Journal of Equine Veterinary Science 32 (2012) 292-296

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Short Communication

A Multiplex Polymerase Chain Reaction Assay for Direct Detection and Differentiation of β-Hemolytic *Streptococci* in Clinical Samples from Horses

Silvia Preziuso DVM, PhD, Vincenzo Cuteri DVM

School of Veterinary Medical Sciences, University of Camerino, Via Circonvallazione 93/95, 62024 Matelica, Italy

A R T I C L E I N F O

Article history:
Received 1 August 2011
Received in revised form
7 October 2011
Accepted 6 November 2011
Available online 14 December 2011

Keywords:
Streptococcus equi subsp. *equi*
S equi subsp. *zooepidemicus*
S dysgalactiae subsp. *equisimilis*
Horse
Multiplex-PCR

A B S T R A C T

Streptococcus equi subspecies *equi*, *S equi* subspecies *zooepidemicus*, and *S dysgalactiae* subspecies *equisimilis* are β-hemolytic *Streptococci*, often isolated from horses with respiratory or genital diseases. The aim of this study was (i) defining and validating a multiplex polymerase chain reaction (PCR) protocol for identifying these *Streptococci* in bacterial cultures and for detecting them directly in equine clinical specimens, and (ii) defining and validating a cheap DNA extraction protocol for clinical specimens. When respiratory and genital samples from symptomatic and asymptomatic horses were tested by bacterial culture and by multiplex PCR, all the 150 samples culture-positive for *S equi*, *S zooepidemicus,* or *S equisimilis* were also positive by PCR. Of 150 culture-negative samples, 143 were negative by PCR. Seven samples were positive by PCR but negative by bacteriology. The multiplex PCR protocol described in this study is proven suitable for a sensitive, specific, and rapid detection and identification of *S equi*, *S zooepidemicus,* and *S equisimilis* in cultured bacterial colonies, as well as in clinical specimens from symptomatic or asymptomatic horses. The inclusion of internal control primers in the PCR protocol excludes false-negative results. A cheap DNA extraction method has been also validated for swabs, tracheal aspirates, bronchoalveolar lavage, and guttural pouches lavage samples.

1. Introduction

Three β-hemolytic *Streptococci* are often isolated from horses with respiratory or genital diseases. *Streptococcus equi* subspecies *equi* (*S equi*) is the agent of strangles, a highly contagious infection of the upper respiratory tract and its associated lymph nodes [1]. *Streptococcus equi* subspecies *zooepidemicus* (*S zooepidemicus*) is an important cause of respiratory disease and metritis, and it has been suggested to play a role in the inflammatory lower airway disease [1-5]. *Streptococcus dysgalactiae* subspecies *equisimilis* (*S equisimilis*) is an infrequent cause of lymphadenitis and placentitis, and it has been isolated from nasal swabs collected from horses with a history of respiratory disease [1,2,6].

Bacterial culture is the standard diagnostic method, but this methodology might be unsuccessful during the incubation and early clinical phases [1]. Furthermore, the colonies of the three bacterial species show the same morphology, and coinfections are difficult to be recognized. The polymerase chain reaction (PCR) technique has been demonstrated to be more sensitive than culture for detecting these *Streptococci* in clinical samples [6,7]. *S equi* guttural pouch carriers might be undetectable for several months by culture of nasal swabs, and repeated PCRs on guttural wash samples is a valid tool for detecting infected animals [8,9]. No data are available on the role of *S zooepidemicus* or *S equisimilis* asymptomatic carriers.

Corresponding author at: Silvia Preziuso, DVM, PhD, School of Veterinary Medical Sciences, University of Camerino, Via Circonvallazione 93/95, 62024 Matelica (MC), Italy.
E-mail address: silvia.preziuso@unicam.it (S. Preziuso).

0737-0806/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jevs.2011.11.001

Table 1
Bacterial culture-positive and -negative samples for *S equi*, *S zooepidemicus*, and *S equisimilis* used for multiplex PCR assay validation

Origin	Bacteriology				Clinical History
	Positive			Negative	
	S equisimilis	*S zooepidemicus*	*S equi*		
Nasal swab	31	15	–	53	Respiratory disease
Tracheal aspirate	7	38	–	37	Respiratory disease
Guttural pouches lavage	–	4	3	15	Respiratory disease
Bronchoalveolar lavage	–	8	–	5	Respiratory disease
Submandibular lymph node	–	–	3	–	Abscessus
Lung	–	3	–	–	Abscessus
Milk	–	5	–	4	Mastitis
Mammary fistula swab	1	–	–	–	Fistula
Cutaneous swab	–	2	–	–	Infected lesion
Uterine swab	6	24	–	40	Endometritis
Total	45	99	6	150	

PCR, polymerase chain reaction.

A multiplex PCR has been suggested to distinguish *S equi* from *S zooepidemicus* [10]. This PCR is based on the evidence that *S equi* genome contains a virulence factor named SeeI, which is not present in *S zooepidemicus*. A real-time PCR has been also developed for identifying *S equi* and *S zooepidemicus* [11]. However, these protocols have been validated on cultured colonies and not directly on clinical specimens so far.

The aims of this study were (i) defining and validating a multiplex PCR for simultaneous detection of *S equi*, *S zooepidemicus*, and *S equisimilis* in both bacterial colonies and clinical samples from horses, and (ii) defining and validating a cheap protocol for DNA extraction from clinical specimens.

2. Materials and Methods

To optimize the reaction conditions, the DNA from *S equi*, *S zooepidemicus,* and *S equisimilis* was used. *S equi* strain CF32 (ATCC 53185) was kindly provided by Dr. J. Alber and Dr. C. Lammler (Justus-Liebig-Universität of Gieβen, Germany). *S zooepidemicus* strain 07/M08 and *S equisimilis* strain 06/M02 were taken from the strain collection of our laboratory and have been previously identified by the API Strep kit (BioMérieux, Roma, Italy) and by PCR [6]. Once the best reaction conditions were found on the single laboratory strain, a mix of the DNA of the three species was used. The multiplex PCR specificity was checked by using *Streptococcus dysgalactiae* subspecies *dysgalactiae* (n = 3), *Streptococcus pneumoniae* (n = 2), *Streptococcus mutans* (n = 3), *Streptococcus pyogenes* (n = 4), *Streptococcus canis* (n = 5), *Streptococcus mitis* (n = 2), *Rhodococcus equi* (n = 5), *Escherichia coli* (n = 3), *Klebsiella pneumoniae* (n = 1), *Pseudomonas aeruginosa* (n = 3), *Staphylococcus aureus* (n = 4), *Staphylococcus intermedius* (n = 4), and *Enterobacter* spp. (n = 4) strains. The strains were obtained from the laboratory collection and were previously identified with biochemical and/or PCR methods.

The bacterial culture has been chosen as gold test to select positive and negative samples. A total of 300 equine clinical specimens have been considered (Table 1), 150 of which were positive and 150 were negative for β-hemolytic *Streptococci* by bacteriology. Every time a horse sample was received for bacterial diagnosis, an aliquot was stored at 4°C for subsequent PCR. The samples have been collected from horses with different clinical signs or with pulmonary abscesses (Table 1).

For bacterial culture, the swab samples were put in 4 mL trypticase soy broth (Oxoid, Milan, Italy) at 37°C to increase the bacterial concentration. After 6 hours incubation, 100 μL of the enrichment broth was spread on 5% horse blood agar added with "*Streptococcus* selective supplement" (Oxoid, Milan, Italy) and incubated at 37°C for 24 hours. In all, 100 μL of the tracheal aspirates (TA), bronchoalveolar lavage (BAL), guttural pouches lavage (GPL), milk samples, or purulent material from abscesses were spread directly on agar plates and incubated as described earlier. For research purpose, only β-hemolytic, catalase-negative *Streptococcus* spp. colonies were subcultured, submitted to a latex agglutination test (BioMérieux, Roma, Italy) to determine the serological group, and identified by the API Strep kit (BioMérieux, Roma, Italy). The colonies from the subculture were also used for DNA extraction and multiplex PCR as described later.

The extraction from colonies was carried out as previously described [10]. In particular, one loop of the β-hemolytic *Streptococci* colonies subcultured on blood agar was suspended in 50 μL of digestion buffer (Tris-HCl: 10 mmol/L, ethylenediaminetetraacetic acid [EDTA]: 1 mmol/L, pH 8.0 containing 5 U/μL lysozyme) (Sigma, Milan, Italy) and incubated at 37°C for 30 minutes. After lysozyme digestion, 0.38 μL proteinase K 20 μg/μL (Eurobio, Courtaboeuf Cedex B, France) was added, and the samples were incubated at 56°C for 30 minutes. The reaction was stopped by boiling the samples for 10 minutes, before cooling them in ice for 3 minutes. After centrifugation at 21,255 × *g* for 10 minutes, the supernatant containing the DNA was used for PCR.

Aliquots (200 μL) of TA, GPL, and BAL samples and of the enrichment broth incubated with the swabs as described earlier in the text were used for DNA extraction by commercial kit (QIAamp Mini Kit, Hilden, Germany) ("kit protocol").

To validate a cheaper protocol for extracting DNA from different clinical specimens, swabs, TA, GPL, and BAL samples were experimentally contaminated with *S equi* and *S equisimilis* colonies and submitted to lysozyme and proteinase K digestion ("L-PK protocol"). In particular,