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New insights into SARS-CoV-2 Lumipulse G salivary antigen testing: accuracy, safety and short TAT enhance surveillance

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Abstract

Objectives: The rapid, accurate and safe detection of SARS-CoV-2 is the key to improving surveillance and infection containment. The aim of the present study was to ascertain whether, after heat/chemical inactivation, SARS-CoV-2 N antigen chemiluminescence (CLEIA) assay in saliva remains a valid alternative to molecular testing.

Methods: In 2022, 139 COVID-19 inpatients and 467 healthcare workers were enrolled. In 606 self-collected saliva samples (Salivette), SARS-CoV-2 was detected by molecular (TaqPath rRT-PCR) and chemiluminescent Ag assays (Lumipulse G). The effect of sample pre-treatment (extraction solution-ES or heating) on antigen recovery was verified.

Results: Salivary SARS-CoV-2 antigen assay was highly accurate (AUC=0.959, 95% CI: 0.943–0.974), with 90% sensitivity and 92% specificity. Of the 254 antigen positive samples, 29 were false positives. We demonstrated that heterophilic antibodies could be a cause of false positive results. A significant antigen concentration decrease was observed after ES treatment (p=0.0026), with misclassification of 43 samples. Heat had a minimal impact, after treatment the correct classification of cases was maintained.

Conclusions: CLEIA SARS-CoV-2 salivary antigen provides accurate, timely and high-throughput results that remain accurate also after heat inactivation, thus ensuring a safer work environment. This supports the use of salivary antigen detection by CLEIA in surveillance programs.

Keywords: chemiluminescence (CLEIA); Lumipulse; N-antigen; saliva; SARS-CoV-2.

Introduction

Since November 2019, when severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was identified as the virus responsible for Coronavirus Disease 2019 (COVID-19), it has been clear that the rapid identification and isolation of infected subjects was crucial to virus spread containment [1]. Later, the SARS-CoV-2 large scale vaccination campaign strongly changed the natural history of the COVID-19 pandemic by reducing the number of hospitalizations and deaths [2]. However, despite vaccination, viral spread seems to be reinforced by the highly mutated and more transmissible SARS-CoV-2 lineages, such as the Omicron variants [3]. It is therefore still of utmost importance to promptly identify and isolate infected subjects, as a protective strategy towards fragile subjects at high risk of developing severe COVID-19 manifestations [4]. Molecular testing on naso-pharyngeal swabs (NPS), the gold standard for SARS-CoV-2 identification, calls for dedicated instrumentation, specialized staff, and has a long turnaround time and low throughput [5, 6]. Several alternatives (e.g., serology, mass spectrometry and imaging) have been proposed as sensitive and specific strategies for the rapid detection of SARS-CoV-2, and a vast array of point of care testing (POCT) and laboratory-based immunoassays have been evaluated with a view to helping laboratories maximize efficiency in this dramatic scenario [7].

Various POCT self-testing devices designed to save healthcare and laboratory resources have been developed

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and commercialized, but their performance has often failed to meet the WHO criteria [8], since their sensitivity is often lower than 80% and specificity lower than 97% in symptomatic individuals [9]. On the contrary, laboratory-based immunoassays for SARS-CoV-2 antigen have proven to be a valid alternative to NPS molecular testing, since their sensitivity and specificity are higher than 80 and 97%, respectively. Moreover, with respect to molecular testing they have a lower turnaround time, a higher throughput and call for a more simple sample management process [10]. To this end, SARS-CoV-2 antigen laboratory-based immunoassays might represent a sustainable solution in large-scale testing for the screening of populations (e.g. schools, hospitals) at a high risk of viral spread. Considering all these aspects, recently, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group on SARS-CoV-2 updated previous indications for diagnosing SARS-CoV-2, recommending the use of different diagnostic strategies in specific clinical settings, such as lab-based molecular testing in high risk populations (e.g. symptomatic patients), POC-based molecular assays or lab-based antigen immunoassays in low risk subjects (asymptomatic, hospital admission/contact tracing) or in epidemiologic surveys and population screening setting, along with tentative indications for identification of new lineages and/or sub-lineages of SARS-CoV-2 [11].

In addition to the better testing strategy to adopt, to ensure sustainability, it is important to also consider aspects such as sample type, collection and handling before the analysis. Saliva, self-collected in a standardized way, has proven a valid alternative to NPS, saving time and resources during the collection phase and ensuring molecular results as accurate as those obtained with NPS [12]. Saliva has been recommended especially in asymptomatic subjects and general population screening or to test symptomatic patients when upper or lower respiratory tract samples cannot be collected or for shortage of molecular tests [11]. However, independently from the type of sample collected, sample handling before analysis is still a concern in terms of staff safety, environmental contamination and accuracy of results [13].

The aim of this prospective study was to evaluate the performance of a high-throughput chemiluminescence assay for the rapid identification of SARS-CoV-2 N antigen on saliva samples, before and after inactivation pre-treatment, with respect to results obtained with molecular testing on the same samples, and also to provide practical solutions in the management of samples, for example in a surveillance program.

Materials and methods

Subjects

A total of 597 subjects were prospectively enrolled from January to March 2022: 139 (63 females, 76 males, mean age ± SD: 60 ± 15 years) were hospitalized patients (HP) with a diagnosis of COVID-19, while 458 (345 females, 113 males, mean age ± SD: 42 ± 13 years) were Padova University-Hospital healthcare workers (HCW) who underwent regular SARS-CoV-2 testing for surveillance. The study was approved by the Local Ethics Committee (Nr. 27444).

Sample collection and methods

Salivary samples were self-collected by both HP and HCW using the Salivette® device (SARSTEDT AG & Co, Nümbrecht, Germany) following the manufacturer's instructions. As some HCW under surveillance provided more than one sample during the study period, a total of 606 salivary samples were analyzed. For 38 HP in addition to salivary samples, NPS were also collected by trained nurses.

Molecular testing: Molecular testing was performed on all samples, saliva and NPS, using TaqPath™ COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher Scientific, USA) targeting ORF1ab, *N* and *S* genes, after RNA extraction (MagNA Pure 96 DNA and Viral NA Small Volume Kit) as described elsewhere [14]. Saliva and NPS samples were considered positive when at least two of three targets had an amplification plot with a Ct value of < 36, in agreement with our previous data [15].

Antigen testing: All salivary samples were tested without any pre-treatment or dilution, and directly from the sampling device, for N antigen quantification using Lumipulse G-SARS-CoV-2 Ag kit chemiluminescent assay on LUMIPULSE G1200 automated analyzer (Fujirebio, Tokjo, Japan), following the manufacturer's instructions.

Pre-analytical treatments (heterophilic blocking tube-HBT and deactivation treatments): Samples that were positive at antigen search but negative for molecular testing (false positive results) and adequate in volume (500 µL) (n=67), were mixed with a lyophilic preparation containing heterophilic immunoglobulin blocking substances (HBT. Scantibodies. Part Number: 3IX762), in order to evaluate any interference from heterophile antibodies. After 30 min incubation, samples were centrifuged and re-tested for SARS-CoV-2 N-antigen.

A series of salivary samples with adequate volumes, were also re-tested for SARS-CoV-2 N-antigen search after the following pre-analytical treatments: a) addition of sample extraction solution (ES) provided by the manufacturer (20% v/v) to the saliva sample followed by incubation at room temperature for 30 min, as indicated by the manufacturer; b) addition of saliva samples to lyophilized ES provided by manufacturer previously prepared in the lab while adhering to the suggested 30 min incubation time; c) saliva sample pre-treatment by heating at 56 °C for 30 min; d) saliva sample pre-treatment by heating at 80 °C for 15 min.

Statistical analysis

The statistical analysis of data was made with GraphPad Prism Software ver. 6.07. Descriptive statistics included mean and standard deviation. The Wilcoxon matched-pairs test was used for comparisons. Non-parametric ROC analyses were used to estimate the area under the ROC curve. Sensitivity and specificity were estimated by means of the user community package "DIAGT". One-way ANOVA and Tukeys multiple comparisons tests were also used.

Results

Diagnostic performances

NPS vs. Saliva: rRT-PCR results

In the series of 38 HP for which both NPS and saliva samples collected at the same time were available, all 38/38 NPS were positive at molecular testing Ct values being lower than 36. Among saliva samples, 36/38 (94.7%) were also positive, the remaining two being false negatives. These two patients had positive NPS results but with Ct values (*N* gene) equal to 35.37 and 35.51, which were very close to the threshold level of 36.

Comparison between antigen and molecular testing in saliva

A total of 606 saliva samples were tested for rRT-PCR and antigen quantification. At molecular testing, 355 samples (58.6%) were negative and 251 (41.4%) positive for SARS-CoV-2. The detection of *N* antigen allowed us to accurately distinguish between SARS-CoV-2 positive and negative subjects (AUC=0.959, 95% CI: 0.943–0.974) classified on the basis of molecular testing (Figure 1).

On using the Lumipulse manufacturer's cut-off (0.67 ng/L), sensitivity and specificity were 90 (95% CI: 85–93%) and 92% (95% CI: 89–95%), respectively. At ROC curve analysis, the best cut-off, of 0.70 ng/L, was very close to the manufacturer's declared threshold; sensitivity (90%; 95% CI: 85–93%) and specificity (92%; 95% CI: 89–93%) were also comparable. Furthermore, Ag levels were significantly correlated ($p<0.0001$) to Ct values obtained at molecular testing, as shown in Figure 2.

The 92% specificity of antigen testing was due to 26/251 false negative antigen test results. All these false negative samples had Ct values higher than 30 (mean ± SD: 32.9 ± 1.7). On the other hand, 29/355 (8.17%) samples from HCW with negative molecular results had false positive antigen test results, values ranging from 0.68 and 20.13 ng/L

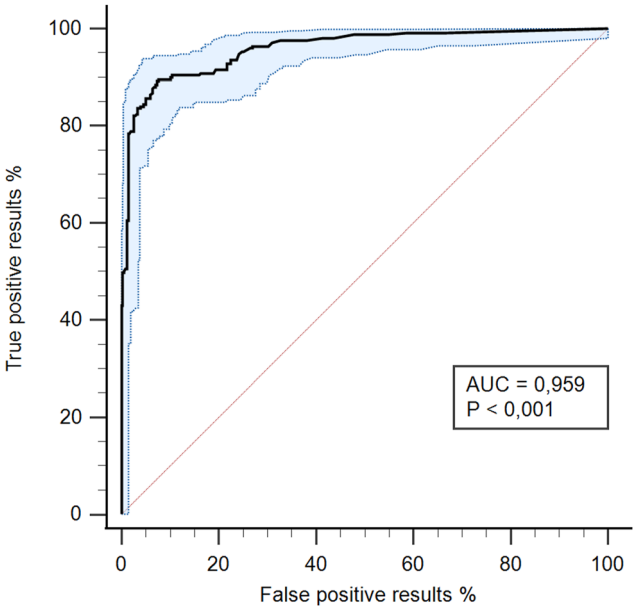

Figure 1: SARS-CoV-2 salivary antigen. ROC curve of Lumipulse salivary SARS-COV-2 antigen assay in distinguishing between positive (n=251) and negative (355) samples at molecular testing.

(mean ± SD: 2.94 ± 4.52 ng/L). These 29 samples were from a total of 23 subjects that included four who collected two or three samples over time in compliance with the surveillance timing (every 7 or 15 days, as requested by the surveillance program, on the basis of risk level in their work area: high or low, respectively). Of interest was the observation that the false positivity appeared to persist in samples collected from the same HCW, also in those repeated after several weeks, and that the single HCW values showed no significant changes in N antigen (Table 1).

To ascertain whether the presence of heterophilic antibodies in saliva might be responsible for the above false positive rate, saliva samples of sufficient volume were re-tested for SARS-CoV-2 antigen after the removal of heterophilic antibodies by HBT treatment. The results obtained are shown in Table 1.

We observed a general decrease in Ag concentrations after HBT treatment in 4/5 samples, the classification changing from positive to negative in 3/5 samples, except for the HCW1.

SARS-CoV-2 deactivation

Extraction solution (ES)

Two hundred and forty-seven salivary samples (168 positive and 79, negative at antigen testing) were re-tested to

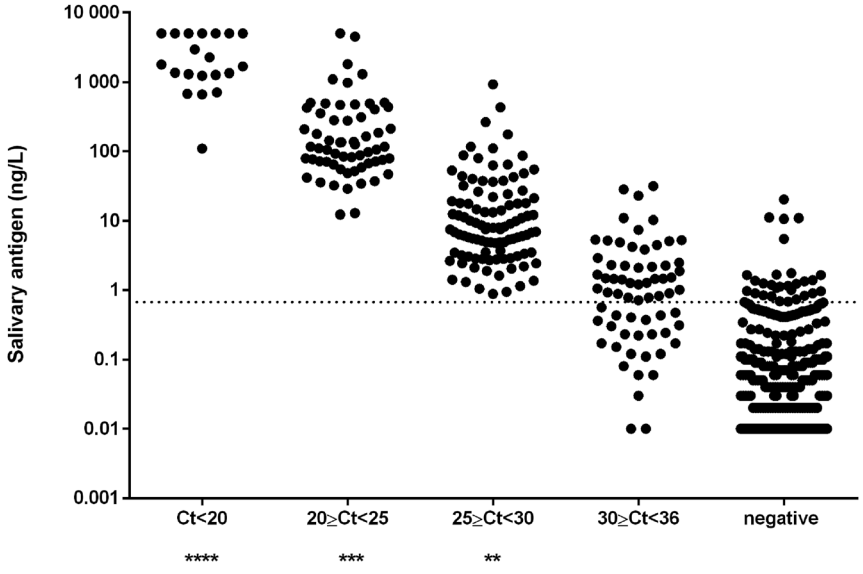

Figure 2: SARS-CoV-2 salivary antigen. Individual levels of salivary SARS-CoV-2 antigen (CLEIA) were grouped on the basis of the corresponding Ct values (*N* gene). The dotted line represents the manufacturer's recommended cut-off (0.67 ng/L). Tukey's pairwise comparisons: ****p<0.0001 with respect to the other groups; ***p<0.0001 with respect to all groups with Ct values higher than 25; **p<0.0001 with respect to groups with Ct values higher than 30.

Table 1: Antigen concentrations before and after HBT pre-treating.

HCW	Date of sample collection	Ag concentration, ng/L before HBT treatment	Classification	Ag concentration, ng/L after HBT treatment	Classification
HCW1	17/02/2022	1.33	Positive		
HCW1	03/03/2022	1.15	Positive	3.47	Positive
HCW2	07/02/2022	10.64	Positive		
HCW2	21/02/2022	11.13	Positive	1.88	Positive
HCW3	17/02/2022	1.75	Positive		
HCW3	24/02/2022	1.19	Positive	0.18	Negative
HCW3	03/03/2022	1.39	Positive		
HCW4	11/02/2022	0.97	Positive	0.19	Negative
HCW4	18/02/2022	1.65	Positive	0.28	Negative
HCW4	25/02/2022	1.00	Positive		

HCW, healthcare worker; HBT, heterophilic blocking tube.

detect Ag before and after pre-treatment with ES supplied by the manufacturer. The results showed an overall agreement of 82.6% (204/247 concordant classification as positive or negative). All 79 negative samples remained negative when analysed after ES treatment. On the contrary, among the 168 SARS-CoV-2 Ag positive saliva samples, 43 were negative after ES treatment (25.6%) with a significant decline in antigen concentration (Wilcoxon matched-pairs: p-value <0.0001) as shown in Figure 3. In ES untreated samples, Ag concentrations ranged from 0.67 to 10 ng/L, and Ct values (*N* gene) were >25 in all cases.

Furthermore, an overall dampening effect of ES on Ag concentrations was found when all tested samples were analysed (p=0.0026).

Lyophilized ES

To test the hypothesis that the decline in SARS-CoV-2 antigen detection after adding the ES might depend on dilution, experiments with lyophilized ES were performed using 67 saliva samples. Ag detection was not possible in 15/67 samples due to excessive viscosity after mixing with lyophilized ES.

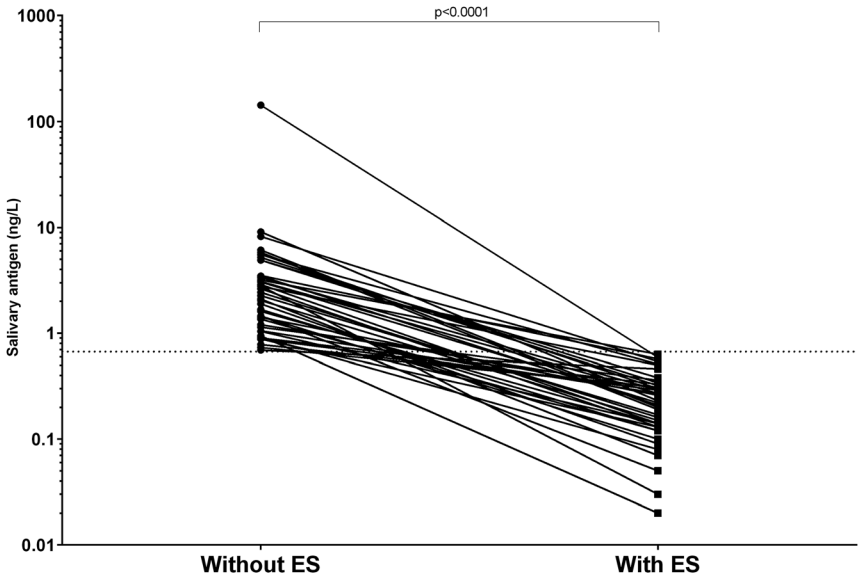

Figure 3: Effect of extraction solution (ES) treatment on antigen concentration. The antigen concentration was determined without and with ES provided by the manufacturer. The results of the 43/168 misclassified samples are reported. The dotted line represents the manufacturer's recommended cut-off (0.67 ng/L).

On considering the remaining 52 samples for which Ag detection was possible, agreement was 73.1% (38/52). One of the 13 negative samples was found to be a false positive after lyophilized ES treatment. On the other hand, 13 of the 39 positive samples were negative after lyophilized ES treatment (33%). Before lyophilized ES treatment, Ag concentrations in the 39 positive samples ranged from 0.78 to 5,000 ng/L, and Ct values from 17.5 to 31.9, spanning from a low to high viral load.

Finally, the comparison between Ag concentrations before and after lyophilized ES treatment in all tested samples highlighted a significant decrease in Ag levels ($p<0.0001$).

Temperature

Based on the above data, considering that ES treatment has a potential effect on test sensitivity, while direct saliva testing offers a highly satisfactory performance, one might embrace the latter option. In this case, however, a strategy to enhance laboratory staff safety measures should be realized. Heating inactivates SARS-CoV-2 virus, but it is not yet known whether it has any impact on antigen testing. We therefore verified any changes under two conditions: a) 56 °C for 30 min, and b) 80 °C for 15 min.

56 °C for 30 min

One hundred and three salivary samples were analyzed to detect Ag before and after incubation at 56 °C for 30 min. No significant reduction in Ag concentration was observed after treatment ($p=0.67$), and an overall agreement equal to 93.2% (96/103) was obtained (Figure 4, upper panel).

80 °C for 15 min

Eighteen salivary samples were analyzed to detect Ag before and after deactivation by temperature (80 °C for 15 min). All samples tested were positive before incubation at high temperature. All results were confirmed (agreement=100%) without there being significant changes in concentration levels after heat inactivation ($p=0.09$) (Figure 4, lower panel).

Discussion

Wide-scale vaccination was crucial to the containment of the SARS-CoV-2 pandemic, significantly reducing the number of hospitalizations [1]. Nevertheless, with the appearance of new highly contagious Omicron variants, the number of infections continue to grow day by day [3]. Virus containment remains a priority, especially for fragile (e.g. elderly or hospitalized) subjects who, even if vaccinated, are at risk of developing severe COVID-19 manifestations [4]. In addition to vaccination campaigns, active surveillance programs in populations (e.g. schools, universities, communities, hospitals) with a high incidence of transmission and contact tracing have proven effective in containing viral spread [15–18]. Currently,

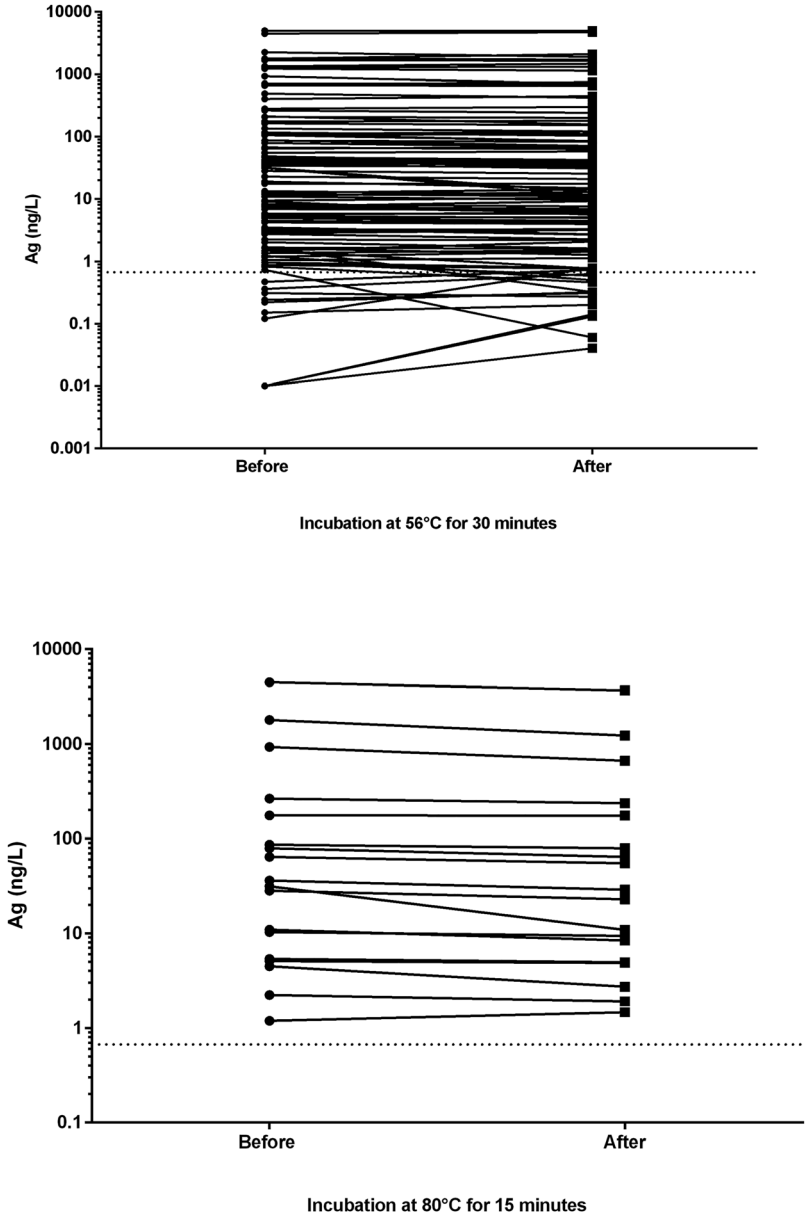

Figure 4: Effect of temperature on antigen concentration. The antigen concentration was determined before and after incubation at 56 °C for 30 min (upper panel) and at 80 °C for 15 min (lower panel). The dotted line represents the cut-off (0.67 ng/L) recommended by the manufacturer. No statistically significant difference before and after treatment was found.

in order to guarantee the prompt identification and isolation of infected subjects, most surveillance programs are conducted by NPS molecular testing, especially in hospitals [19–22]. However, this poses some limitations concerning both matrix and technique used. NPS collection calls for trained staff, and swab sample collection is uncomfortable for subjects; moreover, molecular testing must be conducted with dedicated instrumentation and by specialized staff and, it takes time to release results also when pooling strategies are used. We evaluated the analytical performances of SARS-CoV-2 Ag by Lumipulse G-SARS-CoV-2 Ag chemiluminescent assay on saliva samples as potentially useful in surveillance in view of the fact that they are rapid, and easily performed on self-collected saliva.

Molecular testing of saliva is known to be a valid alternative to NPS, with a comparable sensitivity and specificity [23, 24]. This has been confirmed in the present study on a group of COVID-19 inpatients, saliva molecular testing having a sensitivity of 94.7%, and the two false negative samples being from patients with NPS Ct values very close to the

limit of 36. We evaluated the analytical performances of SARS-CoV-2 Ag by Lumipulse G-SARS-CoV-2 Ag chemiluminescent assay on 606 pure saliva samples self-collected by HP and HCW with respect to those obtained by molecular testing. CLEIA antigen testing in saliva enabled a highly accurate distinction to be made between positive and negative samples, with an area under the ROC curve greater than 0.9, and a high sensitivity (90%) and specificity (92%), in agreement with previously reported data [25, 26].

Then, these data confirm that SARS-CoV-2 Ag detection by Lumipulse G-SARS-CoV-2 Ag chemiluminescent assay on pure saliva allows accurate results to be obtained in a short time interval (about 120 samples per hour, the first result being available after 30 min). We therefore support the premise that CLEIA antigenic testing in saliva should be adopted as a valid alternative to NPS molecular testing in surveillance programs.

The percentage of missed positive cases was very similar to that reported by Favresse et al. [26] on NPS, and in agreement false negative findings were almost exclusively recorded among samples with Ct values at molecular testing higher than 30, expected to be less infectious. The differences between the two analytical principles may explain these data (e.g. Target amplification for molecular testing but not for Ag searching). However high Ct values suggest a low viral load, generally observed after seven or more days from symptoms onset, or in the very early phases of infection [14]. Despite this limitation, CLEIA saliva antigen testing has a sensitivity higher than the 80% level recommended by the WHO [8]. In agreement with Kobayashi et al. [27], we found antigen false positive results in saliva samples. False positive results were obtained among HCW, and in four of them who collected more than one saliva sample, the antigen concentrations were comparable also one week or more later, thus suggesting the potential presence of interfering molecules. We then tested the hypothesis that heterophilic antibodies might be the interfering molecule. The decrease in Ag concentration found after HBT treatment in all (but one) HCW saliva samples, could support this hypothesis. However, other factors such as pre-analytical variables (i.e. particulates or insufficient volume), can affect results [27]. The issue of interferences deserves more investigations to avoid the lowering of assay performance, in particular of the specificity.

In the light of these findings, to limit the potential risk of under-diagnosis due to false negative results, we suggest scheduling SARS-CoV-2 antigen detection by Lumipulse G-SARS-CoV-2 Ag CLEIA with a week's frequency, which is compatible with a diagnostic system that is easy-to perform, fast and high throughput. Considering the results obtained in this study, to minimize the risk of releasing false positive results and reaching the level of specificity required by WHO [8], it is extremely important to provide molecular confirmation of 'positive' samples. Of great interest in this field, is also the quantification of N antigen in blood. Recently, the N antigen detection in plasma by chemiluminescent immunoassay was shown to be useful not only for diagnosing SARS-CoV-2 infections but also to obtain a better discrimination of patients regarding the severity grade if compared to RT-PCR [28, 29].

While ensuring accurate results is the priority of clinical laboratories, it is equally important to guarantee staff safety during sample handling. Although laboratories must comply with safety requirements, using personal protective equipment and disinfectant solutions, and handle samples under laminar flow cabinets thus allowing operators to work safely, it is important to bear in mind that the risk of contagion is significantly lowered if samples are inactivated before handling. In this study we also evaluated the analytical performance of SARS-CoV-2 antigen detection by Lumipulse G-SARS-CoV-2 Ag CLEIA testing in saliva samples after deactivation pre-treatment by means of chemical (extraction) solution and heating (at 56 °C for 30 min and at 80 °C for 15 min). The results obtained demonstrate that the use of extraction solution to deactivate sample has disadvantages, as follows.

- It is time consuming, especially in the case of saliva testing. As the sample obtained after centrifugation has no fixed value (as NPS), the staff are required to measure the sample volume and then add the correct volume of ES in order to guarantee the addition of ES 20% v/v as recommended by manufacturer; this delays the release of results.
- The sample handling (cap and tube uncorking) can generate sample contamination and droplets dissemination.
- It generates Ag decrease, probably due to sample dilution and/or assay interference. In fact, also when ES was lyophilized, it was not possible to detect Ag in all cases.

The deactivation of samples by temperature, whether 56 °C for 30 min or 80 °C for 15 min, is rapid, easy to perform and ensures safer handling of samples. Nor does it expose the sample to possible contamination, or it significantly impacts on results release times and on accuracy of results.

Conclusions

In conclusion, Lumipulse G-SARS-CoV-2 Ag CLEIA testing in saliva samples has satisfactory diagnostic sensitivity

and specificity, thus representing a valid alternative to NPS molecular testing for the prompt identification of SARS-CoV-2 infections in clinical practice, and allowing the identification of most positive samples, albeit with the potential loss of those with very low viral loads. Moreover, the non-invasiveness and simplicity of saliva sample collection, combined with the automation and high-throughput of Lumipulse G-SARS-CoV-2 Ag CLEIA testing make this solution effective and sustainable when a large number of tests are performed. CLEIA antigen testing in saliva, at least once a week, might be suggested for large scale surveillance programs. Antigen positive results should, however, be confirmed with molecular testing. Samples are readily inactivated by heating before their handling without impacting on the accuracy and timeliness of the release of test results.

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Informed consent: Informed consent was obtained from all individuals included in this study.
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