

## Development of a Specimen-Sparing Multichannel Bead Assay to Detect Antiparasite IgG4 for the Diagnosis of *Schistosoma* and *Wuchereria* Infections on the Coast of Kenya

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**Abstract.** To better delineate the impact of parasitic coinfection in coastal Kenya, we developed a novel specimen-sparing bead assay using multiplex flow immunoassay (MFI) technology to simultaneously measure serum or plasma immunoglobulin G4 (IgG4) against *Brugia malayi* antigen (BMA) and *Schistosoma haematobium* soluble worm antigen (SWAP). Properties of the bead assay were estimated by latent class analysis using data from *S. haematobium* egg counts/filarial rapid diagnostic cards (RDTs), parasite-specific enzyme-linked immunosorbent assays (ELISAs), and the multichannel IgG4 assay. For schistosomiasis, the bead assay had an estimated sensitivity of 81% and a specificity of 45%, and it was more sensitive than ELISA or urine egg counts for diagnosing infection. For filariasis, it had a sensitivity of 86% and a specificity of 39%, and it was more sensitive than ELISA or RDT. Measuring antibody by MFI is feasible and may provide more accurate epidemiological information than current parasitological tests, especially in the setting of low-intensity infections.

### INTRODUCTION

The impact of parasitic infections on the health of low-income countries has been regularly underestimated. This underestimation is, at least in part, because of a lack of fully sensitive diagnostic testing for helminth infections, particularly when infections are of low intensity.<sup>1</sup> Misclassification bias caused by inaccurate gold standard laboratory diagnostics has resulted in an underappreciation of the effects of chronic helminth infections in early childhood, especially in terms of such subtle morbidities as growth retardation, wasting, and anemia. New interest has developed in the disabling effects of multiple concurrent parasitic infections—polyparasitism—including the possible deleterious interaction of mixed protozoan and helminthic infections on human development.<sup>2–6</sup>

On the Kenyan coast, coinfection by parasites is common, where up to 55% of rural survey participants have had two or more concurrent parasite infections detected.<sup>7</sup> This finding has been confirmed in other endemic regions,<sup>2–5,7</sup> and in some settings, the detrimental effects of combined parasite infections have been shown to be synergistic as opposed to merely additive.<sup>6,8,9</sup> The study of the health complications of concurrent parasitic infections calls for tools that are able to accurately characterize infection status while efficiently screening for multiple parasites. In areas of low prevalence, sensitive serological testing can help increase the efficiency of detection for these infections, because traditional methods, such as egg counting for schistosomiasis, often miss infections when helminth intensity is low.<sup>10,11</sup> Improved testing can, therefore, allow for more accurate patterning of infection prevalence, which can then be used in the development of more effective control programs.<sup>10</sup>

Multiplex flow immunoassay (MFI) technology is a relatively recent innovation that has been used for polymerase chain reaction (PCR) readouts, serological testing, cytokine

detection, and other biological assays.<sup>12</sup> MFI uses a liquid suspension array of unique microspheres (5–6 μm in diameter) that are conjugated to different capture molecules. These beads are internally labeled with a fluorescent dye and confer a unique fluorescence when a secondary reporter molecule binds to the antigen–bead combination. In serological testing for antiparasite antibodies, a mixture of differently colored beads (each bead conjugated to a different parasite antigen) is combined with a minimal aliquot of patient serum followed by the addition of a fluorescently labeled (phycoerythrin [PE]) reporter secondary antibody. Specific antibody binding response to each test antigen is then detected (according to bead color) and quantified in a flow-based detector.<sup>12</sup> The platform has been used successfully for detection of viral infections (such as Epstein Barr virus [EBV] and herpes simplex virus [HSV]), bacterial infections, and malaria.<sup>13–18</sup> There has been limited experience with helminth infections, although MFI technology has been used in a study on the risk of reinfection of *Schistosoma japonicum*.<sup>19</sup> Most importantly, in these studies, the MFIs have been shown to be comparable with more traditional immunoassays in detecting antibodies to various infections.<sup>13,14,17,18</sup> Although the technology remains complex and not readily available for field use in diagnosing infection, it could be very useful in research and epidemiologic surveys, given the ability to test for many infections simultaneously.

During parasitic infections or allergic conditions, immunoglobulin G4 (IgG4) can account for more than 50% of IgG, making it a good candidate target for a multiplex helminthic infection diagnostic platform.<sup>20,21</sup> Furthermore, studies of human isotypic antibody responses to *S. haematobium* and *Wuchereria bancrofti* infections have shown that IgG4 levels are quite sensitive and specific for detecting current or very recent infections.<sup>22–24</sup>

Immune responses to *Schistosoma* species are well-known to vary by age, although evidence shows that these result may be more from cumulative exposure in older individuals than strict age variations in immune response.<sup>25</sup> IgG4 to *Schistosoma* antigens has been shown to drop in the weeks to months after treatment, making it a good candidate to differentiate active infection from a prior treated infection.<sup>26–28</sup>

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For lymphatic filariasis (LF), antibody responses occur before antigenemia and thus, prove to be useful for detecting early infection.<sup>29</sup> Furthermore, IgG4 to LF parasites has been shown to be elevated in active infections and decline after treatment.<sup>21,30–32</sup>

The present paper describes the development and preliminary testing of a multichannel fluorescent IgG4 antibody detection assay for the study of LF and urogenital schistosomiasis among resident populations in Coast Province, Kenya.

## METHODS

**Study population.** Individuals from the three villages of Mililani, Nganja, and Vuga in Kwale County, Coast Province, Kenya were surveyed as part of a population-based, longitudinal research project.<sup>33</sup> Subjects were eligible if they were over 5 years old, local residents for more than 2 years, and able to provide urine, stool, and blood specimens. Overall, 2,501 participants were enrolled in the three villages. Blood (1 mL) was collected by finger prick or heel stick, with small aliquots taken for hemoglobin determination and rapid card testing for filaria antigen (Binax, Portland, ME). Plasma was separated, stored in labeled Eppendorf tubes, and kept at  $-80^{\circ}\text{C}$  until processed. Urine and stool were collected by participants and brought for evaluation by standard egg detection parasitological testing on site.<sup>34–36</sup> Presence of *S. haematobium* infection was screened for by Nuclepore filtration of two 5-mL aliquots of a single midday urine specimen. Demographic data collected in the field were entered in handheld devices (Dell Axim, Round Rock, TX) using Visual CE 10 (Cambridge, MA). Data were then transferred into ACCESS 2007 (Microsoft, Seattle, WA). Parasitology and anthropometric data were then entered to complete the database.

**Target antigen selection for novel assay.** Seroresponse with IgG4 to *Brugia malayi* antigen (BMA) was used as the marker for filarial infection, because this antigen is preserved across species and can be used to detect exposure to *W. bancrofti*, the filarial worm endemic to Kenya.<sup>37,38</sup> The antigen preparation to detect *S. haematobium* infection was soluble adult worm antigen (SWAP), which has been used frequently in diagnostic enzyme-linked immunosorbent assays (ELISAs) and can be readily prepared.<sup>39</sup> In past studies, sensitivity for diagnosing *Schistosoma* infection using SWAP (by ELISA for total IgG) has been shown to be comparable with sensitivity using soluble egg antigens.<sup>39,40</sup>

**Multichanneled fluorescent antibody detection assay.** SWAP antigen (crude extract) was provided courtesy of the laboratory of Christopher King (Center for Global Health and Diseases, Case Western Reserve University, Cleveland, OH). The crude extract for BMA was prepared as described below, with male and female worms obtained through the Filariasis Research Reagent Resource Center (<http://www.filariasiscenter.org/>).

BMA extract was prepared from live adult *B. malayi* worms (30 females and 30 males). They were washed five times in  $1 \times$  phosphate-buffered saline (PBS). The wash buffer was removed, and the worms were transferred to autoclaved glass mortars, where they were manually homogenized on ice until there was no intact tissue visible and the homogenate was milky in appearance. The preparation was then sonicated on an ice bath for 10 minutes and left overnight at  $4^{\circ}\text{C}$  on a rotor. After centrifuging the mixture at 3,000 rpm for 20 minutes,

the supernatant was removed and transferred to microfuge tubes. This solution was then centrifuged for 30 minutes at 14,000 rpm at  $4^{\circ}\text{C}$ , and the supernatant was removed.

IgG4 antibody responses to SWAP and BMA were assessed using a high-throughput bead-based platform (Bioplex; Biorad, Hercules, CA), allowing the simultaneous detection of the panel of antigens with a single serum sample;  $50 \mu\text{g}$  SWAP or  $1 \mu\text{g}$  BMA was covalently coupled to differently colored beads (bead 52 for SWAP and bead 53 for BMA), with each bead having a unique dye that allowed for automated discrimination of fluorescence values in the multiplex assay using  $6.125 \times 10^5$  beads per sample. Pooled plasma samples from areas endemic for schistosomiasis, filariasis, and hookworm were initially used to optimize the working dilutions of sample and secondary antibodies in the assay. Sera from non-exposed North American donors were used as negative controls in the development of the assay. The above protein content of each antigen coupled to the beads was chosen as the lowest concentration that produced the maximal fluorescence with positive sera. Furthermore, because the use of crude antigen extracts could predispose beads to aggregate, the lowest effective antigen-bead concentrations were necessary to minimize any aggregation that would interfere with accurate measurement of the fluorescence. Beads were lyophilized for storage after conjugation.

In the standardized assay, patient sera and control sera were diluted in the assay buffer of PBS with 1% bovine serum albumin (BSA) plus 0.05% Tween 20 (PBT) at 1:50 and incubated with beads for 30 minutes while shaking at room temperature. Plates were then washed two times with  $100 \mu\text{L}$  assay buffer followed by incubating with  $100 \mu\text{L}$  1:100 R-phycoerythrin (RPE) -conjugated anti-human IgG4 (Southern Biotech) for 30 minutes at room temperature with shaking. Beads were then washed two times with  $100 \mu\text{L}$  assay buffer, resuspended in  $100 \mu\text{L}$  assay buffer, and passed through the Bioplex analyzer to detect specific antibody binding. Individual beads were counted and scored for type (by fluorescence color) and PE fluorescence. The number and intensity of PE-positive beads for each color bead were used to determine the presence or absence of antigen-specific IgG4 in the patient sample. Cutoff values for all of the samples were set at 2 SDs above the mean fluorescent reading of 20 samples run with control sera from an area endemic for multiple species of parasites but not endemic for schistosomiasis or filariasis (courtesy of Christopher King Laboratory). This process minimized potential false positivity of the assay from any low-level cross-reactivity to non-study parasites.

**Protocols for comparator anti-SWAP and anti-BMA ELISAs.** A subset of 111 subjects was tested by ELISA. They were chosen from all three villages in group-wise fashion from three strata that had low, medium, and high fluorescence on the multiplex assay to evaluate all levels of fluorescence; 96-well plates were coated with  $50 \mu\text{L}$   $10 \mu\text{g/mL}$  SWAP or BMA diluted in ELISA coating buffer and allowed to incubate at  $4^{\circ}\text{C}$  overnight. The plates were then blocked with ELISA blocking buffer and washed with ELISA wash buffer as described previously.<sup>41,42</sup> Serum samples prepared in ELISA diluent buffer at a dilution of 1:50 were then allowed to incubate on the antigen-coated plate at  $4^{\circ}\text{C}$  overnight.<sup>41</sup> The plates were washed six times and incubated with  $50 \mu\text{L}$  1:2,000 dilution mouse anti-human IgG4 (Jackson ImmunoResearch, West Grove, PA) for 2 hours. The plates

were washed and incubated with 50 µL 1:1,000 dilution goat anti-mouse alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour. The plates were washed and incubated with alkaline phosphatase substrate in buffer (Sigma Aldrich, St. Louis, MO) for 15 minutes. After 15 minutes, the reaction was stopped with 50 µL 5% (ethylenedinitrilo)tetraacetic acid (EDTA). Bound IgG4 was then measured by determination of optical density at 415 nm. Like the multiplex assay, the cutoff for positivity was set at 2 SDs above the mean optical density of 20 negative controls from an area in northern Kenya, an area endemic for multiple species of parasites but not endemic for schistosomiasis or filariasis (courtesy of Christopher King Laboratory). This cutoff value was set at the outset and not repeated with each plate.

**Statistical analysis.** Initial statistical analysis was performed with SPSS (v.20; IBM Corp, Armonk, NY.), including linear correlation of bead assay results with the respective ELISA tests. Because of the known insensitivity of standard parasitological testing, the sensitivity and specificity of the multiplex assay for parasite diagnosis were estimated using latent class analysis based on the data from all completed tests with the use of Bayes Latent Class Models software (BLCM software<sup>43</sup>; available at <http://www.med.mcgill.ca/epidemiology/Joseph/Bayesian-Software-Diagnostic-Testing.html>). In the modeling of *S. haematobium* infection, the *a priori* specificity of egg detection was set high (at 95–99%) based on very low expected false positivity rates for this test. For other estimates, including all of the filarial testing, the initial inputs specified a uniform distribution for Gibbs sampling across a range of possible values from 50% to 99% for sensitivity or specificity. All models tested converged to consistent values within 10,000 iterations, independent of initial values entered.

**Ethical considerations.** Approval for this study was obtained from the Institutional Review Boards for Human Studies at Case Western Reserve University and Emory University. Approval was also obtained from the Kenya Medical Research Institute National Ethical Review Committee. Written informed consent was obtained from adult participants and the parents or legal guardians of participating children.

## RESULTS

**Study population.** Of 2,525 people canvassed and consented in three villages in coastal Kenya, 24 people were excluded

because of improper data labeling or missing data, yielding 2,501 participants in the parent study. There were 721 participants from Mililani, 1,262 participants from Vuga, and 518 participants from Nganja. Age ranged from 1 to 90 years, with a median of 17 years of age and an interquartile range of 14 years old. Based on initial screening (urine filtration of a single urine and rapid diagnostic card test [RDT] card for circulating filarial antigen), study villages showed some variation of prevalence of filariasis and schistosomiasis: Nganja with 41.1% *S. haematobium* infection and 9.5% filaria infection, Vuga with 19.5% *Schistosoma* and 15.2% filaria, and Mililani with 43.7% *Schistosoma* and 13.0% filaria. Table 1 summarizes the baseline characteristics of the total study population as well as the subset of subjects who had triple testing (MFI, ELISA, and egg count or RDT) done. The prevalence of the different parasitic infections tested is also described. There were no apparent differences in the sex and age distribution of the subset compared with the overall group. Furthermore, there did not seem to be an overrepresentation of one age group over another age group ( $N = 1,051$  ages 0–15 years,  $N = 691$  ages 16–30 years, and  $N = 758$  over 30 years). In terms of the village representation in the triple test subset (not shown), the subjects sampled were distributed evenly among the three study villages.

**Multiplex assay performance versus ELISA and standard urine filtration or filaria RDT.** Antiparasite IgG4 values were determined for all subjects using the multiplex bead assay. After completion of this testing, the results of the bead assay were further compared with ELISA results among a selected subsample of 111 subjects. Tables 2 and 3 summarize the positive and negative scoring of the three individual tests (MFI, ELISA, and filtration or card test) for each parasite for 111 persons in the test comparison group. Table 4 shows the IgG4 positivity rates by MFI broken down by age in those participants who were positive for schistosomiasis and filariasis by traditional methods (egg count and MDT card, respectively). For *Schistosoma* testing, this rate was relatively uniform across age groups. For IgG4 to BMA, this rate of IgG4 positivity seemed to increase with age in RDT-positive individuals.

Overall, for *Schistosoma* diagnosis, 35 of 111 (32%) individuals in the test group were egg-positive, 51 of 111 (46%) individuals were anti-SWAP IgG4 ELISA-positive, and 76 of 111 (68%) individuals were anti-SWAP multiplex bead assay-positive (Table 2). There was strong concordance for positive ELISA and SWAP multiplex bead assay results; among

TABLE 1  
Study demography and infection prevalence: subset that had ELISA testing versus all study participants

|   | ELISA subset |           |           |                | Study group |              |              |                |
|---|--------------|-----------|-----------|----------------|-------------|--------------|--------------|----------------|
|   | Total        | Male      | Female    | <i>P</i> value | Total       | Male         | Female       | <i>P</i> value |
| Count   | 111          | 53 (47.7) | 58 (52.3) |                | 2,501       | 1,137 (45.5) | 1,364 (54.5) |                |
| Mean age (years)                                  | 30.85        | 30.30     | 31.34     | 0.786          | 25.19       | 23.78        | 26.38        | 0.000          |
| Schistosome positivity by urine egg count         | 31.5%        | 34.0%     | 29.3%     | 0.534          | 30.9%       | 32.7%        | 29.5%        | 0.085          |
| Anti-SWAP IgG4 positivity by assay                | 68.5%        | 73.6%     | 63.8%     | 0.379          | 64.1%       | 67.5%        | 61.1%        | 0.001          |
| Anti-SWAP IgG4 positivity by ELISA                | 46.8%        | 45.3%     | 48.3%     | 0.744          |             |              |              |                |
| Filariasis positivity by circulating antigen card | 16.2%        | 9.4%      | 22.4%     | 0.014          | 13.4%       | 14.4%        | 12.5%        | 0.240          |
| Anti-BMA IgG4 positivity by assay                 | 67.6%        | 71.7%     | 63.8%     | 0.475          | 30.4%       | 32.7%        | 28.4%        | 0.020          |
| Anti-BMA IgG4 positivity by ELISA                 | 42.3%        | 37.7%     | 46.6%     | 0.307          |             |              |              |                |
| Malaria positivity by card                        | 7.2%         | 7.5%      | 6.9%      | 0.868          | 8.0%        | 9.0%         | 7.1%         | 0.081          |
| Hookworm egg positivity                           | 33.3%        | 28.3%     | 37.9%     | 0.213          | 23.3%       | 26.3%        | 20.7%        | 0.001          |

*P* values compare the sexes, with  $P < 0.05$  considered significant.

TABLE 2

Overall summary of *S. haematobium* diagnostic test results in 111 subjects included in the triple test comparison

| Egg filtration | ELISA for anti-SWAP IgG4 | Bead assay for anti-SWAP IgG4 | Number in group |
|----------------|--------------------------|-------------------------------|-----------------|
| Positive       | Positive                 | Positive                      | 24              |
| Positive       | Positive                 | Negative                      | 0               |
| Positive       | Negative                 | Positive                      | 3               |
| Positive       | Negative                 | Negative                      | 8               |
| Negative       | Positive                 | Positive                      | 26              |
| Negative       | Positive                 | Negative                      | 1               |
| Negative       | Negative                 | Positive                      | 23              |
| Negative       | Negative                 | Negative                      | 26              |

51 SWAP ELISA-positive subjects, 50 (or 98%) subjects were also bead assay-positive. However, 26 of 60 ELISA-negative subjects (43%) were bead assay-positive. Among those subjects with positive urine egg counts, 27 of 35 (77%) subjects were bead assay-positive, whereas 24 of 35 (69%) subjects were ELISA-positive. Among subjects who were positive by urine egg count, any ELISA positivity was always matched by a positive bead assay result. In the study cohort, the SWAP multiplex assay had a negative predictive value of 77% for egg positivity compared with an 82% negative predictive value of the SWAP ELISA. In comparing each assay's quantitative detection of anti-SWAP IgG4, our scatterplot of the results of traditional ELISA to fluorescence detection in the new multiplex assay (Figure 1) indicated a strong linear correlation between the results of the two anti-SWAP IgG4 assays ( $R^2 = 0.644$ ,  $P < 0.01$ ).

For filarial diagnosis, 18 of 111 (16%) individuals in the test comparison group subjects were RDT-positive for circulating adult filarial antigen, 47 of 111 (42%) individuals were anti-BMA IgG4 ELISA-positive, and 75 of 111 (68%) individuals were anti-BMA multiplex bead assay-positive. Concordance between ELISA and bead assay was almost as good for BMA as SWAP—44 of 47 (94%) ELISA positives were bead-positive, but 31 of 75 (41%) bead positives were ELISA-negative. Among RDT card-positive subjects, 16 of 18 (89%) subjects were anti-BMA IgG4 multiplex bead assay-positive, whereas 12 of 18 (67%) subjects were ELISA-positive. Correlation between anti-BMA IgG4 fluorescence on bead assay and the BMA ELISA results was significant but much less strong ( $R^2 = 0.11$ ,  $P < 0.01$ ) (Figure 2) than for the anti-SWAP assays.

Absent a true gold standard for laboratory diagnosing of these helminth infections (*i.e.*, near-perfect sensitivity and

specificity), we used Bayesian latent class analyses for each infection to help determine the most likely diagnostic performance of each individual test for either infection, taking none of them as a gold standard (Figure 3). Results are shown in Tables 5 and 6. For both pathogens (*S. haematobium* and *W. bancrofti*), the bead assay was estimated to have greater sensitivity but less specificity for detection of antiparasite IgG4 than ELISA. By extension, sensitivity for detection of the presence of infection (as represented by the latent class of true infection in latent class analysis models) was better for the bead assay than ELISA or the reference diagnostic (*i.e.*, egg filtration for *S. haematobium* or circulating antigen detection for *W. bancrofti*). As is typical for many diagnostics, increased sensitivity of the bead assay was offset by a decreased specificity in detection of antiparasite IgG4 or the infection state, which was estimated in the latent class analysis.

## DISCUSSION

For epidemiological studies of parasitism, there is a clear need for better diagnostic tools to supplement our traditional parasitological techniques. It is particularly true when it comes to the chronic infections of schistosomiasis and filariasis. In our study, both the SWAP and BMA components of the multiplex bead assay performed well (compared with traditional ELISAs) in the detection of circulating antiparasite IgG4, which is taken to be a marker of current or recent infection.<sup>22–24</sup> Increased levels of anti-BMA IgG4 levels have been correlated with higher levels of circulating microfilaria,<sup>31</sup> and there is evidence that IgG4 does not cross-react with several other parasitic infections (protozoan and helminths) that are often coendemic with *Wuchereria*.<sup>31</sup> Likewise, antischistosome IgG4 levels have been correlated with infection intensity and seem to peak early in infection.<sup>24,28,44,45</sup> Whereas studies have shown lower IgG4 levels in lighter infections, the correlation between IgG4 positivity and infection status is high (with greater than 90–95% positivity in infected subjects), making it a useful marker for current infection.<sup>26,39,40,46</sup> Treatment effects on antischistosome IgG4 have been varied across studies and can be difficult to interpret given the possibility of reinfection, differences in methodology, and the ideal time post-treatment to test.<sup>45</sup> Grogan and others<sup>28,47</sup> showed a persistence of IgG4 levels against *Schistosoma* adult worm antigens in both adults and children 5 weeks and 2 years after treatment, whereas IgG4 levels against egg antigens declined in all groups at these intervals post-treatment.<sup>28,47</sup> Other studies have supported the decline in IgG4 levels against egg antigens.<sup>26</sup> Several studies have also shown a decrease in IgG4 levels against adult worm antigens months after treatment, despite the fact that other studies did not.<sup>24,27,48</sup> Overall, the data seem to support the use of IgG4 levels against *Schistosoma* antigens as representative of active, untreated infection, regardless of subject age, as long as there has been an ~6- to 12-month window since treatment.

Isotype and subtype antibody profiles after helminth infection differ with patient age,<sup>28</sup> most likely because of an increase in cumulative exposure to helminth infection over time.<sup>44</sup> IgG4 has been found to be correlated with infection intensity (as mentioned above). Therefore, it is likely to peak in the age groups in which infection is most common. Favoring the hypothesis of current exposure-driven generation of IgG4 levels, Naus and others<sup>25</sup> did not find an independent

TABLE 3

Overall summary of *W. bancrofti* diagnostic test results in 111 subjects included in the triple test comparison

| Adult antigen RDT | ELISA for anti-BMA IgG4 | Bead assay for anti-BMA IgG4 | Number in group |
|-------------------|-------------------------|------------------------------|-----------------|
| Positive          | Positive                | Positive                     | 12              |
| Positive          | Positive                | Negative                     | 0               |
| Positive          | Negative                | Positive                     | 4               |
| Positive          | Negative                | Negative                     | 2               |
| Negative          | Positive                | Positive                     | 32              |
| Negative          | Positive                | Negative                     | 3               |
| Negative          | Negative                | Positive                     | 27              |
| Negative          | Negative                | Negative                     | 31              |

TABLE 4  
Total age distribution and MFI assay positivity by age group among RDT-positive and *S. haematobium* egg-positive subjects

| Age groups   | 0–15 years (total <i>N</i> = 1,051) | 16–30 years (total <i>N</i> = 691) | > 30 years (total <i>N</i> = 758) |
|--|-------------------------------------|------------------------------------|-----------------------------------|
| Anti-BMA IgG4 MFI-positive among RDT card-positive                   | 22% ( <i>N</i> = 28)                | 36% ( <i>N</i> = 30)               | 58% ( <i>N</i> = 72)              |
| Anti-SWAP IgG4 MFI-positive among <i>S. haematobium</i> egg-positive | 81% ( <i>N</i> = 351)               | 89% ( <i>N</i> = 217)              | 91% ( <i>N</i> = 87)              |

correlation between age and IgG4 levels in an immigrant population that was exposed to *S. mansoni* for the first time. Again, current infection intensity was related to IgG4 level. In summary, although the level of antiparasite IgG4 tends to peak in younger age groups, it is also found to be elevated in active *Schistosoma* infections among adults.<sup>40,48</sup> In our evaluation, although our sampling strategy and small sample size of the triple test group did not allow for a breakdown of the test characteristic analysis by age, it does not seem that IgG4 positivity differed greatly depending on age in those participants positive for *S. haematobium* infection by egg counting (Table 4). Furthermore, age distribution was fairly uniform across both the ELISA subset and the group as a whole. Therefore, it does not seem that age had an appreciable influence on the results and use of IgG4 as a marker for active/recent infections for schistosomiasis. However, the rate of IgG4 positivity did seem to go up with age (Table 4) in those individuals positive for filaria by RDT; however, this group was overall small, and therefore, it is difficult to make a conclusion from these age-stratified values.

Although cross-reactivity between schistosome species and other helminths has been documented, anti-SWAP has been found to be parasite-specific in standard assays.<sup>22,24,25,44</sup> To further reduce cross-reactivity, we calibrated our assays using control sera from an area endemic to multiple helminths but without schistosomiasis and filariasis to define the cutoff values.

MFI fluorescence correlated with ELISA optical density, and because the two bead assays could be run concurrently with much smaller volumes of patient serum, the MFI seems to offer an attractive alternative to ELISA for screening large

populations, especially if infection prevalence declines under the pressure of mass treatment campaigns. Within the limits of our small pilot study patient sample, the SWAP and BMA MFI assays yielded greater sensitivity compared with the corresponding ELISAs, albeit with a decrease in specificity. Latent class analysis was used for estimation of test performance values because of the known insensitivity of standard testing for active infection.<sup>3,49,50</sup> The standard egg filtration test performed on a single daily urine is estimated to miss up to 40% of infections detected by more intensive sampling over 10 consecutive days.<sup>49</sup> Similarly, the rapid diagnostic card for filarial infection detects adult worm antigen but may not reflect the status of microfilaremia or immune activation related to more clinically active filariasis.<sup>50</sup> The lower sensitivity of anti-BMA ELISA could be caused by several factors: early stage infection with a delayed immune response, low-level infection that does not elicit a strong IgG4 immune response, or technical limitations of the assay. The villages surveyed in the present study underwent lymphatic filariasis treatment in the 2 years preceding initiation of the survey. Subjects may have had circulation of adult filarial worm antigens (as detected by the card tests) but reduced IgG4 because of markedly reduced microfilaremia. Thus, additional test evaluations will be needed to define the MFI performance in detection of low-level or occult infections. Furthermore, because this pilot study did not have the numbers to stratify by age or sex, these findings will need to be validated in larger-scale studies.

The lower specificity of the multiplex assay for both SWAP and BMA may have been an artifact of the lower sensitivities of the ELISA tests, because the use of the northern Kenyan

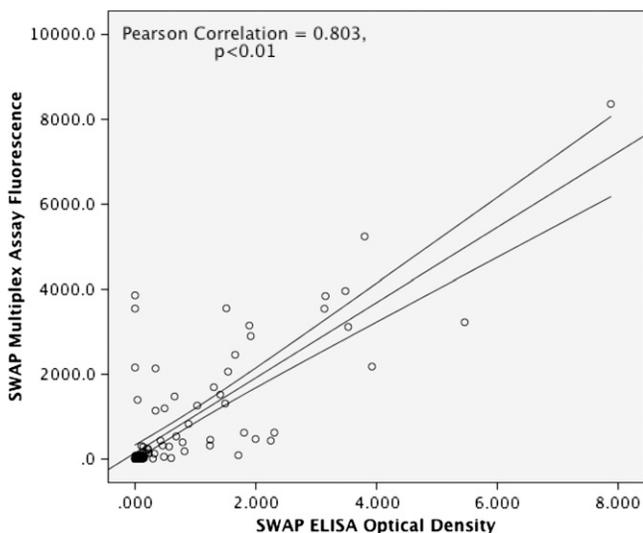


FIGURE 1. Relationship between SWAP IgG4 multiplex assay fluorescence and SWAP IgG4 ELISA optical density.

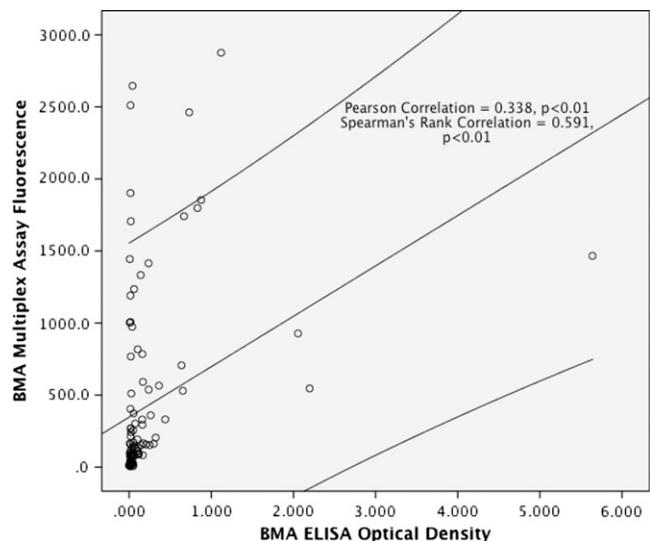


FIGURE 2. Relationship between BMA IgG4 multiplex assay fluorescence and BMA IgG4 ELISA optical density.

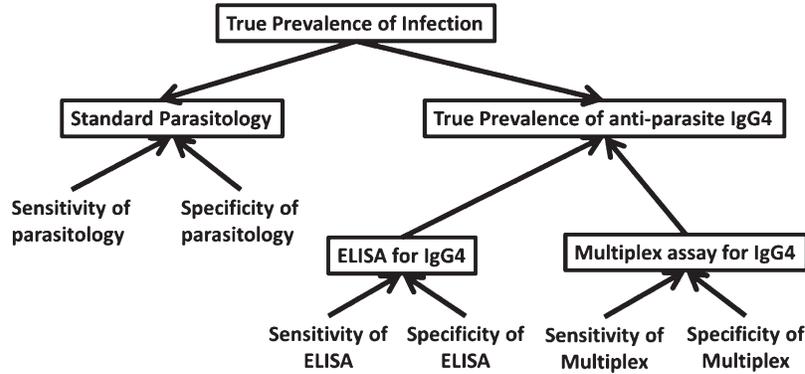


FIGURE 3. Two-class model for estimation of the diagnostic performance of the novel antiparasite IgG4 multiplex assays compared with standard parasitology and classical ELISA technique.

controls (where neither schistosomiasis nor filariasis is endemic) to determine cutoffs should have decreased the cross-reactivity of the assay. Although lower specificity of IgG4 for lymphatic filariasis compared with PCR has been attributed to likely cross-reactivity, the fact that there are no other major filarial parasites endemic to this area makes cross-reactivity less of an issue in our study.<sup>51</sup> Lastly, the subset that had the ELISA testing was similar to the overall group with respect to age (mean age was 30.85 years for the ELISA subgroup and 25.19 years overall) and sex (31.34% female for the ELISA subgroup and 26.38% female overall), which is evidenced in Table 1. Therefore, the makeup of the subpopulation does not seem to have shaped the observed performance characteristics of the tests. Furthermore, although village makeup of the ELISA subset versus the overall group was slightly different (with Vuga having more participants in the overall group and the ELISA group spread evenly over the villages), it is unlikely that this difference had an impact on the performance characteristics of the tests.

One of the potential advantages of the MFI assay is that a very small amount of serum or plasma can be simultaneously tested for antibodies to several parasite infections, thus maximizing efficiency and minimizing the participants' risk from larger blood draws. There are limitations, however, on the use of MFI for antiparasite antibody testing. In our hands, beads prepared using hookworm excretory/secretory antigens proved unable to detect antihookworm IgG4, although the antigens performed well when detecting antihookworm total IgG and when used in standard ELISAs. It is possible that conformational changes created by covalent linkage of the antigen to the beads may have masked its immune epitopes.

However, we believe that, for many other pathogens, conjugated antigen-beads could be added to our multiplex assay for linked serologic diagnosis. Because MFI can be used for measuring levels of other biomarkers, such as cytokines and iron metabolism markers, the possibilities for concurrently assessing the morbidity of parasitic coinfections with one small sample of blood are numerous.

Future studies with MFI should examine the use of soluble egg antigen preparation for detection of IgG4 associated with *S. haematobium* infection, because it has been more consistently associated with declines in antiparasite IgG4 levels post-treatment.<sup>28,47</sup> There are other promising tools for highly sensitive diagnosis of helminth infections, such as the recently reported urine DNA test for *S. haematobium*.<sup>52,53</sup> However, these tests are single-species tests at present, and therefore, the benefits of multiplex MFI techniques in epidemiologic studies remain. Furthermore, because there is not yet a gold standard for diagnosis of *Schistosoma* or lymphatic filariasis infection, improvements in existing means of serologic testing will expand the epidemiological tools for measuring burden of disease in vulnerable populations.

In summary, where concurrent transmission of parasite infections still occurs, sensitive and efficient detection will be required. Although serological testing with IgG4 does have limitations (as outlined above), novel multiplex serological assays could prove to be useful epidemiologic tools for effective mapping and surveillance. The current MFI assay performs in a fashion similar to traditional ELISAs for detection of antiparasite IgG4, but it is able to obtain the information more efficiently (*i.e.*, simultaneously with minimal amounts of serum). Our future work will focus on optimizing

TABLE 5  
Estimated sensitivity and specificity for *S. haematobium* infection by latent class analysis of three laboratory tests

| Diagnostic test      | Sensitivity for anti-SWAP IgG4 detection* |         | Specificity for anti-SWAP IgG4 detection |         | Sensitivity for <i>S. haematobium</i> infection† |         | Specificity for <i>S. haematobium</i> infection |         |
|----------------------|---|---------|--|---------|--|---------|---|---------|
|                      | Median estimate (%)                       | 95% CI‡ | Median estimate (%)                      | 95% CI‡ | Median estimate (%)                              | 95% CI‡ | Median estimate (%)                             | 95% CI‡ |
| Egg filtration       | –   | –       | –  | –       | 62   | 38–95   | 98  | 95–99   |
| ELISA                | 89  | 68–99   | 95                                       | 84–99.7 | 65   | 47–81   | 72  | 55–93   |
| Multiplex bead assay | 97  | 89–99.8 | 61                                       | 46–83   | 81   | 68–90   | 45  | 31–65   |

Values are based on combined results from (1) egg detection in subject's urine, (2) anti-SWAP IgG4 ELISA, and (3) anti-SWAP IgG4 fluorescent bead assay for 111 study participants in Kwale County, Kenya.

\* True prevalence of anti-SWAP IgG4 in the study subsample was estimated by the Bayesian Latent Class model to be 49.8%.

† True prevalence of *S. haematobium* infection in the study subsample was estimated to be 49.5%.

‡ 95% credible interval.

TABLE 6  
Estimated sensitivity and specificity for *W. bancrofti* infection by latent class analysis of three laboratory tests

| Diagnostic test      | Sensitivity for anti-BMA IgG4 detection* |         | Specificity for anti-BMA IgG4 detection |         | Sensitivity for <i>W. bancrofti</i> infection† |         | Specificity for <i>W. bancrofti</i> infection |         |
|----------------------|--|---------|---|---------|--|---------|---|---------|
|                      | Median estimate (%)                      | 95% CI‡ | Median estimate (%)                     | 95% CI‡ | Median estimate (%)                            | 95% CI‡ | Median estimate (%)                           | 95% CI‡ |
| RDT card             | –  | –       | –                                       | –       | 82   | 36–99   | 96  | 87–99   |
| ELISA                | 80                                       | 56–99   | 94                                      | 83–99   | 66   | 43–90   | 62  | 51–77   |
| Multiplex bead assay | 96                                       | 87–99   | 65                                      | 47–96   | 86   | 66–97   | 39  | 29–52   |

Values are based on combined results from (1) circulating antigen detection on rapid diagnostic cards, (2) anti-BMA IgG4 ELISA, and (3) anti-BMA IgG4 fluorescent bead assay for 111 study participants in Kwale County, Kenya.

\* True prevalence of anti-BMA IgG4 in the study subsample was estimated by the Bayesian Latent Class model to be 49.3%.

† True prevalence of *W. bancrofti* infection in the study subsample was estimated to be 17%.

‡ 95% credible interval.

the discrimination of the MFI assay and comparing the mapping of parasitic disease distribution in coastal Kenya by standard testing and this novel multiplex assay.

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