TITLE

Multiplexed flow cytometric approach for detection of anti-SARS-CoV-2 IgG, IgM and IgA using beads covalently coupled to the nucleocapsid protein

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ABSTRACT

Flow cytometry has emerged as a promising technique for detection of SARS-CoV-2 antibodies. In this study, we described a new methodology to detect simultaneously IgG, IgM and IgA of SARS-CoV-2 nucleocapsid protein in human serum by flow cytometry. The Nucleocapsid protein was covalently bound on functional beads surface applying sulfo-SMCC chemistry. BUV395 anti-IgG, BB515 anti-IgM, biotinylated anti-IgA1/IgA2 and BV421 streptavidin were used as fluorophore conjugated secondary antibodies. Serum and antibodies reaction conditions were optimized for each antibody isotype detection and a multiplexed detection assay was developed. This new cell-free multiplex approach based on flow cytometry was able to efficiently discriminate COVID-19 negative and positive samples. The simultaneous detection of IgG, IgM and IgA showed a sensibility of 88.5-96.2% and specificity of 100%. The combined detection of antibody isotypes offers greater spectrum for detection and monitoring of COVID-19 vaccines and seroconversion. This novel strategy opens a new avenue for flow cytometry-based diagnosis.

Keywords. SARS-CoV-2, COVID-19, IgG, IgM and IgA antibodies, flow cytometry, CBA functional beads, multiplex immunoassay

INTRODUCTION

The first case of the novel coronavirus, SARS-CoV-2, which causes a disease known as COVID-19, was reported in Wuhan, China, on December 31, 2019. The World Health Organization (WHO), on 11 March 2020, declared COVID-19 a pandemic. The course of the COVID-19 pandemic is a consequence of the rapid spread of this virus and, more recently, the emergency of novel variants. The value of diagnostic alternatives in the management of COVID-19 is high[1].

The understanding of immune response to SARS-CoV-2 infection is critical, especially in discrimination of disease severity and vaccine efficacy. Although the antibody consequences of COVID-19 are not fully characterized, is well-known that seroconversion for IgG and IgM occurs typically within 3 weeks, being simultaneously or sequentially, initiating 5 days after symptom onset[2], with a median day of seroconversion of 13 days post symptom onset for both IgG and IgM[3]. The isotype IgA forms have gained attention in COVID-19[4]. The secretory form would primarily act at the virus entry site[5] and the circulating IgA has been revealed as neutralizing antibody and correlated with disease severity[6,7]. IgA seroconversion appears as early as IgG and IgM[8], or slightly early than IgG and IgM[2,9].

These multiple antibody isotypes target viral proteins, including spike and its receptor-binding, and nucleocapsid (or nucleoprotein)[10]. Several studies described the detection of SARS-CoV-2-specific IgG and IgM[3,11–19], while the detection of SARS-CoV-2-specific IgA has been less reported[8,9,13,20–22]. In most cases, antibody detection is based on ELISA or chemiluminescent assays.

Recent studies exploited flow cytometric to develop assays to detect COVID-19 seroconversion in humans. In three studies, the spike protein was overexpressed on the surface of cells, allowing the detection of antibodies in patient samples using fluorescent secondary anti-antibodies[23–25]. In another study, SARS-CoV-2 antigens tagged with biotin were non-covalently bound to beads coated with streptavidin[18]. Here, we report the production of cytometric bead array (CBA) functional beads covalently linked to SARS-CoV-2 nucleocapsid protein which allowed accurate multiplexed detection of IgG, IgM and IgA isotypes using flow cytometry.

MATERIALS AND METHODS

Chemicals and antibodies

The cytometric bead array (CBA) polystyrene beads (cat n° 560037), coupling buffer BD™ (cat n° 51-9004756), storage buffer BD™ (cat n° 51-9004758) and wash buffer BD™ (cat n° 51-9003798) were purchased from BD Biosciences. Bovine serum albumin (BSA, A8022), 4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt - sulfo-SMCC (M6035), N-ethylmaleimide (E3876) and dithiothreitol (DTT, 10197777001) were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS) 10x pH 7.2 (70013-032) was purchased from Gibco. Antibodies brilliant ultraviolet (BUV395) mouse anti-human IgG (cat n° 564229), brilliant blue (BB515) mouse anti-human IgM (cat n° 564622), biotin mouse anti-human IgA1/IgA2 (cat n° 555884) and brilliant violet (BV421) streptavidin (cat n° 563259) were purchased from BD Biosciences.

Antigen preparation and beads conjugation

Expression and purification of recombinant SARS-CoV-2 nucleocapsid protein was performed as described previously[15]. The coupling reaction was performed as described by the manufacturer with modifications. Initially, beads and antigen were prepared. CBA

E5 beads were resuspended by vortex for 30 seconds. Then 75 µL of E5 beads were collected and sonicated for 60 seconds. After that, 1.9 µL of DTT 1 mol/L was added, mixed with vortex and placed on horizontal shaker for 1 hour at room temperature. Then, 1 mL of coupling buffer BD™ was added, mixed, centrifuged at 2000xg for 3 minutes and the supernatant discarded. This washing step was repeated three times. Finally, the CBA beads were resuspended in coupling buffer to next step. In parallel, 90 µg of protein in PBS (1x) was mixed with 2 µL of Sulfo-SMCC 2 mg/mL. The mixture was placed on horizontal shaker for 1 hour at room temperature (25 °C).

The maleimide-activated nucleocapsid protein was transferred to the tube containing the prepared beads. The components were mixed in vortex and incubated under agitation for 1 hour at room temperature. After this period, 2 µL of N-ethylmaleimide 2 mg/mL were added and kept under agitation for 15 minutes at room temperature. Then, 1 mL of storage buffer BD™ was added, mixed, centrifuged at 2000xg for 3 minutes and the supernatant discarded. This washing step was repeated three times. After this, the conjugated beads were resuspended in 500 µL of storage buffer and kept at 4°C. The functionalized beads were stable for, at least, 2 months.

Samples

Human serum and EDTA-plasma were collected at Hospital Erasto Gaertner (HEG), a cancer reference center where both oncological and non-oncological COVID-19 positive patients have been admitted. The samples consisted of 10 pre-pandemic, considered as COVID-19 negative or control, and 26 COVID-19 positive, being 18 from oncologic patients and 8 from non-oncologic patients. COVID-19 positive samples were diagnosed by the detection of SARS-CoV-2 RNA using RT-PCR from nasopharyngeal sample swabs by two independent laboratories. COVID-19 positive samples were collected 14 days after hospitalization, with varied time of the appearance of first symptoms (14 to 31 days). This study was approved by the Ethics Committee of HEG (CEP/HEG: 31592620.4.1001.0098).

Staining and analysis

A suspension of conjugated beads was prepared following the proportion of 1µL of stock suspension conjugated beads to 50 µL of wash buffer BD™. Then, 50 µL of diluted beads were mixed with serum or EDTA-plasma (pure or diluted with PBS 1x containing BSA 0.5%) and incubated at room temperature for 90 minutes. Then, beads were centrifuged at 4000xg for 5 minutes. The supernatant was removed and 300 µL of PBS/BSA 0.5% was added and mixed with vortex followed by centrifugation at 4000xg for 5 minutes (this step was performed twice). After that, 50 µL of diluted antibody was added and incubated for 90 minutes (the antibody range used was 1:100; 1:200, 1:300, 1:400, 1:500, 1:1000, 1:2000, 1:3000 and 1:5000). After this time, beads were centrifuged at 4000xg for 5 minutes and washed twice with 300 µL of PBS/BSA 0.5%. For IgG and IgM detection, the samples were resuspended in PBS and analyzed by flow cytometry using UV450/50 filter for IgG and B530/30 filter for IgM. For IgA analysis, a further incubation was performed with 50 µL of streptavidin 1:100 for 90 minutes at room temperature (25 °C). The samples were centrifuged and washed twice with PBA/BSA 0.5%, resuspended in PBS and analyzed by flow cytometry using V450/50 filter.

For multiplex analysis, diluted beads were incubated with serum 1:1000 for 90 minutes and washed as previously described. Then a single solution was made with IgG (1:100), IgM (1:100) and IgA (1:1000). A volume of 50 µL of antibody mixture was incubated with beads for 90 minutes and washed. After that, the incubation with streptavidin (1:100) was performed for 90 minutes and washed. The beads were resuspended in PBS and analyzed using a BD FACS Celesta™ equipped with 3 lasers (355nm, 405nm and

488nm) using UV450/50, V450/50 and B530/30 filters. The data were expressed as the percentage of positive fluorescent beads (PPFB), as previously described[26]. Statistical analysis based on receiver operating characteristic (ROC) curve was used to determine cutoff, sensibility, specificity and area under curve (AUC) using MedCalc v.7.12.7.2.0 (MedCal Software bvba).

RESULTS

Strategy at a glance

Cytometric bead array (CBA) is called functional beads by the producer and compatible with flow cytometry. These fluorescent beads have been widely used to investigate antigens in serum samples. There are several commercially available beads which are covalently covered by antibodies recognizing specific targets, detection of the analyte using flow cytometry is performed using a fluorochrome-conjugated secondary antibody. Despite this well-stablished approach, the application of these CBA beads to covalently bind antigens and investigate the antibodies is poorly studied[27]. In this work, thiol groups of the commercial fluorescent polystyrene naked CBA beads were reduced to the active sulfhydryl form by DTT. The recombinant 6xHis-tagged SARS-CoV-2 nucleocapsid protein solubilized on PBS was covalently bound to CBA beads by sulfo-SMCC chemistry (Fig. 1A). These functionalized beads were named as CBA-N. CBA-N was further used as proof of concept to investigate the presence of IgG, IgM and IgA isotypes in COVID-19 positive serum samples. To allow antibodies detection, specific anti-human IgG and IgM conjugated with BD Horizon BUV395 and BD Horizon BB515 fluorochromes, respectively, were used. Biotinylated anti-human IgA1/IgA2 and streptavidin conjugated with BD Horizon BV421 was used as a second-step reagent to improve the sensitivity of IgA detection (Fig. 1B).

The prepared CBA-N was homogeneously distributed with a diameter size of 7.5 µm, which allowed recognition by flow cytometry according to the forward (FSC) and side scatter (SSC) parameters and using a specific gate (Fig. 1C). All fluorochromes were rationally chosen to attenuate the spillover of fluorescence in a multiplex system, since each fluorochrome is excited by a different laser. This combination of fluorochromes was optimized for a flow cytometer equipped with at least three lasers, including a 355, 405 and 488 nm lasers. As shown in Fig. 1C, the fluorescence was recorded simultaneously using three different channels.

To achieve the best standardization for multiplexed detection of IgG, IgM and IgA response to SARS-CoV-2 infection, the conditions for each antibody response were optimized.

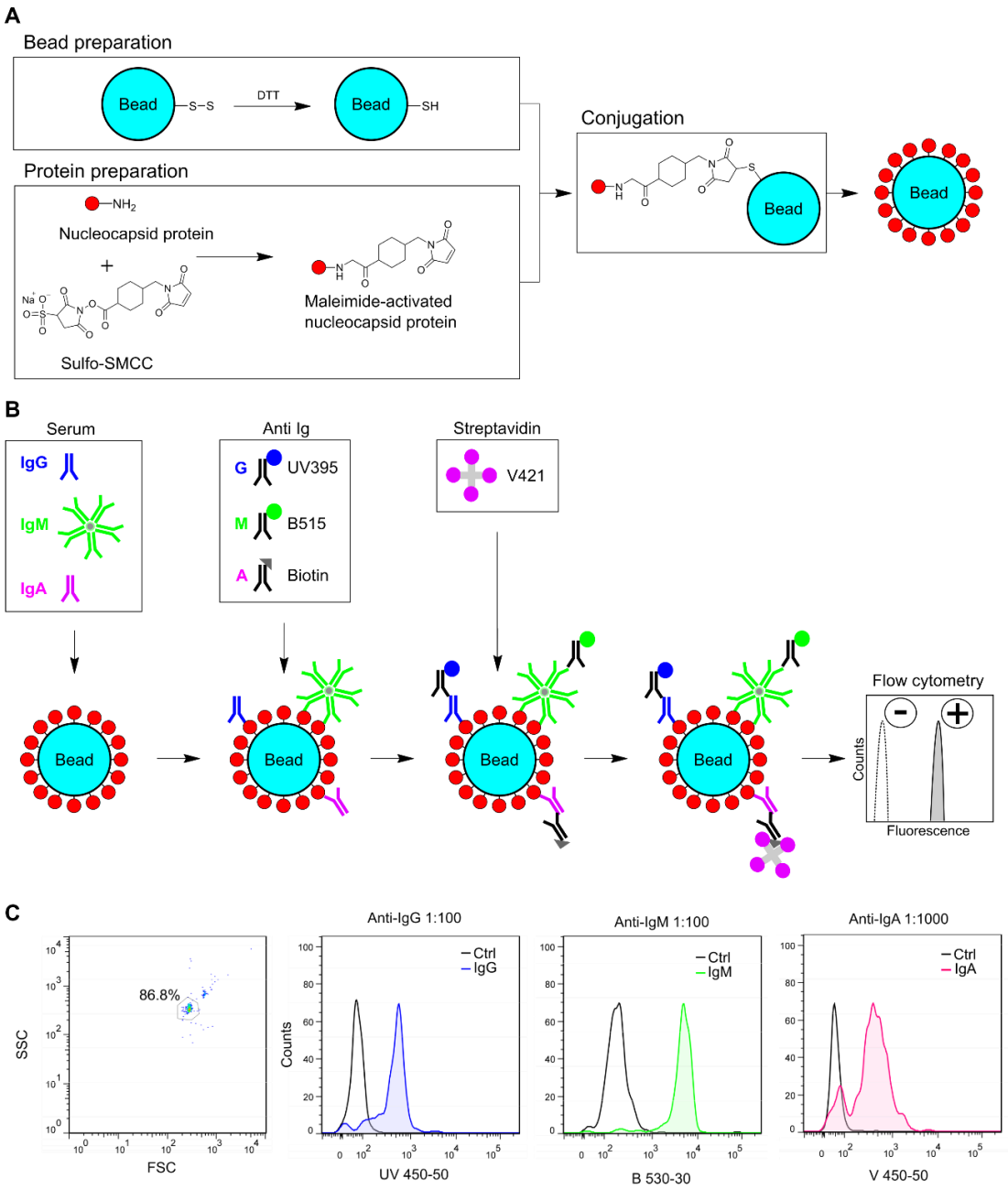

Figure 1: Rational strategy overview. (**A**) Fluorescent polystyrene beads of 7.5 µm reduced by DDT. SARS-CoV-2 nucleocapsid protein solubilized in PBS covalently bound on beads surface by sulfo-SMCC chemistry to originate functionalized beads named as CBA-N. (**B**) Schematic representation of the multiplex assay. (**C**) Gate selection based on bead size. Representative histograms of COVID-19 positive samples.

Optimization of conditions

Negative samples were obtained before the pandemic and were called as control (Ctrl). COVID-19 positive samples were obtained of patients from an oncological hospital (HEG) at Curitiba after 14 days of hospitalization, which correspond to approximately 19 days after the symptom onset (Fig. S1). These patients were diagnosed with COVID-19 by RT-PCR positive results from two independent laboratories (data not shown), and most samples are from oncologic patients. For standardization of antibody response, we used a mixture of, at least three, negative and positive samples.

The data were expressed as the percentage of positive fluorescent beads (PPFB) (Fig. S2). As shown in Fig. 2A, a serum dilution curve revealed that the discrimination of positive *vs* negative IgG samples was achieved at a serum dilution of 1000-fold. After setting the appropriate serum dilution, a range of fluorochrome conjugate (anti-IgG UV395) dilutions were evaluated. The data showed that the best results were obtained using the anti-IgG diluted 100-fold (Fig. 2B). The same rational strategy was carried out for IgM and IgA detection. The IgM detection was only able to discriminate between COVID-19 positive and negative samples when a serum dilution of 1000-fold was applied (Fig. 2C). For IgM detection, the optimized combination was with serum diluted 1000-fold and the anti-IgM B515 diluted 100-fold (Fig. 2C and D). IgA detection showed a different pattern, probably because the biotin-streptavidin system used. As shown in Fig. 2E, the best serum dilutions were between 10 and 100-fold. However, the serum 1000-fold dilution was also able to discriminate between negative and positive samples. Envisioning a multiplexed antibody detection, we decided to use 1000-fold serum dilution for the evaluation of anti-IgA curve. The anti-IgA showed similar results with secondary antibody used at dilution between 100 to 1000-fold (Fig. 2F). In addition, the anti-IgA dilution result was confirmed using the serum 10-fold diluted (Fig. S3).

An estimation of analytical coefficient of variation (CVa) for imprecision (inter-assay; n=12) was calculated using PPFB (%) values from serum pools of controls (mean 2.0±2.0) and COVID-19 positive (IgG, IgM and IgA combined; mean 69.7±19.2) samples, showing a CVa values of 100% and 27.5%, respectively.

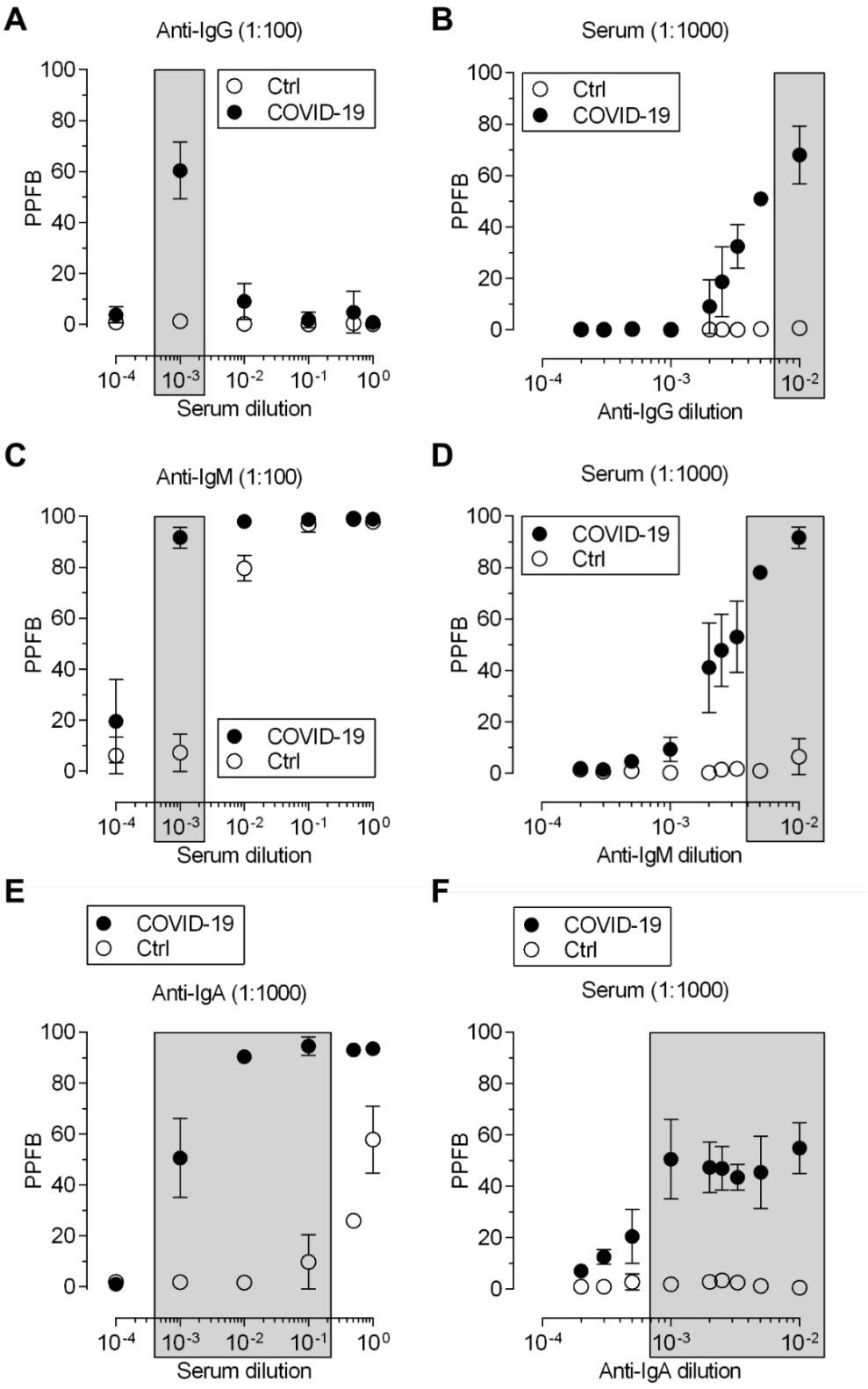

Figure 2: Serum and antibody dilution. Determination of Percentage of Positive Fluorescent Beads (PPFB) by flow cytometry using CBA-N beads. (**A**) Serum dilution using anti-IgG diluted 100-fold. (**B**) Anti-IgG dilution using serum diluted 1000-fold. (**C**) Serum dilution using anti-IgM diluted 100-fold. (**D**) Anti-IgM dilution using serum diluted 1000-fold. (**E**) Serum dilution using anti-IgA diluted 1000-fold. (**F**) Anti-IgA dilution using serum diluted 1000-fold. A mix of three samples were used. Data are representative of at least two independent experiments and values are expressed in mean ± SD.

Proof of concept

After characterization of the optimized conditions for single antibody isotype detection using dilution of serum and secondary antibodies, double and triple staining were performed. The results demonstrated the absence of fluorescence spillover (Fig. S4), confirming the feasibility of the experimental design for multiplex detection. The combination of lasers UV355 nm, V405 nm and blue 488 nm with UV395, V421 and B515 fluorochromes, allowed discriminating negative *vs* positive samples for the simultaneous detection of IgG, IgA and IgM, respectively, without any compensation, as represented by the histograms (Fig. S4). Three independent beads preparations were tested, showing similar results (data not shown).

Finally, the multiplexed approach was used to evaluate a panel of negative (*n=10*) and COVID-19 positive (*n=26*) samples (Fig. 3, S5 and S6). ROC-established cutoff was used for discrimination of negative and COVID-19 positive samples. The specitivity of 100% was achieved for all isotypes. The sensivity was 88.5% for the detection of IgG, 92.3% for IgM and 96.2% for IgA . The area under the ROC curve was calculated as 0.946, 0.950 and 0.962 for IgG, IgM and IgA, respectively (Fig. 3 and Table S1). Venn diagram analysis showed that 22/26 presented IgG, IgM and IgA, 1/26 presented only IgG and IgA, and 2/26 presented only IgM and IgA. None of the positive samples presented only IgG and IgM, and 1/26 did not present any of the antibodies studied (Fig 3D).

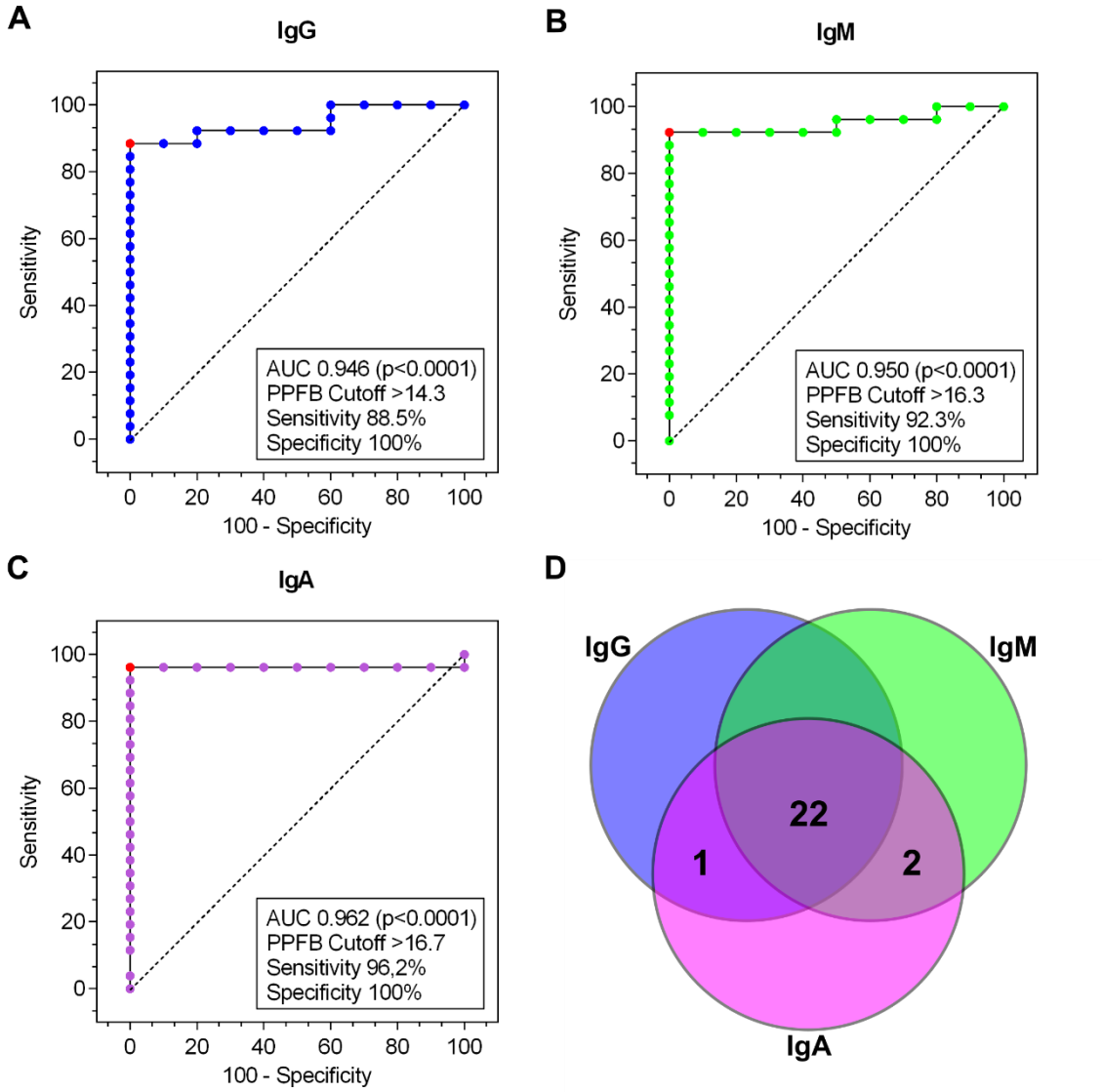

Figure 3. Receiver operating characteristic (ROC) curves and Venn diagram. COVID-19 negative controls (*n=10*) and positive (*n=26*). ROC curve for (**A**) IgG. (**B**) IgM. (**C**) IgA. AUC, area under de curve; PPFB, percentage of positive fluorescent beads (%); red point, highest Youden index (cutoff). (**D**) Venn diagram of COVID-19 positive samples simultaneously compared with the detection of IgG, IgM and IgA. The statistical calculations were performed using MedCalc v.7.12.7.2.0 (MedCal Software bvba).

DISCUSSION

Flow cytometry is described as an important analytical tool for immunological assays. Commercially available CBA functional beads are widely used for the evaluation of multiple analytes in a single sample. For instance, several papers have described the use of CBA bound to specific antibodies for the detection and quantification of a large variety of human cytokines[27] using a non-competing secondary antibody for detection. The application of flow cytometry in COVID-19 has exploited the inverse format to evaluate the humoral antibody response. Lapuente *et al*. (2020)[23], Priya Anand et al (2020)[25] and Simard et al. (2021)[24] used cell lines expressing the SARS-CoV-2 spike protein for the detection of IgG and IgM, IgG and IgG+IgA+IgM pool in human serum samples, respectively. To the best of our knowledge there is only one study employing a cell-free flow cytometric approach to evaluate the humoral antibody response[18]. The

authors used C19BA fluorescent beads coated with streptavidin which allow the binding to biotinylated SARS-CoV-2 antigens to detect IgG and IgM simultaneously.

In our study, the binding of SARS-CoV-2 nucleocapsid protein to CBA functional beads was covalent (Fig.1), which offers advantages of more robust surface, higher density of epitopes and orientation to maximize epitopes exposing and complementary binding[28]. We provided a proof of concept of a cell-free multiplex assay based on flow cytometry for immunological diagnosis of COVID-19 (Fig 1). The novel method allowed accurate discrimination between COVID-19 positive samples and pre-pandemic negative controls using standardized conditions (Fig. 2 and 3). Specificity of 100% and sensitivity of 88.5, 92.3 and 96.2% for IgG, IgM and IgA, respectively, were determined (Fig. 3 and Table S1). The results of our method for IgG and IgM showed both sensitivity and specificity similar to the FDA-approved tests (sensitivity 61-98% and specificity 90-100%)[29]. The detection of IgA showed increased sensitivity and specificity in comparison to a commercial test based on ELISA (Euroimmun™, 82.9% and 82.2%)[30]. Interestingly, the combined IgG, IgM and IgA analysis improved the serological diagnosis. Most of the RT-PCR positive samples presented the three antibody isotypes and, at least, two antibody isotypes were detected from 25/26 (Fig. 3D and S6). It should be considered that the small number of samples may affect the statistical analysis. Hence, a large cohort will be necessary to confirm the results.

A special strength of the present study is the availability of a large repertoire of combinations of multiple antigens covalent bound to each set of fluorescent CBA beads, allowing detection of unique optical signatures. Therefore, the multiplexed flow cytometric-based tool presented here provided a blueprint for rapid development of antibody evaluation to others emerging infections.

CONCLUSIONS

Our data present the first flow cytometric bead-based approach to determination of IgG, IgM and IgA response in COVID-19 as a proof concept for further studies.

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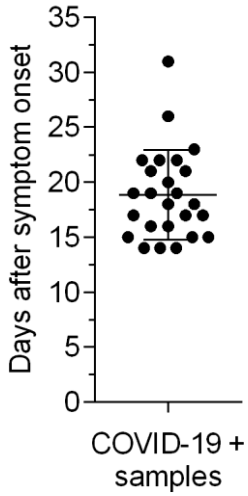

Figure S1: Representation of symptoms onset for the COVID-19 positive samples (*n*=26) used in this work. Samples were collected from patients after 14 days of hospitalization.

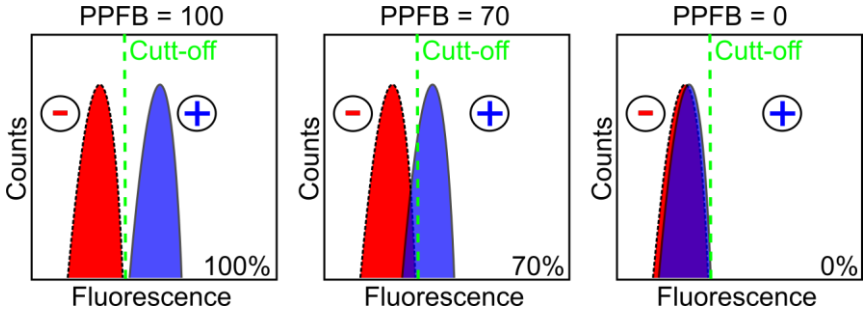

Figure S2: Representative image of the determination of Percentage of Positive Fluorescent Beads (PPFB).

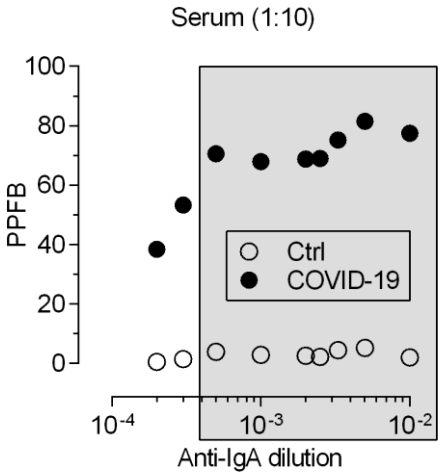

Figure S3: Determination of Percentage of Positive Fluorescent Beads (PPFB) by flow cytometry using CBA-N beads. Anti-IgA dilution using serum 10-fold diluted.

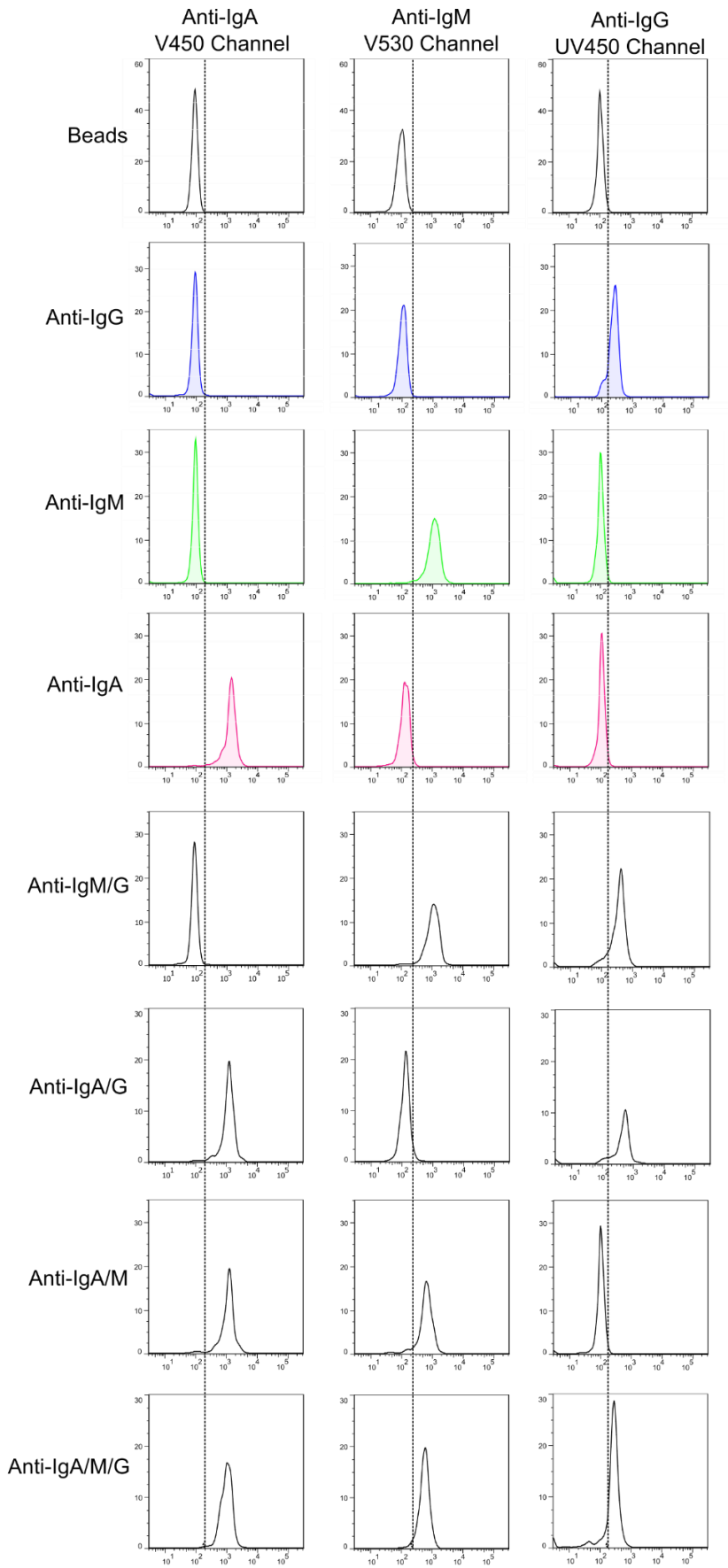

Figure S4: Representative histograms of single, double and triple staining for positive COVID-19 samples.

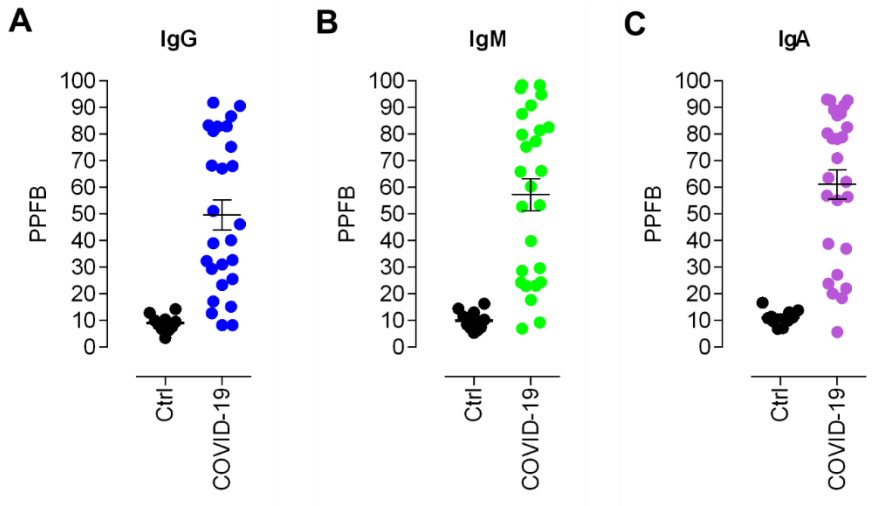

Figure S5: Simultaneous identification of IgG, IgM and IgA using the multiplex approach. Control (Ctrl, negative COVID-19; *n*=10) and COVID-19 positive (*n*=26) samples were analyzed. Determination of Percentage of Positive Fluorescent Beads (PPFB) by flow cytometry using CBA-N beads. (**A**) IgG. (**B**) IgM. (**C**) IgA.

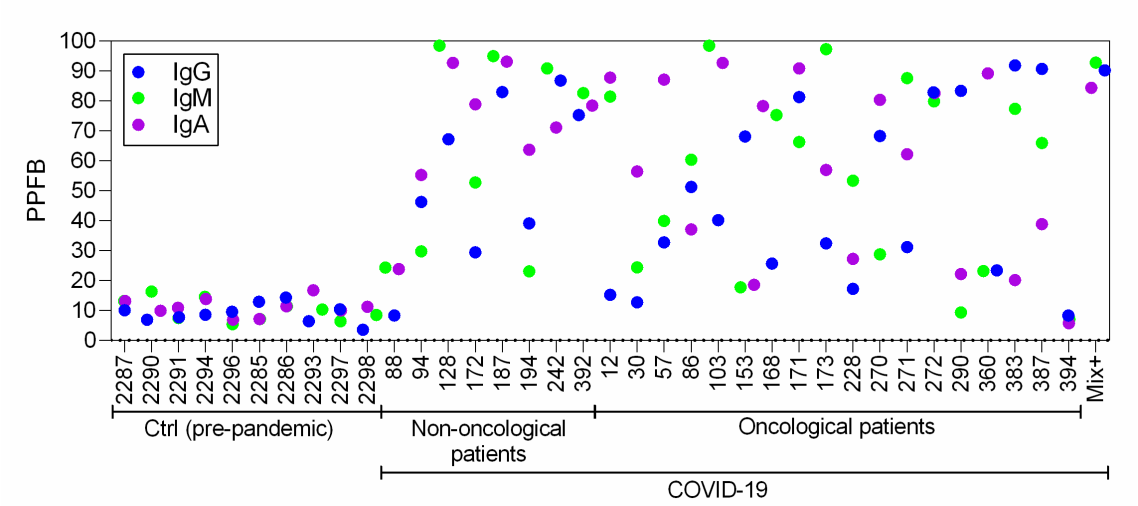

Figure S6: Antibodies profile of each sample. Determination of Percentage of Positive Fluorescent Beads (PPFB) using the multiplex approach based on flow cytometry.

Table S1. ROC curve analysis using MedCalc.

Parameters	IgG	IgM	IgA
AUC (P-value)	0.946 (<0.0001)	0.950 (<0.0001)	0.962 (<0.0001)
Youden index J (95%CI)	0.885 (0.73-0.96)	0.923 (0.77-1.0)	0.962 (0.79-1.0)
Association criterion (95%CI)	>14.3 (>12.9 to >14.3)	>16.3 (>14.5 to >16.3)	>16.7 (>13.8 to >16.7)
Sensitivity, % (95%CI)	88.5 (69.8-97.6)	92.3 (74.9-99.1)	96.2 (80.4-99.9)
Specificity, % (95%CI)	100.0 (69.2-100.0)	100.0 (69.2-100.0)	100.0 (69.2-100.0)
Accuracy, % (95%CI)	91.7 (77.5-98.3)	94.4 (81.3-99.3)	97.2 (85.5-99.9)
Positive Predictive value, % (95%CI)	100.0	100.0	100.0
Negative Predictive value, %	76.9 (53.5-90.6)	83.3 (56.9-94.0)	90.9 (59.4-98.6)