Indian J Med Res 151, May 2020, pp 483-485
DOI: 10.4103/ijmr.IJMR_1256_20

Quick Response Code:

Correspondence

Evaluation of *RdRp* & *ORF-1b-nsp14*-based real-time RT-PCR assays for confirmation of SARS-CoV-2 infection: An observational study

Sir,

COVID-19 caused by SARS-CoV-2 has spread to most countries across the globe including India[1]. Laboratory diagnosis depends on the detection of viral RNA in nasopharyngeal and/or oropharyngeal swabs using real-time reverse transcription polymerase chain reaction (qRT-PCR)[2]. In India, the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV) at Pune, adopted a two-step strategy for the diagnosis of COVID-19 using qRT-PCR. Primers and probes from two different protocols were combined, and initial screening was performed for *E* (envelope) gene specific to Sarbeco sub-genus. Samples positive in the screening test were further subjected to a confirmatory test targeting two genes, one SARS-CoV-2 specific *RdRp* (RNA dependent RNA polymerase) gene and other Sarbeco sub-genus *ORF-1b-nsp14* gene[3,4]. The samples positive for either of the two genes were confirmed as positive for SARS-CoV-2.

To declare a sample positive, four reactions, each for the genes, *E*, *RdRp*, *ORF-1b-nsp14* and *RNaseP* (internal control), are run. This strategy ensures the quality of the clinical sample as well as the testing process and identification of true positives. This two-step diagnostic protocol for SARS-CoV-2 detection is followed by the Virus Research and Diagnostic Laboratory Network (VRDLN)[5,6]. However, with increase in the number of suspected cases, testing for two confirmatory genes would utilize more time, consumables and workforce. Thus, there is an urgent need to revisit the strategy of using two confirmatory genes. In this context, we analyzed the qRT-PCR data of 313 SARS-CoV-2-positive cases tested at ICMR-NIV to find out the sensitivity of *RdRp* and *ORF-1b-nsp14b* gene-based assays to confirm SARS-CoV-2 infection.

All the 313 cases were positive by *E* gene screening [cycle threshold (Ct) values for *E* gene were ≤35] and were also positive by qRT-PCR for either *RdRp* or *ORF-1b-nsp14* or for both genes (Ct value cut-off ≤36). Among the 313 samples, 79.2 per cent (n=248) were positive for both *RdRp* and *ORF-1b-nsp14* genes. *ORF-1b* was exclusively positive in 8.2 per cent (n=57) samples, whereas *RdRp* was exclusively positive in 2.6 per cent (n=8) samples. The sensitivity with 95 per cent confidence interval (95% CI) for the detection of SARS-CoV-2 by *ORF-1b*-based assay was 97.4 per cent (95.0-98.7). The sensitivity with 95 per cent CI for the detection of SARS-CoV-2 by *RdRp*-based assay was 81.8 per cent (77.1-85.7). For comparing the Ct values of the *RdRp* and *ORF-1b-nsp14* gene assays of all 313 positive samples, those showing undetermined Ct values were assigned a Ct value of 45, which was the maximum cycle number of qRT-PCR. The mean Ct value of *ORF-1b-nsp14* gene assay was 28.8 and that of *RdRp* gene assay was 32.6. The mean Ct value was significantly lower in *ORF-1b* assay (P<0.001) using Student's t test.

The results of this observational study suggested that *ORF-1b-nsp14*-based assay performed well as a confirmatory assay as compared to *RdRp*-based assay. A recent study has also reported that the *RdRp*-based assay has missed 35 per cent of SARS-CoV-2 positive cases compared to a novel *RdRp*/helicase-based qRT-PCR assay[7]. A couple of studies posted in preprint servers observed that the primer-probe set reported by Corman *et al*[3] for *RdRp* (with SARS-CoV-2-specific probe) assay had lower sensitivity compared to that of *ORF-1b-nsp14*-based assay, suggesting that it might be due to the presence of a degenerate base at the 12th position of reverse primer[8,9]. The results of these studies are summarized in the Table[7-12]. With these observations, negative results from commercial

Table. Summary of different published studies and studies in preprint servers comparing the *RdRp* based assay[3] with other assays

Reference	Primer probe sets	Analytical sensitivity	Clinical sensitivity
Nalla *et al*[10], 2020	Corman *et al*[3], *RdRp*	63 viral genomic equivalents/reaction	-
	Corman *et al*[3], *E*	6.3 viral genomic equivalents/reaction	
	CDC N2[12]	6.3 viral genomic equivalents/reaction	
Chan *et al*[7], 2020	Corman *et al*[3], *RdRp*	-	Detected 28% positivity from 273 specimens from 15 patients
	RdRp/Hel[7]	-	Detected 43% positivity from 273 specimens from 15 patients
Barra *et al*[11], 2020	Corman *et al*[3], *RdRp*	350 viral genomic equivalents/reaction	-
	Corman *et al*[3], *RdRp* modified primer/probe concentrations	33.7 viral genomic equivalents/reaction	-
Vogels *et al*[8], 2020	*ORF-1b-nsp14*	Detected 10 viral genomic equivalents/µl	-
	Corman *et al*[3], *RdRp*	Could not detect ≤100 genomic equivalents/µl	-
Lim *et al*[9], 2020	Corman *et al*[3], *RdRp*	7-43 times less sensitive than CDC	

qRT-PCR kits which use primers and probe targeting *RdRp* gene as described earlier[3] should be treated with caution and supported with confirmatory assays. We suggest the following diagnostic algorithm for SARS-CoV-2 detection, first screening with *E* gene-based assay[3] followed by confirmation with *ORF-1b-nsp14* gene-based assay[4]. Though both assays would detect all viruses from the Sarbeco sub-genus, as there is no current circulation of SARS CoV-1, positive results in both assays would mean SARS-CoV-2 positivity. Further, when a sample tests positive in *E* gene assay and negative in *ORF-1b-nsp14* assay, testing in a third assay can be considered. Under the current situation, the third assay can be the existing *RdRp*-based assay. Alternatively, highly conserved regions in the *S* (spike glycoprotein) and *N* (nucleocapsid) genes can be explored for developing a confirmatory assay and used for the samples that test negative in the *ORF-1b-nsp14*-based confirmatory assay. The modified algorithm involving three-stage assay strategy will lead to a reduction in the number of reactions required for a positive sample. Instead of the four reactions required for a positive sample (screening, internal control and 2 confirmatory assays), only three might be required. Only the samples positive by *E* gene screening and negative by *ORF-1b-nsp14* assay would require the fourth reaction. Such a strategy will save cost and time. Before implementing the strategy pan India during the current pandemic, data from all the testing centres may be analyzed to make an informed decision.

As the *ORF-1b-nsp14*-based assay detects both SARS-CoV-1 and SARS-CoV-2, it is hypothesized that even within the 132 base pair region amplified by the primers reported by Chu *et al*[4], there is a possibility of presence of SARS-CoV-2 specific region to be used as a probe. When the 132 base pair region was analysed, a 24 base pair region downstream of the forward primer was identified, which could be targeted for designing a probe in reverse orientation, specific to SARS CoV-2. This region is highly conserved among SARS-CoV-2 genomes reported in Genbank and has multiple mismatches with SARS-CoV-1 genome. Because *ORF-1b-nsp14* primer set has been reported to be more sensitive, adding an additional probe specific to SARS-CoV-2 tagged with a fluorescent dye (which is different from the dye used for tagging the already available probe which can detect both SARS-CoV-1 and CoV-2) might provide more information. Thus, a modified duplex *ORF-1b-nsp14*-based assay with an additional probe might be able to add more specificity to the assay in detecting SARS-CoV-2.

To conclude, the present study suggests a modified diagnostic algorithm for qRT-PCR-based diagnosis of SARS-CoV-2 in public health laboratories in India which will be cost-effective. This study also reports the design of a modified duplex *ORF-1b-nsp14*-based assay for discriminating SARS-CoV-1 and SARS-CoV-2 based on *in silico* analysis.

***Financial support & sponsorship*:** The authors acknowledge the Department of Health Research, Ministry of

Health & Family Welfare, Government of India, New Delhi, for financial support.

***Conflicts of Interest*:** None.

K. Alagarasu[1], M.L. Choudhary[2], K.S. Lole[3], Priya Abraham[†], Varsha Potdar[2]* & NIC Team[#]
[1]Dengue & Chikungunya Group, [2]Influenza Group, [3]Hepatitis Group, [†]ICMR-National Institute of Virology, Pune 411 001, Maharashtra, India
[*]*For correspondence*: potdarvarsha9@gmail.com

[#]**National Influenza Centre Team:** V. Vipat, S. Jadhav, U. Saha, H. Kengale, A. Awhale, A. Jagtap, A. Gondhalekar, V. Malik, N. Shrivastava, S. Digraskar, P. Malasane, S. Hundekar, K. Patel, Y. Balakarthick, M. Kakade, Santosh Jadhav, R. Gunjikar, V. Autade, S. Bhorekar, P. Shinde, S. Salve, B. Minhas, S. Bhardwaj, H. Kaushal, Y. Gurav, S. Tomar

References

1. Tandon PN. COVID-19: Impact on health of people & wealth of nations. *Indian J Med Res* 2020; *151* : 140-5.
2. Zhai P, Ding Y, Wu X, Long J, Zhong Y, Li Y. The epidemiology, diagnosis and treatment of COVID-19. *Int J Antimicrob Agents* 2020 March 28; doi: 10.1016/j.ijantimicag. 2020.105955.
3. Corman VM, Landt O, Kaiser 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*. pii: 2000045.
4. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, *et al*. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. *Clin Chem* 2020; *66* : 549-55.
5. Choudhary ML, Vipat V, Jadhav S, Basu A, Cherian S, Abraham P, *et al*. Development of *in vitro* transcribed RNA as positive control for laboratory diagnosis of SARS-CoV-2 in India. *Indian J Med Res* 2020; *151* : 251-4.
6. Gupta N, Potdar V, Praharaj I, Giri S, Sapkal G, Yadav P, *et al*. Laboratory preparedness for SARS-CoV-2 testing in India: Harnessing a network of Virus Research & Diagnostic Laboratories. *Indian J Med Res* 2020; *151* : 216-25.
7. Chan JF, Yip CC, To KK, Tang TH, Wong SC, Leung KH, *et al*. Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-polymerase chain reaction assay validated *in vitro* and with clinical specimens. *J Clin Microbiol* 2020. pii: JCM.00310-20.
8. Vogel CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, *et al*. Analytical sensitivity and efficiency comparisons of SARS-COV-2 qRT-PCR primer probe sets. *medRxiv* 2020; doi: https://doi.org/10.1101/2020.03.30.20048108.
9. Lim J, Kim CM, Lee KH, Seo JW, Yun NR, Lee YM, *et al*. Insufficient sensitivity of RNA dependent RNA polymerase gene of SARS-CoV-2 viral genome as confirmatory test using Korean COVID-19 cases. *Preprints* 2020. doi:10.20944/preprints202002.0424.v1.
10. Nalla AK, Casto AM, Huang MW, Perchetti GA, Sampoleo R, Shrestha L, *et al*. Comparative performance of SARS-CoV-2 detection assays using seven different primer/probe sets and one assay Kit. *J Clin Microbiol* 2020; pii: JCM.00557-20.
11. Barra GB, Rita THS, Mesquita PG, Jacomo RH, Nery LFA. Analytical sensibility and specificity of two RT-qPCR protocols for SARS-CoV-2 detection performed in an automated workflow. *medRxiv* 2020. doi: 10.1101/2020.03.07.20032326.
12. Centers for Disease Control and Prevention. *2019-novel coronavirus (2019-nCoV) real-time rRT-PCR panel primers and probes. 2020*. Available from: *https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf,* accessed on May 12, 2020.