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Isolation of SAT2 Foot and Mouth Disease Virus in Iraq

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A B S T R A C T

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals, causing significant economic losses in livestock industries. The causative agent, Foot-and-Mouth Disease Virus (FMDV), consists of seven serotypes: O, A, C, South African Territories (SAT) 1, SAT2, SAT3, and Asia1. These serotypes are lack of cross-protection even among variants of the same serotype. This study aimed to isolate and identify the SAT2 serotype from Iraqi cattle, using molecular techniques and cell culture methods. A total of 100 samples, including the tongue epithelial tissue and vesicular fluid, were collected from suspected FMD cases in Diyala, Baghdad, Kirkuk, Babylon, and Salahaddin provinces between July 2023 and October 2024. The RNA was extracted, and real-time quantitative polymerase chain reaction (RT-qPCR) was conducted. Primers targeting the 3D gene were used to detect FMDV, while specific primers targeting the 1D gene were employed for the SAT2 serotype identification. The virus isolation was performed on secondary sheep testis (SST) cell cultures, and then the cytopathic effects (CPE) were reported with calculating the tissue culture infectious dose 50% ($TCID_{50}$) was calculated using the Reed and Muench method. The FMDV was detected in 85 samples (85%), among which 30 (35.3%) were positive for the SAT2 serotype. The virus isolation from five representative samples revealed characteristic CPE, including cell rounding, degeneration, and syncytia formation. The RT-qPCR confirmed presence of the SAT2 serotype. The $TCID_{50}$ ranged from $Log_{10}^{6.8}$ $TCID_{50}$/mL to $Log_{10}^{7.05}$ $TCID_{50}$/mL. The present study successfully isolated the SAT2 serotype of the FMDV in Iraq for the first time. It underscores the importance of molecular diagnostics and virus isolation in surveillance and highlights the need for targeted vaccination strategies against SAT2.

Keywords: foot-and-mouth disease virus, SAT2 serotype, molecular detection, virus isolation, secondary sheep testis, Iraq

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals, including cattle, buffalo, sheep, goats, and pigs. It is caused by the Foot-and-Mouth Disease Virus (FMDV), which is classified within the genus Aphthovirus of the family Picornaviridae (1). The virus has a single-stranded RNA genome with linear and positive-sense features. It is categorized into seven serotypes: O, A, C, Southern African Territories (SAT) 1, SAT2, SAT3, and Asia1. A key challenge in controlling FMD is the lack of cross-protection among serotypes and the limited protection between subtypes of the same serotype, necessitating serotype-specific vaccines for effective immunity (2, 3). Clinically, FMD is characterized by fever, vesicular lesions in the oral cavity, interdigital spaces, coronary bands, and mammary glands, along with high mortality in young animals due to myocarditis, commonly referred to as tiger heart (4).

FMD poses a significant threat to livestock production globally, with severe economic repercussions due to reduced productivity, trade restrictions, and costly control

measures. In Iraq, livestock farming, particularly cattle and sheep rearing, is a vital component of the agricultural economy (5, 6). Historical records reveal frequent FMD outbreaks, with serotypes O, A, and Asia1 being the most commonly reported. Particularly, between 1998 and 2000, outbreaks among Holstein cattle were attributed to serotype O (7), while an outbreak in 2009 identified a subtype related to Turkish FMD isolates (8).

Recent reports indicate the emergence of the SAT2 serotype in Iraq, a strain traditionally confined to sub-Saharan Africa (9). SAT2 has been implicated in outbreaks in neighboring regions, including Egypt, Turkey, Jordan and other parts of the Middle East raising concerns about its spread to new areas (9). Despite ongoing vaccination programs in Iraq, cases of FMD in vaccinated animals have been documented. These failures are attributed to antigenic mismatches between circulating strains and the vaccines in use, as well as inadequate vaccination practices (10, 11). These developments highlight the increasing transboundary nature of FMD outbreaks and the challenges they pose for disease control (12). FMD cases in Iraq have primarily been reported during the months of April and May, with endemic serotypes O, A, Asia-1, and SAT2/XIV confirmed (13).

Two recent studies provide insights into the emergence of SAT2 in Iraq. Al-Kalabadi and Al-Thwani (14) confirmed the molecular detection of SAT2 in cattle and buffalo, highlighting its spread but did not perform virus isolation, which is critical for comprehensive characterization. Similarly, Enad and Mansour (15) reported the molecular detection of SAT2 in buffaloes, identifying a genetic relationship with Egyptian strains. However, their study also did not include virus isolation. The absence of virus isolation in these studies limits the ability to fully characterize the strain and assess its potential impact on vaccination efficacy and regional epidemiology.

The emergence of SAT2 presents significant diagnostic challenges, as Iraqi laboratories have limited capacity to differentiate endemic FMDV serotypes from emerging strains (16). Virus isolation, the gold standard for virus detection (17), is essential for understanding strain characteristics and developing targeted vaccines.

This study aimed to address these gaps by isolating the SAT2 serotype of FMDV from the Iraqi cattle on sheep testis for the first time and confirming the findings using molecular techniques. By combining virus isolation and RT-qPCR, this research provides critical insights into the epidemiology of SAT2 in Iraq, contributing to improved diagnostic and control measures.

MATERIALS AND METHODS

Ethical Approval

The experimental design and protocols used in this study were reviewed and approved by the local Animal Care and Use Committee of the College of Veterinary Medicine, University of Baghdad, Iraq, under Animal Utilization Protocol Certificate P.G./1322, dated July 11, 2024.

Animals and Sample Collection

Dairy cows showing clinical symptoms suggestive of FMD were identified. All sampled cows had been previously vaccinated with a trivalent FMD vaccine (JSC, Russia, containing A/Iran 05, O/pan-Asis2, and Asia1 serotypes). Representative samples were collected from five provinces in Iraq: Babylon (10 tongue tissue samples and 1 vesicular fluid sample), Baghdad (15 tongue tissue samples and 1 vesicular fluid sample), Diyala (35 tongue tissue samples and 3 vesicular fluid samples), Kirkuk (25 tongue tissue samples and 3 vesicular fluid samples), and Salahaddin (15 tongue tissue samples and 2 vesicular fluid samples). A total of 100 samples, including 90 tongue tissue samples and 10 vesicular fluid samples, were collected for the study.

Sample Preparation

All samples were collected and processed following the World Organization for Animal Health (OIE) and Bonbon guidelines (18, 19). Epithelial tissues were collected from unruptured or recently ruptured vesicles and placed in virus transport medium (phosphate-buffered saline (PBS) with 50% glycerol) supplemented with antibiotics (penicillin and streptomycin, Lonza, Switzerland). Vesicular fluid samples were aseptically aspirated using sterile needles (BD PrecisionGlide, USA) and stored in sterile containers. Samples were transported in an ice box until reaching the laboratory (Alnahda Veterinary Laboratories and Research Department, Veterinary Director, Iraqi Agricultural Ministry).

Tissue samples were homogenized using sterile sand with mortar and pestle (20). They were centrifuged at 3,000 rpm for 10 minutes at 4°C, and the supernatant was filtered through a 0.2 µm syringe filter (CHMLAB Group, China). The filtered solution was incubated at room temperature for three hours with 1% antibiotic solution (as mentioned above) and stored at -80°C in a deep freezer (Thermo Fisher, USA) until further analysis.

RNA Extraction and Quantification

Total RNA was extracted from the prepared samples using the Total RNApure Extraction Reagent (Catalog ZP401, Beijing Zomanbio Biotechnology Co., China) according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher, USA).

Primers

The primers were supplied by the Alnahda Veterinary Laboratories and Research Department, Veterinary Director, Iraqi Agricultural Ministry. These primers were designed by Pirbright institute (UK) using Primer3 Plus software and synthesized by Alpha ADN, S.E.N.C. (Montreal, Quebec, Canada).

The two sets of primers targeting conserved and variable sequences of the 3D and 1D genes, respectively, were used in this study. Primers and a probe specific to the 3D gene were used to detect the FMDV, while primers and probe specific to 1D gene were applied to detect the SAT2 serotype of the same virus, as mentioned in Table 1.

Table 1. Primer and probe sequences specific for the 3D and 1D genes used in this study

Gene		Sequence (5'-3')
3D	Forward	5'-CACYTYAAGRTGACAYTGRTACTGGTAC-3'
	Reverse	5'-CAGATYCCRAGTGWCICITGTTA -3'
	Probe	5'-CCTCGGGGTACCTGAAGGGCATCC-3'
1D	Forward	5'-CCACACCAACAAGACCACCT-3'
	Reverse 1	5'-RATCTCCAGGTCACAGAAGT -3'
	Reverse 2	5'- GCRATCTCCACCTCACAGAAGTAG-3'
	Probe	5'-ACCTCATGGACACRAAGGAGAAGGC-3'

RT-qPCR

All samples were tested for the presence of the 3D gene, and then, the positive samples were subjected to the primers specific to the 1D gene of the SAT2 serotype. The FMDV field isolates were detected using the one-step real time RT-PCR according to the manufacturer's instructions. The reactions were conducted using a one-step Reverse Transcriptase Enzyme Kit (write the manufacturer, origin or state) and RT-2× PCR Master Mix (abm, Canada). Each 20 µL reaction contained 4 µL RNA template (200 ng/µL), 10 µL RT-PCR Probe Master Mix (1×), 0.4 µL RT-PCR Enzyme Mix (1×), 1 µL of each forward and reversed primers (50 picomoles), and 3.6 µL nuclease-free DEPC-treated water.

The amplification program included cDNA synthesis at 45°C for 45 minutes, heat inactivation at 85°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds and extension at 60°C for 60 seconds. Samples with Ct values <40 were considered positive. Positive samples were categorized by Ct values, where lower Ct values indicated higher viral loads.

Preparation Cell Culture

The FMDV was isolated from the samples positive for the RT-qPCR assay according to (21) and (22) by using primary and secondary sheep testis cell culture. The primary sheep testis (PST) cell culture was prepared from four clinically healthy lambs (3–6 months old). The testes were surgically removed, minced into small pieces, washed thoroughly with PBS, and enzymatically digested with 0.25% trypsin (Gibco, Invitrogen Co., UK). The digested tissue was cultured in Minimum Essential Medium (MEM, Sigma-Aldrich, USA) supplemented with fetal calf serum (Gibco, Life Technologies, USA) into 75 cm^2 disposable flasks (Corning, USA) and maintained at 37°C in a CO2 incubator.

For preparing the secondary sheep testis (SST) cell culture, confluent primary monolayer cultures were trypsinized using a Trypsin-Versin solution (0.05% Trypsin-0.025% Versin, Gibco, Invitrogen Co., UK) and subcultured into 25 cm^2 disposable flasks (Corning, USA) containing 10 mL MEM (21, 22).

Five SAT2-positive samples with high viral loads were inoculated onto confluent SST monolayers (0.5 mL sample/flask). Control flasks were inoculated with maintenance medium only. The inoculated flasks were incubated at 37°C for 1 hour to allow virus adsorption. The cells were washed three times with maintenance medium and then incubated with 10 mL fresh maintenance medium at 37°C. Cultures were observed daily under an inverted microscope (Leica, Germany) for cytopathic effects (CPE). Infected cultures were harvested after 60 hours post-infection (P.I.) by freeze-thawing, and the supernatant was used for subsequent passages. Each sample was passaged four times before being considered negative (1).

Confirmation by RT-qPCR

The RNA extracted from infected tissue cultures was subjected to the RT-qPCR assay targeting the same genes mentioned above. This was performed to confirm the FMDV presence, following the same procedure used for the field isolates.

Virus Titration

The harvested FMDV was 10-fold serially diluted in infection media (MEM supplemented with 2% fetal calf serum). Confluent monolayers in 96-well plates were inoculated with 100 µL of each virus dilution and incubated at 37°C in a CO2 incubator for 24 hours. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed and Muench (23) method

$$Proportionate\ distance\ (PD) = \frac{\%positive\ at\ or\ above\ 50\% - 50}{\%\ positive\ at\ or\ above\ 50\% - \%positive\ below\ 50\%}$$

$$TCID50\ (per\ unit\ volume) = log\ dilution\ above\ 50\% + (PD \times log\ dilution\ factor)$$

Results were expressed as $\log_{10}$ $TCID_{50}$/mL to quantify the concentration of infectious virus particles.

Statistical Analysis

Chi-square (χ^2) tests were used to analyze differences in gene detection among the regions (Babylon, Baghdad, Diyala, Kirkuk, and Salahaddin). Statistical significance was set at $P < 0.05$. Analyses were conducted using JMP Pro 16.0.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

The RNA concentration of the samples ranged between 40 and 100 ng/µL, with purity (A260/A280 ratio) between 1.8 and 2.0, indicating high-quality RNA suitable for downstream applications.

The results of the RT-qPCR assay were evaluated based on Ct values. Out of 100 samples tested using pan-FMD primers and probes targeting the 3D gene, 85 samples (85.0%) were positive for the FMDV (Figure 1A). Subsequently, among the 85 positive samples, 30 samples only (35.3%) were positive for the SAT2 serotype (Ct < 40.0) (Figure 1B).

To confirm the presence of the virus in the SST cell cultures, the RT-qPCR assay confirmed the detection of both the 3D and 1D genes in these samples (Figure 1C).

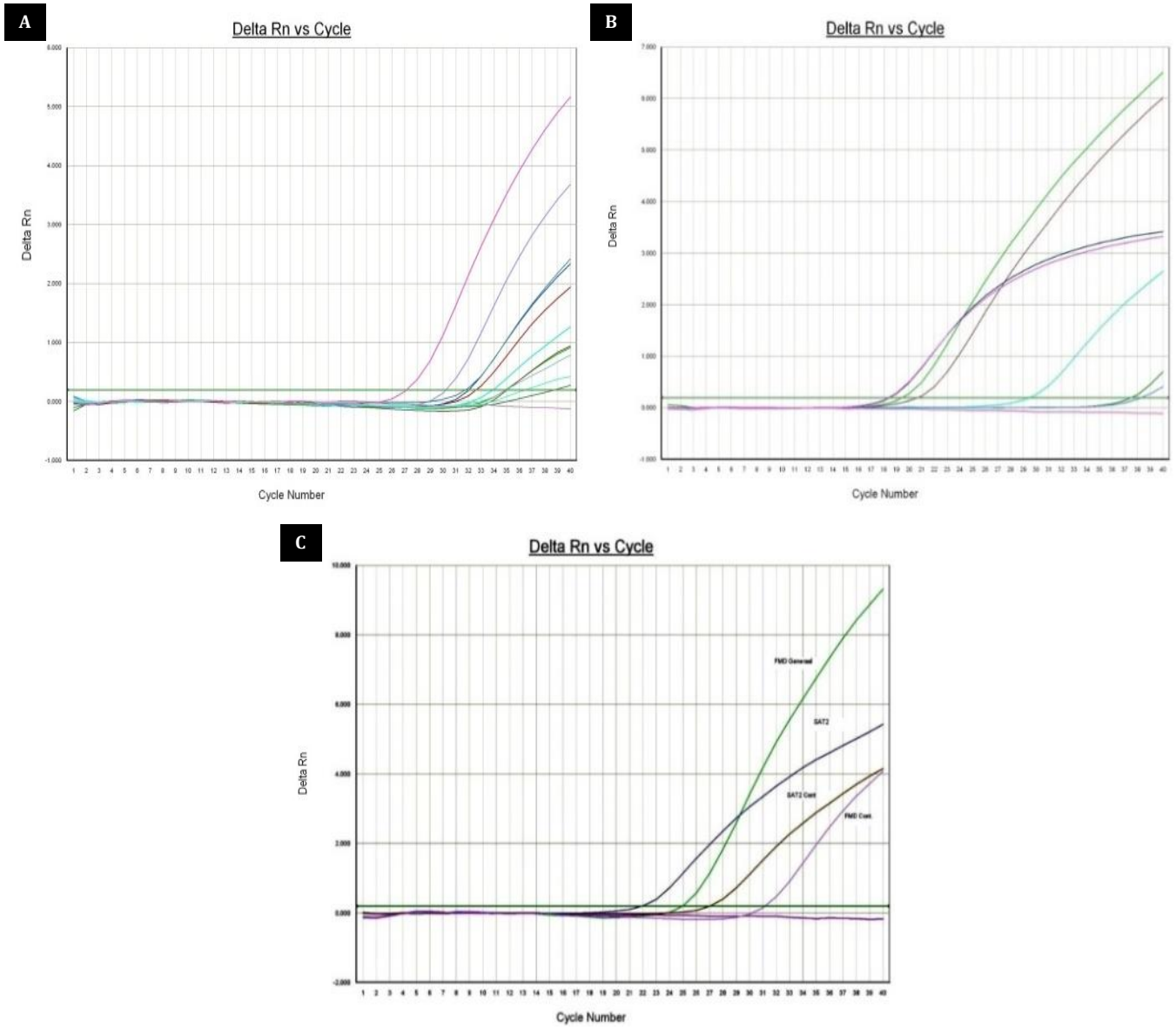

Figure 1. Amplification plots from RT-qPCR showing the detection of FMDV-specific genes. **(A)** Amplification curve for the 3D gene from prepared clinical samples, indicating general FMDV positivity. **(B)** Amplification curve for the 1D gene from prepared clinical samples, confirming the presence of the SAT2 serotype. **(C)** Amplification curves for both the 1D and 3D genes from tissue culture samples, demonstrating successful virus isolation and propagation, with 1D confirming SAT2 specificity and 3D indicating general FMDV detection

Overall Prevalence Analysis

The prevalence of the 3D gene (Table 2) across all samples was 85.0% (95% CI: 76.7%–90.7%), while the prevalence of the 1D gene was 35.3% (95% CI: 25.9%–45.9%). A chi-square analysis confirmed a statistically significant difference between the detection rates of the 3D and 1D genes (χ^2 = 48.26, P < 0.0001). This indicates that the SAT2 serotype (1D gene) was less frequently detected compared to the general FMDV marker (3D gene) across all regions and tissue types.

Virus Isolation

The five FMDV samples that had the highest DNA levels in the RT-qPCR assay exhibited morphological changes characteristic of FMDV CPE on SST cells, including rapid sloughing of monolayer cells, cell rounding, and swelling. These changes became evident within 48 hours post-inoculation (Figure 2A, B).

Over time, sloughing increased, with the detachment of the monolayer from the cell-culture flask and the formation of syncytia. By 72 hours post-inoculation, widespread cell death was observed, confirming the presence of FMDV (Figure 2C, D). Control cultures did not exhibit any morphological changes and retained their normal shape (Figure 2E, F).

Titration of Isolated Virus

The biological titer of the harvested FMDV SAT2 serotype was determined by observing CPEs in 50% of

infected wells as the endpoint. The results ranged from $Log_{10}^{6.8}$ $TCID_{50}$/mL to $Log_{10}^{7.05}$ $TCID_{50}$/mL.

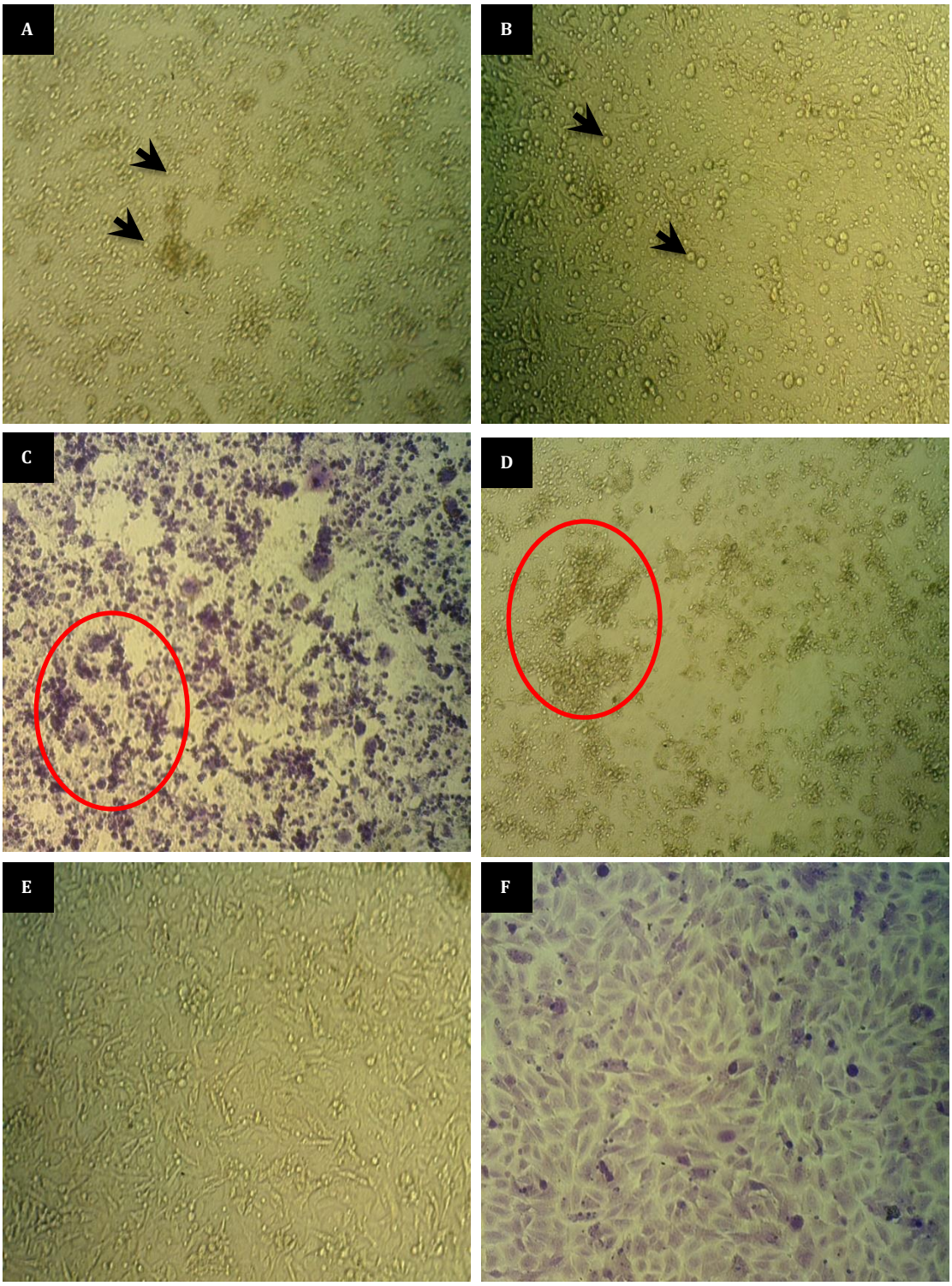

Figure 2. The Morphological characterization of secondary sheep testis (SST) cell culture under light microscopy (100×). **A, B:** 48 h incubation showing round, swelling, cells singly in shape and detachment of SST cell culture (black arrows). **C, D:** 72 h incubation showing severe damage with clamping, large syncytia, and complete detachment of SST cell culture (red circles). **E, F:** Control SST cell culture (normal cells)

DISCUSSION

The FMD remains a significant global threat to livestock due to its high transmissibility and severe economic repercussions. This study represents the first documented isolation on cell culture of the SAT 2 serotype of FMDV in Iraqi cattle, highlighting a critical development in the epidemiological studies of FMD in the region.

The presence of the FMDV SAT2-specific 1D gene in 35.3% of the samples, alongside the pan-FMDV 3D gene in 85%, underscores the growing presence of the SAT2 serotype in Iraq. These findings are consistent with recent studies in the region, including the study of (15), who reported the same serotype in buffaloes with a significantly higher prevalence of 84.31%, using semi-nested RT-PCR and ELISA in the provinces of Wassit and Dhi-Qar. The variation in prevalence could be attributed to differences in species susceptibility, regional outbreaks, or sampling methods.

The detection of SAT2 in this study marks a variation from earlier studies in Iraq, where the serotypes O, A, and Asia1 were predominantly reported (24-26). The aforementioned researchers identified these serotypes using virus isolation and serological methods across various regions in Iraq, but did not report SAT2. This shift suggests a recent introduction or emergence of SAT2, possibly linked to transboundary animal movements or trade, as suggested by (15), who identified genetic similarities between Iraqi SAT2 isolates and Egyptian strains. This transboundary nature of SAT2 underscores the importance of regional collaboration in surveillance and control efforts.

The methodology of this study employed RT-qPCR for gene detection, which offers high sensitivity and specificity. The use of Ct values provided quantitative insights into viral load, differentiating high-load samples for successful virus isolation. This approach contrasts with semi-nested reverse transcription (RT)-PCR, which focuses on genetic characterization of SAT2-specific sequences (15). While both methods are robust, RT-qPCR's quantitative capacity adds an important layer of diagnostic detail, allowing for targeted isolation efforts. The use of SST cell culture in this study demonstrated consistent CPE, such as cell rounding, detachment, and syncytia formation, validating its utility as a reliable system for FMDV propagation. This aligns with the findings of another study (24), which documented similar CPE during virus isolation in various primary cell cultures. Similarly, successful virus isolation was reported in bovine-derived cultures with similar CPE in different fetal cell cultures, but those authors did not use SST cells in their study (25). This comparison underscores the versatility of SST cells in supporting a wide range of FMDV serotypes, including the less common SAT2.

The geographical coverage of this study across five provinces (Babylon, Baghdad, Diyala, Kirkuk, and Salahaddin) complements findings from (15), who focused on southern provinces. Together, these studies highlight the widespread presence of SAT2 in Iraq, emphasizing its adaptability to diverse ecological zones. The findings call for comprehensive national surveillance to map the serotype's full extent and its impact on livestock populations.

The emergence of SAT2 raises critical concerns regarding the effectiveness of existing vaccination programs. The trivalent vaccines commonly used in Iraq target serotypes O, A, and Asia1 but do not provide cross-protection against SAT serotypes. This gap in vaccine coverage could exacerbate the risk of outbreaks. Similar concerns were raised by (14), who emphasized the genetic diversity of circulating FMDV strains and the need for updated vaccine formulations. Future vaccination strategies should incorporate SAT2 antigens to address this emerging threat effectively.

While SST cells provided robust results in this study, their use requires technical expertise and may not be readily available in all laboratories. This contrasts with the more commonly used BHK-21 cell line, which offers easier handling but may not replicate all serotypes equally well. For example, SAT2 isolates were noticed to be propagated well in BHK-21 cells but exhibited slightly different CPE characteristics compared to primary testis cells (27). These differences highlight the importance of selecting the appropriate cell culture system based on the research objective and the specific FMDV serotype under investigation.

The $TCID_{50}$ results also provide valuable insights into the infectivity and replication potential of the isolated SAT 2 viruses. The high viral titers observed in this study suggest that the SAT 2 serotype is well-adapted to the host cells used, which may reflect its potential for rapid spread in natural infections. This aligns with findings from (24), who reported similarly high infectivity for serotypes O, A, and Asia 1 in sheep and bovine testis cultures. The choice of SST cells in this study also highlights their potential as a diagnostic tool for FMDV isolation, particularly in regions where primary and specialized cell lines are unavailable. As observed in this study and by (24), the characteristic and rapid CPEs in SST cells make them a reliable indicator of FMDV presence. These findings emphasize the need to preserve and optimize such systems for future virological research, particularly for emerging serotypes like SAT2.

In conclusion, this study provides the first isolation of the SAT 2 serotype from the Iraqi cattle on SST cells followed by the molecular confirmation, marking a critical advancement in understanding the epidemiology of FMD in the region. The findings underscore the importance of continuous surveillance, regional collaboration, and updated vaccination programs to mitigate the risks posed by this emerging serotype. Future research should focus on genetic characterization, vaccine efficacy, and transboundary risk assessment to develop comprehensive strategies for controlling FMD.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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عزل النمط المصلي الافريقي (SAT2) لفايروس الحمى القلاعية في العراق

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الخلاصة

مرض الحمى القلاعية هو مرض فيروسي شديد العدوى يصيب الحيوانات ذات الحوافر المشقوقة، مما يتسبب في خسائر اقتصادية كبيرة في صناعة الثروة الحيوانية. العامل المسبب، فيروس الحمى القلاعية (FMDV)، يتكون من سبعة أنماط A، O، C، النمط الجنوب أفريقي (SAT) 1- SAT 2، SAT 3، وكذلك النمط الاسيوي Asia 1، والتي تفتقر إلى الحماية المتصالبة حتى بين المتغيرات من نفس النمط. هدفت هذه الدراسة إلى عزل وتأكيد النمط SAT2، وهو سلالة جديدة متداولة في الماشية العراقية، باستخدام تقنيات جزيئية وطريقة زراعة الخلايا. تم جمع ما مجموعه ١٠٠ عينة، بما في ذلك الأنسجة الظهارية من آفات اللسان والسائل الحويصلي، من حالات مشتبه بإصابتها بحمى القلاع في محافظات ديالى وبغداد وكركوك وبابل وصلاح الدين بين يوليو ٢٠٢٣ وأكتوبر ٢٠٢٤. تم استخراج الحمض النووي الريبي (RNA)، وأجريت تفاعل البلمرة المتسلسل للنسخ العكسي في الوقت الحقيقي (RT-qPCR). تم استخدام بادئات عامة تستهدف الجين(D٣) للكشف عن فايروس الحمى القلاعية، بينما تم استخدام بادئات محددة تستهدف الجين (D١) لتحديد النمط (SAT2). تم إجراء عزل الفيروس على خلايا خصية الاغنام الثانوية (SST)، وتم ملاحظة التأثيرات المرضية. (CPE) تم حساب جرعة العدوى في الأنسجة المزروعة

٥٠٪ (TCID50) باستخدام طريقة Reed and Muench تم الكشف عن فايروس الحمى القلاعية في ٨٥ عينة (٨٥٪) باستخدام بادئات (D٣) العامة، و٣٠ عينة (٣٥,٣٪) اختبرت إيجابية لصنف SAT2 باستخدام بادئات (D١) المحددة. كشف عزل الفيروس من خمس عينات ممثلة التي أظهرت تأثيرات مرضية مميزة، بما في ذلك تدور وانتفاخ الخلية، والانحلال، وتكوين الخلايا متعددة الأنوية. أكد تفاعل البلمرة المتسلسل للنسخ العكسي(RT-qPCR) وجود النمط SAT2 في الخلايا التي عزل عليها الفايروس. تراوحت TCID50 من $Log_{10}^{7.05}$ $TCID_{50}$ للمل الواحد الى $Log_{10}^{6.8}$ $TCID_{50}$. نجحت هذه الدراسة في عزل النمط SAT2 من فايروس الحمى القلاعية في العراق لأول مرة. تؤكد هذه الدراسة على أهمية التشخيص الجزيئي وعزل الفيروس في المراقبة وتسلط الضوء على الحاجة إلى استراتيجيات تستهدف اللقاحات ضد النمط الافريقي SAT2.

الكلمات المفاحية: فايروس الحمى القلاعية، النمط المصلي الافريقي٢، التحديد الجزيئي، عزل الفايروس، خلايا خصية الاغنام الثانوية، العراق