



Foot-and-Mouth Disease Serotype SAT2 Outbreak in Iraqi *Buffaloes*: Molecular and Epidemiological Analysis

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Abstract | Foot-and-mouth disease (FMD) is a viral infection that results in considerable economic losses among livestock. This article presents a molecular and epidemiological investigation of FMD serotype SAT2 in Iraqi *buffalo*. The study involved 102 buffaloes from two provinces, Wassit and Dhi-Qar. Clinical examination within the herd revealed classic FMD signs, including salivation, oral and nasal mucosal erosions/ulcerations lesions and laminae of affected animals. The proportions of older animals were more likely to show infection ($p > 0.05$), but there was no significant difference. The female was the predominant sex infected ($p > 0.05$). However, this observation did not reach statistical significance. By semi-nested RT-PCR was used on epithelial tissue samples, 84.31% of samples were positive SAT2 serotypes. Those sequences were deposited into GenBank with accession numbers (PP898191-PP898192). At the same time, the seropositive rate was 80.39% on the ELISA test, which was used on serum samples for antibody detection. Genetic analysis revealed a close genetic relationship between the Iraqi SAT2 isolates and the Egyptian strains, suggesting a possible transmission route. This study outlines an increased risk of disease from SAT2 form and significant biosecurity challenges to Iraqi livestock, indicating requirements for focused at-risk surveillance and control efforts.

Keywords | *Buffaloes*, Epidemiological semi nested RT-PCR, SAT2, Foot-and-Mouth disease, Molecular

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INTRODUCTION

Foot-and-mouth disease (FMD) persists as a global challenge due to its high transmissibility and substantial economic repercussions for the livestock industry. Although the disease is endemic in several regions of the world, the continued emergence of new serotypes poses challenges for control and prevention efforts. The serotypes O, A, and ASIA1 have historically caused FMD outbreaks

in the past in Iraq (Al-Salihi, 2019). However, recent reports indicate the emergence of a new serotype, SAT2 (McLaws *et al.*, 2023). The addition of such a serotype is dangerous to animal life and raises concerns about the effectiveness of existing vaccination programs. The SAT strain of FMDV had remained geographically restricted to certain regions within Africa for many decades, due in part to specific environmental conditions. Nevertheless, recent reports of SAT2 detection in Libya, Egypt, the Palestin-

ian Autonomous Territories, and Bahrain signify a notable change. This expansion is becoming a growing threat to livestock industries-not only in North Africa but also in neighboring Middle Eastern countries (Li *et al.*, 2023).

The SAT2 serotype, characterized by its rapid dissemination and potential for severe clinical manifestations, has triggered outbreaks in neighbouring countries, including Turkey, Jordan, Bahrain, and Oman (Commission and Disease, 2024). Given the transboundary nature of FMD, generating comprehensive epidemiological and molecular data on the newly emerging Iraqi SAT2 strain is crucial for informing control strategies. The FMD virus in Iraq has been studied by many researchers on cows (Abd Hatem *et al.*, 2022; Mansour *et al.*, 2018), sheep (Dawood and Alsaad, 2018; Muhammed Saleh *et al.*, 2013) and camels (Al-Husseiny *et al.*, 2020). However, there is a lack of sufficient research on FMD in *buffaloes*, and no studies specifically address the SAT-2 strain in Iraq.

We used clinical examination, epidemiological surveys, and molecular diagnostic assays (RT-PCR, semi-nested RT PCR ELISA) to characterize the disease presentation, infection rates, and genetic relationships of the virus. The findings of this study will contribute to a better understanding of the present FMD situation in Iraq, help with risk assessment, and inform targeted intervention efforts needed to minimize negative effects caused by emergence or reemergence threats such as the one described here.

This study aims to bridge this critical knowledge gap by conducting the first comprehensive analysis of FMD serotype SAT2 in Iraqi *buffaloes*. To this end, the study used semi-nested PCR technology, genetic analysis, genetic sequencing, and ELISA to confirm the causative agent of FMDV.

MATERIAL AND METHODS

ETHICS APPROVAL

This study has been accepted and all procedures were carried out under the guidelines of the Scientific Committee of the College of Veterinary Medicine at Al-Qadisiyah University.

SAMPLE COLLECTION

SERUM SAMPLES: Samples for this investigation were collected from 102 water *buffalo* suspected of have an FMD. The buffalo exhibited specific clinical signs upon clinical examination. Ten milliliters of blood were drawn from the jugular vein of each animal using sterile syringes and needles. The venipuncture site was cleaned with 70% ethyl alcohol and allowed to dry before sample collection. Blood samples were collected into non-anticoagulant gel tubes.

In a centrifuge speed at 3,000 rpm for 10 minutes, serum was separated. Following that, the serum was collected in 1.5 Eppendorf tubes and stored at -20 °C until a sandwich enzyme-linked immunoassay was performed. Sample methods were done according (Nielsen *et al.*, 2021).

TISSUE SAMPLES

Epithelial tissue samples (n = 102) were collected from *buffaloes* in two regions that exhibited clinical signs of FMD. The epithelium samples were collected and placed in a plastic container containing Trizol® (Amirouche *et al.*, 2021). All samples were then frozen at -20 °C for the time of the PCR test.

EXTRACTION OF VIRAL RNA

Following the manufacturer's protocol, we employed the AccuZol™ Total RNA Extraction Kit. (Bioneer, Korea) to isolate viral RNA from our samples. The extracted RNA was subsequently assessed using a Nano-Drop instrument. This purified RNA then served as the starting material for cDNA synthesis, which was subsequently stored until ready for RT-PCR analysis as shown in the following Figure 1.

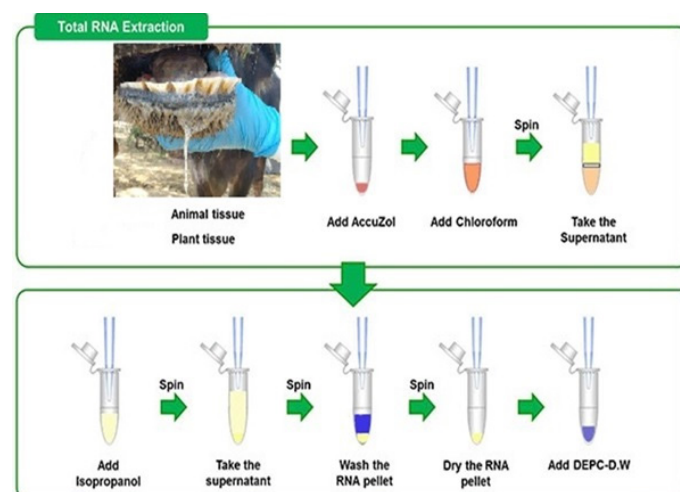


Figure 1: Steps of viral RNA extraction.

cDNA SYNTHESIS

Complementary DNA (cDNA) was synthesized from the extracted RNA, specifically targeting mRNA transcripts. This was achieved by employing the HiSenScript™ RH (-) RT Premix Kit, following the directions provided by the manufacturer.

SEMI NESTED RT-PCR

The RT-PCR product was further amplified using semi-nested PCR with SAT2-specific primers targeting a 506 bp amplicon within the VP1 gene. The primers were designed in this study using the NCBI database and Primer3Plus software and were synthesized by Macrogen, Korea, as shown in the following Table 1.

Table 1: Primer sequences of SAT-2 utilized in (snRT-PCR).

Primer	Sequence	PCR amplicon
Universal FMD	F GCCTGGTCTTTCCAG-GTCT	328
	R CCAGTCCCCTTCT-CAGATC	
FMD SAT2	F CACACATGTCCACACAG-GGA	715
	R GCGCGTCGAATCT-GTCTCTA	
Nested PCR Universal FMD	F GTACTGTGTTTGGCTC-CACG	239
	R GCATCCTTAGCCTGT-CACCA	
Semi-nested PCR FMDV SAT2	F CACACATGTCCACACAG-GGA	506
	R GACTGGCTTGTGCGACG-GTTA	

SEMI NESTED PCR

THERMOCYCLING CONDITIONS

PCR thermal conditions were conducted by using Semi nested PCR thermocycler system as mentioned in the following Table 2.

Table 2: Semi Nested PCR thermal cycling conditions.

PCR step	conditions	cycle
Pre-template denature	5min at 95°C	1
template denature	30sec at 95°C	30 cycles
Primer anneals	30sec at 59°C	
Primer extension	30 sec at 72°C	
Final extension	5min at 72°C	1
Inactivation	4 C	-

RESULTS AND DISCUSSION

CLINICAL EXAMINATION

The clinical investigation results, depending on semi-nested RT-PCR, are listed in Table 3. The important clinical manifestations observed are profuse salivation hanging in long ropy strings up to the ground (Figure 2A). In addition, there are erosions and ulcers on the nostrils and muzzle (Figure 2B). There were erosions, or ulcers on the gums, dental pad, hard palate, tongue, and inside of the mouth (Figure 3A). There were also skin sores in the coronary region and interdigital cavity of the hoof (Figure 3B).

The results of study of recurrent clinical signs, showed the highest rate was observed in Anorexia was seen in 79 buffaloes, accounting for 77.45% of the total, whereas 23 buffaloes

(22.55%) did not show this symptom. Salivation was seen in 73 buffaloes (71.57%), while 29 buffaloes (28.43%) did not exhibit this indication. Oral lesions were present in 71 buffaloes (69.61%), while 31 buffaloes (30.39%) lacked this symptom. Nostril lesions afflicted 46 buffaloes (45.10%), whereas 56 buffaloes (54.90%) did not show this symptom. Lameness was seen in 43 buffaloes (42.16%), while 59 buffaloes (57.84%) did not exhibit this symptom.

Table 3: Clinical signs of FMD in Buffalo results.

Clinical signs	Observed with signs		No signs	
	N	%	N	%
Anorexia	79	77.45%	23	22.55%
Saliva	73	71.57%	29	28.43%
Oral lesion	71	69.61%	31	30.39%
Nostril lesion	46	45.10%	56	54.90%
Lameness	43	42.16%	59	57.84%
Interdigital lesion	32	31.37%	70	68.63%
Calculated χ^2	32.744			
Calculated P value	0.0000042 HS			

HS: High significant difference between groups ($p < 0.01$).

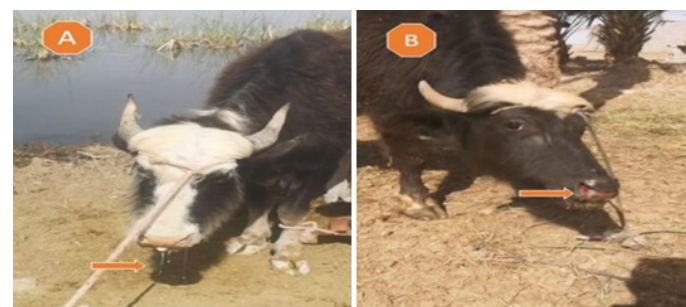


Figure 2: Shows clinical signs of FMD; A: Saliva hanging in long ropy strings; B: Nostril lesion.

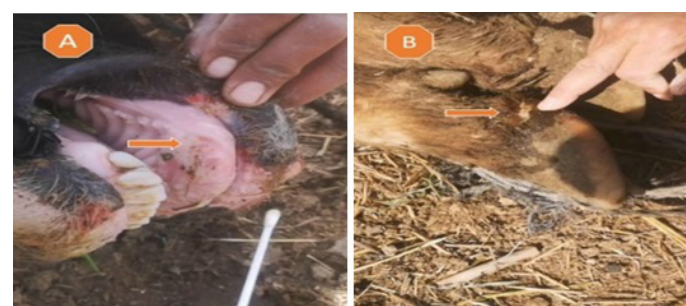


Figure 3: Clinical signs of FMD; A: lesion of the oral cavity; B: lesion of coronary band.

Finally, interdigital lesions were seen in 32 buffaloes (31.37%), whereas 70 buffaloes (68.63%) were clear of this condition. The chi-square statistic of 32.744 and p-value of 0.0000042 show a significant difference ($p < 0.01$). This major finding shows that the incidence of these clinical indications varies greatly across buffaloes, with some being more prevalent than others. Table 3, shown in Figure 4.

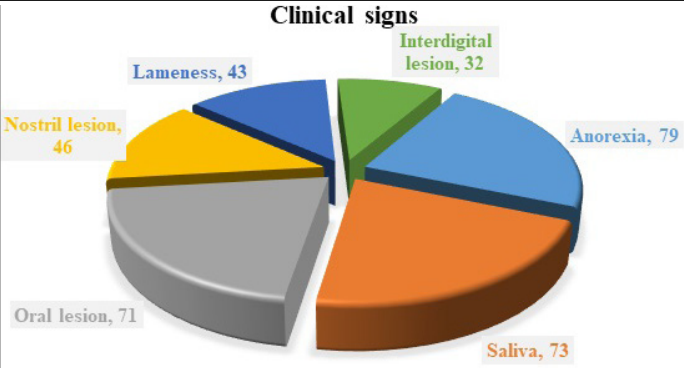


Figure 4: Clinical signs of FMD in buffalo results.

INFECTION RATE OF FMD ACCORDING TO AGE

The results of the epidemiological study according to age groups showed that the youngest age groups (6-18 months and 18-36 months) had the lowest incidence of FMD, with 82.05% and %82.14 Furthermore, the age groups with the highest incidence of FMD virus were those 36 months and above, with percentages of (88.57 %) Table (4), shown in Figure (5).

Table 4: FMD infection rate according to age.

Age	Buffaloes	Infected buf- faloes N (%)	Non-Infected buffaloes N(%)
6-18 (months)	39	32 (82.05%)	7 (17.95%)
18-36 (months)	28	23 (82.14%)	5 (17.86%)
36 months and above	35	31 (88.57%)	4 (11.43%)
Total	102	86 (84.31%)	16 (15.69%)
Calculated χ^2	0.542		
P value	0.763 NS		

NS: Non significant difference between groups (p > 0.05).

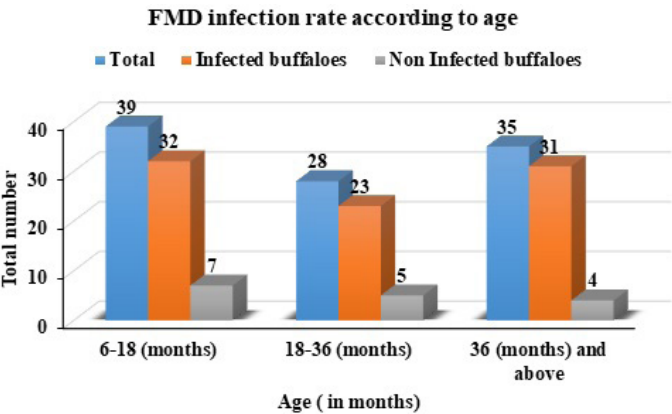


Figure 5: FMD infection rate according to age.

INFECTION RATE OF FMD ACCORDING TO GENDER

The result of incidence of FMD according to age revealed that the male at percentage (80.55%) had less affected than female had percentage (86.36%) Table 5, shown in Figure 6.

DETECTION OF FMD USING ELISA AND RT-PCR

The percentage of incidence of FMD confirmed using

multiple methods and two different types of samples, including blood and tissue samples, revealed 72/102 (70.58), 82 (80.39%), 86/102 (84.31) for conventional RT-PCR, ELISA, and semi-nested RT-PCR respectively as shown in Table 6, shown in Figure 7. The study findings indicated that there was statistically significant variation observed among the different tests used, as determined by the Chi-Square evaluation.

Table 5: FMD infection rate according to gender.

Gender	Buffaloes	Infected buffaloes N (%)	Non-Infected buffaloes N (%)
Male	66	57 (82.14%)	9 (17.86%)
Female	36	29 (88.57%)	7 (11.43%)
Total	102	86 (84.31%)	16 (15.69%)
Calculated χ^2	0.594		
P value	0.441 NS		

NS: Non significant difference between groups (p > 0.05).

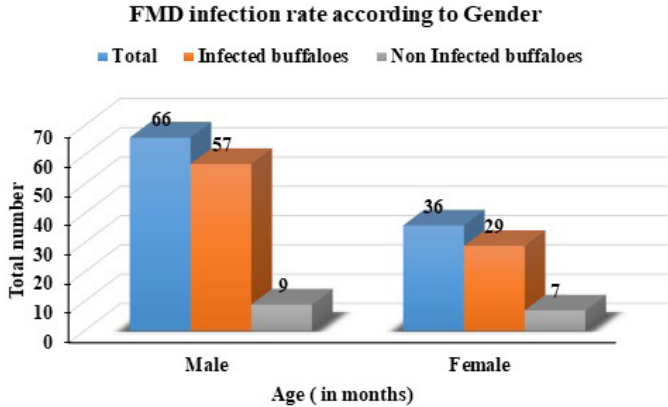


Figure 6: FMD infection rate according to gender.

Table 6: FMD infection rate in buffaloes based on the different test.

Tests	Infected buffaloes N (%)	Non- Infected buffaloes N (%)	Calcu- lated χ^2	P value
RT-PCR	Conventional 72 (70.58%)	30 (29.42%)	6.027	0.0491 S
	Semi-nested 86 (84.31%)	16 (15.69%)		
ELISA	82 (80.39%)	20 (19.61%)		
Total	102 (100%)			

S: Significant difference between groups (p < 0.05).

MOLECULAR DETECTION

DETECTION OF FMD USING RT-PCR: A total of 102 buffalo samples were analysed using conventional reverse transcription-polymerase chain reaction (RT-PCR). Initially, a universal FMDV primer targeting the VP1 gene was used, yielding a 328 bp amplicon in positive samples.

Subsequent testing with a serotype-specific primer for SAT2 FMDV, targeting a 715 bp amplicon within the VP1 gene, revealed 86 out of the initial 102 samples (84.31%) to be positive for this serotype. No samples tested positive for serotypes O or A (Figure 8).

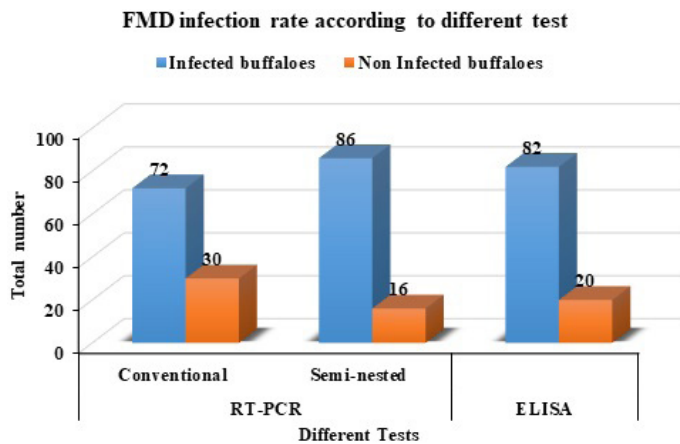


Figure 7: FMD infection rate in *buffaloes* based on the Different test.

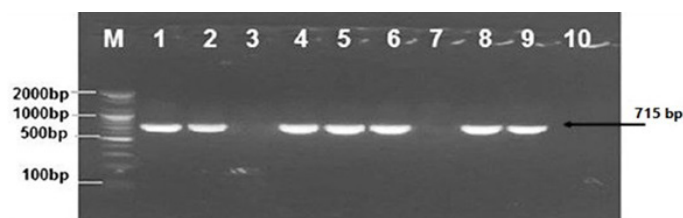


Figure 8: The agarose electrophoresis for FMD SAT 2 (VP1) gene amplicon; lane 1-10 positive samples, the marker M (2000-100 bp). The amplicon size is 715 bp. Revers transcripts PCR.

SEMI-NESTED RT-PCR RESULT: The results of using a specific primer for the viral protein 1 gene (VP1) of SAT-2 of FMD at (506 pb) were amplified by semi-nested RT-PCR. The results showed that 86 (84.31%) *buffalo* out of 102 community isolates tested positive for SAT-2 serotypes (Figure 9).

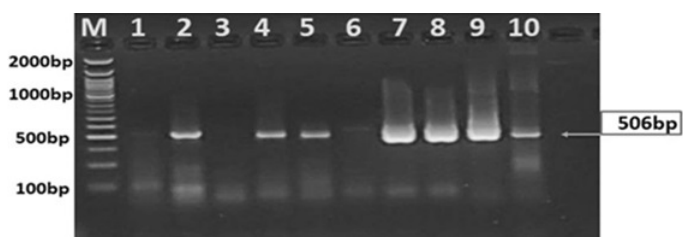


Figure 9: The agarose electrophoresis of FMD (VP1) gene amplicon; lane 1-10 positive samples, the marker M (2000-100 bp); the amplicon size is 506 bp.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The result of serological study showed that 80.39% (82/102) positive results for FMDV as shown in the following Table 6.

EPIDEMIOLOGICAL STUDY OF FMD

CLINICAL MANIFESTATION: The clinical study showed that animals infected with FMD suffered from anorexia, saliva, oral lesion, nostril lesion, lameness and interdigital lesion the results of this study agree with the findings of previous studies on FMD in *buffalo* (Hashem *et al.*, 2018). Depression and anorexia are common early signs of the disease, while lesions of the mouth, nose, and feet appear later. The lameness can be so severe that affected animals are unable to move (Mohebbi *et al.*, 2017). Vesicles occur when cytokines, cellulose, other proteins, and inflammatory cells from blood vessels enter the tissue, manifesting as oedema (Zhang *et al.*, 2022). The body temperature may have increased due to the release of endogenous pyrogens including interleukins and tumour necrosis factor- α in response to antigen interaction (Jafarsab, 2022).

INFECTION RATE OF FMD ACCORDING TO AGE: The infection rate of FMD according to age the results of this study from suggest a possible reduction in FMD in young cattle (6-18 months and 18-36 months) compared to adult animals (greater than 36 months) but do not have statistical significance differences at ($p > 0.05$). The results agreed with many other epidemiological studies on FMD that found no significant differences between different age groups and FMDV infection rates (Awel *et al.*, 2021). The lack of significant differences in the high incidence of infection between different age groups might be due to the attributed of rearing by not separating young animals from their mothers as well as the communal grazing practices (Al-Ajeeli *et al.*, 2018). However, it's important to note that our study may have been underpowered to detect subtle differences due to the relatively small sample size. On the other hand, other researchers (Salim *et al.*, 2020) mentioned that all age groups infected with FMD were equal.

INFECTION RATE OF FMD ACCORDING TO GENDER:

The results did not find statistically significant differences between the infection rates with the FMD virus between males and females, although the infection rate in females was slightly higher. The results of this study agreed with (Dubie *et al.*, 2021). This may be attributed to physiological factors like lactation, pregnancy, and estrus (Aghaalkhani, Hossein and Ahmadi, 2018). However, it's worth noting that other studies have reported different findings, with some showing a higher prevalence in males (Chowdhury *et al.*, 2020) and others finding no difference (Belina *et al.*, 2016). These discrepancies could be due to variations in study populations, diagnostic methods, or other factors.

MOLECULAR DETECTION

The high prevalence (70.58%) of SAT2 FMDV in the conventional PCR-tested *buffalo* population and the detection rate was 84.31% by semi-nested RT-PCR aligns with previous studies (El Damaty *et al.*, 2021) reported 100% prev-

alence of SAT2 FMDV in 54 examined *buffalo* samples using RT-PCR targeting the VP1 gene. Similarly (Hagag *et al.*, 2019) found 96% of 100 *buffalo* samples to be positive for FMDV using VP1 gene-based primers. In Iran, (Fs *et al.*, 2022) reported a 73% prevalence of FMDV in cattle using PCR. However, the high prevalence observed in this study contrasts with lower rates reported in other countries (Abd El-Rahim *et al.*, 2016) found a 26% seroprevalence of FMD in Saudi Arabia. These differing prevalence rates might be due to several factors, including differences in diagnostic methods, animal breeds, disease stage, vaccination programs, and geographic/climatic variations (Ullah *et al.*, 2023).

Furthermore, a wide range of genetic variants of FMD virus and its presence in 7 different serotypes present significant difficulties in identifying the virus but this study used a semi-nested RT-PCR method which proved successful in detecting SAT-2 (Chen *et al.*, 2022; Wong *et al.*, 2020).

ENZYME-LINKED IMMUNOSORBENT ASSAY

In this study, use of ELISA as a diagnostic tool to detect FMDV antibodies in *buffalo* serum samples showed its effectiveness and reliability (Wong *et al.*, 2020; Yan *et al.*, 2023). The results of serological tests showed the presence of antibodies to FMDV virus in 80.39% of the samples, indicating a high prevalence of the disease among the study population. The high seropositivity in our results from this investigation is agreed with high seropositivity reported (Dickmu *et al.*, 2022).

The high seropositivity in our results from this investigation agrees with a high seropositivity reported in Al-Qadisiyah and Basrah (Ajeeli *et al.*, 2012; Al-Rodhan, 2018). while the infection rates in Al-Najaf and Diyala were 34% and 25.33%, respectively (Abd Hatem *et al.*, 2022; Al-Ajeeli *et al.*, 2018).

This variation in seroprevalence across different regions of Iraq may be attributed to differences in vaccination coverage, animal husbandry practices, or the presence of other confounding factors. Differentiation between vaccinated and infected animals is important for prevention planning. ELISA is used to detect infected animals by detecting non-structural proteins such as 3AB, 3ABC, 2C,2B and other targets. Antibodies occurred on days 4-10 post-infection in all animals with no history of prior exposure to FMDV antigens (Wong *et al.*, 2020).

The high distribution suggests that the SAT-2 serotype is responsible for the outbreak, consistent with previous reports of an SAT-2 outbreak in Iraq in 2023 (McLaws *et al.*, 2023).

This study has several limitations. The convenience sam-

pling method may not be representative of the entire *buffalo* population in Iraq. The relatively small sample size limits the generalizability of the findings. Additionally, the study may have lacked data on certain potential confounding factors, such as vaccination history and animal movement patterns, which could influence the observed infection rates. Future studies should aim to address these limitations by using more representative sampling methods, increasing sample sizes, and collecting more comprehensive data on potential confounding factors. Further research is also needed to investigate the transmission dynamics of FMDV SAT2 in Iraqi *buffalo* populations, evaluate the effectiveness of current vaccination strategies against this serotype.

CONCLUSIONS AND RECOMMENDATIONS

The SAT2 serotype is responsible for the foot and mouth disease outbreaks in Iraq in 2023. which were not previously documented in the country. The study verifies previously documented clinical indications of FMD in *buffaloes*, such as fever, decreased appetite, ulcers in the mouth and foot, and restricted movement. While we found no statistically significant differences in infection rates between age groups or genders, it is important to note that our study may have been underpowered to detect subtle differences due to the relatively small sample size. There were no significant differences in infection rates between age groups or genders. Compared to ELISA, semi-nested RT-PCR demonstrated higher effectiveness and accuracy in SAT-2 serotype detection. This study represents the first comprehensive analysis of FMD serotype SAT2 in Iraqi *buffaloes* and provides crucial insights into the epidemiology and molecular characteristics of this newly emerged strain in the region. Based on our findings, we recommend that surveillance and control efforts be intensified and specifically targeted towards the SAT2 serotype. Existing vaccination strategies should be evaluated for their effectiveness against this new strain, and novel vaccine formulations may need to be developed to ensure adequate protection for Iraqi *buffalo* populations.

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NOVELTY STATEMENT

The study's novelty is its focus on diagnosing the SAT 2 strain of FMD recently introduced to Iraq and its effect on

the Iraqi *buffalo* using different field and laboratory methods. It is considered the first study in Iraq to study this strain in detail. We have registered the first Iraqi SAT 2 strain in the International Gene Bank.

AUTHORS' CONTRIBUTIONS

Both of these authors contributed equally.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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