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# An Ecotype of *Neorickettsia risticii* Causing Potomac Horse Fever in Canada

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## ABSTRACT

***Neorickettsia* (formerly *Ehrlichia*) *risticii* is an obligatory intracellular bacterium of digenetic trematodes. When a horse accidentally ingests aquatic insects containing encysted trematodes infected with *N. risticii*, the bacterium is transmitted from trematodes to horse cells and causes an acute and often fatal disease called Potomac horse fever (PHF). Since the discovery of *N. risticii* in the United States in 1984, using immunofluorescence and PCR assays, PHF has been increasingly recognized throughout North America and South America. However, so far, there exist only a few stable *N. risticii* culture isolates, all of which are from horses within the United States, and the strain diversity and environmental spreading and distribution of pathogenic *N. risticii* strains remain poorly understood. This paper reports the isolation of *N. risticii* from the blood of a horse with acute PHF in Ontario, Canada. Intracellular *N. risticii* colonies were detected in P388D$_1$ cells after 47 days of culturing and 8 days after the addition of rapamycin. Molecular phylogenetic analysis based on amino acid sequences of major surface proteins P51 and Ssa1 showed that this isolate is distinct from any previously sequenced strains but closely related to midwestern U.S. strains. This is the first Canadian strain cultured, and a new method was developed to reactivate dormant *N. risticii* to improve culture isolation.**

## IMPORTANCE

***Neorickettsia risticii* is an environmental bacterium that lives inside flukes that are parasitic to aquatic snails, insects, and bats. When a horse accidentally ingests insects harboring flukes infected with *N. risticii*, the bacterium is transmitted to the horse and causes an acute and often fatal disease called Potomac horse fever. Although the disease has been increasingly recognized throughout North and South America, *N. risticii* has not been cultured outside the United States. This paper reports the first Canadian strain cultured and a new method to effectively culture isolate *N. risticii* from the horse blood sample. Molecular analysis showed that the genotype of this Canadian strain is distinct from previously sequenced strains but closely related to midwestern U.S. strains. Culture isolation of *N. risticii* strains would confirm the geographic presence of pathogenic *N. risticii*, help elucidate *N. risticii* strain diversity and environmental spreading and distribution, and improve diagnosis and development of vaccines for this dreadful disease.**

*Neorickettsia* spp. are Gram-negative bacterial endosymbionts of parasitic flukes (trematodes Digenea, phylum Platyhelminthes) that also have the potential to infect leukocytes of vertebrate hosts of flukes and cause systemic diseases (1, 2). *Neorickettsia risticii* was initially isolated from horses with acute Potomac horse fever (PHF), or equine monocytic ehrlichiosis, and experimentally demonstrated to cause PHF in the United States in 1984 (3, 4). PHF is an acute and potentially fatal equine disease that occurs frequently in the United States and is occasionally reported in Canada, Brazil, Uruguay, and Europe. In regions of the United States where PHF is endemic, *N. risticii* DNA is found in freshwater snails (first intermediate host of the trematodes) in the genus *Juga* of the family *Pleuroceridae* in California and lymnaeid snails (*Stagnicola* sp.) in Oregon. As well, *N. risticii* DNA had been found in *Elimia* (*Goniobasis*) species of the *Pleuroceridae* family, including *Elimia livescens* in Ohio, *E. virginica* in Pennsylvania, and *E. laqueata* snails in Kentucky, and trematodes (cercariae) released from the snails (5, 6). The cercaria identified is a virgulate xiphidiocercaria that has a stylet to penetrate the chitinous wall of aquatic insects (the second intermediate hosts of trematodes) (5). *N. risticii* DNA has been detected in 13 species of immature and adult caddisflies (Trichoptera), mayflies (Ephemeroptera), damselflies (Odonata, Zygoptera), dragonflies (Odonata, Anisoptera), and stoneflies (Plecoptera) (second intermediate hosts) (7, 8). Gravid trematodes and trematode eggs containing *N. risticii* DNA and *N. risticii* bacteria were found from the intestines of *Eptesicus fuscus* (big brown bats) and *Myotis lucifugus* (little brown bats) from various sites in Pennsylvania where PHF is endemic, indicating that these bats are the definitive host of the trematodes. Based on morphological keys, the trematode identified is *Acanthatrium oregonense* (9). Further, 43.4% (23 of 53) of tested isolated *E. fuscus* and *M. lucifugus* bats are infected with *N. risticii* in their blood, based on PCR indicating the horizontal transmission of *N. risticii* from adult trematodes to bats (9). Subsequent molecular studies detected *A. oregonensis* trematodes and *N. risticii* DNA in every stage (egg, sporocyst, cercaria, metacercaria, and adult) of *A.*

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*oregonensis*, proving intrastadial and vertical transmission of *N. risticii* in the trematode host (9). *Acanthatrium* sp., *Lecithodendrium* sp. of trematodes, and *Myotis yumanensis* bats and tree swallows (*Hirundo rustica* and *Tachycineta bicolor*) were also reported to be infected with *N. risticii* (10). In addition, *N. risticii* DNA fragments have been detected in various other digenean trematodes having life cycles typical of freshwater/terrestrial ecosystems (11, 12). However, due to limited molecular analysis, the relatedness of these *Neorickettsia* organisms to *N. risticii* strains infecting horses, and whether they can infect horses and cause PHF, is unknown. These studies indicate a complex interplay among *N. risticii*, its trematode hosts, and definitive and intermediate hosts of trematodes and the broad distribution of *N. risticii* in nature (2).

The horse is an accidental host of the digenetic trematodes. During the summer season, when a horse ingests adult aquatic insects containing encysted *N. risticii*-infected trematodes, the bacterium causes PHF. Horizontal transmission of *N. risticii* from trematodes to horse blood monocytes was shown by feeding *N. risticii* PCR-positive caddisflies from the region where PHF is endemic (8, 13). These horses developed clinical signs of PHF, and live *N. risticii* was isolated from the horses' blood, fulfilling Koch's postulates (8). The signs of PHF include depression, anorexia, fever, dehydration, abortion, watery diarrhea, and laminitis (14). The only effective treatment is the administration of tetracycline, a broad-spectrum antibiotic, in the early stages of the disease (14).

Currently, only a single inactivated whole-cell vaccine based on the 1984 strain of *N. risticii* from Maryland, USA, is commercially available. Since 1984, strains of *N. risticii* have been isolated by culture from horses with PHF only in the United States, which show genetic and antigenic strain variation (15–19). Although vaccination has been reported to protect 78% of horses experimentally infected with the same strain, it has been marginally protective in the field (20). Vaccine failure has been attributed to antigenic and genomic heterogeneity among the different strains of *N. risticii* (17). Thus, isolation and characterization of *N. risticii* strains from diverse geographic regions help in the development of more effective preventive measures for PHF.

Although PHF disease was likely first described as early as in 1924 in the Province of Ontario, Canada, by Frank Schofield (21), the first report of PHF in Ontario was in 1995. From 1995 to 2010, 20 PHF cases were confirmed at the Ontario Veterinary College Veterinary Teaching Hospital (OVC-VTH), based on clinical signs, indirect fluorescent antibody (IFA) seroconversion, PCR on the blood and/or feces, and histopathology. Most cases were diagnosed near Lake Ontario, Lake Erie, and Lake St. Clair (22). No cell culture isolation of *N. risticii* has been reported from Canada. In this study, we report the successful isolation of the first *N. risticii* strain from a horse with acute PHF in Canada and the use of a new method to facilitate bacterial isolation in cell culture.

## MATERIALS AND METHODS

**Case history.** The Thoroughbred colt was born in Ontario, Canada, on 10 February 2013 and when 3 weeks old was transported to Kentucky, where he resided until 22 May 2015. The horse went to North Carolina for 3 months and then was transported to a farm in Egbert, Ontario, where he remained for 60 days prior to being moved to a racetrack in Toronto, Canada, on 21 July 2015. On 3 August, equine influenza and rhinopneumonitis vaccine (Calvenza equine influenza virus [EIV]/equine herpesvirus [EHV]; Boehringer-Ingelheim, St. Joseph, MO) and an anthelmintic, moxidectin-praziquantel (Quest Plus; Zoetis Canada, Kirkland, Quebec, Canada), were administered. Since arriving at the racetrack, compounded omeprazole liquid had been administered each day.

On 13 August 2015, the colt was examined by a veterinarian at the racetrack in Toronto, Ontario, due to fever (39.4°C/103°F). No other abnormalities were noted on physical examination. A complete blood count (CBC) was taken, and mild leukopenia ($5.30 \times 10^9$/liter; reference range, $5.40 \times 10^9$ to $14.30 \times 10^9$/liter) and mild lymphopenia ($1.03 \times 10^9$/liter; reference range, $1.50 \times 10^9$ to $7.70 \times 10^9$/liter) were the only hematological abnormalities. The serum amyloid A level was normal. The colt was treated with phenylbutazone (2 g intravenous [i.v.]). Fever was present again on the mornings of 14 August (39.2°C/102.6°F) and 15 August (38.8°C/101.9°F). Phenylbutazone (2 g *per os*) was administered on both days. On the morning of 16 August, the rectal temperature was normal, but the colt had developed watery diarrhea. The veterinarian treated the horse with flunixin meglumine (1.1 mg/kg of body weight i.v.) and referred the horse to the OVC-VTH for further diagnostics and treatment.

**Culture isolation of *N. risticii*.** Approximately 175 ml of citrated blood was collected from the horse on 18 August 2015, and it arrived 48 h later at the Rikihisa laboratory at The Ohio State University, Columbus, OH, for the isolation of *N. risticii*. This horse sample was positive by a PCR test at OVC-VTH. The blood was centrifuged at $500 \times g$ for 10 min to obtain buffy coat. After lysis of red blood cells with ammonia chloride solution, peripheral blood lymphocytes (PBLs) were obtained. The buffy coat or PBLs were inoculated into $P388D_1$ cells cultured in RPMI 1640 with 5% fetal bovine serum (FBS) (18). Signs of cell infection were monitored weekly by light microscope after Diff-Quik staining. Approximately 0.5 ml of cell cultures was harvested for DNA extraction for a nested-PCR test using specific *N. risticii* primers targeting the *N. risticii* 16S RNA gene, 1st primer pair Er-5-3 and Er-3-2, and 2nd primer pair Eris-1 and Eris-2 (19). Over 75% of the growth medium was replaced with fresh RPMI 1640 with 5% FBS every week. On day 39, rapamycin (100 ng/ml) (Sigma, St. Louis, MO) was added to the cell cultures, and culture was continued until *N. risticii* microcolonies (inclusions) were identified in the cytoplasm of host cells under light microscope after Diff-Quik staining. The result was confirmed by nested-PCR and immunofluorescence labeling using the *N. risticii* positive-control antibody (18). For immunofluorescence labeling, cultured cells were fixed in 4% paraformaldehyde at room temperature for 15 min and blocked in PGS buffer (phosphate-buffered saline [PBS] [pH 7.4] containing 0.4% bovine serum albumin [BSA], 0.1% gelatin, and 0.3% saponin) for 30 min at room temperature. The cells were then incubated with PHF-positive-control antibody (1:100 dilution) in PGS buffer for 60 min at 37°C, followed by incubation with Cy3-conjugated anti-horse IgG antibody (diluted 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. For photography, cells were then washed and observed using a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA). Images were processed using SoftWoRX software (Applied Precision) and Adobe Photoshop software CS2 (Adobe Systems, Mountain View, CA).The positive cell cultures were frozen in RPMI 1640 freezing medium containing 10% dimethyl sulfoxide (DMSO) and 30% FBS at −80°C.

**IgM and IgG antibody tests.** In order to determine the anti-*N. risticii* IgM titer in the horse serum, IgG was removed by incubating 20 μl of horse serum (diluted in 200 μl of PBS) with 50 μl of protein G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C with rotation. After IgG removal, the serum sample was serially diluted and spotted on the 12-well *N. risticii* antigen slides. The original serum was used to detect the IgG titer. Fluorescein isothiocyanate (FITC)-labeled goat anti-horse IgM (KPL) and Cy3-conjugated anti-horse IgG antibody (Jackson ImmunoResearch Laboratories) were used as second antibodies to detect horse IgM and IgG, respectively.

**PCR, sequencing, and sequence analysis.** DNA was purified from the buffy coats or $P388D_1$ cells cocultured with horse blood samples using the QIAamp DNA blood minikit (Qiagen, Valencia, CA), according to the manufacturer's instructions. PCR amplification was then performed us-

ing extracted genomic DNA as the template and *Taq* DNA polymerase (New England BioLabs, Ipswich, MA). P51 primer pair 51K-F7/51K-R5 and Ssa1 primer pair 838-1a/838-4 were used to amplify variable regions of P51 containing external loop2 and Ssa1, respectively (17). The PCR products were sequenced at The Ohio State University Comprehensive Cancer Center Nucleic Acid Shared Resource facility. Gene fragments were translated, and phylogenic analysis was performed through the CLUSTAL V method in the MegAlign program (DNAStar, Madison, WI).

**Western blot.** *N. risticii*-infected P388D$_1$ cells ($1 \times 10^6$) with and without rapamycin treatment were resuspended in PBS and lysed by mixing with 2× Laemmli sample buffer (135 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol). Samples were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to a nitrocellulose membrane with a semidry blotter (WEP, Seattle, WA). The membrane was blocked with 5% (wt/vol) skimmed milk (Meijer, Grand Rapids, MI) in Tris-buffered saline (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 0.1% Tween 20, incubated with rabbit anti-*N. risticii* P51 (diluted 1:1,000) (23) and anti-actin (Sigma) antibodies at 4°C for 12 h, and subsequently incubated with peroxidase-conjugated secondary antibodies (KPL) (diluted 1:1,000) at room temperature for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence (Thermo Scientific, Waltham, MA) using the Fuji LAS-3000 imaging system (Fujifilm Medical Systems USA, Stamford, CT), and band density was measured using the Fujifilm Multi Gauge software (Fujifilm).

**Accession number(s).** The GenBank accession numbers of Canadian *N. risticii* P51 and strain-specific antigen 1 (Ssa1) are KX189633 and KX189634, respectively. P51 and Ssa1 sequences used for the phylogenic analysis in this study are listed in Table 1.

**TABLE 1** P51 and Ssa1 sequences used for the phylogenic analysis in this study

| Sample ID[a] | Accession no. |
|---|---|
| P51 | |
| *N. risticii* Illinois | WP_015816118.1 |
| PA-1 | AAM18377 |
| PA-2 | AAM18376 |
| Eclipse | AAC01597 |
| OH07-1 | AEL03838 |
| OH07-4 | AEL03848 |
| OH10-2 | AEL03850 |
| OH07-2 | AEL03839 |
| TW2-1 | AAR22503 |
| TW2-2 | AAR22504 |
| 25-D | AAB46983 |
| 90-12 | AAB46982 |
| CM1-1 | AAR22501 |
| 081 | AAG03354 |
| OV | AAG03353 |
| Doc | AAC01595 |
| Oregon | AAC01600 |
| 11908 | AAL79561 |
| SF Hirose | AAL12490 |
| SF Oregon | AAR23988 |
| Dr. Pepper | AAC01596 |
| Ms. Annie | AAC01599 |
| SHSN-1 | AAB95417 |
| MN | AEK71074 |
| Snail 2121 | AAF20073 |
| Shasta-horse | AAF43112 |
| Siskiyou horse-1 | AAF20069 |
| Juga-1 | AAC01598 |
| Herodia | AEK71065 |
| KY03-1 | AEL03827 |
| *N. sennetsu* Miyayama | WP_011451642.1 |
| Kawano | AAR23991 |
| Nakazaki | AAR23990 |
| Ssa1 | |
| *N. risticii* Illinois | WP_015816716.1 |
| 90-12 | AAC31428 |
| 25D | AAC31427 |
| OH07-1 | AEL03836 |
| PA-1 | AEK71056 |
| *N. sennetsu* Miyayama | WP_011452276.1 |
| MI | Patent (28) |
| OR | Patent (28) |
| NY | Patent (28) |

[a] ID, identification.

## RESULTS

**Clinical course.** On admission to the OVC-VTH on 16 August 2015, the colt was in good body condition (457 kg), tachycardic (60 bpm), tachypneic (24 bpm), and had congested mucous membranes with a prolonged capillary refill time (3 s). The horse had profuse watery diarrhea, a prolonged skin tent, and was assessed to be approximately 6 to 8% dehydrated. Increased gut sounds were auscultated over all four quadrants. Digital pulses were assessed to be normal. Venous blood-gas analysis showed metabolic acidosis (pH 7.278; $HCO_3^-$, 17.6 mmol/liter; base deficit, −7.8), hyponatremia (124 mmol/liter; reference range, 136 to 144 mmol/liter), hypochloremia (89 mmol/liter; reference range, 95 to 104 mmol/liter), and mild hyperlactatemia (3.1 mmol/liter; reference range, <2 mmol/liter). Blood was submitted to the Animal Health Laboratory, University of Guelph, for a CBC, serum biochemical profile, and PCR test for *N. risticii* (24). A feces sample was submitted for bacteriological culture, enzyme-linked immunosorbent assay (ELISA) testing for *Clostridium difficile* toxins A and B and *Clostridium perfringens* enterotoxin, and PCR testing for *N. risticii*.

The CBC showed an elevated hematocrit (0.59%; reference range, 0.28 to 0.44%), red cell count ($14.4 \times 10^{12}$/liter; reference range, $6.9 \times 10^{12}$ to $10.7 \times 10^{12}$/liter), hemoglobin (222 g/liter; reference range, 112 to 169 g/liter), leukocytosis ($11.8 \times 10^9$/liter; reference range, $5.1 \times 10^9$ to $11.0 \times 10^9$/liter), elevated band neutrophils ($1.65 \times 10^9$/liter; reference range, 0 to $0.2 \times 10^9$/liter), and monocytosis ($1.77 \times 10^9$/liter; reference range, $0.1 \times 10^9$ to $0.8 \times 10^9$/liter). The serum biochemical profile showed hyponatremia (124 mmol/liter; reference range, 136 to 144 mmol/liter), hyperkalemia (5.6 mmol/liter; reference range, 3.1 to 4.3 mmol/liter), hypochloremia (81 mmol/liter; reference range, 95 to 104 mmol/liter), hypoalbuminemia (28 g/liter; reference range, 30 to 37 g/liter), azotemia (urea, 26.1 mmol/liter; reference range, 4.2 to 8.9 mmol/liter; and creatinine, 349 μmol/liter; reference range, 80 to 130 μmol/liter), and elevated serum amyloid A (1,276.6 mg/liter; reference range, 0 to 20 mg/liter).

Treatment was commenced with intravenous lactated Ringer's solution with a 10-liter bolus, followed by double maintenance rate, penicillin G sodium (22,000 U/kg of body weight i.v.), gentamicin sulfate (6.6 mg/kg i.v. every 24 h [q24h]), and flunixin meglumine (1.1 mg/kg i.v. q12h). Ten hours after admission, the colt was reluctant to move and had increased digital pulses palpable in both fore limbs. Digital hypothermia was induced by cryotherapy with ice in plastic bags over all limbs. The initial antibiotic regime was discontinued, and oxytetracycline (6.6. mg/kg i.v. q24h for 5 days) was instituted. No *Salmonella* spp. were isolated

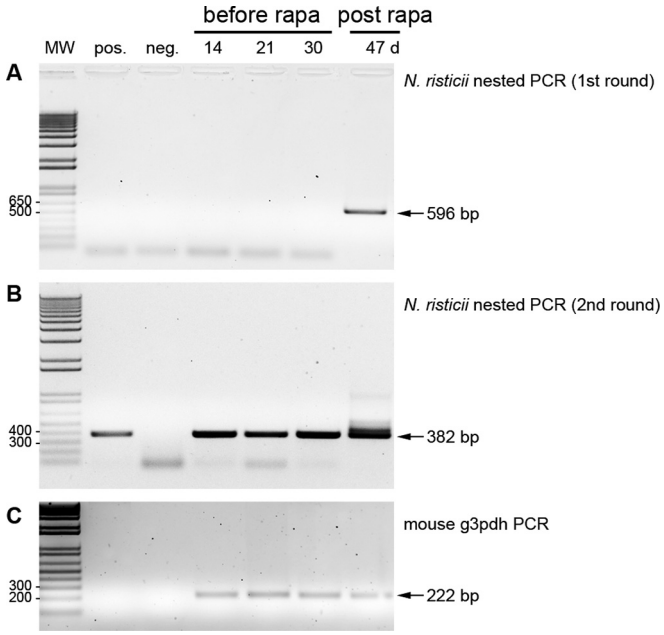


**FIG 1** Detection of *N. risticii* DNA in the isolation culture. DNA samples from cell cultures at day 14, 21, 30 (prior to rapamycin treatment), and 47 (8 days after rapamycin treatment) after initial inoculation of infected horse buffy coat were subjected to nested-PCR test for *N. risticii* 16S rRNA gene. (A and B) The first-round PCR products (target size, 596 bp) (A) and the second-round PCR products (target size, 382 bp) (B) showing significant increase of *N. risticii* DNA after rapamycin (rapa) treatment. (C) Sample DNA input was normalized by mouse g3pdh DNA levels derived from P388D$_1$ cells. MW, molecular weight of marker (in thousands); pos., positive control (DNA from *N. risticii*-infected horse buffy coat); neg., negative control; d, days.

from the feces, and the ELISAs for *C. difficile* toxins A and B and *C. perfringens* enterotoxin were both negative. Over the next 7 days, the horse continued to be reluctant to move because of the acute laminitis. Radiographs of all 4 feet were taken to assess whether any rotation or sinking of the third phalanx was occurring. Phenylbutazone (2 g *per os* q12h) and morphine (0.1 mg/kg i.m. q6h) were instituted on day 3 in an attempt to alleviate the horse's discomfort. By day 4 of hospitalization, the diarrhea had resolved, and the horse had a normal appetite and was drinking normally. Corrective podiatry was conducted following abaxial nerve blocks. The horse was discharged with a guarded prognosis on day 10 with recommendations for stall rest and close monitoring. The horse was euthanized on 30 September 2015 due to pain associated with the acute laminitis. On admission to OVC-VTH, the blood and fecal PCR for *N. risticii* were both positive, and the anti-*N. risticii* IgG and IgM titers were 1:10,240 and 1:2,560, respectively. This indicated that the horse acquired *N. risticii* infection in Canada.

**Culture isolation of *N. risticii*.** The cell culture remained *N. risticii* PCR positive throughout the culture period by nested-PCR (Fig. 1). However, after 39 days of culture, bacterial growth was not evident, as microcolonies (inclusions) of *N. risticii* were not observed under light microscope following Diff-Quik staining (data not shown). Rapamycin (100 ng/ml) was added to the culture medium at day 39, and 8 days later (day 47 post-initial culture), even the first round of nested-PCR showed the specific band of *N. risticii* DNA (Fig. 1B), and the second round of nested-PCR showed increased band intensity of PCR product (Fig. 1C). Furthermore, *N. risticii* inclusions were visible under light microscopy (Fig. 2A to C). *Neorickettsia risticii* inclusions appear to show two different morphological forms: some inclusions were large and compact, while others were small and dispersed (Fig. 2A and B). The result was confirmed by immunofluorescence labeling (Fig. 2C).

The addition of rapamycin to the culture medium appeared to be the key to the successful isolation of this strain of *N. risticii*. To confirm this, we thawed a Canadian isolate of *N. risticii* and tested whether rapamycin enhances its growth in the host cells. Our data showed that rapamycin treatment significantly increases *N. risticii* growth in P388D$_1$ cells, indicated by Western blot analysis detecting *N. risticii* major surface-exposed outer membrane protein P51 (Fig. 2D).

**Sequence variation in P51 and Ssa1.** Previous studies from our laboratory show that the *N. risticii* 51-kDa antigen is the major surface-exposed outer membrane protein, and the P51 amino acid sequence, especially external loops 2 and 4, is highly strain variable (17). We amplified the P51 DNA fragments containing external loop 2 and compared the amino acid sequence with those of other *N. risticii* geographic strains. Phylogeny analysis based on P51 protein (amino acids [aa] 133 to 214) showed that Canadian *N. risticii* strain belongs to the cluster of eastern and midwestern United States and is very close to OH, KY, and PA strains, with a similarity of over 98% (Fig. 3A and B). Strain-specific antigen (Ssa) has been suggested as an immunogenic surface protein with potential use in vaccine development (15, 25–27). We amplified the Ssa1 DNA fragment of the Canadian strain and compared the deduced the amino acid sequence of Ssa1 with those of other *N. risticii* strains, including three recently sequenced strains, NY, MI, and OR (named for the states from which they were isolated) (28). Phylogeny analysis based on the Ssa1 sequence revealed that the Canadian *N. risticii* strain is distinct from these three strains. The similarities to these strains were less than 65% (Fig. 4).

## DISCUSSION

In this study, we have reported the isolation of new *N. risticii* strain from a PHF case in Ontario, Canada. Bioinformatics analysis on the major surface proteins P51 and Ssa1 suggested that the genotype of the Canadian strain is different from previously sequenced *N. risticii* strains.

Although culture isolation of causative pathogens has been considered to be the gold standard of disease diagnosis, it remains challenging for certain intracellular microorganisms, like *Neorickettsia* spp., because it is more technically demanding, expensive, and usually takes longer time than IFA or PCR. Furthermore, *N. risticii* lives only inside live eukaryotic host cells, and it takes a few days for fresh blood specimens to reach the laboratory for culture isolation. Therefore, even if bacteria are still alive, they are dormant. Thus, one of the keys to the successful isolation is to reactivate dormant bacteria to start to grow in cultured cells. Rapamycin is a FDA-approved and safe anticancer drug originally isolated from *Streptomyces hygroscopicus*. The drug is known to induce cellular autophagy and inhibit eukaryotic cell growth by regulating several processes, such as transcription, translation, protein degradation, and ribosome biogenesis (29). Rapamycin increases *Anaplasma phagocytophilum* infection in host cells by inducing autophagy and enhancing nutrition acquisition by *A. phagocytophilum* (30). In the present study, *N. risticii* remained dormant after 39 days postinoculation with the infected blood. Therefore,

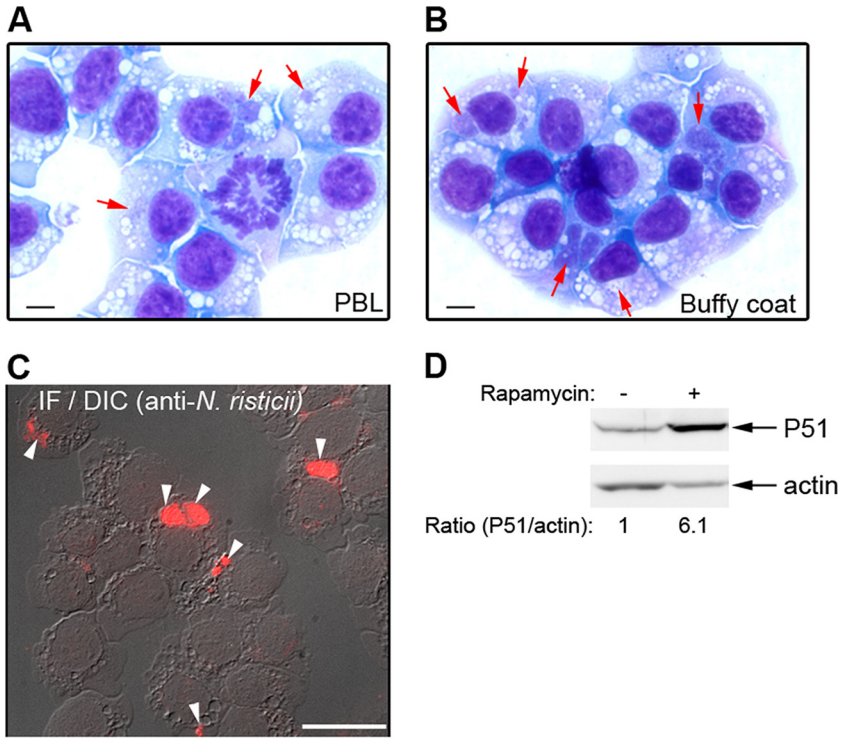


**FIG 2** *N. risticii* Canadian isolate following rapamycin treatment. Peripheral blood leukocytes (PBLs) (A) and buffy coat (B) derived from the horse blood were used to inoculate P388D$_1$ cells. *N. risticii* inclusions (red arrows) were seen in the cytoplasm of host cells after Diff-Quik staining on day 47 of culture (day 8 after rapamycin treatment). Bar = 5 μm. (C) Immunofluorescence labeling of Canadian isolate (white arrowheads) by using horse anti-*N. risticii* serum. IF/DIC, immunofluorescence image merged with differential interference microscopy. Bar = 15 μm. (D) Rapamycin was added at 1 h before inoculation with *N. risticii* Canadian isolate and cultured for 3 days until *N. risticii* infection was examined by Western blotting using rabbit *N. risticii* P51 antibody. Actin was used as the protein loading control. The data are representative of the results from three independent experiments. Rapamycin treatment increased the band density of *N. risticii* P51 >6-fold.

we added rapamycin to the culture media and 8 days after *N. risticii* microcolonies were visible. Both *N. risticii* and *A. phagocytophilum* belong to the same family, *Anaplasmataceae*. Although the mechanism of rapamycin resulting in enhanced *N. risticii* intracellular growth is currently unknown, this approach is potentially applicable for isolating other members of the family *Anaplasmataceae* (31).

Determining variation among different clinical and field isolates is crucial in understanding pathogenicity, diagnosis, vaccine development, and disease migration and evolution. At present, a variety of approaches are used to measure strain variation, such as protein profiles, serological assays, and molecular methods targeting highly variable loci or whole-genome sequencing. Due to the important roles played by surface-exposed proteins during different stages of infections, they are linked to strain antigenic variation and also recognized as a potential candidate for diagnosis and vaccine development. Therefore, these proteins were the focus for analyzing the strain variations. Based on the molecular evidences of genetic variation of two major surface-exposed proteins of *N. risticii* P51 and Ssa1, the Canadian *N. risticii* isolate appears to be a new strain. Previous results depicted that *N. risticii* P51 is a major surface-expressed protein and strongly associated with geographical distribution. *In silico* analysis showed that P51 has 18 transmembrane domains and 9 external loops, of which external loop 2 is the most variable region. Based on the amino acid sequences of external loop 2, 43 different *N. risticii* strains are clustered into two groups: East/Midwest U.S. and Pacific Coast (17). A comparison of external loop 2 sequence fragments revealed that the Canadian isolate fits in the East/Midwest U.S. cluster. *N. risticii* Ssa proteins were originally described in *N. risticii* 25-D (50-kDa antigen) (isolated in 1984 in Maryland) and 90-12 (85-kDa antigen) (isolated in 1990 in Maryland) strains (15, 25, 27). The association of Ssa1 sequence with temporal variation of *N. risticii* strains has been suggested (17). At present, Ssa1 has been sequenced in very few *N.*

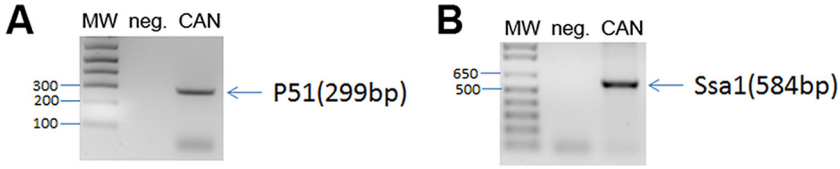


**FIG 3** PCR of P51 and Ssa1 genes of the Canadian strain of *N. risticii*. A single-step PCR detected P51 and Ssa1 genes of Canadian strain of *N. risticii*. The PCR products were sequenced for phylogenetic analysis. MW, molecular weight of marker (in thousands); neg., negative control; CAN, Canadian isolate.

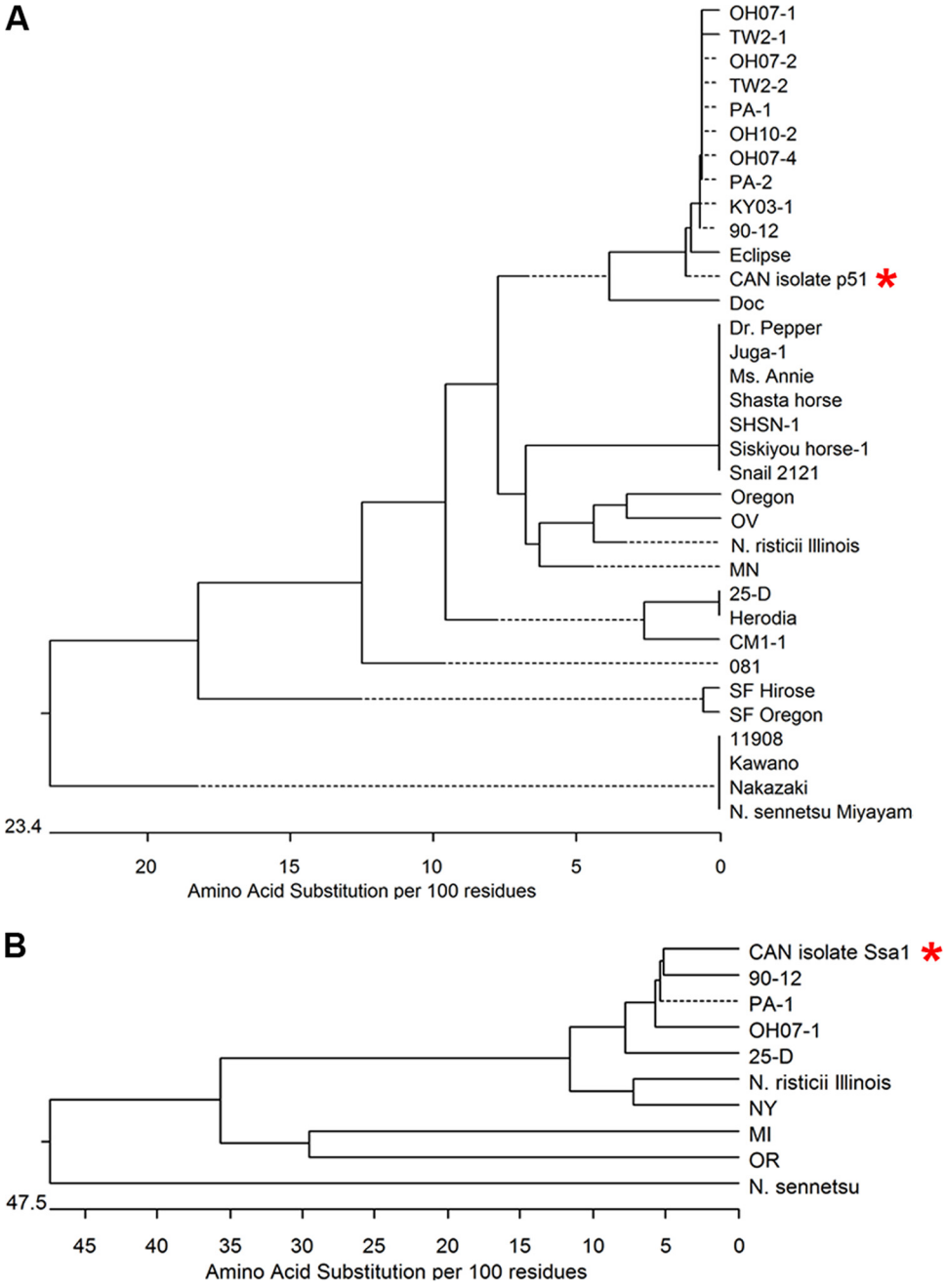


**FIG 4** Phylogenetic analysis of the Canadian strain of *N. risticii* P51 (A) and Ssa1 (B) sequences. Deduced amino acid sequences of P51 and Ssa1 fragments of the Canadian strain of *N. risticii* were compared with those of other *N. risticii* isolates in tree view A by CLUSTAL V method in the MegAlign program of the DNAStar software. The Canadian isolate (CAN) is highlighted with a red asterisk.

*risticii* isolates, including NY, OR, and MI strains. Of note, our data had shown that the Canadian isolate is different from any of these *N. risticii* isolates.

The course of PHF disease without therapeutic intervention is short, usually 5 to 10 days. The mortality rate of clinical cases of PHF ranges from 17 to 36%. Due to this short window for disease diagnosis and the big impact on the horse industry, an effective PHF vaccine is greatly needed in the field. Currently, commercially available inactivated whole-cell vaccines are based on a single strain of *N. risticii*. However, these PHF vaccines provide only limited protection, likely due to their antigenic and genomic heterogeneity, as demonstrated among the >14 different *N. risticii* strains isolated from naturally occurring cases, including this new Canadian strain (17). This study emphasizes the need for a PHF vaccine that protects against various strains of *N. risticii*.

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