



## Clinical and Molecular Detection of Foot and Mouth Disease in Buffalo, Iraq



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### Abstract

**F**OOT-AND-MOUTH disease is a transboundary, highly contagious viral disease. The A, Asia 1, and O are the main serotypes reported in Iraq over the last 60 years, however, a recent outbreak revealed a different phenomenon. So, this study aims to detect the serotype(s) of FMDV that caused the most recent disease outbreak in buffaloes in Wassit and Dhi-Qar, Iraq. This will be done by using both clinical and molecular methods, and the confirmed serotype will come from phylogenetic analysis. In total, 70 buffaloes of different genders and ages were selected for the current study, performed during August and September 2023. All animals in this study were examined clinically to determine the suspected infected animals and then subjected to the collection of epithelial tissue samples. Clinically, the infected buffaloes showed different signs, such as fever, reduced appetite, ulcers in the mouth and foot, and impaired mobility. This study used (Semi-Nested RT-PCR) to detect the SAT2 serotype in buffaloes. The findings indicated that 61 out of 70 local isolates (87.14 percent) tested positive for SAT2. These isolates were subsequently submitted to GenBank and assigned accession numbers ranging from (OR576814.1 - OR576820.1). As the first recorded Iraqi SAT2 isolate. The Iraqi isolates were clustered in the same branch as the Egyptian isolate (MZ097480.1). This study is the first to implement the Semi-nested RT-PCR technique for identifying the SAT2 serotype in buffaloes in Iraq. The findings of this study have the potential to provide valuable insights into the formulation and implementation of FMD protection programmers in Iraq.

**Keywords:** Buffaloes, Semi Nested RT-PCR, SAT2.

### Introduction

A historical account of an epidemic of cattle in Italy in 1546 exists through the writings of Italian physician and scholar Girolamo Fracastoro. His detailed account of the disease's symptoms and transmission strongly suggests that he was observing FMD [1]. Later, the first animal disease to be recognized as a viral infection was FMD, affecting cloven-hoofed animals like cows and pigs. Though the disease itself was observed and documented centuries before, it wasn't until 1897 that scientists identified the cause as a filterable agent, later classified as a virus. This marked a significant milestone in understanding the role of viruses in animal diseases [2]. In the picornavirus family, FMDV is found in seven different strains, called A, C, O, Asia 1, SAT 1, SAT 2, and SAT 3 [3-5]. Each strain has unique immune characteristics. Surface FMDV exhibits a spherical shape, typically characterised by a smooth outer surface [6]. The

FMDV capsid contains 60 copies each of the four viral polypeptides [7,8], namely VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A) [9], which are arranged into classes of increasing complexity [10]. Sequencing the VP1 gene is considered the primary method for the genetic characterization of FMDV. This process involves analyzing the genetic sequence of the VP1 gene to understand viral evolution, disease outbreaks, and the relationship among different virus strain. The VP1 gene sequence analysis is essential for molecular epidemiological studies and is frequently used to identify genetic connections between FMDV isolates, estimate genetic variation, and create topotypes that are connected both geographically and genetically [11]. The VP1 gene is highly variable and is widely used for genotyping due to its serotype specificity and its importance in host cell binding [12]. Therefore, The VP1 gene sequencing protocol is the standard for genetic characterization of FMDV [13]. Scientists analyses genetic material to understand how different

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individuals within a population or species are related and how they vary from one another. They can further classify each serotype by identifying the specific proteins that make up the virus's outer shell. Tracking the origins of novel strains, genotypes, and strains is crucial in order to effectively prevent their dissemination.

The unique sequence of the GH loop region on the VP1 protein can be used to identify the source of FMDV outbreaks. Through genomic comparison, researchers can ascertain the origins and relationships between various FMDV strains [14]. This information is crucial for developing effective control and prevention strategies [15]. Cross-protection between serotypes of FMDV is lacking, and protection is only partially or not at all observed between subtypes within the same serotype [16,17]. The animals frequently exhibit fever, ruby salivation, and blister formation on the lips, tongue, mouth, nostril, interdental space, and occasionally the teats, in addition, milk production declines [18,19]. The present study aimed to investigate FMDV in buffaloes in Wassit and Dhi-Qar provinces, Due to suspicions about the entry of a new serotype into Iraq, which is the Sat2 serotype, and confirming the causative agent of FMDV by using the Semi-Nested PCR technology, and genetic analysis, causal genetic sequencing.

## Material and Methods

### Ethical approvals

This study has been accepted by the Scientific Committee of the College of Veterinary Medicine at Al-Qadisiyah University in Iraq.

### Collection of samples

In this study, 70 water buffalo (*Bubalus bubalis*) suspected of FMD were used as subjects for specimen collection. The buffaloes underwent a

clinical examination using the methodology outlined by [20]. Animals exhibit characteristic clinical manifestations. The samples were collected from skin lesions on the mouth and muzzle, and the samples were stored in a transit medium that consisted of Trizol [21]. The samples were appropriately labelled and then transported, under refrigeration, to the laboratory for subsequent processing.

### 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.

### cDNA synthesis

The RNA samples were used in the cDNA synthesis process, specifically targeting mRNA transcripts. This was achieved by employing the HiSenScript™ RH (-) RT PreMixKit, following the directions provided by the manufacturer.

### PCR Primers

The PCR primers used for direct detection and genotyping of FMD were designed according to [22]. The design of Semi-Nested PCR primers was conducted in this study using the NCBI Database and primer3 plus. These primers were then produced. were provided by Macrogen. Company, Korea, as shown in the following Table 1.

### Polymerase Chain Reaction (PCR)

PCR thermocycler settings were established using the optimize protocol and in accordance with the following Table (2) for primer annealing temperature

TABLE 1. The RT-PCR primers:

Primer	Sequence	PCR amplicon	Location
Universal FMD	F CCAGTCCCCTTCTCAGATC	328	VP1
	R GCCTGGTCTTTCCAGGTCT		
O genotype	F AGCTTGTACCAGGGTTTGGC	402	VP1
	R GCTGCCTACCTCCTTCAA		
A genotype	F GACATGTCCTCCTGCATCTG	866	VP1
	R TACCAAATTACACACGGGAA		
SAT2 genotype	F ACAGCGGCCATGCACGACAG	715	VP1
	R CCACATACTACTTTTGTGACCTGG		

**TABLE 2. RT- Polymerase Chain Reaction (RT-PCR)**

Step	Condition		Cycle No.
	Temp. °C	Time	
Pre denaturation	95°C	5 (minute)	1
Denaturation	95°C	5 seconds	30
Annealing	57°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 (minute)	1

**Semi Nested RT-PCR:**

After the results of the polymerase chain reaction appeared positive for the strain SAT2. The RT-PCR product was further amplified by Semi-Nested PCR, using SAT2 primer (F: ACAGCGGCCATGCACGACAG)

(R, GACTGGCTTGTCGACGGTTA) at size product (506 bp) were designed in this study using NCBI Data base and primer 3plus

**TABLE 3. Semi -Nested PCR thermal cycling conditions**

Step	Condition		Cycle No.
	Temp. °C	Time	
Pre denaturation	95°C	5 (minute)	1
Denaturation	95°C	5 seconds	30
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 (minute)	1

**Results****Clinical Results**

In our study, we observed clinical signs suggestive of FMD, such as fever, dullness, and loss of appetite. Animals that were drooling and lame were confined so that a thorough examination could be conducted and samples could be collected. We observed saliva hanging in long ropy strings up to the ground (Fig. 1-A). Erosions, ulcers on the nostrils and muzzle (Fig. 1-B) The mouths of the water buffalo suggestive lesions of FMD. The gums, dental pad, hard palate, tongue, and interior of the oral cavity contained ruptured vesicles, erosions, or ulcers (Fig. 1-C), and skin lesions develop in the coronary region and interdigital cavity of the hoof (Fig. 1-D).

**Semi-Nested RT-PCR result**

The result of Semi-nested RT-PCR amplification of VP1 of sat2 of FMD at product size 506 pb showed that there were 61 out of 70 (87.14%) positive samples (Fig. 2).

**Phylogenetic tree DNA Sequence results**

Semi-nested PCR end products were sent to Bioneer, Korea, for sequencing using a specific

sequencing system. To analyses the genetic relationship between the viral protein 1 (VP1) gene of FMDV SAT2 and other country-related isolates identified by NCBI-Blast, DNA sequencing was utilized. By looking at the phylogenetic tree, it is clear that we calculated evolutionary distances using the maximum complex likelihood approach in MEGA 6.0. At total genetic changes ranging from 0.08% to 0.02%, the FMDV *Buffalo* No.1–No.7 isolates exhibited close genetic relatedness to the NCBI-BLAST FMDV-type SAT 2 Egypt isolates (MZ097480.1). The homology sequence identity between local FMDV SAT2 No.1–No.7 isolates and NCBI-BLAST-related Egypt, Cameroon, Libya, Ghana, Saudi Arabia, Ethiopia, and Mauritania isolates ranged from (95.77–96.10), (93.10–93.25), (92.57–92.73), (87.53–88.02), (85.71–85.97), (84.47–84.68), and (84.70–84.94), respectively. The genetic variation (mutation analysis) the viral protein 1 genes in FMDV SAT2 No.1–No.7 isolates and NCBI-GenBank country-related isolates showed single nucleotide polymorphisms at genetic variations ranging from (%15.43-%3.90). Finally, the local FMDV SAT2 No.1- No.7 isolates were submitted to GenBank and recorded as accession numbers (OR576814.1-OR576820.1)

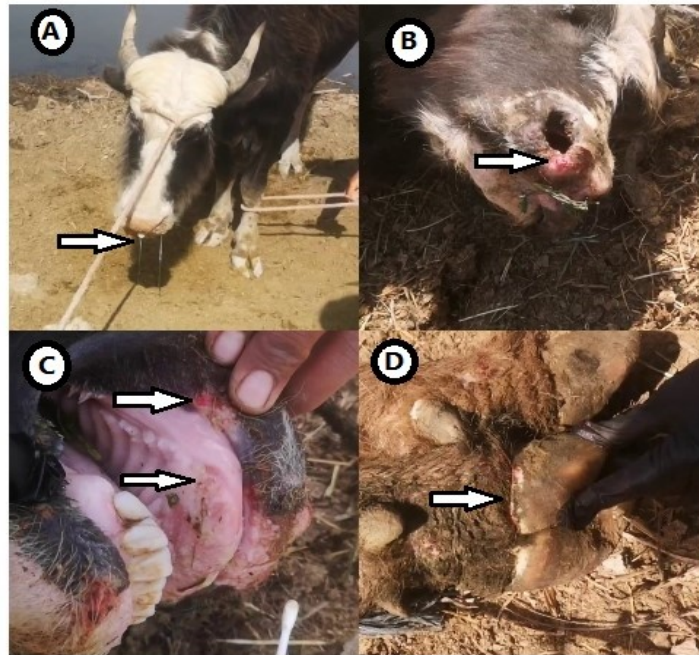


Fig. 1. Shows clinical signs of FMD, A: saliva hanging in long ropy strings, B: nostril lesion C: The gums, dental pad, hard palate, and interior of the oral cavity contained ruptured vesicles, erosions, or ulcers D: lesion around the coronary band.

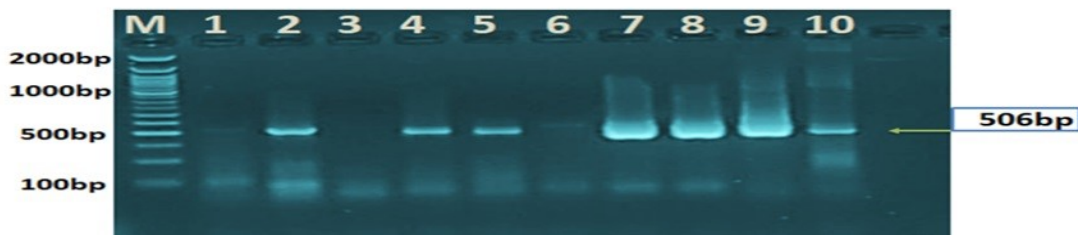


Fig. 2. The image presented here is an agarose gel electrophoresis result illustrating the Semi-Nested PCR product analysis of the viral VP1 gene in *Buffalo* samples of FMD virus SAT 2. Lane 1-10 contained Semi-nested PCR-positive FMD virus SAT 2 genotype amplification samples at a 506 bp PCR product, denoted by the marker M (2000–100 bp).

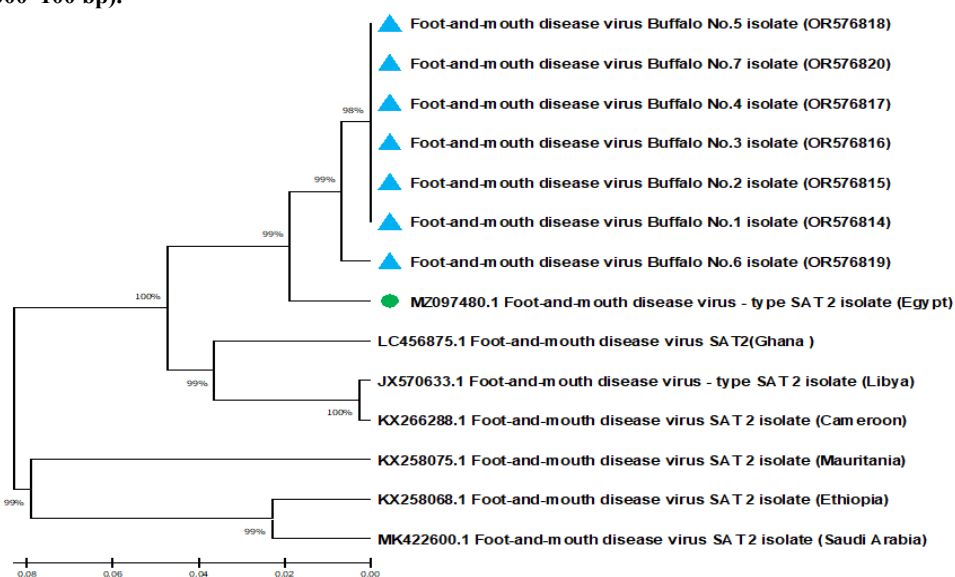


Fig. 3. A phylogenetic tree was employed to analyze the partial sequence of the viral VP1 gene and determine the genetic relationships among the FMDV SAT2 isolates.

## Discussion

The clinical examination of the *buffaloes* in this study revealed the presence of typical signs of FMD, including fever, reduced appetite, ulcers in the mouth and foot, and impaired mobility. These signs are consistent with previous studies and provide further evidence of the presence of FMDV in the studied population [23]. Based on clinical and molecular methods, the serotype was confirmed after phylogenetic analysis. The results revealed that the cause of the FMDV outbreak was serotype SAT2. The Pirbright Institute's epidemiological report on FMD, which said that FMD serotype SAT-2 was brought into Iraq [24], and the WOA's report, which said that serotype SAT2 had spread in Iraq, Jordan, Turkey, and Oman [25], agree with this. The detection rate was 87.14% in the positive samples. It is similar to the infection rates in Egypt and Turkey [26,27]. When used with Semi-nested RT-PCR and the VP1 gene, Semi-nested PCR is hitting the target specifically and quickly. Researchers can depend on it to identify the incriminated strain, especially with viruses such as RNA Viruses, which contains many different strains [28]. This is the first reported use of Semi-nested RT-PCR for identifying the SAT2 serotype in *buffaloes* in Iraq, further highlighting its potential as a valuable diagnostic tool in FMD surveillance and control programs. The phylogenetic analysis of the VP1 sequencing products showed that the Iraqi isolates are clustered in the same branch as the Egyptian isolate (MZ097480.1). This finding suggests a close genetic relationship between the Iraqi and Egyptian isolates and indicates a possible

source or transmission route between the two countries [29]. The genetic homology sequence identity between the local FMDV SAT2 isolates and related isolates from different countries ranged from 84.47% to 96.10%. These results demonstrate a significant level of genetic variability within the SAT2 serotype of FMDV, highlighting the need for continued surveillance and monitoring to track the emergence and spread of different strains. The mutation analysis also found a number of different single nucleotide polymorphisms between the Iraqi isolates and related isolates from other countries. This shows that the SAT2 serotype is genetically variable and has changed over time. The findings of this study have important implications for the formulation and implementation of FMD control measures in Iraq. The identification of the SAT2 serotype in the last outbreak provides valuable insights into the circulating strains of FMDV in the country. This information can be used to inform vaccination strategies and the development of new vaccines that specifically target the prevalent serotypes. It also underscores the importance of maintaining a high level of surveillance and monitoring to detect and respond to any changes in the prevalence and distribution of different FMDV

serotypes. A comprehensive control programme that includes active surveillance, vaccination, strict biosecurity measures, and public awareness campaigns is crucial to effectively controlling and preventing the spread of FMD in Iraq.

## Conclusions

This study identified the SAT2 serotype as the main cause of the recent FMD outbreak in *buffaloes* in Wassit and Dhi-Qar provinces in Iraq. The use of clinical and molecular methods, including Semi-nested RT-PCR and phylogenetic analysis, provided valuable insights into the serotype and genetic relationships of the FMDV SAT2 strains. These findings can contribute to the development of effective control measures for FMD in Iraq and the region. Further studies are needed to investigate the source and transmission dynamics of the FMDV SAT2 strains in order to enhance disease surveillance and prevention efforts.

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## Conflicts of Interest

The authors declare there is no conflict of interest.

## Funding statement

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### الكشف السريري والجزيئي لمرض الحمى القلاعية في الجاموس، العراق

ثائر وداد عناد\* و خليفة علي منصور

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مرض الحمى القلاعية وهو مرض فيروسي شديد العدوى يصيب الحيوانات ذات الظلف المشقوق ويسببه فيروس الحمى القلاعية. تعتبر A و Asia 1 و O هي الأنماط المصلية الرئيسية التي تم الإبلاغ عنها في العراق على مدى السنتين سنة الماضية؛ ومع ذلك، كشف تفشي المرض مؤخرًا عن ظاهرة مختلفة. لذلك، أجريت هذه الدراسة لتحديد النمط المصلي الرئيسي لفيروس الحمى القلاعية في التفشي الأخير للمرض في الجاموس في محافظتين عراقيتين، واسط وذي قار، استناداً إلى الطرق السريرية والجزيئية، مع تأكيد النمط المصلي بعد التحليل التطوري. في المجمل، تم اختيار 70 جاموساً من مختلف الأعمار والجناس للدراسة الحالية، التي أجريت خلال شهري اب ايلول 2023. وتم فحص جميع حيوانات الدراسة سريريًا لتحديد الحيوانات المريضة المشتبه بها ثم إخضاعها لجمع عينات الأنسجة الظهارية. تم استخدام تفاعل (Semi-Nested RT-PCR) بهدف تضخيم منطقة من جين VP1 لمعرفة الانماط المصلية للفيروسات المعزولة، كانت 61 من 70 عينة (87.14%) موجبة ل FMD SAT2. تم تقديم 7 عازلات إلى بنك الجينات وتم تسجيلها بأرقام انضمام (OR576814.1-OR576820.1) كأول عزلة عراقية مسجلة. وأظهر التحليل التطوري لمنتجات تسلسل VP1 أن العازلات العراقية تتجمع في نفس فرع العزلة المصرية. (MZ097480.1). ولأول مرة، تم استخدام تقنية Semi-Nested RT-PCR في هذه الدراسة لتحديد النمط المصلي SAT2 في الجاموس في العراق، وأظهرت نتائج هذه الدراسة قدرتها على تقديم رؤى قيمة في صياغة وتنفيذ تدابير مكافحة مرض الحمى القلاعية المنتجة في العراق.

**الكلمات الدالة:** الجاموس، تفاعل البلمرة المتسلسل شبه المتداخل، عترة سات2.