product at 422 bp. (B): second step of semi-nested PCR for the OvHV-2 of the same gene with amplified product at 238 bp.

Sequencing of the PCR product and phylogenetic analysis

The sequencing result and phylogenetic analysis of the PCR product of the Egyptian strain of OvHV-2 (Assiut, Accession number: OR819791), Fig. 2 revealed 98.36% -100% identity with OvHV-2 strains of different governorates (Beni-suef, Giza and Fayoum) of Egypt, India, Turkey, Iraq, Italy, Brazil and United Kingdom.

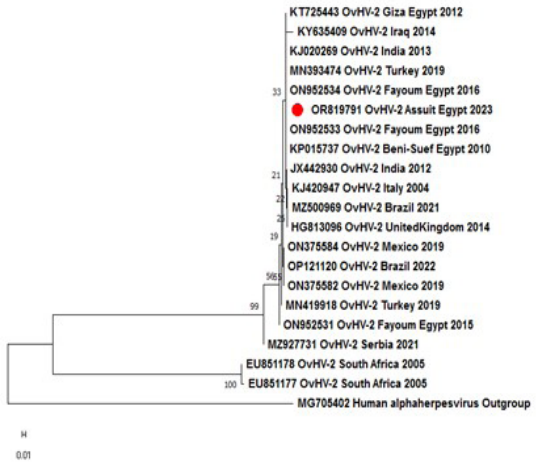

Fig. 2. Phylogenetic tree with 422 bp amplicon with Egyptian (Assiut) OvHV-2 of MCF. The tree was constructed using neighbor-joining and Kimura two-parameter as a nucleotide substitution model. The numbers adjacent to nodes represent percentage of bootstrap support (1,000 replicates) for the clusters. The Egyptian (Assiut) OvHV-2 is marked with a filled circle. Human alpha herpesvirus was used as outgroup (MG705402).

Table 2. Clinical manifestations of MCF diseased cattle (n= 47).

Clinical signs	No. of examined diseased animals	No. of diseased animals infected with MCF (%)	Chi-square test	
			Pearson chi-square (P-value)	Probability (p< 0.05)
Fever and oral lesions	12	2 (16.67%)		
Fever, oral lesions and enlarged superficial lymph nodes	10	5 (50%)		
Fever, oral lesions, bloody diarrhea and respiratory signs	8	4 (50%)		
Fever, oral lesions, enlarged superficial lymph nodes, bloody diarrhea and respiratory signs	7	3 (42.86%)	7.529	0.184
Fever, oral lesions, enlarged superficial lymph nodes, corneal opacity and ulcerative skin lesions	2	2 (100%)		
Fever, oral lesions, enlarged superficial lymph nodes, bloody diarrhea, respiratory signs and corneal opacity	8	5 (62.50%)		
Total	47	21 (44.68%)		

Epidemiological findings

Percentage of MCF infection

According to the current study, 44.68% (21/47) of the cattle that were investigated had MCF infection (Table 3).

Effect of sex

The analytical findings showed that there was no discernible variation in MCF infection percentage between the male and female tested cattle (Table 3).

Age susceptibility

The prevalence of MCF infection was tested in cattle at the ages of 4 months-1 year, >1-3 years, and >3-5 years, with results showing that, out of 47 cattle examined, 43.75%, 37.50%, and 71.43% had the infection. Age groups >3-5 years had the mathematically greatest rate of MCF infection (Table 3).

Breed susceptibility

The percentage of MCF infection in native and mixed breed cattle in the present investigation did not significantly differ, despite the higher infection in mixed breeds being more accurate numerically (Table 3).

Seasonal variation

The percentage of MCF infection did not differ significantly between the cold and hot months, according to our findings (Table 3).

Discussion

MCF typically manifests as sporadic cases; however, some outbreaks linked to both the sheep-associated type and the wildebeest-associated types have been recorded (Holliman *et al*., 2007; Sawai *et al*., 2013; Zaki *et al*., 2016; Avcı *et al*., 2020). MCF has been identified as new hazards to countries in the southern Mediterranean and the Middle East, particularly those that are related to sheep (Zaki *et al*., 2016). MCF can be tentatively diagnosed based on the characteristic clinical symptoms of the disease and a history of interaction with sheep, goats, or wildebeest (Bratanich *et al*., 2012; Zaki *et al*., 2016). Long before Bastawecy discovered the etiological agent for the first time in Egypt in 2012, MCF-like symptoms had been noted in Egypt for many years, but the disease had never been diagnosed (Bastawecy and Abd El-Samee, 2012). In our study, the observed clinical signs associated with suspected clinical cases of MCF are the same as those described in veterinary clinical textbook and previous studies recorded by O'Toole *et al*. (1997); Radostits *et al*. (2007); Bastawecy and Abd El-Samee (2012); Zaki *et al*. (2016); Khudhair *et al*. (2019) and Abd El Rahman *et al*. (2020). The typical clinical signs of MCF were found in our findings, such as persistent fever, oral lesions, enlargement of superficial lymph nodes, bloody diarrhea, respiratory signs, bilateral corneal opacity, and skin lesions. Fever and oral lesions such as erosions and ulcers were the most common symptoms. These findings may be attributed to MCF, which is a multisystemic disease characterised by lymphoid proliferation and infiltration, widespread vascular epithelial lesions, and involvement of the vascular adventitia, which accounts for the development of gross lesions, including epithelial erosions, that are associated with lymphoid cells such as CD8+ T lymphocytes, which are the predominant cells associated with the vascular lesions, and replication of OvHV-2 in lung tissue (Radostits *et al*., 2007; Zaki *et al*., 2016). The lymph node enlargement is caused by abnormal proliferation of sinusoidal cells, which are the favoured predilection seats for replication of gamma herpesviruses like OvHV-2 in lymphoid tissue (Radostits *et al*., 2007; Zaki *et al*., 2016). The thin grey edge that often forms at the limbus of MCF's corneal opacity expands to the center and eventually covers the entire cornea (Zaki *et al*., 2016). Detailed pathophysiological alterations result from this disease and are caused by interactions between the virus and cells directly or potentially by immune-mediated reactions against infected cells (Radostits *et al*., 2007). Asymptomatic sheep or goats in contact with infected cattle may be due to the disease is reported to occur in cattle kept in the same environment as sheep, and sheep act as a potential source of the infection in that they shed the virus in nasal secretions (Bratanich *et al*., 2012; Khudhair *et al*., 2019; Avcı *et al*., 2020). Several publications claimed that when cattle are isolated from sheep or goats by at least 70 m to 5 Km, the transmission of the OHV-2 virus occurs (Giangaspero *et al*., 2013; Sood *et al*., 2014; Khudhair *et al*., 2019; Riaz *et al*., 2021). In Egypt, it is normal practise to raise sheep, goats, and cattle in mixed farming, and the various species are housed next to one another. Additionally, the animals frequently share food sources.

However, since the 2012 report by Bastawecy and Abd El-Samee (2012) who were the ones to initially record the MCF in Egypt, no reports on this disease in Assiut governorate have been published. Consequently, the current study is the first to record the prevalence of OvHV-2 in the Assiut governorate with PCR confirmation. The OvHV-2 tegument gene was detected in 21 (44.68%) of the 47 buffy coat samples of the studied cattle that were molecularly diagnosed by semi-nested PCR for the identification of MCF infection. When this tegument gene was attempted to be amplified in DNA samples of MCF, each reaction produced a distinct single band of the anticipated size, which is in accordance with Hristov *et al.* (2016) and Khudhair *et al.* (2019). The semi-nested PCR technique, which is still regarded by many studies as the best and most sensitive and specific molecular assay for detecting SA-MCF in clinical samples, was used for the confirmatory identification (Baxter *et al*., 1993; Li *et al*.,

Table 3. Prevalence of MCF viral infection in examined cattle according to the sex, age, breed susceptibility, and seasonal variation by using PCR.

Variable		No. of examined animals	No. of positive	Prevalence %	Chi-square test	
					Pearson chi-square (P-value)	Probability (p< 0.05)
Sex	Male	33	14	42.42	0.23	0.63
	Female	14	7	50		
	Total	47	21	44.68		
Age	4 months – 1 year	16	7	43.75	2.53	0.28
	>1 - 3 years	24	9	37.5		
	>3 - 5 years	7	5	71.43		
	Total	47	21	44.68		
Breed	Native	32	13	40.63	0.67	0.41
	Mixed	15	8	53.33		
	Total	47	21	44.68		
Season	Cold months*	10	5	50	0.15	0.70
	Hot months**	37	16	43.24		
	Total	47	21	44.68		

No significant variation at p<0.05; *: Cold months (from October to February); **: Hot months (from March to September).

1995; OIE, 2013; Zaki *et al*., 2016; Riaz *et al*., 2021). Detection primers for a fragment of OvHV-2's open reading frame 75 (ORF75), which codes for the tegument gene (Zaki *et al*., 2016). One of the primers, which binds at a region of low homology between OvHV-2 and Alcelaphine herpesvirus 1 (AlHV-1), is responsible for this PCR's specificity for OvHV-2 (Li *et al*., 1994; Sood *et al*., 2014).

Neocleotide sequence from the current study was confirmed as OvHV-2 when it was submitted to GenBank. The phylogenetic analysis of the PCR product of the Egyptian strain (Assiut) of OvHV-2 revealed 98.36% -100% identity with OvHV-2 strains of of different governorates (Beni-suef, Giza and Fayoum) of Egypt, India, Turkey, Iraq, Italy, Brazil and United Kingdom. This finding may be due to OvHV-2 having a low mutational rate; this condition is quite common in DNA viruses (Coradduzza *et al*., 2022). This result indicates that the OvHV-2 detected in examined diseased cattle was genetically similar to that found in other countries.

Epidemiologically, the current study indicated that 44.68% of the studied cattle in Assiut Governorate, Egypt had MCF infection. A lower percentage of MCF infection was reported by Zaki *et al.* (2016) and Abd El Rahman *et al*. (2020) who found that 4%, 4%, 8%, 8%, 12%, 20%, and 4.22% of examined animals had MCF infection in Fayoum, El-Gharbia, El-Sharkia, El-Dakahlia, Giza, Kafr El-Shiekh, and the Delta region of Egypt, respectively. While a nearly similar rate of infection was noted by Zaki *et al*. (2016) who mentioned that 44% of investigated animals showed MCF infection in El-Menia, Egypt. Such variations may be attributable to variances in sample collection times, sample sizes, sanitary conditions, ambient conditions, and the use of various diagnostic procedures. In terms of sex susceptibility, there was no statistically significant difference in the rate of MCF infection between the male and female studied cattle. This may suggest that MCF is a non-sex-related disease, so male and female cattle do not differ in their resistance to MCF infection, but there are many environmental and management factors that can affect the rate and severity of MCF infection in both sexes of cattle at the same or different stages of life. These factors include the amount of virus exposure, stress exposure, and many others. Concerning age susceptibility, age groups >3-5 years had the mathematically highest rate of MCF infection. This finding was supported by Abd El Rahman *et al.* (2020). This could be explained by the fact that as animals age, their risk of MCF infection rises. Although there was no appreciable difference in the rate of MCF infection between native and mixed breeds in the current study, mixed breeds mathematically had a greater rate than native breeds, this result was agreement with Radostits *et al.* (2007) that concluded that all breeds of animals are equally susceptible. Mixed breeds had higher numerically; this might be because mixed breeds have weaker immune systems than native breeds. Seasonal changes and the frequency of MCF infection were studied; the percentage of MCF infection did not considerably change between the cold and hot months. This disease can be seen at any time of year. There have been suggestions that environmental stressors during all months of the year may predispose animals to the manifestation of the disease (Radostits *et al*., 2007).

Conclusion

The current study illustrated that OvHV-2 is responsible for MCF infection in cattle in Assiut governorate in a sheep-related form. This suggests that raising sheep in the same grazing area as susceptible species can increase the risk of catching infection, which is still a popular practise in Egypt. Therefore, our advice is the separation of grazing areas for cattle from those for sheep, and extensive research on sheep of various ages must be conducted to determine the most proper age for transmitting MCF infection to susceptible animals. Also, all clinical suspicions of MCF must be validated by laboratory testing to rule out clinically similar diseases such as mucosal disease, infectious bovine rhinotracheitis, and foot and mouth disease.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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