

Molecular Diagnosis of COVID-19

Roshan Niloofa¹, Suranjith L Seneviratne², Akila Cooray¹, Nilanthi P Senanayake³,

Ishan De Zoysa²

¹ Department of Zoology and Environment Sciences, Faculty of Science, University of Colombo, Sri Lanka

² Department of Surgery, Faculty of Medicine, University of Colombo, Sri Lanka.

³ Department of Microbiology, Faculty of Medicine, University of Colombo, Sri Lanka.

Abstract – The SARS-CoV-2 coronavirus causes COVID-19. Nucleic acid amplification tests (NAAT), could be used to detect the presence of SARS-CoV-2 viral RNA in clinical samples. Several real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) assays are being used by many laboratories for confirming COVID-19. We have outlined the characteristics of the available SARS-Cov-2 rRT-PCR assays and discussed its clinical utility.

Keywords – SARS-Cov-2; Covid-19; RT-PCR, Molecular Diagnosis.

I. INTRODUCTION

The SARS-CoV-2 coronavirus causes COVID-19 [1]. As of 15th April 2020, more than two million persons from over 200 countries have been reported to be infected with the virus with more than 126,000 deaths. It is postulated that the estimated number of infected cases could be much higher due to two factors, namely: the high percentage of asymptomatic individuals and restrictions with testing due to a shortage of test kits. It is reported that a significant proportion of individuals with COVID-19 are asymptomatic and thus might not be tested [2]. Shortages of testing kits have been reported by several countries. For instance, India has reported 11,000 confirmed patients (until 15th April 2020) despite its high population density, with only 132 tests per million population, being carried out. In such situations, viral transmission may be higher than estimated and thus lead to unexpected increases in the rates of infection. Robust and widespread laboratory confirmatory testing is essential for more optimal control of the COVID-19 pandemic.

The SARS-CoV-2 is a RNA virus belonging to *Coronaviridae* family (genus: *Betacoronavirus*). Nucleic acid amplification tests (NAAT), could be used to detect the presence of SARS-CoV-2 viral RNA in clinical samples. A

real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) is used by many laboratories for confirming COVID-19. So far, several versions of the rRT-PCR test have been developed by different laboratories and companies. Both multiplex and simplex rRT-PCR test kits are available. The multiplex assay amplifies a panel of viral RNAs at the same time, whereas the simplex assay detects a single RNA sequence. Some studies suggest the simplex RT-PCR has a slightly higher analytical sensitivity than a multiplex assay [3], but multiplex assays are also able to test for the presence of other viruses (including related coronaviruses). The RT-PCR tests for SARS-CoV-2 virus could be either qualitative (only detects the presence of the virus) or quantitative (where viral loads could be assessed). Currently, 263 companies have registered their RT-PCR test kits with the World Health Organization (WHO). So far, the WHO has recommended the use of seven different in-house RT-PCR protocols. As of 15th April 2020, the FDA has given Emergency Use Authorization (EUA) for 34 RT-PCR test kits (all for qualitative assessment). The US Centre for Disease Control (CDC) initially developed a test and this was subsequently revised.

II. MAJOR TESTS DEVELOPED

The presently used RT-PCR tests for SARS-CoV-2, amplifies and detects regions of the nucleocapsid (N), envelope (E), spike (S), open reading frame-1b (ORF1b) or ORF1ab genes. The ORF1ab region is highly conserved

among sarbecoviruses, and thus target sequences specific for SARS-CoV-2 need to be carefully selected. Positive rates and the limit of detection (LOD) varies between different gene target sequences (Table 1). The RdRp/Hel gene targeting RT-PCR, had a lower LOD and higher rates of positivity.

Table 1: Performance Characteristics of RRT-PCR Assays Using Different Target Genes

Target	number (Respiratory tract)	samples limit of detection	Positive results (%)	Reference
RdRp-P2	297	3.8 c/r	95	[4]
RdRp/Hel	120	11.2 c/r 1.8X10 ⁰ TCID ₅₀ /ml	85	[6]
RdRp-P2	120	1.8X10 ¹ TCID ₅₀ /ml	61	[6]
E	297	5.2 c/r	95	[4]
	88	3.35 c/r	95	[12]
N	120	21.3 c/r	NG	[6]
ORF1ab& N	323	11.1 c/r	30	[8]
nsp-2	59	1.8 TCID ₅₀ /ml	100	[7]

NG: Not given, N: nucleocapsid, E: envelope, RdRp-P2: RNA-dependent-RNA-polymerase probe-2, RdRp/Hel: RNA-dependent-RNA-polymerase/Helicase ORF1ab: open reading frame 1ab, nsp-2: non-structural protein-2

In January 2020, Corman et al [4] developed three rRT-PCR assays targeting the E, N and RdRp genes. Further validation of the N gene was discontinued due to its lower sensitivity. The genes E and RdRp detecting rRT-PCR assays showed a high sensitivity (95%) when analyzed using SARS-CoV virions. Two primer/probe pairs were used for the RdRp gene. RdRp-P1 was able to detect SARS-CoV-2, SARS-CoV and other bat-SARS-related CoVs whereas RdRp-P2 was specific to SARS-CoV-2. The authors tested for cross-reactivity with other corona viruses using virus cell culture supernatants and found none. However, as the viral titre was not measured in the supernatant, this may have impacted the result. This test has formed the basis for test kits distributed by the WHO. Chu and colleagues [5] developed two 1-step rRT-PCR assays targeting the ORF1b and N genes of SARS-CoV-2. The assay was designed to identify multiple sarbecoviruses that are able to infect humans. A positive result was interpreted as confirmation of infection with SARS-CoV-2 or related viruses. The N gene assay was found to be around 10 times more sensitive than the ORF-1b assay. Thus, the E and N gene-targeted SARS-Cov-2 assays have been recommended for screening, and the RdRp or ORF1ab assays for confirmatory testing [4, 5].

The initial test developed by the CDC, included three separate rRT-PCR reactions targeting the N gene. One primer-probe pair was able to detect all betacoronaviruses, while the other two would specifically detect SARS-CoV-2. A positive with all three reactions were required to reach a presumptive positive result. However, due faulty reagents many inconclusive results were obtained and thus the CDC revised and redeveloped the original assay. The current assay, amplifies two sequences from the N gene (N1 and N2) and the human RNase P gene as a control. In addition to the control sequence, both N1 and N2 sequences need to be positive for a positive result. Positivity in only one of the N sequences is considered as inconclusive and a repeat test recommended.

Recently, Chan and colleagues [6] developed three rRT-PCR assays targeting the RdRp/Hel, S and N genes and compared its performance with RdRp-P2 targeted assay previously established by Corman et al [4]. They found the RdRp/Hel assay to have the lowest LOD, at 11.2 RNA copies/reaction. When compared to the RdRp-P2 assay, the RdRp/Hel assay had higher positivity rates for both respiratory and non-respiratory samples (85% vs 61%). The authors have suggested the RdRp/Hel assay may help with reducing false-negative results due to low viral loads. Furthermore, the high analytical sensitivity of RdRp/Hel

gene-targeted RT-PCR tests, may perform better with samples having lower viral loads such as saliva or plasma. The RdRp/He1 gene amplification did not show any cross-reaction with other human-pathogenic coronaviruses in cell cultures or clinical specimens, but the RdRp-P2 assay had shown some cross-reactivity with SARS-CoV culture lysates [6]. The authors suggest the newer test has higher analytical sensitivity. Further studies would help clarify these findings.

A previously untargeted species-specific nsp2 region in the SARS-CoV-2 genome was selected and optimized as a probe-free rRT-PCR assay: named as COVID-19-non-structural protein 2 (nsp2) assay [7]. This assay did not amplify other human-pathogenic respiratory or coronaviruses and had a low LOD of 1.8 TCID₅₀/ml. A 100% analytical sensitivity was reported on testing 59 specimens from confirmed cases. The reaction time (one hour) was also shorter than the other assays. An important limitation when using SARS-Cov-2 rRT-PCR assays, is that the virus may mutate at a relatively high rate of 10⁻⁴ nucleotide substitutions per site per year. This possibility needs be considered with all the rRT-PCR assays that are being developed [7].

III. DETECTION WINDOW

The diagnostic detection window for the SARS-Cov-2 rRT-PCR assays may be from a few days before the onset of symptoms to the time of recovery [8]. However, viral loads may be low at the early and late phases of the infection. Subclinical or asymptomatic patients may show positive rRT-PCR results and should not be excluded from testing. Furthermore, if clinical and epidemiological pointers are high in individual subjects, repeat testing should be undertaken even if the initial test result is negative. Viral loads could also be measured at different time points of the illness. Significantly higher viral loads have been noted during the early and progressive phases of the illness compared to the recovery phase [8].

IV. POSSIBLE ERRORS IN TESTING

1. Preanalytical errors

The pre-analytical stage may play an important role in the results obtained from molecular diagnostics. Several authors have suggested that false-negative results might be due to the method of sampling and the type of sample. Viral shedding has been identified at the nasal and pharyngeal levels during the first week of symptoms and thereafter within the lower respiratory tract. In a study from China, at an early phase of the pandemic, higher positivity rates were

found in Broncho alveolar lavage fluid compared to nasal/pharyngeal swabs [9]. Technical difficulties with obtaining suitable nasopharyngeal swabs have been noted, but are still more convenient and practical than the more invasive techniques for sampling the lower respiratory tract. Techniques used for RNA extraction may also affect the test result. The CDC has recommended seven RNA extraction kits (from QIAGEN, Roche and BioMerieux) for use with their rRT-PCR assay.

2. Analytical errors

Technical errors in performing an assay may also produce false-negative results. Presently, due to the high demand for widespread molecular testing, minimally trained staff may be carrying out the testing in some laboratories. This may result in mishandling of reagents and instruments. Overuse of assay instruments could also cause errors, due to lack of calibration. Some authors have suggested the combined use of other diagnostic modalities such as a chest CT scans with the results from molecular diagnostic tests in specific patients [10].

V. RAPID DETECTION

The currently used rRT-PCR assays take between three and eight hours to produce a result. Obtaining an early result is very useful for appropriate patient management. Once travel restrictions are relaxed, rapid tests may also be helpful with testing persons at airports or country borders. Thus several groups are trying to develop more rapid molecular testing protocols. The FDA has issued EUAs for tests developed by Cepheid and Abbott Laboratories that takes 45 and 13 minutes respectively for obtaining results. However, these tests require specific instruments such as the 'Gene Expert' and the Abbott 'ID Now' platform, which are not currently available in many countries. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays have been developed by some laboratories. Lamb and colleagues [11] developed a SARS-Cov-2 specific RT-LAMP test that can produce a result in less than 30 minutes. It is still to be compared with the other currently available molecular tests and on clinical samples. RT-LAMP assays could be developed as point-of-care tests. This would be very useful as it does not require a thermocycler, results could be obtained in 15 – 45 minutes and various sample types could be used.

VI. CONCLUSION

rRT-PCR is recognized as the standard confirmatory laboratory diagnostic test for SARS-CoV-2. Several

research groups and companies are working towards further improvements in the currently used assays.

REFERENCE

- [1] Seneviratne SL, Abeysuriya V, de Mel S, De Zoysa I, Niloofa R. Favipiravir in Covid-19. *International Journal of Progressive Sciences and Technologies* 2020; 19: 143 - 145
- [2] Day M. Covid-19: four fifths of cases are asymptomatic, china figures indicate. *BMJ* 2020;369:m1375. Published 2 April 2020. doi: 10.1136/bmj.m1375
- [3] Lv DF, Ying QM, Weng YS, Shen CB, Chu JG, Kong JP, *et al.* Dynamic change of target genes by RT-PCR testing of SARS-CoV-2 during the course of a Coronavirus Disease 2019 patient. *Clinica Chimica Acta*. Published online ahead of print: 27 March 2020. Doi: 10.1016/j.cca.2020.03.032.
- [4] Corman VM, Landt O, Kaise M, Molenkamp R, Meijer A, Chu Dk, *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill.* 2020;25(3):2000045. doi:10.2807/1560-7917.ES.2020.25.3.2000045
- [5] Chu DK, Pan Y, Cheng SM, Hui KP, Krishnan P, Liu Y, *et al.* Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem.* 2020; 66 (4): 549-555. Doi: 10.1093/clinchem/hvaa029
- [6] Chan JFW, Yip CCP, To KKW, Thang THC, Wong SCY, Leung KH, *et al.* Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/HeI real-time reverse transcription-polymerase chain reaction assay validated and with clinical specimens. *J Clin Microbiol.* 2020. Published online ahead of print: 04 March 2020. doi: 10.1128/JCM.00310-20.
- [7] Yip CCY, Ho CC, Chan JFW, To KKW, Chan HSY, Wong SCY, *et al.* Development of a novel, genome subtraction-derived, SARS-CoV-2-specific COVID-19-nsp2-real time RT-PCR assay and its evaluation using clinical specimens. *Int J Mol Sci.* 2020; 21: 2574. Doi: 10.3390/ijms21072574
- [8] Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, *et al.* Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis.* Published online ahead of print: 28 March 2020. Doi: 10.1093/cid/ciaa345
- [9] Liu R, Han H, Liu F, Lv Z, Wu K, Liu Y, *et al.* Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clinica Chimica Acta*. Published online ahead of print: 2020 Mar 7. doi: 10.1016/j.cca.2020.03.009
- [10] Long C, Xu H, Shen Q, Zhang X, Fan B. Diagnosis of the Coronavirus disease (COVID-19): rRT-PCR or CT? *Radiology.* 2020. Published online ahead of print: 25 March 2020. doi: 10.1016/j.ejrad.2020.108961
- [11] Lamb LE, Bartolone SN, Ward E, Chancellor MB. Rapid Detection of Novel Coronavirus (COVID19) by Reverse Transcription-Loop-Mediated Isothermal Amplification. *The Lancet.* Published online ahead of print: 2020 February 14. doi:10.2139/ssrn.3539654
- [12] Pfefferle S, Rvenja R, Nors D, Lutghetmann M. Evaluation of a quantitative of a RT-PCR assay for the detection of the emerging coronavirus SARS-CoV-2 using a high throughput system. *Euro Surveill.* 2020;25(9):pii=2000152. Doi: 10.2807/1560-7917.ES.2020.25.9.2000152