Clinical assessment of FREND COVID-19 Ag test in an unselected population referred for routine SARS-CoV-2 testing

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ABSTRACT

Background: this observational retrospective study was aimed at evaluating the clinical performance of the novel microfluidic fluorescence immunoassay FREND COVID-19 Ag test in a population of unselected individuals undergoing routine SARS-CoV-2 (severe acute respiratory coronavirus 2) testing.
Methods: the study population consisted of a series of outpatients referred to the Service of Laboratory Medicine of Pederzoli Hospital (Peschiera del Garda, Verona, Italy) between April 12 and 30, 2021, for SARS-CoV-2 testing for being either symptomatic or having had close contact with one or more COVID-19 cases. A routine nasopharyngeal sample was collected at hospital admission and analyzed with both molecular (Altona Diagnostics RealStar® SARS-CoV-2 RT-PCR Kit) and antigen (FREND COVID-19 Ag) tests.
Results: the area under the curve (AUC) of FREND COVID-19 Ag in all nasopharyngeal samples compared to molecular testing was 0.69 (95%CI, 0.64-0.75). At the ≥1.0 $TCID_{50}$/mL manufacturer's cut-off, accuracy, sensitivity, specificity, negative (NPV) and positive (PPV) predictive values were 61.3%, 0.27, 1.00, 0.55 and 1.00, respectively. The AUC of FREND COVID-19 Ag in samples with cycle threshold (Ct) values of both SARS-CoV-2 S and E genes <29.5 was 1.00. At ≥1.0 $TCID_{50}$/mL (median tissue culture infective dose per mL) manufacturer's cut-off, accuracy, sensitivity, specificity, NPV and PPV values were 99.2%, 1.00, 0.99, 1.00 and 0.95, respectively.
Conclusions: FREND COVID-19 Ag could not replace routine molecular testing for achieving a definitive diagnosis of SARS-CoV-2 infection, but can be used as a surrogate test for identifying patients with higher nasopharyngeal viral load and thus greater infectious potential.

Keywords: *COVID-19; SARS-CoV-2; diagnosis*

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a severe infectious disease which emerged at the end of 2019, and reached pandemic proportions, causing over 3.2 million of casualties to-date (1). Several lines of evidence now attest that the mainstays for preventing or limiting the dramatic impact of COVID-19 on human health, society and economy encompass the establishment of a series of physical preventive measures (2), accompanied by systematic testing of symptomatic cases and individuals with high risk of transmission or exposure to SARS-CoV-2 (severe acute respiratory syndrome coronavirus disease 2), thus enabling their timely identification, isolation and/or clinical management (3-5).

Many challenges still remain for clinical laboratories that are still handling a dramatic number of tests required for screening or diagnosing SARS-CoV-2 infections. A recent worldwide survey performed by the American

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Association of Clinical Chemistry (AACC) has revealed that over half of responders were currently unable to obtain supplies necessary to sustain the COVID-19 testing volume, with over three-forth admitting that no further increase in the local testing capacity would be possible in the near future (6). In this difficult situation, the availability of decentralized, easy, fast, reliable and possibly inexpensive tests is vital to overcoming the shortages and challenges currently faced by clinical laboratories.

A number of so-called SARS-CoV-2 antigen rapid detection tests (Ag-RDTs) have recently become available in the diagnostic market, though their reliability remains a matter of concern. As recently endorsed by the Task Force on COVID-19 of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), the use of these rapid antigen tests may support global screening and surveillance initiatives, provided that the assays are selected in the context of the intended clinical use and accurate clinical verification/validation is conducted before introducing the test into clinical practice (i.e., for screening, diagnosis and/or longitudinal monitoring of SARS-CoV-2 infection) (7). Therefore, this observational retrospective study was aimed at verifying the clinical performance of the microfluidic fluorescence immunoassay FREND COVID-19 Ag in a population of unselected individuals undergoing routine SARS-CoV-2 testing.

METHODS

Study population

The study population consisted of a series of outpatients referred to the Service of Laboratory Medicine of the Pederzoli Hospital (Peschiera del Garda, Verona, Italy) between April 12 and 30, 2021, for SARS-CoV-2 testing for being either symptomatic or having had close contact with one or more COVID-19 cases. A routine nasopharyngeal sample (Virus swab UTM™, Copan, Brescia, Italy) was collected at hospital admission and further conveyed to the local Service of Laboratory Medicine for both SARS-CoV-2 molecular and antigen testing.

Molecular testing

SARS-CoV-2 nucleic acid amplification testing (NAAT) was carried out using the Altona Diagnostics RealStar® SARS-CoV-2 RT-PCR Kit (Altona Diagnostics GmbH, Hamburg, Germany). This real-time reverse transcription polymerase chain reaction (rRT-PCR) entails two separate amplifications and detections, independently targeting the SARS-CoV-2 E and S gene sequences. A probe and a primer set for internal control is also included in the test kit, for detecting possible rRT-PCR inhibition. The test was carried out on a Bio-Rad CFX96Deep Well Dx Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Results were considered positive when the cycle threshold (Ct) values of both S and E SARS-CoV-2 genes were <45.

Antigen testing

The FREND COVID-19 Ag (NanoEntek, Seoul, South Korea) is a single-use fluorescence immunoassay, belonging to the so-called “third generation microfluidic” tests (8), which has been specifically developed for detecting the presence of SARS-CoV-2 nucleocapsid (N) protein in nasopharyngeal swab specimens. Briefly, a lysis buffer is used for extracting the viral particles from the swab and for releasing viral proteins. The functioning principle of the FREND COVID-19 Ag cartridge encompasses micro-fluidics lateral flow technology, where the analyte in the sample forms immune complexes while moving through the fluidics pathway. Therefore, released SARS-CoV-2 nucleocapsid proteins within the cartridge are captured by specific antibodies and then detected with additional antibodies conjugated to fluorescent micro-particles by the FREND cartridge reader. This system measures the fluorescence intensity generated in both a test zone (for revealing the presence and quantifying the amount of SARS-CoV-2 nucleocapsid protein) and in a control zone (for assuring test validity), displaying the final test results on the screen within 3-5 minutes. The final concentration of SARS-CoV-2 nucleocapsid protein in the test sample is calculated according to the ratio of fluorescent intensity of test and reference zones. According to the manufacturer’s declarations, the instrument uses 1 drop of sample (i.e., 35 µL) and is well suited for being connected to the hospital or laboratory information system. The test is basically semi-quantitative, positive (i.e., “presence of SARS-CoV-2 nucleocapsid protein”) when the concentration of the analyte is ≥1.0 $TCID_{50}$/mL (median tissue culture infective dose per mL). No specific analytical performance (i.e., analytical sensitivity, linearity and imprecision) details were found in the package insert, nor are previous studies assessing the clinical or analytical features of this system currently available, to the best of our knowledge.

Statistical analysis

Results of testing were presented as median and interquartile range (IQR). The diagnostic performance of FREND COVID-19 Ag compared to the reference molecular analysis was assessed with Spearman’s correlation, by constructing receiver operating characteristic (ROC) curves and calculating the diagnostic accuracy, sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) at two diagnostic cut-off of rRT-PCR positivity (Ct values of both SARS-CoV-2 S and E genes <45) and higher infectivity (Ct values of both SARS-CoV-2 S and E genes <29.5), as earlier ascertained by Gniazdowski et al. using the same RealStar® SARS-CoV-2 RT-PCR test (9). Statistical analysis was carried on with the software Analyse-it (Analyse-it Software Ltd, Leeds, UK).

This observational retrospective study was based on clinical laboratory operations for routine SARS-CoV-2 diagnostics at the local facility, and thereby patient informed consent and Ethics Committee approval were

unnecessary. All test results were anonymized prior to statistical analysis. The study was conducted in accordance with the Declaration of Helsinki, under the terms of relevant local legislation.

RESULTS

The final study population consisted of 256 patients (median age 48 years and IQR 29-58 years; 143 women, 55.9%), 136 (53.1%) testing positive with RealStar® SARS-CoV-2 RT-PCR Kit (Ct values of both SARS-CoV-2 S and E genes <45) and 36 (14.1%) with high viral load (Ct values of both SARS-CoV-2 S and E genes <29.5).

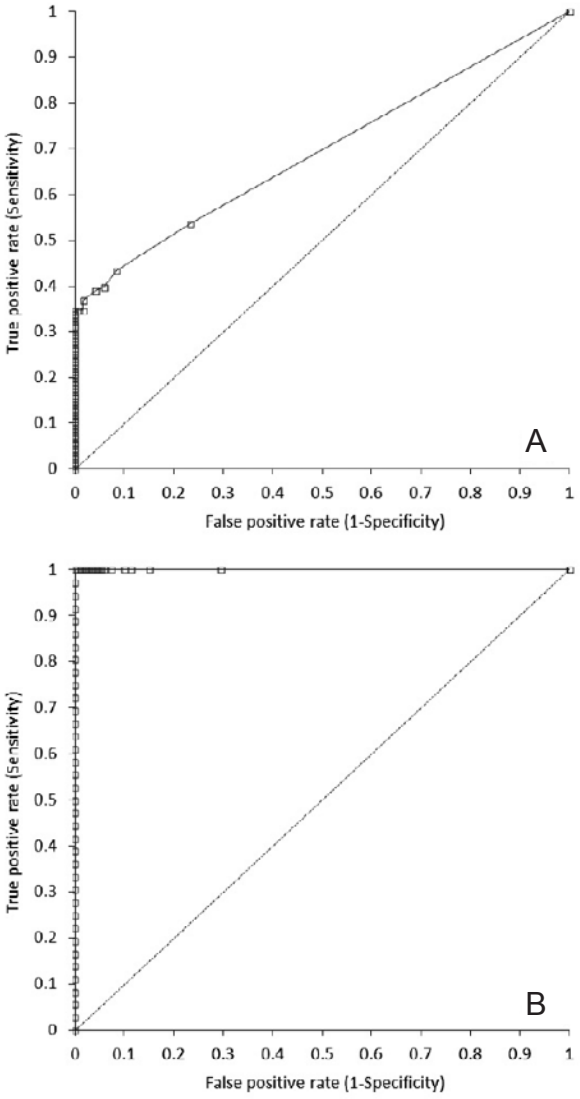

Figure 1
Receiver operating characteristic (ROC) curve of FREND COVID-19 Ag test for (a) identifying positive nasopharyngeal samples at molecular testing (i.e., cycle threshold values of both SARS-CoV-2 S and E genes <45) or (b) discriminating nasopharyngeal specimens associated with higher infectious risk (i.e., cycle threshold values of both SARS-CoV-2 S and E genes <29.5).

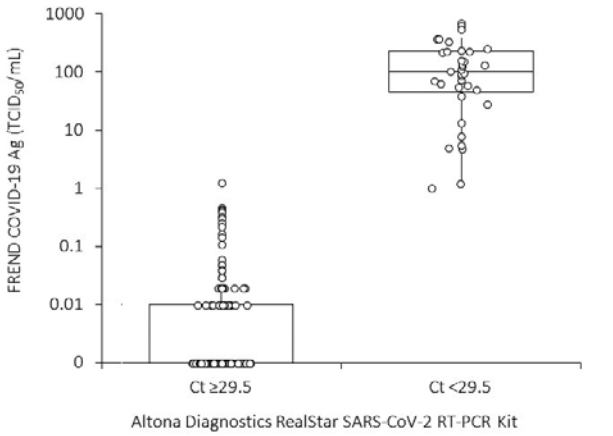

Figure 2
Distribution of FREND COVID-19 Ag test values according to the cycle threshold (Ct) values of both SARS-CoV-2 S and E genes ≥ or < 29.5.

The median Ct values of RealStar® SARS-CoV-2 RT-PCR Kit positive samples were 35.1 (IQR, 27.9-38.4) and 35.6 (IQR, 28.3-38.3) for SARS-CoV-2 S and E genes, respectively. The correlation between molecular assay and FREND COVID-19 Ag in positive samples was -0,50 (95%CI, -0.61 to -0.38; p<0.001) for both SARS-CoV-2 S and E genes, and 0.98 (95%CI, 0.98-0.99; p<0.001) between the Ct values of the two SARS-CoV-2 S and E genes.

The cumulative diagnostic performance of FREND COVID-19 Ag, as well as that for detecting nasopharyngeal samples with higher viral load is shown in Figure 1. The area under the curve (AUC) in all nasopharyngeal samples was 0.69 (95%CI, 0.64-0.75) (Figure 1A). At the ≥1.0 $TCID_{50}$/mL manufacturer's cut-off, the values of accuracy, sensitivity, specificity, NPV and PPV were 61.3% (95% CI, 55.1-67.3%), 0.27 (95% CI, 0.20-0.35), 1.00 (95% CI, 0.97-1.00), 0.55 (95% CI, 0.2-0.57) and 1.00 (95% CI, 1.00-1,00) respectively. Lowering the cut-off to ≥0.02 $TCID_{50}$/mL, the corresponding values of accuracy, sensitivity, specificity, NPV and PPV values were 66.0% (95% CI, 60.0-71.8%), 0.43 (95% CI, 0.35-0,52), 0.92 (95% CI, 0.85-0.96), 0.59 (95% CI, 0.55-0.63) and 0.86 (95% CI, 0.76-0.92), respectively.

The distribution of FREND COVID-19 Ag test values according to Ct values of RealStar® SARS-CoV-2 RT-PCR Kit is summarized in Figure 2. The median nucleocapsid antigen concentration in samples with Ct values of both SARS-CoV-2 S and E genes <29.5 (102.0 $TCID_{50}$/mL, IQR, 44.2-232.8 $TCID_{50}$/mL) was found to be significantly higher than that of samples with higher Ct values (0 $TCID_{50}$/mL; IQR, 0-0.01 $TCID_{50}$/mL; p<0.001) (Figure 2).

The AUC of FREND COVID-19 Ag in nasopharyngeal samples with Ct values of both SARS-CoV-2 S and E genes <29.5 was 1.00 (95% CI, 1.00 to 1.00; p<0.001) (Figure 1B). At the ≥1.0 $TCID_{50}$/mL

manufacturer's cut-off, the values of accuracy, sensitivity, specificity, NPV and PPV were 99.2% (95% CI, 97.2-99.9%), 1.00 (95% CI, 0.90-1.00), 0.99 (95% CI, 0.97-1.00), 1.00 (95% CI, 1.00-1.00) and 0.95 (95% CI, 0.81-0.99), respectively.

DISCUSSION

The pandemic proportion reached by the ongoing SARS-CoV-2 outbreak calls for the establishment of reliable, trustable and effective counteractive strategies, which encompass widespread availability of COVID-19 diagnostics for anticipating or limiting viral spread within specific populations or social settings (10). The increasing availability of SARS-CoV-2 antigen immunoassays, still largely based on Ag-RDTs, is seen as a potentially viable surrogate of molecular tests under highly specific circumstances. In fact, these rapid antigen immunoassays may also be available as a point of care (POC) devices that do not require specific staff or dedicated instrumentation to be run and are characterized by a shorter turnaround time compared to routine nucleic acid amplification tests (NAATs) (11). Nonetheless, their placement within SARS-CoV-2 diagnostic protocols should be clearly defined and validated, thus preventing any misuse that may cause unwarranted consequences, such as misdiagnosis of COVID-19 or misidentification of SARS-CoV-2 cases with high viral load and larger infective potential, as recently highlighted by Goyal et al. (12).

The results of our observational retrospective study, where the clinical performance of a "third-generation" microfluidic fluorescence immunoassay has been compared with that of a reference NAAT, confirm previous evidence summarized by the Cochrane COVID-19 Diagnostic Test Accuracy Group (13), that the sensitivity of SARS-CoV-2 antigen immunoassays depends largely on the viral load present in the test specimens. It is hence not surprising that we found a cumulative 61% accuracy for definitive diagnosis of SARS-CoV-2 infection, since these types of tests are not suited, nor should be used, for this specific purpose. The adoption of a much lower cut-off than that recommended by the manufacturer (i.e., ≥0.02 *versus* ≥1,0 $TCID_{50}$/mL) was substantially effective at improving the diagnostic accuracy in our cohort of unselected outpatients undergoing routine SARS-CoV-2 diagnostic testing (i.e., 66% *versus* 61%). However, when we limited our analysis to nasopharyngeal specimens with high viral load (i.e., Ct values of both SARS-CoV-2 S and E genes <29.5), the diagnostic accuracy at the ≥1.0 $TCID_{50}$/mL manufacturer's cut-off became optimal, with remarkably high accuracy (i.e., 99.2%) and virtually perfect NPV (i.e., 1.00). This would translate into the concept that a negative FREND COVID-19 Ag test result (i.e., <1.0 $TCID_{50}$/mL) would enable exclusion, with near 100% accuracy, of nasopharyngeal viral loads of both SARS-CoV-2 S and E genes <29.5. As a matter of fact, Gniazdowski et al. found that the SARS-CoV-2 virus grew in cell culture was in <3% of all cases when the Ct value of RealStar SARS-CoV-2 RT-PCR Kit test was >30, whilst tests following negative results always had Ct values >29.5 and were not associated with positive virus culture. This strongly suggests that the likelihood of bearing viable viral particles, and thereby the risk of being a sustained SARS-CoV-2 spreader, would be very limited when FREND COVID-19 Ag test result is negative (for the presence of SARS-CoV-2 nucleocapsid protein). This remarkable NPV for detecting high viral load was also found to be associated with very high PPV (i.e., 0.95), which hence allows to reliably assume that a FREND COVID-19 Ag positive test results would efficiently permit the identification of subjects with high viral load, thus allowing immediate isolation and/or appropriate clinical management (if symptomatic).

Evidence has also been published that a higher nasopharyngeal viral load may be associated with adverse clinical progression, more specifically with greater risk of developing severe and/or critical forms of COVID-19 illness (14,15). The use of rapid antigen tests, enabling identification rapidly and with high accuracy of this subset of high-risk patients could be a valuable resource for risk stratification and for longitudinal monitoring of COVID-19 patients, both at hospital admission (16) and/or during their stay in sub-intensive and intensive care units (17).

In conclusion, the findings of this observational retrospective study suggest that FREND COVID-19 Ag could not replace routine molecular testing for achieving a definitive diagnosis of SARS-CoV-2 infection, but can be used as a surrogate assay for identifying patients with higher nasopharyngeal viral load and thereby with greater infectious potential.

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CONFLICT OF INTEREST

None.

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