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Research Article

Comparison of Nucleocapsid Antigen, ORIF 1ab and RdRp (RNA-Dependent RNA Polymerase) in the Diagnosis of COVID-19

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A B S T R A C T

Introduction: Severe acute respiratory syndrome coronavirus disease 2019 (COVID-19) is responsible for a global pandemic, causing respiratory or flu-like illnesses. This study prompted the urgent need for simple, rapid, and accurate diagnostic tests for COVID-19. It evaluates the diagnostic performance of RT-PCR (real-time reverse transcriptase-polymerase chain reaction), targeting the *ORF1ab, RdRp*, and N genes of SARS-CoV-2, and compares these results with rapid antigen detection tests.

Method: This study was conducted at Saveetha Medical College and Hospital, Chennai, India, from April to September 2022. A total of 100 suspected COVID-19 samples were analyzed. RT-PCR performance was assessed in terms of sensitivity and specificity, focusing on the *ORF1ab, RdRp,* and N genes. Two RT-PCR kits, KIT-1 and KIT-2, were evaluated for diagnostic accuracy. The study also examined c-reactive protein (CRP) and procalcitonin (PCT) levels as potential COVID-19 diagnosis and management biomarkers.

Results: RT-PCR demonstrated a sensitivity of 83.8% and a specificity of 59.3% compared to rapid antigen tests. Detection of the ORF1ab gene showed higher sensitivity (89.7%) than the *RdRp* gene, which had a higher specificity (84.3%). Among the two RT-PCR kits, KIT-2 outperformed KIT-1 in both sensitivity and specificity. The study also found statistically significant p values for CRP and PCT levels among suspected COVID-19 cases, indicating their potential diagnostic value.

Conclusion: This study supports the use of RT-PCR as the primary diagnostic tool for COVID-19, with the *ORF1ab* and *RdRp* genes proving effective targets. Rapid antigen tests serve as useful supplements in high-prevalence settings. Further research on viral gene dynamics, especially *ORF1ab*, is recommended for a better understanding of COVID-19 pathogenesis and for enhancing diagnostic approaches.

Keywords: COVID-19, SARS-CoV-2, RT-PCR, CRP, PCT, Rapid Antigen Test, *ORF1ab, RdRp*, N Gene

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Introduction

SARS-CoV-2 is a novel coronavirus that originated in Wuhan, China in December 2019. It rapidly spread across the globe, leading to a severe public health crisis known as COVID-19.[1] To diagnose the virus and confirm the COVID-19 disease, a reliable and precise method called Reverse Transcriptase Real-Time Polymerase Chain Reaction (rtRT-PCR) is utilised. This molecular diagnostic technique is quick, sensitive, repeatable, and has a low risk of contamination. The test involves taking swab samples from the upper respiratory tract, specifically the nasopharyngeal and oropharyngeal areas.

Coronaviruses are positive-stranded RNA viruses that derive their replication and transcription complex, as well as their RNA-dependent RNA polymerase (*RdRp*), from a single large open reading frame known as *ORF1ab*. The production of subgenomic messenger RNAs, which significantly outnumber (anti)genomic RNAs at particular stages of the replication cycle, results in the coronavirus structural proteins, such as the spike (S), nucleocapsid (N), and envelope (E) proteins. When using RT-PCR for SARS-CoV-2 detection, the *ORF1ab*/ *RdRp*, E, N, and S genes are the most frequently used targets.[2]

The primer–probe technology is designed to identify various parts of the SARS-CoV-2 virus, including the ORF1 (a, b), *envelope* (E), *nucleocapsid* (N), spike (S), and RNA-*dependent* RNA *polymerase* (*RdRp*) genes, which are all crucial for RT-PCR tests. These tests reveal that each gene has unique sensitivity and specificity, making them effective for both detection and confirmation of SARS-CoV-2 across the globe. In the early stages of the COVID-19 (SARS-CoV-2) pandemic, the World Health Organization (WHO) advised using RT-PCR as the primary screening method, starting with an E-gene test followed by a *RdRp* test, due to the highest analytical sensitivity of *RdRp* tests. Additionally, research has shown that employing two primer–probe sets aimed at the *nucleocapsid* genes (N1 and N2) as recommended by the Centers for Disease Control and Prevention (CDC) can enhance the accuracy and reliability of detecting SARS-CoV-2 in the future.[3]

The SARS-CoV-2 genome consists of several genes, the most important of which is the *ORF1ab* gene, located at the 5' end. It accounts for more than two-thirds of the entire genome. Additionally, six more ORF genes, including *ORF3a, ORF6, ORF7a, ORF7b, and ORF8*, are involved.[4] At the near 3' end, there is a gene known as the N gene, which contains 908 nucleotides and encodes nucleocapsid, a specific type of structural protein that the US Centres for Disease Control and Prevention (CDC) recommends as a viable target for virus detection.[5] It is usually preferable to use two molecular targets simultaneously to avoid the genetic drift of SARS-CoV-2 and possible cross-reaction with other coronaviruses.

When diagnosing a viral infection using RT-PCR, the Ct value of a target gene provides information about the virus's presence and concentration during infection.[6] However, laboratory complications may limit the clinical significance of this value. The Ct value is the cycle number at which the sample fluorescence exceeds a set threshold. A lower Ct value indicates a higher concentration of the gene in the sample, and therefore, a higher viral load. RT-PCR is the most sensitive and specific assay for viral detection, making it the recommended method.[7]

Addressing the existing gap in the literature regarding the comparative effectiveness of RT-PCR targeting specific viral genes against rapid antigen tests for COVID-19, this study is pivotal. By comparing the sensitivity and specificity of RT-PCR targeting specific viral genes against rapid antigen tests, the research aims to identify the most reliable diagnostic methods, thereby guiding healthcare practices and public health policies. This study not only supports the primary use of RT-PCR, supplemented by rapid tests in high prevalence areas but also contributes to our knowledge of SARS-CoV-2 pathogenesis, informing further research and the development of effective treatments and vaccines. This study aimed to comprehensively examine the behaviour of the *ORF1ab* and *N* genes of the SARS-CoV-2 virus, to assess their viability as reliable targets for the Real-Time Polymerase Chain Reaction (RT-PCR) diagnostic process in the detection of COVID-19.

Methodology

This cross-sectional study was conducted between April 2022 and September 2022 at the Saveetha Medical College and Hospital in Chennai, Tamil Nadu, India. One hundred COVID-19-suspected samples were obtained from participants of all ages and genders after obtaining prior informed consent from the patients. All specimens received in the molecular diagnostic and research laboratory that were suspected of carrying COVID-19 were included in the study. The confirmation of positive cases was done according to the guidelines set by the Indian Council of Medical Research (ICMR), Govt. of India. RT-PCR profiling was carried out for all samples, and their Ct values were recorded.

For the collection of clinical specimens, the fever clinic was asked to obtain nasopharyngeal and oropharyngeal swabs, ensuring a comprehensive approach to sample collection for respiratory analysis. Each sample was meticulously labelled to preserve the integrity of the specimen and transported under controlled temperatures between 4–8 °C to the molecular diagnostics and research laboratory for subsequent processing.

In addition to virological testing, blood samples that were received at our clinical microbiological laboratory were

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correlated to measure the levels of c-reactive protein (CRP) and procalcitonin (PCT) as part of the comprehensive diagnostic assessment for COVID-19. CRP was measured using a high-sensitivity assay suitable for detecting low levels of inflammation, while PCT was measured using an immune-luminometric assay, both conducted as per the manufacturer's instructions and under appropriate quality control measures.

The inclusion criteria for the study were specifically designed to ensure the validity and reliability of the test results. Only samples collected from the nasopharynx and oropharynx, stored in Viral Transport Medium, and transported under the specified temperature conditions were considered for testing. This rigorous selection process was essential for maintaining the quality and viability of the samples.

Conversely, the exclusion criteria were established to maintain the study's integrity. Samples lacking essential identification details such as the patient's name, age, sex, or hospital number were excluded from the study. Additionally, any samples compromised due to leakage in packing or failure to maintain the required transportation temperature were also disqualified and flagged for re-sampling. This comprehensive study design was instrumental in ensuring the accuracy and reliability of our findings in assessing the diagnostic performance of RT-PCR and rapid antigen detection tests for COVID-19.

RT-PCR for SARS-CoV-2 Detection by ARGENE® SARS-COV-2 R-GENE® and PathoDetect COVID-19 Qualitative PCR Kit

These assays are designed to detect the presence of viral nucleocapsid antigens in patient samples using two real-time polymerase chain reaction amplification kits. The procedure was adapted as per the manufacturer's instructions as follows.

The ARGENE® SARS-COV-2 R-GENE®, BioMérieux, France [Realtime detection kit (Product REF: 423720)] was considered as kit 1 and is specifically designed for the detection of SARS-CoV-2 RNA in respiratory samples utilising a real-time RT-PCR method. This procedure begins with the collection of appropriate respiratory specimens for analysis. Following sample collection, RNA is extracted using systems that have been validated according to the kit's protocols. The extracted RNA is then subjected to real-time RT-PCR amplification, targeting specific viral genes such as the N gene for nucleocapsid, *RdRp* gene for RNA-dependent RNA polymerase, and E gene for envelope, to identify the presence of SARS-CoV-2 RNA. The kit includes various controls to ensure the accuracy of the test results. The analysis of results is conducted by examining the fluorescence signals emitted during the amplification process, which indicate the presence or absence of viral RNA. The interpretation of results by the Argene kit is explained in Table 1 as follows:

This comprehensive approach ensures reliable detection of COVID-19, focusing on critical viral genes, incorporating stringent controls, and adhering to validation steps for accurate diagnostics, making it suitable for high-complexity laboratories.

The Patho-detect, COVID-19 Qualitative PCR-Mylab discovery solutions, India was considered as kit 2 and it provides a tailored procedure for the accurate identification of SARS-CoV-2 RNA using real-time RT-PCR. The process starts with the collection and transport of respiratory or serum samples, which are maintained at a controlled temperature of 4–8 °C during transport to the laboratory. Upon receipt, RNA is extracted from these samples in preparation for amplification. The kit employs specific primers and probes targeting the E gene for initial screening and N and *RdRP* genes for confirmation of the SARS-CoV-2 genome, including an internal control to verify the extraction and amplification process's efficacy. The reaction mix, containing the extracted RNA, undergoes thermal cycling under defined conditions to amplify the targeted viral genes. Data interpretation is based on control samples and the amplification curves generated, indicating the presence of SARS-CoV-2 RNA, providing a precise and reliable method for detecting SARS-CoV-2 in patient samples. The interpretation of results by the PathoDetect (Mylab) kit is explained in Table 2.

Table 1.RT-PCR Diagnostic Panel Result Interpretation by Argene Kit

Gene/Sample	**Ct Target or Δ Ct [IC1sample-IC1W0]**				
N gene (530 nm)	+	+	-	-	
IC1 sample -IC1W0 (560 nm)	≤ 3 Ct or > 3 Ct			≤ 3Ct	≥ 3Ct
RdRp gene (670 nm)	+	-	+	-	
Interpretation	SARS-CoV-2 detected	Equivocal result (perform PCR2 and/ or retest PCR1)*		SARS-CoV-2 NOT detected (or< LOD)*	Invalid result (inhibition/ poor extraction)

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Table 2.RT-PCR Diagnostic Panel Result Interpretation by PathoDetect Kit

RdRp/N (FAM)	E(Cy5)	RNaseP (VIC/ HEX)	Results
+ve Ct value ≤ 40	+ve Ct value ≤ 40	Ct value ≤ 38	COVID-19 RNA is detected
-ve	-ve	Ct value ≤ 38	COVID-19 virus NOT detected
+ve Ct value ≤ 40	-ve	Ct value ≤ 38	COVID-19 RNA is detected
-ve	*+ve Ct value ≤ 40	Ct value ≤ 38	COVID-19 presumptive positive re-extraction and retest recommended
Ct value ≤ 40 - ≤ 45	Ct value ≤ 40 - ≤ 45	Ct value ≤ 38	Re-extraction and retest recommended
-ve	-ve	-ve	Inhibition re-extraction and retest recommended

Ethics Statement

This study was approved by the Institutional Ethics Committee.

Results

Of the total participants in this study, 52 were female, while 48 were male. The sex and age-wise distribution is depicted in Table 3.

The study aimed to investigate the presence of COVID-19 infection by RT-PCR and immunochromatography card test. Among the study participants, 67% (n = 67) tested positive for COVID-19 infection, while 33% (n = 33) tested negative. The present study further assessed the dynamics of rapid antigen testing, *ORF1ab, RdRp*, and N genes from nasopharyngeal and oropharyngeal swabs of all 100 participants. The CT value for the N gene was observed to range from 20.76 to 34.37, while the *RdRp* gene ranged from 22.91 to 36.52 and the ORF gene ranged from 17.81 to 37.21. The findings of this study provide valuable insights into the dynamics of COVID-19 infection and highlight the importance of targeted testing and monitoring to control its spread. The correlation between RT-PCR and rapid antigen detection test is explained in Table 4.

It shows that the sensitivity and specificity of RT-PCR, as compared to the rapid antigen detection test, are 83.8% and 59.3%. The positive and negative predictive values of RT-PCR compared to the rapid antigen detection test are 81.4% and 63.3%.

The comparison of *RdRP* and *ORF1ab* sensitivity, specificity, positive predictive value, and negative predictive values are 89.7%, 84.3%, 92.4%, and 79.4%; and it is explained in Table 5. We conclude that *RdRP* is more sensitive and *ORF1ab* is more specific.

This analysis (Table 6) presents the evaluation of CRP and PCT levels in 100 suspected cases of COVID-19. The mean CRP level among the suspected COVID-19 cases was found to be 47.92 mg/L, with a standard deviation of 18.16 mg/L. The statistical analysis showed a p value of 0.01, indicating that the observed CRP levels are significantly different. This suggests that CRP could be a valuable biomarker in the diagnostic process for COVID-19, highlighting an inflammatory response associated with the infection. For procalcitonin, the mean level was observed at 0.504 ng/mL with a standard deviation of 0.191 ng/mL. The p value associated with PCT levels was 0.03, also indicating a statistically significant difference. Procalcitonin, known to elevate in response to bacterial infections, shows significance in this viral context, possibly reflecting severe or systemic infection including secondary bacterial infections in COVID-19 suspected cases.

Table 3. Age and Sex-wise Distribution of Participants

Age Group (Years)	Male (N = 48)	Female (N = 52)	Total
0–14	6	12	18
15–59	17	21	38
≥ 60	25	19	44
Total	48	52	100

Table 4. Comparison of RT-PCR and Rapid Antigen Detection Test Reports

Rapid Antigen Detection	RT-PCR	RT-PCR	Total
	Positive	Negative	
Positive	57	13	70
Negative	11	19	30
Total	68	32	100

RT-PCR: Real-Time Polymerase Chain Reaction

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Table 5.Comparison of *RdRp* and ORF1ab Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Values

RdRP	ORF1ab	ORF1ab	Total
	Positive	Negative	
Positive	61	5	66
Negative	7	27	34
Total	68	32	100

Table 6. Statistical Analysis of C-Reactive Protein and Procalcitonin Levels

Marker	Mean	SD	p Value
CRP (mg/L)	47.92	18.16	0.01
PCT (ng/mL)	0.504	0.191	0.03

The statistically significant p values for both CRP and PCT levels among COVID-19 suspected cases underscore their potential utility in diagnosing and managing COVID-19. CRP, with its higher mean level and significant deviation, along with PCT, supports the inflammatory and infection response suspicion in patients. These findings advocate for the inclusion of CRP and PCT level monitoring in the clinical assessment of patients suspected of having COVID-19, not only to support the diagnosis but also to guide treatment strategies based on the severity of infection and inflammation.

Table 7 delineates the relationship between CRP levels and RT-PCR outcomes in suspected COVID-19 cases. The Phi coefficient, a measure of association for two binary variables, is calculated from the Chi-square statistic and is 0.122819 in this case, suggesting a weak positive correlation between CRP levels and RT-PCR results. However, with a p value of 0.219, this correlation is not statistically significant.

Table 8 illustrates the association between PCT levels and RT-PCR outcomes in the evaluation of suspected COVID-19 cases. The Phi coefficient value of 0.400193 signifies a moderate positive association between PCT levels and positive RT-PCR test results, indicating that higher PCT levels might correspond with a higher likelihood of a positive RT-PCR result for COVID-19. The Chi-square statistic of 16.015441 with a highly significant p value of 0.000063 robustly supports this association. These results suggest that PCT could be a reliable biomarker for identifying individuals with active COVID-19 infection and that PCT testing may serve as a valuable adjunct to RT-PCR in diagnosing COVID-19.

Table 7.Correlation of C-Reactive Protein (CRP) Levels with RT-PCR Outcomes

CRP	RT-PCR Positive	RT-PCR Negative	Total	Measure	Value
CRP positive	50	21	71	-	-
CRP negative	16	13	29	-	-
Total	66	34	100	-	-
Correlation	-	-	-	Phi coefficient	0.122
-	-	-	-	Chi-square statistic	1.508
-	-	-	-	p value	0.219

Table 8.Correlation of Procalcitonin (PCT) Levels with RT-PCR Outcomes

PCT Test	RT-PCR Positive	RT-PCR Negative	Total	Phi Coefficient (PCT)	Chi-Square Statistic (PCT)	p Value (PCT)
PCT Positive	47	10	57	-	-	-
PCT Negative	18	25	43	-	-	-
Total	65	35	100	-	-	-
Correlation	-	-	-	0.400	16.0154	0.000063

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Discussion

In COVID-19 diagnosis, three distinctive targets have been identified: the *nucleocapsid* (N) gene, *ORF1ab*, and *RdRp* gene.[8] These targets are located in different regions of the virus genome and play distinct roles in the viral life cycle.

The N gene encodes the nucleocapsid protein, which is a structural component of the virus that safeguards the viral RNA. The *ORF1ab* region encodes a large polyprotein that is subsequently cleaved into several non-structural proteins, which are fundamental to viral replication. Finally, the *RdRp* gene encodes the RNA-dependent RNA polymerase, which a key enzyme is required for the virus to replicate its RNA genome.[9] The efficacy of each target varies depending on the type of test employed. For instance, various studies have shown that the *RdRp* gene may be more sensitive than the N gene in certain types of tests, while others have demonstrated the converse.[10]

Healthcare professionals must meticulously examine the available options and select the most suitable target for each patient based on factors such as the test type, the availability of reagents, and the prevalence of different viral strains in the population. By doing so, they can ensure an accurate and timely diagnosis of COVID-19, which is crucial for proper patient management and infection control.

Molecular testing has emerged as the standard laboratory diagnosis for SARS-CoV-2 infection. It involves the use of RT-PCR assays that are widely used in COVID-19 diagnostic laboratories to detect SARS-CoV-2 RNA in clinical specimens.[11] In a recent study, 100 COVID-19 suspected patients were randomly selected, out of which 52 were female, and 48 were male. Among them, 67 (67%) tested positive for COVID-19 infection, and 33 (33%) tested negative for Real-Time Polymerase Chain Reaction.[12]

To speed up disease screening, rapid antigen immunoassays with equivalent sensitivity and specificity to real-time RT-PCR assays have been developed.[10] In the study, the commercially available rapid SARS-CoV-2 antigen detection method was compared with the RT-PCR assay to detect SARS-CoV-2 infection. For the master mix step in RT-PCR, two kits were processed separately-KIT-1 and KIT-2. KIT-1 showed the N gene and ORF1ab, while KIT-2 showed *RdRp* and N-gene during analysis for the final reports in the RT-PCR machine.[13,14] When tested with KIT-1, 66 samples tested positive, and 34 tested negative, while KIT-2 reported 68 positives and 32 negative cases. In comparing the sensitivity and specificity between KIT-1 and KIT-2, we observed a sensitivity of 89.7% and a specificity of 84.3%. The positive and negative predictive values were 92.4% and 79.4%. Thus, it was concluded that KIT-2 is more sensitive and specific when compared to KIT-1 and can be preferred for comparative studies.

The study done by Visseaux and co-workers observed that the performance of the RealStar® SARS-CoV-2 RT-PCR assay was assessed and found to slightly outperform the reference WHO assay. The comparative analysis between RT-PCR and rapid antigen detection test results revealed that both tests identified 57 positive and 19 negative cases correspondingly. It was determined that the RT-PCR method exhibits a higher sensitivity of 83.8% and a specificity of 59.3% in comparison to the rapid antigen detection approach. Consequently, due to its superior sensitivity and specificity, RT-PCR is regarded as the benchmark diagnostic procedure over the rapid antigen detection kit.[15]

Similarly, Chaimayo and team studied the rapid assay for SARS-CoV-2 antigen detection and concluded that it showed comparable sensitivity (98.33%; 95% CI, 91.06 99.96%) and specificity (98.73%; 95% CI, 97.06 99.59%) with Real-Time PCR assay. The author believes that this rapid and simple SARS-CoV-2 antigen detection test is a potential screening assay, especially in a high prevalence area.[16] Another clinical study on assessment of SARS CoV-2 antigen rapid detection compared with RT-PCR assay for emerging variants at a high throughput community testing site in Taiwan by Jian et al. discussed that considering the short turnaround times and lower costs, this simple SARS-CoV-2 antigen detection test for rapid screening, combined with RT-PCR as a double confirmatory screening tool, can facilitate the prevention of community transmission during COVID-19 emergencies.[17]

In addition to molecular testing for viral detection, evaluating the levels of biomarkers such as CRP and PCT in suspected COVID-19 cases has emerged as a significant aspect of the diagnostic process.[18,19] The analysis presented here involves the evaluation of CRP and PCT levels in 100 suspected cases of COVID-19. These findings highlight the importance of CRP and PCT as biomarkers in the COVID-19 diagnostic process, offering insights into the infection's severity[20] and the patient's inflammatory response.[21] The analysis also reveals a weak and non-significant correlation between CRP levels and RT-PCR outcomes, while PCT levels exhibit a moderate and statistically significant correlation with RT-PCR results, suggesting PCT as a potential biomarker for active COVID-19 infection.

Conclusion

To conclude, while molecular testing using RT-PCR assays is the gold standard for detecting SARS-CoV-2 infection, rapid antigen immunoassays serve as valuable tools for quick disease screening. The findings from evaluating CRP and PCT levels among suspected COVID-19 cases add another layer to the diagnostic process, offering insights into the inflammatory response and potential secondary infections associated with the disease. Combining molecular testing with biomarker evaluation can enhance diagnostic accuracy,

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guide treatment decisions, and contribute to better patient management during the COVID-19 pandemic.

The commercially available rapid SARS-CoV-2 antigen detection method is a potential screening tool, especially in high-prevalence areas. However, it is recommended to use RT-PCR as the primary testing method and rapid antigen detection as a complementary tool. Incorporating both tests can aid in preventing community transmission during COVID-19 emergencies and emerging variants. Despite numerous studies being conducted on SARS-CoV-2, there still exists a knowledge gap regarding the viral gene dynamics, particularly the ORF gene's specificity and its relation to the pathogenesis and pathophysiology of COVID-19. Therefore, it is crucial to conduct comprehensive research on the persistence of the ORF gene to provide a better understanding of controlling and managing this pandemic. Such research will shed light on the ORF gene's dynamics and provide insights into its effective use in diagnosing and managing COVID-19. In addition to molecular testing for viral detection, evaluating the levels of biomarkers such as CRP and PCT in suspected COVID-19 cases has emerged as a significant aspect of the diagnostic process.

Nonetheless, samples with Ct values greater than 35 displayed inconsistent outcomes across various RT-PCR testing kits, so caution must be exercised in interpreting these results. Moreover, as the variety of commercial COVID-19 testing kits is introduced, it becomes essential for scientists to exchange details like the comparison methods for multi-centre kits and the detection capabilities of different commercial RT-PCR diagnostic tests on various samples.

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