Article

Validation of a point-of-care polymerase chain reaction assay for detection of *Streptococcus equi* subspecies *equi* in rostral nasal swabs from horses with suspected strangles

Andrew T. Willis, Samantha Barnum, Nicola Pusterla

Abstract — This study aimed to validate a point-of-care polymerase chain reaction (PCR) assay for detection of *Streptococcus equi* subsp. *equi (S. equi)* in rostral nasal swabs from horses with suspected acute strangles and to compare the results against the molecular gold standard of quantitative polymerase chain reaction (qPCR). Two hundred thirty-two individual swabs of rostral nasal passages were characterized by qPCR as *S. equi* positive, *S. equi* subsp. *zooepidemicus (S. zooepidemicus)* positive, or *S. equi* and *S. zooepidemicus* negative. The specificity and sensitivity of the point-of-care PCR assay were 89% and 84%, respectively. The limits of detection of the qPCR assay and the point-of-care PCR analyzer were 3 and 277 *eqbE* target genes of *S. equi,* respectively. Overall agreement and short turnaround time make the point-of-care PCR assay a potential molecular diagnostic platform that will enhance the capability of equine veterinarians to timely support a diagnosis of strangles and institute proper biosecurity protocols.

Résumé — Validation d'une épreuve d'amplification en chaîne par la polymérase au point de service pour la détection de *Streptococcus equi* sous-espèce *equi* dans des écouvillons nasaux rostraux de chevaux suspectés d'avoir la gourme. La présente étude visait à valider une épreuve d'amplification en chaîne par la polymérase (PCR) au point de service pour la détection de *Streptococcus equi* ssp. *equi* (*S. equi*) à partir d'écouvillons nasaux rostraux de chevaux suspectés être atteints de gourme aiguë et de comparer les résultats à ceux de l'épreuve étalon de la réaction d'amplification en chaîne par la polymérase quantitative (qPCR). Deux cent trente-deux écouvillons individuels des voies nasales rostrales furent caractérisés par qPCR comme étant *S. equi* positif, *S. equi* ssp. *zooepidemicus* (*S. zooepidemicus*) positif ou *S. equi* et *S. zooepidemicus* négatifs. La spécificité et la sensibilité de l'épreuve PCR au point de service étaient de 89 % et 84 %, respectivement. Les limites de détection de l'épreuve par qPCR et de l'analyseur PCR au point de service étaient de 3 et 277 copies du gène cible *eqbE* de *S. equi*, respectivement. L'accord général et le court temps de réponse font du PCR au point de service une plate-forme de diagnostic moléculaire potentielle qui augmentera les capacités des vétérinaires équins à appuyer adéquatement un diagnostic de gourme et d'instituer les protocoles de biosécurité appropriés.

(Traduit par Dr Serge Messier)

Can Vet J 2021;62:51–54

Introduction

Strangles is a bacterial infection of the upper respiratory tract of equids, caused by *Streptococcus equi* subsp. *equi (S. equi)* (1). Clinical disease involves bacterial colonization of the patient's tonsils and pharynx resulting in upper respiratory catarrh and abscessation of the mandibular and retropharyngeal lymph nodes. The incubation period of strangles is up to 2 wk and signs will be evident within 1 to 2 d of the onset of fever. It is essential to isolate any horse with signs of strangles to prevent population outbreaks. Current diagnostic testing requires confirmation of the presence of *S. equi* detected by conventional bacterial culture and/or polymerase chain reaction

William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, 1 Garrod Drive, Davis, California, USA (Willis); Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, California, USA (Barnum, Pusterla).

Address all correspondence to Dr. Nicola Pusterla; e-mail: npusterla@ucdavis.edu

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Table 1. Characterization of 232 rostral nasal swabs based on qPCR results used for the validation of a point-of-care PCR testing platform specific for *S. equi* subspecies *equi*.

Sample group	*S. equi* qPCR cycle threshold value	Copies of *eqbE* gene/μL	Minimum	Maximum	Median	Interpretation	Number of samples
1	None	—	—	—	—	Negative *S. equi* and *S. zooepidemicus*	42
2	None	—	—	—	—	Negative *S. equi* and positive *S. zooepidemicus*	40
3	< 32	> 828	897	32 083	8087	Strong positive (abundant amount of *S. equi* DNA present)	42
4	32 to 35	92 to 828	128	828	346	Moderate positive (moderate amount of *S. equi* DNA present)	49
5	> 35	< 92	5	91	15	Weak positive (small amount of *S. equi* DNA present)	59

(PCR) (2). The latter analytical platform is considered to be the gold standard for detection of the streptococcal organism (2,3). The current workflow for PCR detection requires access to a laboratory for testing, often with turnaround times between 24 and 72 h from sample collection. Although same-day reporting of a molecular result is possible with current diagnostic advancements, timely diagnosis of strangles, isolation of index cases, and institution of proper biosecurity protocols are critical to prevent rapid spread of disease in at-risk populations. Recent advances in diagnostic technology have allowed for patient-side PCR tests, such as influenza virus and respiratory syncytial virus testing in humans, to be developed with turnaround times within 1 h of sample collection (4).

Advantages of a point-of-care PCR platform for the equine practitioner would be to initiate timely treatment and biosecurity protocols, to offer immediate and weekend testing when central laboratories are closed, to test for *S. equi* at multiple time points during clinical disease, to initiate timely outbreak testing protocols in at-risk populations, and to screen high-risk horses entering *S. equi*-free herds or facilities. The objective of this study was to validate a point-of-care PCR assay for the detection of *S. equi* in rostral nasal swabs from horses with strangles and to compare the results against the molecular gold standard of quantitative PCR (qPCR). The authors hypothesized that the point-of-care PCR assay would generate comparable results in terms of sensitivity, specificity, and overall agreement compared with the gold standard of qPCR for the detection of *S. equi* in rostral nasal swabs from horses with suspected acute strangles.

Materials and methods

Study samples

A total of 232 individual swabs collected from the rostral nasal passages from horses were tested for *S. equi* in parallel with the gold standard qPCR system (7900 HTA; Applied Biosystems, Foster City, California, USA) and the point-of-care PCR analyzer (Fluxergy, Irvine, California, USA). Testing was performed in the investigators' laboratory and not patient-side. Rostral nasal secretions were collected from horses with acute respiratory signs compatible with strangles using rayon-tipped swabs. The swabs were immersed in 1 mL of phosphate-buffered saline solution (PBS).

Sample testing

The gold standard methodology included nucleic acid purification followed by qPCR analysis, while the POC PCR methodology combined sample preparation (including DNA extraction) and amplification into a microfluidic test card. Nucleic acid was extracted from 200 μL of the clinical samples in PBS using an automated nucleic acid extraction protocol (QIAcube HT; Qiagen, Valencia, California, USA) and analyzed for the presence of *S. equi* and *S. zooepidemicus* using gold standard qPCR. In brief, purified nucleic acids were assayed for the presence of selected streptococcal-specific genes (*eqbE* gene of *S. equi* and *ITS* gene of *S. zooepidemicus*) according to previously published protocols (5). The samples were amplified in a combined thermocycler/fluorometer (7900 HTA; Applied Biosystems) with the standard thermal cycling protocol: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The qPCR reactions were composed of a commercially available mastermix (Universal TaqMan Mastermix with AmpErase UNG; Applied Biosystems), containing 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM dUTP, 0.625 U of AmpliTaq Gold per reaction, 0.25 U AmpErase UNG per reaction, 400 nM of each primer (*eqbE* forward primer: CTATTGTTGTCGCTATGGGTGG, *eqbE* reverse primer: GAATGGAAATCCAATCTTTCGG; *ITS* forward primer: GAGAGCGCCTGCTTTGCA, *ITS* reverse primer: GGTAAACCGAACCGTCTGTTAGTATC) and 80 nM of the respective TaqMan probe (*eqbE* probe: FAM-CAGAAGCATCTATTTGGTC-MGB; *ITS* probe: TAMRA-CAGGAGGTCAGCGGTTCGATCCC) and 1 μL of DNA sample for a total volume of 12 μL. An aliquot of the samples in PBS was available for testing using the point-of-care PCR analyzer (Fluxergy). For each test, 36 μL of nasal secretions in PBS were mixed with 84 μL of rehydration buffer and pipetted into a microfluidic test card targeting the *eqbE* gene of *S. equi* (forward primer: ATGTAGCTATGGCAAATGTGGC, reverse primer: AACACCCTTAGGAACACCTG). The test card was inserted into the device and the *S. equi* PCR test was initiated. Fluxergy's proprietary microfluidic system handles sample preparation (including DNA extraction) by employing a sample type specific buffer system. Specifically, the inhibition-resistant buffer along with a mixture of surfactants and dispersants allows

Table 2. Overall agreement for 232 rostral nasal swabs between the gold standard of qPCR and the point-of-care PCR testing platform for the detection of *S. equi* subspecies *equi.*

Point-of-care PCR platform	qPCR platform		
	Positive *S. equi*	Negative *S. equi*	Total
Positive *S. equi*	126	0	126
Negative *S. equi*	16	73	89
Indeterminate[a]	8	9	17
Total	150	82	232

[a] The point-of-care PCR assay reports indeterminate results when the housekeeping gene *(eGAPDH)* is not detected in a biological sample and reflects a sample that does not pass quality control.
Sensitivity 84% (126/150).
Specificity 89% (73/82).

for rapid sample dispersion and lysis of raw sample matrix. The cycling conditions of the point-of-care PCR device were 94°C for 5 min followed by 45 cycles of 10 s at 94°C and 25 s at 56°C. The total time for each test was 50 min.

Each clinical sample was assigned to one of 5 groups based on the gold standard qPCR results (Table 1). *Streptococcus equi* qPCR-positive samples were further divided into 3 arbitrarily chosen groups based on absolute quantitation of the target gene and cycle threshold value. Absolute quantitation of the target gene *(eqbE)* of *S. equi* was performed using a standard curve as previously reported (5). The cycle threshold is defined as the number of heat cycles required for the fluorescent signal to cross a threshold that confirms presence of the specific target gene. The upper cycle threshold cut-off value for both readers was set at a cycle threshold value of 40. The outcome for the point-of-care PCR testing platform was either negative (absence of the target gene and presence of the housekeeping gene *eGAPDH*), positive (presence of the target gene and housekeeping gene *eGAPDH*) or indeterminate. The latter result was reported when the point-of-care PCR analyzer was unable to detect the housekeeping gene *eGAPDH*. Unfortunately, indeterminate results could not be repeated due to the inability to resample and retest the clinically affected horses.

Limit of detection (LOD) of the qPCR device and the point-of-care PCR assay was tested using 10-fold dilutions of a plasmid containing the *eqbE* target sequence. Analytical sensitivity for the 2 PCR platforms was assessed in order to explain potential discrepant test results. While both PCR platforms used the same specimens (swabs in PBS), the sample input to generate the respective results was different. The PCR POC platform used 36 μL of specimen while the qPCR used 1 μL of purified DNA from 200 μL of specimen. Differences in volume and dilution were considered when determining the LOD for each of the 2 platforms.

Once the validation had been completed, the results for both testing platforms were available to determine sensitivity, specificity, and overall agreement.

Data analysis

Descriptive statistics were used to determine sensitivity, specificity, and overall agreement for both testing platforms. For analytical purposes, all indeterminate results were classified as negative results.

Results

The overall agreement between the 2 PCR platforms was 85.8% (Table 2). For strongly positive and moderately positive bacterial loads (cycle threshold values < 32 and cycle threshold values 32 to 35, respectively), the point-of-care PCR assay showed 100% agreement with the qPCR analyzer and for weak bacterial loads (cycle threshold values < 35), the point-of-care PCR assay showed 71% agreement with the qPCR device. The rate of indeterminate samples was 17/232 (7.4%). Among the indeterminate results 9/17 were negative by the qPCR device and 8/17 were positive by the qPCR device. Indeterminate samples were not retested due to the unavailability of horses for further sampling. When indeterminate samples were included in the calculations, the specificity and sensitivity of the point-of-care PCR assay was 89% and 84%, respectively. The limits of detection of the qPCR assay and the point-of-care PCR analyzer were 3 and 277 *eqbE* target genes of *S. equi,* respectively.

Discussion

The point-of-care PCR assay showed strong agreement with the qPCR assay and detected *S. equi* in most study samples in less than 1 h. The strong agreement and short turnaround time make the point-of-care PCR device a potential molecular diagnostic platform allowing detection of *S. equi*. The availability of an accurate point-of-care device for the detection of *S. equi* will enhance the diagnostic capability of equine veterinarians to timely support a diagnosis of strangles and institute proper biosecurity protocols. Furthermore, the point-of-care PCR device was able to differentiate between *S. zooepidemicus* and *S. equi*, which is relevant for the equine practitioner, as false positive results can have undesirable consequences. False negative results are equally concerning, especially when they directly impact biosecurity decisions. Seventeen samples yielded indeterminate results, meaning that the nasal secretions did not pass quality control for reasons such as not enough nucleic acids present in the biological sample or presence of organic inhibitors. Unfortunately, horses with indeterminate results were unavailable for resampling and retesting. Considering that almost half of the indeterminate results tested PCR-positive for *S. equi* using the gold standard qPCR system, such horses should always be treated as a potential risk and isolated whilst awaiting the results of a follow-up test.

This study also confirmed that rostral nasal swabs from acute cases were a suitable sample for the point-of-care PCR assay. Validation of other biological samples, such as lymph node aspirates, nasopharyngeal washes, nasopharyngeal swabs, and guttural pouch washes using the point-of-care PCR assay is needed since deeper respiratory tract samples have been shown to be more sensitive in the detection of *S. equi* compared to rostral nasal secretions (2). While the point-of-care PCR assay offers much convenience for testing, the limit of detection must be considered when testing clinical samples. The point-of-care PCR assay has a 100-fold lower limit of detection compared to the qPCR platform. The point-of-care PCR instrument uses 36 times the volume of material compared to the qPCR (36 μL *versus* 1 μL). The difference in volume was taken into account

when the limit of detection was calculated, meaning that for the point-of-care PCR instrument, the limit of detection is 277 *eqbE* target genes in 36 μL of template, while for the qPCR it is 3 *eqbE* target genes in 1 μL of purified nucleic acid. The reduced limit of detection of the point-of-care PCR assay likely relates to a faster nucleic acid extraction protocol and a shorter nucleic acid purification step. The 16 samples that gave a false negative *S. equi* result by the point-of-care PCR assay were all below its analytical sensitivity. Unfortunately, the horses from which samples were submitted were unavailable for retesting. It is the authors' recommendations that if a horse is presented with clinical signs consistent with strangles, the first step is to isolate the index case and to institute proper biosecurity protocols in order to reduce potential pathogen spread. Furthermore, if rostral nasal swabs test negative by the point-of-care PCR assay, the patient should either be retested 12 to 24 h later when a greater number of *S. equi* are present in nasal secretions, a deeper biological sample (e.g., nasopharyngeal swab or guttural pouch wash) should be collected for diagnostic testing or the sample should be shipped to a diagnostic laboratory and tested using the gold standard qPCR.

When considering the use of a novel point-of-care assay, the end-user must consider advantages and limitations of the technology. The performance of the point-of-care PCR was evaluated in a controlled laboratory setting, which potentially could facilitate workflow and reduce the risk of environmental contamination. The point-of-care PCR is easy to perform and suitable as a patient-side assay with low risk of contamination considering minimal sample handling following collection and closed-tube microfluidic card for analysis. While the format of the point-of-care PCR and the quick turnaround time are appealing factors, one also needs to consider that, at the present time, the point-of-care PCR can only analyze 1 sample at the time, is limited to the testing of a single pathogen, and is less sensitive than laboratory-based qPCR technologies. Furthermore, based on the limit of detection of the point-of-care PCR, testing should be restricted to horses with clinical signs compatible with acute strangles and future studies are required to determine the efficacy of this technology to detect subclinical infections using more sensitive samples such as guttural pouch washes. While the *S. equi* point-of-care PCR assay is not cheaper ($50 US for test card and reagents) than a similar assay offered by a commercial diagnostic laboratory, the lack of shipping costs should make this assay cost-effective to the client. Sample preparation buffers, PCR reagents, and the test card consumables must be purchased individually. Veterinarians can gain access to the laboratory platform through the company's pilot program and can build and validate their own PCR tests for use in their practice. The company's device, reagents, and consumables are not currently USDA approved.

In conclusion, the point-of-care PCR assay yielded acceptable results in terms of sensitivity, specificity, and overall agreement when compared to a qPCR platform for the detection of *S. equi* in rostral nasal swabs of horses with signs compatible with strangles.

Acknowledgment

The authors thank Fluxergy for providing access to PCR readers and providing the test cards to run the point-of-care PCR.

CVJ

References

1. Sweeney CR, Timoney JF, Newton JR, Hines MT. *Streptococcus equi* infections in horses: Guidelines for treatment, control, and prevention of strangles. J Vet Intern Med 2005;19:123–134.
2. Boyle AG, Timoney JF, Newton JR, Hines MT, Waller AS, Buchanan BR. *Streptococcus equi* infections in horses: Guidelines for treatment, control, and prevention of strangles — Revised consensus statement. J Vet Intern Med 2018;32:633–647.
3. Waller A. New perspectives for the diagnosis, control, treatment, and prevention of strangles. Vet Clin N Am Equine Pract 2014;30:591–607.
4. Basile K, Kok J, Dwyer DE. Point-of-care diagnostics for respiratory viral infections. Expert Rev Mol Diagn 2018;18:75–83.
5. Pusterla N, Leutenegger CM, Barnum SM, Byrne BA. Use of quantitative real-time PCR to determine viability of *Streptococcus equi* subspecies *equi* in respiratory secretions from horses with strangles. Equine Vet J 2018; 50:697–700.