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Positivization time of a COVID-19 rapid antigen self-test predicts SARS-CoV-2 viral load: a proof of concept

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Abstract

Objectives: This proof of concept study was aimed to validate the hypothesis that the time of positivization of SARS-CoV-2 self-performed rapid diagnostic tests (RDTs) may reflect the actual viral load in the specimen.

Methods: A SARS-CoV-2 positive sample with high viral load was diluted and concomitantly assayed with molecular assay (Xpert Xpress SARS-CoV-2) and RDT (COVID-VIRO ALL IN RDT). The (mean cycle threshold; Ct) values and RDT positivization times of these dilutions were plotted and interpolated by calculating the best fit. The parameters of this equation were then used for converting the positivization times into RDT-estimated SARS-CoV-2 Ct values in routine patient samples.

Results: The best fit between measured and RDT-estimated Ct values could be achieved with a 2-degree polynomial curve. The RDT-estimated Ct values exhibited high correlation (r=0.996) and excellent Deming fit (y=1.01 × x − 0.18) with measured Ct values. In 30 consecutive patients with positive RDT test, the correlation between RDT positivization time and measured Ct value was r=0.522 (p=0.003). The correlation of RDT-estimated and measured Ct values slightly improved to 0.577 (Deming fit: y=0.44 × x + 11.08), displaying a negligible bias (1.0; 95% CI, −0.2 to 2.2; p=0.105). Concordance of RDT-estimated and measured Ct values at the <20 cut-off was 80%, with 0.84 sensitivity and 0.73 specificity.

Conclusions: This proof of concept study demonstrates the potential feasibility of using RDTs for garnering information on viral load in patients with acute SARS-CoV-2 infection.

Keywords: COVID-19; diagnosis; immunoassay; laboratory medicine; SARS-CoV-2.

Introduction

The burden of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections is still progressing on an unprecedented scale, with cases (either new infections or re-infection, along with “breakthrough” infections) sustained by the most recent Omicron lineages, occurring even in environmental conditions that were extremely unfavourable for transmission of former variants (i.e., high temperature, open spaces) [1]. Considering that the real end of this pandemic cannot be predicted and is likely not to occur in the near future [2], the paramount number of coronavirus disease 2019 (COVID-19) tests that will need to be performed for diagnosing both symptomatic and asymptomatic cases in the comings months and years will continue to place significant pressure on healthcare and clinical laboratories around the world, thus forcing scientists, policymakers and healthcare administrators to cooperate for identifying sustainable policies encompassing faster and possibly cheaper solutions to widespread SARS-CoV-2 molecular testing [3].

In the effort to address the still immense number of SARS-CoV-2 cases that will need to be diagnosed, it is hence not surprising that the World Health Organization (WHO) has recently published its forthcoming strategy, underpinning once more the pivotal role played by *in vitro* diagnostic testing. More specifically, the agency has advocated that all countries should reinforce their laboratory capacities in order to ensure accurate and rapid SARS-CoV-2 detection, coupled with generalized

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diffusion of rapid diagnostic tests (RDTs) [4]. This suggestion is mainly justified by the fact that several factors related to traditional SARS-CoV-2 molecular testing are compounding to encumber large population screenings, including relatively low throughout and long turnaround time, the need of dedicated healthcare personnel equipped with specific protective equipment for collecting the samples and so forth [5]. Thus, the use of self-collected samples to be assayed with rapid, simple and relatively inexpensive immunoassays represents a valuable alternative for diagnosing SARS-CoV-2 infections [6]. The WHO itself, in its updated document "Antigen-detection in the diagnosis of SARS-CoV-2 infection" [7], recognizes that direct assessment of SARS-CoV-2 viral proteins using nasal swabs or other respiratory samples by means of RDTs may represent a quicker and relatively inexpensive strategy for surrogating molecular testing in some specific circumstances, especially for diagnosing COVID-19 in symptomatic people or in asymptomatic subjects at enhanced risk of being infected (i.e., contacts and health workers). The Working Group of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) on SARS-CoV-2 variants [8] has also provided guidance that SARS-CoV-2 RDTs could be reliably used for purposes of population screening and/or in epidemiologic surveys. In keeping with these indications, the widespread diffusion of SARS-CoV-2 RDTs has now become an integral part of the ongoing strategy to address COVID-19 in several countries, such as the US [9], where isolation guidelines for patients with SARS-CoV-2 infection could be based on RDTs [10]. This is mainly due to the fact that consolidated evidence has now been provided that duration of SARS-CoV-2 RNA shedding is substantially longer than infectivity, and negative results of SARS-CoV-2 RDTs will reflect a high likelihood of obtaining negative viral culture [11].

The three leading and well-recognized limitations of SARS-CoV-2 RDTs include lower diagnostic performance compared to molecular testing for detecting SARS-CoV-2 RNA and laboratory-based SARS-CoV-2 antigen immunoassays, as recently documented by two large and comprehensive meta-analyses [12, 13], the lower diagnostic yield when self-administered [14], along with the fact that they have been developed for providing qualitative results (i.e., negative/positive).

As concerns the last limitation, several lines of evidence now attest that quantification of viral load, either in terms of measuring RNA cycle threshold (Ct) value or SARS-CoV-2 antigen(s) concentration, may provide valuable epidemiological and clinical information [15]. More specifically, viral load assessment in routine specimens may help forecasting both emergence and progression of local SARS-CoV-2 outbreaks [16, 17], could be used to define subjective infectiveness and risk of being contagious [18], and may be employed for predicting the risk of developing severe/critical COVID-19 illness [19, 20]. To this end, interesting evidence has emerged from two previous studies, showing that accurately measuring the positivization time of some SARS-CoV-2 RDTs may provide a roughly estimation of viral load in nasal and/or nasopharyngeal specimens [21, 22]. We have hence planned this proof of concept study to validate the hypothesis that measuring the time of positivization of SARS-CoV-2 RDTs may reflect the actual viral load in nasal samples. A SARS-CoV-2 positive sample with high viral load was serially diluted and concomitantly assayed with a molecular assay and an RDT, to obtain a sort of "calibration" of the RDT, with could be used to translate the time of positivization of the device into RDT-estimated viral load in routine patient samples.

Materials and methods

SARS-CoV-2 RNA detection and quantification

SARS-CoV-2 RNA was assayed with Xpert Xpress SARS-CoV-2 (Cepheid, Sunnyvale, CA, USA). This test is a real-time reverse transcription polymerase chain reaction (RT-PCR) that has been specifically developed for quantitative detection of SARS-CoV2 nucleocapsid (N2) and envelope (E) RNA in a vast array of clinical specimens (i.e., nasopharyngeal and/or nasal swabs, nasal wash/aspirates) collected with specific transport media (Viral Transport Medium (VTM), Universal Transport Medium (UTM)). The assay, encompassing automated/integrated sample preparation, RNA extraction and amplification and target sequence detection was performed on a GeneXpert GX-XVI Processing Unit (Cepheid, Sunnyvale, CA, USA). Test results are automatically interpreted by the GeneXpert System. The limit of detection is 0.02 plaque forming unit (PFU)/mL for both target SARS-CoV-2 genes. Samples are considering positive when the signal for both nucleic acid targets N2 and E displays a Ct value within a valid range and endpoint above the minimum proprietary setting. The hands-on and turnaround time for positive samples are around 1 and 50 min, respectively. According to manufacturer's claims, the test displays 0.98 (95% confidence interval (CI), 0.88–1.00) diagnostic sensitivity and 0.96 (95% CI, 0.85–0.99) diagnostic specificity, respectively. On-field evaluation of this method carried out by Zhen et al. confirmed optimal diagnostic performance, reporting 98.3% (95% CI, 91–100%) percent positive agreement and 100% (95% CI, 93–100%) percent negative agreement for diagnosing SARS-CoV-2 infection, respectively [23]. The excellence performance of this test was more recently confirmed by another clinical study, reporting sensitivity as high as 0.99 for detecting SARS-CoV-2 RNA [24]. According to a recent claim of the US Food and Drug Administration (FDA), the clinical performance of this test seems unaffected by different SARS-CoV-2 variants, including Omicron linages [25]. The viral load in each diagnostic sample included in this study was finally expressed as

mean Ct value of SARS-CoV-2 *E* and *N2* genes, as follows: [SARS-CoV-2 *E* Ct value + SARS-CoV-2 *N2* Ct value]/2.

COVID-VIRO ALL IN RDT description

Rapid SARS-CoV-2 antigen screening was conducted using COVID-VIRO ALL IN (AAZ-LMB, Boulogne-Billancourt, France), a "vertical" (rather than lateral) flow immunoassay encompassing the use of sensitive monoclonal antibodies against the SARS-CoV-2 core antigen in nasal specimens, to be used as a single-use device for *in vitro* diagnostics of COVID-19 by lay people in private settings (i.e., self-testing) or accompanied by healthcare professionals (Figure 1). Briefly, colloidal gold-labelled monoclonal antibodies against the core protein of SARS-CoV-2 are fixed in the test area of a nitrocellulose strip. When SARS-CoV-2 and its antigen are present in the test sample, coloured immune-complexes are generated with anti-SARS-CoV-2 monoclonal antibodies, and then captured by other membrane-bound monoclonal anti-SARS-CoV-2 antibodies during capillarity-induced migration to the test (T) line, thus producing a coloured band. A control window present as internal quality check, must always appear when the test has worked properly. The complete self-testing procedure, as suggested by the manufacturer, encompasses some sequential steps, briefly summarized in Figure 1. The whole procedure takes nearly 1 min and results should be read within 15 min. A recent clinical evaluation of this SARS-CoV-2 RDT revealed that sensitivity, specificity and accuracy compared to a reference molecular test on nasopharyngeal samples were as high as 0.93, 1.00 and 0.98, respectively, reporting also more favourable comments in terms of being comfortable and easier to use compared to other SARS-CoV-2 RDTs currently available in the market [26].

Calibration of COVID-VIRO ALL IN RDT

The calibration of COVID-VIRO ALL IN RDT was performed by serially diluting with UTM a SARS-CoV-2 positive nasopharyngeal sample known to have substantially high viral load (SARS-CoV-2 *E* gene Ct: 16.5, SARS-CoV-2 *N2* gene Ct: 17.4, mean SARS-CoV-2 Ct, 17.0) at ratios of 1:2.5, 1:5; 1:10; 1:25 and 1:50. The serial dilutions were simultaneously assayed with Xpert Xpress SARS-CoV-2 and COVID-VIRO ALL IN RDT. As concerns the manual assay, 50 µL of each serial dilution were pipetted on the sponge of the test kit, followed by activation of device and registration of positivization time (i.e., time measured from device activation to band appearance in the "T" window) (Figure 1). The paired values (i.e., positivization time of COVID-VIRO ALL IN RDT vs. mean SARS-CoV-2 Ct values measured with Xpert Xpress) were then plotted and interpolated by calculating the best fit. The parameters obtained by constructing the best possible equation were finally used for converting the positivization time of clinical samples into estimated SARS-CoV-2 viral load (i.e., RDT-estimated Ct values).

Study population

The study population consisted of a series of patients presenting to Hospital Pederzoli of Peschiera del Garda (Verona, Italy) for SARS-CoV-2 diagnosis (for presence of suggestive symptoms and/or for recent contact with SARS-CoV-2 positive patients), between August 2 and September 3, 2022 (Omicron BA.5 >90% prevalence). Two separate nasopharyngeal (healthcare-collected; Virus swab UTM Copan, Brescia, Italy) and nasal (self-collected; COVID-VIRO ALL) swabs were collected upon patient presentation. The nasopharyngeal swab was transported to the Laboratory Medicine service, for being assayed with the molecular test (as described before), according to locally defined standard operating procedures, and with a turnaround time of around 6 h. The self-collection of a second nasal sample and performance of RDT using COVID-VIRO ALL IN was offered on voluntary basis to all patients presenting for SARS-CoV-2 testing at the facility, as a faster screening assay. The patient was rapidly instructed to use the device by providing a detailed utilization notice [27], and test results, along with positivization time, were finally interpreted in the presence of a healthcare professional. Only patient's samples testing positive with COVID-VIRO ALL IN RDT were used for this proof of concept study, since assessment of the clinical performance of COVID-VIRO ALL IN RDT was already performed in a previous evaluation and hence outside the scope of the present investigation [26].

Statistical analysis

All test results were finally reported as median values and interquartile range (IQR). The best fit between positivization time of COVID-VIRO ALL IN RDT and Ct values measured with Xpert Xpress SARS-CoV-2 was calculated using Microsoft Excel (Microsoft, Redmond, WA, United States). The correlation between viral load estimated from the positivization time of COVID-VIRO ALL IN RDT (i.e., RDT-estimated Ct) and the mean Ct value measured with Xpert Xpress SARS-CoV-2 was analyzed using linear regression analysis (with Pearson's correlation), Deming fit and Bland and Altman plots, whilst concordance between RDT-estimated and mean measured SARS-CoV-2 Ct values was tested with Kappa statistics. The statistical analysis was carried out with Analyse-it software (Analyse-it Software Ltd, Leeds, UK).

This study was part of routine clinical laboratory operations for SARS-CoV-2 screening and diagnosis at the local facility. The investigation was conducted in accordance with the Declaration of Helsinki, under the terms of relevant local legislation, and was part of broader study protocol cleared by the Ethical Committee of the Provinces of Verona and Rovigo (971CESC; Approved July 25, 2016).

Results

Calibration of COVID-VIRO ALL IN RDT

The plots of test results obtained with Xpert Xpress SARS-CoV-2 (mean Ct values), and COVID-VIRO ALL IN RDT (positivization time) are shown in Figure 2A. Briefly, the best fit between viral load measured with Xpert Xpress SARS-CoV-2 and positivization time of COVID-VIRO ALL IN RDT could be achieved using a 2-degree polynomial curve (r=0.996). The resulting calibration curves obtained transforming the positivization time of COVID-VIRO ALL IN RDT into estimated viral load using the correlation parameters of the 2-degree polynomial curve is shown in Figure 2B, displaying an adjusted correlation

Preparation

1. Blow the nose before testing
2. Place the device on a plane layout
3. Remove the device from its holder by pulling upwards
4. Remove the protective cap

Performing the test

1. Insert gently the swab into the left nostril until (approx. 1-2 cm)
2. Rotate the swab in one nostril for 15 sec by wiping the sponge against the inside of the nose
3. Repeat the same operation in the other nostril
4. Put the device with the swab in face down in the holder on the plane layout
5. Press down firmly on the device for breaking the buffer capsule and push it to the bottom of the holder

Test result interpretation

1. A pink band must appear in the control (C) window within 1 min to validate test result
2. Wait up to 15 min and then check:
   - No bands: test FAILED
   - Only one band in the (C) window: NEGATIVE
   - Two bands in (C) and (T) windows: POSITIVE

Figure 1: Description of COVID-VIRO ALL IN self-performed rapid diagnostic test (RDT).

between RDT-estimated and mean measured SARS-CoV-2 Ct of r=0.996, and a Deming fit as follows: $y=1.01\times x - 0.18$. The parameters from the calibration procedure were then employed for transforming the RDT positivization time into estimated Ct values for the study population, as described below.

Sample comparison

Our final study population of consisted of 30 voluntary patients displaying COVID-VIRO ALL IN RDT positive test results (median age 46 years, IQR 37–52 years; 22 women), after excluding 6 samples with positivization time >3 min (median Ct: 26.3; IQR, 24.6–29.0), and thus outside the range of our calibration curve.

The median Ct (and interquartile range; IQR) of the SARS-CoV-2 *E* gene and *N2* gene measured with the Xpert Xpress SARS-CoV-2 in the 30 study samples were 21.7 (IQR, 17.2–24.0; range, 14–27), and 23.2 (IQR, 19.3–25.4; range, 16–30), respectively. The (mean of both genes) median Ct was 22.6 (IQR, 18.3–24.6; range, 15–29), The median positivization time of COVID-VIRO ALL IN RDT was 61 s (IQR, 32–81; range, 20–180 s).

The linear regression analysis between mean measured SARS-CoV-2 Ct and positivization time of COVID-VIRO ALL IN RDT is shown in Figure 3A. The correlation was r=0.522 (p=0.003). The linear regression analysis between RDT-estimated viral load (obtained by transforming the positivization time into estimated Ct values) and mean Ct values measured with Xpert Xpress SARS-CoV-2 is shown in Figure 3B. The correlation slightly improved to r=0.577 (z-statistic, −0.290; p=0.772), and the Deming fit was as follows: $y=0.44 \times x + 11.08$. The bias between RDT-estimated and measured SARS-CoV-2 Ct values was 1.0 (95% CI, −0.2 to 2.2), failing to achieve statistical significance (p=0.105). The overall concordance of RDT-estimated and measured Ct values at the Ct <20 cut-off (i.e., around 46 s of positivization time of COVID-VIRO ALL IN RDT, which designates samples with very high viral load [10]), was 80% (95% CI, 61–92%; p=0.002), associated with 0.84 (95% CI, 0.60–0.97) sensitivity and 0.73 (95% CI, 0.39–0.94) specificity.

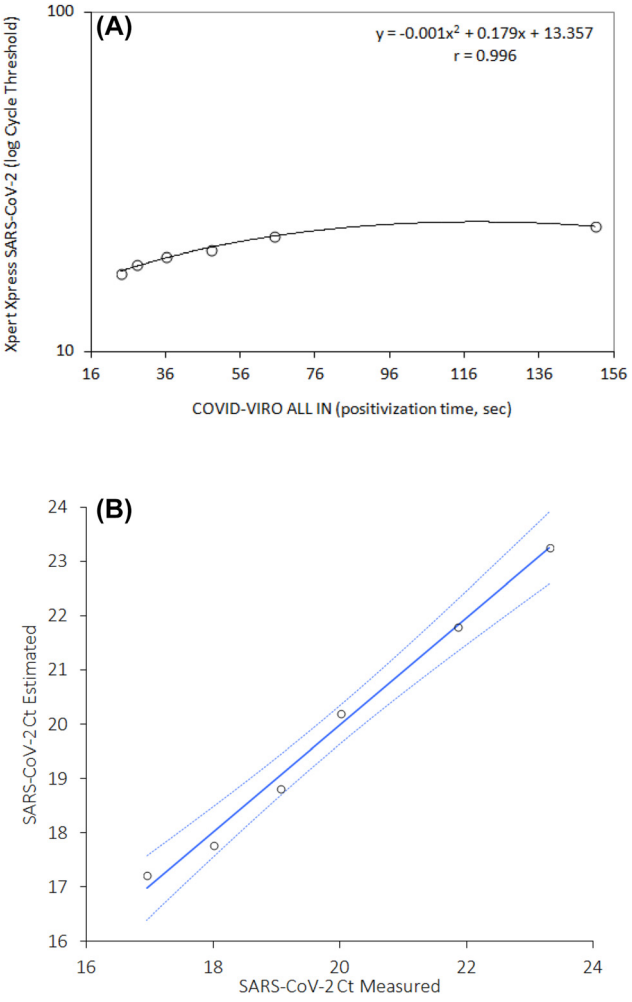

Figure 2: Calibration of COVID-VIRO ALL IN RDT. (A) Calibration curve obtained by plotting the positivization time of COVID-VIRO ALL IN self-performed rapid diagnostic test (RDT) vs. the viral load (i.e., mean SARS-CoV-2 cycle threshold value of the E and N2 genes) measured with Xpert Xpress SARS-CoV-2 obtained by serially diluting a patient sample with high nasopharyngeal viral load; (B) Correlation between RDT-estimated and Xpert Xpress measured SARS-CoV-2 cycle threshold values in the calibration samples.

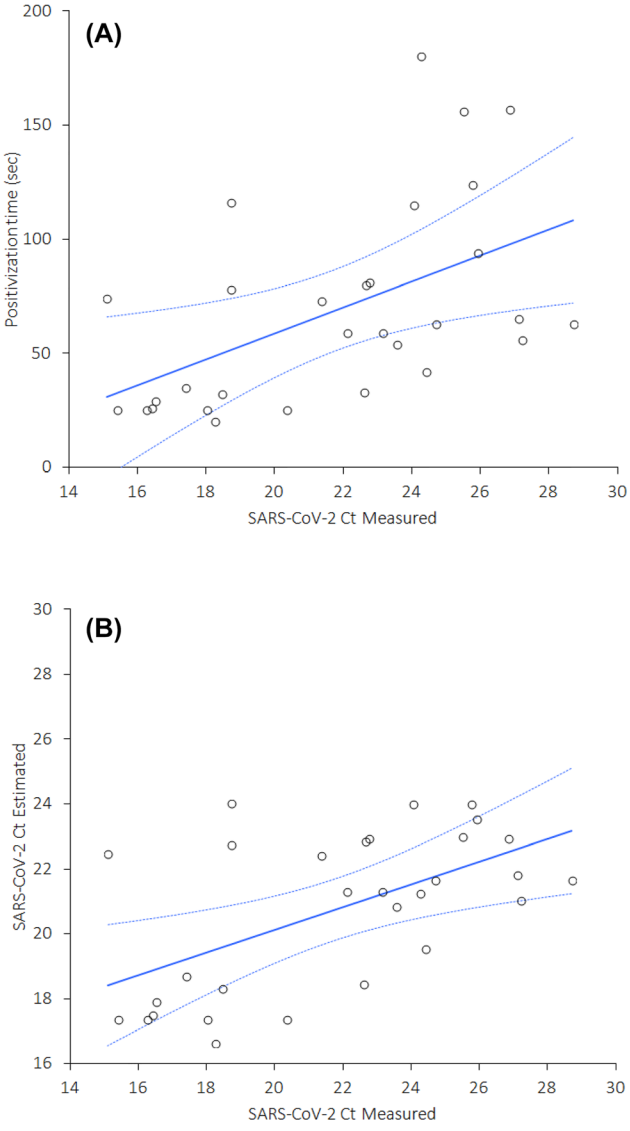

Figure 3: Linear regression analysis between (A) positivization time of COVID-VIRO ALL IN self-performed rapid diagnostic test (RDT) and viral load (i.e., mean SARS-CoV-2 cycle threshold value of E and N2 genes) measured with Xpert Xpress SARS-CoV-2 in 30 routine patient's samples; and (B) RDT-estimated and Xpert Xpress measured cycle threshold values in 30 routine patient's samples.

Discussion

It is now undeniable that diagnosing SARS-CoV-2 infection by measuring specific viral proteins (e.g., nucleocapsid or core) in nasopharyngeal and/or nasal swabs represents a valuable alternative – in specific circumstances – to support or even replenish the paramount number of SARS-CoV-2 molecular tests still needed for facing the unremitting COVID-19 pandemic [28]. The current situation is further worsened by an almost incessant emergence of new and highly mutated sublineages, such as Omicron BA.4, BA.5, BA.2.75 and BA.2.12.1, which not only seem to have higher infectious potential compared to previous SARS-CoV-2 variants of concern (VOCs), but are also capable to efficiently evade the humoral immunity developed after previous infections or vaccination [29], thus fostering a greatly enhanced burden of diagnostic work-up due to re-infections (even in patients originally infected by Omicron BA.1/2) and breakthrough infections [30].

One of the major shortcomings of SARS-CoV-2 RDTs, along with subjective interpretation of test results and lower diagnostic performance (especially limited analytical and diagnostic sensitivity compared to molecular

methods and laboratory-based immunoassays), is represented by the generation of qualitative test results (i.e., negative/positive), typically defined by the absence or presence of a coloured band within a test ("T") window of the portable device. This inherent characteristic would hence preclude their usage for obtaining accurate information on viral load, which is an important clinical parameter for predicting individual infectivity and contagiosity, along with the risk of worsening and developing unfavourable disease progression [15]. As concerns the instance of low diagnostic performance when self-used, a recent study demonstrated that providing reliable instructions, encompassing for example brief videos and handouts containing text and images for self-swab collection, was effective to consistently improve the performance of self-performed SARS-CoV-2 RDT, achieving diagnostic values comparable to those typically characterizing swab samples collected by skilled health care personnel, even in children aged 14 years or younger [31]. The use of fast and relatively less expensive self-performed assays for SARS-CoV-2 diagnostics, that would be capable to allow estimating the actual viral load, shall hence be seen as a potential breakthrough in this otherwise challenging scenario.

Two previous studies have preliminary assessed the possible clinical significance of measuring the positivization time of SARS-CoV-2 RDTs for predicting the viral load [21, 22], thus paving the way to further investigations aimed at establishing accuracy and reliability of this intriguing diagnostic strategy. The results of this proof of concept study thus confirm the potential viability of using validated SARS-CoV-2 RDTs for garnering information on viral load in patients with acute SARS-CoV-2 infection. More specifically, besides demonstrating that (i) the positivization time of the RDT used in this study was significantly associated with SARS-CoV-2 viral load measured with an accurate and reliable molecular test (i.e., Xpert Xpress SARS-CoV-2; Figure 3A), (ii) the positivization time could be straightforwardly converted into RDT-estimated viral load (i.e., Ct values; Figure 3B), we also showed that (iii) a cut-off of positivization time could be specifically calculated for identifying samples with very high SARS-CoV-2 viral load (e.g., Ct values <20). These results are even more convincing given that they may have been inevitably plagued by the bias arising from specimen self-collection and RDT self-performance.

Despite a relatively limited sample size, the findings of our study may have important practical implications, since the use of RDT that can be easily self-performed outside healthcare environments and without specific supervision like COVID-VIRO ALL IN RDT, may provide extremely useful information for improving the managed care of COVID-19 during the ongoing pandemic. In fact, knowing the extent of RDT-estimated viral load, even in outpatient settings, (i) could help stratifying the baseline risk of SARS-CoV-2 positive subjects to progress towards severe and/or critical COVID-19 illness, thus deciding on the need of more aggressive home care or hospitalization earlier before clinical deterioration, (ii) could reliably detect patients at major risk of spreading the infection (i.e., the so-called "super-spreaders", those with Ct <20), and (iii) may also be used by patients for home monitoring of viral load and thus predicting the possible end of their infectivity and – eventually – the length of their isolation and/or quarantine periods. Further studies, using different types of RDTs, a larger number of samples and even using different sample matrices (e.g., saliva) [32], should be planned to validate the preliminary findings emerged from this proof of concept study.

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Ethical approval: The investigation was conducted in accordance with the Declaration of Helsinki, under the terms of relevant local legislation, and was part of broader study protocol cleared by the Ethical Committee of the Provinces of Verona and Rovigo (971CESC; Approved July 25, 2016).

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