

## Serologic screening of United States blood donors for *Babesia microti* using an investigational enzyme immunoassay

Andrew E. Levin,<sup>1</sup> Phillip C. Williamson,<sup>2</sup> Evan M. Bloch,<sup>3,4</sup> Joan Clifford,<sup>1</sup> Sherri Cyrus,<sup>2</sup> Beth H. Shaz,<sup>5</sup> Debra Kessler,<sup>5</sup> Jed Gorlin,<sup>6</sup> James L. Erwin,<sup>1</sup> Neil X. Krueger,<sup>1</sup> Greg V. Williams,<sup>1</sup> Oksana Penezina,<sup>1</sup> Sam R. Telford IV,<sup>7</sup> John A. Branda,<sup>8</sup> Peter J. Krause,<sup>9</sup> Gary P. Wormser,<sup>10</sup> Anna M. Schotthoefer,<sup>11</sup> Thomas R. Fritsche,<sup>11</sup> and Michael P. Busch<sup>3</sup>

**BACKGROUND:** The tick-borne pathogen *Babesia microti* has become recognized as the leading infectious risk associated with blood transfusion in the United States, yet no Food and Drug Administration–licensed screening tests are currently available to mitigate this risk. The aim of this study was to evaluate the performance of an investigational enzyme immunoassay (EIA) for *B. microti* as a screening test applied to endemic and nonendemic blood donor populations.

**STUDY DESIGN AND METHODS:** The study aimed to test 20,000 blood donors from areas of the United States considered endemic for *B. microti* and 10,000 donors from a nonendemic area with the investigational *B. microti* EIA. Repeat-reactive samples were retested by polymerase chain reaction (PCR), blood smear, immunofluorescent assay (IFA), and immunoblot assay. In parallel, serum samples from symptomatic patients with confirmed babesiosis were tested by EIA, IFA, and immunoblot assays.

**RESULTS:** A total of 38 of 13,757 (0.28%) of the donors from New York, 7 of 4583 (0.15%) from Minnesota, and 11 of 8363 (0.13%) from New Mexico were found repeat reactive by EIA. Nine of the 56 EIA repeat-reactive donors (eight from New York and one from Minnesota) were positive by PCR. The specificity of the assay in a nonendemic population was 99.93%. Among IFA-positive clinical babesiosis patients, the sensitivity of the assay was 91.1%.

**CONCLUSION:** The *B. microti* EIA detected PCR-positive, potentially infectious blood donors in an endemic population and exhibited high specificity among uninfected and unexposed individuals. The EIA promises to provide an effective tool for blood donor screening for *B. microti* in a format amenable to high-throughput and cost-effective screening.

**T**ransfusion-transmitted babesiosis caused by the tick-borne parasite *Babesia microti* has emerged in recent years as the foremost infectious risk of blood transfusion in the United States for which

**ABBREVIATIONS:** CTS = Creative Testing Solutions; IFA = immunofluorescent assay; S/CO = signal/cutoff.

From <sup>1</sup>ImmuneNetics, Inc., Boston, Massachusetts; <sup>2</sup>Creative Testing Solutions, Tempe, Arizona; <sup>3</sup>Blood Systems Research Institute, San Francisco, California; <sup>4</sup>Johns Hopkins University School of Medicine, Baltimore, Maryland; <sup>5</sup>New York Blood Center, New York, New York; <sup>6</sup>Innovative Blood Resources/Memorial Blood Centers, St Paul, Minnesota; <sup>7</sup>Tufts University Cummings School of Veterinary Medicine, North Grafton, Massachusetts; <sup>8</sup>Massachusetts General Hospital, Boston, Massachusetts; <sup>9</sup>Yale School of Public Health and Yale School of Medicine, New Haven, Connecticut; <sup>10</sup>New York Medical College, Valhalla, New York; and <sup>11</sup>Marshfield Clinic, Marshfield, Wisconsin.

Address reprint requests to: Andrew E. Levin, PhD, ImmuneNetics, Inc., 27 Drydock Avenue, Boston, MA 02210; e-mail: alevin@immunetics.com.

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no test licensed by the US Food and Drug Administration (FDA) is available.<sup>1-3</sup> Of the *Babesia* species that have been shown to cause human disease, *B. microti* is the overwhelmingly predominant species in the United States.<sup>4</sup> As *B. microti* is transmitted by the same *Ixodes* ticks that transmit *Borrelia burgdorferi* (the agent of Lyme disease), the two pathogens have similar endemic foci in the Northeast and Upper Midwest. Acquisition of *B. microti* through a tick bite most often results in transient, viral-like symptoms in healthy individuals, but those with compromised immune systems, including the very young, those of advanced age, and those without a spleen or with cancer or human immunodeficiency virus (HIV), are at risk for severe illness.<sup>4,5</sup> The ability of the parasite to establish asymptomatic infection in immunocompetent hosts, coupled with its survival for lengthy periods of time in blood donations puts transfusion recipients at risk.<sup>1,5</sup> More than 150 cases of transfusion-transmitted babesiosis have been described since 1979, approximately one-fifth of which were fatal.<sup>6</sup>

At present there is no effective means to identify and remove infected blood donors from the donor pool, as risk factors for *Babesia* infection are insufficiently specific to be incorporated into donor health questionnaires and no blood screening tests for *B. microti* have been licensed by the FDA. Acute babesiosis has traditionally been diagnosed through examination of Giemsa-stained blood smears as for malaria.<sup>4</sup> More recently, polymerase chain reaction (PCR) tests have been developed which detect *B. microti* DNA.<sup>7-10</sup> Individuals who are infected with *B. microti* also develop an immune response which is detectable by serologic assays such as immunofluorescent assay (IFA), enzyme-linked immunosorbent assay, and immunoblot.<sup>11-13</sup> Antibody titers and direct markers of infectivity in asymptomatic blood donors, however, have only recently been evaluated.<sup>14-20</sup>

Previously, we described the development of an enzyme immunoassay (EIA) for human IgG and IgM antibodies to *B. microti* based on a combination of immunodominant peptide antigens.<sup>20</sup> A study of approximately 27,000 US blood donors was carried out under an FDA-approved investigational new device exemption (IND) to determine the seroprevalence of *B. microti* in both endemic and nonendemic donor populations using the investigational *B. microti* EIA to support licensure of the assay. This report describes the results of the blood donor study and performance of the EIA on sera from clinical cases of babesiosis.

## MATERIALS AND METHODS

### Study design and donor populations

The donor study was designed to measure *B. microti* seroprevalence with the investigational EIA in endemic and nonendemic regions of the United States. Donors at least

18 years of age in Suffolk and Nassau Counties, New York—highly and moderately endemic areas, respectively—were enrolled by New York Blood Center. Donors in Minneapolis, Minnesota—a moderately endemic area—were enrolled by Memorial Blood Centers division of Innovative Blood Resources. Donors in New Mexico, a nonendemic area, were enrolled by United Blood Services division of Blood Systems, Inc. All donor protocols were approved by the respective institution's institutional review board. Donors were provided factual information on this research study and enrollment was contingent upon signing a general informed consent permitting use of the donor's blood samples for *Babesia* research. Samples collected for the study represented routine donor collections at the study sites. Other than excluding some D- and platelet donors, autologous donors, and all donors under 18 years of age, no other selective criteria were applied. A serum sample was obtained from each donor for testing by EIA, IFA, and immunoblot, and a whole blood sample, for PCR and blood smear. EIA, IFA, and blood smear testing were carried out by Creative Testing Solutions (CTS) in Tempe, Arizona, Chicago, Illinois, and Bedford, Texas, while PCR and immunoblot were carried out at Blood Systems Research Institute (San Francisco, CA) and Immunetics (Boston, MA), respectively. Per study design (Fig. 1), donor serum samples underwent initial testing by *B. microti* EIA at CTS. Initially reactive (defined as signal/cutoff [S/CO]  $\geq 1$ ) and gray zone ( $0.934 \leq S/CO < 1.0$ ) samples were retested by EIA in duplicate at CTS. The purpose of the gray zone was to identify any donor samples that were reactive below the provisional cutoff (see below) and were positive by either PCR or blood smear, to determine a final cutoff based on study data. Repeat-reactive samples were defined as those with at least two of three reactive ( $S/CO \geq 1.0$ ) results. Repeat gray zone samples were defined as those with an initially reactive or gray zone result and at least one subsequent gray zone result. Samples that were initially nonreactive ( $S/CO < 0.934$ ) as well as those with an initially reactive or gray zone result that were nonreactive in both duplicate retests were considered nonreactive. Serum aliquots from donors with repeat reactive or repeat gray zone EIA results were tested by IFA and immunoblot, while processed whole blood-derived aliquots from the same donors were tested in parallel by PCR and blood smear. Donors that were found repeat reactive by EIA received notice within 1 week of donation and were deferred indefinitely from further blood donations. The associated blood products were not released for transfusion, but aliquots were retained by CTS for further research use.

### Clinical babesiosis study

Serum samples were obtained from 129 symptomatic patients residing in endemic areas of the Northeast and

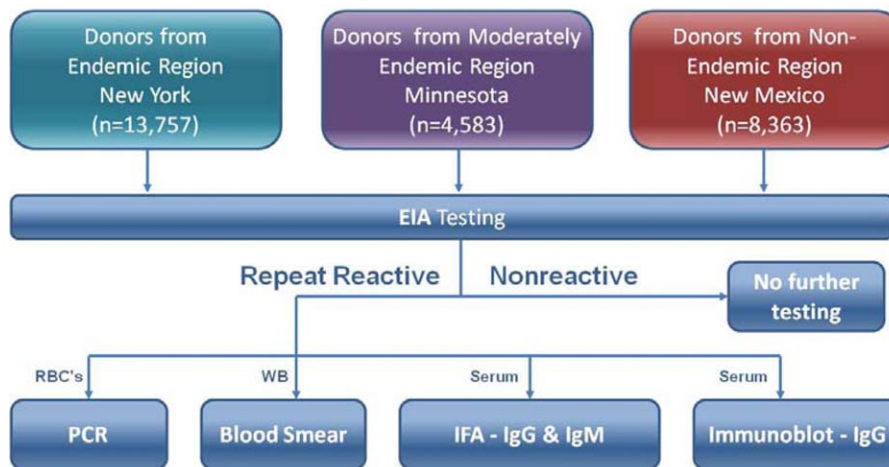


Fig. 1. Flow chart showing the study populations tested and the scheme for initial testing by EIA followed by retesting of repeat-reactive samples by PCR, blood smear, IFA, and immunoblot.

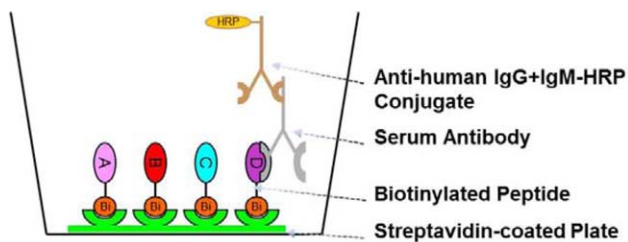


Fig. 2. Schematic of EIA principle. Four biotinylated peptides representing immunodominant sequences from *B. microti* antigens BMN1-9 and BMN1-17 were combined and immobilized to the microplate well. The assay detected both IgG and IgM antibodies in a single well.

Midwest that had been diagnosed with babesiosis based on clinical presentation, history, and a positive PCR or blood smear result, irrespective of serologic status. Samples were tested with the *B. microti* EIA at CTS, by EIA and immunoblot at Immunetics, and by IFA at Tufts University.

**Cross-reactivity, interfering substances, and reproducibility studies**

The EIA was evaluated for cross-reactivity with other disease conditions by testing serum samples from patients with *Trypanosoma cruzi*, *Leishmania*, influenza, cytomegalovirus, Epstein-Barr virus, hepatitis C, HIV, *Plasmodium falciparum*, *Plasmodium vivax*, *Toxoplasma gondii*, *B. burgdorferi*, and *Anaplasma phagocytophilum* infections. Performance of the assay in the presence of rheumatoid factor and 18 other potentially interfering substances was also evaluated. Reproducibility was measured by testing replicates of a set of moderately reactive, weakly reactive, and nega-

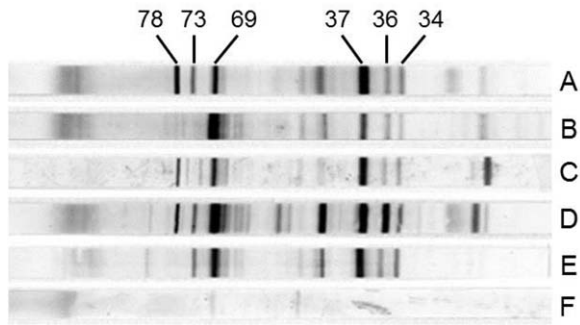
tive samples across different EIA kit lots, operators, days, and sites.

**EIA**

The *B. microti* EIA used in the study has been described previously.<sup>20</sup> In brief, the EIA uses four peptide antigens derived from two members of the BMN1 gene family, BMN1-9 (also termed BmSA1) and BMN1-17, which have been shown to be immunodominant in prior studies.<sup>21-23</sup> The four peptide antigens were identified by screening candidate peptides against a library of sera from patients with clinically diagnosed babesiosis as well as healthy controls. The biotinylated peptides were immobilized on streptavidin-coated microplates (Fig. 2). Serum samples diluted 100-fold were incubated for 30 minutes on the microplate, after which unbound antibodies were removed by washing with buffer. Bound antibodies were detected by incubation with an anti-human IgG/IgM horseradish peroxidase (HRP) conjugate for 30 minutes, followed by further buffer washes. Microplates were incubated with a soluble peroxidase substrate (tetramethylbenzidine) for 10 minutes, after which a stop reagent was applied, and absorbance was read at 450 nm. A provisional cutoff based on prior studies<sup>20</sup> was calculated by adding a fixed value (0.355) to the mean absorbance in three negative control wells.

**IFA**

Blood donor samples were tested by IFA as described by Krause and colleagues<sup>11</sup> using *B. microti* substrate slides obtained from Fuller Laboratories. IgG and IgM antibodies were detected separately using corresponding fluorescein isothiocyanate-labeled goat anti-human antibody conjugates (Jackson ImmunoResearch). Slides were analyzed with a microscope (Model BX43, Olympus) equipped with



**Fig. 3. Representative *B. microti* immunoblots.** Molecular weights (kDa) of the six bands on which interpretive criteria are based are shown at top. Samples tested were (A) positive control; (B) PCR-positive blood donor from New York; (C) PCR-positive blood donor from Minnesota; (D) PCR-positive clinical babesiosis patient from Massachusetts; (E) blood smear-positive clinical babesiosis patient from Connecticut; (F) EIA-nonreactive blood donor from Arizona, a nonendemic region.

rhodamine and fluorescein filters at 400 $\times$  magnification. IFA results were interpreted as positive or negative based on a cutoff of 1:128, which was selected based on studies with previously characterized clinical and blood donor samples (data not shown).

### Immunoblot

Parasites harvested from infected hamster blood and enriched by differential centrifugation were lysed and extracted in sodium dodecyl sulfate buffer. The lysate was separated by polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. After incubation of membrane strips with human serum samples, bound IgG antibodies were detected using an anti-human IgG HRP conjugate. Immunoblot interpretive criteria were developed using panels of well-characterized sera from clinical babesiosis patients and controls. A positive result was defined as antibody reactivity with a 37-kDa antigen band previously identified as BMN1-9 plus reactivity with at least one of a set of bands migrating at 34, 36, 69, 73, and 78 kDa. All other band patterns were interpreted as negative (Fig. 3). With these scoring criteria, 87.3% (96/110) of PCR-positive or blood smear-positive clinical case sera and 97.6% (82/84) of IFA-positive sera, some of which were used in assay development, were immunoblot positive. Conversely, 100% (106/106) of a set of EIA nonreactive sera from low-risk blood donors in Arizona were immunoblot negative.

### Peripheral blood smear

Peripheral blood smears were prepared from EDTA-preserved whole blood samples using the method of Houwen<sup>24</sup> and allowed to air dry completely before staining. A

minimum of two microscope slides were prepared as thin smears from each donor sample collected from *B. microti*-endemic areas. Smears were stained using Wright's-Giemsa stain (Easy I, Azer Scientific). Microscopic examination to detect the presence of *B. microti* or other blood parasites, and quantification of the organisms detected, was performed according to the method of Blevis and coworkers<sup>25</sup> utilizing NCCLS-recommended standards (300 fields at 1000 $\times$  per slide). Any positive or inconclusive results were confirmed by independent review before the final interpretation.

### PCR

*B. microti* PCR was carried out according to an algorithm which employed two separate PCR assays, a screening assay followed by a confirmatory assay (Figure 4). The screening assay, based on primers Bab2 and Bab3, has been described previously.<sup>9</sup> The 95% limit of detection for this assay was approximately 13 parasites per 2 mL of whole blood. The confirmatory assay was adapted from a second previously described assay<sup>7</sup> and used a different primer pair, Bab8F/Bab8R. This assay was found to be similar in limit of detection to the screening assay (data not shown). EIA repeat-reactive samples were initially tested by the screening assay in triplicate; if any one of the triplicate results was positive (meeting both quantification cycle and melting temperature criteria), the sample was retested by the confirmatory assay in duplicate. Testing by both screening and confirmatory assays was repeated on those samples found positive by the initial screening assay run. A PCR result was considered as a confirmed positive when both of the confirmatory duplicates were positive (both duplicates meeting quantification cycle and melting temperature criteria) in either the initial or the repeat assay runs.

## RESULTS

Donor enrollment began July 29 and ended November 15, 2013, yielding a total of 28,615 samples. After exclusion of samples that did not meet inclusion criteria, the total numbers of donor samples tested were 13,757 (New York), 4583 (Minnesota), and 8363 (New Mexico). The exact distribution of donors between Suffolk and Nassau Counties, New York, was not determined, but Nassau County was expected to account for the majority based on blood center collection practices.

### EIA and PCR reactivity at the provisional cutoff

Of the 26,703 donor samples tested by EIA, 122 were found repeat reactive and 12 were in the gray zone at the provisional EIA cutoff. Further testing of repeat-reactive samples yielded 37 that were positive by IFA and 40 that were positive by immunoblot (Table 1). Among the 12 EIA



**TABLE 1. Seroprevalence measured by *B. microti* EIA in endemic and nonendemic blood donor populations and clinical babesiosis patients at the provisional cutoff and revised cutoff, compared with detection by PCR, blood smear, IFA, and immunoblot\***

Study population	<i>B. microti</i> EIA repeat reactive		IFA positive, Out of (A)	Immunoblot positive		PCR positive	Blood smear positive
	(A) Provisional C/O	(B) Revised C/O (×1.6)		Out of (A)	Out of (B)		
High-risk endemic (New York), n = 13,757	0.54 (74)	0.28 (38)	0.22 (30)	0.24 (33)	0.15 (21)	0.06 (8)	0 (0)
Moderate-risk endemic (Minnesota), n = 4583	0.35 (16)	0.15 (7)	0.04 (2)	0.02 (1)	0.02 (1)	0.02 (1)	0 (0)
Nonendemic (New Mexico), n = 8363	0.38 (32)	0.13 (11)	0.06 (5)	0.07 (6)	0.06 (5)	0 (0)	0 (0)
Clinical babesiosis PCR/BS positive, n = 129	84.5 (109/129)	77.5 (100/129)	78.3 (101/129)	87.3 (96/110)	87.3 (96/110)	98.1 (102/104)	89.2 (74/83)
IFA positive, n = 101	98.0 (99/101)	91.1 (92/101)	100 (101/101)	97.6 (82/84)	97.6 (82/84)	97.4 (74/76)	86.8 (59/68)

\* Figures indicate percent and total number of reactive or positive samples in each category. Denominators indicate total number of samples tested; in some cases in the clinical babesiosis group, sample volume did not permit the test to be carried out. C/O = cutoff

gray zone samples, three were IFA positive and two were immunoblot positive.

PCR testing of the 134 repeat-reactive and gray zone donor samples yielded six donors who met both screening and confirmatory PCR criteria for positivity, while three donors met screening criteria but were positive by only one of the confirmatory replicates and were therefore identified as probable PCR positives (Table 2). All PCR-positive and probable-positive samples were from endemic regions. IFA titers for eight of the nine samples were at least 1024 and one was 512. All nine samples were positive by immunoblot (Fig. 3). At the provisional cutoff, the EIA S/CO values for the nine samples ranged from 1.62 to 5.75; none were in the gray zone. The frequencies of EIA repeat-reactive and PCR-positive samples were distributed randomly over the 16-week study period, with no obvious pattern. The distribution of EIA absorbance values for donors in the endemic versus nonendemic populations compared with EIA absorbance values for clinical babesiosis patients is shown in Fig. 5. None of the EIA repeat-reactive or gray zone samples were positive by blood smear examination.

**EIA reactivity at a revised cutoff**

An analysis of seroreactivity in blood donors versus EIA cutoff indicated that the cutoff could be increased up to 1.6-fold (the “revised cutoff”) without sacrificing detection of any of the nine PCR-positive or probable positive donor samples. Concurrently, the rate of seroreactivity in the subset of donor samples that were PCR negative declined by 56%, to 0.20% in endemic areas, and by 66%, to 0.13% in the nonendemic area. Results calculated with the

revised cutoff are shown in Table 2. Based on the revised cutoff, the repeat-reactive rate in the endemic population in New York was 0.28%, which was approximately twice the repeat-reactive rate in the endemic Minnesota population (0.15%) or the nonendemic New Mexico population (0.13%). At the revised cutoff, the difference between the frequency of EIA repeat-reactive samples in the endemic New York population and that in the nonendemic New Mexico population was significant (p = 0.03), while at the provisional cutoff, the difference was not significant (p = 0.1). In the nonendemic (New Mexico) population, 32 of 8363 (0.38%) donors were repeat reactive at the provisional cutoff, decreasing to 11 of 8363 (0.13%) at the revised cutoff; of these 11, four were positive by IFA at titers of at least 256 and five were positive by immunoblot, leaving 6 of 8363 (0.07%) as the rate of EIA repeat reactivity among immunoblot-negative donors, presumed to be the EIA false-positive rate.

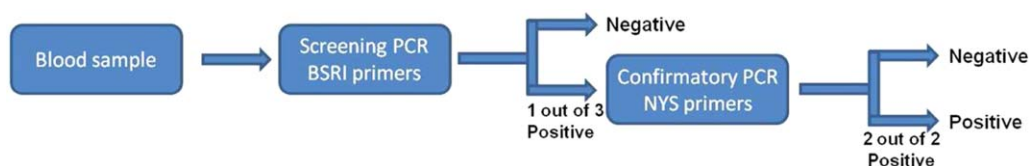
**EIA reactivity in clinical babesiosis patients**

Testing of serum samples from symptomatic patients who were diagnosed with babesiosis, with positive PCR or blood smear results, yielded 109 of 129 samples (84.5%, 95% confidence interval [CI], 77.1%-90.3%) with repeat-reactive results at the provisional EIA cutoff. By comparison, 101 of 129 (78.3%) were positive by IFA. Of the 20 EIA nonreactive clinical samples, 18 were available for further testing. Eleven of these 18 samples were negative by both IFA and immunoblot, while five samples yielded positive EIA results upon retesting. Including these five samples, 98% of IFA-positive samples were reactive by EIA and the correlation between EIA results and IFA or immunoblot was 86

**TABLE 2. Characteristics of the PCR-positive blood donor samples identified in the donor study**

Sample #	Origin	Date Tested	Screening PCR	Confirmatory PCR	EIA S/CO*	IFA IgG	IFA IgM	Immunoblot
1	Nassau County, NY	8/2/2013	Pos	Equiv	1.21	1024		Pos
2	Suffolk County, NY	8/1/2013	Pos	Pos	1.17	1024	128	Pos
3	Suffolk County, NY	9/12/2013	Pos	Pos	2.06	1024	1024	Pos
4	Suffolk County, NY	9/20/2013	Pos	Pos	1.02	1024		Pos
5	Suffolk County, NY	10/24/2013	Pos	Pos	1.05	1024		Pos
6	Suffolk County, NY	11/9/2013	Pos	Pos	2.94	1024		Pos
7	Suffolk County, NY	11/12/2013	Pos	Equiv	3.20	1024		Pos
8	Suffolk County, NY	11/14/2013	Pos	Equiv	2.02	1024		Pos
9	Hennepin County, MN	8/30/2013	Pos	Pos	3.24	512	256	Pos

\* At revised EIA cut-off.



**Fig. 4. Scheme utilized for PCR testing of EIA repeat-reactive blood donors.**

and 96%, respectively. Among the 101 IFA-positive patient sera, increasing the cutoff value 1.6-fold resulted in reclassification of nine samples (7%) as nonreactive, yielding 92 of 101 (91.1%) EIA repeat-reactive samples (Table 1 and Fig. 5).

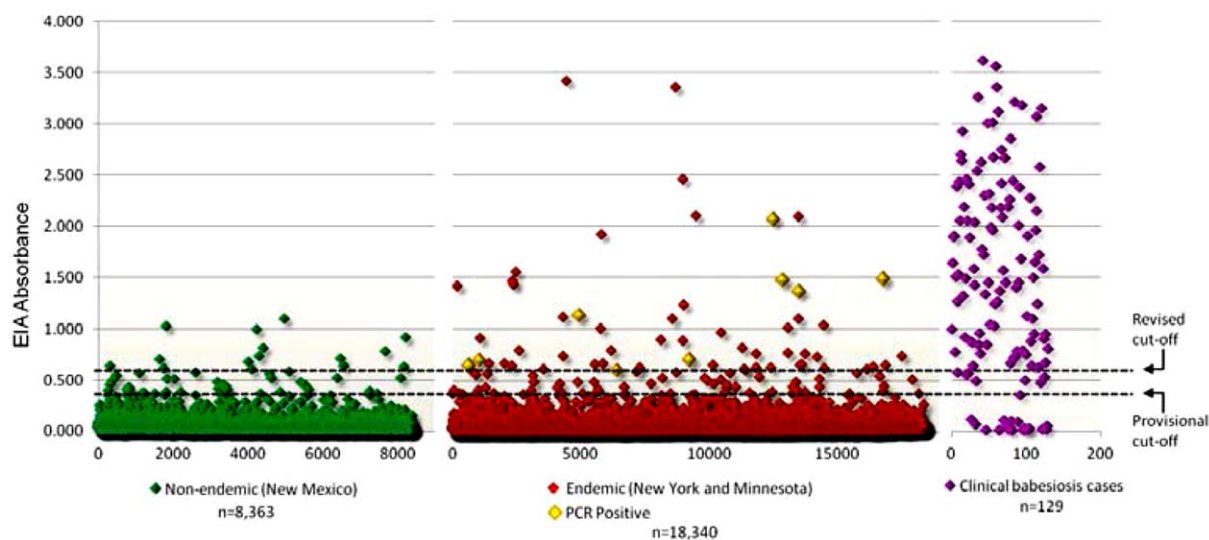
**Reproducibility, interfering substances, and cross-reactivity studies**

Reproducibility of the EIA as measured by replicate testing yielded coefficients of variation (CV) averaging less than 10% across kit lots, operators, days, and sites with no

significant variance due to these factors (not shown). Likewise, none of the interfering substances tested produced any effect on EIA reactivity. Among potentially cross-reactive conditions, only anaplasmosis and Lyme disease yielded one of 20 and four of 20 samples that were reactive in the EIA, respectively; all others were nonreactive.

**DISCUSSION**

A principal aim of this study was to determine the seroprevalence of *B. microti* in blood donors residing in endemic



**Fig. 5. Distribution of absorbance values in the *B. microti* EIA for blood donors from nonendemic (n = 8363) and endemic areas (n = 18,340) and for clinical babesiosis patients (n = 129). The provisional and revised EIA cutoffs are shown as horizontal dashed lines.**

and nonendemic regions of the United States using an investigational EIA. While serologic assays based on IFA have been used for detection of antibodies in clinical patients and blood donors, few EIAs have been developed for this purpose,<sup>13,17,26</sup> and none are currently licensed for blood screening. As the investigational EIA makes use of synthetic peptide antigens rather than whole *B. microti* cells obtained from hamster inoculation as in IFA methods, it offers the benefit of reproducible manufacture and performance, substantiated by the CV values in the reproducibility component of this study. The peptide sequences selected for use in the EIA were derived from known immunodominant antigens BMN1-9 and BMN1-17. EIA testing of well-characterized clinical babesiosis cases from endemic regions in the US Northeast and Midwest yielded high sensitivity of detection with these peptides, suggesting that the sequences are relatively conserved among *B. microti* strains that infect humans. Approximately 80% of PCR- or blood smear-positive clinical case sera were reactive in the peptide EIA, similar to the 81.6% sensitivity reported for an IFA evaluated in blood donor studies by Moritz and colleagues.<sup>19</sup> Clinical case sera that were EIA negative were divided principally between those that showed no detectable antibody by IFA or immunoblot, potentially representing early-stage infections, and a subset in which spurious results were overturned by subsequent retesting. The strong overlap between clinical samples detected by EIA and those detected by IFA further suggests that the peptide antigens present in the EIA recapitulate the dominant antigenic components of the organism. How diverse *B. microti* sequences are in nature, and whether sequence diversity affects antigenicity, infectivity, or pathogenicity, are still unknown.

One of the aims of the study was to optimize the EIA cutoff based on study results. Unlike blood-borne viruses for which antibodies are markers of chronic infection, babesiosis in healthy individuals is typically a transient infection, in which serum antibodies persist long after the infection is resolved.<sup>27</sup> Furthermore, exposure to *B. microti* through tick bites appears to be relatively frequent in endemic regions of the United States as evidenced by seroprevalence rates ranging from 1% to 2.5%.<sup>14,28</sup> This is consistent with our study results that showed that 90% of EIA repeat-reactive donors in endemic areas and 100% in nonendemic areas were PCR and blood smear negative, indicating that the majority of EIA-reactive donors are not likely to be infectious. Such EIA repeat-reactive donors, many of whom have antibodies to *B. microti* that can be verified by IFA or immunoblot, likely represent individuals with prior, resolved infections and with lingering but waning antibody as demonstrated in other studies.<sup>15,29</sup> However, exceptions may occur among seropositive donors with low levels of parasitemia that are detected inconsistently by currently used PCR assays, as shown in a recent study by Leiby and colleagues.<sup>15</sup> As high titers of antibody

have been shown to correlate with active infection,<sup>27,29</sup> an optimal EIA cutoff would desirably discriminate active from prior, resolved infections. Study results indicated that a 1.6-fold increase in EIA cutoff above the provisional value substantially decreased the number of donors that were classified as EIA reactive and would hence be deferred, with no loss in detection of those EIA-reactive donors who were PCR positive and a very modest impact on detection of clinical babesiosis cases. This revised cutoff has been adopted for analysis of the study data for licensure by FDA and for ongoing use of the EIA in prospective screening under IND.

With the revised cutoff, the rate of seroreactivity in endemic areas of New York was 0.28%, which was more than twice the rate of 0.13% measured in the nonendemic area (New Mexico). By comparison, the seroprevalence rate measured in a previous study carried out in New York using the same EIA during the previous year (2012) was approximately 0.91%,<sup>20</sup> and a rate of 0.75% was reported in a study of donors sampled in another endemic region in 2011 and tested by an IFA method.<sup>19</sup> The lower seroreactivity rate in endemic areas in this study reflects in part the application of a higher EIA cutoff, but more significantly, it likely reflects the New York blood donor population that was tested. This population comprised a large fraction of donors from Nassau County, where the prevalence of babesiosis reported in 2013 was approximately 24-fold lower than in Suffolk, a much higher risk area.<sup>30</sup>

The causes of EIA reactivity in the 11 donors residing in the nonendemic region are unknown. A total of five of these 11 EIA-reactive donors had positive immunoblots, which may be due to exposure to either *B. microti* or a similar organism or may represent cross-reactivity from unrelated sources. Enzootic *B. microti* has been reported in Colorado,<sup>31</sup> and pathogenic *Babesia* species have been described in Mexico;<sup>32</sup> thus, exposure in or near New Mexico may be a possibility, in addition to the possibility of exposure during travel to other states. Sera from patients with Lyme disease and anaplasmosis were found to yield low levels of reactivity in the *B. microti* EIA, an expected result based on the known occurrence of coinfections by ticks carrying *B. microti* along with *B. burgdorferi* or *A. phagocytophilum*,<sup>33,34</sup> or alternatively of serial infections by these organisms leaving overlapping antibody titers in the human host.

One question that pertains to donor screening policy is seasonality. Given that clinical babesiosis cases peak in midsummer and decline rapidly in autumn,<sup>35</sup> one option would be to limit screening for *B. microti* to those months when the risk is highest. Based on the apparently random distribution of PCR-positive and serologically positive donor samples detected each week between August and November, however, our study results are not consistent with seasonal screening limited to the summer months. This finding suggests that subclinical infection persists in

at least some donors for months if not longer, consistent with evidence presented in prior studies.<sup>14,29</sup> On the other hand, culling of EIA-reactive donors has been shown to decrease their prevalence in the donor population,<sup>36</sup> which may conceivably leave a preponderance of donors with new, seasonal infections.

A shortcoming of all serologic assays including the present EIA is the inability to detect window period infections, during which parasitemia precedes the appearance of circulating antibodies. Given the study design, which relied on EIA as the first-step screening test, the number of window period infections that were not detected is not known. Other studies have reported window phase infections in clinical patients<sup>37</sup> and, in one instance, in a blood donor.<sup>28</sup> Statistically, window period infections should account for a minor fraction of all donor infections given their presumed short duration and temporal limitation to seasonal periods of active transmission. Detection of window period infections would require methods other than serology, such as PCR or other direct parasite detection techniques, with the attendant relatively significant incremental cost.<sup>38,39</sup> However, even PCR is subject to limitations in sensitivity dependent on the target selected and on sample volume, which may vary between assays, and is capable of yielding false-negative results, in particular at low levels of parasitemia.<sup>15</sup> Volumes of whole blood utilized in published *B. microti* PCR protocols range from 200  $\mu$ L to 2.0 mL, with corresponding analytical sensitivities ranging between 10 and 100 gene copies per PCR procedure.<sup>7-10</sup>

Using immunoblot as a second step assay to define serological true and false positives in the nonendemic donor population, as was done in a previous study by Moritz and colleagues<sup>19</sup> with IFA as the screening assay, yielded a net specificity of 99.93% for the EIA (95% CI, 99.84%-99.97%). The ratio of PCR-positive, presumed infectious donors to EIA repeat-reactive donors in the combined endemic areas was 9:45 or 20% (21% in New York alone). This value is similar to, although slightly higher than, the 13% ratio of PCR-positive to IFA-positive donors in an endemic area reported by Moritz and coworkers.<sup>19</sup> Based on its efficiency of detection of potentially infectious donors in endemic areas, the EIA promises to be useful as a blood screening assay for *B. microti*. As an EIA in standard microplate format, it should be amenable to high-throughput processing and promises to meet cost-effectiveness criteria as set forth in recent analyses.<sup>40</sup>

The deferral rate of donors that are seropositive but uninfected, and by what means such donors might be reinstated, are issues that remain to be considered. Studies are ongoing to elucidate the kinetics of the antibody response in seroreactive donors over time, measured by the same *B. microti* EIA. Eventually, given the typically transient course of infection with *B. microti*, it may be important to determine whether a decrease in EIA reactivity could be one criterion in an algorithm for reentry of seroreactive, deferred donors. Given the recent recom-

mendation by the Blood Products Advisory Committee for universal serologic screening of blood donors for *B. microti*, the seroprevalence data resulting from this study with an EIA intended for this application may be useful in informing further decisions on screening policy including timing and geography.

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#### CONFLICT OF INTEREST

AEL, JC, JLE, GVW, OP, and NXX are employees of Immunetics, Inc. GPW has received research grants from Immunetics, Inc., Institute for Systems Biology, Rarecyte, Inc., and bioMérieux SA. He owns equity in Abbott, has been an expert in malpractice cases involving babesiosis, and is an unpaid board member of the American Lyme Disease Foundation. JAB has received research grants from Immunetics, Inc., Diasorin, Inc., Alere, Inc., and bioMérieux, Inc. The other authors have disclosed no conflicts of interest.

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