Molecular Identification of *Streptococcus equi* subspecies *equi* in Horses

[1]S. H. Mahmood[@]; [1]Abd M.H. Al_Judi and [2]Haidar Khalid Mosstafa
[1]Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, Baghdad University.Iraq.
[2]Ministry of Science and Technology, Baghdad, Iraq.
E-mail:sad_766@yahoo.com

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Summary

The objective of this study is to evaluate the existence of *Streptococcus equi* subspecies *equi* as probable agents of naturally occurring infection of the equine upper respiratory disease from the Equestrian club in Baghdad city. Nasal swabs and pus samples from 141 horses with upper respiratory tract infections were collected. Results indicated that different microorganisms were isolated and identified *S. equi subsp equi* (30 isolates), *S. equi subsp zooepidemicus* (14 isolates), *S. equisimilus* (9 isolates), *Enterococcus. fecalis* (17 isolates), *Pasteurella spp.* (29 isolates), *Staphylococcus spp.* (25 isolates), *Bacillus spp.* (24 isolates), *Pseudomonas spp*.(16 isolates), and *E. coli* (21 isolates). All 30 isolates of *S. equi* were characterized by biochemical tests. For molecular identification of the subspecies *S. equi* one genomic region SeM was amplified.

Keywords: Respiratory disease, Horse, PCR, *Streptococcus equi.*

Introduction

Streptococcus equi is the causative agent of strangles, a contagious inflammatory disease of the upper respiratory tract the outcome is only rarely fatal due to complications (1). Strangles is a common infection among the horse population. It is a notifiable disease based on laboratory confirmation or clinical symptoms speed of diagnosis is of great importance to prevent spreading of the disease, as morbidity is high (2). Identification of *Streptococcus* species is customarily based on biochemical tests including Lancefield grouping (3). *S. equi* shed in nasal discharges and pus from lymph nodes of affected animal. A routine laboratory detection of the organism involving culture of nasal swabs and pus from abscesses is often difficult because of background contamination. Culture and identification usually takes 2-3 days, an excessively lengthy interval given the highly contagious nature of strangles and the need to quickly identify the shedding animals so that they may be isolated in the early stages of an outbreak (4). The symptoms become apparent after an incubation period of 3 to 8 days, and the clinical course usually lasts 3 to 4 weeks (5-8). Marked fever (38.4-40.5˚C) develops during the acute phase and may subside until formation of lymph nodes abscess, at which time a second wave of fever may develop. Affected horses typically become anorexic, depressed, and develop bilateral, serous to mucoid nasal discharge within 24 hours of onset of fever. The discharge becomes mucopurulent as the disease progresses, and a moist cough may develop in some cases.

Plasma fibrinogen concentration and leukocyte counts usually increase at this time. The submandibular lymph nodes are involved most often and become enlarged, firm, and painful. The retropharyngeal lymph nodes may also be affected, and can induce dysphagia if they become markedly enlarged. The abscessed lymph nodes typically rupture 7 to 10 days after the onset of clinical signs and, in uncomplicated cases; recovery is complete 1 to 2 weeks thereafter (9). Due to rapidity, sensitivity, and specificity, Polymerase Chain Reaction (PCR) techniques are increasingly being used to diagnose bacterial diseases of equines (10 and 11). A PCR method targeting the *S. equi*-specific gene SeM (a cell wall associated protein) known to be a major virulence factor and protective antigen of *S. equi* (12-14), has been developed to overcome the drawbacks of the bacterial isolation method, and its usefulness in the diagnosis of strangles has been reported (4,15 and 16). The primer for this PCR test was designed on the variable portion of the SeM gene (17). This study aimed to molecular identification of

Streptococcus equi subspecies *equi* from horses with upper respiratory tract infections inthe Equestrian club in Baghdad city in Iraq.

Materials and Methods

A total of 141 horses aged 1-5 years, of both sexes located in the Equestrian club in Baghdad city were enrolled in the study between November 2012 to mid of April 2013. Clinical signs of respiratory tract disease were detected such as fever, cough, purulent nasal discharge, and enlargement of sub-mandibular lymph nodes, increased respiration rate, loss of appetite and congestion of mucous membrane.

A total of 121 samples of nasal discharge and 20 pus samples from affected sub-mandibular lymph nodes were collected from diseased horses for bacteriological examination. Samples were collected from nasal cavities of horses by using sterile swabs, pus swabs from open lymph node by using sterile swabs, and pus samples by using sterile disposable syringe which 18G from un-open lymph node, Samples were transported to the laboratory under sterile condition, where the required tests were done or stored at -20˚C. At least three cultures were made for each specimen. All cultures were incubated at 37˚C for 24-48 hours aerobic and un-aerobic condition, isolation of β-hemolytic streptococci-like colonies which identified by characteristic colony morphology, Gram staining and biochemical tests including catalase, isolates identified as ***S.equi*** fermented salicin but not sorbitol, lactose, raffinose, inulin, trehalose, or mannitol. The isolates hydrolysed starch but not aesculin (3).

The DNA from ***S. equi*** samples was extracted using the Wizard® Genomic DNA Purification Kit (Promega #A1120, USA).1ml of an overnight culture to a 1.5ml micro centrifuge tube, and centrifuge at 13,000-16,000 ^{x}g for 2 minutes to pellet the cells, remove supernatant and take the Pellet, re-suspend the cell in 480µl. of 50 mM EDTA, add the appropriate lytic enzymes to the resuspended the cell pellet in total volume 120µl, and incubate the sample at 37˚C for 30-60 min, and centrifuge for 2 minutes at 13,000-16000^{x}g, and add 600µl. of Nuclei Lysis solution, and incubate at 80˚C for 5 minutes to lyse the cell; then cool to room temperature, and add 3µl of RNase Solution to the cell lysate, and incubate at 37˚C for 15-60 minutes, then cool to room temperature, and add 200µl of Protein Precipitation Solution, and incubate the sample on ice for 5 minutes, and centrifuge at 13,000 -16,000^{x}g for 3 minutes, and transfer the supernatant containing the DNA to a clean 1.5 micro centrifuge tube containing 600µl of room temperature isopropanol, and centrifuge at 13,000-16,000^{x}g for 2 minutes, and carefully pour off the supernatant and drain the tube on clean absorbent paper, then add 600µl of room temperature 70% ethanol, and centrifuge at 13,000-16,000^{x}g for 2 minutes, then carefully aspirate the ethanol, and remove supernatant and drain the tube on clean absorbent paper and allow the pellet to air-dry for 10-15 minutes, and add 100µl of DNA rehydration solution to the tube and rehydrate the DNA by incubating at 65˚C for 1 hour, then periodically mix the solution by gently tapping the tube, and store the DNA at 2-8˚C, and the extracted DNA sample was kept at -20˚C.

Table, 1: Oligonucleotide primers used in the PCR and PCR product size.

Target gene	Sequence 5'-'3		Product size (base pairs)
SeM (PCR1)	**F**	**TGCATAAAGAAGTTCCTGTC**	677bp
	R	**GATTCGGTAAGAGCTTGACG**	
SeM (PCR2)	**F**	**CATACCTATCTCCATCAGCA**	325bp
	R	**CGAACTCTGAGGTTAGTCGT**	

Two separate PCR mixtures, largely based on that previously described (4, 18, 19 and 20), were used but with modification. Two reactions of PCR were used in ongoing attempts to improve sensitivity and reliability with further nested set of primers. Two separate PCR tests, based on published primers were used to detect *S. equi*, the primers SeM were used for the amplification of a 677 bp sequence and the primers SeM

were used for the amplification of a 325 bp sequence of the 16SrRNA region. The primers for these PCRs are shown in (Table, 1). The PCR-1. reactions were conducted with 10.5 µl of template DNA and 25 µl of reaction mix contained 12.5 µL of Taq PCR mastermix (Promega), 2 µL each primer DNA template prepared from samples according its concentration. Then water was added to complete the volume of reaction to (25 µL). In order to check out reagent contamination, one tube was prepared with ddH2O instead of template DNA (21).

PCR was conducted with the following program: one cycle 95 ℃ for 1 min, 35 cycles at 94 ℃ for 1 min, at 58 ℃ for 1.5 min, and at 72 ℃ for 1 min, followed by a final extension at 72 ℃ for 10 min and hold at 4˚C. PCR-2 was conducted in the same ways as PCR-1 with the exception of the primers. In parallel experiments, the SeM and SeM2 primers were used in a duplex PCR using the PCR mixture and the protocol described above. The duplex PCR for detection *S. equi* by using SeM and SeM2 genes primers, reactions were conducted with 8.5 µl of template DNA and 25 µl of reaction mix contained 12.5 µL of Taq PCR mastermix (Promega), 2 µL from SeM primer, 2 µL from SeM2 primer, DNA template was prepared from samples according its concentration then water was added to complete the volume of reaction to (25 µL), and it was conducted in the same way as PCR-1. The presence of PCR products were detected by electrophoresis of 7 µl of sample mixed with 3 µl loading buffer in 2% agarose gel which was prepared in 1X Tris base (TBE) buffer containing 108 g Tris base (pH 8.3), 55 g Boric acid, 0.5 mM Ethylene diamine tetra acetic acid (EDTA) Its was prepared according to (22). Six µL of Ethidium Bromide were added to agarose gel solution and mixed well.The apparatus was settled at 110 volts for 45min, product sizes were determined by comparison with the relative mobilities of the 1 kb DNA standard ladder. The DNA can be visualized on a UV transilluminator and photographed (21-23).

Results and Discussion

The clinical sings of the infected horses were an increase in temperature 38.4 - 40.5˚C, an increase in pulse rate 40 -52 bit/min and respiratory rate 15-22, with cough. Serous and mucopurulent nasal discharge and ocular discharge, enlargement of the submandibular lymph node, some of it was un-open and some was open and excreted pus which was creamy in color, congestion of mucous membrane, and in one case there was abscess in neck and legs of horse.

The bacteriological culture results of samples were shown in (Table, 2), from 141 horses enrolled in thus study. 185 isolates were obtained. The bacterial isolates were *S. equi subsp equi* (30 isolates), *S. equi subsp zooepidemicus* (14 isolates), *S. equisimilus* (9 isolates), *Enterococcus. fecalis* (17 isolates), *Pasteurella spp.* (29 isolates), *Staphylococcus spp.* (25 isolates), *Bacillus spp.* (24 isolates), *Pseudomonas spp.*(16 isolates), and *E. coli* (21 isolates), and other (40) samples had a respiratory infection of unknown etiology. 101 horses, 30 (29.70%) were found positive for *S. equi* and the strangles were observed in all age groups from November to mid of April (Table, 2).

Table, 2: Results of bacterial isolates obtained from nasal swabs and pus samples cultures of diseased horses.

Type of organisms	No. of isolates	% of bacterial isolates
Streptococcus equi subsp. equi	**30**	**16.21%**
Streptococcus equi subsp zooepidemicus	**14**	**7.56%**
S. equisimilus	**9**	**4.86%**
Enterococcus. fecalis	**17**	**9.18%**
Pasteurella spp.	**29**	**15.67%**
Staphylococcus spp.	**25**	**13.51%**
Bacillus spp.	**24**	**12.97%**
Pseudomonas spp.	**16**	**8.64%**
E. coli	**21**	**11.35%**
Total	**185**	**100%**

Al-Judi, (24) and Al-Kabae, (25) who isolated *S. equisimilus* and *Enterococcus. Fecalis*, present findings agree with the results of (19) who isolated S. *equi subsp equi*, *S. equi subsp zooepidemicus*, *Pasteurella spp.*, *Staphylococcus spp.*, *Bacillus spp.*, *Pseudomonas spp*., and *E. coli*. and (26) as they isolated *S. equi*. and (27) isolated *S. equi subsp equi*, *S. equi subsp zooepidemicus*, and *S. equisimilus*. and (28) reported that specificity was verified again in their study on different streptococci and equine respiratory bacteria obtained, that the cultures included *Streptococcus equi* subsp. *equi*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, *Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus canis*, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Rhodococcus equi*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginos*.

These results were consistent with those of (29) who was reported 33.2% strangles of less than 2 years of age and 35.4% of more than 2 years of age. And (30) they reported 82% strangles <1 years of age, 78% in those from 1-2 years, 20% from 2-3 years, 12% from 3-4 years, and 6% from 4-5 years. And (31) who found rates of *S. equi* infections of the upper respiratory tract and lymph nodes (strangles) in horses to be 47.5% in 1 year old horses, and 37.5% in foals. *S. equi* subsp. *equi* was isolated from nasal, pharyngeal, or lymph node specimens in 31 (60.8%) of 51 sick horses.

Foals are highly susceptible to developing strangles following *S. equi* s;ubsp. *equi* exposure as shown by rates of 86% (19/22) and 91% (10/11), respectively (32).

In bacterial isolation. It was found that the percentages of isolated *S. equi* in male were higher than that in female, and there was no reason for these results because there was no relationship between the sex and incidence of disease (Table, 3).

Table, 3: *Streptococcus equi* isolated from males and females horses.

Sex	No.	Number positive for *S. equi*	Total (%)
Male	**45**	**17**	**56.66%**
Female	**56**	**13**	**43.33%**

PCR done on genomic DNA extracted from the samples. As shown in (Figure, 1 and 2). PCR-mediated identification based on the M-like protein gene had been described for *S. equi* (4 and 15). A total of 101 samples collected from 56 mares and 45 stallions were subjected to a conventional bacterial isolation method and PCR. Results obtained are summarized in (Table, 4). PCR using SeM primers resulted in a 677 bp segment in the *S. equi*, from the 101 equine nasal swabs and pus samples collected. Products 677 bp in size were obtained from the SeM as shown in (Figure, 1), and in the same samples by PCR using SeM2 primers as shown in (Figure, 2). Results of culture and PCR for *S. equi* were concordant in 27 out of 30 samples. Furthermore, PCR was positive not only in the 27 that were positive by bacteriology, but also in 24 out of the 71 samples that were negative for *S. equi* by culture. As shown in (Table, 4).

The 30 samples bacteriologically positive for *S equi*, there were just three samples negative by PCR due to it was not obtained by DNA extraction. But other samples were positive by PCR, using DNA obtained by the "Wizard protocol" and the "Wizard kit protocol." From 101 samples which DNA extracted there was 57 samples examined by PCR (Table, 4). Forty-seven samples of seventy-one samples negative for *S equi* at the bacterial culture were also negative by PCR as shown in (Table, 4). Twenty-four samples negative by bacteriology were positive by PCR for *S equi*. (Fig.1 and 2), that agreement with (27) who found seven samples were positive by PCR but negative by bacteriology. And (33) they found the real-time PCR method detected *S. equi* in two cases and *S. zooepidemicus* in four cases that were negative by conventional culture. PCR was able to detect *S. equisimilis* DNA in 6 samples more than bacterial culture (28). Anza, *et al.,* (10)

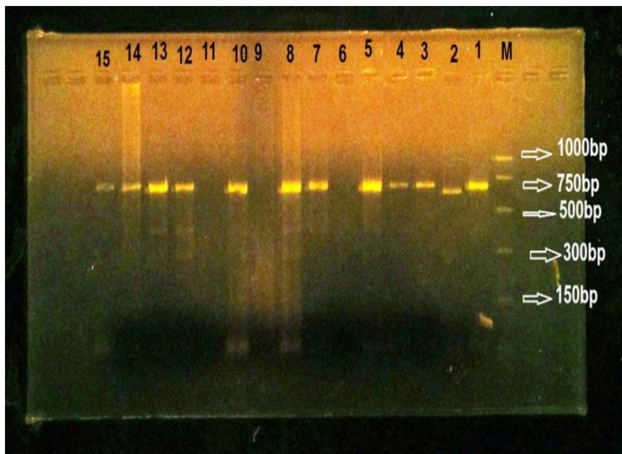
who found that PCR could detect *Taylorella equigenitalis* from not only 2 samples that were positive by bacterial culture but also from 10 samples that were negative by bacterial culture, suggesting that the PCR test was more sensitive than bacterial isolation from field samples; two of the PCR positive mares appeared to be negative by previous bacteriologic and serologic analyses. These results suggest that PCR may be more effective in detecting carrier animals with chronic infection.

Table , 4: Comparative between the bacterial isolation and PCR test.

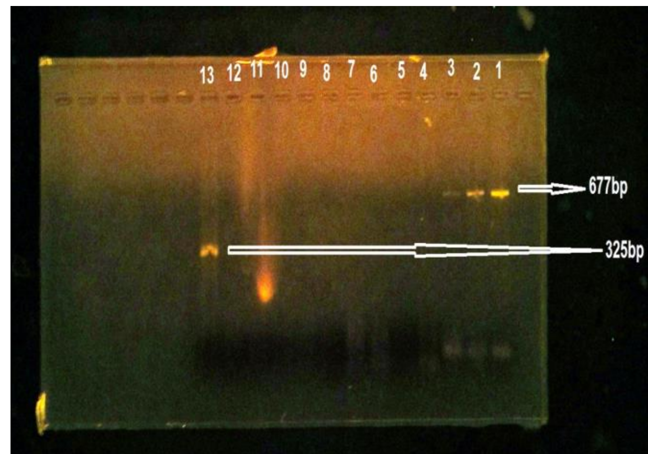
Test	Positive Samples	Negative Samples	Total of samples
bacterial isolation	**30** **29.70%**	**71** **70.29%**	**101** **100%**
PCR Test	**51** **89.47%**	**6** **10.52%**	**57** **100%**

Table , 5: The percentage of PCR reaction.

Tests	Total of samples	Positive samples	Negative Samples
DNA extraction	**101 100%**	**51 50.49%**	**50 49.50%**
PCR Test	**57 100%**	**51 89.47%**	**6 10.52%**



Figure, 1: Show typical PCR product of S. equi subsp. equi. with size of 677 bp using the SeM lane 1,2,3,4,5,7,8,10,12,13,14,15. Negative reactions of S. equi with primers of SeM lanes 6,9,11. Lane M , 100bp ladder served as size marker.



Figure, 2: typical PCR products of *S. equi* subsp. *equi* with specific oligonucleotide primers respectively lanes 1,2,3 show the PCR products of *S. equi* with sized 677bp by using SeM primers. Lanes 13, band of 325bp length amplified using SeM2.

This study, sought to implement a PCR assay for the rapid and inexpensive diagnosis of *S equi* infections directly from equine clinical specimens. PCR enhanced the sensitivity but it was not clear when the PCR was applied directly from the agar plate. Further studies are necessary to evaluate the real-time PCR system developed in our study directly on clinical samples (33).

Comparison between two genes used in PCR *S. equi* subsp. *equi* with specific oligonucleotide primers the PCR products of *S. equi* with sized 677bp by using SeM primers, and band of 325bp length amplified by using SeM2 primers (figure, 2). The PCR protocols to detect a common sequence of the species *S. equi* has been previously described so far to identify *S equi* (18, 33), and may be used as a control for species identification based on DNA extracted from bacteria. With such protocols, the sensitivity of PCR is limited by the sensitivity of the bacterial culture. The data on occurrence of *S. equi* is shown (Table, 5) of 101 horses, 51 (50.49%) were found positive for *S. equi*.

Present findings were in agreement with the results of (15) who was reported 3 protracted 'strangles' outbreaks on different kinds of establishments in which between 29 and 52% of sampled horses were infected as detected by culture and/or PCR. Of the infected horses,

between 9 and 44% were identified as carrying *S. equi* after clinical signs had disappeared and the predominant site of carriage was the guttural pouch. Prolonged carriage of *S. equi*, which lasted up to 8 months did not cease spontaneously before treatment was initiated to eliminate the infections. Comparing PCR and culture, many more swabs were found to be positive using PCR (56 vs. 30% of 61 swabs). Similar results were obtained for guttural pouch samples from 12 established carriers (PCR 76% and culture 59%).

These results from repeated samples from relatively few animals need confirming using more long-term carriers. These results confirm that PCR was more sensitive than traditional culture methods. Here in this study that PCR has been shown to be more sensitive in the detection of *S. equi*. The PCR assay demonstrated high sensitivity and specificity in detecting *S equi* directly in equine specimens. The PCR method has advantages and disadvantages compared with bacteriology. In particular, bacterial culture is fundamental for detecting other concomitant nonstreptococcal infections, for testing the antibiotic sensitivity, and for storing the strains in the laboratory collections for further studies. The PCR does not differentiate the vaccine strain from the field strains and could result in a false positivity if samples are collected too early after vaccination (27).

In the human and veterinary medical literature there were many examples that demonstrate that PCR is more sensitive than traditional culture methods, PCR has been shown to be more sensitive in the detection of *Mycoplasma pneumoniae*, *Neiserria gonorrhea* and *Streptococcus pneumoniae* in human clinical samples, *Rhodococcus equi* from foals, and *Mycoplasma bovis* from cattle (34-38). Traditional PCR was more sensitive than bacterial culture of equine nasopharyngeal wash samples (4). later described a real time PCR assay to detect *S. equi* and *S. zooepidemicus* in nasopharyngeal swabs and transtracheal washes from horses that showed increased sensitivity compared to culture (33).

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التحري الجزيئي عن المكورات السبحية الخيلية في الخيول

صدام حسين محمود[1] وعبد المناف حمزة جودي و[2]حيدر خالد مصطفى

[1]فرع الطب والعلاج الباطني، كلية الطب البيطري، جامعة بغداد، العراق.

[2]وزارة العلوم والتكنلوجيا، العراق.

E-mail:sad_766@yahoo.com

الخلاصة

الهدف من هذه الدراسة تقييم وجود جراثيم المكورات السبحية الخيلية كمسبب طبيعي محتمل لحدوث الاصابة في الجزء العلوي للجهاز التنفسي للفصيله الخيلية من نادي الفروسية في مدينة بغداد. تم جمع المسحات الانفية و وعينات القيح من 141 حصان كانت تعاني من اصابات للجهاز التنفسي العلوي .عزلت وصنفت اجناس وانواع مختلفه من الجراثيم هي كالاتي المكورات السبحية الخيلية *S. equi subsp equi* (30 عزلة)**,** *S. equi subsp zooepidemicus* (14 عزلة), *S. equisimilus* (9 عزلات), *Enterococcus. Fecalis* (17 عزلة), *Pasteurella spp.* (29 عزلة), *Staphylococcus spp.* (25 عزلة), *Bacillus spp.* (24 عزلة), *Pseudomonas spp***.** (16 عزلة) و *E. coli* (21 عزلة). وكل عزلات المكورات السبحية الخيلية قد ميزت بواسطة الاختبارات الكيميوحياتية. ولغرض التحري الجزيئي لجرثومة المكورات السبحية الخيلية فقد تم تضخيم منطقة واحدة من الجين SeM الخاص بها.

الكلمات المفتاحية: الجهاز التنفسي العلوي, الخيل, تفاعل البلمرة المتسلسل, المكورات السبحية الخيلية.