1   **Validation of a commercially available SARS-CoV-2 serological Immunoassay**

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3   **Benjamin Meyer[#,1], Giulia Torriani[#,2], Sabine Yerly[3], Lena Mazza[3], Adrien Calame[4], Isabelle**

4   **Arm-Vernez[3], Gert Zimmer[5,6], Thomas Agoritsas[7,8], Jérôme Stirnemann[7], Hervé**

5   **Spechbach[9], Idris Guessous[9], Silvia Stringhini[9,10], Jérôme Pugin[11], Pascale Roux-**

6   **Lombard[12], Lionel Fontao[13], Claire-Anne Siegrist[1], Isabella Eckerle[2,4,14], Nicolas**

7   **Vuilleumier*[,#,12,15], Laurent Kaiser*[,#,3,4,14] for the Geneva Center for Emerging Viral**

8   **Diseases**

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10   #contributed equally

11   *corresponding authors

12

13   **Affiliations:**

14   *[1]Centre for Vaccinology, Department of Pathology and Immunology, University of Geneva,*

15   *Geneva, Switzerland*

16   *[2]Department of Microbiology and Molecular Medicine, University of Geneva, Geneva,*

17   *Switzerland*

18   *[3]Laboratory of Virology, Geneva University Hospitals, Geneva, Switzerland*

19   *[4]Division of Infectious Disease, Geneva University Hospitals, Geneva, Switzerland*

20   *[5]Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*

21   *[6]Department of Infectious Diseases and Pathobiology (DIP), Vetsuisse Faculty, University of*

22   *Bern, Bern, Switzerland*

23   *[7]Division of General Internal Medicine, Department of Medicine, Geneva University Hospitals,*

24   *Geneva, Switzerland*

25   *[8]Department of Health Research Methods, Evidence, and Impact, Hamilton, Ontario, Canada*

26   *[9]Division and Department of Primary Care Medicine, Geneva University Hospitals, Geneva,*

27   *Switzerland*

28   *[10]Unit of Population Epidemiology, Division of Primary Care, Geneva University Hospitals,*

29   *Geneva, Switzerland*

30   *[11]Division of Intensive Care, Geneva University Hospitals, Geneva, Switzerland*

31   *[12]Division of Laboratory Medicine, Department of Diagnostics, Geneva University Hospitals and*

32   *Geneva University, Geneva, Switzerland*

33   *[13]Division of Dermatology and of Laboratory Medicine, Geneva University Hospitals, Geneva,*

34   *Switzerland*

35   *[14]Geneva Centre for Emerging Viral Diseases, Geneva University Hospitals, Geneva,*

36   *Switzerland*

37   *[15]Division of Laboratory Medicine, Department of Medicine, Faculty of Medicine, Geneva,*

38   *Switzerland*

39

40   Address for correspondence:

41   Laurent Kaiser: Laurent.Kaiser@hcuge.ch

42   Nicolas Vuilleumier: Nicolas.Vuilleumier@hcuge.ch

Abstract:

Objectives: To validate the diagnostic accuracy of a Euroimmun SARS-CoV-2 IgG and IgA immunoassay for COVID-19.

Methods: In this unmatched (1:1) case-control validation study, we used sera of 181 laboratory-confirmed SARS-CoV-2 cases and 176 controls collected before SARS-CoV-2 emergence. Diagnostic accuracy of the immunoassay was assessed against a whole spike protein-based recombinant immunofluorescence assay (rIFA) by receiver operating characteristic (ROC) analyses. Discrepant cases between ELISA and rIFA were further tested by pseudo-neutralization assay.

Results: COVID-19 patients were more likely to be male and older than controls, and 50.3% were hospitalized. ROC curve analyses indicated that IgG and IgA had high diagnostic accuracies with AUCs of 0.992 (95% Confidence Interval [95%CI]: 0.986-0.996) and 0.977 (95%CI: 0.963-0.990), respectively. IgG assays outperformed IgA assays (p=0.008). Taking an assessed 15% inter-assay imprecision into account, an optimized IgG ratio cut-off > 1.5 displayed a 100% specificity (95%CI: 98-100) and a 100% positive predictive value (95%CI: 97-100). A 0.5 cut-off displayed a 97% sensitivity (95%CI: 93-99) and a 97% negative predictive value (95%CI: 93-99). Substituting these thresholds for the manufacturer's, improved assay performance, leaving 12% of IgG ratios indeterminate between 0.5-1.5.

Conclusions: The Euroimmun assay displays a nearly optimal diagnostic accuracy using IgG against SARS-CoV-2 in patient samples, with no obvious gains from IgA serology. The optimized cut-offs are fit for rule-in and rule-out purposes, allowing determination of whether individuals in our study population have been exposed to SARS-CoV-2 or not. IgG serology should however not be considered as a surrogate of protection at this stage.

Introduction:

67

68 High throughput and reliable serological assays detecting antibodies against SARS-CoV-2 are

69 essential to determine the proportion of SARS-CoV-2 infected individuals and estimate the

70 current seroprevalence in the general population or in high-risk groups, such as health care

71 workers. Serological assays can complement diagnostic strategies focusing on the identification

72 of the infectious agent during the acute phase of disease. Unlike RT-PCR, they can identify

73 infected individuals that remained asymptomatic or undiagnosed, which are both frequent

74 conditions during SARS-CoV-2 infection, long after the initial infection. Validated serological

75 assays are also key to understanding the (immuno)-pathophysiology of COVID-19 in various

76 patients' groups and will be critical to characterize responses elicited by the numerous vaccine

77 candidates in development (1).

78 Designing serological testing strategies with high sensitivity and specificity and with acceptable

79 positive (PPV) and negative predictive values (NPV) is far from trivial, and requires taking two

80 major analytical aspects into account: analytical specificity and sensitivity (2),(3). The former

81 may largely be determined by the degree of cross-reactivity with other CoVs, which frequently

82 cause common colds in humans (i.e. HCoV-229E, -NL63, -OC43 and -HKU1) (4) resulting in

83 seroprevalence rates usually above 90% in adults (5).

84 This cross-reactivity occurs when virus-specific antigenic epitopes are highly similar and

85 recognized by the same B cells. It is best defined by the proportion of "false-positive" SARS-

86 CoV-2 results in individuals who were never exposed to this pathogen. In contrast to common

87 cold CoVs, the seroprevalence for MERS-CoV is low even in endemic countries (6). Therefore,

88 cross-reactivity between MERS-CoV and SARS-CoV-2 is not a critical factor when assessing

89 population seroprevalence. Previous studies have shown that antibodies against common cold

90 CoVs can cause considerable cross-reactivity in serological assays, depending on the type of

91 assay and antigens used. Particularly, whole virus- or nucleocapsid protein-based assays

92 showed a higher cross-reactivity compared to whole spike or S1 domain-based assays resulting

93 in lower specificity (4). During the early phase of an outbreak, when SARS-CoV-2

94 seroprevalence is low, serological testing strategies must have a very high specificity to reach a

95 high positive predictive value (PPV) and avoid false positive results.

96 On the other hand, analytical sensitivity is strongly influenced by the epidemic course, the

97 disease biology, and numerous analytical factors. All these interrelated items are primarily

98 influenced by the intrinsic immunogenicity of the SARS-CoV-2 antigens and the magnitude and

99 duration of B cell responses elicited by infection, be it asymptomatic, benign, moderate or

100 severe (2),(3).

4

101   Last but not least in the context of a pandemic, the availability of high throughput and reliable
102   diagnostic platforms is key for health care systems to effectively handle the testing demand,
103   while respecting clinically compatible diagnostic turnaround times (TAT). In this study, we
104   performed an extensive validation of a high throughput SARS-CoV-2 commercial serological
105   platform quantifying both serum IgG and IgA against the S1 protein. As reference method, we
106   used a whole spike-based recombinant immunofluorescence assay (rIFA) (4)·(7)·(8). Selected
107   sera from SARS-CoV-2-infected patients were assessed for their neutralization capacity using a
108   pseudovirion assay (see below).
109
110   **Methods:**
111   **Aims**
112   The aim of this study is to validate and define the operational cut-off values of a commercially
113   available ELISA-based SARS-CoV-2 serological assay that could be applied at large scales to
114   reliably determine the presence of specific IgG as a marker of SARS-CoV-2 infection. This study
115   used RT-PCR confirmed cases, but the goal was to be able to identify exposure to SARS-CoV-2
116   by immunoassay alone. Therefore, the ELISA results were compared against recombinant
117   immunofluorescence assay (rIFA), which was considered as the reference method due to its
118   demonstrated high specificity for serology of other CoVs such as MERS-CoV (6). The
119   secondary goal is to assess the potential added value of IgA in patients recently infected with
120   SARS-CoV-2.
121
122   **Study population**
123   Negative control serum samples (n=176) were collected for various serological testing in our
124   routine laboratory and stored for analytical validation. These sera were collected in 2013, 2014
125   and 2018 before the start of the outbreak and thus have not been exposed to SARS-CoV-2.
126   Sera (n=181) of PCR-confirmed COVID-19 patients were collected at the University Hospitals of
127   Geneva from hospitalized patients (n=91) as well as from patients from the outpatient clinic
128   (n=90). Ethical approval for all sera used in this study was waived by the local ethics committee
129   of the HUG that approves usage of leftover of patient serum collected for diagnostic purposes.
130   The number of days from symptom onset to blood collection was based on patient history
131   whenever this information was available or could be retrieved in a reliable way; otherwise, we
132   used the date of RT-PCR positivity as a surrogate for onset of symptoms. Serum samples from
133   unmatched PCR-confirmed COVID-19 hospitalized patients were collected for routine diagnostic
134   purposes under a general informed consent and outpatients were asked if they were willing to

135 return to the hospital after their symptoms had subsided and to donate a serum sample under
136 written general informed consent.
137

Blood sample collection

139 We used serum samples collected on patient admission, during hospitalization or, when
140 needed, left over sera used for other routine investigations. Samples were immediately
141 processed and then frozen and stored at −20°C until ELISA, recombinant immunofluorescence
142 analyses, and pseudoneutralization were performed (see below). Patients were sampled at
143 different times after onset of symptoms (dpos) or according to days post RT-PCR diagnosis
144 (dpd) if the onset was not known. To compare the seropositivity between different time points,
145 we grouped patients in the following dpos/dpd categories: 0-10 (n=8, 4.4%), 11-20 (n=112,
146 61.9%) and 21-39 (n=61, 33.7%).
147

SARS-CoV-2 RT-PCR analyses

149 SARS-CoV-2 RT-PCR was performed according to manufacturers' instructions on various
150 platforms, including initially in house method using eMAG (bioMérieux, France) and Charité RT-
151 PCR protocol(9), then BD SARS-CoV-2 reagent kit for BD Max system (Becton, Dickinson and
152 Co, US) and Cobas 6800 SARS CoV2 RT-PCR (Roche, Switzerland).
153

Serum IgG and IgA ELISA

155 Both IgG and IgA enzyme-linked immunosorbent assays (ELISA) used the S1-domain of the
156 spike protein of SARS-CoV-2 as antigen. Sera were diluted at 1:101 and assessed with the IgG
157 and IgA CE-marked ELISA (Euroimmun AG, Lübeck, Germany # EI 2606-9601 G and # EI
158 2606-9601 A) according to the manufacturer's instructions. They were run on Dynex Agility
159 (Ruwag, Switzerland) according to the manufacturer's protocol. After adding the conjugate,
160 samples' immunoreactivity was measured at an optical density of 450nm (OD450) and then
161 divided by the OD450 of the calibrator provided with each ELISA kit to minimize inter-assay
162 variation(8). The quantitative results obtained were then expressed in arbitrary units and
163 interpreted as follows: OD ratio: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive.
164 Inter-assay variation was 15.6% for IgG at a ratio of 2.09 (n=17) and 17.7% for IgA at a ratio of
165 4.85 (n=7).
166

Recombinant immunofluorescence assay

6

168   The IgG antibody response against the spike protein of SARS-CoV-2 was assessed by rIFA as

169   described and previously validated for MERS-CoV (6)·(7). Briefly, Vero B4 cells were

170   transfected with the mammalian expression vector pCG1-SCoV2-S (kindly provided by M.

171   Hoffmann and S. Pöhlmann, DPZ, Göttingen, Germany) using Fugene HD (Promega, #E2311).

172   After 24h of incubation cells were detached and residual trypsin was removed by centrifugation

173   at 300x g for 5min. 50µl of transfected cells were seeded at a density of $2x10^5$/ml on multi-test

174   glass slides (DUNN Labortechnik GmbH #40-412-05) and incubated for 6h at 37°C, 5% CO_2.

175   Afterwards slides were washed 2x with PBS and fixed for 10min using ice-cold

176   Acetone/Methanol (ratio 1:1). For rIFA staining, slides were rehydrated for 10min using PBS +

177   0.1% Tween20 (PBS-T) and blocked with 5% milk in PBS-T for 30min at RT. Sera were diluted

178   1:40 in blocking buffer, 30µl were applied on each spot and incubated for 60min at 37°C or RT.

179   After 3x washing with PBS-T, secondary goat anti-human-IgG antibody conjugated with

180   Alexa488 (Jackson ImmunoResearch, #109-545-088) was diluted 1:200 in PBS and 25µl were

181   applied to each spot. The secondary antibody was incubated for 45min at 37°C and slides were

182   washed 3x with PBS-T afterwards. Slides were briefly rinsed with dH_2O and mounted using

183   glycerol. rIFA results were judged by 3 observers independently and the inter-observer kappa

184   correlation was 0.59 (95%CI: 0.31-0.87) between observer 1 and 2, 0.62 (95%CI: 0.33-0.91)

185   between observer 2 and 3 as well as 0.75 (95%CI: 0.52-0.99) between observer 1 and 3, which

186   could be considered a substantial level of agreement(10).

187

188   **VSV-based pseudo-neutralization assay**

189   VeroE6 cells were seeded in 96-well plates at $2 x 10^4$ cells per well and grown into confluent

190   monolayer overnight. Sera from patients were inactivated at 56°C for 30 minutes and diluted 1:5

191   in 120 µl DMEM 2% FCS in the first column of a 96-well plates (in duplicate). Remaining wells

192   were filled with 60 µl of DMEM 2% FCS. Two-fold dilutions were performed until 1:80 was

193   reached by moving 60 µl from one well column to the following one. VSV-based SARS-CoV-2

194   pseudotypes (generated according to Berger, Rentsch, and Zimmer (11) and Torriani et al. (12))

195   expressing a 19 amino acids C-terminal truncated spike protein (13) (NCBI Reference

196   sequence: NC_045512.2) were diluted in DMEM 2% FCS in order to have MOI=0.01 in 60 µl

197   volume and added on top of serum dilutions (final serum dilutions obtained were from 1:10 to

198   1:160). The virus-serum containing plate was incubated at 37°C, for 2h. Vero E6 were then

199   infected with 100µl of virus-serum mixtures. After incubation at 37°C for 1.5h, cells were washed

200   once with 1X PBS and DMEM 10% FCS was added. After 16-20h of incubation at 37°C, 5%

201   CO_2 cells were fixed with 4% formaldehyde solution for 15 min at 37°C and nuclei stained with

202 1µg/ml DAPI (AppliChem #A4099) solution. GFP positive infected cells were counted with

203 ImageXpress® Micro Widefield High Content Screening System (Molecular Devices) and data

204 analyzed with MetaXpress 5.1.0.41 software.

205

206 **Statistical analyses**

207 Analyses were performed with Graph Pad Prism version 8.3.1 software using Fisher's bilateral

208 exact test and Mann–Whitney *U*-test where appropriate. ROC analyses were performed

209 using ANALYSE-IT™ software for Excel (Microsoft, Redmond, WA, USA) to: (i) confirm the cut-off

210 values, prospectively proposed by the manufacturer, when compared to IFA results as the

211 reference method (samples with very weak fluorescence signal just above background were

212 deemed putative negatives, and were considered as negative for the purpose of this analysis);

213 (ii) compare the diagnostic accuracy of IgG alone, IgA alone, or both combined; and (iii)

214 determine the optimal rule-in cut-off (PPV of 100%), a rule-out cut-off with a NPV above 95%,

215 and an indeterminate interval, taking into account the analytical imprecision-derived least

216 significant change (LSC). The LSC represents the smallest significant detectable difference

217 between two measurements based given the analytical imprecision, and is conventionally

218 defined as $1.96 * \sqrt{2} * VC$ (14)(15). AUC comparisons were performed according to the

219 nonparametric approach proposed by DeLong *et al.* (16). Sensitivity (SE), specificity (SP), PPV

220 and NPV with the respective 95% CIs are given. A value of *p*<0.05 was considered statistically

221 significant.

222

223 **Results:**

224 The baseline demographic characteristics of the participants are summarized in table 1. There

225 was a higher proportion of males (61.3%) vs females (38.7%) in COVID-19 patients compared

226 to controls (Fisher's exact test, p=0.002). Among COVID-19 patients, we included an equal

227 representation of hospitalized patients (n=91, 50.3%) and outpatients (n=90, 49.7%).

228

229 **Prevalence of IgG seropositivity according to rIFA**

230 An in-house developed recombinant immunofluorescence assay (rIFA) using the whole spike

231 protein of SARS-CoV-2 as antigen was used to assess spike-specific serum IgG. Since rIFA

232 was used as a confirmatory assay, we maximized its specificity by interpreting putative

233 negatives as negative to avoid false positive results. Among negative control samples, we found

234 no positive, five putative negative (2.8%) and 171 (97.2%) negative samples indicating a

235 specificity of 100%. Among COVID-19 samples there were 165 (91.2%) positive, two (1.1%)

8

236   putative negative and 14 (7.7%) negative samples, indicating an overall detection rate of 91.2%.

237   We found that rIFA seropositivity was low at 0-10 dpos/dpd (12.5%) but increased to 92.0% and

238   100% in sera collected at 11-20 and 21-39 dpos/dpd, respectively (table 2). Similar results were

239   found in hospitalized and outpatients. Thus, in our sample of 181 COVID-19 samples, rIFA

240   proved a robust method for the detection of SARS-CoV-2 spike-specific IgG.

241

242   **Diagnostic accuracies of IgG and IgA ELISA against rIFA**

243   Overviews of OD ratios for IgG and IgA are shown in Figure 1 A and B, respectively. In control

244   sera, we found no positive, five (2.8%) indeterminate and 171 (97.2%) negative samples for

245   IgG, and 14 (7.7%) positive, 10 (5.7%) indeterminate and 157 (86.7%) negative samples for IgA

246   according to the manufacturer defined cut-off (EI cut-offs) (table 2). Considering the

247   indeterminate values as positive resulted in a specificity of 97.3% (IgG) and 86.4% (IgA),

248   respectively. Analysis of COVID-19 samples revealed 154 (85.1%) positive, one (0.6%)

249   indeterminate and 26 (14.4%) negative samples for IgG, and 164 (90.6%) positive, four (2.2%)

250   indeterminate and 13 (7.2%) negative samples for IgA. This resulted into an overall significantly

251   higher seropositivity for IgA (90.6%) than IgG (85.1%) in the S1-based ELISA (p= 0.041).

252

253   Next, we analysed the seropositive rates of both ELISAs at different dpos or dpd (Figure 1E)

254   according to the EI cut-offs. Sera collected 21 dpos/dpd had a similarly high seropositivity for

255   both IgG (96.7%) and IgA (96.7%) (p >0.99). A higher seropositivity was observed for IgA

256   compared to IgG for sera collected at 11-20 dpos/dpd (91.1% vs 84.8%, p= 0.0264) but not 0-10

257   dpos/dpd (37.5% vs 0%, p= 0.2). No significant difference was found between hospitalized and

258   outpatients in IgG or IgA ELISA (p= 0.833, and p= 1.000, respectively).

259

260   ROC curve analysis (Fig 1; C and D) indicated that overall, both IgG and IgA had a high

261   diagnostic accuracy with respective AUCs of 0.992 (95%CI: 0.986-0.996) and 0.977 (95%CI:

262   0.963-0.99), respectively. Although modest, this AUC difference was found to be significant

263   according to the de Delong method (p=0.008). Combining IgG and IgA ELISAs together (AUC

264   0.986; 95%CI: 0.976-0.995) tended to decrease the diagnostic accuracy of the model when

265   compared to IgG alone (p=0.080), but outperformed IgA alone (p<0.001). Although these

266   differences were modest, our results indicate that IgG ELISA displays the optimal fit with IgG

267   rIFA and that IgA did not improve diagnostic value. In a subgroup analysis considering only sera

268   harvested before 21 days after symptoms onset (n=120), ROC curve analyses displayed similar

9

269   results - with an AUC of 0.981 for the IgG ratio and of 0.965 for the IgA ratio. At these early

270   time-points, the AUC difference between IgG and IgA ratio was not significant (p=0.19).

271

272   Thirteen patient samples had discrepant or potentially discrepant results, i.e. were negative or

273   indeterminate in ELISA and putative negative or positive in rIFA. To investigate this

274   discordance, we performed a VSV-based pseudovirus neutralization assay (pseudo-NT) for 1)

275   these 13 samples (n=13), 2) five negative control samples, including one sample that showed

276   putative negative results in rIFA, and 3) seven patient samples that were positive by ELISA IgG,

277   IgA and rIFA (table 3). All samples that showed negative and putative negative rIFA results

278   were confirmed negative, whereas all rIFA positive samples were confirmed as positive by the

279   pseudo-NT assay (table 3). These findings support the accuracy of the rIFA and our decision to

280   count putative negative rIFA results as negative.

281

282   **Optimizing the threshold for IgG serology**

283   Taking into account the variation coefficient (VC) of 15% at a low positivity IgG ratio (2.09), and

284   assuming that such VC could at least be transposed to the 1.1 cut-off ratio, the minimal LSC

285   would be 0.42. Added to the 1.1 ratio, an IgG ratio cut-off of 1.5 (1.1 + 0.42) would be the lowest

286   able to secure 100% PPV while taking into account the analytical imprecision. Furthermore, an

287   LSC of 0.42 also indicates that the indeterminate zone proposed by the manufacturer (between

288   0.8 and 1.1) should be reconsidered, as it falls within the analytical imprecision range. At the 1.5

289   IgG ratio cut-off, ROC curve analyses indicated that the SE was 86%, the SP 100%, and the

290   NPV 89%. Thus, selecting a 1.5 cut-off, rather than the recommended 1.1 cut-off for IgG

291   seropositivity, allows the securing of a PPV of 100% despite a 15% imprecision. Higher VCs

292   would translate into a higher seropositivity cut-off to secure an identical PPV. For rule-out

293   purposes (i.e. the seronegativity lower cut-off), the best trade-off IgG ratio cut-off was found to

294   be < 0.5. At this value, the SE was 97%, the SP 87%, the NPV 97% and the PPV 86% (table 4).

295   This defines an indeterminate range between IgG ratios of 0.5 and 1.5, which represented 43

296   cases (12%) of our samples (including 20 control and 23 COVID-19 samples). In this

297   indeterminate zone, all 20 sera from controls were confirmed as negative (n=19) or putative

298   negative (n=1) by rIFA. For indeterminate samples of COVID-19 patients, five were negative,

299   one was a putative negative and 18 were positive by rIFA.

300   In the subgroup of patients whose samples were taken before 21 dpos, using the manufacturer

301   IgG ratio seropositivity cut-off (1.1), the SE was 91%, the SP 100%, the PPV 100%, and the

302   NPV 62%. At the manufacturer seronegative cut-off (<0.8), the SE was 91%, the SP 100%, the

303   PPV 100%, and the NPV 64% (table 4). Importantly, no patient displayed a ratio between 0.8

304   and 1.1 in this subgroup, preventing us from estimating the importance of the indeterminate

305   cases.

306   Using the aforementioned optimized IgG ratio cut-off for IgG seropositivity (1.5), the SE was

307   82%, the SP 100%, the PPV 100%, and the NPV 46%. At the cut-off for IgG seronegativity

308   (<0.5), the SE was 96%, the SP 69%, the PPV 95%, and the NPV 73% (table 4). The number of

309   undetermined cases between 0.5 and 1.5 IgG ratio values represented 17% (20/120) of the

310   subgroup. Taken together, these results indicate that if the seropositivity cut-off of 1.5 for the

311   IgG ratio would be suitable for patients with sample taken <21 days post symptoms onset, the

312   seronegative cut-off of 0.5 would display a non-optimal NPV for rule-out purposes. The optimal

313   seronegative (NPV 100%) cut-off would have been <0.4, at the cost of a modest increase of

314   indeterminate cases to 20% (24/120). Thus, this assay performs best for convalescent samples

315   taken as of 21 dpos.

316

317   **Discussion:**

318   The key finding of this validation study, derived from a large cohort of laboratory-confirmed

319   SARS-CoV-2 cases and unmatched negative controls, is that the present CE-IVD marked

320   immunoassay for IgG has a good diagnostic accuracy with an AUC of 0.99 and outperformed

321   IgA according to ROC curve comparisons. These results parallel the ones obtained by two other

322   smaller studies (8)·(17), which tested the non CE-marked ELISA version and showed potentially

323   better diagnostic performances of IgG compared to IgA ELISA but lower AUCs compared to this

324   study. Our improved results might result from the higher number of negative controls and

325   COVID-19 patient sera tested, and from the decreased non-specific background relative to that

326   observed in the earlier studies. Despite the greater performance of the IgG based ELISA, it was

327   reassuring to see that the IgA results correlated well with the IgG results and gave very similar

328   AUCs upon ROC analyses. However, knowing if and how IgA could be used in clinical practice

329   to further refine rule-in our rule-out strategies still remains elusive.

330

331   The second notable finding of this study is that the current manufacturer cut-offs are prone to

332   misinterpretations and should not be used without proper evaluation before routine testing.

333   Keeping in mind the challenges of developing a serological assay for a new viral disease in an

334   emergency situation; securing both rule-in and rule-out cut-offs is key to mitigate the

335   unavoidable risk of false positive and false negative results due to the combination of a highly

336   dynamic pre-test probability variation, with a suboptimal seroconversion time when studies are

337   undertaken at the peak of an epidemic. Our analyses revealed the following limitations of the

338   manufacturer's seropositivity cut-off. First, with an inter-assay imprecision of 15% assessed at

339   an IgG ratio of 2.09 (above than the 1.5 two cut-off value selected) translating into a LSC of 0.42

340   IgG ratio, our results indicate that the analytical imprecision is higher than the range of the

341   indeterminate zone proposed by the manufacturer, which encompasses a delta of 0.3 IgG ratio.

342   This implies that any result within the 0.8-1.1 IgG ratio range could be randomly either above,

343   within or below these values just because of analytical imprecision. Secondly, with a LSC of

344   0.42 our results indicated that a higher IgG ratio cut-off value was needed to secure an optimal

345   specificity and PPV. Adding the 0.42 LSC to the 1.1 cut-off yielded a 1.5 ratio as the IgG

346   seropositivity cut-off with a PPV of 100%, the lower end of the 95%CI still being compatible with

347   a 97% rule-in strategy. Notably, using this cut-off in the subgroup of patients under 21 dpos/dpd,

348   the SP and PPV were still 100%, however, with broader confidence intervals. Similarly, in an

349   attempt to maximize the negative predictive value at the rule-out cut-off, the cut-off had to be

350   decreased from 0.8 to 0.5 IgG ratio in order to reach an overall NPV of 97%, with a 93% at the

351   lower end of the 95CI. In the subgroup of patients under 21 dpos/dpd this interval was found to

352   be substantially larger (95%CI: 45-91). Taken together, these results indicate that at this stage

353   the optimal rule-in cut-off should be set at >1.5 of IgG ratio for seropositivity and at <0.5 of IgG

354   ratio for rule-out purposes (seronegativity). With these optimized "Geneva's" cut-offs, the

355   indeterminate zone represented 12% of the cases overall (20 controls and 23 COVID-19

356   samples), representing a volume easily amenable to further confirmation tests like rIFA.

357

358   In this respect, the 13 discordant cases between ELISA and rIFA were tested using the pseudo-

359   NT assay. This indicated that all non-positive samples (negative and putative negative) by rIFA

360   were negative in the pseudo-NT assay, and that all positive samples by rIFA (but ELISA

361   negative) were positive in the pseudo-NT assay. One sample that was indeterminate in ELISA

362   and positive in rIFA was also positive in pseudo-NT. These findings may indicate an earlier IgG

363   antibody response, which could better mediate virus neutralization, against the S2 domain of the

364   spike protein, as previously suggested for SARS-CoV-1 neutralizing antibodies targeting the S2

365   domain (18)·(19)·(20)·(21). Although based upon a limited number of observations which

366   prevents us from drawing final conclusions, these results suggest that rIFA may be an

367   appropriate confirmatory assay in the future.

368   Regarding potential limitations, we need to highlight several additional points. We evaluated an

369   ELISA assay measuring antibodies against the S1 domain of the spike protein and not against

370   the full protein, which may contain other highly relevant epitopes from the S2 domain. Such

371   factors could potentially explain why some samples were negative by ELISAs but positive by

372   rIFA, as the whole-spike protein is used in rIFA. Secondly, we strongly emphasize two important

373   cut-off limitations related to the analytical imprecision. At the level of the analytical imprecision,

374   the SARS-CoV-2 IgG intra-individual biological variation being unknown, we had to use the LSC

375   instead of the reference change value (22), which most likely would have translated into a

376   higher seropositivity cut-off. Along the same line, because the intra-lot imprecision of the

377   reagents is still undetermined, but expected to be higher than 15%, this could have a similar

378   impact on cut-off determination. Nevertheless, these cut-offs implemented in routine testing at

379   the Geneva University Hospitals (GE-cut-offs) proved useful in the management of a number of

380   clinically compatible COVID-19 patients with negative PCR results.

381   Importantly, this study is a diagnostic accuracy validation study and not a seroprevalence study.

382   This implies that the current seropositivity cut-off has to be considered with caution in population

383   studies. Indeed, the 100% PPV achieved in this study was due to combined effect of a 100%

384   specificity and a 1:1 distribution of control and samples form patients with positive SARS-CoV-2

385   PCR. Therefore, in population seroprevalence studies with a lower expected proportion of

386   COVID-19, the PPV at the 1.5 cut-off will likely decrease. In this context, increasing the rule-in

387   IgG cut-off to a higher value or using a secondary specific confirmatory assay may become

388   necessary. Due to this limitation, at this stage we recommend the confirmation of all positive

389   ELISA results (including ratios above 1.5) using a second serological assay such as rIFA for

390   seroprevalence studies with a low pre-test probability, and the confirmation of doubtful ELISA

391   results in settings with a pre-test probability around 50%. A summary of our proposed testing

392   strategy is given in figure 2.

393

394   In conclusion, in this validation study performed on 357 sera, of which 50.7% came from

395   patients with COVID-19, we demonstrate a close to optimal diagnostic accuracy of IgG SARS-

396   CoV-2 serology of the Euroimmun assay, without any obvious gains from IgA serology. Taking

397   analytical imprecision into account, we propose optimized cut-offs allowing a PPV of 100% and

398   a NPV of 97% to be secured with an indeterminate zone comprising about 12% of the results,

399   for which additional rIFA analyses are currently necessary. Ongoing seroprevalence studies will

400   be instrumental to further refine the optimal rule-in and rule-out cut-offs, as well as the optimal

401   testing strategy, which may require a highly specific confirmatory assay. For the time being, this

402   assay seems to be fit for the purpose of enabling authorities to make informed decisions

403   regarding measures to be taken in order to manage the SARS-CoV-2 pandemic. Using the GE

404 cut-offs could also allow the determination of whether individuals have been exposed or not to

405 SARS-CoV-2 with high confidence. As a final caution, at this time simple IgG levels determined

406 by ELISA or rIFA cannot be considered as a surrogate of protection for individual patients. This

407 implies that risk mitigation decisions may not yet be based only on SARS-CoV-2 ELISA/rIFA

408 serology.

409

420

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Figure legends:

Figure 1: A and **B**: OD ratio of negative control samples (n=176) and PCR-confirmed CoVID-19 patients (n=181) were determined using an IgG and IgA ELISA. **C** and **D**: ROC curve analysis for IgG and IgA OD ratio results including the area under the curve (AUC) and 95% confidence intervals. **E** IgG OD ratios of COVID-19 patients at different days post onset of symptoms (dpos, dots) or days post diagnosis (dpd, triangles). Red dots or triangles show samples that are confirmed by whole spike recombinant immunofluorescence analysis. Dotted and dashed lines indicate the Euroimmun- (EI) or Geneva- (GE) cut-offs for negative, indeterminate and positive samples, respectively.

Figure 2: Graphical overview or serological testing strategies in low (<10%) and high (>50%) pre-test probability settings. PPV: positive predictive value; NPV: negative predictive value; rIFA: recombinant immunofluorescence assay; 95%CI: 95% confidence intervals.

Tables

Table 1

	Negative Controls			COVID-19 Patients		
	No (%)	Median (IQR)	Range	No (%)	Median (IQR)	Range
Age (years)						
All	176 (100)	39.2 (23.8)	18.0-89.5	181 (100)	53.1 (29.2)	20.1-86.9
18-40	100 (56.8)			61 (33.7)		
41-65	61 (34.7)			70 (38.7)		
66+	15 (8.5)			50 (27.6)		
Gender						
Female	97 (55.1)			70 (38.7)		
Male	79 (44.9)			111 (61.3)		
Hospitalized				91 (50.3)		
Outpatient				90 (49.7)		
DPOS/DPD						
0-10				8 (4.4)	7.0 (1.75)	0-8
11-20				112 (61.9)	15.0 (2.0)	11-20
21-39				61 (33.7)	28.0 (5.0)	21-39

DPOS days post onset of symptoms, DPD days post diagnosis, IQR inter quartile range

17

Table 2

Samples	Total No. (%)	S-rIFA IgG No. (%)			ELISA IgG No. (%) EI cut-offs[1]			ELISA IgG No. (%) GE cut-offs[2]		
		Negative	Putative Neg.	Positive	Negative	Indeterminate	Positive	Negative	Indeterminate	Positive
Negative Controls	176 (100)	171 (97.2)	5 (2.8)	0 (0)	171 (97.2)	5 (2.8)	0 (0)	156	20 (11.4)	0 (0)
COVID-19 Patient										
All	181 (100)	14 (7.7)	2 (1.1)	165 (91.2)	26 (14.4)	1 (0.6)	154 (85.1)	15 (8.3)	23 (12.7)	143 (79.0)
0-10 DPOS / DPD	8 (4.4)	6 (75.0)	1 (12.5)	1 (12.5)	8 (100)	0 (0)	0 (0)	7 (87.5)	1 (12.5)	0 (0)
11-20 DPOS / DPD	112 (61.9)	8 (7.1)	1 (0.9)	103 (92.0)	17 (15.2)	0 (0)	95 (84.8)	7 (6.3)	19 (17.0)	86 (76.8)
21-39 DPOS / DPD	61 (33.7)	0 (0)	0 (0)	61 (100)	1 (1.6)	1 (1.6)	59 (96.7)	1 (1.6)	3 (4.9)	57 (93.4)
COVID-19 Outpatients										
All	90 (100)	8 (8.9)	0 (0)	82 (91.1)	14 (15.6)	0 (0)	76 (84.4)	7 (7.8)	14 (15.6)	69 (76.6)
0-10 DPOS / DPD	5 (5.6)	4 (80)	0 (0)	1 (20)	5 (100)	0 (0)	0 (0)	4 (80.0)	1 (20.0)	0 (0)
11-20 DPOS / DPD	41 (45.6)	4 (9.8)	0 (0)	37 (90.2)	8 (19.5)	0 (0)	33 (80.5)	2 (4.9)	11 (26.8)	28 (68.3)
21-39 DPOS / DPD	44 (48.8)	0 (0)	0 (0)	44 (100)	1 (2.3)	0 (0)	43 (97.7)	1 (2.3)	2 (4.6)	41 (93.2)
COVID-19 Hospitalized										
All	91 (100)	6 (6.6)	2 (2.2)	83 (91.2)	12 (13.2)	1 (1.1)	78 (85.7)	8 (8.8)	9 (9.9)	74 (81.3)
0-10 DPOS / DPD	3 (3.3)	2 (66.7)	1 (33.3)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
11-20 DPOS / DPD	71 (78.0)	4 (5.6)	1 (1.4)	66 (93.0)	9 (12.7)	0 (0)	62 (87.3)	5 (7.0)	8 (11.3)	58 (81.7)
21-39 DPOS / DPD	17 (18.7)	0 (0)	0 (0)	17 (100)	0 (0)	1 (5.9)	16 (94.1)	0 (0)	1 (5.9)	16 (94.1)

DPOS days post onset of symptoms, DPD days post diagnosis, rIFA recombinant immunofluorescence assay

1:    EI cut-offs: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive

2:    GE cut-offs: <0.5 = negative; ≥0.5 and <1.5 = indeterminate; ≥1.5 = positive

Table 3

Sample Code	DPOS/ DPD	Type	Result			Reciprocal Endpoint Titre	
			ELISA IgG	S-rIFA IgG	ELISA IgA	Pseudo-NT 90	Pseudo-NT 50
8	NA	Neg. Ctrl.	0.79	NEG	1.29	<10	<10
9	NA	Neg. Ctrl.	0.31	NEG	0.56	<10	<10
10	NA	Neg. Ctrl.	0.57	NEG	0.79	<10	<10
11	NA	Neg. Ctrl.	0.97	NEG	2.72	<10	<10
56	NA	Neg. Ctrl.	0.81	Putative NEG	0.47	<10	<10
209	8	COVID-19 Pat.	0.73	POS	3.66	20	≥160
30181704	22	COVID-19 Pat.	1.08	POS	0.77	20	≥160
30189617	16	COVID-19 Pat.	0.60	POS	9.99	20	80
30186069	14	COVID-19 Pat.	0.55	POS	2.94	20	80
30193397	11	COVID-19 Pat.	0.56	POS	4.21	10	80
30193613	11	COVID-19 Pat.	0.51	POS	0.92	80	≥160
30193717	11	COVID-19 Pat.	0.41	POS	1.02	10	40
30197451	14	COVID-19 Pat.	0.57	POS	3.85	≥160	≥160
30197527	16	COVID-19 Pat.	0.46	POS	0.67	<10	20
30202986	18	COVID-19 Pat.	0.58	Putative NEG	1.53	<10	<10
30208864	14	COVID-19 Pat.	0.47	POS	0.33	20	80
30208885	7	COVID-19 Pat.	0.39	Putative NEG	0.48	<10	<10
30251875	27	COVID-19 Pat.	0.36	POS	0.51	10	80
202	16	COVID-19 Pat.	6.02	POS	10.70	160	≥160
205	11	COVID-19 Pat.	13.08	POS	10.93	≥160	≥160
30175134	15	COVID-19 Pat.	14.64	POS	10.41	≥160	≥160
30175147	15	COVID-19 Pat.	13.90	POS	10.41	≥160	≥160
30175152	18	COVID-19 Pat.	13.67	POS	10.41	≥160	≥160
30175157	15	COVID-19 Pat.	9.74	POS	9.65	≥160	≥160
30193442	11	COVID-19 Pat.	1.40	POS	6.48	20	80

DPOS days post onset of symptoms, DPD days post diagnosis, rIFA recombinant immunofluorescence assay,
NA not applicable, Pseudo-NT 90 and 50: pseudovirus neutralization test using 90% or 50% reduction as cut-off

502

Table 4

	Cut-Offs	Sensitivity (95%)	Specificity (95%)	Positive predictive value (95%)	Negative predictive value (95%)
All samples (n=357) IgG ratio AUC: 0.992 (95%CI: 0.986-0.996)	GE cut-offs[1] >1.5 (rule-in) <0.5 (rule-out)	86% (80-91) 97% (93-99)	100% (98-100) 87% (81-91)	100% (97-100) 86% (81-90)	89% (84-93) 97% (93-99)
	EI cut-offs[2] >1.1 (rule in) <0.8 (rule out)	93% (87-96) 94% (88-94)	100% (98-100) 98% (94-99)	100% (97-100) 97% (92-99)	94% (90-97) 95% (91-97)
<21DPOS/DPD(n=120) IgG ratio AUC: 0.981 (95%CI: 0.961-1.00)	GE cut-offs[1] >1.5 (rule-in) <0.5 (rule-out)	82% (73-88) 96% (90-99)	100% (76-100) 69% (41-88)	100% (95-100) 95% (89-98)	46% (29-63) 73% (45-91)
	EI cut-offs[2] >1.1 (rule in) <0.8 (rule out)	91% (83-95) 91% (84-96)	100% (76-100) 100% (76-100)	100% (95-100) 100% (95-100)	62% (41-79) 64% (43-81)

DPOS days post onset of symptoms, DPD days post diagnosis, AUC area under the curve
1: GE cut-offs: <0.5 = negative; ≥0.5 and <1.5 = indeterminate; ≥1.5 = positive
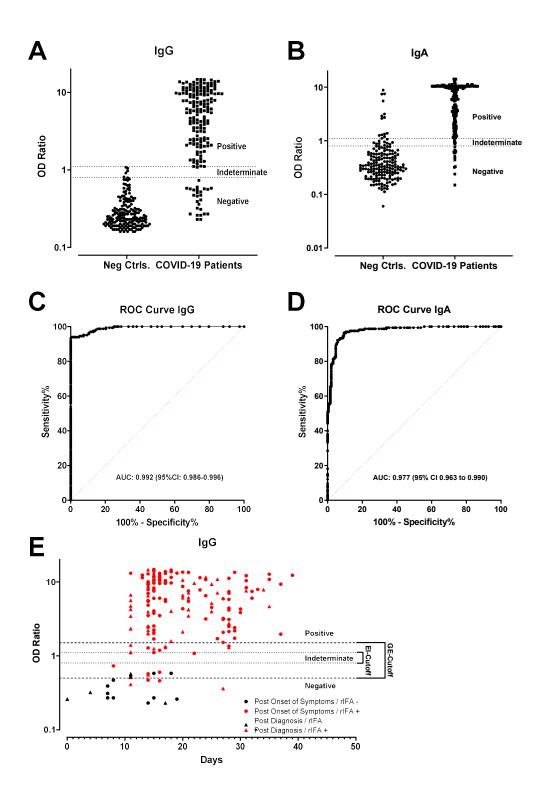2: EI cut-offs: <0.8 = negative; ≥0.8 and <1.1 = indeterminate; ≥1.1 = positive

503

504 **Figure 1**

505

506

507

508 **Figure 2**

Serological Testing Strategy SARS-CoV-2

Low pre-test probability (<10%) | High pre-test probability (>50%)

Individual testing (prevalence <10%)
- IgG ratio ≥1.5 → rIFA to confirm
- IgG ratio 0.5-1.5 → Indeterminate: rIFA to confirm or repeat ELISA in 3 weeks
- IgG ratio <0.5 → Seronegativity confirmed

Individual testing (prevalence >50%)
- IgG ratio ≥1.5 → Seropositivity confirmed PPV: 100% (95%:CI: 97-100)
- IgG ratio 0.5-1.5 → Indeterminate: rIFA to confirm
- IgG ratio <0.5 → Seronegativity confirmed NPV: 97% (95%CI: 93-99)

509