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Short communication

# A probe-based real-time PCR assay for the detection of *Neospora caninum* in clinical samples from cattle

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ABSTRACT

*Neospora caninum* is an apicomplexan protozoan parasite that is a leading cause of abortion in cattle. Detection of parasite-specific DNA by PCR is a highly sensitive method for identifying the presence of *N. caninum* in a variety of tissues. We developed and validated a probe-based real-time PCR assay targeting the conserved Nc5 gene of *N. caninum*. Using *N. caninum* strain Nc-1 genomic DNA and a synthetic gene fragment as amplification standards, we determined the PCR amplification efficiency and the limit of detection to be 95.60% and 3 copies, respectively. Five pathogens frequently associated with bovine abortions, namely bovine viral diarrhea virus types I and II, bovine alphaherpesvirus-1, *Chlamydia,* and *Leptospira*, were tested to ensure analytical exclusivity. A total of 103 clinical samples from aborted fetuses were tested concurrently with a standard conventional PCR and the new probe-based real-time PCR assay. All tested samples showed 100% agreement between these two assays. In conclusion, the probe-based real-time PCR assay facilitates accurate and rapid detection of *N. caninum* from abortions in cattle.

## 1. Introduction

*Neospora caninum* is an apicomplexan protozoan parasite that infects a wide range of domestic and wild animal hosts including dogs, cattle, sheep, goats, deer, rodents, coyotes, foxes and raccoons. *N. caninum* causes neonatal neuromuscular disease in dogs and abortions in cattle. *N. caninum*-induced abortion in dairy cows was first reported in New Mexico in 1989, and *N. caninum* continues to be one of the major causes of abortions in cattle (Thilsted and Dubey, 1989). The organism can be transmitted either by endemic transplacental transmission to the fetus or by epidemic transmission by ingestion of contaminated mixed rations or water. Although neosporosis is a leading cause of economic losses in the dairy and beef industries in the US and worldwide, an effective vaccine does not exist and management practices have not been able to eradicate the disease (McAllister, 2016; Reichel et al., 2013).

Commonly used methods to diagnose natural *N. caninum* infection include histological, molecular, and serological assays (Sinnott et al., 2017). *N. caninum* may be diagnosed by detection of parasite-specific serum antibodies in the live adult population and by characteristic lesions in brain, heart, and liver in aborted fetuses combined with antigen-specific immunohistochemical examination. However, immunohistochemistry (IHC) is not a highly sensitive method for detecting *N. caninum* in host tissues due to low parasite numbers and variable quality of tissues, especially when the fetal tissues are autolyzed, mummified, or desiccated. Nucleic acid detection by PCR is a very useful tool for the detection of *N. caninum* in bovine aborted fetuses since even small amounts of parasite DNA can be amplified to detectable levels even from poor-quality tissue. A well-established conventional PCR assay is able to reliably detect ≥3 genome copies (Baszler et al., 1999). Real-time PCR (rtPCR) assays employing amplicon-specific probes are highly sensitive and precise for the rapid and accurate detection of pathogens from clinical samples that often contain infectious agents in low quantities. Use of a dsDNA-binding dye to label and detect rtPCR amplicons is an alternative method which relies on melt-curve analysis to ensure specificity of well-optimized primers. Probe-based rtPCR depends on the binding of a fluorescently-labeled amplicon-specific oligonucleotide probe to indicate amplification of the

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desired target sequence. For regions that are unique in a target species yet highly conserved within variants of that species, probe-based rtPCR permits detection of multiple strains and offers the option for multiplexing to detect more than one organism in the same assay. Compared with conventional PCR and dsDNA-binding dye-based rtPCR, probe-based rtPCR can provide enhanced exclusivity through the use of a fluorescently-labeled amplicon-specific oligonucleotide probe (Ghalmi et al., 2008). Although probe-based rtPCR for *N. caninum* detection has been described (Almería et al., 2016; Pereira et al., 2014), validation of such an assay for detecting the parasite in clinical samples from naturally infected aborted bovine fetal tissue has not been reported.

The *N. caninum* genome encodes around 7121 protein-coding genes across 14 chromosomes (Reid et al., 2012). Common targets of molecular assays include internal transcribed spacer 1 (ITS1) of rRNA and the Nc5 gene. The Nc5 region is a repeated sequence in the DNA genome which has been shown to be highly specific for *N. caninum* (Kaufmann et al., 1996) and shows ≥95% identity across the 105 publicly-available sequences of *N. caninum*, thus making this region an attractive target for novel molecular diagnostic assays. Given the lack of vaccine and effective treatment for control of neosporosis, novel specific and sensitive assays to diagnose the presence of *N. caninum* are urgently needed. Here we provide validation of a novel assay for detection of *N. caninum* in natural infections of aborted bovine fetal tissue using probe-based rtPCR, which can detect three copies of parasite genomic DNA.

## 2. Materials and methods

### 2.1. Specimens

Brain or heart/brain pooled tissue samples from aborted fetuses submitted to the Pennsylvania Animal Diagnostic Laboratory System during 2016 through 2018 were used as sources for nucleic acid to compare an existing conventional PCR assay with the new rtPCR assay. Fresh tissue samples were frozen and stored at −20 °C until use for nucleic acid extraction within one week.

Genomic DNA from *Neospora caninum* strain Nc-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and resuspended to a concentration of 4 µg/mL (equivalent to $6.07 \times 10^4$ genomic copies/mL based on estimated molecular weight of *N. caninum* genome of $3.97 \times 10^{10}$ g/mol). Bovine viral diarrhea virus type I strain Singer, bovine viral diarrhea virus type II strain 125, and bovine alphaherpesvirus type 1 strain Colorado were obtained from National Veterinary Services Laboratories (NVSL, Ames, IA) and propagated in Madin-Darby Bovine Kidney cells. *Chlamydophila abortus* strain B-577 (ATCC VR-656) and *Leptospira interrogans* type strain (ATCC 23,581) were obtained from ATCC. *C. abortus* was propagated in McCoy cells. L. *interrogans* was propagated in polysorbate 80-bovine albumin liquid.

### 2.2. Nucleic acid isolation

Homogenates of 0.5 *g* thawed fresh-frozen tissue samples were prepared in 3 mL of 20 mM Tris−HCl with 20 mM EDTA and 0.5% sodium dodecyl sulfate, pH 8.0, using a handheld tissue homogenizer. Homogenates were incubated at 56 °C for ≥30 min until sample was completely lysed as evidenced by clarification of digestion buffer. The homogenate was vortexed to combine with an equal volume (3 mL) of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and then centrifuged at 9000 x g for 10 min. The aqueous phase was collected, and DNA was precipitated using ethanol in 3 M sodium acetate. After centrifuging at 14,000 x g for 10 min, the ethanol was completely removed, and the pellet washed with 1 mL of cold ethanol. The wash was removed, and the dried DNA pellet was resuspended in a total volume of 200 µL nuclease-free water.

Nucleic acid was extracted from bovine viral diarrhea virus types 1 and 2, bovine alphaherpesvirus type 1, and *C. abortus* using a QIAamp MinElute Virus Spin Kit (Qiagen, Redwood City, CA) following the manufacturer's instructions. DNA was extracted from *L. interrogans* using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions.

### 2.3. Conventional PCR

Conventional PCR was performed on 10 µL of extracted DNA to amplify a 275 bp region of the Nc5 genomic region as previously described, with modifications (Baszler et al., 1999). Briefly, primers Np4 (5′−CCTCCCAATGCGAACGAAA -3′) and Np7 (5′-GGGTGAACCGAGG GAGTTG-3′) were used at 400 nM in 50 µL reactions containing 2 U Fisher BioReagents™ *Taq* DNA polymerase (Fisher Scientific, Hampton, NH), 100 µM dNTP mix (Promega, Madison, WI), 500 µM magnesium chloride (Promega) in 1x assay buffer A (final concentration Tris−HCl 10 mM, pH 9, Fisher Scientific). Reactions were carried out using a 9600 GeneAmp PCR system (Perkin Elmer, Waltham, MA) with the following conditions: 94 °C 4 min; 40 cycles of 95 °C 45 s, 62 °C 1 min, 72 °C 1 min; 72 °C 10 min. Reaction product (20 µL) was visualized on a 1.5% agarose gel containing 0.5 mg ethidium bromide per mL of agarose solution.

### 2.4. Real-time PCR

Using Primer Express 3.0.1 software (Applied Biosystems, Foster City, CA), probe and primers were designed to amplify an 85 bp region of Nc5 (GenBank X84238.10). Purified forward primer (5′-CTGTGCT CGCTGGGACTTC-3′), reverse primer (5′-CGATTTACGACATACGGTGT TCA-3′), and probe (5′-[FAM]CATCGGAGGACATCGCTCACTGA CTG[BHQ1]-3′) were purchased from Integrated DNA Technologies (IDT) (Skokie, IL). VetMAX-Plus™ qPCR Master Mix (Applied Biosystems) was used, with each 25 µL reaction consisting of 1x qPCR Master Mix, 400 nM of each primer, 120 nM probe, and 5 µL of nucleic acid template. Reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following thermal cycling conditions: 95 °C 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 45 s (data collection step).

### 2.5. Nc5 region sequencing

The Nc5 region of five clinical isolates from aborted bovine fetal tissue was amplified using Np1 and Np2 primers (Yamage et al., 1996) utilizing Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, MA) according the manufacturer's instructions. Amplification products were gel-purified using E.N.Z.A. Gel Extraction Kit (Omega Bio-tek, Norcross, GA) and sequenced at the Penn State Genomics Core Facility - University Park using an 3730XL instrument (Applied Biosystems).

### 2.6. Data analysis

DNA encoding the 85 bp region of GenBank accession number X84238.10 targeted by the rtPCR primers was synthesized by IDT to serve as a quantitation template for evaluating PCR amplification efficiency and limit of detection. To determine the PCR amplification efficiency, Prism 7.03 (GraphPad, San Diego, CA) was used to determine the slope of linear regression between Ct values resulting from rtPCR assay of serial dilutions of the 85 bp template tested in triplicate and corresponding $\log_{10}$ transform of number of template copies. Three independent assays were performed on serial dilutions, and the mean PCR amplification efficiency was calculated. Similarly, linear regression was conducted between Ct values resulting from rtPCR assay of *N. caninum* genomic DNA tested in triplicate and corresponding $\log_{10}$ transform of mass. PCR amplification efficiency, E, was defined as $E = 10^{(-1/\text{slope})} - 1$. Graphical representations and *p*-value (two-tailed unpaired homoscedastic parametric t-test) analyses were generated in

Prism 7.03. Mean values ± standard deviations are reported.

## 3. Results

A primer-probe set for rtPCR was identified using PrimerExpress based on the Nc5 hybridization probe region from strain NC-1 (GenBank accession X84238.1). The targeted sequence amplifies bases 199 through 283, the sequences for which are lacking in the majority of *N. caninum* Nc5 sequences in GenBank. The five sequences covering this region from three distinct isolates were compared with the primer and probe sequences. The primers and probe demonstrated 95–100% identity with the isolate sequences. This level of identity is in the range of variation found within replicate sequencing results of the Nc5 region of individual *N. caninum* strains (96–98%) and was thus deemed acceptable. Three additional sequences available in GenBank covered a portion of this region (bases 199 to 248). For these sequences, the forward primer showed 17–18 of 19 (89.5%–94.7%) nucleotide identity, and the portion of the probe area for which sequence was available showed 100% nucleotide identity. Critically, the three terminal nucleotides at the 3′ end of each primer matched all available sequences 100%.

To confirm that the region identified through primer/probe design was an acceptable target for screening clinical samples of aborted bovine fetal tissue, *N. caninum* genomic DNA from five conventional PCR-positive clinical specimens was sequenced in the rtPCR-targeted area. These isolates matched the rtPCR probe with 86.4–100% identity and the forward primer and reverse primers at 89.5–100% and 91.8–100% identities, respectively (Fig. 1A). The 3′ clamp region demonstrated 100% homology in both primers for all isolates. When assayed using the new probe-based rtPCR assay, all isolates tested positive. Overall, the primers and probe were found to have sufficient homology to the targeted region of the *N. caninum* genome, and they successfully identified multiple isolates despite known mismatches.

For comparison, 65 GenBank natural isolates of *N. caninum* sequenced through bases 550–568 were evaluated for homology to primer Np7 (Yamage et al., 1996), which is commonly used to screen for *N. caninum* including in the standard diagnostic conventional PCR at our laboratory (McAllister, 2016). Eleven (16.9%) of these isolates contained one base mismatch against the Np7 primer, with nucleotide identity therefore 94.7% for these isolates. Two of the isolates demonstrated base mismatch within the 3′ clamp end of the Np7 primer.

To evaluate the analytical sensitivity of the new rtPCR, serial dilutions of synthesized DNA encoding the targeted sequence were used as template for the reaction. Based on the generated standard curves, PCR amplification efficiency was calculated to be 92.48% (Fig. 1B). As few as 5 copies of target could be consistently amplified. A limit of detection of 200 fg was obtained when using genomic DNA from *N. caninum* strain Nc-1 as template (Fig. 1C). This mass corresponds to genomes from approximately 3 tachyzoites. Based on an input of DNA from 12.5 mg of tissue into each rtPCR reaction, the limit of detection is 400 Nc5 region copies (or approximately 240 tachyzoites) per gram starting tissue.

Repeatability and precision of the rtPCR assay were tested by assaying triplicate repeats of 3 concentrations of genomic DNA in 6 separate rtPCR setups over 6 days. Across all replicates throughout the 6 runs, the standard deviation was no more than 1.03% of the mean (Table 1). To determine the exclusivity of the rtPCR for *N. caninum*, 5 organisms known to cause abortions in cattle and other livestock were tested. Genomic nucleic acid samples from bovine viral diarrhea virus type 1, bovine viral diarrhea virus type 2, bovine alphaherpesvirus type

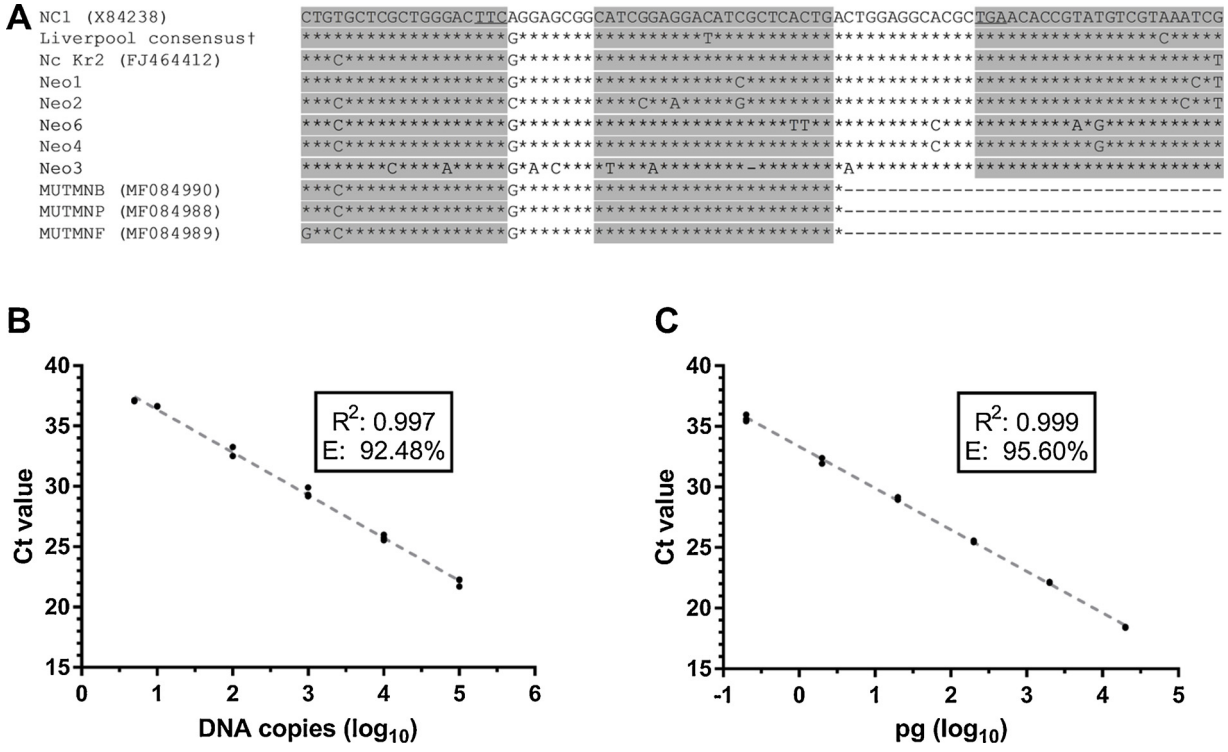


**Fig. 1. Sufficient homology to primers and probes for *N. caninum* probe-based real-time PCR produce high amplification efficiency in clinical specimens.** The primers and probe (shaded) identified by primer/probe design were aligned with sequences from laboratory strains and clinical isolates of *N. caninum* with 86.4–100% homology (A). Consensus sequence of Liverpool strain was computed from the three entries of this isolate, GenBank accession numbers FR823382, LN714476 and LN14488. The 3′ clamp section of each primer is underlined. Graphs of serial dilutions of *N. caninum* Nc5 region bases 199 to 283 (B) or *N. caninum* genomic DNA (C) are shown, with linear regression correlating log-transformed copy number (B) or mass in pg (C) with Ct value displayed as a dashed line. The corresponding $R^2$ value and calculated PCR amplification efficiency E (where $E = 10^{(-1/slope)} - 1$) are shown in inset. Each serial dilution was tested in triplicate in each rtPCR run. Two or three rtPCR runs were conducted to produce standard curves, and a representative graph is shown. Replicates are shown as circles. Ct = threshold cycle.

**Table 1**
Precision and repeatability of N. caninum Nc5 gene real-time PCR assay, with results expressed as threshold cycle (Ct) mean from 6 separate replicates performed on multiple days.

| dilution ($log_{10}$) | mean | standard deviation | |
|---|---|---|---|
| | | absolute | percent of mean |
| −1 | 22.40 | 0.21 | 0.94% |
| −2 | 25.77 | 0.26 | 1.03% |
| −3 | 29.35 | 0.27 | 0.93% |

1, *Chlamydophila abortus* and *Leptospira interrogans* were used as input in the rtPCR reaction. Nucleic acid from all 5 organisms showed no Ct in the assay.

A set of 103 genomic DNA samples from aborted fetuses were tested using both a standard conventional PCR assay and the new rtPCR assay for *N. caninum*. Sixteen of the samples were positive, and 87 of these samples were negative (Ct ≤ 35.66) using the rtPCR assay. Overall, the same result (positive vs. negative) was determined for all 103 samples tested in both the conventional PCR and rtPCR assays, despite the use of only half as much DNA input in the rtPCR assay compared with the conventional PCR.

A subset of 30 samples that included 14 negative and the 16 positive specimens were selected for further examination. First, the samples were blinded and independently tested by a separate laboratory using the new rtPCR assay. The same results (positive vs. negative) were obtained in both laboratories, indicating the reproducibility of the new rtPCR assay. Of the 16 rtPCR-positive specimens, 13 were derived from good-quality tissue, while the remaining 3 were from poor-quality tissue demonstrating autolysis and/or desiccation. To investigate whether tissue quality affected Ct values of the rtPCR assay, the Ct results from these samples were compared (Table 2). Overall, the mean Ct values of DNA from poor-quality tissue were significantly higher compared with those of DNA from good-quality tissue (poor: 33.07 ± 2.47, good: 28.50 ± 2.47, $p = 0.012$). Furthermore, we evaluated whether presence of lesions consistent with *N. caninum* infection in brain tissue correlated with lower Ct values. Using the Mann-Whitney U-test, no significant difference of Ct values was found between tissue with lesions and those without.

## 4. Discussion

Various rapid molecular methods for detecting *Neospora caninum* from a multiple sample types have been described, including recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and rtPCR using dsDNA binding dyes (such as SYBR Green and EvaGreen) or amplicon-specific probes. An RPA assay demonstrated similar ability to detect the *N. caninum* Nc5 region from aborted bovine fetal tissue as compared with semi-nested conventional PCR (Tian et al., 2018). *N. caninum*-specific LAMP assays evaluated mainly with canine samples have shown similar detection limits and presence/absence results as EvaGreen-based rtPCR (Mahittikorn et al., 2017) and higher sensitivity as compared with semi-nested conventional PCR (Ramos et al., 2017). Multiple probe-based rtPCR approaches have been evaluated using canine and murine samples (Klein et al., 2019; Müller et al., 2002), including one demonstrating a limit of detection of 800 genomic copies of *N. caninum* per gram tissue, similar to the value estimated here of 240 copies per gram tissue.

Other rtPCR assays specifically aimed at detecting *N. caninum* in bovine samples include a SYBR Green assay to evaluate bovine blood (Okeoma et al., 2005) and a probe-based assay to study experimentally-infected bovine fetal tissue (Almería et al., 2016). Like the current study, Collantes-Fernández et al. evaluated bovine clinical fetal abortion samples from naturally infected animals (Collantes-Fernández et al., 2002). The SYBR Green rtPCR assay had a limit of detection of ≤ 2900 tachyzoite genomic copies per gram tissue. Similar to our findings, this group also observed *N. caninum* DNA detection in brain tissue samples without any corresponding brain tissue lesions. However, whereas Collantes-Fernández et al. found a statistically significant higher amount of DNA in tissues with lesions consistent with *N. caninum* infection compared with those without, in our analysis the Ct levels were not different. This may be due to differing quality of tissue in the two studies, a larger number of samples in evaluated in the current study, or other reasons.

Probe-based rtPCR in this report offers equivalent order of magnitude of LOD to the LAMP, RCA and SYBR Green rtPCR assays previously described and the lowest reported detection limit per gram of tissue. There is the possibility of adapting this probe-based rtPCR assay to multiplex to enable screening of multiple pathogens in a single run, as has been done by others. For example, a 3-component multiplex has been described targeting the same region of the Nc5 region as described here (Reisberg et al., 2013). A related multi-screening real-time PCR to detect seven bovine abortive pathogens was recently reported with a limit of detection of 12,000 copies of cloned *N. caninum* target per gram tissue, whereas in the current report detecting a single organism the limit of detection was 400 copies per gram.

Very similar approaches to the probe-based rtPCR described here have been successfully used to investigate *N. caninum* presence in more than seven animal species (Almería et al., 2016; Constantin et al., 2011; Reisberg et al., 2013). In the current study, we validated the assay using clinical samples from naturally-infected aborted bovine fetal tissue and assessed potential effects of tissue quality on Ct values. The significantly higher Ct values in tissues from mummified, autolyzed, and/or desiccated fetuses highlight the fact that future investigations of diagnostic molecular assays should include evaluation of samples of poor quality such as these.

**Table 2**
Influence of tissue source and quality on *N. caninum* Nc5 gene real-time PCR assay outcome in samples positive by conventional PCR. Results are expressed as mean threshold cycle (Ct) from 3 replicates.

| tissue source | tissue quality | Ct value |
|---|---|---|
| brain | poor: autolyzed, mummified | 35.32 |
| brain | good | 25.46 |
| brain | good | 25.46 |
| brain | good | 25.73 |
| brain | good | 26.71 |
| brain | good | 26.8 |
| brain | good | 28.05 |
| brain | good | 31.22 |
| brain | good | 31.91 |
| heart/brain | poor: highly autolyzed | 30.42 |
| heart/brain | poor: autolyzed, desiccated | 33.46 |
| heart/brain | good | 27.25 |
| heart/brain | good | 28.75 |
| heart/brain | good | 30.44 |
| heart/brain | good | 31.09 |
| heart/brain | good | 31.62 |

## 5. Conclusion

The rtPCR assay described here is expected to be able to detect most of the known *N. caninum* strains given the similarity of the Nc5 gene that is targeted. This validation was performed using genomic DNA isolated from fresh fetal brain tissue or a mixture of fetal brain and heart tissues. It is expected that the assay would be useful to test for *N. caninum* in other bovine tissues types and samples from other animals.

## Competing interests

The authors declared no potential conflicts of interest with respect

to the research, authorship, and/or publication of this article.

## Authors' contributions

SVK conceived and supervised the study; SVK, RB and RHN designed this study; RB conducted validation assays; WF and NT conducted cross-laboratory testing; RB and RHN analyzed data; RHN, RB and SVK wrote the manuscript; DT and BMJ contributed to the study design and provided funding acquisition, access to samples, and laboratory oversight. All authors read and approved the manuscript.

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