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Evaluation of a new simultaneous anti-SARS-CoV-2 IgA, IgM and IgG screening automated assay based on native inactivated virus

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ABSTRACT

In addition to molecular testing, there is evolving interest for anti-SARS-CoV-2 antibodies serologic assays. Majority of them focus on IgM/IgG despite IgA important role in mucosal immunity.

A simultaneous anti-SARS-CoV-2 IgA/IgG/IgM immunoassay, performed on an automated instrument by ELISA kit coated with native inactivated SARS-CoV-2, was detected on two control groups (negative swab healthcare workers; pre-pandemic healthy or with other viral infections individuals) and on two COVID-19 patient groups (early and late infection).

Specificities were 100% in all groups, indicating no cross-reactivity with other infectious or pre-pandemic sera. Sensitivities were 94% in early infection group and 97% in total positive patient group, reaching 100% in late infection group.

To our knowledge, this is the first technique based on native SARS-CoV-2. It is able to identify more positive samples than kits using recombinant antigens, therefore virus native epitopes as well as simultaneous anti-SARS-CoV-2 IgA/IgM/IgG detection could help to contain COVID-19 spreading.

1. Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, has resulted in a global pandemic with hundreds of thousands of deaths and millions of illnesses. Diagnosis of SARS-CoV-2 infection is principally dependent on RT-PCR using nasal and throat swabs, which is not ideally suited to mass population testing [1]. Moreover, it can give false negative results, depending on sampling and extraction methods or on the presence of a low viral load, causing an underestimation of SARS-CoV-2 infection prevalence as well as serious consequences due to contagious individuals circulation [2,3].

In addition to molecular testing, there is increasing interest for use of serologic assays to detect antibodies against SARS-CoV-2. Unlike molecular testing, the immune response to the virus is an indirect marker of infection and it should be exploited to determine the true number of infections using a surveillance approach, essential to maintain safe

patient care standards and support public health efforts [4].

Timing of immunoglobulin production (from 4 days after symptoms onset, to 10–14 days) can limit its applicability in the acute phase diagnosis [5,6], but in general IgM and IgA anti-SARS-CoV-2 antibodies are rapidly formed in response to infection and their detection may significantly increase COVID-19 patients diagnostic sensitivity when combining serological tests with molecular tests [7]. Most serological assays rely on IgM and IgG antibodies although IgA antibodies play an important role in mucosal immunity. Besides typical respiratory symptoms, digestive symptoms have been frequently reported. IgA assays could be useful, along with IgG and IgM, in patients with atypical symptoms, in pauci-symptomatic cases or when specific SARS-CoV-2 RT-PCR remains negative in suspected subjects. A complete serological screening comprising IgA, IgM and IgG detection could be more consistent as a security strategy to prevent virus spreading [8].

SARS-CoV-2 is a single-stranded RNA virus with four major structural proteins: envelope protein (EP), membrane protein (MP),

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nucleocapsid protein (NP) and spike protein (SP). The NP is the most abundant viral protein shed during infection. The SP consists of 2 subunits, called S1 and S2: S1 contains the receptor-binding domain (RBD) required for binding to the host angiotensin-converting enzyme 2 (ACE2) receptor [9]; S2 is involved in membrane fusion [10]. The NP, SP proteins and RBD domain appear to be the main humoral immune response targets in coronavirus infections, including SARS-CoV-2, but detection of antibodies directed against spike proteins or their subunits seems to have a neutralizing activity and could better describe the immunization state.

Nevertheless, many steps in the antibodies manufacturing process focus on the use of purified antigens, which are often from recombinant sources and may present non-native epitopes, while it should be essential to recognize the native, physiologically occurring epitopes [11].

Current literature describes over 200 immunoassays available worldwide, highlighting results discrepancies. They can differ in: specimen type (whole blood, finger-stick whole blood, serum, plasma); antibody classes detected (IgA, IgM, IgG); format (enzyme-linked immunosorbent assays, ELISA; chemiluminescent immunoassays, CLIA; lateral flow immunoassays, LFIA); SARS-CoV-2 antigens used in the assays (recombinant nucleocapsid protein, spike glycoprotein subunit 1 or spike glycoprotein receptor binding domain) [4].

These findings point out a great variability among different serological kits, suggesting the importance of accredited laboratories validations and the requirement of an international consensus on methodologies and SARS-CoV-2 antigens used.

Such validations must include specificity assessment with samples from healthy people, or with non-infectious diseases, and individuals with other common infectious pathogens antibodies, collected prior to the outbreak start [4].

We formerly proposed two flowcharts where serological tests, integrated with nasopharyngeal RT-PCR swab, were included to help social and work activities implementation after the pandemic acute phase. In the first flowchart anti-SARS-CoV-2 IgG and IgM detection, was considered as a tool for a safe readmission at work [12]; then, with these new evidences, we added anti-SARS-CoV-2 IgA measurement for a complete and more reliable screening on general population [13].

From this point of view, we report the results of a simultaneous anti-SARS-CoV-2 IgA/IgM/IgG detection, performed on an automated instrument by ELISA kit coated with native inactivated SARS-CoV-2 antigens.

The combined assay was carried out on a control group (healthcare workers with negative nasopharyngeal swabs); on a secondary control group (pre-pandemic healthy individuals screened for routine analysis or pre-pandemic patients with other viral infections) and on two different COVID-19 patient groups: early infection time patients (ranging from 1 to 9 days from first access to Emergency Department and from first positive nasopharyngeal swab); late infection time patients (ranging from 19 to 41 days from first access to Emergency Department and from first positive nasopharyngeal swab).

2. Materials and methods

2.1. Patients and serum specimens

Serum samples were recovered, in accordance with local ethical approvals (R.S.44.20), from “Tor Vergata” University COVID-Hospital of Rome as follows: 45 hospitalized patients with positive SARS-CoV-2 RT-PCR (mean age 67.5 years \pm 16.5 years; 26 males and 19 females), collected on days 1 to 9 from first access to Emergency Department and from first positive nasopharyngeal swab (early infection time group); 42 hospitalized patients with positive SARS-CoV-2 RT-PCR (mean age 71.1 years \pm 12.8 years; 23 males and 19 females), collected on days 19 to 41 from first access to Emergency Department and from first positive nasopharyngeal swab (late infection time group); 44 negative SARS-CoV-2 RT-PCR subjects (mean age 41.7 years \pm 11.1 years; 23 males

and 21 females) collected from Tor Vergata Hospital physicians and healthcare workers screened for internal surveillance (control group) and 74 pre-pandemic individuals screened on 2018 for routine serology analysis (mean age years 44.9 \pm 13.9 years; 42 males and 32 females), including 54 healthy subjects, 10 patients positive to Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection and 10 patients positive to Human Immunodeficiency Virus (HIV) infection (pre-pandemic control group).

2.2. Real time polymerase chain reaction (RT-PCR)

Nasopharyngeal swabs were tested for SARS-CoV-2 infection with Seegene AllplexTM2019-nCoV Assay (Seegene, Seoul, South Korea), according to the manufacturer protocols. Automated RNA extraction and PCR setup were carried out using Seegene NIMBUS, a liquid handling workstation. RT-PCR was run on a CFX96TMDx platform (Bio-Rad Laboratories, Inc., CA, USA) and subsequently interpreted by Seegene Viewer Software. The Seegene AllplexTM2019-nCoV Assay identifies the virus by multiplex real-time PCR targeting three viral genes (E, RdRP and N), thus complying with international validated testing protocols.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Immunoenzymatic assay for the combined determination of IgA, IgM and IgG class antibodies to SARS-CoV-2 in human serum, performed with single-test, ready-to-use disposable devices on the automated CHORUS Instrument (DIESSE Diagnostica Senese spa, Siena, Italy), at 37 °C. The “Chorus SARS-CoV-2 Screen Serum” commercial kit (DIESSE Diagnostica Senese spa, Siena, Italy) is based on the principle of competitive enzyme immunoassay and the antigen bound to the solid phase is the inactivated native SARS-CoV-2, as declared by manufacturer. Anti-SARS-CoV-2 immunoglobulins present in serum samples compete with anti-SARS-CoV-2 spike protein monoclonal antibody conjugated to horseradish peroxidase (HRP). After washing to eliminate unbound conjugate proteins, a chromogenic solution with HRP substrate (tetramethylbenzidine; TMB) is added, developing a blue color. Optical density (OD) is inversely proportional to the quantity of anti-SARS-CoV-2 antibodies present in the samples. Results are calculated semi-quantitatively by a ratio between a cut-off OD value and samples OD values (cut-off OD/sample OD) and are expressed as Cut Off Index (COI).

According to the manufacturer’s instruction, the cut-off index is negative for all the values < 0.9 COI; equivocal for all the values between 0.9 and 1.1 COI; positive for all the values > 1.1 COI. This test is CE approved.

2.4. Statistical analysis

Specificity and sensitivity were calculated by Receiver Operating Characteristic Curves (ROC Curve). All data were analyzed using Med Calc Ver.18.2.18 (MedCalc Software Ltd, Ostend, Belgium). The investigators were blinded to the group allocation during the experiment.

2.5. Ethical statement

The study was performed according to “Tor Vergata” University COVID-Hospital of Rome local ethical approvals (protocols no. R. S.44.20). Informed consent was obtained from all subjects enrolled in the study. The study was in accordance with the Helsinki Declaration, as revised in 2013.

3. Results

Anti-SARS-CoV-2 IgA/IgM/IgG levels in four sets of serum samples are shown in Fig. 1; the analytical parameters (area under curve (AUC), sensitivity and specificity) are summarized in Table 1. They have been

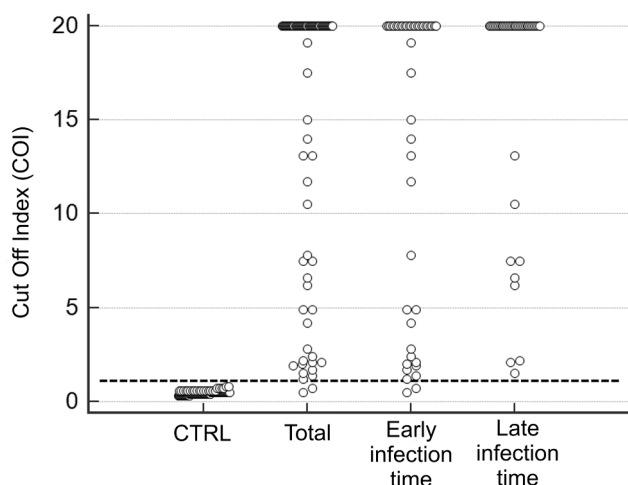


Fig. 1. Anti-SARS-CoV-2 IgA/IgM/IgG levels in control group, total positive patients group, early infection time group and late infection time group, respectively. The dotted line represents the manufacturer’s cut-off index (1.1 COI).

Table 1
Sensitivity, specificity and area under curve (AUC) values of the simultaneous anti-SARS-CoV-2 IgA/IgM/IgG screening assay.

Control Negative Group N = 118	Total (1–41 days) N = 87	Early infection time (1–9 days) N = 45	Late infection time (19–41 days) N = 42
Sensitivity (%)	97	94	100
Specificity (%)	100	100	100
Kit Cut-off	>1.1 COI	>1.1 COI	>1.1 COI
Area under the ROC curve (AUC); 95% Confidence interval	0.996; 0,974 to 1,000	0,992; 0,963 to 1,000	1; 0,977 to 1,000
Laboratory Cut-off	>0.8 COI	>0.8 COI	>0.8 COI
Sensitivity (%)	98	96	100
Specificity (%)	100	100	100

correlated to the manufacturer’s cut-off and recalculated on a best fit cut-off that emerged from our data analysis.

The ROC curves display excellent AUC values between groups, especially for the late infection patient group (0.992 and 1, respectively); recalculating the ROC curve combining all positive patients into one group, an equally excellent AUC value of 0.996 was obtained (Fig. 2).

Specificities are 100% in all groups, thus indicating no cross-reactivity with antibodies from other infectious diseases (such as HBV, HCV and HIV) or sera from pre-pandemic individuals. Sensitivities are very high for the early infection group (94%) and the total positive patient group (97%), reaching 100% in the late infection time group.

Interestingly, our previous results on the same samples [13] detecting separately anti-SARS-CoV-2 IgA, IgM and IgG with three different ELISA plates, showed lower sensitivities (82%, 80%, 94% in the early infection group, respectively; 90%, 92%, 98% in the late infection group, respectively) probably due to a limitation generated by the presence of only specific recombinant antigens coated on the microwells (NP, S1 and S2). Moreover, patients not detected on those assays are now detected with this simultaneous assay, sometimes with a low cut-off index, suggesting the presence of anti-SARS-CoV-2 antibodies not directed against nucleocapsid or spike proteins or rather recognizing N or S proteins native epitopes.

To notice, sensitivities and specificities results have been calculated with the manufacturer’s positive cut-off index of 1.1. Our best-fit positive cut-off from experimental data was 0.8, giving a further sensitivity increase from 94% to 96% in early infection group and from 97% to 98%

in total positive group, which could probably allow to identify a higher number of positive individuals on a massive testing.

4. Discussion

To estimate the extent of virus circulation and the protection likelihood against a re-infection, there is a crucial need to add serology to the testing algorithms. The required performance of a serological assay will depend on specific aims, which may be either population screening (in the general population or at-risk populations) or diagnostic support [14]. As asymptomatic cases exist, the real percentage and how long they carry the virus is still not known, therefore screening for virus-specific IgA, IgM and IgG antibodies could be an informative and decisive factor to control the pandemic as it is the main indicator of population immunity development [7].

For serological tests, manufacturers have often demonstrated very good performances in terms of sensitivity and specificity [15,16]. However, for antibody testing in acute disease, the sensitivity is highly dependent on the kinetics of antibody development. Similarly, specificity is dependent on the type of samples selected to evaluate cross-reactions [17].

Sensitivity problems can derive from several conditions: early antibody assessment during infection, especially for methodologies detecting only IgG; SARS-CoV-2 infection with asymptomatic or mild forms, where IgG production could be absent or low while IgM and IgA are more frequently observed; the antigens used in the assay, most of them are recombinant specific SARS-CoV-2 proteins, therefore native epitopes or other virus proteins cannot be identified.

Furthermore, manufacturers have made new devices available in record time, probably preferring specificity over sensitivity in order to prevent bad publicity in case of false positive reactions [17]. Currently, some countries recommend the use of rapid antigen detection as support for COVID-19 diagnosis, based on the investigation of SARS-CoV-2 proteins in respiratory samples. The sensitivity substantially decreases when the viral load decreases, leading to false negative results, which can have great consequences during this time of pandemic [18].

We have previously published a flowchart in which the combined anti-SARS-CoV-2 IgA/IgM/IgG detection could be considered for a screening on general population and serological positivity would be an “alert” to better investigate whether subjects are currently infectious or not, in order to avoid and contain new outbreaks [13].

Our data, obtained on a simultaneous IgA/IgM/IgG assay with native inactivated SARS-CoV-2 antigens, support and corroborate the flow-chart. In fact, specificities and sensitivities results are excellent, giving values of 100% for both parameters, leading to a small number of false positive or false negative individuals, that is crucial for a broad population screening. Moreover, the choice to use native viral antigens proved to be much more effective than recombinant specific antigens and it should also be noted that the assay is performed on a fully automatic instrument able to send results to Laboratory Information System, limiting manual errors and technologist exposition to biologic samples.

Furthermore, since a sensitivity of 100% was found on the late infection group, this denotes a persistent antibody positivity over time, useful for the use of serological screening to understand the overall infection rate in communities, including the rate of asymptomatic infections.

Nevertheless, our study has several limitations related to the small number of patients analyzed. Besides, in patients hospitalized for COVID-19, the humoral response to SARS-CoV-2 can be exacerbated giving high sensitivities values, whereas specificities should be assessed considering also cross-reactions to other viruses of coronavirus family.

5. Conclusions

As expected, serological testing analytical performance is becoming more efficient compared to the early stages of this pandemic but kit

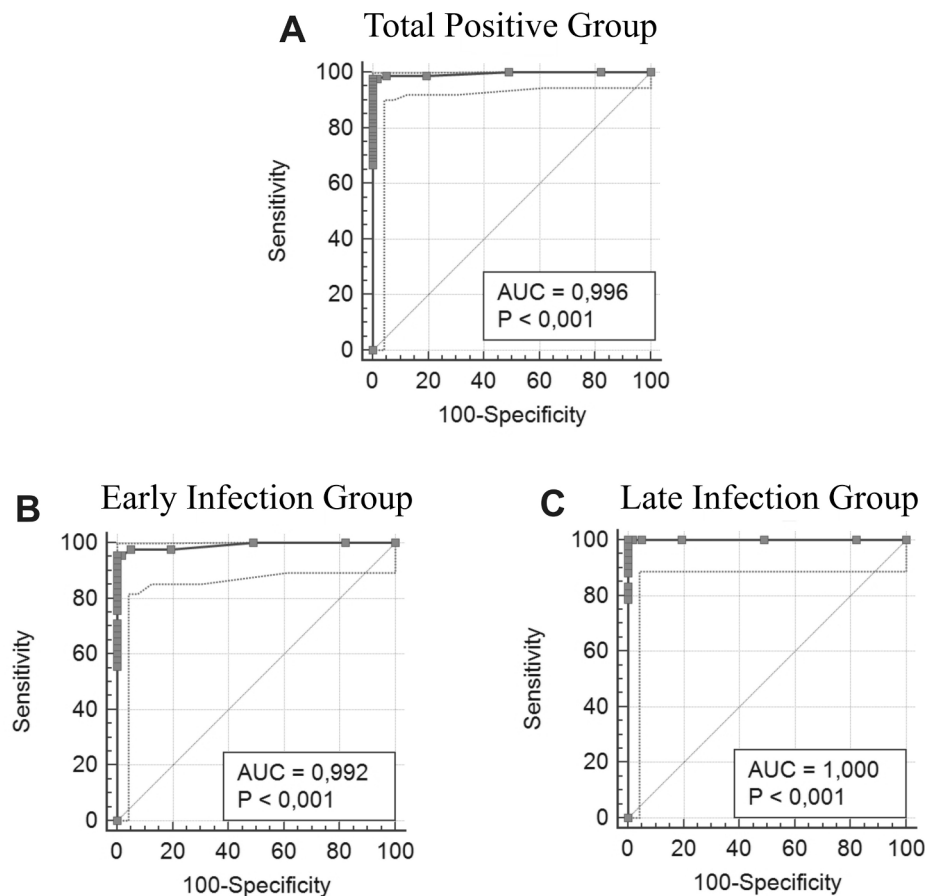


Fig. 2. Simultaneous anti-SARS-CoV-2 IgA/IgM/IgG screening assay ROC curves. AUC values are shown in panel A for the total positive patients group, in panel B for the early infection time group and in panel C for the late infection time group (0.996, 0.992 and 1.000, respectively; p value < 0.001).

evaluations have never been done with the same panel of samples. Hundreds of immunoassays are available around the world, showing a great variability of the results mainly depending on the methodology or on the nature of the antigens.

To our knowledge, this is the first technique based on the use of native inactivated virus as antigen recognized by anti-SARS-CoV-2 antibodies. We highlight the ability of this kit to identify more positive samples than those using recombinant antigens, therefore virus native epitopes and simultaneous anti-SARS-CoV-2 IgA/IgM/IgG detection could be a better strategy to understand the extent of COVID-19 spread.

CRedit authorship contribution statement

Marzia Nuccetelli: Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Massimo Pieri:** Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Francesca Gisone:** Formal analysis, Methodology, Visualization. **Serena Sarubbi:** Formal analysis, Methodology, Visualization. **Marco Ciotti:** Formal analysis, Methodology, Visualization. **Massimo Andreoni:** Conceptualization, Visualization, Supervision. **Sergio Bernardini:** Conceptualization, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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