Specificity of the IgG antibody response to *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* MSP1$_{19}$ subunit proteins in multiplexed serologic assays

Jeffrey W. Priest$^{1*}$, Mateusz M. Plucinski$^{2,3}$, Curtis S. Huber$^7$, Eric Rogier$^2$, Bunsoth Mao$^4$, Christopher J. Gregory$^5$, Baltazar Candrinho$^6$, James Colborn$^7$ and John W. Barnwell$^2$

Abstract

**Background:** Multiplex bead assays (MBA) that measure IgG antibodies to the carboxy-terminal 19-kDa sub-unit of the merozoite surface protein 1 (MSP1$_{19}$) are currently used to determine malaria seroprevalence in human populations living in areas with both stable and unstable transmission. However, the species specificities of the IgG antibody responses to the malaria MSP1$_{19}$ antigens have not been extensively characterized using MBA.

**Methods:** Recombinant *Plasmodium falciparum* (3D7), *Plasmodium malariae* (China I), *Plasmodium ovale* (Nigeria I), and *Plasmodium vivax* (Belem) MSP1$_{19}$ proteins were covalently coupled to beads for MBA. Threshold cut-off values for the assays were estimated using sera from US citizens with no history of foreign travel and by receiver operator characteristic curve analysis using diagnostic samples. Banked sera from experimentally infected chimpanzees, sera from humans from low transmission regions of Haiti and Cambodia (N = 12), and elutions from blood spots from humans selected from a high transmission region of Mozambique (N = 20) were used to develop an antigen competition MBA for antibody cross-reactivity studies. A sub-set of samples was further characterized using antibody capture/elution MBA, IgG subclass determination, and antibody avidity measurement.

**Results:** Total IgG antibody responses in experimentally infected chimpanzees were species specific and could be completely suppressed by homologous competitor protein at a concentration of 10 μg/ml. Eleven of 12 samples from the low transmission regions and 12 of 20 samples from the high transmission area had antibody responses that were completely species specific. For 7 additional samples, the *P. falciparum* MSP1$_{19}$ responses were species specific, but various levels of incomplete heterologous competition were observed for the non-*P. falciparum* assays. A pan-malaria MSP1$_{19}$ cross-reactive antibody response was observed in elutions of blood spots from two 20–30 years old Mozambique donors. The antibody response from one of these two donors had low avidity and skewed almost entirely to the IgG3 subclass.

**Conclusions:** Even when *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* are co-endemic in a high transmission setting, most antibody responses to MSP1$_{19}$ antigens are species-specific and are likely indicative of previous infection history. True pan-malaria cross-reactive responses were found to occur rarely.

*Correspondence: j_priest@cdc.gov
$^1$ Division of Foodborne, Waterborne, and Environmental Diseases at the Centers for Disease Control and Prevention, Atlanta, GA, USA
Full list of author information is available at the end of the article

© The Author(s) 2018. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background
Approximately 2.5 billion people, or one-third of the world’s estimated 2018 population live in regions of stable or unstable malaria transmission, and are at risk for infection [1, 2]. In sub-Saharan Africa, Plasmodium falciparum has been the major focus of treatment and intervention strategies because of the high mortality associated with infection. Three additional species of human malaria, two Plasmodium ovale sub-species and Plasmodium malariae, share much of the same geographic range in Africa yet are considered less important because prevalence estimates based on microscopic detection of parasites in blood films are generally low [3]. However, mounting clinical evidence suggests that malaria infection with species other than P. falciparum is not benign and that infection prevalence may be increasing in children, even in areas where anti-malarial drug therapy is regularly administered [4–8]. Similarly, the risk of Plasmodium vivax infection in sections of sub-Saharan and central Africa has been considered to be nil because large fractions of the populations in these regions lack the Duffy receptor used by the parasite for red blood cell invasion [9–12]. New evidence, however, suggests that low levels of P. vivax transmission in Africa may be occurring in susceptible Duffy-positive residents and that some level of infection is also occurring in Duffy-negative individuals by another reticulocyte invasion mechanism [13–16]. Because these non-P. falciparum infections are frequently sub-patent and their symptoms may be masked by the overwhelming levels of P. falciparum parasitaemia, accurate mapping and the estimation of prevalence levels in this population are difficult using traditional microscopic or PCR methods.

Serologic assays that detect IgG antibodies to specific P. falciparum and P. vivax antigens have been used in multiple studies in many parts of the world to estimate infection incidence and immunity levels (reviewed in [17–19]). Antibody data from cross-sectional surveys can be used to calculate the community-level seroconversion rates [20–27], and longitudinal and cross-sectional data provide similar estimates of community seroconversion rates [28]. Serologic assays using species-specific antigens could identify individuals who either are currently infected or have been previously infected with different malaria species, even if the infections were sub-patent [29]. Advances in multiplex assay technology make serologic antibody assays for multiple malaria antigens more attractive because antibody responses to a range of malaria antigens can be detected in a single well from a small volume of blood or serum and because malaria-specific assays can be integrated with assays for other antibody responses of public health interest [30–33].

One target antigen frequently used in malaria serologic antibody studies is the 19-kDa carboxy-terminal sub-unit of the merozoite surface protein 1 (MSP119) [34–36], a glycosylphosphatidylinositol-anchored fragment of the larger MSP1 protein that is found in abundance on the parasite surface (reviewed in [37]). Although the MSP119 proteins from P. falciparum, P. malariae, P. ovale, and P. vivax share 48–58% identity at the amino acid level [38], many of the conserved residues are cysteines and other hydrophobic amino acids that are unlikely to be exposed to the immune system [39]. Despite the sequence similarity, Cook et al. [24] were able to demonstrate unique sero prevalence curves for the P. falciparum and P. vivax MSP119 antibody responses in areas of reduced transmission in Vanuatu. Similarly, Bousema et al. [40] did not observe any correlation between the P. falciparum and P. vivax MSP119 antibody responses in ELISA studies of sera from a population living in a Somalian region of low endemicity for both parasites. In a study of malaria antibody responses in adult Cambodian women, Priest et al. [33] found that 79% of sera from women who were positive for antibodies to malaria reacted with the MSP119 antigen from only one species. However, all of these three studies were conducted in regions of relatively low transmission, and it is important to determine whether the MSP119 antigen-based assays will be species specific in regions of high transmission with multiple circulating species of malaria parasite.

During a bed net intervention study in a high malaria transmission region of northern Mozambique [41], numerous samples from individuals were assayed and found to have very high IgG antibody responses to multiple malaria species MSP119 antigens, including the P. vivax antigen. These samples and samples from two low transmission regions in a multiplex assay format to expand on the MSP119 competition ELISA studies of Amanfo et al. [42].

Methods
Human sample sets
Anonymous serum samples (N=88), collected prior to 2000 from US citizens with no history of foreign travel, were presumed to be negative for antibodies to Plasmodium spp. and were used to define the cut-off values for the various assays. Anonymized, residual sera submitted to the Centers for Disease Control and Prevention

Keywords: Serology, Malaria, MSP119, Multiplex, Specificity
between 1995 and 2011 for malaria diagnostic testing were used for the assessment of multiplex assay sensitivity. The panel included sera from patients having microscopically confirmed and/or PCR confirmed infections with *P. falciparum* (*N* = 33), *P. malariae* (*N* = 6), *P. ovale* (*N* = 7), or *P. vivax* (*N* = 35) [43]. The timing of sample collection relative to malaria infection or symptom development was not known. In addition to a pan-*Plasmodium* spp. immunofluorescence assay (IFA) positive serum pool (CDC Lot 8), mono-specific infection IFA serum controls were available for *P. falciparum* (CDC Lot 6) and *P. malariae* (CDC Lot 2).

Sera or dried blood spots previously identified by multiplex assay as having high levels of IgG antibodies to one or more MSP1<sub>19</sub> proteins were selected for the specificity studies. This set included: 3 anonymous, adult blood donor samples collected in 1998 from a region of Haiti with a low prevalence of *P. falciparum* infection [43]; 9 sera from an integrated serologic study of immune status to vaccine-preventable diseases and neglected tropical diseases conducted in 2012 among women 15–39 years of age in Cambodia [33, 44, 45]; and, 20 dried blood spots from participants (±60 years of age) in a long-lasting insecticide-treated bed net impact study conducted in 2013–2014 in a high malaria transmission province of northern Mozambique [41]. The sample set from Mozambique was biased towards individuals with a positive antibody response to the *P. vivax*, *P. ovale* and *P. malariae* antigens.

**Ethics statement**

Residual malaria diagnostic sera were made anonymous under a protocol approved by the CDC Institutional Review Board. Written informed consent was obtained prior to enrolment and participation in the Cambodian sero-survey, and the study protocol was reviewed and approved by the National Ethics Committee in Cambodia [33, 44, 45]. Written informed consent was obtained prior to enrolment and participation in the Mozambique bed net study and sero-survey, and the study was approved by the National Bioethics Committee in Mozambique. For both of these studies, CDC researchers had no access to personal identifiers, and CDC staff were not considered to be engaged with human research subjects.

**Banked chimpanzee sera**

Banked sera from malaria studies conducted in experimentally infected chimpanzees prior to 2000 were included in this report. Chimpanzees Bit and Klimatis were infected with the Uganda I strain of *P. malariae* [46, 47], Alpert was infected with the Nigeria I strain of *P. ovale* [48], and Duff was infected with the Salvador I strain of *P. vivax* [49]. As previously described, all animals had been splenectomized before they were inoculated intravenously with heparinized, infected blood.

**Antigens for multiplex assay**

The cloning of the 3D7 strain *P. falciparum* MSP1<sub>19</sub> in pGEX 4T-2 plasmid (GE Healthcare, Piscataway, NJ, USA) as a fusion protein with *Schistosoma japonicum* glutathione-S-transferase (GST) and the purification of the MSP1<sub>19</sub>-GST fusion protein have been described elsewhere [50].

Using the protocol of Priest et al. [33] and a new reverse PCR primer, a *P. vivax* MSP1<sub>19</sub> clone lacking the carboxy-terminal, hydrophobic anchor sequence was generated in pGEX 4T-2 plasmid (GE Healthcare), and the MSP1<sub>19</sub>-GST fusion protein was purified. The target sequence was amplified from Belem strain DNA using a reverse deoxyoligonucleotide PCR primer with the following sequence: 5’-CGG GAA TTC TTA GCT GGA GGA GCT ACA AAG ACC C-3’. The underlined sequence reverse primer identifies an *Eco*RI restriction endonuclease site used in directional cloning, and the italicized bases identify an introduced in-frame stop codon. All other cloning conditions remained as previously described [33]. The clone was sequenced using BigDye Terminator V3.1 chemistry (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA).

Cloning, expression and purification of a *P. ovale* MSP1<sub>19</sub>-GST fusion protein from Nigeria I strain DNA was accomplished using the strategy described in Priest et al. [33] with the following deoxyoligonucleotide primers: forward, 5’-CGC GGA TCC TTA GCT GGA TCT AAA CAT AAA TGT-3’ and reverse, 5’-CGC GAA TTC TTA ACT TGA TGA GCC ACA GAA AAC ACC C-3’. The underlined sequence in the forward primer identifies a *Bam*HI restriction endonuclease site used in directional cloning. These primer sequences were based on the sequence of the Cameroon OMA1A *P. ovale* isolate sequence (GenBank accession number FJ824670) described by Birkenmeyer et al. [38].

Cloning of the *P. malariae* MSP1<sub>19</sub> coding sequence from China I strain DNA required two PCR reactions. The first reaction used long PCR primers (forward, 5’-AAT ATT AGC GCA AAA CAT GCA TGT ACC GAA ACA-3’; reverse, 5’-ACT TGA AGA ACC ACA GAA AAC ACC TTC AAA TAT AG-3’) and the amplification conditions previously described [33]. These primer sequences were based on the sequence of the Cameroon MM1A *P. malariae* isolate sequence (GenBank accession number FJ824669) described by Birkenmeyer et al. [38]. A total of 5% of the purified primary product (StrataPrep PCR purification kit, Stratagene, LaJolla, CA, USA) was used in a second amplification reaction with the following primers: forward, 5’-CGC GGA TTC AAT ATT AGC
GCA AAA CAT GCA TGT-3'; reverse, 5'-GCG GAA TTC TTA ACT TGA AGA ACC ACA GAA AAC ACC-3'. This final PCR product was cloned in pGEX 4T-2 plasmid (GE Healthcare), and a GST fusion protein was expressed and purified using the protocol of Priest et al. [33].

Expression and purification of the control GST protein with no fusion partner has been described elsewhere [51]. A synthetic 20 amino acid peptide, (NANP)_5-amide, corresponding to the carboxy-terminal repeat of the *P. falciparum* circumsporozoite protein (PICSP peptide) [52, 53] was cross-linked to GST using the glutaraldehyde protocol of Benitez et al. [54]. Tetanus toxoid antigen from Massachusetts Biologic Laboratories (Boston, MA, USA) was exchanged into buffer containing 10 mM Na_2HPO_4 and 0.85% NaCl at pH 7.2 (PBS) [44].

**Comparison of *Plasmodium malariae* MSP1<sub>19</sub> sequences from other geographic locations**

Ten nanograms of DNA from *P. malariae* strains Greece I, Guyana, and Uganda I were PCR amplified using the forward and reverse long deoxyoligonucleotides described above and the Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN, USA). Cycle conditions were as follows: 94 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, and a final extension step of 68 °C for 5 min. Products were purified (StrataPrep PCR purification kit, Stratagene) and sequenced as described above.

**Antigen coupling and multiplex bead assays**

Antigens were coupled in 1.0 ml of buffer containing 25 mM 2-(N-morpholino)-ethanesulfonic acid (MES) at pH 5.0 with 0.85% NaCl using the following amounts of protein for 12.5 x 10<sup>6</sup> SeroMap microspheres (Luminex Corp, Austin, TX, USA): MSP1<sub>19</sub>-GST fusion proteins, 30 μg; GST control protein, 15 μg; PICSP peptide-GST, 30 μg; and tetanus toxoid, 12.5 μg. The coupling protocol and bead storage buffer have been described previously [55].

Blood spots were collected on filter paper disks (Cel-labs, Sydney, Australia). A single tab containing 10 μl whole blood (approximately 5 μl of serum) was eluted overnight at 4 °C in 200 μl of buffer containing PBS with 0.05% Tween-20 and 0.05% NaN_3 for a 1:40 serum protein dilution [56]. Samples were further diluted 1:10 (final 1:400 serum dilution) in PBS buffer (pH 7.2) containing 0.3% Tween-20, 0.02% NaN_3, 0.5% casein, 0.5% polyvinyl alcohol (PVA), 0.8% polyvinylpyrrolidone (PVP), and 3 μg/ml *Escherichia coli* extract (Buffer A) [33, 57]. Test sera were diluted 1:400 in Buffer A. BSA was not included in the dilution buffer as it was found to be unnecessary for blocking when casein was present.

The multiplex bead assay protocol for total IgG has been described elsewhere [55, 58]. Assays were run in duplicate wells, and each plate included a buffer only blank. The reported “median fluorescent intensity minus background” value (MFI-bg) is the average of the 2 median fluorescent intensity values minus background blank values from two replicate wells. Negative MFI-bg values were set to 0.

In the multiplex IgG sub-class assays, serum antibodies were bound to beads using the previously described multiplex assay protocol [55, 58]. Washed beads were then incubated for 45 min at room temperature with 50 μl/well of a 1:500 dilution in Buffer B (0.5% BSA, 0.05% Tween-20, and 0.02% NaN_3 in PBS at pH 7.2) of biotinylated monoclonal mouse anti-human IgG subclass secondary antibody to IgG<sub>1</sub> (clone HP6025), IgG<sub>2</sub> (clone HP6002), IgG<sub>3</sub> (clone HP6047), or IgG<sub>4</sub> (clone HP6025) (all from Zymed/Invitrogen, South San Francisco, CA, USA). Wells were developed with R-phycocerythrin-labelled streptavidin and read on a BioPlex 200 instrument (BioRad, Hercules, CA, USA) as described above.

**Assessment of coupling efficiency**

To determine whether the *Plasmodium* spp. GST-MSP1<sub>19</sub> fusion proteins were coupled to the SeroMap beads with similar efficiencies, multiplex assays were run using a serial dilution of a goat anti-GST polyclonal IgG antibody (GE Healthcare) as the primary antibody to detect the fusion protein on the bead. The initial dilution of anti-GST antibody was 1:1000 in modified Buffer A lacking the *E. coli* extract (50 μl/well), and the final dilution was 1:1.0 x 10<sup>7</sup>. Bound anti-GST antibody was detected with 50 μl/well of a biotinylated rabbit anti-goat IgG secondary antibody (1:500 dilution in Buffer B; Invitrogen) and wells were developed with R-phycocerythrin-labelled streptavidin and read on a BioPlex 200 instrument (BioRad) as described above.

**MSP1<sub>19</sub> competition assays**

Serial dilutions of purified MSP1<sub>19</sub>-GST competitor proteins were generated from a 0.5 mg/ml stock solution using PBS buffer at pH 7.2. A 96-well incubation plate (V-bottom, Fisher Scientific) was set up such that wells contained 3 μl of the competitor MSP1<sub>19</sub>-GST fusion protein dilution and 147 μl of serum dilution in Buffer A for 1:50 dilution of competitor protein and a negligible further dilution of the serum. Final competitor protein concentrations in the serum dilution ranged from 10 μg/ml to as low as 0.025 μg/ml. The plate was incubated at room temperature for 1 h, and then each well of the incubation plate was used to load duplicate multiplex bead assay wells at 50 μl each. The standard total IgG assay protocol was then followed as described above. The
standard MSP1₁₉ competition assay used a final competitor protein concentration in diluted serum of 10 μg/ml, and a ≥30% reduction in multiplex assay signal was considered to be evidence of antibody cross-reactivity.

MSP1₁₉-specific antibody binding and elution
Using the standard coupling protocol [55], individual MSP1₁₉-GST fusion proteins were coupled to magnetic beads (region 14, Luminex) in 100 μl of MES/NaCl buffer at pH 5.0 at 4.5 μg for 1.25 × 10⁹ microspheres (a 50% increase in protein compared to SeroMap bead amounts). Coupled beads were re-suspended in 120 μl of storage buffer with protease inhibitors [55]. A 1:200 dilution of serum in Buffer A or a 1:5 dilution of blood spot eluate in Buffer A (approximately 1:200 serum dilution) was incubated for 1 h at room temperature with 20 μl of coupled beads (washed 1× with 0.05% Tween-20 in PBS prior to use). Beads were collected by magnetic capture, the used serum or blood spot dilution was removed, and the beads were washed 4× with 200 μl 0.05% Tween-20 in PBS. To elute the bound antibodies, beads were re-suspended for 10 min at room temperature in 100 μl of buffer containing 3 parts of 4 M MgCl₂ in 100 mM N-hydroxethylpiperazine-N′-2-ethanesulfonic acid (HEPES) at pH 8.0 and 1 part ethylene glycol [59]. The beads were collected by magnetic capture, and the supernatant was removed and diluted into 0.9 ml of buffer containing 50 mM tris(hydroxymethyl)-aminomethane (Tris) at pH 7.5 and 0.85% NaCl. The antibody elution process was repeated once. The 2 ml of eluted antibody in Tris/NaCl buffer was concentrated to 50 μl using a Centricron-50 centrifugal filter device as directed by the manufacturer (Millipore, Bedford, MA, USA). The concentration procedure was repeated following a 2 ml Tris/NaCl buffer dilution and again after a 1 ml PBS buffer dilution. The final 30–50 μl of concentrate was diluted with >3 volumes of Buffer A, and duplicate multiplex assays were performed using half of the eluted antibody per well.

Antibody avidity determinations
To determine the avidity of IgG antibody binding, MSP1₁₉-GST fusion protein coated SeroMap beads incubated for 1 h with 1:400 serum dilutions were immediately washed with 100 μl of 6 M urea in PBS for 5 min at room temperature [60]. The urea wash was repeated once followed by three 100 μl washes with 0.05% Tween-20 in PBS. The normal total IgG development protocol was then followed. An avidity index was calculated by dividing the 6 M urea-treated MFI-bg value by the untreated MFI-bg value.

Data analysis
Protein sequences were aligned using COBALT [61]. The means plus 3 standard deviations of the MBA responses from 88 adult US citizens with no history of foreign travel were used to define potential cutoffs for the MSP1₁₉ protein and CSP peptide assays. Receiver-operating characteristic (ROC) curves were also used to generate potential cut-offs for the MSP1₁₉ assays. The ROC analysis [62] and Spearman rank order correlation analysis were performed using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA, USA). The J-index [63] was calculated from the sensitivity and specificity values.

Results
MSP1₁₉ sequences
The DNA sequence of the P. malariae China I strain MSP1₁₉ clone (deposited in GenBank as MH577182) differed from the Cameroon sequence of Birkenmeyer et al. [38] at 3 nucleotide base positions, leading to 2 amino acid substitutions in the deduced amino acid sequence: G41E and Q51K (numbering based on mature MSP1₁₉ protein sequence). As shown in Fig. 1, the deduced amino acid sequence of the China I strain was identical to that reported for the Brazil I11 strain [64] and was also identical to that of the Greece I strain (GenBank MH577183). Compared to the Cameroon strain, the Uganda I strain of P. malariae contained only a G41Q amino acid substitution (GenBank MH577184), while the Guyana strain contained only a G41E substitution (GenBank MH577185). The DNA sequence of the P. ovale Nigeria I clone (GenBank MH577181) matched the GenBank sequence reported for the Cameroon OMA1A P. ovale isolate (FJ824670) by Birkenmeyer et al. [38]. The Nigeria I strain likely belongs to the newly identified Plasmodium ovale curti species as the MSP1₁₉ predicted protein sequence has a Ser at position 23 rather than a Pro [65]. The sequence of the P. vivax strain of the Cameroon 3D7 strain [67], P. falciparum 3D7 strain [67], P. vivax Belem strain [66, 68], and P. ovale Nigeria I strain proteins in Fig. 1 showed conservation of 32 amino acids among the four species including 10 cysteines and 5 hydrophobic residues.

Assessment of coupling efficiency
The multiplex response titration curves using dilutions of the anti-GST antibody as the primary antibody in the multiplex reaction were similar for all 4 proteins (Fig. 2). In contrast, the multiplex response titration curves for the GST control bead (coupled at half the protein concentration of the MSP1₁₉-GST reactions) and for the cross-linked P. falciparum CSP peptide-GST bead were
| P. malariae Cameroon | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae Brazil 23PA | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae Guyana | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae Uganda I | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae Brazil II | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae Greece I | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. malariae China I | NISAKHACTETKPENAGC(GLHAE)KVEVWRCNNP. falciparum 3D7 | P. vivax Belem | P. ovale Nigeria I |
|---------------------|----------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| ...H.C.....P.N..C.R..D..E...C.LL..K.....CV......C.....NNNG | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | CAPEANCTKGDDN----KIVCACPYPSEIPFEGVFGSSS | TMSSEHTCIDTVIPDNAACYRLEWRLTTFKEGGKCVPAVSICDKNNNG | SMGSHKHCDITYPDAGCGRFSDGREERCLNFKEVCTVPNNNPTCAENNNG |

**Fig. 1** Alignment of predicted *Plasmodium* spp. MSP119 protein sequences using COBALT [61]. Residues in the *P. malariae* sequence that differ from the Cameroon sequence of Birkenmeyer et al. [38] are shaded. Predicted protein sequences resulting from the oligonucleotides used in PCR amplification are underlined. The positions of residues conserved among all the presented MSP119 protein sequences are indicated in the consensus with divergent residues indicated by a dot. GenBank accession numbers are MH577181, *P. ovale* Nigeria I strain; MH577182, *P. malariae* China I strain; MH577183, *P. malariae* Greece I strain; MH577184, *P. malariae* Uganda I strain; and MH577185, *P. malariae* Guyana strain.
indicative of lower amounts of bound target protein compared to the MSP1<sub>19</sub> beads.

Cut-off determinations

One outlier from the group of 88 US citizens with no history of foreign travel with a MBA response of 8690 MFI-bg units was censored from the *P. vivax* cut-off calculation, and one outlier with a MBA response of 10,377 MFI-bg units was censored from the *P. falciparum* CSP peptide calculation. The cut-offs in MFI-bg units were: *P. falciparum* CSP peptide, 1351; *P. falciparum* MSP1<sub>19</sub> 313; *P. malariae* MSP1<sub>19</sub> 397; *P. ovale* MSP1<sub>19</sub> 175; and, *P. vivax* MSP1<sub>19</sub>, 203.

**MSP1<sub>19</sub> multiplex assay specificity**

If closely related antigens coupled on different beads share common epitopes and compete for the same pool of antibodies, the values from a multiplexed assay would be expected to be lower than the values from assays using a single bead only. To test this hypothesis, each *Plasmodium* spp. MSP1<sub>19</sub> was assayed in isolation (individual monoplex), and the results were compared to values obtained when all of the beads were included in the routine multiplex format. As shown in Table 1, responses from 2 defined sera (Pan *Plasmodium* spp. Lot 8 and *P. malariae* Lot 2), 3 elutions from individual Mozambique blood spots, and one high-titre elution from a combination of Mozambique blood spots were essentially identical regardless of the bead complexity of the assay (Spearman rank order correlation coefficient = 0.999; *P* < 0.001). The results from this limited panel of samples suggest that a response dilution effect in the multiplex assay format is not a universal feature of the MSP1<sub>19</sub> protein family and that the multiplex assay may be useful in infection species determinations.

That some MSP1<sub>19</sub> antibody responses are species specific can also be demonstrated using sera from experimentally infected chimpanzees (Table 2). Sera from chimpanzees Klimatis (*P. malariae* infection) and Duff (*P. vivax* infection) had high antibody response values to the corresponding species-specific MSP1<sub>19</sub> protein and had no responses to MSP1<sub>19</sub> antigens from other species. In contrast, other animals such as chimpanzees Alpert (*P. ovale* infection) and Bit (*P. malariae* infection) reacted strongly with the MSP1<sub>19</sub> antigen corresponding to the species of the infecting parasite, but they also had weak responses to *P. vivax* MSP1<sub>19</sub> and strong responses to *P. falciparum* MSP1<sub>19</sub>. The wild-caught chimpanzees used in the experimental infection studies were never exposed to *P. falciparum* sporozoites in the laboratory and were never experimentally infected with *P. falciparum*. Thus, the presence of a *P. falciparum* CSP peptide response suggests that the *P. falciparum* MSP1<sub>19</sub> response likely arose by natural infection with a closely related species.
of chimpanzee malaria, such as *Plasmodium reichenowii* [69], rather than with an experimentally-induced cross-reactivity. Similarly, the weak *P. vivax* antibody responses in these 2 chimpanzees may also reflect prior exposure to *P. vivax*-like parasites in the wild. These unexpected responses highlight the difficulty of differentiating historic infection from true antibody cross-reaction.

**Specificity analysis using antigen competition**

An alternative approach to assess the specificity of the MSP119 antibody response relies on the ability of soluble antigen to saturate the antibody in a pre-incubation step so as to prevent antibody binding to MSP119 coated beads during the multiplex assay. To determine the concentration of competitor protein necessary to prevent antibody binding to MSP119 coated beads, sera that had high antibody responses were incubated with 0.025–10 μg/ml of competitor protein prior to multiplex assay as described in “Methods”. The GST control, PfCSP peptide-GST, and all 4 MSP119-GST protein-coated beads were included in the multiplex assay, but only the homologous MSP119 was

**Table 1 Impact of bead complexity on multiplex bead assay response values using beads coated with MSP119 proteins from four malaria species**

| Sample                          | Assay type      | Pf MSP119 (MFI-bg) | Pm MSP119 (MFI-bg) | Po MSP119 (MFI-bg) | Pv MSP119 (MFI-bg) | Pf CSP-peptide (MFI-bg) | GST (MFI-bg) |
|---------------------------------|-----------------|--------------------|--------------------|--------------------|--------------------|------------------------|--------------|
| Pan *Plasmodium* Lot 8 serum    | Individual monoplex | 28,537             | 355                | 721                | 23,449             | N/A                    | N/A          |
|                                 | Multiplex       | 28,442             | 351                | 724                | 23,688             | 2281                   | 41           |
| *P. malariae* Lot 2 serum       | Individual monoplex | 19                 | 10,413             | 6                  | 2                  | N/A                    | N/A          |
|                                 | Multiplex       | 20                  | 10,878             | 6                  | 2                  | 170                    | 11           |
| Mozambique donor 15             | Individual monoplex | 28,088             | 1437               | 16,504             | 260                | N/A                    | N/A          |
|                                 | Multiplex       | 28,242             | 1446               | 17,352             | 272                | 26,039                 | 53           |
| Mozambique donor 7              | Individual monoplex | 9396               | 734                | 9566               | 78                 | N/A                    | N/A          |
|                                 | Multiplex       | 9484               | 798                | 10,623             | 86                 | 22,359                 | 11           |
| Mozambique donor 8              | Individual monoplex | 28,034             | 23,220             | 11                 | 45                 | N/A                    | N/A          |
|                                 | Multiplex       | 28,438             | 23,378             | 14                 | 47                 | 15,819                 | 11           |
| Mozambique elution mix          | Individual monoplex | 28,842             | 27,229             | 20,193             | 24,113             | N/A                    | N/A          |
|                                 | Multiplex       | 28,911             | 27,082             | 21,585             | 24,048             | 17,682                 | 279          |

N/A assay not performed

**Table 2 Multiplex bead assays results using sera from chimpanzees experimentally infected with a single species of malaria parasite**

| Chimpanzee name | Lab. infection species | Pm MSP119 (MFI-bg) | Po MSP119 (MFI-bg) | Pv MSP119 (MFI-bg) | Pf MSP119 (MFI-bg) | Pf CSP-peptide (MFI-bg) | GST (MFI-bg) |
|-----------------|------------------------|--------------------|--------------------|--------------------|--------------------|------------------------|--------------|
| Klimatis        | *P. malariae*          | 23,289             | 1                  | 2                  | 119                | 14                     | 0            |
| Duff            | *P. vivax*             | 41                 | 51                 | 26,588             | 64                 | 156                    | 3            |
| Alpert          | *P. ovale*             | 71                 | 22,581             | 724                | 25,757             | 4002                   | 0            |
| Bit             | *P. malariae*          | 24,223             | 49                 | 664                | 5247               | 23,270                 | 19           |

Fig. 3 Antibody competition titration assays using homologous MSP119 proteins. Dilutions (1:400) of *P. falciparum* Lot 6 defined human serum or of sera from chimpanzees experimentally infected with either *P. malariae* (Klimatis), *P. ovale* (Alpert) or *P. vivax* (Duff) were incubated with the indicated concentrations of the homologous MSP119 competitor protein for 1 h at room temperature. Multiplex bead assays were performed as described in “Methods”, and the multiplex responses in MFI-bg units are plotted versus the competitor concentration.
used as competitor. Thus, the *P. falciparum* Lot 6 defined human serum was competed using soluble *P. falciparum* MSP119-GST fusion protein, while sera from chimpanzees experimentally infected with either *P. malariae* (Klimatis), *P. ovale* (Alpert) or *P. vivax* (Duff) was incubated with the indicated concentrations of the MSP119 competitor protein for 1 h at room temperature. Competitor proteins used were: a *P. falciparum* MSP119, b *P. malariae* MSP119, c *P. ovale* MSP119, d *P. vivax* MSP119. Multiplex bead assays were performed as described in “Methods” and the multiplex response in MFI-bg units are plotted versus the competitor concentration. Multiplex responses are presented as a percentage of the assay results for the PBS control.

**Fig. 4** Antibody competition titration assays using MSP119 proteins from four *Plasmodium* species. A combined dilution (1:400 of each serum) containing sera from chimpanzees experimentally infected with either *P. malariae* (Klimatis), *P. ovale* (Alpert) or *P. vivax* (Duff) was incubated with the indicated concentrations of the MSP119 competitor protein for 1 h at room temperature. Competitor proteins used were: a *P. falciparum* MSP119, b *P. malariae* MSP119, c *P. ovale* MSP119, d *P. vivax* MSP119. Multiplex bead assays were performed as described in “Methods” and the multiplex response in MFI-bg units are plotted versus the competitor concentration. Multiplex responses are presented as a percentage of the assay results for the PBS control.

Next, sera from chimpanzees Klimatis (*P. malariae*), Alpert (*P. ovale*), and Duff (*P. vivax*) (1:400 dilution of each serum) were combined, and the competitor titration assays were repeated. Figure 4 shows the multiplex responses in the presence of various concentrations of the 4 MSP119 competitor proteins and expressed as a percentage of the PBS control. In Panel A, addition of the *P. falciparum* MSP119 competitor protein had no effect on the *P. malariae*, *P. ovale* or *P. vivax* multiplex responses. Similarly, heterologous MSP119 competitor proteins had no effect on multiplex responses (Fig. 4b–d), while multiplex response curves for the homologous species of competitor MSP119 proteins in Fig. 4b–d resemble the individual curves previously shown in Fig. 3. The chimpanzee multiplex responses in the presence of homologous species competitor protein showed >97% suppression at 2.5 µg/ml and were below the respective cut-off values at 5 µg/ml of competitor.
Finally, combined human sera (pan-Plasmodium spp. positive serum pool and *P. malariae* mono-specific infection serum control, each at 1:400 dilution) competitor studies showed similar heterologous and homologous titration profiles except that the *P. malariae* response was reduced by approximately 27–29% at the 10 μg/ml of heterologous MSP1<sub>19</sub>-GST competitor protein concentrations (Additional file 1). For the human multiplex responses in the presence of homologous species competitor protein, values were below the respective cut-off values at 2.5 μg/ml of competitor. Based on these studies, a competitor concentration of 10 μg/ml was selected to maximize suppression of antibody binding in the multiplex assay.

### Table 3  Representative MSP1<sub>19</sub> competition assay results using sera from low incidence settings

| Sample       | Competitor added       | Pf MSP1<sub>19</sub> (MFI-bg) | Pm MSP1<sub>19</sub> (MFI-bg) | Po MSP1<sub>19</sub> (MFI-bg) | Pf CSP (MFI-bg) | GST (MFI-bg) |
|--------------|------------------------|-------------------------------|-------------------------------|-------------------------------|----------------|--------------|
| Haiti 1      | PBS buffer only        | 25,260                        | 15                            | 21                            | 9              | 65           | 1            |
|              | GST                    | 25,473                        | 16                            | 21                            | 11             | 65           | 4            |
|              | Pf MSP1<sub>19</sub>   | 13<sup>a</sup>                | 14                            | 21                            | 9              | 70           | 3            |
|              | Pm MSP1<sub>19</sub>   | 25,058                        | 6                             | 17                            | 8              | 63           | 3            |
|              | Po MSP1<sub>19</sub>   | 25,344                        | 12                            | 6                             | 7              | 62           | 2            |
|              | Pv MSP1<sub>19</sub>   | 25,181                        | 11                            | 18                            | 4              | 65           | 3            |
| Cambodia 2   | PBS buffer only        | 35                            | 21                            | 15                            | 23,836         | 64           | 1            |
|              | GST                    | 37                            | 22                            | 17                            | 23,623         | 66           | 1            |
|              | Pf MSP1<sub>19</sub>   | 28                            | 24                            | 11                            | 21,057         | 70           | 1            |
|              | Pm MSP1<sub>19</sub>   | 40                            | 13                            | 17                            | 23,348         | 61           | 2            |
|              | Po MSP1<sub>19</sub>   | 41                            | 23                            | 13                            | 23,662         | 67           | 1            |
|              | Pv MSP1<sub>19</sub>   | 36                            | 22                            | 14                            | 9              | 61           | 2            |
| Cambodia 3   | PBS buffer only        | 29,763                        | 3177                          | 10                            | 2871           | 0            |
|              | GST                    | 30,461                        | 3443                          | 12                            | 2909           | 1            |
|              | Pf MSP1<sub>19</sub>   | 32                            | 3428                          | 11                            | 2821           | 1            |
|              | Pm MSP1<sub>19</sub>   | 30,233                        | 9                             | 14                            | 2817           | 0            |
|              | Po MSP1<sub>19</sub>   | 30,352                        | 3480                          | 10                            | 3041           | 1            |
|              | Pv MSP1<sub>19</sub>   | 30,129                        | 3043                          | 12                            | 2879           | 2            |
| Cambodia 4   | PBS buffer only        | 15                            | 6                             | 13                            | 20,899         | 17           | 0            |
|              | GST                    | 15                            | 7                             | 13                            | 20,930         | 13           | 1            |
|              | Pf MSP1<sub>19</sub>   | 9                             | 6                             | 14                            | 15,929         | 14           | 0            |
|              | Pm MSP1<sub>19</sub>   | 12                            | 2                             | 12                            | 20,382         | 16           | 0            |
|              | Po MSP1<sub>19</sub>   | 11                            | 5                             | 2                             | 20,177         | 15           | 0            |
|              | Pv MSP1<sub>19</sub>   | 13                            | 6                             | 12                            | 1              | 13           | 0            |
| Cambodia 9   | PBS buffer only        | 1882                          | 21                            | 17                            | 12,439         | 22           | 3            |
|              | GST                    | 2023                          | 23                            | 21                            | 13,095         | 21           | 4            |
|              | Pf MSP1<sub>19</sub>   | 31                            | 22                            | 23                            | 9347           | 21           | 4            |
|              | Pm MSP1<sub>19</sub>   | 1877                          | 13                            | 23                            | 12,075         | 20           | 4            |
|              | Po MSP1<sub>19</sub>   | 1854                          | 24                            | 17                            | 12,679         | 21           | 2            |
|              | Pv MSP1<sub>19</sub>   | 1725                          | 22                            | 21                            | 4              | 22           | 3            |
| Cambodia 5   | PBS buffer only        | 28,405                        | 743                           | 39                            | 24,051         | 1072         | 800          |
|              | GST                    | 28,084                        | 799                           | 44                            | 24,307         | 1143         | 911          |
|              | Pf MSP1<sub>19</sub>   | 50                            | 439<sup>b</sup>              | 34                            | 23,510         | 1160         | 881          |
|              | Pm MSP1<sub>19</sub>   | 26,098                        | 31                            | 34                            | 23,646         | 1136         | 839          |
|              | Po MSP1<sub>19</sub>   | 27,871                        | 737                           | 32                            | 23,808         | 1103         | 861          |
|              | Pv MSP1<sub>19</sub>   | 27,620                        | 617                           | 33                            | 19             | 1090         | 828          |

<sup>a</sup> Multiplex bead assay responses that were completely suppressed by homologous protein competition are indicated in italics cells. These responses were below the respective cut-off values.

<sup>b</sup> Multiplex bead assay responses that, as a result of heterologous competition, decreased more than 30% compared to the PBS control but remained above the respective cut-off values are indicated in bold italics cells. These values indicate some level of cross-reactivity.
Assay specificity in low malaria incidence settings

Representative competition assay results for a serum sample set from two regions of low malaria incidence (Haiti and Cambodia) are presented in Table 3. Additional results from this sample set can be found in Additional file 2. Most of the samples chosen from these areas had positive antibody responses to only one or two MSP1<sub>19</sub> proteins, and only one person (Cambodia 5) reacted with MSP1<sub>19</sub> proteins from three malaria species. Antibody responses to the *P. falciparum* CSP peptide were mostly negative, and, when present, were < 4000 MFI-bg units (median = 64.5; range 14–3930). Addition of GST control protein to the competition assay at a concentration of 10 μg/ml had no effect on any of the antibody responses. One person had an antibody response to the GST coupled control bead, but the response was not inhibited by pre-incubation with soluble GST protein. This response was probably unrelated to the presence of the GST protein as the *P. ovale* MSP1<sub>19</sub>-GST response was consistently < 50 MFI-bg units.

In 9 of the 12 serum samples tested, all of the malaria MSP1<sub>19</sub> antibody responses appeared to be species specific as only homologous competitor MSP1<sub>19</sub> protein completely eliminated the antibody response (highlighted in italics in Table 3 and Additional file 2). For two of the tested sera (Table 3) multiplex assay response values to the *P. vivax* antigen demonstrated a weak heterologous competition effect with *P. falciparum* MSP1<sub>19</sub> competitor protein, but the effect did not meet the 30% threshold definition (approximately 25% reduction; Cambodia 4 and 9). Interestingly, the sample from Cambodia donor 4 had no antibodies to either *P. falciparum* antigen by MBA. Only one donor had a heterologous competition assay response reduction of > 30%; for Cambodia 5 (indicated in bolditalics in Table 3), addition of *P. falciparum* MSP1<sub>19</sub> competitor protein reduced a weak *P. malariae* response by 41% while completely eliminating a strong homologous *P. falciparum* MSP1<sub>19</sub> response (> 28,000 MFI-bg units). The MFI-bg value for the heterologous competition assay remained above the respective *P. malariae* cut-off.

**Assay specificity in a high malaria incidence setting**

Representative competition assay results for a sample set from a high malaria incidence region of Mozambique are presented in Table 4 with additional values shown in Additional file 3. These 20 samples were selected from the parent study [41] because they exhibited high IgG antibody responses to one or more MSP1<sub>19</sub> antigens by multiplex bead assay, and the selection was biased towards samples that had a strong positive responses to the *P. vivax*, *P. ovale* or *P. malariae* proteins. Historically, rates of vivax malaria have been expected to be low in East African populations lacking the Duffy antigen [12], and an antibody response to the *P. vivax* MSP1<sub>19</sub> might be indicative of antibody cross-reactivity. In contrast to the samples from low prevalence areas described above, eluted blood spot samples from Mozambique often had strong antibody responses to the *P. falciparum* CSP peptide (median = 23,407; range = 2833–27,033).

Species-specific anti-MSP1<sub>19</sub> antibody responses, as indicated by the presence of complete homologous competition and the absence of heterologous competitor effects, were observed in 10 of the 20 samples tested (Table 4 and Additional file 3). In one additional sample (Mozambique 20 in Table 4), species-specific responses were observed, but the suppression of the *P. malariae* MSP1<sub>19</sub>-specific antibody responses was incomplete: values remained above the 397 MFI-bg assay cut-off threshold in the presence of 10 μg/ml *P. malariae* competitor protein. Given the very high *P. malariae* MSP1<sub>19</sub> control antibody responses for these samples (> 27,000 MFI-bg units), it is possible that the competitor protein concentration was insufficient for complete antibody blocking. As previously demonstrated, addition of GST control protein to the competition assay at a concentration of 10 μg/ml had no effect on the antibody responses.

One sample (Mozambique 1, Table 4) demonstrated a partial loss (40% reduction) of anti-*P. malariae* MSP1<sub>19</sub> antibody response in the presence of *P. falciparum* competitor protein, but antibodies to the other 3 species antigens were specific. Four samples, represented by Mozambique 16 in Table 4, demonstrated some combination of incomplete homologous response suppression and heterologous assay inhibition. In the case of Mozambique 16, the *P. falciparum* competitor protein partially inhibited the heterologous *P. malariae* MSP1<sub>19</sub> antibody response (68% reduction), and the *P. vivax* competitor protein had a major impact on the *P. ovale* heterologous response (99% reduction), but, in the same reaction, this competitor did not completely block the homologous *P. vivax* MSP1<sub>19</sub> antibody response (90% reduction). Reciprocal heterologous competition was only observed between *P. ovale* and *P. vivax* MSP1<sub>19</sub> antigens and only in two donors represented by Mozambique 19 (Table 4).

Heterologous competition assays leading to response values below the cut-off were observed for both *P. malariae* and *P. vivax* MSP1<sub>19</sub> proteins and the *P. ovale* antigen partially reduced the *P. vivax* antibody response.

As shown in Table 5, what appeared to be a true, pan-malarial MSP1<sub>19</sub> antibody response was observed in blood spot elutions from two 20–30 years old Mozambique donors, numbers 3 and 12. In contrast to assays described above, control response values to MSP1<sub>19</sub> proteins for 3 of the malaria species (*P. malariae*, *P. ovale*, *P. vivax*) were
Table 4 | Representative MSP1<sub>19</sub> competition assay results using sera from a high incidence setting

| Sample          | Competitor added | Pf MSP1<sub>19</sub> (MFI-bg) | Pm MSP1<sub>19</sub> (MFI-bg) | Po MSP1<sub>19</sub> (MFI-bg) | PvMSP1<sub>19</sub> (MFI-bg) | FcSP (MFI-bg) | GST (MFI-bg) |
|-----------------|------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|---------------|--------------|
| Mozambique 6    | PBS buffer only  | 28,163                        | 93                            | 75                            | 83                            | 24,603        | 22           |
|                 | GST              | 28,217                        | 79                            | 57                            | 56                            | 24,181        | 21           |
|                 | PF MSP1<sub>19</sub> | 45<sup>a</sup>               | 76                            | 54                            | 48                            | 24,313        | 27           |
|                 | Pm MSP1<sub>19</sub> | 28,133                        | 16                            | 54                            | 42                            | 24,467        | 25           |
|                 | Po MSP1<sub>19</sub> | 28,237                        | 71                            | 32                            | 47                            | 24,533        | 21           |
|                 | Pv MSP1<sub>19</sub> | 27,881                        | 71                            | 57                            | 17                            | 24,367        | 25           |
| Mozambique 13   | PBS buffer only  | 29,635                        | 24,263                        | 660                           | 28,869                        | 26,738        | 6            |
|                 | GST              | 29,480                        | 24,168                        | 656                           | 28,481                        | 26,414        | 6            |
|                 | PF MSP1<sub>19</sub> | 38                            | 24,276                        | 594                           | 28,799                        | 26,792        | 6            |
|                 | Pm MSP1<sub>19</sub> | 29,729                        | 395                           | 618                           | 29,039                        | 26,728        | 6            |
|                 | Po MSP1<sub>19</sub> | 29,522                        | 24,142                        | 29                            | 28,411                        | 26,736        | 7            |
|                 | Pv MSP1<sub>19</sub> | 29,724                        | 24,182                        | 637                           | 122                           | 26,670        | 7            |
| Mozambique 17   | PBS buffer only  | 27,423                        | 1082                          | 23,326                        | 87                            | 23,363        | 2            |
|                 | GST              | 27,420                        | 1164                          | 23,551                        | 100                           | 23,351        | 5            |
|                 | PF MSP1<sub>19</sub> | 25                            | 1189                          | 23,599                        | 104                           | 23,481        | 3            |
|                 | Pm MSP1<sub>19</sub> | 27,280                        | 18                            | 23,389                        | 97                            | 23,514        | 2            |
|                 | Po MSP1<sub>19</sub> | 27,239                        | 1157                          | 16                            | 94                            | 23,468        | 4            |
|                 | Pv MSP1<sub>19</sub> | 27,199                        | 1173                          | 23,387                        | 29                            | 23,329        | 4            |
| Mozambique 1    | PBS buffer only  | 29,855                        | 5009                          | 4945                          | 27,283                        | 22,826        | 10           |
|                 | GST              | 29,797                        | 5191                          | 4942                          | 27,173                        | 22,987        | 9            |
|                 | PF MSP1<sub>19</sub> | 66                            | 3017<sup>b</sup>              | 4998                          | 26,901                        | 22,818        | 11           |
|                 | Pm MSP1<sub>19</sub> | 29,636                        | 169                           | 4841                          | 26,949                        | 22,820        | 8            |
|                 | Po MSP1<sub>19</sub> | 29,597                        | 4578                          | 44                            | 26,592                        | 22,866        | 9            |
| Mozambique 20   | PBS buffer only  | 26,789                        | 27,032                        | 491                           | 1227                          | 23,870        | 24           |
|                 | GST              | 26,841                        | 26,991                        | 536                           | 1384                          | 24,149        | 11           |
|                 | PF MSP1<sub>19</sub> | 98                            | 27,258                        | 459                           | 1301                          | 24,083        | 30           |
|                 | Pm MSP1<sub>19</sub> | 26,668                        | 408<sup>c</sup>               | 388                           | 1084                          | 23,921        | 20           |
| Mozambique 16   | PBS buffer only  | 27,518                        | 6425                          | 17,816                        | 24,437                        | 21,029        | 25           |
|                 | GST              | 27,782                        | 6588                          | 18,263                        | 24,430                        | 21,019        | 17           |
|                 | PF MSP1<sub>19</sub> | 25                            | 2040                          | 18,892                        | 24,275                        | 21,320        | 20           |
|                 | Pm MSP1<sub>19</sub> | 27,399                        | 80                            | 18,126                        | 24,324                        | 20,318        | 20           |
| Mozambique 19   | PBS buffer only  | 26,777                        | 2904                          | 353                           | 16,473                        | 23,808        | 5            |
|                 | GST              | 2977                          | 3230                          | 371                           | 18,213                        | 23,981        | 5            |
|                 | PF MSP1<sub>19</sub> | 12                            | 3187                          | 366                           | 17,993                        | 24,120        | 4            |
| Mozambique 2    | PBS buffer only  | 2950                          | 40                            | 151<sup>d</sup>              | 19,599                        | 23,715        | 5            |
|                 | GST              | 2931                          | 2605                          | 40                            | 8192                          | 24,276        | 5            |

<sup>a</sup> Multiplex bead assay responses that were completely suppressed by homologous protein competition are indicated in italics cells. These responses were below the respective cut-off values.

<sup>b</sup> Multiplex bead assay responses that, as a result of heterologous competition, decreased more than 30% relative to the PBS control but remained above the respective cut-off values are indicated in bold italics cells. These values indicate some level of cross-reactivity.

<sup>c</sup> Multiplex bead assay responses that were only partially reduced by homologous protein competition are indicated in underlined cells. These responses remained above the respective cut-off values and likely represent incomplete antibody blocking.

<sup>d</sup> Multiplex bead assay responses that were completely suppressed by heterologous protein competition are indicated in underlined italics cells. These responses were below the respective cut-off values.
reduced by about 50% upon addition of GST control protein. The reason for the observed signal suppression by GST is not understood, but it was probably not related to the presence of the GST component of the MSP119-GST fusion proteins since no antibody bound to the GST control bead and there was no decrease in the PfCSP peptide-GST response. For both donors, the response to the Pf MSP119 decreased in the serum dilution after exposure to magnetic beads (Table 6), and antibodies eluted from the magnetic bead only reacted with the Pf vivax bead in the multiplex assay. Thus, the cross-reactivity previously observed in the competition assays was confirmed for this sample. Plasmodium falciparum MSP119-GST-coated magnetic beads were used to affinity purify antibodies from two additional Mozambique samples: sample 15, previously shown to have specific responses to all 4 MSP119 proteins (Table 4); and sample 12, previously shown to have a pan-malaria cross-reactive response (Table 5). As expected for a species-specific antibody response, only the P. falciparum MSP119 antibody response decreased in the serum dilution following exposure to magnetic beads, and the elution only contained antibodies that recognized the P. falciparum protein in the multiplex assay (Table 6). Incubation of Mozambique sample 12 with the P. falciparum MSP119-GST-coated magnetic beads drastically decreased the antibody responses to proteins from all 4 species in the post-treatment serum dilution, but

### Table 5 Mozambique blood spot elutions that demonstrate multiple cross-reacting MSP119 antibody responses

| Sample          | Competitor added | Pf MSP119 (MFI-bg) | Pm MSP119 (MFI-bg) | Po MSP119 (MFI-bg) | Pv MSP119 (MFI-bg) | PfCSP (MFI-bg) | GST (MFI-bg) |
|-----------------|------------------|--------------------|--------------------|--------------------|--------------------|----------------|--------------|
| Mozambique 3    | PBS buffer only  | 24,960             | 13,703             | 11,965             | 21,795             | 23,643         | 4            |
|                 | GST              | 19,926             | 6961               | 6418               | 12,687             | 23,442         | 2            |
|                 | Pf MSP119        | 40                 | 232                | 53                 | 232                | 23,742         | 1            |
|                 | Pm MSP119        | 221                | 17                 | 40                 | 210                | 23,283         | 2            |
|                 | Po MSP119        | 2487               | 183                | 27                 | 227                | 23,919         | 3            |
|                 | Pv MSP119        | 2506               | 222                | 60                 | 40                 | 23,882         | 1            |
| Mozambique 12   | PBS buffer only  | 24,121             | 13,890             | 9743               | 10,178             | 25,839         | 20           |
|                 | GST              | 22,343             | 6172               | 4284               | 4944               | 25,547         | 10           |
|                 | Pf MSP119        | 62                 | 62                 | 36                 | 175                | 25,874         | 10           |
|                 | Pm MSP119        | 1420               | 22                 | 31                 | 156                | 25,062         | 6            |
|                 | Po MSP119        | 1488               | 54                 | 31                 | 172                | 25,492         | 7            |
|                 | Pv MSP119        | 1726               | 75                 | 40                 | 30                 | 25,447         | 8            |

* Multiplex bead assay responses that were completely suppressed by homologous protein competition are indicated in italics cells. These responses were below the respective cut-off values

* Multiplex bead assay responses that were completely suppressed by heterologous protein competition are indicated in underlined cells. These responses were below the respective cut-off values

* Multiplex bead assay responses that, as a result of heterologous competition, decreased more than 30% relative to the PBS control but remained above the respective cut-off values are indicated in bold italics cells. These values indicate some level of cross-reactivity

### Affinity purification of MSP119 antibodies

Another potential method to detect antibody cross-reactivity is to affinity purify antibody using an MSP119 protein from a single malaria species and then assess the reactivity of the eluted antibody using MSP119 coated beads from all 4 species. Tetanus toxoid, a protein lacking GST, was included in the multiplex panel as an additional assay control.

*Plasmodium vivax* MSP119-GST-coated magnetic beads were used to affinity purify antibodies from two Mozambique samples: sample 13, previously shown to have specific responses to all 4 MSP119 proteins (Table 4); and sample 12, previously shown to have a pan-malaria cross-reactive response (Table 5).
positive responses were not observed in the MBA analysis of the eluted antibodies. *Plasmodium ovale* MSP1$_{19}$ –GST-coated magnetic beads were also used for antibody capture from sample 12 with results similar to those described above (Additional file 4).

**Sub-class and avidity studies**

The inability to affinity purify and recover antibodies from a highly cross-reactive sample suggested that the antibody response in Mozambique 12 might have some unique features relative to responses that were species specific or weakly cross-reactive. Table 7 shows that, while the Mozambique sample 13 anti-MSP1$_{19}$ antibody responses were predominantly of the IgG$_1$ sub-class, Mozambique sample 16 and 15 responses were a combination mainly of IgG$_1$, IgG$_2$, and IgG$_3$ sub-classes. Of particular interest, the *P. falciparum* and *P. malariae* MSP1$_{19}$ responses for both donors had strong IgG$_3$ components, but the *P. ovale* and *P. vivax* responses for sample 16 were largely of the IgG$_2$ sub-class. Further, the MSP1$_{19}$ antibody responses observed in Mozambique 13, 16 and 15 samples appeared to be a mixture of high avidity and low avidity antibodies as determined by the 6 M urea treatment, with responses to the *P. falciparum* MSP1$_{19}$ having a high avidity (≥ 0.98) and responses to the *P. vivax* MSP1$_{19}$ being mainly low avidity (ratio ≤ 0.12). This low avidity, however, did not prevent antibody capture and elution using *P. vivax* MSP1$_{19}$-coated beads shown in Table 6.

The pan-malaria cross-reactive response of Mozambique 12 was completely different. The response to MSP1$_{19}$ proteins from all four malaria species was almost exclusively of the IgG$_3$ sub-class, and the entire IgG response was low avidity with avidity index values of 0.01–0.03 (Table 7). The donor was clearly capable of making high avidity IgG antibodies of other sub-classes as evidenced by the responses to the *P. falciparum* CSP and tetanus toxoid (Table 7). Unfortunately, this observation could not be confirmed using the other highly cross-reactive sample (Mozambique 3) as no additional antibody eluate was available for testing.

**Discussion**

Serologic antibody responses to malaria MSP1$_{19}$ antigens are increasingly used to map geographic distributions and transmission intensities of malaria infection, but questions about the specificity of the responses remain incompletely explored [17–19]. Genomic sequence analysis demonstrates limited allelic variability within species (often only 2–3 amino acids), but considerable sequence heterogeneity between species (this work [35, 38, 65, 66, 67]). In a recent serologic IgG antibody survey of two
communities in northern Mozambique [41], a non-trivial 2–4% prevalence for IgG antibodies to *P. vivax* MSP119 antigen was observed in a population that is expected to be ≥95% negative for the Duffy marker used for RBC invasion [10–12, 70]. The current study was undertaken to determine whether these unexpected responses represented antibody cross-reactivity resulting from the transmission of *P. malariae* and *P. ovale* in the context of intense *P. falciparum* infection or whether they represented true *P. vivax* infections [41].

First, monoplex bead assays using a single MSP119 antigen were compared to multiplex bead assays that included beads coated with antigens from all 4 species as well as GST control and PfCSP peptide coupled to GST. The monoplex versus multiplex results for all 4 MSP119 antigens using a panel of 2 sera and 4 blood spot elutions with a range of antibody response values were virtually identical, and no response dilution effect was detected. Similar results were previously reported by Kerkhof et al. [31] using the *P. falciparum* and *P. vivax* MSP119 antigens and 3 different positive control serum dilutions. However, the observation that a two-fold increase in the number of beads used per assay well had only marginal effects on the measured *P. falciparum* and *P. vivax* MSP119 antibody responses [31] suggests that this technique may not be a sensitive method for identifying partial cross-reactivity.

Second, banked sera from chimpanzees infected with a single species of malaria in a controlled laboratory setting were tested by MBA. While all 4 animals had homologous antibody responses to the laboratory-administered antigens using a panel of 2 sera and 4 blood spot elutions with a range of antibody response values were virtually identical, and no response dilution effect was detected. Similar results were previously reported by Kerkhof et al. [31] using the *P. falciparum* and *P. vivax* MSP119 antigens and 3 different positive control serum dilutions. However, the observation that a two-fold increase in the number of beads used per assay well had only marginal effects on the measured *P. falciparum* and *P. vivax* MSP119 antibody responses [31] suggests that this technique may not be a sensitive method for identifying partial cross-reactivity.

### Table 7 IgG sub-class and antibody avidity index for samples from Mozambique

| Sample       | Antibody detected | Description | Pf MSP1<sub>19</sub> (MFI-bg) | Pm MSP1<sub>19</sub> (MFI-bg) | Po MSP1<sub>19</sub> (MFI-bg) | Pv MSP1<sub>19</sub> (MFI-bg) | PfCSP (MFI-bg) | GST (MFI-bg) | Tet (MFI-bg) |
|--------------|-------------------|-------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|----------------|--------------|--------------|
| Mozambique 13| IgG<sub>1</sub>    | No treatment| 25,380                        | 7236                          | 369                           | 23,068                       | 7081            | 1            | 700          |
|              | IgG<sub>2</sub>   | No treatment | 1255                          | 214                           | 36                            | 778                          | 21,283         | 3            | 17           |
|              | IgG<sub>3</sub>   | No treatment | 404                           | 441                           | 50                            | 281                          | 7555            | 0            | 10           |
|              | IgG<sub>4</sub>   | No treatment | 26                            | 4                             | 3                             | 9                            | 0               | 0            | 28           |
| Total IgG    | No treatment      |             | 31,255                        | 26,542                        | 1198                          | 30,077                       | 28,809          | 12           | 1861         |
| Total IgG    | 6 M urea wash     |             | 31,009                        | 5724                          | 623                           | 2297                         | 28,005          | 3            | 2253         |
| Total IgG    | Avidity index<sup>a</sup> | 0.99 | 0.22                          | 0.52                          | 0.08                          | 0.97                         | N/D             | 1.21         |
| Mozambique 16| IgG<sub>1</sub>    | No treatment| 2425                          | 627                           | 89                            | 190                          | 3722            | 9            | 5100         |
|              | IgG<sub>2</sub>   | No treatment | 107                           | 41                            | 6003                          | 22,534                       | 205             | 4            | 168          |
|              | IgG<sub>3</sub>   | No treatment | 30,533                        | 3401                          | 43                            | 55                           | 5654            | 2            | 107          |
|              | IgG<sub>4</sub>   | No treatment | 52                            | 6                             | 7                             | 7                            | 13              | 0            | 1206         |
| Total IgG    | No treatment      |             | 30,369                        | 59,12                         | 16,287                        | 23,777                       | 20,145          | 35           | 24,367       |
| Total IgG    | 6 M Urea wash     |             | 29,850                        | 3456                          | 1689                          | 223                          | 7332            | 4            | 24,683       |
| Total IgG    | Avidity index<sup>a</sup> | 0.98 | 0.58                          | 0.10                          | 0.01                          | 0.36                         | N/D             | 1.01         |
| Mozambique 15| IgG<sub>1</sub>    | No treatment| 14,698                        | 416                           | 4196                          | 89                           | 11,752          | 1            | 2348         |
|              | IgG<sub>2</sub>   | No treatment | 573                           | 18                            | 58                            | 26                           | 578             | 4            | 67           |
|              | IgG<sub>3</sub>   | No treatment | 30,026                        | 2400                          | 48                            | 64                           | 19,941          | 0            | 86           |
|              | IgG<sub>4</sub>   | No treatment | 3271                          | 9                             | 4                             | 3                            | 281             | 0            | 2934         |
| Total IgG    | No treatment      |             | 30,749                        | 1652                          | 18,046                        | 302                          | 28,269          | 8            | 14,066       |
| Total IgG    | 6 M Urea wash     |             | 30,298                        | 1183                          | 6032                          | 35                           | 25,232          | 2            | 13,336       |
| Total IgG    | Avidity index<sup>a</sup> | 0.99 | 0.72                          | 0.33                          | 0.12                          | 0.89                         | N/D             | 0.95         |
| Mozambique 12| IgG<sub>1</sub>    | No treatment| 269                           | 45                            | 35                            | 57                           | 7043            | 2            | 4212         |
|              | IgG<sub>2</sub>   | No treatment | 28                            | 15                            | 26                            | 15                           | 468             | 5            | 188          |
|              | IgG<sub>3</sub>   | No treatment | 24,515                        | 11,988                        | 8263                          | 8259                         | 22,011          | 3            | 33           |
|              | IgG<sub>4</sub>   | No treatment | 6                             | 1                             | 2                             | 3                            | 9               | 0            | 177          |
| Total IgG    | No treatment      |             | 23,607                        | 6741                          | 4437                          | 4162                         | 26,444          | 16           | 21,638       |
| Total IgG    | 6 M Urea wash     |             | 719                           | 36                            | 29                            | 61                           | 19,765          | 4            | 19,375       |
| Total IgG    | Avidity index<sup>a</sup> | 0.03 | 0.01                          | 0.01                          | 0.01                          | 0.75                         | N/D             | 0.90         |

<sup>a</sup> The total IgG antibody avidity index, indicated in italic cells, was calculated by dividing the total IgG response after 6 M urea wash by the total IgG response with no treatment

N/D not determined
parasite infection, 2 of the animals also had weak heterologous antibody responses to the P. vivax antigen and strong heterologous responses to the P. falciparum antigen despite the fact that they had never been infected with either of these parasites in the laboratory. Responses to the PfCSP antigen were also observed despite the lack of laboratory exposure to P. falciparum sporozoites. The presence of the P. falciparum MSP119 and CSP responses strongly suggested that infections had occurred in the wild, perhaps with one of the Laveranian great ape malaria species that are genetically very similar to P. falciparum [69]. Muerhoff et al. came to the same conclusion regarding a P. falciparum MSP119 response observed in chimpanzee sera by ELISA [36]. The absence of pre-exposure baseline sera for the chimpanzees meant that it was impossible to discriminate between cross-reactive responses resulting from the laboratory infections and pre-existing responses from infections acquired in the wild prior to capture.

Suppression of antibody binding by pre-absorption with excess heterologous or homologous MSP119 antigen should be a sensitive method for the identification of cross-reactive antibody responses in MBA. Amanfo et al. [42] used 2 sera with a competition ELISA technique to demonstrate a lack of cross-reactivity between P. falciparum, P. ovale and P. malariae MSP119 antigens (the P. vivax antigen was not included in their analysis). In the third part of this current study, 12 samples from low transmission areas in Haiti and Cambodia and 20 samples from a high transmission area in Mozambique were used to assess cross-reactivity by antigen competition MBA. Eleven of 12 sera from residents of the low transmission areas had MSP119 antibody responses that were completely species specific. Only one individual had a heterologous competition response decrease that met the >30% reduction definition. In the Mozambique sample set, antibody responses for 12 of the 20 samples tested were totally species specific, and 6 of these 12 samples were positive for antibodies to all 4 malaria parasite species. For 6 additional Mozambique samples, the P. falciparum MSP119 responses were species specific, but various levels of incomplete heterologous competition were observed for the non-P. falciparum assays ranging from a 31 to 99% response reduction. The high specificity of the P. falciparum assay may reflect the affinity maturation of the immune response upon repeated infection with P. falciparum in the high intensity transmission setting of Mozambique. Most heterologous competition was non-reciprocal, suggesting that infection with one malaria species elicited both specific and cross-reactive antibodies while infection with the other malaria species resulted in only specific antibodies. Most commonly, P. malariae responses cross-reacted with P. falciparum antigen. Two examples of reciprocal heterologous competition were also identified, and both of these involved P. vivax and P. ovale responses. Whether higher concentrations of competitor MSP119-GST protein (>10 μg/ml) might have resulted in more complete heterologous competition of these responses was not determined.

Two individuals were identified who had very high responses to all 4 MSP119 antigens (>9000 MFI-bg units) and who appeared to have pan-malaria MSP119 antibody responses by antigen competition MBA. However, perhaps because of the very high levels of antibodies generated by intense levels of P. falciparum transmission, heterologous antigens could only partially suppress the P. falciparum antibody response. These 2 samples represent only 15% of the 13 samples that were positive for antibodies to all four malaria species in the high transmission area sample set, and it should be noted that the sample set was not randomly selected from the overall Mozambique bed net study population. In fact, samples with high responses to the non-P. falciparum MSP119 antigens were intentionally chosen in an attempt to identify those ‘worst case scenario’ samples where cross-reactivity might be observed. Because only 40 samples from the overall Mozambique bed net study set (N=2408) had responses above the cut-off values to all 4 MSP119 antibodies [41], the number of potential pan-malaria reactive individuals in Mozambique is likely quite low (<0.3%).

Finally, an antibody capture/elution technique was used with the MBA to confirm the results of the competition assays described above. While species specific and partially cross-reactive MSP119 antibodies could be eluted from capture beads, appreciable quantities of captured antibodies could not be recovered from the pan-malaria responsive DBS elution despite repeated attempts with multiple capture antigens. Further analysis of the samples from the species specific and partially cross-reactive donors revealed IgG responses of varying avidity dominated by the IgG1 and IgG3 sub-classes. Others have reported that exposure to P. falciparum MSP119 elicits a mixed pattern of IgG1 and IgG3 antibodies and that repeated infection causes a shift towards an IgG1-dominated response [35, 71–78]. The species specific and partially cross-reactive results presented here are consistent with those reports. In contrast, the pan-malaria response from Mozambique sample 12 exhibited very low avidity binding to MSP119 antigens from all 4 malaria species and was skewed entirely to the IgG3 sub-class. Low avidity responses to the P. falciparum MSP119 are relatively rare [74], and only one previously reported example of a mixed IgG1/IgG3 response that skewed almost entirely to an IgG3 response upon repeat infection with P. falciparum was found in the literature [78]. At present, it cannot be determined whether these
observations result from host-specific factors or are a universal feature of pan-malaria responses, nor can any definitive conclusions be drawn about the impact of such responses on malaria immunity or potential malaria pathology.

Previous studies on allele-specific antibody responses to *P. falciparum* MSP119 and apical membrane antigen 1 (AMA1) suggested that children develop allele-specific responses upon primary infection and that the prevalence of cross-reactive antibodies to conserved epitopes increases with age and increasing experience of infection [79, 80]. The number of samples in this study was too small to definitively address the issue of age as a proxy for infection experience and the development of cross-reactive antibody responses. However, two of the partially cross-reactive samples from Mozambique were from 5-years old donors, and 5 of the donors with specific antibody responses to all 4 malaria species were >50 years of age. Simultaneous infection with multiple malaria species, which is known to occur in Mozambique [81], might play a larger role in the development of antibody responses against shared MPS119 epitopes than total infection experience [82]. Thus, even in a high transmission setting with multiple co-endemic malaria species, most antibody responses to *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* MSP119 antigens are likely indicative of previous infection history with those parasite species.

**Conclusions**

Globally, most areas of malaria transmission are seldom mono-specific. In sub-Saharan Africa, *P. falciparum* is the most prevalent infection with the highest intensity of transmission, but significant transmission attributable to *P. malariae*, *P. ovale*, and, in some areas, *P. vivax* occurs. In South and Central America, *P. malariae*, *P. falciparum*, and *P. vivax* are transmitted endemically whereas in Asia all four human malaria species can be transmitted. MSP-119 is a major antigen recognized by the IgG antibody response of a majority of exposed individuals in an endemic population. Malaria control efforts would likely benefit from being able to rapidly and easily monitor immune responses not only for the main targeted species such as *P. falciparum* and *P. vivax*, but also the lesser species, *P. malariae* and *P. ovale*. The analyses presented in this work indicate that the multi-species MSP-119 multiplex bead assay will be a useful tool in future malaria epidemiologic surveillance and control program studies.

**Footnotes**

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

**Additional files**

Additional file 1. Human antibody competition titration assays using MSP119 proteins from four *Plasmodium* species. A combined dilution (1:400 of each serum) containing sera from pan *Plasmodium* Lot 8 and *P. malariae* Lot 2 defined human sera was incubated with the indicated concentrations of the MSP119 competitor protein for 1 hr at room temperature. Competitor proteins used were: Panel A, *P. falciparum* MSP119; Panel B, *P. malariae* MSP119; Panel C, *P. ovale* MSP119; Panel D, *P. vivax* MSP119. Multiplex bead assays were performed as described in “Methods” and the multiplex response in MFI-bg units are plotted versus the competitor concentration. Multiplex responses are presented as a percentage of the assay results for the PBS control.

Additional file 2. Additional MSP119 competition assay results using sera from low malaria incidence settings.

Additional file 3. Additional MSP119 competition assay results using sera from a high malaria incidence setting.

Additional file 4. Additional MSP119 antibody binding and elution assays using beads coated with *Plasmodium ovale* (Po) or *Plasmodium malariae* (Pm) antigens.

**Abbreviations**

MBA: multiplex bead assay; MSP119: 19 kDa subunit of merozoite surface protein 1; CSP: circumsporozoite protein; IFA: immunofluorescence assay; PVA: polyvinyl alcohol; PVP: polyvinylpyrrolidone; PBS: buffer containing 10 mM Na2HPO₄ and 0.85% NaCl at pH 7.2; Buffer A: PBS buffer (pH 7.2) containing 0.3% Tween-20, 0.02% NaN₃, 0.5% casein, 0.5% PVA, 0.8% PVP and 3 µg/ml E. coli extract; Buffer B: PBS buffer (pH 7.2) containing 0.5% BSA, 0.05% Tween-20, and 0.02% NaNO₃; Tris: tris(hydroxymethyl)-aminomethane; GST: *E. coli* glutathione-S-transferase; MES: 2-(N-morpholino)-ethanesulfonic acid; HEPES: N-hydroxyethylpiperazine- N’-2-ethanesulfonic acid; MFI-bg: median fluorescent intensity minus background, MES: 2-(N-morpholino)-ethanesulfonic acid; Pm: *P. malariae*; Po: *P. ovale*; Pv: *P. vivax*; Pf: *P. falciparum*; AMA1: apical membrane protein 1.

**Authors’ contributions**

JWP planned and designed the study. JWP, MMP, CSH, ER, BM, CJG, BC, JC and JWB participated in data collection and analysis. JWP performed the final analysis and drafted the manuscript. MMP, ER, and CSH provided edits to the manuscript. All authors read and approved the final manuscript.

**Author details**

1 Division of Foodborne, Waterborne, and Environmental Diseases at the Centers for Disease Control and Prevention, Atlanta, GA, USA. 2 Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, Atlanta, GA, USA. 3 US President’s Malaria Initiative, Centers for Disease Control and Prevention, Atlanta, GA, USA. 4 University of Health Sciences, Phnom Penh, Cambodia. 5 Division of Vector-borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA. 6 National Malaria Control Programme, Maputo, Mozambique. 7 Clinton Health Access Initiative, Boston, MA, USA.

**Acknowledgements**

The authors gratefully recognize Patrick Lammie for contributions to the Cambodia serosurvey project.

**Competing interests**

The authors declare they have no competing interests.

**Availability of data and materials**

All data is available under reasonable request.
Consent for publication
Not applicable.

Ethics approval and consent to participate
Residual malaria diagnostic sera were made anonymous under a protocol approved by the CDC Institutional Review Board. Written informed consent was obtained prior to enrollment and participation in the Cambodian serosurvey, and the study protocol was reviewed and approved by the national ethics committee in Cambodia. Written informed consent was obtained prior to enrollment and participation in the Mozambique bed net study and serosurvey, and the study was approved by the National Bioethics Committee in Mozambique. For both of these studies, CDC researchers had no access to personal identifiers, and CDC staff were not considered to be engaged with human research subjects.

Funding
MP was supported by US President’s Malaria Initiative.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 11 September 2018   Accepted: 1 November 2018

Published online: 09 November 2018

References
1. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, et al. A long neglected world malaria map. Plasmodium vivax endemicity in 2010. PLoS Negl Trop Dis. 2012;6:e1814.
2. Gething PW, Patil AP, Smith DL, Guerra CA, Elyazar IR, Johnston GL, et al. A new world malaria map: Plasmodium falciparum endemicity in 2010. Malar J. 2011;10:378.
3. Rutledge GG, Bohme U, Sanders M, Reid AJ, Cotton JA, Maiga-Ascofare O, et al. Plasmodium malariae and P. ovale genomes provide insights into malaria parasite evolution. Nature. 2017;542:101–4.
4. Rutledge GG, Marr I, Huang GKL, Auborn S, Mafurt J, Sanders M, et al. Genomic Characterization of recrudescence Plasmodium malariae after treatment with artemether/lumefantrine. Emerg Infect Dis. 2017;23:1390–7.
5. Betson M, Clifford S, Stanton M, Kabatereine NB, Stothard JR. Emergence of non falciparum Plasmodium infection despite regular artemisinin combination therapy in an 18-month longitudinal study of Ugandan children and their mothers. J Infect Dis. 2018;217:1099–109.
6. Roucher C, Rogier C, Sokhna C, Tall A, Trape JF. A 20-year longitudinal study of Plasmodium ovale and Plasmodium malariae prevalence and morbidity in a West African population. PLoS ONE. 2014;9:e87169.
7. Langford S, Douglas NM, Lampah DA, Simpson JA, Kenangalem E, Sugianto P, et al. Plasmodium malariae infection associated with a high burden of anemia: a hospital-based surveillance study. PLoS Negl Trop Dis. 2015;9:1309–7.
8. Livingstone FB. The Duffy blood groups, vivax malaria, and malaria selection in human populations: a review. Hum Biol. 1984;56:413–25.
9. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, et al. The international limits and population at risk of Plasmodium vivax transmission in 2009. PLoS Negl Trop Dis. 2010;4:e774.
10. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FYN. N Engl J Med. 1976;295:302–4.
11. Menard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, Ratimbasoa A, et al. Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci USA. 2010;107:5967–71.
12. Howes RE, Reiner RC Jr, Battle KE, Longbottom J, Mappin B, Ordanovich D, et al. Plasmodium vivax transmission in Africa. PLoS Negl Trop Dis. 2015;9:e0004222.
13. Poirier P, Doderer-Lang C, Atchade PS, Lemoine JP, de l’Isle MC, Aboubacar A, et al. The hide and seek of Plasmodium vivax in West Africa: report from a large-scale study in Beninese asymptomatic subjects. Malar J. 2016;15:570.
14. Ngassa Mbenda HG, Das A. Molecular evidence of Plasmodium vivax mono and mixed malaria parasite infections in Duffy-negative native Cameroonians. PLoS ONE. 2014;9:e103262.
15. Cutts JC, Powell R, Aguiss PA, Beeson JG, Simpson JA, Fowkes FJ. Immunological markers of Plasmodium vivax exposure and immunity: a systematic review and meta-analysis. BMC Med. 2014;12:50.
16. Folegatti PM, Siqueira AM, Monteiro WM, Lacerda MV, Drakeley CJ, Braga EM. A systematic review on malaria sero-epidemiology studies in the Brazilian Amazon: insights into immunological markers for exposure and protection. Malar J. 2017;16:107.
17. Corran P, Coleman P, Riley E, Drakeley C. Serology: a robust indicator of malaria transmission intensity? Trends Parasitol. 2007;23:575–82.
18. Drakeley CJ, Corran PH, Coleman PG, Tongren JE, McDonald SL, Carneiro J, et al. Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proc Natl Acad Sci USA. 2005;102:5108–13.
19. Cunha MG, Silva ES, Sepulveda N, Costa SP, Saboia TC, Guerreiro JF, et al. Serologically defined variations in malaria endemicity in Para state, Brazil. PLoS ONE. 2014;9:e113357.
20. Wong J, Hamel MJ, Drakeley CJ, Kariuki S, Shi YP, Lal AA, et al. Serological markers for monitoring historical changes in malaria transmission intensity in a highly endemic region of Western Kenya, 1994–2009. Malar J. 2014;13:431.
21. Cook J, Reid H, Javo J, Kuwahata M, Talego G, Clements A, et al. Using serological measures to monitor changes in malaria transmission in Vanuatu. Malar J. 2010;9:169.
22. Rosas-Aguirre A, Llanos-Cuentas A, Sepybroeck N, Cook J, Contras-Mancilla J, Soto V, et al. Assessing malaria transmission in a low endemicity area of north-western Peru. Malar J. 2013;12:339.
23. Rosas-Aguirre A, Sepybroeck N, Llanos-Cuentas A, Rosanas-Urgell A, Carrasco-Escober G, Rodriguez H, et al. Hotspots of malaria transmission in the Peruvian Amazon: rapid assessment through a parasitological and serological survey. PLoS ONE. 2015;10:e0137458.
24. Stewart L, Gosling R, Griffin J, Gesase S, Campo J, Hashim R, et al. Rapid assessment of malaria transmission using age-specific sero-conversion rates. PLoS ONE. 2009;4:e6083.
25. Arnold BF, Priest JW, Hamlin KL, Moss DM, Colford JM Jr, Lammie PJ. Serological measures of malaria transmission in Haiti: comparison of longitudinal and cross-sectional methods. PLoS ONE. 2014;9:e93684.
26. Metcalf CJ, Farrar J, Cutts FT, Basta NE, Graham AL, Lessier J, et al. Use of serological surveys to generate key insights into the changing global landscape of infectious disease. Lancet. 2016;388:278–30.
27. Jepsen MR, Rosier D, Christiansen M, Olsen Larsen S, Cavanagh DR, et al. Development and evaluation of a multiplex screening assay for Plasmodium falciparum exposure. J Immunol Methods. 2012;384:62–70.
28. Kerkhof K, Canier L, Kim S, Heng S, Sochantha T, Sovannaroth S, et al. Implementation and application of a multiplex assay to detect malaria-specific antibodies: a promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas. Malar J. 2015;14:338.
29. Koff D, Toure AO, Varela ML, Vigan-Womas I, Beourou S, Brou S, Ehoun MF, et al. Analysis of antibody profiles in symptomatic malaria in three sentinel sites of Ivory Coast by using multiplex, fluorescent, magnetic, bead-based serological assay (MAGPIX). Malar J. 2015;14:509.
30. Priest JW, Jenks WH, Moss DM, Mao B, Buth S, Wannamuehler K, et al. Implementation of multiplex bead assays for parasitic diseases into a national, population-based serosurvey of women 15–39 years of age in Cambodia. PLoS Negl Trop Dis. 2016;10:e004699.
34. Soares IS, Levitus G, Souza JM, Del Portillo HA, Rodrigues MM. Acquired immune responses to the N- and C-terminal regions of Plasmodium vivax merozoite surface protein 1 in individuals exposed to malaria. Infect Immun. 1997;65:1606–14.

35. Egan AF, Chappel JA, Burghaus PA, Morris JS, McBride JS, Holder AA, et al. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1(19), the carboxy-terminal fragment of the major merozoite surface protein of Plasmodium falciparum. Infect Immun. 1995;63:456–66.

36. Muehoff AS, Birkenmeyer LG, Coffey R, Dille BJ, Barnwell JW, Collins WE, et al. 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. Clin Vaccine Immunol. 2010;17:1631–8.

37. Holder AA. The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. Parasitology. 2009;136:1445–56.

38. Birkenmeyer L, Muehoff AS, Dawson GJ, Desai SM. Isolation and characterization of the MSP1 genes from Plasmodium malarium and Plasmodium ovale. Am J Trop Med Hyg. 2010;82:996–1003.

39. Babon JJ, Morgan WD, Kelly G, Eccleston JF, Feeney J, Holder AA. Structural studies on Plasmodium vivax merozoite surface protein-1. Mol Biochem Parasitol. 2007;153:31–40.

40. Boureau T, Yousuff RM, Cook J, Cox J, Alegana VA, Amran J, et al. Serologic markers for detecting malaria in areas of low endemicity, Somalia. Emerg Infect Dis. 2010;16:392–9.

41. Plucinski MM, Candrinho B, Chambe G, Muchanga J, Muguande O, Almeida WM, et al. Immunogenicity of synthetic peptides from circumsporozoite protein of Plasmodium falciparum. Science. 1984;225:593–9.

42. Holder AA. The carboxy-terminus of merozoite surface protein: structure, specific antibodies and immunity to malaria. Parasitology. 2009;136:1445–56.

43. Priest JW, Moss DM, Won K, Todd CW, Henderson L, Jones CC, Wilson M. Detection of merozoite surface protein-1 p19 antibodies in human malaria patients and experimentally infected nonhuman primates. Clin Vaccine Immunol. 2010;17:1631–8.

44. Scobie HM, Mao B, Buth S, Wannemuehler KA, Sorensen C, Kannarath C, et al. Detection of merozoite surface protein-1 p19 antibodies in human malaria patients and experimentally infected nonhuman primates. Clin Vaccine Immunol. 2010;17:1631–8.

45. Prince HE, Wilson M. Simplified assay for measuring Toxoplasma gondii immunoglobulin G avidity. Clin Diagn Lab Immunol. 2001;8:904–8.

46. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem. 1993;39:561–77.

47. Youden WI. Index for rating diagnostic tests. Cancer. 1950;3:32–5.

48. Guimaraes LO, Wunderlich G, Alves JM, Bueno MG, Rohe F, Catalo-Dias JL, et al. Merozoite surface protein-1: genetic diversity in Plasmodium malariae and Plasmodium brasilianum from Brazil. BMC Infect Dis. 2015;15:529.

49. Putaporntip C, Hughes AL, Jongsuwytes S. Low level of sequence diversity at merozoite surface protein 1 locus of Plasmodium ovale curtisi and P. ovale wailkeni from Thai isolates. PLoS One. 2013;8:e83962.

50. Putaporntip C, Jongsuwytes S, Sakihama N, Ferreira MI, Kho WG, Kaneko A, et al. Mosaic organization and heterogeneity in frequency of allelic recombination of the Plasmodium vivax merozoite surface protein-1 locus. Proc Natl Acad Sci USA. 2002;99:16348–53.

51. Scobie HM, Mao B, Buth S, Wannemuehler KA, Sorensen C, Kannarath C, et al. Tetanus immunity among women of child-bearing age in a national population-based serosurvey, 2012. Clin Vaccine Immunol. 2016;23:546–56.

52. Prince HE, Wilson M. Simplified assay for measuring Toxoplasma gondii immunoglobulin G avidity. Clin Diagn Lab Immunol. 2001;8:904–8.

53. Youden WI. Index for rating diagnostic tests. Cancer. 1950;3:32–5.
76. Scopel KK, Fontes CJ, Ferreira MU, Braga EM. Factors associated with immunoglobulin G subclass polarization in naturally acquired antibodies to *Plasmodium falciparum* merozoite surface proteins: a cross-sectional survey in Brazilian Amazonia. Clin Vaccine Immunol. 2006;13:810–3.

77. Nebie I, Diarra A, Ouedraogo A, Soulama I, Bougouma EC, Tiono AB, et al. Humoral responses to *Plasmodium falciparum* blood-stage antigens and association with incidence of clinical malaria in children living in an area of seasonal malaria transmission in Burkina Faso, West Africa. Infect Immun. 2008;76:759–66.

78. Cavanagh DR, Dobano C, Elhassan IM, Marsh K, Elhassan A, Hviid L, et al. Differential patterns of human immunoglobulin G subclass responses to distinct regions of a single protein, the merozoite surface protein 1 of *Plasmodium falciparum*. Infect Immun. 2001;69:1207–11.

79. Cortes A, Mellombo M, Masciantonio R, Murphy VJ, Reeder JC, Anders RF. Allele specificity of naturally acquired antibody responses against *Plasmodium falciparum* apical membrane antigen 1. Infect Immun. 2005;73:422–30.

80. Kusi KA, Dodoo D, Bosomprah S, van der Eijk M, Faber BW, Kocken CH, Remarque EJ. Measurement of the plasma levels of antibodies against the polymorphic vaccine candidate apical membrane-antigen 1 in a malaria-exposed population. BMC Infect Dis. 2012;12:32.

81. Oki M, Asai S, Saito-Nakano Y, Nakayama T, Tanaka Y, Tachibana H, et al. A case of quadruple malaria infection imported from Mozambique to Japan. Am J Trop Med Hyg. 2014;90:1098–101.

82. Kusi KA, Manu EA, Manful Gwira T, Kyei-Baafour E, Dickson EK, Amponsah JA, et al. Variations in the quality of malaria-specific antibodies with transmission intensity in a seasonal malaria transmission area of Northern Ghana. PLoS ONE. 2017;12:e0185303.