



## Clinical, Bacteriological, and Molecular Study of *Streptococcus equi* Isolated from Horses in Baghdad, Iraq

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### A B S T R A C T

This study addresses the limited research on *Streptococcus equi* subsp. *equi* (*S. equi*) infections in horses in Baghdad, Iraq, a highly contagious disease known as strangles. The objective was to investigate the clinical, bacteriological, and molecular characteristics of these infections. A total of 115 samples were collected from 100 racehorses, consisting of 85 nasal swabs from horses without abscesses and 30 samples (15 nasal swabs and 15 abscess samples) from 15 horses with abscesses. The bacterium was identified based on traditional bacteriological diagnostic methods and confirmed using PCR amplification of the *seel* gene. PCR detected a 34% infection rate. The findings demonstrated the PCR was more sensitive than bacterial cultivation in relation to the diagnosis of strangles. Clinical signs included fever, dyspnea, mucopurulent nasal discharge, submandibular lymph node abscessation, dysphagia, and significant respiratory distress. This study concluded that strangles significantly impact equine health, manifesting in moderate to severe clinical signs that can lead to mortality in affected horses. This underscores the necessity of studying the causative agent, *S. equi*, through advanced genetic methodologies to achieve accurate diagnosis and facilitate comprehensive epidemiological research. Furthermore, investigating the *seel* gene expressed by *S. equi* is crucial to understanding its role in the disease process and developing effective control measures.

**Keywords:** horse, PCR, *seel* gene, *S. equi*, strangles

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## INTRODUCTION

Streptococcal infections are widespread in veterinary medicine, affecting a range of animal species with significant health and economic impacts (1). Studies from Iraq have explored various aspects of streptococcal infections in horses (2-7).

Strangles is a highly contagious respiratory disease caused by the highly pathogenic equine specific *Streptococcus equi* subsp. *equi* (*S. equi*) affecting equids worldwide, with outbreaks being reported in donkeys (8), horses and Arabian horses (9-11). The disease has high morbidity and low mortality in susceptible populations, with the younger ones being more susceptible (12).

*S. equi* is a Gram-positive cocci arranged in chains, pus-forming microorganisms, and a member of the Lancefield group C. Colonies of virulent *S. equi* are always mucoid, glossy, translucent, produce hemolysis on the blood agar, catalase negative, and ferment maltose but not lactose, sorbitol, and trehalose, and the bacterium is one of the most common infectious agents of the horse (13-17).

Transmission of *S. equi* infection occurs through both direct and indirect routes and involves horse-to-horse contact and sharing of contaminated equipment. Transmission can also occur via other animal species (18, 19).

Clinical signs include abrupt pyrexia, pharyngitis, nasal discharge, cough, lymphadenopathy, abscess formation,

upper respiratory tract obstruction, neurological complications, dysphagia, bastard strangles, lymphangitis, and rarely, cases of *S. equi* pneumonia has been reported (20-23). However, the clinical presentation can vary depending on the horse's immunity and exposure to *S. equi* (18). Some horses may show milder signs like coughing, whereas others can develop complications like guttural pouch empyema, where the lymph nodes rupture and form abscesses (24, 25). It is even more imperative to elucidate the true clinical strangles cases due to *S. equi* by using the most sensitive and rapid diagnostic approaches (26).

Complications of strangles include empyema of the guttural pouch and its carrier state persistence, secondary cellulitis at external abscessation sites, emergency tracheostomies, metastatic abscessation, purpura hemorrhagica, and unusually secondary *S. equi* pneumonia or myositis (27).

In *S. equi*, several superantigen-encoding genes have been identified, including *seeI*, *seeH*, *seeL*, and *seeM*, which share similarities with superantigens from *Streptococcus pyogenes*, the human pathogen (28-31). *seeI* gene encoding the pyrogenic mitogen SeeI (SePE-I), which has been shown to stimulate the proliferation of equine peripheral blood mononuclear cells (PBMC) in vitro and exhibits a pyrogenic activity (fever-inducing) in ponies. Horses that have recovered from strangles or have been immunized against SePE-I develop antibodies against it, which can neutralize its effects, suggesting its role in the pathogenicity of *S. equi* (29, 32).

A needle aspirate from an enlarged or abscessed lymph node is the optimal sample for confirmation of *S. equi* infection; although ruptured lymph node swabs, moistened nasopharyngeal swabs, as well as nasopharyngeal and guttural pouch washes can also be used to collect samples for culture and PCR (18, 33).

In Iraq, there are limited studies investigating strangles and its causative agent. Despite the growing application of molecular assays in clinical studies on various animal species (34-39), their use in investigating equine diseases remain notably limited. Therefore, this study aims to identify the infection rate of *S. equi* in horses within Baghdad city, Iraq, along with phenotypic and genotypic characterization of the bacterium.

## MATERIALS AND METHODS

### Ethical Approval

This study was approved by the ethical and research committee of Veterinary Medicine of College, University of Baghdad, Ministry of Higher Education and Scientific Research (issue No.2278/H.E. on 16/10/2023).

### Animals and Clinical Examination

This study included 100 horses from various areas of Baghdad province, Iraq, 80 of which had a range of clinical signs related to strangles, and 20 horses were apparently normal, examined during the period between October 15, 2023 and March 28, 2024. The study included horses ranging in age from 1-6 years, with 49 females and 51

males. We subjected all animals to routine clinical examination, recording systemic reaction, assessing the presence or absence of nasal discharge, examining lymph nodes, and identifying other signs associated with strangles.

### Sample Collection and Bacteriological Examination

A total of 115 samples consisting of 85 nasal swabs were collected from horses without abscesses, and 30 samples (15 nasal swabs + 15 abscess samples) from 15 animals. Furthermore, disposable sterile swabs with Amies transport medium were used, and all samples were transported to the lab in an insulated medical icebox and promptly processed for bacterial isolation.

Direct smears of all samples (nasal swabs and lymph node abscess samples) were prepared and stained with Gram's stain for microscopic examination (17).

All 115 clinical samples were cultured on the Columbia blood agar (Oxoid, UK) supplemented with 5% sheep blood for 24 h at 37°C. The growing colonies were examined for their size, shape, color, and hemolysis type.  $\beta$ -hemolytic streptococci-like colonies were subcultured again on the Columbia blood agar. The recovered isolates were microscopically examined using Gram's stain and subsequently identified biochemically according to the catalase test and their sugar fermentation abilities of lactose, sorbitol, trehalose, and maltose.

### Molecular Detection

Genomic DNA was extracted from 59 bacterial isolates using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan), following the manufacturer's protocol. The extracted DNA was eluted and stored at -20°C until further use. Quantification of the purified DNA was performed using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific™, USA) to ensure suitable concentrations for downstream applications.

For the molecular identification of *S. equi*, partial amplification of the *seeI* gene was performed using specific primers previously described by Alber (2004). The primer sequences used were: Forward primer (*seeI*-F): 5'-GAAGGTCCGCCATTTTCAGGTAGTTTG-3', The reverse primer (*seeI*-R): 5'-GCATACTCTCTGTACCATGTCCTG-3'. These primers were designed to amplify a 520 base pair (bp) fragment of the target gene.

Polymerase chain reaction (PCR) was carried out using the MultiGene™ OptiMax Thermal Cycler (Labnet, USA). The thermal cycling conditions for amplifying the *seeI* gene included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 64°C for 30 sec, and extension at 72°C for 30 sec. A final extension step was performed at 72°C for 7 min, followed by a hold at 10°C for 10 min.

Following amplification, agarose gel electrophoresis was employed to verify the presence and correct size of the PCR products. A 1.2% agarose gel (MarLiJu, Korea) was prepared in 1X TBE buffer (MarLiJu, Korea) and stained with SafeView™ Classic Stain (abm®, Canada). The gel was poured into a casting tray, allowed to solidify, and then

submerged in TBE buffer within the electrophoresis chamber (Labnet, USA). The amplified PCR products were loaded alongside a 100 bp DNA ladder (BIONEER, Korea), and electrophoresis was conducted at 100 volts for 75 min. The resulting DNA bands were visualized using a gel documentation system (Major Science, Taiwan) to confirm the expected 520 bp fragment.

## RESULTS

### Clinical Signs of Strangles

The clinical signs observed in the infected horses were fever, polypnea, dyspnea, and tachycardia. One animal was hypothermic. In some cases, the mucous membrane was

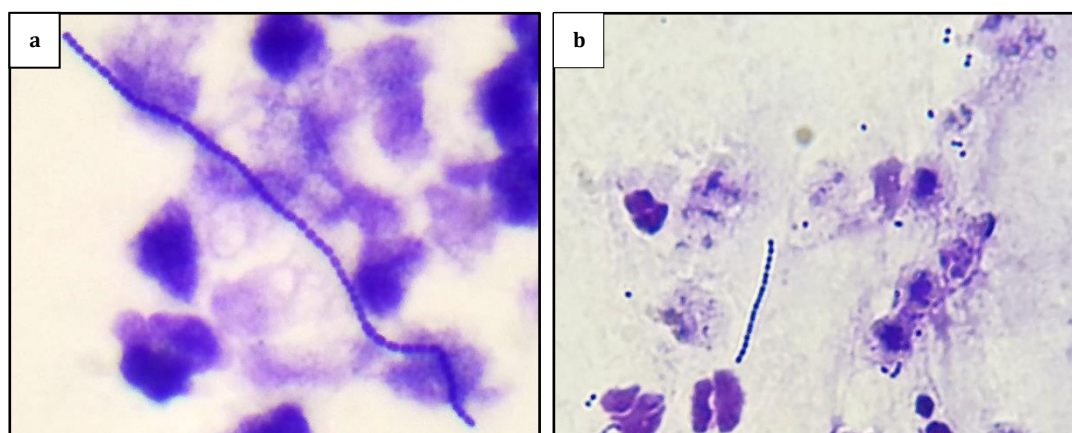
congested with or without petechial hemorrhage (Figure 1a).

Bilateral and unilateral submandibular lymph node enlargement with corresponding bilateral and unilateral mucopurulent nasal discharge, and some were stained with blood upon swabbing (Figure 1b). Some horses had intact submandibular lymph node abscessation, and in other animals the abscess was ruptured (Figures 1c and 1d). Productive coughing was observed in some animals. Whole face swelling was observed in some animals.

One horse died due to severe dyspnea and exhibited head extension, facial swelling, eye infection with purulent discharge, tongue protrusion, and complete inappetence (Figure 1e and 1f).



**Figure 1.** Strangles clinical signs in infected horses, (a) congested mucus membrane of the eye, (b) bilateral nasal discharge, (c) intact submandibular lymph node abscessation, (d) ruptured submandibular lymph node abscess with multiple openings, (e) head extension signaling difficulty in breathing, (f) infection of the eye with yellow mucopurulent discharge



**Figure 2.** Direct smears of *S. equi* appear as Gram-positive cocci arranged in chains, pairs, and singles under the light microscope, (a) prepared from a submandibular lymph node abscess sample, (b) prepared from a nasal swab



### Bacteriological Characteristics of *S. equi* Isolates

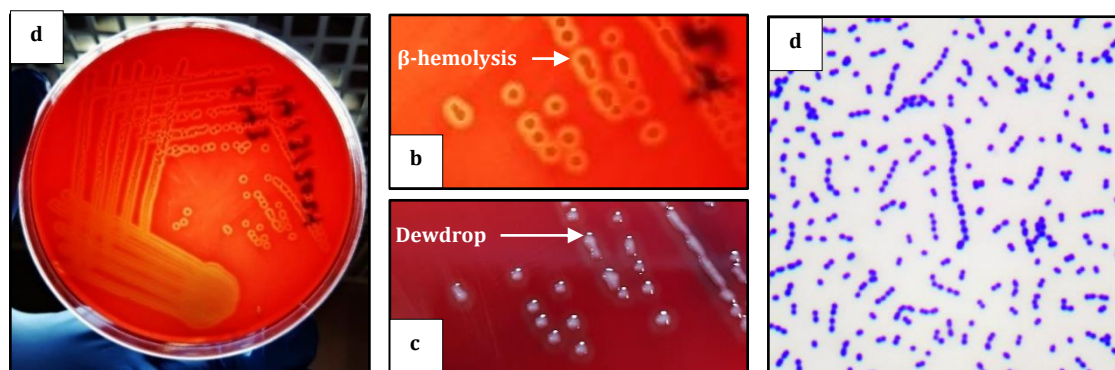
Direct smear examination of nasal swabs and submandibular lymph node abscess samples showed the typical Gram-positive long-chain cocci under the light microscope (Figure 2).

The *S. equi* isolates cultured on the Columbia blood agar showed colonies of different morphologies. The colonies were small to medium sized, circular, smooth, glistening, transparent-gray, convex, even margin, mucoid, and dewdrop-like colonies with  $\beta$ -hemolytic zones, and when

staining with Gram's stain, *S. equi* appeared as Gram-positive cocci arranged in chains, pairs, and singles (Figure 3).

### Biochemical Identification of *S. equi*

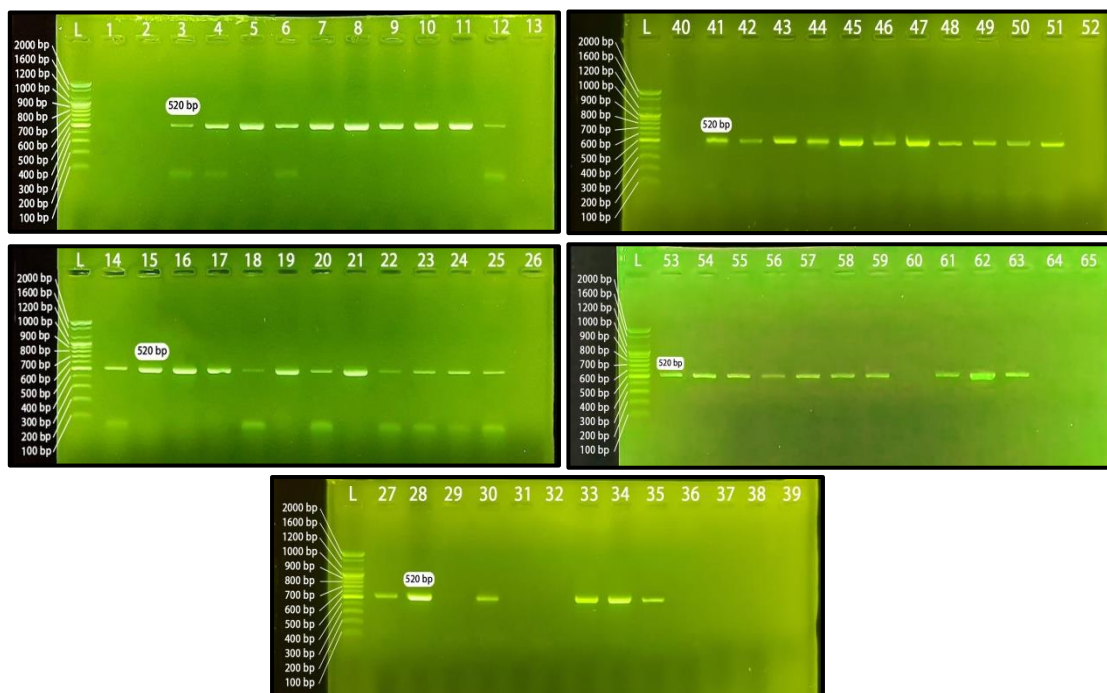
According to the results of the traditional bacteriological method, 30 out of 115 clinical samples were identified as *S. equi*, while 29 identified as  $\beta$ -hemolytic *Streptococcus* spp. with variable sugar fermentation results (Table 1).



**Figure 3.** Colonies of *S. equi* isolated from a horse with strangles show (b)  $\beta$ -hemolysis and (c) dewdrop-like morphology on the Columbia blood agar, and (d) a smear showing *S. equi* as gram-positive cocci arranged in chains, pairs, and singles

**Table 1.** Isolation of *Streptococcus* spp. by bacteriological method

Group	Isolates No.	Sorbitol	Lactose	Trehalose	Maltose	Hemolysis	Catalase
<i>S. equi</i>	30	-	-	-	+	Beta	Negative
	2	-	-	+	-		
<i>Streptococcus</i> spp.	18	-	-	+	+		
	2	-	-	-	-	Beta	Negative
	7	+	+	-	+		
Total	59						



**Figure 4.** Electrophoreses of agarose gel (1.2%) show the amplification of 520 bp of the *seeI* gene of *S. equi* subsp. *equi*. Lane L shows PCR ladder: lanes 13, 26, 39, 52, and 64 are negative controls (no DNA sample); lanes 1, 2, 29, 31, 32, 36-38, 40, and 60, are negative samples; all the remaining lanes are positive samples; lane 65 is left empty

## Molecular Detection

The extracted DNA concentration and purity ranged between 88.8 - 141.5 ng/μl and 1.72 - 1.83 at absorbance 260/280 nm, respectively.

Forty-nine isolates were positive for the *seel* gene out of the 59 isolates, which produced 520 bp, 19 of them were negative by the traditional bacteriological method, this was confirmed by gel electrophoresis (Figure 4) which corresponds to an infection rate of 34% among the 100 horses sampled. Notably, three animals without clinical signs were among the positive results and were considered "asymptomatic carriers".

## DISCUSSION

Strangles is a highly contagious respiratory disease that infects members of the family Equidae. It is caused by the host-specific *S. equi*, which is a Streptococcus from Lancefield group C. Clinical signs and laboratory diagnosis are the methods for confirming the infection (41, 42).

Clinical examination revealed typical signs of strangles, such as fever, nasal discharge, lymph node abscessation, and dysphagia, consistent with previous reports (11, 14, 18). One horse died due to severe respiratory distress, pointing to severe pharyngitis and lymphadenopathy of the head and neck region, leading to asphyxiation, hence the name strangles, this is compatible with the study of Christmann and Pink (43), who reported the death of two horses in the late stage of the disease due to severe respiratory symptoms even after tracheotomy. Boyle (27) stated that horses may develop severe respiratory distress requiring immediate tracheotomy. Also, Slater (44) mentioned that *S. equi* can spread systemically that lead to abscess formation in different organs "bastard strangles" which usually lead to horse death.

The detection of three asymptomatic carriers in this study highlights the significant challenges for disease control and biosecurity, which is consistent with previous studies (10, 45-48). Shi (49) mentioned that asymptomatic carriers continue to shed the bacteria, thus complicating disease management by causing new outbreaks. Furthermore, Weller (42) stated that the identification of these cases by diagnostic testing programs is important, as it exposes a higher prevalence of infection than clinical signs alone suggest. Pringle (50) reported carrier rates of 15-37% based on qPCR in two separate outbreaks, sampling fully recovered horses at least six months after the index cases using nasal swabs, nasopharyngeal lavage, and guttural pouch visualization and lavage.

In this study, the infection rate of *S. equi* in 100 horses was 34% tested positive using the PCR amplification of the *seel* gene. These findings align with those of Newton (51), who documented a comparable rate of 33%. However, these results were lower than those reported in a previous study in Iraq by Mahmood (2), who found an infection rate of 50.49%. Similarly, they were lower than the 44.8% infection rate reported by Osman (52). On the other hand, the infection rates in this study were higher than those reported by Patty and Cursons (53) and Neamat-Allah and

Damaty (25), who documented lower rates of 20.83% and 24.48%, respectively.

In the current study, a PCR assay targeting the *seel* gene was applied to characterize *S. equi*. The results showed that this gene was present in 49 isolates. These findings are consistent with the research by Holden (15), who reported that the genome of *S. equi* subsp. *equi* strain 4047 contains four specific genes: *seeH*, *seel*, *seeL*, and *seeM*. Similarly, Alber (40) demonstrated the differentiation of *S. equi* subspecies using a PCR assay, they employed the *sodA* gene to identify *S. equi* species and the specific genes *seeH* and *seel* to detect *S. equi* subsp. *equi* but not *S. equi* subsp. *zooepidemicus*. Furthermore, Artiushin (29) highlighted the presence of virulence genes encoding the pyrogenic mitogens *SeeH* (SePE-H) and *SeeI* (SePE-I) in *S. equi* subsp. *equi*. Paillot (28) also found that *S. equi* produces four bacterial superantigens encoded by *seeH*, *seel*, *seeL*, and *seeM* genes, which play a significant role in the pathogenesis of *S. equi* subsp. *equi*.

In this study, the PCR was more sensitive for the detection of *S. equi*, which surpasses that of the traditional bacteriological method. This aligned with previous studies that utilized the *seel* gene as a target to enhance diagnostic sensitivity for strangles (40, 53, 54) demonstrating higher sensitivity compared to culture methods. Also, Newton (51) concluded in their study on strangles that PCR testing is a potentially useful adjunct to the culture of nasal swabs for detecting *S. equi*, particularly in identifying asymptomatic carriers following a strangles outbreak.

The presence of 19 *S. equi* isolates with variable sugar fermentation results, such as fermentation of trehalose and maltose, points out the drawbacks of conventional biochemical diagnostic techniques. These findings align with previous studies that highlight the variability in biochemical profiles of *S. equi*, Silva and Webb (55, 56) both agreed that culture and biochemical identification methods can't be regarded as the gold-standard tests for *S. equi* because of low sensitivity, the increased turnaround time, failure to detect *S. equi* in some cases and atypical strain can lead to misdiagnosis. Furthermore, Grant (57) documented atypical isolates of *S. equi* from suspected strangles cases that have shown variable fermentation patterns, including the fermentation of lactose and trehalose.

In conclusion, this study sheds light on the clinical presentation of strangles with bacteriological and molecular diagnosis of *S. equi* infecting horses in Baghdad, Iraq, and emphasizes the need for strong biosecurity programs and better diagnostic methods. The *seel* gene identified in our study, which is one of the major virulence factors produced by *S. equi*, plays a key role in its pathogenicity, as supported by its involvement in pyrogenic activities and immune responses. These findings add new information to our understanding of *S. equi* in Iraq and globally and highlight the need for ongoing study and surveillance in the fight against this serious equine illness. Furthermore, effective management must account for the asymptomatic carriers to prevent outbreaks and ensure equine health.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## دراسة سريرية وبكتريولوجية وجزيئية لبكتيريا المكورات السبحية الخيلية المعزولة من الخيول في بغداد، العراق

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### الخلاصة

تتناول هذه الدراسة ندرة الأبحاث حول الإصابات بالمكورات السبحية الخيلية في خيول السباق في بغداد، العراق، وهي مرض معد للغاية يُعرف بخناق الخيل. هدفت الدراسة إلى التحقيق في الخصائص السريرية والبكتريولوجية والجزيئية لهذه الإصابات. تم جمع ١١٥ عينة من ١٠٠ خيل سباق، تضمنت ٨٥ مسحة أنفية من خيول بدون خراجات، و ٣٠ عينة (١٥ مسحة أنفية و ١٥ عينة خراج) من ١٥ حصانًا تعاني من خراجات. تم تحديد البكتيريا بناءً على الطرق التشخيصية البكتريولوجية التقليدية وتم تأكيدها باستخدام تفاعل البلمرة المتسلسل لاستهداف جين *sef*. كشف تفاعل البلمرة المتسلسل عن معدل إصابة بنسبة ٣٤٪ من ١٠٠ خيل سباق. أظهرت النتائج أن تقنية تفاعل البلمرة المتسلسل كانت أكثر حساسية مقارنة بزراعة البكتيريا فيما يتعلق بتشخيص مرض الخناق. تضمنت العلامات السريرية الحمى، وضيق التنفس، وإفرازات أنفية قيحية، وخراجات في العقد الليمفاوية تحت الفك السفلي، وعسر البلع، واعتلالات تنفسية شديدة. نستنتج من هذه الدراسة تأثير مرض الخناق بشكل كبير على صحة الخيول، حيث يظهر بأعراض سريرية تتراوح بين المعتدلة والشديدة والتي قد تؤدي إلى نفوق الخيول المصابة. يؤكد ذلك على ضرورة دراسة العامل المسبب المكورات السبحية الخيلية، باستخدام منهجيات جينية متقدمة لتحقيق تشخيص دقيق وتعزيز البحث الوبائي الشامل. علاوة على ذلك، فإن دراسة جين *sef* الذي تعبر عنه المكورات السبحية الخيلية بعد أمراً بالغ الأهمية لفهم دوره في عملية المرض وتطوير استراتيجيات فعالة للسيطرة عليه.

الكلمات المفتاحية: الخيول، تفاعل البلمرة المتسلسل، جين *sef*، المكورات السبحية الخيلية، الخناق