Sensitive detection of SARS-CoV-2 seroconversion by flow cytometry reveals the presence of nucleoprotein-reactive antibodies in Covid-19-naive individuals

AUTHORS

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ABSTRACT

We have developed a novel multiplexed flow cytometric bead array (C19BA) for the detection of SARS-CoV-2 seroconversion that allows sensitive identification of IgG and IgM antibodies against three immunogenic proteins: the spike receptor-binding domain (RBD), the spike protein subunit 1 (S1) and the nucleoprotein (N) simultaneously. This assay is more sensitive than ELISA, and the combination of three antigens allows for the interrogation of full seroconversion. Importantly, we have detected N-reactive antibodies in COVID-19-negative individuals.

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global spread has resulted in an ongoing pandemic. To date, most immunoassays to determine seroconversion and measure antibody responses are based on enzyme-linked immunosorbent assay (ELISA). Serological assays are important to detect previously infected individuals and perform epidemiological seroconversion studies. Moreover, they have important implications in the development of antibody-based therapeutics (i.e. convalescent serum or monoclonal antibodies) and vaccines (i.e. selection of non-immunised individuals and follow-up). For these reasons, there is a need of developing fast and sensitive serology assays that can be deployed at a large scale.

SARS-CoV-2 contains several structural proteins, among them the Spike (S) and the Nucleoprotein (N) are the most immunogenic viral antigens and are used in serologic assays. The S protein contains the receptor-binding domain (RBD) that binds to its cognate receptor angiotensin converting enzyme 2 (ACE2) expressed by host cells. The S protein is comprised of two subunits: S1 and S2. S1 includes the RBD domain and its sequence is specific for SARS-CoV-2, often generating neutralising antibodies in seropositive individuals. Given their specificity, both RBD and S are considered ideal for serology assays, especially in the form of recombinant proteins produced in mammalian cell systems that reflect a physiological glycosylation pattern. In general, ELISAs have an acceptable specificity and sensitivity profile, but have important limitations including cost, time and throughput. ELISAs require optimisation and the use of individual plates for each antigen or antibody to be tested. Moreover, the antigen is immobilised to the plate, which can hide epitopes or increase the background noise. For these reasons, ELISAs are not well suited to detect low antibody titers and often give undetermined values that are close to the cut-off with difficult interpretation of results.

We have developed a novel flow cytometry assay based on multiplexed microbeads with different intrinsic fluorescence intensities coated with different viral antigens. The coupling was
based on the interaction between microbeads functionalized with streptavidin and proteins tagged with a unique terminal biotin, which allows for the orientation of the antigen on the surface of the bead. The bead array (C19BA) is incubated with serum samples to allow the binding of anti-SARS-CoV-2 antibodies and then stained with anti-IgG and anti-IgM secondary antibodies labelled with different fluorochromes (Fig. 1a and Supplementary Fig.1). In order to fully assess the specific seroconversion against SARS-CoV-2, we have chosen RBD, S1 and N as target antigens. A previously developed ELISA includes a S1 confirmatory assay after positivity against RBD\(^6\). In our method, the redundancy of RBD as a sequence included in S1 allows for the confirmation of intra-assay specificity on different microbeads simultaneously. The N protein was also included in the assay because of its immunogenicity. N is predicted to be less specific for SARS-CoV-2 based on the analysis of the sequence alignment with other coronavirus family members (Supplementary Fig.2). We reasoned that fully seroconverted individuals would present antibodies against the three chosen antigens. We first tested the ability of this assay to identify recombinant IgG antibodies against RBD and N. As can be seen on Fig. 1b, the microbead array clearly identified the binding of these antibodies. Importantly, the sensitivity of C19BA was superior to ELISA (Fig. 1c) when their performance was compared in serial dilutions of recombinant anti-RBD and anti-N IgG antibodies. C19BA presented a better linear range and identified low antibody concentrations that were not detected by ELISA.

We then applied C19BA to interrogate serum samples from a cohort of 43 individuals who tested positive by PCR for SARS-CoV-2 infection that were obtained at the time of hospital admission (COVID cohort). As a control, sera from 50 individuals collected before the pandemic (2018-2019) were analysed (preCOVID cohort). Both ELISA and C19BA were able to discriminate both cohorts based on the presence of IgG and IgM antibodies against RBD, S1, and N. At this early stage of infection, not all samples from the COVID cohort presented reactivity against viral antigens (Supplementary Fig.3). Serial dilutions of 10 COVID and 10 preCOVID samples were performed to further compare the sensitivity of C19BA versus ELISA.
Fig. 2a shows the dilution curves corresponding to the presence of IgG antibodies by these two methods. C19BA was superior to ELISA separating both cohorts and identifying the presence of antibodies at lower concentrations. This was confirmed by plotting the area under the curve (AUC) and determining its statistical significance (Fig. 2b). The titers of IgM antibodies were lower than IgG as measured by both methods (Supplementary Fig. 4), in line with previous studies⁹.

Importantly, our assay identified the presence of N-reactive IgG antibodies in preCOVID samples, although in general at lower titers than in the COVID cohort (Fig. 2a,b). Fig. 2c shows representative dot plot profiles corresponding to preCOVID and COVID samples. While the reactivity against RBD and S1 was specific for COVID samples, cross-reactivity against N was observed in some preCOVID individuals that contained IgG, but not IgM antibodies. This fact raises concerns about the specificity of the use of N protein for serological assays, given its high homology with N proteins of other coronavirus. In the COVID cohort, several samples that presented full seroconversion against RBD, S1 and N for both IgG and IgM were identified. A minority of COVID samples presented only N-reactive IgG and IgM antibodies, testing negative for RBD or S1. This suggests that these individuals mounted secondary antibody responses against N after mobilization of memory B cells generated as a result of a previous coronavirus infection. Recently, in a similar fashion, N-reactive memory T cells have been also identified in COVID-19-negative individuals¹⁰. Together, these data provide evidence that cellular and humoral immune responses against SARS-CoV-2 exist as a result of crossreactivity against the N protein originated by previous coronavirus infections. The impact of these pre-existing T cell and antibody responses in the control and pathogenesis of COVID-19 requires further investigation.

In summary, we have developed a novel multiplexed method with higher sensitivity than traditional serology assays, using a triple combination of antigens that exploits the specificity of the Spike and RBD together with the less-specific N protein for the detection of antibodies against SARS-CoV-2.
METHODS

Serum samples. All serum samples were provided by the Basque Biobank (www.biobancovasco.org) after approval from the corresponding ethics committee (CEIC-E 20-26, 1-2016). All participants in the study provided informed consent and were anonymized. The COVID cohort corresponds to 43 patients presenting COVID-19 symptomatology and diagnosed by PCR. The preCOVID cohort (50 serum samples) was obtained during the yearly medical check-up of the working population of the Basque Country in 2018-2019 in collaboration with Osarten Kooperatiba Elkartea from Mondragon Corporation.

Microbead coating. PMMA (polymethyl methacrylate) 8.2 μm microbeads coated with streptavidin were purchased from PolyAn (#10652009). Each microbeads presented a different fluorescence intensity (Red4 dye, Excitation: 590-680 nm/Emission: 660-780 nm). First, microbeads were washed with cold PBS pH 7.2 (Gibco #14190-094) by centrifugation at 2000 rpm for 5 min and resuspended in PBS. Then, biotinylated recombinant RBD, S1 and N (Acrobiosystems #SPD-C82E9, #S1N-C82E8 and #NUN-C81Q6, respectively) were added to the tubes (RBD at 11 μg/mL, S1 at 30 μg/mL, N at 19.5 μg/mL) and kept at 4°C on rotating head over tail for an hour, protected from light. Positive control beads were coated with biotinylated human IgG (Novus Biologicals #NBP1-96855) and IgM (Novus Biologicals #NBP1-96989) on the same microbead at 15 μg/mL each. Negative control beads were not coated with protein. After the coupling reaction, microbeads were washed three times with PBS. Then, D-biotin (2 μM) (Sigma-Aldrich #8512090001) was added and incubated for 15 min at room temperature (RT) to inactivate residual streptavidin. After three additional washes, equal amounts of each microbead were combined in the same vial.

C19BA assay. Antigen-coupled microbeads were added to protein LoBind 1.5 mL Eppendorf tubes (Eppendorf #525-0133) in a volume of 50 μL of PBS containing a total of 5000-6000 beads. After centrifugation (2000 rpm, 5 min), microbeads were resuspended with 100 μL of pre-diluted (PBS) serum samples or serially diluted commercial antibodies against RBD (GenScript #A02038) or N (Acrobiosystems #NUN-S41) starting from a 1 mg/mL
concentration. Negative control samples were prepared with PBS. After a 30 minute incubation (RT protected from light), samples were washed three times in PBS. Secondary antibodies were diluted in 100 µL of PBS containing 5% FBS: anti-human IgG-PE (1:50) (BD Biosciences #555787) and anti-human IgM-BV421 antibodies (1:1000) (BD Biosciences #555783). The mix was incubated with the samples for 15 min at RT protected from light. One final wash was performed, and microbeads were resuspended in 200 µL of PBS supplemented with 5% FBS for acquisition. At least 600 events for each type of microbead were acquired in a FACSsymphony flow cytometer (BD Biosciences) and geometric mean fluorescence intensities (gMFI) were obtained. Results were analyzed using FlowJo version 10 (BD Biosciences).

ELISA. The protocol was adapted from a previously established immunoassay. Briefly, 96-well ELISA plates (Nunc Maxisorp) were coated overnight at 4°C with 50 µL of biotinylated RBD, S1 or N protein (Acrobiosystems), at 2 µg/mL (for RBD and S1) or 1.4 µg/mL (N) in PBS (Gibco). Then, the coating solution was removed and plates were blocked with 3% non-fat milk in PBST (PBS plus 0.1% Tween-20) for 1 hour at RT. Serum samples were pre-diluted in 1% non-fat milk in PBST, and incubated for 2 hours at RT. After three washes with 250 µL of PBST in a plate washer (Biotek), each well was incubated with an anti-human IgG-horseradish peroxidase (HRP) conjugated secondary antibody (1:5000) (GenScript #A01854) or anti-human IgM-HRP (Novus Biologicals #NBP1-75014) for 1 hour at RT. Plates were washed three times with PBST, and 100 µL of TMB substrate (Thermo Scientific) were added to each well, incubated for 2 min and the reaction was stopped with 50 µL of stop solution (Thermo Scientific). The optical density (OD) was measured at 450 nm in a VictorNivo multimode plate reader (PerkinElmer).

Data analysis

To calculate the sample ODs and gMFIs, the values corresponding to the negative controls were subtracted from all samples. For values below 0.11, the OD or gMFI values were set at 0.11 in order to calculate the AUC and generate the graphs. Statistical analyses comparing
the preCOVID and COVID cohorts were performed with an unpaired two-tailed Student’s t-test. Data were analyzed using Prism 8 (GraphPad).

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AUTHOR CONTRIBUTIONS

AP conceived and administered the project. LEM and AB performed all experiments and analysed data. AB, LEM, NGAA and EP performed computational and statistical analyses. AGDR, SYL, BJL and AAV contributed ideas. OM, JMM, CB, MB and NE provided patient samples. AP and EP wrote the manuscript with help from all co-authors.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Fig. 1. Workflow and performance of C19BA. 

a, Overview of the detection of SARS-CoV-2 seroconversion by flow cytometric bead array. Streptavidin-coated microbeads with different fluorescence intensities are conjugated with recombinant biotinylated viral antigens (RBD, S1, N).
and N) and mixed and incubated with pre-diluted serum samples together with control beads. After incubation, microbeads are washed and stained with anti-human IgG and IgM secondary antibodies, washed and acquired on a flow cytometer for downstream analysis. **b,** Representative dot plots showing unstained beads corresponding to each control or antigen (left), and the specificity of the staining pattern of recombinant anti-RBD IgG (middle) or anti-N IgG (right) antibodies. **c,** Comparison of the titration of anti-RBD (blue and green) and anti-N (orange) IgG antibodies against recombinant RBD, S1 and N proteins by C19BA (top) and ELISA (bottom). The mean value of two replicates is shown, error bars represent SD.
Fig. 2. Identification of SARS-CoV-2 seroconversion by C19BA. a, Titration curves of the reactivity of individual serum samples against RBD, S1 and N proteins measured by flow cytometry against IgG by C19BA (top) and ELISA (bottom) for each cohort: preCOVID (red, n=10) and COVID (black, n=10). b, AUC values for the experiment shown for C19BA (top) and ELISA (bottom). Statistical analyses were performed using an unpaired two-tailed Student’s t-test (*** represent p<0.001). Horizontal lines represent median values. c, Dot plots
showing IgG (top) and IgM (bottom) reactivity against RBD, S1 and N for representative preCOVID and COVID samples.
Supplementary Fig. 1. Overview of the setup of control microbeads for C19BA assay. a, Dot plots showing the staining patterns of positive (red) and negative (black) control beads with secondary anti-IgG-PE, anti-IgM-BV421 or both. The signal corresponding to IgG-PE (top) and IgM-BV421 (bottom) is shown for each column. b, Histogram showing the distribution of the microbeads based on their intrinsic fluorescence on the APC channel. Each colour represents the coating for each microbead.
Supplementary Fig. 2. Conservation of the N protein sequence across different members of the Coronaviridae family. a, Alignment of the sequences (Uniprot codes: SARS-CoV-2, P0DTC9; SARS-CoV-1, P59595; MERS-CoV-1, R9UM87; OC43, P33469; 229E, P15130; NL63, Q6Q1R8; and UKU1, Q5MQC6) using Clustal Omega and represented using ESPRIPT 3.0 software using the 70% of equivalent residues calculated per columns, considering physico-chemical properties; on top of the sequences the secondary structural elements derived from the 3D structures determined for the RNA-binding domain (PDB ID: VYO) and the C-terminal dimerization domain (PDB ID: 6WJI) and corresponding to the most conserved regions. b, Phylogram generated from the FASTA alignment file using FastTree (https://www.genome.jp/). The two columns on the right represent the sequence identity and similarity in a pairwise alignment with the SARS-CoV-2.
**Supplementary Fig. 3.** Serological responses against RBD, S1 and N measured by C19BA and ELISA. a,b, gMFI values obtained by C19BA (top) and OD values obtained by ELISA (bottom) for the same samples for IgG (a) and IgM (b). Statistical analyses were performed using an unpaired two-tailed Student's t-test to compare the preCOVID (red, n=50) and COVID (black, n=43). Asterisks represent p values (***, p<0.001, *, p<0.05, ns: not significant). Horizontal lines represent median values.
Supplementary Fig. 4. Identification of SARS-CoV-2 IgM seroconversion by C19BA. 

**a,** Titration curves of the reactivity of individual serum samples against RBD, S1 and N proteins measured by flow cytometry against IgM by C19BA (top) and ELISA (bottom) for each cohort: preCOVID (red, n=10) and COVID (black, n=10). **b,** AUC values for the experiment shown for C19BA (top) and ELISA (bottom). Statistical analyses were performed using an unpaired two-tailed Student’s t-test. Asterisks represent p values (***, p<0.001, *, p<0.05, ns: not significant). Horizontal lines represent median values.