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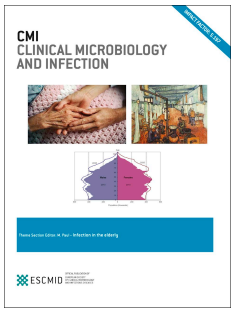

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Detection of SARS-CoV-2 N-antigen in blood during acute COVID-19 provides a sensitive new marker and new testing alternatives

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Keywords: COVID-19; SARS-CoV-2; Antigen; Antigenemia; Diagnostic; Serum; Plasma; Blood

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ABSTRACT

Objectives. Molecular assays on nasopharyngeal swabs remain the cornerstone of COVID-19 diagnostic. The high technicalities of nasopharyngeal sampling and molecular assays, as well as scarce resources of reagents, limit our testing capabilities. Several strategies failed, to date, to fully alleviate this testing process (e.g. saliva sampling or antigen testing on nasopharyngeal samples). We assessed the clinical performances of SARS-CoV-2 nucleocapsid antigen (N-antigen) ELISA detection in serum or plasma using the COVID-19 Quantigene® (AAZ, France) assay.

Methods. Performances were determined on 63 sera from 63 non-COVID patients and 227 serum samples (165 patients) from the French COVID and CoV-CONTACT cohorts with RT-PCR confirmed SARS-CoV-2 infection, including 142 serum (114 patients) obtained within 14 days after symptoms' onset.

Results. Specificity was 98.4% (95% confidence interval [CI], 95.3 to 100). Sensitivity was 79.3% overall (180/227, 95% CI, 74.0 to 84.6) and 93.0% (132/142, 95% CI, 88.7 to 97.2) within 14 days after symptoms onset. 91 included patients had a sera and nasopharyngeal swabs collected in the same 24 hours. Among those with high nasopharyngeal viral loads, i.e. Ct value below 30 and 33, only 1/50 and 4/67 tested negative for N-antigenemia, respectively. Among those with a negative nasopharyngeal RT-PCR, 8/12 presented positive N-antigenemia; the lower respiratory tract was explored for 6 of these 8 patients, showing positive RT-PCR in 5 cases.

Conclusion. This is the first evaluation of a commercially available serum N-antigen detection assay. It presents a robust specificity and sensitivity within the first 14 days after symptoms onset. This approach provides a valuable new option for COVID-19 diagnosis, only requiring a blood draw and easily scalable in all clinical laboratories.

INTRODUCTION

Molecular assays on nasopharyngeal swabs remain the cornerstone of COVID-19 diagnostic. Despite massive efforts, the high technicalities of nasopharyngeal sampling and molecular assays, as well as scarce resources of reagents, limit our testing capabilities. Several strategies failed, to date, to fully alleviate this testing process, *e.g.* saliva sampling [1,2] or antigen testing on nasopharyngeal samples [3,4]. Nucleocapsid-antigen (N-antigen) has been detected in the serum of SARS-CoV-infected patients and, recently, it has been demonstrated in SARS-CoV-2 infected patients by a single study with a global sensitivity of 41/64 patients [5,6].

In this work, we assessed the performances of N-antigen sera detection in a large patients' population using the first commercially available assay, the COVID-19 Quantigene® (AAZ France) providing a low limit of detection at 2.98 pg/mL.

METHODS

Patients and ethics. Negative samples are composed of 50 pre-pandemic samples (collected between December 2, 2019 and January 13, 2020) and 13 pandemic samples from SARS-CoV-2 non-infected patients that tested positive for other microbial antigens (*i.e.* NS1 antigen, HBs antigen, HIV-1 p24 antigen, HKU1 coronavirus or malaria antigens). Positive samples were collected between January 25, 2020 and September 2, 2020 from study participants included in the French COVID (clinicaltrials.gov NCT04262921) and CoV-CONTACT cohorts (clinicaltrials.gov NCT04259892). We selected the first serum samples available after COVID-19 diagnosis (cf. Supplementary Figure 2). The following serum samples of those patients, when collected at physician discretion, were also included. They have provided written informed consent for the use of their samples for research. Ethics approval was given by the French Ethics Committee CPP-Ile-de-France 6 (ID RCB: 2020-A00256-33 and ID RCB: 2020-A00280-39) and the French National Data Protection Commission (approval #920102).

For COVID-19 patients, available sera samples were classified into different categories according to the delay since symptom onset: sera collected ≤14 days post-symptoms onset (142 sera from 114 patients), samples collected >14 days post-symptoms onset (81 samples from 72 patients), samples collected from asymptomatic patients (3 sera from 3 patients), and patient without date of symptom onset (1 sera from 1 patient). Distribution of sera samples according to date of sampling and hospitalization status is detailed in Supplementary Figure 2.

N-antigen levels assessment. Prior to analysis, sera samples were stored at -80°C. N antigenemia levels were determined with a being marketed CE-IVD ELISA microplate assay, COVID-Quantigene® (AAZ), according to manufacturer recommendations. Briefly, in each well of 96-wells microplates

previously coated with anti-SARS-CoV-2 N-antibodies, 50 µL of a solution containing biotinylated anti-SARS-CoV-2 N antibodies and 50 µL of sera were added. After incubation at 37°C for 60 minutes, plates were washed 5 times with a phosphate buffer solution. Then, 100 µL of a solution containing HRP-conjugated streptavidin were added, followed by incubation for 30 minutes at 37°C. Plates were washed 5 times with the phosphate buffer solution, then 50 µL of a solution containing the substrate (3,3',5,5'-tetramethylbenzidine (TMB)) and 50 µL of a second solution containing urea were added. After 15 minutes at 37°C, the colorimetric reaction was stopped by adding 50 µL of H_2SO_4. Absorbance values were measured at 450nm, with a reference set at 630nm. In each plate, standards made of recombinant N antigens were tested, to quantify the N antigenemia levels for each patient's sample. As the purpose of this study was to assess the sensitivity of this new assay, samples with titers above 180 pg/mL were not diluted for precise quantification.

RT-PCR assays. For all patients included in this study, diagnosis of SARS-CoV-2 infection was performed in the virology department of Bichat-Claude Bernard University Hospital by RT-PCR on naso-pharyngeal swabs, as recommended. Different techniques were performed throughout the study period for nasopharyngeal samples, due to frequent shortages issues and requirements for fast turnaround time: RealStar® SARS-CoV-2 (Altona, Hamburg, Germany), Cobas® SARS-CoV-2 (Roche Diagnostics, Branchburg, NJ, USA), Simplexa® COVID-19 Direct (DiaSorin, Gerenzano, Italy), BioFire® SARS-CoV-2 (BioMerieux, Salt Lake City, UT, USA), QIAstat-Dx® Respiratory SARS-CoV-2 (Qiagen, Hilden, Germany) and NeumoDX® (QIAgen, Hilden, Germany) using the IP2 Institute Pasteur and the WHO E gene primers[7]. E gene cycle threshold (Ct) values, available for all techniques except Simplexa® COVID-19 Direct and BioFire® SARS-COV-2, were used as a proxy for viral load for 104 samples from 91 patients with paired naso-pharyngeal swabs and sera (*i.e.* collected in the same 24 hours).

For a subset of 146 samples, corresponding to 89 patients included in the French COVID-19 cohort, paired sera and plasma samples were available, allowing to determine the presence of viral RNA in plasma. Briefly, viral nucleic acids were extracted from 200 µL of plasma with the MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume (Roche Diagnostics, Branchburg, NJ, USA) and eluted in 50 µL. RT-PCR was performed on 10 µL of eluate using the RealStar® SARS-CoV-2 assay (Altona, Germany), according to the manufacturer recommendations. Samples with RT-PCR cycle threshold values above 40 were considered negative.

Detection of anti-SARS-CoV-2 nucleocapsid IgG. For a subset of 85 sera, corresponding to 80 patients (ICU patients: n=21, ward patients: n=36 and outpatients: n=23), we performed a chemiluminescent microparticle immunoassay detecting anti-N immunoglobulins G (Architect SARS-

CoV-2 IG Assay, Abbott). Results were reported as a signal to cut-off (S/Co) value. The positivity threshold was set to 1.4, as recommended by the manufacturer.

Data availability. A file compiling all data used in this article is available on Mendeley Data public repository (https://data.mendeley.com/datasets/fjz6zbkxvm/1).

RESULTS

Specificity of the COVID-19 Quantigene® was 98.4% (95% confidence interval [CI], 95.3 to 100), as N-antigenemia was negative for 62 samples out of 63 non-COVID-19 patients.

N-antigenemia sensitivity was determined on 227 sera, obtained from 165 patients included in the French COVID and CoV-CONTACT cohorts with RT-PCR confirmed SARS-CoV-2 infection. Among them, 180/227 sera tested positive, leading to a sensitivity of 79.3% (95% CI, 74.0 to 84.6). When restricting sensitivity analysis to samples collected in two first weeks after symptoms onset, 132 out of 142 samples tested positive for N-antigenemia, leading to a sensitivity of 93.0% (95% CI, 88.7 to 97.2) (Figures 1A and 1B). Patients with positive RNAaemia (viremic patients) exhibited higher N-antigen sera levels (Figure 1-C). In sera collected more than 14 days after symptoms onset, N antigenemia frequently declined and was undetectable in 84.6% (11/13), 42.1% (8/19) and 32.7% (16/49) samples of outpatients, ward, and ICU patients, respectively. The lower detection in late-stage samples appears linked with the apparition of anti-N IgG (figure 1A and Supplementary Appendix 1A).

For 91 patients, 104 paired samples of sera and nasopharyngeal swabs collected in the same 24 hours were available, allowing us to compare N-antigen detection with E gene Ct values for those patients (Figure 1-D). Among patients with E gene Ct value below 30 and below 33 on their nasopharyngeal swab, only 1/50 and 4/67 tested negative for N-antigenemia, respectively (Supplementary Figure 1B). For patients with positive NP samples with Ct values ≥33, only 15/25 (60%) were positive for N-antigenemia. Interestingly, 8 out of 12 patients with a negative nasopharyngeal RT-PCR presented positive N-antigenemia. The lower respiratory tract was explored for 6 of these 8 patients either the same day or in the 5 following days. RT-PCR on the lower respiratory tract sample was positive in 5 of these 6 patients.

DISCUSSION

This is the first evaluation of a commercially available SARS-CoV-2 N-antigen serum or plasma detection. This assay presented a low detection limit at 2.98 pg/mL and a sensitivity above 90% during the acute phase of the disease (*i.e.* <14 days after symptoms onset in PCR confirmed COVID-

19 patients). In the first two weeks, N-antigen negativity was associated with anti-N IgG detection (6/10) and/or low nasopharyngeal viral load in the same 24 hours (7/7, Ct value >30). This sensitivity could allow its use for COVID-19 diagnostic and is in line with RT-PCR on NP samples whose reported sensitivity rates ranged between 71 and 98%, based on negative RT-PCR tests which were positive on repeat testing [8].

Detection of viral antigens in the blood of COVID-19 patients has been recently described by Ogata and collaborators, who detected N and S1 antigens in the blood of 41 out of 64 COVID-19 patients [5]. If antigens blood circulation is not uncommon in infectious diseases, antigenemia tests usually target blood-borne pathogens, notably Dengue, CMV, HBV or HIV. In respiratory diseases, antigen circulation into non-respiratory body fluids is usually not considered, even if likely, because of the focal nature of the infection or possible pre-existence of antibodies. Antigen detection in non-respiratory fluids is still used for two respiratory bacteria: *Streptococcus pneumonia* and *Legionella pneumophila*. Interestingly, it has also been reported in SARS-CoV-1 infection [6]. Whether the circulation of free viral antigens has an impact on disease physiopathology should be assessed in future studies.

N-antigenemia was also detectable in outpatients but the decrease seems to occur earlier in our study. Detection of viral antigens in this population was not evaluated in the study by Ogata and collaborators. Detection of N-antigenemia was higher for patients with either high NP viral loads, i.e. Ct below 30, or active replication in the lung, i.e. high Ct values (>30) in NP samples but positive RT-PCR in lower respiratory tract samples.

This innovative marker may also be of help for prognostic evaluation of patients. An association between high N-antigen levels and higher ICU admission rates has been reported by Ogata et al. [5]. In our study, we observed higher N-antigen levels in serum of viremic patients, in line with the possible association of viremia, or RNAemia, with disease severity and/or immunosuppression [9–11].

Our study presents several limitations. We included a very few number of outpatients and this population needs to be explored in larger cohorts, ideally with longitudinal samples of paucisymptomatic and asymptomatic patients. The case-control design is another limitation and the test performances could be slightly deteriorated in real life condition.

In conclusion, sensitive N-antigen detection in serum or plasma provides a valuable new marker for COVID-19 diagnosis, only requiring a blood draw, scalable in all clinical laboratories. It allows potential new developments to design rapid antigen blood test or combined ELISA assays, detecting both antigens and antibodies. It also raises new questions about the physiological mechanisms at play explaining blood circulation of this antigen and its potential correlation to disease severity.

CONFLICT OF INTEREST

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AUTHORS CONTRIBUTION

QLH, BV, CCha, DD, NHF conceptualized the study and its methodology. QLH, HI, FD, NB, MB performed the experiments. CL, ST, CB, CCho, XD, JFT, LB, JG, YY collected data and participated to the validation of the study. QLH, BV wrote the first draft. All authors reviewed and edited the final manuscript.

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FIGURE LEGEND

Figure 1: (A) Evolution of N-antigen sera levels in SARS-CoV-2-infected patients according to hospitalization status (N=227 serum samples from 165 patients); sequential samples are connected with a gray line, while the positivity threshold value for N-antigen (2.97 pg/mL) is indicated with a dashed red line. (B) N-antigenemia levels according to delay since symptoms onset. (C) N-antigen sera levels according to positive and negative RNAaemia status (n=146 sera, N=89 patients). (D) N-antigen sera levels according to E-gene cycle threshold value of 104 nasopharyngeal swabs collected within 24 hours (N=91 patients).

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Figure 1