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Molecular Identification of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* in Nasal Swabs Samples from Horses Suffering Respiratory Infections in Iran

[1,3]A.A. Jannatabadi, [1]G.R. Mohammadi, [2]M. Rad and [2]M. Maleki
[1]Department of Clinical Sciences, [2]Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Department of Veterinary Medicine, Islamic Azad University of Sabzevar, Sabzevar, Iran

Abstract: The objective of this study was to evaluate the existence of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* as probable agents associated with naturally occurring infection of the equine upper respiratory disease in Mashhad area. Nasal swabs samples from thirty horses with upper respiratory tract infections were collected. The bacteria isolated and identified were *Streptococcus equi* subsp. *equi* (1 isolate), *Streptococcus equi* subsp. *zooepidemicus* (25 isolates), *Pasteurella* sp. (11 isolates), *Staphylococcus* sp. (17 isolates), *Bacillus* sp. (4 isolates), *Pseudomonas* sp. (4 isolates), *Proteus* sp. (1 isolate), *Neisseria* sp. (1 isolate) and *E. coli* (1 isolate). All 25 isolates of *Streptococcus equi* subsp. *zooepidemicus* and the isolate of *Streptococcus equi* subsp. *equi* were characterized by biochemical tests and molecular techniques. For molecular identification of the subspecies *S. equi* and *S. zooepidemicus* two genomic region *SeM* and *sod*A were amplified. This study is the first report of molecular identification of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* in Iran.

Key words: *Streptococcus equi*, *Streptococcus zooepidemicus*, upper respiratory disease, horse, PCR, Iran

INTRODUCTION

Streptococcus equi subsp. *equi* is worldwide known as the causative agent of strangles, a contagious inflammatory disease of the Upper Respiratory Tract (UPR) and associated lymph nodes of equids. The outcome is only rarely fatal due to complications (Sweeney *et al*., 2005). 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. There is a great demand by clinicians and horse owners for earlier laboratory confirmation than is provided with conventional cultivation. *S. equi* subsp. *zooepidemicus* is also an important pathogen of equines being associated with respiratory tract infections of foals and with uterine infections in mares (Radostits *et al*., 2007). *S. equi* subsp. *zooepidemicus* as a member of Lance field's serogroup C is regarded as archetypal species of the closely related species *S. equi* subsp. *equi* (Chanter *et al*., 1997; Harrington *et al*., 2002; Timoney, 2004). *Streptococcus* species identification is customarily based on biochemical typing schemes, including Lancefield grouping (Quinn *et al*., 1994).

So far there has not been any report of molecular identification of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* from horses with upper respiratory tract infections in Iran. Therefore, the present study was designed to investigate the different methods for identification of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* and on the basis of this knowledge devise a Polymerase Chain Reaction (PCR) system that can quickly detect *S. equi* and *S. zooepidemicus* and distinguish between them.

MATERIALS AND METHODS

Clinical investigations: Thirty horses aged 20 months to 22 years, located at 5 racing clubs or referred to the clinic of the Ferdowsi School of Veterinary Medicine were enrolled in the study between January 1st, 2006 and June 1st, 2007. The group of animals investigated was mainly composed of thoroughbred, turkomanbred and mixed breed horses.

Corresponding Author: G.R. Mohammadi, Department of Clinical Sciences, School of Veterinary Medicine, Ferdowsi University of Mashhad, P.O. Box 91775-1793, Mashhad, Iran
Tel: +989153156494 Fax: +985118763852

Table 1: Oligonucleotide primers used in PCR

Target gene	Test	Primer (5'→3')	Product (bp)	Annealing T(°C)	Species
SeM	PCR-1	TGCATAAAGAAGTTCCTGTC			
		GATTCGGTAAGAGCTTGACG	677	58	*S. equi*
SeM	PCR-2	CATACCTATCTCCATCAGCA			
		CGAACTCTGAGGTTAGTCGT	325	58	*S. equi*
*sod*A	PCR-3	CAGCATTCCTGCTGACATTCGTCAGG			
		CTGACCAGCCTTATTCACAACCAGCC	235	70	*S. zooepedemicus*

Clinician diagnosed respiratory tract disease was by detection of abnormal clinical signs related to the respiratory tract such as fever, cough, nasal discharge and the enlargement of lymph node, change in respiratory rate, abnormal auscultation of the trachea and thoracic cavity and loss of appetite.

Bacteriological examination: Samples for bacteriological examination were collected from nasal cavities of horses using sterile swabs (NS) taken from distant part of the nasal cavity after careful cleaning of the nares and anterior part of nasal mucosa with disinfectant. All samples were collected in duplicated and processed for bacterial isolation as soon as possible, usually within 0.5-4 h after collection. At least two cultures were made for each specimen. Samples were streaked into trypticase soy agar with 5% (v/v) ox blood. All cultures were incubated in 5% (v/v) CO_2 at 37°C for 24-48 h. Typical β-haemolytic streptococci-like colonies were detected on blood agar and identified by characteristic colony morphology, Gram staining and biochemical tests including catalase. Isolates identified as *S. equi* fermented salicin and sucrose but not sorbitol, lactose, raffinose, inulin, trehalose, glycerine, or mannitol. The isolates hydrolysed starch but not aesculin. Isolates identified as *S. zooepidemicus* gave the same biochemical results but fermented sorbitol and lactose (Quinn *et al*., 1994).

Polymerase chain reaction: The DNA extraction was performed according to the method described previously (Alber *et al*., 2004), with some modifications. For this three colonies of the strain to be investigated were suspended in 50 mL TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 8.0) containing 1 mL mutanolysin (5 U mL^{-1}) and incubated for 60 min at 37°C in a water bath. The suspension was subsequently treated with 1 mL proteinase K for 120 min at 56°C. After boiling for 15 min at 100°C the suspension was centrifuged (10, 000 g, 5 min) and the supernatant cooled before use.

Three separate PCR mixtures, largely based on that previously described (Timoney and Artiushin, 1997; Newton *et al*., 2000; Alber *et al*., 2004), were used but with modification. Three versions of PCR were used in ongoing attempts to improve sensitivity and reliability with further nested set of primers. The primers for these PCRs are shown in Table 1. In brief, PCR-1 was conducted by mixing 2 μL of extracted sample with 3 μL of 10x PCR buffer, 0.9 μL (50 mmol L^{-1}) magnesium chloride, 0.6 μL (10 mmol L^{-1}) dNTP mix, 0.6 μL each first round primer at 25 pmol μL^{-1}, 0.5 μL AmpliTaq, 21.8 μL water. During the first round of the reaction, the mixture was heated at 95°C for 1 min followed by 35 cycles of 94°C for 1 min, 58°C for 1.5 min, 72°C for 1 min followed by 10 min at 72°C.

PCR-2 was conducted in the same way as PCR-1 with the exception of the primers. Also, PCR-3 mediated identification based on the superoxide dismutase A encoding gene (*sod*A) was conducted in the same way as PCR-1 but annealing temperature was 70°C.

The presence of PCR products were detected by electrophoresis of 5 μL of sample mixed with 1 μL loading buffer in 2.0% (w/v) agarose gel containing 0.04 mol L^{-1} Tris-acetate (pH 8.3), 0.001 mol L^{-1} ethylene diamine tetra acetic acid in GNA 100 apparatus at 110 V for 45 min. Product sizes were determined by comparison with the relative mobilities of the 1 kb DNA standard ladder. Gels were examined on a UV transilluminator after immersion in 0.5 μg mL^{-1} ethidium bromide for 15 min.

RESULTS AND DISCUSSION

The bacteriological culture results of samples from the nasal swabs are shown in Table 2. From the 30 horses enrolled in this study, 65 isolates were obtained. The bacterial strains were *Streptococcus equi* subsp. *equi* (1 isolate), *Streptococcus equi* subsp. *zooepidemicus* (25 isolates), *Pasteurella* sp. (11 isolates), *Staphylococcus* sp. (17 isolates), *Bacillus* sp. (4 isolates), *Pseudomonas* sp. (4 isolates), *Proteus* sp. (1 isolate), *Neisseria* sp. (1 isolate) and *E. coli* (1 isolate). Isolation of most bacterial species from the Upper Respiratory Tract (URT) swabs (NS) has not been shown to be associated with respiratory disease in horses, with the exceptions of *S. equi* and possibly *Pasteurella* sp. and *S. zooepidemicus* are generally considered to be normal URT commensal organisms and considerable care is needed in interpreting their presence in URT swabs taken during clinical disease.

Streptococcus equi subsp. *equi* was isolated from nasal swabs of five year old horse with submandibular lymphadenitis. She was referred to the Ferdowsi School

Table 2: Results of bacterial isolates obtained from nasal swabs cultures of diseased horses

Type of organisms	No. of isolates
Streptococcus equi subsp. *equi*	1
Streptococcus equi subsp. *zooepidemicus*	25
Pasteurella sp.	11
Staphylococcus sp.	17
Bacillus sp.	4
Pseudomonas sp.	4
Proteus sp.	1
Neisseria sp.	1
E. coli	1

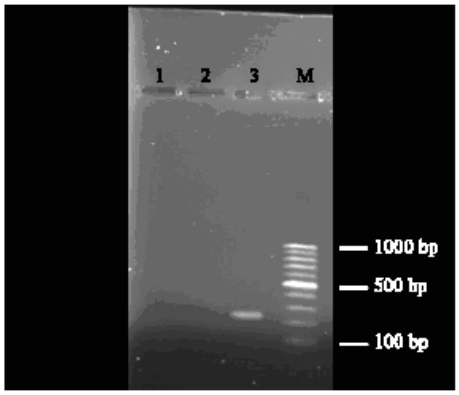

Fig. 1: Typical PCR product of *S. equi* subsp. *zooepidemicus* with size of 235 using the *Sod*A lane (3). Negative reactions of *S. equi* subsp. *zooepidemicus* with the primers of *SeM* lanes (1 and 2). Lane M, 100 bp ladder (Gene craft) served as size marker

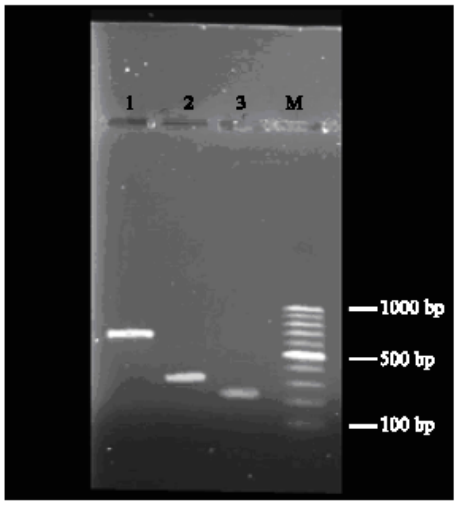

Fig. 2: Typical PCR products of *S. equi* subsp. *equi* with specific oligonucleotide primers respectively lanes 1, 2 show the PCR products of *S. equi* with sized 677 and 325 bp using *SeM* primers. Lanes 3, band of 235 bp length amplified using *Sod*A. Lane M, 100 bp ladder (Gene craft) served as size marker

clinic of Veterinary Medicine with complains of sever dyspnea. However, *S. equi* subsp. *zooepidemicus*, the opportunistic pathogen, was isolated from 25 horses according to Timoney (2004) produce disease in situation of virus infection or heat stress and it was therefore of interest to investigate in samples from horses with respiratory disease.

Polymerase chain reaction done on genomic DNA extracted from the bacterial colonies confirmed the bacteriological results (Fig. 1, 2).

The closely related Streptococcal species *S. equi* subsp. *equi* and subsp. *zooepidemicus* were identified by polymerase chain reaction using oligonucleotide primers designed according to species-specific parts of the super oxide dismutase A encoding gene (*sod*A). Further differentiation of both sub species was be performed by amplification of the gene encoding the protein M which could be isolated from *S. equi* subsp. *equi* but not from *S. equi* subsp. *zooepidemicus*.

The identification and differentiation of *S. equi* subsp. *equi* and subsp. *zooepidemicus* traditionally relies on phenotypic characteristics including serological properties by use of lancefield group C specific antisera and biochemical properties such as the fermentation of lactose, trehalose and sorbitol.

A positive lactose and sorbitol reaction is typical for *S. equi* subsp. *zooepidemicus*.

While negative reaction in these entire tests is typical for *S. equi* subsp. *equi* (Lammler and Hahn, 1994; Harrington *et al*., 2002). However, atypical *S. equi* subsp. *equi* that ferment one or both of lactose and trehalose, have been described by Grant *et al*. (1993).

Resent developments in nucleic acid technology such as PCR have resulted in new method that can be used for identification of bacteria.

PCR-mediated identification base on the M-like protein gene had been described for *S. equi* subsp. *equi* (Timoney and Artiushin, 1997; Newton *et al*., 2000).

However, *S. equi* subsp. *equi* possesses genes encoding two M-like proteins one of which is *S. equi* subsp. *equi* while the other M-like protein is a homologue to the M-like protein of *S. equi* subsp. *zooepidemicus* (Timoney *et al*., 1997).

In addition, truncated forms of M proteins might occur (Chanter *et al*., 1997). In the present study species-specific segments of an internal part of superoxide dismutase A encoding gene (*sod*A) was used for specific identification of both subspecies of *S. equi*.

The species-specific sequence variation of this gene had already been used for identification of various Gram-positive bacteria also including bacteria of genus *Streptococcus* (Poyart *et al*., 1998, 2002; Whatmore *et al*.,

2001). The *sod*A gene sequences were published previously by to Poyart *et al*. (1998). However, because of the close relationship of the *sod*A gene sequences of both subspecies of *S. equi*, the designed oligonucleotide primers did not allow a differentiation of *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi* since both belong to the pyogenic group of genus *Streptococcus*.

A differentiation of both subspecies could be performed by detection of the genes encoding antiphagocytic factor of *S. equi* subsp. *equi* protein. The occurrence of the antiphagocytic factor seems to be a constant characteristic of *S. equi* subsp. *equi* but not of *S. equi* subsp. *zooepidemicus* and could be used for differentiation of both subspecies.

The positive *sod*A gene reaction of the investigated *S. equi* strains and the positive M-protein reaction of the *S. equi* subsp. *equi* strains could be confirmed by PCR are shown in Fig. 1 and 2.

A major concern in this study is the presence of false negatives due to either sequence mutations or a lack of the toxin mediated identification and differentiation of both subspecies. Larger number of strains of different origin should be reinvestigated.

According to our best knowledge, this study is the first report of molecular detection of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* in Iran.

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