NARRATIVE REVIEW

PCR to CRISPR: Role of Nucleic Acid Tests (NAT) in detection of COVID-19

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Abstract

COVID-19 infection has emerged as an unparalleled pandemic with morbidity and mortality tolls challenging diagnostic approaches and therapeutic interventions, and raising serious questions for healthcare policy-makers. From the diagnostic perspective, Reverse transcriptase polymerase chain reaction remains the gold standard. However, issues associated with gene primer variation in different countries, low analytical sensitivity, cross-reactivity with certain human coronaviruses have raised serious concerns within the scientific community. Alongside longer turnaround times, requirements of sophisticated equipment and trained technicians are the other challenges for conventional reverse transcriptase polymerase chain reaction testing. The recent biotechnological boom has now allowed newer nucleic acid testing options for diagnosing severe acute respiratory syndrome Coronovairus 2 (SARS-CoV2) with much better diagnostic efficiency, reduced turnaround times and possible benefit for use as a point-of-care test. Isothermal techniques with simple equipment requirements along with uniform temperature for analysis have emerged to be more sensitive and specific with turnaround times as low as 10-15 minutes. Similarly, Cluster Regularly Interspaced Short Palindromic Repeats have also been seen to play a very decisive role in COVID-19 diagnostics with much superior diagnostic efficiency and feasibility as a point-of-care test and its possible use for sequencing. The current narrative review was planned to consolidate data for all possible nucleic acid testing options under research/clinical use, and to provide a comparative assessment from the perspective of both the clinician and the laboratory.

Keywords: Nucleic acid test, NAT, COVID-19, SARS-CoV-2, Reverse transcriptase polymerase chain reaction, RT-PCR, CRISPR Cas technology, Specific high-sensitivity enzyme reporter unlocking technology, SHERLOCK, Isothermal amplification methods, Loop-mediated isothermal amplification, Lamp.

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Introduction

The world almost paralysed and reached a real-time "lock-down" by the newcomer Severe Acute Respiratory Syndrome-Corona Virus-2 (SARS-CoV-2), also called the coronavirus disease-2019 (COVID-19), as people started to talk about the "New Normal". At the time of writing, there have almost been over 15,945,330 people having already suffered from the virus, with more than 0.64 million deaths.[1] While the race for developing therapeutic agents and an efficacious vaccine is underway at an unprecedented pace, it will be some time before it becomes available in clinical settings. From the laboratory's perspective, we are witnessing multiple dimensions emerging to diagnose the disease, including clinical criteria, serological biomarkers, nucleic acid tests (NATs) and sequencing technologies. The current gold standard is NAT with Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) which allows the highest possible diagnostic sensitivity to detect SARS-CoV-2 in various specimen types and has been utilised for isolation strategies. RT-PCR, though considered the most dependable tool for COVID-19 diagnostics, does have issues like the need for sophisticated instrumentation as well as technical and human expertise, and is therefore limited only to large-scale laboratories. Furthermore, the diagnostic sensitivity of currently marketable RT-PCR for COVID-19 infection remains up to 83% with false negative (FN) rate slightly non-affordable during contact tracing with reasons attributable to both sampling technique and PCR methodology.[2] Moreover, in real-time clinical settings, an investigative marker with rapid turnaround time (TAT) is needed where the best of PCR methods in use struggle to provide early results within 3-5 hours.[3] From a technical perspective bias may be introduced due to international non-standardisation of primers where different PCR systems target E-gene, RdRp or N and Orf1ab genes and others.[4] Newer molecular techniques, like isothermal amplification methodologies, have also emerged to address some of the PCR shortcomings and to reduce TATs.[5] Further enhancement in NAT techniques is also being explored with Cluster Regularly Inter-Spaced Palindromic Repeats (CRISPR) technology, which is considered one of the revolutionising concepts in modern-day molecular sciences with slight modification to diagnose COVID-19 infection.[6] Without doubt, the bio-technology boom is rising to the occasion by developing

multiple versatile molecular techniques for the clinical setting to deal with issues like delayed and wrong diagnoses for COVID-19 infections. However, with technology there is also a real need to understand the underlying technology, limitations, cost-effectiveness, TATs, limit of detections (LOD), performance characteristics, like diagnostic sensitivity, clinical usage, and, most importantly, the real gene targets.

The current narrative review was planned to consolidate the rapidly emerging data on NAT with the elaboration of various gene targets used in PCR methods, and to compare and contrast various NAT techniques available to date for COVID-19 detection.

Review Methodology

Search was planned on PubMed, Cochrane Library, Google Scholar databases, and extended search included specific references within the articles found. Initial search words were selected with one-year filters ending June 15, 2020. "NAT and COVID-19" led to 5022 articles; 81(1.6%) on PubMed, 1(0.01%) on Cochrane Library, and 4940(98.3%) on Google Scholar. Search with "PCR and COVID-19" yielded 8778 results; 127(1.4%) on PubMed, 1(0.01%) on Cochrane Library, and 8650(98.5%) on Google Scholar. Search was extended by exploring specific references within the initial data. Studies which did not directly deal with NAT details or were perspective or containing only generalised information without details were excluded, and so were those only dealing with sequencing information/phylogenetic trees or considered non-relevant. Of the 13,800 articles initially searched, 758(5.5%) made the cut; 23(3.03%) on PubMed, 1(0.13%) on Cochrane Library and 734(96.83%) on Google Scholar. Overlapping studies and, wherever possible, free-access articles were preferred. Studies in any language other than English were excluded. The selected studies were downloaded and reviewed to see relevance to the current review's objectives.

PubMed, Cochrane Library and Google Scholar were searched separately for "CRISPR and COVID" and "Isothermal Amplification and COVID". Of the 1562 studies found, 7(0.44%) were on PubMed, 1(0.06%) on Cochrane Library and 1554(99.5%) on Google Scholar. Those found on Google Scholar were highly non-specific and multiple studies were excluded after abstract skimming. Wherever needed, attempt was made to reach the primary reference and cross-referencing was avoided.

Results

Initial focus of the reviewed data was to discuss the genetic structure, conserved and specific genes between human coronaviruses (hCoVs) across species and selection of gene targets with associated advantages and issues. Literature review suggested few of the regions to be well-conserved across species and there remained specific gene region differentiating SARS-CoV-2 from other hCoVs (Figure-1).

SARS-CoVs have multiple sub-genomic regions where literature identified the role of 4 regions specifically encoding structural proteins, and while the functions of other regions are yet to be fully characterised, phylogenetic studies have identified loss of Orf4 with gain of Orf3a and 8 to be related with SARS-CoV appearance over time.[7] The understanding of these genes is important to the NATs as they help in designing specific primers for detecting various virus genes. Earlier experiences from SARS-CoV and Middle East Respiratory Syndrome Corona Virus (MERS-CoV) allowed us a real-time insight into developing NAT methods for this recent COVID-19 pandemic. Previous gene amplification targets for various RT-PCR platforms developed for MERS-CoVs, as

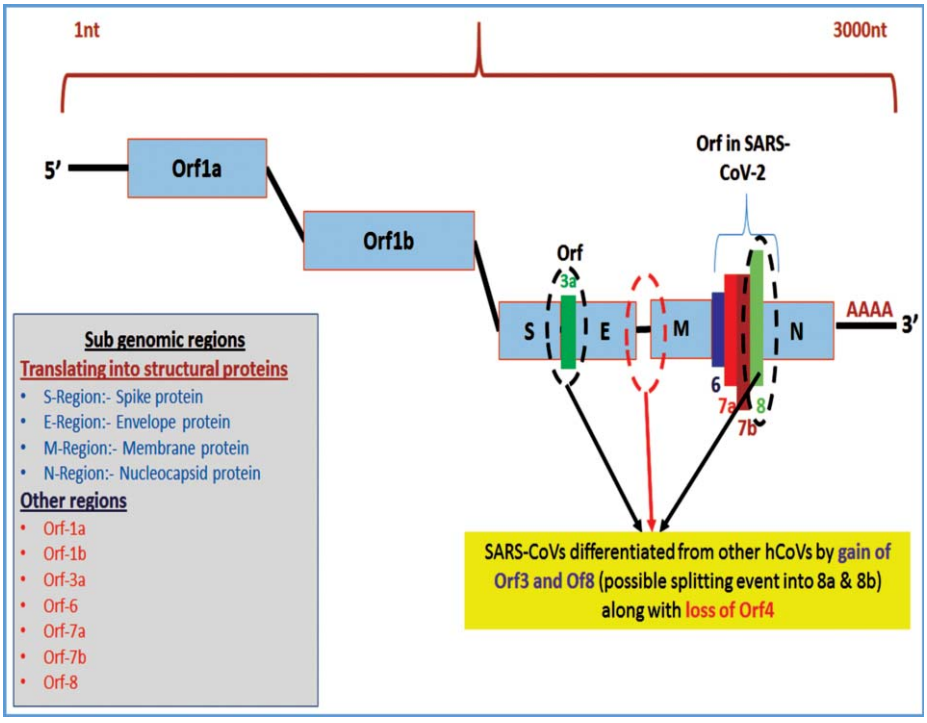
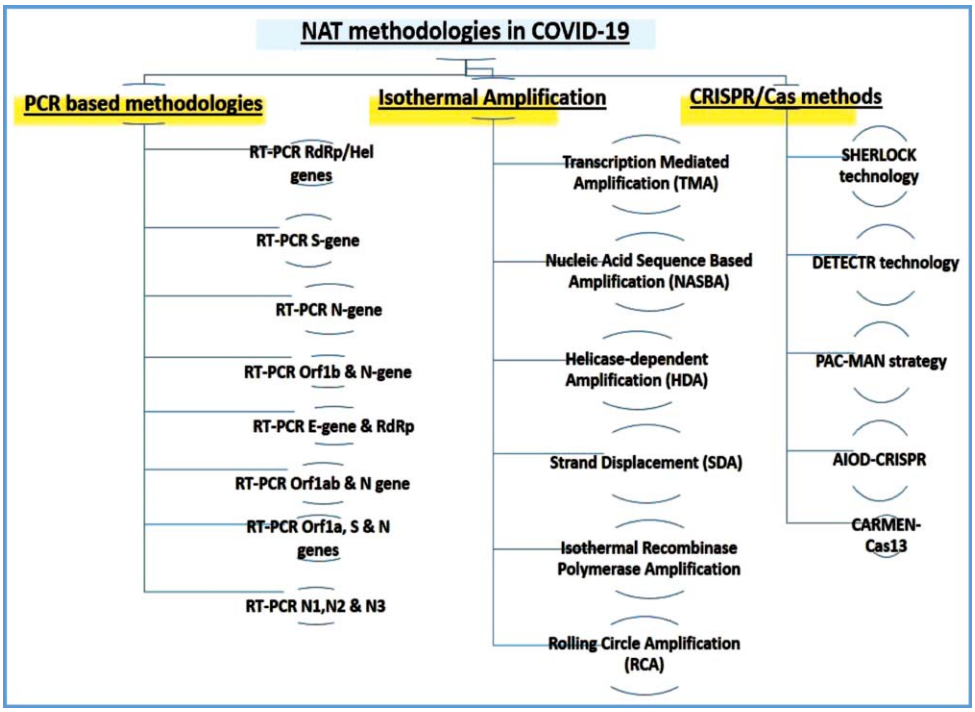


Figure-1: Schematic representation of major genes and overview of genetic structure of corona ribonucleic acid (RNA).



PCR: Polymerase chain reaction, CRISPR: Cluster regularly interspaced short palindromic repeats, DETECTR: Deoxyribonucleic acid Endonuclease Targeted CRIPSR Trans Reporter, SHERLOCK: Specific High-Sensitivity Enzyme Reporter Unlocking, PAC-MAN: Prophylactic Anti-viral CRISPR human, AIOD-CRISPR: All-in-one dual CRISPR, CARMEN: Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids.

Figure-2: Various nucleic acid testing (NAT) techniques used for detection of coronavirus disease-2019 (COVID-19) infection.

recommended by the World Health Organisation (WHO), include upE, Orf1b and Orf1a with variable analytical sensitivities.[8,9] The Food and Drug Administration (FDA), on the other hand, suggested N-gene along with Orf1a for detecting MERS-CoV infection by RT-PCR method.[10] As of now, the selection of molecular techniques, especially for RT-PCR methodology, varies between different set-ups and manufacturers requiring an unmet need for standardising methods and gene targets for PCR and other NATs (Figure-2).

The different methods include:

a. PCR Methods: While multiple methodological differences exist between PCR methods, the focus is around the gene targets in COVID-19-specific assays. Three crucial biotechnological aspects prevail while designing a PCR analysis, including primer designing (sequence of codons for forward and reverse primers), optimisation of analytical approach (Diagnostic LOD, performance, reporting) and validation with standardised protocols. PCR platforms, though time-tested, are affected by the differences in primers for amplification. Recently Wang et al. performed whole genome sequencing of various SARS-CoV-2 strains, suggesting Orf1a and Orf8 to have more mutations without clinical significance, implying that genomic information may be carefully used to avoid primer selection from regions with high mutation rates.[11] Generally, Orf1a and Orf1b genes appear to be conserved across most hCoV. Recent data identified gene targets in RT-PCR for SARS-CoV-2 to include ribonucleic acid (RNA) dependent RNA polymerase (RdRp), Helicase (Hel), Spike (S) gene, gene for nucleocapsid (N) protein and Envelope (E). Different set-ups and countries are relying upon different sets of platforms, protocols for nucleic acid extraction and analytical techniques, but major differences arise from the gene targets. Chan et al. compared various gene targets, including RdRp/Hel, RdRp-p2, S-gene and N-gene to find the RdRp/Hel to have the lowest cross-reactivity with hCoVs and showed better diagnostic efficiency for SARS-CoV-2 RNA detection.[12] Reviewing specific literature related with primer selection from targeted genes, the differences in primers and targeted genes are quite obvious. Chinese Center for Disease Control and Prevention (CDC) authorities suggest primer selection from Orf1ab and N-gene.[13] German scientists have suggested use of 2 primers from RdRp, E and N genes.[14] United States authorities selected all the primers and probes by selecting three regions within the N-gene i.e., N1, N2 and N3.[15] Similarly, other countries have defined their own primer and probe protocols which differ in many ways from referenced data.[16,17] Important in the selection of forward or reverse primer and probe is the idea of both conserved gene regions within all SARS viral genome (Pan CoV markers), including RdRp gene in Orf1ab region, nucleocapsid (N) gene and Envelope (E) gene. Also relevant is the assay optimisation indices, including diagnostic aspects where RdRp and E-genes have increased sensitivity in comparison to N-gene.[14] The conduct of analysis also varies from one-step methodology with all genes tested together as done by the US CDC protocol, followed by a 2-step design where a pan hCoV primer/probe panel is followed by a specific SARS-CoV-2 primer.[18] Primers using RdRp-P2, spike and nucleocapsid genes had lower diagnostic sensitivity and

specificity and were found to cross-react with other hCoVs.[12] Son et al. reported 100% specificity and 95% sensitivity by utilising RdRp with E-gene with restriction enzymes being Tsp45I, AlUl and EcoRI, to differentiate SARS-CoV-2 from the earlier SARS-CoV.[19] Park et al. identified false positive (FP) results in non-optimised primer sets and recommended through in vitro and in silico validation of a primer set from RdRp, Spike, Nucleocapsid and Envelope genes demonstrating better specificity.[20]

b. Isothermal Amplification: These are nucleic acid amplification techniques which work at constant temperatures and thus obviate the need of a thermal cycler. In contrast to needing a high melting temperature to separate deoxyribonucleic acid (DNA) strands, isothermal amplification methods utilise specialised DNA polymerases which separate DNA strands and synthesise alongside a complementary strand.[21] The time taken to amplify DNA is therefore less than PCR methods which employ alternate high-low temperature cycles. Examples of isothermal amplification methodologies tested in COVID-19 include:

• **Loop-Mediated Isothermal Amplification (LAMP):** Normally employed as Reverse Transcriptase-LAMP, it has shown an improved TAT with higher diagnostic sensitivity and specificity for detecting SARS-CoV-2 infection and similar outbreaks in the past.[21,22] The technique can be easily be multiplexed to detect multiple respiratory conditions at the same time.[23-25] This is one of the most utilised isothermal amplification techniques.

• **Isothermal Recombinase Polymerase Amplification (RPA):** This isothermal amplification variant demonstrated hands-on run-time of 7-20 min with a very high detection analytical sensitivity with 7.74 copies/reaction.[26] This technique uses a set of three enzymes which constitute a recombinase enzyme with a strand displacing polymerase enzyme and single-stranded DNA binding proteins. This is identified as one of the most rapid isothermal amplification methods, using a single tube and currently has been studied for diagnosis of SARS-CoV-2.[27,25] In the reverse-transcriptase RPA variant it does not need to create complementary DNA (cDNA) which further reduces TAT. Another advantage of this methodology is the operating temperature which is just close to room temperature and, thus, can easily be deployed for viral detection in field epidemiology.[26]

• **Nucleic Acid Sequence Based Amplification (NASBA):** This is a slightly different isothermal amplification technology which incorporates reverse transcriptase enzyme, an Avian Myeloblastosis Virus (AMV), a T7 RNA polymerase and a ribonuclease (RNase) H having 2 oligonucleotide primers. Apart from other advantages of isothermal amplification, NASBA suffers the least by various inhibitory substances and is suitable enough for use as a point-of-care test (POCT) option.[28] Chantratita et al. have demonstrated this technique to be more sensitive and speedy in comparison to RT-PCR methods in detecting SARS-CoV infection.[29]

• **Helicase-dependent Amplification Isothermal Amplification (HDA):** Another variant of isothermal amplification that utilises a heat-stable helicase enzyme to open the DNA strand, followed by primer annealing and polymerase-based elongation of DNA.[30] Choi et al described a paper-based POCT format utilizing HDA NAT technology for possible COVID-19 detection, which can reduce TAT to minutes.[31]

• **Transcription Mediated Amplification (TMA):** This isothermal amplification method slightly differs from RPA as it uses only two enzymes, including RNA polymerase and reverse transcriptase. However, it is less sensitive than RT-RPA and RT-Loop methods.[32] Currently, TMA technique developed by Hologic Aptima has shown higher analytical sensitivity in comparison with RT-PCR method for diagnosing SARS-CoV-2 infection with short TAT.[33]

• **Strand Displacement (SDA):** This technique is used both to detect DNA or RNA and utilises a slightly different mode of amplification by using primers termed "Bum" primers on the outside with two primers on the inside. It also requires a 5"-tail region containing a nicking enzyme. During the process the bumper primer attaches with the template strand upstream of amplification site, leading to further amplification of strand. Once amplification is complete, the newly-formed strand is displaced. This new strand is recognised by the nicking enzyme, an exonuclease which only creates a nick in the newly-formed strand, which is again recognised by the polymerase. The cycle of DNA strand nicking, amplification, displacement takes place in repetition to exponentially amplify DNA.[32,34] Though we could not find a reference utilising SDA for SARS-CoV-2 infection, the technique has been used in the detection of certain RNA viruses.[35] We believe this technique could also be used for detecting COVID-19 infection in future.

• **Rolling Circle Amplification (RCA):** This methodology has a very low LOD, implying detection of infective agents from a very small amount of nucleic acid. RCA method targets circular DNA or RNA molecules. The technique employs nicking the circular nucleic acid, allowing elongation of 3'-end by the non-nicked DNA (Leading Strand). This results in displacement of the 5'-end

(Lagging Strand). Later, both the leading and the lagging strands replicate to form DNA circularisation.[36-38]

Over time, various modifications are emerging on the scientific front mainly to address the clinical diagnostic feasibility, TATs and most significantly their utility as POCT.[39-41]

c. CRISPR/Cas methods- CRISPR/Cas technology is adopted from archeal and bacterial immune-based system where CRISPR acted to guide Cas nucleases for recognising and destroying the foreign antigens.[42] Various CRISPR/Cas platforms have been recommended for use in COVID-19 diagnosis:

i. SHERLOCK Technology: Specific High sensitivity Enzyme Reporter unLOCKing (SHERLOCK) technology is a CRISPR modification. The innovations added in SHERLOCK include the use of a prior recombinase-derived polymerase amplification of nucleic acid, followed by Cas-12 or Cas-13 mediated fluorescent detection. The usual TAT is upto 15-60 min.[43] Young et al. deployed this as POCT option for COVID-19 infection by using a slightly modified variation, obviating the need of numerous fluid handling steps. They termed this method "STOP", or SHERLOCK Testing in One Pot, which not only allowed use in field epidemiological set-ups, but provided TAT of up to one hour.[44]

ii. DETECTR Technology: This type-V CRISPR technology is unique in the sense that it combines the use of Cpf1 (Cas12a) nuclease with isothermal amplification. Cpf1 is programmed to create double-stranded DNA breaks which in combination with isothermal amplification provide a very specific and sensitive method for detecting various pathogens. This technique is termed DNA Endonuclease Targeted CRIPSR Trans Reporter (DETECTR).[45] Broughton et al. highlighted 100% negative predicative values, rapid TATs along with visual data interpretation for COVID-19 infection.[46,47]

iii. PAC-MAN Strategy: Prophylactic Anti-viral CRISPR (PAC) huMAN (PAC-MAN) employs Cas-13, which can screen specific CRISPR RNAs (crRNAs) meant for targeting conserved regions within viral genomes.[48] The technique is also currently being considered for its therapeutic potential.[49]

a. AIOD-CRISPR: The All In One Dual (AIOD) CRISPR utilizes Cas-12a in a single pot. The technique is a rapid and highly sensitive method for SARS-CoV-2 and similar infections which relies upon visual mode of detection. Its potential as POCT testing is also being explored.[50]

b. CARMEN-Cas-13: Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic Acids (CARMEN) isa CRISPR/Cas type-IV approach which combines CRISPR in nano-sised droplets containing the required genome detection payloads for amplification in small microarray wells with crRNA. Cas-13 is usually employed as a nuclease.[51] Recently the technique has been used by Ackerman et al to conclude that CARAMEN-Cas-13 is able to scale up testing in COVID-19, with possible multiplexing in POCT formats and cost-effectiveness.[52]

The CRISPR/Cas technologies represent a rapidly evolving field modifying the traditional landscape of laboratory investigation and therapeutics.

Table-1 and 2 provides a comparative analysis between three different technologies explained above for diagnosing COVID-19 infections.

Table-1: Comparative clinical application parameters among various nucleic acid testing (NAT) techniques.

Ser	Comparative index	PCR	Isothermal amplification techniques	CRISPR/Cas technology
1	Temperature variability	From 95°C, to 65°C to 72°C	From 60°C to 65°C[21,26,25]	No mandatory condition
2	Market availability	++++	++	+
3	Cost of equipment (max to min)	High	Low[32,33]	Higher[53]
4	Turnaround times (TAT)	> 60-90 min	15-45 min[21,31-35]	15-60 min [46,47,50,51]
5	Use as point-of-care test (POCT)	+	+++ [21,31,39-41]	+++ [50-52]
6	Data interpretation	+[18-20]	+++ [21,32,34-36]	++ [48,50]
7	Multiplexing	+++[54,55]	+++ [21,23-25]	++ [51,52]
8	Therapeutic potential	-	-	++++[48,49]
9	Future trend	+	+++	++++

++++ > Commonest
+++ > Commoner
++ > Common
+ > Less common
- > Not associated
PCR: Polymerase chain reaction, CRISPR: Cluster regularly interspaced short palindromic repeats.

Table-2: Diagnostic performance comparison among various NAT techniques.

Ser	Comparative index	PCR	Isothermal amplification techniques	CRISPR/Cas technology
1	Limit of detection (LOD)	+	+++ [21,28-36]	+++ [46,47,50,52]
2	Diagnostic sensitivity	+++ [21,13]	++++ [25-34,21]	+++ [46,47]
3	Diagnostic specificity	++ [21,13]	+++ [24-33,36-38]	++++ [46-52]
4	hCoVs Cross-reactivity	++++ [21,13]	+[21]	- [46,47]
5	Analytical sensitivity	Up to nano gram [21,12,13,19]	Up to femto gram [21,26,33]	Need evaluation [46]
6	Analytical specificity	+ [12,21,13]	+++ [21,29,35]	++++ [46,47]
	• Non-specific amplification	+++ [57]	+ [21,22,29]	Not documented
	• Primer-dimers	+++ [57]	+	Not documented
	• Cross-contamination	+++ [56,12,13,19]	+	+
7	DNA template preparation	Needs purification step	Not needed	Not needed
8	Loop primers	Not needed	Needed [29]	Not needed
9	Whole genome sequencing	-	+++ [59,60]	+++ [58]
10	Primer numbers	+	+++ [21,29]	-
11	Primer optimization need	+	+++ [21,25,29]	-

++++ > Highest
+++ > Higher
++ > High
+ > Possible
- > Not associated
PCR: Polymerase chain reaction, CRISPR: Cluster regularly interspaced short palindromic repeats, hCoVs: Human coronaviruses, DNA: Deoxyribonucleic acid.

Discussion

The challenges associated with the COVID-19 pandemic remains unparalleled with other such pandemics in recent times. The foremost ingredient for a management recipe remains accurate, precise and timely diagnosis for disease surveillance, appropriate isolation, disease management and control. Apart from the labour-intensive and time-consuming viral cultures, serological diagnostics suffer a great deal in terms of accuracy due to variability in the time course of appearance of antibodies, leading to FN results. NAT has somewhat overcome these shortcomings, being more accurate and precise, in a timely manner. From PCR to CRISPR, biotechnology has allowed very promising diagnostic modalities where each NAT technique has emerged as a sensitive and specific diagnostic modality with very low TATs.

The aforementioned review highlights PCR techniques to be robust, cost-effective, well-interpreted and able to provide a reasonably sensitive and specific result within a manageable timeframe.[12,19] However, the data also highlights the issues in terms of lack of standardisation which are related to platform selection and the targeted genes.[13,14,20] RdRp with helicase or P2 seems to be more precise targets for primer/probe selection with N-gene, and E-gene being suggested as equally comparable.[12,19] US authorities have suggested testing 3 different regions from the N-gene.[15] Provided the clinical market across the globe is almost taken over by RT-PCR methods, issues like detection of dead viral particles, inter-lab variations and limited diagnostic sensitivity have caused major concerns among laboratories and clinical set-ups.[61,20]

These loopholes have allowed newer NAT modalities to enter into the research and clinical arena. Isothermal techniques, due to their simplistic methodology and shortest possible TATs with higher analytical sensitivity and clinical specificity via use of multiple primers, have given a booster to field epidemiological testing.[25,29,35] While multiple varieties of isothermal amplification techniques have appeared in literature, LAMP, TMA and SDA have emerged as better established methods over time.[22,33,35] This technology has not only prevented cross-reactant hCoV-associated-positivity, but allowed for minimal equipment requirement providing cost-effective testing in resource-limited set-ups.[29,12] CRISPR modifications, especially using nucleases like Cas-12 and Cas-13, are now available for timely and more accurate clinical diagnostic testing. The CRISPR promise is greater in terms of both miniaturised technologies, such as SHERLOCK, CARMEN and AIOD-CRISPR, where lowest possible analytical LODs have become thinkable without any cross-reactivity or requiring multiple Loop primers in a very reasonable timeframe.[44,52,50] Apart from newly-emerged RT-PCR set-ups, both isothermal amplification techniques and CRISPR are mostly becoming available as a POCT option, thus taking the COVID-19 war arsenal to field settings.[50,44] CRISPR technology, like PAC-MAN, has also been employed for therapeutic purposes.[49]

The current narrative review has certain limitations.

Firstly, the diagnostic technology boom has actually followed the clinical needs in real time with modifications, joint-ventures and newer NAT concepts. The data relating to NAT and COVID-19 is fast growing with mushrooming of PCR primer targets and varying NAT modifications along with volumes of literature appearing by the hour. Therefore, this review may not encompass all possible aspects of the innovative research work on the subject. Secondly, standardisation between techniques in terms of analytical format, gene selection, isothermal amplification and CRISPR technique validations are much-needed areas to be taken care of by the validating authorities. Finally, most of the research work associating NAT with COVID-19 is of preliminary nature and bigger and better quality trials are needed to fill in some of the unanswered queries.

Despite the limitations, however, the current review of NAT data is expected to be valuable to those undertaking further research.

Conclusion

RT-PCR technologies, being the most marketable and currently well-understood by laboratory technologists, are hindered due to variable diagnostic and analytical sensitivity, equipment installation constraints, and cross-reactivity of some gene primers with other hCoVs. Isothermal techniques probably will become a POCT option in the near future, with better analytical and diagnostic efficiency and improved specificity. CRISPR market is emerging in the diagnostic domain and has multiple benefits in terms of aforementioned diagnostic parameters and also pave the way forward for sequencing and therapeutic options.

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