Real-Time PCR Differential Detection of Neorickettsia findlayensis and N. risticii in Cases of Potomac Horse Fever

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ABSTRACT

Potomac horse fever (PHF) is an acute and potentially fatal enterotyphlocolitis of horses with clinical signs that include anorexia, fever, diarrhea, and laminitis. Its incidence is increasing despite a commercially available vaccine. PHF is caused by Neorickettsia risticii, and the recently rediscovered and classified N. findlayensis. PHF diagnosis is currently accomplished using serology or nested PCR. However, both methods cannot distinguish the two Neorickettsia species that cause PHF. Further, the current N. risticii real-time PCR test fails to detect N. findlayensis. Thus, in this study, two Neorickettsia species-specific real-time PCR assays based on Neorickettsia ssa2 and a Neorickettsia genus-specific real-time PCR assay based on Neorickettsia 16S rRNA gene were developed. The ssa2 real-time PCR tests differentiated N. findlayensis from N. risticii in the field samples for which infection with either species had been verified using multiple other molecular tests and culture isolation, and the 16S rRNA gene real-time PCR detected both Neorickettsia species in the samples. These tests were applied to new field culture isolates from three Canadian provinces (Alberta, Quebec, Ontario) and Ohio as well as archival DNA samples from suspected PHF cases to estimate the prevalence of N. findlayensis in different geographic regions. The results suggest that N. findlayensis frequently causes PHF in horses in Alberta and Quebec. The development of these tests will allow rapid, sensitive, and specific diagnosis of horses presenting with clinical signs of PHF. These tests will also enable rapid and targeted treatment and help develop broad-spectrum vaccines for PHF.

KEYWORDS

Neorickettsia findlayensis, N. risticii, Potomac horse fever, real-time PCR, culture isolation, enterotyphlocolitis

Potomac horse fever (PHF) is an acute and potentially fatal enterotyphlocolitis of horses characterized by fever, lethargy, anorexia, dehydration, diarrhea, laminitis, and occasional abortion (1). PHF was originally described in the early 1980s as a new disease (“acute equine diarrhea syndrome”) on farms along the Potomac River in Virginia and Maryland. Since then, PHF has been diagnosed throughout the USA and several provinces in Canada and has been occasionally identified in Brazil, Uruguay, and Europe (1–5). In the early 1980s, electron microscopy analyses of samples from horses revealed distinct rickettsia-like bacteria mostly in the wall of the large colon with clinical signs of PHF (6, 7). In parallel, a rickettsial organism was isolated from the peripheral blood mononuclear cells of affected horses using the U937 human monocyte cell line (8, 9) or primary canine blood monocytes (10) and was named Ehrlichia
ricticii (11). Later, the bacterium was reclassified as Neorickettsia ricticii (12). The most effective treatment for PHF is administration of oxytetracycline during the early stages of the disease (1). PHF typically occurs in the warm-weather months of late spring to early fall (1). Trematodes are natural reservoirs and vectors of N. ricticii. Horses are infected by accidental ingestion of N. ricticii-infected trematodes. Koch’s postulates were fulfilled following the oral administration of N. ricticii-infected trematodes, which are endoparasites of aquatic insects (e.g., mayflies and caddisflies) to horses. N. ricticii was culture isolated from blood mononuclear cells of horses that developed PHF (13, 14). Importantly, PHF occurs despite horses being vaccinated with a commercially available vaccine (N. ricticii–based bacterin). This lack of protection is likely attributable to insufficient immunological response and various antigenic variants (15–17).

From 1984 to 2020, it was assumed that only N. ricticii causes PHF, despite the finding that a molecularly and antigenically unique Neorickettsia sp. named 081 was cultured from a blood sample from a horse with PHF in Findlay, Ohio in 1991 (15, 18). Neorickettsia sp. 081 was recently classified as a new species named N. findlayensis along with two other new strains cultured from blood samples from horses with PHF in Ontario, Canada. This was based on whole genome sequencing of the type-strain Fin17 and by experimental infection that fulfilled Koch’s postulates (19, 20). Cell-culture isolation of Neorickettsia spp. from horses suffering with PHF provides a highly sensitive and definitive diagnosis (19, 21). However, owing to technical, economic, and time constraints, cell-culture isolation has succeeded only in a few laboratories in the USA (9, 10, 15, 19–22). Instead, PHF diagnosis is mainly accomplished by nested PCR that detects both N. ricticii and N. findlayensis (19, 21) or N. ricticii real-time PCR (quantitative PCR) (23) (both tests are based on the Neorickettsia 16S rRNA sequence) or serology (indirect immunofluorescence microscopy using N. ricticii or N. findlayensis–infected P388D1 cells as antigens) (19, 21, 24). However, these methods cannot distinguish the two Neorickettsia species that cause PHF. Further, the current N. ricticii real-time PCR test based on 16S rRNA gene fails to detect N. findlayensis, giving false negative test result (19). In addition, antibody tests cannot distinguish current from previous infection/exposure or vaccination, and the nested PCR test is cumbersome for routine clinical diagnosis which requires rapid turnover time. Thus, in this study, by comparing the genome sequences of N. findlayensis and N. ricticii (19, 25) and other known Neorickettsia spp. (26–28), we developed a simple and rapid diagnostic method to distinguish N. findlayensis from N. ricticii in addition to a method that detects both Neorickettsia spp. using well-defined clinical specimens. These tests were applied to the new field culture isolates from three Canadian provinces (Alberta, Quebec, Ontario) and OH, USA as well as archival DNA samples from suspect PHF cases to estimate the prevalence of N. findlayensis in different geographic regions. The results suggest that N. findlayensis frequently causes PHF in horses in Quebec and Alberta, encouraging future epidemiologic and ecological studies. These new rapid diagnostic methods will improve the current laboratory diagnosis of PHF.

MATERIALS AND METHODS

Study design. First, new primers were designed for Neorickettsia species-specific and genus-specific real-time PCR assays. Second, validity of these real-time PCR assays was experimentally tested using known N. findlayensis- and N. ricticii-positive clinical specimens which are culture-positive, Neorickettsia 16S rRNA nested PCR-positive, and multiple Neorickettsia sequence analysis-positive (19). Third, the new real-time PCR methods were applied to new clinical specimens including those from new geographic regions, which were culture-positive and Neorickettsia 16S rRNA nested PCR-positive, but Neorickettsia species unknown. Lastly, the new real-time PCR methods were applied to PHF-suspected clinical specimens, which were Neorickettsia 16S rRNA nested PCR-positive or –negative but had no other test results available.

Real-time PCR primer design, real-time PCR. To design real-time PCR targets and primers for N. findlayensis and N. ricticii differential assay, we first compared whole genome sequences of all Neorickettsia species (N. findlayensis Fin17 - GenBank accession no. NZ_CP047224; N. ricticii Illinois - NC_013009; N. sennetsu Miyayama - NC_007798; N. helminthoeca Oregon - NC_0007481) (19, 25, 26, 29) by synteny analysis using MUMmer program (30). For phylogenetic analysis of 16S rRNA gene and SSAs, we also included Neorickettsia sp. SF (Stellantchasmus falcatus) stain sequences (27, 31) by aligning nucleotide or protein sequences using CLUSTAL Omega (32) in MegAlign Pro program of DNASTar Lasergene 17.
Taq Standard and ER-3-2 were used in the
been using a nested PCR method developed previously (21) using two primer pairs: the primers ER-5-3
Cancer Center Genomics Shared Resource Facility.
amplicons (176 bp for
250 nM (Thermo Fisher Scienti
Thermo Fisher Scienti
and the 16S rRNA gene were cloned into the TOPO vector (Zero Blunt TOPO PCR Cloning kit;
products for species-specifc ssa2 and the 16S rRNA gene were cloned into the TOPO vector (Zero Blunt TOPO PCR Cloning kit; Thermo Fisher Scientific), and the plasmids were purifed using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientifc). The plasmids were validated by sequencing. Using 10-fold serially diluted plasmids as the template (from 10⁸ copies/μL of plasmid to 10⁰ copies/μL), the minimum number of the plasmid detectable in each real-time PCR was estimated as (X × 6.0221 × 10²=no molecules/mole) divided by (N × 660 g/mole) × 10³, where X is the number of nanograms of the plasmid DNA and N is the size (in bp) of the plasmid with the insert.
DNA was purifed directly from the buffy coats of the clinical horse blood samples or from Neorickettsia in the P388D, cell cultures using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). All real-time PCR assays were performed using extracted DNA (approximately 150 ng) as the template, Maxima SYBR green/ROX qPCR Master Mix (2×; Thermo Fisher Scientifc, Waltham, MA), and 250 mM (final concentration) of each primer in a 20-μL reaction mixture with a thermal cycle of 95°C for 30 sec followed by 35 cycles of 95°C for 30 sec, 58°C for 1 min, and 68°C for 30 sec in a Mx3000P Multiplex Quantitative PCR system (Stratagene, La Jolla, CA). All real-time PCR tests were conducted in parallel with positive control templates (N. fi 17 or N. risticii PA-1 DNA) and negative controls (buffer only).
To determine sensitivities of the real-time PCR assays, the PCR products of N. fi ndlayensis and N. risticii ssa2 and the 16S rRNA gene were cloned into the TOPO vector (Zero Blunt TOPO PCR Cloning kit; Thermo Fisher Scientifc), and the plasmids were purifed using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientifc). The plasmids were validated by sequencing. Using 10-fold serially diluted plasmids as the template (from 10⁸ copies/μL of plasmid to 10⁰ copies/μL), the minimum number of the plasmid detectable in each real-time PCR was estimated as (X × 6.0221 × 10²=no molecules/mole) divided by (N × 660 g/mole) × 10³, where X is the number of nanograms of the plasmid DNA and N is the size (in bp) of the plasmid with the insert.
Sequencing PCR products. Approximately 300 ng DNA template was added to 0.625-unit Taq DNA polymerase with 10× Standard Taq reaction buffer (New England BioLabs, Ipswich, MA), (2.5 μL), 2 mM MgCl₂ (Thermo Scientifc), 0.2 mM each dNTPs, with 400 mM (each) the appropriate primers in 25-μL reaction volume in a GeneAmp PCR System 9700 Thermal Cycler (Applied System, Foster City, CA). The PCR products were analyzed with a 1% agarose gel containing 0.5
reaction mixture with a thermal cycle of 95°C for 5 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, and 68°C for 1 min, followed by extension at 68°C for 7 min. For each set of reactions, a buffer control and positive control containing N. risticii PA-1 DNA were conducted in parallel. The amplicons were analyzed with a 1% agarose gel containing ethidium bromide and visualized under UV light using Amersham Imager 680 QC.
PHF cases. The PHF cases comprised newly cultured isolates from 11 horses diagnosed in 2018–2020.

| Target gene          | Primer name     | Direction | Sequence (5′–3′)                  | References                        |
|----------------------|-----------------|-----------|----------------------------------|-----------------------------------|
| N. fi ndlayensis ssa2 | Nfn_Ssa2F       | Forward   | GAAACCGGCGCTAAAGATAAGG           | This study                        |
| N. risticii ssa2     | Nris_Ssa2R      | Reverse   | TCTCGTATGCTATCTTGTAATGACC        | This study                        |
| Neorickettsia 16S rRNA (qPCR) | Neorick16S_F | Forward   | GTGTTGAAATCGTGGCTTTAACCC         | This study                        |
| Neorickettsia 16S rRNA (nested PCR, 1st round) | ER-5-3 | Forward   | ATTGGAGAAGTTATGCTGTTGCG          | Chaichanasiriwithaya et al., (15) |
| Neorickettsia 16S rRNA (nested PCR, 2nd round) | Eris-1 | Forward   | GGAATCCGAGGTCTGGTGACCT           | Mott et al., (21)                |
| Neorickettsia 16S rRNA (nested PCR, 2nd round) | Eris-2 | Reverse   | TGGTGGTACCCCTCATTCTCCTTCCA       | Mott et al., (21)                |

(Madison, WI). To estimate confdence levels in phylogenetic analysis, bootstrap values for 1,000 replicates were calculated by Maximum likelihood using RAxML option by MegaAlign Pro program (DNASTar).

The sequence distance is calculated by dividing the numbers of nucleotide or amino acid differences by the total numbers of nucleotides and amino acids among homologous sequences. Furthermore, N. risticii and N. fi ndlayensis strain-variable and -conserved sequences of clinical specimens (15, 19, 33) were taken into consideration. Based on these analyses, two pairs of primers unique to each Neorickettsia sp.: Nfn_Ssa2F/Nfn_Ssa2R (for N. fi ndlayensis) and Nris_Ssa2F/Nris_Ssa2R (for N. risticii) were designed (Table 1). Although the 16S rRNA genes from all Neorickettsia spp. are quite conserved, currently used N. risticii 16S rRNA gene-based real-time PCR assay, 16S rRNA genes of all Neorickettsia spp. including N. fi ndlayensis and N. risticii were aligned, and primers: Neorick16S_F/Neorick16S_R were designed to amplify 16S rRNA genes of all Neorickettsia spp. except N. helminthoeca (Table 1). All primer sequences were blasted against the entire GenBank database to assure the specifcity.

(2020. Molecular diagnostic testing for other common enteropathogens using a commercial equine...
diarrhea panel at IDEXX Laboratories, Inc. (West Sacramento, CA) was negative for Salmonella spp., Clostridium difficile toxin genes A and B, C. perfringens, and equine Coronavirus in these horses. Some N. findlayensis samples were tested by N. risticii real-time PCR at IDEXX Laboratories, and at the Animal Health Laboratory (AHL), University of Guelph.

Culture isolation of Neorickettsia. For each horse, approximately 50 mL of blood was collected into EDTA-coated tubes that were then transported within 48 h to the Rikihisa laboratory, The Ohio State University (Columbus, Ohio), for culture. Blood from each horse was centrifuged at 500 × g for 10 min to obtain the buffy coat, and the remaining red blood cells in the buffy coat were lysed with an ammonium chloride solution to yield peripheral blood leukocytes (15). The leukocyte preparations were individually inoculated into P388D1, cell preparations (15), which were then cultured in RPMI 1640 medium containing 5% fetal bovine serum (21). Samples of the cultured cells were examined weekly for signs of infection under a light microscope after Diff-Quik staining (15). When infection was detected, ~0.5 mL of the culture was harvested for the isolation of DNA that was then used in a nested PCR test directed to the Neorickettsia 16S rRNA gene to amplify a 382-bp sequence (21) (Table 1). At least 75% of each culture medium was replaced with fresh RPMI 1640 containing 5% fetal bovine serum weekly until infection was seen or the experiment was ended by freezing the infected cells.

GenBank accession numbers. GenBank accession numbers for the N. findlayensis ssa2 sequences from horses Alb20, Cin20, Dom20, Zig20, Til20, and Bul20 are MZ161203 to MZ161206, OK491930, and OK491931, respectively. GenBank accession numbers for the N. risticii ssa2 sequences from horses Bla20, Chu18, Oreo20, Rog18, Whi18, and PA-1 are OL362023 to OL362027, and OL657171, respectively.

RESULTS

Development of new real-time PCR methods. Based on comparison of all available whole genome sequences of Neorickettsia species and other available Neorickettsia spp. sequences at GenBank, and N. risticii and N. findlayensis strain-variable and -conserved sequences of clinical specimens, the real-time PCR target and species-specific primers were designed. For example, P51 is a 51-kDa Neorickettsia outer membrane β-barrel protein composed of 18 transmembrane segments which are Neorickettsia species conserved, and an variable extracellular domain containing nine loops (34). In particular the surface-exposed loop 2 of p51 is highly variable among isolated N. risticii strains. Thus, using primers designed for the conserved flanking transmembrane domains it is possible to amplify the surface-exposed loop 2 of all Neorickettsia species and sequence the PCR product for initial confirmation and comparison of Neorickettsia isolates (27, 34, 35). However, high strain variability is not suitable to design species-specific real-time PCR primers within the surface-exposed loop 2. Another variable Neorickettsia genomic locus consists of two to three tandem ssa genes encoding “strain-specific antigens” (SSAs), as shown by whole genome synteny alignment (Fig. S1). Among them, ssa1 DNA sequences are highly variable among Neorickettsia strains (19), which precludes the development of species-specific PCR tests. In addition, ssa1 lacks the conserved sequences suitable for designing primers to amplify ssa1 of all N. risticii or N. findlayensis strains. Although our previous study found ssa3 is useful to distinguish N. findlayensis from N. risticii by regular PCR (19), extensive intramolecular base sequence repeats made it impossible to design real-time PCR primers. However, based on comparison of whole-genome sequences of all available Neorickettsia spp., only N. findlayensis and N. risticii have ssa2 encoding “strain-specific antigen” 2 (Fig. 1). Although Neorickettsia sp. 179522 from Fasciola hepatica is most closely related to N. findlayensis based on 16S rRNA gene sequence comparison (Fig. S2) (19), and the assembled genome of Neorickettsia sp. 179522 contains two truncated ssa1 genes, it lacks ssa2 (Fig. 1). Alignment of the ssa2 sequences of the N. findlayensis Fin17 strain (19) and the N. risticii Illinois strain (25) revealed unique sequence regions for each species (Fig. S3). Thus, we designed two sets of primer pairs based on ssa2 to specifically amplify N. findlayensis and N. risticii, respectively, by real-time PCR (Table 1).

Although the 16S rRNA genes from all Neorickettsia spp. are quite conserved, currently used N. risticii 16S rRNA gene-based real-time PCR (23) cannot detect N. findlayensis (19). Thus, we also designed a pair of real-time PCR primers based on the 16S rRNA gene (Table 1) that can detect all Neorickettsia spp. except N. helminthoeca. Fig. 2 shows the real-time PCR product sizes and specificity of Neorickettsia ssa2–based PCR using the N. findlayensis Fin17 and N. risticii PA-1 strains as templates (19). N. risticii Illinois and PA-1, a year 2000 isolate in Pennsylvania have identical 16S rRNA gene sequence (33, 34). The PCR product sequences of the ssa2 gene of PA-1 was identical to ssa2 of N. risticii Illinois type strain. Using serially diluted N. findlayensis ssa2, N. risticii
and Neorickettsia 16S rRNA gene–encoding plasmids, we determined that the real-time PCR primer pairs could detect as few as 21 copies of ss2a of N. findlayensis, 210 copies ss2a of N. risticii, and 24 copies of 16S rRNA gene, respectively.

**Specificity of the new real-time PCR of known clinical isolates.** Three culture-positive clinical samples of N. findlayensis (two from Ontario, Canada, and one from Ohio), and 10 culture-positive clinical samples of N. risticii previously analyzed by multigene sequence analysis (19) were tested by the real-time PCR primer pair. The ss2a real-time PCR tests detected all three N. findlayensis and all 10 N. risticii strains, respectively, and there was no cross-amplification between two Neorickettsia species (Table 2). Further, the new 16S rRNA gene–specific pan Neorickettsia real-time PCR test detected all three N. findlayensis and all 10 N. risticii strains of known culture-positive clinical specimens (Table 2). As negative control, 15 clinical samples that were negative by the Neorickettsia 16S rRNA gene–specific nested PCR test during 2020 and 2021 at the Ohio State University, were tested by ss2a-based PCR assays and the new 16S rRNA-based real-time PCR assay. All assays for these specimens were negative.

**Application of the new real-time PCR tests to new clinical isolates.** Next, these new real-time PCR tests were applied to three 2018 and eight 2020 culture isolates.
from horses with PHF. The real-time PCR results were also compared to those obtained from an established method using nested primers directed to the 16S rRNA gene (Table 3). The last two numbers in each horse ID indicate the year (2018 or 2020) of stable culture isolation. The horses resided near a river, stream, or lake in four geographic locations: Quebec, Alberta, and Ontario in Canada, and OH, USA (Table 3). The horses resided along the St. Lawrence River in Ontario and Quebec, along the Richelleu River in Quebec, near many lakes, rivers, and creeks, such as “Nose Creek” and Red Deer River near Calgary, Alberta, near Lake Simcoe in Ontario, Canada, and along the Tuscarawas River, Ohio. Near Calgary, Dom20 and Alb20 resided near many lakes. Zig20 resided near “Nose Creek” and small lakes, and Cin20 resided near Red Deer River, and many PHF cases from Alberta were previously reported based on serological test results (36). Table 3 presents data concerning the number of days each horse was observed to be sick by the owner before the attending veterinarian first examined the horse and collected blood samples, the clinical signs, and the vaccination status of each horse. The following clinical signs were recorded: lethargy, anorexia, fever, color of mucous membranes (buccal and conjunctival), nature of diarrhea [mild diarrhea (softer than normal), moderate (“cowpie”), severe (watery, profuse, projectile)], and laminitis (hoof pain). The horses showed typical clinical signs of PHF, including fever (8/11), lethargy (11/11), anorexia (11/11), diarrhea (9/11), abnormal mucous membrane color (11/11), and laminitis (0/11) (Table 3). All 11 samples tested positive using both nested PCR for Neorickettsia spp. and Neorickettsia spp.–specific real-time PCR directed to the 16S rRNA gene (Table 3). At least eight of the 11 horses were not vaccinated for PHF. Using ssA2-directed real-time PCR, four of four (100%) Neorickettsia isolates from Alberta were identified as Neorickettsia findlayensis, and two isolates from three cases (66%) from Quebec were identified as Neorickettsia ndlayensis. The remaining cases, i.e., three from Ontario, and one each from Quebec and Ohio, were identified as Neorickettsia risticii (Table 3). The PCR product sequences of the ssA2 gene of all six Neorickettsia findlayensis isolates were identical to ssA2 of Find17 type strain (19) except for a single base-pair difference in Dom20. The PCR product sequences of the ssA2 gene of all five Neorickettsia risticii isolates were identical to ssA2 of Illinois type strain (25), but distinct from those of Neorickettsia ndlayensis (GenBank accession Numbers are shown in the Material and Methods section). A previous study reported currently used Neorickettsia risticii–specific real-time PCR (23) fails to detect culture-positive Neorickettsia ndlayensis in clinical specimens (19, 20). In the present study, blood samples of all four culture-positive Neorickettsia findlayensis samples from Alberta were negative by IDEXX Neorickettsia risticii real-time PCR test (Table 3). Out of total six culture-positive Neorickettsia ndlayensis strains, five were tested by Neorickettsia risticii real-time PCR test at AHL-University of Guelph, and the results were all negative (Table 3).

### Table 2: Validation of ssA2 real-time PCR assay using known Neorickettsia findlayensis and Neorickettsia risticii clinical isolates

| Horse ID | NFini, verified | NRIi, verified | Neorickettsia findlayensis spp. (nested PCR) | Neorickettsia findlayensis spp. (qPCR) | NFIi (qPCR) | NRI (qPCR) |
|----------|-----------------|-----------------|----------------------------------------|-----------------------------------|-------------|-----------|
| 081 Ohio | +               | –               | +                                      | –                                 | +           | –         |
| Fin17    | +               | –               | +                                      | +                                 | +           | –         |
| Tom16    | –               | –               | –                                      | +                                 | –           | –         |
| May17    | –               | +               | +                                      | –                                 | +           | +         |
| Luc17    | –               | –               | –                                      | +                                 | –           | –         |
| Cup17    | –               | +               | –                                      | +                                 | –           | –         |
| Lad17    | –               | +               | +                                      | –                                 | –           | +         |
| Dun17    | –               | –               | –                                      | +                                 | –           | –         |
| Jan17    | –               | +               | +                                      | –                                 | –           | –         |
| Gab17    | –               | +               | +                                      | –                                 | –           | +         |
| Dai17    | –               | +               | –                                      | +                                 | –           | –         |
| Too16    | –               | –               | –                                      | +                                 | –           | –         |
| Reg16    | –               | +               | +                                      | –                                 | –           | +         |

a+, positive; –, negative.

bNFini, Neorickettsia findlayensis; NRI, Neorickettsia risticii.

cTeymournejad O, et al. 2020. (19)

dqPCR, real-time PCR (this study).
| Horse ID | Neorickettsia spp. (nested PCR) | Neorickettsia spp. (qPCR) | NFIN<sup>c</sup> (qPCR) | NRI<sup>c</sup> (qPCR) | AHI | IDEXX | Region | Age (yrs) | Stable at night | Sick (days) | PHF vaccinated | Lethargy | Anorexia | Fever | Diarrhea | Mucous membrane<sup>e</sup> |
|----------|-------------------------------|--------------------------|------------------------|------------------------|-----|-------|--------|-----------|----------------|------------|----------------|---------|---------|-------|---------|---------------------|
| Whi18    | +                             | -                        | +                      | NT<sup>d</sup>          | NT  | NT    | Ontario| 14       | No             | 0           | Yes             | Yes     | Mild     | Yes   | Yes   | Pale pink, cow pie, pink |
| Rog18    | +                             | -                        | +                      | NT                      | NT  | NT    | Ontario| 14       | No             | 0           | Yes             | Yes     | Transient | Yes   | No     | Pale pink |
| Chu18    | +                             | -                        | +                      | NT                      | NT  | NT    | Ontario| 7        | No             | 5           | No              | Yes     | Yes     | Yes   | Yes   | Pale pink |
| Bul20    | +                             | -                        | +                      | NT                      | NT  | NT    | Quebec | 5        | Yes            | 1           | No              | Yes     | Yes     | Yes   | Yes   | Pale pink |
| Bla20    | +                             | -                        | +                      | NT                      | NT  | NT    | Quebec | 14       | Yes            | 1           | No              | Yes     | Yes     | Yes   | Yes   | Pale pink |
| Til20    | +                             | +                        | -                      | NT                      | NT  | NT    | Quebec | 10       | No             | 5           | No              | Yes (mild) | Yes     | Yes   | No     | Pale pink |
| Ore20    | +                             | +                        | -                      | NT                      | NT  | NT    | Ohio   | 21       | Yes            | 4           | Not known       | Yes     | Yes     | No     | Yes   | Dark, pink, toxic line |
| Alb20    | +                             | +                        | -                      | -                      | -   | -     | Alberta| 13       | No             | 2           | No              | Yes     | Yes     | No     | Yes   | Dark pink, projectile, toxic line |
| Cin20    | +                             | +                        | -                      | -                      | -   | -     | Alberta| 4        | No             | 4           | No              | Yes     | Yes     | No     | Yes   | Dark pink, toxic line |
| Dom20    | +                             | +                        | -                      | -                      | -   | -     | Alberta| 9        | No             | 3           | No              | Yes     | Yes     | No     | Yes   | Dark pink, toxic line |
| Zig20    | +                             | +                        | -                      | -                      | -   | -     | Alberta| 7        | No             | 0           | No              | Yes     | Yes     | Yes   | Yes   | Dark pink |

<sup>a</sup> +, positive; -, negative.
<sup>b</sup> The last two numbers in each horse ID indicate the year (2018 or 2020) of stable *Neorickettsia* species culture isolation.
<sup>c</sup>NFIN, *N. findlayensis*; NRI, *N. risticii*.
<sup>d</sup>NT, not tested.
<sup>e</sup>None of horses developed laminitis, and all had been treated and recovered.

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**TABLE 3** PCR test results of blood samples, region of origin, clinical information, vaccination status, and treatment outcome of horses from which *Neorickettsia* species were isolated in 2018 and 2020<sup>a</sup>.

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Application of the new real-time PCR to archival DNA samples from suspected clinical PHF cases. The ssa2 real-time PCR was applied to stored DNA of blood samples from horses suspected of having PHF in 2019 and 2020. Of 67 suspected PHF cases from Ontario, 33 tested positive by nested Neorickettsia 16S rRNA gene–based PCR (2 were identified as Neorickettsia findlayensis, and 31 were identified as N. risticii by ssa2 real-time PCR), and the remaining 34 were negative by all three PCR tests, implying 34 animals were not PHF cases (Table 4). Of 11 suspected PHF cases from The Ohio State University Veterinary Teaching Hospital from 2011 to 2020, 10 were positive for Neorickettsia by nested PCR directed to the 16S rRNA gene. All of the positive samples were identified as N. risticii, but not Neorickettsia findlayensis by ssa2 real-time PCRs (Table 4).

DISCUSSION

Currently there is no rapid method available to diagnose Neorickettsia infection. This study describes the development and validation of Neorickettsia findlayensis–specific and Neorickettsia risticii–specific real-time PCR tests directed to ssa2. Known Neorickettsia findlayensis and Neorickettsia risticii–culture-positive clinical specimens were used to validate the tests. Recent (19, 20) and current studies showed currently used Neorickettsia risticii 16S rRNA gene–specific real-time PCR fails to detect Neorickettsia findlayensis in clinical specimens. However, the new 16S rRNA gene–based Neorickettsia genus–specific real-time PCR developed in the present study detected Neorickettsia findlayensis in all clinical specimens from diverse geographic regions in 1991–2020. Interestingly, there was no co-infection of two Neorickettsia species in any of PHF cases. This assay is expected to reduce false negative PCR diagnosis of PHF due to Neorickettsia findlayensis infection. Moreover, compared with the classic 16S rRNA gene–based nested PCR, the newly developed pan-Neorickettsia real-time PCR is similarly effective in the molecular diagnosis of PHF. Using plasmids containing ssa2s or the 16S rRNA gene, the ability of these assays to detect Neorickettsia findlayensis, Neorickettsia risticii and the Neorickettsia genus are shown for the first time and informs us of the PCR detection threshold (PCR assay negative if bacterial numbers are below the threshold). The current result is expected to encourage future investigation to analyze PHF diagnostic sensitivities and specificities for the new real-time PCR tests with a larger number of clinical specimens.

The utility of the developed assays was evaluated with clinical isolates submitted from new geographic regions as well as with stored DNA samples from suspected PHF cases. Although we could assess only a limited number of culture–positive cases, the current study reveals that PHF caused by Neorickettsia findlayensis infection is widespread in Canada, and this pathogen is potentially a major cause of PHF in certain geographic regions (Alberta and Quebec), but a minor cause of PHF in other geographic regions (Ontario and Ohio). Fig. 3 depicts geographic distribution of Neorickettsia findlayensis in Canada confirmed by culture isolation and/or the current real-time PCR. Using both the Neorickettsia risticii and Neorickettsia findlayensis real-time tests directed to ssa2, the proportions of PHF caused by Neorickettsia findlayensis in other geographic regions can be investigated. Combining these tests with the Neorickettsia genus–specific real-time PCR test would facilitate the discovery of other Neorickettsia species that may cause PHF.

Neorickettsia spp. are Gram–negative obligatory intracellular bacteria of digenean trematode flukes, which are transmitted through all developmental stages of the trematodes and vertically through generations of trematodes (37–41). Several Neorickettsia

| Yr                        | Samples (N) | NFIn (ssa2 qPCR) | NRI (ssa2 qPCR) | Neorickettsia spp. nested PCR | Negative |
|---------------------------|-------------|------------------|-----------------|------------------------------|----------|
| 2019 (Ontario, Canada)    | 26          | 1                | 7               | 8                            | 18       |
| 2020 (Ontario, Canada)    | 41          | 1                | 7               | 8                            | 16       |
| Totals                    | 67          | 2                | 31              | 33                           | 16       |
| 2011–2020 Ohio            | 11          | 0                | 10              | 10                           | 1        |

*NFIn, Neorickettsia findlayensis; NRI, Neorickettsia risticii.
spp. are horizontally transmitted from infected trematodes to humans, horses, and dogs and cause severe diseases in these accidental hosts (42). Thus, the geographic distribution and prevalence of PHF in areas where horses are raised likely corresponds to the distribution of *Neorickettsia* spp.—infected trematodes and the intermediate and definitive hosts of the trematodes. In the case of *N. risticii*, each developmental stage of the trematode species naturally infected with this bacterium in Pennsylvania was morphologically and molecularly identified as *Acanthatrium oregonense* (33). Big brown bats (*Eptesicus fuscus*) and little brown bats (*Myotis lucifugus*) are infected with *N. risticii* as confirmed by 16S rRNA nested PCR and *p51* (encoding the major outer membrane protein P51 of porin activity unique to *Neorickettsia* spp. (27, 43)) PCR-positive bat liver, spleen and/or blood samples, followed by sequencing the PCR products (37). The intestinal lumen of these bats harbors adult gravid trematodes infected with *N. risticii* and individual eggs isolated from them were also infected with *N. risticii* (37). It is of great interest to uncover the trematode species that carry *N. findlayensis* and hosts of trematodes in Canada, and whether the trematode’s natural hosts are infected.

The course of PHF is usually 5–10 days with a mortality rate of 17–36%; however, PHF cases respond well to early medical intervention. Currently, the efficacy of inactivated whole-cell vaccines is debatable. Commercially available vaccines provide only limited or no protection (17), and this lack of protection has been clearly demonstrated in previous (19) and current study (Table 3). The inclusion of immune-protective antigens derived from contemporary *Neorickettsia* species including *N. findlayensis* could improve the efficacy of vaccines against PHF.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

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*FIG 3* Geographic locations of *N. findlayensis*-infected horses with PHF. Red dots represent the geographic locations of clinical cases from which *N. findlayensis* was isolated from blood of horses and verified by real-time PCR: four in Calgary, Alberta and two in Montreal, Quebec from this study, and two in Toronto, Ontario were from previous study (19). Blue dots represent clinical cases from which *N. findlayensis* was detected by real-time PCR of blood specimens (two in Toronto, Ontario from this study).
Y.R., L.G.A., and J.D.B. designed the research; K.B. performed the research; M.L.I. designed the primers; Q.Y. and R.C. performed the PHF diagnostic tests; K.B., and Y.R., and M.L.I. analyzed data; G.H. M. Le, and W.W. performed sample collection and clinical case management; and Y.R. and K.B. wrote the paper.

We have no conflicts of interest to disclose.

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