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SEROPREVALENCE, ISOLATION, AND MOLECULAR DETECTION OF FOOT-AND-MOUTH DISEASE VIRUS (FMDV) IN CATTLE IN El-MINIA GOVERNORATE, EGYPT

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ABSTRACT

A cross-sectional study has been undertaken in El-Minia province to survey an outbreak of foot-and-mouth disease (FMD) in cattle and identify the associated risk factors. From November 2021 to October 2022, researchers collected 100 blood samples, ten tongue epithelium specimens, and saliva swabs from animals of various ages and sexes in different locations. Additionally, the animals were examined for FMD signs. The overall seroprevalence was tested via SNT, which was 67%. Tissue samples and swabs were isolated and examined using conventional RT-PCR for detection of the FMD serotype, revealing serotype A. Statistically, no significant variation ($P > 0.05$) was observed in the prevalence of FMD in relation to age, sex, season, or locality. The findings of this study indicated that the recent FMD outbreak has been caused by serotype A in the studied locations and that FMD is widespread in cattle populations in El-Minia province. These findings underline the necessity for immediate attention and future research to detect FMDV strains spreading in the region that aid in executing effective prevention strategies.

***Keywords*:** FMD, RT-PCR, Risk Factors, Seroprevalence, Egypt.

INTRODUCTION

Foot-and-mouth disease (FMD), a viral infection of cloven-hooved animals caused by the foot-and-mouth disease virus (FMDV), is one of the diseases with the greatest economic impact due to its extremely contagiousness, ability to induce chronic infections and protracted consequences concerning the health and production of several animal hosts it influences (Ko *et al.,* 2009). Foot-and-Mouth Disease Virus (FMDV) is the causal organism, which is a small positive-sense single-stranded RNA (ssRNA) virus (approximately 8.5 kb) that pertains to the family Picornaviridae, genus Aphthovirus (Belsham, 1993). There are seven antigenically different serotypes of FMDV (A, O, C, South African territories (SAT) types 1, 2, 3, and Asia 1) and several subtype variants (i.e., topotypes). Vaccination or infection with one serotype of FMD does not offer immunity against other serotypes or,

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occasionally, subtypes inside the same serotype; this antigenic diversity hampers the control of the disease (Paton *et al.,* 2006). FMD is spread by direct contact with secretions or excretions from acutely infected animals and possibly by indirect contact with contaminated fomites (Gloster *et al.,* 2010). Identification of FMDV may be attained by isolating the virus or detecting the presence of FMDV nucleic acid or antigen in suspected samples. Virus-specific antibody detection could be utilized for diagnosis, and regardless of vaccination status, antibodies against nonstructural proteins (NSPs) can serve as helpful markers of infection (Biswal *et al.,* 2022). Since 1950, numerous epidemics have documented the endemic nature of the disease in Egypt. Researchers identified the FMD serotypes A, O, and SAT2 in Egypt (Shawky *et al.,* 2013). In Egypt, vaccination is the only way to manage FMD. The generation of the FMD virus in tissue culture, its stability following virus inactivation procedures, and the manufacturing of the vaccine all have a major role in the immunogenicity of FMD vaccination (Parida, 2009). Currently, FMD is partly unregulated throughout the country, despite vaccination campaigns. Understanding the factors influencing the illness's susceptibility as well as the associated serotypes of FMD virus are essential inputs needed to launch a control campaign; however, there is not recently published FMD data in Minya governorate. As a result, this investigation aims to determine the circulating serotypes and assess the seroprevalence of bovine foot-and-mouth disease.

MATERIALS AND METHODS:

1. Ethical approval:

The board of the Faculty of Veterinary Medicine at El-Minia University in Egypt has granted this study ethical approval (approval no. IRB-FVM-MU-2023-105). The study's objective was to reduce animal discomfort during sample collection, and it was carried out in compliance with global ethical guidelines for animal care and usage.

2. Study area and population:

The survey has been conducted to evaluate the individual seroprevalence of FMD among cattle and detect the circulating serotypes responsible for the FMD outbreak in 2021. The study was accomplished in 12 months, from November 2021 to October 2022, in five localities (Mallawi, Abo-Korkas, Minya, Bany-Mazar, and Maghagha) in El-Minia governorate in Upper Egypt. These regions were chosen according to FMD history and animal population density. During the period of investigation, a total of 100 cattle of different ages, sexes, and regions were examined for FMD infection, and the data of each examined animal (case history and clinical examination) was documented in a separate case sheet.

3. Sampling method and sample preparation:

A random selection approach has been implemented to choose the animals to be utilized for the study of the prevalence and detection of FMD in the study area. One hundred whole blood samples and ten samples of saliva swabs and tongue epithelium were collected from suspected and in-contact animals. The blood samples were drawn from the animals and placed in plain vacutainer tubes (without anticoagulant). The samples were kept at 4 °C overnight and then centrifuged at 1000 rpm for 10 minutes. The sera were removed from the tubes, placed into Eppendorf tubes, and stored at -20 °C until used for serodiagnosis. Five tissue samples were obtained from suspected cattle using sterile forceps, then ground, frozen, and thawed before being clarified by centrifugation. The supernatants were collected for virus isolation in cell cultures and PCR techniques, ensuring aseptic conditions were maintained. Furthermore, five salivary swabs were collected and placed directly into sterile tubes containing transport media, then transferred to cryovials to be stored at -80 °C until used for virus isolation and PCR assays.

4. Serological examination:
In order to determine the animals' antibody status against FMDV, serum samples were submitted to serum neutralization test (SNT), which was conducted following OIE (2009). FMD virus strains O PanAsia-2, A Iran-5, and SAT2/EGY/2012, of cattle origin, were graciously provisioned by VSVRI, Abasia, Cairo, Egypt, and used as a positive control in SNT. All sera had been inactivated for 30 minutes at 56◦C in a water bath before being serially diluted in microtiter plates. Equal volumes of FMDV (100 TCID50/50 µl) were added to each well. Controls were added and used to calculate the virus titer in the test. The covered plates had been placed for one hour at 37 °C in the incubator. Afterward, a volume of 50 µl of cell suspension (10^6 cells/ml) was added to the wells. The plates were incubated for two days at 37 ˚C and microscopically examined after 48 hours for the occurrence of a cytopathic effect (CPE). A serum/virus mixture titer of 1/45 or higher of the final serum dilution is considered positive. A titer of less than 1/16 is deemed negative.

5. Virus isolation:
The prepared epithelium samples and saliva swabs were inoculated in confluent BHK-21 flasks and incubated in a CO2 incubator at 37°C for 24–72 hours before being examined microscopically for the presence of CPE. This method of isolating FMDV was carried out using BHK-21 cell culture, as previously prescribed by OIE (2009).

6. Viral RNA extraction:
The total RNA was extracted from the FMDV isolates using a QIAamp® Viral RNA Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted RNA was eluted in 60 µl of AVE kit elution buffer and kept at -80 °C until used.

7. Molecular detection of the FMD virus by RT-PCR:
Conventional one-step RT-PCR was followed up according to the manufacturer's directions for serotyping the FMD virus field isolates. The viral VP1-RNA was amplified by RT-PCR using specific primers for serotypes O, A, and SAT-2, according to El-Khabaz and Al-Hosary (2017). Amplicons were analyzed by Agarose Gel Electrophoresis, and their size was estimated with a size marker DNA of 2000 bp before being observed by a gel UV-transilluminator.

Table 1: Oligonucleotide primers used for detection of FMD virus via RT-PCR

Primer		Sequence (5′ to 3′)	Expected size
Universal primer	P1 P2	5′- CCTACCTCCTTCAACTACGG-3′ 5′-GAAGGGCCCAGGGTTGGACTC-3′	216-bp
Serotype O 1D/2B region	Ph1 Ph2	5′-AGC TTG TAC CAG GGT TTG GC-3′ 5′-GCT GCC TAC CTC CTT CAA-3′	402-bp
General SAT	SAT-ID209F FMD2B208R	5′-CCACATACTACTTTTGTGACCTGGA-3′ 5′-ACAGCGGCCATGCACGACAG-3′	≥700-bp
Serotype SAT2	P1 VP3-AB	5′-GAA GGG CCC AGG GTT GGA CTC-3′ 5′-CAC TGC TAC CACTCR GAG TG-3′	880-bp
Serotype A	PH9 PH10	5′-TAC CAA ATT ACA CAC GGG AA-3′ 5′-GAC ATG TCC TCC TGC ATC TG-3′	863-866 bp

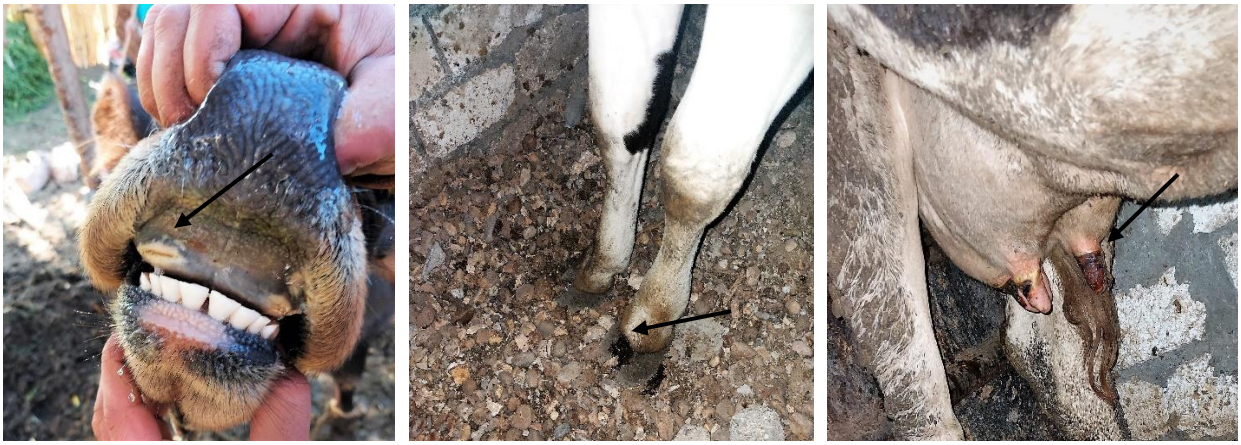
8. Statistical analysis:
The epidemiological data was analyzed with SPSS, version 26, 2017. A Chi-square test of independence was used, and tests were judged significant at $P < 0.05$.

RESULTS:

1. Field diagnosis of clinical cases infected with FMD:
The diagnosis of FMD in cattle was made in the field through clinical examination and the identification of distinctive clinical signs. The study's animals exhibited the usual clinical symptoms of FMD, including fever, dullness, oral lesions, foot lesions, udder lesions, and cardiac arrhythmia. The clinical examination revealed that 32 out of 45 FMD-infected animals were lethargic, 20 were febrile, 39 had oral lesions, 18 had foot lesions, 12 suffered from udder lesions, and seven animals exhibited cardiac arrhythmia (Table 1 & figure 1).

Table 2: Clinical findings in examined cattle.

Examined	Clinically diseased	FMD-Seropositive	Clinical signs in Seropositive animals	Diseased	
				No.	%
100	57	45	Fever	20	44.4
			Dullness	32	71.1
			Oral lesions and signs	39	86.6
			Foot lesions and signs	18	40
			Udder lesions and signs	12	26.7
			Cardiac arrhythmia	7	15.6



Oral erosions **Foot erosions** **Udder erosions**

Figure 1. Clinical findings of FMD–affected cattle.

2. Association between infection rate of FMD and potential risk factors according to SNT results:

2.1. Percentage of FMD-infected animals:
100 serum samples were obtained from cattle and tested against the three FMDV serotypes (A, O, and SAT2) via SNT. The titers' positive cut-off value was 1.65 log10 serum titer (i.e., ≥ 1/45). The results revealed that 67 cattle out of 100 (67%) animals were detected as seropositive for serotype A alone. There were no positive samples for serotypes O or SAT-2.

2.2. Age susceptibility:
The findings revealed that FMD was noticeably higher in cattle between the ages of 1-2 years (70.2%), then in 2-3 years (66.7%), followed by animals less than a year (63.6%), and the lowest prevalence was in cattle above 3 years (60%). However, no statistical association was established (Table 2).

2.3. Effect of sex:
According to the results, females had a somewhat greater prevalence of infection (69.8%) than males (62.1%). Statistical research, however, revealed no correlation between the disease's prevalence and sex (Table 2).

2.4. Seasonal variation:
Winter had the highest rate of FMD infection (75%), followed by fall (68.57%), summer (60.7%), and finally spring (50%). Nonetheless, no statistical correlation between the season and FMD infection was found (Table 2).

2.5. Effect of location:
Bany-mazar had the highest prevalence of FMD infection (76.5%), followed by Abo-korkas (72.2%), Minya (68.4%), and Maghagha (64.7%). The lowest rate of infection was in Mallawi (58.6%). Similarly, there was no association between FMD infection and animals' locations
(Table 2).

Table 3: Relation of rate of FMD in cattle by SNT to some risk factors according to SNT results.

Variable		No. of examined animals	No. of positive	Infection rate %	Chi-square test	
					χ2 value	P value
Age	<1 year	22	14	63.6	0.555	0.907
	>1-2years	47	33	70.2		
	>2-3 years	21	14	66.7		
	>3 years	10	6	60		
	Total	100	67	67		
Sex	Female	63	44	69.8	0.622	0.430
	Male	37	23	62.1		
	Total	100	67	67		
Season	Winter (Dec. to Feb.)	44	33	75	3.365	0.339
	Spring (Mar. to May)	12	6	50		
	Summer (June to Aug.)	28	17	60.7		
	Fall (Sep. to Nov.)	16	11	68.75		
	Total	100	67	67		
Locality	Abo-korkas	18	13	72.2	1.890	0.756
	Bany-mazar	17	13	76.5		
	Maghagha	17	11	64.7		
	Mallawi	29	17	58.6		
	Minya	19	13	68.4		
	Total	100	67	67		

No significant variation at $p > 0.05$.

3. FMDV isolation and serotyping using RT-PCR assay:

Trials for isolating FMDV from suspected samples were conducted by inoculating them onto BHK-21 cell cultures. Cytopathic effects (CPE) were observed in tongue epithelium and saliva swabs after 48 hours post-inoculation. Out of the ten samples, six exhibited CPE in the form of rounding, granulation, and detachment (Figure 3).

The six samples that exhibited CPE were examined using the RT-PCR assay. Results showed that all six samples were positive for serotype A, with amplicons of the expected size. No amplicons were detected for serotypes O or SAT-2 (Figure 4).

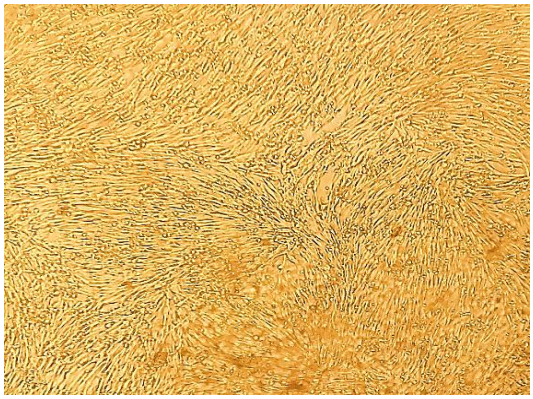


Figure 2. Normal BHK-21 cells (after 48 hrs.)

Figure 3. BHK-21 cells showing CPE

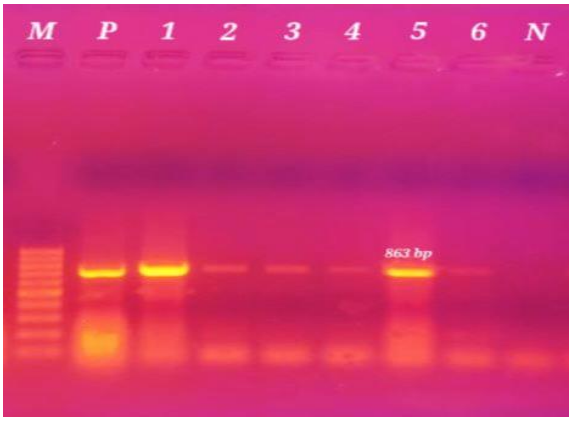


Figure 4: The PCR results show that the gel contains 863 bp bands (serotype A).
Lane M: Marker, lanes 1, 2, 3, 4, 5, and 6 show a positive response, lane N shows negative control, and lane P shows positive control.

DISCUSSION

Foot-and-mouth disease (FMD) is a contagious disease affecting almost exclusively cloven-hooved animals, including domesticated (cattle, buffalo, sheep, and goats) and wild ones (approximately 70 species of wild animals). Pyrexia, vesicular sores on the foot, buccal mucosa, and female mammary glands distinguish the condition. There have been seven antigenically dissimilar serotypes: O, A, C, SAT-1, SAT-2, SA-T-3, and Asia-1, each with a huge number of subtype variations. The OIE lists FMD as one of the infectious illnesses of animals, and it has been acknowledged as the main obstacle to global commerce in animals and animal products (OIE, 2022). FMD has been widespread in broad parts of Africa, Asia, and South

America, and it spreads readily across country lines to cause outbreaks in places that were once virus-free. The disease is not enzootic in Europe, Australia, New Zealand, or North America; nonetheless, occasional outbreaks have occurred in Europe, primarily as a result of Middle Eastern transmission (Abdul-Hamid *et al.,* 2011). The most effective way to stop FMD outbreaks is vaccination against the ailment. One FMDV serotype infection or vaccine may not offer complete protection against other serotypes or different subtypes of the same serotype (Paton *et al.,* 2006). In Egypt, FMDV was first detected in 1950 when serotype SAT2 caused an outbreak; then, A and O types were also discovered in later epidemics, and ever since, the three serotypes have continued to circulate in livestock, causing periodic outbreaks and serious losses. Egypt's vaccination program is primarily reliant on locally produced polyvalent inactivated vaccines (Ahmed *et al.,* 2012). In this study, seroprevalence of FMD has been assessed in suspected and seemingly healthy in-contact animals, and FMDV was isolated, molecularly diagnosed, and characterized in suspected cases. SNT was employed to estimate antibodies against FMDV infection and to calculate the predicted proportion of protection. Regarding the negative/positive cut-off value, various laboratories interpret the SNT results differently; however, the OIE recommends that a titer of 1.65 $\log^{10}$ or greater of the final serum dilution in the serum/virus mixture be deemed positive. The OIE (2012) considers titers 1.2 $\log^{10}$ to 1.5 $\log^{10}$ as questionable, and 0.9 $\log^{10}$ or less as negative. In our results, clinical examination for signs of FMD in cattle revealed that 45 out of 100 animals exhibited signs of FMD infection and were seropositive. The results showed that 32 (71.1%) of the infected animals were lethargic, 20 (44.4%) cattle were febrile, 39 (86.6%) animals had oral lesions and signs, and 18 (40%) animals showed foot lesions. 12 (26.7%) cattle exhibited udder lesions, and only 7 (15.6%) animals suffered from cardiac arrhythmia. Some of our study's findings were somewhat similar to those of Sobhy *et al.* (2018) in Egypt, who recorded that the clinical manifestations associated with FMD were: 52 out of 108 (48.1%) FMD-positive cases suffered from pyrexia; 20 (21.6%) animals showed cardiac abnormalities; 81 (87.4%) cattle had oral lesions; and 34 (31.4%) animals exhibited panting. However, other findings were inconsistent, as 49 (45.4%) animals were dull, and 71 (76.7%) animals suffered from foot lesions and lameness. A variety of study design factors, such as the time and location of the studies, the tests used for animal diagnosis, and variations in the study object, such as sample size, may contribute to differences in the findings of the two studies. In terms of the seroprevalence study of FMD using SNT, the findings indicated that among the 100 cattle tested, 67 (67%) had antibodies against serotype A of FMD virus, while no sample had antibodies against any other type that tested positive. FMD prevalence was not sufficiently reported in the Minya governorate; however, this percentage is considered to be significantly high. The high overall prevalence of FMD could be attributed to the outbreak that occurred in Egypt, which lasted from 2021 to 2022 and affected a large number of animals. Our result was lower than that of Nthiwa *et al.* (2020) in Kenya, who reported an overall seroprevalence of FMD, which was 83.8% (980 out of 1170 tested animals were seropositive). Nonetheless, our obtained findings were substantially higher than those of Tolawak *et al.* (2023) in Ethiopia, who reported that FMD overall seroprevalence was 20.3% (54 out of 266 animals were seropositive). This variance in seroprevalence among regions might be attributed to differences in the time and place of the study, methods utilized, cattle density, herd size, and biosecurity, all of which could be key risk factors in FMDV transmission and persistence. Studying the seroprevalence of FMD in cattle according to age, the results showed that antibodies were noticeably higher in cattle aged between 1-2 years (70.2%), notwithstanding that statistical significance was not attained, followed by those aged between 2-3 years (66.7%), after those cattle less than one year (63.6%), and

finally cattle above 3 years (60%). Our findings concur with those of Aidaros *et al.* (2016) in Egypt, who recorded that the highest prevalence was noticed in cattle aged between 1-2 years (52.8%), followed by those less than 1 year (8.3%), and finally old cattle aged between 3-5 years (5.6%). Increased viral exposure and the fact that recovered animals have protective antibodies for several years following natural infection or vaccination may explain the lower seroprevalence with increasing age. As a result, the older the animal, the more likely it is to have been infected over its lifetime. Furthermore, young animals (less than one-year-old) are more likely to succumb to death due to FMD infection, whereas senile ones may be immunocompromised with low levels of protective antibodies (FAO, 2012). Concerning the seroprevalence of FMD in cattle according to sex, the findings showed that the prevalence of infection has been slightly higher in females (69.8%) than in males (62.1%). However, statistical analysis found that there was no association between sex and the prevalence of the disease. Our results partially disagree with those of Abdulahi Mohamoud (2011) in Ethiopia, who recorded a higher occurrence of FMD in male cattle (19.4%) than in female ones (13.6%). Nevertheless, both studies concluded that sex and the rate of the disease were independent. Seasonal differences in the seroprevalence of FMD in cattle showed that winter had the highest rate of FMD infection (75%), followed by fall (68.57%), summer (60.7%), and lastly, spring (50%). Despite the lack of statistical significance, historical occurrences characterized comparable chronological FMDV distributions, with serotype A tending to produce epidemics between October and February that can last until June, as demonstrated in Egypt's 2006 outbreak. On the other hand, serotype O usually causes outbreaks between February and August, with the most severe epidemic reported between April and June as the virus shows some heat tolerance. Meanwhile, SAT-2 outbreaks usually appear in March and April (WRLFMD, 2022). Our values coincide with those of Aidaros *et al.* (2016) in Egypt, who recorded a higher rate of the disease in the winter (71.4%) than in the summer (50%). Nonetheless, they disagree with those of Ullah *et al.* (2023) in Pakistan, who noticed the highest incidence of the disease in the autumn (83.3%). Seasonal variation across different studies may be attributed to differences in climatic conditions in different countries or regions of the same country. The statistical relationship between locality and the rate of FMD was studied during the period of investigation. Among the five regions under study, Bany-Mazar had the highest prevalence of FMD infection at 76.5%, followed by Abo-korkas at 72.2%, Minya at 68.4%, and Maghagha at 64.7%. The lowest rate of infection was in Mallawi (58.6%). Despite the data variations, we were unable to establish a link between location and disease prevalence. As far as we are aware, earlier epidemiological research on FMD in these areas has not been conducted, but changes in disease prevalence can be attributed to factors like herd size, management, and biosecurity measures. Trials for the isolation of the virus were conducted. Suspensions of field samples (epithelial tissue and saliva swabs) suspected to contain FMD virus were inoculated onto a baby hamster kidney (BHK-21) cell culture, and the cell culture was examined for CPE after 48 hours. The CPE appeared in five epithelial tissue samples and one saliva swab sample after 48 hours post-inoculation in the form of cell rounding, granulation, and cell detachment (Borca *et al.,* 2012). Conventional RT-PCR was used for the typing of five epithelial tissue samples and one saliva swab sample by using specific primers for the A serotype (after they gave negative results with the Oligonucleotide O primers) to amplify the VP1 coding region fragment of FMDV. It was observed that all six FMDV-positive samples yielded positive results for serotype A. Our results align with the findings of Shahein *et al.* (2023) in Egypt, who also investigated the FMD outbreak in 2022 and discovered that the causative serotype was A. Furthermore, strain A (Africa topotype) genotype IV was discovered after sequencing field isolates and conducting phylogenetic analysis. Therefore, to attain the

highest level of protection against FMD infection, they suggested that the current isolates be incorporated into the locally made FMD vaccine.

CONCLUSION

Minya province has a significantly high rate of FMD among cattle, which poses a significant challenge for farmers and results in extensive economic losses. This finding demonstrates the importance of FMDV immunity in cattle protection and reemphasizes the need to address an improved FMDV vaccination program among livestock. To provide maximum protection against circulating FMDV viruses, further studies of the serotype (A) immunogenic relationship to the vaccine strains are necessary. Furthermore, factors such as susceptibility, sex, age, and immune response differences should be considered when developing an effective mass vaccination program.

CONFLICT OF INTERESTS

The authors declared that no conflict of interest exists.

RECOMMENDATIONS

Implementing a national bovine FMD control and eradication plan. Investing in the research and development of new and improved bovine FMD vaccines. Raising awareness of bovine FMD among farmers and veterinarians.

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الانتشار المصلي ، وعزل ، والكشف الجزيئي عن فيروس مرض الحمى القلاعية في الأبقار في محافظة المنيا ، مصر

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تم إجراء دراسة مقطعية لمسح تفشي مرض الحمى القلاعية (FMD) بين الأبقار وتحديد عوامل الخطرالمرتبطة به. تم تجميع ١٠٠ عينة دم وعشر عينات من نسيج اللسان بالإضافة إلى مسحات اللعاب من الحيوانات المختلفة خلال الفترة من نوفمبر ٢٠٢١ إلى أكتوبر ٢٠٢٢. تم فحص الحيوانات بحثا عن أعراض المرض وكذلك تم تحديد معدل انتشار المرض باستخدام اختبار تحييد المصل (SNT) والذي بلغ ٦٧% . بالإضافة إلى ذلك، تم عزل عينات الأنسجة والمسحات وفحصها باستخدام تفاعل البلمرة المتسلسل العكسي (RT-PCR)للكشف عن النمط المصلي للحمى القلاعية والذي كشف عن النمط المصلي A. إحصائياً ، لم يُلاحظ أي اختلاف جوهري ($P > 0.05$) في معدل انتشار الحمى القلاعية بالنسبة للعمر أو الجنس أو الموسم أو المكان. وأشارت نتائج هذه الدراسة إلى أن تفشي الحمى القلاعية الأخير كان ناتجًا عن النمط المصلي A في المواقع التي تمت دراستها وأن مرض الحمى القلاعية منتشر على نطاق واسع بين قطعان الأبقار في محافظة المنيا. تؤكد هذه النتائج على ضرورة الاهتمام الفوري وإجراء المزيد من الأبحاث لتحديد سلالات الفيروس المنتشرة في المنطقة والتي ستساعد في تنفيذ استراتيجيات وقاية فعالة.