Optimization of an in vitro assay to detect *Streptococcus equi* subsp. *equi*

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**A B S T R A C T**

*Streptococcus equi* is the etiologic agent of a highly infectious upper respiratory disease of horses known as strangles. Bacterial culture methods and polymerase chain reaction (PCR) of nasopharyngeal washes and guttural pouch lavages are used routinely to test clinical and carrier animals for the presence of *S. equi* but no definitive or gold standard test method has been shown to be optimal. We hypothesized that (i) a flocked swab submerged in ten-fold serial dilution suspensions of *S. equi* prepared in 0.9% NaCl would detect more colony forming units (CFU) than a rayon swab when used to inoculate a blood agar plate, (ii) centrifugation of a 1 ml aliquot of each suspension would improve the limit of detection (LOD) by bacterial culture and PCR compared to the culture or PCR of submerged swab samples, (iii) PCR of the centrifuged samples from each suspension would be more sensitive than aerobic culture alone, and (iv) PCR of a 1 ml aliquot directly from a sample would be more sensitive than PCR of a sample following submersion of a flocked swab in 1 ml saline. Using 7 ten-fold serial dilutions of *S. equi* in 0.9% NaCl, the LOD for 4 bacterial culture methods and 3 PCR methods were compared. The LOD of direct PCR and flocked swab culture was determined at 1 cfu/ml. All PCR methods were equivalent to each other and were more sensitive than any of the culture methods at the lower dilutions. At higher cell densities (>100 cfu/ml) flocked swab culture was not statistically better than rayon swab culture, but it was superior to all other methods tested.

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1. Introduction

*Streptococcus equi* is the etiologic agent of a highly infectious upper respiratory disease of horses known as strangles (Sweeney et al., 2005). Asymptomatic carriers in the population may result in the spread of disease via introduction of *S. equi* to naïve populations. Bacterial culture and polymerase chain reaction (PCR) of nasopharyngeal (NP) washes and guttural pouch (GP) lavages have been used for both diagnostic testing and for the detection of *S. equi* in carrier animals but no definitive or gold standard test method has been shown to be optimal (Holland et al., 2006; Timoney and Artiushin, 1997; Sweeney et al., 2005; Verheyen et al., 2000). The sensitivity and specificity of *S. equi* PCR from NP swabs have been documented to range from 45% to 50% and 71%, respectively and the sensitivity and specificity of culture from NP swabs range from 18% to 45% and 94%, respectively (Gronbaek et al., 2006; Newton et al., 1997, 2000).

Recently, flocked swabs have been introduced in human diagnostics and have been shown to increase the sensitivity of bacterial culture and PCR via improved specimen collection and efficient release of the specimen material (Chernesky et al., 2006; Goldfarb et al., 2009; Van Horn et al., 2008). We hypothesized that (i) a flocked swab submerged in ten-fold serial dilution suspensions of *S. equi* prepared in 0.9% NaCl would detect more colony forming...
Table 1
4 Culture methods and 3 PCR methods tested in preliminary study to determine the optimization of an in vitro assay to detect Streptococcus equi subsp. equi in 0.9% NaCl.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Culture: Rayon swab submerged directly in the fluid dilution</td>
</tr>
<tr>
<td>2</td>
<td>Culture: Flocked swab submerged in the fluid dilution</td>
</tr>
<tr>
<td>3</td>
<td>Culture: 1 ml fluid dilution centrifuged for 1 min at 20,000 × g, supernatant decanted, and remaining pellet sampled with a 1 μl bacteriology loop</td>
</tr>
<tr>
<td>4</td>
<td>PCR: 1 ml fluid dilution centrifuged for 1 min at 20,000 × g, supernatant decanted, and remaining pellet sampled with a rayon swab</td>
</tr>
<tr>
<td>5 and 7</td>
<td>PCR: 1 ml fluid dilution centrifuged for 3 min at 20,000 × g, supernatant decanted, and remaining pellet resuspended in 100 μl of 0.9% NaCl extracted and DNA amplified (direct PCR ran two separate times)</td>
</tr>
<tr>
<td>6</td>
<td>PCR: Rayon swab submerged in the fluid dilution, immersed in 1 ml of 0.9% saline, centrifuged for 3 min at 20,000 × g, supernatant decanted, and remaining pellet resuspended in 100 μl of 0.9% NaCl extracted and DNA amplified</td>
</tr>
<tr>
<td>8</td>
<td>PCR: Flocked swab submerged in the fluid dilution, immersed in 1 ml of 0.9% saline, centrifuged for 3 min at 20,000 × g, supernatant decanted, and remaining pellet resuspended in 100 μl of 0.9% NaCl extracted and DNA amplified</td>
</tr>
</tbody>
</table>

units (CFU) than a rayon swab when used to inoculate a blood agar plate (Method 2 > Method 1) (Table 1), (ii) centrifugation of a 1 ml aliquot of each suspension would improve the limit of detection (LOD) by bacterial culture and PCR compared to the culture or PCR of submerged swab samples respectively (Methods 3 and 4 > Method 1; Methods 5 and 7 > Method 6), (iii) PCR of the centrifuged samples from each suspension would be more sensitive than aerobic culture alone. (Methods 5 and 7 > Methods 1 and 2), and finally (iv) PCR of a 1 ml aliquot directly from a sample would be more sensitive than PCR of a sample following submerison of a flocked swab in 1 ml saline (Methods 5 and 7 > Method 8). In this in vitro study, our goal was to optimize and determine the limit of detection of S. equi by aerobic culture and PCR in 0.9% NaCl using 4 bacterial culture techniques and 3 DNA amplification techniques.

2. Materials and methods

All testing was performed at the University of Pennsylvania New Bolton Center Microbiology Laboratory. This lab is fully accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD).

2.1. Validation and verification of assay

The S. equi PCR assay has previously been validated (Baverud et al., 2007) and has been verified in-house for use as a screening test (results not shown).

2.2. DNA extraction

DNA was extracted from 1 ml of each suspension using PrepMan Ultra as described by the manufacturer (Applied Biosystems, Foster City, CA). Briefly, 1 ml was centrifuged (Eppendorf Centrifuge Model 5417C) for 3 min at 20,000 × g, the pellet was resuspended in 100 μl of PrepMan Ultra and incubated for 10 min at 100 °C. The boiled extract was then diluted 1/100 in nuclelease-free water (Fisher Scientific, Pittsburgh, PA).

2.3. S. equi subsp. equi DNA amplification

Realtime PCR to specifically detect S. equi subsp. equi was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The assay detected the seel gene and used the following primers and probe to amplify a 520 bp fragment as described previously (Baverud et al., 2007).

Seel-F: 5'-CGGATACGGGTATGGTTAAAGA-3'
Seel-R: 5'-TTCCTTCTCAAAAGCAGA-3'
Seel probe: 5'-TTGGCCGCCTCTCTAGATTTCAA-3'

OmniMix HS (Takara Bio, Inc.) lyophilized beads were reconstituted to a final volume of 40 μl and contained: 37 μl H2O, 2 μl each of 100 μM forward and reverse primers and 1 μl of 5 μM probe. Each PCR reaction consisted of 20 μl OmniMix Mastermix as described above plus 5 μl of extracted DNA for a final concentration of 3 U Taq, 200 μM dNTPs, 4 mM MgCl2 and 25 mM HEPES. Amplification began with 8-min at 95 °C, followed by 45 cycles of 20 s at 95 °C and 60 s at 60 °C.

2.4. Preparation of standard curve

ATCC 33398 S. equi subsp. equi was grown from a frozen inoculum in 10 ml of Brain Heart Infusion (BHI) broth at 35 °C overnight. The 10 ml broth was centrifuged (Eppendorf Centrifuge Model 5417C) at 3000 × g for 10 min and the supernatant fluid was discarded. 0.9% sterile saline was added to the cell pellet until the suspension was equivalent to a 0.5 McFarland standard (approximately 1 × 10⁸ cfu/ml) using a Turbidity Meter (Siemens, Deerfield, IL). Six 10-fold serial dilutions were prepared and colony counts were performed from each dilution in triplicate. DNA was extracted from 1 ml of each of these standard dilutions in triplicate, PCR was performed and the cycle threshold (CT) values were recorded. To determine the technical accuracy of the dilutions the average cfu/ml were compared to the CT value of each dilution. The most accurate dilution was used to prepare bacterial suspensions that contained 1, 3, 10, 30, 100, 300, and 1000 cfu/ml for use in the study.

2.5. S. equi culture methods: 5 replicates tested at each dilution

Method 1: Rayon swab (BBI™ CultureSwab™ BD, Franklin Lakes, NJ) immersed in 1 ml of standard bacterial suspensions, plated directly to blood agar (Tryptic soy agar (TSA) with 5% sheep blood) (Becton, Dickinson & Co., Cockeysville, MD) and incubated at 35 °C overnight.

Method 2: Flocked swab (Copan Diagnostics, Inc., Corona, CA) immersed in 1 ml of standard bacterial
 suspensions, plated directly to blood agar and incubated at 35 °C overnight.

Method 3: 1 ml of standard bacterial suspensions centrifuged for 1 min at 20,000 × g, supernatant fluid removed, the pellet plated directly to blood agar using a 1 μl bacteriology loop and incubated at 35 °C.

Method 4: 1 ml of standard bacterial suspensions centrifuged for 1 min at 20,000 × g, supernatant fluid removed, the pellet plated directly to blood agar using a rayon swab.

2.6. S. equi PCR methods: 5 replicates done at each dilution

2.6.1. DNA extraction

DNA was extracted from a 1 ml sample using PrepMan Ultra as described above. The rayon and flocked swabs were dipped once into each dilution and then transferred to 1 ml of fresh sterile PBS and rotated thoroughly to remove any organisms. The 1 ml PBS containing the specimen was processed as described below.

Method 5: 1 ml of standard bacterial suspensions, DNA extracted and amplified as described above.

Method 6: Rayon swab immersed in 1 ml of standard bacterial suspensions DNA extracted and amplified.

Method 7: 1 ml of standard bacterial suspensions, DNA extracted and amplified as described above (same as Method 5, but repeated at a later date when Method 8 was run).

Method 8: Flocked swab immersed in 1 ml of standard bacterial suspensions, DNA extracted and amplified.

2.7. Statistical analysis

Poisson regression (Fassina and Corradin, 2009) was used to determine the interaction between laboratory method and the detection of colony forming units. A P-value of 0.05 was used to determine significance of each statistical test. Limit of detection (LOD) was determined. The methods of Collett, Hosmer, and Lemeshow, and Campbell were followed to confirm the robustness of the analyses. Specifically, overall model significance was corroborated using Fisher’s exact test, aptness of the Poisson links were tested with reference to the outcome susceptibility to alternate link functions, and susceptibility of our findings to data configurations (Hosmer and Lemeshow, 2000) were judged with the aid of robust methods (Stata 11.1, College Station, TX) (Campbell, 2006; Collett, 1991; Hosmer and Lemeshow, 2000).

3. Results

The ability of each method to detect S. equi of known cfu/ml in 0.9% saline by eight (seven different) laboratory methods was examined (Table 1). Each method was replicated five times for each ten-fold serial dilution tested. The LOD for direct PCR (Methods 5 and 7) and flocked swab culture (Method 2) was determined at 1 cfu/ml. The LOD for assays, except for rayon culture swab (Method 1) and flocked swab PCR (Method 8), were reproducible at 30 cfu/ml or higher. LOD for Methods 1 and 8 were reproducible at 100 cfu/ml or higher.

<table>
<thead>
<tr>
<th>Method</th>
<th>Coefficient</th>
<th>Std. error</th>
<th>P value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.208</td>
<td>2.898</td>
<td>0.268</td>
<td>-2.472–8.887</td>
</tr>
<tr>
<td>3</td>
<td>2.915</td>
<td>2.996</td>
<td>0.501</td>
<td>-3.856–7.886</td>
</tr>
<tr>
<td>4</td>
<td>-1.862</td>
<td>4.642</td>
<td>0.688</td>
<td>-10.960–7.236</td>
</tr>
<tr>
<td>5</td>
<td>7.905</td>
<td>2.799</td>
<td>0.005</td>
<td>2.419–13.392</td>
</tr>
<tr>
<td>6</td>
<td>7.925</td>
<td>2.808</td>
<td>0.005</td>
<td>2.420–13.430</td>
</tr>
<tr>
<td>7</td>
<td>7.925</td>
<td>2.799</td>
<td>0.005</td>
<td>2.440–13.411</td>
</tr>
<tr>
<td>8</td>
<td>7.961</td>
<td>2.799</td>
<td>0.004</td>
<td>2.476–13.446</td>
</tr>
</tbody>
</table>

Flocked swab culture (Method 2) was not statistically better than the rayon culture swab (Method 1) (P = 0.268) (Table 2), but was superior to all other methods tested including PCR methods at higher concentrations (>100 cfu/ml) (Fig. 1). Centrifugation/bacteriology loop culture and centrifugation/rayon swab culture (Methods 3 and 4) were not statistically better than rayon culture swab (Method 1) (P = 0.501, 0.688, respectively) (Table 2). Methods 1, 3, and 4 (non-flocked swab culture methods) were the least sensitive (Table 2, Fig. 1). All PCR methods (Methods 5–8) were more sensitive than any of the culture methods at detecting the presence of S. equi at the lower concentrations (1, 3, 10, and 30 cfu/ml) (P = 0.004–0.005) (Table 2, Fig. 1). All PCR methods (Methods 5–8) were equivalent to each other (Table 2, Fig. 1). The overall rate of detection of the PCR (Methods 5–8) did not improve with increasing cfu/ml compared to detection rates at the lower concentrations (Fig. 1). Direct PCR (Methods 5 and 7) were statistically better than flocked swab culture (Method 2) (P < 0.001) and rayon culture swab (Method 1) (P = 0.005, Table 1). As expected, the sensitivity of each culture method to detect S. equi improved as the number of cfu/ml increased.

4. Discussion

This preliminary in vitro study was performed to determine the optimal method for culture and/or PCR for NP and GP lavage washes from horses. The sensitivity and specificity of flocked swab culture and direct PCR are currently being compared in an funded field trial to assess how each of these assays perform with NP and GP wash samples from horses.

In the human and veterinary medical literature there are many examples that demonstrate that PCR is more sensitive than traditional culture methods. We confirm these results here. PCR has been shown to be more sensitive in the detection of Mycoplasma pneumoniae, Neisseria gonorrhoea and Streptococcus pneumoniae in human clinical samples, Rhodococcus equi from foals, and Mycoplasma bovis from cattle (Brugger et al., 2009; Hjelmevoll et al., 2008; leven et al., 1996; Sachse et al., 2010; Sellon et al., 2001). Limited human studies have examined the use of flocked swab PCR. Using serial dilutions of reference strains of Chlamydia trachomatis and Neisseria gonorrhoea, Chernesky et al. (2006) found
improved sensitivity using flocked swab PCR. Flocked swab PCR was equivalent to conventional culture for the detection of Group A Streptococcus (GAS) in human throat samples (Slinger et al., 2011). Timoney and colleagues showed that traditional PCR was more sensitive than bacterial culture of equine NP wash samples. Techniques were compared using NP washes from horses versus known concentrations of S. equi in 0.9% saline but only one pre-processing technique was examined: centrifugation of the sample prior to traditional culture and PCR (Timoney and Artiushin, 1997). Baverud et al. (2007) later described a real time PCR assay to detect S. equi and S. zooepidemicus in nasopharyngeal swabs and transtracheal washes from horses that showed increased sensitivity compared to culture when the cfu/ml was low.

The present study did not demonstrate an increased sensitivity of PCR with increased numbers of S. equi in the dilution tested once the number of organisms exceeded 100 cfu/ml. We do not have an explanation for this other than that the dilutions used in this study were extremely low in order to identify the LOD. Given that these very low organism numbers are likely not representative of the number of organisms expected in washes from clinically ill horses with strangles this would suggest that PCR is a very sensitive method to detect S. equi in washes from clinical animals. A sample from a clinically ill equine with strangles could be expected to contain S. equi numbers in the range of $1 \times 10^5$ to $1 \times 10^6$ cfu/ml (Bingen et al., 1990).

Surprisingly, bacterial culture with a flocked swab (Method 2) outperformed all PCR methods at the higher concentrations (>100 cfu/ml). To our knowledge, this has not been examined previously in the human or veterinary literature. The flocked swab culture was accurate and reproducible when the number of organisms in suspension was >30 cfu/ml. The flocked swab has a higher surface area to absorb the liquid in which it is submerged. Instead of a traditional woven swab, the flocked swab is made of individual perpendicular strands of synthetic material which enables the swab to absorb and then completely release its contents (Goldfarb et al., 2009).

The flocked swab/PCR technique (Method 8) was equivalent to centrifuging a 1 ml aliquot prior to DNA amplification (Method 5 and 7). In Methods 5 and 7, a 1 ml aliquot of sample was centrifuged and the pellet resuspended directly in 100 μl of PrepMan Ultra. Despite the extra steps of the fluid dilution adhering to the flocked swab and then released again into 1 ml PBS Method 8 performed equivalently and has the potential to perform better in a field trial situation with background “noise” (commensal bacterial, epithelial cells, and white blood cells).

Centrifugation prior to bacterial culture actually decreased the sensitivity of culture (Methods 3 and 4). We had anticipated an increased sensitivity by concentrating the sample into a pellet. Since these samples were pure cell suspensions, they did not contain the additional commensal bacteria and inflammatory and epithelial cells that would be found in samples from an equine patient, resulting in a less visible pellet (Chiesa et al., 1999). Therefore, the pellet may have been mistakenly decanted or not sampled.

Limitations to the in vitro study include the following: pipetting errors were possible in the production of the original cell dilutions and would be more apparent in lower dilutions. Randomization of the preparations of the dilutions would have provided more objectivity. The dilutions tested did not represent the number of organisms expected in NP wash or GP lavage samples from clinical horses that are truly positive for S. equi. The dilutions tested were more representative of the number of bacterial cells expected in NP wash or GP lavage samples from

Fig. 1. Poisson regression analysis of colony forming units by measurement method and dilution. Method 1 (rayon swab culture) = ●, Method 2 (flocked swab culture) = ◆, Method 3 (loop culture) = ▲, Method 4 (spin, rayon swab culture) = ■, Method 5 (direct PCR) = ★, Method 6 (rayon swab PCR) = x, Method 7 (direct PCR) = ○, and Method 8 (flocked swab PCR) = ◇. The seven dilutions considered here are plausible reflections of colony forming units in nonclinical animals. A sample from a clinically ill equine horse with strangles would be expected to contain S. equi numbers in the range of $1 \times 10^5$ to $1 \times 10^6$ cfu/ml (Bingen et al., 1990).
nonclinical carrier horses (Bingen et al., 1990). The reasons for this are: (1) the study was designed to determine the detection limit of S. equi and (2) quantification becomes increasingly difficult at high concentrations.

5. Conclusion

To the author’s knowledge, the pre-processing techniques for bacterial culture and PCR of S. equi in 0.9% saline had not been previously examined. A funded field trial will compare Method 2 (flocked swab culture), Method 8 (flocked swab PCR) with the current “gold standard” used in the lab (as represented by Methods 5 and 7) 1 ml aliquot PCR in the presence of background “noise” (commensal bacteria, epithelial cells, and white blood cells). This field trial will test NP wash and GP lavage samples from clinical and asymptomatic (but potentially S. equi carrier) animals to verify the previous in vitro results. The LODs determined in this in vitro study (1 CFU for Methods 2, 5, and 7; >30 CFU for Method 8), are highly sensitive going into this field trial. No previous LODs have been determined for PCR and bacterial culture of equine NP and GP wash samples, other than a reference to unpublished data by Timoney and Artiuishin (1997) suspected to be at 10 or fewer CFU for centrifugation prior to PCR. The final method will be submitted to the Clinical and Laboratory Standards Institute for consideration as a national standard operating procedure.

Conflict of interest statement

None. There is no relationship between the authors and Copan Diagnostics, Incorporated, manufacturers of Copan™ flocked swabs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2012.04.014.

References


