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

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**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 *sodA* 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 Lancefield’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.
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
sulcin and sucrose but not sorbitol, lactose, raffinose,
rafinil, trehalose, glycerine, or mannitol. The isolates
hydrolysed starch but not asulcin. 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
protease 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 Ariushshin, 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 but
with the exception of the primers. Also, PCR-3 mediated
identification based on the superoxide dismutase A
encoding gene (soda) 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 commensals 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 Ferndowsi School
Table 2. Results of bacterial isolates obtained from nasal swabs cultures of diseased horse

<table>
<thead>
<tr>
<th>Type of organism</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus equi</em> subsp. <em>equirum</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Streptococcus equi</em> subsp. <em>zooepidemicus</em></td>
<td>25</td>
</tr>
<tr>
<td>Peptostrept</td>
<td>11</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp.</td>
<td>17</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.</td>
<td>4</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>4</td>
</tr>
<tr>
<td><em>Proteus</em> sp.</td>
<td>1</td>
</tr>
<tr>
<td><em>Haemophilus</em> sp.</td>
<td>1</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1</td>
</tr>
</tbody>
</table>

Fig 1: Typical PCR product of *S. equi* subsp. *zooepidemicus* with size of 235 bp using the *SodA* lane (5). Negative reactions of *S. equi* subsp. *zooepidemicus* with the primers of *SodM* 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 *SodM* primers. Lanes 3, band of 235 bp length amplified using *SodA*. Lane M, 100 bp ladder (Gene craft) served as size marker.

clinic of Veterinary Medicine with complaints of severe 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 (*sodA*). Further differentiation of both sub species was 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 antiser a 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* (Lamm 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).

Recent 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 Aitkhan, 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 (*sodA*) 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.,...
The sodA gene sequences were published previously by to Poyart et al. (1998). However, because of the close relationship of the sodA 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 sodA 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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REFERENCES


