Letter to the Editor

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Evaluation of an ELISA for SARS-CoV-2 antibody testing: clinical performances and correlation with plaque reduction neutralization titer

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To the Editor,

The pandemic of coronavirus disease 2019 (COVID-19), caused by severe respiratory syndrome coronavirus 2 (SARS-CoV-2), continues to spread all around the world, with an exponential increase in the number of cases and deaths. An accurate and timely detection of SARS-CoV-2 is essential not only for diagnosing the infection, but also for establishing of infection control measures and preventing further contagions [1]. Although real-time reverse transcription polymerase chain reaction (rRT-PCR) using nasopharyngeal swabs is considered the reference method for diagnosis of acute SARS-CoV-2 infection, especially in asymptomatic and mildly symptomatic individuals, this technique is biased by several pre-analytical and analytical vulnerabilities [2]. Serological assays for SARS-CoV-2 are principally aimed to measure the antibody (Ab) response against the virus, thus allowing to estimate the prevalence of infection in a general population or in subpopulations of high-risk subjects (e.g., healthcare workers), as well as for screening potential convalescent donors and for complementing nucleic acid amplification tests (NAATs) in patients with indefinite results [3]. Both laboratory-based (chemiluminescent [CLIA] and enzyme-linked immunosorbent assays [ELISA]) and point-of-care tests (lateral flow assays [LFA]) are performed using recombinant antigens, typically encompassing the spike protein and/or its receptor binding domain (RBD), or the viral nucleoprotein (N) [4]. The purpose of this study was to evaluate an ELISA (ENZY-WELL SARS-CoV-2) detecting specific anti-SARS-CoV-2 IgM, IgA, and IgG antibodies based on native antigen obtained from Vero E6 cells infected with SARS-Cov-2, strain "2019-nCoV/Italy-INMI1" and developed by Diesse Diagnostica Senese [5]. According to the manufacturer's claims, imprecision and repeatability vary between 2.6 and 14.7% for all antibodies (IgA, IgM, and IgG) across a media index ranging from 0.5 to 1.9.

A total of 233 leftover serum samples from 152 COVID-19 patients (nine asymptomatic/mildly symptomatic, who recovered at home with supportive care and isolation, and 143 hospitalized, classified with moderate or severe disease following WHO interim guidance [6]), 81 SARS-CoV-2 negative subjects (60 healthcare workers, 13 autoimmune patients, eight pregnant women) were included in the study. All subjects underwent nasopharyngeal swab testing, analyzed by rRT-PCR as described elsewhere [7]. Briefly, RNA was extracted using an automated platform (Magna Pure 96 Instrument, Roche Diagnostics, USA) and then used for rRT-PCR, which was performed in separate reactions with SARS-CoV-2 gene E and RNaseP analyses by ABI prism® AB 7900 or 7900HT or QuantStudio™ 5 real-time PCR Systems (Applied Biosystems, USA). The threshold cycle (Ct) of SARS-CoV-2 gene E and of RNaseP was obtained after standardization of the rRT-PCR instruments software settings as follows: baseline calculated in the cycle range 3–15; fixed threshold at 0.2. Healthcare workers were considered negative (Neg-HW) on the basis of at least three negative

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Table 1: Clinical performances of ENZY-WELL SARS-CoV-2 IgA, IgM and IgG, obtained using 1.1 Signal/Cut-off index (S/CO) (claimed by manufacture's insert) and 0.8 S/CO as thresholds.

Assay	Threshold	Time-frame	Sensitivity[a] (95% CI)	Specificity[a] (95% CI)	PLR (95% CI)	NLR (95% CI)
SARS-CoV-2 IgA	1.1 S/CO	<12 d	45.2 (27.3–64.0)	98.8 (93.3–100.0)	36.6 (5.0–266.6)	0.56 (0.40–0.76)
		≥12 d	79.2 (71.0–85.9)		64.1 (9.1–450.9)	0.21 (0.15–0.30)
		Overall	73.0 (65.2–79.9)		59.1 (8.4–415.8)	0.27 (0.21–0.36)
	0.8 S/CO	<12 d	64.5 (45.4–80.8)	96.3 (89.6–99.2)	17.4 (5.6–54.5)	0.37 (0.23–0.59)
		≥ 12 d	86.4 (79.1–91.9)		23.3 (7.7–71.0)	0.14 (0.09–0.22)
		Overall	82.2 (75.2–88.0)		22.2 (7.3–67.6)	0.18 (0.13–0.26)
SARS-CoV-2 IgM	1.1 S/CO	<12 d	25.8 (11.9–44.6)	98.8 (93.3–100.0)	20.9 (2.7–160.3)	0.75 (0.61–0.93)
		≥12 d	56.0 (46.8–64.9)		45.3 (6.4–320.1)	0.45 (0.37–0.54)
		Overall	50.7 (42.4–58.9)		41.0 (5.8–289.6)	0.50 (0.42–0.59)
	0.8 S/CO	<12 d	25.8 (11.9–44.6)	97.5 (91.4–99.7)	10.4 (2.3–46.5)	0.76 (0.62–0.94)
		≥12 d	75.2 (66.7–82.5)		30.5 (7.7–120.1)	0.25 (0.19–0.35)
		Overall	66.4 (58.3–73.9)		26.9 (6.8–106.3)	0.34 (0.27–0.43)
SARS-CoV-2 IgG	1.1 S/CO	<12 d	48.4 (30.2–66.9)	100.0 (95.5–100.0)	79.4 (4.9–288.6)	0.52 (0.37–0.73)
		≥12 d	93.6 (87.8–97.2)		152.9 (9.6–2425.4)	0.07 (0.04–0.13)
		Overall	84.2 (77.4–89.6)		137.7 (8.7–2185.4)	0.16 (0.11–0.23)
	0.8 S/CO	<12 d	54.8 (36.0–72.7)	100.0 (95.5–100.0)	89.7 (5.6–1447.5)	0.46 (0.31–0.67)
		≥12 d	95.2 (89.8–98.2)		155.5 (9.8–2466.5)	0.05 (0.02–0.11)
		Overall	86.8 (80.4–91.8)		142.0 (8.9–2253.1)	0.13 (0.09–0.20)

PLR, positive likelihood ratio; NLR, negative likelihood ratio; d, days.
Subjects included in the analyses: Overall, n=233; time frame <12 d, n=112 (31 positive and 81 negative to SARS-CoV-2 rRT-PCT); time frame ≥ 12 d, n=206 (125 positive and 81 negative to SARS-CoV-2 rRT-PCR).
[a]Sensitivities and specificities values are expressed as percentages.

sequential rRT-PCR results obtained between February 26th and May 29th, 2020. For SARS-CoV-2 positive patients, the median time from symptoms onset and sample collection was 24 days (interquartile range [IQR], 14–29 days; overall range 4–93 days). For a subgroup of 52 samples collected from SARS-CoV-2 positive subjects, plaque reduction neutralization test (PRNT) was also performed, according to a slightly modified protocol from Suthar et al. [8]. In this assay, neutralization titer was defined as reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% ($PRNT_{50}$). Stata v 16.1 (StataCorp, LakeWay Drive, TX) was used for the statistical analyses. The study protocol (number 23307) was approved by the Ethics Committee of the University-Hospital of Padova (Padova, Italy).

The diagnostic performance of ENZY-WELL SARS-CoV-2, evaluated by calculating the area under the receiver operating characteristics curve (AUC) over the entire study period (overall data), revealed comparable results for IgA (AUC 0.935 [95%CI: 0.904–0.967]), IgM (AUC 0.921 [95%CI: 0.887–0.954]) and IgG (AUC 0.942 [95%CI: 0.911–0.972]). Overlapping confidence intervals indicated that AUC results were not significantly different. Diagnostic sensitivity and specificity were then assessed on the overall data, in two period (<12 days and ≥12 days) and with two different cut-offs, as shown in Table 1. Specificity was found to be optimal for all immunoglobulin classes assays and for each period, being always >96%. The lowest sensitivity was found for IgMs <12 days, whilst the highest sensitivity was observed for IgGs ≥12 days. Positive and negative likelihood ratios were also calculated, and the best results were found for IgG. According to Colavita et al., performance significantly varies considering the two period <12 days and ≥12 days, especially for IgM and IgG [5]. This is expected, since several studies demonstrated that, although immunoglobulin rise could be considered significant from 6–7 days after symptom onset, the vast majority of patients had positive IgGs after day 11th [9, 10]. As concerns the neutralization $PRNT_{50}$ values, the highest correlation was found with IgMs (rho = 0.732; p<0.001), followed by IgG (rho = 0.541; p<0.001) and IgA (rho = 0.480; p<0.001). These results are in keeping with data published by Perera et al., who also showed highly significant correlation between $PRNT_{50}$ and IgM ELISA results [11].

For an additional series of seven SARS-CoV-2 positive patients with severe/moderate disease, time kinetics of IgAs, IgMs, and IgGs were evaluated. Up to five repeated samples were available for these patients (n=4 for one and n=5 for six patients), collected in a period of time ranging between 4 and 24 days (Figure 1). Although comparable findings were found for the kinetics of IgAs and IgGs, evidence suggests that IgAs responds seemingly earlier than IgGs in moderate/severe SARS-CoV-2 patients (Figure 1). These findings are aligned with those previously published by our group, using a different immunoassay [10]. Notably, IgM titers appeared lower compared to those of IgA and IgG

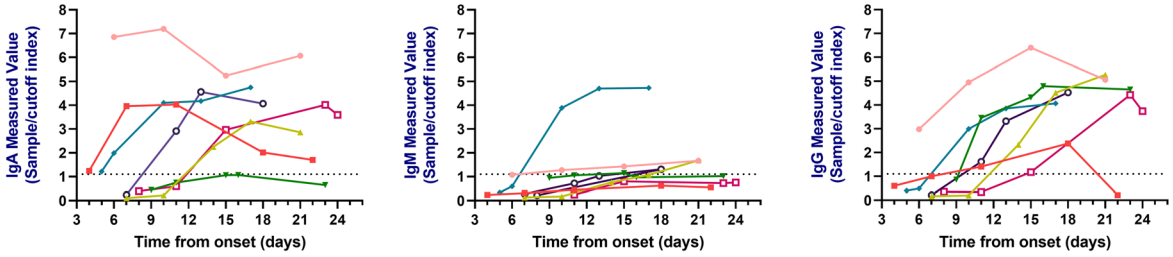

Figure 1: Spaghetti plot of IgA, IgM, and IgG levels of the seven patients with up to five serial antibody determinations after the onset of symptoms. Dotted lines showed the manufacturer's cut-off (1.1 S/CO).

values. Moreover, IgM titer remained below 1.1 S/CO (i.e., manufacturer's cut-off) in two of seven patients.

In conclusion, ENZY-WELL SARS-CoV-2 immunoassay displayed excellent performance, especially for the IgGs, showing high sensitivity (lower 95% confidence interval >89%) and specificity (lower 95% confidence interval >95%). Neutralization titers were instead strongly correlated with IgM assays values. These results might be attributable to the assay design, and the use of native antigen, obtained from Vero E6 cells infected with the SARS-Cov-2, strain "2019-nCoV/Italy-INMI1". Finally, time kinetics suggests that a detectable IgA immune response may develop early during the infection, though the clinical role of this class of antibodies requires further scrutiny.

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