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Streptococcus equi M-like Protein Gene Detection from Clinical Cases of Strangles and Apparently Healthy Indian Equines

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ABSTRACT

Strangles is a highly contagious bacterial infection affecting the upper respiratory tract of equids, caused by *Streptococcus equi* subsp. *equi*, a β-hemolytic, gram-positive bacterium classified under Lancefield group C. Early and precise identification of the pathogen is crucial for effective disease control. This study focused on the molecular detection of *Streptococcus equi* M like Protein (*SeM)* gene, a surface protein from clinical cases of Strangles in Indian equines. A total of 45 isolates obtained from abscess material and nasal swabs of clinically diagnosed and apparently healthy Indian equines were analyzed. DNA was extracted and amplified using gene-specific primers targeting a 541 bp region of the *SeM* gene. PCR results confirmed the presence of the *SeM* gene in the isolates, demonstrating the effectiveness of this molecular approach for the detection and identification of *S. equi* and serving as a highly specific molecular marker. PCR was also employed to direct nasal swabs by boil and chill method with successful amplification of *SeM* gene in positive cases (16.7%). The study highlighted the effectiveness of PCR-based diagnostics in enhancing the detection of strangles and advocating the broader implementation of molecular techniques in routine diagnostics, surveillance and epidemiological investigations throughout India.

Key words: Strangles, *Streptococcus equi, SeM* gene, PCR, *Streptococcus* species

INTRODUCTION

Strangles is an important bacterial infection affecting the upper respiratory system of equines. The etiology of the disease is a “spherical, gram positive”, beta hemolytic and pus forming bacterium *Streptococcus equi* subspecies *equi* (*S. equi*) belonging to “Lancefield group C”. It is a widely reported disease throughout the world. The disease is marked by fever, mucopurulent nasal discharge, and the development of abscesses in the lymph nodes of the head and neck region in horses (Neamat-Allah *et al.,* 2016; Chhabra *et al*., 2023; Mclinden *et al*., 2023). Early and accurate detection of the pathogen is crucial for effective disease control for outbreak management, and prevention of transmission, especially in equine-dense regions.

Traditional diagnostic methods, such as microbial culture, remain the gold standard for confirming *S. equi* infections. However, culture techniques are time-consuming, may lack sensitivity-especially in chronic or partially treated cases and require well-preserved specimens. In contrast, molecular methods such as PCR have gained prominence for their speed, specificity and sensitivity.

The *SeM* gene, which encodes the M-like surface protein of *S. equi*, is considered a highly specific molecular marker for the bacterium. Targeting the *SeM* gene in PCR assays allows for the precise identification of *S. equi*, making it an ideal candidate for diagnostic purposes. Many researchers conclude that *S. equi* shows the evolutionary relationship with *S. zooepidemicus* and evolved from them. Conventional assays like isolation and biochemical characterization of the organism are cumbersome and confounded by the presence of other beta-hemolytic bacteria like *S. zooepidemicus*. The M protein, which is considered as a virulence factor, possesses a variable region at N terminal suggesting it as a good candidate (Javed *et al*., 2016; Bekele *et al*., 2024) for analysis at molecular level in isolates collected from strangles cases in equines in India. Despite the high incidence of strangles in India, research in this area remains limited; therefore, the present study

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focused on the molecular identification of *Streptococcus equi* from India through *SeM* gene amplification using PCR.

MATERIALS AND METHODS

A total of 101 samples were examined; out of them 45 *Streptococcus* cultures were available in Central Instrumentation Laboratory (CIL) of National Research Centre on Equines, Hisar and 56 nasal swabs were collected from organized and unorganized farms, health camps both from healthy as well as clinically affected suspected animals from above mentioned Indian states. Appropriate biosafety and biosecurity measures were followed throughout the specimen collection and transportation process. Random sampling was done from organized and un-organized farms in Haryana and Rajasthan from symptomatic and asymptomatic animals. For Haryana the latitude ranged from 27°39 to 30°35 N and its longitude ranged from 74°28 to 77°36 E, for Rajasthan the latitude and longitude ranges were 27°00'N, 74°00'E.

All isolates were cultured on Columbia Blood Agar (COBA), an enriched medium supplemented with 5% defibrinated sheep blood. Sheep blood was collected in a sterile flask containing silica beads, which served as an anticoagulant. The isolates were streaked on solidified blood agar plates and incubated at 37°C under aerobic conditions supplemented with 5-10% CO_2 for a period of 24 to 48 h.

The bacterial colonies which showed zones of β-haemolysis with typical *Streptococcus* appearance were further Gram stained and microscopically examined. The colony's haemolytic pattern, size and morphology were all studied along with catalase test. Selected colonies were sub-cultured on CSBA and enriched in Todd Hewitt Broth (THB) supplemented with various supplements like yeast extract and 2% horse serum at 37°C. The isolates were preserved by freezing at -40°C as 50% glycerol stocks (Veiga *et al*., 2024).

Genomic DNA was extracted from 45 *S. equi* cultures preserved in glycerol-supplemented Todd Hewitt Broth (THB). Prior to DNA extraction, each isolate was sub-cultured twice on 5% Columbia Blood Agar to ensure purity and viability. A loopful of bacterial growth was inoculated into 5 ml of THB supplemented with 2% horse serum and incubated at 37°C to facilitate bacterial proliferation. DNA extraction was performed using the Quick Bacterial and Fungal "DNA Extraction Kit (Zymo Research)", following the manufacturers protocol. Bacterial suspensions were centrifuged at 10,000 × g for 1 min using a Spin win MC03 centrifuge (Tarsons), and the resulting eluted DNA was used as a template for PCR and stored at -20°C until further use. Additionally, 5 µl of each DNA sample was subjected to electrophoresis on a 1.5% agarose gel prepared in TAE buffer containing 0.5 µg/ml ethidium bromide to verify DNA integrity.

A total of 56 nasal swab samples were collected randomly from equines of Haryana and Rajasthan. DNA extraction was done using boil-chill method. Nasal swab was dipped to a new, sterile vial containing 200 µl nuclease free water and boiled for 10 min in water bath and chilled at -20°C. After chilling, 2 µl of supernatant was used as a DNA template either immediately for PCR reaction and rest stored at -20°C (Zu *et al*., 2024).

The concentration of genomic DNA was quantified by assessing absorbance at 260 nm with an instrument Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies). Samples exhibiting an A260/A280 absorbance ratio between 1.7 and 1.8 were considered acceptable for further analysis.

The *SeM* gene of *S. equi* was amplified through PCR using the primers as described previously (Chhabra *et al*., 2024). A gradient PCR was performed out using a thermal cycler (Bio-Rad) to optimize precisely the annealing temperature ranging from 54.2°C to 55.2°C employing a known positive DNA sample of *S. equi*. The forward primer and reverse primer of *SeM* gene were used to amplify a 541 bp fragment corresponding to the 5' region of the *SeM* gene. The PCR reaction was performed in a total volume of 25 µl, consisting of 12.5 µl dream Taq Green Master Mix (Thermo Fisher Scientific), 1.0 µl of the forward primer (final concentration: 10 µM), 1.0 µl of the reverse primer (final concentration: 10 µM), 2.0 µl of DNA template (102 ng/µl), and 8.5 µl of nuclease-free water. Following optimization of the annealing temperature, PCR amplification was performed on all samples using the following PCR cycling conditions: An "initial denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 40 sec; followed by a final extension

at 72°C for 5 min". The amplified PCR products were analyzed by agarose gel electrophoresis using a 1.5% agarose prepared in 1× TAE buffer. Electrophoresis was conducted at 80 V for 90 min. Agarose gel was stained with ethidium bromide and visualized under Ultra violet (UV) trans-illumination. A 100 bp DNA ladder (Promega) was used as a molecular size marker for fragment size estimation.

Statistical analysis was performed to compare the diagnostic performance between direct nasal swab testing and culture-based detection of *S. equi*. Sensitivity, specificity, true positives (TP), true negatives (TN), and false positives (FP) were calculated for each method. Comparisons of detection rates and specificity between nasal swabs and cultures, which involved categorical variables (positive/ negative results), were analyzed using Fisher's Exact Test due to the relatively small sample sizes and the presence of low expected frequencies in some groups.

RESULTS AND DISCUSSION

The present study was undertaken on the 101 isolates comprising 45 *Streptococcus* cultures available in the laboratory and 56 nasal swabs collected from Haryana and Rajasthan, were examined for the presence of *S. equi.* Nasal swabs were cultured on solidified blood agar and incubated at 37°C for 24 to 48 h in aerobic conditions. In a total of 29 samples, beta haemolysis was detected using blood agar. Beta-hemolytic colonies were sub-cultured on 5% Columbia blood agar by inoculation and incubation at 37°C for 24 h. After 24 h the growth of colonies was observed and characterisation was undertaken. The colony diameters ranged from 0.5 to 2.0 mm. The colour varied from grey to whitish, though they were typically amber or appeared mucous-like without any pigmentation (Fig. 1). Clear zones were observed around the colonies, indicating haemolysis of red blood cells (Manuja *et al*., 2025).

Morphology of colonies of isolates of *S. equi* was checked by Gram's staining. Gram staining results showed that *S. equi* were gram positive cocci in the form of pairs or long chain (Fig. 2). DNA of cultures and collected samples was isolated by quick-DNA bacterial Microprep kit (Zymo research). The confirmation of DNA was done on 1.5% of agarose gel (Fig. 3).

Gradient PCR revealed no amplification band at 54.2°C; however, a distinct and specific band

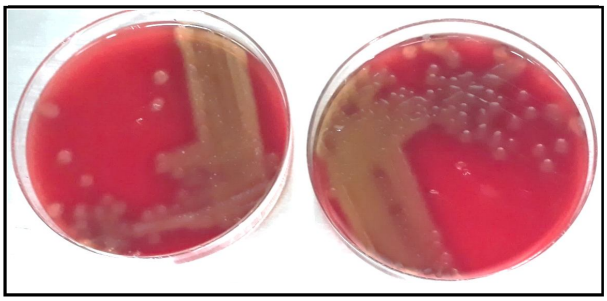

Fig. 1. Colonies of *S. equi* on 5% sheep blood agar.

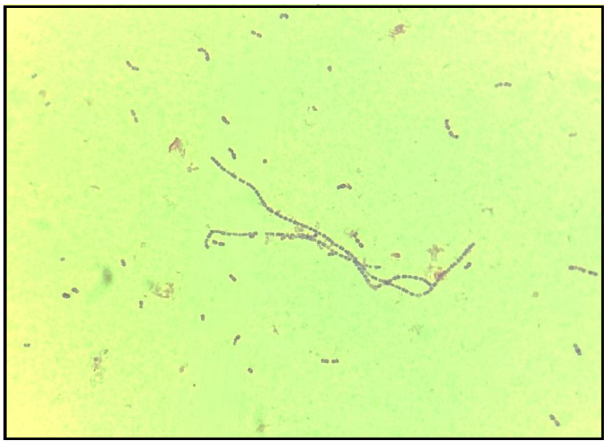

Fig. 2. Gram staining and cell morphology of *Streptococcus equi* under compound microscope.

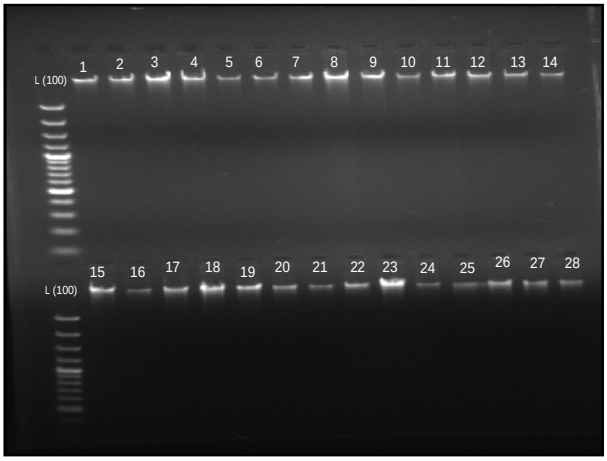

Fig. 3. Representative gel image of genomic DNA of *S. equi.*

was successfully obtained at 55°C, which was subsequently selected as the optimal annealing temperature for further reactions (Fig. 4). All 45 *Streptococcal* isolates yielded positive results for *Streptococcus* spp. through direct DNA analysis, indicating a 100% detection rate. Among these, 34 isolates were already identified as *S. equi* and further confirmed with conventional PCR. Additionally, out of 56 nasal swab samples collected from

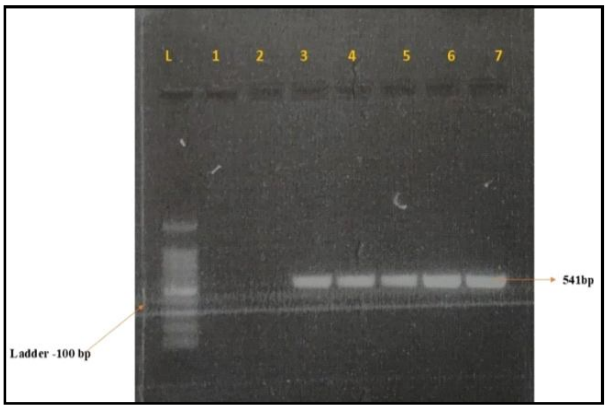

Fig. 4. Representative gel image of gradient PCR for *SeM* gene amplification. No band was observed at the annealing temperature of 54.2°C, whereas a clear and specific amplification band was obtained at 55°C, indicating the optimal annealing temperature for the assay.

the field, nine (16.7%) samples were confirmed as positive for *S. equi* through PCR targeting the *SeM* gene. The PCR product of *SeM* gene yielded 541 bp products when subjected to PCR conditions. The primary PCR products were generated from *S. equi* samples of different horses using gene specific primers (Fig. 5). The results are in conformity with the previous findings of gene expression using gene specific primers of *S. equi* (Dharvi *et al*., 2024). PCR-positive results should, therefore, be regarded as presumptive in the absence of confirmation by bacterial culture (Laus *et al*., 2016).

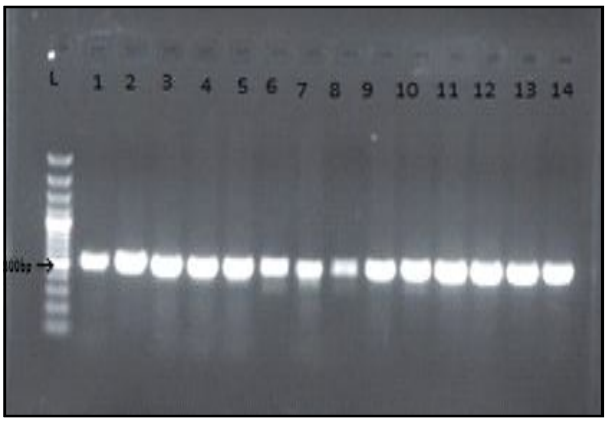

Fig. 5. Representative image depicting the amplification of the *SeM* gene of *S. equi* demonstrates the successful amplification of 541 bp fragment.

In the study, the detection of *S. equi* was evaluated using two different methods: direct nasal swab analysis and detection after bacterial culture. The detection rate for *S. equi* was significantly higher in samples processed through culture compared to direct nasal swabs (Fig. 6). Specifically, *S. equi* was detected in 34 out of 45 cultures (75.6%) compared to only nine out of 56 nasal swabs (16.1%). Statistical analysis using Fisher's Exact Test demonstrated a highly significant difference between the two methods ($P < 0.0001$), indicating that culturing substantially improved the likelihood of detecting *S. equi* compared to direct swab testing. However, there was no statistically significant difference in specificity between the two methods ($P = 0.192$), suggesting both methods were similarly effective.

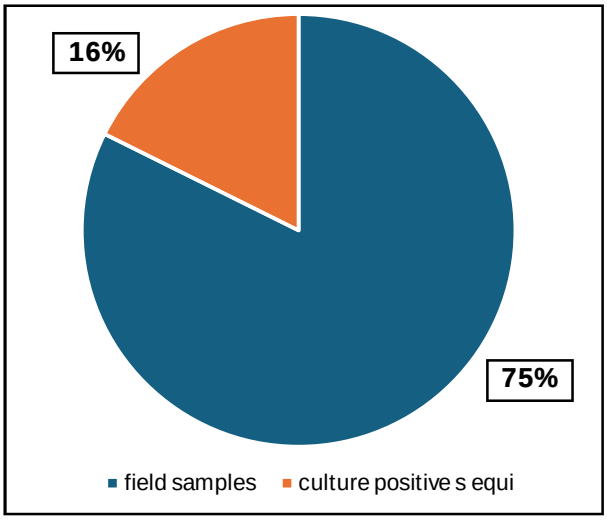

Fig. 6. Showing positive results of *Streptococcus equi* from field samples compared with available *Streptococcus* cultures deposited in the laboratory.

The PCR demonstrated a sensitivity of 100% and a specificity of 87.23%. Of the 56 nasal swab samples collected from field, nine were confirmed "positive" for *S. equi* by both *SeM* and traditional culture method. Forty-seven samples were negative by *SeM,* while 41 were found negative in gold standard method (Table 1). Additionally, six more samples were detected positive by gene specific *SeM* PCR which could not be confirmed by conventional method.

CONCLUSION AND FUTURE PROSPECTS

The present study highlighted the significance of PCR-based molecular identification of *S. equi* using the *SeM* gene as a specific genetic marker. Given the limitations of traditional culture methods, especially in chronic or partially treated cases, PCR targeting the *SeM* gene offered specific diagnostic approach. This study underscored the need for wider implementation

Table 1. Sensitivity and specificity PCR of field samples and 45 cultures as compared with traditional method for detection of *S. equi*

	Total 101 samples	56 Nasal swabs	45 cultures
Streptococcus species	74	29	45
Streptococcus equi	43	9	34
True positive	43	9	34
True negative	52	41	11
False positives/ extra detected	6	6	0
Sensitivity	100%	100%	100%
Specificity	89.65%	87.23%	100%

of molecular diagnostics in India, where strangles remained a prevalent and economically impactful disease in equines. PCR analyses validated the existence of the SeM gene within the isolates, thereby illustrating the efficacy of this molecular methodology for the detection and characterization of *S. equi*, and functioning as a highly precise molecular biomarker. Furthermore, PCR was utilized to analyze nasal swabs utilizing the boil and chill technique, resulting in successful amplification of the *SeM* gene in a subset of positive cases (16.7%). Enhanced molecular surveillance and detection of *S. equi* isolates can contribute significantly to better disease management, outbreak control and preventive strategies in the equine population. Future applications include the development of rapid, field-deployable diagnostics, more precise molecular epidemiological tracking, and potential insights for vaccine development. Integrating advanced molecular tools like qPCR and whole genome sequencing could further improve early detection and control of outbreaks, advancing targeted and effective management strategies for strangles in equine populations.

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