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V. Båverud, S.K. Johansson, A. Aspan. Real-time PCR for Detection and Differentiation of subsp. e and subsp.. *Veterinary Microbiology*, 2007, 124 (3-4), pp.219. 10.1016/j.vetmic.2007.04.020 . hal-00532249

**HAL Id: hal-00532249**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

Title: Real-time PCR for Detection and Differentiation of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus*

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PII: S0378-1135(07)00187-3  
DOI: doi:10.1016/j.vetmic.2007.04.020  
Reference: VETMIC 3664

To appear in: *VETMIC*

Received date: 3-10-2006  
Revised date: 27-3-2007  
Accepted date: 5-4-2007

Please cite this article as: Båverud, V., Johansson, S.K., Aspan, A., Real-time PCR for Detection and Differentiation of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus*, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.04.020

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**Real-time PCR for Detection and Differentiation of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus***

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**Keywords**

*Streptococcus equi*, *Streptococcus zooepidemicus*, *Streptococcus ruminatorum*, real-time PCR, 16S rRNA, *sodA*

1 **Abstract**

2 Strangles is a contagious equine disease caused by *Streptococcus equi* subsp. *equi*. In this  
3 study, clinical strains of *S. equi* (n=24) and *S. equi* subsp. *zooepidemicus* (n=24) were  
4 genetically characterized by sequencing of the 16S rRNA and *sodA* genes in order to devise a  
5 real-time PCR system that can detect *S. equi* and *S. zooepidemicus* and distinguish between  
6 them.

7

8 Sequencing demonstrated that all *S. equi* strains had the same 16S rRNA sequence, whereas  
9 *S. zooepidemicus* strains could be divided into subgroups. One of these (n=12 strains) had 16S  
10 rRNA sequences almost identical with the *S. equi* strains. Interestingly, four of the strains  
11 biochemically identified as *S. zooepidemicus* were found by sequencing of the 16S rRNA  
12 gene to have a sequence homologous with *S. equi* subsp. *ruminatorum*. However, they did not  
13 have the colony appearance or the biochemical characteristics of the type strain of *S.*  
14 *ruminatorum*. Classification of *S. ruminatorum* may thus not be determined solely by 16S  
15 rRNA sequencing.

16

17 Sequencing of the *sodA* gene demonstrated that all *S. equi* strains had an identical sequence.  
18 For the *S. zooepidemicus* strains minor differences were found between the *sodA* sequences.

19

20 The developed real-time PCR, based on the *sodA* and *seeI* genes was compared with  
21 conventional culturing on 103 cultured samples from horses with suspected strangles or other  
22 upper respiratory disease. The real-time PCR system was found to be more sensitive than  
23 conventional cultivation as two additional field isolates of *S. equi* and four of *S.*  
24 *zooepidemicus* were detected.

1

**2 1. Introduction**

3 Strangles is a highly contagious and serious worldwide infectious disease in horses. The  
4 causative organism is *Streptococcus equi* subsp. *equi* (*S. equi*). Typical clinical signs are  
5 purulent nasal discharge, pyrexia, anorexia and swollen submandibular and retropharyngeal  
6 lymph nodes which frequently form abscesses. The outcome is only rarely fatal due to  
7 complications (Sweeney et al., 2005).

8

9 Strangles is a common infection among the Swedish horse population; with 76 reported  
10 outbreaks in the year 2005 (Swedish Board of Agriculture). It is a notifiable disease based on  
11 laboratory confirmation or clinical symptoms. Speed of diagnosis is of great importance to  
12 prevent spreading of the disease, as morbidity is so high. There is a great demand by  
13 clinicians and horse owners in Sweden for earlier laboratory confirmation than is provided  
14 with conventional cultivation.

15

16 *Streptococcus* species identification is customarily based on biochemical typing schemes,  
17 including Lancefield grouping (Quinn et al., 1994). Closely related streptococcal species have  
18 a high sequence similarity in 16S rRNA (Kawamura et al., 1995), and thus it can be difficult  
19 to use sequence data from this gene for speciation. An alternative approach is the sequencing  
20 of the gene for manganese-dependent superoxide dismutase A (*sodA*), which encodes for an  
21 enzyme that contributes to the bacterium's defence against oxidative stress. This gene has  
22 been sequenced for a large number of streptococcal species, including *S. equi*, and been found  
23 to be suitable for species identification of streptococci (Poyart et al., 1998).

24

1 The pyrogenic mitogen SePE-I (*seeI*), was characterized and found to be consistently present  
2 in *S. equi* and the human pathogen *Streptococcus (S.) pyogenes* but absent from the closely  
3 related *Streptococcus equi* subsp. *zooepidemicus (S. zooepidemicus)* (Artiushin et al., 2002;  
4 Hynes, 2004). The *sod A* and *seeI* genes have earlier been used as targets in PCR (Alber et al.,  
5 2004).

6  
7 The purpose of this study was to genetically characterize a set of Swedish clinical strains of *S.*  
8 *equi* and *S. zooepidemicus* and on the basis of this knowledge devise a real-time PCR system  
9 that can quickly detect *S. equi* and *S. zooepidemicus* and distinguish between them.

10

## 11 **2. Materials and Methods**

### 12 **2.1 Bacterial strains**

13 Altogether, 24 clinical strains of *S. equi* subsp. *equi* and 24 *Streptococcus equi* subsp.  
14 *zooepidemicus* obtained from the Institute's strain collection (National Veterinary Institute,  
15 (SVA)) were used in this study. The clinical strains had been isolated from horses showing  
16 symptoms of strangles or other upper respiratory diseases and typed by applying standard  
17 biochemical tests (see 2.2). In addition, the following type and reference strains were included  
18 in the study: *Streptococcus agalactiae* CCUG 39325, *Streptococcus canis* CCUG 37323,  
19 *Streptococcus dysgalactiae* subsp. *dysgalactiae* CCUG 27436, *S. equi* subsp. *equi* ATCC  
20 33398/CCUG 23255<sup>T</sup>, *S. equi* subsp. *equi* CCUG 27367, *S. equi* subsp. *ruminantium* CCUG  
21 47 520<sup>T</sup>, *Streptococcus equi* subsp. *zooepidemicus* ATCC 43079/CCUG 23256<sup>T</sup>,  
22 *Streptococcus pyogenes* ATCC 12344/CCUG 12701<sup>T</sup> and *Streptococcus uberis* CCUG  
23 27444, all obtained from the Culture Collection, University of Göteborg (CCUG), Sweden.

24

25

1

2 **2.2 Culture and biochemical identification**

3 Nasal swab samples and tracheal wash fluid or swabs were transported to the laboratory at  
4 ambient temperature. Fluid samples were centrifuged at 600 g for 10 min. The pellets and the  
5 swabs were cultured within 24h on COBA (Colistin Oxolinic Blood Agar) plates (Columbia  
6 agar base [Oxoid CM331], 39g/L; Streptococcus selectatab, [Mast Diagnostic], 4 tablets/L;  
7 citrated horse blood, 50ml/L [SVA]);  
8 two horse blood agar plates (blood agar base no. 2, [LabM lab 15], 39.5 g/L; citrated horse  
9 blood [SVA], 50ml/L) and on bromcresolpurpur lactose agar plates (balanced peptone, 10g/L  
10 [LabM MC4]; NaCl, 5g/L [Merck]; sodium ammoniumphosphate, 1g/L [Merck 1.06682];  
11 Lab Lemco Powder, 4g/L [Oxoid L29]; Agar no. 2, 10g/L [LabM MC6]; lactose solution  
12 20%, 50ml/L [SVA]; bromcresolpurpur solution 1.6%, 1ml/L [SVA]). One of the horse blood  
13 agar plates was together with the COBA plate, incubated in an atmosphere containing 5%  
14 CO<sub>2</sub> at 37°C, while the other agar plates were incubated in aerobic atmosphere at 37°C. All  
15 agar plates were read after 24 and 48h. Typical β-haemolytic streptococci-like colonies were  
16 subcultured on 5% horse blood agar and identified by characteristic colony morphology,  
17 Gram stain, and biochemical tests including catalase. Isolates identified as *S. equi* fermented  
18 salicin and sucrose but not sorbitol, lactose, raffinose, inulin, trehalose, glycerine or mannitol.  
19 The isolates hydrolysed starch but not aesculin. Isolates identified as *S. zooepidemicus* gave  
20 the same biochemical results but fermented sorbitol and lactose. Isolates identified as  
21 *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*), also a Lancefield group C  
22 streptococcus, gave as well the same biochemical results but fermented trehalose but not  
23 sorbitol, while lactose varied.

24

### 1 **2.3 DNA preparation**

2 All clinical strains from the strain collection were recovered from frozen condition ( $-70^{\circ}\text{C}$ ) in  
3 serum broth with 15% glycerol and were subcultured twice on 5% horse blood agar plates  
4 prior to DNA preparation. A loopful of bacteria was suspended in 450  $\mu\text{l}$  of 0.1M Tris-HCl,  
5 pH 8.5, supplemented with 0.05% Tween 20 and 0.24 mg/ml proteinase K, and incubated at  
6  $60^{\circ}\text{C}$  for one hour. Then the samples were incubated at  $90^{\circ}\text{C}$  for 15 min, and immediately  
7 placed on ice for 10 min. The suspension was centrifuged at 10,000g for 5 min. and the  
8 supernatant was used as template in the PCR reactions.

9

### 10 **2.4 Investigation of strains for the presence of *sodA* and *seel* genes by PCR**

11 The clinical strains of *S. equi* ( $n=24$ ) and *S. zooepidemicus* ( $n=24$ ), as well as all reference  
12 strains ( $n=9$ ) used in this study, were investigated by the multiplex PCR which used the *sodA*  
13 and the *seel* genes as targets as described by Alber et al. (2004).

14

### 15 **2.5 Molecular characterization of strains by sequencing of 16S rRNA and *sodA* genes**

16 All clinical strains of *S. equi* ( $n=24$ ) and *S. zooepidemicus* ( $n=24$ ), as well as the reference  
17 strains of *S. equi* and *S. zooepidemicus* used in this study, were sequenced for parts of the 16S  
18 rRNA and *sodA* genes (PCR and sequencing primers are listed in Table 1). PCR before  
19 sequencing was carried out in a 50- $\mu\text{l}$  reaction mixture of 50 mM KCl, 10mM Tris-HCl (pH  
20 8.3), 1.5 mM  $\text{MgCl}_2$ , 0.2 mM each of deoxynucleoside triphosphates, 0.2  $\mu\text{M}$  of each primer  
21 and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif., USA).  
22 The reaction mixture was amplified with a GeneAmp 9700 Thermocycler (Applied  
23 Biosystems). The amplification reaction was started by heating at  $95^{\circ}\text{C}$  for 8 min. A  
24 procedure comprising 35 cycles of  $95^{\circ}\text{C}$  for 30 sec,  $55^{\circ}\text{C}$  for 30 sec and  $72^{\circ}\text{C}$  for 1 min. was  
25 performed. Negative controls with the DNA template replaced by water in the reaction



1 mixture were included in each PCR run. Amplicons were visualized on 1.5% agarose gels  
2 with a 100 base-pair ladder (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as molecular  
3 weight markers. DNA sequencing reactions using fluorescently labelled dideoxynucleotide  
4 technology were applied. The PCR products were diluted 1:2 and cycle sequencing reactions  
5 were carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)  
6 according to the manufacturer's recommendation. The nucleotide sequences were determined  
7 by separating the dideoxy fragments on the ABI PRISM 3100 Genetic Analyzer (Applied  
8 Biosystems). The sequence was fully determined for both strands of each DNA template to  
9 ensure maximum accuracy of the data. Sequences were edited, assembled and analyzed using  
10 Vector NTI Advance 9.0 (InforMax Inc., Bethesda, Md, USA).

11

12 *S. zooepidemicus* 16S rRNA and *sodA* sequences were aligned with ClustalW Multiple  
13 Sequence Alignment tool and rooted phylogenetic trees were constructed by the use of  
14 ClustalW Multiple Sequence Alignment, using default settings. This was performed via  
15 Biology WorkBench 3.2 (<http://workbench.sdsc.edu/>). We also included one example of the  
16 *S. equi* sequences, as well as the sequences of the type strains of *S. equi*, *S. zooepidemicus*,  
17 and *Streptococcus equi* subsp. *ruminatorum*. *S. canis* (ATCC 43 498) was used as outgroup  
18 (Fig. 1-2).

19

## 20 **2.6 Extended typing of *S. zooepidemicus***

21 The clinical strains biochemically identified (see 2.2) as *S. zooepidemicus* ( $n=24$ ) were  
22 subjected to typing with additional tests, hippurate and ribose so as to compare them with the  
23 typing scheme described for *S. ruminatorum*. Lancefield grouping was performed with the  
24 commercial Streptococcal grouping kit to identify streptococcal serogroups DR 0585A

1 (Oxoid, Basingstoke, Hampshire, England). The results of this procedure were then compared  
2 to the sequence data obtained from the 16S rRNA (Table 2) and *sodA* genes.

3

#### 4 **2.7 Design of species-specific real-time PCR system**

5 To design real-time PCR primers and probes, the following GenBank accession numbers (acc.  
6 no.) were used: *sodA* Z95901; Z95902 and *seel* AF186180. For primers and probes, see Table  
7 1. The final optimized PCR reaction mixture contained 50 mM KCl, 10mM Tris-HCl (pH  
8 8.3), 0.6  $\mu$ M of each primer for *sodA* and 0.9  $\mu$ M of each primer for *seel*, 0.20  $\mu$ M of each  
9 probe, 6mM MgCl<sub>2</sub>, 8mM dNTP and 1.25 U of AmpliTaq Gold (Applied Biosystems) in a  
10 total volume of 23  $\mu$ l.

11 Finally, 2  $\mu$ l of template DNA was added to each reaction tube. The tubes were then subjected  
12 to 95°C for 8 min followed by a program of 45 cycles of 95°C for 20 sec and 60°C for 60 sec,  
13 on a real-time PCR thermal cycler RotorGene 3000 (Corbett Research, Sydney, Australia).

14

#### 15 **2.8 Comparison of real-time PCR from agar plates for detection and subspecies**

##### 16 ***determination of S. equi and S. zooepidemicus versus conventional culture combined with*** 17 ***biochemical identification***

18 During a 6-week period, all clinical specimens sent to our lab from horses suspected of  
19 suffering from strangles or other upper respiratory tract infections were included in this  
20 comparative study. The nasal swab samples or tracheal wash fluid were cultured on agar  
21 plates as described in 2.2 above. In samples from 103 horses, typical  $\beta$ -haemolytic  
22 streptococci-like colonies grew on the agar plates, and one colony was selected for further  
23 confirmation and was subcultured overnight. Next day, this culture was analysed by  
24 biochemical testing (see 2.2 above) to verify the presence of *S. equi*, *S. zooepidemicus* or *S.*  
25 *equisimilis*. Simultaneously, from each primary culture, a DNA preparation (see 2.3) was

1 made from the primary streak, and also from the same colony as was subcultured for  
2 biochemical confirmation. These two DNA preparations were subjected to real-time PCR  
3 analysis, as described above.

4

### 5 **3. Results**

#### 6 **3.1 Investigation of strains for the presence of *sodA* and *seeI* genes by PCR**

7 The *sodA* PCR (Alber et al., 2004) gave amplicons for all clinical strains biochemically typed  
8 to *S. equi* ( $n=23$ ) and *S. zooepidemicus* ( $n=24$ ) except for two strains. One of these strains  
9 was later confirmed by biochemical tests and Lancefield grouping (Group G) to be *S. canis*  
10 and not *S. equi*. The second strain (Bd 11725/03) was confirmed to be *S. zooepidemicus* but  
11 had a deviant *sodA* gene sequence in the region where the reverse primer should have  
12 annealed (see 3.3 and 3.7). The reference strains of *S. equi* and *S. zooepidemicus* used in this  
13 study were also positive by PCR but not the reference strains of the other streptococcal  
14 species.

15 The *seeI* gene-based PCR (Alber et al., 2004) gave amplicons for all clinical strains ( $n=23$ )  
16 and reference strains of *S. equi*, but not the *S. zooepidemicus* strains or the *S. canis* strain. For  
17 the other reference strains used it gave also an amplicon for *S. pyogenes*.

18

#### 19 **3.2 Molecular characterization of strains by sequencing of 16S rRNA gene**

20 All clinical strains of *S. equi* ( $n=23$ ) had identical 16S rRNA sequence. The 24<sup>th</sup> strain had a  
21 16S rRNA sequence which indicated that this strain was *S. canis* and not *S. equi*. The 24  
22 clinical strains biochemically typed to *S. zooepidemicus* could be divided into subgroups  
23 according to their 16S rRNA gene sequences (Fig. 1). One of these groups ( $n=12$ ; 16S rRNA  
24 genogroup I) had 16S rRNA sequences identical or almost identical with the *S. equi* strains;  
25 another group ( $n=4$ ; 16S rRNA genogroup II) had 16S rRNA sequences that matched the

1 published sequence of the recently described subspecies *S. ruminatorum*. Yet other variants  
2 were intermediates between *S. equi* and *S. ruminatorum* ( $n=5$ ; 16S rRNA). The sequences  
3 were deposited in GenBank under acc.no. EF405986-EF406029. Even more interesting, three  
4 clinical strains carried two variants of the 16S rRNA gene, one similar to *S. equi* and one  
5 more similar to *S. ruminatorum* (Table 2).

### 7 **3.3 Molecular characterization of strains by sequencing of *sodA* gene**

8 Part of the *sodA* gene was sequenced for all clinical strains, to better establish the genetic  
9 relationship between the strains. The 435 base pair sequences of the 23 clinical strains of *S.*  
10 *equi* were all alike, and also identical with the two *S. equi* reference strains. Minor differences  
11 were observed between the *sodA* sequences of the clinical strains of the 24 *S. zooepidemicus*  
12 (Fig. 2). However, these differences were not consistent with the grouping made by the 16S  
13 rRNA sequencing data (Fig. 2, Table 2). Also, one clinical strain of *S. zooepidemicus*  
14 (Bd 11 725/03) differed substantially in one part of the *sodA* sequence. The sequences were  
15 deposited in GenBank under acc. no. EF406036-EF406082.

### 17 **3.4 Extended typing of *S. zooepidemicus***

18 All clinical strains ( $n=24$ ) of *S. zooepidemicus* were Lancefield group C. The four clinical  
19 strains with a 16S rRNA sequence (16S rRNA genogroup II) resembling *S. ruminatorum* did  
20 not have the colony appearance or biochemical characteristics of hippurate, salicin, and  
21 sucrose of the type strain according to tests performed, see 2.2 and 2.7. Their *sodA* sequences  
22 did not cluster with that of the type strain of *S. ruminatorum* (Table 2, Fig 2).

### 24 **3.5 Design of species-specific real-time PCR system**

1 The real-time PCR system developed in this study could assign all clinical strains of *S. equi*  
2 ( $n=23$ ) and *S. zooepidemicus* ( $n=24$ ) from the strain collection and the reference strains of *S.*  
3 *equi* and *S. zooepidemicus* to the expected subspecies. The other streptococcal reference  
4 strains were not detected by the system, except for *S. pyogenes*. However, *S. pyogenes* could  
5 be readily distinguished from *S. equi* and *S. zooepidemicus*, as only an amplicon for *seeI* and  
6 not for the *sodA* gene was produced. The clinical strain of *S. canis* from the strain collection  
7 proved negative by real-time PCR for both the *sodA* and the *seeI* genes.

8

### 9 ***3.6 Comparison of real-time PCR from agar plates for detection and subspecies***

#### 10 ***determination of S. equi and S. zooepidemicus versus conventional culture combined with*** 11 ***biochemical identification***

12 From 103 clinical samples a comparison was made between real-time PCR and conventional  
13 culture techniques in combination with biochemical typing. The results of this comparison are  
14 presented in Tables 3-5. All field isolates ( $n=15$ ) identified by biochemical tests as *S. equi* and  
15 all ( $n=77$ ) identified as *S. zooepidemicus*, except two (see 3.7), were detected by real-time  
16 PCR. Two additional field isolates of *S. equi* and four of *S. zooepidemicus*, that were negative  
17 by cultivation with biochemical typing, were detected by real-time PCR.

18

### 19 ***3.7 S. zooepidemicus isolates with an aberrant sodA gene sequence***

20 One clinical strain (Bd 11 725/03, GenBank acc.no. EF406076, Fig. 1) from the strain  
21 collection (see 3.3) and two field isolates (Bd 16498/05, acc.no. EF406083 and Bd 4116/06,  
22 acc.no. EF406084) among the samples analysed as described in 3.6, had an aberrant *sodA*  
23 gene sequence in a few bases (Fig. 2). These three strains biochemically identified as *S.*  
24 *zooepidemicus* were confirmed to be *S. zooepidemicus* by 16S rRNA sequencing (Fig. 1), but  
25 proved negative by real-time PCR for both the *sodA* and the *seeI* genes. As we have chosen to

1 regard these strains as *S. zooepidemicus*, we decided to include an additional reverse primer  
2 for the *sodA* gene in the final real-time PCR protocol, at a concentration of 0.3  $\mu$ M (primer  
3 *SodA*-Bd 4116/06-R). This extended real-time PCR system is currently being evaluated at the  
4 SVA diagnostic laboratory.

5

## 6 **Discussion**

7 In the present investigation, a set of clinical strains from the institute's strain collection were  
8 genetically characterized by sequencing of the 16S rRNA and *sodA* genes in order to devise a  
9 real-time PCR for detection and differentiation of *S. equi* and *S. zooepidemicus*. *S.*  
10 *zooepidemicus* was included in the PCR method as it is an opportunistic commensal and may  
11 according to Timoney et al. (2004) produce disease in situations of e.g. virus infection or heat  
12 stress, and it was therefore of interest to investigate in samples from horses with respiratory  
13 disease. The 16S rRNA sequences for all clinical strains and type strains of *S. equi* included in  
14 this study were identical. However, the 16S rRNA sequence obtained in this study for one of  
15 the type strains, *S. equi* ATCC 33398 (acc.no. EF406032) differed in a segment of 13  
16 nucleotides from the sequence for this type strain earlier deposited in GenBank (acc. no.  
17 AB002515). Interestingly in our study, 50% (12/24) of the clinical strains and the type strain  
18 (ATCC43079) of *S. zooepidemicus* had almost identical 16S rRNA sequence with the  
19 sequence obtained for all the *S. equi* strains. We therefore conclude that the 16S rRNA  
20 sequence seems to be conserved in *S. equi* and in one 16S rRNA genogroup of *S.*  
21 *zooepidemicus*. For the other clinical strains of *S. zooepidemicus* we found variations and the  
22 strains could be divided into genogroups. In GenBank, 16S rRNA variants of *S.*  
23 *zooepidemicus* can be found deposited (acc. no. AF073802-10, AF073813-14). However,  
24 these sequences are shorter than ours and a complete comparison can, therefore, not be made.  
25 Abdulmawjood and Lammler (2000) demonstrated variations of the V2 region of the 16S

1 rRNA gene between *S. zooepidemicus* strains. This tallies with the results of different  
2 genogroups of *S. zooepidemicus* presented in our study.

3

4 In this study, part of the *sodA* gene was sequenced and the sequences confirmed that *S. equi*  
5 strains are closely related, whereas *S. zooepidemicus* has a greater variability between strains.  
6 However, the phylogenetic relationship between the clinical strains of *S. zooepidemicus*  
7 suggested by 16S rRNA gene sequencing was not confirmed by the *sodA* sequencing data.

8

9 A novel and interesting finding in our study was that four of the clinical strains biochemically  
10 identified as *S. zooepidemicus* were found by sequencing of the 16S rRNA gene to have a  
11 sequence homologous with the newly described subspecies *S. ruminatorum* (Fernandez et al.,  
12 2004). However, those clinical strains did not have the biochemical characteristics or the  
13 colony appearance of the type strain of *S. ruminatorum*. Surprisingly, their *sodA* sequences  
14 did not cluster with the *sodA* sequence of the type strain. We suggest that to establish  
15 definitely whether or not a strain should be classified as *S. ruminatorum* cannot be determined  
16 solely by 16S rRNA sequencing.

17 Moreover, we found three *S. zooepidemicus* strains that had an aberrant *sodA* gene sequence  
18 where the reverse primer in our real-time PCR system did not anneal. As this primer site  
19 overlaps with that described by Alber et al. (2004), neither of our PCR systems detected these  
20 strains. To overcome this problem an additional reverse primer for the *sodA* gene was  
21 designed and added to our real-time PCR system.

22

23 PCR detection of *S. equi* with the *SeM* gene as a target has been used in previous studies to  
24 enhance the sensitivity in the diagnostics of stranglers (Timoney and Artiushin 1997; Newton  
25 et al., 2000; Gronbaek et al., 2006). Alber and colleagues (2004) developed a multiplex PCR

1 in order to distinguish *S. equi* from *S. zooepidemicus*. We have used the same gene targets  
2 (*sodA* and *seeI*) as in their study. However, their PCR is in a conventional format, in contrast  
3 to the real-time PCR described in this paper.

4  
5 Our real-time PCR system was compared with conventional culturing techniques, on clinical  
6 samples sent to our laboratory from horses with suspected strangles or other upper respiratory  
7 tract infection. Agar plates with alleged growth of  $\beta$ -haemolytic streptococci were analysed in  
8 parallel by real-time PCR and biochemical typing. In 6 cases out of 103, real-time PCR was  
9 more sensitive than conventional culturing with biochemical typing. The real-time PCR  
10 method detected *S. equi* in two cases and *S. zooepidemicus* in four cases that were negative by  
11 conventional culture. The probability of detecting of *S. equi* was therefore enhanced by 13%  
12 (2/15) by PCR from agar plates, in comparison with conventional culture techniques. In  
13 studies by Gronbaek et al. (2006), PCR was performed both using DNA extracted from  
14 growth on the agar plate and by extracting DNA directly from clinical samples. PCR  
15 enhanced the sensitivity but it was not clear when the PCR was applied directly from the agar  
16 plate. Further studies are necessary to evaluate the real-time PCR system developed in our  
17 study directly on clinical samples. An advantage with PCR for detection and identification of  
18  $\beta$ -haemolytic streptococci from an agar plate can be that only viable bacteria are detected.  
19 Direct PCR from e.g., a guttural pouch sample may detect dead as well as living bacteria and  
20 needs to be subjected to further investigation (Sweeney et al., 2005). In samples PCR positive  
21 for *S. equi*, the bacterium should always be isolated in order to be able to perform further  
22 characterization and antimicrobial susceptibility testing (Keller and Hendrix, 2005).

23

24 *Streptococcus equisimilis* was demonstrated by biochemical typing to be present in 5 of the  
25 103 samples. As our real-time PCR system does not detect *S. equisimilis*, the PCR of the



1 individual colony of these samples was negative. However, one of these samples was PCR  
2 positive for *S. equi* and three for *S. zooepidemicus* from the primary streak. Interestingly, this  
3 finding demonstrates that in 80% of the samples with *S. equisimilis* other  $\beta$ -haemolytic  
4 streptococci such as *S. equi* and *S. zooepidemicus* were also present in the same sample. *S.*  
5 *equisimilis* is an infrequent opportunist in respiratory disease according to Timoney (2004).  
6 For three samples, PCR of a single colony proved positive for *S. zooepidemicus* but negative  
7 concerning the primary streak. We therefore recommend that the PCR is performed both on  
8 individual colonies and the primary streak.

9

#### 10 **In conclusion**

11 The real-time PCR for detection and differentiation of *S. equi* and *S. zooepidemicus* was  
12 found to be reliable, and shortened the turnaround time at the laboratory. Further studies are  
13 needed concerning differentiation of *S. zooepidemicus* into genogroups and of their possible  
14 correlation to different clinical signs of disease.

15

#### 16 **Acknowledgements**

17 Yvonne Andreasson, Helena Ljung and Karin Malm are acknowledged for excellent  
18 laboratory support. This research has been supported by the Swedish Foundation for Equine  
19 Research and the Agria Foundation for research.

20

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22

1 Figure 1

2 Phylogenetic tree showing the relationship among clinical and reference strains  
3 of *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equi* and the type  
4 strain of *Streptococcus equi* subsp. *ruminatorum* based on 16S rRNA sequences.

5 *Streptococcus canis* was used as outgroup. Genogroups defined according to this paper.

6 Note that strains of *S. equi* had identical 16S rRNA sequences whereas strains of *S.*  
7 *zooepidemicus* could be divided into genogroups. Four strains of *S. zooepidemicus* had 16S  
8 rRNA sequences (16S rRNA genogroup II) that matched the sequence of the reference strain  
9 of *S. ruminatorum*.

10 \*Two field isolates (Bd 16 498/05 and Bd 4116/06) for which an additional reverse primer for  
11 the *sodA* gene was designed and added to our real-time PCR system.

12

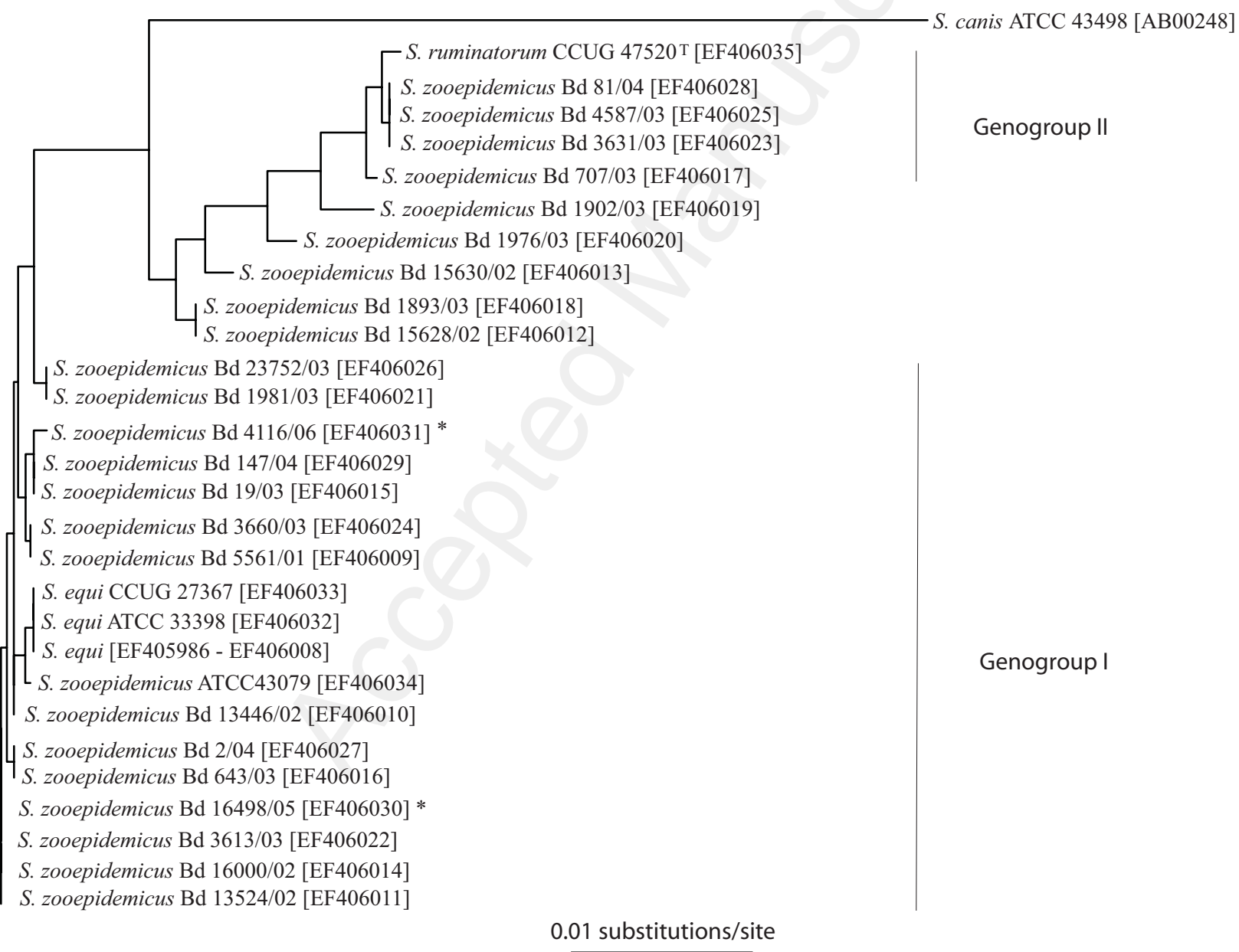
13

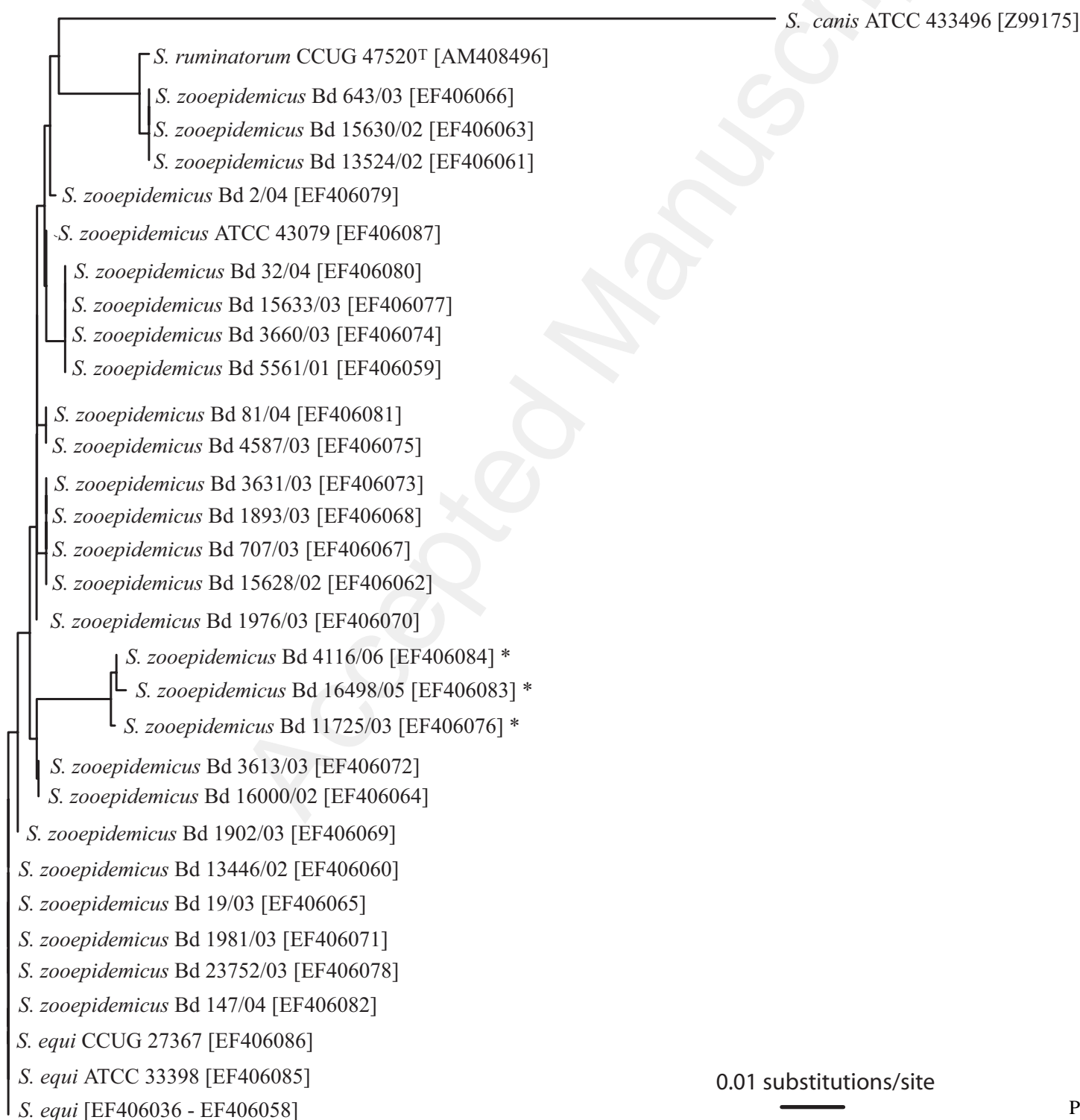
14 Figure 2

15 Phylogenetic tree showing the relationship among clinical and reference strains  
16 of *Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus equi* subsp. *equi* and the type  
17 strain of *Streptococcus equi* subsp. *ruminatorum* based on *sodA* sequences. *Streptococcus*  
18 *canis* was used as outgroup.

19 Note that the *sodA* sequences of all *S. equi* strains were all alike whereas minor differences  
20 were observed between the *sodA* sequences of the strains of *S. zooepidemicus*.

21 \*One clinical strain (Bd 11 725/03) and two field isolates (Bd 16 498/05 and Bd 4116/06) for  
22 which an additional reverse primer for the *sodA* gene was designed and added to our real-time  
23 PCR system. They were confirmed to be *S. zooepidemicus* by 16S rRNA sequencing and  
24 biochemical typing.





1 Table 1  
2 Primers used for PCR and sequencing in this study

Target gene	Oligonucleotide primer	Sequence (5'→3')	Application	Reference
<b>16SrRNA</b>	fD1	AGAGTTTGATCCTGGCTCAG	PCR & sequencing	Weisburg et al., 1991
	rP2	ACGGCTACCTTGTTACGACTT	PCR & sequencing	Weisburg et al., 1991
	16S 349F	AGGCAGCAGTGGGGAAT	Sequencing	This study
	16S 357R	CTGCTGCCTYCCGTA	Sequencing	This study
	16S 800F	AATAGATACCCTGGTAG	Sequencing	Drancourt et al., 1997
	16S 800R	CTACCAGGGTATCTAAT	Sequencing	Drancourt et al., 1997
	16S 1050F	TGTCGTCAGCTCGTG	Sequencing	Drancourt et al., 1997
	16S 1050R	CACGAGCTGACGACA	Sequencing	Drancourt et al., 1997
<i>sodA</i>	d1	CCITAYICITAYGAYGCIYTIGARCC	PCR & sequencing	Poyart et al., 1998
	d2	ARRTARTAIGCRTGYTCCCAIACRT	PCR & sequencing	Poyart et al., 1998
<i>sodA</i>	sodAequi/zooep-F	CAGCATTCTGCTGACATTCGTCAGG	PCR	Alber et al., 2004
	sodAequi/zooep-R	CTGACCAGCCTTATTCACAACCAGCC	PCR	Alber et al., 2004
<i>seeI</i>	seeI-F	GAAGGTCCGCCATTTTCAGGTAGTTT	PCR	Alber et al., 2004
	seeI-R	GCATACTCTCTGTCCACCATGTCCTG	PCR	Alber et al., 2004
<i>sodA</i>	SodA-For	AGAGCAATTCACAGCAGCA	Realtime PCR	This study
	SodA-Rev	ACCAGCCTTATTCACAACCA	Realtime PCR	This study
	SodA-Bd 4116/06-R	ACCGGCTTGGTTAACCCTA	Realtime PCR	This study
	SodA probe	CAGGCCCAACCTGAGCCAAA	Realtime PCR	This study
<i>seeI</i>	SeeI-For	CGGATACGGTGATGTTAAAGA	Realtime PCR	This study
	SeeI-Rev	TTCCTCCTCAAAGCCAGA	Realtime PCR	This study
	SeeI probe	TTTGCCGCTCCTCTAGATTTCAA	Realtime PCR	This study

3

Table 2

Comparison of biochemical typing and 16SrRNA sequencing results between clinical strains ( $n=24$ ) of *Streptococcus equi* subsp.

*zooepidemicus*, and with type strains of *Streptococcus equi* subsp. *ruminatorum*, *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp.

*zooepidemicus*

Strain (no of strains)	16S rRNA genogroup*	Hippurate	Salicin	Sucrose	Sorbitol	Lactose	Trehalose	Ribose
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 3613/03, 2/04 (2)	I	-	+	+	+	+	-	-
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 16000/02 (1)	I	-	+	+	+	+	-	(+)
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 5561/01, 13446/02, 13524/02, 19/03, 643/03, 1981/03, 3660/03, 23752/03, 147/04 (9)	I	-	+	+	+	+	-	+
<i>S. equi</i> subsp <i>zooepidemicus</i> ATCC 43079/CCUG 23256 <sup>T</sup> (1)	I	-	+	+	+	+	-	+
<i>S. equi</i> subsp <i>equi</i> CCUG 27367 and ATCC 33398/CCUG 23255 <sup>T</sup> (2)	I	-	+	+	-	-	-	-
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 1976/03 (1)	Intermediate	-	+	+	+	+	-	-
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 15628/02, 15630/02, 1893/03, 1902/03 (4)	Intermediate	-	+	+	+	+	-	+
<b><i>S. equi</i> subsp <i>zooepidemicus</i> Bd 707/03, 3631/03, 4587/03, 81/04 (4)</b>	<b>II</b>	-	+	+	+	+	-	+
<b><i>S. equi</i> subsp <i>ruminatorum</i> CCUG 47520<sup>T</sup> (1)</b>	<b>II</b>	+	-	-	+	+	-	+
<i>S. equi</i> subsp <i>zooepidemicus</i> Bd 11725/03**, Bd 15663/03, Bd 32/04 (3)	Mix***	-	+	+	+	+	-	+

\*Numbering of genogroups is in concordance with the numbering in Figure 1.

\*\*This isolate required an additional reverse primer for the *sodA* gene to be designed and added to the real-time PCR system.

\*\*\*Three clinical strains of *S. equi* subsp. *zooepidemicus* carried two variants of the 16S rRNA gene, one similar to *S. equi* subsp. *equi* and one more similar to *S. equi* subsp. *ruminatorum*.



Table 3

Comparison of real-time PCR from agar plates for detection and subspecies determination of *Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus*, versus conventional culture in combination with biochemical identification for nasal swab samples or tracheal wash fluid from 103 horses with suspected strangles or other upper respiratory tract infection

No. of samples	Results from conventional biochemical identification	Results from real-time PCR analysis of primary streak from the agar plate	Results from real-time PCR analysis of one colony from the agar plate
74	<i>S. zooepidemicus</i>	<i>S. zooepidemicus</i>	<i>S. zooepidemicus</i>
3	<i>S. zooepidemicus</i>	Negative	<i>S. zooepidemicus</i>
2*	<i>S. zooepidemicus</i>	Negative	Negative
15	<i>S. equi</i>	<i>S. equi</i>	<i>S. equi</i>
1	<i>S. equisimilis</i>	<i>S. equi</i>	Negative
3	<i>S. equisimilis</i>	<i>S. zooepidemicus</i>	Negative
1	<i>S. equisimilis</i>	Negative	Negative
1	Negative	<i>S. equi</i>	<i>S. equi</i>
1	Negative	<i>S. zooepidemicus</i>	Negative
2	Negative	Negative	Negative
103	Total		

\*An additional reverse primer for the *sodA* gene was designed and added to our real-time PCR system, after which the two samples proved positive by PCR.

Table 4

*Streptococcus equi* subsp. *equi* biochemical identification from colony, versus real-time PCR from colony and/or primary streak from culture of nasal swab samples or tracheal wash fluid from 103 horses with suspected strangles or other upper respiratory tract infection

	Real-time PCR +	Real-time PCR -	Total
Biochemical tests +	15	0	15
Biochemical tests -	2	86	88
Total	17	86	103

Table 5

*Streptococcus equi* subsp. *zooepidemicus* biochemical identification from colony, versus real-time PCR from colony and/or primary streak from culture of nasal swab samples or tracheal wash fluid from 103 horses with suspected strangles or other upper respiratory tract infection

	Real-time PCR +	Real-time PCR -	Total
Biochemical tests +	77	2*	79
Biochemical tests -	4	20	24
Total	81	22	103

\* An additional reverse primer for the *sodA* gene was designed and added to our real-time PCR system, after which the two samples proved positive by PCR.