Veterinary Microbiology 152 (2011) 126–130

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Isolation and characterization of β-haemolytic-Streptococci from endometritis in mares

P. Casagrande Proietti [a,*], A. Bietta [a], G. Coppola [b], M. Felicetti [b], R.F. Cook [c], M. Coletti [b], M.L. Marenzoni [b], F. Passamonti [b]

[a] Department of Biopathological Science and Hygiene of Food and Animal Production, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy
[b] Pathology, Diagnostic and Veterinary Clinic Department, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy
[c] Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546, USA

ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 6 April 2011
Accepted 8 April 2011

Keywords:
β-Haemolytic Streptococci
Endometritis
Horses
Bacteriological method
PCR
Superantigens

ABSTRACT

The objective of this manuscript was to validate published PCR-based methods for detection of β-haemolytic Streptococci by comparison with established bacteriological techniques using 85 clinical isolates recovered from uterine swabs of mares with clinical signs of endometritis and to determine the distribution of SeeL/SeeM and SzeL/SzeM superantigens in isolates of *Streptococcus equi* subsp. *equi* (*S. equi*) and *S. equi* subsp. *zooepidemicus* (*S. zooepidemicus*). The conventional bacteriological techniques showed the vast majority of these isolates (78) were *S. zooepidemicus* with just 5 *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*) and 2 *S. equi* strains detected. The PCR analyses confirmed the bacteriological results demonstrating the reliability of the 16S rRNA PCR assay for detecting *Streptococci*, the multiplex PCR for differentiating between *S. zooepidemicus*, and *S. equi*, and PCR assays based on streptokinase genes for identification of *S. equisimilis*. PCRs for genes encoding superantigens revealed *seeL* and *seeM* specific amplicons with size of approximately 800 and 810 bp respectively for the *S. equi* strains and for 2 *S. zooepidemicus* strains. To our knowledge, this is the first report of *szeL* and *szeM* possession by *S. zooepidemicus* isolates derived from endometritis in mares.

1. Introduction

Endometritis in mares is an important disease that often causes conception or gestational failure resulting in significant economic losses for the horse industry (Widders et al., 1995; Watson, 2000). *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is considered to be a major cause of endometritis because it is isolated from 22 to 54% of all cases in mares (Bain, 1966; Wingfield Digby and Ricketts, 1982; Welsh, 1984; Silva et al., 1999; Watson, 2000; Blanchard et al., 2003). Furthermore, this organism is a significant contributor to foetal loss being identified as the primary aetiological agent in 15–20% of equine abortions (Roberts, 1971; Welsh, 1984). However, *S. zooepidemicus* is not the only β-haemolytic Streptococcus associated with endometritis as other species including *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*) and *S. equi* subsp. *equi* (*S. equi*) (Albihn et al., 2003) have been isolated from clinical uterine samples collected from mares. Although these bacteria all belong to Lancefield serogroup C, *S. equisimilis* is genetically distinct (Timoney, 2004) while *S. zooepidemicus* is regarded as the archetype of the very closely related *S. equi* species (Chanter et al., 1997; Harrington et al., 2002; Timoney, 2004). At present detection of β-haemolytic Streptococci in equine clinical samples is generally reliant on time consuming bacterial culture methods with identification of different species accomplished using biochemical and/or serological techniques. Differentiation of the closely related *S. equi* and *S.*

* Corresponding author. Tel.: +39 0755857666; fax: +39 0755857738.
E-mail address: patrizia.casagrandeproietti@unipg.it
(P. Casagrande Proietti).

0378-1135/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2011.04.009

zooepidemicus can be difficult and has traditionally relied on biochemical properties such as the fermentation of lactose, trehalose and sorbitol. Positive lactose and sorbitol reactions are typical for *S. zooepidemicus* whereas a negative reaction in all of these tests is usually indicative of *S. equi* (Lämmler and Hahn, 1994; Harrington et al., 2002). However, strains of *S. equi* that ferment lactose or trehalose or both, have been described (Grant et al., 1993).

Although bacterial culture methods provide valuable material for subsequent epidemiological analysis, more rapid methods of detection and identification are required for accurate diagnosis of β-haemolytic Streptococci infections in horses. A polymerase chain reaction (PCR) based technique has been described for detection of *S. equisimilis* in formalin-fixed human pulmonary tissue samples (Nakamura et al., 2001) and adapted for identification of this organism in cultures obtained from equine nasopharyngeal swabs (Laus et al., 2007). Furthermore, Alber et al. (2004) have described a multiplex PCR protocol for detection and differentiation of *S. equi* and *S. zooepidemicus*. This technique is based on amplification the *sodA* (superoxide dismutase A) gene and the superantigenic exotoxins (*see*) *H* and *I* genes. *S. equi* and *S. zooepidemicus* are both positive for the *sodA* gene but only *S. equi* is positive for *seeH* and *seeI* genes. Bacterial superantigens (SAgs) are major virulence factors produced by some species of Streptococci that have the unique property of interacting simultaneously with the major histocompatibility complex (MHC) class II molecules of antigen presenting cells and the T cell receptor. This interaction stimulates T lymphocytes causing massive systemic release of pro-inflammatory cytokines potentially resulting in fever and toxic shock (Van Den Bussche et al., 1993; Alouf and Müller-Alouf, 2003). Interestingly two novel SAg genes, pyrogenic exotoxin L (*seeL*) and pyrogenic exotoxin M (*seeM*) have been identified by analysis the *S. equi* genome database at the Sanger Center (Proft and Fraser, 2003). Although these genes were clearly present in the sequenced prototype strain they could not be detected in 8 clinical *S. equi* isolates despite the fact that in the same series of experiments orthologous *speL* and *speM* genes were detectable in some isolates of *S. pyogenes* (Proft and Fraser, 2003). However, in a separate survey, *seeL* and *seeM* were detected in all 17 *S. equi* isolates along with orthologs of these genes (*szeL*, *szeM*) in 1 of 31 *S. zooepidemicus* isolates (Alber et al., 2005) and more recently Holden et al. (2009) found that the genes encoding SeeL and SeeM were present in all strains of *S. equi* and 4 of 140 isolates of *S. zooepidemicus* tested. These contrasting results suggest the distribution of these phage-associated SAg genes may vary in isolates of both *S.equi* and *S. zooepidemicus*. As the proteins encoded by *seeL* and *seeM* have significant mitogenic activity in cultures of equine peripheral mononuclear cells (Paillot et al., 2010) possession of these genes (or the closely related *szeL*/*szeM* orthologs) could have a significant impact on pathogenicity. In view of the considerable health problems caused by endometritis and the consequent urgent need for rapid detection and identification of β-haemolytic *Streptococci* isolates, this study was conducted to validate published PCR-based methods by comparison with established bacteriological techniques using an extensive panel of clinical isolates. In addition to these important validation experiments the availability of large numbers of clinical samples enabled investigations to determine the distribution of *seeL*/*seeM* and *szeL*/*szeM* in isolates of *S. equi* and *S. zooepidemicus* that are at present circulating in the Italian horse population.

2. Materials and methods

2.1. Bacteriological examination

A total of 85 β-haemolytic *Streptococci* isolates were included in this study. All isolates were recovered from uterine swabs of mares with clinical signs of endometritis. Bacteria were isolated from clinical specimens on Columbia blood agar plates (Oxoid, Italy) supplemented with colistin sulphate (10 mg/l) and nalidixic acid (15 mg/l) (CNA plates). CNA plates were incubated aerobically at 37 °C for 48 h. Biochemical identification was performed using the commercial API 20 Strep System (BioMérieux, Milan, Italy) according to the manufacturer's instructions. Haemolytic activity was determined on Columbia blood agar plates after 24 h at 37 °C under aerobic conditions. The Lancefield serological groups were determined using the Slidex Strepto-kit (BioMérieux, Milan, Italy) following the manufacturer's instructions.

2.2. DNA extraction and polymerase chain reaction (PCR)

For PCR analyses DNA was extracted from bacterial colonies following growth on blood agar using the Charge Switch gDNA Kit (Invitrogen, UK) according to the manufacturer's recommendations. Gene specific primers (Invitrogen, UK) and PCR protocols for amplification of 16S rRNA, *sodA*, *seeI*, *seeL*, *seeM* and streptokinase genes have been described previously (Nakamura et al., 2001; Alber et al., 2004, 2005). Detection and differentiation of *S. equi* and *S. zooepidemicus* were performed using the *sodA*–*seeI* multiplex PCR protocol reported by Alber et al. (2004), whereas *S. equisimilis* was identified by PCR amplification of streptokinase (Nakamura et al., 2001).

The 16S rRNA, *seeL* and *seeM* PCR reaction mixtures contained 0.7 μl of each primer (10 μM), 0.8 μl dNTPs mix (10 mM), 1× PCR buffer, 1.5 mM of $MgCl_2$ 1.6 U of Taq DNA polymerase (all reagents from Invitrogen, UK) with distilled water to a final volume of 18 μl and 2 μl of each DNA sample preparation. Reaction conditions included an initial denaturation step of 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s; 54 °C for 30 s and 72 °C for 1 min with a final extension of 72 °C for 5 min. The PCR reactions were conducted using a Gene Amp PCR System 2400 thermal cycler (Applied Biosystems, USA) with PCR products resolved by electrophoresis in 1–2% agarose gel containing 4 μl of GelRed (Nucleic Acid Gel Stain, 10,000× in water) per 100 ml (Biotium, USA). All stained PCR products were visualized by transillumination (Euroclone, Italy).