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Eric Rogier, Centers for Disease Control and Prevention
Delynn M. Moss, Centers for Disease Control and Prevention
Anna N. Chard, Emory University
Victoria Trinies, Emory University
Seydou Doumbia, University of Sciences, Techniques and Technologies of Bamako (USTTB)
Matthew Freeman, Emory University
Patrick Lammie, Emory University

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Evaluation of Immunoglobulin G Responses to \textit{Plasmodium falciparum} and \textit{Plasmodium vivax} in Malian School Children Using Multiplex Bead Assay

Eric Rogier,1* Delynn M. Moss,2 Anna N. Chard,3 Victoria Trinies,3 Seydou Doumbia,4 Matthew C. Freeman,3 and Patrick J. Lammie1

1Division of Parasitic Diseases and Malaria, Centers for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia; 2Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; 3Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, Georgia; 4Malaria Research and Training Center, Faculty of Medicine and Odontostomatology, University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali

Abstract. Malaria serology through assaying for IgG against \textit{Plasmodium} spp. antigens provides evidence into the infection history for an individual. The multiplex bead assay (MBA) allows for detection of IgG against multiple \textit{Plasmodium} spp., and can be especially useful in many regions where \textit{Plasmodium falciparum} is of primary clinical focus, but other species are co-endemic. Dried blood spots were collected from 805 Malian children attending 42 elementary schools in the regions of Mopti, Sikasso, Koulikoro, and Bamako capital district, and IgG assayed by MBA. As southern Mali is known to be holoendemic for \textit{P. falciparum}, merozoite surface protein 1 19-kDa subunit (MSP-142) and apical membrane antigen 1 (AMA-1) antigens were included for serology against this parasite. Responses to these antigens both provided high estimates for lifetime exposure, with 730 (90%) children with IgG antibodies for MSP-142, 737 (91%) for AMA-1, and 773 (96%) positive for either or both. Also included was the antigen \textit{Plasmodium vivax} MSP-119, against which 140 (17.4%) children were found to have antibodies. Increases in antibody titers with older age were clearly seen with the \textit{P. falciparum} antigens, but not with the \textit{P. vivax} antigen, likely indicating more of a sporadic, rather than sustained transmission for this species. The MBA provides effective opportunities to evaluate malaria transmission through serological analysis for multiple \textit{Plasmodium} species.

INTRODUCTION

Malaria is caused by parasites within the protozoan genus \textit{Plasmodium} that are transmitted by the mosquito genus \textit{Anopheles}. The blood stages of both \textit{Plasmodium vivax} and \textit{Plasmodium falciparum} can cause severe illness and death by invading and destroying red blood cells (RBCs), and appropriate parasite identification and surveillance at the species level is needed to implement appropriate antimalarial drug strategies for a region. \textit{Plasmodium falciparum} is the most prevalent and clinically relevant malaria on the African continent while \textit{P. vivax} is generally found in populations more likely to carry the Duffy erythrocyte receptor that allows \textit{P. vivax} attachment to RBCs.1 In 2000, 2.4 billion people in 106 countries and territories were at risk of malaria infection; however, because of growing resistance to drugs and insecticides, environmental changes, and human migration, these numbers in 2015 have increased to 3.2 billion people at risk in only 97 countries.2 Effective control efforts through insecticide-treated nets, indoor residual spraying, artemisinin-based combination therapies, and other interventions have substantially reduced \textit{P. falciparum} cases and deaths, but have uncovered problems with a stubbornly persistent \textit{P. vivax}, which has different biodynamics of infection.2,3

Mali, an economically poor country traversing Saharan and sub-Saharan Africa, is holoendemic for falciparum malaria in the southern part of the nation.4,5 In 2015, it was estimated that the parasite prevalence in some regions of southern Mali exceeded 80%,6 representing a large clinical burden for the area. As with other areas in Africa,7 infection with \textit{P. vivax} is becoming more documented in Mali, and recent studies have shown infection with this parasite as both a single and mixed infection with \textit{P. falciparum}.8,9 Serology can provide valuable information on the prevalence of exposure to malaria and can be helpful for characterizing parasite transmission dynamics in an area, and identifying where interventions are needed the most. As some \textit{Plasmodium} spp. antigens are known to elicit an IgG response that can be detected for a long period, serological analysis of children can provide an estimate of lifetime exposure for these young individuals.10 To this end, we included \textit{P. falciparum} and \textit{P. vivax} antigens in a serology study that evaluated IgG responses by a multiplex bead assay (MBA), which has been used in other serological studies.11-13 Recombinant antigens included the \textit{P. vivax} merozoite surface protein 1 19-kDa subunit (\textit{P. vivax} MSP-142), the 42-kDa subunit of \textit{P. falciparum} MSP-1 (MSP-142), and \textit{P. falciparum} apical membrane antigen 1 (AMA-1).

MATERIALS AND METHODS

Study population. The Ethics Committee of the National Institute of Public Health Research in Mali (02/2014/CE- INRSP) and the Institutional Review Board of Emory University reviewed and approved this study (IRB00060756). The trial was registered at ClinicalTrials.gov (NCT01787058). Data come from a cross-sectional serological study evaluating Ig G responses to antigens from a range of pathogens and vaccine-preventable diseases, which was nested within a longitudinal impact evaluation of a school-based water, sanitation, and hygiene (WASH) program in Mali. Detailed methods and results from the impact evaluation are found elsewhere.14 Laboratory staff from the Centers for Disease Control and Prevention had no contact with children enrolled in the study nor any access to personal identifiers. A total of 805 Malian children, age range 4–17 years, in 42 elementary schools in the regions of Mopti, Sikasso, Koulikoro, and Bamako capital

*Address correspondence to Eric Rogier, Centers for Disease Control and Prevention, Division of Parasitic Diseases and Malaria, 1600 Clifton Road, Atlanta, GA 30329. E-mail: wwx6@cdc.gov
Successful coupling for ethanesulfonic acid, 0.85% NaCl, pH 5.0. As a control, a dif-

After collection and drying at room temperature, DBSs were stored at −20°C. Samples were collected between January and June 2014, which is the dry season in Mali.

**Antigen coupling to beads.** The recombinant antigen \( \text{P. vivax MSP-1}_{19} \) was fused with glutathione-S-transferase (GST) to allow purification. The external domain of AMA-1 and \( \text{MSP-1}_{42} \) antigens were produced at Walter Reed Army Institute of Research under previously published conditions. To couple antigens to polystyrene beads, the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide method was used to convert carboxyl groups on the surface of specifically classified spectral magnetic polystyrene microspheres (MagPlex Beads; Luminex Corporation, Austin, TX) to reactive esters. The esters readily react with available primary amino groups on the antigens to form covalent amide bonds between antigen and microspheres. The recombinant antigens were coupled to differently classified spectral activated beads (12.5 million) using 23 \( \mu \text{g} \) \( \text{P. vivax MSP-1}_{19}\text{GST} \), 23 \( \mu \text{g} \) AMA-1, and 17 \( \mu \text{g} \) \( \text{MSP-1}_{42} \) in 50 mM 2-(N-morpholino)ethanesulfonic acid, 0.85% NaCl, pH 5.0. As a control, a different bead classification was coupled with 15 \( \mu \text{g} \) GST using the same conditions to ensure no nonspecific binding of human IgG to the GST fusion protein on the \( \text{P. vivax} \) antigen. Successful coupling for \( \text{P. vivax MSP-1}_{19} \) (fused to GST) was determined by test runs using an in-house polyclonal IgG anti-GST. In addition, completed couplings of \( \text{P. falciparum} \) and \( \text{P. vivax} \) antigens to beads were validated by reactivity to known positive sera pools. Blank wells and positive and negative sera were included on each assay plate for the study as controls.

**DBS elution and serology data acquisition.** One filter paper extension (10 \( \mu \text{L} \) dried whole blood) from each child was placed in 0.5 mL of elution buffer consisting of phosphate-buffered saline (PBS) with 0.5% bovine serum albumin, 0.3% Tween 20, 0.1% sodium azide, 0.5% polyvi-

The \( \text{E. coli} \) extract adsorbs \( \text{E. coli} \)-specific IgG that could react with residual \( \text{E. coli} \) proteins coupled to beads. After overnight storage at 4°C, the final dilutions, representing a serum dilution of approximately 1:400, were exposed to anti-
gen species for children within each of the 42 schools as shown in Figure 1B and C. Of 42 schools, 20 (47.6%) had 100% of children positive for either or both antigens. This was also reflected in mapping of the percent positive children within each of the 42 schools shown in Figure 1B and C. Of 42 schools, 20 (47.6%) had 100% of children with \( \text{MSP-1}_{42} \) antibodies and 16 (38.1%) had 100% of children with AMA-1 antibodies. Overall, 32 (76.2%) of the 42 schools had 100% of children positive for \( \text{MSP-1}_{42} \) or AMA-1 antibodies. Due to the saturation of his-
torical \( \text{P. falciparum} \) infection, it was difficult to assess any geospatial patterning for the transmission intensity of this parasite. However, a few schools around the urban center of Bamako showed relatively lower percentages of children having IgG antibodies against \( \text{MSP-1}_{42} \), with 28 schools having exactly 20 children sampled.

RESULTS

Study area and schools. As shown in Figure 1A, 42 schools were included in the study, representing a large part of southern Mali. Four Mali regions were included: Mopti with four schools, Sikasso with 16, Koulikoro with 20, and the Bamako capital district with two schools. An average of 19 children were sampled from each school (range: 14–21), with 28 schools having exactly 20 children sampled.

Prevalence of antigen positivity by school. Of all children in the study, a high percentage were found to be positive for \( \text{P. falciparum} \) antibodies with 90% of all children having IgG antibodies against \( \text{MSP-1}_{42} \). 91% of children with antibodies to AMA-1, and 96% positive for either or both antigens. This was also reflected in mapping of the percent positive children within each of the 42 schools as shown in Figure 1B and C. Of 42 schools, 20 (47.6%) had 100% of children with \( \text{MSP-1}_{42} \) antibodies and 16 (38.1%) had 100% of children with AMA-1 antibodies. Overall, 32 (76.2%) of the 42 schools had 100% of children positive for \( \text{MSP-1}_{42} \) or AMA-1 antibodies. Due to the saturation of his-
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positive for *P. falciparum* antigens. In addition, elevation of the school was plotted against antibody prevalence, but no correlation was observed (data not shown).

Serology for *P. vivax* revealed much different findings, with only 17.5% of all Malian children sampled having antibodies against this parasite. As shown in Figure 1D, this varied widely, with school positivity rates of 0–36%, and showed a higher number of positive children in the southernmost region of Sikasso. Interestingly, only two of the 42 schools showed a complete absence of children seropositive for *P. vivax*, indicating low-level and dispersed transmission for this parasite. Again, plotting school elevation against antibody prevalence within the school was found to provide no correlation (data not shown).

**Relationship of MFI-bg signals from different antigens.**

The *P. falciparum* antigens MSP-1 and AMA-1 have been used extensively in malaria serological studies, and are both thought to induce IgG antibody responses which would be present for long periods of time following infection. For this cohort of Malian children, we found the MFI-bg signal intensities for each of these antigens to be highly skewed, with the distinct possibility of having a high titer for one of these antigens and not for the other (Figure 2). However, the antibody signal intensities for these two antigens were found to be correlated for the overall population of children (Spearman’s rank correlation coefficient: $\rho = 0.493$, $P < 0.0001$). Testing for the relationship between *P. falciparum* MSP-1$_{42}$ and *P. vivax* MSP-1$_{19}$ found a statistically significant rank correlation ($\rho = 0.299$, $P < 0.001$), but antibody intensity varied, with the *P. vivax* antigen showing fewer high titers.
the MBA, the serology for *P. falciparum* antigens showed a strong positive skew, with many of the observations on the upper end of the Bio-Plex machine's fluorescence detection capacity (Figure 3). As explained in Methods section, sero-positivity cutoff determination for the *P. falciparum* antigens provided a cutoff value that was well outside the highest density of the MFI-bg distribution, and showed strong evidence for very few children in this Malian population as not having IgG antibodies against *P. falciparum*. In contrast, the distribution of the *P. vivax* MSP-119 MFI-bg signal provided a much more symmetric distribution, and indicated the majority of children as not having antibodies against *P. vivax*. Overall, antibody titers were lower for the children considered positive for *P. vivax* antibodies, but some children showed MFI-bg values in the high thousands (Figures 2 and 3), possibly indicating recent, or multiple lifetime, *P. vivax* infections for these few individuals.

**Malaria antibody levels in relation to child age.** At birth, children in malaria-endemic regions tend to have significant titers of maternal antimalarial antibodies, but these generally are lost by approximately the 6th month of life. In high-transmission settings, children are almost inevitably exposed to malaria at a young age, and begin developing IgG antibodies based on their own adaptive immune responses. As shown in Figure 4 for this sample of Malian children aged 5–16 years, we found a substantial increase in the mean *P. falciparum* antibody levels as age increased. Older children in this setting have an almost certainty of being exposed to *P. falciparum* multiple times throughout their lives, and this is reflected by the increase in titers for these antigens. Modeling for antibody increase over time revealed an increase for every year of life of 756 MFI-bg units for MSP-142 (Wald $\chi^2 = 9.69, P = 0.002$) and 616 MFI-bg units for AMA-1 (Wald $\chi^2 = 6.46, P = 0.011$). This same trend was not observed in the antibody levels for the *P. vivax* MSP-119 antibodies, likely due to the overall low probability of exposure. Modeling provided an estimated increase of 14 MFI-bg units for every year of life, but this was nonsignificant (Wald $\chi^2 = 0.18, P = 0.67$). Throughout this age range of children, high antibody levels appeared to be random across ages, suggesting stochastic exposure and largely the absence of repeated exposures as children age.

**DISCUSSION**

Many areas of Africa still remain holoendemic for *P. falciparum*, and this parasite represents an enormous
Serological studies for malaria assess the concept of historical transmission rather than prevalence estimates through active case detection. In areas of the world co-endemic for multiple human malarias, the MBA provides a cost-effective and practical way to estimate transmission histories for multiple species. In addition, by the nature of having serology data on all antigens collected simultaneously, the MBA provides malaria research groups increased potential to gather data from studies conducted by other disease groups. In this study, we found that schoolchildren in southern Mali showed nearly ubiquitous carriage of IgG antibodies against *P. falciparum* antigens MSP-142 and AMA-1, typically at high titers which would indicate both repeated and recent lifetime exposures. In a geospatial context, no differences were noted in the prevalence of children in schools having antibodies against *P. falciparum*, with the exception of a few schools around the urban area of Bamako. As a data sample, the relationship between *P. falciparum* MSP-142 and AMA-1 antibody carriage was found to be significant, but a high titer was occasionally seen in a child for one antigen but a negligible titer for the other (Figure 2). For southern Mali as a whole, levels of antibodies against the *P. falciparum* proteins MSP-142 and AMA-1 were found to be very high, concordant with previous studies showing high parasite prevalence in this area.4,25 These data revealed a clear increase in the levels of antibodies to the *P. falciparum* antigens as children mature, as repeated exposure is likely. This aligns with previous studies showing IgG against *P. falciparum* antigens as an important factor in the development of clinical immunity.26,27

Within this southern part of Mali, serological evidence for the transmission profile of *P. vivax* was quite different, with a much smaller percentage of children harboring IgG antibodies against the *P. vivax* antigen MSP-119. Overall, evidence for previous *P. vivax* exposure was shown in 17.3% of all children sampled, and displayed a much more heterogeneous endemicity pattern among the schools. Schools in the southern most region of Sikasso tended to show higher percentages of children with *P. vivax* antibodies, with multiple schools in this region showing over 25% of children with lifetime exposure to this parasite. As the mosquito vector would be shared between *P. vivax* and *P. falciparum*, previous reports showing high *P. falciparum* parasite rates among young children in this area4 would indicate a robust regional vector population for the lifecycle of both parasites to persist. It was interesting to find only two of 42 schools sampled in southern Mali with no children harboring anti-*P. vivax* antibodies. This would allude to widespread low-transmission but a persistent rate of *P. vivax* that has been found by other studies.28,29 Evidence for this notion was also supported by the finding that IgG antibody levels for the *P. vivax* antigen did not increase with age, indicating a more stochastic nature of *P. vivax* exposure, rather than repeated exposures as one ages.

With the predominance of *P. falciparum*, asymptomatic nature of *P. vivax*, potential intra-host competition between the two parasites, and paucity of the Duffy receptor, it is not surprising that *P. vivax* infections have been overlooked in this area of Mali. Though not as clinically relevant in many settings, it is becoming more appreciated that *P. vivax* lifecycle can persist in some Duffy-negative African populations,7,9,30 and serological surveillance through the MBA offers a prime opportunity for inexpensive and robust identification of regions where this parasite may be endemic.
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Note: Supplemental table appears at www.ajtmh.org.

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Authors’ addresses: Eric Rogier and Patrick J. Lammie, Division of Parasitic Diseases and Malaria, Centers for Global Health, Centers for Disease Control and Prevention, Atlanta, GA, E-mail: vwx6@cdc.gov and pj1@cdc.gov. Delynn M. Moss, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, E-mail: dmm3@cdc.gov. Anna N. Chard, Victoria Triniés, and Matthew C. Freeman, Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, GA, E-mails: acharld@emory.edu, vtriniess@gmail.com, and mcfreemi@emory.edu. Seydou Doumbia, Malaria Research and Training Center, Faculty of Medicine and Odontostomatology, University of Sciences, Techniques and Technologies of Bamako (USTTB), Bamako, Mali, E-mail: sdoumbi@icemail.org.

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