EVALUATION OF CURRENTLY AVAILABLE MOLECULAR ASSAYS AND PERFORMANCE OF SAMPLING APPROACHES FOR DETECTION OF SARS-COV-2 RNA

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How to cite this article

Asad MT, Khattak AA, Awan UA, Iqbal W, Ahmad Z, Abbas M. Evaluation of Currently Available Molecular Assays and Performance of Sampling Approaches for Detection of Sars-Cov-2 RNA. J Gandhara Med Dent Sci. 2024;11(1):21-25

Date of Submission: 09-06-2023
Date Revised: 27-11-2023
Date Acceptance: 28-11-2023

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ABSTRACT

OBJECTIVES

This study aims to identify the essential characteristics of diagnostic tests for SARS-CoV-2 and to discuss the limitations of currently available tests and their impact on the test selection process.

METHODOLOGY

The current study was conducted at Mardan Medical Complex (MMC). One hundred nasopharyngeal-positive samples were collected from February to March 2021. Oropharyngeal swab OPS, sputum, and blood samples were collected from the participants to detect SARS-CoV-2 RNA. RNA extraction of SARS-CoV-2 was done using a BigFish auto extractor. A Qiagen Thermal Cycler was used for genome amplification. Five different molecular assays, namely COVSIGN (N gene) Spain, BGI (ORF1ab gene) China, Maccura(ORF1ab, E and N gene) China, R-GENE (RdRp and N genes) France and Genuru (N gene, S gene and ORF ab/1) were used.

RESULTS

100 % positivity was recorded in the sputum of all individuals, followed by 91 % OPS and 21% blood. The highest positivity rate for different genes was observed. ROC (Receiver operating characteristic curve) was developed through SPPS version 26.00 to compare the sensitivity and specificity.

CONCLUSION

By comparing the results of different diagnostic kits, it was found that BGI and Maccura are the most sensitive and specific for diagnostic purposes against COVID-19.

KEYWORDS: *Molecular Assays, Sars-Cov-2 RNA, Sampling Approaches COVID-19*

INTRODUCTION

The name corona was derived from a Latin word which means a crown, as its structure is spherical with a core-shell and surface projection. Coronaviruses are single-stranded RNA viruses that cause infection in animals and humans. Coronaviruses are zoonotic viruses that can pass from animals to humans and are transmitted through aerosols and droplets in the air once they are adopted by humans.[1] Coronavirus infections were initially identified as a cause of the common cold in 1960. In 2002, the first lethal severe acute respiratory syndrome (SARS-CoV) causing coronavirus was discovered. SARS-CoV was reemerged in China similarly to SARS-CoV-2.[2] The outburst of the novel coronavirus SARS-CoV-2 that started in the Hubei Province, China, resulted in a global pandemic. The World Health Organization's (WHO) Emergency Committee declared a worldwide health emergency on January 30, 2020, citing rising case notification rates in China and internationally.[3] A high number of mutations occurs within the spike's protein of the SARS-CoV-2-2, causing variance that helps the virus protect against the vaccine-induced.[4,5] These mutations change the virus's properties, i.e., transmittance, morbidity, vaccine performance, treatment mechanism and diagnostics. SARS-CoV-2 can be transferred both directly and indirectly (through droplets and person-to-person transmission) (via airborne contagion and contaminated objects).[6,7] Individuals with COVID-19 experience a broad range of symptoms. Acute respiratory distress syndrome and multi-organ failure can range from asymptomatic to acute respiratory distress syndrome.[8,9] As a result, obtaining an accurate COVID-19 diagnosis is difficult. Routine diagnosis of COVID-19 is based on clinical signs, epizootiology

history and laboratory diagnostic methods such as nucleic acid amplification test (NAAT), computed tomography (CT) scans and serological approaches.[10] Several investigations have argued that pre-analytical and analytical factors were to blame for the failure to detect SARS-CoV-2.[11,12,13] Low viral load associated with disease phase, recombination or mutation of viral genes, and a lack of standardisation of specimen type, sampling time, sample storage conditions, contamination, and insufficiently validated and verified assays were all cited as potential causes for inaccurate results.

METHODOLOGY

This study was carried out at the Medical Lab Technology Department of the University of Haripur in collaboration with Mardan Medical Complex (MMC). An ethical Approval certificate was obtained from MMC Mardan NO. 2516/MMC. Individuals from varied demographic backgrounds and geographical areas were eligible to participate, as were individuals of either gender between the ages of 18 and 60 who had a suspected or confirmed SARS-CoV-2 infection. Pulmonary specimens, including sputum, bronchoalveolar lavage, nasopharyngeal and oropharyngeal swabs, and other similar samples, were taken within a certain period following the symptoms beginning. People not tested for or diagnosed with SARS-CoV-2 are not eligible to participate. Subjects whose medical history or current medications could compromise the reliability of molecular tests, samples taken before or after the allotted time, and specimens lacking sufficient quantity or quality to permit testing. The test samples were collected at the Medical Teaching Institution, Mardan Medical Complex (MTI, MMC) Mardan. One hundred nasopharyngeal-positive samples were collected from February 2021 to March 2021. Those positive patients were further sampled for oropharyngeal, sputum, blood, and faecal samples to diagnose SARS-CoV-2 RNA. The specimens were put forward for routine 2019-nCoV diagnosis. Nasopharyngeal swab NPS, Oropharyngeal swab OPS, sputum and blood were obtained from each patient and mixed with 3mL of viral transport medium. Samples were aliquoted and kept at 80°C after initial testing. RNA extraction of SARS-CoV-2 was done using the BigFish auto extractor. Using a viral extraction kit (DNA/RNA), 200µL viral RNA were extracted. Automatic NA extraction system BigFish auto extractor was used following manufacturer guidelines. One elution volume per sample was sufficient to execute all RT-qPCR reactions in this investigation. Elutes were kept at a temperature of 80°C. About 200ul samples were obtained from VTM and poured into the BigFish auto extractor (RNA extraction kit). Afterwards, this healthy plate was placed in an extraction machine (BigFish Auto Extractor Machine), and after 30 minutes, the extracted RNA was obtained.[14] Qiagen Thermal Cycler (RT-PCR amplification machine) was used to amplify the extracted RNA. Five amplification kits were used: master mix, enzyme mix, positive control and negative control. 19ul of master mix and enzymes mix 1ul were taken in a tube and mixed thoroughly. Afterwards, 5ul of extracted RNA in the corresponding tube was placed in the thermal cycler. After selecting fluorochrome, the Thermal cycler was run within 2 hours of the amplification report. Different molecular diagnostic assays used in the current study were frequently employed in the designated COVID-19 lab of MTI, MMC, for SARS-CoV-2 diagnosis, for the qualitative diagnosis of Covid-2019 Qiagen RT-PCR thermal cycler was used for amplification of five different molecular detection kits. (COVSIGN RT-PCR (Fluorescence probe for detection of N gene) Spain, BGI RT-PCR (ORF1ab gene) China, Maccura RT-PCR (ORF1ab, E and N gene) China, R-GENE (for the detection of RdRp and N genes) France, Genuru (for the detection of N gene, S gene and ORF ab/1). Positive and negative were declared based on CT values. Patients with CT values lower than 35 were considered Positive, while those with more than 35 were negative. Each assay's negative, positive, and internal controls were employed for quality assurance and quality control. The Data had a normal distribution, and numerical data was represented as the arithmetic mean with standard deviation. The investigation was carried out using IBM SPSS Version 26.0.

RESULTS

The current study was designed to evaluate different specimens (nasopharyngeal, oropharyngeal, blood and sputum) and different diagnostics assays/kits (Maccura, BGI, Genru, Argene and Covsign) used in real-time reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of SARS-CoV-2 RNA.

Different Diagnostic Specimen Comparison

Different specimens were collected from the same patients whose nasopharyngeal samples were positive, i.e., oropharyngeal, blood and sputum. The present study collected separate samples for each specimen (oropharyngeal, blood, nasopharyngeal and sputum). These samples were then run in 5 diagnostics assays /kits (Maccura, BGI, Genru, Argene and Covsign). All these kits/assays targeted different genes for amplification. BGI (China) amplify the ORF1ab1 gene whereas Maccura amplifies the ORF1ab1, E gene and N gene, Genru amplify FAM N and S gene, Argene amplifies RdRP and Green FAM N gene, and Covsign

amplifies the ORF1ab1, E and N gene. After running the thermal cycler (RT PCR) machine, CT (cycle threshold) values of the different genes were obtained, which were used to interpret the results (positive or negative). The CT value represented the number of cycles necessary to identify the virus. The CT value of genes equal to or greater than ≥35 was considered negative, and those with CT values less than <35 were positive.

Null and Alternate Hypothesis were as follows:

Ho: "There is no significant difference between the different used diagnostic kits sensitivities and specificities."

Ha: There is a significant difference between the different diagnostic kits used, sensitivities, and specificities.

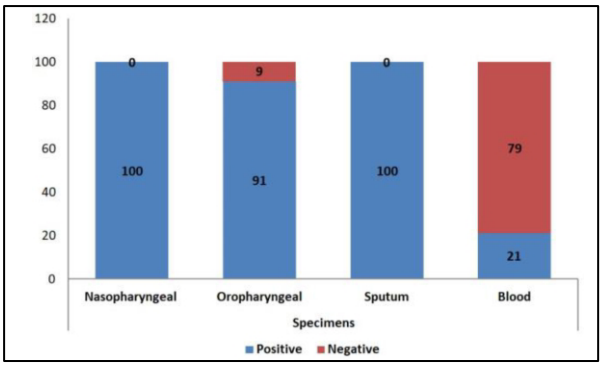

Figure 1: Percentage Distribution of the Range of the Covid-19 Sample Sources from Different Patients

One hundred nasopharyngeal COVID-19-confirmed positive individuals were included in the study. Afterwards, these patients were further sampled for oropharyngeal, blood and sputum. Each individual was sampled and analysed for these stated specimens (oropharyngeal, blood and sputum). Sputum was observed (100%) to be positive in all individuals for finding RNA of SARS-CoV-2 detection, followed by oropharyngeal (91%) and blood (21%), respectively.

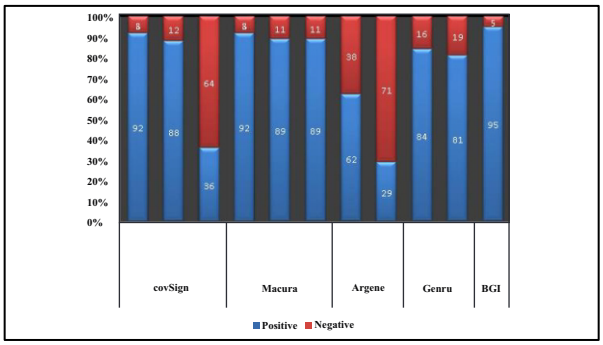

Figure 2: Different Diagnostic Kits Gene Amplification

Using the CovSign diagnostic kit, maximum ORF1ab1 gene amplification (92%) was observed, whereas 88% and 36% positive results were observed for E and N genes, respectively. On the other hand, the highest ORF1ab1 gene amplification (92%) was observed in the Maccura kit. Both genes, i.e., E and N, were 89% positive. Green FAM N gene's amplification was 62% positive using the Argene kit, while RdR1 (cy5) was only positive for 29% of cases. By using Genru FAM (N) and VIC (S), it was found that 84% and 81% were positive. About 95% gene amplification (FAM ORF ab/1) was observed by using a BGI diagnostic kit. By comparing the results of different diagnostic kits, it was found that BGI and Maccura were the most sensitive and specific assays for diagnostic purposes against COVID-19. ROC curve (receiver operating characteristic curve) was developed through SPSS. Positive (+ve) was considered 1, and negative (-ve) was 0. Data was analysed, and Ties produced diagonal segments

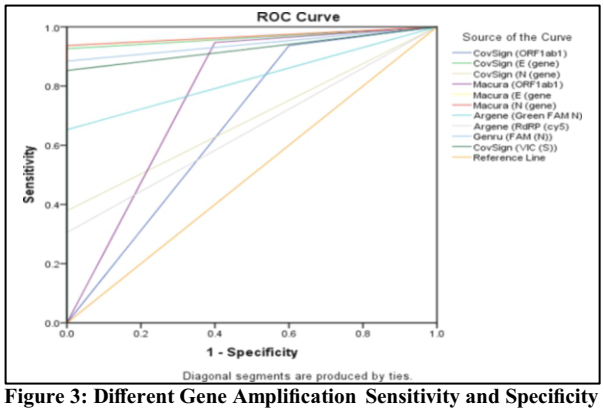

Figure 3: Different Gene Amplification Sensitivity and Specificity

The graph shows that area under the curve (AUC) was significantly (0.0000) higher for Maccura E gene ((96.8%) followed by CovSign E gene (96.3%), Genru FAM N gene (94.2%), Covsign VIC S gene (92.6%), Argene Green FAM N gene (82.6%), Maccura ORF1ab1 (77.4%), Covsign N (68.9%), Covsign ORF1ab1 (66.8%) and Argene RdRP (65.3). This shows that the Maccura E gene has better sensitivity and specificity than the other kits, as given in the table below.

Table 1: Area Under the Curve

Test Result Variable (s)	Area	Sig Level	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
CovSign (ORF1ab1)	0.668	0.206	0.382	0.955
CovSign (E) (gene)	0.963	0.001	0.926	1.000
CovSign (N) (gene)	0.689	0.155	0.517	0.862
Maccura (ORF1ab1)	0.774	0.040	0.508	1.000
Maccura (E) (gene)	0.968	0.000	0.935	1.000
Maccura (N) (gene)	0.968	0.000	0.935	1.000
Argene (Green FAM N)	0.826	0.014	0.719	0.934
Argene (RdRP) (cy5)	0.653	0.252	0.463	0.842
Genru (FAM) (N)	0.942	0.001	0.893	0.991
CovSign (VIC) (S)	0.926	0.001	0.869	0.984

The test result variable(s): CovSign (ORF1ab1), CovSign (E (gene), CovSign (N (gene), Maccura (ORF1ab1), Maccura (E (gene, Maccura (N (gene), Argene (Green FAM N), Argene (RdRP (cy5), Genru (FAM (N)), CovSign (VIC (S)) has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Based on nonparametric test and results as given in the table "frequencies" and "Test Statistic" also given in "Hypothesis Test Summary".

Table 2: Frequencies of Different Diagnostic Assays

	Value	
	00	**00**
CovSign (ORF1ab1)	08	92
CovSign (E) (gene)	12	88
CovSign (N) (gene)	64	36
Maccura (ORF1ab1)	08	92
Maccura (E)(gene)	11	89
Maccura (N) (gene)	11	89
Argene (Green FAM N)	38	62
Argene (RdRP) (cy5)	71	29
Genru (FAM) (N)	16	84
CovSign (VIC) (S)	19	81
BGI (FAM ORF ab/1)	05	95
CovSign (ORF1ab1)	08	92

Test Statistics

N	**100**
Cochran's Q	401.729a
Degree of Freedom	10
Asymp. Sig.	0.000

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of CovSign (ORF1ab1), CovSign (E (gene), CovSign (N (gene), Macura (ORF1ab1), Macura (E (gene, Macura (N (gene), Argene (Green FAM N), Argene (RdRP (cy5), Genru (FAM (N)), CovSign (VIC (S)) and BGI (FAM ORF ab/1) are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

DISCUSSION

The current study was designed to evaluate different specimens (nasopharyngeal, oropharyngeal, blood and sputum) and different diagnostics assays/kits (Maccura, BGI, Genru, Argene and Covsign) used in real-time reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of SARS-CoV-2 RNA. One hundred nasopharyngeal COVID-19-confirmed positive individuals were included in the study. Afterwards, these patients were further sampled for oropharyngeal, blood and sputum. Sputum was observed (100%) to be positive in all individuals for finding RNA of SARS-CoV-2, followed by oropharyngeal (91%) and blood (21%), respectively. The outcome of this study is in line with the finding of Yang et al. (2020), in which the researchers concluded that the highest positivity rate was observed by sputum (87.5 % and 82.6 %) followed by oropharyngeal swabs (72.7 % and 53.2 %).[15] According to their study results, sputum shows greater sensitivity for finding RNA of SARS-CoV-2, followed by OPS. Sputum production was seen in a modest percentage of COVID-19 individuals (28 to 33.7%), according to the researchers (Yang et al., 2020). Similarly, in another study by Bwire et al. (2021), it was noted that nasopharyngeal and sputum samples show the highest detection rate, 91 %, followed by oropharyngeal samples. The lowest detection rate was observed in blood, and zero detection from urogenital specimens.[16] A study by Ling et al. (2021) concluded that NPS showed the highest positivity rate compared to other specimens. The same study also concluded that the virus may harm the gastrointestinal tract in later stages, which may also be detected in faecal specimens or rectal swabs. The study also recommends saliva and oropharyngeal swabs for detecting SARS-CoV2 as they showed a high detection rate.[17] Using the CovSign diagnostic kit, maximum ORF1ab1 gene amplification (92%) was observed, whereas 88% and 36% positive results were observed for E and N genes, respectively. On the other hand, the highest ORF1ab1 gene amplification (92%) was observed in the Maccura kit. Both genes, i.e., E and N, were 89% positive. Green FAM N gene's amplification was 62% positive using an Argene kit, while RdR1 (cy5) was only positive for 29% of cases. By using Genru FAM (N) and VIC (S), it was found that 84% and 81% were positive. About 95% gene amplification (FAM ORF ab/1) was observed by using a BGI diagnostic kit. By comparing the results of different diagnostic kits, it was found that BGI and Maccura were the most sensitive and specific assays for diagnostic purposes against COVID-19. The outcome of this study is in line with the results of Eberle et al. (2021), who concluded that using different assays showed contrasting results. When dealing with the COVID-19 epidemic, quick and accurate results are critical. The study's findings suggested that using assay which targeted two different regions will robust the recognition of SARS CoV-2, which rapidly changes its genome. According to the researchers, the BGI (China kit) showed 95%ensitivity.[18] The result of this study is also in line with the findings of Iglói et al. (2020), who concluded that using thirteen different assays showed comparatively better sensitivity for detecting SARS-CoV-2. Four assays showed the highest sensitivity rate, i.e., Maccura, Altona diagnostics, XABT and Sansure Biotech. In the current study, Maccura showed the highest sensitivity rate.[17,19]

LIMITATIONS

The main strength of this research was its protocol, in which every inpatient was screened by a SARS-CoV-2 rapid antigen test on admission and at the time of discharge. The study was not without limitations. A rapid antigen test was used due to its quicker result and lower cost, but it might have underestimated the burden of nosocomial COVID-19 due to its lower sensitivity compared to RT–PCR, especially in asymptomatic patients. The study protocol did not include follow-up after discharge, and some patients may have been infected during the hospital stay and developed symptoms after discharge. Some patients failed to complete the study protocol because they died or were discharged before being tested. The source of SARS-CoV-2 was not studied.

CONCLUSIONS

The present study concluded that all the RT-PCR kits reviewed in this study can be used routinely by molecular diagnostic laboratories to diagnose COVID-19 in patients. The current study also recommended that public health professionals and policymakers employ the Maccura kit in standard diagnostic laboratories for more sensitive and accurate findings.

CONFLICT OF INTEREST: None

FUNDING SOURCES: None

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