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Screening of patient blood samples for babesiosis using enzymatic assays

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Abstract

Human babesiosis is an emerging tick-borne disease in the United States and Europe. Transmitted by Ixodes ticks, the causative agent *Babesia microti* is an intraerythrocytic parasite that causes mild to deadly disease. Transmission of *B. microti* can also occur by transfusion of infected blood and blood products resulting in transfusion-transmitted babesiosis (TTB), which carries a high risk of fatality. To effectively manage this rise in *B. microti* infections, better screening tools are needed, which require minimal manipulation of the samples before testing. To this end, we tested two enzymatic assays, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), for efficacy in diagnosis of babesiosis. The results show that AST and ALT activity is significantly higher in the plasma of *B. microti*-infected patients. Moreover, statistical analysis revealed that these assays have high sensitivity and positive predictive values, which highlights their usefulness as diagnostics for babesiosis. These standardized enzymatic assays can be used to perform high-throughput, large-scale screens of blood and blood products before they are certified safe for transfusion.

Keywords

Babesia microti; Babesiosis; Aspartate aminotransferase; Alanine aminotransferase; Molecular beacons; Diagnostic test

1. Introduction

Tick-borne infections have been increasing steadily over the last few decades. *Ixodes* tick species transmit a variety of human pathogens, including *Borrelia* species, the causative agents of Lyme disease and *Babesia* species, protozoan parasites which cause human babesiosis (reviewed in (Diuk-Wasser et al., 2016)). In the United States, *B. microti* is the main cause of babesiosis and has been reported in the Northeastern states as well as the Midwestern states of Wisconsin and Minnesota. *Babesia duncani* infections have also been reported in Western, coastal states (Centers for Disease and Prevention, 2012). Reported *B. microti* infections increased from a few sporadic cases in the 1960's to over 1000 new cases

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Appendix A. Supplementary data

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in 2011 when the CDC declared it as a notifiable disease (Centers for Disease and Prevention, 2012). The increasing infection of *Ixodes* tick species by multiple pathogens (Aliota et al., 2014; Eshoo et al., 2014; Franke et al., 2010; Hersh et al., 2014; Lommano et al., 2012) is thought to enhance the transmission of *B. microti* to reservoir hosts and humans (Diuk-Wasser et al., 2014; Diuk-Wasser et al., 2016; Dunn et al., 2014).

Babesia species infects red blood cells. Active infection results in lysis of infected red blood cells, which can lead to hemolytic anemia. In addition to tick-mediated transmission, *Babesia* can also be transmitted by transfusion of infected blood and blood products, resulting in transfusion-transmitted babesiosis (TTB) (Herman et al., 2010; Herwaldt et al., 2011; Sinski et al., 2011). Clinical manifestations of babesiosis in previously healthy individuals range from asymptomatic to influenza-like symptoms; however, in elderly and immunocompromised individuals, babesiosis has serious consequences and can be fatal (Chiang and Haller, 2011; Holler et al., 2013; Hunfeld et al., 2008; Kjemtrup and Conrad, 2000; Poisnel et al., 2013; Setty et al., 2003; Van Vugt et al., 2011; Yoshinari et al., 2003).

The currently used diagnostic methods for babesiosis are inefficient and labor-intensive. Microscopic evaluation of Giemsa-stained blood smears is a confirmatory diagnostic method; however, it is limited in its application, since low levels of parasitemia are difficult to detect. Serology-based diagnosis can also be used (Foppa et al., 2002; Lempereur et al., 2015; Ryan et al., 2001), but this is ineffective until the patient produces a humoral immune response. In addition, serological tests cannot distinguish past infections from active infections. Importantly, these methods are not ideal for testing a large number of samples for active disease, as the former is time-consuming and requires specific expertise, and neither can reliably detect acute disease. This is especially important for testing donated blood for *B. microti* infection. In fact, the FDA has recommended babesiosis testing of blood donated for transfusion, but there is presently no approved test available for large-scale application (BPAC, 2015).

In this report, we describe the evaluation of two enzymatic assays, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as diagnostics for *B. microti* infection. Previous studies have determined that these enzymes show elevated plasmatic activity associated with liver injury due to genetic disorders, microbial/viral infections and veterinary babesiosis (Dkhil et al., 2010; Furlanello et al., 2005; Shmagel et al., 2016; Zulfiqar et al., 2012; Zygnier et al., 2007). Our results show that in the absence of any known predisposition of patients to liver disease, AST and ALT assays can screen for Babesia infection in patients.

2. Materials and methods

2.1. Human samples

Results from blood samples from 108 patients collected in 2015 from three different counties in New Jersey (Monmouth, Ocean, and Morris counties) are reported in this study. In Monmouth and Ocean counties, samples were collected by the Meridian hospitals/Jersey Shore University Medical Center (JSUMC), while the Gedroic Center collected samples from Morris County. At each medical facility, patients presenting with different clinical

symptoms were sent for evaluation for tick-borne illnesses. Consequently, differential diagnosis for tick-borne illnesses was performed for each patient at the admitting medical facility. Evaluation procedure for *B. microti* infection was described previously (Akoolo et al., 2017). Blood samples from symptomatic patients were sent for testing. Testing for *B. microti* infection by microscopic evaluations (at diagnostic laboratories), and using qPCR (by our laboratory) was described previously (Akoolo et al., 2017).

Samples collected at Gedroic Center and at JSUMC were serially numbered to protect patient confidentiality, beginning with the initials KG and J, respectively. Aliquots of these were sent to our laboratory for analyses, in a coded manner such that the patients remained unidentified to us.

All patients provided informed consent for blood collection in Ethylenediaminetetraacetic acid (EDTA) containing tubes for testing for infectious diseases. After retrieval of aliquots to send to the testing laboratories (IGeneX or JSUMC), the remaining samples were provided to our laboratory in a coded, de-identified manner to preserve anonymity. Enzymatic assays and qPCR with human blood were conducted under the exempt protocol at New Jersey Medical School (NJMS), in accordance with the ethical standards of the responsible committee on human experimentation, the Institutional Review Board (IRB) 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.

2.2. AST/ALT enzymatic assays

AST and ALT enzymatic assays were performed following manufacturer's instructions to detect abnormal liver function known to be associated with veterinary babesiosis (Abcam: ALT kit: ab105134; AST kit: ab105135). Patient plasma samples were used at optimized 1:15 dilutions, and enzymatic activity measured when colorless probes were converted to colored/fluorescent products to detect glutamate produced by AST and pyruvate by ALT. Kinetic analyses were conducted at three-minute intervals and data analyzed according to manufacturer recommendations. To establish threshold values for each assay, we used two patient samples that were negative for *B. microti* infection by three alternative tests. AST and ALT values for these samples were used to establish a reference range for normal enzymatic activity, such that an abnormal level is defined as a value exceeding the upper reference limit.

2.3. Statistical analyses

Donald Alderson at Rutgers University Biostatistics and Epidemiology Services Center conducted statistical analyses for the assays. Calculations for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were carried out as previously described (Akoolo et al., 2017).

3. Results and discussion

TTB is a growing threat due to the transfusion of blood donated by asymptomatic but *Babesia*-infected individuals. Blood transfusion usually involves already sick and weakened

patient recipients, and thus it carries a high risk of increased morbidity and mortality. In fact, the FDA reported that *B. microti* is the most frequently transmitted pathogen by blood transfusion in the U.S.A. (Fang and McCullough, 2016; Levin et al., 2016), and results in a high percentage (27%) of fatalities caused by babesiosis due to blood transfusion during 2010–2014). An even higher rate of 71% (5 out of 7) transfusion-associated deaths was reported due to babesiosis in 2008. Donated blood is not currently screened for babesiosis; however, due to the increasing TTB-risk, the FDA has recommended that blood supplies be tested for infection by *B. microti*.

One possible strategy to screen for *B. microti* infection on a large scale is to use a high-throughput method, which requires limited sample manipulations, to prescreen all samples that are evidently infected. AST and ALT enzymatic assays detect elevated plasmatic activity associated with liver injury observed in veterinary babesiosis (Dkhil et al., 2010; Furlanello et al., 2005; Shmagel et al., 2016; Zulfikar et al., 2012; Zygner et al., 2007). AST also detects hemolytic anemia, an important symptom associated with babesiosis. We determined ALT and AST activities in patient plasma and compared results with our qPCR, and microscopic examination of blood smears either after Giemsa-staining or after FISH. We previously showed a high agreement in babesiosis diagnosis using qPCR and microscopic examination of Giemsa-stained blood smears or FISH (Akoolo et al., 2017).

For this study, we retrospectively tested 108 patient plasma samples to assess the usefulness of AST and ALT as a diagnostic for babesiosis. We found that samples diagnosed positive for babesiosis displayed significantly higher enzymatic activity compared to babesiosis negative samples (Figs. 1 and 2). Interestingly, the increase in AST activity was significantly higher in patient samples that were positive by qPCR ($P = 0.0275$) as well as in those positive by microscopy ($P = 0.0021$) (Fig. 1A and B). Further analysis of this data showed an overall positive correlation between elevated AST activity and increasing *B. microti* levels measured by qPCR (Supplementary Fig. 1A). This suggests that the increase in AST activity was likely due to *Babesia*-mediated abnormal liver function. For the ALT enzymatic assays, plasmatic activity of ALT was also higher in patients who tested positive by qPCR, but the difference between positive and negative samples was not statistically significant ($P = 0.2466$) (Fig. 2A), neither was there a strong correlation between *B. microti* levels by qPCR and increase in ALT activity (Supplementary Fig. 1B). However, ALT showed significantly higher activity ($P = 0.0203$) in patient samples tested positive by microscopic examination of Giemsa stained blood smears (Fig. 2B). When compared to qPCR, the sensitivity of AST and ALT assays were 63% and 48.9% and specificity 50% and 56.3%, respectively. When compared to the microscopic tests, however, both assays show a dramatic increase of up to 30 points in sensitivity, while ALT also has a higher specificity (Table 1). Additionally, the positive predictive value for both AST and ALT are high, 87.9% and 86.5%, respectively (compared to qPCR). Altogether, these results show a high agreement of positive babesiosis diagnosis among all three methods (Fig. 3, Supplementary Table 1), and demonstrate the usefulness of AST and ALT as prescreening enzymatic assays for babesiosis.

Asymptomatic but *Babesia*-infected individuals are the main cause of transfusion-transmitted babesiosis. To investigate the usefulness of AST and ALT enzymatic assays to

detect infection in asymptomatic patients, we tested forty-nine plasma samples from asymptomatic patients. Interestingly, we found that 47% had elevated AST activity, while ALT activity was elevated in 33% of these samples (Fig. 4). This suggests that some of those asymptomatic patients may have been infected by *B. microti*. Moreover, the difference in enzymatic activity levels between elevated and normal samples was highly significant, with a P-value of 0.0004 for ALT and $P < 0.0001$ for AST. This suggests that very few samples had borderline enzymatic activity, either just above or below the cutoff, lending further credence that this may be the result of active infections in these asymptomatic patients.

As babesiosis often remains asymptomatic in both acute and chronic stages of infection, donated blood samples are not tested (Krause et al., 2008; Krause et al., 1998). Microscopic examination of blood smears, although confirmatory, works well only with fresh blood and not with refrigerated samples. Our work here shows that it is possible to conduct AST and ALT enzymatic assays with blood and plasma samples after cold-storage; furthermore, these tests can be used to effectively screen blood samples on a large scale, since minimum sample processing is required. These assays both have a high positive predictive value relative to qPCR, which indicates that the tests have high predictive ability for active Babesia infection and hence, even asymptomatic babesiosis. Indeed, elevated enzymatic activity was also detected in asymptomatic patient samples (Fig. 4). The assays had a very low negative predictive value (16% and 19%) when compared to qPCR (Supplementary Table 2), however, which could be attributed to our testing of only a few true negative samples, and potentially high falsenegative outcomes suggesting higher enzyme activities may not persist when parasitemia is reduced.

Although we tested AST and ALT in patient samples as complementary tests for babesiosis, these assays can be used as preliminary screening to test donated blood samples for babesiosis because they do not require any preparation and can be used as a high-throughput method. The power of this method is further improved by the ability of these enzymatic assays to detect abnormal activity levels in asymptomatic patients (Fig. 4). Additionally, there was no significant variation in AST and ALT activity among adult patients of different age groups (Supplementary Fig. 2), suggesting that the test can be reliably applied to any person of legal blood-donor age. While this assay shows great promise for streamlining the testing process to detect babesiosis in donated blood samples, a caveat should be considered that these enzymes are associated with liver malfunction and increase in their activities can be due to other infectious diseases and even non-infectious diseases such as fatty liver disease. The presence of such diseases may explain why some patient samples show no parasite infection by qPCR, but still produce high enzymatic activity (Supplementary Fig. 1). Therefore, prescreened positive blood samples can be confirmed by qPCR, if needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

qPCR	quantitative PCR
TTB	transfusion-transmitted babesiosis
AST	aspartate aminotransferase
ALT	alanine aminotransferase
FISH	fluorescence in situ hybridization
CDC	Centers for Disease Control and Prevention
FDA	US Food and Drug Administration
PPV	positive predictive value
NPV	negative predictive value

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Fig. 1.

Aspartate aminotransferase (AST) activity is elevated in plasma of *B. microti*-infected human samples. (A) Comparison of AST activity in selected patient samples that were previously diagnosed as positive or negative for *B. microti* infection by qPCR, or (B) microscopic examination of blood smears. AST activity is significantly higher in samples that tested positive for *B. microti* infection by qPCR or microscopy, compared to the negative samples. Significance determined by two-tailed unpaired *t*-test (* $P < 0.05$, ** $P < 0.01$).

Fig. 2.

Alanine aminotransferase (ALT) activity is elevated in plasma of *B. microti*-infected human samples. (A) ALT activities are shown in selected patient samples that were previously diagnosed as positive or negative for *B. microti* infection by qPCR. ALT activity increased in *B. microti*-infected samples detected by qPCR but the difference is not statistically significant. (B) ALT activity is significantly higher in samples tested positive by microscopic examination of blood smears as compared to the negative samples. Significance determined by twotailed unpaired *t*-test (**P* < 0.05).

Fig. 3.

Venn diagram summarizing the agreement in positive diagnosis of babesiosis in patients using different diagnostic methods. Diagnosis of *B. microti* infection by ALT and AST enzymatic assays together overlaps with the positive results by qPCR and microscopic methods.

Fig. 4. Elevated AST and ALT activity in plasma of asymptomatic human samples. (A) AST activity is significantly higher in 47% of asymptomatic patient samples. (B) ALT activity is significantly higher in 33% of asymptomatic patient samples. Significance determined by two-tailed unpaired *t*-test (****P* < 0.001, *****P* < 0.0001).

Table 1

Diagnosis of babesiosis by two plasmatic/liver enzymatic activities and microscopy.

Diagnostic test	Microscopy		Total	Standard Error	95% CI (Lower, Upper Bound)
	Positive	Negative			
AST Positive	32	11	43		
AST Negative	5	11	16		
Sensitivity	86.5%			5.6%	(75.5%, 97.5%)
Specificity	50.0%			10.7%	(29%, 71%)
PPV	74.4%			6.7%	(61.3%, 87.5%)
NPV	68.8%			11.6%	(46.1%, 91.5%)
ALT Positive	29	7	36		
ALT Negative	8	15	23		
Sensitivity	78.4%			6.8%	(65.1%, 91.7%)
Specificity	68.2%			9.9%	(48.6%, 87.4%)
PPV	80.6%			6.6%	(67.6%, 93.4%)
NPV	65.2%			9.9%	(45.6%, 84.4%)

CI, Confidence Interval

Sensitivity: No. of test positives/No. of microscopy positives

Specificity: No. of test negatives/No. of microscopy negatives