Comparison of Sampling Sites and Laboratory Diagnostic Tests for *S. equi* subsp. *equi* in Horses from Confirmed Strangles Outbreaks

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Background: Strangles is a contagious equine-specific disease caused by *Streptococcus equi* subsp. *equi*. Unfortunately, detection of *S. equi* can fail in up to 40% of horses with strangles. Whereas recent molecular biologic methods and sampling techniques have improved recovery of *S. equi* optimal sampling methods and laboratory analyses remain ill-defined.

Objectives: To determine the yield of *S. equi* from horses with acute strangles in confirmed outbreaks by field-sampling methods subjected to culture and biochemical identification, and real-time PCR directly and after culture.

Animals: Fifty-seven horses of varying breeds and ages from 8 strangles outbreaks.

Methods: Prospective study. Culture with biochemical identification and real-time PCR directly, and from culture, were performed on nasal swabs, nasopharyngeal swabs, and nasopharyngeal lavages.

Results: Real-time PCR directly from samples identified the highest number of infected horses, with 45/57 nasal swabs, 41/57 nasopharyngeal swabs, and 48/57 nasopharyngeal lavages *S. equi* positive. Biochemical identification (highest positives 22/57) was inferior to real-time PCR for *S. equi* recovery regardless of sampling method. Real-time PCR of nasopharyngeal lavage directly and after culture yielded 52/57 positives whereas direct real-time PCR of nasopharyngeal lavage combined with either nasopharyngeal swabs or nasal swabs yielded 53/57 positives. Three horses were negative on all samples.

Conclusions and Clinical Importance: Nasopharyngeal lavage analyzed by a combination of real-time PCR directly and after culture or, alternatively, real-time PCR directly on a nasopharyngeal lavage and a nasal/nasopharyngeal swab can identify *S. equi* in over 90% of acute strangles cases.

Key words: ESwab; Nasal lavage; Real-time PCR; *S. equi* detection.

The highly contagious upper respiratory disease strangles in horses has been known for over a millennium, having been mentioned by military veterinary surgeons in the Roman Empire. Its causal agent, *Streptococcus equi* subsp. *equi* (*S. equi*) is a pathogen solely of the horse. Verification of strangles requires detection of *S. equi* by bacterial culture, PCR, or both on samples from the upper respiratory tract. Various sampling methods to detect *S. equi* are suggested, including use of nasal swabs, nasopharyngeal swabs, nasopharyngeal lavages, or even lavages from the guttural pouches. However, regardless of method chosen detection of *S. equi* fails in a substantial proportion of horses with clinical signs of strangles. A maximum of 60% of clinically ill horses are *S. equi* culture-positive. Moreover, samples from abscesses in strangles horses are culture- and PCR-positive in 30% and 80% of cases, respectively, whereas as few as 18% of nasal passage samples might be culture-positive. Whereas PCR is reportedly more sensitive than bacterial culture for detection of *S. equi*, the anticipated recovery rate from various upper respiratory samples in acute strangles remains unclear.

Reasons for failure to identify *S. equi* presence in clinical strangles include low-bacterial shedding because of stage of the clinical disease, variation in sampling method (and hence anatomical location) of sample collection from the horse, and potential overgrowth of other bacteria, in particular *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*), cloaking the presence of *S. equi*. On the other hand, as there is increasing evidence that *S. zooepidemicus* can also act as a contagious upper respiratory pathogen in horses, it is all the more important to clarify, by a panel of the most current diagnostic methods and techniques, what proportion of true clinical strangles horses will be positive for *S. equi*.

In suspected strangles outbreaks, sensitive and rapid diagnostic methods are needed to identify the presence of *S. equi*. However, when laboratory tests fail to detect the presence of *S. equi*, the clinical diagnosis of strangles is invariably brought into question. Importantly, this lack of laboratory confirmation may weaken vigilance of stable owners to maintain biosecurity and quarantine measures during outbreaks.

The aims of this study were to evaluate the ability to detect *S. equi* in individual horses in confirmed outbreaks of strangles in field practice conditions, and to determine the relative success in *S. equi* recovery from commonly used clinical sampling and processing methods, including biochemical identification after culture, real-time PCR after culture, and real-time PCR directly from samples.

Abbreviation:

PCR: polymerase chain reaction
Materials and Methods

Clinical Samples

The study included 57 horses with clinical signs of strangles from 8 stables (5 stables with individual stalls for each horse and 3 loose housing stables) where acute S. equi infection had recently been identified by bacterial culture in at least 1 horse. Thus, the inclusion criteria were the presence of characteristic clinical signs of strangles in horses from stables where S. equi infection had been previously confirmed by culture. Breeds varied and horses ranged in age from 1 to 26 years (51 horses were ≤ 4 years old and 6 horses 7–26 years old). Upper airway samples were collected between November 2008, and December 2009, from horses in contact with other S. equi infected horses in the stable, and having 1 or more clinical signs of strangles, including swollen or abscessed lymph nodes, serous to purulent nasal discharge, fever, anorexia, cough, and depression. None of the horses had been treated with antibiotics before sampling. The study was approved by the Swedish Ethical Committee on Animal Experiments (C199/8).

All clinically affected horses in each of the outbreaks were sampled on a single herd visit. Initial sampling included use of two types of nasal swabs; a rayon swab with Amies agar gel (108C Amies Agar Gel) and a nasopharyngeal swab with Amies transport medium. Subsequently, a nasopharyngeal swab sample was obtained to allow one to be processed for real-time PCR directly from the sample and the other for culture. A sterile cotton swab with Amies transport medium was initially intended to be coanalyzed for detection of DNA and real-time PCR directly from sampling material without charcoal (108C Amies Agar Gel) and then immediately transferred to a sterile 50-mL Falcon® tube. The same veterinarian (SL) collected all samples. The veterinarian and the assistant used disposable gloves during sampling, which were discarded after sampling of each horse. The sampling was conducted systematically and carefully to minimize the risk of cross-contamination of samples, both within and between horses. All samples were transported to the laboratory and held at room temperature (approximately 20°C) overnight until further processing to mimic field sample submissions via mail. Once at the laboratory the samples were handled with the same rigor as during sampling to reduce the risk of cross-contamination in the laboratory during culturing of the samples. Preparation of DNA, both from culture on agar plates and directly from samples, was performed in a laboratory separate from the laboratory used to prepare samples for real-time PCR, according to standard procedures at the National Veterinary Institute Diagnostic Laboratory.

Culture and Biochemical Identification

Nasopharyngeal lavage fluids were centrifuged at 3000 × g for 10 min and the pellets collected. Swab samples and aliquots (1 µL) of the pellets from the nasopharyngeal lavage samples were cultured on selective colistin oxaline acid blood agar (COBA) plates (Columbia agar base [Oxoid CM331], 39 g/L; Streptococcus selectatab, [Mast Diagnostic], 4 tablets/L; citrated horse blood, 50 mL/L) and incubated in 5% CO2 atmosphere at 37°C for 24 h. Beta-hemolytic colonies were subcultured on 5% horse blood agar (blood agar base no. 2, [LabM lab 15], 39.5 g/ L; citrated horse blood, 50 mL/L) and identified by biochemical methods.13

DNA Preparation and Real-Time PCR

Extraction of DNA from Cultures on Agar Plates. The DNA from cultures was extracted from the primary streak on the agar plate and also from a single colony of those plates where β-hemolytic colonies appeared. Bacteria from the primary streak were suspended in 450 µL of lysis buffer (0.1M Tris-HCl, pH 8.5, 0.05% (v/v) Tween 20, 100 U mutanolysin, and 0.4 mg/mL lysozyme) and incubated at 37°C in a thermomixer at 300 rpm for 20 min. Bacteria from a single colony were suspended in 50 µL of lysis buffer and incubated at 37°C in a similar manner. Proteinase K (0.24 mg/mL) was added to each sample (10 µL for primary streak samples and 1 µL for single colony samples). All samples were incubated at 56°C for 10 min, then 96°C for 10 min, and thereafter immediately placed on ice for 15 min. The suspensions were centrifuged at 13000 × g for 5 min, and the supernatant was used as template in real-time PCR reactions.

Extraction of DNA Directly from Swabs and Nasopharyngeal Lavage Fluid. Swab tips were put in 1 mL of 0.86–0.90% NaCl (except ESwabs, which were kept in their liquid Amies transport medium) and then placed in a thermomixer at room temperature at 750 rpm for 5 min. The swabs were then discarded and the

Horses were restrained with a lip-twitch during sampling, and when required, light sedation by detomidine (Domosedan®). Between each horse the lip-twitch handle was disinfected and the rope replaced. Nasal swabs were inserted into 1 nostril with the full length of the swab shaft (approximately 12 cm). The nasopharyngeal swabs were inserted into the contralateral nostril. The nasopharyngeal lavage was performed similar to previous workers by a single-use sterile 50-cm soft feeding catheter inserted via the ventral nasal meatus to the nasopharyngeal region, and an aliquot of sterile NaCl (NaCl Irriflex® irritation bottle) slowly flushed through the catheter. The fluid exiting the nostrils was collected from both nostrils in a sterile glove (VGS3090®) and then immediately transferred to a sterile 50-mL Falcon® tube. The same veterinarian (SL) collected all samples. The veterinarian and the assistant used disposable gloves during sampling, which were discarded after sampling of each horse. The sampling was conducted systematically and carefully to minimize the risk of cross-contamination of samples, both within and between horses. All samples were transported to the laboratory and held at room temperature (approximately 20°C) overnight until further processing to mimic field sample submissions via mail. Once at the laboratory the samples were handled with the same rigor as during sampling to reduce the risk of cross-contamination in the laboratory during culturing of the samples. Preparation of DNA, both from culture on agar plates and directly from samples, was performed in a laboratory separate from the laboratory used to prepare samples for real-time PCR, according to standard procedures at the National Veterinary Institute Diagnostic Laboratory.

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NaCl solutions and the ESwab transport media were centrifuged at 6000 × g for 5 min. Nasopharyngeal lavage fluid was centrifuged at 3000 × g for 10 min (see Culture and Biochemical Identification). Supernatants were discarded and pellets (except 1 μL of the nasopharyngeal lavage fluid pellet used for culture) were resuspended in 100 μL of lysis buffer (see Extraction of DNA from Cultures on Agar Plates) and incubated at 37°C, 300 rpm for 20 min. Five μL of Proteinase K (0.24 mg/mL) and 100 μL of buffer G2 (DNA extraction kit for BioRobot EZ1™) were added to each sample followed by incubation at 56°C, 600 rpm for 10 min. Final extraction of the DNA was performed on a BioRobot EZ1™ automated workstation.

**Real-Time PCR for S. equi.** Real-time PCR, as previously described by sodA and seeI as target genes, was performed on culture from agar plates and directly from samples. Negative controls were added for every 5 samples in the DNA preparation procedure and the real-time PCR analysis. Samples were considered positive by real-time PCR from culture either from the single colony, the primary streak, or both.

**Statistical Analysis**

McNemars test for correlated proportions was used to determine if there were differences in recovery of S. equi between sampling methods having the same lab analysis, and between lab analyses having the same sampling method of each sample. In addition, individual results were compared to the total sum of all samples combined for each lab analysis and for all laboratory analyses for each sampling method (Tables 2 and 3).

Because of the lack of normality of distribution of number of days from initial disease outbreak that sampling took place non-parametric methods were used to assess whether time from initial outbreak to sampling was related to bacterial recovery by culture and biochemical testing. Results are provided as mean ± SD, with P < .05 considered significant.

**Results**

Nasal swab, nasopharyngeal swab, and nasopharyngeal lavage samples yielded only 21 or 22/57 (Table 1) S. equi positive cultures. When all sampling methods were combined, there were 36/57 horses (63%) that had at least 1 positive sample by bacterial culture, whereas all samples were culture positive in only 11 of the 57 horses (19%, data not shown).

Real-time PCR from cultures on agar plates detected significantly more positive samples than biochemical identification of culture for all sampling methods (P = .001–.001, Table 2).

Real-time PCR directly from the clinical sample had the highest detection rate of S. equi for each sampling method, with the exception of the cotton nasal swab (P = .093), being significantly higher than results from culture and biochemical identification (P < .001) (Table 2). When compared with results of real-time PCR performed after culture there was no significant difference for rayon nasal swab or ESwab, but there was a tendency for increased recovery of S. equi by real-time PCR directly for the nasopharyngeal swab and a significantly higher recovery for the nasal lavage at 48/57 (84%) positivity (P = .065, P = .012, respectively, Table 2).

When combining results of individual samples from real-time PCR after culture and directly the nasopharyngeal lavage yielded 52/57 (91%) positives (Table 1), detecting 4 additional positive horses over those by direct real-time PCR alone. Alternatively, similar numbers of positives (53/57) were detected if results for the nasopharyngeal lavage of the same horse were combined with direct real-time PCR results of either the nasal ESwab or the nasopharyngeal swab (data not shown). Combining results from direct real-time PCR testing for all samples and all sampling methods detected only 1 additional horse (Table 1).

When comparing S. equi yield between the various sampling methods only the dry cotton swab (that was used as a substitute for the rayon nasal swab for the direct real-time PCR) was statistically inferior (P = .001–.007) compared with all other sampling methods (Table 3).

Horses that were positive for S. equi by cultivation in combination with biochemical identification were sampled 7.8 ± 6.4 days after the initial outbreak date, whereas those identified as S. equi positive solely by real-time PCR directly from the sample were examined significantly earlier (4 ± 2.3 days; P = .01; Mann-Whitney) in the outbreak (data not shown). Of the 3 horses from which S. equi could not be detected from any sample, two were sampled on day

### Table 1. Detection of S. equi by bacterial culture and biochemical identification (culture), real-time PCR after culture (PCRac) and PCR directly from samples (PCRd) from rayon nasal swabs (RNS), nasal ESwab (ES), nasopharyngeal swab (NP), and nasopharyngeal lavage (NL) obtained from clinically ill horses (n = 57) in 8 confirmed outbreaks of strangles.

<table>
<thead>
<tr>
<th>Laboratory Method</th>
<th>RNS</th>
<th>ES</th>
<th>NP</th>
<th>NL</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture and biochemical identification</td>
<td>21/57&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; (37%)</td>
<td>22/57&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; (39%)</td>
<td>21/57&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; (37%)</td>
<td>22/56&lt;sup&gt;c&lt;/sup&gt; (39%)</td>
<td>36/57 (63%)</td>
</tr>
<tr>
<td>PCRac</td>
<td>38/57&lt;sup&gt;d&lt;/sup&gt; (67%)</td>
<td>40/57&lt;sup&gt;d&lt;/sup&gt; (70%)</td>
<td>34/57&lt;sup&gt;d&lt;/sup&gt; (60%)</td>
<td>36/57&lt;sup&gt;d&lt;/sup&gt; (63%)</td>
<td>43/57 (75%)</td>
</tr>
<tr>
<td>PCRd</td>
<td>30/57&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (53%)</td>
<td>45/57&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (79%)</td>
<td>41/57&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (72%)</td>
<td>48/57&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (84%)</td>
<td>54/57 (95%)</td>
</tr>
<tr>
<td>All</td>
<td>38/57 (67%)</td>
<td>45/57 (79%)</td>
<td>43/57 (75%)</td>
<td>52/57 (91%)</td>
<td>54/57 (95%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dry cotton swab.  
<sup>b</sup>Entries within each column from individual sample types having different superscripts differ significantly (P < .05; see Table 2 for actual P values).  
<sup>c</sup>Entries in rows from specific laboratory methodology with differing superscripts differ significantly between sample methods (P < .05; see Table 3 for actual P values).
2 of the outbreak whereas the remaining horse was sampled on day 9 after the initial outbreak occurred.

### Discussion

Current diagnostic methods frequently fail to detect *S. equi* in horses with clinical signs of strangles. This lack of identification of the organism in up to 40% or more of the cases has been a key obstacle in management and control of this strictly equine pathogen. The key finding from this study was that real-time PCR analysis on combinations of samples or processing methods can detect *S. equi* on a single sampling occasion in over 90% of horses with clinical signs of strangles.

The diagnostic samples in this study group were obtained from the various recommended anatomic sites of the upper respiratory tract of individual horses with presumed acute *S. equi* infection. The guttural pouches were not included in our sample sites as that location is of more importance when detecting subclinical bacterial shedding and carrier status, whereas horses in this study had acute clinical strangles. As sampling was performed in a predefined order, the influence of successive sampling on the results cannot be discounted. However, the fixed order was considered important to minimize the potential cross-contamination of the more rostral nasal swabs if the nasopharyngeal lavage or nasopharyngeal swab were performed first.

Nasopharyngeal lavage has been suggested as being the optimal sampling method for recovery of *S. equi* as it samples a large nasal and nasopharyngeal surface area. However, the basis for this recommendation appears to originate from a report of 5 strangles

### Table 2. Comparison of yield of *S. equi* (Pos = positive; Neg = negative) from individual sampling techniques (RNS, ES, NP, NL) using culture and biochemical identification, real-time PCR after culture (PCRac), or real-time PCR directly (PCRd) from samples. For example, RNS culture vs PCRac is compared using McNemars square. The PosNeg (0) and NegPos (17) numbers dictate whether results are significantly different (based on the four first numbers in the PCRac column).

<table>
<thead>
<tr>
<th>Sample &amp; Analysis</th>
<th>PCRac</th>
<th>PCRd</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNS culture</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRac, P &lt; .001</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs PCRd, P = .93</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>ES culture</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRac, P &lt; .001</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs PCRd, P = .93</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NL culture</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRac, P &lt; .01</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs PCRd, P = .01</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NP culture</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRac, P &lt; .1</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRd, P = .14</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>RNS PCRac</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRd, P = .134</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs ES PCRac</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRd, P = .125</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NP PCRac</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRd, P = .06</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NL PCRac</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs PCRd, P = .012</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

### Table 3. Comparison of yield of *S. equi* (Pos, positive; Neg, negative) between individual sampling techniques (RNS, ES, NP, NL) based on culture and biochemical identification, real time PCR after culture (PCRac), and real time PCR (PCRd) directly from samples.

<table>
<thead>
<tr>
<th>Sample &amp; Analysis</th>
<th>ES</th>
<th>NP</th>
<th>NL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNS culture</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs ES, P = 1</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs NP, P = .789</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>vs NL, P = 1</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>ES culture</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs NP, P = 1</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs NL, P = 1</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>NP culture</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>vs NL, P = 1</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

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RNS, rayon nasal swab; ES, ESwab; NP, nasopharyngeal swab; NL, Nasal lavage.

Dry cotton swab.
outbreaks of which nasopharyngeal lavage was performed only one of the outbreaks, which coincidentally had the highest recovery of *S. equi* DNA positive horses (8/13 [61%]) of all farms investigated. Unfortunately, epidemiologic differences between herd outbreaks could have influenced recovery of *S. equi* regardless of sampling method. Nonetheless, our current study supports and strengthens their conclusions, as we were able to show that nasopharyngeal lavage subjected to real-time PCR directly from samples also provided the highest single yield (48/57) for *S. equi*, and if processed both directly and after culture detected over 90% (52/57) of the presumably true positive cases. Alternatively, when time is of the essence to confirm the diagnosis of *S. equi* infection, simply performing direct real-time PCR on 2 differing upper airway samples from an individual horse, providing one is a nasopharyngeal lavage, can identify equally as many *S. equi* positives, but without the delay imposed by culture. Thus, when obtaining diagnostic samples from a strangles suspect, obtaining at least 1 additional airway sample such as a nasal swab (ESwab) along with the nasopharyngeal lavage for the subsequent laboratory analyses may be sufficient to approach optimal recovery of *S. equi*.

Regarding results, the various swabs used for sampling texture differences may have affected bacterial recovery, as nylon-flocked swabs (such as the ESwab) have been shown to release more bacteria than rayon swabs. This factor may explain why the ESwab was the most effective of all of the swabs in recovering *S. equi* by real-time PCR directly from the sample. Moreover, the transport medium included for the ESwab is suitable for preparation of DNA directly, as well as for culturing of the swab. The sterile cotton swab in this study for the direct real-time PCR was included to enable detection of viral upper respiratory pathogens as well as bacteria by real-time PCR. However, recovery of DNA from *S. equi* on the dry cotton swab was significantly lower than by direct real-time PCR from an ESwab and nasopharyngeal lavage, and moreover not significantly different from only culture and biochemical identification.

Detection of *S. equi* by cultivation and biochemical identification, regardless of sampling method used yielded highly similar recovery rates of slightly less than 40% positive. Use of culture with biochemical identification to detect *S. equi* is inarguably the gold standard method to confirm active infection. However, even when results from all different swabs and the lavage sample were combined, providing 4 sequential cultures for each horse, only 36/57 (67%) horses were positive for *S. equi*. Of those *S. equi* culture positive samples, all nasal ESwabs and nasopharyngeal swabs were positive by direct real time PCR whereas culture positive rayon swabs from 7 horses and nasopharyngeal lavages from 2 horses were negative by real-time PCR directly from the samples (data not shown). This suggests that real-time PCR directly from samples captures almost all possible culture positive horses, with the exception of real-time PCR directly from dry cotton swabs, a sampling material found unsuitable for detection of *S. equi*.

Several mucoid β-hemolytic colonies selected for subcultivation and biochemical identification were found to be *S. equi* subsp. *zooepidemicus* (*S. zooepidemicus*), a closely related bacterium often found in horse respiratory samples and that can interfere with detection of *S. equi*. The presence of *S. zooepidemicus* on the agar plates makes selection of *S. equi* colonies for further identification more difficult.

Performing PCR after culture from clinical samples can also be considered a more current gold standard as it will reflect recovery of live bacteria. However, based on the results of this study it appears that even this approach will miss some positive animals as, apart from the dry cotton nasal swab, real-time PCR directly rather than after culture from samples detects even more of the true positives in acute infection (Table 1). For nasal ESwab and nasopharyngeal swab there were marginal to no improvements in detection when real-time PCR directly from the sample was combined with real-time PCR from culture (Table 1). On the other hand, even though *S. equi* recovery from nasopharyngeal lavage by real-time PCR from culture was significantly (*P = .012*) lower than real-time PCR directly from the sample, 4 horses that were negative by direct real-time PCR were identified when real-time PCR from culture was also performed (Tables 1 and 2).

Given the extreme sensitivity of PCR methods, one must consider that contamination via other horses in the stable or of the sampling instruments at the stable may have confounded our findings. Measures such as glove and twitch rope change and twitch handle cleaning were performed between horses to minimize the risk of cross-contamination of swabs. As most horses were PCR positive on differing swab types and sampling sites, and given that three of the horses were completely negative on all samples, we believe that the positive real-time PCR results were indeed indicative of those individuals being infected by *S. equi*.

Sampling early in the progression of the disease outbreak is a variable that can impair recovery of *S. equi*, particularly based on traditional bacterial culture methods. Indeed, those animals sampled earliest at 2 days into the outbreak (n = 12) included 2 horses completely negative on all samples. Our findings that the group of horses that were *S. equi* positive based on both culture and biochemical identification was sampled significantly later in the outbreak than those culture negative/PCR positive is consistent with this earlier study. Thus, similar to recent findings in other horse bacterial infections, PCR is of value early in disease when more traditional diagnostic methods can be falsely negative.

Regarding the 3 horses completely negative to *S. equi*, two were sampled on day 2 of their clinical signs occurring, and being presumably early in the disease may have had minimal detectable shedding. The remaining *S. equi* negative horse was sampled after 9 days of the initial outbreak, which is beyond the early stages when shedding is reportedly low. Given that these 3 horses...
were housed in close contact with confirmed strangles cases and were clinically ill at the time of sampling, we chose to classify all three as false negatives on our testing panel. From the aspect of evaluating diagnostic tests, this study population was assumed to contain only true strangles-positives and only sensitivity could be evaluated. Determination of specificity and predictive values of the method for strangles diagnosis will require its application to a population of horses that also includes many noninfected animals.

Recent studies have highlighted the importance of verification of S. equi infection not only in horses with clinical signs of disease as in acute outbreaks, but also in horses that after an outbreak become subclinical carriers and can shed viable bacteria for a long period of time after the clinical signs have resolved. Detection of carrier horses is of great importance in the overall management of S. equi infection, but has so far proven difficult and labor intensive. Development of a readily available test will be most valuable in the management of this important equine disease.

Our findings suggest that for the individual horse where acute strangles is suspected, a single nasopharyngeal lavage analyzed by real-time PCR directly from the sample and in combination with real-time PCR from culture appears to be the optimal sampling and analyses methods. On the other hand, performing real-time PCR directly on nasopharyngeal lavage and a single additional sample such as a nasal swab (ESwab) appears to be equally effective in recovery of S. equi to confirm a diagnosis of strangles, but without the time delay imposed by culture.

Footnotes

a Copan Innovation Ltd, Brescia, Italy  
b EquiVet, Kruuse, Marslev, Denmark  
c Selefa Trade, Spånga, Sweden  
d Orion Pharma Animal Health, Solllentuna, Sweden  
e Swevet Piab AB, Sjöbo, Sweden  
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