RESEARCH ARTICLE

Efficient detection of symptomatic and asymptomatic patient samples for \textit{Babesia microti} and \textit{Borrelia burgdorferi} infection by multiplex qPCR

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Abstract

Background

Tick-borne infections have been increasing steadily over the years, with co-infections with \textit{Borrelia burgdorferi} and \textit{Babesia microti}/\textit{divergens} emerging as a serious health problem. \textit{B. burgdorferi} is a spirochetal bacterium that causes Lyme disease while protozoan pathogens belonging to \textit{Babesia} species are responsible for babesiosis. Currently used serological tests do not always detect acute Lyme disease or babesiosis, and fail to differentiate cured patients from those who get re-infected. This is a major problem for proper diagnosis particularly in regions endemic for tick-borne diseases. Microscopy based evaluation of babesiosis is confirmatory but is labor intensive and insensitive such that many asymptomatic patients remain undetected and donate blood resulting in transfusion transmitted babesiosis.

Results

We conducted multiplex qPCR for simultaneous diagnosis of active Lyme disease and babesiosis in 192 blood samples collected from a region endemic for both diseases. We document qPCR results obtained from testing of each sample three times to detect infection with each pathogen separately or together. Results for Lyme disease by qPCR were also compared with serological tests currently used for Lyme disease when available. Considering at least two out of three test results for consistency, 18.2% of patients tested positive for Lyme disease, 18.7% for co-infection with \textit{B. burgdorferi} and \textit{B. microti} and 6.3% showed only babesiosis.

Conclusions

With an 80% sensitivity for detection of Lyme disease, and ability to detect co-infection with \textit{B. microti}, multiplex qPCR can be employed for diagnosis of these diseases to start appropriate treatment in a timely manner.
Introduction

Tick-borne infections have shown an alarming increase in the last decade. According to the CDC, approximately 300,000 cases of Lyme disease and 2,000 cases of babesiosis occur in the United States per year [1, 2]. Lyme disease caused by the spirochete group _Borrelia burgdorferi_ sensu lato is also prevalent in Europe. _Ixodes scapularis_ ticks transmit several pathogens including _B. burgdorferi_ and _Babesia microti_. In the U.S.A, Lyme disease is endemic in the Northeastern and upper Midwestern states with some cases also reported in northern California, Oregon, and Washington [2]. _Babesia_ species are protozoan parasites that cause malaria like febrile illness with most cases of babesiosis attributed to _B. microti_, while some patients are also infected by _B. duncanii_ in Western states [3, 4]. _B. burgdorferi_ is an extracellular, highly adherent pathogen while _Babesia_ species are intracellular parasites of erythrocytes. _B. burgdorferi_ and _B. microti_ are the most common tick-borne co-infections in the Eastern United States accounting for 40–80% of concurrent infections in different years [5, 6]. Recently, Diuk-Wasser summarized outcomes of several studies, which demonstrated that up to 40% of Lyme disease patients are also infected with _B. microti_, and 2/3rd of babesiosis patients were also infected with _B. burgdorferi_ in the Northeastern USA [6]. These results emphasize the importance of development and testing of a more efficient and high throughput assays to examine _B. burgdorferi_ and _B. microti_ simultaneously among patients particularly in the endemic regions of North America and Europe.

Upon transmission of _B. burgdorferi_ by ticks, Lyme disease usually starts with non-specific flu like manifestations. Lyme spirochetes disseminate to various organs including the heart, joints and central nervous system using the blood as a conduit, and colonize different tissues resulting in systemic disease. Inability to diagnose and treat Lyme disease early in infection can lead to severe symptoms that can persist long after the conclusion of antibiotic treatment [7–12]. Patients suffering from this post-treatment Lyme syndrome show subjective manifestations such as chronic fatigue, musculoskeletal pain and malaise, memory loss, and inability to concentrate, all of which significantly reduce the patient’s quality of life [10]. Therefore, it is important to detect _B. burgdorferi_ during the early stages of infection to treat the disease successfully.

Currently available FDA approved serological tests for _B. burgdorferi_ infection cannot be used to detect acute infection before the adaptive immune response is induced [13]. The positive predictive value (PPV) of the two-step testing procedure approved by the FDA depends both on the prevalence of the disease in the region, and on the sensitivity and specificity of the commercial kit used [14–16]. Overall, the sensitivity of serological tests for the diagnosis of Lyme disease is reported to vary between 50–97%, and is highly dependent on the stage and disease manifestations of the patient [17]. In addition, antibodies to _B. burgdorferi_ persist even after spirochete clearance, making it difficult to ascertain when the disease has been cured. Variation in the adaptive immunological response to different strains may also affect the sensitivity of the test. Furthermore, re-infection cannot be determined using these tests, a serious problem in the endemic regions. Therefore, there is a desperate need for a technically simple, rapid, and accurate test that can be readily automated to unequivocally diagnose active, acute, and post-treatment Lyme disease.

_Babesia_ species are harbored by the same reservoir host, white footed mouse, as _B. burgdorferi_ and can also be transmitted by blacklegged ticks. Indeed, co-infections of _Ixodes_ species ticks with _B. burgdorferi_ and _B. microti_ and of the reservoir hosts have been increasing steadily over the years [18–22], and have resulted in an associated increase of co-infected individuals [5, 6, 23, 24]. Babesiosis manifestations in humans can range from asymptomatic in immunocompetent hosts to life threatening in immunocompromised, splenectomized and elderly
patients [25]. Transfusion of blood containing *B. microti* infected erythrocytes from asymptomatic patients can result in transfusion-transmitted babesiosis (TTB) [26, 27]. Previous studies have shown that patients co-infected with *B. burgdorferi* and *B. microti* display more extensive symptoms that persist longer and also exhibit more severe disease than in Lyme disease patients [28–30]. Additionally, antibiotics used for treatment of Lyme disease are ineffective against babesiosis, further complicating the treatment of co-infections. Thus, accurate early diagnosis of co-infected individuals is imperative to provide the most effective treatment, and decrease the chance of long-lasting deleterious effects.

Diagnosis of both *B. microti* and *B. burgdorferi* infection in patients is subject to many challenges. One method used is the microscopic evaluation of Giemsa-stained blood smears. Though confirmatory, this is a time-consuming method that requires specific expertise, which limits its use for large-scale testing. This method is also limited in its application since intermittent or low levels of parasitemia are difficult to detect. Specific antibody-dependent methods can also be used for diagnosis of babesiosis, but the typical problems associated with serological tests also apply to their use for babesiosis diagnosis. Several laboratories have started using more sensitive and cost-effective PCR tests for diagnosis of babesiosis in both Europe and the United States and have found that real-time PCR assays are highly sensitive for detection of infection by *Babesia* spp. in patients [31–45]. We report here that our published real-time PCR assay [46] is also effective in diagnosing patients infected with *B. burgdorferi*.

In this report, we describe an accurate, sensitive multiplex quantitative PCR (qPCR) assay, that uses specific molecular beacon probes [46] to detect the presence *B. burgdorferi* and *B. microti* DNA in patient samples. This method detected a higher percentage of co-infected samples than were diagnosed using other methods. Thus, our qPCR assay can be effective for diagnosis of Lyme disease and babesiosis in patients even at the acute stage, whether the causative agents are present separately or together. This multiplex assay would be especially useful in the Northeastern and Upper Midwestern states, which are endemic for both pathogens [1, 2] as well as for the endemic regions of Europe for tick-borne infections. A qPCR-base test also could provide invaluable information about infected blood donated by patients asymptomatic for babesiosis.

**Materials and methods**

**Human samples**

During a visit to either Gedroic Center or the Jersey Shore University Medical Center (JSUMC), physicians ordered blood samples collection from 192 patients from three different counties in New Jersey for blood tests, including that for various infections [45]. Remaining unused aliquots of samples after clinical testing were provided to Dr. Parveen’s laboratory for this study. Physicians ordered blood collection only requires verbal, and not written, consent from patients. Patients presenting with different clinical symptoms were recommended for testing for tick borne diseases for initial evaluation or follow-up care. At the Gedroic Center, a history of tick bite, and patients presenting with high fever (>102 °F) eight weeks after noticing a tick-bite were suspected of suffering from babesiosis. Furthermore, if a patient reported a history of erythema migrans indicating potential tick-borne infection, or exhibited two of the three symptoms, night sweats, shortness of breath and frontal headaches, a high index of *Babesia* infection was considered and samples were sent to IGeneX. *B. microti* presence on the air-dried blood smears on slides was examined by Fluorescence in situ hybridization (FISH) using 18S rDNA/rRNA target. Blood samples from patients, who reported a tick bite, or presented with migratory joint pain, mild to moderate fatigue, mental confusion/cognitive dysfunction and occipital headaches, were sent for serological testing for Lyme disease. Testing at Stony
Brook Laboratory was conducted using two-step serological test for Lyme disease. Blood samples from 106 patients were collected at the Gedroic Center that also included patients who were considered asymptomatic for babesiosis and Lyme disease and were not sent for testing for tick-borne diseases.

Patient identification criteria for testing at JSUMC for babesiosis included either a history of exposure to a tick bite or patients who did not recall a tick bite but had: fever, plus or minus rash; malaise, fatigue, joint pain; anemia, with or without neutrophilia, and decrease platelet counts. Blood testing for babesiosis was conducted by microscopic examination of Giemsa-stained smears at JSUMC. Blood samples from patients with either a tick-bite and erythema migrans, or those having fever, arthritis; malaise, fatigue and headaches when tick-bite history, were tested using Zeus Lyme V1sE1/pepC10 IgM/IgG antibodies at JSUMC. Among 86 samples collected at Meridian hospitals, samples not tested for either Lyme disease or babesiosis were also included. Some of these served as healthy cohorts when neither the results for tick-borne diseases were positive nor symptoms associated with these diseases were observed. Thus, results from a total of 192 samples conducted by clinics at two different locations are presented in this study.

**Ethics statement**

Patients were not recruited for this study. Blood samples collection was ordered by physicians at two locations for blood analyses and/or for testing for various infections during patients’ visit to the clinic/hospital. Therefore, the Institutional review board (IRB) approved protocol or patients consent were not needed for blood collection. Blood collected in Ethylenediaminetetraacetic acid (EDTA) containing tubes was sent to clinical laboratories for testing for infectious diseases. After retrieval of aliquots to send to the testing laboratories, the remaining samples were provided to conduct this study at Parveen’s laboratory at the New Jersey Medical School (NJMS). Samples were provided in a coded, de-identified manner to preserve patient anonymity. Our qPCR experiments with human blood were conducted under the exempt IRB protocol of Dr. Parveen at NJMS, in accordance with the ethical standards of the responsible committee on human experimentation, of Rutgers-NJMS, and in agreement with the Helsinki Declaration of the World Medical Association. Department of Health and Human Services Federal Wide Assurance is provided to NJMS for work involving human samples.

**B. burgdorferi and B. microti quantitation by qPCR**

Multiplex qPCR was conducted using primers and molecular beacon probes for recA amplicon for *B. burgdorferi* and Bmtpk amplicon for *B. microti* amplification and detection as previously described [46]. We used whole blood from each patient for isolation of DNA and determined the presence of both pathogens by qPCR using the respective standard curves [45]. The presence of leukocytes in whole blood allowed us to include human actA1 amplicon as an internal control to ensure that DNA quality is suitable for PCR.

**Lyme C6 enzyme-linked immunosorbent assay (ELISA)**

Lyme C6 ELISA was performed and data analyzed per manufacturer’s instructions (Immunetics: *B. burgdorferi* (Lyme) ELISA kit: DK-E352-096) using patient plasma samples diluted at 1:50. The assay calibrators were used to calculate the Lyme index for each patient sample, which directly classifies each sample as positive, negative or equivocal for *B. burgdorferi* infection. Lyme Index was determined according to manufacturer’s guidelines. Briefly, A450 of \( \leq 0.90 \) was considered negative, 0.91–1.09 equivocal and \( \geq 1.10 \) as positive result in the assay.
Statistical analysis

We determined the sensitivity of qPCR as a measure of the proportion of subjects that tested positive for *B. burgdorferi* infection as compared to the disease state of the subject based upon clinicians’ determination and according to tests conducted by clinical laboratories and our laboratory in this study, i.e., two-tier serological tests and C6 ELISA for Lyme disease. Specificity measure in our study indicated the proportion of subjects that tested negative by qPCR given that the subjects did not have Lyme disease by these two types of serological tests. Detailed analysis of data and formulae used for various parameters is shown in results section.

Results

Testing of patient samples by two-tier serological tests and C6 ELISA

Of the 192 patient samples, 36 were asymptomatic for Lyme disease, while 156 presented with symptoms consistent with possible tick-borne infection likely due to *B. burgdorferi*. Blood samples from all symptomatic patients were sent to diagnostic laboratories to be tested for Lyme disease by the CDC recommended two-tier serological tests. After testing samples by either enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence assay (IFA) as primary tests, samples were further tested by the standardized Western blotting method to detect antibodies against specific *B. burgdorferi* protein markers. Forty-nine of the 156 samples (31%) tested positive for Lyme disease by these two-tier serological tests, while 107 samples (69%) tested negative.

C6 Lyme ELISA uses a synthetic conserved peptide (C6 peptide) derived from the antigenic VlsE protein of *B. burgdorferi*, and has been reported to be more sensitive for diagnosis of Lyme disease compared to the recommended two-tier testing for patients in North America [15, 47–49]. Testing of all 192 samples detected 67 (35%) to be positive for antibodies against *B. burgdorferi*, while 118 (61%) tested negative (Fig 1). Seven samples (4%) produced an equivocal result. Of the 156 symptomatic patient samples tested by two-tier serology, 49 samples (31%) tested positive for Lyme disease. Thus, C6 serology corroborates Lyme disease diagnosis by two-tier serology. Combined results from all serological tests with the respective samples are documented in S1 Table.

![Histogram showing the Lyme Index distribution produced by C6 Lyme ELISA conducted on all 192 patient samples. An index ≤ 0.90 is a negative Lyme diagnosis (118 samples), an index of 0.91–1.09 is an equivocal result (7 samples), and an index ≥ 1.10 is a positive Lyme diagnosis (67 samples).](https://doi.org/10.1371/journal.pone.0196748.g001)
Comparison of qPCR assay for Lyme disease with serological test results

Patient samples were tested for tick-borne infections either at JSUMC or through commercial laboratories by Gedroic Center. To determine the efficacy of our previously optimized qPCR assay for diagnosis of Lyme disease in patient samples [46], we compared the qPCR results with two-tier serological test results provided by Drs. Gedroic and Rojtman. We found that 114 of 156 samples tested were positive by qPCR at least once and PCR cycle number for detection was below 40 (Table 1 and S1 Table). Nine samples positive by two-tier serological tests and negative by qPCR indicate the likely absence of spirochetes in blood at the time of sample collection. This is not surprising because B. burgdorferi is known to present only transiently in blood during acute phase of infection. We also cannot rule out the possibility that the level of spirochetes was below the level of detection of qPCR. In fact, a significant number of samples tested positive only once among three repeats of the test out of 146 qPCR positive samples indicating that the presence of spirochetes in blood is often at the lower end of detection limit of qPCR. We also used C6 ELISA as a complementary test for diagnosis of patient samples for Lyme disease to resolve this problem. These results also emphasize the caveat that an efficient detection of B. burgdorferi in blood is dependent on collection of sample during active dissemination phase of infection.

Data analysis for Table 1.

\[
\text{Sensitivity} = \frac{\# \text{ of True Positives}}{\# \text{ with Disease}} = \frac{40}{49} = 0.8163 = 81.63% \\
\text{Standard Error of Sensitivity} = \sqrt{\frac{\text{Sensitivity} \times (1 - \text{Sensitivity})}{\# \text{ with Disease}}} = \sqrt{\frac{0.816 \times (1 - 0.816)}{49}} = 0.0553 = 5.53% \\
\text{Specificity} = \frac{\# \text{ of True Negatives}}{\# \text{ without Disease}} = \frac{33}{107} = 0.3084 = 30.84% \\
\text{Standard Error of Specificity} = \sqrt{\frac{\text{Specificity} \times (1 - \text{Specificity})}{\# \text{ without Disease}}} = \sqrt{\frac{0.3084 \times (1 - 0.3084)}{107}} = 0.0446 = 4.46% \\
\]

Table 1. Diagnosis of Lyme disease by qPCR and two-tier serology.

| qPCR | Two-tier Serological test results | Total |
|------|----------------------------------|-------|
|      | Positive            | Negative |       |
| Positive (at least 1/3 repeats) | 40       | 74     | 114   |
| Negative                      | 9        | 33     | 42    |
| Total                          | 49       | 107    | 156   |

qPCR metrics Standard Error 95% CI (Lower, Upper Bound)

|          | Sensitivity | Specificity | PPV  | NPV  |
|----------|-------------|-------------|------|------|
| Sensitivity | 81.6%       | 30.8%       | 35.1%| 78.6%|
| Specificity | 4.5%        | 4.5%        | 6.3% | 6.3% |
| PPV       | 5.5%        | 4.5%        | 4.5% | 4.5% |
| NPV       | 5.5%        | 4.5%        | 4.5% | 4.5% |

a CI: Confidence Interval

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PPV = \# of True Positives / \# of Positive Calls = \frac{40}{114} = 0.3509 = 35.09% 

\text{Standard Error of PPV} = \sqrt{\frac{PPV \times (1 - PPV)}{\# of Positive Calls}} = \sqrt{\frac{0.3509 \times (1 - 0.3509)}{114}} = 0.0447 = 4.47% 

NPV = \# of True Negatives / \# of Negative Calls = \frac{33}{42} = 0.7857 = 78.57% 

\text{Standard Error of NPV} = \sqrt{\frac{NPV \times (1 - NPV)}{\# of Negative Calls}} = \sqrt{\frac{0.7875 \times (1 - 0.7875)}{42}} = 0.0633 = 6.3%

Sensitivity of qPCR was approximately 82% and Negative Predictive Value (NPV) 79%. The low specificity and Positive Predictive Value (PPV) observed are attributable to the unavailability of a Gold standard test for Lyme disease for comparison and due to the inability of serological tests to detect acute disease in a significant number of samples tested.

For further analysis of our qPCR, we compared results with total output from all serological tests including C6 ELISA. We found that 146 samples (76%) were positive in at least one of the three qPCR repeats conducted for each sample, such that sensitivity of the assay was approximately 80%, and specificity 29.1% when compared with all serological tests (Table 2 and S1 Table). Thus, sensitivity and specificity of qPCR compared to 2-tier serological tests remained almost unchanged when we included results from C6 ELISA. Again, somewhat lower PPV for qPCR is because the serological tests, which are used for comparison here, are not the Gold standard tests particularly for acute Lyme disease.

Some samples (21 in total), were serologically positive but negative by qPCR (Table 2), suggesting that patients had persistent antibodies after clearance of infecting spirochetes and thus, affect NPV values. Alternatively, due to transient presence of Lyme spirochetes in blood, B. burgdorferi were either absent in blood at the time of samples collection or their numbers were below the detection limit of qPCR. Forty-six serologically positive samples were positive by

Table 2. Diagnosis of Lyme disease by qPCR and serological tests.

| qPCR metrics | Total |
|--------------|-------|
| Sensitivity  | 79.8% | 3.9% | (72.1%, 87.5%) |
| Specificity  | 29.1% | 4.9% | (19.5%, 38.7%) |
| PPV          | 57.6% | 4.1% | (49.6%, 65.7%) |
| NPV          | 54.4% | 7.3% | (40%, 68.7%) |

*CI: Confidence Interval. Two samples gave equivocal results by C6 ELISA and were not tested by 2-tier serological tests.

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qPCR only once; indicating that these samples possibly had spirochete numbers at, or close to the lowest detection limit of qPCR.

Positive test results for Lyme disease are further summarized in the form of Venn diagram (Fig 2) for ease of comparison, with 67 samples (40.1%) positive by C6 ELISA, 49 samples (29.4%) positive by two-tier serological tests and highest, 146 samples (76%) positive by qPCR. Thus, 37.7% samples were positive only by qPCR compared to 7.2% and 5.4% samples that tested positive only by C6 ELISA or 2-tier serological tests, respectively. Samples that showed positive results by serological tests and negative by qPCR may depict either a past infection detectable by antibodies despite spirochete clearance or that patients were on the verge of cure of Lyme disease. In all these situations, there is likelihood of no to low *B. burgdorferi* presence in blood that could be below the detection limit of qPCR. The lack of overlap between 92 positive samples by either two-tier tests or C6 ELISA was surprising and highlights the problem associated with the use of different kits/tests for serological evaluation and the absence of ideal standards for diagnosis of Lyme disease. Two-tier serological test positive results for 24%
samples also showed positive reaction with qPCR. A higher agreement was obtained between samples positive by C6 ELISA and qPCR with 32.9% samples positive by both tests.

Testing of samples for babesiosis
As we reported previously [45], clinical samples were also tested either by IFA, FISH or microscopic examination of Giemsa-stained blood smears for the presence of \textit{B. microti} (results are summarized here in S1 and S2 Tables). Based upon the lack of symptomatology, 131 samples were not tested by the clinical laboratories using any test for \textit{B. microti}. Our qPCR testing of these samples showed that 38% were positive for \textit{Babesia} presence [45]. A high congruency of DNA based assays, qPCR and FISH was observed because 27 of 28 FISH positive samples (96%) were also positive by qPCR. Interestingly, of 78 samples untested through Gedroic Center due to the absence of symptoms, 22 were positive by our qPCR. Direct detection of \textit{Babesia} by labor-intensive microscopic examination of patient blood smears was used only for a few J samples. By considering individuals positive for \textit{Babesia} infection when results from currently available microscopy, FISH or serological tests were positive, we found that our qPCR is highly sensitive (96.4%) and showed a specificity of 70.5% for \textit{Babesia} presence [45]. Somewhat lower specificity of qPCR could be because serological tests may not depict active infection in all cases. Samples without a known history of tick bite and negative by all tests for both Lyme disease and babesiosis served as healthy, uninfected cohorts for tick-borne diseases here.

Detection of \textit{B. burgdorferi-} \textit{B. microti} co-infections by qPCR
Assessing the efficacy of our qPCR as a multiplex assay for detection of co-infections with \textit{B. burgdorferi} and \textit{B. microti} showed it to be very effective (Table 3 and S1 Table). Our results show co-infection rate of approximately 39% among our 192 patient samples, while single infection with \textit{B. microti} was almost 10%. We found that qPCR could uniquely detect the presence of both infections simultaneously, which is not yet possible by other standard diagnostic tests. Furthermore, it is possible to detect active infection by both tick-borne pathogens using multiplex qPCR particularly when bacteremia/parasitemia is low and patients are asymptomatic for babesiosis as commonly observed in healthy but \textit{B. microti} infected individuals [Table 3, S1 Table and [45]].

**Discussion**
Symptoms-based diagnosis of various tick-borne diseases is problematic due to similar early clinical manifestations. Although it is possible to use serological tests in a high throughput manner during the post-acute phase of different infectious diseases, problems associated with these tests are well-established and hinder diagnosis of active \textit{B. burgdorferi} infection using

**Table 3. Detection of single and co-infections of \textit{B. burgdorferi} and \textit{B. microti} using qPCR.**

|            | Uninfected | Single infection | Co-infection |
|------------|------------|------------------|--------------|
|            |            | \textit{B. burgdorferi} | \textit{B. microti} | |
| qPCR ++ or +++<sup>a</sup> | 0 | 35 (18.2%) | 12 (6.3%) | 36 (18.7%) |
| qPCR +<sup>b</sup> | 0 | 37 (19.3%) | 6 (3.1%) | 38 (19.8%) |
| qPCR Negative | 28 (14.6%) | 0 | 0 | 0 |
| Total: 192 | 28 (14.6%) | 72 (37.5%) | 18 (9.4%) | 74 (38.5%) |

<sup>a</sup>qPCR ++ or +++: Samples positive two or three times of the 3 qPCR assays done
<sup>b</sup>qPCR +: Samples positive one of the 3 qPCR assays done
FDA approved two-tier serological tests [50, 51]. Subjective interpretation of Western blot results as the second-tier test further diminishes accuracy with an average of 70–80% serological test efficiency noted for diagnosis of Lyme disease. However, accuracy of a single C6 ELISA test sensitivity is reported to be slightly higher for Lyme disease than the two-tier serological test [49]. PCR-based assays have had only a limited level of success for Lyme disease diagnosis due to the presence of small numbers of spirochetes often transiently present in blood, and because of the design imperfections [52–54]. Our multiplex qPCR, with a much improved design that use of molecular beacon probes tagged with different fluorophores was highly sensitive and specific in detection of both B. burgdorferi and B. microti spiked blood [46].

We used this qPCR assay for retrospective diagnosis of Lyme disease and babesiosis in patient samples, collected from a region endemic for tick-borne diseases during transmission season. Our results highlight the advantages of qPCR-based diagnosis of Lyme disease. This method is independent of an immune response to infection, and is able to detect acute infection. In the absence of confirmatory tests available for Lyme disease, the PPV of the qPCR was relatively low though still above average, 58% when we compared it with all serological test results combined, suggesting that many serologically negative samples that showed positive results by qPCR are likely from patients with acute disease who lack antibodies against B. burgdorferi antigens. Although we cannot rule out false negative detection by qPCR, an NPV of 54.4% suggests that serologically positive samples that were negative by qPCR (Fig 2 and S1 Table) could be attributable to persistent antibodies in some patient samples, even though these patients no longer have Lyme disease or to the absence/low levels of spirochetes in blood below the detection limit of qPCR at the time of sample collection.

A major advantage of our multiplex qPCR is its ability to detect different tick-borne infections simultaneously. Indeed, we were able to detect a surprisingly high rate of B. burgdorferi-B. microti co-infections (~39%) in patient samples collected from a state endemic for tick-borne diseases. These results could be attributable to a high rate of infections with both pathogens in the counties where this study was undertaken. Antibiotic treatment used for Lyme disease is ineffective for babesiosis, which is often asymptomatic in immunocompetent individuals. Patients with severe babesiosis need hospitalization and the disease can even cause death of such patients due to multi-organ failure [55]. Therefore, diagnosis by a qPCR method gives the advantage of timely and appropriate treatment of patients and thus, could help in the prevention of TTB. Serum contains nucleases such that pathogenic nucleic acids do not persist long in the host after the disease has been cured. This allows for the use of nucleic acids-based assays as a test of cure for a variety of infectious diseases [31]. In fact, in addition to our studies, a comparative analysis conducted by the CDC last year further emphasized the importance of molecular beacon probes-based qPCR tests for babesiosis [44, 45], Success of such assays in other well-known laboratories highlights the significance of our test. Multiplex assays have not been employed yet by any other laboratory for tick-borne diseases further emphasizing the importance of our assay in the simultaneous detection of tick-borne pathogens in endemic regions.

Conclusions

Our multiplex qPCR offers a unique advantage for simultaneous diagnosis of Lyme disease and babesiosis in co-infected patients. Additionally, our qPCR can be used to efficiently detect B. microti presence in donated blood.

Supporting information

S1 Table. Master table for patients test data. (PDF)
S2 Table. Summary of results of all tests used for Lyme disease and babesiosis.

(PDF)

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References

1. Linnavuo M, Ylilaakko P, Mattila MJ, Maki M and Seppala T (1987) A new device to measure drug-induced changes on reactive and coordinative skills of human performance. Pharmacol Toxicol 61: 142–147. PMID: 2890156

2. Hautanen A, Gailit J, Mann DM and Ruoslahti E (1989) Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. J Biol Chem 264: 1437–1442. PMID: 252482

3. Takada Y, Ohta Y, Kawasaki S, Maki M, Hiroe M, Kusakabe K, et al. (1987) [Radioiodide therapy in pulmonary metastasis of differentiated thyroid cancer—factors affecting the success of I-131 therapy]. Nippon Igaku Hoshasen Gakkai Zasshi 47: 962–970. PMID: 3658666

4. Conrad PA, Kjemtrup AM, Carreno RA, Thomford J, Wainwright K, Eberhard M, et al. (2006) Description of Babesia duncani n.sp. (Apicomplexa: Babesiidae) from humans and its differentiation from other piroplasms. Int J Parasitol 36: 779–789. https://doi.org/10.1016/j.ijpara.2006.03.008 PMID: 16725142

5. Swanson SJ, Neitzel D, Reed KD and Belongia EA (2006) Coinfections acquired from Ixodes ticks. Clin Microbiol Rev 19: 708–727. https://doi.org/10.1128/CMR.00011-06 PMID: 17041141

6. Diuk-Wasser MA, Vannier E and Krause PJ (2016) Coinfection by Ixodes Tick-Borne Pathogens: Ecological, Epidemiological, and Clinical Consequences. Trends Parasitol 32: 30–42. https://doi.org/10.1016/j.pt.2015.09.008 PMID: 26613664

7. Krupp LB, Hyman LG, Grimson R, Coyle PK, Melville P, Ahnn S, et al. (2003) Study and treatment of post Lyme disease (STOP-LD): a randomized double masked clinical trial. Neurology 60: 1923–1930. PMID: 12921734

8. Steiner I (2003) Treating post Lyme disease: trying to solve one equation with too many unknowns. Neurology 60: 1888–1889. PMID: 12921728

9. Aucott JN, Crowder LA and Kortte KB (2013) Development of a foundation for a case definition of post-treatment Lyme disease syndrome. Int J Infect Dis 17: e443–449. https://doi.org/10.1016/j.ijjid.2013.01.006 PMID: 23462300

10. Aucott JN, Rebman AW, Crowder LA and Kortte KB (2013) Post-treatment Lyme disease syndrome symptomatology and the impact on life functioning: is there something here? Qual Life Res 22: 75–84. https://doi.org/10.1007/s11136-012-0126-6 PMID: 22294245
Krause PJ, Telford SR, Spielman A, Sikand V, Ryan R, Christianson D, et al. (1996) Concurrent Lyme disease and babesiosis. Clin Rheumatol 34: 585–589. https://doi.org/10.1027/s10067-014-2706-2 PMID: 24924604

Krause PJ, McKay K, Thompson CA, Sikand VK, Lenzt R, Lepore T, et al. (2002) Disease-specific diagnosis of coinfecting tickborne zoonoses: babesiosis, human granulocytic ehrlichiosis, and Lyme disease. Clin Infect Dis 34: 232–238. https://doi.org/10.1016/j.clinmic.2004.04.002 PMID: 15358654

Brown SL, Hansom SL, Langone JJ (1999) Role of serology in the diagnosis of Lyme disease. Jama 282: 62–66. PMID: 10404913

Fang DC and McCullough J (2016) Transfusion-Transmitted Babesia microti Infection in Nymphal Ixodes dammini. J Clin Microbiol 24: 446–447. PMID: 3760136

Hersch MH, Ostfeld RS, McHenry DJ, Tibbetts M, Brunner JL, Killilea ME, et al. (2014) Co-infection of Babesia microti and Borrelia burgdorferi is higher than expected and acquired from small mammal hosts. PLoS One 9: e99348. https://doi.org/10.1371/journal.pone.0099348 PMID: 24940999

Schulze TL, Jordan RA, Healy SP and Roegner VE (2013) Detection of Babesia microti and Borrelia burgdorferi in host-seeking Ixodes scapularis (Acari: Ixodidae) in Monmouth County, New Jersey. J Med Entomol 50: 379–383. PMID: 23540127

Tokarz R, Tagliafierro T, Cucura DM, Rochlin I, Sameroff S and Lipkin WI (2017) Detection of Anaplasma phagocytophilum, Babesia microti, Borrelia microti, Borrelia miyamotoi, and Powassan Virus in Ticks by a Multiplex Real-Time Reverse Transcription-PCR Assay. mSphere 2.

Diuk-Wasser MA, Liu Y, Steeves TK, Folsom-O’Keefe C, Dardick KR, Lepore T, et al. (2014) Co-infection of blacklegged ticks with Babesia microti and Borrelia burgdorferi promotes the establishment of Babesia microti in the northeastern United States. PLoS One 9: e115494. https://doi.org/10.1371/journal.pone.0115494 PMID: 25545393

Genda J, Negron EA, Lottipour M, Balabhadra S, Desai DS, Craft DW, et al. (2016) Severe Babesia microti Infection in an Immunocompetent Host in Pennsylvania. J Investig Med High Impact Case Rep 4: 2324709616663774.

Fang DC and McCullough J (2016) Transfusion-Transmitted Babesia microti. Transfus Med Rev 30: 132–138. https://doi.org/10.1016/j.tmrv.2016.04.002 PMID: 27260107

Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D and Wilson M (2011) Transfusion-associated babesiosis in the United States: a description of cases. Ann Intern Med 155: 509–519. https://doi.org/10.7326/0003-4819-155-8-20110108-00362 PMID: 21893613

Krause PJ, McKay K, Gadgebaw J, Christianson D, Closter L, Lepore T, et al. (2003) Increasing health burden of human babesiosis in endemic sites. The American journal of tropical medicine and hygiene 68: 431–436. PMID: 12875292

Krause PJ, McKay K, Thompson CA, Sikand VK, Lenzt R, Lepore T, et al. (2002) Disease-specific diagnosis of coinfecting tickborne zoonoses: babesiosis, human granulocytic ehrlichiosis, and Lyme disease. Clin Infect Dis 34: 1184–1191. https://doi.org/10.1086/339813 PMID: 11941544

Krause PJ, Telford SR, Spielman A, Sikand V, Ryan R, Christianson D, et al. (1996) Concurrent Lyme disease and babesiosis. Evidence for increased severity and duration of illness. Jama 275: 1657–1660. PMID: 8637139
31. Teal AE, Habura A, Ennis J, Keithly JS and Madison-Antenucci S (2012) A new real-time PCR assay for improved detection of the parasite Babesia microti. Journal of clinical microbiology 50: 903–908. https://doi.org/10.1128/JCM.05848-11 PMID: 22170915

32. Young C, Chawla A, Berardi V, Padbury J, Skowron G and Krause PJ (2012) Preventing transfusion-transmitted babesiosis: preliminary experience of the first laboratory-based blood donor screening program. Transfusion 52: 1523–1529. https://doi.org/10.1111/j.1537-2995.2012.03612.x PMID: 22452654

33. Bloch EM, Hernaldt BL, Leiby DA, Shaieb A, Herron RM, Chervenak M, et al. (2012) The third described case of transfusion-transmitted Babesia duncanii. Transfusion 52: 1517–1522. https://doi.org/10.1111/j.1537-2995.2011.03467.x PMID: 22168221

34. Rolland L, Bent SJ, Krause PJ, Usmani-Brown S, Steeves TK, States SL, et al. (2013) Quantitative PCR for detection of Babesia microti in xodae scapularis ticks and in human blood. Vector Borne Zoonotic Dis 13: 784–790. https://doi.org/10.1089/vbz.2011.0935 PMID: 24107203

35. Johnson ST, Van Tassell ER, Tonnetti L, Cable RG, Berardi VP and Leiby DA (2013) Babesia microti real-time polymerase chain reaction testing of Connecticut blood donors: potential implications for screening algorithms. Transfusion 53: 2644–2649. https://doi.org/10.1111/trf.12125 PMID: 23445322

36. Tonnetti L, Thorp AM, Deisting B, Bachowski G, Johnson ST, Wey AR, et al. (2013) Babesia microti seroprevalence in Minnesota blood donors. Transfusion 53: 1698–1705. https://doi.org/10.1111/j.1537-2995.2012.03948.x PMID: 23145838

37. Wang G, Villafuerte P, Zhuge J, Visintainer P and Wormser GP (2015) A comparison of a quantitative PCR assay with peripheral blood smear examination for detection and quantitation of Babesia microti infection in humans. Diagn Microbiol Infect Dis 82: 109–113. https://doi.org/10.1016/j.diagmicrobio.2015.03.010 PMID: 25861873

38. Wang G, Wormser GP, Zhuge J, Villafuerte P, Ip D, Zeren C, et al. (2015) Utilization of a real-time PCR assay for diagnosis of Babesia microti infection in clinical practice. Ticks Tick Borne Dis 6: 376–382. https://doi.org/10.1016/j.ttbdis.2015.03.001 PMID: 25819568

39. Wilson M, Glaser KC, Adams-Fish D, Boley M, Mayda M and Molestina RE (2015) Development of droplet digital PCR for the detection of Babesia microti and Babesia duncanii. Exp Parasitol 149: 24–31. https://doi.org/10.1016/j.exppara.2014.12.003 PMID: 25500215

40. Ohmori S, Nagano-Fujii M and Saito-Ito A (2016) Development of absolute quantification method for genotype-specific Babesia microti using real-time PCR and practical experimental tips of real-time PCR. Parasitol Int 65: 567–571. https://doi.org/10.1016/j.parint.2016.03.003 PMID: 26972181

41. Bish EK, Moritz ED, El-Amir H, Bish DR and Stramer SL (2016) Cost-effectiveness of a Babesia microti blood donation intervention based on real-time prospective screening in endemic areas of the United States. Transfusion 56: 775–777. https://doi.org/10.1111/trf.13453 PMID: 26954455

42. Mareeud N, Schottohofer AM, Tompkins J, Hall MC, Fritsche TR and Frost HM (2017) Risk Factors for Severe Infection, Hospitalization, and Prolonged Antimicrobial Therapy in Patients with Babesiosis. Am J Trop Med Hyg 97: 1216–1225. https://doi.org/10.4269/ajtmh.17-0146 PMID: 28722598

43. Schlachter S, Chan K, Marras SAE and Parveen N (2017) Detection and Differentiation of Lyme Spirochetes and Other Tick-Borne Pathogens from Blood Using Real-Time PCR with Molecular Beacons. Methods Mol Biol 1616: 155–170. https://doi.org/10.1007/978-1-4939-7037-7_10 PMID: 28600768

44. Souza SS, Bishop HS, Sprinkle P and Qvarnstrom Y (2016) Comparison of Babesia microti Real-Time Polymerase Chain Reaction Assays for Confirmatory Diagnosis of Babesiosis. Am J Trop Med Hyg 95: 1413–1416. https://doi.org/10.4269/ajtmh.16-0406 PMID: 27928088

45. Akoolo L, Schlachter S, Khan R, Alter L, Rojzman AD, Gedroc K, et al. (2017) A novel quantitative PCR detects Babesia infection in patients not identified by currently available non-nucleic acid amplification tests. BMC Microbiol 17: 16. https://doi.org/10.1186/s12866-017-0929-2 PMID: 28088177

46. Chan K, Marras SA and Parveen N (2013) Sensitive multiplex PCR assay to differentiate Lyme spirochetes and emerging pathogens Anaplasma phagocytophilum and Babesia microti. BMC microbiology 13: 295. https://doi.org/10.1186/1471-2180-13-295 PMID: 24359556

47. Steere AC, McHugh G, Damle N and Sikand VK (2008) Prospective study of serologic tests for Lyme disease. Clin Infect Dis 47: 188–195. https://doi.org/10.1086/589242 PMID: 18532885

48. Burbano PD, Issa AT, Ching KH, Cohen JI, Iadarola MJ and Marques A (2010) Rapid, simple, quantitative, and highly sensitive antibody detection for Lyme disease. Clin Vaccine Immunol 17: 904–909. https://doi.org/10.1128/CVI.00476-09 PMID: 20392886

49. Wormser GP, Schriever M, Aguero-Rosenfeld ME, Levin A, Steere AC, Nadelman RB, et al. (2013) Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease. Diagn Microbiol Infect Dis 75: 9–15. https://doi.org/10.1016/j.diagmicrobio.2012.09.003 PMID: 23062467

50. Bakken LL (2002) Role of experience and context in learning to diagnose Lyme disease. J Contin Educ Health Prof 22: 131–141. https://doi.org/10.1002/chp.1340220302 PMID: 12227235
51. Wright WF, Riedel DJ, Talwani R and Gilliam BL (2012) Diagnosis and management of Lyme disease. Am Fam Physician 85: 1086–1093. PMID: 22962880

52. Aguero-Rosenfeld ME, Wang G, Schwartz I and Wormser GP (2005) Diagnosis of Lyme borreliosis. Clinical microbiology reviews 18: 484–509. https://doi.org/10.1128/CMR.18.3.484-509.2005 PMID: 16020686

53. Wilske B, Fingerle V and Schulte-Spechtel U (2007) Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol Med Microbiol 49: 13–21. https://doi.org/10.1111/j.1574-695X.2006.00139.x PMID: 17266710

54. Joss AW, Evans R, Mavin S, Chatterton J and Ho-Yen DO (2008) Development of real time PCR to detect Toxoplasma gondii and Borrelia burgdorferi infections in postal samples. J Clin Pathol 61: 221–224. https://doi.org/10.1136/jcp.2007.048801 PMID: 17496189

55. Martinez-Balzano C, Hess M, Malhotra A and Lenox R (2015) Severe babesiosis and Borrelia burgdorferi co-infection. QJM 108: 141–143. https://doi.org/10.1093/qjmed/hcs100 PMID: 22685248