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Development and Characterization of a 13-Plex Binding Assay to Detect *Shigella* Antibodies in Human Samples

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Shigella is the leading bacterial cause of diarrhea worldwide, with increasing levels of antibiotic resistance. The greatest burden is among children aged <5 years in low- and middle-income countries, and efforts are ongoing to develop vaccines against this pathogen. One of the challenges associated with the development of a vaccine against *Shigella* is the need for a multivalent vaccine covering the most prevalent *Shigella* serotypes. Epidemiologic studies to better understand the prevalence of the *Shigella* serotypes and inform vaccination schedules are very useful, with clinical data showing the ability of vaccines to elicit cross-reactive antibodies. Here, we set up a Luminex-based method able to reproducibly measure antibodies specific to 13 *Shigella* antigens in human sera. This method will allow the rapid collection of large amounts of data based on the analysis of serum samples from vaccinated individuals or people naturally exposed to *Shigella*, supporting the development of a vaccine against this disease.

Keywords. antibodies; human serology; multiplex; *Shigella* screening.

Shigella is a gram-negative bacterium that causes shigellosis, a severe and often life-threatening gastrointestinal disease [1]. *Shigella* infections remain a pervasive global health threat, marked by their high infectivity and tendency for pathogenesis, especially in low- and middle-income countries [2]. Furthermore, the rise of antimicrobial resistance makes the disease an even greater global concern [3]. Within the *Shigella* genus, *S sonnei* and *S flexneri* are the primary causative agents of this disease [4, 5]. While the former has only 1 serotype, *S flexneri* is composed of 15 serotypes and subserotypes, each exposing a distinct O-antigen (OAg) on its surface [6]. The OAg represents the outermost component of the lipooligosaccharide (LPS), a key factor of *Shigella*-induced disease [7]. The majority of candidate vaccines under development target the OAg, which has been widely recognized as the main immunogenic target [8–10]. However, other approaches of vaccination against *Shigella* consider conserved proteins, such as invasion plasmid antigen (Ipa) proteins [11]. Determining the prevalence of *Shigella* serotypes in different geographic areas is crucial for understanding the epidemiology of these bacteria [12]; similarly, it is crucial to assess the quantity of vaccine-induced antigen-specific antibodies [13]. Traditionally, methods based on enzyme-linked immunosorbent assay are the most commonly used to detect antibodies specific to these antigens, as they generally require low-tech equipment, offer high throughput, and can be easily automated, although they allow quantification of humoral responses against only 1 antigen at a time [14].

Nonetheless, in recent times, multiplex assays offering the unique ability to concurrently detect and differentiate multiple analytes have emerged [15]. About a decade ago, Kaminsky et al developed a multiplex *Shigella*-specific assay with Ipa proteins and *S sonnei*, *S flexneri* 2a, and *S boydii* LPS [16]. In this study, we present the development of an assay enabling the simultaneous detection of 9 *S flexneri* serotypes-specific OAg, in addition to *S sonnei* OAg and relevant conserved *Shigella* proteins [17]. We assessed assay performance in terms of reproducibility, linearity, sensitivity, and specificity. The assay's design, the selection of pertinent molecular targets, and the assay's potential application within epidemiologic settings, as well as large vaccine trials, are all discussed.

MATERIALS AND METHODS

Antigen Purification

OAgs were extracted and purified from generalized modules for membrane antigens (GMMA) [18]. GMMA mutated not to display OAg chains were produced and used as coating antigens. *Shigella* strains used are listed in Supplementary Table 1. Briefly, *Shigella* mutant strains were grown at 30 °C in liquid Luria-Bertani medium in a rotary shaker for 16 hours.

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For GMMA production, overnight cultures were diluted in high-throughput media complex medium, supplemented with appropriate antibiotics, to a 0.3 OD_{600} (optical density at 600 nm) and grown at 30 °C in a rotary shaker for 8 hours with a liquid to air volume ratio of 1:5. GMMA were purified by ultracentrifugation and resuspended in water.

OAg extraction was performed by acetic acid hydrolysis as previously described [19]. Gel filtration chromatography (HiPrep 16/60 Sephacryl S100 HR column) eluting with phosphate-buffered saline (PBS) buffer at 0.5 mL/min was used to collect OAg populations pure from residual proteins and DNA [20]. The purity of polysaccharides was >99% in terms of protein, as estimated by microbicinchoninic acid assay, and nucleic acid impurities, as estimated by absorbance at 260 nm per a spectrophotometer (PerkinElmer Lambda 25).

Recombinant clones expressing the Ipa of interest were kindly provided by Wendy Picking of the University of Oklahoma [21]. *Escherichia coli* strains BL21(DE3) and BLR harboring the IpaD-HisTagged pET15b and IpaB pACYCDuet/IpgC-HisTagged plasmid constructs were grown at 37 °C in Terrific Broth with appropriate antibiotics. Both recombinant strains were induced at an OD_{600} of 1.0 with 1mM isopropyl-β-d-thiogalactopyranoside, incubated at 37 °C for 18 hours, and harvested for further processing. Clarified supernatants of sonicated cells containing IpaD-HisTagged and IpaB/IpgC-HisTagged complex were loaded into a 5-mL HisTrap high-performance immobilized metal affinity chromatography column for His-tag purification (GE Healthcare) in 10mM Tris-HCl, 150mM NaCl, 30mM imidazole, and pH 7.2. Bound complexes were eluted in the same buffer with 500mM imidazole concentration. Fractions containing IpaD-HisTagged and IpaB/IpgC-HisTagged were pooled and concentrated with buffer exchanged in PBS with Amikon 10k and 30k for the IpaD-HisTagged and IpaB/IpgC-HisTagged complex, respectively. Purified proteins were quantified by microbicinchoninic acid assay, following the manufacturer's instructions, and stored at −80 °C.

OAg Biotinylation

Biotinylation reaction was performed through OAg activation by CDAP chemistry (1-cyano-4-dimethylaminopyridinium tetrafluoro borate) following the protocol of Nappini et al [22]. Polysaccharide activation was performed in the following conditions: 4.4 mg/mL of polysaccharide (1.1 mg/mL for *S sonnei* OAg) in 50mM DABCO buffer at pH 9 with 0.88 mg/mL of CDAP (0.55 mg/mL for *S sonnei* OAg) for 15 minutes at 0 °C in an ice bath. Biotin hydrazide solution (B7639; Sigma-Aldrich) was added at 20 mg/mL in reaction, and the mixture was kept under stirring for 2 hours at room temperature (22 ± 2 °C). Biotin-OAgs were purified through 2 consecutive G25 disposable columns (28918008; Cytiva) against 1M NaCl and water, respectively, to ensure the removal of reactants in excess. Determination of OAg biotinylation level was performed through biotin assay based on the use of the HABA (4-hydroxyazobenzene-2-carboxylic acid)/avidin reagent (H2153-1VL; Sigma-Aldrich), by its change in absorption at 500 nm, due to the displacement of HABA on avidin mediated by biotin.

MagPlex Beads Coupling to Antigen of Interest

To couple MagPlex beads to antigens of interest, 2 strategies were utilized, depending on whether they were proteins or polysaccharides. The proteins were linked to the beads through activation of the beads' carboxyl groups, which rely on N-hydroxysulfosuccinimmide (24500; Thermo Fisher) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (22980; Thermo Fisher) and the subsequent reaction of free ammines in the proteins of interest with these activated groups, following the manufacturer's instructions. The OAgs were first biotinylated and then linked to beads by streptavidin (434301; Thermo Fisher), as coupled to them with the same conditions as for proteins. The most suitable quantity of antigens to couple to each bead utilized in the assay was selected by testing concentrations of antigens from 0.01 to 50 μg per 1.25×10^6 beads. Mice polyclonal sera generated against each antigen or a rabbit polyclonal antibody specific for IpaD and IpaB (MyBioSource) was used to select the optimal condition of each antigen to use in the panel.

Multiplex and Monoplex Protocols

A bead-based assay was run in multiplex format and monoplex format with beads of different regions (Supplementary Table 2). To run the assay in multiplex format, 1.25×10^6 beads of each region were mixed with the same number of others, resulting in a final 1/13 dilution of each region. Therefore, when the assay was run in monoplex format, each region was diluted 1/13 in PBS 0.05% Tween 20 (assay buffer). Human pooled sera were diluted according to condition to test, and 40 μL of diluted sera was incubated with 10 μL of bead mix for 1 hour, in the dark, under shaking at 750 rpm at room temperature. Beads were washed in assay buffer and incubated with 25 μL of 1:100 secondary antibody (R-Phycoerythrin AffiniPure Goat Anti-Human IgG, 109-115-098; Jackson Immunoresearch) for 45 minutes, in the dark, under shaking at 750 rpm at room temperature. The beads were washed and resuspended in PBS. Phycoeritrin signals were acquired by a Bio-Plex 200 (Bio-Rad) instrument and analyzed with Bio-Plex Manager software (Bio-Rad). Signals were subtracted from the background, as considered the median fluorescent intensity (MFI) of the corresponding beads incubated with PBS alone.

Serum Samples

Pooled sera from participants enrolled in 2 clinical trials were used in this study: H03_03TP (NCT03527173; "A Study to Evaluate the Efficacy, Safety and Immunogenicity of a Vaccine Designed to Protect Against Infection With *Shigella*

sonnei in Healthy Adults") and H01_02TP (NCT01229176; "Safety and Immunogenicity of Vi-CRM197 Vaccine Against *S typhi* in Adults, Children, Older Infants and Infants") performed in Pakistan and India, endemic areas for *Shigella*.

Precision

The precision of the method expresses the closeness of agreement among multiple analyses of the same sample tested under the prescribed conditions. Repeatability (intra-assay variation), reproducibility, and intermediate precision (interassay variation) were evaluated. For the monoplex and multiplex methods, samples were run in duplicate, on 3 days by 2 operators. Results were analyzed with Minitab 18 (Minitab Inc) by applying a mixed effects model considering the day as a random factor to determine the repeatability and intermediate precision of the assay, as measured by the coefficient of variation (percentage).

Linearity

To assess linearity, serum samples were prediluted in PBS (neat, 1.5-fold dilution curve for a total of 8 points) before analysis. PBS was chosen as a diluent because the contribution of the matrix in this assay was considered irrelevant, as it was observed to be under the detection limit of the assay, similar to the one obtained by PBS alone. Predilutions were prepared independently. MFI signals were plotted against theoretical signals, as represented by the signal obtained at the first dilution point (neat) divided by the corresponding dilution factor. A regression analysis was performed by Minitab 18.

Specificity

To assess specificity, serum was incubated with different quantities of LPS or recombinant proteins (range, 0.01–10 μg/mL) overnight, in shaking, at 4 °C. PBS was used as negative control. Percentages of depletion were calculated as follows: (MFI of beads incubated with the antigen of interest / MFI of beads depleted with PBS) × 100.

Statistical Analysis

Statistical analyses were performed with Minitab 18 and Prism version 9.5.3 (GraphPad). Analysis of variance with variance component analysis (mixed effect model with random factors) was used to estimate the intermediate precision (defined as the variability among days and operators) and repeatability (defined as the variability under the same operating conditions over a short interval of time) and to evaluate the contributions of the day of analysis and operator to the variability. Regression analysis was used to evaluate linearity.

RESULTS

The aim of the study was to develop a multiplex binding assay to detect the presence of antibodies targeting *Shigella*-specific polysaccharide and protein antigens in serum samples of human or animal origin. We purified and characterized protein antigens and OAg from different *Shigella* serotypes (Supplementary Table 1), derivatized them to enable their reaction with beads, set up and optimized the assay's conditions, and fully characterized the assay in multiplex format with human and animal sera.

Antigen Generation, Characterization, and Coupling to Beads

OAgs were purified from GMMA-producing strains, and identity, purity, and molecular weight were characterized. OAgs from *Shigella* serotypes were biotinylated for coupling to MagPlex beads. Table 1 reports the biotinylation percentages for each purified OAg with its relative molecular weight. Recombinant conserved IpaD and IpaB were produced in *E coli*, purified, and characterized. Also GMMA from OAg– *S sonnei* and *S flexneri* strains were used as antigens to detect antibodies against *Shigella* surface-exposed proteins. Antigen concentration was optimized for each coupling to beads (Supplementary Table 2).

Method Setup

The assay was run in monoplex format for each antigen to screen for positive signal of human pooled samples. Limit of blank for each antigen was determined as the signal derived from incubation of antigen-coated beads with PBS and PE-conjugated antibody in 6 replicates, plus 2.447 times the SD of these measurements. Limit of detection was found by adding the SD of the diluted positive controls to the limit of blank, as reported by Holstein et al [23] (Supplementary Table 3). To assess positivity to selected *Shigella* antigens, human pooled serum from H01_02TP was diluted 1/100, 1/500, 1/2000, and 1/8000. Signals obtained were subtracted from blank values, and 1/500 dilution was selected as the one that allowed the minimal use of the sample to maintain the highest signal on different antigens; therefore, it was selected for

Table 1. Characterization of All Biotinylated OAgs Used as Coating Antigens

OAg-Biotin	Biotin/Repeating Unit, %	Polysaccharide Molecular Weight, kDa
Shigella flexneri		
1b	13	13.5
2a	12	41.5–13.4
3a	11	69.0–15.7
4a	9	17.2
5b	2	74.7–17.8
X	6	66.9–16.4
Y	6	60.0–15.0
6	6	22.9
Shigella sonnei	3	38.6

For each antigen, the percentage of biotinylation per repeating unit and the molecular weight of the polysaccharide are reported.

Abbreviation: OAg, O-antigen.

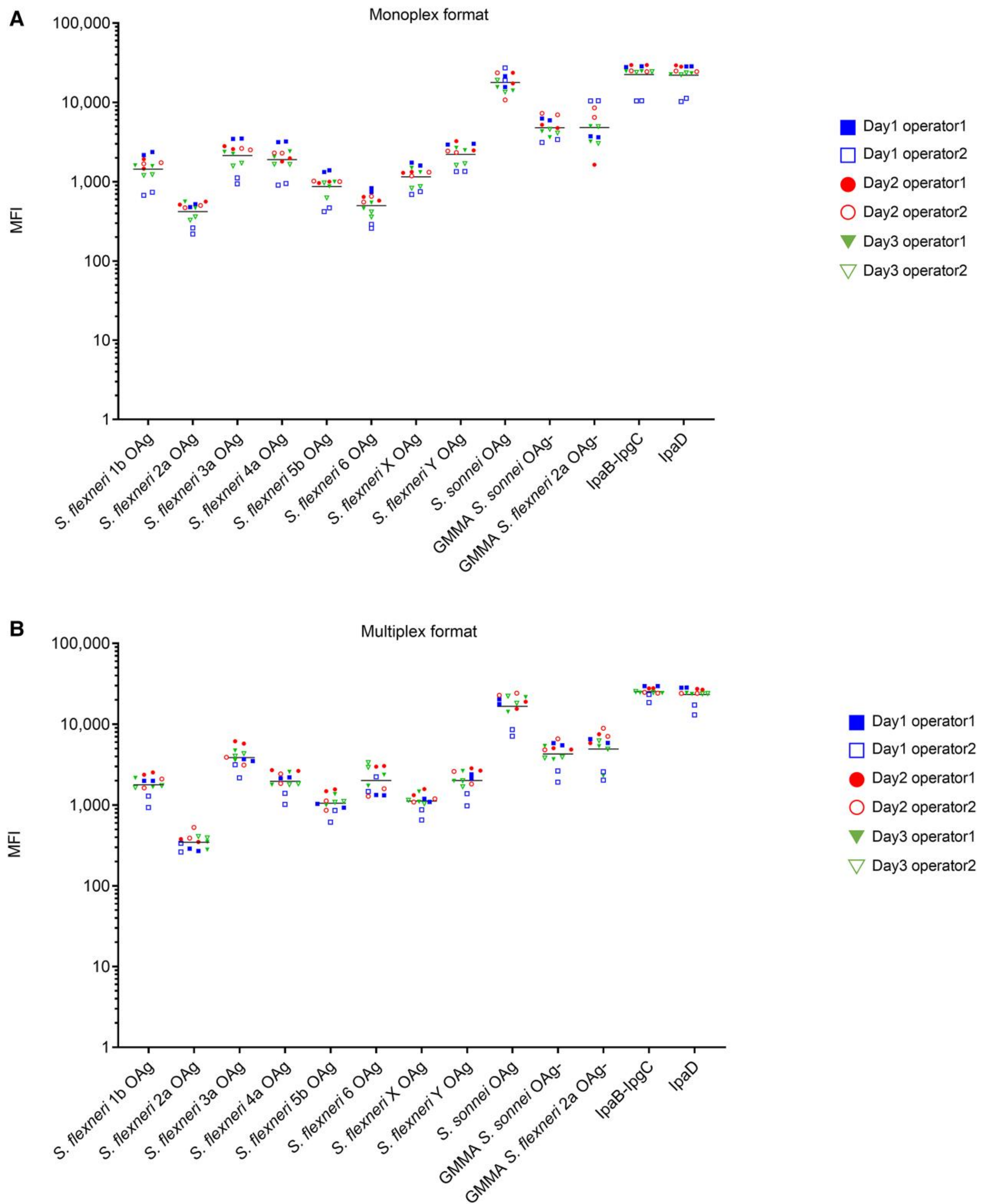

Figure 1. Median fluorescent intensities (MFIs) are reported for 6 independent measures in duplicate, as performed by 2 operators on 3 days at a serum dilution of 1/500: *A*, monoplex format; *B*, multiplex format. Measures performed on the same day by both operators are reported in the same color and with the same symbol; solid symbols are for measures of operator 1 and empty symbols for those of operator 2. The line corresponds to the geometric mean of signals for each antigen. GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; OAg, O-antigen.

Table 2. Repeatability and Intermediate Precision Values for Each Antigen Performed in Monoplex and Multiplex Format

	Monoplex			Multiplex		
		CV, %			CV, %	
	Average	Repeatability	Intermediate Precision	Average	Repeatability	Intermediate Precision
Shigella flexneri OAg						
1b	1532	9.79	*35.09*	1839	12.38	25.36
2a	437	8.71	27.76	353	14.41	21.86
3a	2291	4.52	*37.52*	4010	12.90	28.98
4a	2034	5.69	*37.29*	2031	14.81	26.22
5b	921	11.73	*33.48*	1086	14.30	26.23
6	528	10.04	*34.99*	2137	15.21	*37.91*
X	1203	6.65	29.96	1147	13.39	22.07
Y	2304	9.93	29.63	2096	16.11	28.01
Shigella sonnei OAg	18 392	26.37	26.37	17 622	15.86	*31.95*
GMMA OAg–						
S sonnei	4967	6.26	29.18	4502	16.64	*30.99*
S flexneri 2a	5562	18.94	*58.23*	5431	22.45	*41.08*
IpaB-IpgC	23 649	1.31	28.86	25 363	5.51	12.52
IpaD	23 081	2.64	28.47	23 747	5.36	19.75

Values outside the selected cutoff (CV, 30%) are reported in italics.

Abbreviations: CV, coefficient of variation; GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; OAg, O-antigen.

further experiments. We do not exclude that this assay could operate at higher dilutions, but we suggest making a dilution scan with representative samples from the ones to screen to select the best dilution, before operating at dilutions higher than 1/500.

Monoplex vs Multiplex, Repeatability, and Intermediate Precision

Since one of the main advantages of bead-based immunoassays is to work in multiplex format, results obtained in multiplex vs monoplex format were initially compared. Therefore, pooled human serum H01_02TP at 1:500 dilution was run, and median fluorescence intensity on the beads tested in monoplex (Figure 1*A*) and multiplex (Figure 1*B*) was assessed. The experiment was performed in 3 replicates, on 3 days, by 2 operators running the same plate layout each day. The *t* test between geometric means obtained by the 2 methods against each antigen demonstrated that results in monoplex were significantly lower than in multiplex only for *S flexneri* 3a and 6 OAg (geometric mean ratio 95% CI, .41–.75 and .18–.34, respectively). For all the other antigens tested, no significant differences were observed (Supplementary Table 4).

Repeatability and intermediate precision of the assays were calculated and results are reported in Table 2. Neither days nor operators contributed significantly to the variability of the assay. Background signals were measured by testing human sera depleted of IgG. Signals were inferior to the lower detection limits for all the antigens displayed in the panel (Supplementary Table 3), demonstrating the ability of the assay to be unaffected by a negative matrix.

Linearity

To assess linearity, pooled human serum was tested at different dilutions in a standard assay in multiplex format. A regression analysis was performed on calculated MFI (y-axis) vs theoretical MFI (x-axis) for each antigen (Figure 2). From the analyses of variance, the linear models were significant (lack of fit, $P > .05$) and the residuals of the linear regression models were normally distributed. For each antigen, the intercept (constant in Table 3) was not significantly different from 0 (95% CI ranges included 0), and the slope (T term in Table 3) was not significantly different from 1 (95% CI ranges included 1), with the only exception being *S flexneri* 1b OAg (Table 3).

Specificity

Specificity refers to the ability of the assay to determine solely the concentration of the analyte that it intends to measure. Initially, homologous specificity was measured by depleting the serum with different concentrations of the homologous antigen in comparison with serum not depleted to obtain the minimal concentration that enabled observation of a signal reduction >70%. Depletion of a homologous signal >70% was observed for all antigens. In particular, loss of a homologous signal was >80% for all antigens except IpaB-IpgC (79.5%), with depletion >95% for GMMA *S sonnei* OAg– (98.65%), GMMA *S flexneri* 2a OAg– (96.9%), and recombinantly expressed IpaD (95.45%). Heterologous specificity was assessed by using the same

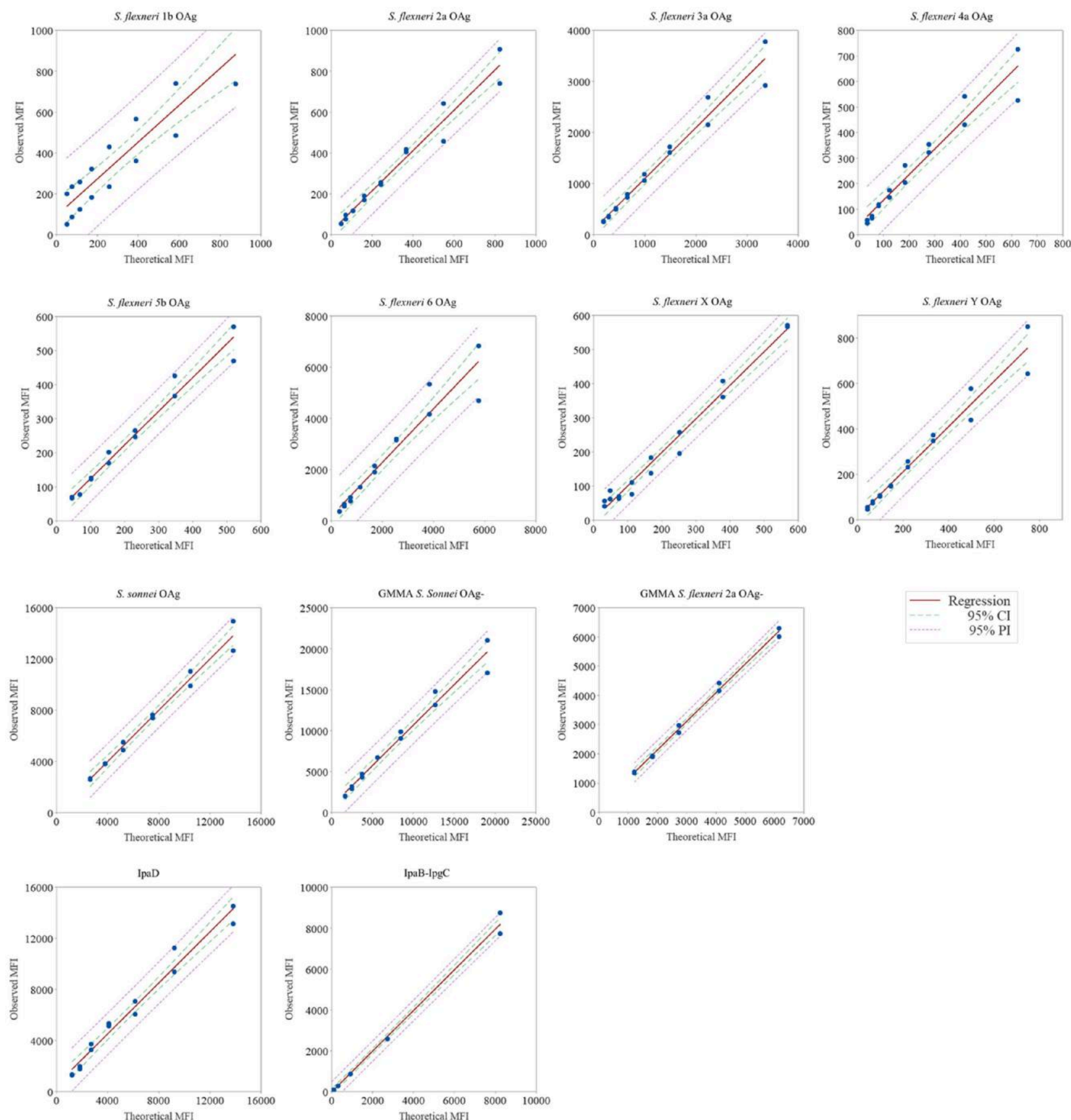

Figure 2. Median fluorescent intensities (MFIs) plotted on the y-axis were experimentally obtained for each antigen in the dilution range selected, as opposed to theoretical ones on the x-axis. Single data points are indicated with blue dots. The solid line represents the linear regression and the internal and external dashed lines the 95% confidence interval (95% CI) and 95% probability interval (95% PI), respectively. GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; OAg, O-antigen.

concentration of antigen for homologous specificity of heterologous antigen, as represented by *S sonnei* LPS for *S flexneri* OAg, *S flexneri* Y LPS for *S sonnei* OAg, *S sonnei* OAg for Ipa proteins, and GMMA OAg– from the different species and *S sonnei* or *S flexneri* Y LPS for beads coupled with *S flexneri* or *S sonnei* OAg– GMMA, respectively (Figure 3).

Depletion with heterologous antigens resulted in a loss of signal that was always <30%, except for *S flexneri* 1b OAg, which gave a loss of signal of about 43.05% when depleted with *S sonnei* LPS. In the case of OAg– GMMA, depletion of the signal after incubation with the GMMA from the other serotype was >30%, in line with similar but not identical compositions in terms of outer

Table 3. Linear Regression Model Performed With All the Antigens Included in the Panel

	MFI Range	T Term	Constants	*P* Value (Lack of Fit)
Shigella flexneri OAg				
1b	51.3–877.0	0.693–1.108	*7.0, 179.3*	>.999
2a	72.3–824.0	0.872–1.099	−27.9, 63.5	.994
3a	196.3–3354.0	0.893–1.104	−66.5, 269.1	.985
4a	36.5–626.0	0.857–1.146	−7.8, 77.9	.874
5b	45.5–521.0	0.881–1.098	−3.1, 54.0	.716
6	337.8–5762.5	0.881–1.218	−278.0, 644.0	.738
X	33.1–569.5	0.906–1.061	−18.9, 23.0	.485
Y	44.0–748.0	0.881–1.109	−27.9, 52.9	.999
Shigella sonnei OAg	2632.5–13 819.5	0.903–1.097	−798.0, 798.0	>.999
GMMA OAg–				
S sonnei	1674.9–19 078.0	0.888–1.088	−176.0, 17.5	.885
S flexneri 2a	1217.0–6161.0	0.925–1.032	−14.8, 378.7	.740
IpaD	1215.0–13 838.0	0.897–1.095	−154.0, 1232.0	.386
IpaB-IpgC	11.0–8254.0	0.948–1.040	−156.3, 146.0	.949

For each antigen, MFI range, slope, intercept, and lack of fit were calculated. Values outside the selected cutoff are reported in italics.

Abbreviations: GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; MFI, median fluorescent intensity; OAg, O-antigen.

membrane proteins of the *Shigella* genus [24]. Apart from the aforementioned *S flexneri* 1b OAg and *S flexneri* 2a OAg, the heterologous inhibition was <20% for all other antigens in the panel and <5% for 5 of these antigens (IpaD, IpaB-IpgC, *S flexneri* 5b OAg, *S flexneri* 4a OAg, and *S flexneri* 3a OAg; Figure 3).

Testing Multiplex Assay Setup for Analysis of Human Sera and Mouse Monoclonal Antibodies

The multiplex assay was tested on pooled human serum from challenged *S sonnei* vaccinees (NCT03527173) and 3 mouse monoclonal antibodies (mAbs) developed by Takis with known specificity (Figure 4) [14].

As expected, the tested mAbs gave positive signals against *S sonnei*, *S flexneri* 2a, and 3a OAgs, respectively (Figure 4*A*). The pooled human serum was positive only on *S sonnei* OAg, IpaB-IpgC protein complex, and *S sonnei* and *S flexneri* GMMA not displaying the OAg (Figure 4*B*). This is in line with the nature of the sample, being serum from a pool of sera from persons vaccinated with *S sonnei* GMMA and challenged with whole bacterium, thereby confirming the suitability of the assay to measure the response to specific vaccine antigens.

DISCUSSION

We set up a method that was able to discriminate antibodies specific to *Shigella* antigens in human sera. The assay can be performed in multiplex format, allowing the simultaneous detection of 13 antigens of protein and polysaccharide nature, with good specificity. This is particularly useful in the context of *Shigella*, which is characterized by >50 disease-causing serotypes with varied prevalence, depending on geographic regions and/or age groups, and for which a multivalent vaccine is recommended. In addition, the target population for a *Shigella* vaccine is children aged <2 years, for which sample availability is limited [25]. Kaminski et al developed a similar multiplex assay with the focus on detection of antibodies specific for *S sonnei*, *S flexneri* 2a, and *S boydii* LPS and IpaB, IpaC, and IpaD recombinant proteins [16]. Here we developed an assay including the same antigens previously described, except for *S boydii*, and 7 additional *S flexneri* OAgs, as well as the GMMA purified from *S sonnei* and *S flexneri* 2a OAg– strains to quantify antibodies specific for membrane proteins of *Shigella*. To our best knowledge, the assay described here has the highest breadth so far in the field of *Shigella*.

The assay will enable determination of antigen-specific antibodies of multicomponent *Shigella* vaccines under development, which in all cases contain as active moieties *S sonnei* and at least 3 *S flexneri* OAgs [13]. This assay will also allow easy and rapid testing of cross-reactivity sera coming from vaccine clinical trials, critical information to be obtained to assess potential vaccine coverage. In addition, the multiplex assay can be applied to the characterization of mAbs to determine their ability to bind *Shigella*-specific antigen and to be cross-reactive. To this extent, the presence of *S sonnei* and *S flexneri* OAgs represents an internal control in our assay, given the diversity of the OAg backbones between these serotypes [26].

It should be noted that the antigen amounts required to perform the assay are very low and permit usage of the same batch of antigen for a long period. To do so, the stability of antigens on beads needs to be checked properly and will be done once the assay is validated. In addition, a very small amount of sample is necessary to perform this assay; in principle, as little as 0.3 μL of serum is sufficient to perform the assay in triplicate at the dilution selected. This is a really important characteristic to consider when thinking about screening of samples deriving from

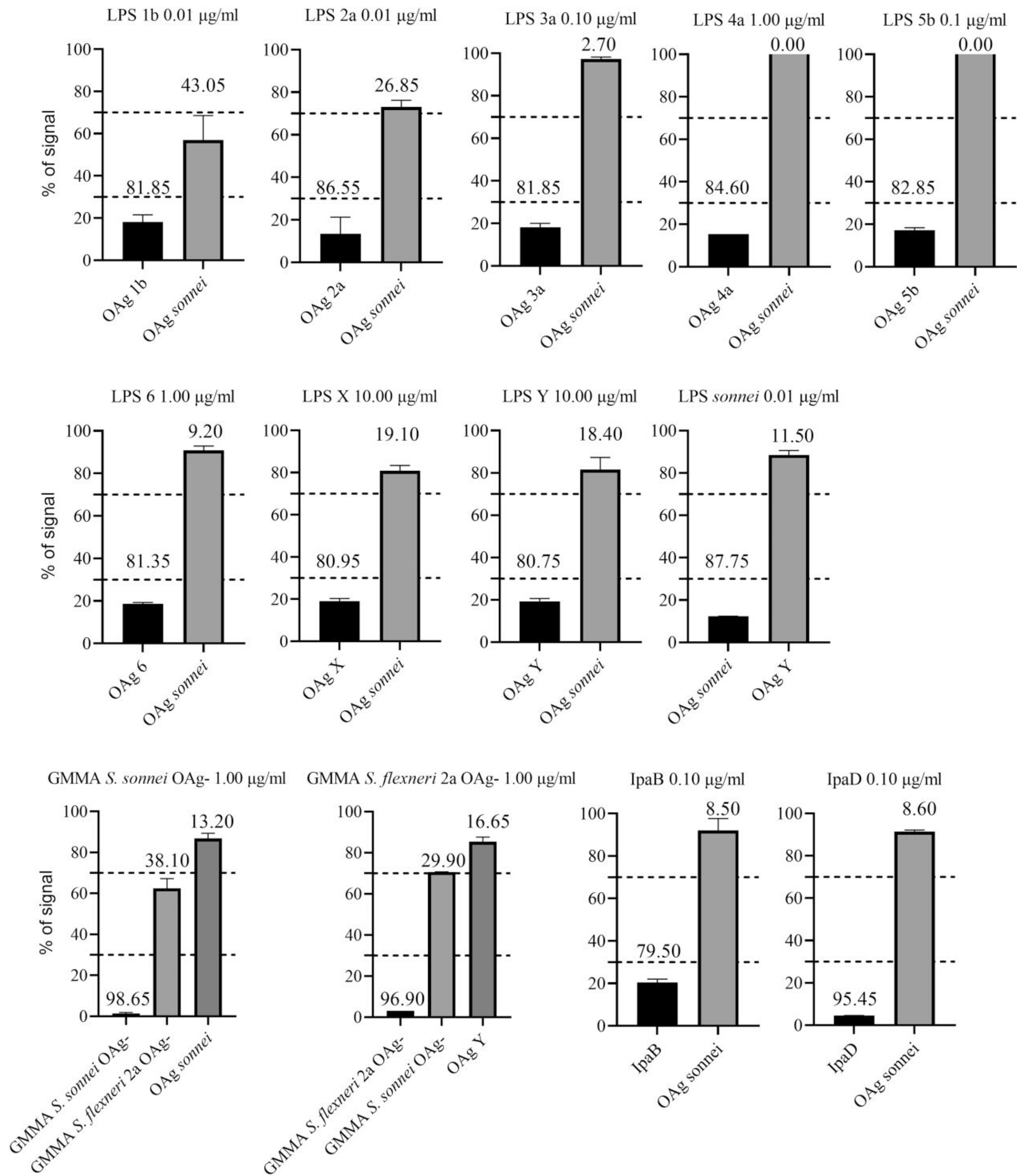

Figure 3. Specificity of signals for each antigen is reported as the percentage of signal decrease on the y-axis; beads were coated with the antigen reported in the x-axis when the human serum was incubated with the antigen indicated on the top of each graph. Dashed lines correspond to the 2 thresholds: the lower to the 30% of inhibition, the upper to 70%. Average of 2 individual percentages of heterologous and homologous depletion are reported on the top of each column. Error bars indicate standard deviation of the measurments. GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; LPS, lipooligosaccharide; OAg, O-antigen.

Shigella-endemic regions or vaccinated participants, as individuals mainly affected by these bacteria are infants and young children.

Reproducibility of the assay was good in terms of repeatability and intermediate precision, with a coefficient of variation <30% for all antigens except for 4. The assay will be further characterized and validated in the near future with the aim to obtain improved reproducibility for all antigens, but results obtained at this stage look promising. In addition, we reported that neither different days nor operators significantly affected the results of the analysis, granting interassay consistency of

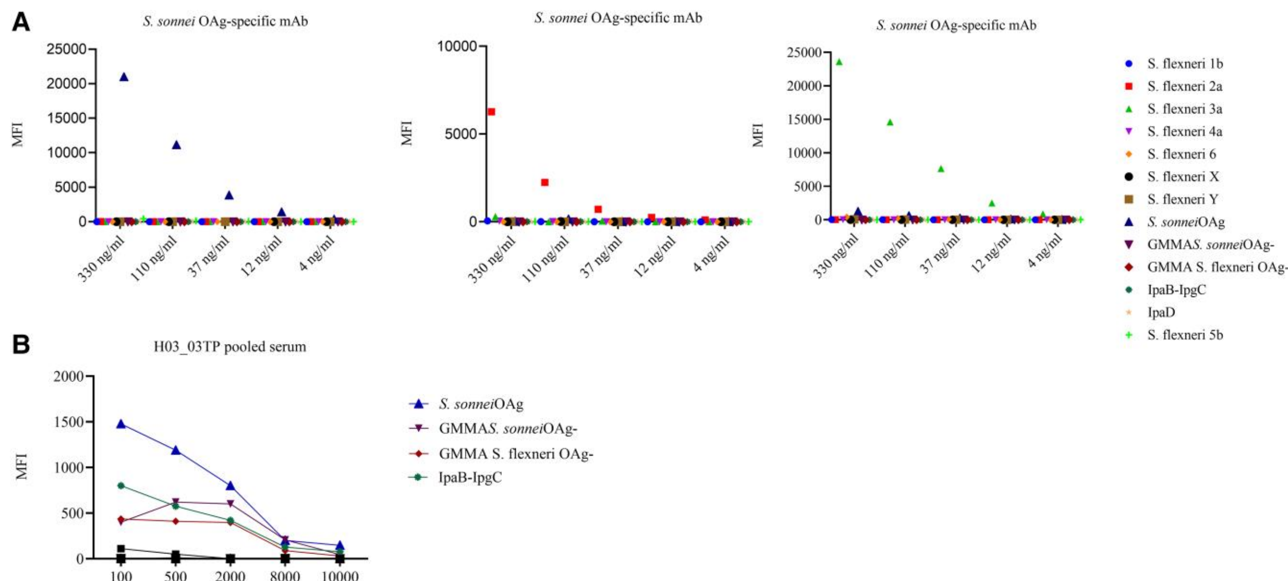

Figure 4. Signals obtained by (*A*) murine monoclonal antibodies (mAbs) and (*B*) H03_03TP pooled sera tested in the panel. Median fluorescent intensities (MFIs) are reported on the y-axis. On the x-axis, either (*A*) the concentration at which each mAb was tested is reported or (*B*) the reciprocal of the serum dilutions utilized. Each symbol represents a signal on the corresponding antigen, as depicted in the legend. GMMA, generalized modules for membrane antigens; Ipa, invasion plasmid antigen; OAg, O-antigen.

measurements. A limitation of our study is the lack of a standard curve. However, the methodology that we presented is really flexible to perform screening of large data sets and, in general, to compare samples qualitatively across visits on the same trials while establishing a proper reference curve. With the reagents available at the moment, it is difficult to obtain a polyclonal curve with antibodies balanced against the different antigens. Also, an internal calibrator can be used only to establish relative units but not absolute values; thus, overall, comparing results at the same dilution will still enable a good comparison of results per se. Besides the fact that efforts are being made, an international reference standard against *Shigella* is not available yet, and only by the implementation of this will a real standardization and bridging of results obtained by different binding assays across laboratories be possible [13].

Taking into account these results, we propose the use of this assay to screen human sera, either from vaccinated participants or infected ones, as well as to investigate the specificity of mAbs. With minimal adjustments, this panel can be used to measure not only antibodies in animal sera, simply by changing the secondary antibody, but also antibody classes and subclasses. By providing a comprehensive and reproducible protocol for concurrent detection and differentiation of antibodies against several *Shigella* serotypes, this research significantly contributes to the diagnostic tools available for *Shigella* infections.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. G. V., O. R., and F. M. took part in conceptualization of the work. G. V. and F. M. took part in data curation and formal analysis. M. M. R., R. N., C. G., and G. G. generate and characterized the proteins and polysaccharides. All authors contributed in drafting the manuscript and agreed to the published version of the manuscript.

Informed consent statement. The studies are registered in ClinicalTrials.gov and were conducted in accordance with all applicable regulatory requirements, the good clinical practice guidelines, and the Declaration of Helsinki. The protocol and study-related documents were approved by the institutional review boards, and all participants provided written informed consent.

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Potential conflicts of interest. G. V., F. M., M. M. R., C. G., R. N., G. G., F. B. S., M. I., F. M., and O. R. are employees of the GSK group of companies. R. R. is an employee of Fondazione Biotecnopolo Siena. F. M., O. R., M. I., and F. B. S. report ownership of GSK shares/share options. GSK Vaccines Institute for Global Health Srl is an affiliate of GlaxoSmithKline Biologicals SA.

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