CLINICAL AND VACCINE IMMUNOLOGY, Oct. 2010, p. 1631–1638 Vol. 17, No. 10
1556-6811/10/$12.00 doi:10.1128/CVI.00196-10

Detection of *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* Merozoite Surface Protein 1-p19 Antibodies in Human Malaria Patients and Experimentally Infected Nonhuman Primates[▽]

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Received 13 May 2010/Returned for modification 3 June 2010/Accepted 30 July 2010

Approximately 3.2 billion people live in areas where malaria is endemic, and WHO estimates that 350 to 500 million malaria cases occur each year worldwide. This high prevalence, and the high frequency of international travel, creates significant risk for the exportation of malaria to countries where malaria is not endemic and for the introduction of malaria organisms into the blood supply. Since all four human infectious *Plasmodium* species have been transmitted by blood transfusion, we sought to develop an enzyme-linked immunosorbent assay (ELISA) capable of detecting antibodies elicited by infection with any of these species. The merozoite surface protein 1 (MSP1), a *P. falciparum* and *P. vivax* vaccine candidate with a well-characterized immune response, was selected for use in the assay. The MSP1 genes from *P. ovale* and *P. malariae* were cloned and sequenced (L. Birkenmeyer, A. S. Muerhoff, G. Dawson, and S. M. Desai, Am. J. Trop. Med. Hyg. 82:996-1003, 2010), and the carboxyl-terminal p19 regions of all four species were expressed in *Escherichia coli*. Performance results from individual p19 ELISAs were compared to those of a commercial test (Lab 21 Healthcare Malaria enzyme immunoassay [EIA]). The commercial ELISA detected all malaria patients with *P. falciparum* or *P. vivax* infections, as did the corresponding species-specific p19 ELISAs. However, the commercial ELISA detected antibodies in 0/2 and 5/8 individuals with *P. malariae* and *P. ovale* infections, respectively, while the p19 assays detected 100% of individuals with confirmed *P. malariae* or *P. ovale* infections. In experimentally infected nonhuman primates, the use of MSP1-p19 antigens from all four species resulted in the detection of antibodies within 2 to 10 weeks postinfection. Use of MSP1-p19 antigens from all four *Plasmodium* species in a single immunoassay would provide significantly improved efficacy compared to existing tests.

More than 3.2 billion people in the world today live in areas where malaria is endemic. The World Health Organization estimates that more than 350 to 500 million malaria clinical disease episodes occur each year worldwide, with more than 1 million deaths occurring annually in sub-Saharan Africa, mostly among children under the age of 5 years (50). The combination of high disease prevalence and high frequency of international travel creates a significant risk for the exportation of malaria to countries where the disease is nonendemic. This risk is accompanied by the potential for introduction of malaria-causing organisms into the blood supplies used for transfusions. All four principal species of *Plasmodium* that infect humans have been transmitted via blood transfusion in the United States (36), France (4), the United Kingdom (23), and Switzerland (19). This has resulted in the implementation of donor deferral policies in many countries that restrict blood donation by those with a history of recent travel to or emigration from regions of endemicity and by those with recent cases of clinical malaria. Recent publications indicate that the prevalence of *Plasmodium knowlesi*, a pathogen of simian origin, in human populations in Southeast Asia (11, 12), Singapore (37), the Philippines (31), and Thailand (21) is much higher than previously believed. However, *P. knowlesi* malaria appears to be a zoonotic disease and to our knowledge has been not implicated in cases of transfusion-transmitted malaria in humans.

The effectiveness of donor deferral programs has previously been questioned (29), and there is concern that many donors are needlessly deferred, since the rates of imported malaria are much lower than the rates of travel to areas of endemicity (17, 35). To prevent erosion of qualified donor populations, some countries have implemented antibody screening such that only individuals who are known to have been exposed to organisms causing malaria are subject to deferral of donations rather than all donors who have traveled to or lived in regions where malaria is endemic. Commercial antibody enzyme-linked immunosorbent assays (ELISAs) are currently in use (in the United Kingdom, France, and Australia), and reinstatement of questionnaire-deferred donors is being discussed in Canada and the United States (16, 24, 42). In these cases, potential donors are tested for antibodies to *Plasmodium*-derived antigens within several months of deferral; when the tested individuals show negative antibody results, donation is allowed.

Antibodies to asexual malaria parasites (i.e., merozoites) appear within days to weeks after the invasion of erythrocytes and can persist for months or even years (14, 49). Historically,

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▽ Published ahead of print on 11 August 2010.

antibodies to parasite antigens have been detected using the immunofluorescence assay (IFA). This assay is not particularly sensitive or specific and is labor-intensive, requiring careful preparation of reagents. Commercially available ELISAs have been developed that use recombinant antigens or *P. falciparum* whole-organism lysates for detection of immunoglobulins (IgG and/or IgM, IgA) in human serum or plasma (Lab 21 Healthcare Laboratories, United Kingdom; Cellabs, Australia; DiaMed AG, Switzerland; LG Chemical Inc., Iksan, South Korea; Green Cross, Inc., Youngin, South Korea [Genedia Malaria Ab Rapid]; and Standard Diagnostics, Suwon, South Korea). These assays are typically easier to perform and exhibit higher throughput and better sensitivity and specificity than IFA (25, 42, 47), though this is not always the case (32). Some ELISAs may be better than others for detection of antibodies against all four *Plasmodium* species that cause malaria in humans (44). However, none of the available commercial assays currently include *P. ovale*- or *P. malariae*-derived antigens. Because these organisms have been implicated in transfusion-transmitted malaria (TTM), it would be advantageous to include antigens from these organisms in an antibody detection assay.

Antigens used in some commercial ELISAs for the capture of antibodies have included vaccine candidates, since their ability to elicit antibody responses in animals and human vaccine recipients has been predetermined and naturally occurring antibodies are measured prior to vaccination. Examples of such antigens include circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1), merozoite surface protein 1 (MSP1), and, in particular, a 19-kDa C-terminal fragment of MSP1 (MSP1-p19) (22, 25, 40). *Plasmodium falciparum* MSP1 has been extensively studied and was one of the very earliest vaccine candidates; it elicits a protective antibody response against severe malaria, and the presence of MSP1 antibodies correlates with protective immunity (45). MSP1 is expressed as an ~200-kDa precursor molecule linked by a glycosyl phosphatidylinositol anchor to the merozoite surface membrane. MSP1 is processed into a complex of polypeptides on the merozoite surface, including N-terminal and central regions of 82, 30, and 38 kDa, as well as the C-terminal region of 42 kDa. At the time of invasion of red blood cells, MSP1-p42 is further processed by proteolytic cleavage into a 33-kDa fragment (MSP1-p33), which is shed with the rest of the complex, and a C-terminal 19-kDa fragment (MSP1-p19). Only the C-terminal MSP1-p19 fragment remains anchored on the merozoite surface and is carried into parasitized red blood cells (RBC) (10). In monkeys, immunization with recombinant *P. falciparum* MSP1-p42 and *P. falciparum* MSP1-p19 has been shown to elicit various degrees of protection against *P. falciparum* challenge (15, 26). MSP1-p19 proteins from both *P. falciparum* and *P. vivax* have been proposed as vaccine candidates (18, 41, 48).

By analogy to *P. falciparum* and *P. vivax* findings, one would predict that the MSP1 genes of *P. ovale* and *P. malariae* would be useful as reagents for vaccination or antibody detection. We recently cloned and expressed the MSP1-p19 proteins of *P. malariae* and *P. ovale* as recombinant antigens in *Escherichia coli* (2). We report here the independent evaluation of these proteins as reagents for antibody detection using sera from human malaria patients and experimentally infected nonhuman primates. In addition, a prototype immunoassay combining MSP1-p19 antigens from all four *Plasmodium* species was evaluated and its performance compared to a commercially available antibody test.

MATERIALS AND METHODS

Specimens. Serum samples from nonhuman primates infected with *Plasmodium* parasites were obtained from John Barnwell, Centers for Disease Control and Prevention (CDC), Atlanta, GA. New World monkeys (*Aotus nancymaae* and *Saimiri boliviensis* spp.) were infected with one of two strains of *P. falciparum* (Vietnam-Oak Knoll or Uganda-Palo Alto). Sera were obtained within 15 to 18 days postinfection. Chimpanzees were infected with *P. ovale* (Nigeria I), *P. malariae* (Uganda I), or *P. vivax* (Salvador I or India VII). Serum samples were obtained 16 to 24 days postinfection from chimpanzees infected with *P. vivax* or *P. ovale* and 16 to 68 days postinfection for the animal infected with *P. malariae*. Animals were infected with cryopreserved blood specimens. Chimpanzees were subjected to splenectomy prior to infection. Malaria infections were treated with chloroquine (10 mg/kg of body weight one time a day) and a single dose of mefloquine (30 mg/kg for 3 days).

Serum samples from microscopy- and IFA-confirmed malaria patients were obtained from Marianna Wilson, Reference Immunodiagnostic Laboratory, CDC. All samples were received frozen as liquid or lyophilized aliquots; lyophilized samples were dissolved in phosphate-buffered saline (PBS) prior to testing. Immunofluorescent antibody titers for each human infectious parasite species for each sample were provided, as was the species identification determined by blood smear examination. All samples were collected prior to 1990 and are considered to represent anonymous residual human specimens, since original records regarding the identity of the donor or patient no longer exist. The times between infection or clinical presentation and sample collection are not known.

Commercial ELISA. All human specimens were tested for the presence of *Plasmodium* antibodies by using a commercially available ELISA (Lab 21 Healthcare Malaria EIA; Lab 21 Healthcare, Cambridge, United Kingdom [formerly produced and distributed by Newmarket Laboratories Ltd., Kentford, United Kingdom]). This direct ELISA detects *P. falciparum*- and *P. vivax*-specific IgG, IgM, and IgA by using a mixture of four recombinant antigens coated onto microtiter plates. The assay was performed as directed by the manufacturer.

MSP1-p19 recombinant antigens. The 19-kDa carboxy-terminal region of merozoite surface protein 1 from *P. falciparum* (Wellcome strain), *P. vivax* (SalI strain), and *P. malariae* and *P. ovale* (both Cameroon strains) (2) was expressed in *E. coli* as (2) carboxyl-terminal fusions to the *E. coli* enzyme CMP-3-deoxy-D-manno-octulosonate cytidyltransferase (CMP-KDO synthetase, or CKS) (3). All antigens included C-terminal hexahistidine tags and were purified via immobilized metal affinity chromatography to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Coating of polystyrene beads. Polystyrene beads (0.25-in diameter) were used for the solid phase for the enzyme immunoassays (EIA). Prior to coating, beads were washed with 15% 1-propanol (in water) at room temperature for 20 min without agitation. The 1-propanol was removed, and the beads were rinsed twice with deionized water. The washed beads were then added to a vial containing recombinant antigen(s) (individual or mixed antigens) diluted to a final protein concentration of 0.25 to 5.0 μg/ml in 0.1 M sodium phosphate (pH 7.0) or 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 6.3) (0.233 ml per bead). Beads were incubated at 40°C for 2 h with gentle mixing. Beads were then washed three times with PBS and then incubated in PBS containing 0.1% Triton X-100 at 40°C for 1 h with gentle mixing. They were again washed three times in PBS and then incubated at 40°C in 5% bovine serum albumin (BSA) in PBS for 1 h with gentle mixing. Beads were washed four times with PBS and then incubated at room temperature in PBS containing 5% sucrose without mixing for 20 min. The sucrose buffer was removed, and the beads were dried in air. Coated beads were stored under desiccated conditions at 4°C.

Enzyme immunoassay method. Serum or plasma was tested for immunoreactivity to antigen-coated polystyrene beads. Specimens were diluted 1:16 in diluent buffer (Tris-phosphate buffer [pH 7.8], comprising 20% goat serum, 10% calf serum, 0.2% Triton X-100, and sodium azide), and 0.010 ml was added to a well of a plastic test tray and then combined with an additional 0.20 ml of the same diluent buffer for a final sample dilution of 1:336. The recombinant protein-coated bead was added to the diluted sample and incubated at 37°C for 90 min with mixing. Beads were then washed with 11 to 14 ml of deionized water followed by the addition of 0.2 ml of peroxidase-labeled goat anti-human IgG (20 ng/ml) or anti-human IgM (250 ng/ml). Beads were incubated at 37°C for 30 min with mixing. Beads were washed with 11 to 14 ml of deionized water and then transferred into plastic tubes to which 0.3 ml of OPD substrate (0.3% *O*-phenyl-

TABLE 1. Specificity of individual *Plasmodium* MSP1-p19 enzyme immunoassays[a]

Antigen	No. of normal U.S. donors tested	IR (S/N ratio > 5.0)	RR (S/N ratio > 5.0)	Specificity (%)
P. ovale MSP1-p19	500	0	0	100
P. vivax MSP1-p19	500	6	5	99.0
P. falciparum MSP1-p19	500	0	0	100
P. malariae MSP1-p19	500	3	0	100

[a] Initial reactive rates (IR) were determined by testing normal blood donors from the United States in singlicate. Initial reactives were retested in duplicate; specimens with a mean S/N ratio > 5.0 or greater were considered to represent repeat reactives (RR). Two of the five *P. vivax* MSP1-p19 repeat reactives were positive by the Lab 21 Healthcare antibody ELISA.

enediamine-2-HCl in citrate buffer containing 0.02% H_2O_2) was added and were incubated in the dark at room temperature for 30 min without mixing. Reactions were quenched by the addition of 1 ml of 1 N H_2SO_4, and the optical density (OD) at 492 nm was determined using 1 N H_2SO_4 as the blank. The OD is directly proportional to the amount of antibody bound to the bead. Signal-to-negative (S/N) ratios were calculated for each test sample by dividing the test sample OD by the mean negative control OD. Specimens with S/N values greater than or equal to 5.00 (anti-IgG) or 10.0 (anti-IgM) were assumed to be immunoreactive.

Testing of New World monkeys for MSP1-p19 antibody assays utilized species-specific horseradish peroxidase goat anti-*Aotus* or anti-*Saimiri* IgG conjugates (kindly provided by John Barnwell, CDC). The signal-to-negative ratio for each postinfection specimen was determined by dividing the OD of the postinfection specimen by the OD of the preinfection specimen.

RESULTS

Assay design and specificity. Indirect EIAs were optimized by coating beads with MSP1-p19 antigens at a variety of protein concentrations followed by testing using a panel of known *Plasmodium* antibody-positive plasma specimens from HIV antibody-negative blood donors from Cameroon (5). The protein coating concentration for assay development was chosen by selecting that which provided OD values in the range of 0.02 to 0.05 upon testing of normal donor specimens from the United States. Beads were then coated in large batches (1,000 to 1,200 beads) and qualified using the Cameroon donor panel. Attempts to improve assay sensitivity by adding reducing agent to the sucrose-containing overcoat buffer were detrimental to performance. However, coating the recombinants with 50 mM MES buffer (pH 6.3) marginally improved sensitivity, but the improvement varied depending on the recombinant being coated (data not shown). Hence, all testing was done using beads coated with 100 mM Na_2PO_4 as described above in Materials and Methods unless otherwise indicated. Specificity for anti-IgG detection was determined for individually coated beads by testing 500 normal U.S. donor sera (Table 1). It was assumed that, since these specimens were from donors whose contributions were not deferred by the questionnaire and therefore represented a population at low risk for malaria infection, EIA-reactive specimens from such a population represent false positives. In general, a provisional cutoff value was set at the population mean value plus 9 times the standard deviation, which equates to a signal-to-negative (S/N) value of approximately 5.00. Specificity was 100% for three of the four anti-IgG EIAs and 99% for the CKS *P. vivax* MSP19 assay. The negative values for the anti-IgM assays conducted on the chimpanzee sera were determined by testing 4 to 6 replicates of a normal human donor serum sample with each experiment. This was done since we did not have a population of control animals available for determination of a provisional population-based cutoff value. While human serum could have been used for this purpose, we felt that using these data and extrapolating for use in chimpanzee assays could have resulted in an artificially high or low cutoff value. Thus, for each experiment in which IgM antibody reactivity to the various antigens was measured, a conservative cutoff value was set at 10 times the average optical density obtained with the normal human donor serum used. This was twice the value used for the interpretation of IgG assay results.

MSP1-p19 IgG antibodies in experimentally infected monkeys. Serum specimens were available from New World monkeys infected with two different strains of *P. falciparum*. These included sera obtained immediately prior to the time of infection. All specimens were tested using each of the four MSP1-p19 EIAs. There was no immunoreactivity to *P. ovale*, *P. vivax*, or *P. malariae* MSP1-p19 antigens in any pre- or postinfection sample (Table 2). All serum specimens collected within 15 to 18 days postinfection exhibited immunoreactivity to *P. falciparum* MSP1-p19. Thus, the *P. falciparum* MSP1-p19 EIA detects antibodies to two different strains of *P. falciparum* in these nonhuman primate models within approximately 2 weeks of infection.

MSP1-p19 antibodies in experimentally infected chimpanzees. Chimpanzee serum samples collected within 16 to 68 days postinoculation with *P. malariae*, *P. ovale*, or *P. vivax* were provided by the CDC for testing (Table 3). Serum specimens were tested for the presence of IgG and IgM antibodies by the use of the four individual MSP1-p19 EIAs. *P. falciparum* MSP1-p19 IgG was detected in only 1 of the 14 chimpanzees. This reactivity to the *P. falciparum* antigen may reflect prior infection with *P. reichenowi*, a great ape malaria species that is closely related to *P. falciparum*. *P. malaria*e MSP1-p19 IgG and IgM antibodies were detected in the single animal infected with *P. malariae*. This animal also exhibited antibodies against other MSP1-p19 antigens, predominantly IgM. *P. ovale* MSP1-p19 IgG antibodies were detected in three of four chimpanzees infected with *P. ovale*, and IgM antibodies were detected in all four (100%). *P. vivax* MSP1-p19 IgG antibodies were detected in 7/9 (78%) chimpanzees infected with *P. vivax*, while IgM antibodies were detected in all 9 (100%). Overall, the antibody detection rate was higher using recombinant antigens corresponding to the infecting *Plasmodium* species.

MSP1-p19 antibodies in human malaria patients. Serum samples from microscopy- and IFA-confirmed malaria patients were made available by the Centers for Disease Control and Prevention. Immunofluorescent antibody titers for each human infectious *Plasmodium* species for each sample were known, as was the species identification determined by blood smear analysis (Table 4). The times between infection or clinical presentation and sample collection were not known; however, the ability to detect parasites in blood smears suggests that they were collected at or near the onset of symptoms (possibly due to initial infection or relapse).

Among the 22 specimens available, 16 were confirmed by microscopy to be from *P. vivax*- or *P. ovale*-infected individuals and 4 to be from *P. falciparum*- and 2 from *P. malariae*-infected individuals (Table 4). The commercial ELISA detected anti-

TABLE 2. Detection of MSP1-p19 IgG in experimentally infected New World monkeys[a]

Animal	Date collected (day-mo-yr)	Infection[b]	IFA titer				MSP1-p19 EIA S/N ratio			
			P. falciparum 3D7	*P. vivax* SalI	*P. malariae*	*P. ovale*	*P. falciparum*	*P. vivax*	*P. ovale*	*P. malariae*
AI 1112	13-12-1995	None	16	<16	16	<16	1.0	1.0	1.0	1.0
	28-12-1995	*P. falciparum* FVO	≥16,384	256	256	256	26.6	3.2	1.0	1.0
AI 1113	13-12-1995	None	16	<16	16	<16	1.0	1.0	1.0	1.0
	28-12-1995	*P. falciparum* FVO	≥16,384	1,024	1,024	256	47.6	2.2	1.0	1.0
AI 2009	13-12-1995	None	64	<16	<16	16	1.0	1.0	1.0	1.0
	28-12-1995	*P. falciparum* FVO	4,096	256	1,024	256	40.8	0.8	1.0	1.0
SI 214	31-10-1997	None	<16	16	<16	<16	1.0	1.0	1.0	1.0
	18-11-1997	*P. falciparum* FUP	1,024	256	1,024	64	6.8	1.3	1.0	1.0
SI 840	31-10-1997	None	<16	16	<16	<16	1.0	1.0	1.0	1.0
	18-11-1997	*P. falciparum* FUP	4,096	256	256	256	154.1	1.4	1.0	1.0
SI 927	31-10-1997	None	16	16	<16	<16	1.0	1.0	1.0	1.0
	18-11-1997	*P. falciparum* FUP	4,096	256	64	64	68.5	1.7	1.0	1.0

[a] Samples exhibiting an S/N ratio ≥ 5.00 were considered antibody positive. Infection shown as "None" indicates serum collected prior to infection.
[b] *P. falciparum* FVO, Vietnam-Oak Knoll strain; *P. falciparum* FUP, Uganda Palo Alto strain.

bodies in all specimens from *P. falciparum*- and *P. vivax*-infected patients. Of the eight individuals with *P. vivax* infections, the *P. vivax* MSP1-p19 EIA detected IgG antibodies in all eight. Three of these individuals were shown to be *P. ovale* antibody positive by IFA; the *P. ovale* MSP1-p19 EIA detected IgG in two of the three. The four *P. falciparum*-infected individuals were shown to be antibody positive by using the commercial ELISA. However, the *P. falciparum* MSP1-p19 EIA detected antibodies in only two. *P. vivax* MSP1-p19 IgG was detected in the other two *P. falciparum*-infected individuals, both of whom were shown to be *P. vivax* antibody positive by IFA. The apparent discrepancy between the Lab 21 Healthcare assay and the *P. falciparum* MSP1-p19 EIA may have been related to the presence of multiple *P. falciparum* antigens in the Lab 21 Healthcare ELISA, allowing for the detection of additional antibodies. In addition, since the latter assay utilizes a direct format, it can detect IgG and IgM or IgA antibodies. IgM antibodies directed against *P. falciparum* MSP1-p19 were not detected in the two discrepant samples when anti-IgM peroxidase conjugate was used (data not shown). The *P. malariae* MSP1-p19 EIA detected IgG antibodies in sera from the two *P. malariae*-infected individuals; however, both of these individuals were shown to be antibody negative by using the commercial ELISA despite the IFA detection of *P. falciparum* antibodies in one of the two. The *P. ovale* MSP1-p19 EIA detected IgG in 8/8 (100%) of individuals with microscopy-confirmed *P. ovale* infection, while the Lab 21 Healthcare ELISA detected antibodies in only 5. Among the eight *P. ovale* infections, 4 were shown to have corresponding IFA titers greater than 1:256 for *P. falciparum* or *P. vivax*—these 4 specimens were positive in the commercial ELISA, suggesting that preexisting *P. falciparum* or *P. vivax* antibodies may

TABLE 3. *Plasmodium* MSP1-p19 IgG and IgM antibodies in experimentally infected chimpanzees

Animal	Infection	Species-specific IgG titer determined by IFA				S/N ratio[a]							
						P. falciparum		*P. vivax*		*P. ovale*		*P. malariae*	
		P. falciparum 3D7	*P. vivax* SalI	*P. malariae*	*P. ovale*	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
Heppie	*P. malariae*	≥16,384	≥16,384	≥16,384	≥16,384	1.3	10.1	7.3	93.4	5.3	20.1	67.8	109.1
Foxy	*P. ovale*	1,024	4,096	1,024	≥16,384	1.0	7.3	1.0	6.2	13.4	109.1	1.3	45.1
Jeanie	*P. ovale*	256	≥16,384	256	≥16,384	2.8	4.5	2.5	2.7	2.3	69.2	0.9	13.0
Marietta	*P. ovale*	1,024	≥16,384	1,024	≥16,384	1.1	2.0	1.1	4.9	22.6	109.1	0.9	24.5
Merv	*P. ovale*	256	≥16,384	1,024	4,096	107.1	74.6	107.1	98.8	131.1	109.1	67.8	109.1
Amanda	*P. vivax*	256	≥16,384	256	≥16,384	1.0	5.0	25.2	107.1	1.0	10.2	0.8	16.4
Arthur	*P. vivax*	4,096	≥16,384	1,024	≥16,384	0.8	6.9	44.9	107.1	1.5	29.5	1.5	47.5
Brandy	*P. vivax*	64	≥16,384	16	≥16,384	1.0	1.7	1.0	16.7	1.0	3.2	0.7	3.8
Brodie	*P. vivax*	64	≥16,384	64	≥16,384	1.0	8.8	19.1	107.1	1.5	7.1	0.9	7.3
Callie	*P. vivax*	256	≥16,384	64	≥16,384	0.9	1.7	14.5	107.1	0.9	5.0	1.1	20.5
Edwina	*P. vivax*	256	4,096	64	≥16,384	0.8	3.8	3.1	103.0	0.8	3.2	0.7	32.0
Luther	*P. vivax*	64	4,096	64	256	1.1	2.2	12.8	107.1	0.7	4.4	0.6	11.0
Mary	*P. vivax*	4,096	≥16,384	1,024	≥16,384	1.2	5.1	107.1	107.1	131.1	83.3	5.6	7.1
Patrick	*P. vivax*	4,096	≥16,384	1,024	≥16,384	1.1	11.1	34.2	107.1	1.4	18.4	1.0	32.3

[a] Samples exhibiting an S/N ratio ≥ 5.00 (IgG assays) and S/N ratio ≥ 10.0 (IgM assays) were considered antibody positive.

TABLE 4. *Plasmodium* MSP1-p19 IgG antibodies among blood smear- and IFA antibody-positive human patients[a]

Sample	Species	IFA result(s)	Commercial ELISA S/CO	MSP1-p19 EIA S/N ratio			
				P. falciparum	*P. vivax*	*P. malariae*	*P. ovale*
Pv-1	*P. vivax*	*P. vivax*	2.41	1.3	62.1	12.3	1.1
Pv-2	*P. vivax*	*P. vivax*	19.20	1.3	67.4	2.7	8.3
Pv-5	*P. vivax*	*P. vivax*, *P. ovale*	19.20	1.3	67.4	2.0	11.0
Pv-9	*P. vivax*	*P. vivax*, *P. ovale*	19.20	0.6	67.4	2.5	13.2
Pv-10	*P. vivax*	*P. vivax*	4.82	1.7	49.5	10.9	1.9
Pv-11	*P. vivax*	*P. vivax*, *P. ovale*	19.20	10.6	67.4	1.6	1.0
Pv-12	*P. vivax*	*P. vivax*	6.69	0.5	41.0	0.9	1.4
Pv-14	*P. vivax*	*P. vivax*, *P. falciparum*	19.20	11.4	67.4	0.8	2.4
Pf-6	*P. falciparum*	*P. falciparum*	19.20	72.3	1.0	45.1	5.3
Pf-16	*P. falciparum*	*P. falciparum*	12.02	7.8[b]	2.9	4.8	7.6
Pf-17	*P. falciparum*	*P. vivax*, *P. falciparum*, *P. ovale*	19.20	1.8	14.6	1.2	0.7
Pf-18	*P. falciparum*	*P. vivax*, *P. falciparum*	19.20	1.0	8.2	0.6	0.5
Pm-2	*P. malariae*	*P. malariae*	0.29	0.8	0.7	67.4	0.9
Pm-15	*P. malariae*	*P. falciparum*, *P. malariae*	0.88	1.3	0.6	8.0	0.6
Po-1	*P. ovale*	*P. falciparum*, *P. ovale*	14.49	34.6	10.2	4.3	100.0
Po-19	*P. ovale*	*P. vivax*, *P. ovale*	19.20	3.8	67.4	6.3	43.7
Po-20	*P. ovale*	*P. ovale*	0.43	0.4	0.7	0.5	11.5
Po-21	*P. ovale*	*P. vivax*, *P. ovale*	16.78	63.7	16.0	24.8	100.0
Po-23	*P. ovale*	*P. falciparum*, *P. ovale*	16.54	43.3	11.1	5.3	100.0
Po-24	*P. ovale*	*P. ovale*	13.88	47.8	1.1	1.2	47.8
Po-25	*P. ovale*	*P. ovale*	0.37	1.2	0.7	0.8	44.3
Po-26	*P. ovale*	*P. ovale*	0.88	13.8	0.8	2.2	69.3

[a] Samples exhibiting an S/N ratio ≥ 5.00 were considered antibody positive. The IFA column entries represent the detection of antibodies to any species with a titer greater than 1:256. The species column entries represent *Plasmodium* species identified by microscopic examination of blood smears. S/CO, signal-to-cutoff ratio; values greater than 1.00 represent positive results.

[b] S/N ratio of 24.1 using beads coated in MES buffer.

have contributed to the observed reactivity in the commercial assay.

In an attempt to develop a single assay capable of detecting MSP1-p19 antibodies from all four *Plasmodium* species (a pan-malaria antibody assay), beads were coated with a mixture of all four antigens. Preliminary experiments had indicated that the use of 50 mM MES buffer (pH 6.3) during the coating process improved individual assay performance; thus, the co-coating of antigens was done using this buffer. The pan-malaria MSP1-p19 assay was performed on the panel of sera from human malaria patients described above; IgG antibodies were detected in 22/22 (100%) of the panel members, whereas the commercial assay lacking *P. ovale* or *P. malariae* antigens detected only 17/22 (77.3%) (Table 5). The specificity of this assay was not determined.

DISCUSSION

Commercial antibody assays are available whose manufacturers claim detection of human antibodies elicited by *P. falciparum*, *P. vivax* and *P. ovale*, and *P. malariae* infection. Many have been evaluated for their ability to detect antibodies in malaria patients and blood donor populations. For example, the manufacturer of a pan-malaria-antibody competitive ELISA (CELISA; Cellabs Pty. Ltd., Brookvale, NSW, Australia) claims detection of antibodies against the four principal *Plasmodium* species that cause malaria in humans with a sensitivity of 94% versus IFA (per package insert). However, Mertens et al. (32) indicated poor sensitivity of the assay compared to IFA for *P. falciparum* (64%) and non-*P. falciparum* (0 to 33%) malaria antibody detection. Evaluation of the assay by She et al. (44) showed an inability of the assay to detect antibodies in some individuals who gave positive results for *P. ovale* or *P. malariae* antibodies by IFA. A study using an assay from DiaMed AG (Switzerland) that utilizes extracts of cultured *P. falciparum* and *P. vivax* recombinant circumsporozoite protein demonstrated poor sensitivity for detection of antibodies among symptomatic individuals with microscopically confirmed *P. vivax* infection (18/24) but evaluated only 4 individuals with *P. ovale* or *P. malariae* infection, though all of the individuals were antibody positive (13). Assessment of the DiaMed assay in Korea, where *P. vivax* malaria predominates, indicated sensitivity of only 53% relative to microscopy and PCR (38). The poor detection of antibodies in cases of *P. vivax*-confirmed infection may be due to the choice of CSP, an antigen of sporozoites that is also found in the liver (39, 46), or possibly to strain differences. The package insert accompanying the malaria antibody assay manufactured by Lab 21 Healthcare Laboratories, Ltd. (Kentford, United Kingdom) claims detection of all four species responsible for human malaria, though the assay contains only *P. falciparum*- and *P. vivax*-derived recombinant antigens. The package insert indicates sensitivities for *P. ovale* and *P. malariae* antibody detection of 80% and 67%, respectively. Independent evaluation of the Lab 21 Healthcare ELISA showed detection of only 9/14 (64%) patients with acute *P. ovale* malaria and only 85% (15/18) of patients with *P. vivax* malaria (25). We found this assay unable to detect antibodies in all of the individuals with microscopy-confirmed *P. ovale* or *P. malariae* infection (Table 4). Our utilization of MSP1-p19 from these species allowed detection of IgG antibodies in these individuals.

Among commercial antibody tests where the source and/or composition of solid-phase antigens is known (e.g., Lab 21 Healthcare [42], DiaMed [16], and several Korean assays [see

TABLE 5. Pan-*Plasmodium* MSP-19 IgG antibodies among blood smear- and IFA antibody-positive human malaria patients[a]

Sample	Species	IFA	Commercial ELISA S/CO	Pan-malaria MSP1-p19 EIA S/N ratio
Pv-1	*P. vivax*	*P. vivax*	2.41	58.35
Pv-2	*P. vivax*	*P. vivax*	19.20	65.93
Pv-5	*P. vivax*	*P. vivax*, *P. ovale*	19.20	65.93
Pv-9	*P. vivax*	*P. vivax*, *P. ovale*	19.20	65.93
Pv-10	*P. vivax*	*P. vivax*	4.82	47.27
Pv-11	*P. vivax*	*P. vivax*, *P. ovale*	19.20	65.93
Pv-12	*P. vivax*	*P. vivax*	6.69	37.62
Pv-14	*P. vivax*	*P. vivax*, *P. falciparum*	19.20	65.93
Pf-6	*P. falciparum*	*P. falciparum*	19.20	65.93
Pf-16	*P. falciparum*	*P. falciparum*	12.02	8.51
Pf-17	*P. falciparum*	*P. vivax*, *P. falciparum*, *P. ovale*	19.20	20.27
Pf-18	*P. falciparum*	*P. vivax*, *P. falciparum*	19.20	12.76
Pm-2	*P. malariae*	*P. malariae*	0.29	65.93
Pm-15	*P. malariae*	*P. falciparum*, *P. malariae*	0.88	5.11
Po-1	*P. ovale*	*P. falciparum*, *P. ovale*	14.49	65.93
Po-19	*P. ovale*	*P. vivax*, *P. ovale*	19.20	65.93
Po-20	*P. ovale*	*P. ovale*	0.43	2.64[b]
Po-21	*P. ovale*	*P. vivax*, *P. ovale*	16.78	65.93
Po-23	*P. ovale*	*P. falciparum*, *P. ovale*	16.54	65.93
Po-24	*P. ovale*	*P. ovale*	13.88	55.35
Po-25	*P. ovale*	*P. ovale*	0.37	23.47
Po-26	*P. ovale*	*P. ovale*	0.88	32.37

[a] Data represent EIA results obtained using beads cocoated with four MSP1-p19 recombinant proteins. The species column entries represent *Plasmodium* species identified by microscopic examination of blood smears. The IFA column entries represent those species detected with an antibody titer greater than 1:256. Samples exhibiting an S/N ratio $\geq$ 5.00 are considered antibody positive.

[b] S/N ratio of 11.5 using *P. ovale* MSP1-p19 antigen-only EIA.

reference 30]), the absence of *P. ovale*- or *P. malariae*-derived antigens suggests that detection of antibodies to these species must rely on antibody cross-reactivity to *P. falciparum* or *P. vivax* antigens—unless reactivity is due to the persistence of *P. vivax* or *P. falciparum* antibodies from previous infections. We detected *P. vivax* MSP1-p19 IgG reactivity among all individuals with microscopy-confirmed *P. ovale* infection for whom IFA exhibited the presence of both *P. ovale* and *P. vivax* antibodies (Table 4). This finding could be explained by recent infection with *P. ovale* (or a relapse) and persistence of *P. vivax* antibodies from a previous infection. The Lab 21 Healthcare ELISA gave positive results for 4/4 individuals with *P. falciparum* malaria, whereas the *P. falciparum* MSP1-p19 EIA gave positive results for only 2 (Pf-17, Pf-18) of 4. Antibodies against the three *P. falciparum* antigens used in the Lab 21 Healthcare assay, when the tests were conducted independently, were not detected (data not shown), suggesting that immunoreactivity in the commercial assay was due to the presence of *P. vivax* antibodies only (both individuals were *P. vivax* antibody positive by IFA). Individual EIAs utilizing recombinant *P. vivax* MSP1-p19 (Table 4) (and *P. vivax* MSP1-p33; data not shown) detected IgG antibodies in these specimens. Thus, it is possible that the inability to detect *P. falciparum* MSP1-p19 antibodies was due to one of the following: (i) the microscopic diagnosis of *P. falciparum* infection was in error; (ii) the inability to detect *P. falciparum* MSP1-p19 antibodies was due to strain differences between the antigen used in the assay and the infecting species; or (iii) the sample was from a patient with an acute infection prior to seroconversion.

Antibody seroconversion profiles among humans naturally infected with *Plasmodium* organisms have previously been determined by using IFA (6–9), but data from longitudinal studies describing the antibody response to individual markers have been limited (1, 33, 34). Animal models for the investigation of human malaria have been established and are ideal for studying the temporal relationship between infection and antibody detection. Among chimpanzees infected with *P. vivax*, *P. ovale*, or *P. malariae*, IgG antibodies were detected in most animals within 16 to 68 days postinfection; IgM antibodies were detected in all chimpanzees by the use of species-homologous MSP1-p19 EIAs (Table 3). Some apparent cross-reactivity among the non-*P. falciparum*-infected chimpanzees was observed with *P. falciparum* MSP1-p19; perhaps this cross-reactivity with *P. falciparum* reflects prior infection with *P. reichenowi*, a species causing great ape malaria that is closely related to *P. falciparum*. However, antibodies to MSP1-p19 were most efficiently detected by using recombinant antigens derived from the infecting species. Using New World monkeys experimentally infected with two different *P. falciparum* strains, we detected *P. falciparum* MSP1-p19 IgG antibodies in sera collected 15 to 18 days postinfection (Table 2). There was no cross-reactivity with the MSP1-p19 antigens from the other three strains despite the apparent cross-reactivity observed by using IFA. Interestingly, in 4 of the 6 monkeys we also detected *P. falciparum* CSP IgG (data not shown). These data indicate that anti-MSP1-p19 antibodies are detectable within days of infection in these experimental animal models. Such models are useful for the evaluation of antigen immunoreactivity during the acute and chronic phases of infection.

Current U.S. blood donor evaluation policy results in deferral of 73,000 to 75,000 donations per year due to travel, residence in an area where malaria is endemic, or a history of malaria (28, 29). Implementation of universal donor screening, or screening of deferred donors for reentry into the donation

pool after a short deferral period (as is done in some countries [20, 43]), could significantly increase the pool of available donors, since most deferrals are imposed on the basis of travel to low-risk areas (e.g., Mexico) and since the rate of post-transfusion-transmitted malaria (PTTM) in the United States is extremely low (28, 36). However, while the rate of PTTM is low, there exists a significant percentage of nondeferred donors who harbor anti-*Plasmodium* antibodies. A recent American Red Cross study (27) identified 18 of 1,227 (1.47%) deferred donors who gave repeated positive results that indicated reactivity to antibodies by using a commercially available test (Lab21 Healthcare). Of interest was that 11 of 3,229 (0.34%) nondeferred donors also gave repeated positive results that indicated reactivity. We tested the samples from the nondeferred donors who gave positive results for anti-*P. falciparum* and anti-*P. vivax* MSP1-p19 in individual assays and found 7 of the 11 to be antibody positive in one or both assays. Of note is that 4 of the 7 individuals that gave positive results admitted having had malaria in the past—and one whose infection had occurred as long ago as 1958 still exhibited significant levels of antibodies against MSP1-p19 (data not shown). Whether such individuals could transmit disease via transfusion is unknown, and the authors of the study suggested that permanent deferral of individuals with a history of malaria should be considered. We found no immunoreactivity to *P. ovale* or *P. malariae* MSP1-p19 among the Lab21 immunoassay-positive deferred or nondeferred donor samples (data not shown). If universal donor screening is implemented, care must be taken to ensure that assay sensitivity and specificity are equivalent to, and preferably better than, the accuracy represented by the current questionnaire.

Our results show the importance of utilizing antigens from all four human *Plasmodium* parasites for accurate detection of antibodies. While recent evidence indicates a wider prevalence and incidence of *P. knowlesi* infection in humans (11, 12, 21, 31, 37), to our knowledge, there have been no reports of post-transfusion *P. knowlesi* infections. However, because the MSP1 gene sequence of *P. knowlesi* is already known, it would be possible to incorporate a fifth antigen into future iterations of an MSP1-based antibody test.

In summary, we have shown use of the MSP1-p19 antigens from *P. malariae* and *P. ovale* to be effective for the detection of antibodies in human malaria patients with IFA- and microscopy-confirmed *P. ovale* or *P. malariae* infection. Further evidence of their serological efficacy is shown by detection of IgG and IgM among experimentally infected chimpanzees. Furthermore, we demonstrated the feasibility of combining the MSP1-p19 antigens from all four human infective *Plasmodium* species into a single prototype antibody assay. Though the specificity of this pan-malaria assay was not determined, the sensitivity was superior to that of the Lab 21 commercial assay. Utilization of MSP1-p19 antigens from *P. ovale* and *P. malariae*, as well as from *P. falciparum* and *P. vivax*, for antibody detection should provide significantly greater efficacy than the use of *P. vivax* and *P. falciparum* antigens alone.

ACKNOWLEDGMENTS

Potential conflicts of interest: A.S.M., L.G.B., R.C., B.J.D., G.J.D., and S.M.D. are employees of Abbott Laboratories.

We thank Gerald Schochetman, Senior Director of Infectious Disease Research, Abbott Diagnostics, for program support and Alla Haller for technical support.

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