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Evaluation of antigen-based fluorescent immunoassay detection test for the diagnosis of SARS-CoV-2 in nasal swab specimens

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ABSTRACT
World Health Organization (WHO) has declared novel corona virus 2019 (COVID-19) as a global pandemic. Various rapid antigen detection test kits are available for the qualitative determination of SARS-CoV-2 antigens for SARS-CoV-2 infections.The purpose of this study was to assess diagnostic accuracy of STANDARD F COVID-19 Ag FIA test with nasal swab specimens compared to standard comparator RT-PCR test for detection of SARS-CoV-2.A total 822 study subjects were recruited to obtain minimum 100 SARS- CoV-2 positive study subjects and 400 SARS- CoV-2 negative specimens. Diagnostic accuracy of STANDARD F COVID-19 Ag FIA test was assessed in comparison to reference standard RT-PCR test for the detection of SARS-CoV-2 infection.We observed that sensitivity and overall specificity of the STANDARD F COVID-19 Ag FIA test was 95.5% and 99.2%respectively. Again, we found that sensitivity of STANDARD F COVID-19 Ag FIA test for 0-3 days post onset symptoms was 91.1% and 100%. The sensitivity and specificity in asymptomatic study group was observed to be 100% and 99.7%, respectively.The sensitivity ofSTANDARD F COVID-19 Ag FIA test in terms of cycle threshold i.e. ≤ 30 CT and >30 CT in positive study subjects was 96.7% and 88.2%, respectively.
The STANDARD F COVID-19 Ag FIA test showed sensitivity and specificity within acceptable limits for rapid detection of SARS-CoV-2 infection in nasal swab specimens of symptomatic and asymptomatic patients. In addition to negative results obtained by rapid immunoassay kits, target gene specific real time PCR test must be performed for the verification of results.
Key words:SARS-CoV-2, COVID-19 Ag FIA test, Rapid antigen test, Fluorescent immunoassay.

I. INTRODUCTION

Coronavirus is a single-stranded positive-sense RNA virus with an envelope of about 80 to 120 nm in diameter. Its genetic material is the largest of all RNA viruses and is an important pathogen of many domestic animals, pets, and human diseases. It can cause a variety of acute and chronic diseases. Common signs of a person infected with a coronavirus include respiratory symptoms, fever, cough, shortness of breath, and dyspnea. In more severe cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure, and even death. The 2019 new coronavirus, or "2019-nCoV", was discovered because of Wuhan Viral Pneumonia cases in 2019, and was named by the World Health Organization (WHO) on January 12, 2020, confirming that it can cause colds and the Middle East Respiratory Syndrome (MERS) and more serious diseases such as severe acute respiratory syndrome (SARS)[1].On 11 Mar 2020, World Health Organization (WHO) has declared novel coronavirus 2019 (COVID-19) as a global pandemic[2]. Real time reverse transcription polymerase chain reaction (RT-PCR)test is the gold standard in the diagnosis of SARS-CoV-2 infection[3]. Nowadays, various rapid antigen detection test kits are available for the qualitative determination of SARS-CoV-2 antigens in context to SARS-CoV-2 infections.A recent study suggested that SARS-CoV-2 testing volume was not able to even reached the no. of cases affected by the infection occurs, including most of the developed countries[4-5].This lacuna may lead to delayed diagnosis and clinical management of the infections, further associated with the spread of viral transmission & progression of disease [6], and also affects contact tracing strategies[7].Due to certain limitations of Nucleic acid based tests, including dedicated turnaround time, requirements of highly skilled professionals and equipment, rapid immunoassay test kits are much useful in testing at a larger scale, also endorsed by

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WHO[8].The diagnostic sensitivities of rapid immunoassay test kits are lower as compared to the RT-PCR; however, their mass scale usage with minimal resources may be an advantage to the early detection of the infection and screening of population[9].

The purpose of this study was toassess diagnostic accuracy of STANDARD F COVID-19 Ag FIA test with nasal swab specimens compared to standard comparator RT-PCR test for detection of SARS-CoV-2.

II. METHODOLOGY

Statistics & Sample Size Calculation

The sample size was calculated using online sample size calculator (calculator.net) software[10].Accordingly, minimum 97 SARS-CoV-2 positive specimen were required to analyse the qualitative results and accuracy of STANDARD F COVID-19 Ag FIA test compared to standard reference assay RT-PCR test with 10% of population proportion and 6% of margin of error (as mentioned in STANDARD F COVID-19 Ag FIA kit insert, by SD Biosensor, Inc., KOREA) at 95% confidence interval. An unpaired't' test was used to compare the mean of two independent groups by GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as a p-value <0.05.

Study population and selection criteria

We have conducted a prospective, randomized, blinded study to evaluate the diagnostic accuracy of STANDARD F COVID-19 Ag FIA test with nasal swab specimens compared to reference standard RT-PCR test for detection of SARS-CoV-2 infection.

A total of 822 study subjects were recruited to obtain minimum 100 SARS- CoV-2 positive study subjects (due to low positivity rate of SARS- CoV-2 subjects) and 400 SARS- CoV-2 negative specimens for the present study. Study subjects were recruited based on inclusion and exclusion criteria, samples were collected from various registered sample collection centers, of Rapidx pathology labs- A unit of Cauro Diagnostics Pvt Ltd, Gurugram (HR)-122001, India, for present study.The Study subjects were recruited on following inclusion and exclusion criteria:

SARS- CoV-2 positive subjects

1) Ct value > 30 - more than 10 subjects
2) Required subject number based on post onset symptom date for nasal swab

	Post onset symptom date	No. of Subject required
Symptomatic	days 0-3	Minimum 40
	days 4-7	Minimum 40
Asymptomatic	No Symptoms	N*
Total	NA	Minimum 100

N*: It means that there is no specific required number of subjects.

3) Study subjects were recruited based on questionnaire indicating symptoms of fever, cough & cold, fatigue, headache, body ache, loss of appetite, taste and smell and throat pain, etc.
4) SARS- CoV-2 symptomatic subjects are divided into two groups (N=40) based upon the onset of symptoms i.e. 0-3 days & 4-7 days. The randomization of subjects was performed by block randomization method using online software (Research randomizer)[11], in study groups with 2 Sets of 40 Unique sample ID per Set & Range: From 1 to 80 as follows:

Set #1 (Group I, Symptomatic):
P4, P23, P54, P78, P34, P17, P38, P13, P6, P75, P63, P25, P71, P58, P20, P7, P28, P56, P67, P64, P9, P26, P41, P51, P1, P62, P31, P29, P76, P16, P52, P59, P19, P35, P12, P18, P22, P24, P5, P2

Set #2 (Group II, Symptomatic):
P11, P14, P73, P53, P74,P26, P24, P49, P20, P80, P50, P71, P19, P40, P23, P32, P33, P29, P45, P43, P75, P66, P68, P38, P15, P35, P13, P7, P67, P64, P52, P56, P2, P8, P69, P42, P76, P5, P58, P34

SARS- CoV-2 Negative subjects

Study subjects were confirmed as SARS- CoV-2 negative with reference assay. The subject was enrolled when "Essential information for the specimen is permitted" and provided consent for enrollment in the study.

Exclusion criteria

- Obstruction of one or more nares.
- Any condition that in the judgment of the investigator precludes participation because it could adversely affect subject safety or data integrity.
- Any patient who does not give consent for participation in this study.
- COVID-19 re-infection patients were excluded from the study.

Sample collection and clinical performance

All the study subjects were recruited

between the duration of 14th February to 28th February, 2021.An approval of ethics committee of Good Society for Ethical Research, New Delhi, was obtained (GSER/2021/BMR/CL/007), and informed consents were obtained from the recruited study subjects prior to study. COVID-19 suspected patients visited clinics (sample collection site), two nasal swabs was collected from single subject by first technician and test has been performed with STANDARD F COVID-19 Ag FIA using one swab, as per manufacturers protocol. The operators performing the Antigen test was not aware of the reference assay result. Second nasal swab was stored in Viral Transport Media (VTM) for RT PCR assay.

STANDARD F COVID-19 Ag FIA test of SARS-CoV-2

STANDARD F COVID-19 Ag FIA is a fluorescent immunoassay for the qualitative detection of the specific nucleocapsid protein antigen from SARS-CoV-2 in nasopharyngeal and nasal swab specimens. STANDARD F COVID-19 Ag FIA should be used with the STANDARD F Analyzers, manufactured by SD Biosensor, Inc., KOREA. The first Swab specimen was placed into the extraction buffer tube. While squeezing the buffer tube, swab was removed while squeezing the sides of the tube to extract the liquid from the swab. The nozzle cap was tightly pressed onto the tube. Patient's information was labeled on the test device and insertedto the test slot of the analyzer. Once it wasinserted, the analyzer read the barcode data and checked the validity of the test device. Subsequently, 4 drops of extracted specimen was applied to the specimen well of the test device and immediately pressed 'TEST START' button.Results are conducted automatically and displayed after 15 minutes.Resultswere interpreted in terms of Cutoff index (COI) value. The COI is a numerical representation of the measured fluorescence signal. COI $\geq$ 1.0 indicates Positive for SARS-COV-2 Antigen, whether COI < 1.0 depicts Negative for SARS-COV-2 Antigen. If COI value is not displayed, it represents the Invalid test;Retesting should be performed with a new test device and specimen.

Reference standard RT-PCR test of SARS-CoV-2:

RNA was extracted from second swab placed in VTM by Zybio automated Nucleic acid isolation system (EXM3000), China, using Zybio Nucleic acid extraction kit. Real time PCR was performed by BIO-RAD CFX96 Real time system (Bio-Rad Laboratories, Inc., CA, USA).The detection of SARS-CoV-2 infection was performed by using TRUPCR® SARS-CoV-2 RT qPCRKIT (Kilpest India Ltd, India), as per manufacturers protocol.The target genes were RdRP+N and E genes. RNaseP gene was served as an internal control. The cutoff of the kit was 35 cycle threshold (CT). Any amplification beyond the cutoff point was not considered for true amplification or positive results.

Data analysis

STANDARD F COVID-19 Ag FIA test results was compared with the results obtained from reference real time PCR assay in terms of diagnostic sensitivity; Positive percent agreement (PPA). PPA was reported as the number of positive samples for the STANDARD F COVID-19 Ag FIA test on the total number of 'true' positive samples tested together with a 2-sided 95% confidence interval in real time assay.

Diagnostic specificity; Negative percent agreement (NPA) of the STANDARD F COVID-19 Ag FIA test was calculated with reference assay as the number of negative samples for the STANDARD F COVID-19 Ag FIA test on the total number of 'true' negative samples tested together with real time assay in nasal swab.

III. RESULTS

A total 822 subjects including Positive (N=110) and Negative (N=712) for SARS- CoV-2 test, has been studied in current study. The distribution of age, gender and general data is described in **Table 1**. Total no. of male subjects (N=521) was higher as compared to female study subjects (N=301)**(Table 1A).**

We observed a significance difference (p=0.002) in the mean age of positive and negative group of study subjects. Similarly, a statistically significant difference was observed between positive and negative group of male (p=0.03) and female study subjects (p=0.01). There was no significant difference (p=0.058) observed in symptomatic study subjects for positive and negative results of SARS- CoV-2 infection. However, we observed a statistically significant difference (p=0.006) between the positive and negative results among total asymptomatic study subjects **(Table 1B).**

We compared the results of STANDARD F COVID-19 Ag FIA test with the reference RT PCR assay. We observed that 105 subjects (out of 110RT PCR positive subjects) were tested positive with STANDARD F COVID-19 Ag FIA assay. Similarly, 706 subjects (out of 712RT PCR negative subjects), were tested negative with STANDARD F COVID-19 Ag FIA assay. Total 6

subjects were found false positive and 5 subjects were found as false negative with STANDARD F COVID-19 Ag FIA assay. **(Table 1C)**

Therefore, we observed that sensitivity and overall specificity of the STANDARD F COVID-19 Ag FIA test kit was95.5% and99.2%,respectively. Again, we found that sensitivity ofSTANDARD F COVID-19 Ag FIA test kit for 0-3 days post onset symptoms was 91.1% and 100%. The excellent sensitivity and specificity was observed for asymptomatic study group as 100% and 99.7%, respectively. **(Table 2A)**

The sensitivity ofSTANDARD F COVID-19 Ag FIA test kit in terms of cycle threshold i.e. ≤ 30 CT and >30 CT in positive study subjects are 96.7% and 88.2%, respectively. **(Table 2B)**

IV. DISCUSSION

We evaluated the diagnostic accuracy of the STANDARD F COVID-19 Ag FIA, a fluorescent immunoassay for the qualitativedetection of SARS-CoV-2 in nasal swab specimens compared to that of RT-PCRas the reference standard. This test is for in-vitro professional diagnostic use and intended as an aid to early diagnosis of SARS-CoV-2 infection in patient with clinical symptoms with SARS-CoV-2 infection.

Although point-of-care antigen-based rapid fluorescent immunoassay tests are less time consuming & easy screening technique for population, their diagnostic sensitivity, specificity and accuracy differs widely, reported by the Cochrane COVID-19 Diagnostic Test Accuracy Group[12].Previous studies reported that rapid antigen assay kits have shown greater extent of detection and lesser levels of sensitivity as compared to qualitative real time PCR tests[13-15].False negative results can also be the matter of discussion for the samples having low viral load in rapid antigen test for SARS-CoV-2 analysis.

In the present study, STANDARD F COVID-19 Ag FIA test showed magnificent specificity (99.2%) and good sensitivity (95.5%) in the studied samples. In asymptomatic study group the sensitivity and specificity of the present kit was found to be excellent i.e. 100% & 99.7%, respectively.Similarly, we observed a higher sensitivity (97.7%) for the present kit in high viral load patients having 4-7 day post onset symptoms. The sensitivity of the assay was higher (96.7%) for the samples having ≤30 CT values as compared to the sensitivity (88.2%) obtained for samples having >30 CTvalues (for target genes RdRp and N) by real time PCR.Therefore, rapid fluorescent immunoassay tests can be helpful for the rapid screening of coronavirus infection in the symptomatic patients with increased viral loads.

We also observed that the rate of positive cases among symptomatic study subjects was found to be high as compared to asymptomatic study subjects. As per WHO 2020, Coronavirus disease 2019 (COVID-19) Situation Report – 73[16],data from various clinical and virological studies suggested that shedding of COVID-19 virus is highest in patients during the initial three days from the onset of symptoms and may be more infectious as compared to later with the day's progression[17-18].In our study, we have recruited unbiased study subjects, based on of inclusion and exclusion criteria and block randomization was used for the segregation of symptomatic patients in two groups.In terms of positive cases, the no. of male subjects (N=63) was found to be 34% more than the no. of female subjects (N=47). Recent study on large population - based cohort suggested that male subjects withlower educational acquirement was independently associated with positive cases for COVID-19, among overall recruited study subjects[19].

Previous study suggested that nasopharyngeal swab samples having CT values >25, is suitable for isolation of virus, although it was also reported for theCT values >35 through thelive virus culture methods to understand the infectious potential of the virus[20].A negative result may occur if the concentration of antigen is below the limit of detection of the test or if the specimen was collected or transported improperly, therefore a negative test result does not eliminate the possibility of SARS-CoV-2 infection, and should be confirmed by molecular assay.In addition to negative results obtained by rapid immunoassay kits, target gene specific real time PCR test must be performed for the verification of results.

In conclusion, the results of our clinical evaluation of STANDARD F COVID-19 Ag FIA test by SD Biosensor Inc. showed excellent sensitivity and specificity within acceptable limits for rapid detection of SARS-CoV-2 infection in nasal swab specimens of symptomatic and asymptomatic patients. Present STANDARD F COVID-19 Ag FIA test by SD Biosensor Inc. is user-friendly test kit with cost-effective and rapid detection excellence for the SARS-CoV-2 infections. The rapid antigen testing might be useful in screening on large-scale population. However, due to its false-negative results, molecular diagnostic assay is suggested to rule out the SARS-CoV-2 infections.Our observations are exploratory; present study was carried out to

ascertain diagnostic accuracy of STANDARD F COVID-19 Ag FIA test in nasal swab specimens.

Conflict of interest

No conflicts of interest to disclose.

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TABLE 1: Results of STANDARD F COVID-19 Ag FIA assay

1A: General data of study subjects

Study subjects	Comparative Results	
	RT-PCR assay	COVID-19 FIA Ag test
Total no. of study subjects	822	822
Positive	110	105
Negative	712	706
False positive	N/A	06
False negative	N/A	05

Table 1B: Distribution of age among study groups

Study group	Average age in study subjects*		p-value#
	Positive	Negative	
Total subjects (N= 822)	39.2 ± 17.6	34.5 ± 14.1	0.002
Total Male subjects (N= 521)	38.60 ± 16.5	34.4 ± 14.1	0.03
Total female subjects (N= 301)	40.36 ± 18.9	34.58 ± 14.1	0.01
Total Symptomatic (N= 97)	38.3 ± 17.8	26.8 ± 6.9	0.058
Symptomatic; 0-3 days (N= 47)	40.3 ± 18.7	27.5 ± 4.9	0.34
Symptomatic; 4-7 days (N= 50)	36.3 ± 16.9	26.5 ± 7.6	0.14
Total Asymptomatic (N= 725)	43.0 ± 16.1	34.6 ± 14.2	0.006

*Results are expressed as age in years &mean ±SD.#p<0.05 is considered to be significant.

1C: Comparative analysis of positive results of COVID-19 FIA Ag test with reference standard RT-PCR assay

Subjects	Age*	RT-PCR	CT Values of target genes		COVID-19 FIA Ag test
			RdRp+N gene	E gene	
Male (N=63)	38.6±16.5	Positive	24.7±4.5	22.8±4.8	Positive = 61 False negative= 2
Female (N=47)	40.36±18.9	Positive	26.1±5.3	24.8±5.8	Positive= 44 False negative= 3
Total (N=110)	39.2±17.6	Positive	25.3±4.9	23.6±5.3	Positive= 105 False negative= 5 Sensiivity= 95.5% Specificity= 99.2%

*Results are expressed as age in years & mean ±SD.

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Table 2: Result analysis of STANDARD F COVID-19 Ag FIA test as compared to reference standard RT-PCR assay

Table 2A: Specificity and sensitivity of STANDARD F COVID-19 Ag FIA

Parameters	**Sensitivity (%)[#]**	**Specificity (%)[#]**
Overall	105/110 X 100 = 95.5	706/712 X 100 = 99.2
All Symptomatic	83/88 X 100 = 94.3	5/9 X 100 = 55.5
0-3 days Post-onset symptoms	41/45 X 100 = 91.1	2/2 X 100 = 100
4-7 days Post-onset symptoms	42/43 X 100 = 97.7	3/7 X 100 = 42.9
All Asymptomatic	22/22 X 100 = 100	701/703 X = 99.7

Data expressed as percentage; no. of antigen test/ no. of total test by referece RT PCR test

Table 2B: Sensitivity of STANDARD F COVID-19 Ag FIA kit based on cycle threshold (CT) cut-off in positive study subjects

Parameters	**Sensitivity (%)[#]**
Subjects ≤ 30 CT value of target genes	15/17 X 100 = 96.7
Subjects> 30 CT value of target genes	83/88 X 100 = 88.2

CT, Threshold cycle; [#]Data expressed as percentage; no. of antigen test/ no. of total test by referece RT PCR test