Evaluating the serological status of COVID-19 patients using an indirect immunofluorescent assay, France.

Running title: SARS-CoV2 serology

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ABSTRACT

An indirect immunofluorescent assay was developed in order to assess the serological status of 888 RT-PCR-confirmed COVID-19 patients (1,302 serum samples) and controls in Marseille, France. Incorporating an inactivated clinical SARS CoV-2 isolate as the antigen, the specificity of the assay was measured as 100% for IgA titre ≥ 1:200; 98.6% for IgM titre ≥ 1:200; and 96.3% for IgG titre ≥ 1:100 after testing a series of negative controls as well as 350 serums collected from patients with non-SARS-CoV-2 Coronavirus infection, non-Coronavirus pneumonia and infections known to elicit false-positive serology. IFA presented substantial agreement (86%) with ELISA EUROIMMUN SARS-CoV-2 IgG kit (Cohen's Kappa=0.61). Seroprevalence was then measured at 3% before a five-day evolution up to 47% after more than 15 days of evolution. We observed that the seroprevalence as well as the titre of specific antibodies were both significantly higher in patients with a poor clinical outcome than in patients with a favourable evolution. These data, which have to be integrated into the ongoing understanding of the immunological phase of the infection, suggest that serotherapy may not be a therapeutic option in patients with severe COVID-19 infection. The IFA assay reported here is useful for monitoring SARS-CoV-2 exposure at the individual and population levels.

INTRODUCTION

The SARS-CoV-2 is a coronavirus belonging to the genus *Betacoronavirus* that emerged in humans in December 2019 [1]. It was first described in China before spreading and being classified as a pandemic [2]. It causes a respiratory disease known as Covid-19 that is usually mild but can result in a severe and even life-threatening pneumonia, particularly in elderly people [2–4]. On 21 September 2020, 31,033,397 SARS-CoV-2 infections and 960,736 associated deaths had been reported worldwide (https://coronavirus.jhu.edu/map.html).

To date, the virological diagnosis of infections by SARS-CoV-2 has been essentially based on real-time reverse transcription PCR [5]. This virus has been shown to elicit specific antibodies during the course of infection [6,7]. This serological response has mainly been analysed using enzyme-linked (ELISA) or chemiluminescence immunoassays among exposed populations in China and neighbouring countries. Previous studies showed that specific IgG, IgM and IgA were produced in response to the infection [7]. The kinetics of these three classes of antibodies have been described, yet correlations with the clinical outcome of the patients has been poorly reported [7].

In this study, we are reporting our experience with an indirect immunofluorescent assay (IFA) for the detection of anti-SARS-CoV-2 antibodies, that we implemented before any other serological test was available in France, further comparison with ELISA. We found significant differences in the seroprevalence and antibody titres between groups of patients depending on their clinical outcome.

PATIENTS AND METHODS

Study design. A cohort of patients with confirmed SARS-CoV-2 infection was studied at the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in Marseille, France, as previously described [4]. All patients presenting symptoms compatible with COVID-19 and contacts of suspected and confirmed COVID-19 cases were tested using a SARS-CoV-2 specific qRT-PCR assay [4,8]. Treatment with hydroxychloroquine (HCQ) associated with azithromycin (AZ) was proposed to all qPCR-positive patients who enrolled on a voluntary basis if they did not present contraindications [4]. Patients were followed-up on an out-patient basis at our day care hospital or were hospitalised in the infectious disease units of the IHU, in intensive care units or in other medical departments of the Assistance Publique-Hôpitaux de Marseille, depending on the severity of the disease. We included in the present study all patients from the previous study by Million *et al.* for whom ≥1 serum sample was available for serological testing as part of the routine care of these patients. All the serums were tested retrospectively using IFA. Also, we further compared the specificity and sensitivity of IFA to a SARS-CoV-2 IgG ELISA which became commercially available three months after we set-up IFA. The time of serum collection was determined relative to the date of the onset of symptoms. The non-interventional nature of this study was approved by the Ethical Committee of the IHU Méditerranée Infection under no. 2020-13.

Case definition. SARS-CoV-2 infection was defined by clinical, radiological, and microbiological criteria as previously reported [3,4]. Briefly, the national early warning score (NEWS) for COVID-19 was used for the classification of clinical presentation of patients. This score was based on 6 physiological parameters including respiratory

rate, oxygen saturations, temperature, systolic blood pressure, heart rate, level of consciousness to predict deterioration risk in acute ill patients. Three risk categories were defined for clinical deterioration: low score (NEWS 0–4), medium score (NEWS 5–6), and high score (NEWS≥7). Virological evidence of the infection was based on a positive qRT-PCR on a nasopharyngeal sample or another respiratory sample. Pulmonary involvement was evaluated by chest low-dose computed tomography for all patients. Five groups of patients were constituted according to the following criteria [4]: (1) Patients with mild disease and good clinical and virological outcome (GO; n= 681); (2) Patients with poor virological outcome defined by persistence at day 10 or more of viral detection in respiratory samples (PVirO; n= 100); (3) Patients who received HCQ + AZ treatment for more than three days, with poor clinical outcome requiring prolonged hospitalisation for 10 days or more despite three days or more of HCQ + AZ treatment (PClinO1; n= 53); (4) Patients who received HCQ + AZ treatment fewer than three days, with poor clinical outcome requiring prolonged hospitalisation for 10 days or more (PClinO2; n = 25); (5) Patients with poor clinical outcome requiring prolonged hospitalisation for 10 days or more leading to death (PClinO3; n= 29). Main characteristics of the patients in each group are summarised in Table 1.

Indirect immunofluorescence assay. Anti-SARS-CoV 2 antibodies were detected using an in house indirect immunofluorescence assay (IFA), as previously described [9]. Vero E6 cells (ATCC CRL-1586, Rockville, MD, USA) infected with the SARS-CoV2 strain IHU-MI2 (full genome sequence of this strain was deposited under the European Molecular Biology Laboratory EMBL project accession no. PRJEB38023) [10] were harvested between 24 hours and 48 hours post-inoculation when cytopathic effect begins to be observed before massive cell lyses begin, washed with

sterile phosphate buffered saline (PBS) (Oxoid, Dardilly, France) and inactivated using 5% paraformaldehyde. This preparation was used as the antigen and 50 nL of antigen were spotted on each well of 18-well microscope glass slides using Echo 525 Liquid Handler instruments (Labcytes, Cannock, United Kingdom) that uses acoustic energy to transfer liquid from a 96-well plate containing the antigen to slides. Fifty nanolitres of uninfected Vero cells were also spotted on each well as a negative control and a clinical isolate of *Staphylococcus aureus* (identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry) [11] was spotted on each well in order to ensure further serum deposition, as previously described [12]. Each slide was air dried, fixed in acetone for 10 minutes and conserved at 4°C in the dark.

In a first step, each serum sample was screened for the presence of anti-SARS CoV-2 antibodies using the IFA, as previously described [9]. Serum samples were heat-decomplemented for 30 minutes at 56°C, diluted in 3% PBS-milk and 25 µL of a 1:50 dilution and a 1:100 dilution were pipetted onto a 18-spot slide then incubated for 30 minutes at 37°C in the dark to be screened for the detection of total immunoglobulin (IgT). After washing thrice, the slides with sterile PBS for 10 minutes, 25 µL of total FITC-conjugated IgT anti-human immunoglobulin (Bio-Rad, Marnes-la-Coquette, France) with 0.5% Evans blue (Bio-Rad) were incubated for 30 minutes at 37°C. After washing, slides were observed under a fluorescence microscope (AxioSkop 40, Zeiss, Marly le Roi, France). In a second step, all the serum samples screened positive at a 1:100 dilution were quantified for IgG, IgM and IgA as reported above, except that serum samples were diluted up to 1:1,600 for IgA and IgM and 1:3,200 for IgG; and anti-IgG, anti-IgM and anti-IgA conjugates were used (bioRad). Serum samples exhibiting positivity at 1:3,200 were further tested up

to 1:25,600. Reading of slide was performed in duplicate by two experienced laboratory technicians or medical microbiologists. In case of discrepancy, a third operator read the slide. A serum sample exhibiting a 1:400 titre collected from one patient who was positive by SARS COV-2 RT-PCR, was anonymised and used as a positive control on each slide for screening and on each run for antibody quantification allowing daily to follow the robustness of the technique and to validate any new lot of antigen. A negative serum collected in December 2019 from a patient and PBS-milk 3% were used as negative controls on each slide screened. In order to interpret the IFA, any serum sample exhibiting IgG ≥ 1:100 with or without IgM and/or IgA ≥1:50 was considered as positive; as well as any serum sample exhibiting isolated IgM or IgA 1:200 (Fig.1).

Serum samples. The specificity of the IFA was evaluated by testing four series of serum samples. Negative control samples (n = 200) had been collected from patients between November and December 2018 (before the COVID-19 epidemics in France). Further, 150 serum known to be associated with nonspecific serological interference were collected from 14 patients diagnosed with Epstein-Barr virus infection; eight patients diagnosed with Cytomegalovirus infection; seven patients diagnosed with A hepatitis virus infection; 10 patients diagnosed with toxoplasmosis and 25 patients diagnosed with E hepatitis virus infection. Serum samples were also collected from 50 patients diagnosed with Coronavirus NL63, OC43, 229E or HKU1; as well as 36 sera collected from patients diagnosed with non-coronavirus pneumonia, including 14 *Mycoplasma pneumoniae* infections, 10 *Legionella pneumophila* infections, and 12 *Chlamydia pneumoniae* infections, in order to assess for potential cross-reactivity. We evaluated repeatability of IFA by testing 5 sera in

triplicate by a same operator and reproducibility testing 5 sera by 2 independent operators.

ELISA. To compare our IFA with commercial ELISA IgG, we randomly selected 70 sera with possible cross reactivity (including 45 sera with possible nonspecific serological interference and 25 sera from patients diagnosed with common others human coronavirus), 30 sera collected before the pandemic and 100 sera from our cohort of SARS-CoV-2 infected patients among all the sera that we tested by IFA. EUROIMMUN® SARS-CoV-2 IgG ELISA (Euroimmun France®, Bussy Saint-Martin, France) was performed using Elispeed DUO system (Euroimmun France®) according to the manufacturer's recommendations. The ratio (AUC sample/AUC calibrator) was interpreted as follows: < 0.8 negative; ≥ 0.8 to <1.0 undetermined (grey zone); ≥ 1.1 positive. We considered results in grey zone as negative for statistical analyses.

Statistical analysis. To avoid bias in data analysis, we studied the serological response according to the time of sampling of the sera related to the date of the onset of symptoms. The analysis of sera was divided into different times (D0-D5, D6-D10, D11-D15 and D16-D38). For the study of seroprevalence and for the comparison of IgG titre, we considered only the sera with the higher IgG titre or with the higher IgM or IgA titre when several sera were available for a same patient. For the data comparisons and statistical analyses, Fisher's exact test, the Chi-squared test, Mann-Witney test and standard statistical software (GraphPad Prism 7) were used. The agreement rate and Cohen's Kappa value were determined for agreement between ELISA and IFA. A p-value < 0.05 was considered statistically significant. ROC curves were calculated using GraphPad Prism 7.

RESULTS

IFA. The results of IFA assay specificity were summarized in Table 2. In the negative control group of 200 serum samples collected from patients in November and December 2018 before the emergence of COVID-19 in France, no IgG and no IgA were detected and three samples exhibited a IgM titre of 1:25 for two samples and 1:100 for one sample. In the group of 64 serum samples known to yield cross-reactivities, four samples exhibited IgG titre ≥ 1:100 and 8 samples exhibited IgM titre ≥ 1:100. Of the 50 serum samples collected from patients diagnosed with another Coronavirus other than COVID-19, none reacted in IgG, none reacted in IgA and 9 reacted in IgM with titre ≥ 1:100. Also, among 36 sera collected from patients diagnosed with non-Coronavirus pneumonia, nine yielded an IgG titre ≥ 1:100 but did not react for IgM and IgA. Overall, 13/350 serum samples yielded a false positivity of IgG ≥ 1:100, yielding a 96.3% specificity for IgG; and 5/350 serum samples yielded a false positivity of IgM ≥ 1:200, yielding a specificity of 98.6% for IgM. Specificity of IgA titre of 1:200 was 100%. Combination of criteria IgG ≥ 1:100 with or without IgM and/or IgA ≥1:50 showed 96% specificity (Fig.S1). Reproducibility and repeatability of the assay was evaluated to 100% and 100% for screening and to 100% and 93% for quantification if we tolerate a deviation of one dilution.

COMPARISON BETWEEN IFA AND ELISA FOR THE DETECTION OF SARS-CoV-2 IgG. Specificity and sensitivity of ELISA IgG was evaluated to 97% and 45% respectively compare to 93% and 41% for our in house IFA among the 200 sera tested with both techniques. Three sera among the negative controls were positive for ELISA and 7 for IFA respectively. Discordant results were found for 15% (31/200) of the sera; 17 sera were positive only by IFA (including 7 false positive) and 14 sera

were positive only by ELISA (including 3 false positive). IFA presented substantial agreement (86%) with ELISA EUROIMMUN SARS-CoV-2 IgG kit (Cohen's Kappa=0.61).If we considered only sera collected at least 14 days after the onset of symptoms of SARS-CoV-2 Infected patients (n=56), sensitivity was 67% with ELISA versus 64% with IFA that is not significantly different.

SEROLOGICAL RESPONSE OF INFECTED PATIENTS.

We then evaluated the serological response in a collection of 1,302 serum samples from 888 patients infected with SARS-CoV-2 between 12 March and 17 April 2020 [4]. SARS –CoV-2 infection was confirmed by positive RT-PCR on respiratory specimens for all these patients with a median Ct value of 25.65 (range 12.5-35). This cohort, which included 408 men (46%), had a median age of 45 years (range, 14–97 years). Median age of patients with poor clinical outcome (PClinO1, PClinO2, PClinO3) were significantly higher than the median age of patients from PVirO and GO group ($p<0.0001$). Most of the patients (778/888, 88%) presented a low NEWS score (≤ 4) and 24 patients were asymptomatic. Patients from the 3 groups with poor clinical outcome presented significant higher NEWS score than patients from PVirO and GO group ($p<0.0001$ and $p<0.0001$, respectively), (Table 1). Serum samples had been collected at a median time of 15 days (range, 0–38 days) after onset of symptoms. Seventy (5.4%) sera were collected between D0-D5, 238 (18.3%) between D6-D10, 395 (30.3%) between D11-D15 and 599 (46%) between D15-D38. At least one positive serology (with our defined criteria) was found in 330 patients, leading to a seroprevalence of 37.2%. The time distribution of positive serum samples was as follows: 3% (2/60) between D0-D5, 13% (26/197) between D6–D10, 27% (97/365) between day D11–D15 and 47% (242/519) after D16.

Multiple sera were available for 299 patients. Among them, we observed 88 (29%) seroconversions with 6 (7%) patients seroconverting between D6–D10, 25 (28%) between D11–D15 and 57 (65%) after D16. Only two patients were observed to be positive within five days after onset of the illness, one patient exhibited IgG titre 1:100 and another patient with IgG titre at 1:1,600 and IgA at 1:100. Seroconversion occurred earlier in patients with poor clinical outcome (PClinO) with a median of 13 days compared to patients with good outcome for whom seroconversion occurred with a median of 19 days (p=0.0009). We evaluated the kinetics of the antibodies in our cohort of 1302 sera. An earlier increase of IgG, IgM and IgA was found in patients with poor outcome compared to patients with good outcome and with virological persistence (Fig. 2).

Detailing the results for each group of patients, the median time of serum sampling was 8, 11, 11, 16 and 16 days after the onset of symptoms for dead patients (PClinO3), poor outcome patients with HCQ+AZ < 3 days (PClinO2), poor outcome patients with HCQ+AZ ≥ 3 days (PClinO1), with persistent viral shedding (PVirO) and with good outcome (GO) respectively. Seroprevalence by group was 28% (8/29) in PClinO3 (dead), 56% (14/25) in PClinO2 (HCQ+AZ < 3 days), 49% (26/53) in PClinO1(HCQ+AZ ≥ 3 days), 44% (44/100) in PVirO (virological persistence) and 35% (241/681) in patients with good outcome (GO)" Higher seroprevalence was observed in group of patients with poor clinical outcome (PClinO3, PClinO2, PClinO1) compared to patients with virological persistent shedding and patients with clinical good outcome (Fig. 3) for each period time but significant results were observed after 10 days. Higher seroprevalence was found in PClinO3 (70%), PClinO2 (71%), PClinO1 (57%) compared to patients with good clinical outcome (GO) (37%), p=0.046, p=0.01 and p= 0.015, respectively (Fig. 3d).

In particular, the five dead patients had exhibited positive serology after day 16. No significant difference was observed between patients with persistent viral shedding (PVirO) and patient with good outcome (GO). We did not observed significant difference of seroprevalence among the asymptomatic patients (7/24, 29%) compared to symptomatic patients with low NEWS score (264/754, 35%), p=0.708.

We did not observe significant difference in the time of occurrence of the different classes of antibodies from the onset of symptoms in our cohort and we noted presence of IgG in most of patients with positive serology (n=333). The median of occurrence of IgG with titer ≥ 1:100 was 18 days (range 2-38) and were detected in sera from 326 patients (98%) (Table S1). IgA seems to have better sensitivity than IgM at the acute phase of the disease. IgM with titer ≥ 1:50 were detected in 42 patients (13%) with a median of 16 days (range 6-31) and IgA with titer ≥ 1:50 were detected in 107 patients (32%) with a median of 16 days (range 5-32). Three patients presented isolated IgA ≥ 1:200 and 8 patients presented isolated IgM ≥ 1:200 in early sera. All the other patients presented concomitant IgG ≥ 1:100. We did not detailed seroprevalence data for IgG because they were very similar to seroprevalence data including all classes of antibodies as described above. However, we observed significant higher prevalence of IgA in patients with poor clinical outcome (27/107,22%) compared to patients with persistent viral shedding (PVirO) (11/100, 11%) and patients with good outcome (GO) (69/681,10%) (p=0.013 and p<0.0001 respectively). For IgM, we found a significant higher prevalence in all patients with poor clinical outcome (19/107,18%) and with virological persistence (13/100,13%) compared to patients with good outcome (45/681,7%) (p=0.0002 and p=0.038 respectively).

We also compared IgG titre between the five groups of patients but we included only sera collected at least 10 days after the onset of symptoms (n=321). We found significant higher IgG titre in patients with a poor clinical outcome (died PClinO3, PClinO2, PClinO1) compared to patients with good outcome (GO) (p=0.0006) (Fig. 4). The median of IgG titer was 1:800 for patients with poor clinical outcome and 1:200 for both patients with viral persistent shedding and patients with good outcome. We did not observed significant difference of IgM and IgA titer between the different groups of patients.

DISCUSSION

To date, many methods exist for both rapid (lateral flow assays) and semi/quantitative (CLIA, ELISA) measurement of SARS-CoV-2 antibodies [13]. However, at the beginning of the pandemic, most of currently commercially available serological tests were not available for several months because. In this context, we developed an in house indirect immunofluorescence assay for the detection of IgG, IgM and IgA anti-SARS CoV-2 antibodies using SARS-CoV-2 antigen produced directly in our biosafety level 3 laboratory. We used it to assess the serological status of hundreds of COVID-19 patients and controls, as such an assay has been only reported on a very small group of patients [14,15]. In order to avoid false negative results, the assay incorporated *S. aureus* as a control of deposition of tested sera, as *S. aureus* protein A and protein M bind non-specifically to any serum antibody [12]. The assay also incorporated non-infected Vero cells on which the viral antigen has been produced, in order to identify false positive reactivities. Reading of both controls was incorporated into the interpretation algorithm. Accordingly, the specificity of the assay was measured at 100% for IgA, 98.5% for IgM and 95.9% for

IgG. Substantial agreement was found between commercial ELISA IgG kit and our IFA technique which attests to the reliability and robustness of our in house IFA assay compared to a standardized commercial test.

Using this assay, we observed low values of seroprevalence, at 37% in RT-PCR confirmed COVID-19 patients, ranging precisely from 3% before five days' evolution to 47% after 15 days' evolution. However, seroconversions of specific IgM and IgG antibodies were observed as early as day four after the onset of symptoms, as previously described [2]. This low seroprevalence is here observed in a population of treated patients with a favourable clinical evolution and outcome in most of these patients. In contrast, we identified that patients with severe disease developed a serological response in most cases (and all patients who died) that was characterised by high levels of IgG as was also observed for SARS-CoV infection [16] and in agreement with others reports about SARS-CoV-2 infection. Most study reported higher antibody levels after a severe and critical infection than after a mild infection [17–20] especially on sera taken 7-10 days after the onset of symptoms. These findings are confirmed for different subtype of antibodies directed against S1, RBD and S2 protein [19] as well as for neutralizing antibody [19,21,22] . Only minority of study did not find this correlation [23,24]. Most of them include few numbers of sera or did not analyzed data according collection samples time after symptoms onset. In fact, no significant difference were mostly found on early sera [17,18]. The discrepancies between the studies can also be explained by the nature and the target of the antigens used. Some other studies also reported an earlier serological response in severe compared to mild SARS-CoV-2 infection [5,20,25] that is consistent with the earlier seroconversion that we found in patients with poor clinical outcome (PClinO). On the other hand, an analysis of patients with mild

symptoms of COVID-19 showed that SARS-CoV-2 can persist in patients who developed specific IgG antibodies for a very long period of time, up to 28 days, whereas only one patient who did not develop an IgG response cleared the virus after 46 days [26].

Higher seroprevalence and antibodies titer found in patients with more severe disease call into question the much hoped-for role for serotherapy in SARS-CoV-2 infection. The use of convalescent plasma with high levels of antibodies planned at the onset of the pandemic for the treatment of severe COVID-19 infections may not be an effective treatment option [27–29].

Detecting anti-SARS CoV-2 antibodies is useful as a marker associated with COVID-19 severity. Serology also assesses exposure to the virus, at the individual level for middle-long term medical monitoring of the patients; and at the population level for monitoring the circulation of the virus, as it is one of the markers contributing to assessing the effectiveness of countermeasures.

AUTHORS' CONTRIBUTIONS.

DR and MD initiated and concept the study. SE, MD, BLS, LT and FD organized and developed the technique. HTD, MM, JCL, AS, PP, PB, PG were the clinicians in charge of the patients. SE, CM collected and interpreted the data. SE and PC performed the statistical analysis. SE, PC, CM and MD drafted the manuscript. All authors amended and commented on the final manuscript.

ACKNOWLEDGEMENTS.

The authors acknowledge the contribution of the technical staff of the IHU Méditerranée Infection Laboratory. This work was supported by IHU Méditerranée Infection, Marseille, France.

FINANCIAL SUPPORT

This study was funded by ANR-15-CE36-0004-01 and by ANR “Investissements d’Avenir” Méditerranée Infection 10-IAHU-03.

CONFLICTS OF INTEREST.

None to declare.

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Figure Legends.

Figure 1. Picture of immunofluorescence assay of serum sample from a COVID-19 Infected patient. Each well of glass slides was spotted with SARS-Cov-2 antigen (A), non-infected VERO cells (B) and *S. aureus* antigen (C). Left panel, patient's serum with anti-SARS-CoV-2 total immunoglobulins detectable at dilution 1:100. Patient presented IgG titer at 1:400, IgM titer at 1:50 and IgA titer at 1:100. Right panel, negative control serum. Slides were observed using Zeiss microscope, objective x40.

Figure 2. Antibody titer of IgG (a), IgM (b), IgA (c) according to delay after the onset of symptoms (n=1302) between the different groups of patients infected with SARS-CoV-2. The curve represents the mean values expected from a third order polynomial regression.

Figure 3. Comparison of seroprevalence among the five groups of patients (a) Between days 6 and 10 (b) Between days 11 and 15 (c) between days 16 and 38 (d) After day 10.

Figure 4. Comparison of median of IgG titre detected at least 10 days after the onset of symptoms between the different groups of patients infected with SARS-CoV-2. (Only the sera with higher IgG titre were considered for this analysis when multiple sera were available for a same patient).

Supplementary figures.

Figure S1. ROC curves for IgG (a), IgM (b) and IgA (c) detection by IFA including the 1,302 sera from patients and the 350 sera from controls. Area under the curve were 0.63, 0.51 and 0.56 respectively.