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Molecular study of *Streptococcus equi* isolated from horses with strangles in Iraq

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ABSTRACT

Background: Strangles is a highly contagious equine respiratory disease caused by *Streptococcus equi subsp. equi*. It is a globally significant pathogen and one of the most common infectious agents in horses. In Iraq, no sequencing data on this pathogen are available, and only two molecular studies have been published to date. This study provides preliminary insights into strain diversity and provides a foundation for future large-scale investigations.

Aim: This study aimed to investigate the molecular characteristics, identify *SeM* gene alleles, and perform a phylogenetic analysis of *S. equi* isolates from horses in Baghdad, Iraq.

Methods: We analyzed 59 *Streptococcus spp*. isolates previously obtained from horses clinical samples. Conventional PCR (Polymerase Chain Reaction) targeting the *SeM* gene was used to identify *S. equi*. Additionally, nine PCR-positive *SeM* gene products were sequenced, followed by phylogenetic analysis and allele identification.

Results: We confirmed 49 isolates as *S. equi* from the 59 isolates according to the molecular assay. Additionally, nine PCR products were used for sequencing and allele typing of the *SeM* gene, which provided the initial report of *SeM*-97 allele identification in Iraq. Phylogenetic analysis along with *SeM* gene typing revealed a close relationship between the Iraqi strains and one Iranian strain with 100% sequence identity, revealing important epidemiological relationships that may indicate regional ties to the strain detected in Iran.

Conclusion: The present study represents the first investigation of *SeM* allele typing in Iraq, identifying the *SeM*-97 allele of *S. equi* along with its unique amino acid variations. The findings highlight genetic similarities between Iraqi isolates and a strain from Iran, suggesting the potential regional dissemination of *S. equi*.

Keywords: Strangles, *Streptococcus equi subsp. equi,* PCR, *SeM* gene, *SeM*-97.

Introduction

Strangles is a highly infectious and contagious bacterial infection of the upper respiratory system of horses and other equids of all ages and breeds, with high morbidity and low mortality in susceptible populations (Jaramillo-Morales *et al.*, 2023). It is characterized by abrupt pyrexia followed by pharyngitis and subsequent abscess formation in the submandibular and retropharyngeal lymph nodes, and typically, young horses are frequently affected (Sweeney *et al.*, 2005; Boyle, 2016; Arafa *et al.*, 2021; Bekele *et al.*, 2024).

The causative organism, *Streptococcus equi subsp. equi* (*S. equi*), is a member of Lancefield Group C. It is a gram-positive, pus-forming bacteria that produces hemolysis on blood agar and is one of the most common infectious agents in horses (Sweeney *et al.*, 1989, 2005; Holden *et al.*, 2009; McGlennon *et al.*, 2021).

Strangles may be misdiagnosed and complicated by other infections, such as pneumonia, guttural pouch empyema, and purpura hemorrhagica (Whelchel and Chaffin, 2009; Arafa *et al.*, 2024).

The organism has several virulence factors shielding it from the host's immune system, such as a hyaluronic acid capsule, superantigens, and M-like proteins *SeM* (Anzai *et al.*, 1999; Artiushin *et al.*, 2002; Proft *et al.*, 2003; Timoney *et al.*, 2014). *SeM* protein is responsible for increased virulence of *S. equi* over its ancestor, *Streptococcus zooepidemicus*, by actively binding to fibrinogen and IgG and inhibiting C3b deposition on the bacterial surface, triggering an antiphagocytic action resembling that of the M proteins of group A streptococci (Boschwitz and Timoney, 1994; Meehan *et al.*, 2000; Boyle *et al.*, 2009; Quinn *et al.*, 2011).

Serological tests, such as *SeM*-based and dual-target Enzyme-Linked Immunosorbent Assays (ELISAs), detect antibodies against *S. equi* and identify recent exposures and complications like purpura hemorrhagica (Boyle *et al.*, 2009; Robinson *et al.*, 2013). However, they cannot confirm active infection or carrier status,

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and cross-reactivity with *S. zooepidemicus* can limit their specificity (Duran and Goehring, 2021; Durham and Kemp-Symonds, 2021).

The culture and biochemical identification of *S. equi* methods is of low sensitivity, whereas the molecular identification of *S. equi* based on the amplification and sequencing of the *SeM* gene has been used previously, with high sensitivity and specificity compared to culture (Newton *et al.*, 1997; Timoney and Artiushin, 1997; Gronbaek *et al.*, 2006).

DNA sequencing of the hypervariable N-terminal region of the *S. equi SeM* gene is a useful tool for strain-typing to determine the source of strangles outbreaks (Kelly *et al.*, 2006; Ivens *et al.*, 2011; Kasuya *et al.*, 2019; Rotinsulu *et al.*, 2023). *Streptococcus equi SeM* allele typing has been well documented in China (Dong *et al.*, 2019; Liu, 2019), Thailand (Tonpitak *et al.*, 2016), New Zealand (Patty and Cursons, 2014), Ireland (Moloney *et al.*, 2013), Brazil (Libardoni *et al.*, 2013), Sweden (Lindahl *et al.*, 2011), and Europe (Kelly *et al.*, 2006).

In Iraq, strangles remain a significant equine health concern, yet studies on their prevalence and molecular characterization are limited. A seroprevalence study in Mosul reported a 12% prevalence of anti-*S. equi* antibodies in horses, with higher rates observed in animals under 3 years old (20%) and those exhibiting respiratory signs (Al-Robaiee *et al.*, 2023). In Baghdad, Mahmood (Mahmood *et al.*, 2014) detected *S. equi* in 30 out of 141 clinical samples from horses with respiratory infections using conventional PCR, whereas Alwan (Alwan, 2014) employed Real-Time PCR targeting the *SeM* gene and identified *S. equi* in 24 out of 30 isolates (80%). Earlier, Jawad (Jawad, 2000) documented clinical cases of strangles in young horses and emphasized the potential of vaccination to control strangles. Similarly, Kaisium (Kaisium and Al-Judi, 2007) demonstrated the efficacy of a live attenuated vaccine in inducing immunity in rabbits.

Despite these efforts, molecular studies on *S. equi* in Iraq remain scarce, with the last investigations conducted nearly a decade ago. Moreover, none of the previous studies examined the genetic diversity of *S. equi* or performed *SeM* gene sequencing, which is critical for strain typing and understanding the epidemiology of strangles.

Strangles in Iraq likely originate from neighboring countries, such as Turkey and Iran, because of the uncontrolled and unsupervised importation of horses. The *S. equi* isolates from Iraq are hypothesized to have close genetic relationships with those from these regions. In addition, the *SeM* gene alleles circulating in Baghdad may exhibit significant variability, reflecting the diverse origins of the imported strains. This study aimed to molecularly identify *S. equi* isolates from horses with strangles in Baghdad, Iraq, sequence the *SeM* gene, identify circulating alleles, and perform phylogenetic comparisons with international strains.

Materials and Methods

Source of Streptococcus isolates

Fifty-nine isolates of *Streptococcus* spp. were obtained from the Department of Internal and Preventive Veterinary Medicine Laboratory, College of Veterinary Medicine, University of Baghdad. These isolates were collected during clinical practice on horses in Baghdad city and were initially identified as *Streptococcus* spp. using bacteriological methods. The identification included culturing on Columbia blood agar (Oxoid, UK) supplemented with 5% sheep blood for 24 hour at 37°C, Gram staining, and finally, suspected colonies purified by sub-culturing again on Columbia blood agar. Subsequently, biochemical identification was performed according to the sugar fermentation abilities of lactose, sorbitol, trehalose, and maltose in addition to the catalase test. These isolates were subsequently used in this study to identify *S. equi* via molecular methods.

DNA extraction

DNA was extracted and purified using Presto™ Minig DNA Bacteria Kit (Geneaid, Taiwan) according to the manufacturer's instructions. The eluted DNA was stored at −20ºC until use.

Polymerase chain reaction

Conventional PCR was used to amplify a 541 bp segment of the N-terminal of the *SeM* gene using the primers *SeM*-Forward ASW73 (5′-CAGAAAACTAAGTGCCGGTG-′3) and *SeM*-Reverse ASW74 (5′-ATTCGGTAAGAGCTTGACGC-′3) designed by (Kelly *et al.*, 2006), in addition to GoTaq® Green Master Mix (Promega, USA), the thermocycler initial denaturation was set at 95°C for 5 minutes, denaturation at 90°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 minutes, and final extension at 72°C for 7 minutes.

Negative control for PCR

To ensure the reliability of the results and monitor for potential contamination during DNA extraction and PCR amplification, a negative control was included in the procedure (Baverud *et al.*, 2007; Bekele *et al.*, 2024; Zu *et al.*, 2024). The negative control was prepared during the DNA extraction process and consisted of only Gram+ buffer from the DNA extraction kit, with no sample added. This control underwent the entire DNA extraction process and PCR thermocycling alongside the test samples. By including this control, any contamination introduced during the extraction or amplification process could be identified and mitigated, ensuring the validity of the results obtained from the experimental samples.

Sequencing of the SeM gene and data analysis

Positive PCR amplifications with *SeM*-Forward primer were submitted for Sanger sequencing with ABI3730XL, an automated DNA sequencing platform, at Macrogen Corporation in Korea. The results were

processed using Geneious Prime® and MEGA11 software.

Results

DNA extraction

The DNA of 59 suspected *Streptococcus* spp. isolates was successfully extracted, which appeared as compact bands on gel electrophoresis. The negative control showed no bands, confirming the absence of contamination during the extraction process.

The concentration and purity of extracted DNA were measured by NanoDrop™ spectrophotometer, and they ranged between 88.8 and 141.5 ng/µl and 1.72 to 1.83 at absorbance 260/280 nm, respectively.

PCR analysis of S. equi

PCR targeting the *SeM* gene identified *S. equi* in 49 out of the 59 isolates (83.1%) based on the expected amplification product of 541 bp. This result confirms the presence of the causative agent of strangles in most of the tested samples (Fig. 1).

Analysis of SeM gene sequences

Nine random positive PCR products of the *SeM* gene were sequenced, and the data were utilized for further analysis.

DNA sequence annotation and quality checks were conducted using Geneious Prime®. Next, nucleotide sequences were compared using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) to the *S. equi SeM* gene, which was available in the GenBank database.

BLAST results gave 188 significant alignments, and only one exact match was found (100%) for our *SeM* sequences query, and it was Iranian in origin, with (GenBank ID OL332314.*1*). Additionally, the nine Iraqi *SeM* gene sequences were submitted to the NCBI database, and accession numbers were assigned (PP726110.1, PP738564.1, PP738563.1, PP738562.1, PP738561.1, PP738560.1, PP738559.1, PP738558.1, PP738557.1).

Eighteen NCBI *SeM* sequences from different countries were selected and downloaded; they had query coverage (100%) and an *E*-value of (0.0) compared to our sequences. Furthermore, the total number of 27 *SeM* sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) multiple alignment method (Edgar, 2004; Katoh and Standley, 2013) in Geneious Prime® 2024.0.5 software. A maximum likelihood tree was generated by PhyML version 3.3.2 (Guindon *et al.*, 2010) using likelihood settings from the best-fit model (T93+G) selected by MEGA11 (Nei and Kumar, 2000; Tamura *et al.*, 2021) and visualized using iTOL (Letunic and Bork, 2024), as shown in (Fig. 2). The result shows that the nine Iraqi strains, in addition to the Iranian strain (OL332314.1) (Moghaddam *et al.*, 2023), had a close relationship with two other previously isolated *S. equi* strains from the UK (AJ249868.1) and Iran (MZ292707.1), with (99.79%) identity for both strains.

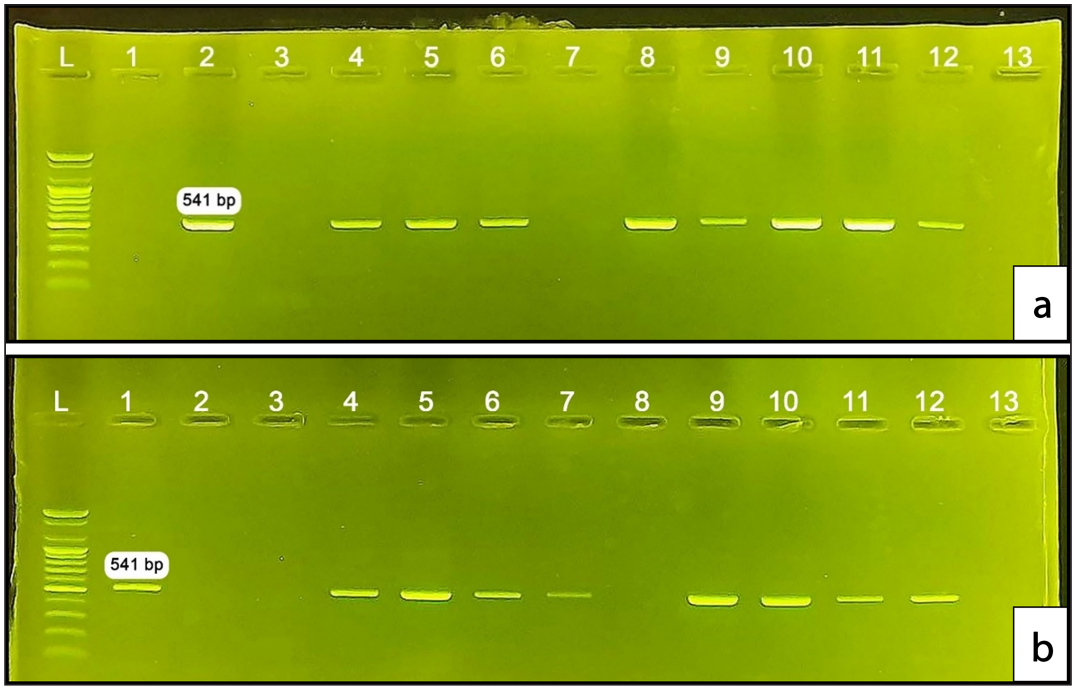

Fig. 1. (a) and (b) Electrophoreses of the agarose gel (1.2%) show the amplification of 541 bp *SeM* gene fragments from *S. equi.* Lane L shows the PCR ladder, and lane 13 shows the negative control.

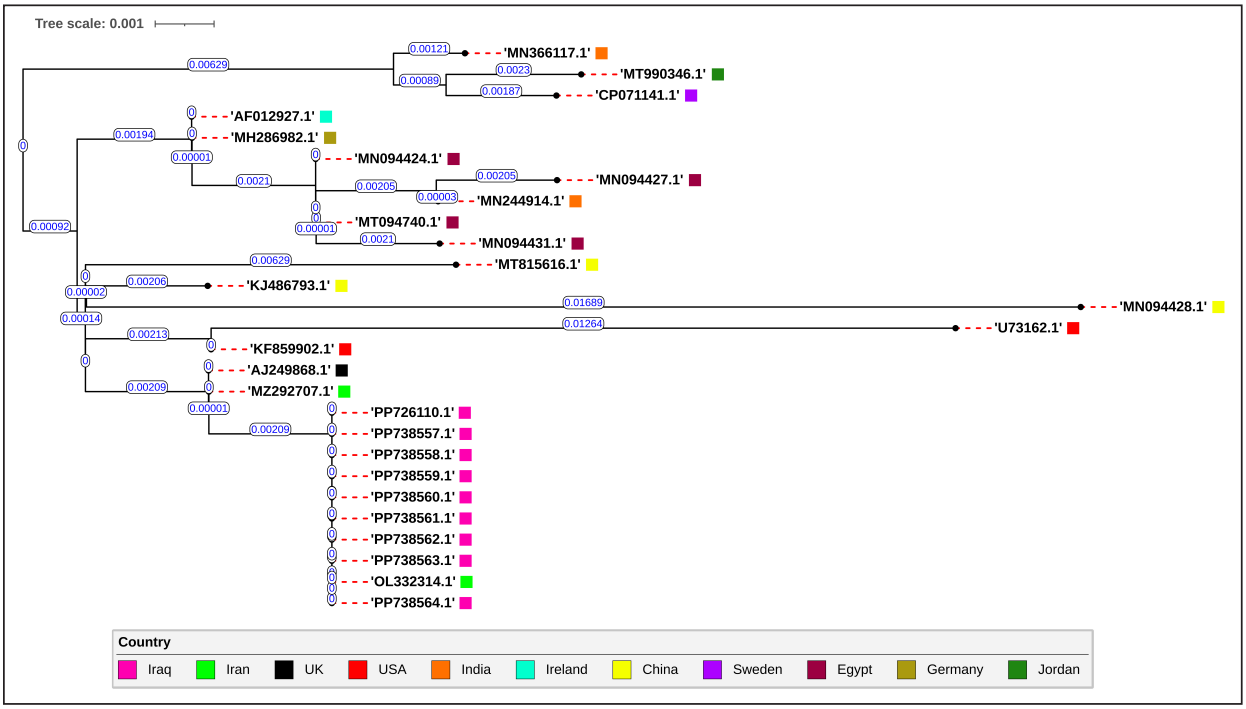

Fig. 2. Maximum likelihood tree illustrating the phylogenetic relationships among 27 different *SeM* gene sequences of *S. equi*, generated using PhyML version 3.3.2. The nine Iraqi strains (highlighted in pink), along with a highly similar Iranian strain, form a distinct cluster. This cluster shows a close evolutionary relationship with another group consisting of UK and Iranian strains. The scale bar represents the number of substitutions per site, indicating the degree of genetic divergence among sequences.

Table 1. Comparison of the predicted amino acid sequences from the transcript of Iraqi *S. equi* and reference strain 4047.

Allele	Strain	Amino acid at codon no. of the *SeM* protein			
		107	92	65	58
SeM-3	(NC_012471.1)	Val	Ser	Ala	Glu
	(PP726110.1)	Met	Pro	Thr	Asp
	(PP738564.1)	*	*	*	*
	(PP738563.1)	*	*	*	*
	(PP738562.1)	*	*	*	*
SeM-97	(PP738561.1)	*	*	*	*
	(PP738560.1)	*	*	*	*
	(PP738559.1)	*	*	*	*
	(PP738558.1)	*	*	*	*
	(PP738557.1)	*	*	*	*

*Similar changes in amino acid.

SeM alleles identification

The nine Iraqi *SeM* sequences were compared to the *SeM* allelic database in PubMLST. A single exact match was discovered, identifying the *SeM*-97 allele, which was previously isolated from a strain in Iran (OL332314.1) (Moghaddam *et al.*, 2023). For evaluation of *SeM* gene alleles, a comparison of the predicted amino acid sequences of *SeM*-97 of the nine Iraqi strains to *SeM*-3 of the NCBI reference *S. equi* strain 4047 (NC_012471.1) (Holden *et al.*, 2009) identified 4 amino acid differences (Table 1).

Discussion

This study provides foundational insights into the genetic variability and strain relationships of *S. equi* in Iraq while acknowledging the need for larger scale

investigations to address this gap fully. PCR targeting of the *SeM* gene successfully identified *S. equi* in 49 (83.1%) out of the 59 isolates. These results were consistent with previous studies by Alwan (Alwan, 2014) in Iraq, who identified *S. equi* 24/30 (80%) isolates using the *SeM* gene with Real-Time Polymerase Chain Reaction (Real-Time PCR), and Tartor (Tartor *et al.*, 2020) in Egypt, who successfully identified *S. equi* 92/100 (92%). Mahmood (Mahmood *et al.*, 2014) identified 51/101 (50.49%) positive samples from equine clinical samples using the *SeM* gene in Iraq. In Colombia, Morales (Jaramillo-Morales *et al.*, 2022) reported a (13.5%) prevalence of *S. equi* in guttural pouch samples. These variations in detection rates may reflect differences in sampling methods, populations, and diagnostic approaches across studies.

Analysis of our *SeM* gene sequences revealed four polymorphisms in comparison to the reference *S. equi* 4047 strain. These polymorphisms led to amino acid changes and may influence the virulence and immunogenicity of *S. equi.* The exact match with an Iranian strain (OL332314.1) (Moghaddam *et al.*, 2023) suggests a potential link in terms of epidemiology, highlighting the need for regional surveillance and comparative genomic studies.

This study provides the first report of *SeM* allele typing in Iraq, identifying the *SeM*-97 allele in *S. equi* isolates. The *SeM*-97 allele was previously reported by (Moghaddam *et al.*, 2023) in Iran, highlighting potential epidemiological links between the regions. The identification of *SeM* alleles aids in differentiating strangles outbreaks and understanding the epidemiology of the disease at regional and national levels (Ivens *et al.*, 2011; Parkinson *et al.*, 2011). *SeM* typing in other regions emphasizes its value in understanding strain movement and epidemiology. In Egypt, *SeM* typing revealed novel alleles (*SeM*-139 and *SeM*-141) linked to strains from donkeys in China and others (*SeM*-140, *SeM*-199, and *SeM*-7) associated with European isolates (Tartor *et al.*, 2020). Similarly, in Thailand, the identification of *SeM*-48, previously reported in UK outbreaks, suggests the importation of infected horses as a potential source of transmission (Tonpitak *et al.*, 2016). These studies demonstrate how *SeM* allele data can be used to trace cross-regional strain connections and inform control measures.

Conclusion

This study provides critical molecular and sequencing insights into the *S. equi* strains responsible for strangles in Baghdad, Iraq. The detection of the *SeM*-97 allele, reported for the first time in Iraq, highlights the utility of molecular diagnostics in identifying and characterizing strains associated with outbreaks. Furthermore, these results support the hypothesis of regional strain connections due to unsupervised horse importation. These findings establish a foundation for future epidemiological research and emphasize the need for targeted biosecurity measures and control strategies to mitigate strangles in Iraq.

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Conflicts of interest

The authors declare no conflicts of interest.

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Authors' contributions

Saif Aldeen Kamal Abdul-Latif: Conceptualization, sample collection, data analysis, methodology, molecular work, manuscript drafting, and final review. Afaf Abdulrahman Yousif: Supervision and critical manuscript review. Both authors reviewed and approved the final manuscript.

Data availability

The *SeM* gene sequences analyzed during this study have been submitted to the NCBI GenBank database under the accession numbers PP726110.1, PP738564.1, PP738563.1, PP738562.1, PP738561.1, PP738560.1, PP738559.1, PP738558.1, PP738557.1.

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