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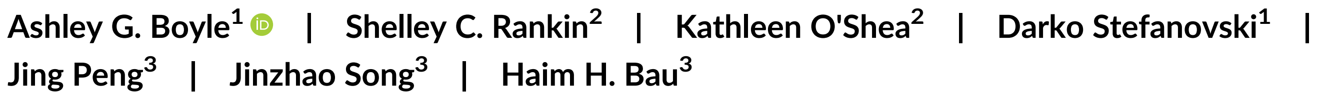
Detection of *Streptococcus equi* subsp. *equi* in guttural pouch lavage samples using a loop-mediated isothermal nucleic acid amplification microfluidic device



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Abstract

Background: Rapid point-of-care (POC) detection of *Streptococcus equi* subsp. *equi* (*S. equi*) would theoretically reduce the spread of strangles by identifying index and carrier horses.

Hypothesis: That the *eqbE* isothermal amplification (LAMP) assay, and the same *eqbE* LAMP assay tested in a microfluidic device format, are comparable to a triplex real-time quantitative polymerase chain reaction (qPCR) assay that is commonly used in diagnostic labs.

Samples: Sixty-eight guttural pouch lavage (GPL) specimens from horses recovering from strangles.

Methods: Guttural pouch lavage specimens were tested for *S. equi* retrospectively using the benchtop *eqbE* LAMP, the *eqbE* LAMP microfluidic device, and compared to the triplex qPCR, that detects 2 *S. equi*-specific genes, *eqbE* and *SEQ2190*, as the reference standard using the receiver operating characteristic area under the curve (ROC).

Results: The 27/68 specimens were positive by benchtop *eqbE* LAMP, 31/64 by *eqbE* LAMP microfluidic device, and 12/67 by triplex qPCR. Using the triplex PCR as the reference, the benchtop *eqbE* LAMP showed excellent discrimination (ROC Area = 0.813, 95% confidence interval [CI] = 0.711-0.915) as did the LAMP microfluidic device (ROC Area = 0.811, 95% CI = 0.529-0.782). There was no significant difference between the benchtop LAMP and LAMP microfluidic device (ROC Area 0.813 ± 0.055 vs 0.811 ± 0.034, *P* = .97).

Conclusions: The *eqbE* LAMP microfluidic device detected *S. equi* in GPL specimens from convalescent horses. This assay shows potential for development as a POC device for rapid, sensitive, accurate, and cost-efficient detection of *S. equi*.

Abbreviations: AUC, area under the curve; CFU, colony forming unit; CT, cycle threshold; GPL, guttural pouch lavage; IQR, interquartile range; LAMP, loop-mediated isothermal nucleic acid amplification; LOD, limit of detection; POC, point-of-care; qPCR, quantitative polymerase chain reaction; ROC, receiver operator characteristic; SZIC, *Streptococcus equi* subsp. *zooepidemicus* internal control.

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KEYWORDS
diagnostics, DNA amplification, equine, point-of-care, strangles, *Streptococcus equi*

1 | INTRODUCTION

There is a need for convenient, rapid, and accurate diagnostic tools for strangles, the highly infectious upper respiratory disease of horses caused by the bacteria *Streptococcus equi* subsp. *equi* (*S. equi*). A low-cost point-of-care (POC) diagnosis of strangles could theoretically reduce the spread of this infectious disease by identifying *S. equi* in index horses earlier. In addition, a POC test could also be used to detect *S. equi* in convalescent, or carrier animals, and thus enable rapid implementation of biosecurity measures. There are currently no validated commercially available stall-side tests to detect *S. equi*. Real-time quantitative polymerase chain reaction (qPCR) has been shown to be approximately 3 times more sensitive than culture.[1,2] Quantitative polymerase chain reaction; tests are typically only offered at veterinary diagnostic laboratories and while the qPCR test can be completed within 1 to 2 hours of the specimen arriving at the lab, results can take longer, 1 to 3 days, if specimens need to be shipped to the lab.

Streptococcus equi qPCR assays have been developed to detect various target genes: a partial DNA sequence of *SeM* (fibrinogen-binding protein gene),[2,3] *seeI* (a superantigen-encoding gene),[4] *eqbE* (equibactin gene involved in iron acquisition),[5] or a combination of *SeeI* and *eqbE*. A triplex qPCR assay has been developed that detects 2 *S. equi*-specific genes (*eqbE* and *SEQ2190*) and an internal control (SZIC) developed from a strain of *Streptococcus equi* subsp. *zooepidemicus* that serves as a DNA extraction and within-assay qPCR control to reduce the risk of false negative reporting.[6] Real-time qPCR is a high complexity test and it is expensive because it requires a laboratory infrastructure and specialized equipment.[7]

Loop-mediated isothermal amplification (LAMP) is a rapid nucleic acid amplification performed at a constant temperature (63°C) using strand displacement amplification. The method has high amplification efficiency, uses 6 specially designed primers, that span 8 distinct sequences of a target gene in a single reaction.[7,8] Amplification products can be detected by agarose gel electrophoresis, use of a turbidimeter, visualization with an intercalating dye, and on lateral flow devices.[7] This technology has been used to detect many veterinary pathogens such as foot-and-mouth disease virus and canine distemper virus.[7]

Loop-mediated isothermal nucleic acid amplification has been successfully demonstrated in a microfluidic device and this will allow for "real time" detection of pathogens at a low estimated cost of $10 per device; with the additional benefit that these assays can be used at the point of care.[9] A LAMP microfluidic device is disposable, water-activated, requires no power and limited external instrumentation (a smartphone; Figure 1).[9,10] Liu et al successfully used a LAMP microfluidic device to detect *Escherichia coli*.[11] Additional organisms, including hepatitis B virus,[12] *Mycobacterium tuberculosis*,[13] human immunodeficiency virus,[14] and Zika virus,[15] have since been detected using similar systems.

Boyle et al showed that guttural pouch lavage (GPL) is the best specimen to collect from horses to detect carriers of *S. equi*.[16,17] The

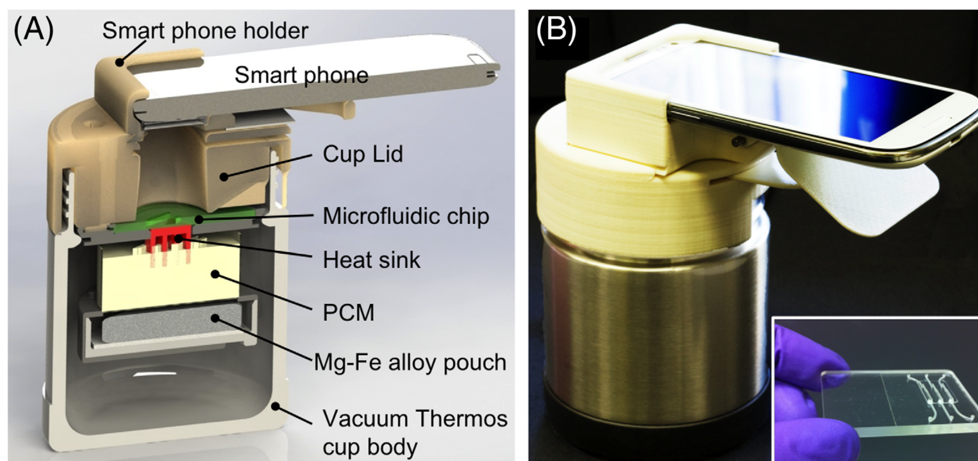

FIGURE 1 The smart cup is a customized Thermos bottle that provides the inserted single-use chip with constant temperature heating. The smart cup has a built-in mount for positioning a smartphone. The smartphone flash excites fluorescence emission, the smartphone CCD camera monitors the reaction progress. Heating is due to an exothermic reaction of Mg-Fe powder initiated by addition of water. A phase-change material (PCM) maintains the chip at a constant ∼65°C temperature for about 60 minutes. A, Cross-section of Smart Cup showing Mg-Fe alloy pouch that is activated by adding water to initiate heating, PCM, heat sink, and slot holding microfluidic chip. Smartphone is held for optimal focus of smartphone CCD camera on chip amplification chamber to measure fluorescence; B, Field unit and companion chip (inset). The smart cup can be optionally made of Styrofoam, making it entirely disposable.[9] Permission obtained from Elsevier for reprint of figure and legend License number 4850770852787, June 16, 2020

same group designed and validated a LAMP assay for the specific detection of *S. equi* using the target gene *eqbE*—the validation data showed 100% sensitivity and 100% specificity.[17] To verify that this assay can detect *S. equi*, the *eqbE* LAMP assay has previously been compared with a qPCR assay that detected the *seeI* gene.[17] The comparison used GPL specimens from strangles convalescent horses and was found to have acceptable discrimination when the *seeI* PCR result was considered as a true measure of outcome (receiver operator characteristic [ROC] 0.78).[17] The performance of the *eqbE* LAMP assay[17] has not been compared to the triplex real-time qPCR assay developed by Webb et al.[6] In this study presented here, we hypothesized that the *eqbE* LAMP assay, and the *eqbE* LAMP assay in a microfluidic device, are both comparable to the triplex real-time qPCR assay using GPL specimens from strangles convalescent horses.[6]

2 | METHODS

2.1 | Samples

For this proof of principle study, 68 frozen GPL specimens obtained from asymptomatic horses recovering from confirmed *S. equi* infection were analyzed with 3 molecular diagnostic methods for the detection of *S. equi* (*eqbE* benchtop LAMP assay,[17] *eqbE* LAMP microfluidic device, and the triplex qPCR[6]). Logistic regression power analysis determined a sample size of 60 GPL specimens were enough for the study to demonstrate a difference between the 3 DNA amplification methods (power of 80% and $\alpha \leq .05$) (STATA 14.0). Use of archived samples was approved by the University of Pennsylvania and client consent was obtained from the original protocol for which they were obtained.

2.2 | Diagnostic tests

Standard laboratory protocols included the following:

DNA extraction was performed in the Veterinary Microbiology Research Laboratory from a 1 mL aliquot of the GPL as previously described.[17] A 5 μL volume of the PrepMan Ultra nucleic acid extraction was used as template for the benchtop LAMP and triplex qPCR assay, in a final volume of 25 μL.

A: The benchtop LAMP assay was performed in the Veterinary Microbiology Research Laboratory and used the following primers and probe as described previously.[17]

eqbE F3	5′- CACATAAAACTACAGTACAAGGT- 3′
eqbE B3	5′- GCGAGTATGAGTAATGCCA- 3′
eqbE FIP	5′TAAAGCTTTTTCCCAAGAAGCTTCTGCTGG TGGTCAATTCTCT- 3′
eqbE BIP	5′ATAGGGCTTGGGCTGATGTTAATGCTAAAAT AACAACGTGGC- 3′
eqbE Loop F	5′- GCGCTTGTCCAACCCGAATA- 3′
eqbE Loop B	5′- AAATAGTTGAACGAGTTTGAGCGGT- 3′

B: *eqbE* LAMP microfluidic devices were created and the assay was performed by the Micro and Nano Fluidics Laboratory at the University of Pennsylvania School of Engineering. Each device housed 3 to 4 reaction chambers for isothermal amplification of nucleic acids. Each chamber is equipped with an integrated, flow-through membrane for the isolation, concentration, and purification of DNA. The nucleic acids captured by the membrane are used directly as templates for amplification.[14] A 200 μL sample of the GPL specimen was added directly to the device.

2.2.1 | Microfluidic device design

The laboratory tested the microfluidic device with samples that contained known quantities of *S. equi* spiked in buffer. Using previously described methods,[14,15] standard amplification curves were obtained for the detection of known concentrations of *S. equi*: 0 (n = 3), 100 (n = 10), 1000 (n = 3), and 10^6 (n = 3) colony forming units (CFU)/mL (Figures 2 and 3). The emission intensity of the intercalating dye was monitored in real time to obtain amplification curves, and the time delay until the signal exceeds a threshold was correlated with target concentration. The amplicons were removed from the reactors and subjected to gel electrophoresis for verification (only during the development work).

2.2.2 | Device and flow control

Rapid prototyping and layered manufacturing was used to form microfluidic cassettes or “chips” (devices). Sheets of poly(methyl methacrylate) were patterned with microfluidic circuits and reaction chambers using a CNC computer-controlled milling machine (HAAS Automation Inc, Oxnard, California) and a laser cutter (Universal Laser Systems, Scottsdale, Arizona). The individual layers were solvent (acetonitrile) bonded at room temperature. Although reagents can be prestored in the cassette,[14] in this work, the reaction mix was inserted into the reaction chamber after nucleic acid isolation and wash steps as previously described.[18] For the purpose of this validation, the majority of the results of the reaction were read with a custom-made reader that utilizes a USB microscope. To demonstrate the ability of the camera to read the fluorescent by-product of the amplification reaction and distinguish a positive vs negative results, some results were also read by a smartphone.[10,19]

C: Triplex qPCR.[6]

The real-time qPCR assay was performed by the Veterinary Microbiology Research Laboratory as the reference standard and used primers and probes to detect *eqbE*, *SEQ2190*, and SZIC as described by Webb et al.[6] DNA extraction was performed on a 1 mL sample of the GPL specimen using a GenElute Kit (Sigma-Alrich, St. Louis, Missouri). Thermal cycling conditions were 3 minutes at 95°C, followed by 40 cycles of 95°C for 3 seconds and 60°C for 10 seconds. The triplex PCR was considered positive

when either *eqbE* or *SeQ2190* or both targets were positive. The limit of detection (LOD) for each target was determined by Webb et al, as 20 copies for *eqbE* and 10 copies for *SEQ2190*. In the absence of a reported positive cycle threshold (CT) cutoff by Webb et al,[6] standard curves were performed and showed that CT values ≤ 30.5/31.5 are positive for the *eqbE* and *SEQ2190* targets, respectively, based on the LODs reported above. These CT values were used in this study to determine whether the GPL specimens were positive or negative.

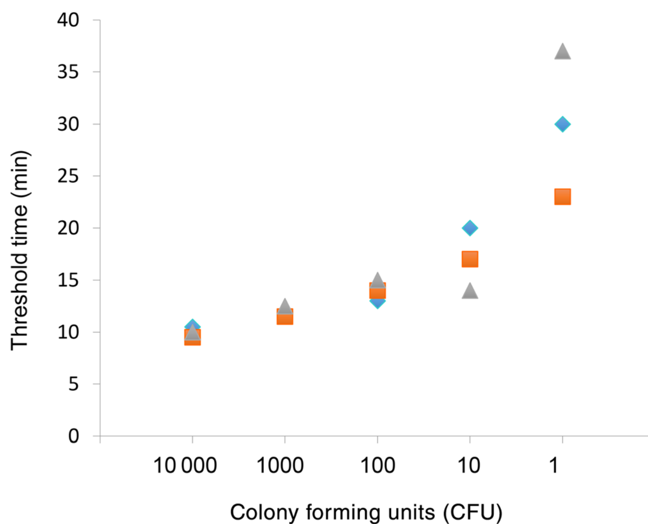

FIGURE 2 Limit of detection of known positive *S. equi* CFU measured in threshold time (minutes) via the LAMP assay on microfluidic device. CFU, colony forming unit; LAMP, loop-mediated isothermal nucleic acid amplification

2.3 | Statistical analysis

Equality between the benchtop LAMP assay, the LAMP microfluidic device, and the gold standard triplex qPCR tests was determined via ROC area under the curve (AUC). All statistical analyses were performed using STATA 14 (StataCorp, College Station, Texas). Readers of each test were blinded to the results of the others.

3 | RESULTS

Sixty-eight frozen GPL specimens obtained from horses recovering from naturally occurring *S. equi* infection were analyzed with the 3 molecular diagnostic methods to detect *S. equi* (bench top *eqbE* LAMP assay,[17] *eqbE* LAMP microfluidic device, and the triplex qPCR[6]). One specimen broke after the bench top LAMP assay and 3 specimens did not have enough volume to perform the LAMP microfluidic device.

The benchtop *eqbE* LAMP assay[17] showed that 27 of the 68 (40%) samples were positive. The *eqbE* LAMP microfluidic device showed that 31 of the 64 (48%) samples tested were positive. The median time to *S. equi* detection on the LAMP device was 15 minutes (range, 11-38 minutes; interquartile range [IQR], 13-22 minutes). Limit of detection of the LAMP microfluidic device was a single CFU at 30 to 40 minutes. Ten CFU were detected at 15 minutes (Figure 2). The intensity of the fluorescence was the same whether 1 CFU or 1000 CFU were detected and the number of minutes to fluorescence was longer with less colony forming units (Figure 3). Equal fluorescence was demonstrated at 40 minutes whether the sample contained 1 CFU or 100 CFU, providing an easily distinguishable positive or negative result for the user (Figure 4). Fluorescent by-product of LAMP distinguished a positive vs negative result of *S. equi* DNA detection on

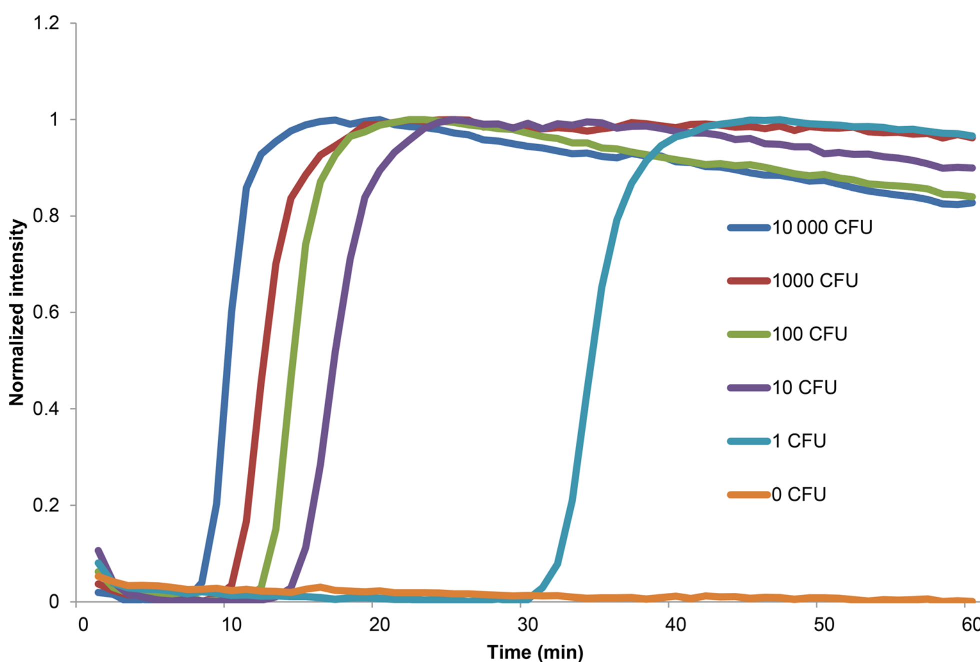

FIGURE 3 Normalized fluorescence intensity of *S. equi* amplicons as a function of time (minutes) LAMP assay on microfluidic device. LAMP, loop-mediated isothermal nucleic acid amplification

dry storage microfluidic device (Figure 5A) as seen via a smartphone screen (Figure 5B).

Of the 67 specimens tested by the real-time triplex qPCR,[6] 12 (18%) were positive for *S. equi*. The SZIC internal control was positive in 63 of 67 specimens. Of these 12 specimens, only 11 were positive for *eqbE* (using a cutoff value of <30.5 CT, 20 DNA copies) and all 12 were positive for *SEQ*2190 (using a cutoff value of <31.5 CT, 10 DNA copies). Median CT value for real-time qPCR targeting *eqbE* was 25 CT (IQR, 20.7-25.9) and median CT value for real-time qPCR targeting *SEQ2190* was 25.2 CT (IQR, 22.2-26.2). The LAMP microfluidic device detected *eqbE* in 1 sample that was negative for *eqbE* and positive for *SEQ2190* by the qPCR triplex assay; the benchtop LAMP did not detect *S. equi* in this sample. An additional 24 samples had a qPCR CT value between 30 and 40 CT for the *SEQ2190* gene (median value was 38.09 CT; IQR, 36.3-39.3) and a simultaneous "undetermined" (negative) value for the *eqbE* target (Table S1).

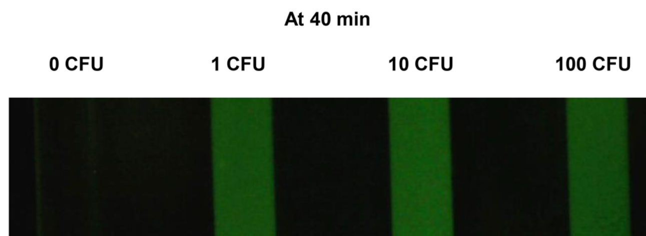

FIGURE 4 Fluorescence emission from the microfluidic device following 40 minutes of *S. equi* DNA LAMP amplification. There is equal fluorescence at 40 minutes whether LAMP detects 1 CFU or 100 CFU. CFU, colony forming unit; LAMP, loop-mediated isothermal nucleic acid amplification

Table 1 shows the sensitivity, specificity, and receiver operator curves comparing the 2 LAMP techniques with the triplex real-time qPCR. In comparison to triplex real-time qPCR as the reference, benchtop LAMP showed excellent discrimination (ROC AUC = 0.813, 95% confidence interval [CI] = 0.711-0.915). The newly developed *eqbE* LAMP microfluidic device also showed excellent discrimination when compared to triplex real-time qPCR (ROC AUC = 0.811, 95% CI = 0.529-0.782). There was no significant difference between the benchtop LAMP and the LAMP microfluidic device (*P* = .97). The LOD for the *eqbE* LAMP microfluidic device was lower than the triplex real-time qPCR[6] (1 CFU for *eqbE* LAMP vs 10 CFU for real-time qPCR using *SEQ2190* and 20 CFU using *eqbE*).[6]

4 | DISCUSSION

This study showed that the *eqbE* LAMP[17] assay was more sensitive than the triplex real-time qPCR[6] for the detection of *S. equi* in GPL

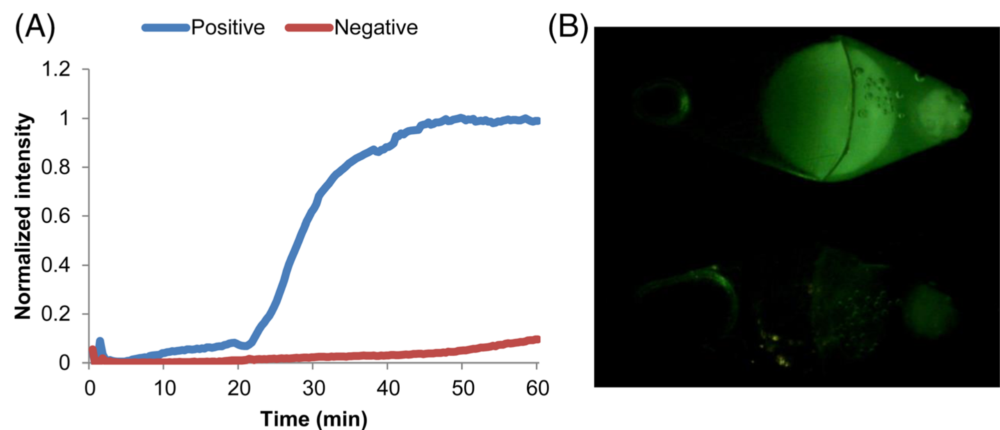

FIGURE 5 (A) Fluorescent by-product of LAMP distinguished a positive vs negative result of *S. equi* DNA detection on dry storage microfluidic device (B) as seen via a smartphone screen. LAMP, loop-mediated isothermal nucleic acid amplification

TABLE 1 Sensitivity and specificity comparing (a) triplex real-time qPCR to the benchtop *eqbE* LAMP and (b) triplex real-time qPCR to the *eqbE* LAMP Microfluidic Device

		Triplex real-time qPCR						
		Positive	Negative	Total	Sensitivity	Specificity	Correctly classified	ROC
Benchtop *eqbE* LAMP	Positive	11	16	27	92%	71%	75%	0.81
	Negative	1	39	40				
	Total	12	55	67				
eqbE LAMP Microfluidic Device	Positive	11	20	31	100%	62%	64%	0.81
	Negative	0	33	33				
	Total	11		64				

Abbreviations: LAMP, loop-mediated isothermal nucleic acid amplification; qPCR, quantitative polymerase chain reaction; ROC, receiver operator characteristic.

specimens from horses convalescing from strangles. The performance of the *eqbE* LAMP assay in a microfluidic device was shown to be equivalent to the benchtop *eqbE* LAMP assay. The microfluidic device was more efficient as there is no need for thermal cycling and LAMP assays use multiple primers (6 instead of 2) from 1 gene target increasing sensitivity and specificity. The LOD of both *eqbE* LAMP assays were lower than the triplex real-time qPCR.

Based on the standard curve of the triplex real-time qPCR,[6] 3 CTs were a log difference in DNA copy number. In the absence of a reported cutoff,[6] the standard curve showed that any CT value less than 30.5/31.5 was positive for *eqbE* based on the LODs reported by Webb et al.[6] Competition of primers may exist in the real-time qPCR. Both targets may be detected when the reactions are single gene assays but when combined, in our hands, the *eqbE* gene in the real-time qPCR was inhibited. Test performance is also dependent on samples tested.[17] All of our samples were from horses that were known to have been historically positive for *S. equi*, but had minimal to no clinical signs suggesting low bacterial loads within the guttural pouch. Webb et al examined horses from all stages of diseases, resulting in varied bacterial loads.[6] Disparate testing performance of the triplex real-time qPCR in these samples from the Mid-Atlantic United States may also be due to difference in strains of *S. equi* samples in different parts of the world.[20] The previous study looking at the sensitivity of the triplex real-time qPCR was performed on samples from the United Kingdom.[6] The risk of environmental contamination causing false positives on the *eqbE* LAMP microfluidic device was very low considering that assay was performed in the engineering lab were the device was made, it was a closed system, and the lab had never previously seen *S. equi*.

Limitations to this study included possible sample degradation because all samples were stored frozen. Reproducibility of the LOD was limited by the amount of clinical specimen stored for each horse. Only a single gene target, *eqbE*, was used. Adding additional targets for LAMP is possible and may improve the test's sensitivity and accuracy, in particular combining with *SEQ2190*.[9,15] In order to provide comparable samples across different testing methodologies, the same DNA extraction of samples was run on the microfluidic device in our study.

Recently, a commercial PCR POC for the detection of *S. equi* has become available with a sensitivity of 89% and specificity of 84% when testing nasal secretions in a laboratory. The LOD reported was higher (277 *eqbE* gene copies) than the device examined in this study (1 CFU),[21] making the PCR POC less ideal for testing convalescent horses with low numbers of DNA in their guttural pouches.

We have shown that the LAMP microfluidic device has high sensitivity as a diagnostic test for the detection of *S. equi* in convalescent animals previously diagnosed with strangles. We have also shown that the previously validated benchtop LAMP and the microfluidic device are comparable at detecting *S. equi* when testing convalescent sample from horses previously diagnosed with strangles. In previous work, the benchtop LAMP was shown to be comparable to SEEI PCR at detecting *S. equi*,[17] also in strangles convalescent samples. In the current study, both LAMP methods were not comparable to triplex qPCR. Further comparisons between various PCR platforms and the LAMP microfluidic device using fresh samples from horses in all stages of disease are needed to explore this difference.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST DECLARATION

The University of Pennsylvania has applied for patent protection for the microfluidic device technology, with H. H. Bau and J. Song named as coinventors. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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