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Streptococcus equi Detection Polymerase Chain Reaction Assay for Equine Nasopharyngeal and Guttural Pouch Wash Samples

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Background: Bacterial culture and polymerase chain reaction (PCR) assays for the detection of *Streptococcus equi* in nasopharyngeal washes (NPW) and guttural pouch lavage (GPL) samples have low sensitivity. In human diagnostics, processing of samples with flocked swabs has improved recovery rates of bacterial agents because of improved surface area and elution factors.

Hypothesis: For *S. equi* subsp. *equi* (*S. equi*) detection in NPW and GPL samples we hypothesized that: direct-PCR would be more reliable than flocked swab culture (FS culture); flocked swab PCR (FS-PCR) would be equivalent to direct-PCR; and FS culture would be more reliable than traditional culture.

Samples: A total of 193 samples (134 NPW and 59 GPL) from 113 horses with either suspected *S. equi* infection, convalescing from a known *S. equi* infection, or asymptomatic horses screened for *S. equi*.

Methods: Prospective study. Samples were submitted for *S. equi* direct-PCR. Using logistic regression, direct-PCR (gold standard) was compared to FS culture, traditional culture, and FS-PCR also performed.

Results: Direct-PCR was statistically more sensitive than FS-PCR, FS culture, and traditional culture ($P < .001$). All methods had sensitivities <70% relative to the direct-PCR. FS culture had a similar sensitivity relative to traditional culture. The odds of GPL samples being positive on direct-PCR ($P = .030$) and FS-PCR were greater than those for NPW samples ($P = .021$).

Conclusions and Clinical Importance: Use of flocked swabs during laboratory preprocessing did not improve detection of *S. equi* via either PCR or bacterial culture from samples. Direct-PCR is the preferred method of detection of *S. equi*.

Key words: Flocked swab; Polymerase chain reaction; Strangles.

Strangles affects a large proportion of at risk animals during an outbreak and carries a high financial burden for the equine industry. Up to 20% of horses might become persistent carriers and these animals are the cause for its continued existence.[1–3] Bacterial culture and polymerase chain reaction (PCR) of nasopharyngeal washes (NPW) and guttural pouch lavages (GPL) are used to detect carrier animals. These tests have low sensitivity requiring veterinarians to obtain multiple samples before comingling animals after an outbreak.[4] The sensitivity and specificity of *Streptococcus equi* PCR from nasopharyngeal (NP) swabs have been documented to range from 45–50 and 71%, respectively. The sensitivity and specificity of culture from NP swabs range from 18–45 and 94%, respectively. When three consecutive NP swab cultures were performed, the sensitivity of culture increased to 85%.[2,5,6] Detection rates of *S. equi* in acutely ill horses are highest in NPW PCR samples (48/57, 84%) when compared to swabbing the rostral nasal passage for culture (21/57, 37%), rostral nasal passage for PCR (30/57, 53%), nasopharynx for culture (21/57, 37%), or nasopharynx for PCR (41/57, 72%).[7]

Sampling human patients and processing samples in the laboratory using flocked nylon swabs has improved recovery rates of bacterial and viral organisms via improved surface area and elution factors.[8,9] Studies evaluating flocked swabs in veterinary diagnostics have been limited. Sampling using flocked nylon swabs does not statistically improve the detection of *S. equi* via PCR or bacterial culture of the rostral nasal passages in acute strangles infection.[7]

In a recent in vitro laboratory-based study,[10] as a means of assessing the detection limit of different methods, various concentrations of *S. equi* diluted in saline

Abbreviations:

CI	confidence interval
CFU	colony forming units
CT	cycle threshold
FS	flocked swab
GPL	guttural pouch lavage
NPW	nasopharyngeal wash
OR	odds ratio
PCR	polymerase chain reaction
ROC	receiver operator characteristic
S. equi	*Streptococcus equi* subsp. *equi*

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were sampled for DNA amplification and culture using flocked swabs. The results showed that direct and flocked swab (FS) PCR were more sensitive than all of the culture methods at lower limits of detection (1 colony forming unit [CFU]/mL), that the FS-PCR was equivalent to the direct-PCR (leading to the assumption that the flocked swab attracts all of the organisms in the original specimen and elutes all the organisms to the 1 mL of physiologic buffered saline [PBS]), and that the flocked swab culture was more sensitive than PCR at dilutions greater than 100 CFU/mL.[10]

The aim of the current field study was to compare FS culture, traditional culture, and FS-PCR methods with the results of a direct-PCR (gold standard). We used NPW and GPL clinical samples from sick, convalescing and asymptomatic horses to verify the previous in vitro results. We hypothesized that: the direct-PCR would detect *S. equi* in NPW and GPL samples more reliably than FS culture and traditional culture; that the FS-PCR method would detect *S. equi* in NPW and GPL samples with equivalent frequency as direct-PCR; and that the FS culture would detect *S. equi* in NPW and GPL samples more reliably than traditional culture.

Materials and Methods

Samples

Nasopharyngeal washes and GPL samples from clinically ill horses with suspected *S. equi* infection, horses convalescing from a known *S. equi* infection, and asymptomatic horses undergoing *S. equi* screening were routinely submitted to the University of Pennsylvania New Bolton Center Clinical Microbiology Laboratory for *S. equi* direct-PCR and traditional bacterial culture. Two additional aliquots of each sample were submitted for FS-PCR and FS culture. More than 1 sample originated from some horses because of the nature of the diagnostic testing for the disease strangles. The protocol was approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

Testing

PCR and aerobic culture were performed on the samples in the following manner as described below.

Direct S. equi PCR

DNA Extraction. DNA was extracted using PrepMan Ultra[a] as described by the manufacturer. Briefly, 1 mL of NPW or GPL sample was centrifuged[b] for 3 minutes at 13,000 × *g* and the pellet was resuspended in 100 μL of PrepMan Ultra and boiled for 10 minutes. The boiled extract was then diluted 1/100 in nuclease-free water[c] and used immediately for PCR or stored at −20°C.

***S. equi* subsp. *equi* DNA Amplification.** Real-time PCR to specifically detect *S. equi* subsp. *equi* was performed on a thermal cycler.[d] The assay detected the *seeI* gene and used the following primers and probe to amplify a 520 bp fragment as described previously.[11] The assay has been validated and verified in-house for use as a screening test (results not shown).

SeeI-F: 5′-CGGATACGGTGATGTTAAAGA-3′
SeeI-R: 5′-TTCCTTCCTCAAAGCCAGA-3′
SeeI probe: 5′-TTTGGCCGCTCCTCTAGATTTCAA-3′.

Lyophilized beads[e] were reconstituted to a final volume of 40 μL and this mastermix contained: 35 μL H_2O, 2 μL each of 100 μM forward and reverse primers and 1 μL of 5 μM probe. Each PCR reaction consisted of 20 μL mastermix as described above plus 5 μL internal amplification control.[f] Amplification began with 8 minutes at 95°C, followed by 40 cycles of 20 seconds at 95°C and 60 seconds at 60°C.

S. equi Culture

A rayon swab[g] was immersed in the vortexed specimen, plated directly to Columbia CNA (colistin, nalidixic acid) blood agar[h] and incubated at 35°C overnight.

FS S. equi PCR

A FS[i] was dipped once into the specimen and then transferred to 1 mL of fresh sterile PBS, rotated thoroughly to remove any organisms and centrifuged for 1 minute at 13,000 × *g*. The pellet was resuspended in 100 μL of PrepMan Ultra and boiled for 10 minutes. The boiled extract was then diluted 1/100 in nuclease-free water[c] and used immediately for PCR or stored at −20°C.

FS S. equi culture

A FS[i] was submerged in the vortexed specimen and was plated directly to a Columbia CNA (colistin, nalidixic acid) blood agar[h] and incubated at 35°C overnight.

Laboratory personnel were blinded to both the results from the direct-PCR (the gold standard for this comparison) and the clinical status of the animal from which samples were obtained.

Statistical Analysis

Descriptive statistics were generated for each variable. Continuous variables were checked for normality using a Shaprio–Wilk test. For normal distributions, two-group comparisons (eg, CT values from direct-PCR and FS-PCR) were by means of parametric *t*-tests (paired or unpaired and corrected for unequal variances where appropriate). Nonparametric sign and signed-rank tests were used for data that were not normally distributed. Binomial (positive or negative) test results for each alternative method were compared to the gold standard direct-PCR. Additional comparisons were made by sample classification (clinical, convalescent, or subclinical), and by sample type (NPW or GPL) for all methods. Simple binomial comparisons were done by using a chi-squared test, or a Fisher's exact test when at least one expected value in a cell was <5, effects were quantified by means of logistic regression. To control for correlations in subjects with repeated measures, cluster-adjusted robust variance estimation methods were used. Sensitivity, specificity, and positive and negative predictive values were calculated for each alternative method relative to the gold standard (direct-PCR). Receiver operator characteristic (ROC) curves were plotted and the area under the curves was used to assess test accuracy. Differences between proportions were evaluated using two-sample tests of proportion. t were performed using a commercial statistical software package.[j] Statistical significance was inferred when P was $\leq .05$.

Results

One hundred and ninety-three samples (134 NPW and 59 GPL) were submitted to the laboratory for detection of *S. equi* via direct-PCR from 113 different

horses. Median horse age was 6 years (range 0.2–29, IQR 3–10 years). Breed information was available for 111 horses and was distributed as follows: 33/111 (30%) Standardbreds, 19/111 (17%) Thoroughbreds, 17/111 (15%) Warmbloods, 16/111 (14%) pony breeds 11/111 (10%) Quarter Horses, 3/111 (3%) Tennessee Walking Horses and 12/111 (11%) other breeds. For those animals where sex was noted, the majority, 53/108 (49%), were female, 14/108 (13%) were male, and 41/108 (38%) were geldings. Of the 193 samples submitted, 192 had a clinical status assigned on the laboratory submission form, 31 of which (16%) were classified as clinical, 69/192 (36%) samples were classified as convalescent, and 92/192 (48%) were classified as asymptomatic (subclinical) at the time of sampling. Of these 192 samples, 59 were from horses of New Bolton Center (5 horses of the Widener Hospital and 54 horses of William Boucher Field Service) and 133 were from horses of ambulatory clinicians of other practices. Of the 113 horses from which samples were obtained, 67 (59%) had a single sample submitted, either 2 or 3 samples were obtained from each of 20/113 horses (18%), and 4 samples from 5/113 horses (4%). The most samples collected was 6 from 1/113 horses (1%).

Thirty-five of the 193 (18%) samples tested were positive for *S. equi* by the gold standard procedure (direct-PCR). Eight of 35 (23%) were clinical, 16/35 (46%) were convalescent, and 10/35 (29%) direct-PCR positive samples were classified as subclinical. Compared to samples classified as subclinical, the odds of detecting a positive with direct-PCR were significantly increased if samples were classified as either clinical (OR = 2.85, 95% CI 1.05–7.74, $P = .040$) or convalescent (OR = 2.68, 95% CI 1.14–6.31, $P = .024$). Similar relationships were observed for the 24 samples that were FS-PCR positive (clinical OR = 7.62, 95% CI 2.16–27.15, $P = .002$ and convalescent OR = 4.63, 95% CI 1.39–15.44, $P = .013$) and the 15 FS culture positive samples (clinical OR = 8.65, 95% CI 1.55–48.39, $P = .014$ and convalescent OR = 5.90, 95% CI 1.13–30.82, $P = .035$). In the case of traditional culture, there were also 15 positive samples, but compared to subclinical samples, the odds of detecting a positive were only statistically significantly increased if the sample was classified as clinical (OR = 10.80, 95% CI 1.99–58.52, $P = .006$, whereas convalescent OR = 5.08, 95% CI 0.99–25.86, $P = .050$).

Table 1 shows the comparison of the laboratory tests based on sample type (NPW versus GPL). With the exception of FS culture where slightly more GPL than NPW samples were found to be positive, for the other tests the number of NPW samples detected as positive was either greater than (direct-PCR, FS PCR) or equal to (traditional culture) the number of positive GPL samples. Despite that, as there were fewer GPL samples overall, compared to NPW (16 ± 3%, 95% CI 10–23) a greater proportion of GPL samples (27 ± 6%, 95% CI 17–40) were identified as positive although this effect did not achieve statistical significance ($P = .198$). For direct-PCR, the odds of detecting a positive on a NPW sample were decreased by 56% compared to the odds of detecting a positive on a GPL sample ($P = .030$). Likewise, the odds of samples tested by FS culture being positive were decreased by 69% for those from NPWs compared to a GPL source ($P = .021$). The odds of detecting a positive from NPW compared to GPL samples were also reduced for FS PCR (53%, $P = .056$) and traditional culture (60%, $P = .059$) but in neither case were the differences statistically significant. There was no effect of age, sex, or breed on *S. equi* detection rates of any of the methods examined in this study.

Table 2 shows the comparison of the different laboratory methods to the gold standard (direct-PCR) based on a binomial (positive or negative) outcome. None of the other testing methods achieved greater than 69% sensitivity when compared to direct-PCR and for culture methods sensitivity was in the 40% range. Moreover, the positive and negative predictive values of culture methods were markedly reduced compared to PCR. Although preliminary statistics (Fisher's exact test) indicated that the direct-PCR and FS-PCR results were significantly different ($P < .001$), the logistic regression model gave an OR = 1 (ie, the results were essentially identical). However, because non-negative FS-PCR results predicted success perfectly (ie, there were no false-negatives), the model could not determine the 95% CI and P value. As a result, the area under the ROC curve was 0.5 suggesting that the diagnostic accuracy of the FS-PCR corresponded to random chance. The results obtained by both culture methods were also significantly different from those of the direct-PCR ($P < .001$) and the odds of identifying a sample as positive by either traditional or FS culture were significantly lower than the odds of detecting a positive with direct-PCR (Table 2). For both traditional (0.694) and

Table 1. Comparison of the ability of laboratory methods to detect positive *Streptococcus equi* samples by sample source; nasopharyngeal wash (NPW) and guttural pouch lavage (GPL);the odds of detecting a positive from a NPW compared to a GPL sample were quantified by means of logistic regression.

Laboratory Method	No. NPW + of All +(%)	No. GPL + of All + (%)	No. of NPW + of All NPW (%)	No. of GPL + of All GPL (%)	OR	95% CI	*P* Value
Direct PCR	19/35 (54)	16/35 (46)	19/134 (14)	16/59 (27)	0.44	0.21–0.92	.030
Flocked swab PCR	13/24 (54)	11/24 (46)	13/134 (10)	11/59 (19)	0.47	0.22–1.02	.056
Flocked swab culture	7/16 (44)	9/16 (56)	7/134 (5)	9/59 (15)	0.31	0.11–0.84	.021
Traditional culture	8/16 (50)	8/16 (50)	8/134 (6)	8/59 (14)	0.40	0.16–1.04	.059

OR, odds ratio; CI, confidence interval.

Table 2. Comparison of the ability of laboratory methods to detect positive *Streptococcus equi* field samples compared to the direct-PCR (gold standard).

Laboratory Method	Number Positive	False Positives	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	CC (%)	OR	95% CI	*P* Value
Direct PCR (gold standard)	35	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Flocked swab PCR	24	0	69	100	100	100	94	1	NA	.001*
Flocked swab culture	13	3[a]	37	98	81	88	87	30.5	8.6–108.8	<.001
Traditional culture	14	2[a]	40	99	88	88	88	52	10.8–250.2	<.001

OR, odds ratio; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; CC, correctly classified; Ref, referent; NA, not available.

[a]Two of these three values represent the same samples.

*Fisher's exact test, *P* value for logistic regression model not calculable.

FS culture (0.676) the area under the ROC curves indicated rather poor diagnostic accuracy relative to direct-PCR. Comparison of direct-PCR with the FS-PCR based on a continuous outcome (CT value) revealed that for the 24 samples positive on both tests, the direct-PCR amplified *S. equi* DNA significantly more rapidly (ie, lower CT value, mean = 32.1, 95% CI 30.3–34.0, $P = .002$) than did FS PCR (mean = 33.7, 95% CI 31.9–35.4). CT values for the 11 samples positive by direct-PCR but not on FS PCR were significantly higher (mean = 37.8, 95% CI 37.0–38.7, $P < .001$) than with those obtained from the 24 samples that were positive on both PCR tests. Similarly, the CT values for those samples identified as positive by direct-PCR but negative on traditional culture (N = 21, mean = 35.8, 95% CI 34.0–37.5, $P = .001$) were significantly higher than the CT values for those positive on both test (N = 14, mean = 31.1, 95% CI 28.8–33.5, $P = .001$), as were the equivalent groups for direct-PCR and FS-culture (PCR positive/FS culture negative N = 22, mean = 35.5, 95% CI 33.7–37.2, $P = .004$; positive on both tests N = 13, mean = 31.3, 95% CI 28.7–33.9). In the case of culture methods, the upper limits of the CIs suggest that samples with a CT value above 37 on direct-PCR are significantly less likely to yield a positive result on culture, and all direct-PCR samples with a CT value >37.9 were negative by culture.

Additional comparisons between laboratory methods include the following: traditional culture identified 13/24 (54%) of the samples positive on the FS-PCR and 3 samples were culture positive that were FS-PCR negative ($P < .001$); FS culture identified 12/24 (50%) of the samples that were positive on FS-PCR and 4 samples were FS culture positive that were FS-PCR negative ($P < .001$). Although the results obtained by FS culture and traditional culture were found to be statistically different from each other ($P < .001$), the differences were in fact marginal (Table 2) and the methods could be considered roughly equivalent with, in this study, traditional culture being very slightly better than FS culture.

Discussion

Direct-PCR more reliably detected *S. equi* in equine samples than did FS culture and traditional culture. None of the other testing methods achieved greater than 69% sensitivity compared to direct-PCR and in the case of culture methods sensitivity was closer to 40% or less (Table 2). Despite being statistically different, the sensitivity of FS culture was similar to traditional culture, with the latter performing only marginally better overall. Our study corroborates the findings of another recent study by Lindahl et al, in which direct real-time PCR had the highest detection rates of *S. equi* from swabs of the rostral nasal passages and NP, and NPW samples collected from acutely ill horses. PCR detection rate improves (reached over 90%) when more than one sample per horse is processed at the same time.[7]

In contrast to previous in vitro findings,[10] the ability of the FS-PCR to detect *S. equi* in NPW and GPL field samples was not equivalent to direct-PCR. The FS-PCR requires additional handling steps in the laboratory which might result in loss of organism in the sample. The inferior performance of the FS-PCR observed in this study was in contrast to reported improvement in the detection of other organisms when the FS was used in preprocessing PCR methods in human medicine.[8] Because the FS sample taken from the original wash is submerged into 1 mL of saline to perform the FS-PCR, the sample is further diluted, resulting in less positive samples via FS-PCR. It follows that direct-PCR amplified *S. equi* DNA faster than the FS-PCR, indicated through statistically lower CT values. This could be avoided if FSs are used at the time of sample collection for *S. equi* and requires further study. Despite showing promise in the in vitro testing,[10] FS culture performed similar to traditional culture with field samples. Relative to direct-PCR, the FS and traditional cultures performed quite poorly with sensitivities of 37 and 40%, respectively. The majority of these samples were from convalescent or subclinical animals, which were evaluated for carrier status. Therefore, the bacterial cell counts are likely considerably lower than they would be in clinical animals, a contention supported by the odds of detecting a positive calculated here on the basis of sample type.[12] This suggests that the poor performance of both culture methods was not the result of incorrect identification and selection of *S. equi* colonies on the plate because of an overgrowth of other organisms, but rather a lack of sensitivity in the culture's ability to grow *S. equi*. In terms of methods, these results showed that direct-PCR was the most sensitive method of detection of *S. equi* carriers.

Guttural pouch lavage samples were more likely to be positive on direct-PCR and FS culture. The guttural pouch is known to harbor *S. equi* for extended periods post infection.[2,4] It is also known that intermittent shedding of *S. equi* into the NP from the guttural pouch makes the NP a less reliable sample despite its ease of access clinically.[4] One limitation of this study was that different anatomical sample sources from the same horse were not compared at the same point in time.

Additional limitations include sampling of animals in both the clinical and potentially carrier state of the disease. Most samples were from convalescent or subclinical horses. Due to the high cost associated with diagnostic testing of large groups of animals during a strangles outbreak, often the index case is sampled at the time of acute infection and subsequent cases are defined as *S. equi* based on clinical signs alone. Most diagnostic testing is performed during the convalescent period of an outbreak to verify negative *S. equi* status and permit release of animals from quarantine. This explains the prevalence of 18% *S. equi* positive samples among this population. Because samples were received from multiple veterinarians at multiple practices, there was no way to effectively standardize the collection methods or volumes of the samples obtained, but, to make them as homogenous as possible, all samples were vortexed at the time of processing. Further characterization of the horse's clinical status beyond the three categories of clinical, convalescent, and subclinical was not feasible in this study. Additional clinical data were not available from majority of the samples with clinical status defined (133/192) for they were not from horses in our hospital or ambulatory unit, rather samples from outside veterinarians who had recorded the clinical status on the laboratory submission form.

This study, along with others,[7,10,13,14] suggests that direct real-time PCR should replace bacterial culture as the gold standard for detection of *S. equi*. In a previous study, dilutions of live *S. equi* in saline were used in vitro to compare laboratory processing methods. The limit of detection for *S. equi* culture was not repeatable until 300 CFU/mL as compared to PCR at 1 CFU.[10] In this study, 21/35 (60%) of the positive direct PCRs had negative traditional bacterial cultures. As a result of the low bacterial counts found in carrier animals, PCR cycle thresholds were high. In the case of culture methods, the upper limits of the CT CIs suggest that samples with a CT value above 37 on direct-PCR are significantly less likely to yield a positive result on culture, and all direct-PCR samples with a CT value >37.9 were negative by culture. Since the majority of samples submitted to our laboratory are from subclinical or convalescent horses, direct real-time *seeI* PCR is used as the gold standard in our laboratory and was used as such in this study. This does not discount the fact that if a sample is culture positive, the sample will be considered positive for *S. equi*. We suggest that both direct-PCR and traditional culture be performed on every sample submitted for the detection of *S. equi* for the highest sensitivity rate. *The seeI* gene target for PCR has recently been argued to be less specific for *S. equi* detection.[13,15] At the time this study was designed, the more specific triplex PCR[13] and the *eqbE* PCR[15] were not yet published.

Use of flocked swabs in conjunction with either PCR or culture did not improve detection of *S. equi* in equine horse specimens. Direct real-time PCR was found to be the most sensitive method and overall a greater proportion of GPL samples were found to be positive indicating the guttural pouch, as opposed to the nasopharynx, to be a more reliable location for detection of *S. equi*. However, further study comparing NPW and GPL sites in the same horse at the same time is warranted.

Footnotes

[a] PrepMan Ultra, Applied Biosystems, Foster City, CA
[b] Eppendorf Centrifuge Model 5417C, Eppendorf North America, Hauppauge, NY
[c] Fisher Scientific, Pittsburgh, PA
[d] 7500 Fast Real-Time PCR System, Applied Biosystems
[e] OmniMix HS lypholized beads, TaKaRa Bio, Inc., Otsu Shiga, Japan
[f] QuantiFast Pathogen PCR and Internal Control (IC) Kit, Qiagen, Inc., Valencia, CA
[g] BBL CultureSwab, Becton Dickinson, Franklin Lakes, NJ
[h] Columbia CNA blood agar plate, Becton Dickinson
[i] Copan Diagnostics, Inc., Corona, CA
[j] STATA version 13.1, Stata Corp., College Station, TX

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Off-Label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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