1 Evaluating the serological status of COVID-19 patients using an indirect
2 immunofluorescent assay, France.
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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 150 serums collected from patients with non-SARS-CoV-2 Coronavirus infection, non-Coronavirus pneumonia and infections known to elicit false-positive serology. 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.

32 ## INTRODUCTION

33 The SARS-CoV-2 is a coronavirus belonging to the genus *Betacoronavirus* that
34 emerged in humans in December 2019 (1). It was first described in China before
35 spreading and being classified as a pandemic (2). It causes a respiratory disease
36 known as Covid-19 that is usually mild but can result in a severe and even life-
37 threatening pneumonia, particularly in elderly people (2, 3). On 24 April 2020,
38 2,699,338 SARS-CoV-2 infections and 188,437 associated deaths had been
39 reported worldwide [https://coronavirus.jhu.edu/map.html].

40 To date, the virological diagnosis of infections by SARS-CoV-2 has been
41 essentially based on real-time reverse transcription PCR (4). This virus has been
42 shown to elicit specific antibodies during the course of infection (1, 5). This
43 serological response has mainly been analysed using enzyme-linked or
44 chemiluminescence immunoassays among exposed populations in China and
45 neighbouring countries. Previous studies showed that specific IgG, IgM and IgA were
46 produced in response to the infection (6). The kinetics of these three classes of
47 antibodies have been described, yet correlations with the clinical outcome of the
48 patients has been poorly reported (6).

49 In this study, we implemented an indirect immunofluorescent assay for the
50 detection of anti-SARS-CoV-2 antibodies, and observed significant differences in the
51 seroprevalence and antibody titres between groups of patients depending on their
52 clinical outcome.

53

54 ## PATIENTS AND METHODS

55 **Study design.** A cohort of patients with confirmed SARS-CoV-2 infection was
56 studied at the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection in

57 Marseille, France, as previously described (7). All patients presenting symptoms
58 compatible with COVID-19 and contacts of suspected and confirmed COVID-19
59 cases were tested using a SARS-CoV-2 specific qRT-PCR assay (7, 8). Treatment
60 with hydroxychloroquine (HCQ) associated with azithromycin (AZ) was proposed to
61 all qPCR-positive patients who enrolled on a voluntary basis if they did not present
62 contraindications (7). Patients were followed-up on an out-patient basis at our day
63 care hospital or were hospitalised in the infectious disease units of the IHU, in
64 intensive care units or in other medical departments of the Assistance Publique-
65 Hôpitaux de Marseille, depending on the severity of the disease. We included in the
66 present study all patients from the previous study by Million *et al.* for whom ≥1 serum
67 sample was available for serological testing as part of the routine care of these
68 patients. The serum samples were tested retrospectively using an indirect
69 immunofluorescence assay (IFA). The time of serum collection was determined
70 relative to the date of the onset of symptoms. The non-interventional nature of this
71 study was approved by the Ethical Committee of the IHU Méditerranée Infection
72 under no. 2020-13.

73

74 **Case definition**. SARS-CoV-2 infection was defined by clinical, radiological, and
75 microbiological criteria as previously reported (3, 7). Briefly, the national early
76 warning score (NEWS) for COVID-19 was used for the classification of clinical
77 presentation of patients. Virological evidence of the infection was based on a positive
78 qRT-PCR on a nasopharyngeal sample or another respiratory sample. Pulmonary
79 involvement was evaluated by chest low-dose computed tomography for all patients.
80 Five groups of patients were constituted according to the following criteria (7): (1)

81 Patients with mild disease and good clinical and virological outcome (GO; n= 681);
82 (2) Patients with poor virological outcome defined by persistence at day 10 or more
83 of viral detection in respiratory samples (PVirO; n= 100); (3) Patients who received
84 HCQ + AZ treatment for more than three days, with poor clinical outcome requiring
85 prolonged hospitalisation for 10 days or more despite three days or more of HCQ +
86 AZ treatment (PClinO1; n= 53); (4) Patients who received HCQ + AZ treatment for
87 fewer than three days, with poor clinical outcome requiring prolonged hospitalisation
88 for 10 days or more (PClinO2; n = 25); (5) Patients with poor clinical outcome
89 requiring prolonged hospitalisation for 10 days or more leading to death (PClinO3; n=
90 29). Main characteristics of the patients in each group are summarised in Table 1.

91 **Indirect immunofluorescence assay.** Anti-SARS-Cov 2 antibodies were detected
92 using an in house indirect immunofluorescence assay (IFA), as previously described
93 (9). Vero E6 cells (ATCC CRL-1586, Rockville, MD, USA) infected with the SARS-
94 CoV2 strain IHU-MI2 (full genome sequence of this strain was deposited under the
95 European Molecular Biology Laboratory EMBL project accession no. PRJEB38023)
96 (10) were harvested between 24 hours and 48 hours post-inoculation when
97 cytopathic effect begins to be observed before massive cell lyses begin, washed with
98 sterile phosphate buffered saline (PBS) (Oxoid, Dardilly, France) and inactivated
99 using 5% paraformaldehyde. This preparation was used as the antigen and 50 nL of
100 antigen were spotted on each well of 18-well microscope glass slides using Echo
101 525 Liquid Handler instruments (Labcytes, Cannock, United Kingdom) that uses
102 acoustic energy to transfer liquid from a 96-well plate containing the antigen to
103 slides. Fifty nanolitres of uninfected Vero cells were also spotted on each well as a
104 negative control and a clinical isolate of *Staphylococcus aureus* (identified by matrix-
105 assisted laser desorption ionization-time of flight mass spectrometry) (11) was

106 spotted on each well in order to ensure further serum deposition, as previously
107 described (12). Each slide was air dried, fixed in acetone for 10 minutes and
108 conserved at 4°C in the dark.

109 In a first step, each serum sample was screened for the presence of anti-
110 SARS CoV-2 antibodies using the IFA, as previously described (9). Serum samples
111 were heat-decomplemented for 30 minutes at 56°C, diluted in 3% PBS-milk and 25
112 µL of a 1:50 dilution and a 1:100 dilution were pipetted onto a 18-spot slide then
113 incubated for 30 minutes at 37°C in the dark to be screened for the detection of total
114 immunoglobulin (IgT). After washing thrice, the slides with sterile PBS for 10
115 minutes, 25 µL of total FITC-conjugated IgT anti-human immunoglobulin (Bio-Rad,
116 Marnes-la-Coquette, France) with 0.5% Evans blue (Bio-Rad) were incubated for 30
117 minutes at 37°C. After washing, slides were observed under a fluorescence
118 microscope (AxioSkop 40, Zeiss, Marly le Roi, France). In a second step, all the
119 serum samples screened positive at a 1:100 dilution were quantified for IgG, IgM and
120 IgA as reported above, except that serum samples were diluted up to 1:1,600 for IgA
121 and IgM and 1:3,200 for IgG; and anti-IgG, anti-IgM and anti-IgA conjugates were
122 used (bioRad). Serum samples exhibiting positivity at 1:3,200 were further tested up
123 to 1:6,400. A serum sample exhibiting a 1:400 titre collected from one patient who
124 was positive by SARS COV-2 RT-PCR, was anonymised and used as a positive
125 control on each slide for screening and on each run for antibody quantification. A
126 negative serum collected in December 2019 from a patient and PBS-milk 3% were
127 used as negative controls on each slide screened. In order to interpret the IFA, any
128 serum sample exhibiting IgG 1:100 was considered as positive; as well as any serum
129 sample exhibiting isolated IgM or IgA 1:200.

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

143 **Statistical analysis.** To avoid bias in data analysis, we studied the serological
144 response according to the time of sampling of the sera related to the date of the
145 onset of symptoms. The analysis of sera was divided into different times (D0-D5, D6-
146 D10, D11-D15 and D16-D38). For the studied of seroprevalence and for the
147 comparison of IgG titre, we considered only the sera with the higher IgG titre or with
148 the higher IgM or IgA titre when several sera were available for a same patient. For
149 the data comparisons and statistical analyses, Fisher's exact test or the Chi-squared
150 test and standard statistical software (GraphPad Prism 5) were used. A p-value <
151 0.05 was considered statistically significant. ROC curves were calculated using
152 GraphPad Prism 5.

153

154 **RESULTS**

155 **IFI assay.** In the negative control group of 200 serum samples collected from
156 patients in November and December 2018 before the emergence of COVID-19 in
157 France, no IgG and no IgA were detected and three samples exhibited a IgM titre of
158 1:25 for two samples and 1:100 for one sample (Figure 1). In the group of serum
159 samples known to yield cross-reactivities, two samples collected from patients
160 diagnosed with Epstein-Barr virus infection (n = 14) exhibited IgG titre 1:200, one to
161 1:100 and two IgM titre ≥ 1:100; two samples collected from patients diagnosed with
162 Cytomegalovirus infection (n = 08) exhibited IgM titre 1:100; one sample collected
163 from patients diagnosed with hepatitis A (n = 07) exhibited an IgM titre at 1:200 and
164 two at 1:100; and one sample collected from patients diagnosed with hepatitis E (n =
165 25) exhibited IgG titre at 1:400; and one serum exhibited IgM titre at 1:100. Of the 50
166 serum samples collected from patients diagnosed with another Coronavirus other
167 than COVID-19, none reacted in IgG, none reacted in IgA and six reacted at 1:100 in
168 IgM, two reacted at 1:200 and one reacted at 1:800 (Table 2). No positivity was
169 observed from 10 serum samples drawn from toxoplasmosis patients. Also, 36 sera
170 collected from patients diagnosed with non-Coronavirus pneumonia yielded an IgG
171 titre at 1:400 (n = 3) and an IgG titre at 1:100 (n = 6). Overall, 13/350 serum samples
172 yielded a false positivity of IgG ≥ 1:100, yielding a 96.3% specificity for IgG; and
173 05/350 serum samples yielded a false positivity of IgM ≥ 1:200, yielding a specificity
174 of 98.6% for IgM. Specificity of IgA titre of 1:200 was 100%.

175 We then evaluated the serological response in a collection of 1,302 serum
176 samples from 888 patients infected with SARS-CoV-2 between 12 March and 17
177 April 2020 (7). This cohort, which included 408 men (46%), had a median age of 45
178 years (range, 14–97 years). Median age of patients from PClinO1, PClinO2, PClinO3

179 group were significantly higher than the median age of patients from PVirO and GO
180 group (p<0.0001). Serum samples had been collected at a median time of 15 days
181 (range, 0–38 days) after onset of symptoms. Seventy (5.4%) sera were collected
182 between D0-D5, 238 (18.3%) between D6-D10, 395 (30.3%) between D11-D15 and
183 599 (46%) between D15-D38. Multiple sera were available for 299 patients. At least
184 one positive serology was found in 330 patients, leading to a global seroprevalence
185 of 37.2%. The time distribution of positive serum samples was as follows: 3%
186 between D0-D5, 13% between D6–D10, 27% between day D11–D15 and 47% after
187 D16. We observed 88 (29%) seroconversions that occurred between D6–D10 in 6
188 (7%) cases, between D11–D15 in 25 (28%) cases and after D16 in 57 (65%) cases.
189 Only two patients were observed to be positive within five days after onset of the
190 illness, one patient exhibited IgG titre 1:100 and another patient with IgG titre at
191 1:1,600 and IgA at 1:100.

192 Detailing the results for each group of patients, the median time of serum
193 sampling was 8, 11, 11, 16 and 16 days after the onset of symptoms for PClinO3,
194 PClinO2, PClinO1, PVirO and GO, respectively. Global seroprevalence by group
195 was 29% in PClinO3, 56% in PClinO2, 49% in PClinO1, 44% in PVirO and 29% in
196 GO patients. Higher seroprevalence was observed in group of PClinO3, PClinO2,
197 PClinO1 compared to GO group between D6-D10 but this was not significant.
198 However, significant higher seroprevalence between patients with poor clinical
199 outcome compared to patients with good clinical outcome was observed after D10
200 (Figure 2). Higher seroprevalence was found in PClinO3 (70%), PClinO2 (71%),
201 PClinO1 (57%) compared to patients with good clinical outcome (GO) (37%),
202 p=0.046, p=0.01 and p= 0.015, respectively. In particular, the five deda patients had

203 exhibited positive serology after day 16. No significant difference was observed
204 between PVirO and GO group.

205 We also compared IgG titre between the five groups of patients on sera
206 collected at least 10 days after the onset of symptoms. We found significant higher
207 IgG titre in patients with a poor clinical outcome (died PClinO3, PClinO2, PClinO1)
208 compared to patients with good outcome (GO) (p=0.0007) (Figure 3).

209

210 DISCUSSION

211 We developed an indirect immunofluorescence assay for the detection of IgG, IgM
212 and IgA anti-SARS CoV-2 antibodies and we used it to assess the serological status
213 of hundreds of COVID-19 patients and controls, as such an assay has been only
214 reported on a very small group of patients (13). In order to avoid false negative
215 results, the assay incorporated *S. aureus* as a control of deposition of tested sera, as
216 *S. aureus* protein A and protein M bind non-specifically to any serum antibody (12).
217 The assay also incorporated non-infected Vero cells on which the viral antigen has
218 been produced, in order to identify false positive reactivities. Reading of both
219 controls was incorporated into the interpretation algorithm. Accordingly, the
220 specificity of the assay was measured at 100% for IgA, 98.5% for IgM and 95.9% for
221 IgG.

222 Using this assay, we observed low values of seroprevalence, at 37% in RT-
223 PCR confirmed COVID-19 patients, ranging precisely from 3% before five days'
224 evolution to 47% after 15 days' evolution. However, seroconversions of specific IgM
225 and IgG antibodies were observed as early as day four after the onset of symptoms,
226 as previously described (2). This low seroprevalence is here observed in a

227 population of treated patients with a favourable clinical evolution and outcome in
228 most of these patients. In contrast, we identified that patients with severe disease
229 developed a serological response in most cases (and all patients who died) that was
230 characterised by high levels of IgG; in agreement with previous reports that antibody
231 levels were higher after a severe and critical infection than after a mild infection (14-
232 16). An immediate antibody response was observed in severe cases while it
233 appeared later in mild cases (15, 16). On the other hand, an analysis of patients with
234 mild symptoms of COVID-19 showed that SARS-CoV-2 can persist in patients who
235 developed specific IgG antibodies for a very long period of time, up to 35 days,
236 whereas a patient who did not develop an IgG response cleared the virus after 46
237 days (17).
238 Thus, high antibody titres were associated with severe disease regardless of
239 age, gender and comorbidities, and there was no correlation between an early
240 adaptive humoral response and improved clinical outcome (14). These results
241 therefore call into question the much hoped-for role for serotherapy in SARS-CoV-2
242 infection. The use of convalescent plasma with high levels of neutralising antibodies
243 planned at the onset of the pandemic for the treatment of severe COVID-19
244 infections may not be an effective treatment option (18-20).

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

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384

385 **Figure Legends.**

386 **Figure 1. Picture of immunofluorescence assay of serum sample from a**
387 **COVID-19 Infected patient**. Each well of glass slides was spotted with SARS-Cov-2
388 antigen (A), non-infected VERO cells (B) and *S. aureus* antigen (C). Left panel,
389 patient’s serum with anti-SARS-CoV-2 total immunoglobulins detectable at dilution
390 1:100. Patient presented IgG titer at 1:400, IgM titer at 1:50 and IgA titer at 1:100.
391 Right panel, negative control serum. Slides were observed using Zeiss microscope,
392 objective x40.

393 **Figure 2.** Comparison of seroprevalence among the five groups of patients (a)
394 Between days 6 and 10 (b) Between days 11 and 15 (c) between days 16 and 38 (d)
395 After day 38.

396 **Figure 3.** Comparison of IgG titre detected at least 10 days after the onset of
397 symptoms between the different group of patients infected with SARS-CoV-2. When
398 multiple sera were available for a same patient, only the sera with higher IgG titre
399 were considered for this analysis.

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