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Review Article

Effectiveness of Different Gene-Target Strategies for SARS-CoV-2 Screening by RT-PCR and Other Modalities: A Scoping Review

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ABSTRACT

Background: The ongoing Corona Virus-2019 (COVID-19) pandemic has seen overwhelming dependence on molecular diagnostics, especially Real-Time Polymerase Chain Reaction (RT PCR) techniques for diagnosis purposes. A sophisticated research/diagnostic technique has almost metamorphosed into a point of care test technique. There is an evolution of the various types of gene targets with time.

Objective: To compile a cohesive literature review of the effectiveness and accuracy of various gene targets for different RT PCR protocols/kits for detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). We also want to analyze other evolving techniques and radiological techniques e.g. Computed Tomography (CT) scan for diagnosis of COVID 19.

Materials and Methods: Medline, google scholar, embase, pre-print servers (e.g. bioRxiv) were searched for literature on molecular techniques and targets for diagnosis of COVID-19.

Results: Seven original articles were retained out of the initial 33 articles/reports. The most commonly employed gene targets were E-gene, RdRp, ORF 1b, S, N, etc. Multiple novel Polymerase Chain Reaction (PCR) protocol with new targets are being tried. Other molecular methods of detection are also evolving. Radiological investigation (e.g. CT scan chest) is found to very useful, especially in RT PCR negative high suspect cases.

Conclusion: Almost all RT PCR kits follow the World Health Organization (WHO) guidelines for two targets. Radiology (CT scan) certainly has a role in COVID diagnosis.

Keywords: COVID-19; RT PCR; SARS-CoV2; Gene targets; Molecular tests; Chest CT

INTRODUCTION

Based on phylogeny, taxonomy and established practices, the Coronaviridae Study Group (CSG) of the International Committee on Taxonomy of Viruses, recognizes this virus as forming a sister clade to the prototype human and bat Severe Acute Respiratory Syndrome Coronaviruses (SARS-CoVs) of the species Severe acute respiratory syndrome-related coronavirus and designated it as SARS-CoV-2 [1]. On 11 February 2020 the World Health Organisation (WHO) announced the name for the disease caused by the new virus to be"COVID-19" i.e. Coronavirus Disease-2019 [2]. In a matter of months it resulted in a major outbreak and reached most of the countries of the world due to inter-state movement of people, rapidly resulting in an acute global health crisis.

In India, initially, the disease progression was slower but by the end of March and the beginning of April, the positive cases started rising exponentially. Initially, only the National Institute of Virology (NIV), Pune, which has a Biosafety Level (BSL) 4 facility, was entrusted with the job of diagnosis of COVID 19 cases by RT qPCR. Later on, other labs of Indian Council of Medical Research (ICMR) and functional state Viral Research and Diagnostic Laboratories (VRDLs) and many private sector labs were roped in for diagnosis of COVID 19.

In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions in the nucleic acid testing assay. The real-time Reverse Transcription-Polymerase Chain Reaction (real-time RT-PCR) is one of the best and accurate laboratory methods for detecting, tracking, and studying the coronavirus. Real-time RT-PCR is a method by which we can detect the presence of specific target genetic material. Nowadays various fluorescent dyes are used as a marker to detect the specific genetic target; earlier radioactive isotopes were used as markers. The most

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important aspect of using real-time RT-PCR assays is that the amplification and analysis will be carried out in a closed system; therefore the chances of false-positive results will be minimized [3]. The Real-time RT-PCR facilitates in analyzing the result in real time even though the process is still ongoing; which makes it more useful than conventional RT-PCR which provides the result at the end. The recent emergence of the Coronavirus (COVID-19) has demonstrated the need for reliable and rapid detection, thus in the present scenario, the real-time RT-PCR is the most widely used method for the detection of coronavirus. The molecular testing is still a "gold standard" for relevant case diagnosis [4]. For conducting the PCR assay the number of the molecular target have been identified within the RNA of Coronaviruses; such as Helicase (Hel), Nucleocapsid (N), Transmembrane (M), Envelope (E) and envelope glycoproteins Spike (S) (23) [5]. Hemagglutinin-Esterase (HE), Open Reading Frames ORF1a and ORF1b and RNA-Dependent RNA Polymerase (RdRp), are some other genes that encode structural proteins can be utilized for the COVID-19 diagnosis [5]. In this real time-PCR assay the viral RNA is measured by the Cycle threshold (Ct), which is defined as the number of cycles required for the fluorescent signal to cross the threshold and becomes detectable. The interpretation of result in real time-PCR is based on Ct values for the specimen; a value less than 40 is clinically reported as PCR positive. In RT-PCR most of the time results are 100% specific but the false-negative result may also occur; which may be due to sampling error inappropriate timing of sampling [3].

Laboratory testing for COVID-19 is an essential component of containment and mitigation strategies, as it allows the appropriate clinical management and public health interventions [6-8]. Nucleic Acid Amplification Tests (NAAT) such as Real-Time Reverse Transcription Polymerase Chain Reaction (RRT-PCR) is the methods of choice for SARS CoV-2 diagnostic testing. With the rapid availability of genome sequences [9,10] Laboratory-Developed Tests (LDTs) for the detection of the SARS-CoV-2 were quickly developed. The first LDTs relied primarily on the detection of SARS-CoV-2 Envelope (E), RNA-Dependent RNA Polymerase (RdRp), and Nucleocapsid (N) genes [11,12], but more recent RRT-PCR method targets include open reading frame 1 a/b (Orf1a/b) and the gene encoding Spike (S) protein. The performance characteristics of PCR methods can vary with reagents, PCR, and instrumentation [13,14].

Objectives

To describe the advantages and loopholes of various gene targets used in testing for COVID 19 and help readers to understand the relative merits and demerits of various targets at different places for the screening of the ongoing pandemic. The role of other molecular techniques and radiological investigations are also to be analyzed.

MATERIALS AND METHODS

Literature search strategy

A literature search was performed on 23rd June 2020 on all the research articles related to real time PCR targets for COVID-19 testing published or accepted to be published between January and 23rd June 2020. Literatures for this review were identified by searching the following online databases: Medline, Embase, Google scholar, as well as CNKI, and Wang Fang data (two primary databases for research in China for abstract). We searched scientific publications from 1st January to 23rd June 2020 using the keywords "(using English MeSH keywords and Emtree terms): [SARS-CoV-2 AND RT PCR] OR, [2019-nCoV AND RT-PCR Targets]" OR "COVID-19 AND PCR targets] OR [New Coronavirus AND RT PCR AND Gene Targets] OR [Wuhan Coronavirus AND RT PCR AND Targets] OR [Coronavirus AND RT PCR AND Targets].

Considering the urgency of the topic and to increase the sensitivity of the search, a gray literature search was performed using the same keywords on Google Scholar, bioRxiv, and medRxiv (pre-print servers) to capture the most recently published articles. WHO/CDC databases of publications on SARS-CoV-2 were also searched for relevant articles. Furthermore, related articles were also retrieved from the reference list and abstracts of published articles.

Inclusion and exclusion criteria

All the retrieved articles were screened for relatedness to the topics under study by both the authors independently. A consensus was drawn between both the researchers in making a strategy for the inclusion of relevant studies and excludes the irrelevant ones. Theoretical articles, commentaries, reports, and news articles were excluded from this analysis. Non-diagnostic articles like economic and mathematical aspects of the pooling of samples were also excluded from the study. Studies that did not provide or mention the appropriate data which are essential in the synthesis of results are excluded from the studies. To eliminate selection bias, two authors selected the articles independently according to the eligibility criteria and the 7 selected articles were unanimously selected by the two authors (Figure 1).

Paper quality evaluation

Newcastle-Ottawa quality assessment scale for cross-sectional studies was used. Table 1 yields the result of this evaluation [15-22].

Data extraction

The following data were extracted from the final selected articles: name of the first author, country/place of study, rate of positivity of COVID 19 in that particular country, test performed for the diagnosis of COVID 19, false positivity and false negativity rate of the test kit, number of PCR targets, sensitivity/specificity of targets (if available), house-keeping gene used, relative merits and demerits of the targets, number of countries targets are used (if available), Level of Detection (LOD) for targets, etc. One of the reviewers performed the data extraction, and the other reviewer assessed the accuracy of the extracted data.

RESULTS

In the initial literature search, 33 articles and reports were found in this topic from different databases. Following this, 13 articles were excluded due to being duplicated in different databases. Of the remaining 20 articles, 6 news articles were removed (screened by reading the abstract and material methods). Out of the remaining articles left, 7 more articles were eliminated after full-text screening as they were more on a different principle of molecular tests rather than on PCR targets. Finally, 7 original research articles were selected to be included in this review (Figure 1).

The WHO recommended that the E gene assay followed by a confirmatory assay using the RdRp gene can be utilized for first-

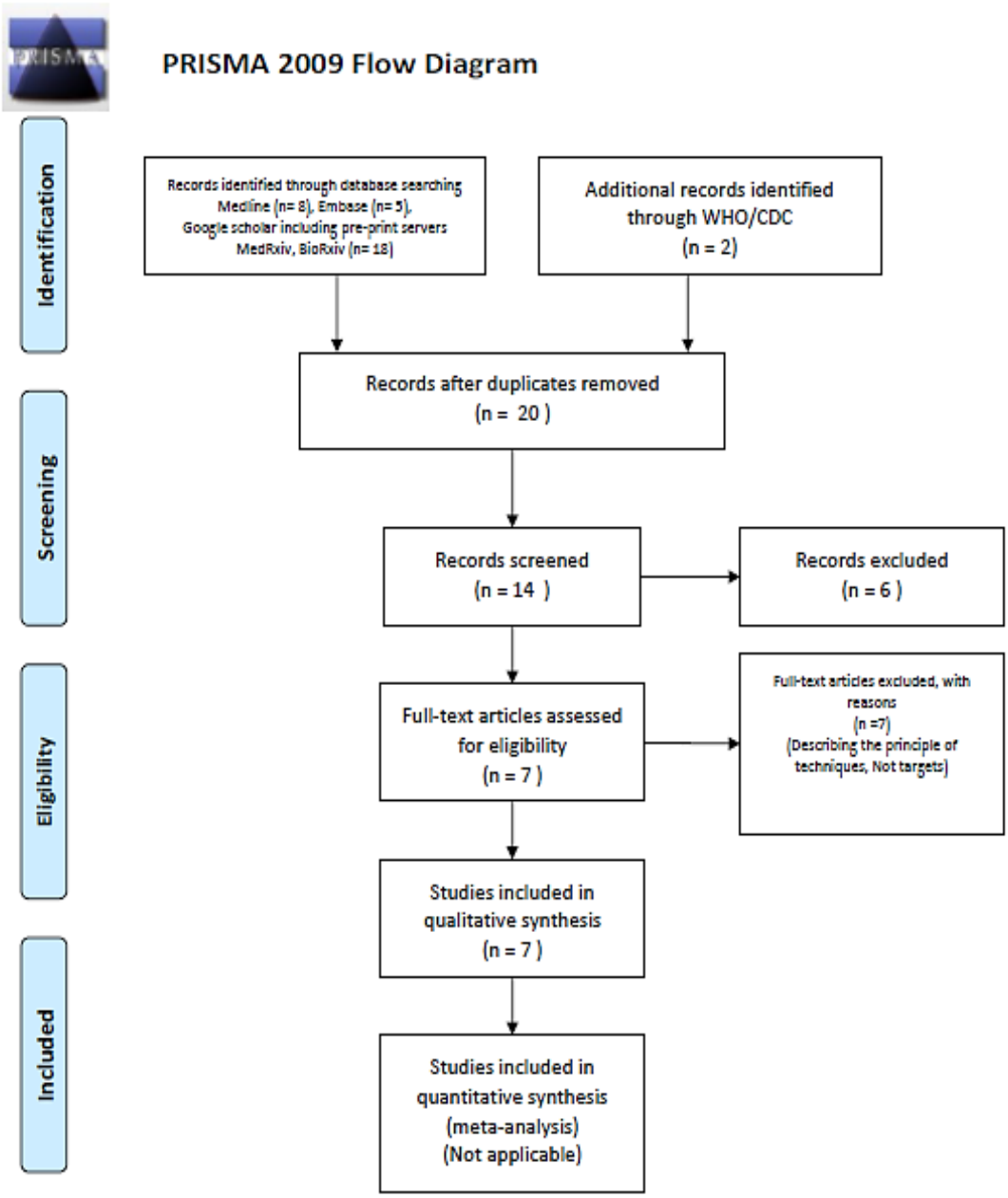

Figure 1: PRISMA flow chart (From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta- Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097. For more information, visit www.prisma-statement.org).

line screening of COVID-19 cases; [23] and in the United States the CDC asked to use two nucleocapsid protein targets [N1 and N2] as a molecular assay [24]. A study published from Hong Kong, China found that RdRp/Hel assay had the lowest limit of detection *in vitro* and have higher sensitivity and specificity among the three developed novel real-time RT-PCR assays targeting the RdRp/Hel, S, and N genes of SARS-CoV-2 [25]. It is advisable to use, at least two molecular targets to avoid the situation of a potential genetic drift of SARS-CoV-2 and the cross-reaction with other endemic coronaviruses as well, However, the ideal design would include at least one conserved region and one specific region to mitigate against the effects of genetic drift, especially as the virus evolves within new populations [5].

PCR targets and sensitivity

In these research articles, the diagnosis of COVID 19 was done by real-time Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-PCR) for the detection of viral RNA of SARS-CoV-2. In the first step, viral Ribonucleic Acid (RNA) is extracted from a

Table 1: Newcastle-Ottawa quality assessment scale for cross-sectional studies [15-22].

Studies (First author)	Selection	Comparability	Outcome	Total score
Wong et al. [16]	*****	***	***	9*
Yip et al. [17]	*****	***	***	9*
He et al. [18]	****	**	**	8*
Fang et al. [19]	*****	**	**	8*
Liu et al. [20]	***	**	**	7*
Ishige et al. [21]	***	**	**	7*
Muenchoff et al. [22]	***	**	**	7*

sample collected from the patient's respiratory tract and purified for reverse transcription. In the second step, this RNA eluate is used as a template for PCR amplification and loaded into the thermal cycler along with specific viral primers. Here cDNA is amplified and annealed to the target sequence. While extending through each PCR cycle, a reporter dye is cleaved or broken from a probe to amplify fluorescence intensity and reveal a positive sample. Detection of the SARS-CoV-2 specific targets like E-gene (for screening), S-gene, ORF1ab (RdRp), and ORF1b in combination implies a positive test.

Wong et al. used RdRp/Helicase gene combination in their study on 64 (51 known positives) patients and found sensitivity at 91% [16]. Yip et al. on the other hand used Non-Structural Protein 2 (nsp2) with as target 100% analytical sensitivity [17]. In both sensitivity could not be measured. He et al. did with ORF 1ab (Open Reading Frame 1ab) 79% sensitivity only 34 patients. The specificity was 100% [18]. Fang et al., studied it in 2 phases. First up they tested with ORF 1ab, envelope gene (e-gene), and Nucleocapsid gene (N) and had a sensitivity of 71%. A study with a solo target (ORF1ab) in a large study had no significant analytical sensitivity/specificity data [19]. A study by Liu et al. with Nucleocapsid Protein (NP) as a target has a similar sample size and outcome [20]. Ishige et al. in their study developed a multiplex PCR targeting 3 genes Sarbeco-e gene, N-gene, and human abl1 as an internal control. This kit results perfectly matched with simplex PCR results with different targets [21]. Muenchoff et al. in a multicenter comparative study (seven laboratories) found RdRp to be lower sensitivity with the need to improve its sensitivity. However, the same study found CDC N1 primer/probe-based kits highly useful and sensitive [22] (Table 2).

Table 2: Overview of the seven studies included in the scoping review [16-22].

First author	Place of study/ Setting	Populations/parameters	Study design/ time horizon	Targets used	Sensitivity	Specificity	Outcome/comment
Wong et al. [16]	University of Hong Kong, Hong/	255 subjects comparison of chest X-ray and RT PCR	Comparative study of chest X-ray and RT PCR	RdRp and Helicase (Hel) gene	91 (83-97)	NA	Chest X-ray findings have lower sensitivity than initial RT-PCR testing (69% versus 91%, respectively)
Yip et al. [17]	Queen Mary Hospital, HKSAR, Hong Kong, China	59 clinical specimens were evaluated (23 positive and 36 negatives)	Comparison of a new target with other established targets (RdRp/Hel)	nsp2 (Nucleotide position 1865–2018)	100% concordance with established targets	NA	The new assay showed 100% concordance with our previously developed COVID-19-RdRp/Hel reference assay. A rapid, sensitive, SARS-CoV-2-specific real-time RT-PCR assay, COVID-19-nsp2, was developed LoD was 1.8 TCID 50/mL.
He et al. [18]	Zhujiang Hospital, Southern Medical University, Guangzhou andThe University of Hong Kong-Shenzhen Hospital, Shenzhen, 518000, China	82 patients admitted to hospital between Jan 10, 2020, to Feb 28, 2020, were enrolled (34 COVID-19 and 48 non-COVID-19 patients)	Comparative study of RT PCR and CT	e- gene and RdRp (as per communication with the author)	79% (27/34)	100% (48/48)	RT-PCR and chest CT had comparable diagnostic performance in the identification of suspected COVID-19 patients outside the epidemic center. To compensate for the potential risk of false-negative PCR, chest CT should be applied for clinically suspected patients with negative initial RT-PCR.
Fang et al. [19]	Taizhou Hospital, Wenzhou Medical University, China	51 patients over 3 days-RT PCR and Chest CT compared	Comparative study between CT-scan and RT PCR	e- gene and RdRp (as per communication with the author)	71%	NA	Support the use of chest CT for COVID 19 patients with clinical and epidemiological feature compatible with COVID 19, in RT PCR negative
Liu et al. [20]	Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China	4880 cases with respiratory symptoms or close contact with COVID-19 patients	Risk factor analysis and intergroup differences (COVID-19 positive and negative cases)	NCV-NP and ORF-1ab	93%	97%	Very essential role of RT PCR in the diagnosis of COVID 19

Ishige et al. [21]	Chiba University Hospital, 1-8-1 Inohana, Chuo-ward, Chiba-city, Chiba 266-8677, Japan	30 known positive samples were tested by established simple PCR (N gene) and new multiplex kit	Comparative study of new multiplex PCR kit with established simplex PCR	Sarbecovirus specific E gene, the SARS-CoV-2 specific N gene	100%	NA	<25 copies/reaction of SARS-CoV-2 RNA 1) the NIID-N set is slightly more sensitive than E_Sarbeco; 2) NIID-N and E_Sarbeco are a good combination for detecting two regions of the SARS-CoV-2 genome with high sensitivity; and 3) the human ABL1 gene is useful as an IC for checking the qualities of the specimen, nucleic acid extraction step, and RT-PCR amplification
Muenchoff et al. [22]	Ludwig Maximilian University, Munich, Germany	RNA extract from stool sample (extracted by QA symphony and frozen) diluted and aliquoted and sent to participating labs	Comparison of different RT PCR assays used in 7 laboratories. Droplet PCR was used as a reference.	SARS-CoV-2 Nucleocapsid gene (N) Envelope gene (E), the RNA-Dependent RNA Polymerase (RdRp) gene	5 copies of RNA (LoD)	NA	The majority of RT-PCR assays for SARS-CoV-2 examined in this study detected ca 5 RNA copies per reaction, reflecting a high sensitivity and their suitability for screening purposes world-wide. A reduced sensitivity was noted for the original Charité RdRp gene confirmatory protocol, which may have impacted the confirmation of some COVID-19 cases in the early weeks of the pandemic. The protocol needs to be amended to improve the sensitivity of the RdRp reaction
NA: Not Available; CT: Computerized Tomogram; Hel: Helicase							

Besides these most of the other studies used at least two target assays in combination for the diagnosis of COVID-19 infections. In a study from Germany, they have chosen envelope and RNA-dependent RNA polymerase [23]. In another study from Hong Kong, China the researcher first used the nucleocapsid for screening followed by confirmation by the open reading frame 1b [25]. Similarly CDC conducted a study in the United States by selecting two loci in nucleocapsid gene and found the good performance for detection of COIVD-19 [24]. Given the critical situations of COVID-19 infections worldwide, various companies attempted to develop commercial kits for detection of SARS-CoV-2 RNA by real-time PCR.

Various institutes of Indian Council of Medical Research, New Delhi (ICMR) to date evaluated the performance of 31 such commercial kits and among these 14 kits were found satisfactory [26]. Even though the RT-PCR is a tool to do a definitive diagnosis of COVID-19, but the sensitivity is reported to be lower than the Chest CT examinations. However; the chest CT alone does not differentiate between COVID-19 pneumonia to other viral Pneumonia [27].

Comparison with radiography

Considerable discussion/studies are being conducted on the role and significance of radiological finding versus COVID diagnosis and its relative accuracy vis-à-vis real-time PCR technique.

Wong et al. opined that chest X-ray abnormalities in COVID-19 mirror those of chest CT-scan, demonstrating bilateral peripheral consolidation. They found that chest X-ray findings have lower sensitivity than initial RT-PCR testing (69% versus 91%, respectively). Chest X-ray abnormalities preceded RT PCR changes in 9% cases. Common X-ray findings were, bilateral, peripheral, ground-glass appearance, consolidation etc [16]. He et al. found similar and good diagnostic performance of RT PCR as well as chest CT in clinically suspected cases [18]. They opined that normal chest CT can be found in RT-PCR positive COVID-19 cases and typical CT manifestation can be found in RT-PCR negative cases. The sensitivity to identify COVID-19 was 79% (95% Confidence Interval [CI], 66%-93%) in initial RT-PCR and 77% (95% CI 62%-91%) in CT. The specificity was 100% (95% CI 100%) in initial RT-PCR and 96% (95% CI 90%-100%) in CT. The accuracy was 92% (95% CI 91%-92%) in initial RT-PCR and 88% (95% CI 88%) in CT. There is no statistical difference in the above-mentioned indicators. They concluded that chest CT should be applied for clinically suspected COVID-19 patients with negative initial RT-PCR [18]. Fang et al. also took a very similar stand by supporting the use of chest CT for the screening of COVID-19 for patients with clinical and epidemiological features compatible with COVID-19 infections particularly when RT PCR is negative [19]. In their study, the sensitivity of chest CT was greater than RT-PCR (98% v/s 71%; $p<0.001$) which was attributed to factors

like immature nucleic acid technology, variation in detection rate depending on kit manufacturer, low viral load, improper clinical samples etc [19].

Novel kit and target development

With the evolution of pandemic various novel techniques and RT-PCR targets are being tried and tested. In the context of this review, novel RT-PCR targets are covered. Yip et al. initially selected four specific regions (of 154, 58, 63 and 93 nt length) longer than 50 nucleotides in the SARS-CoV-2 genome [17]. Finally, stable primers were designed to target the longest (154 nt) and previously untargeted nsp2 region and optimized as a probe-free real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay. The new COVID-19-nsp2 assay had a Limit of Detection (LOD) of 1.8 TCID50/mL and did not amplify other human-pathogenic coronaviruses and respiratory viruses. Evaluation of the new assay using 59 clinical specimens from 14 confirmed cases showed 100% concordance with our previously developed COVID-19-RdRp/Hel reference assay [17]. Ishige et al. also initiated a novel multiplex RT PCR protocol using Sarbecovirus specific E gene, the SARS-CoV-2 specific N gene, and the human ABL1 gene as an internal control [21]. Very good correlation of cycle threshold values was observed between the simplex and novel multiplex RT-PCR methodologies with the same targets. Low copies (<25 copies/reaction) of SARS-CoV-2 RNA were detected by the novel multiplex RT-PCR method. This kit has the potential for highly sensitive detection of SARS-CoV-2RNA, reducing reagent use and cost, and time required [21].

Other technique

Another molecular method that is Loop-Mediated Isothermal Amplification (LAMP) reaction, could serve as an alternative method to the RT-qPCR to detect COVID-19. The LAMP is a nucleic acid amplification technique, which amplifies the DNA in isothermal conditions with rapidity and high specificity. This method can be utilized for the diagnosis of COVId-19 without the need for specialized equipment and trained analysts. Shortly the point-of-care device based on LAMP can be a potential diagnostic tool for the diagnosis of COVID-19 infected individuals [28]. Recently; Prof. Feng Zhang et al. gave a Clustered Regularly Interspaced Short Palindromic Repeats and Associated Cas Proteins13 (CRISPR-Cas13) based Specific High-Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) protocols, which is claimed to be an accurate and most rapid method for novel Coronavirus (COVID-19) [29]. Kim et al. in their study reported that they have constructed the Next Generation Sequencing (NGS) library by amplifying the full-length genes of the isolates using the synthesized cDNA and primers specific for SARS-CoV-2 [12].

Further development and inclusion of these new technologies for the diagnosis of COVID-19 can provide a better, accurate, and rapid tool. These developments may also reduce the need for sophisticated equipment and specific training; this will help us to reach to a wide community for screening as well as for diagnosing them.

CONCLUSION

From this review, we can conclude that WHO guidelines of RT PCR targets for SARS CoV-2 detection i.e. at least 2 targets namely, one sarbecovirus specific e-gene and other SARS CoV-2 specific gene (N, RdRp or ORF1b, etc.) positivity is most essential and followed by most of the kits available. Being in the early phase of kit maturity, essential diagnostic parameters (analytical sensitivity, analytical specificity, Limit of detection i.e. LoD, etc.) are yet to be settled down. The utility of radiological investigation (Chest CT) as a complementary to RT PCR, especially in PCR negative clinically high suspect cases is immense. New RT PCR protocols/assays with newer targets are evolving and it can be predicted that in future much reformed and stable kit may be available. Similarly, alternative nucleic acid amplification techniques or other molecular methods (different than RT PCR) are also evolving.

CONFLICT OF INTEREST

None of the authors had any conflict of interest to declare.

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