Article

Relationship between quantitative real-time PCR cycle threshold and culture for detection of *Streptococcus equi* subspecies *equi*

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Abstract

Objective
To compare PCR and culture results for the detection of *Streptococcus equi* subspecies *equi (S. equi)*.

Animals
Respiratory tract samples (N = 158) from horses being tested for *S. equi*.

Procedure
Bacterial culture was carried out on samples from which *S. equi* was detected by quantitative real-time PCR.

Results
S. equi was isolated from 12 (7.6%) samples: 4/9 (44%) samples when the PCR cycle threshold (C_T) was ≤ 30, 7/30 (23%) when the C_T was 30.1 to 35, and 1/119 (0.8%) when the C_T was 35.1 to 40. The highest C_T sample from a sample that yielded a positive culture was 36.9. The optimal Youden's J value was at a C_T of 34.2, the same value as determined by number needed to misdiagnose when the cost of a false negative is deemed to be either 5 or 10 × that of a false positive.

Conclusions
Viable *S. equi* was only detected in a minority of quantitative PCR (qPCR) positive samples. A qPCR C_T of 34.2 was a reasonable breakpoint for likelihood of the presence of culturable *S. equi.*

Clinical relevance
Evaluation of C_T values may be useful as a proxy to indicate the likelihood of cultivable *S. equi* being present and could be useful as part of risk assessments.

Résumé

Relation entre le seuil du cycle de PCR quantitatif en temps réel et la culture pour la détection de *Streptococcus equi* sous-espèce *equi*

Objectif
Comparer les résultats de PCR et de culture pour la détection de *Streptococcus equi* sous-espèce *equi* (*S. equi*).

Animaux
Échantillons des voies respiratoires (N = 158) de chevaux testés pour *S. equi*.

Procédure
La culture bactérienne a été réalisée sur des échantillons à partir desquels *S. equi* a été détecté par PCR quantitatif en temps réel.

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Résultats
S. equi a été isolé à partir de 12 échantillons (7,6 %) : 4/9 (44 %) échantillons lorsque le seuil du cycle de PCR (C_T) était ≤ 30, 7/30 (23 %) lorsque le C_T était de 30,1 à 35 et 1/119 (0,8 %) lorsque le C_T était de 35,1 à 40. L'échantillon C_T le plus élevé d'un échantillon ayant donné une culture positive était de 36,9. La valeur J optimale de Youden était à un C_T de 34,2, la même valeur que celle déterminée par le nombre nécessaire pour un mauvais diagnostic lorsque le coût d'un faux négatif est estimé à 5 ou 10 × celui d'un faux positif.

Conclusion
Du *S. equi* viable n'a été détecté que dans une minorité d'échantillons positifs pour le PCR quantitatif (qPCR). Un C_T qPCR de 34,2 était un seuil raisonnable pour la probabilité de la présence de *S. equi* cultivable.

Pertinence clinique
L'évaluation des valeurs C_T peut être utile comme approximation pour indiquer la probabilité de présence de *S. equi* cultivable et pourrait être utile dans le cadre d'une évaluation des risques.

(Traduit par D[r] Serge Messier)

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Introduction

Streptococcus equi subspecies *equi (S. equi)* is a horse-adapted *Streptococcus* species that causes strangles, a highly infectious and high-profile disease of equids that may be subclinical as well as chronic (1). This bacterium is endemic in the horse population internationally and is spread mainly through direct contact. Because of the highly infectious nature of *S. equi,* strict control measures are sometimes applied; these include testing of in-contact horses, routine testing of apparently healthy horses at the time of entry to a new facility, isolation of infected horses, quarantine of exposed horses or facilities, and testing for release from quarantine. Since some horses may shed *S. equi* or have positive PCR tests for prolonged periods of time (2), control measures can be severely disruptive if affected horses or facilities are subjected to prolonged restrictions. Control of *S. equi* requires balancing disease prevention and disruption. Challenges and knowledge gaps with diagnostic testing and poor understanding of the infectious dose of *S. equi* make it difficult to conduct proper risk assessment.

Culture and PCR are the main tests used for clinical diagnosis or surveillance. Culture detects viable *S. equi,* whereas PCR may detect DNA from viable or non-viable bacteria. Polymerase chain reaction testing is more sensitive than culture (2–4), and quantitative PCR (qPCR) can provide an estimate of the bacterial load — not just a binary positive or negative result — through reporting of the PCR cycle threshold (C_T), which is the PCR cycle at which point the fluorescent signal crosses the threshold. Increased sensitivity can be beneficial, but if it detects horses that are not infectious, then PCR testing could result in unnecessary restrictions or concerns. The objective of this study was to compare PCR and culture results for the detection of *S. equi* in equine upper respiratory tract samples.

Materials and methods

Consent was requested to perform culture on upper respiratory tract samples submitted to the University of Guelph Animal Health Laboratory (Guelph, Ontario) for *S. equi* qPCR.

Real-time qPCR assay amplifying 110 bp of the *eqbE* gene was performed for 45 cycles (5). A PCR reaction with a clear amplification curve and $C_T < 40$ was considered positive. Samples that yielded a PCR $C_T < 40$ were subjected to bacterial culture. Two milliliters of each sample were centrifuged for 5 min at 10 000 rpm. The pellet was plated on Columbia and phenylethyl alcohol blood agar plates and incubated anaerobically at 37°C in 5% CO_2, 10% H_2, and 85% N_2 atmosphere. Plates with hemolytic bacterial colonies resembling *S. equi* were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper; Bruker, Bremen, Germany) using a commercially available reference library. Bacterial species identification was achieved based on the best matching spectra with a score ≥ 2. Protein extract of *Escherichia coli* DH5 α was used for every MALDI-TOF run as a quality control.

Medians, ranges, and interquartile ranges (IQR) were calculated. The Wilcoxon test was used to evaluate the association between C_T and culture. An ANOVA was used to evaluate the association between sample number and C_T. Negative and positive predictive values were calculated. A receiver operating curve (ROC) was plotted. Area under the ROC (AUROC) was calculated. The optimal C_T cutoff was applied using Youden's J statistic (sensitivity + specificity − 1), which uses equal weighting of sensitivity and specificity. The C_T with the highest Youden's value was considered the optimal cutoff. Number needed to misdiagnose (NNM) was also calculated (6), with the C_T highest NNM value considered the optimal cutoff. However, given the potential infection-control implications of false negative results, cutoffs were also determined using weighted NNM (6), assigning the cost of false negatives as 2, 5, and 10 × that of false positives.

Results

A total of 158 PCR positive samples ($C_T < 40$) from 121 horses were tested. The median number of samples tested per horse was 1 (range: 0 to 6). Because limited metadata were available, associations between variables such as age, sex, and sample type were not evaluated. Information about clinical status (*e.g.,* testing in the presence of clinical signs of strangles, post-exposure surveillance, routine surveillance, post-treatment testing) was typically lacking.

The overall median C_T was 37.1 (range: 22.3 to 40; IQR: 3.1). *Streptococcus equi* subsp. *equi* was isolated from 12 (7.6%) samples: 4/9 (44%) samples when the C_T was ≤ 30, 7/30 (23%)

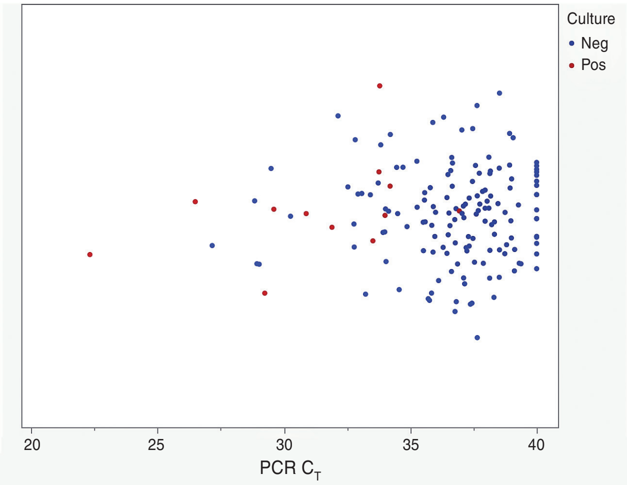

Figure 1. Scatterplot of culture results and PCR cycle threshold (C_T) values for *Streptococcus equi* subsp. *equi (S. equi)* PCR positive equine respiratory tract samples, depicting the range of PCR C_T results for specimens from which *S. equi* was (red) or was not (blue) isolated by culture.

Neg — Negative; Pos — Positive.

when the C_T was 30.1 to 35, and 1/119 (0.8%) when the C_T was 35.1 to 40.

There was a significant inverse association between PCR C_T and culture results ($P < 0.0001$). The median C_T for samples from which *S. equi* was isolated was 32.7 (range: 22.3 to 36.9; IQR: 4.6), compared to 37.2 (range: 27.2 to 40; IQR: 2.7) for negative samples. Figure 1 depicts PCR C_T and culture results. The highest C_T sample from a sample that yielded a positive culture was 36.9.

Table 1 displays the positive predictive value (PPV) and negative predictive value (NPV) for different qPCR C_T thresholds for detection of viable *S. equi.* The ROC is presented in Figure 2. The AUROC was 0.90. The optimal Youden's J value was 0.77 at a C_T of 34.2, with a sensitivity of 92% and specificity of 85%. Based on NNM, the optimal cutoff would be 26.5; however, when the weight (cost) of false negatives is calculated at twice that of a false positive, the optimal cutoff increases to 31.9. When the cost is deemed to be either 5 or 10 × that of a false positive, the optimal C_T is 34.2.

More than 1 sample was collected at different time points from 25 horses. Three of those had a positive culture. In 2 of those 3, *S. equi* was isolated from the 1st sample, followed by a single negative sample. For the other, *S. equi* was isolated only from the 3rd of 6 PCR positive samples collected over 82 d. Overall, there was no apparent association between sample time point (sample 1, sample 2, …) and C_T ($P = 0.09$).

Discussion

Determining optimal approaches to testing requires determining the goals of testing. These goals may vary between situations, creating a need for more than 1 method of interpretation. In some situations, the goal may be ultimate sensitivity; *i.e.,* ensuring that no potentially infected horses are missed. However, this can be disruptive if infection control measures, treatments, and other interventions are unnecessarily applied in situations

Table 1. Positive predictive value (PPV) and negative predictive value (NPV) of quantitative PCR (qPCR) for detection of viable *Streptococcus equi* subsp. *equi (S. equi)* shedding from samples with a qPCR cycle threshold (C_T) < 40, based on testing of respiratory tract samples from horses being tested for *S. equi.*

C_T value cutoff	PPV (%)	NPV (%)
≤ 27.5	67	94
≤ 30	44	95
≤ 32.5	46	96
≤ 35	28	99
≤ 37.5	13	100
< 40	7.6	100

where there was little or no chance that the horse was infectious, or where the population and population management are such that the horse would likely pose limited additional risk. Consideration of the individual horse; the local population and other populations that might be affected (*e.g.,* other horses at a racetrack, patients at a veterinary hospital); the likely *S. equi* status of other, untested horses in the group; and the local epidemiology of the organism (*e.g.,* degree of endemicity) is required. Risk aversion and the implications of both false positives (unnecessary restrictions or responses) and false negatives (risk of transmission to other horses) must factor into the decision-making process. The sample method and number must also be considered when designing a surveillance or control program or acting on the results (7).

The discrepancy between culture and PCR was expected based on the higher sensitivity of qPCR (2–4,8). The results of this study were similar to those of a previous study that compared culture and qPCR from different sample types (9). In that study, samples with a C_T < 37 were significantly more likely to have positive culture results. The highest C_T that yielded a positive culture was 37.9, which is similar to the 36.9 value reported here.

The goal of this study was not to determine a universal C_T cutoff, as interpretation criteria will vary based on the laboratory methods, study population, and goals of testing. Rather, the goal was to provide data to guide the interpretation of C_T cutoffs for samples processed at a specific laboratory and to provide preliminary information to assist with risk assessment. Based on both ROC analysis and weighted NNM, a C_T of 34.2 was suggested as optimal. However, the weighting of false positive and false negative results could affect the decision, and the cost-benefit of false positives *versus* false negatives must be considered when determining cutoffs. With a C_T of 34.2, only 1 (0.6%) horse that had detectable bacterial shedding would have been missed: an indication of low, but non-zero, risk. Consideration of the likelihood of shedding of viable bacteria can be useful for risk assessment after exposure and when assessing potential responses (*e.g.,* active surveillance, environmental cleaning and disinfection, closure of areas).

Ultimately, the risk of transmission is dependent on shedding of adequate numbers of viable bacterium. Testing always provides a somewhat artificial assessment of this because the bacterium may not be homogenously distributed in the upper respiratory tract. Further, nasopharyngeal or guttural pouch specimens are not from sites that are directly exposed to other

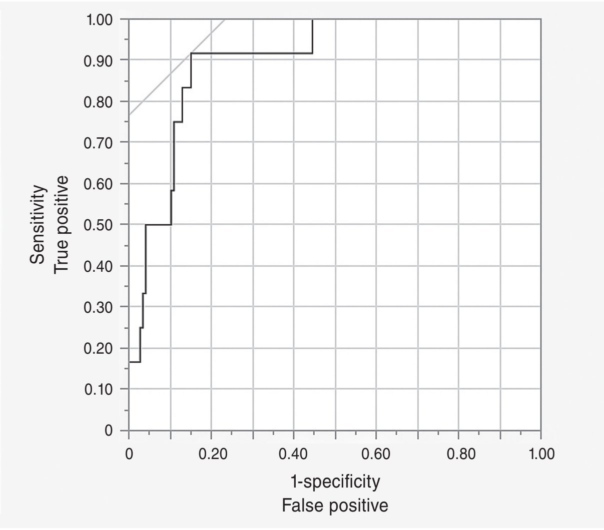

Figure 2. Receiver operating curve (ROC) evaluating bacterial culture for the detection of *Streptococcus equi* subsp. *equi (S. equi)* from PCR positive equine respiratory tract samples.

horses or surfaces, shedding is likely dynamic, and the infectious dose is unknown. Yet, it is reasonable to assume that horses from which viable bacteria cannot be cultured pose lesser or limited (but non-zero) risk. The lack of information about infective dose and variability in *S. equi* shedding over short periods of time hampers determination of the relevance of PCR positive/culture negative results.

Combining culture and PCR can be valuable to provide more insight into the likely bacterial load and transmission risk. However, the added cost can be a barrier. In 2018, the last year for which data are available, $<$ 10% of samples submitted to the University of Guelph Animal Health Laboratory for *S. equi* testing were for both culture and PCR (10) — although paired culture and PCR may be more commonly conducted in some other areas (8).

Various limitations must be considered. The results of this study apply to this specific population and may differ in other populations. Limited metadata were available, but it is unlikely these would affect the test comparison and interpretation — apart from antimicrobial therapy, which would likely have a differential effect of antimicrobial treatment on qPCR *versus* culture results. Anecdotally, very few horses had been treated before sampling, but clear antimicrobial exposure history for most horses was lacking. The potential for loss of *S. equi* viability before culture must also be considered, as culture was typically done after PCR testing results were available, which itself could have been 1 or more days from sample collection. Samples for PCR were typically submitted in saline and, although they were refrigerated, a storage medium was not used. The effect of this is unclear; however, *S. equi* is a relatively hardy bacterium and no effect of short-term (1-week) storage was noted if samples were kept refrigerated (11), as was the case here. Sample type (*e.g.,* nasopharyngeal wash, nasopharyngeal swab, guttural pouch lavage) can also affect sensitivity of sampling (9) but should have less effect on the comparison between culture and PCR. A larger sample size would also have provided more confidence and precision in potential interpretative breakpoints.

Results from this study should not be interpreted as indicating horses that have qPCR positive results with high C_T values pose no risk. Rather, the results provide information that can be used to help assess the degree of risk that might be posed, based on the likelihood that viable *S. equi* is present. In some situations, qPCR positive results with high C_T values may indicate the need for action or restrictions. However, in other situations, the likely lower risk of infectivity might influence the actions or communications that ensue.

In order to balance reducing the risk of *S. equi* transmission with the ramifications of identifying clinically irrelevant shedding of this endemic pathogen, the testing goals and responses should be considered to identify optimal cutoff points and when interpreting qPCR C_T values. Paired culture and qPCR testing (or molecular assessment of bacterial viability, if available) are ideal for assessing the potential risk for transmission. However, evaluation of C_T values may be useful as a proxy to indicate the likelihood of cultivable *S. equi* being present, and could be useful as part of risk assessments.

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