

Two *Anaplasma phagocytophilum* Strains in *Ixodes scapularis* Ticks, Canada

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We developed PCR-based assays to distinguish a human pathogenic strain of *Anaplasma phagocytophilum*, Ap-ha, from Ap-variant 1, a strain not associated with human infection. The assays were validated on *A. phagocytophilum*-infected black-legged ticks (*Ixodes scapularis*) collected in Canada. The relative prevalence of these 2 strains in *I. scapularis* ticks differed among geographic regions.

The gram-negative bacterium *Anaplasma phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA) in the United States (1). More than 90% of HGA cases occur in the Upper Midwest and Northeast (2). In these regions, black-legged ticks (*Ixodes scapularis*) are the vectors of a human pathogenic strain (Ap-ha) and a variant strain (Ap-variant 1) of *A. phagocytophilum* (3–7), the latter of which appears not to be associated with human infection (1,3). HGA represents an emerging disease in southern Canada because populations of *I. scapularis* ticks have become established or are in the process of becoming established (8,9). However, there is limited information on the occurrence of *A. phagocytophilum* in these ticks (10–12) and the proportion of ticks infected with the Ap-ha strain.

The most commonly used method to distinguish the human pathogenic strain of *A. phagocytophilum* from those not associated with human infection is to sequence the 16S rRNA gene (3–7,13). The Ap-ha strain differs from the Ap-variant 1 strain by 2 nucleotide differences at the 5' end of the gene sequence (1,3). However, there is a need for an alternative to currently used PCR-based methods for strain identification that are reliable, but faster and more cost-effective. Therefore, the objectives of the current study were to determine the proportion of black-legged ticks infected with *A. phagocytophilum* in different geographic regions of

Canada, and to develop restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP) genotyping assays, targeting the 16S rRNA gene, to differentiate the Ap-ha strain from the Ap-variant 1 strain. We assessed the usefulness of these assays and determined the prevalence of the Ap-ha strain in *I. scapularis* ticks from different geographic regions of Canada.

The Study

We conducted real-time PCR analyses targeting the *msp2* gene on genomic (g) DNA of 12,606 *I. scapularis* ticks collected across Canada (online Technical Appendix 1, <http://wwwnc.cdc.gov/EID/article/20/12/14-0172-Techapp1.pdf>). Of these, 169 (1.3%) ticks were PCR-positive (Table 1). There were significant differences ($\chi^2_5 = 129.7$, $p < 0.001$) in the proportions of ticks infected with *A. phagocytophilum* among geographic regions; with a greater proportion of PCR-positive ticks in Manitoba than in Ontario, Quebec, and the Atlantic provinces.

We developed PCR-based assays to distinguish the Ap-ha strain from the Ap-variant 1 strain of *A. phagocytophilum* based on DNA sequence comparisons of the 16S rRNA gene of each strain (online Technical Appendix 1) over a much larger region (875 bp) than in previous studies (1,3). In addition to the 2 nucleotide differences described previously (1,3), a third difference (at position 536) was detected in the aligned sequences (online Technical Appendix 2, <http://wwwnc.cdc.gov/EID/article/20/12/14-0172-Techapp2.pdf>). This nucleotide alteration in the DNA sequence of the Ap-variant 1 strain was associated with a restriction site for the endonuclease *Kpn2I* (T/CC-GGA) that was absent in the sequence of the Ap-ha strain. Two PCR-RFLP assays (online Technical Appendix 1), developed at different laboratories, were designed on the basis of this sequence difference and tested on 22 amplicons derived from *A. phagocytophilum*-infected ticks collected in Minnesota, USA ($n = 17$) and Manitoba ($n = 5$). Identical results were obtained for both assays. Three different RFLP patterns were produced. Eighteen amplicons remained undigested (i.e., a single band of ≈ 920 bp), and 3 amplicons had 2 bands (≈ 360 and ≈ 550 bp), representing the expected patterns for the Ap-ha strain and Ap-variant 1 strain, respectively (Figure 1). These results were confirmed on the basis of comparisons of the DNA sequences of representative samples. The RFLP pattern of 1 amplicon, derived from the gDNA of a female tick from Itasca State Park, Minnesota, consisted of 3 bands (≈ 360 , ≈ 550 , and ≈ 920 bp) (online Technical Appendix 2), suggesting a mixture of the 2 strains. Additional analyses of another 125 amplicons from *A. phagocytophilum*-infected ticks revealed that 79 (63%) had RFLP patterns consistent with the Ap-variant 1 strain, and 46 (37%) had RFLP patterns of the Ap-ha strain. The DNA sequences of a subset of these samples ($n = 58$)

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Table 1. *Anaplasma phagocytophilum* PCR-positive black-legged ticks collected from various provinces during 2007–2010, Canada

Location	2007		2008		2009		2010		2007–2010	
	No. ticks	No. +	No. ticks	No. +	No. ticks	No. +	No. ticks	No. +	No. ticks	No. + (%)
Prairie Provinces										
Alberta*	9	0	25	0	13	0	42	3	89	3 (3.4)
Saskatchewan*	5	2	3	1	1	1	1	0	10	4 (40.0)
Manitoba	35	2	156	4	119	6	260	20	570	32 (5.6)
Central Canada										
Ontario	1,187	4	1,402	3	856	3	962	3	4,407	13 (0.3)
Quebec	982	13	1,687	26	1,026	8	1,002	24	4,697	71 (1.5)
Atlantic Provinces										
New Brunswick	129	1	174	0	189	4	271	10	763	15 (2.0)
Nova Scotia	201	2	394	4	378	4	676	9	1,649	19 (1.2)
Prince Edward Island	54	3	76	1	107	3	122	3	359	10 (2.8)
Newfoundland*	11	0	9	0	15	1	27	1	62	2 (3.2)
Total	2,613	27	3,926	39	2,704	30	3,363	73	12,606	169 (1.3)

*Data from these provinces were not included in the statistical analyses because sample sizes (2007–2010) were <100 ticks.

showed 100% agreement between RFLP pattern and strain type of *A. phagocytophilum* (i.e., 24 of the Ap-ha strain and 34 of the Ap-variant 1 strain).

We also designed a custom TaqMan SNP genotyping assay (<https://www.lifetechnologies.com/order/custom-genomic-products/tools/genotyping>) to differentiate the two *A. phagocytophilum* strains on the basis of a nucleotide difference at the 5' end of the 16S rRNA gene sequence (online Technical Appendix 1). The SNP assay clearly discriminated between ticks infected with the Ap-ha and/or Ap-variant 1 strains (Figure 2). Of the 142 amplicons tested by this assay, 82 (58%) contained the Ap-variant 1 strain, 59 (42%) contained the Ap-ha strain, and 1 contained a mixture of both strains, which was in 100% agreement with the results of the RFLP analyses and DNA sequencing. The results of the SNP analyses also revealed a significant difference ($\chi^2_2 = 40.48, p < 0.001$) in the proportions of *I. scapularis* ticks infected with the Ap-ha strain among geographic regions (Table 2). A smaller proportion of ticks from Central Provinces were infected with the Ap-ha strain when compared with those from the Prairie and Atlantic Provinces.

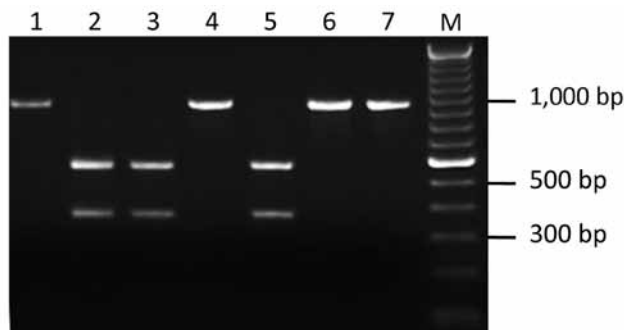


Figure 1. Restriction fragment length polymorphism patterns of 16S DNA for 7 *Anaplasma phagocytophilum* PCR-positive *Ixodes scapularis* ticks. All amplicons were produced by semi-nested PCR and digested with the restriction enzyme *Kpn2I*. Lane M, molecular mass marker.

Conclusions

A small proportion (1.3%) of *I. scapularis* ticks collected in Canada were infected with *A. phagocytophilum*. The prevalence of *A. phagocytophilum*-infected ticks differed among geographic regions, but the potential significance of this finding needs to be explored further. Although knowledge of the prevalence of *A. phagocytophilum*-infected *I. scapularis* ticks provides some information for determining the public health risks for HGA, the prevalence of the Ap-ha strain of *A. phagocytophilum* in black-legged

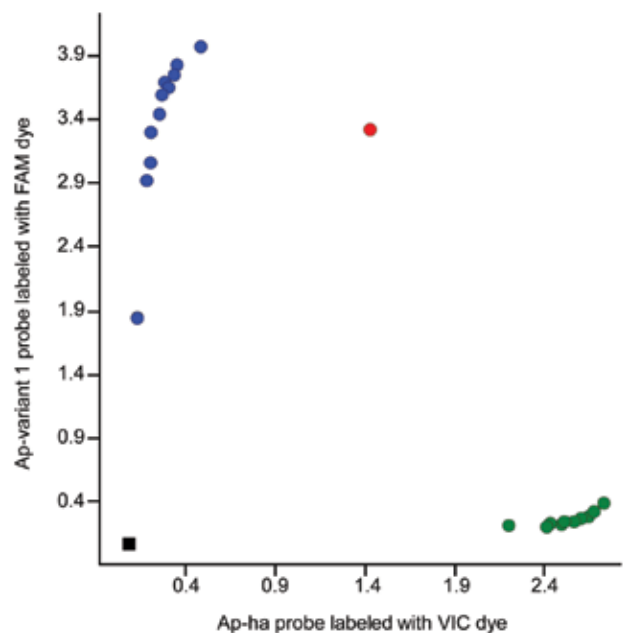


Figure 2. Allelic discrimination plot for the *Anaplasma* single nucleotide polymorphism assay based on the 16S RNA gene. Blue circles represent samples that contain the Ap-ha strain; green circles represent samples that contain the Ap-variant 1 strain. The red circle represents a sample containing a mixture of both strains. The black square represents the control (no template).

Table 2. Black-legged ticks infected with the Ap-ha or Ap-variant 1 strain of *Anaplasma phagocytophilum* based on analyses using the TaqMan SNP genotyping assay,* Canada

Province	No. ticks	Ap-ha (%)	Ap-variant 1 (%)
Prairie Provinces			
Alberta	1	1 (100)	0 (0)
Saskatchewan†	2	2 (100)	0 (0)
Manitoba	17	15 (88.2)	2 (11.8)
Central Canada			
Ontario	24†	2 (8.3)	22 (91.7)
Quebec	47	9 (19.1)	38 (80.9)
Atlantic Provinces			
Prince Edward Island	7	3 (42.9)	4 (57.1)
New Brunswick	10	6 (60.0)	4 (40.0)
Nova Scotia	17	8 (47.1)	9 (52.9)
Total	125	46 (36.8)	79 (63.2)

*<http://www.lifetechnologies.com/search/global/searchAction.action?query=SNP&resultPage=1&resultsPerPage=15&autocomplete>.

†Includes 13 ticks listed in Table 1 and an additional 11 A.

phagocytophilum-infected ticks collected by drag sampling at 2 sites in Ontario.

ticks should be considered for risk assessment, since the Ap-ha strain, and not the Ap-variant 1 strain, is most often associated with clinical cases of HGA (4).

For the current study, 3 PCR-based assays were developed to distinguish the Ap-ha strain from the Ap-variant 1 strain. DNA sequencing of representative samples confirmed the reliability of these assays. Each of the 3 assays detected the presence of both strains in the gDNA of a female tick. Mixed infections of both strains in *I. scapularis* ticks have been reported (4), but appear to be uncommon. Results of these assays also were 100% concordant with the results of 2 RFLP assays (developed at different laboratories) that were tested on the same gDNA samples. Similarly, there was total concordance in the identifications of the *A. phagocytophilum* strains present within 125 infected ticks collected across Canada by using the seminested PCR-RFLP assay and the TaqMan SNP genotyping assay. The results of these analyses revealed significant differences in the proportion of black-legged ticks infected with the Ap-ha strain among geographic regions ($p < 0.001$). The public health implications of these findings need to be examined in more detail, using the molecular assays developed in this study.

The TaqMan SNP genotyping assay is ideal for clinical and epidemiologic use where it may be essential to distinguish between the 2 strains of *A. phagocytophilum* in *I. scapularis* to assess the potential risk for human infection. However, in a clinical setting, it remains to be established how this assay would be incorporated into or supplement the current diagnostic approach for detecting *A. phagocytophilum* infections in humans. This test is less technically demanding and takes less time to perform than nested/semi-nested PCR-RFLPs and DNA sequencing analyses. Moreover, this method eliminates the need for postamplification manipulations and technical problems that are sometimes

associated with RFLP analyses of amplicons produced by nested PCR (14,15). Nonetheless, given the 100% concordance in the results of the different analytical methods, the PCR-RFLP assays provide a reliable and cost-effective approach for distinguishing the Ap-ha strain from the Ap-variant 1 strain of *A. phagocytophilum*. The PCR-RFLP assays will be particularly useful in research laboratories that lack the capacity to conduct real-time PCRs providing an independent and relatively inexpensive method to confirm the results of the SNP assay.

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Technical Appendix 1. Samples, DNA extraction and prevalence of *A. phagocytophilum* in *I. scapularis*

Samples, DNA extraction and prevalence of *A. phagocytophilum* in *I. scapularis*

Blacklegged ticks ($n=12,606$) were collected over four years (2007-2010) as part of a national passive tick surveillance program conducted in Canada by the National Microbiology Laboratory (NML) using the methods described previously (1). Adult *I. scapularis* were also collected by drag sampling: in 2008 from Itasca State Park, Camp Ripley and St. Croix State Park ($n=56$ per locality) in Minnesota (USA), in 2010 from within Pembina Valley Provincial Park ($n=46$), and along the Stanley Trail ($n=44$), in southern Manitoba, and in 2009 from the Wainfleet Bog Conservation area and St. Lawrence Islands National Park in southern Ontario ($n=11$). Total genomic (g) DNA was extracted from each tick at the NML or the University of Saskatchewan (U of S) using either the QIAamp DNA Mini Kit or DNeasy 96 Blood and Tissue Kit (QIAGEN, Ontario, Canada) as per the manufacturer's instructions, but with the modifications described previously (1,2). The gDNA of *A. phagocytophilum* was also extracted (1) from an equine isolate (MN-93) propagated in the HL-60 promyelocytic cell line (ATCC CCL-240). This sample was used as a positive control in PCR assays conducted at the NML.

The gDNA of all 12,606 *I. scapularis* from the passive surveillance program were screened by real-time PCR targeting the *msp2* gene to determine if they were infected with *A. phagocytophilum*. Real-time PCR was conducted using a 7500 or 7900HT SDS Real-time PCR System (Applied Biosystems; California, USA), and the conditions described by Courtney et al. (3). All PCR-positive samples were confirmed using a real-time PCR targeting the 16S rRNA gene of *A. phagocytophilum* (4). Contingency (χ^2) tests were used to determine if the relative number (i.e. percentage) of *A. phagocytophilum*-infected ticks differed among geographical regions.

Comparison of the 16S rRNA sequences of the Ap-ha and Ap-variant 1 strains

The DNA sequences of a part (870 bp) of the 16S rRNA gene were determined for *A. phagocytophilum* present in *I. scapularis* adults collected from Minnesota. These tick samples were selected because both the Ap-variant 1 and Ap-ha strains have been reported in *I. scapularis* from the Upper Midwest of the United States (5). Initially, the presence/absence of *A. phagocytophilum* in each tick was determined by targeting part of the bacterial 16S gene using nested (n) PCR. Primers EC12 (5'-TGATCCTGGCTCAGAACGAACG-3') and EC9 (5'-TACCTTGTTACGACTT-3') (6) were used in phase 1, while primers HGE1F (5'-GGATTATTCTTTATAGCTTGCT-3') and HGE3R (5'-TTCCGTTAAGAAGGATCTAATCTC-3') (7) were used phase 2 of the nPCR. All PCRs were conducted in 25 µl reaction volumes containing 3 mM MgCl₂, 200 µM of each dNTP, 25 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Bio-Rad, Ontario, Canada), and 1 µl of gDNA (for phase 1) or 1 µl of purified amplicon (for phase 2). Amplicons (10 µl) were purified by adding exonuclease I (3 U) (New England BioLabs, Ontario, Canada) and shrimp alkaline phosphatase (0.15 U) (Fermentas, Ontario, Canada) and PCR buffer (1 µl) prior to incubation at 37°C for 15 min, and 80°C for 15 min. PCRs were performed using the following conditions; for phase 1: 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min, and for phase 2: 95°C for 5 min, then 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Negative (i.e., no gDNA) controls were included in each set of reactions. The amplicons of seven of 17 PCR-positive samples were purified (as described above) and sequenced using primers HGE1F and HGE3R (in separate reactions). Sequencing was conducted at the National Research Council (Saskatoon) on an ABI 3730xl capillary sequencer using BigDye™ Terminator version 3.1 cycle sequencing kits (Applied Biosystems). The sequences of the two strains have been deposited in GenBank under the accession numbers HG916766 and HG916767. Sequence data were compared to the 16S rRNA sequences of *A. phagocytophilum* on GenBank using BLAST.

The analyses of the Blast searches revealed that the DNA sequences of five amplicons were 100% identical to the sequence of the 16S rRNA gene of the Ap-ha strain (accession no. U02521), whereas those of the other two amplicons differed at three nucleotide positions (alignment positions 3, 11, and 536) compared to the sequence of the Ap-ha strain (Table 1). The first 375 bp of these two amplicons were identical in sequence to the 16S rDNA sequence of the

Ap-variant 1 strain (accession no. AY193887); however, a comparison over the 870 bp was not possible since there is no sequence data available for this part of the gene for the Ap-variant 1 strain.

PCR-RFLP

Data analyses using the program Webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2>) revealed that the nucleotide alteration in the DNA sequence of the Ap-variant 1 strain was associated with a restriction site for the endonuclease *Kpn2I* (T/CCGGA) that was absent in the sequence of the Ap-ha strain. A nested PCR-RFLP assay, developed (at the U of S) based upon this restriction site was tested on the *A. phagocytophilum*-positive amplicons derived from the ticks collected in Minnesota. RFLP digests were performed in 30 µl volumes containing 15.5 µl of nuclease free H₂O, 2.5 µl of the restriction enzyme *Kpn2I* (FastDigest®, Fermentas), 2 µl of 10x FastDigest® Green Buffer and 10 µl of unpurified PCR product from the second phase of the nested PCR (described above). Digests were performed at 37°C for 2 hrs and then at 80°C for 15 mins prior to loading (10 µl of product) on 1% agarose-TBE gels that were subjected to electrophoresis (100 volts for 2 hrs). A nested PCR was then conducted on the total gDNA of the 90 ticks collected from southern Manitoba, and the RFLP assay applied to all PCR-positive samples. The undigested products of three PCR-positive samples were purified and sequenced using primers HGE1F and HGE3R (in separate reactions) to confirm that there was 100% concordance between RFLP patterns and stain type of *A. phagocytophilum*.

A second PCR-RFLP assay was developed and tested (at NML) using amplicons of the bacterial 16S gene produced by semi-nested PCR. This modification was introduced because the semi-nested PCR produced better quality amplicons from a few gDNA samples compared to those produced by nested PCR. In the first phase of the semi-nested PCR, part (932 bp) of the 16S gene was amplified using primers Ge3a (5'-CACATGCAAGTCGAACGGATTATTC-3') and Ge10r (5'-TTCCGTTAAGAAGGATCTAATCTCC-3') and the cycling conditions of Massung et al. (8). Then, a smaller fragment (919 bp) was amplified using primers Ge9f (5'-AACGGATTATTCTTTATAGCTTGCT-3') (8) and Ge10r, and the same cycling conditions as for phase 1, except that 30 cycles were used, and the initial denaturation period was 5 min. PCRs were conducted in 50 µl reaction volumes containing 3 mM MgCl₂, 200 µM of each dNTP, 0.2 µM of each primer, 2.5 U of *iTaq* DNA polymerase (Bio-Rad), and 3 µl of gDNA (for phase 1)

or 2 µl of unpurified amplicon (for phase 2). The 919 bp amplicons were digested with *Kpn2I* as described above. This PCR-RFLP assay was tested on the gDNA of 114 of the 169 ticks from the passive surveillance program that were positive for *A. phagocytophilum* (i.e., by *msh2* and 16S rRNA gene real-time PCRs) and 11 *A. phagocytophilum*-infected ticks collected by drag sampling at two sites in Ontario. These 125 gDNA samples were selected based on their cycle threshold (Ct) value in the *msh2* real-time PCR assay; all samples had Ct values ≤ 35 . A subset ($n=58$) of undigested amplicons were purified using Montage filter units (Millipore) and sequenced using primers Ge9f and Ge10r to confirm 100% concordance between RFLP pattern and strain type of *A. phagocytophilum*. The gDNA of all *A. phagocytophilum*-positive ticks from Minnesota previously tested by nested PCR-RFLP at U of S were also subjected to the semi-nested PCR-RFLP assay (at NML) to test for concordance. Sequencing was conducted on an ABI 3130xl Genetic Analyzer using BigDye™ Terminator version 3.1 cycle sequencing kits (Applied Biosystems). Sequence data was analyzed using DNASTAR Lasergene 9 Software and compared to those on GenBank using BLAST.

SNP assay

A custom TaqMan® SNP Genotyping Assay (Applied Biosystems, California, USA) was designed to determine the *A. phagocytophilum* strains present in the gDNA of 125 *I. scapularis* from the passive surveillance program. These representative samples were all confirmed to be positive for *A. phagocytophilum* by real-time PCR (*msh2* and the 16S gene), and typed as either Ap-ha or Ap-variant 1 strain by PCR-RFLP. They also covered the geographical range of the tick submissions, different collection years, and a range of Ct values. The 17 *A. phagocytophilum*-infected ticks from Minnesota were also used to validate the SNP assay. The SNP Genotyping Assay mix contained two primers (Ap Forward: 5'-ACATGCAAGTCGAACGGATTATTCT-3' and Ap Reverse: 5'-GCTATCCCATACTACTAGGTAGATTCCT-3') that flank the region (50 bp) containing two SNP sites (alignment positions 3 and 11; Table 2), and two TaqMan probes, one that matched the Ap-ha strain (Ap-ha probe: 5'-CTGCCACTAACTATTCT-3') labelled with VIC, the other designed for the Ap-variant 1 strain (Ap-var 1 probe: 5'-CTGCCACTAATTATTCT-3') labelled with 6-carboxy-fluorescein (FAM) and a non-fluorescent quencher with a minor groove binder at the 3' end. Reaction mixtures (25 µl) contained 1.25 µl of 20X Custom TaqMan® SNP Genotyping Assay, 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 6.25 µl nuclease-free water and 5 µl of gDNA (or

water for the negative controls). Real-time PCRs were performed in a 7500 Real-time PCR System (Applied Biosystems) using the following amplification conditions; 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Strain assignment was performed using the AB 7500 version 2.0.5 software. A strain discrimination plot was generated using the R_N (fluorescence) from the Ap-ha probe versus the R_N from the Ap-var 1 probe. Contingency (χ^2) tests were used to determine if the proportions of ticks infected with the Ap-ha and Ap-variant 1 strains differed among geographical regions.

Extracts containing DNA from *Borrelia burgdorferi*, *B. miyamotoi*, *Ehrlichia chaffeensis* and *Babesia microti* were used to test the specificity of the SNP assay. In each case, detectable amplification products were not produced for further genotyping. In order to determine the limit of detection, serial dilutions of DNA from *A. phagocytophilum* propagated in HL-60 cells were used to generate a standard curve. The dynamic range of the assay spanned from 90 ng/ μ l DNA to 28 pg/ μ l DNA which corresponded to Ct values of 28 and 37, respectively.

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Two *Anaplasma phagocytophilum* Strains in *Ixodes scapularis* Ticks, Canada

Technical Appendix 1. Samples, DNA extraction and prevalence of *A. phagocytophilum* in *I. scapularis*

Samples, DNA extraction and prevalence of *A. phagocytophilum* in *I. scapularis*

Blacklegged ticks ($n=12,606$) were collected over four years (2007-2010) as part of a national passive tick surveillance program conducted in Canada by the National Microbiology Laboratory (NML) using the methods described previously (1). Adult *I. scapularis* were also collected by drag sampling: in 2008 from Itasca State Park, Camp Ripley and St. Croix State Park ($n=56$ per locality) in Minnesota (USA), in 2010 from within Pembina Valley Provincial Park ($n=46$), and along the Stanley Trail ($n=44$), in southern Manitoba, and in 2009 from the Wainfleet Bog Conservation area and St. Lawrence Islands National Park in southern Ontario ($n=11$). Total genomic (g) DNA was extracted from each tick at the NML or the University of Saskatchewan (U of S) using either the QIAamp DNA Mini Kit or DNeasy 96 Blood and Tissue Kit (QIAGEN, Ontario, Canada) as per the manufacturer's instructions, but with the modifications described previously (1,2). The gDNA of *A. phagocytophilum* was also extracted (1) from an equine isolate (MN-93) propagated in the HL-60 promyelocytic cell line (ATCC CCL-240). This sample was used as a positive control in PCR assays conducted at the NML.

The gDNA of all 12,606 *I. scapularis* from the passive surveillance program were screened by real-time PCR targeting the *msp2* gene to determine if they were infected with *A. phagocytophilum*. Real-time PCR was conducted using a 7500 or 7900HT SDS Real-time PCR System (Applied Biosystems; California, USA), and the conditions described by Courtney et al. (3). All PCR-positive samples were confirmed using a real-time PCR targeting the 16S rRNA gene of *A. phagocytophilum* (4). Contingency (χ^2) tests were used to determine if the relative number (i.e. percentage) of *A. phagocytophilum*-infected ticks differed among geographical regions.

Comparison of the 16S rRNA sequences of the Ap-ha and Ap-variant 1 strains

The DNA sequences of a part (870 bp) of the 16S rRNA gene were determined for *A. phagocytophilum* present in *I. scapularis* adults collected from Minnesota. These tick samples were selected because both the Ap-variant 1 and Ap-ha strains have been reported in *I. scapularis* from the Upper Midwest of the United States (5). Initially, the presence/absence of *A. phagocytophilum* in each tick was determined by targeting part of the bacterial 16S gene using nested (n) PCR. Primers EC12 (5'-TGATCCTGGCTCAGAACGAACG-3') and EC9 (5'-TACCTTGTTACGACTT-3') (6) were used in phase 1, while primers HGE1F (5'-GGATTATTCTTTATAGCTTGCT-3') and HGE3R (5'-TTCCGTTAAGAAGGATCTAATCTC-3') (7) were used phase 2 of the nPCR. All PCRs were conducted in 25 µl reaction volumes containing 3 mM MgCl₂, 200 µM of each dNTP, 25 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Bio-Rad, Ontario, Canada), and 1 µl of gDNA (for phase 1) or 1 µl of purified amplicon (for phase 2). Amplicons (10 µl) were purified by adding exonuclease I (3 U) (New England BioLabs, Ontario, Canada) and shrimp alkaline phosphatase (0.15 U) (Fermentas, Ontario, Canada) and PCR buffer (1 µl) prior to incubation at 37°C for 15 min, and 80°C for 15 min. PCRs were performed using the following conditions; for phase 1: 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min, and for phase 2: 95°C for 5 min, then 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Negative (i.e., no gDNA) controls were included in each set of reactions. The amplicons of seven of 17 PCR-positive samples were purified (as described above) and sequenced using primers HGE1F and HGE3R (in separate reactions). Sequencing was conducted at the National Research Council (Saskatoon) on an ABI 3730xl capillary sequencer using BigDye™ Terminator version 3.1 cycle sequencing kits (Applied Biosystems). The sequences of the two strains have been deposited in GenBank under the accession numbers HG916766 and HG916767. Sequence data were compared to the 16S rRNA sequences of *A. phagocytophilum* on GenBank using BLAST.

The analyses of the Blast searches revealed that the DNA sequences of five amplicons were 100% identical to the sequence of the 16S rRNA gene of the Ap-ha strain (accession no. U02521), whereas those of the other two amplicons differed at three nucleotide positions (alignment positions 3, 11, and 536) compared to the sequence of the Ap-ha strain (Table 1). The first 375 bp of these two amplicons were identical in sequence to the 16S rDNA sequence of the

Ap-variant 1 strain (accession no. AY193887); however, a comparison over the 870 bp was not possible since there is no sequence data available for this part of the gene for the Ap-variant 1 strain.

PCR-RFLP

Data analyses using the program Webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2>) revealed that the nucleotide alteration in the DNA sequence of the Ap-variant 1 strain was associated with a restriction site for the endonuclease *Kpn2I* (T/CCGGA) that was absent in the sequence of the Ap-ha strain. A nested PCR-RFLP assay, developed (at the U of S) based upon this restriction site was tested on the *A. phagocytophilum*-positive amplicons derived from the ticks collected in Minnesota. RFLP digests were performed in 30 µl volumes containing 15.5 µl of nuclease free H₂O, 2.5 µl of the restriction enzyme *Kpn2I* (FastDigest®, Fermentas), 2 µl of 10x FastDigest® Green Buffer and 10 µl of unpurified PCR product from the second phase of the nested PCR (described above). Digests were performed at 37°C for 2 hrs and then at 80°C for 15 mins prior to loading (10 µl of product) on 1% agarose-TBE gels that were subjected to electrophoresis (100 volts for 2 hrs). A nested PCR was then conducted on the total gDNA of the 90 ticks collected from southern Manitoba, and the RFLP assay applied to all PCR-positive samples. The undigested products of three PCR-positive samples were purified and sequenced using primers HGE1F and HGE3R (in separate reactions) to confirm that there was 100% concordance between RFLP patterns and stain type of *A. phagocytophilum*.

A second PCR-RFLP assay was developed and tested (at NML) using amplicons of the bacterial 16S gene produced by semi-nested PCR. This modification was introduced because the semi-nested PCR produced better quality amplicons from a few gDNA samples compared to those produced by nested PCR. In the first phase of the semi-nested PCR, part (932 bp) of the 16S gene was amplified using primers Ge3a (5'-CACATGCAAGTCGAACGGATTATTC-3') and Ge10r (5'-TTCCGTTAAGAAGGATCTAATCTCC-3') and the cycling conditions of Massung et al. (8). Then, a smaller fragment (919 bp) was amplified using primers Ge9f (5'-AACGGATTATTCTTTATAGCTTGCT-3') (8) and Ge10r, and the same cycling conditions as for phase 1, except that 30 cycles were used, and the initial denaturation period was 5 min. PCRs were conducted in 50 µl reaction volumes containing 3 mM MgCl₂, 200 µM of each dNTP, 0.2 µM of each primer, 2.5 U of *iTaq* DNA polymerase (Bio-Rad), and 3 µl of gDNA (for phase 1)

or 2 µl of unpurified amplicon (for phase 2). The 919 bp amplicons were digested with *Kpn2I* as described above. This PCR-RFLP assay was tested on the gDNA of 114 of the 169 ticks from the passive surveillance program that were positive for *A. phagocytophilum* (i.e., by *msh2* and 16S rRNA gene real-time PCRs) and 11 *A. phagocytophilum*-infected ticks collected by drag sampling at two sites in Ontario. These 125 gDNA samples were selected based on their cycle threshold (Ct) value in the *msh2* real-time PCR assay; all samples had Ct values ≤ 35 . A subset ($n=58$) of undigested amplicons were purified using Montage filter units (Millipore) and sequenced using primers Ge9f and Ge10r to confirm 100% concordance between RFLP pattern and strain type of *A. phagocytophilum*. The gDNA of all *A. phagocytophilum*-positive ticks from Minnesota previously tested by nested PCR-RFLP at U of S were also subjected to the semi-nested PCR-RFLP assay (at NML) to test for concordance. Sequencing was conducted on an ABI 3130xl Genetic Analyzer using BigDye™ Terminator version 3.1 cycle sequencing kits (Applied Biosystems). Sequence data was analyzed using DNASTAR Lasergene 9 Software and compared to those on GenBank using BLAST.

SNP assay

A custom TaqMan® SNP Genotyping Assay (Applied Biosystems, California, USA) was designed to determine the *A. phagocytophilum* strains present in the gDNA of 125 *I. scapularis* from the passive surveillance program. These representative samples were all confirmed to be positive for *A. phagocytophilum* by real-time PCR (*msh2* and the 16S gene), and typed as either Ap-ha or Ap-variant 1 strain by PCR-RFLP. They also covered the geographical range of the tick submissions, different collection years, and a range of Ct values. The 17 *A. phagocytophilum*-infected ticks from Minnesota were also used to validate the SNP assay. The SNP Genotyping Assay mix contained two primers (Ap Forward: 5'-ACATGCAAGTCGAACGGATTATTCT-3' and Ap Reverse: 5'-GCTATCCCATACTACTAGGTAGATTCCT-3') that flank the region (50 bp) containing two SNP sites (alignment positions 3 and 11; Table 2), and two TaqMan probes, one that matched the Ap-ha strain (Ap-ha probe: 5'-CTGCCACTAACTATTCT-3') labelled with VIC, the other designed for the Ap-variant 1 strain (Ap-var 1 probe: 5'-CTGCCACTAATTATTCT-3') labelled with 6-carboxy-fluorescein (FAM) and a non-fluorescent quencher with a minor groove binder at the 3' end. Reaction mixtures (25 µl) contained 1.25 µl of 20X Custom TaqMan® SNP Genotyping Assay, 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 6.25 µl nuclease-free water and 5 µl of gDNA (or

water for the negative controls). Real-time PCRs were performed in a 7500 Real-time PCR System (Applied Biosystems) using the following amplification conditions; 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Strain assignment was performed using the AB 7500 version 2.0.5 software. A strain discrimination plot was generated using the R_N (fluorescence) from the Ap-ha probe versus the R_N from the Ap-var 1 probe. Contingency (χ^2) tests were used to determine if the proportions of ticks infected with the Ap-ha and Ap-variant 1 strains differed among geographical regions.

Extracts containing DNA from *Borrelia burgdorferi*, *B. miyamotoi*, *Ehrlichia chaffeensis* and *Babesia microti* were used to test the specificity of the SNP assay. In each case, detectable amplification products were not produced for further genotyping. In order to determine the limit of detection, serial dilutions of DNA from *A. phagocytophilum* propagated in HL-60 cells were used to generate a standard curve. The dynamic range of the assay spanned from 90 ng/ μ l DNA to 28 pg/ μ l DNA which corresponded to Ct values of 28 and 37, respectively.

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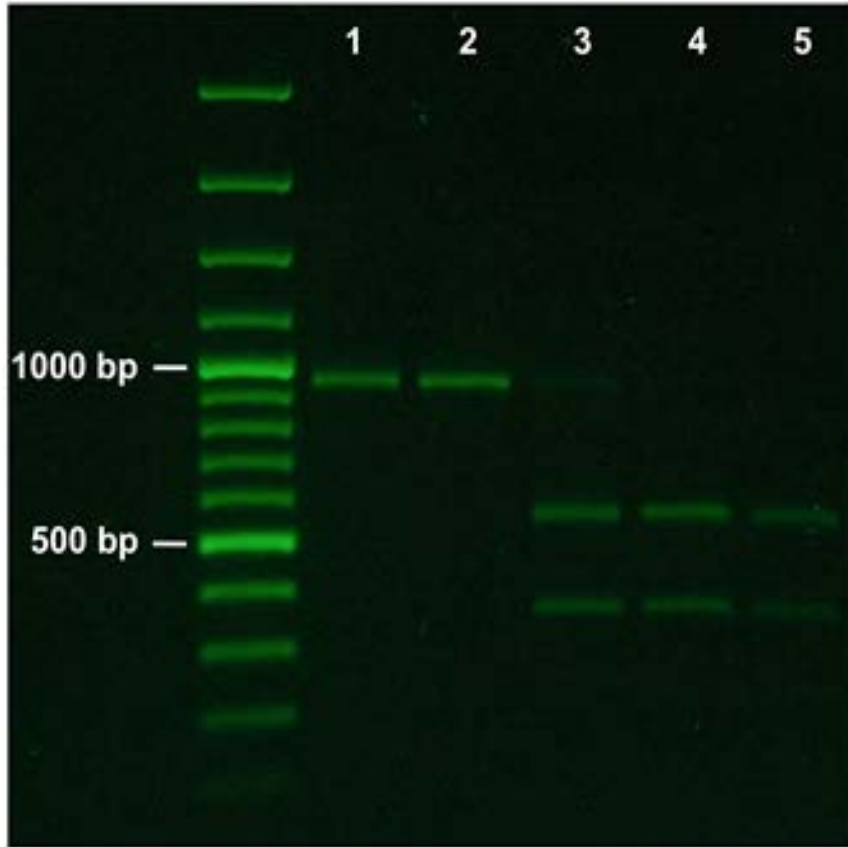
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Technical Appendix 2 Table. Variable positions in the aligned 16S rRNA gene sequences of representative samples of *A. phagocytophilum* from the genomic DNA of 7 *I. scapularis* (CS-F-21, CR-M-7, CS-F-23, CS-F-24, CS-M-23, IS-F-4 & IS-M-2) compared with the sequences of the Ap-variant 1 and Ap-ha strains of *A. phagocytophilum* from GenBank (AY193887 and U02521)

Strain	Alignment position		
	3	11	536
Ap-variant 1 strain (AY193887)	G	A	*
Ticks CS-F-21 & CR-M-7	G	A	A
Ap-ha strain (U02521)	A	G	G
Ticks CS-F-23, CS-F-24, CS-M-23, IS-F-4 & IS-M-2	A	G	G

*Unknown nucleotide.



Technical Appendix 2 Figure. RFLP patterns of the 16S ribosomal DNA for 5 *A. phagocytophilum* PCR-positive *I. scapularis*. Amplicons were produced by nested PCR. Note the sample in lane 3 is a mixture of 2 strains.