Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Review Article

COVID diagnostics by molecular methods: A systematic review of nucleic acid based testing systems

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ABSTRACT

Background: The selection of appropriate kit and PCR equipment for the detection of SARS CoV-2 is critically important in view of many options available in the diagnostic market. Since last year many molecular products are available for COVID-19 diagnostics., some of these diagnostics have become commercially available for health-care workers and clinical laboratories. However, the diagnostic technologies have specific limitations and reported several false-positive and false-negative cases, especially during the early stages of kit development and use.

The current article addresses these and other relevant questions important to the medical microbiologists running or aspiring to run COVID diagnostic services using PCR and related technologies.

Methods: In this Systematic Review we follow Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA). A total of 258 citations retrieved, among those 77 peer reviewed articles was assessed for eligibility, and 181 studies were excluded. Based on inclusion criteria final data extraction was done.

Results: The question of diagnostic dilemma has also been addressed in view of discordant results between assays, inter-test variability, repeat testing requirements in specific settings and inconclusive or indeterminate results. Kit efficiency was satisfactory for all assays and the estimates varied within sample types and technology. Using clinical samples, we observed some variations in detection rate between kits. Importantly, none of the assays showed cross-reactivity with other respiratory (corona) viruses, except as expected for the SARS-CoV-1 E-gene.

Conclusions: We conclude SARS CoV-2 related molecular assays differed considerably in performance. Hence we need to understand importance of molecular diagnostics test interpretation in light of the latest pandemic virus.

1. Introduction

The COVID pandemic which started in 2019 has been defined by extensive use of reverse transcriptase real time polymerase chain reaction as a diagnostic modality. Since many infections are pauci-symptomatic and asymptomatic accurate testing is very important [1]. Early accurate diagnosis of COVID facilitates better management in terms of timely hospital admission, initiation of specific anti-viral agents such as remdesivir and infection control measures. The symptoms of COVID are quite similar to that of common flu (influenza); [2] therefore, it is difficult to distinguish the difference between SARS-CoV-2 and common flu. Although the number of COVID tests done in India have steadily increased there is a need to test more considering the enormity of India's population (1.3 billion) and surge of cases due to the second wave of the pandemic in the country. This article mainly concentrates on the current scenario in India regarding molecular based testing for COVID-19 infection. It covers the types of RT-PCR based molecular tests, test kits. The objective of this review is to help and inform health care service providers and health administrators about RT-PCR based COVID tests and optional strategies for selection of select equipment for such test.

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2. Materials and methods

2.1. Types of studies

This Systematic Review was conducted following Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA) requirement and Cochrane Collaboration recommendations.

2.2. Search strategy

The literature search criteria for this study was divided in two category. In Step-1 we searched PubMed and Google Scholar regarding abstracts and manuscripts using key words: "COVID-19 detection" OR "COVID-19" AND "COVID-19 CBNAAT," and "COVID-19" AND "RT-PCR" AND "COVID-19" AND "PCR". In Step- 2: public web portal based information (ICMR- Indian Council of Medical Research, Government of India, World Health Organization, CDSCO (Central Drugs Standard Control Organization), FIND (Foundation for Innovative New Diagnostics) web portal) were searched. In this case we only considered company based validation data because in some cases there was no proper study published till date. All data were considered between January to September 2020. We searched the website of Foundation for Innovative New Diagnostics (FIND)- a global non-profit organization website. FIND is a WHO Collaborating Centre for Laboratory Strengthening and Diagnostic Technology Evaluation platform. Information based on FIND's all Real-Time PCR kits which are US-FDA can be used for lab diagnosis in India after due marketing approval from DCGI (The Drugs Controller General of India). Search criteria (key words) for searching FIND web portal https://www.finddx.org/included: "commercialized", "Test format", "manual NAAT" or "automated", "POC", "NAT" or "POC" "NAAT", "Regulatory", "CE IVD" or "INDIA", "CDSCO" or "US FDA" or "RUO" (Research use only). This study also searched performance evaluation of commercial kits for real time PCR for COVID as done by ICMR validation centres whose report was published by Indian Council of Medical Research, Government of India. https://www.icmr.gov.in/pdf/covid/kits/RT_PCR_Tests_Kits_Evaluation_Summ_21012021.pdf.

2.3. Types of participants

Record identified through searching **Step-1** we searched Pubmed and Google Scholar #In **Step- 2** public web portal based information (ICMR Govt of India, WHO, DCGI recognized kits, FIND web portal).

2.4. Study selection

The eligibility criteria for included studies were as follows:

1. All retrospective studies reporting diagnostic outcomes for COVID – 19 RT-PCR or COVID-19 RT-PCR validation or COVID-19 RT PCR based kit comparisons or severe acute respiratory syndrome coronavirus or SARS CoV-2 molecular diagnostic techniques AND
2 Human subjects of all ages.
3 Published in English language.

Inappropriate technology, duplicate studies, sample size under 10, retracted manuscripts, Kit not approved by CE IVD or US FDA or DCGI, manuscripts without PCR data, editorials, and PCR RUO data were excluded.

2.5. Types of outcome measures

2.5.1. Primary

1. The comparison studies reveal high specificity and no cross-sensitivity for different assays as well as comparable sensitivities in Indian scenario.

2.5.2. Secondary

1. Optimization of best molecular diagnostics assay for COVID-19.

2.6. Data extraction

Data extraction was done using a data extraction form that was designed and pilot tested a priori. Two authors (PD and SB) independently extracted the following information from each study: author year, country, study design, setting (hospital or diagnostic laboratory), method of recruitment, inclusion criteria, risk of bias, participants (technology, sample tested, final outcome), intervention (turnaround time, cost, throughput, and co-intervention if any), outcomes, loss to followup and key conclusions. Any disagreements between the two review authors were resolved through discussion with the third author (SM).

3. Results

3.1. Description of studies

Of 258 total citations retrieved, the full text of 77 papers was assessed for eligibility, and 181 studies were excluded [supplementary].

3.2. Genomic organization of SARS CoV-2

Coronaviruses are un-segmented single-stranded RNA viruses ranging from 26 to 32 kilobases in length, belonging to the subfamily Coronavirinae of the family Coronaviridae of the order Nidovirales [3]. The genome of Coronaviruses, includes a variable number of open reading frames (ORFs) [4]. The SARS-CoV-2 genome was reported to possess 14 ORFs encoding 27 proteins [5]. The spike surface glycoprotein (S) plays an essential role in binding to receptors on the host cell and is crucial for determining host tropism and transmission capacity, mediating receptor binding and membrane fusion [6]. Generally, the spike protein of Coronaviruses is functionally divided into the S1 domain, responsible for receptor binding, and the S2 domain, responsible for cell membrane fusion [7].The eight accessory proteins (3a, 3b, p6, 7a, 7b, 8b, 9b, and orf14) and four major structural proteins, including the spike surface glycoprotein (S), small envelope protein (E), matrix protein (M), and nucleocapsid protein (N), are located in the 3′-terminus of the SARS-CoV-2 genome [5].

3.3. Present strategy of laboratory diagnosis of COVID-19

Sensitive, specific, precise and accurate reliable diagnostic kits and reagents are of paramount importance for combating the ongoing COVID-19 pandemic. The World Health Organization has recommended reverse transcription-polymerase chain reaction (RT-PCR) for screening and confirmation of COVID-19 Table 1 [8]. A study by Corman et al. have reported RT-PCR assays based on the RNA-dependent RNA polymerase (RdRp) gene, envelope (E) gene and nucleocapsid (N) gene for the beta-coronaviruses, including SARS-CoV-2. Chu et al. have reported two other gene target based assays based on ORF 1b and N gene that are highly preserved among Sarbeco viruses. On the other hand Indian Council of Medical Research and National Institute of Virology (ICMR-NIV), at Pune in India developed a real-time RT-PCR assay for screening (E gene) and confirmation (RdRp, N and ORF gene) along with a housekeeping RNase P gene to verify sample quality, RNA extraction and rule out PCR inhibition [9]. Most of the national and international commercial kit manufacturers select these genes for different platform of RT-PCR. Table 2 shows different gene targets available in different PCR kits.

3.4. General testing methodologies

There are currently 4 testing methodologies to clinically detect viral disease:

Table 1
Country wise summary of COVID RT-PCR protocols published by public health and research labs.

Country	Institute	Gene targets	Reference
Germany	Charité	RdRP, E, N	https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2
China	China CDC	ORF1ab and N	http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html
Hong Kong SAR	HKU	ORF1b-nsp14, N	https://www.who.int/docs/default-source/coronaviruse/peiris-protocol-16-1-20.pdf?sfvrsn=af1aac73_4
Japan	National Institute of Infectious Diseases, Department of Virology III	Pancorona and multiple targets, spike protein	https://www.who.int/docs/default-source/coronaviruse/method-niid-20200123-2.pdf?sfvrsn=fbf75320_7
Thailand	National Institutes of Health	N	https://www.who.int/docs/default-source/coronaviruse/conventional-rt-pcr-followed-by-sequencing-for detection-of-ncov-rirl-nat-inst-health-t.pdf?sfvrsn=42271c6d_4
India[a]	Principal Scientific Adviser to the Government of India	RdRp, E and N.	http://psa.gov.in/sites/default/files/pdf/Handbook_COVID19_Research_Institutions.pdf
USA[b]	US CDC	Three targets in N	https://www.fda.gov/media/134922/download
France	Institut Pasteur, Paris	Two targets in RdRP	https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2

CDC, Centres for Disease Control and Prevention; ORF, open reading frame.
[a] Principal Scientific Adviser to the Government of India; update effective from April 11, 2020.
[b] CDC updates effective from March 15, 2020.

1. **Real-Time PCR: manual or Cartridge Based Nucleic Acid Amplification Test (CBNAAT) format**
2. Isothermal Amplification
3. **CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats)**
4. **Serological IgM/IgG Antibody Detection (not discussed in this review)**
5. **Viral Antigen Detection (not discussed in this review)**

3.5. Major advantages of RT-PCR

- Directly detects for viral RNA by molecular methods
- High specificity, sensitivity, positive and negative predictive values

3.6. Limitations of RT-PCR

- Instrument, consumable and reagent costs are much higher than most laboratory assays
- Needs personnel trained in molecular testing.
- Labour intensive and less automated in comparison to other laboratory tests (e.g. serology)
- Turn Around Time: Conventional RT-PCR testing may take up to 4–6 h to obtain results including time for nucleic acid extraction
- Requires additional lab equipment, consumable supply, and reagents for nucleic acid extraction.
- Conventional RT-PCR requires separate rooms, equipment, and safety cabinets to prevent nucleic acid contamination between setup, amplification, and reading.
- Sensitivity is greatly affected by proper specimen collection
- False negative may occur with: inadequate swabbing of the appropriate target region where virus resides; timing of sample collection; improper shipping/storage conditions; mutation in the region of gene target

3.7. Isothermal amplification

In Table 3 examples of isothermal amplification techniques for nucleic acids include:

- Loop mediated isothermal amplification
- Strand displacement amplification
- Nucleic Acid Sequence Based Amplification
- Helicase Dependent Amplification
- Recombinase Polymerase Amplification
- SMART- Simple Method to Amplify RNA Targets

3.8. Advantages of isothermal nucleic acid amplification methods

- High specificity and sensitivity.
- Instrumentation has a small footprint and are inexpensive and simple to use in comparison to real-time PCR equipment
- Extra equipment like DNA/RNA extraction not required
- Rapid method: Denaturation/annealing/amplification steps are performed at one temperature instead of the heating and cooling steps required in real-time PCR. Thus, reducing the time-to-result for many assays. Results can be as rapid as within 30 min.
- Requires less manual labor than most other molecular assays.

3.9. Limitations isothermal nucleic acid amplification methods

- Reagent costs per sample are much higher than most real-time PCR assays
- Not recommended for high-throughput environments as batch testing is not available; most instrumentation can only run 1 sample at a time.
- Limited versatility – only short gene targets can be amplified; thus, variety of different assays available to run on these technologies are very narrow and limited in scope in comparison to real-time PCR
- Sensitivity is greatly affected by proper specimen collection.

3.10. CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats)

- The technology is based on gene editing
- It has programmable ability to detect specific sequences of DNA within a gene of interest
- The method can subsequently cut the gene at a specific sequence using an enzyme which act as a molecular scissors
- CRISPR can operate as a diagnostic tool for detecting specific sequences of DNA/RNA such as those that uniquely exist in the SARS-CoV-2 virus
- CRISPR-based POCT (point of care tests), specifically for rapid Covid-19 diagnosis has been developed by SHERLOCK biosciences

3.11. CBNAAT and TRUENAT

The rapid diagnosis of coronavirus disease 2019 (COVID-19) is a significant step towards the containment of the virus. Cartridge Based Nucleic Acid Amplification Test (CBNAAT) platforms like Cepheid Xpert Xpress SARS CoV-2, that employ real time RT PCR technology, are in use for COVID-19 testing in India. TRUENAT SARS CoV-2 is a chip-based Real-time Reverse transcription Polymerase Chain Reaction (RT-PCR)

Table 2
List of conventional SARS CoV-2 RT-PCR kits.

Manufacturer Details	Country of manufacturer	Regulatory status	Target Gene	Single tube multiplex/ Multitube multiplex	PCR time	Instrument Compatibility	Pack Size	Reporter dye
International Manufacturer								
Altona Diagnostics	Germany	USA FDA EUA; CE-IVD	E gene, S gene with IC	SINGLE	2.15 h	ABI 7500, Roche LightCycler® 480, RGQ, QuantStudio 5 R, CFX96, Mx 3005P™ QPCR System, L	384 Rxn	FAM, JOE, Cy5
Seegene	South Korea	USA FDA EUA; CE-IVD; Korea; Singapore, Australia; Canada	E gene, RdRP gene, N gene with IC	SINGLE	1.30 h	CFX 96 (BioRad)	50/100 Rxn	FAM, CalRed 610, Quasar 670, HEX
SD Biosensor	Republic of Korea	USA EUA; CE-IVD; Korea; Brazil, CDSCO India	ORF1ab,E gene with IC	SINGLE	1.30 h	LightCycler 480 or CFX96Dx System, ABI 7500	96 Rxn	FAM, JOE/VIC/HEX, Cy5
BGI	China	USA EUA; CE-IVD; China; Singapore; Canada	ORF1ab region, β-Actin	SINGLE	3 h	ABI 7500,Roche LightCycler 480,QuantStudio 5	50 Rxn	FAM/VIC (NOTspecified)
ABI (Applied bio systems)	USA	No web information	ORF1ab,S,N with IC	SINGLE	1.10hrs	ABI 7500,QuantStudio 5	96 Rxn	FAM, VIC, ABY, JUN
ADT Biotech SdnBhd	Malaysia	RUO	E gene, RdRP gene with IC	SINGLE	2.15 h	Rotor-Gene 3000/ 6000,Rotor-Gene Q5/6 plexPlatform,ABI 7500,CFx96	48 Rxn	FAM,HEX
OSANG Health Care	Republic of Korea	FDA EUA,CE-IVD	E, RdRP, N with IC	SINGLE (Single gene)	2.15 h	CFX96Dx System, ABI 7500	100 Rxn	FAM,TexasRed,JOE/ VIC,Cy5
Gene Matrix	California, USA	USA FDA EUA	N, RdRP	SINGLE	3 h	CFX96Dx, ABI 7500	96 Rxn	FAM, HEX
Accelerate Technologies Pte Ltd	Singapore	RUO	Not Specified	SINGLE	2.30 h	CFX96Dx	200 Rxn	FAM, HEX
Daan Gene Co. Ltd.,	China	CE-IVD; China	ORF1ab, N	SINGLE	1.30 h	LightCycler480 II, ABI 7500	24/48/ 96 Rxn	FAM,VIC,Cy5
JN MedsysPte Ltd,	Singapore	USA EUA, CE IVD Philippines	N1, N2 and RNase P	SINGLE	1.35 Hrs	QS3	100 RX	FAM
Kogene Biotech	Korea	CE-IVD	E, RdRP	TWO	No web information	CFX 96, ABI 7500, PowerAmp 96	50/ 100Rxn	FAM, JOE (VIC/HEX)
LabGenomics,	South Korea	USA FDA EUA	E, RdRP	TWO	2.30Hrs	CFX 96, ABI 7500	100 RX	:FAM and HEX (VIC), Cy5
OSANG Health Care	South Korea	CE IVD, USA FDA EUA	E, RdRP, N	SINGLE	2 Hrs	CFX 96, ABI 7501	100 RX	FAM,Texas Red, JOE/ VIC,Cy5
Primer Design,	UK	CE IVD/RUO*	N, RdRP	SINGLE	No web information	Roche LightCycler 480 II,CFX 96, ABI 7500	96 Rxn	FAM, VIC/HEX
Sansure Biotech Inc.,	China	CE IVD, USA FDA EUA	ORF1ab, N	SINGLE	2.30 Hrs	ABI 7500	24/48 Rxn	FAM, ROX, Cy5
ZyBioInc,	China	USA FDA EUA	No web information	No web information	No web information	No web information	32 Rxn	No web information
National Manufacturers								
MY LAB	India	DGCI India	RdRp, E gene RNAseP as IC	THREE	2 h	ABI 7500, RGQ, QS 5 R, CFX instrument (Bio-Rad)	50 and 100 Rxn	FAM (RdRP) VIC/HEX (E and RNAseP)
KILPEST (BLACKBIO)	India	DGCI India	E, RdRP, N with IC	SINGLE/TWO	1.10hrs	ABI 7500, RGQ, QuantStudio 3	100 Rxn	FAM, HEX
Helini Biomolecules	India	CE IVD, ICMR	RdRp and ORF gene	SINGLE	1.35 Hrs	Agilent, Bio-Rad, Roche Lightcycler-96, RocheZ480/Cobas-480, ABI 7500, Thermo-Piko-Real, Rotor gene 5/6plex, Alta-96, Cepheid Real time PCR machines	25/50/ 100 Rxn	FAM & HEX & Cy5
GCC Biotech	India	ICMR	RdRP gene, N gene with IC	SINGLE	1.35 Hrs	RGQ,QuantStudio 3	100/ 200/500 Rxn	No web information

Table 3
Isothermal DNA Amplification Technologies compared to PCR (a non isothermal method).

Property	PCR	NASBA	SMART	SDA	RCA	LAMP	HDA	SPLA
DNA amplification	+	+	+	+	+	+	+	+
RNA amplification	+	+	+	+	+	+	+	+
Temperature in degree Centigrade (Celsius)	94, 55–60, 72	37–42	41	37	37	60–65	Room temp, 37, 60-65	45,50
Primer design	Simple	Simple	Complex	Complex	Simple	Complex	Simple	Simple
Number of enzyme	1	2–3	2–3	2	1	1	2	3
Multiplex amplification	+	+	–	–	–	–	+	–
Product detection	Gel electrophoresis, Real-time	Gel electrophoresis, Real-time, ECL	Gel electrophoresis, Real-time,	Gel electrophoresis, Real-time,	Gel electrophoresis, Real-time,	Gel electrophoresis, Real-time, turbidity	Gel electrophoresis, Real-time, ELISA	Bioanalyzer

• PCR: polymerase chain reaction; NASBA-nucleic acid sequence based amplification; SMART- Simple Method to Amplify RNA Targets; SDA-strand displacement amplification; RCA-recombinase polymerase amplification; LAMP- loop mediated isothermal amplification; HDA- Helicase Dependent Amplification; SPLA - Single-primer-limited amplification.

Table 4
Potential reasons for COVID RT-PCR test discrepancy.

1. Differences in pre-analytical variables:
   a. Sample collection kit
      i. Viral transport media
      ii. Swab type
   b. How diligently the sample is collected
      i. Sometimes the collector is afraid to collect sample properly; sometime the patient gags or does not cooperate; sometimes the tonsil and posterior pharyngeal wall or mid inferior portion of inferior turbinate cannot be easily reached)
   c. Sample transport conditions
   d. Sample storage conditions
   e. Timing of the test (samples taken in relation to symptom; sample taken in relation to sub-clinical infection duration)
2. Differences in analytical variables:
   a. RNA extraction method
      i. Spin column extraction
      ii. Magnetic bead extraction
      iii. Automated RNA extraction instrument and kit type
   b. PCR kit used: we use three different kits: Seegene, Altona, True PCR; Every kit may have differences in
      i. Genes detected
      ii. Analytical sensitivity (Limit of detection)
      iii. Clinical sensitivity and specificity
3. Differences in Post analytical variables:
   a. CT cut-off used to call positive (True PCR: 35; Seegene 40; Altona 45)
   b. Number of genes and type of genes for calling a sample positive
   c. Sometimes the borderline cases just over the cut-off may be called positive or vice versa

The discrepancy is likely to be less with low CT values (CT < 35); and significantly more after that (CT > 35 or low viral load situation)

Also note CT values may be affected by all the factors mentioned as above.

Some experts are of the view that as COVID is a new disease with no proven anti-viral treatment or vaccine, reporting borderline situation as positive is perhaps safer than doing it otherwise

test for the semi-quantitative detection of SARS CoV-2 RNA. TRUENAT system has been validated by ICMR and Cepheid Xpert Xpress SARS CoV-2 has been approved by US FDA for use under an emergency use authorization. Initially TRUENAT Beta CoV was used for screening assay and TRUENAT SARS-CoV-2 used for confirmation assay as a PoC basis for the detection of SARS-CoV-2. Subsequently a single assay has been developed by TRUENAT however with a CT cut-off of 32.

4. Discussion

4.1. Summary of evidence

4.1.1. SARS-CoV- 2 molecular test kit validation guidelines

For CE-IVD approved/Non US-FDA approved/USA EUA/Indigenous Kits: First batch of kits will require validation from any of nine direct ICMR validation centres or any other 15 DBT/CSIR/other affiliated institute prior to DCGI approval. In the post marketing phase additional two batches should be tested as per medical device rule in four months time [10]. In India, as per ICMR guideline any RT-PCR kits approved by US-FDA need not require additional validation.

4.2. Post-analytical issues

(i) **Interpretation of molecular results.**

As per CDC initial protocol the laboratory confirmatory criteria for COVID-19 positive cases was detection of both of two targets in the CDC assay (nucleocapsid proteins N1 and N2 had to be positive) [11]. A cycle threshold (CT) value of less than 40 was defined as a positive test, while a CT value of 40 or more was defined as a negative test. A CT value of less than 40 for only one of the two nucleocapsid protein (N1 and N2) was defined as indeterminate (inconclusive) and required confirmation by retesting the test [11]. In triplex assays with three targets (Table 1), positives for two or more targets are considered positive [12]. Viral loads

determined by real-time RT-PCR assays should not be used to indicate COVID-19 severity or to monitor therapeutic response [13–15]. However, low RT-PCR CT values signifying high viral loads may be used as an indication of transmissibility.

(ii) **COVID infectivity: time dependence, symptom dependence, clinical phenotype dependence and RT-PCR CT value dependence**

Infectivity duration of symptomatic and asymptomatic COVID patients is a matter of concern for patients, care givers, healthcare providers and public health. In a study from the virus reference laboratory at Colindale UK it was reported that the probability of culturing virus declined to 8% in samples with CT > 35 and to 6% 10 days after onset; it was found to be similar in asymptomatic and symptomatic persons [16]. A study from the University of Nebraska reported that, viable virus was rarely cultured at CT values > 30 on or after 14 days of illness, suggesting that the probability of infectivity decreased with increasing CT values [17]. A study from the virus reference lab at Ireland reported that COVID-19 patients with mild-to-moderate illness were highly unlikely to be infectious beyond 10 days of symptoms. SARS-CoV-2 was isolated beyond day 10 for approximately 3% of included patients. Two studies identified immunocompromised patients from whom SARS-CoV-2 was isolated for up to 20 days. Three virus culture studies included patients with severe or critical disease; SARS-CoV-2 was isolated in these critically ill patients for up to day 32 in one study [18]. A study from the Harvard University reported that SARS-CoV-2 appeared to be most contagious around the time of symptom onset, and infectivity rapidly decreased thereafter to near-zero after about 10 days in mild/moderately ill patients and 15 days in severely-critically ill and immunocompromised patients. The longest interval associated with replication-competent virus thus far is 20 days from symptom onset [19]. An European study looking at correlation between successful isolation of virus in cell culture and CT value of quantitative RT-PCR targeting E gene suggested that patients with CT above 33–34 using RT-PCR system were not contagious and thus may be discharged from hospital care or strict confinement for non-hospitalized patients [20].

(iii) Inconclusive RT-PCR results

The qualitative real-time PCR interpretation of SARS-CoV-2 from patient is mainly relying on three possible reporting outcomes: Positive, Negative and Inconclusive. If the internal control for extraction/PCR (IC) was not amplified repeat extraction and RT-PCR should be considered. Inconclusive results may also happen if only the E gene is positive. There are multiple reasons which may cause inconclusive results for COVID-19 RT-PCR assay (Table 3). In this review we are try to highlight some of the possible causes for inconclusive result with respect to COVID-19 RT-PCR assay.

- Improper or inadequate sample
- RNA Extraction failure
- Presence of PCR inhibitors in the sample
- Mutation of the virus in the target region
- Timing of the sample in relation to the clinical course of the disease

4.3. Using COVID RT-PCR CT cut-off as 35: the pros and cons

In a communication dated April 05, 2021 ICMR issued an advisory stating that with regard to RT_PCR assays for SARS-CoV-2 a CT value cut-off of 35 with a good sigmoidal real-time RTPCR curve is acceptable. All patients with a Ct value < 35 may be considered as positive while those with Ct value > 35 may be considered as negative. All samples with CT value < 35 with poor sigmoidal curves should be essentially re-tested. The advantages and disadvantages of this advisory may be summarized as follows:

4.3.1. Pros

1. Simple to interpret
2. Pragmatic for any level of diagnostic or healthcare setup
3. Supported by viral culture data (no virus cultivable if RT-PCR CT > 35)
4. CT cut-off of 35 increases assay specificity but decreases sensitivity

4.3.2. Cons

1. It assumes all RT-PCR systems are equal; actually all Rt-PCR systems are different > actually not because in differences in kit chemistry, thermal profile, PCR kinetics, etc (System includes PCR kit, PCR machine and PCR software; different kits are compatible with different machines and they have specific versions and software; these factors are generally different in different labs) (Table 1)
2. Pre-analytical variables like sample quality, RNA extraction efficiency not taken into consideration (CT or viral load also depends on sample transport and storage conditions, phase of illness)
3. A PCR kit is optimized based on chemistry, machine specifications and software. This implies that an RT-PCR optimized to run till CT 45 can detect one copy of RNA at 45 CT; a PCR kit optimized to CT35 detects one RNA copy at 35 CT
4. With difference of CT of 3.3 the change in viral load is 10 times (1 log). Therefore if the reaction is specific and the kit is optimized at CT35 the viral load of a 45 cycle PCR is 1000 copies/mL
5. CT cut-off of 35 increases assay specificity but decreases sensitivity

4.4. Importance of RNA extraction systems on PCR

RNA extraction protocol is also very crucial step in any RT-PCR overall performance [21]. According to ICMR guideline CE-IVD approved RNA extraction kit like QIA amp VIRAL RNA MINI KIT by Qiagen, Germany. 2. PureLink RNA Mini Kit (Invitrogen, USA), 3. GenElute Total RNA Purification Kit (Sigma-Aldrich, USA) 4. ReliaPrep RNA Miniprep System (Promega, USA) 5. RNASure Virus Kit (Magnetic extraction) by Trivitron Health Care, India, needs to be used for viral RNA extraction purpose [22]. Faced with sudden increase in sample throughput demand combined with unprecedented urgency, the challenges of scaling up nucleic acid isolation kits can often become overwhelming. Till date there are few comparative analysis done for COVID-19 RNA extraction and performance with respect to robotic automated systems and manual system (see Table 4).

4.5. CBNAAT systems

Table 5 compares most of the self-enclosed systems (CBNAAT) integrating nucleic acid extraction, amplification and detection which play a major role in point-of-care testing for hospitals and clinics without the need of a comprehensive molecular biology laboratory [23]. QIAstat-Dx by Qiagen, Germany is a multiplex syndromic cartridge based detection system which differentiates 22 respiratory targets, including SARS-CoV-2 from Nasopharyngeal swabs.

4.6. PCR kits made in India: a comparison of two kits

The present commercial kit available in the Indian market may be categorized based on the number of tube per sample reaction, number of gene targets, number of samples which may be tested within a given run (this may be determined by the number of tubes per sample), RT-PCR instrument compatibility, reporter dye for each of the gene target, PCR time, pack size of the kits, reaction volume, price of the kit, supply chain flow. Among the made in India kits for COVID, Mylab Discovery Solutions (PathoDetect) was the first to be available commercially. This kit comprised of RdRp, E gene and RNAseP as internal control. The major disadvantage of this version 1 kit from MyLab was the fact that it was not

Table 5
List of cartridge-based molecular test for detection of COVID-19.

Manufacturer	Test kit	Diagnostic equipment	Instrument throughput	Separate Viral RNA extraction required?	COVID 19 viral gene targets	Test per kit	Time to results
Moderate Complexity and available to Hospital Laboratories							
BioFire	BioFire COVID-19 Test	1) Film Array 2.0 2) FilmArray Torch	Low	No	3	6-test kit or 30-test kit	0.83 h
GenMarkDx	ePlex SARSCoV-2 Test	GenMarkePlex	Low	No	Not disclosed	12 Tests	2 h
DiaSorin Molecular	Simplexa COVID-19 Direct	LIAISON MDx	Low	No	2	24 Tests	1.5 h
Roche Molecular	cobas SARSCoV-2	Options: 1) cobas 6800 2) cobas 8800	High	No	2	192 Tests	3.5 h
Qiagen	Respiratory SARS-CoV-2 Panel	QIAstat-Dx	Low	No	2	6 tests	1.10 h
BD	BioGX SARSCoV-2 Reagent	BD Max	Low to Moderate	No	2	24tests	3 h
Luminex	ARIES SARSCoV-2	Luminex ARIES M2 or M1	Low	No	2	24tests	2 h
Moderate Complexity available to Out patientClinics and Hospital Laboratories							
Mesa Biotech	Accula SARSCov-2 Test	**Options:** 1) Accula Dock 2) Silaris Dock	Low	No	1	25 Tests	0.5 h
Cepheid	Xpert Xpress SARS-COV-2 test	**Options:** 1) GeneXpertDx 2) GeneXpert Infinity 3) GeneXpert Xpress II (POC) 4) GeneXpert Xpress IV (POC)	Low to High	No	2	10 Tests	0.75 h
Abbott Diagnostics	ID NOW COVID-19	ID NOW	Low	No	1	24 Tests	0.25 h
High Complexity with Full Walk-away Automation							
Hologic	Panther Fusion SARS-COV-2	Panther System + Fusion Module	Moderate to High	No	2	96 Test	2.5 h
NeuMoDx	NeuMoDx SARS-CoV-2 Assay	Options: 1) NeuMoDx 96 2) NeuMoDx 288	Moderate to High	No	2	96 Test	1.5 h

a single tube test. One could process only 30 samples at a given time in a typical 96 well plate format. This increased the workload and time significantly, as well as possibility of error. On the other hand a kit developed by BlackBio Biotech (True-PCR) which has a single tube format required significantly less time (1.3hrs) to produce PCR results.

4.7. Limitations

The studies were variable in many aspects (blinding of participants and outcome assessment, technology selection, sensitivity/specificity of the assay, kit format, number of targeted genes, chemistry of assay, timing of assay etc.

5. Conclusion

Early detection of symptomatic as well as asymptomatic SARS-CoV-2 infections and reduction of transmission rates is critical to prevent and manage any outbreaks, and is pivotal for the prevention of COVID-19 pandemic. Real-time polymerase chain reaction (RT-PCR) is the gold standard among the early detection methods for COVID-19. In this systematic review we have attempted to collate the information about molecular based detection of COVID-19. Our success to tackle the pandemic effectively will depend on our ability to use the kits, machines and protocols effectively.

CRediT authorship contribution statement

Parijat Das: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Sudipto Mondal:** Methodology, Visualization. **Soumik Pal:** Methodology, Visualization. **Samadrita Roy:** Methodology, Visualization. **Anju Vidyadharan:** Visualization. **Rajneesh Dadwal:** Writing – original draft, Writing – review & editing, Visualization. **Sanjay Bhattacharya:** Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Deepak Kumar Mishra:** Visualization, Supervision, Project administration. **Mammen Chandy:** Visualization, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijmmb.2021.05.012.

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