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A targeted immunomic approach identifies diagnostic antigens in the human pathogen *Babesia microti*

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Abstract

BACKGROUND—*Babesia microti* is a protozoan parasite responsible for the majority of reported cases of human babesiosis and a major risk to the blood supply. Laboratory screening of blood donors may help prevent transfusion-transmitted babesiosis but there is no Food and Drug Administration–approved screening method yet available. Development of a sensitive, specific, and highly automated *B. microti* antibody assay for diagnosis of acute babesiosis and blood screening could have an important impact on decreasing the health burden of *B. microti* infection.

STUDY DESIGN AND METHODS—Herein, we take advantage of recent advances in *B. microti* genomic analyses, field surveys of the reservoir host, and human studies in endemic areas to apply a targeted immunomic approach to the discovery of *B. microti* antigens that serve as signatures of active or past babesiosis infections. Of 19 glycosylphosphatidylinositol (GPI)-anchored protein candidates (BmGPI1-19) identified in the *B. microti* proteome, 17 were

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

successfully expressed, printed on a microarray chip, and used to screen sera from uninfected and *B. microti*-infected mice and humans to determine immune responses that are associated with active and past infection.

RESULTS—Antibody responses to various *B. microti* BmGPI antigens were detected and BmGPI12 was identified as the best biomarker of infection that provided high sensitivity and specificity when used in a microarray antibody assay.

CONCLUSION—BmGPI12 alone or in combination with other BmGPI proteins is a promising candidate biomarker for detection of *B. microti* antibodies that might be useful in blood screening to prevent transfusion-transmitted babesiosis.

Human babesiosis is an emerging tick-borne infection endemic in the northeastern and midwestern United States and primarily caused by *Babesia microti*.¹ The disease has also been reported in Europe, Africa, Asia, Australia, and South America.^{2,3} In addition to tick transmission, *B. microti* is also the most commonly reported pathogen transmitted through the blood supply in the United States.^{4,5} *B. microti* infection may cause asymptomatic infection, mild flu-like illness, or severe illness with serious complications and death.¹ Confirmation of recent or past babesiosis infection depends on laboratory diagnosis. Although different assays have been used to detect current and past infections of *B. microti*,^{6–11} the indirect immunofluorescence assay (IFA) remains the most widely used antibody assay for determining exposure to *B. microti*. However, this assay is subjective and labor-intensive, requires highly trained personnel, and lacks the sensitivity and specificity necessary for optimal screening of the blood supply to prevent transfusion-transmitted babesiosis.¹²

To develop more sensitive and specific assays for detection of *B. microti* antigens and antibodies in human sera, which can be used in large-scale blood screening, we have taken advantage of the completed genome sequence, annotation, and mapping of one *B. microti* clinical isolate named R1.^{13,14} Studies in other protozoan parasites such as *Plasmodium falciparum*, *P. vivax*, and *Theileria* sp. have identified surface antigens that are critical for cell adhesion and are the primary targets of host immunity.^{15–17} Several of these antigens contain a glycosylphosphatidylinositol (GPI) anchor^{17–19} and are among the most antigenic and abundant proteins expressed during the life cycle of many parasites.^{20–22}

Identification of the full set of GPI-anchored proteins (GPI-proteome) in an organism can be determined using both bioinformatics and experimental techniques. For example, the *P. falciparum* merozoite GPI-anchored proteome was identified using a combination of experimental and bioinformatics analyses,²¹ whereas that of *P. vivax* was determined using prediction programs and homology searches.²³ The in silico analysis aims to identify the presence of specific N- or C-terminal targeting motifs, recognition by the GPI-anchor transamidase, topology, and localization.^{21,24–29}

Herein we describe the development of a reverse-phase, antigen down, protein microarray for *B. microti* consisting of 17 of the 19 predicted GPI-anchored proteins (BmGPIs) encoded by the parasite genome and the screening of this array using sera from uninfected and infected laboratory mice and sera from patients with babesiosis and healthy control subjects.

We used the 17-plex microarray to profile the immunoglobulin (Ig)G and IgM responses to 17 GPI-anchored proteins and evaluate their utility as a biomarker for exposure. This study shows that three *B. microti* proteins, BmGPI6, BmgGPI12, and BmGPI17, appear to be specific and sensitive markers of *B. microti* infection. We identify BmGPI12 as a potentially useful biomarker for detection of past or current infection with *B. microti* and present preliminary data suggesting high sensitivity and specificity of this antigen.

MATERIALS AND METHODS

In silico analyses

The proteome of *B. microti* was obtained from the annotated genome at piroplasmaDB database repository. Proteins were submitted to TMPred server (http://www.ch.embnet.org/software/TMPRED_form.html) based on a script and a local Web server using Apache 2.4.4 and PHP 5.4.12 with php_curl extension. Results were filtered using the command (`$val[1] ['to'] < $limit_pos_deb && $val[1] ['score'] > $limit_score`) && (`$val[$nbhelice] ['to'] > ($val[$nbhelice] ['long_tot'] - $limit_pos_fin)`) && (`$val[$nbhelice] ['score'] > $limit_score`). The parameters were `$limit_pos_deb = 36`; `$limit_pos_fin = 11`; `$limit_score = 900`; `$data["max"] = '29'` and `$data["min"] = '12'`. We keep only proteins presenting and transmembrane domain beginning in the first 36 residues and one finishing within the 11 last residues, with a score more than 900 and a size between 12 and 29. Three different programs for detection of a GPI anchor signal peptide were selected to validate these candidates: FragAnchor (<http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html>), GPI-SOM (<http://gpi.unibe.ch/>), and PredGPI (<http://gpcr.biocomp.unibo.it/predgpi/>). Homology searches were performed using BLAST at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Motif detection was performed using InterPro (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). Phylogenetic analysis was performed at phylogeny.fr website using one click option, but without Gblock option.³⁰ Proteins from the BMN1 family were recovered from NCBI protein database after BLAST analysis with the following entries: BMN1-2, AAF68235.1; BMN1-3, AAF68236.1; BMN1-6, AAF68242.1; BMN1-7, AF68243.1; BMN1-9, AAF68245.1; BMN1-13, AAF68250.1; BmSA1, ADK22856.1; BmP32, BAM10993.1; N1-21a, AAO18094.1; N1-21b, AAO18095.1.

RNA-seq data were obtained from ENA database (study ID PRJNA218917 to PRJNA218922). Bam files were generated using Bowtie and TopHat.^{31,32} The second *B. microti* release was used as a reference genome (IDs FO082871, FO082872, LN871598, and LN871599 for Chromosomes I, II, III, and IV, respectively). Genes without read (four in total) were not included in the quantitative analysis. The filtering threshold was estimated at 4.26 reads per genes and genes not meeting this selection were removed (66 genes). The "Relative Log Expression" (RLE) method was used to normalize data.³³ The level of expression of each gene was calculated as the mean of expression among the six strains.

Serum collection

Sera used in this study were obtained using protocols described in detail in the Supplemental Data (available as supporting information in the online version of this paper) were obtained from five infected Swiss Webster mice before and at different periods after infection with

10^7 *B. microti*-infected red blood cells (RBCs). All mice were anesthetized using 50% isoflurane and blood collection (approx. 100 mL) was performed via retroorbital bleeding. The blood was collected in 1.5-mL microcentrifuge tubes and left at room temperature for 3 hours. Once sera separation occurred, samples were spun down at 4°C for 10 minutes at $15,700 \times g$. The sera were then separated from the coagulated blood, transferred into new 1.5-mL microcentrifuge tubes, and stored at -80°C until used. All animal experimental protocols followed Yale University institutional guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University.

Sera were also obtained from 60 wild-caught white-footed mice (*Peromyscus leucopus*) in the towns of North Branford, Redding, and Storrs, Connecticut. Mice were trapped using Sherman live traps (H.B. Sherman Traps, Inc.) from July to September 2007. All mice were anesthetized using isoflurane and blood collection (approx. 100–200 mL) was performed via cardiac puncture. Blood was collected in 1.5-mL microcentrifuge tubes, immediately placed in an insulated container with cold packs, and transported to the laboratory the same day. Once in the laboratory, samples were centrifuged for 4 minutes at $1677 \times g$. Sera were then separated from whole blood, put into new 1.5-mL microcentrifuge tubes, and stored at -80°C . Mouse capture and handling protocols were approved by the Wildlife Division of the Connecticut Department of Environmental Protection and the Connecticut Agricultural Experiment Station's Institutional Animal Care and Use Committee in accordance with the American Society of Mammalogists guidelines for the use of wild animals in research.³⁴

Human serum samples were obtained from 20 patients during initial evaluation of fever and viral-like illness from Nantucket, Massachusetts, in 2011, 18 of whom were clinically diagnosed and laboratory confirmed by blood smear and/or *B. microti* polymerase chain reaction (PCR) as having babesiosis. We also obtained sera from 112 healthy residents from Block Island, Rhode Island, who participated in a semiannual serosurvey between 1995 and 2006. Ten of these subjects had no history of tick-borne illness and their sera were nonreactive by IFA for *B. microti* antigen and by enzyme-linked immunosorbent assay (ELISA) and Western blot for *B. burgdorferi* antigen; five of these subjects also had a negative *B. microti* PCR. The blood was drawn from subjects into serum collection tubes and subjected to centrifugation at $1000 \times g$ at the study sites. The samples were then shipped to Yale where they were aliquoted into cryotubes and frozen at -80°C . Blood collections followed Yale University Human Investigation Committee guidelines. All personal identifiers of study subjects were removed from the samples before storage and testing for this study. A specific informed consent was required to obtain blood for a related project, which included information pertaining to the potential use of data and residual donor blood for research and development.

Additional methods

Protocols for chip fabrication and probing are provided in detail in the Supplemental Data.

Expression and localization of BmGPI12

BmGPI12 cDNA was PCR amplified and cloned into the pIVEX 2.4a vector and expressed in *Escherichia coli* BL21(DE3). Recombinant protein was purified by affinity purification using a Ni-charged NTA column. Antisera against BmGPI12 were raised in mice. For IFA analyses infected RBCs were fixed in a methanol:acetone mixture (1:4) for 20 minutes at -20°C , washed three times in phosphate-buffered saline (PBS)-Tween 0.1%, and incubated for 1 hour with sera (dilutions between 1:64 and 1:1024). Immunoreaction was detected using an antimouse antibody coupled to fluorescein isothiocyanate (FITC). IFAs using human sera were performed using goat anti-human FITC as the secondary antibody to detect *B. microti* and rat anti-Ter119 monoclonal antibody (MoAb) and secondary goat anti-rat Alexa 594 (red) to detect mouse RBCs. Hoechst dye was used for nuclear staining.

BmGPI12 RBC binding assay

Human and gerbil RBCs were washed with $1\times$ PBS and incubated with 0.2 mg/mL of recombinant protein dissolved in PBS for 1 hour at room temperature. Cells were collected and deposited on silicon oil. After centrifugation for 5 minutes at $4000\times g$, the proteins that remained attached to the RBCs were recovered at high salt concentration (0.5 mol/L NaCl) and detected by Western blotting using an anti-HisTag MoAb conjugated with peroxidase (Qiagen).

Data and statistical analyses

The individual CSV files were compiled and organized using R into the “raw” data files. The raw data are normalized by dividing the IVTT protein spot intensity by the sample specific median of the 33 IVTT control spots printed throughout the array and then taking the base-2 logarithm of the ratio. The normalized data provide a relative measure of the specific antibody binding to *B. microti* proteins compared to the antibody binding to the *E. coli* proteins in the IVTT master mix (a.k.a. background). With the normalized data a value of 0.0 means the intensity is no different than background and a value of 1.0 indicates a doubling with respect to background.

Normalized data are assessed for antigen reactivity, antibody breadth, and magnitude. t tests (or Wilcoxon tests) and multivariate linear regression models were used for group comparisons where appropriate. For biomarker performance characteristics, area under the curve of the receiver operating characteristics (ROC) curve allowed sensitivity and specificity to be computed. The mean of the IgG and IgM data was calculated to generate the IgG + IgM data for the ROC curve analysis. All statistical tests were two-sided. A global alpha level of 0.05 was used to assess statistical significance, and adjustment of significance levels for the false discovery rate was performed using the Benjamini-Hochberg (BH) method.³⁵ The Clopper-Pearson exact confidence intervals (CIs) were calculated using computer software (SAS, SAS Institute).

RESULTS

Identification of the *B. microti* GPI proteome

The availability of the genome sequence of *B. microti* made it possible to perform thorough in silico analyses to search for putative GPI-anchored proteins, which could play a role in parasite host adaptation and evasion. We searched the predicted proteome of the parasite for proteins harboring an N-terminal signal peptide and a C-terminal signature for GPI transamidase based on their hydrophobic properties (Fig. S1, available as supporting information in the online version of this paper). With the *P. falciparum* GPI-Proteome as a training set,²¹ a threshold of 14 amino acid residues in the N-terminal or C-terminal ends of the proteins was defined as the minimal size for the hydrophobic helix to be used for transmembrane domain prediction. Using these criteria, 32 CDS were identified and were subjected to FragAnchor, GPISOM, and PredGPI program analysis to select those predicted to be true-positive GPI proteins (Table S1, available as supporting information in the online version of this paper). A total of 19 putative GPI protein-encoding genes, *BmGPI1*-*BmGPI19*, were thus selected (Table S2, available as supporting information in the online version of this paper). Most of these genes lack introns (Table S1) and some encode members of previously described families of surface antigens including the BMN family.³⁶⁻³⁸ Several of the 19 predicted GPI-anchored proteins of *B. microti* are unique to this parasite and only a few of them share sequence homology with each other (Table S2). Based on sequence homologies, the presence of specific sequence motifs and gene clustering pattern on individual chromosomes, these 19 genes were divided into five groups. Group 1 encompasses *BmGPI2*, *BmGPI3*, and *BmGPI4* genes, which are clustered on Chromosome I along with *BmGPI1* (Fig. 1A). They encode proteins with significant sequence similarity. Multigene Family 2 (Group 2) includes *BmGPI05*, *BmGPI08*, *BmGPI15*, and *BmGPI19* genes, which are distributed on all four chromosomes and encode proteins related to the s48/45 sexual stage antigens found in *Plasmodium* species and harbor the conserved 6-cysteine s48/45 domain. In *P. falciparum*, these GPI-anchored proteins are expressed throughout the parasite life cycle³⁹ and on the surface of gametes and have been suggested to play a role in gamete recognition and fertilization.⁴⁰ The presence of the 6-cysteine s48/45 domain in *B. microti* *BmGPI5*, *BmGPI8*, *BmGPI15*, and *BmGPI19* suggests a possible role for these proteins in sexual development in the tick vector. The third group of GPI-anchored protein encoding genes of *B. microti* includes *BmGPI9*, *BmGPI10*, and *BmGPI12* genes encoding proteins that share 19% to 40% sequence identity between each other. They belong to a subgroup of the *B. microti* BMN multigene family of seroreactive antigens previously described in the MN1 and Gray strains (Figs. 1B and 1C).^{36,37,41} *BmGPI10* and *BmGPI12* are identical to N1-21a and BMN1-9 BMN antigens, respectively.³⁶ However, no orthologs of *BmGPI9* were found among known BMN antigens. The N21-1b antigen of the BMN family shares significant homology with an incomplete CDS from the R1 isolate located on Chromosome III (BBM_03g00005). This protein, however, lacks the C-terminal domain containing the GPI recognition motif. *BmGPI13* gene is located on Chromosome III adjacent to and in the same orientation as *BmGPI12* and encodes a protein with a lysine-rich region similar to that found in the BMN proteins (Fig. S2, available as supporting information in the online version of this paper) although no homology could confirm its membership to the BMN multigene family. The fourth group of

B. microti GPI-anchored protein encoding genes includes two genes *BmGPI6* and *BmGPI11*, which are conserved among apicomplexa but their function remains unknown. Group 5 contains unique genes that are specific to *B. microti*.

RNAseq analysis revealed that among the 19 *BmGPI* genes, *BmGPI5*, 12, and 13 showed high expression levels during the intraerythrocytic phase of the parasite life cycle (Table S2). The transcript levels of *BmGPI12* and *BmGPI13* are the highest among *B. microti* genes, consistent with the high level of protein expression previously reported by Luo and colleagues for BmSA1.⁴¹

Antibody kinetics after experimental infection in mice

A protein array consisting of 17 of the 19 BmGPI proteins was developed as previously described^{42–59} as a tool to measure antibodies in serum. BmGPI1 and BmGPI4 could not be cloned and thus were not included in the array. To study the kinetics of the humoral immune response associated with *B. microti* infection, sera were collected from wild type Swiss Webster outbred mice on Days –1, 4, 8, 12, and 16 post–intrapertoneal injection with *B. microti* LabS1. The sera collected were used to probe the BmGPI protein array to detect IgG and IgM antibodies (Fig. 2). Whereas no antibodies could be detected with preimmune sera (naïve sera), an early IgM response could be detected 4 days postinfection (parasitemia less than 0.1%) to multiple proteins and peaked between Day 8 and Day 12 (Fig. S4, available as supporting information in the online version of this paper). The IgG response to the BmGPI proteins increased continuously until Day 16. The increase in the IgM and IgG response was concomitant with the increase in parasitemia (0.03% at Day 4 to 6%–8% at Day 16; Figs. 2A and 2B). Quantitative analyses showed strong IgM response for BmGPI12 and moderate IgM response for BmGPI5, BmGPI16, and BmGPI17. The IgG response for BmGPI12 was first detected on Day 8 and reached a plateau by Day 12. Other BmGPI proteins showed a similar IgG profile and included BmGPI6, BmGPI9, BmGPI10, BmGPI17, BmGPI18, and BmGPI19.

Antibody profile of babesiosis patients

The BmGPI protein array was then screened using a collection of 28 human sera. Eighteen of these sera (positive sera) were isolated from Nantucket residents who had an acute symptomatic illness consistent with babesiosis and laboratory evidence of acute babesiosis that included a positive thin blood smear and/or positive *B. microti* PCR in 16 subjects and a positive *B. microti* IFA antibody in two subjects (Table S3, available as supporting information in the online version of this paper). The remaining 10 sera were isolated from healthy Block Island serosurvey donors who denied ever having had babesiosis. The sera from these negative controls were nonreactive by conventional *B. microti* IFA antibody assay and *B. burgdorferi* ELISA and Western blot assay combination. Five of the negative sera were negative by PCR. Using protein arrays, the babesiosis patient group had 35.6-fold more BmGPI12 IgG reactivity and 16.3-fold more IgM reactivity than the control group (Fig. 3). These differences were statistically different after BH correction: p values of 1.64×10^{-8} and 2.02×10^{-6} , respectively (Table S4, available as supporting information in the online version of this paper). One of the negative samples had detectable levels of IgG antibodies and none had detectable IgM levels against BmGPI12 (Fig. 3). A threshold for

positive sera was fixed at twice the value of background intensity or a value of 1.0 in the normalized data. Under these conditions, 18 of 18 of the positive sera had IgM and IgG responses to BmGPI12. Weaker but still significant responses were seen for BmGPI17. Anti-BmGPI17 antibodies are at least twofold greater for IgG (2.9-fold) and IgM (2.3-fold) in the babesiosis patient group than in the control group and the differences are statistically significant for both IgG (5.8×10^{-3}) and IgM (2.6×10^{-4}). There was a more varied and less robust response to other BmGPI proteins. BmGPI06 (2.1-fold), BmGPI09 (1.8-fold), and BmGPI13 (2.0-fold) are more IgG reactive in the babesiosis group than the control group. These differences are significant with BH corrected p values of 5.8×10^{-3} . ROC curves were generated for BmGPI6, BmGPI12, and BmGPI17 (Fig. 5) for the IgG, IgM, and IgG + IgM (combined in silico) data. To determine the performance of these three potential biomarkers using the human data we calculated the Youden index, or J statistic (Table S5, available as supporting information in the online version of this paper), which describe the measurement cutoff value that yields the best performance of the biomarker as a binary classifier. As expected based on the group statistics calculated above, BmGPI12 performed the best with a sensitivity of 100% (18/18; 95% CI, 81.47%–100%) and a specificity of 100% (10/10; 95% CI, 69.15%–100%) for IgG alone, IgM alone, or a combination for IgG and IgM. BmGPI17 performed best when used as an IgG + IgM biomarker (18/18; 95% CI, 81.47%–100%; and 9/10; 95% CI, 55.50%–99.75%, respectively) or as an IgM test (17/18; 95% CI, 72.71%–99.86%; and 9/10; 95% CI, 55.50%–99.75%, respectively), depending on the need for maximum specificity or sensitivity. BmGPI6 performed best as an IgG + IgM biomarker (7/10, 95% CI, 34.75%–99.33%; 10/10 95% CI, 69.15%–100%, respectively). No false-positive detection was observed as the negative control sera had little to no reactivity to BmGPI proteins and there was a clear break in the responses between negative and positive samples. Immunofluorescence analysis on *B. microti*-infected RBCs using these sera to label the parasite, Ter119 to label mouse RBCs, and Hoechst to stain parasite DNA confirmed the seropositive and seronegative characteristics of these sera as revealed by the array data (Fig. 5).

BmGPI12 antibodies as a signature of babesiosis infection

The data collected from positive and negative control mice and humans indicate that BmGPI12 and other BmGPI proteins are recognized by the immune system of the reservoir host whereas in humans both the IgM and the IgG responses are in large part centered on BmGPI12. Immunoblot analyses using specific antibodies against BmGPI12 showed high level expression of the protein inside and on the surface of parasites. Binding assays using a GFP-tagged BmGPI12 protein showed strong binding of BmGPI12 to mammalian RBCs, whereas a control GFP alone did not bind to these cells (Fig. 6). The expression of BmGPI12 on the surface of parasites and its specific binding to host RBCs suggest a possible role of this protein in merozoite binding to its target RBC during invasion.

The strong immunoreactivity of BmGPI12 in both mice and humans suggests that it might be an ideal candidate antigen for screening human blood. Accordingly, we screened 99 sera from healthy adults participating in a serosurvey conducted on Block Island, Rhode Island, between 1995 and 2006 against BmGPI12. Using the optimal cutoff value of 2.75 for IgG (or fourfold above the mean for the negative control sera) and 1.76 for IgM (or 2.74-fold

above the mean of the negative control serum) our analysis of the 99 human sera resulted in 6% of the samples being classified as seropositive for IgG and 2% of the samples being classified as seropositive for IgM. Only one of the IgM positives was also IgG positive (Fig. 7).

We also examined the extent of BmGPI12 response in naturally infected animals using 60 sera obtained from wild mice captured in Connecticut. These sera were previously tested using an IFA as previously described⁶⁰ and divided into 30 IFA-positive and 30 IFA-negative sera (Fig. 8). Our analysis, however, showed that few sera previously considered to be negative by IFA were positive for IgM and IgG response. The IgM response for BmGPI12 was overall weaker than the IgG response but the frequency of recognition was actually higher. Thirty-nine of the mice had detectable levels of anti-BmGPI12 antibodies and 47 mice had detectable levels of anti-BmGPI12 IgM antibodies. There is a positive correlation between intensity of the spot on the array (proportional to antibody titer) and IFA titers for BmGPI12 for the IgG data ($p = 1.20 \times 10^{-5}$; Fig. S4), but there are multiple samples with a negative IFA result that gave intensities of at least double the background (value of 1 on the y-axis) for the IgG assay and samples with high IFA and low intensity. In contrast to the IgG data, the IgM data did not show significant correlation with the IFA titer data ($p = 0.22$).

DISCUSSION

Prevention of transfusion-transmitted babesiosis is a major health priority and can be successfully achieved if cost-effective, highly specific, and sensitive assays are developed to screen the blood supply. An initial *B. microti* screening program relied primarily on the use of PCR and IFA-based assays.¹² However, these assays are not optimal for screening the blood supply because they are not yet highly automated and/or have limited screening specificity or sensitivity.¹² More recently an investigational enzyme immunoassay based on four synthetic peptides selected from the *B. microti* BMN1 family of antigens was used to assess seroprevalence in blood donor populations.¹¹ Considering the diversity in the BMN family of antigens and the location of their encoding genes in chromosomal areas associated with major recombination events,^{13,14} these antibody assays may not capture all antibody responses caused by different isolates of *B. microti*.

Using the fully annotated genome of *B. microti*^{13,14} we identified 19 proteins with GPI-anchored protein signatures that we call BmGPI1-BmGPI19. Successful expression and printing on a microarray of 17 of these proteins led us to screen sera from laboratory mice, as well as sera from wild mice and humans from endemic areas. We identified BmGPI12 as the most immunodominant GPI protein that provided excellent sensitivity and specificity when used in a microarray assay platform. Similar results were obtained with BmGPI12 in ELISAs (not shown). It is noteworthy that BmGPI12 identified in the R1 strain of *B. microti* is nearly identical to the BmSA1 antigen reported by Luo and colleagues in the Gray strain⁴¹ (Fig. S5, available as supporting information in the online version of this paper). BmSA1 was used in a multiplex assay to detect *B. microti* IgG antibodies and showed specificity and sensitivity levels similar to those reported in this study for BmGPI12.^{10,38,41,61} Our studies revealed that *B. microti* expresses a smaller set of GPI-anchored proteins than that of *P. falciparum*.²¹ The predicted GPI-anchored proteins of *B. microti* expressed in the blood

stage of the parasite are different from those of *P. falciparum* and *Toxoplasma gondii* parasites. In *Toxoplasma*, specific antigens such as SAG and BSR are expressed at different stages of development, whereas in *Plasmodium*, about half of the predicted GPI-anchored proteins are transcribed during blood stage development. Recently, the GPI-anchored proteome of *B. bovis* was predicted using a similar in silico analysis and was shown to be organized in three gene families.⁶²

Based on the findings of this study, we believe that an optimal diagnostic assay for *B. microti* should include BmGPI12 as a bait to detect anti-BmGPI12 antibodies or as an antigen target in a direct detection assay using high-affinity anti-BmGPI12 antibody or a combination of both. Interestingly, this protein is unique to *B. microti* and available data suggest that it undergoes little to no variation between different isolates. The BmGPI12 protein is encoded by one of the most highly expressed genes in the *B. microti* genome and is a member of the BMN multigene family whose antigenicity has been conserved in many *Babesia* sp. and hosts. BmGPI12 is therefore a prime candidate for a direct detection sandwich assay. Two other proteins, BmGPI12 and BmGPI17, could also be combined with BmGPI12 for the detection of *B. microti* as they were positive in a significant number of human sera. These antigens may potentially enhance the sensitivity of detection when studying a much larger cohort of blood donors or babesiosis patients, especially for early-stage infections.

Although the data identified BmGPI12 as a promising marker for babesiosis diagnosis, the assay performance was based on a very small data set comprising 18 true positives and 10 true negatives. Based on the very small sample size used to determine the cutoff, the cutoff has not yet been optimized, and consequently the sensitivity and specificity and the balance between the frequency of true positives and false positives remains to be determined. We detected past infections in 99 sera from healthy residents of Block Island using the BmGPI12 microarray antibody assay as a screening tool. *B. microti* IgG and IgM seropositivity values were 6 and 2%, respectively, which are somewhat higher than previously have been found in southern New England using *B. microti* IFA testing.^{63–66} Due to the fact that no comparator assays for the data reported from the 99 Block Island sera were conducted and considering that the preliminary cutoffs were based on a small number of positive and negative controls, large-scale population studies will be needed to validate our assay in low and high *B. microti*-endemic settings to confirm that BmGPI12-based antibody assays are highly sensitive and specific.

This study is the first to evaluate 17 GPI-anchored proteins as antigens for evaluation of *B. microti* seroprevalence in human and mouse sera from endemic areas. A more specific and sensitive assay based on BmGPI12, and possibly in combination with other BmGPI proteins, could be an invaluable tool in large-scale screening assays to estimate babesiosis seroprevalence and in blood donor screening to help reduce the number of cases of transfusion-transmitted babesiosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

BH	Benjamini-Hochberg
GPI	glycosylphosphatidylinositol
IFA	indirect immunofluorescence assay
ROC	receiver operating characteristics

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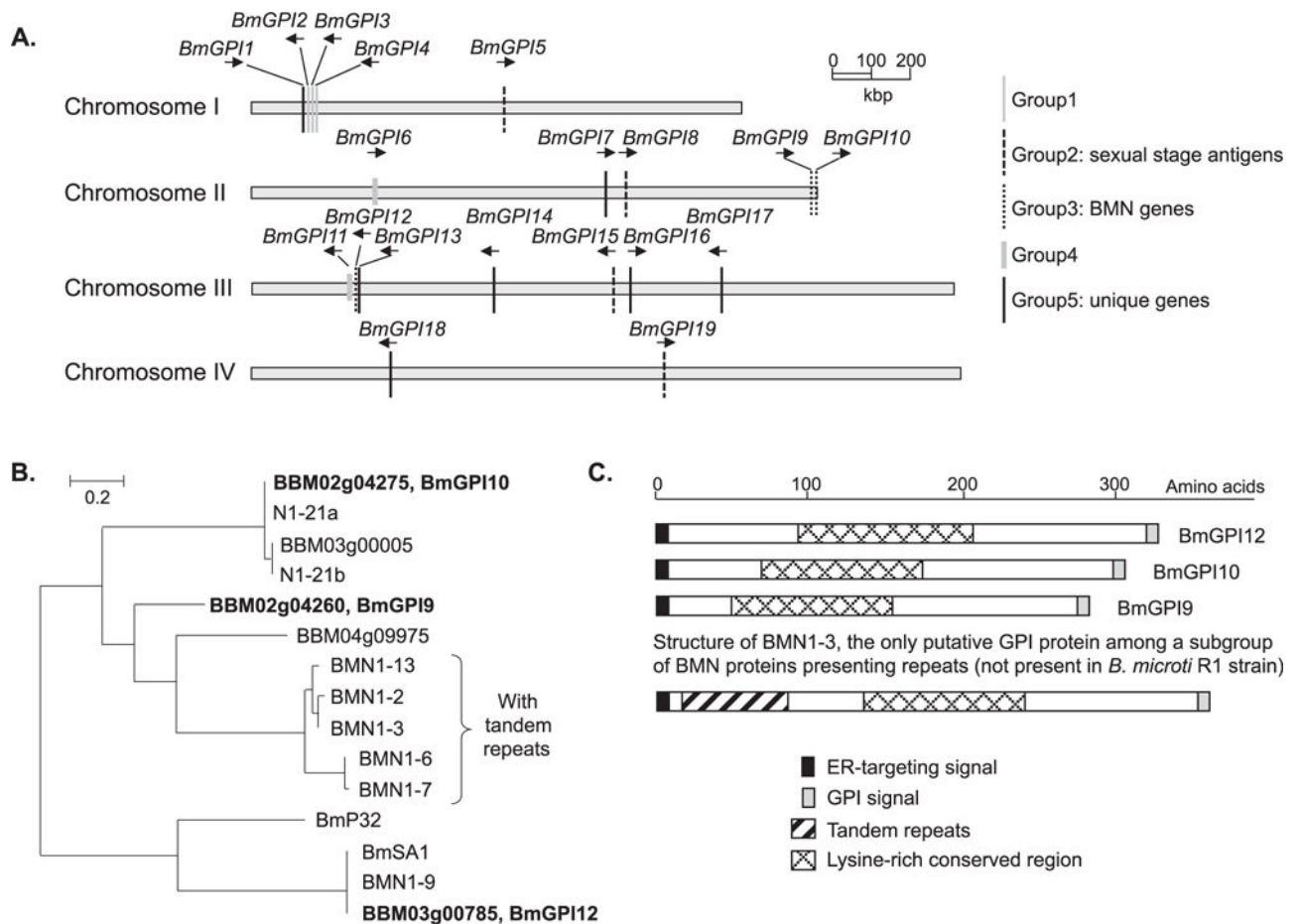


Fig. 1. Genome distribution of *BmGPI* genes and comparison with proteins from the BMN multigene family. (A) Distribution of the 19 *bmGPI* genes on the four chromosomes of the *B. microti* R1 isolate. Dashed, dotted, or gray vertical bars are used to indicate position of genes from Groups 1 to 4. Dark plain bars correspond to unique genes (Group 5). (B) Phylogenetic analysis of *BmGPI* proteins including homologs from the BMN family. Maximum likelihood analysis was performed using “phylogeny.fr” with default options. Scale bar indicates the number of substitutions per site. (C) Description of conserved domains in *BmGPI* protein from the BMN family. *BmGPI*9, 10, and 12 were characterized from the *B. microti* genome. BMN1-3 protein is the only BMN antigen isolate from the MN1 strain with a conserved GPI anchoring signal and a tandem array of a degenerate six-amino-acid repeat in the N-terminal end.²⁶

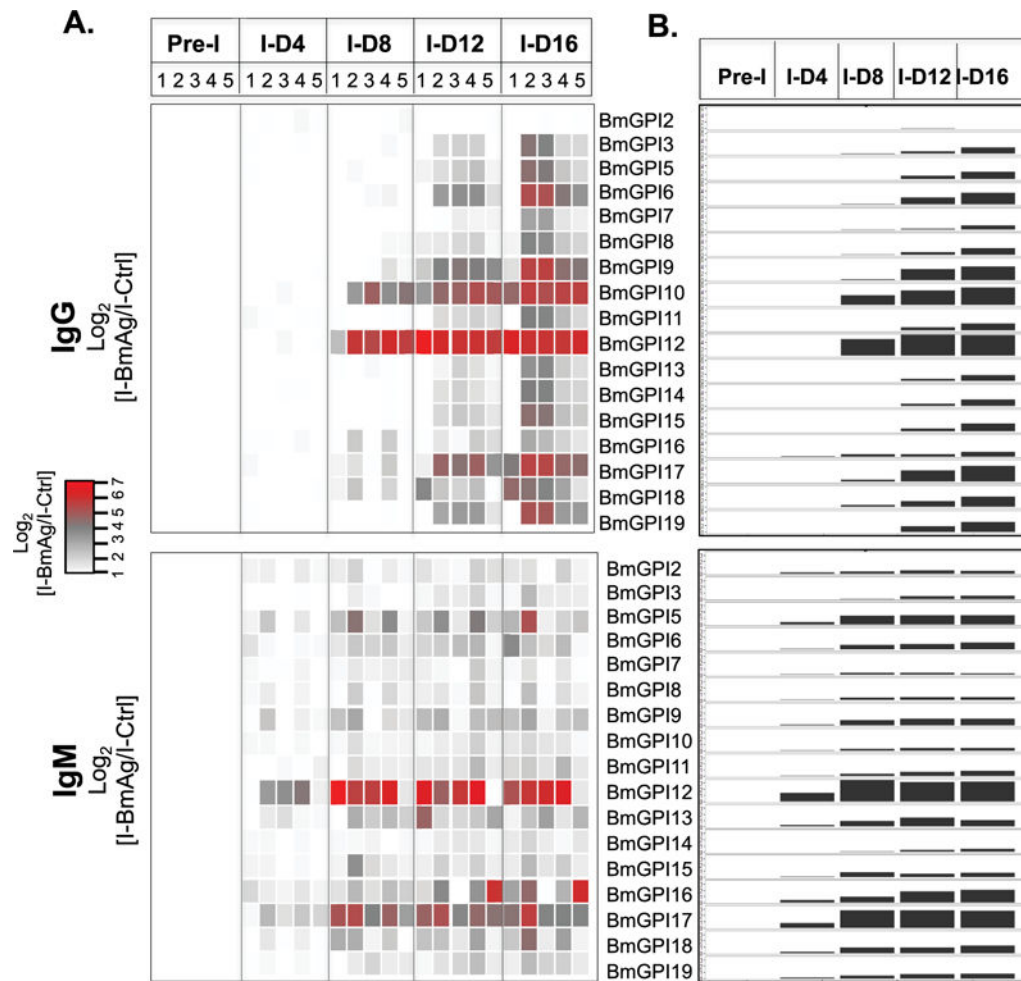


Fig. 2. Time course data obtained from five mice at five time points. The data generated with the BmGPI protein microarray were visualized using a heat map (A) for 17 BmGPI proteins (rows) and 25 individual samples (columns) for the detection of IgG (top) and IgM (bottom). Samples were grouped based on time postinfection; each square is the data from one protein and one sample. The color scale goes from white > gray > red to represent low > mid > high antibody titers. The mean value for each protein was calculated at each time point and bar graphs were generated (B) to follow the group trends.

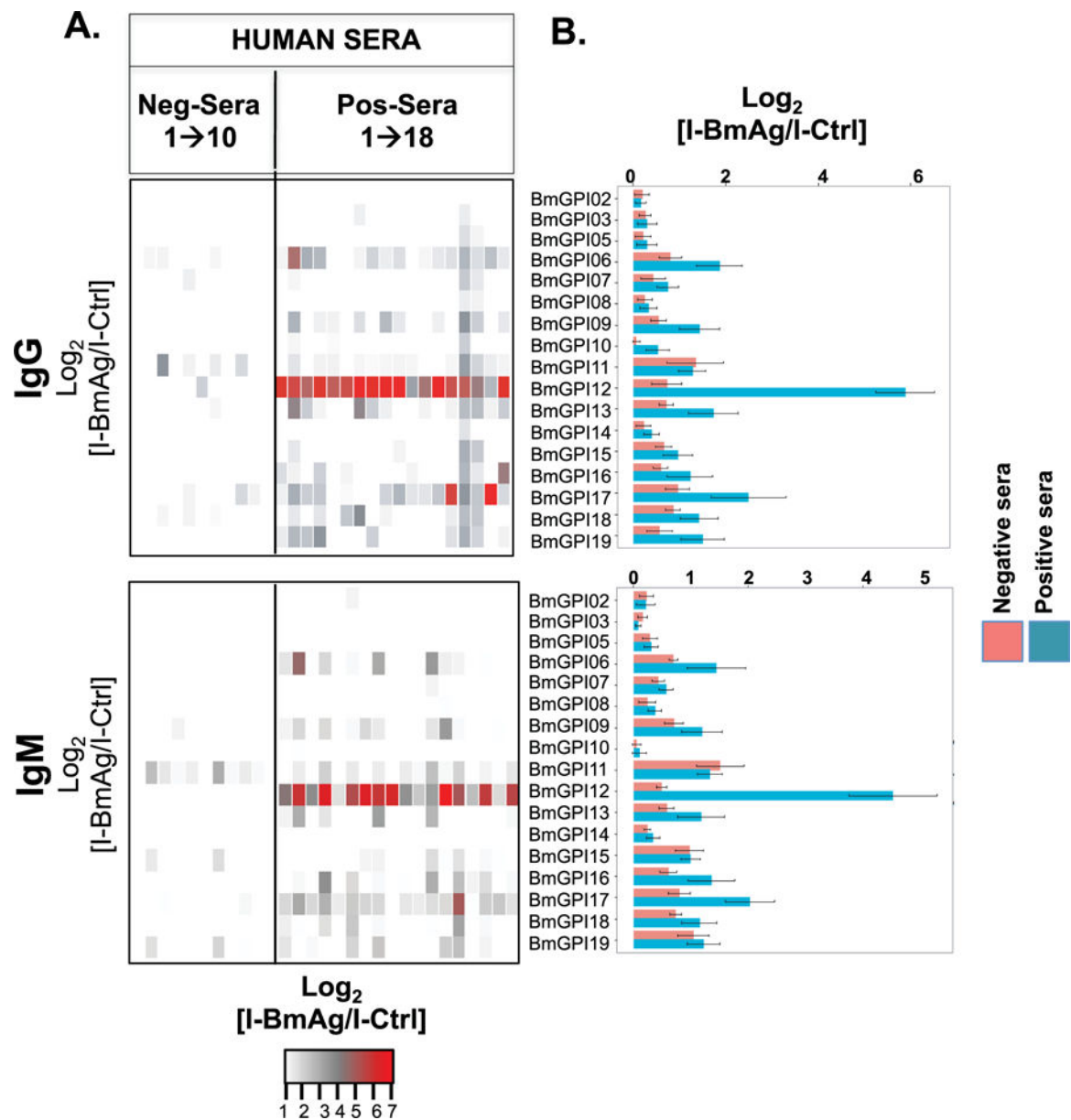


Fig. 3. Samples from 18 patients who were clinically diagnosed and laboratory confirmed (smear and/or PCR) as having babesiosis and 13 nonexposed individuals were probed on the BmGPI protein microarray. Normalized data were used to generate a heat map from the IgG (A, top) and IgM data (A, bottom) from a matrix in which the proteins are in rows and samples are in columns. The color scale goes from white > gray > red to represent low > mid > high antibody titers. Univariate statistics were calculated, and the group means and 95% CIs were plotted for each protein. The nonexposed, or Neg-Sera, are represented by the salmon colored bars and the babesiosis patient group, or Pos-Sera, is represented by the turquoise bars.

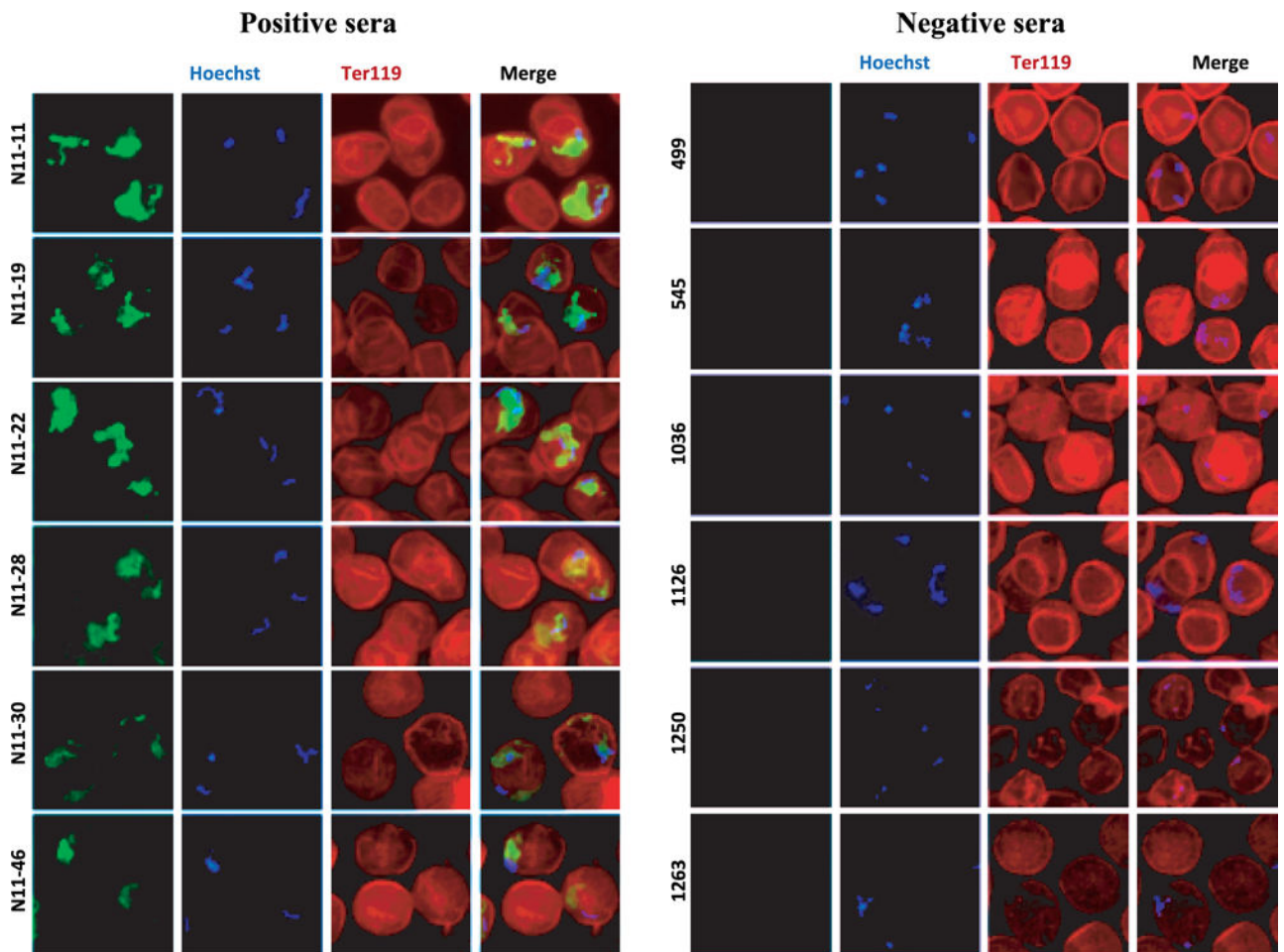


Fig. 4. Validation of the array data by IFA for a set of six positive and negative sera. IFA was performed using mouse RBCs infected with *B. microti* LabS1 strain with 50% parasitemia. Green = positive and negative sera labeled with fluorescein; blue = parasite nuclei labeled with Hoechst dye; red = mouse blood cells labeled with anti-Ter119 coupled to rhodamine.

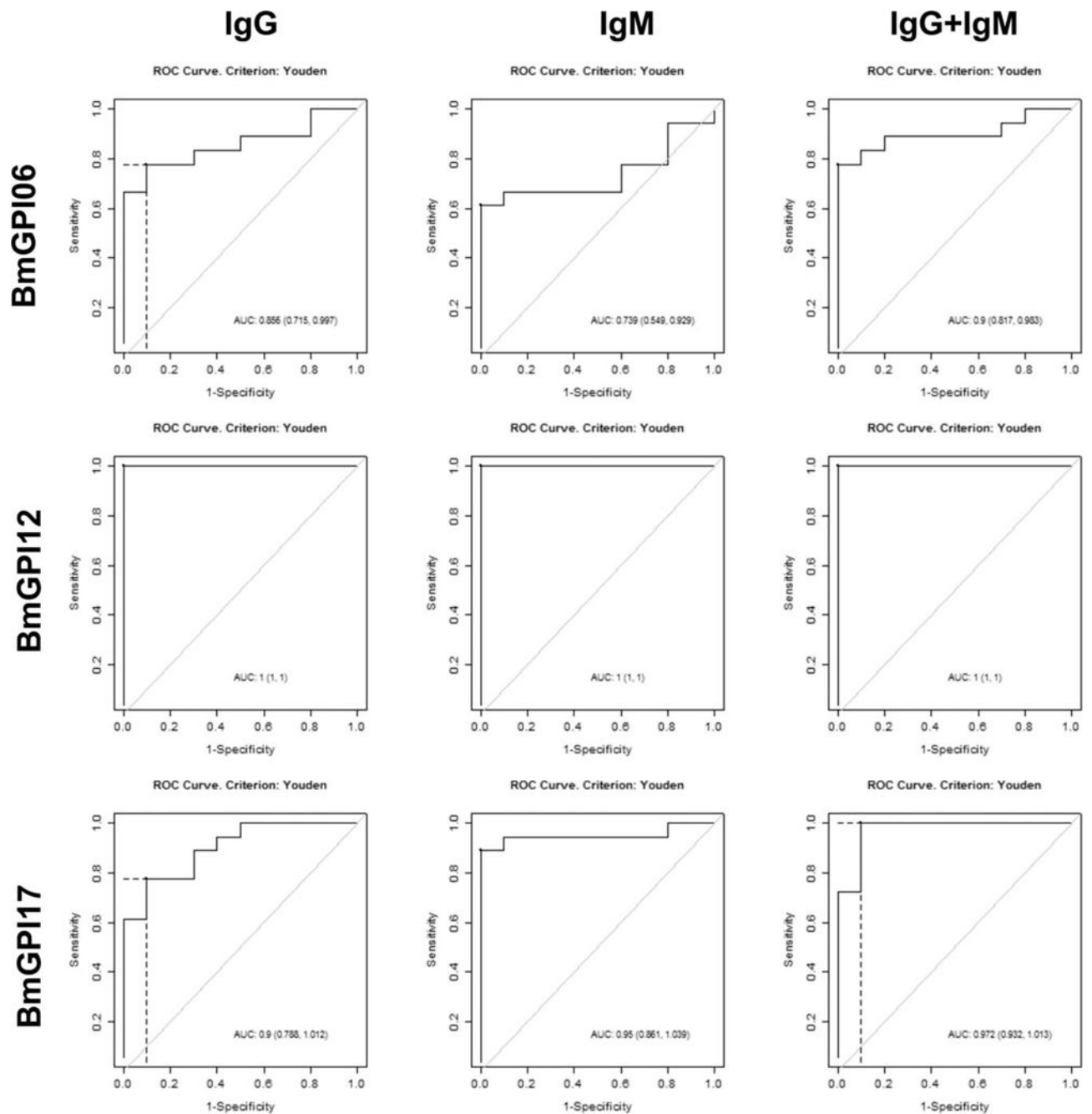


Fig. 5. ROC curves were generated for BmGPI6 (top), BmGPI12 (middle), and BmGPI17 (bottom) with sensitivity (y-axis) and 1 – specificity (x-axis) using the OptimalCutpoints package in the statistical programming environment R using the data from the 18 *Babesia* patient samples and 13 nonexposed donor samples from the IgG probing (left), the IgM probing (middle), and the sum of the IgG and IgM data for each protein (right). All plots have a diagonal line with a y-intercept of 0 and a slope of 1 that represents a binary classifier with results equivalent to “random” chance and the area under the curve (AUC).

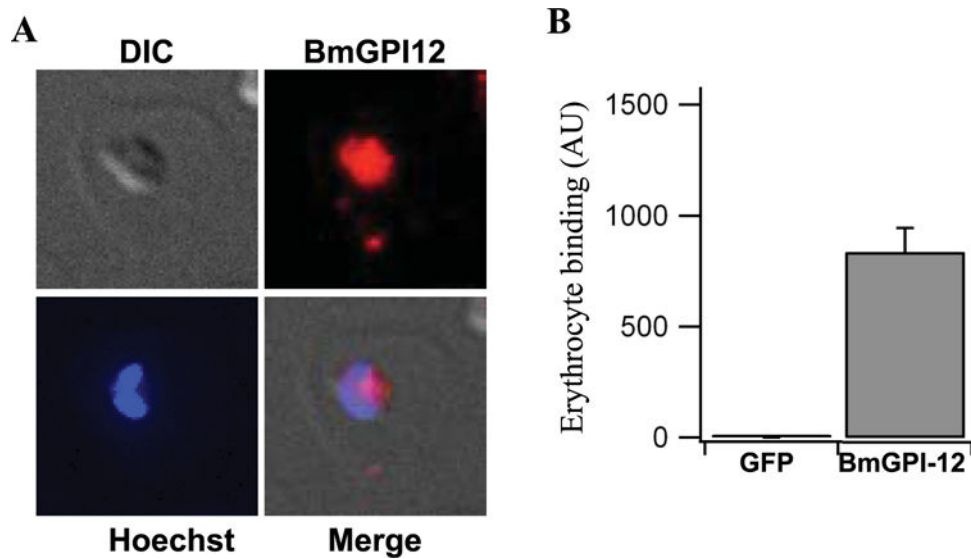
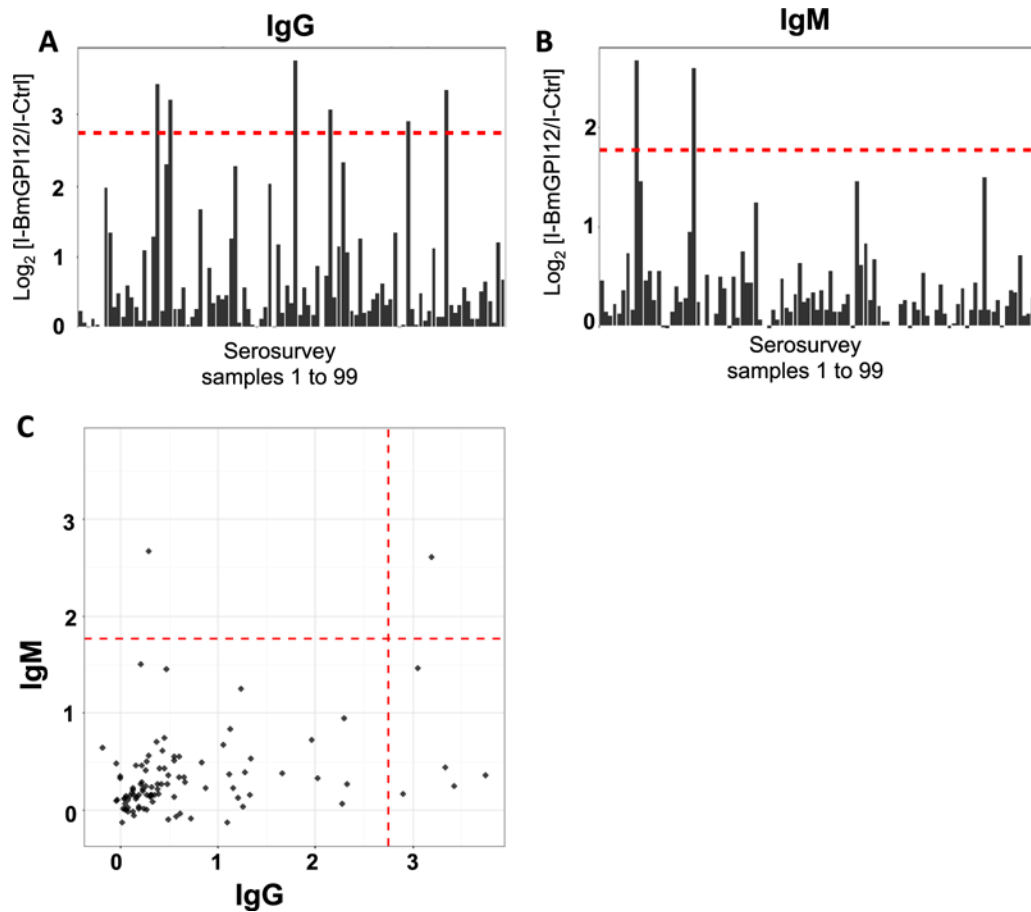
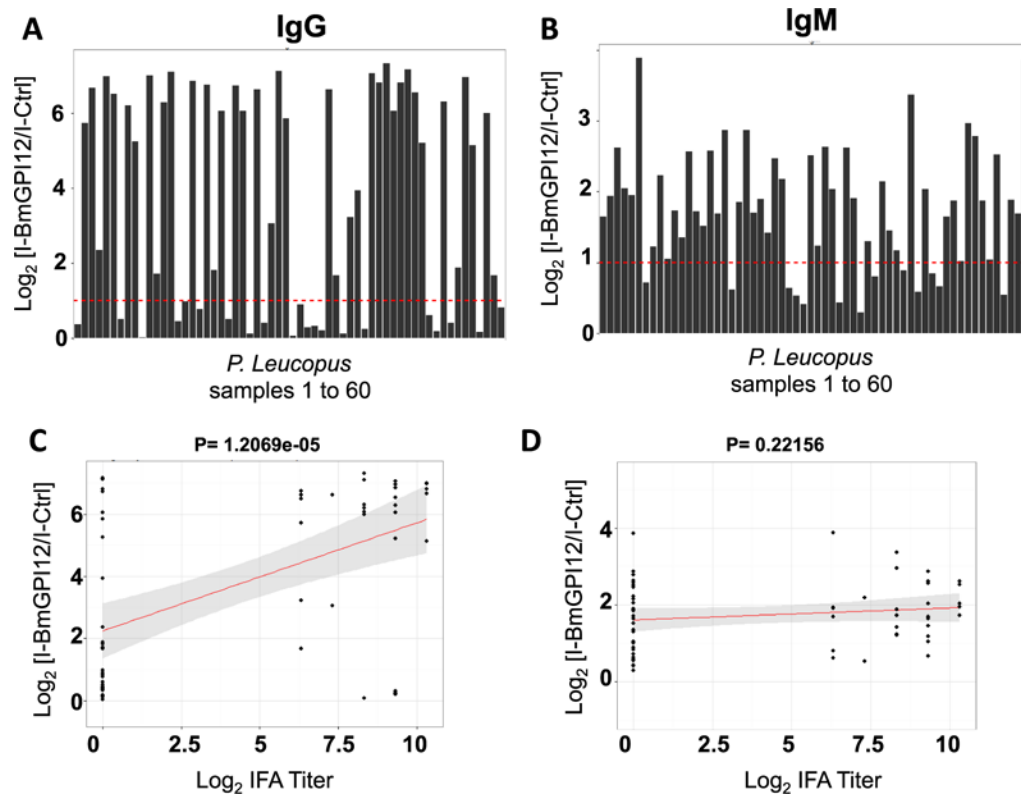


Fig. 6. Cellular location and RBC binding properties of BmGPI12 surface antigen. (A) Immunolocalization of BmGPI12 in *B. microti*-infected RBCs. RBCs were fixed and stained using mouse polyclonal antibodies (red) against BmGPI12. Nuclear staining was achieved using Hoechst. DIC = differential interference contrast. (B) RBC binding properties of BmGPI12. Purified recombinant His6-tagged BMG proteins and His6-GFP control were examined for their ability to bind RBCs as described under Materials and Methods. Binding was determined by immunoblotting and quantified using CCD camera. Values are normalized to amount of input proteins.

**Fig. 7.**

Serum samples from 99 healthy residents from Block Island, Rhode Island, who participated in a semiannual serosurvey between 1995 and 2006 were probed on the BmGPI protein microarray and evaluated for reactivity against BmGPI12. Bar graphs were generated for both IgG (A) and IgM (B) with the normalized intensity values on the y-axis and the samples on the x-axis. The red dotted line crosses the y-axis at 2.75 for IgG and 1.76 for IgM, the cutoff values calculated using the 28 well-characterized human samples. (C) Scatterplots of the IgG (x-axis) and IgM (y-axis) data and associated cutoffs, x-intercept, and y-intercept, respectively, were plotted to identify samples that were positive for IgG, IgM, or both.

**Fig. 8.**

Serum samples from 60 wild mice with known IFA titers (A, B) were probed on the BmGPI protein microarray and evaluated for reactivity against BmGPI12. Bar graphs were generated for both IgG (A) and IgM (B) with the normalized intensity values on the y-axis and the samples on the x-axis. A value of 1 (one doubling) was considered positive. The red dotted line crosses the y-axis at 1, the cutoff value.