Comparison of a Real-time PCR Method with Serology and Blood Smear Analysis for Diagnosis of Human Anaplasmosis: Importance of Infection Time Course for Optimal Test Utilization

Running title: Laboratory diagnosis of Human Anaplasmosis

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Abstract

Anaplasmosis and ehrlichiosis are emerging tick-borne diseases with clinically similar presentations, caused by closely related pathogens. Currently, laboratory diagnoses rely predominantly on blood smear analysis for detection of intracellular morulae and serologic tests, both of which have recognized limitations. We compared the performance of a published real-time PCR assay that incorporates melt-curve analysis for differentiation of Anaplasma and Ehrlichia species with blood smear and serologic methods in an upper Midwest population. Overall, 38.5% of the specimens selected for evaluation, had one or more tests positive for anaplasmosis. PCR positivity for all specimens was maximal (21.2%; 29/137) during the early acute phase of illness (0-4 days since illness onset) and significantly less frequent (11.5%, 20/174) during later phases (> 4 days since illness onset). All positive specimens were A. phagocytophilum; no Ehrlichia species were identified. The real-time PCR detected 100% of infections that were detected by blood smear analysis (14/14) and broadened the detection window from a maximum of 14 days for smear positivity to 30 days for PCR. Additional infections were detected by real-time PCR in 12.9% (11/85) of smear-negative patients. There was poor agreement between the real-time PCR assay and serologic test results: 19.8% (19/96) and 13.7% (29/212) of seropositive and -negative patients, respectively, were PCR positive. Seropositivity increased with days of illness, demonstrating that serologic detection methods are best utilized during presumed convalescence. Our results indicated that optimal performance and utilization of laboratory tests for diagnosis of anaplasmosis requires knowledge regarding symptom onset or days of illness.
Members of the genera *Anaplasma* and *Ehrlichia* (order Rickettsiales, family Anaplasmataceae) are obligate intracellular α-proteobacteria that are transmitted to vertebrate hosts by ticks of the family Ixodidae and cause clinically similar febrile diseases in humans and domestic animals. In the United States most human infections are caused by *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, and produce the nationally reportable diseases referred to as human anaplasmosis (human granulocytic anaplasmosis) and ehrlichiosis (human monocytic ehrlichiosis), respectively (18, 21). Although some differences in clinical presentation have been reported for the diseases, they are largely indistinguishable.

Symptoms may be mild to severe and frequently include fever, chills, headache, myalgia, nausea and cough, with laboratory features of leukopenia, thrombocytopenia, and elevated hepatic transaminases (21, 32). Differences in disease presentation include rashes frequently being reported in ehrlichiosis, but rarely for anaplasmosis patients (14, 18). Ehrlichiosis may also have higher rates of severe manifestations including disseminated intravascular coagulopathy and meningoencephalitis (18).

Although most anaplasmosis and ehrlichiosis patients resolve their infections, mortality occurs in 1-2% of cases (18). In addition, although anaplasmosis and ehrlichiosis are both effectively treated with doxycycline and tetracycline antibiotics, not all antibiotics are successful at treating both infections (24, 32).

Given the non-specific and similar presentations of the illnesses associated with *A. phagocytophilum* and *E. chaffeensis*, diagnostic tests that reliably detect and differentiate infections are needed to adequately diagnose patients and deliver prompt, appropriate treatment. At present, serologic diagnostic methods are most frequently used, but these tests have been shown to have poor sensitivity during the acute phases of infection (< 1 week since symptom onset) when tests are most likely to be performed (7). A single positive titer result also is unable to distinguish between current infection and evidence of previous exposure to these pathogens, as IgG antibodies may persist in patients for several years (4, 32, 34). Thus, evidence of seroconversion or a four-fold increase in titers between acute and convalescent serologic testing is recommended for confirming diagnoses (12); however, physicians rarely test a patient twice (18). Moreover, serologic tests developed for one specific agent (e.g., *A. phagocytophilum*) may not cross-react with human ehrlichioses.
phagocytophilum) may be unable to detect infections with the other closely related species, thereby
limiting the ability to adequately identify infections in areas where the pathogens overlap in distribution or
where they may be just emerging. Conversely, cross-reactivity between serologic tests for A.
phagocytophilum and E. chaffeensis has been demonstrated in about 15% of patients, suggesting testing
for both pathogens should be performed in geographic areas where both pathogens may be present to
differentiate between the diseases (16, 17).

Detection of the intracellular clusters of bacteria, called morulae, microscopically via blood smear
analyses is also possible and may provide presumptive differentiation of anaplasmosis versus ehrlichiosis
infections, as A. phagocytophilum preferentially infects neutrophils, whereas E. chaffeensis infects
monocytes (21, 32). Another pathogen closely related to E. chaffeensis, E. ewingii, may also cause
infections in susceptible humans, but rather than infect monocytes this Ehrlichia species infects
granulocytes (11). In geographic regions where A. phagocytophilum, E. chaffeensis, and E. ewingii co-
extist (e.g., the central Midwest and southeastern United States; [9, 18]), visualization of morulae in
granulocytes would be insufficient for differentiation of A. phagocytophilum and E. ewingii infections.
Blood smear analysis also has poor sensitivity due to the transient nature of the bacteremia and provides
only supportive evidence of infection because of the potential for misinterpreting toxic granulation, various
cytoplasmic inclusions and staining artifacts on slides as bacterial morulae (4, 12, 32).

The use of PCR-based diagnostic tests for detection of these infections offers several advantages
over the traditional serologic and blood smear tests. PCR tests have sensitivity and specificity rates that
approach 100% (20), they tend to have a higher degree of sensitivity during the acute phase of illness (7,
22), and they have the potential to detect co-infections simultaneously when configured in multiplexed
reactions (20). A real-time assay developed by Bell and Patel (10), in particular, represents an attractive
alternative to serologic and blood smear analyses in clinical diagnosis because of its ability to detect and
differentiate at least 4 species, including A. phagocytophilum and E. chaffeensis, in a single reaction
assay. Therefore, in areas where the distribution of these organisms may overlap, use of this test can be
expected to improve time to diagnosis with greater accuracy. Analytical sensitivity of the test was
demonstrated to be high, with the ability to detect between 5 and 10 copies of E. chaffeensis or A.
phagocytophilum target DNA, respectively; however, evaluation of this test in detecting infections in
clinical samples was limited to a comparison of the real-time PCR test results with a conventional PCR method (10). Here we report on the performance of the real-time PCR assay compared to serologic and blood smear methods in detecting infections in clinical samples obtained from patients within an area of high *A. phagocytophilum* incidence rates in the upper Midwest.

**MATERIALS AND METHODS**

**Patient specimens.** Remainder EDTA-preserved and serum blood samples were collected from patients for whom diagnostic tests (blood smear analysis, serology) for human anaplasmosis were ordered between April and December 2011 by health-care providers at a largely rural healthcare system in central and northern Wisconsin, an area known to be endemic for *A. phagocytophilum* (15, 18). Upon completion of routine testing, specimens were aliquoted into 1.5 ml cryovial tubes and frozen at -80°C. Chart reviews, approved by the Institutional Review Board of the Marshfield Clinic, were performed and clinical data abstracted including patient age, gender, date of symptom onset, and the signs and symptoms associated with patient illness. The CDC case definition for human anaplasmosis (12) was used as a guide to categorize patients into those with clinically compatible and incompatible signs and symptoms for anaplasmosis. Patients (n=311) that presented with at least two of the following: fever, chills and/or sweats, headache, myalgia, anemia, leukopenia, thrombocytopenia, or elevated hepatic transaminases were considered to be cases consistent with anaplasmosis. An additional 50 control patients with symptoms inconsistent with anaplasmosis were also included to evaluate the specificity of the assay; these patients either reported no symptoms or reported fatigue, myalgia or joint pain without evidence of fever. In total, specimens from 361 patients were tested using the real-time PCR assay.

When available, results from physician ordered serologic and blood smear tests were also recorded for patients. The serologic test used was an indirect immunofluorescence-antibody assay (IFA) that detected IgG antibodies specific for *A. phagocytophilum* using a polyvalent anti-human conjugate, with a titer ≥1:64 considered positive (Focus Diagnostics, Cypress, CA). Blood smear slides were made from whole blood, stained by the Wright’s method and examined microscopically under oil immersion for leukocytic intracellular morulae by trained clinical staff.
Although CDC guidelines require a four-fold rise in titers between acute and convalescent serologic tests for a confirmed case of anaplasmosis (13), only 1.6% of our patients were tested more than once by physicians, therefore, we considered patients with a positive serologic test as positive for anaplasmosis, but recognizing that a single positive titer cannot be considered sufficient evidence of current infection. Therefore, in this study, we did not have a true reference method that the performance of the PCR assay and blood smear analysis could be compared. By limiting our analysis to patients with clinical symptoms compatible with anaplasmosis, however, the positive predictive value of the serologic test may be elevated, and thus, errors comparing the serologic test with the PCR assay or blood smear analysis may be reduced.

**DNA extraction.** Frozen blood samples were thawed at 37°C, and DNA was extracted from 200 µl using the QIAamp DNA blood minikit (Qiagen, Valencia, CA). DNA was then eluted into 200 µl in AE buffer as recommended by the manufacturer.

**Real-time PCR.** The extracted DNA was amplified by using a previously described real-time PCR (10). This assay was designed to amplify and detect a segment of the heat shock protein operon groEL in three closely related, target organisms: *A. phagocytophilum*, *E. chaffeensis*, and *E. ewingii*. Recently, the assay also was demonstrated to detect a novel *Ehrlichia* agent identified in Minnesota and Wisconsin patients (28). The assay incorporates the primers ESp-F (5'-TACTCAGAGTGCTTCTCAATGT-3') and ESp-R (5'-GCATACCATCAGTTTTTTCAAC-3') and fluorescence resonance energy transfer (FRET)-labeled probes. A single acceptor probe (ESp-RD) hybridizes to the amplified DNA of all 4 species. Two separate donor probes were designed to allow for differentiation of the organisms. Specifically, donor probe Aph-FL hybridizes to amplified product from *A. phagocytophilum* and donor probe Ec/e-FL hybridizes to amplified product from *E. chaffeensis*, *E. ewingii*, and the new *Ehrlichia* sp. Wisconsin. Mismatches in the sequences of the corresponding donor probe regions of the 4 organisms permit their differentiation using melting curve analysis (10, 28).

For the assay, 5 µl of extracted DNA was added to 15 µl of master mix that contained 10 µl of 2x Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 0.4 µl of 50 mM MgCl₂, 0.5 µl of each of the primers, 0.8 µl of the acceptor probe, 0.4 µl of each of the donor probes, and 2 µl of sterile water. DNA was then amplified using a 480 LightCycler instrument (Roche Applied Sciences) under the
following conditions: 1 cycle for UNG treatment at 50°C for 2 min (4.4°C/s) and 95°C for 3 min (4.4°C/s),
followed by 45 amplification cycles of 95°C for 10 s (4.4°C/s), 55°C for 25 s (2.2°C/s), and 72°C for 22 s (4.4°C/s), a single melting curve cycle of 95°C for 1 min (4.4°C/s), 40°C for 2 min (1.0°C/s), and 80°C for 0 s (0.11°C/s), and a single cooling cycle of 40°C for 30 s (1.5°C/s). A negative control of sterile water and 2 positive controls, one containing pooled, positive plasmid DNA for *A. phagocytophilum*, *E. chaffeensis*, and *E. ewingii* and a second containing positive plasmid DNA for only *Ehrlichia* sp. Wisconsin, were included in each run of the assay. The positive controls were generated by inserting amplified products from each species into plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA). We confirmed analytical sensitivity of the assay by testing dilutions of 5 to 1 x 10^6 copies of each positive control and obtained detection limits consistent with those described previously (10).

**Statistical methods.** Only patients that had an illness that was clinically compatible with the anaplasmosis case definition were included in statistical analyses. Categorical descriptive variables were compared between PCR-positive and PCR-negative patients using 2x2 contingency tables, whereas continuous variables were compared between the groups using Wilcoxon rank sum tests. Test outcomes were also compared across time intervals termed early acute and later phases (≤ 4 days versus > 4 days since illness onset, respectively), and agreement among the PCR, serologic and blood smear tests were evaluated using sets of 2x2 contingency tables and the kappa statistic (29). A p-value of ≤ 0.05 was considered significant.

**RESULTS**

**Detection of *A. phagocytophilum* and *Ehrlichia* spp. in clinical samples.** Of the total 361 patient specimens that were included in our evaluation of the real-time PCR assay, a positive PCR result for *A. phagocytophilum* was obtained in 49 (13.6%) patients. An additional 90 patients were negative by the PCR, but were seropositive for *A. phagocytophilum*; this gave an overall positivity rate of 38.5% in our sample population. Positive blood smear results were only found in patients that also had either a positive PCR or serologic test result. Among the patients that had serologic test results (n=358), 109
(30.4%) were seropositive; blood smear analyses were positive in 14 of 106 (13.2%) patients with blood smear results. No infections with Ehrlichia spp. were detected in our study.

**Demographic and clinical characteristics of PCR-positive patients.** Only patients with clinical signs and symptoms compatible with anaplasmosis were PCR-positive. Moreover, the majority of PCR-positive patients (48 of 49) had symptoms consistent with the CDC case definition for anaplasmosis (12). One PCR-positive patient reported headache and myalgia, consistent with anaplasmosis, but failed to report the occurrence of fever, chills, or sweats, and had no evidence of fever upon presentation at the clinic, although the patient was leukopenic and thrombocytopenic, and had elevated liver enzymes, consistent with anaplasmosis.

Comparison of clinical characteristics of PCR-positive and PCR-negative patients indicated that positive patients tended to be male, in the acute phase of infection (≤1 week since onset of symptoms), and required hospitalization. They were also more likely to present with fever, sweats, or chills, urinary tract-related complaints, leukopenia, thrombocytopenia, and elevated liver enzymes, and to have reported tick exposure (Table 1). Although being 50 years or older was not associated with being PCR-positive (Table 1), PCR-positive patients did tend to be slightly older (Wilcoxon rank sum test, p = 0.029), with their median (range) age of 57.8 (13.7-85.8) versus 53.7 (2.2-96.1) for PCR-negative patients.

Twelve of our 361 patients tested for anaplasmosis/ehrlichiosis using the PCR assay had evidence of Lyme disease exposure; eight of these patients could be considered seropositive based on the Lyme disease CDC two-tier testing algorithm (13) and 6 patients had a physician diagnosed erythema migrans rash. Four of the seropositive Lyme disease patients were included in the 49 PCR-positive A. phagocytophilum patients identified in our study, suggesting these patients had evidence of either co-infection or were recently sequentially infected with both A. phagocytophilum and Borrelia burgdorferi.

Two other patients that were PCR-positive for A. phagocytophilum also had antibody titers consistent with Babesia microti exposure. Because these patients had symptoms consistent with our inclusion criteria, and seropositivity does not necessarily mean patients were currently infected with more than one tick-borne disease, they were retained in our analysis.

**Comparison of positivity among detection methods in anaplasmosis compatibly-ill patients.** Of the patients with clinical symptoms compatible with anaplasmosis, the real-time PCR assay detected A.
phagocytophilum infections in patients that had reported being ill beginning on the date of clinic visit (days since illness onset = 0) through one month prior to clinic visit (days since illness onset = 30) (Figure 1a). The median number of days PCR-positive patients reported being ill prior to testing was 4 days. This was in contrast to blood smear positive patients that had a range of detection between days 2 and 14 post-illness onset (Figure 1b), although the median number of days ill for blood smear-positive patients was also 4 days. Serology-positive patients reported being ill between 0 and 81 days prior to testing (Figure 1c), with a median number of days ill of 6. Based on these results, we constructed two groups to compare the abilities of the 3 detection methods in identifying anaplasmosis-positive patients: patients tested in the early acute phase of infection (0-4 days since illness onset) and patients tested in the later phase of infection (> 4 days since illness onset).

Timing of testing was significantly associated with a PCR-positive test results. Patients tested in the early acute phase of infection were more likely to be PCR-positive than patients tested later (29 of 137 [21.2%] versus 20 of 174 [11.5%], $\chi^2 = 7.26, p = 0.020$). Positivity for blood smear analyses was also higher in the early acute phase of illness, although patients tested during this phase were not significantly more likely to be smear positive than those tested later (8 of 46 patients, 17.4% versus 6 of 53 [11.3%], $\chi^2 = 0.74, p = 0.387$) (Figure 1b). Patients with a positive serologic test tended to be in the later stage of infection (days since illness onset > 4; $\chi^2 = 3.35, p = 0.067$), with maximum positivity observed for patients tested between 22 and 28 days post-illness onset (6 of 11, 54.5%) (Figure 1c). In addition, antibody titers for seropositive patients were significantly higher in patients that were in the later phase of illness (Wilcoxon rank sums statistic = 1419.5, $p = 0.029$).

Patients that were seropositive and PCR-positive tended to have higher antibody titers than seropositive patients that were PCR-negative (Wilcoxon rank sums statistic = 1194, $p = 0.0096$). Titers for both groups of patients ranged between 1:64 and >1:512, though those that were sero- and PCR-positive had a median titer of 1:512 and geometric mean titer of 1:355, whereas those that were seropositive and PCR-negative had a median titer of 1:128 and geometric mean titer of 1:170. The highest median and geometric mean titers were observed in patients (n=15) that were PCR-positive and in the later phase of illness (median titer of >1:512, geometric mean titer of 1:512), and these titers were significantly higher.
than the titers for the 4 patients that were both seropositive and PCR-positive in the early acute phase of illness (2 had titers of 1:64 and 2 had titers of 1:128; Wilcoxon rank sums statistic = 15, p = 0.0078).

There was moderate agreement between the PCR and blood smear analysis test results (kappa = 0.66, 95% CI 0.48-0.84). All of the blood smear positive patients were PCR-positive (Table 1, Figure 2a). Among smear negative patients, an additional 11 were PCR-positive, representing 11.1% of 99 patients that had blood smear analysis results. Compared to the PCR assay, the sensitivity of blood smear analysis was 56%. There was little agreement between the PCR and serologic test results (kappa = 0.071, 95% CI -0.036-0.18) (Figure 2b). Of the 96 patients that were seropositive, only 19 (19.8%) had a PCR-positive result. Likewise, the majority of PCR-positive patients (60.4%, 29/48) were seronegative.

Disparity between the PCR and serologic test results was particularly high during the early acute phase of infection (Figure 2b); only 4 of the 28 PCR-positive patients (14.3%) in this phase were seropositive and 31 of 35 (88.6%) seropositive patients were PCR-negative. Agreement between the two tests improved during the later phases of infection (Figure 2b), as 75% (15/20) of the PCR-positive patients were also PCR-negative. Overall, about 31% (96/308) of the patients that were considered to have an illness compatible with anaplasmosis and had serologic test results available were seropositive. Of the 103 patients that had all 3 test results, the PCR assay detected 7 additional *A. phagocytophilum* infections that were not detected by either blood smear or serologic tests, representing 29.2% of the 24 patients in this sample that had a positive PCR test.

**Patients with anaplasmosis-incompatible illness**

All of the 50 patients with incompatible symptoms had a negative PCR test result. Likewise, all blood smear tests for these patients were negative. Twenty-six percent (13/50) of these patients, however, were seropositive.

**DISCUSSION**

Although serologic assays and microscopic examination of stained blood smears are practical and reliable diagnostic methods to detect infections with *A. phagocytophilum* and *Ehrlichia* spp., the tests do have recognized limitations (1, 33). Blood smear analysis is insensitive due to the small number of circulating cells that can be practically examined during routine microscopy, rarity of infected cells, lack of
expertise among personnel performing smear examination and the occurrence of intracellular artifacts that may mimic morulae (3, 5). Serologic tests have limited utility in the early acute phase of infection when the majority of patients seek care, prior to the production of detectable antibodies. The available assays also suffer from lack of specificity and may have considerable turnaround times (7, 32). Importantly, IgG-only assays lack the ability to distinguish between active and resolved infections, as IgG antibodies may persist in patients for long periods of time after infections have resolved (2, 7).

Based upon recognized improvements in sensitivity, specificity and turn-around times for PCR-based assays (26, 35), we evaluated the performance of a real-time PCR assay (10) to detect infections with *A. phagocytophilum* in comparison to serologic and blood smear examination tests. Timing of specimen collection relative to patient reported onset of symptoms was subsequently examined for each diagnostic method to better assess optimal test utilization by stage of infection. The real-time PCR assay performed similarly to other PCR assays that have been developed for *A. phagocytophilum* detection (20, 21). As with other studies, we found that maximum positivity was obtained in the early phase of illness, when morulae were likely to be visible upon blood smear analysis (50% of our PCR-positives in the early phase were also smear positive), and before an effective antibody response had been mounted by patients (74% of these PCR-positives were seronegative). The positivity rate for the real-time PCR assay also declined with the numbers of days patients reported being ill (Figure 1a) (4). This decline likely reflects the natural clearance of morulae from circulating neutrophils that has been described with the simultaneous appearance of infection-specific neutralizing antibodies (3). Despite this decline in positivity with length of patient illness, sensitivity of the PCR assay was 100% compared to blood smear analysis and detected infections over a wider window of time (0-30 days versus 2-14 days post-illness onset, respectively), suggesting it would provide more reliable results for anaplasmosis diagnosis in the early stage of infection. Improving diagnosis during acute infection has clinical relevance as the majority of patients seek care within the first few days of an acute, febrile illness. Of the 361 patients included in our study, 219 (60.7%) visited a clinic within 7 days of illness onset. Thus, a more sensitive diagnostic method during this phase would ensure proper diagnosis and initiation of antibiotic treatment in more patients at a time when failure to treat may lead to more severe manifestations of anaplasmosis (4, 14).
In the absence of paired acute and convalescent serology tests to confirm *A. phagocytophilum* infection, it is difficult to compare the sensitivity of the PCR assay with the IFA serologic test as used in our study. Overall, a higher percentage of patients were seropositive than PCR-positive, although the seropositivity rate is indicative of antibody responses to active infections as well as evidence of previous exposure. Seroprevalence studies previously performed in Wisconsin have demonstrated background seropositivity rates around 15% (6). In our study, we included 50 control patients with incompatible symptoms for anaplasmosis; none of these patients were PCR-positive, although 13 (26%) of them were seropositive, suggestive of prior exposure. This background seropositivity may be higher than what was previously reported because we were selecting patients from a pool that had been tested for anaplasmosis, rather than from a pool of completely healthy individuals. Thus, although their clinical symptoms were not compatible with the case definition for anaplasmosis, they presented with symptoms that caused enough suspicion to be tested by their healthcare providers. It may be that some of these control patients were actually still recovering from a recent anaplasmosis infection.

Characteristics of patients with PCR-positive test results were largely consistent with what has been reported previously for anaplasmosis. The PCR assay detects pathogen DNA in the blood of infected patients. Anaplasmosis patients are most likely to be bacteremic during acute infection, and our data supported this observation, with 78.6% of our positive blood smears detected during the first week of patient illness. This period has also been shown to coincide with hematologic abnormalities, including leukopenia and thrombocytopenia that are associated with infections (1, 8). In our study, the association between urinary-tract complaints and positive PCR status has not previously been reported. An abnormal urinalysis result indicating hemoglobinuria/myoglobinuria (the laboratory urinary test used did not distinguish between them) was reported for 15 of 49 (30.6%) patients that were PCR-positive for *A. phagocytophilum* infection, and 6 additional patients reported dark urine, which may be a sign of hemoglobinuria/myoglobinuria, as well as dehydration. Hemoglobinuria/myoglobinuria, in particular, may result from cell lysis or the pro-inflammatory immune response that occurs in *A. phagocytophilum* infection (19, 23). In more severe cases, acute renal failure may also occur, such that hemoglobinuria/myoglobinuria may be a precursor to renal dysfunction. Additionally, rhabdomyolysis has been described as a complication in association with some cases of anaplasmosis (30);
hemoglobinuria/myoglobinuria may be supportive evidence of this condition. Whether hemoglobinuria/myoglobinuria is another clinical sign that will be consistently supportive of an anaplasmosis diagnosis or be indicative of the severity of infection should be examined in future studies.

Although we did not detect any *Ehrlichia* spp. infections in our study population, the ability of the real-time PCR assay to detect these pathogens represents a significant advantage over traditional serologic and blood smear tests and will permit species specific surveillance in the future.

Currently, the geographic distributions and life cycles of the two most common pathogens, *A. phagocytophilum* and *E. chaffeensis*, are largely distinct, allowing for presumptive diagnoses of infections based upon geographic location of patient exposure. *A. phagocytophilum* is transmitted primarily by black-legged ticks (*Ixodes scapularis* in the eastern United States and *Ixodes pacificus* in the western United States) with highest incidence rates reported in the northern Midwest and northern Atlantic seaboard states, whereas *E. chaffeensis* is transmitted by the Lone Star tick (*Amblyomma americanum*) and most frequently causes infections in the central Midwest and southeastern states (18). Range expansions of the black-legged and Lone Star ticks present increasing risks of exposure to these infections in new areas and the potential for misdiagnoses as the distributions of the pathogens become more overlapping (25, 27). Having the ability to detect and differentiate among them will be important for their accurate diagnosis and treatment, as all antibiotic therapies are not effective against both pathogen groups (14, 32). In particular, *E. chaffeensis* appears to be resistant to fluoroquinolone antibiotics (24).

Moreover, we now know that Wisconsin is endemic for not only *A. phagocytophilum*, but also the newly described *Ehrlichia* sp. Wisconsin (28). To date, PCR is the only known method available for its detection. There have not been any diagnoses of *Ehrlichia* sp. Wisconsin infections by blood smear analysis (B. Pritt, personal communication). A diagnostic method capable of detecting this new pathogen will aid in understanding its epidemiology. In addition, evidence suggests the species is transmitted by the black-legged tick (28, 31), and therefore, the potential for co-infections with *A. phagocytophilum* exists. The use of this real-time PCR assay enables the detection of co-infections in a single run of the assay.

In summary, utilization of the described real-time PCR assay has enabled us to rapidly and accurately identify infections with *A. phagocytophilum* and potentially other species of Anaplasmataceae.
known to occur in our geographic area. The assay is more sensitive than blood smear analysis and extends the window of direct organism detection from 14 to 30 days from onset of symptoms. Serologic detection, not surprisingly, correlates poorly with PCR or smear analysis and more accurately reflects the collective exposure history occurring from late in the acute infection period into convalescence. Depending upon patient history, symptomatology, and hematologic and chemical metabolic profiles, the PCR assay may be an appropriate diagnostic adjunct. Proper selection of currently available diagnostic assays for maximal diagnostic potential does depend, however, upon the taking of a detailed clinical history that identifies the time interval from onset of symptoms to submission of clinical specimens.

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References


**Table 1.** Demographic and clinical characteristics of patients that were either positive or negative by the PCR assay.

<table>
<thead>
<tr>
<th></th>
<th>PCR positive</th>
<th>PCR negative</th>
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<tr>
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<td><strong>N (%)</strong></td>
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<td>(15.7)</td>
<td>262 (86.4)</td>
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<td><strong>Serology positive</strong></td>
<td>48</td>
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<td>77 (29.6)</td>
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<td><strong>Smear positive</strong></td>
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<td><strong>Gender (% male)</strong></td>
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<td><strong>Acute infections</strong></td>
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<td>52 (19.9)</td>
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<td></td>
</tr>
<tr>
<td><strong>Positive for symptoms:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fever/sweats/chills</strong></td>
<td>49</td>
<td>262</td>
<td>0.046</td>
</tr>
<tr>
<td>(95.9)</td>
<td>224 (85.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Headache</strong></td>
<td>49</td>
<td>262</td>
<td>0.893</td>
</tr>
<tr>
<td>(57.1)</td>
<td>147 (56.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Myalgias</strong></td>
<td>49</td>
<td>262</td>
<td>0.557</td>
</tr>
<tr>
<td>(63.3)</td>
<td>154 (58.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cold symptoms</strong>³</td>
<td>49</td>
<td>262</td>
<td>0.670</td>
</tr>
<tr>
<td>(36.7)</td>
<td>88 (33.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GI symptoms</strong>⁴</td>
<td>49</td>
<td>262</td>
<td>0.986</td>
</tr>
<tr>
<td>(44.9)</td>
<td>118 (45.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urinary symptoms</strong>⁵</td>
<td>49</td>
<td>262</td>
<td>0.049</td>
</tr>
<tr>
<td>(44.9)</td>
<td>80 (30.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR Positive</td>
<td>PCR Negative</td>
<td>Statistic</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>44</td>
<td>26 (59.1)</td>
<td>238</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>44</td>
<td>37 (84.1)</td>
<td>237</td>
</tr>
<tr>
<td>Elevated liver enzymes</td>
<td>33</td>
<td>22 (66.7)</td>
<td>168</td>
</tr>
<tr>
<td>Reported tick exposure</td>
<td>49</td>
<td>27 (55.1)</td>
<td>262</td>
</tr>
</tbody>
</table>

* based on chi-square analysis; significant variables (P<0.05) are indicated in bold

Sample sizes refer to the numbers of patients that were PCR positive or PCR negative and had data available on the various factors listed, and therefore, could be included in the comparisons.

- includes cough, sore throat, sinus congestion
- includes nausea, vomiting, diarrhea, abdominal pain
- includes positive culture for urinary tract infection, urinary incontinence, urgency, frequency, or dysuria, hematuria, hemoglobinuria/myoglobinuria
Figure legends.

**Figure 1.** Frequency histograms showing the number of patients with positive and negative test results and percentages of positive tests (± 95% confidence intervals) by intervals of time since illness onset for A) real-time PCR assay, B) blood smear analysis, and C) serologic test.

**Figure 2.** Comparisons of A) blood smear and B) serologic tests with that of the real-time PCR assay for detecting *A. phagocytophilum* infection in all patients, and for those in the early acute (0-4 days since illness onset) and later (>4 days since illness onset) phases of infection.