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P. Casagrande Proietti, A. Bietta, G. Coppola, M. Felicetti, R.F. Cook, M.  
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Authors: P. Casagrande Proietti, A. Bietta, G. Coppola, M. Felicetti, R.F. Cook, M. Coletti, M.L. Marenzoni, F. Passamonti



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1 **Original research paper**

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3 **Isolation and characterization of  $\beta$ -haemolytic-Streptococci from**  
4 **endometritis in mares**

5

6 P. Casagrande Proietti,<sup>a\*</sup> A. Bietta,<sup>a</sup> G. Coppola,<sup>b</sup> M. Felicetti,<sup>b</sup> R.F. Cook,<sup>c</sup> M. Coletti,<sup>b</sup>  
7 M. L. Marenzoni,<sup>b</sup> F. Passamonti<sup>b</sup>

8

9 <sup>a</sup>Department of Biopathological Science and Hygiene of Food and Animal Production,  
10 University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy

11 <sup>b</sup>Pathology, Diagnostic and Veterinary Clinic Department, University of Perugia, Via  
12 San Costanzo 4, 06126 Perugia, Italy

13 <sup>c</sup>Department of Veterinary Science, Gluck Equine Research Center, University of  
14 Kentucky, Lexington, KY 40546, USA

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18

19

20

21

22 <sup>\*</sup>*Corresponding author.* Tel: +390755857666; fax: +390755857738

23 E-mail address: patrizia.casagrandeproietti@unipg.it (P. Casagrande Proietti)

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25

26 **Abstract**

27 The objective of this manuscript was to validate published PCR-based methods for  
28 detection of  $\beta$ -haemolytic Streptococci by comparison with established bacteriological  
29 techniques using 85 clinical isolates recovered from uterine swabs of mares with clinical  
30 signs of endometritis and to determine the distribution of SeeL/SeeM and SzeL/SzeM  
31 superantigens in isolates of *Streptococcus equi* subsp. *equi* (*S. equi*) and *Streptococcus*  
32 *equi* subsp. *zooepidemicus* (*S. zooepidemicus*). The conventional bacteriological  
33 techniques showed the vast majority of these isolates (78) were *S. zooepidemicus* with  
34 just 5 *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*) and 2 *S. equi* strains  
35 detected. The PCR analyses confirmed the bacteriological results demonstrating the  
36 reliability of the 16SrRNA PCR assay for detecting *Streptococci*, the multiplex PCR for  
37 differentiating between *S. zooepidemicus*, and *S. equi*, and PCR assays based on  
38 streptokinase genes for identification of *S. equisimilis*. PCRs for genes encoding  
39 superantigens revealed *seeL* and *seeM* specific amplicons with size of approximately  
40 800 and 810 bp respectively for the *S. equi* strains and for 2 *S. zooepidemicus* strains.  
41 To our knowledge, this is the first report of *szeL* and *szeM* possession by *S.*  
42 *zooepidemicus* isolates derived from endometritis in mares.

43

44 **Keywords:**  $\beta$ -haemolytic Streptococci; endometritis; horses; bacteriological method;  
45 PCR; superantigens.

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## 52 1. Introduction

53 Endometritis in mares is an important disease that often causes conception or  
54 gestational failure resulting in significant economic losses for the horse industry  
55 (Widders et al., 1995; Watson, 2000). *Streptococcus equi* subsp. *zooepidemicus* (*S.*  
56 *zooepidemicus*) is considered to be a major cause of endometritis because it is isolated  
57 from 22 to 54 per cent of all cases in mares (Bain, 1966; Wingfield Digby and Ricketts,  
58 1982; Welsh, 1984; Silva et al., 1999; Watson, 2000; Blanchard et al., 2003).  
59 Furthermore, this organism is a significant contributor to foetal loss being identified as  
60 the primary aetiological agent in 15 to 20 per cent of equine abortions (Robert, 1971;  
61 Welsh, 1984). However, *S. zooepidemicus* is not the only  $\beta$ -haemolytic Streptococcus  
62 associated with endometritis as other species including *Streptococcus dysgalactiae*  
63 subsp. *equisimilis* (*S. equisimilis*) and *Streptococcus equi* subsp. *equi* (*S. equi*) (Albihn  
64 et al., 2003) have been isolated from clinical uterine samples collected from mares.  
65 Although these bacteria all belong to Lancefield serogroup C, *S. equisimilis* is  
66 genetically distinct (Timoney, 2004) while *S. zooepidemicus* is regarded as the  
67 archetype of the very closely related *S. equi* species (Chanter et al., 1997; Harrington et  
68 al., 2002; Timoney, 2004). At present detection of  $\beta$ -haemolytic Streptococci in equine  
69 clinical samples is generally reliant on time consuming bacterial culture methods with  
70 identification of different species accomplished using biochemical and/or serological  
71 techniques. Differentiation of the closely related *S. equi* and *S. zooepidemicus* can be  
72 difficult and has traditionally relied on biochemical properties such as the fermentation  
73 of lactose, trehalose and sorbitol. Positive lactose and sorbitol reactions are typical for  
74 *S. zooepidemicus* whereas a negative reaction in all of these tests is usually indicative of  
75 *S. equi* (Lämmle and Hahn, 1994; Harrington et al., 2002). However, strains of *S. equi*  
76 that ferment lactose or trehalose or both, have been described (Grant et al., 1993).

77 Although bacterial culture methods provide valuable material for subsequent  
78 epidemiological analysis, more rapid methods of detection and identification are  
79 required for accurate diagnosis of  $\beta$ -haemolytic Streptococci infections in horses. A  
80 polymerase chain reaction (PCR) based technique has been described for detection of *S.*  
81 *equisimilis* in formalin-fixed human pulmonary tissue samples (Nakamura et al., 2001)  
82 and adapted for identification of this organism in cultures obtained from equine  
83 nasopharyngeal swabs (Laus et al., 2007). Furthermore, Alber et al. (2004) have  
84 described a multiplex PCR protocol for detection and differentiation of *S. equi* and *S.*  
85 *zooepidemicus*. This technique is based on amplification the *sodA* (superoxide  
86 dismutase A) gene and the superantigenic exotoxins (*see*) *H* and *I* genes. *S. equi* and *S.*  
87 *zooepidemicus* are both positive for the *sodA* gene but only *S. equi* is positive for *seeH*  
88 and *seeI* genes. Bacterial superantigens (SAGs) are major virulence factors produced by  
89 some species of Streptococci that have the unique property of interacting  
90 simultaneously with the major histocompatibility complex (MHC) class II molecules of  
91 antigen presenting cells and the T cell receptor. This interaction stimulates T  
92 lymphocytes causing massive systemic release of pro-inflammatory cytokines  
93 potentially resulting in fever and toxic shock (Van Den Bussche et al., 1993; Alouf and  
94 Muller-Alouf, 2003). Interestingly two novel SAg genes, pyrogenic exotoxin L (*seeL*)  
95 and pyrogenic exotoxin M (*seeM*) have been identified by analysis the *S. equi* genome  
96 database at the Sanger Center (Proft et al., 2003). Although these genes were clearly  
97 present in the sequenced prototype strain they could not be detected in 8 clinical *S. equi*  
98 isolates despite the fact that in the same series of experiments orthologous *speL* and  
99 *speM* genes were detectable in some isolates of *S. pyogenes* (Proft et al., 2003).  
100 However, in a separate survey, *seeL* and *seeM* were detected in all 17 *S. equi* isolates  
101 along with orthologs of these genes (*szeL*, *szeM*) in 1 of 31 *S. zooepidemicus* isolates  
102 (Alber et al., 2005) and more recently Holden et al. (2009) found that the genes

103 encoding SeeL and SeeM were present in all strains of *S. equi* and 4 of 140 isolates of *S.*  
104 *zooepidemicus* tested. These contrasting results suggest the distribution of these phage-  
105 associated SAg genes may vary in isolates of both *S. equi* and *S. zooepidemicus*. As the  
106 proteins encoded by *seeL* and *seeM* have significant mitogenic activity in cultures of  
107 equine peripheral mononuclear cells (Paillot et al., 2010) possession of these genes (or  
108 the closely related *szeL/szeM* orthologs) could have a significant impact on  
109 pathogenicity. In view of the considerable health problems caused by endometritis and  
110 the consequent urgent need for rapid detection and identification of  $\beta$ -haemolytic  
111 *Streptococci* isolates, this study was conducted to validate published PCR-based  
112 methods by comparison with established bacteriological techniques using an extensive  
113 panel of clinical isolates. In addition to these important validation experiments the  
114 availability of large numbers of clinical samples enabled investigations to determine the  
115 distribution of *seeL/seeM* and *szeL/szeM* in isolates of *S. equi* and *S. zooepidemicus* that  
116 are at present circulating in the Italian horse population.

117

## 118 **2. Materials and methods**

### 119 *2.1 Bacteriological examination*

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

128 The Lancefield serological groups were determined using the Slidex Strepto-kit  
129 (BioMérieux, Milan, Italy) following the manufacturer's instructions.

### 130 2.2 DNA extraction and Polymerase Chain Reaction (PCR)

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

140 The 16S rRNA, *seeL* and *seeM* PCR reaction mixtures contained 0.7 µl of each primer  
141 (10 µM), 0.8 µl dNTPs mix (10 mM), 1X PCR buffer, 1.5 mM of MgCl<sub>2</sub> 1.6 U of Taq  
142 DNA polymerase (all reagents from Invitrogen, UK) with distilled water to a final  
143 volume of 18 µl and 2 µl of each DNA sample preparation. Reaction conditions  
144 included an initial denaturation step of 94 °C for 3 min followed by 30 cycles of 94 °C  
145 for 30 sec; 54 °C for 30 sec and 72 °C for 1 min with a final extension of 72 °C for 5  
146 min. The PCR reactions were conducted using a Gene Amp PCR System 2400 thermal  
147 cyclor (Applied Biosystems, USA) with PCR products resolved by electrophoresis in 1-  
148 2 % agarose gel containing 4 µl of GelRed (Nucleic Acid Gel Stain, 10,000X in water)  
149 per 100 ml (Biotium, USA). All stained PCR products were visualized by  
150 transillumination (Euroclone, Italy).

### 151 2.3 Cloning and sequencing

152 *SodA*, *seeI* and streptokinase PCR products were purified using Jet Quick gel extraction  
153 spin kit (Genomed GmbH) according to the manufacturer's instructions and subjected to



154 direct sequencing whereas amplicons derived from the *seeL* and *seeM* genes of either *S.*  
155 *equi* or *S. zooepidemicus* were cloned into a pCR2.1-TOPO vector (TOPO TA Cloning  
156 Kit for subcloning, Invitrogen, UK) as described by the manufacturer. Gel extracted and  
157 purified plasmid DNA (DNA plasmid purification kit, Invitrogen, UK) products were  
158 submitted for nucleotide sequencing (PRIMM srl Milan, Italy). All gel purified PCR  
159 products were sequenced using gene specific primers whereas those products that had  
160 been molecularly cloned into pCR2.1-TOPO were sequenced with M13 universal  
161 primers. The final sequences were aligned using AlignX (Vector NTI) and their identity  
162 confirmed by conducting a BLAST search against prokaryotic databases (NCBI, USA).

163

### 164 **3. Results**

165 The conventional bacteriological techniques were conducted independently of the  
166 molecular analyses and revealed that all 85 isolates were  $\beta$ -haemolytic Streptococci  
167 with Lancefield group C antigens. Furthermore, the API 20 Strep System showed the  
168 vast majority of these isolates (78) were *S. zooepidemicus* with just 5 *S. equisimilis* and  
169 2 *S. equi* strains detected. The PCR analyses matched these results perfectly. All isolates  
170 were confirmed to be members of the *Streptococcus* genus by 16SrRNA PCR (data not  
171 shown) with 78 producing positive reactions with primers for *sodA* (235 bp product)  
172 and negative for *seeI* consistent with *S. zooepidemicus*, 2 giving positive reactions with  
173 both *sodA* and *seeI* (520 bp product) as predicted for *S. equi* (Figure 1a) and 5 positive  
174 for the streptokinase (279 bp product) of *S. equisimilis* (Figure 1b). Nucleotide sequence  
175 analysis confirmed the identity of each PCR product demonstrating a minimum 99%  
176 homology with *sodA*, *seeI* and streptokinase gene sequences registered in NCBI  
177 database (GenBank<sup>TM</sup> accession numbers NC011134; AE006546; AF104301). In the  
178 case of primers designed to detect the *seeL* and *seeM* superantigen genes, specific  
179 amplicons with sizes of 800 and 810 bp respectively, were observed in both *S. equi*

180 isolates and in 2 (2.5%) of the *S. zooepidemicus* strains (Figure 2). The PCR amplified  
181 products were cloned in preparation for nuclear sequence analysis. Comparison of these  
182 sequences demonstrated both genes were extensively (99% homology) conserved both  
183 between individual isolates and between the two species (data not shown). In addition,  
184 *seeL/seeM* from *S. equi* and *szel/szeM* from *S. zooepidemicus* isolates were homologous  
185 (99% homology) to *seeL*, *seeM*, *szel*, and *szeM* sequences registered in the NCBI  
186 database (GenBank<sup>TM</sup> accession numbers AJ583527, AJ583528, AJ851369 AJ851826)  
187 along with those published by Alber et al. (2005).

188

#### 189 **4. Discussion**

190 Although conventional culture methods will always be valuable for the isolation and  
191 characterization of bacterial strains, molecular diagnostic techniques can have  
192 considerable advantages in terms of speed, specificity and sensitivity. Furthermore,  
193 molecular techniques are not limited to the detection of pathogenic organisms but can  
194 be used to differentiate closely related species and identify potential virulence factors.  
195 However, before any new technique can become part of a routine diagnostic procedure  
196 it must be carefully validated against the established techniques using an extensive  
197 number of clinical specimens, also considering that PCR does not distinguish between  
198 dead and live bacteria.

199 In this study PCR-based methods for detection of  $\beta$ -haemolytic *Streptococci*,  
200 differentiation of *S. equi* from *S. zooepidemicus* and identification of *S. equisimilis* were  
201 compared with conventional bacterial culture, biochemical and antigenic techniques  
202 using a panel of 85 uterine swabs from confirmed cases of endometritis. There was  
203 100% concordance between conventional and molecular techniques with both assay  
204 formats demonstrating the sample panel comprised 92% *S. zooepidemicus*, 6% *S.*  
205 *equisimilis* and 2% *S. equi*. These results are in agreement with previously published

206 studies of conventional bacteriological techniques that showed that  $\beta$ -haemolytic  
207 *Streptococcus* group C (Frontoso et al. 2008) and in particular *S. zooepidemicus* (Albihn  
208 et al. 2003) are the main cause of fertility problems in mares. In addition, they support  
209 conclusions reached by Albihn et al. (2003) that  $\beta$ -haemolytic *Streptococci* other than *S.*  
210 *zooepidemicus* can be isolated from the mare reproductive tract; in fact Albihn et al.  
211 (2003) identified 31  $\beta$ -haemolytic *Streptococci* isolates, and *S. zooepidemicus* in 68% of  
212 cases with 13% and 3% comprising of *S. equisimilis* and *S. equi* respectively. These  
213 authors were unable to identify the species by biochemical analysis in the remaining  
214 16% of the samples. The demonstration that multiple  $\beta$ -haemolytic *Streptococci* species  
215 are associated with endometritis in the horse is an important finding and it is  
216 particularly interesting that *S. equi*, an organism that is traditionally associated with  
217 lymph nodes of the upper respiratory tract, may also affect equine reproductive health.  
218 The results of the comparative validation assays demonstrate the reliability of the  
219 16SrRNA PCR assay for detecting *Streptococci*, the multiplex PCR for differentiating  
220 between *S. zooepidemicus*, and *S. equi*, and PCR assays based on streptokinase genes  
221 for identification of *S. equisimilis*. The reproducibility of these molecular assays makes  
222 them ideal candidates for inclusion in routine diagnosis where they can be used to  
223 corroborate results of conventional culture, biochemical and serological techniques.  
224 However, they could be used to provide a very rapid initial diagnosis and therefore  
225 facilitate the formulation of early treatment options, by adapting them for use on  
226 material extracted directly from uterine swabs. In addition to validation experiments,  
227 access to large numbers of samples containing  $\beta$ -haemolytic *Streptococci* from cases of  
228 endometritis in horses has also enabled important studies to determine the prevalence of  
229 the SeeL and SeeM SAGs. As stated above, *seeL* and *seeM* genes were initially  
230 identified from data generated during the whole genome sequencing of *S. equi* at the  
231 Sanger Centre (Proft et al., 2003). However, these authors were unable to detect these

232 genes in any of the 8 *S. equi* isolates they had available for analysis. In contrast, *seeL*  
233 and *seeM* were present in all 17 *S. equi* isolates examined by Alber et al (2005)  
234 suggesting these genes occur frequently in this highly pathogenic *Streptococcal* species.  
235 Furthermore, Alber et al. (2005) discovered very closely related orthologs of these SAg  
236 genes in 1 of 31 *S. zooepidemicus* (*szel*, *szem*) isolates and Holden et al. (2009) found  
237 that the genes encoding SeeL and SeeM were present in all strains of *S. equi* and 4 of  
238 140 isolates of *S. zooepidemicus* tested. The results presented in this report are  
239 consistent with those of Alber et al. (2005) and with those of Holden et al. (2009) in that  
240 *seeL* and *seeM* were present in the *S. equi* isolates. In addition, we confirm observations  
241 that orthologs of these SAg genes are present in a minority of *S. zooepidemicus*  
242 isolates. However, to our knowledge, this is the first report of *szel* and *szem* possession  
243 by *S. zooepidemicus* isolates derived from different outbreaks of endometritis in mares.  
244 In fact the *S. zooepidemicus* strain described by Alber et al. (2005) derived from a cow  
245 with mastitis and the ones described by Paillot et al. (2010) from equine respiratory  
246 disease. The *S. zooepidemicus* isolates described by Holden et al. (2009) were recovered  
247 from the same outbreak of equine respiratory disease; moreover they analyzed 45 *S.*  
248 *zooepidemicus* isolated from uterine infection/abortion and none was reported to  
249 possess *seeL* and *seeM* genes. The exact role of sAgs in the pathogenicity of  $\beta$ -  
250 haemolytic-Streptococci infections remains to be elucidated. Paillot et al. (2010)  
251 demonstrated that proteins encoded by *seeI*, *seeL* and *seeM* possess significant  
252 mitogenic activity including the induction of gamma interferon expression, in equine  
253 peripheral mononuclear cell cultures. Superantigen-induced proliferation was dose  
254 dependent, with activity observed at very low concentrations. In particular SeeM  
255 possessed the most potent activity. Therefore, it is proposed these antigens may play an  
256 important role in *S. equi* pathogenicity by stimulating an overzealous and inappropriate  
257 Th1 response. Considering the extent of homology between the *seeL* and *szel* plus

258 *seeM* and *szeM* genes, it is very likely the proteins they encode will have very similar  
259 mitogenic properties and might have an important role in the pathogenicity of  
260 endometritis. Therefore, further studies are required to determine the prevalence of *szeL*  
261 and *szeM* in *S. zooepidemicus* strains isolated from different host species and to  
262 investigate if possession of these SAg genes enhances pathogenicity.

263

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336 **Figure Captions.**

337 Figure 1. Molecular characterization of  $\beta$ -haemolytic-Streptococci

338 Figure 1a – Multiplex PCR.

339 Figure 1b – *S. equisimilis* PCR.

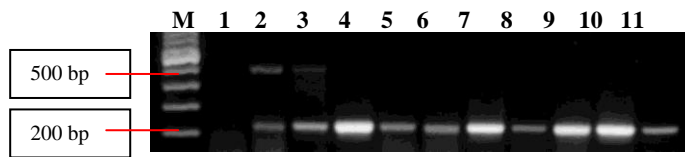
340 Figure 2. *SeeL* and *seeM* PCR products

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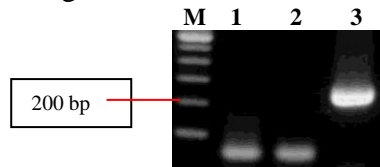
Figure 1

Figure 1a.



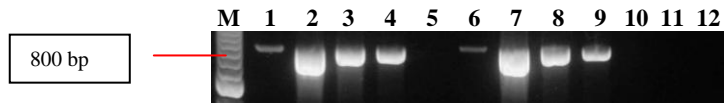
M, 100 bp DNA ladder;  
1, PCR negative control;  
2 and 3, *S. equi* strains positive for *sodA* and *seel*;  
4-11, *S. zooepidemicus* strains positive for *sodA* and negative *seel*.

Figure 1b



M, 100 bp DNA ladder;  
1, PCR negative control;  
2  $\beta$ -haemolytic-*Streptococcus* negative strain;  
3, *S. equisimilis* strain positive

Figure 2.



M, 100 bp DNA ladder; 1 and 4, *seeL S. equi*;  
2 and 3, positive *seeL S. zooepidemicus*;  
5, negative *seeL S. zooepidemicus*; 6 and 9, *seeM S. equi*;  
7 and 8, positive *seeM S. zooepidemicus*;  
10, negative *seeM S. zooepidemicus*;  
11 and 12, *seeL* and *seeM* PCRs negative controls.