Journal of Immunological Methods 499 (2021) 113148

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Development of a new peptide-bead coupling method for an all peptide–based Luminex multiplexing assay for detection of *Plasmodium falciparum* antibody responses

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ARTICLE INFO

Keywords:
Malaria
Plasmodium falciparum
Multiplex
Peptides
Coupling
Serology

ABSTRACT

Using a recombinant protein antigen for antibody testing shows a sum of antibody responses to multiple different immune epitopes existing in the protein antigen. In contrast, the antibody testing to an immunogenic peptide epitope reflects a singular antibody response to the individual peptide epitope. Therefore, using a panel of peptide epitopes provides an advantage for profiling multiple singular antibody responses with potential to estimate recent malaria exposure in human infections. However, transitioning from malaria immune epitope peptide-based ELISA to an all peptide bead-based multiplex Luminex assay presents some challenges including variation in the ability of different peptides to bind beads. The aim of this study was to develop a peptide coupling method while demonstrating the utility of these peptide epitopes from multiple stage antigens of *Plasmodium falciparum* for measuring antibodies.

Successful coupling of peptide epitopes to beads followed three steps: 1) development of a peptide tag appended to the C-terminus of each peptide epitope consisting of beta-alanine–lysine (x 4)–cysteine, 2) bead modification with a high concentration of adipic acid dihydrazide, and 3) use of the peptide epitope as a blocker in place of the traditional choice, bovine serum albumin (BSA). This new method was used to couple 12 peptide epitopes from multiple stage specific antigens of *P. falciparum*, 1 *Anopheles* mosquito salivary gland peptide, and 1 Epstein-Barr virus peptide as an assay control. The new method was applied to testing of IgG in pooled samples from 30 individuals with previously repeated malaria exposure in western Kenya and IgM and IgG in samples from 37 U.S. travelers with recent exposure to malaria.

The new peptide-bead coupling method and subsequent multiplex Luminex assay showed reliable detection of IgG to all 14 peptides in Kenyan samples. Among 37 samples from U.S. travelers recently diagnosed with malaria, IgM and IgG to the peptide epitopes were detected with high sensitivity and variation. Overall, the U.S. travelers had a much lower positivity rates of IgM than IgG to different peptide epitopes, ranging from a high of 62.2% positive for one epitope to a low of only 5.4% positive for another epitope. In contrast, the travelers had IgG positive rates from 97.3% to 91.9% to various peptide epitopes. Based on the different distribution in IgM and IgG positivity to overall number of peptide epitopes and to the number of pre-erythrocytic, erythrocytic, gametocytic, and salivary stage epitopes at the individual level, four distinct patterns of IgM and IgG responses

Abbreviations: ADH, adipic acid dihydrazide; BSA, bovine serum albumin; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; HPLC, high-performance liquid chromatography; IFA, immunofluorescence assay; IgG, immunoglobulin G; IgM, immunoglobulin M; MES, 2-(N-morpholino)ethanesulfonic acid hydrate; MFI, mean fluorescence intensity; NHP, normal human plasma; OD, optical density; PBS, phosphate-buffered saline; RT, room temperature; Sulfo-NHS, N-hydroxysulfosuccinimide; TFA, trifluoroacetic acid..

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https://doi.org/10.1016/j.jim.2021.113148
Received 10 May 2021; Received in revised form 16 August 2021; Accepted 15 September 2021
Available online 21 September 2021

among the 37 samples from US travelers were observed. Independent peptide-bead coupling and antibody level readout between two different instruments also showed comparable results.

Overall, this new coupling method resolves the peptide-bead coupling challenge, is reproducible, and can be applied to any other immunogenic peptide epitopes. The resulting all peptide bead-based multiplex Luminex assay can be expanded to include other peptide epitopes of *P. falciparum*, different malaria species, or other diseases for surveillance, either in US travelers or endemic areas.

1. Introduction

Malaria poses a significant public health threat throughout the world, as 2.5 billion people live in regions of stable or unstable transmission (World Health Organization, 2018). The potential for such wide exposure results in approximately 219 million cases and 435,000 deaths annually, with a particularly large burden in sub-Saharan Africa (World Health Organization, 2018). With such a large burden comes the need to implement and efficiently evaluate large-scale prevention, control, and elimination measures. Currently, assessment of these efforts is often conducted by quantifying prevalence, incidence, mortality, entomological inoculation rate, and more recently by seropositivity, or the seroconversion rate of antibodies to malaria antigens (Drakeley et al., 2005; Ambrosino et al., 2010; Shaukat et al., 2010; Tusting et al., 2014; World Health Organization. Global Malaria Programme, 2014; Roth et al., 2016).

Upon initial exposure to malaria, an anti-malarial antibody response is triggered within the body; this response is initiated by IgM response followed by switching from IgM to IgG antibodies and by increasing IgG levels upon repeated exposure (Healer et al., 2018; Ly and Hansen, 2019). Although antibody production primarily serves to protect a host from disease, these immune molecules have also been used in serological assays for more than 50 years to estimate malaria exposure rates within human populations (Kagan et al., 1969a; Kagan et al., 1969b). An advantage of measuring serological responses is that some antibodies persist past the initial infection, allowing for measurement of transmission and exposure history in the absence of parasite (Arnold et al., 2017; Priest et al., 2018). The utility of serological assays for surveillance has been demonstrated in many malaria endemic or pre-elimination countries such as Kenya, Zambia, Haiti, Ethiopia, Uganda, Peru, and Myanmar (Kobayashi et al., 2012; Rosas-Aguirre et al., 2013; Rogier et al., 2015; Sepulveda et al., 2015; Kulkarni et al., 2017; Weber et al., 2017; Nyunt et al., 2018; Assefa et al., 2019).

Historically, serological studies have employed the enzyme-linked immunosorbent assay (ELISA) that detects an antibody response to a single antigen (Perraut et al., 2005). Although this assay has been utilized efficiently for years, recently there has been a shift in the field toward multiplexing. During malaria infection, the human immune system mounts an immune response to thousands of malaria antigens. Bead-based flow cytometric assays can simultaneously detect up to one hundred antibody response to these different antigens in a single sample, doing so even when the levels of antibody differ substantially by antigen. In addition, a multiplex assay might include antigens not only from multiple stages of an infectious agent but also multiple diseases, saving time and money during study design, sample collection, and data analysis (Arnold et al., 2017).

Since antibodies can last for several years after pathogen clearance, it is difficult to determine when exposure, infection, or transmission occurred. Many serological tests, including the singleplex ELISA and the bead-based multiplex Luminex assay, use recombinant protein antigens (Katz et al., 2012; Anderson et al., 2015; Rascoe et al., 2015; Materniak-Kornas et al., 2017). Although useful for testing antibody responses, using recombinant protein antigens consisting of multiple different immune epitopes has several major drawbacks compared with using a panel of individual immune epitope peptides within an antigen. First, production of recombinant protein is more time-consuming than peptide synthesis, with greater potential for batch-to-batch variability, increases cost, and reduces stability (Palomares et al., 2004; Duong-Ly and Gabelli, 2014; Ahmad et al., 2018). Second, the use of recombinant protein antigens introduces a greater risk of cross-reactivity of antibody responses to other malaria species as well as non-malaria infections (Kijanka et al., 2009). Finally, using a panel of individual peptide immune epitopes within an antigen has the ability to test a singular antibody response to individual epitopes, and profiling of multiple singular antibody responses has the potential to distinguish between recent and past exposures (Forsstrom et al., 2015). For these reasons, it was determined best to use an all peptide-based multiplexing Luminex serological assay for profiling multiple singular antibody responses for potential application in the assessment of recent malaria exposure or infection. However, there are challenges with transitioning peptides from ELISA to an all peptide bead-based Luminex assay mostly due to challenges associated with substantial variation in coupling of some small peptides to the beads.

Given the necessity of an all peptide bead-based Luminex malaria serological assay for our research and the challenges presented with coupling small peptides, the objectives of this study were: 1) to develop a bead coupling method for small peptide epitopes from multiple stages of *Plasmodium falciparum* infection to be used in multiplex serological assays through testing for IgG in samples collected from individuals with previously repeated malaria infections from Kenya and 2) to validate the all peptide bead-based multiplex serological assay through testing for IgM and IgG in samples from United State travelers with recent malaria exposure and through testing of assay reproducibility. The methods developed in this study can be applied to serological monitoring of malaria both in endemic countries as well as travelers in non-endemic settings. Additionally, the new bead-coupling method developed can be broadly applied to any other immunogenic peptide epitopes and can be expanded to additional peptide epitopes of *P. falciparum*, other malaria species, and other diseases of interest.

2. Materials and methods

2.1. Study design and sample source

This study was divided into two stages: development and validation. For the method development stage, plasma samples from 30 individuals demonstrating a high level of anti-malarial IgG antibody by immunofluorescence assay (IFA) (Sulzer et al., 1969; Manawadu and Voller, 1978). These samples were from children and adults (pregnant women excluded) who participated in a malaria immunology study conducted in rural communities near Kisumu city, a high malaria transmission area of western Kenya. The samples from this immunology study were approved for serological testing by the Ethics Review Committee of the Kenya Medical Research Institute and the Institutional Review Board of the CDC. For method development as well as positive antibody controls during method validation, three plasma pools were made, each from 10 IFA-positive individuals, and used throughout the study.

For the method validation stage, individual plasma samples from United States travelers returning from malaria endemic areas were employed. These samples were collected as part of routine CDC domestic malaria surveillance (National Malaria Surveillance System, NMSS; Mace et al., 2021), and serological testing on these samples were approved by the Institutional Review Board of the CDC. The samples

from travelers with IFA positivity for *P. falciparum* malaria infection were selected. Additionally, samples from frequent international travelers were excluded, based on the information from the NMSS. Using these criteria, stored plasma samples (at −20 degree) from 37 individuals from May 2010–2014 were available. These samples obtained from 15 females, 18 males, and 4 individuals of unknown sex, between the ages of 3 and 67 with a median age of 30 years. Although all plasma samples were obtained from hospitals in the United States, brief clinical information was available for only 20 of the 37 travelers. Information regarding the onset date of malaria infection was not available for any traveler, however all samples were provided for diagnostic purposes. Personal identifiers were removed from the samples, which were randomly assigned with laboratory codes for testing.

In addition, plasma samples from 30 blood donors from a Tennessee community blood bank were used as negative controls. These donors had no international travel within the past six months and were assumed to have no recent malaria infection in the past six months. These samples were used throughout the study.

2.2. Peptide design and synthesis with unique tag

2.2.1. Peptide selection and design

Peptides previously used in singleplex ELISA assays were selected for this study (Table 1) (Kaur et al., 1990; Fidock et al., 1994; Kumar et al., 1995; Ploton et al., 1995; Udhayakumar et al., 1995; Bottius et al., 1996; Theisen et al., 2000; Udhayakumar et al., 2001; Cortes et al., 2003; Klutts et al., 2004; Poinsignon et al., 2008; Mahajan et al., 2010). These peptides represent 12 *P. falciparum* antigens: 4 from the pre-erythrocytic stage antigens, 5 from the erythrocytic stage, 1 appearing in both of these stages, and 2 from the gametocytic stage. In addition, 2 peptides from non-malaria antigens were used, an *Anopheles* salivary gland peptide for testing antibody response to mosquito bites and a peptide from the Epstein-Barr virus (EBV) viral capsid protein, which has a high serological response and infection rate, was used as an internal quality control (Ooka et al., 1991; Poinsignon et al., 2008).

2.2.2. Peptide synthesis

Peptides were synthesized either in their standard epitope form as listed in Table 1 or with the addition of a unique C-terminal tag to aid in the bead coupling process. This universal tag added to the C-terminus consisted of βAKKKKC. For example, the tagged peptide Pf GLURP (P3), from N-terminus to C-terminus, would have the following sequence: EPLEPFPTQIHKDYK-βAKKKKC.

The peptides were assembled using Fmoc solid-phase peptide synthesis via model 433A (Applied Biosystems, Foster City, CA, USA), model Liberty Blue (CEM Corporation, Matthews, NC, USA), model Tribute (Protein Technologies, Tucson, AZ, USA) or model CSBio (CSBio, Menlo Park, CA, USA) automated peptide synthesizers followed by cleavage in trifluoroacetic acid (TFA)/phenol/thioanisole/ethanedithiol/water mixture at 25 °C for 120 min with shaking. The crude products were precipitated with dry diethyl ether and incubated for 60 min at -20 °C. Crude peptides were then washed 3 times with cold diethyl ether, and the precipitate was dissolved in water and/or acetonitrile before lyophilization.

2.2.3. Peptide purification

The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC), typically on a Zorbax SB-C_{18} reversed-phase column (9.4 mm × 250 mm, 5 mm particle size, 300 Å pore size), using either a model Delta 600 HPLC system (Waters, Milford, MA, USA) or Dionex UltiMate 3000 HPLC System (ThermoFisher Scientific, Waltham, MA, USA). The column was equilibrated against 5% v/v aqueous acetonitrile in 0.1% trifluoracetic acid (TFA), and the peptides eluted at 55 °C at a flow rate of 2 mL/min using a linear gradient of solvent B (80%, v/v, in acetonitrile containing 0.08% aqueous TFA) in solvent A (0.1%, v/v, aqueous TFA). The final peptide purity was confirmed by analytical RP-HPLC, and their masses were confirmed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) using the Bruker UltrafleXtreme MALDI-TOF/TOF system. Following lyophilization, the peptides were obtained in the form of their trifluoroacetate salts.

2.3. Coupling of peptides to beads

2.3.1. Luminex standard bead coupling protocol

For standard coupling of peptides to Luminex beads, previously published methods outlined by the "Carbodiimide Coupling Protocol for Antibodies and Proteins" in the Luminex Xmap Cookbook v 4.0 were followed (Stephen Angeloni et al., 2018). Briefly, 5.0 × 10^6 bead stock microspheres were washed in water and activated for 20 min at room temperature (RT, ~25 °C) in the dark with mixing in 80 μL of 1 M sodium phosphate, pH 6.2, activation buffer (ThermoFisher Scientific, Waltham, MA, USA) plus 10 μL of 50 mg/mL N-hydroxysulfosuccinimide (Sulfo-NHS, ThermoFisher Scientific, Waltham, MA, USA) and 10 μL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, ThermoFisher Scientific, Waltham, MA, USA). The beads were then washed three times with 250 μL of coupling buffer containing 50 mM 2-(N-morpholino) ethanesulfonic acid hydrate (MES, Sigma Aldrich, St. Louis, MO, USA), pH 5.0, and resuspended in 100 μL of coupling buffer. The peptide was then added at an appropriate concentration (determined by titration, data not shown) to the resuspended bead mixture and incubated at RT for 2 h. The peptide-coupled beads were then washed

Table 1
Peptide name, sequence, and source. For each of the 14 peptides used in this study, a sequence, beginning at the N-terminus, as well as a source (i.e., stage of malarial infection, mosquito saliva, or Epstein-Barr virus) is listed. The C-terminal universal tag sequences (βAKKKKC) are not shown.

Peptides			
Name	N-C Sequence	Source	Citation
Pf SALSA-1 (23–49)	SAEKKDEKEASEQGEESHKKENSQESA	Pre-erythrocytic	Bottius E., 1996
Pf SALSA-2 (50–83)	NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPK	Pre-erythrocytic	Bottius E., 1996
Pf LSA-J (S-12)	ERRAKEKLQEQQRDLEQRKADTKK	Pre-erythrocytic	Fidock DA., 1994
Pf CSP (Repeat)	NANPNVDPNANPNVDPNANPNANPNANPNANP	Pre-erythrocytic	Kaur P. 1990
Pf GLURP (P3)	EPLEPFPTQIHKDYK	Pre-erythrocytic Erythrocytic	Theisen M., 2000
Pf MSP-3b (S-14)	AKEASSYDYILGWEFGGGVPEHKKEEN	Erythrocytic	Mahajan B., 2010
Pf AMA-1 (PL169)	DGNCEDIPHVNEFSAIDL	Erythrocytic	Udhayakumar V., 2001
Pf AMA-1 (446–490)	YKDEIKKEIERESKRIKLNDNDDEGNKIIAPRIFISDDKDSLKC	Erythrocytic	Cortes A., 2003
Pf AMA-1 (PL173)	GNAEKYDKMDEPQHYGKS	Erythrocytic	Udhayakumar V., 2001
Pf MSP-1 (PL97)	NSGCFRHLDEREECKCLLN	Erythrocytic	Udhayakumar V., 1995
Pfs48/45 (98–109)	LPEKCFQKVYTD	Gametocytic	Kumar N., 1995 and Ploton IN., 1995
Pfg27 (P5)	KPLDKFGNIYDYHYEH	Gametocytic	Kumar N., 1995 and Ploton IN., 1995
gSG6-p1	EKVWVDRDNVYCGHLDCTRVATF	Mosquito Salivary	Poinsignon A., 2008
EBV VCA p-18	AVDTGSGGGGQPHDTAPRGARKKQ	Epstein-Barr Virus	Klutts JS., 2004

three times in blocking buffer containing PBS-TBN with 1% BSA (1× phosphate buffered saline [PBS, GE Healthcare Life Sciences, Pittsburgh, PA, USA], 0.02% Tween-20 [Sigma-Aldrich, St. Louis, MO, USA], 1% bovine serum albumin [BSA, ThermoFisher Scientific, Waltham, MA, USA], 0.05% sodium azide [Sigma-Aldrich, St. Louis, MO, USA]) and were further blocked in blocking buffer for 30 min at RT. Finally, beads were washed three times with storage buffer (PBS-TBN with 0.1% BSA), resuspended in 500 μL of storage buffer, and stored at 4 °C protected from light.

2.3.2. Luminex modified ADH bead coupling protocol

For adipic acid dihydrazide (ADH, Santa Cruz, Dallas, TX, USA) modified coupling of peptides to Luminex beads, the previously published "Modification of Microspheres with ADH" protocol was used with important changes (Stephen Angeloni et al., 2018). Changes included incubation with high concentration ADH, blocking with the peptide of interest, and removal of BSA from all solutions. To start, 1.25×10^6 Luminex beads were washed with 1 mL of 0.1 M MES, pH 6.0, wash buffer I. Beads were then resuspended in 180 μL of 35 mg/mL ADH diluted in wash buffer I. After, 36 μL of 200 mg/mL EDC diluted in wash buffer I was added to each tube. Tubes were incubated at RT for 2 h. Then, beads were washed three times with 500 μL of wash buffer II (0.1 M MES, pH 4.5) and were resuspended in 175 μL of wash buffer I.

Unlike the standard protocol where the optimal peptide concentrations varied depending on the peptide (data not shown), it was found that 10 μg of peptide was consistently sufficient for effective coupling in this modified protocol. Hence, 10 μg of a given peptide was added to the ADH-modified beads at this point in the protocol. Then 10 μL of 50 mg/mL EDC and 10 μL of 50 mg/mL Sulfo-NHS, each resuspended in wash buffer I, was added. Beads were then incubated at RT for 2.5 h and then washed twice with wash buffer I.

Unlike standard blocking with BSA, beads were blocked with 500 μL wash buffer I containing 300 μg of whichever peptide had previously been coupled and incubated for 1 h at RT, washed twice with wash buffer I and then resuspended in 100 μL of modified storage PBS-TN buffer also without BSA. Coupled beads were then stored at 4 °C protected from light. This protocol is only adapted for 1.25×10^6 beads and has not been scaled up. Therefore, bulk production is accomplished by following this protocol and combining end products.

2.4. Luminex serological assay

The Luminex multiplex assay was adopted from the "Indirect (serological) immunoassay" Luminex Xmap Cookbook v 4.0 (Stephen Angeloni et al., 2018). First, total volume needed for 50 μL per sample of PBS-TM assay buffer was determined (1× PBS, pH 7.2, 0.3% Tween-20, 5% non-fat, dry milk (ThermoFisher, Waltham, MA, USA)). Approximately 1000 beads per a region per a sample well was then added to the assay buffer and 50 μL of bead mixture per a well was aliquoted into a black, round-bottomed 96-well plate (Corning, Tewksbury, MA, USA). Plasma test samples, positive, and negative controls were diluted 1:25 in assay buffer and 50 μL of the sample was plated in duplicate to the 96 well plate containing the bead mixture. The plate was rocked on a microplate shaker (Lab-Line Instruments, Mansfield, TX, USA) at 700 rpm for 1.5 h at RT then washed three times in wash buffer (1× PBS, 0.3% Tween-20) using an Elx50 plate washer (BioTek, Winooski, VT, USA).

After washing the 50 μl of secondary antibody mouse anti-human IgG Fc-BIOT (SouthernBiotech, Birmingham, AL, USA) diluted 1:2500 in 1× PBS was added to each well and shaken for 1 h at RT. The plate was then washed 3 times and 50 μL Streptavidin, R-phycoerythrin conjugate (SAPE) (ThermoFisher Scientific, Waltham, MA, USA) diluted 1:250 in 1× PBS was added to each well and shaken 30 min at RT, washed 3 times and 100 μL of 1× PBS added to each well. The plate was then read either on a Luminex 100/200 (Luminex, Austin, TX, USA) or a Bioplex MAGPIX Multiplex Reader (Luminex, Austin, USA, USA) instrument using Bio-Plex Manager 6.1 software (BioRad, Hercules, CA, USA) for data acquisition. A minimum of 50 beads per a bead region were acquired and readout was shown as mean fluorescence intensity (MFI) minus background MFI as the average of two duplicates.

The testing for IgM serological response followed the same protocol except the secondary antibody mouse anti-human IgM FC-Biot (SouthernBiotech, Birmingham, AL, USA) diluted 1:2500 was used.

2.5. ELISA serological assay

All fourteen peptides have previously been successfully used in enzyme-linked immunosorbent assays (ELISA) (Kaur et al., 1990; Fidock et al., 1994; Kumar et al., 1995; Ploton et al., 1995; Udhayakumar et al., 1995; Bottius et al., 1996; Theisen et al., 2000; Udhayakumar et al., 2001; Cortes et al., 2003; Klutts et al., 2004; Poinsignon et al., 2008; Mahajan et al., 2010). In this study, we applied a common ELISA protocol for testing 4 peptide antigens: Pf AMA-1 (446–490), Pf AMA-1 (PL173), Pf MSP-1 (PL97), and gSG6-p1. First, the peptide was diluted to a concentration of 5 μg/mL in coating buffer containing 0.3% sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), 0.16% sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA), 0.02% sodium azide in water. Then 50 μL diluted peptide was added to each well of a 96 well ELISA plate (ThermoFisher, Waltham, MA, USA), covered, and incubated overnight at 4 °C. The plate was washed with 300 μL /well PBS-T and then 3 times with distilled water followed by adding 100 μL/well blocking buffer (2.5% non-fat milk, 0.05% Tween-20 in 1× PBS) incubating at RT for 3 h with 700 rpm shaking. After washing the plate 3 times as described previously, Kenyan samples and malaria negative normal human samples, diluted 1:25 in blocking buffer, were added to the plate at 50 μL/well in triplicate. The plate was shaken for 2 h at RT and then washed as previously described. One hundred μL of goat anti-human IgG HRP conjugate (Promega, Madison, WI, USA) diluted 1:5000 in PBS containing 1% milk, was added to each well and incubated 1 h and, washed 3 times, then 100 μL/well SureBlue TMB Microwell Peroxidase Substrate (KPL) (ThermoFisher, Waltham, MA, USA) was added. Color was developed for 15 min at RT in the dark and stopped with the addition of 100 μL/well of 1 M phosphate acid (ThermoFisher, Waltham, MA, USA). Plates were read at 450 nm using an Infinite M200 microplate reader (Tecan, Mannedorf, Switzerland) and readout was recorded as optical density (OD) minus blank.

2.6. Data processing and analysis

Quality control (QC) of individual runs was conducted by assessing average positive control MFI and average negative control MFI for each antigen, as well as individual sample EBV results. All plates as well as samples passed the QC. The IgG and IgM serological cutoffs were calculated based on the mean MFI plus 2.96 standard deviation respectively for each individual peptide antigen using 30 NHP samples. These different cutoffs were subtracted from the Kenya pooled and NMSS traveler individual results for each test antigen. Anything above zero was considered as positive while any negative value was declared as the negative that was converted to 1 for further log transformation. Serological data was transformed using natural log for comparison purposes. Comparisons among or between bead couplings and instruments were conducted using one-way ANOVA statistical test and *t*-test, respectively, with significance p value <0.05. Individual results were also plotted for comparison between independent couplings and instruments where Pearson's r correlations were performed to determine the strength of the correlations as well as the statistical significance (Mazhari et al., 2020). Using the same criteria established from this paper, Pearson's r values >0.7 were considered strong correlations, 0.3–0.7 moderate correlations, and < 0.3 weak.

3. Results

3.1. Development of all peptide-based multiplexing assay

3.1.1. Challenge

During the process of assay development, 12 peptides that represent pre-erythrocytic, erythrocytic, and gametocytic stages of the *P. falciparum* parasite, a peptide from *Anopheles* mosquito salivary gland and a peptide of EBV as an internal quality control were used (Table 1). These peptides ranged in amino acid length from as small as 12 amino acids (Pfs48/45 (98–109)) to as large as 44 amino acids (Pf AMA-1 (446–490)) and had all previously been used in ELISA assays where they were shown to have a high frequency of IgG responses detected in humans from malaria endemic areas (Kaur et al., 1990; Fidock et al., 1994; Kumar et al., 1995; Ploton et al., 1995; Udhayakumar et al., 1995; Bottius et al., 1996; Theisen et al., 2000; Udhayakumar et al., 2001; Cortes et al., 2003; Klutts et al., 2004; Poinsignon et al., 2008; Mahajan et al., 2010). To confirm the antibody responses to these peptides, malaria positive samples from Kenya as described in the methods were used in both ELISA and singleplex Luminex Assay using the standard protocol. However, measured antibody levels to some peptide antigens were substantially different between the ELISA and Luminex Assay with no antibody responses detected for some of the peptides in the Luminex Assay (data not shown). This presented challenges in transitioning all peptides to a multiplex bead-based Luminex Assay as it has been previously demonstrated by others using recombinant proteins (Stephen Angeloni et al., 2018).

3.1.2. Solutions

Several different potential causes and solutions for the challenge were explored to successfully transition the peptide-based ELISA assay to the peptide bead-based Luminex assay. The exploration resulted in one method consisting of three modifications that allowed a successful transition of all peptides from ELISA to Luminex. Table 2 shows the outcomes of stepwise modifications using four individual peptide antigens gSG6-P1, Pf AMA-1 (446–490), Pf MSP-1 (PL97), and Pf AMA-1 (PL173). The first modification was the addition of a peptide tag (βAKKKKC) to the C-terminus of the antigenic sequence. The Luminex standard bead coupling protocol with the tagged peptides (Table 2, column 3) resulted in the detection of positive IgG to Pf AMA-1 (PL173) with an MFI of 1086 in the positive Kenya sample compared to MFI of 21 without the peptides tagged (Table 2, column 2). Despite the detection of antibody to this peptide, the assay failed to detect antibodies against the remaining 3 peptides compared to the ELISA assay (Table 2, column 3 vs 1). Therefore, the second modification was to modify the Luminex bead surface with the addition of a large concentration of ADH, to allow for more efficient peptide binding. The third modification was to modify the coupling procedure itself where the beads were blocked in remaining procedures with the same peptide that had already been coupled to the bead, i.e. Pf MSP-1 (PL97) coupled bead blocked in blocking buffer containing Pf MSP-1 (PL97) peptide. This requires the preparation of individual blocking buffer for each peptide that is coupled instead of standard blocking and storage buffer that use the large protein molecule bovine serum albumin (BSA). After using all three modifications in combination, significant antibody responses to all four peptides in Kenya positive samples with 100 times higher than normal control was shown (Table 2, column 4). In addition, the new method with 3 modifications together also increased the antibody detection level of the peptide antigen Pf AMA-1 (PL173) from an average MFI of 1086 to an average MFI of 2902 (Table 2, column 3 vs 4). These results clearly demonstrate the difference between the new method with 3 modifications compared to the standard Luminex protocol, resolving the challenge in detection of antibody responses in peptide-based Luminex assay.

3.1.3. Transition to all peptide-based multiplexing assay

The new coupling method was used to couple all fourteen peptides (Table 1). To verify that the peptide coupling method was successful for all 14 peptides, known IgG antibody positive samples from individuals with repeated malaria infections in Kenya were used to test the IgG response for each peptide epitope antigen (Fig. 1). The Kenyan pooled samples, after NHP cutoff subtraction, showed high IgG responses to each peptide antigen with the average MFI from approximately a high of 25,000 for pre-erythrocytic stage SALSA-1 (23–49) peptide to a low MFI of 400 for gametocytic stage Pfs48/45 (98–109) peptide (Fig. 1). The all peptide multiplexing Luminex assay included the mosquito salivary gland antigen gSG6-p1 to measure exposure to a transmission vector. It

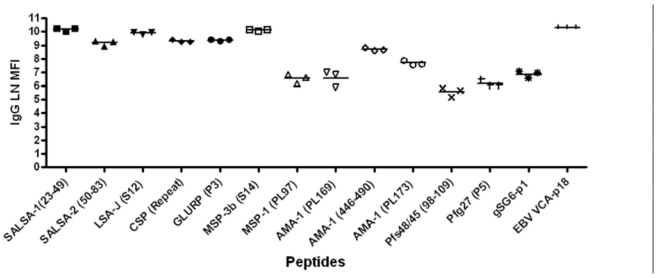

Fig. 1. Tagged peptide ADH coupled and peptide epitope as blocking agent all peptide-based multiplexing Luminex platform for *P. falciparum*. IgG response was measured using 3 Kenya pooled serum samples tested in duplicate, with 10 individuals per a pool. The IgG response to 14 peptides is read out as LN MFI after subtracting the IgG NHP antigen cutoff, a line indicates the average IgG MFI between the 3 pooled samples for a given peptide.

Table 2
Comparison among ELISA, Luminex standard protocol, Luminex standard protocol with tagged peptides, and Luminex ADH protocol with tagged peptides for four different peptides. A malaria positive IgG pool and normal human plasma (NHP) pool were used for testing. Three malaria peptide antigens and one mosquito salivary gland peptide antigen were coated to an ELISA plate, coupled to Luminex beads using the standard Xmap protocol, peptide-tagged and coupled to Luminex beads using the standard Xmap protocol, or peptide-tagged and coupled to Luminex beads using an ADH modified protocol. ELISA results are read out as the optical density (OD) from three triplicate replicates. Luminex results are read out as mean fluorescence intensity (MFI) of duplicate samples.

Antigen	ELISA (OD)		Luminex standard protocol (MFI)		Luminex standard protocol tagged peptides (MFI)		Luminex ADH protocol tagged peptides (MFI)	
	Malaria positive IgG pool	Normal human plasma	Malaria positive IgG pool	Normal human plasma	Malaria positive IgG pool	Normal human plasma	Malaria positive IgG pool	Normal human plasma
gSG6-p1	2.1062	0.5666	17	15	24	11	1446*	11
Pf AMA-1 (446–490)	1.4859	0.7819	253	8	132	26	6508*	52
Pf MSP-1 (PL97)	1.041	0.6066	28	17	18	7	840*	12
Pf AMA-1 (PL173)	3.3351	0.2517	21	16	1086*	1	2902*	41

* Values represent a positive assay response.

showed an IgG response with an average MFI of 1091 (Fig. 1) to this vector peptide. The results of IgG responses to various malaria peptides confirmed that the new coupling method is universal for bead-peptide coupling. In addition, antibody response to an EBV peptide was used as an internal quality control as EBV exposure is almost universal at a population level with approximately 97% seroconversion in adult populations (Tzellos and Farrell, 2012; Smatti et al., 2017). This internal control antibody response allows us to examine and determine if low immune responses or no antibody responses to other peptide antigens are caused by issues of sample collection/preparation or assay failure. Fig. 1 shows there is a strong antibody response to EBV peptide in each sample with an average MFI of almost 30,000, suggesting the all peptide based multiplexing Luminex platform is reliable.

3.2. Validation of all peptide-based multiplexing assay

3.2.1. Testing with domestic traveler samples

To validate and evaluate the limit of detection for this new serological protocol using peptides, we used 37 samples from NMSS; these are diagnostic samples obtained from patients experiencing an acute malaria illness. First, an IgG response was tested for all 13 peptide antigens displayed as LN MFI minus IgG NHP cutoff (Fig. 2A). There was an IgG response detected for each antigen, though varying by individual. With the samples representing acute malaria cases one would expect there to be a primary IgM response as well. IgM in the 37 NMSS samples was tested for each peptide antigen and the results displayed as LN MFI minus IgM NHP cutoff (Fig. 2B). While both IgG and IgM responses were detected, the positivity rates to different peptide antigens varied. Overall, IgG showed a high percent positive for each antigen varying between a high of 97.3% positive for SALSA-2 (50–83), CSP (Repeat), GLURP (P3), MSP-3b (S-14), AMA-1 (446–490), and AMA-1 (PL173) to a low of 91.9% positive for Pfg27 (P5) (Fig. 2A). On the contrary, IgM had a much lower percent positive than IgG and varied between a high of 62.2% positive for gSG6-P1 to a low of only 5.4% positive for SALSA-1 (23–49) (Fig. 2B).

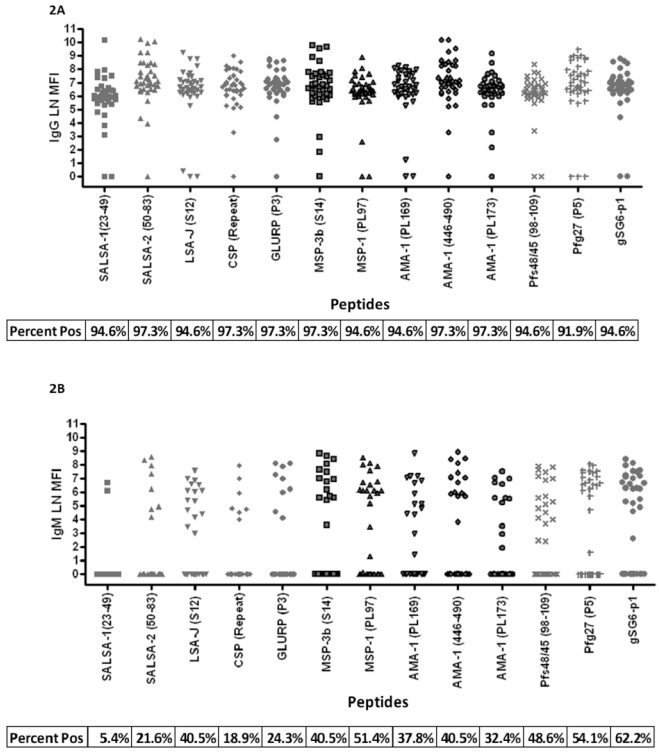

Fig. 2. Antibody responses to 13 peptide antigens in 37 NMSS samples tested in duplicate. (A) Individual IgG response to 13 peptides readout as LN MFI after subtracting the IgG NHP antigen cutoff. The percent positivity for each peptide antigen is indicated below. (B) Individual IgM response to 13 peptides readout as LN MFI, after subtracting the IgM NHP antigen cutoff. The percent positivity for each peptide antigen is indicated below.

Using the data described above, we further looked at the seropositivity patterns of IgG and IgM to the 12 *P. falciparum* peptides and the *Anopheles* saliva peptide antigens at individual level among specimens from NMSS (Fig. 3). All the samples tested had IgG responses to at least one *P. falciparum* peptide antigen, with the majority of samples having a positive response to at least 11 peptide antigens (Fig. 3), the exception being samples M3 and M21 which only had positive IgG responses to 2 and 6 peptide antigens respectively (Fig. 3). Unlike IgG, the IgM response had a much more diverse distribution of positivity to each peptide antigen, with some samples having an IgM positive response to all 13 antigens while others having no response to any of the peptide antigens (Fig. 3).

IgM antibody responses are produced first during an initial infection and rapidly wane over time in comparison to long lived IgG antibodies (Salonen et al., 1985). Specific to malaria infection, IgM antibodies have been shown to rise to peak levels 6–10 days after infection, have a serum half-life of less than 25 days, represent 50%–80% of initial antibody response, and are barely detectable by two months (Gysin et al., 1982; Brown et al., 1988; Oyong et al., 2019). Therefore, it is important to explore the number of singular IgM and IgG positive to peptide antigens at an individual level. Using arbitrary positive antibody responses to 7 peptides representing about 50% positivity within singular IgM and IgG to the 13 peptides tested as criteria in a sample, we categorized the 37 traveler samples into four groups. The samples in the first group (Fig. 4A, group 1) had antibody responses to fewer than 7 peptide epitopes for both IgM and IgG, as seen in samples M3 and M21. The second group (Fig. 4A, group 2) was classified as having both IgM and IgG antibody responses to 7 or more peptide epitopes. The third group were samples IgG-positive for at least 7 peptides and IgM-positive for one to six peptide epitopes (Fig. 4A, group 3). Finally, the last group had IgG positive responses to at least 7 peptides but had no IgM positive response to any peptide antigens (Fig. 4A, group 4). Among the 37 NMSS samples, 2 samples could be classified as group 1, 13 samples as group 2, 15 samples as group 3, and 7 samples as group 4 (Fig. 4A).

To further examine the IgM response, the number of peptide antigens found to be IgM positive within each parasite stage in individual samples was displayed (Fig. 4B). Within group 2, 11 of 13 samples were IgM positive for at least 2 or more pre-erythrocytic peptide antigens, and 13 of 13 samples were IgM positive for at least 3 or more erythrocytic peptide antigens. Additionally, all 13 samples had a response to the salivary gland antigen and 10 of 13 samples were IgM positive for both gametocyte peptide antigens (Fig. 4B). For group 3, all samples had 1 or no IgM response to the pre-erythrocytic peptide antigens, 12 of 15 samples had 2 or less IgM responses to the erythrocytic peptide antigens, and 11 of 15 samples had 1 or no response to the gametocyte peptide antigens. Additionally, 6 of 15 samples had no IgM response to saliva gland antigen (Fig. 4B).

3.2.2. Reproducibility of peptide coupling and assay

While a Luminex 100/200 instrument is more accurate and sensitive, many times a MAGPIX instrument is used, especially in the field as MAGPIX is cheaper and is easier to maintain. Therefore, comparison of differences in MFI readout between running the assay on a Luminex 100/200 instrument and a MAGPIX instrument was conducted. The same coupled beads for all 13 antigens and the same set of traveler samples were first run on a Luminex 100/200 instrument and then on MAGPIX. The natural log mean MFI IgG response from all 37 NMSS samples was calculated for each individual antigen (Table 3, column 1 vs column 2). There was no statistically significant difference in MFI IgG levels for each peptide antigen between the Luminex instrument and the MAGPIX instrument. Additionally, individual results for the 37 NMSS samples run on the Luminex 100/200 instrument were plotted against

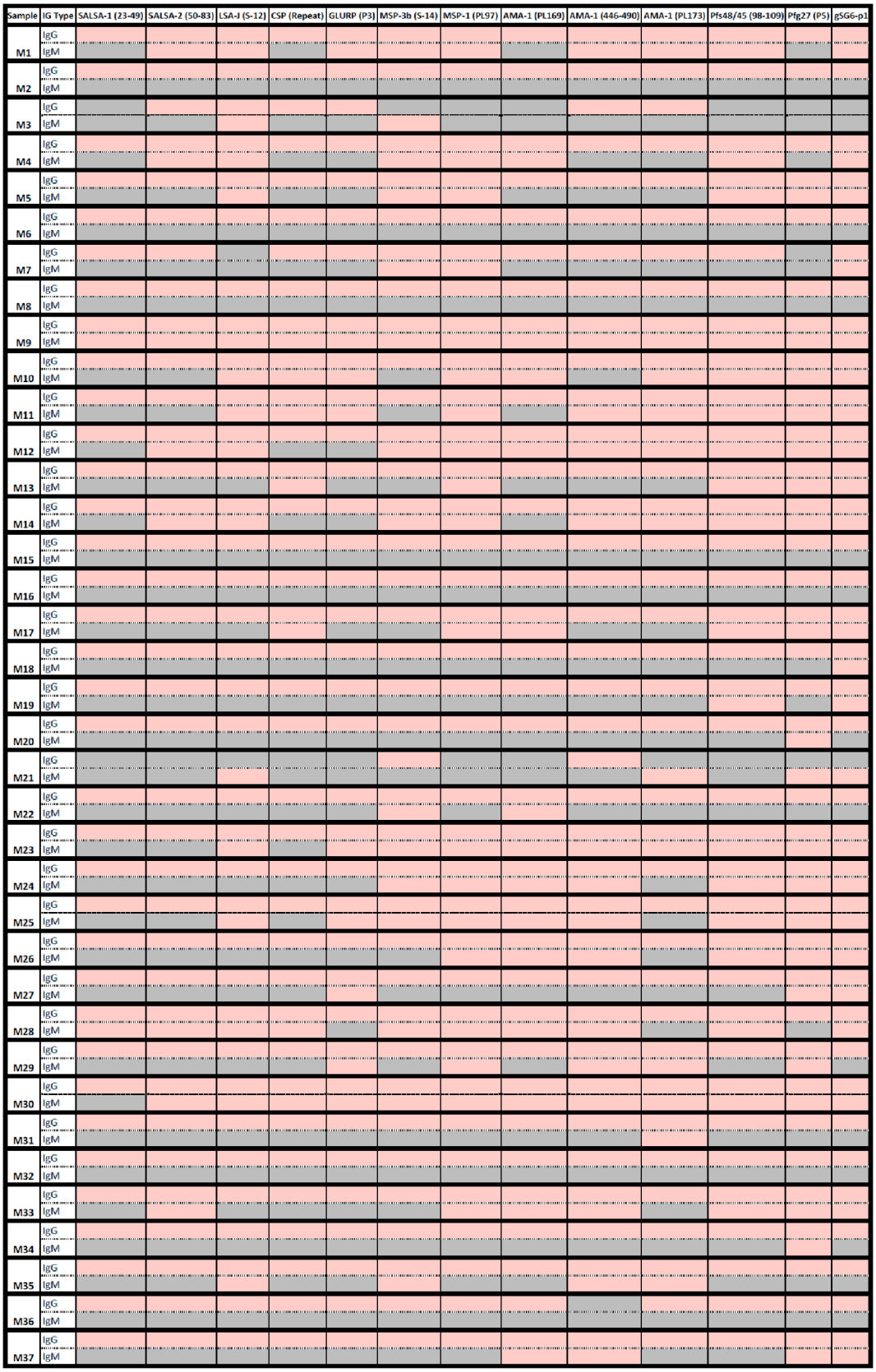

Fig. 3. Individual patterns of IgM and IgG seropositivity to 13 *P. falciparum* peptides in 37 NMSS samples. Antigens are displayed on the x-axis in order of peptides from pre-erythrocytic, erythrocytic, gametocytic, and salivary gland while the y-axis contains each individual sample M1-M37. Each individual antibody response is color coded pink (positive), grey (negative) based on the calculated NHP cutoffs for each antigen run on the Luminex 100/200 in duplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

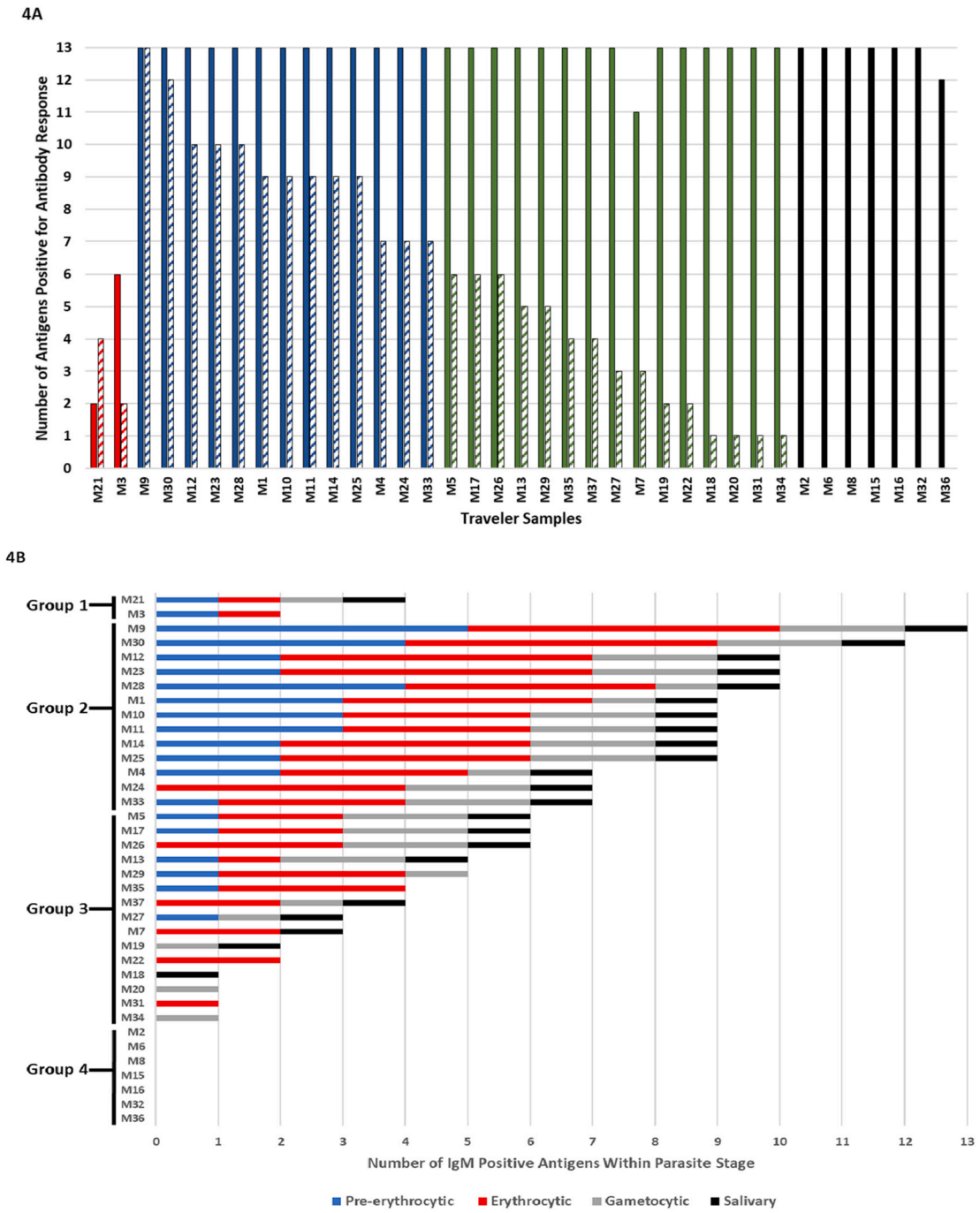

Fig. 4. Exploratory profiling of singular IgG and IgM response to peptide antigens from different parasitic stages. (A) Number of positive IgG and IgM antibody response to peptide antigens observed in each traveler sample. A solid bar represents number of IgG positive and a striped bar represents number of IgM positive. The samples are grouped into four categories based on the number of IgG and IgM positivity in individual samples ■ Group 1 (IgG and IgM both less than 7 positive), ■ Group 2 (IgG and IgM both greater than 7 positive), ■ Group 3 (IgG greater than 7 positive, IgM less than 7 positive but at least 1), ■ Group 4 (only IgG positive, no IgM positive). (B) Number of IgM positive response to peptide antigens from different parasitic stages within each categorized group. The stages of peptides are displayed as ■ Pre-erythrocytic ■ Erythrocytic ■ Gametocytic ■ Salivary stage and the samples are ordered as in (A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the results of the same samples run on the MAGPIX instrument (Fig. 5A). There was strong correlation between the two machine runs, with 11 of the 13 peptides showing a Pearson's r value greater than 0.85, $p < 0.005$ (Fig. 5A). The other two peptides showed moderate correlation with a Pearson's r value >0.43, $p < 0.005$ (Fig. 5A).

To ensure that the bead coupling method was reproducible, two independent couplings were conducted, and the IgG response tested in the 37 NMSS samples between two runs of coupling were compared on a MAGPIX machine. The natural log mean MFI IgG response to each antigen was calculated for two couplings (Table 3, columns 2 and 3). There

Table 3
Comparison between two independent bead couplings and between a MAGPIX and Luminex 100/200 instrument. The 37 NMSS samples in duplicates were used for the comparison and average LN MFI [95% CI] is displayed. Luminex 1 and MAGPIX 1 represent one independent bead coupling run on different instruments, while MAGPIX 2 represents a second independent bead coupling run on the same instrument as MAGPIX 1. There was no statistical difference among three groups using one-way ANOVA statistical test $p > 0.05$ for each peptide antigen, and no statistical difference in pair comparison (T-test) between instruments (Luminex 1/MAGPIX 1) $p > 0.05$ for each antigen and between bead couplings (MAGPIX 1/MAGPIX 2) respectively $p > 0.05$ for each antigen.

	Average LN MFI [95% CI] (N = 37)		
	Luminex 1	MAGPIX1	MAGPIX 2
SALSA-1 (23–49)	6.09 [5.66,6.52]	5.98 [5.54,6.42]	6.05 [5.59,6.52]
SALSA-2 (50–83)	7.72 [6.76,7.77]	7.24 [6.72,7.77]	7.27 [6.78,7.76]
LSA-J (S-12)	6.68 [6.32,7.04]	6.69 [6.28,7.10]	6.79 [6.41,7.17]
CSP (Repeat)	6.74 [6.36,7.12]	6.69 [6.29, 7.09]	6.75 [6.34, 7.15]
GLURP (P3)	6.94 [6.60,7.28]	6.86 [6.47,7.24]	6.88 [6.48,7.27]
MSP-3b (S-14)	6.97 [6.57,7.36]	6.89 [6.45,7.32]	6.99 [6.58,7.39]
MSP-1 (PL97)	6.51 [6.20, 6.84]	6.44 [6.06, 6.82]	6.54 [6.16, 6.93]
AMA-1 (PL169)	6.72 [6.38, 7.06]	6.61 [6.22, 6.99]	6.73 [6.35, 7.11]
AMA-1 (446–490)	7.68 [7.29, 8.06]	7.56 [7.13, 7.99]	7.6- [7.24, 7.97]
AMA-1 (PL173)	6.46 [6.12, 6.81]	6.40 [6.00, 6.81]	6.51 [6.09, 6.93]
Pfs48/45 (98–109)	6.51 [6.24, 6.77]	6.41 [6.08, 6.75]	6.48 [6.18, 6.78]
Pfg27 (P5)	7.61 [7.28, 7.93]	7.53 [7.18, 7.88]	7.50 [7.11, 7.88]
gSG6-p1	7.00 [6.72, 7.28]	6.84 [6.49, 7.18]	6.89 [6.58, 7.20]

was no statistically significant difference between two couplings for MFI IgG levels to each antigen tested. Additionally, individual results for the 37 NMSS samples run between coupling 1 and coupling 2 were plotted against each other to compare coupling reproducibility (Fig. 5B). There was strong correlation between the independent couplings, with 11 of the 13 peptides showing a Pearson's r value greater than or equal to 0.89, $p < 0.005$ (Fig. 5B). The other 2 peptides showed moderate correlation with a Pearson's r value >0.43, $p < 0.005$ (Fig. 5B).

4. Discussion

This study describes the development and validation of an all peptide-based *P. falciparum* serological Luminex multiplex assay. A panel of 12 peptide antigens from *P. falciparum* parasite pre-erythrocytic, erythrocytic and gametocytic stages, a salivary gland peptide from *Anopheles* mosquito and an EBV peptide as an internal assay quality control were used in this study for detection of antibody responses in malaria infected humans. Due to the challenge in transitioning malaria peptides from ELISA assays to bead-based Luminex assays, a 3-step solution was used for peptide-bead coupling which included peptide-tagging, bead modification with high concentrations of ADH and blocking modification through blocking with already coupled peptide. This resulted in a reliable and sensitive all peptide-based Luminex multiplexing assay for the detection of *P. falciparum* IgG and IgM responses. The study further showed that the new coupling method was reproducible between bead coupling batches and the final readouts of antibody responses were comparable between Luminex and MAGPIX machines. Finally, the all peptide-based Luminex multiplexing assay was able to detect malaria-specific IgG and IgM in malaria-exposed travelers, allowing us to further explore potential utility of profiling singular antibody responses to assess recent malaria exposure in malaria endemic areas.

Among the 3 step solutions for peptide-bead coupling, the first addition of a tag (βAKKKKC) to a peptide epitope improves coupling; being a small epitope, the peptide requires an area that allows for access to this epitope by corresponding antibody (Potocnakova et al., 2016; Stephen Angeloni et al., 2018). The tag creates a natural peptide extender and moves the peptide epitopes slightly away from the beads surface providing greater exposure for the antibody to bind without interference. Second, the tag with four lysine residues provides four additional epsilon-amino groups that are available for attachment to the carboxyl groups on the bead providing more potential sites for coupling the peptide epitope to the bead. Finally, the C-terminal universal tag improved aqueous solubility of the peptides during HPLC purification and coupling to the beads. As a second step of solutions, the addition of

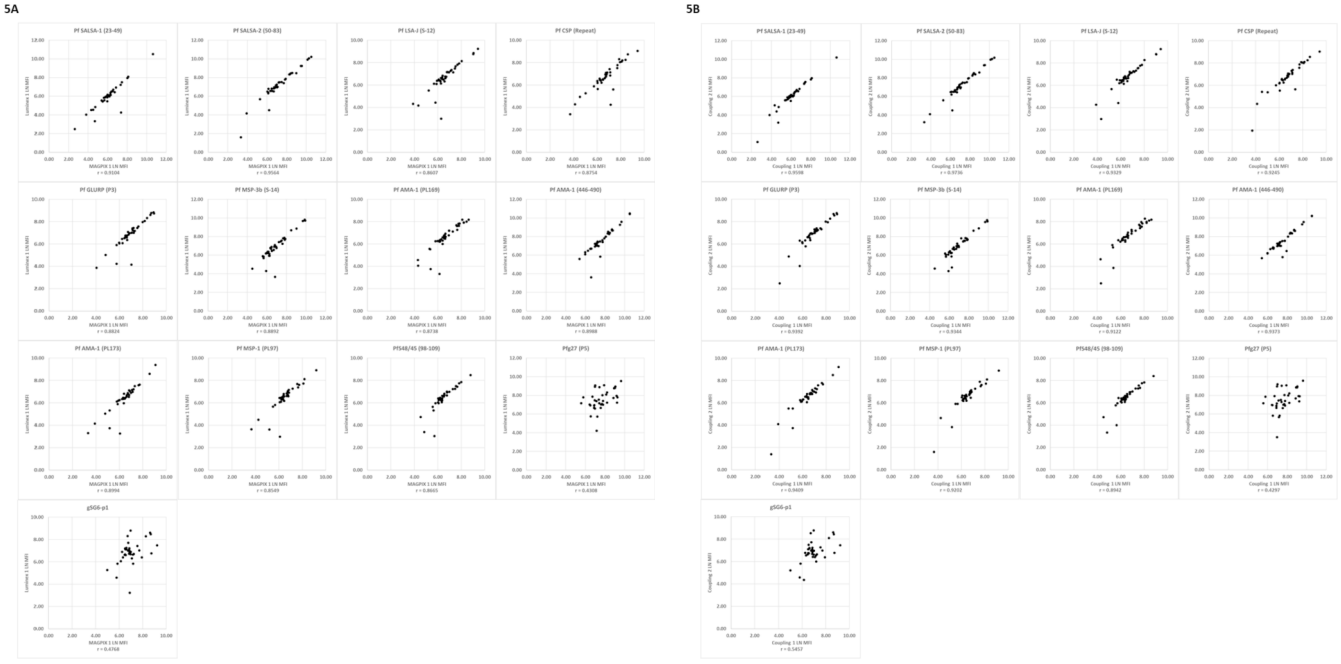

Fig. 5. Comparison between a MAGPIX and Luminex 100/200 instrument as well as two independent bead couplings. (A) LN MFI IgG levels measured against 13 peptides in 37 NMSS samples using a Luminex 100/200 instrument compared to using a MAGPIX instrument. (B) LN MFI IgG levels measured against 13 peptides independently coupled one time compared to the same 13 peptides independently coupled a second time in 37 NMSS samples using a MAGPIX instrument. Pearson's r correlations are displayed under each peptide comparison $p < 0.005$ for all, $r > 0.7$ strong correlation, 0.3–0.7 moderate correlation, <0.3 weak correlation (Mazhari et al., 2020).

high concentration of ADH to the beads works as a modification of the surface of bead allowing for more efficient binding. The final critical step was the addition of a peptide blocker representing the same peptide being coupled. The use of this peptide as a blocker instead of the common blocking agent BSA is because a large molecule like BSA could shield the bead-coupled-peptide epitope from exposure to antibodies. Utilizing coupled peptide as a blocker resulted in an exposed peptide epitope, allowing for antibodies to bind. In addition, for this same reason BSA was eliminated from the entirety of the Luminex assay.

It is important to mention that a few other potential solutions for improving peptide-bead coupling such as using BSA as a molecular linker (Lateef et al., 2007) or using 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH) (Stephen Angeloni et al., 2018) were tested. However, the other solutions either introduced high non-specific background and caused inconsistent results or completely failed in the Luminex assay in our study (data not shown). In contrast, the 3-step solutions developed in this study resolves the peptide-bead coupling issue and produced reliable results in all peptide bead-based multiplexing Luminex assay for the detection of *P. falciparum* antibody responses. This was demonstrated by testing samples from people with repeated malaria infection from western Kenya (Table 2 and Fig. 1).

This study also shows that the new peptide-bead coupling method and resulting *P. falciparum* multiplexing Luminex serological assay is sensitive and reproducible as demonstrated by testing samples for IgG and IgM in NMSS specimens, by independent peptide-bead couplings and by running the assay on different machines (Table 3, Figs. 2, 3, and 5). These results have three practical implications. First, the ability to detect antibody responses in travelers suggests that the multiplexing serological assay with the current peptide epitope panel has the potential to be utilized for malaria surveillance. Second, the high reproducibility between peptide-bead coupling batches and comparable MFI readouts between two Luminex instruments provide researchers the flexibility to include other peptide epitopes of *P. falciparum*, different malaria species or other diseases for surveillance. Third, less cost and more stability for peptide synthesis plus adaptability of MAGPIX (cheaper compared to the 100/200 instrument) make the all peptide-based multiplexing serological assay field usable in resource-limited countries.

Testing IgM is important as IgM is a class of antibody that is associated with recent exposure or infection because it has a shorter half-life than IgG and is often the first responder during initial infection (Gronwall et al., 2012; Liu et al., 2019). Recently it has been shown that IgM response is immediate following repeat exposure to malaria, indicating IgM may be useful antibody marker for recent exposure even in areas of high transmission (Krishnamurty et al., 2016). Although IgM has 5-mer structure compared to IgG monomer, seropositivity at one-time point is mainly influenced by the time of sampling after exposure/infection and the half-life of IgM and IgG when appropriate cutoffs are applied for IgM and IgG respectively. In the current study, profiling singular IgM and IgG positivity among specimens from travelers returning from malaria endemic areas were explored. The results showed that an IgM response to different peptide antigens can be detected (Fig. 2B), but unlike IgG there was a much more diverse distribution of positivity to each peptide epitope at the individual level (Fig. 3). Previous immunological studies demonstrate that while IgG responses have been shown to linger, there is no indication that IgM has the same lingering half-life (Wipasa et al., 2010). Keeping this knowledge in mind, 4 different and distinct groupings were observed in this study based on the number of IgM positivity combined with IgG positivity in a sample using the criteria of about 50% peptide epitope positivity (Fig. 4A). In addition, the study further explored the patterns of IgM singular responses to 12 *P. falciparum* peptide epitopes representing three stages of parasite life cycle and mosquito saliva gSG6-p1 peptide in the groups categorized above. It illustrates the variability in IgM expression patterns in relation to different parasite stage peptides along with the gSG6-p1 peptide (Fig. 4B). It is important to point out that although the current study designed for method development is unable to directly link the results from exploratory analysis above to recent exposure due to lack of information regarding the time of infection (see sample source in method section), the unique patterns observed in the travelers indicate the potential of using IgM along with IgG testing and profiling singular IgM response to different stage peptides for determining recent malaria exposure. Further investigation using a large sample size from US travelers with known malaria exposure history, symptom onset date and time of infection is warranted to demonstrate the utility of profiling singular IgM along with IgG response to peptide epitopes as an indicator of recent malaria exposure. Currently, large ongoing studies are also being conducted by us to utilize the all peptide bead-based multiplexing Luminex assay for singular IgG and IgM response detection to help in assessing recent exposure and transmission in both low and high malaria transmission areas.

5. Conclusions

The new peptide coupling method developed in this study resolves the peptide-bead coupling challenge, is reproducible, and can be applied to any other difficult small immunogenic peptide epitopes. In the future this method and the resulting all peptide-bead based multiplexing Luminex assay can be expanded to include other peptide epitopes of *P. falciparum*, different malaria species or other diseases for surveillance, either in travelers in non-malaria settings or in endemic areas.

Authors contributions

WBS designed and conducted the experiments,performed the data analysis and drafted the manuscript. SP, MMA, WJ, SP, and PJ investigated chemistry of peptides, designed the additional tag for peptide epitopes and synthesized the peptides. MMK participated in invesigation and conducted experiments at earlier stage of the study. MK was responsible for domestic malaria surveillance investigation and EE, RH, and QY conducted lab testing for domestic malaria surveillance investigation, and retrieved and sorted the historical samples from travelers. SYP conceived, designed, and supervised the current study, and edited and finalized the manuscript. KS and SYP served as co-PIs for the malaria immunology study conducted in western Kenya from which samples were used for this study. All authors reviewed the draft manuscript, provided comments and suggestions, and approved of the final manuscript.

Ethics approval and consent to participate

The samples for assay development from western Kenya were approved for serological tests by the Ethics Review Committee of the Kenya Medical Research Institute and the Institutional Review Board of the CDC. Traveler samples were obtained from routine domestic malaria surveillance which was approved for serological testing by the Institutional Review Board of the CDC.

Availability of data and material

All data is available upon reasonable request.

Funding

Funding for this project was provided by the Malaria branch within the Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention.

Consent for Publication

All authors approve of final submission of this manuscript.

Declarations

The authors declare that they have no competing financial interests.

Declaration of Competing Interest

The authors declare they have no competing interests.

Acknowledgements

B.S. Wakeman was supported by WILLIAMS CONSULTING LLC, Baltimore, Maryland, USA. We thank Malaria Branch, Division of Parasitic Diseases and Malaria (DPDM), Center for Global Health (CGH), Center for Disease Control and Prevention (CDC), USA for providing funding support to this study. Peptide synthesis was supported by CDC LaSSI #201706. We also thank Henry Bishop DPDM, CGH, CDC for comments and suggestions on this manuscript. The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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