Molecular Evaluation of COVID-19 in Pandemic Era

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Authors' contributions

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ABSTRACT

COVID-19 (SARS-CoV-2) has recently emerged as an ongoing pandemic disease or probably syndemic and became the latest threat to global health. The main route of transmission of virus droplet start with respiratory problems coughing, sneezing, fever during infection period, which may end up with death, if not treated. Many emerging epidemiological factors have made COVID-19 as global alarming disease. To control the outbreak of COVID-19, a critical management along with quarantine measure is applicable, which requires early detection of infection. The early detection of COVID-19 infection is playing a vital role in controlling of progression of illness and limiting viral spread within the population. Therefore, reliable, highly specific and sensitive diagnosis techniques

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are necessary for its early detection. Among several detection techniques, qRT-PCR is the rapidly employed, sensitive and widely accepted technique. In present review, different molecular techniques with emphasis on qRT-PCR for early diagnosis of emerging COVID-19 will be discussed.

Keywords: COVID-19; SARS-CoV-2; qRT-PCR.

1. INTRODUCTION

COVID-19 (SARS-CoV-2) has recently emerged as an enduring pandemic disease or probably syndemic and became the latest threat to global health. Not surprisingly, COVID-19 pandemic also continuously destroying socioeconomic condition of world as well as creating pathetic condition. The journey of COVID-19 started from china and by this time it travel to most of part of world. This was originated from animal belonging to order Nidovirales, family Coronaviridae, subfamily Coronavirinae, genus Betacoronavirus, subgenus Sarbecovirus, and found to be similar with MERS and SARS coronaviruses [1]. The COVID-19 structure comprised with pleomorphic or spherical envelope shape containing single stranded RNA (+ssRNA) having 30 kb genome, which encodes 9,860 amino acids [2,3,4,5]. The COVID-19 structural components are composed of spike (S), membrane (M), envelope (E), and nucleocapsid (N) and organized as 5'-replicase (ORF1a/b)-spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)-poly(A)-3' (Fig. 1) [2]. The COVID-19 is a communicable disease so the ideal response to check spread of disease start with early detection.

COVID-19 predominately using ACE2 (angiotensin converting enzyme 2) as entry receptor, it presents on host susceptible cells, ACE2 can bind substrate binding domain that found in viral spike which facilitate its attachment to the host cellular system as well as cause infection followed by its multiplication inside host system [1]. Recent studies and epidemiological data attribute COVID-19 race may be mutated strain of bat coronavirus. However, its intermediate host or reservoir is still unknown [4]. Similar to SARS and MERS, COVID-19 transmits through respiratory droplets produced by an infected individual during sneezing or cough (WHO 2020). The COVID-19 spreads primarily among people during close contact, most often through small droplets formed by coughing; sneezing, speaking [6]. It is not yet known that virus can spread to the fetus via placenta during pregnancy or breast feeding, and is also inadequate knowledge on coronavirus transmission during baby delivery [7,8]. Genetic material of coronavirus have been isolated from different specimen nasopharyngeal swabs (NPS), sputum, urine, blood, rectal swab and stool from infected person showing symptoms of COVID -19 [2]. COVID-19 popular symptoms include fever, cough, tiredness, shortness of breath, and loss of smell and taste. The time required for occurrence of symptoms varies from person to person, which usually takes 5 to 14 days from its exposure. However it varies from patient to patient [9]. Reports indicates that number of asymptomatic patients are also increasing surprisingly, which can equally spread the infection without any notice [3]. Therefore, a large scale testing of humans and isolating them is the only solution to check the spread of COVID-19. There are several methods adopted for detection of deadly COVID-19, like quantitative Real-time reverse transcription polymerase chain reaction (qRT PCR), loop-mediated isothermal amplification, multiplex isothermal amplification followed by microarray detection, and CRISPR (clustered regularly interspaced short palindromic repeats)-based assay, Digital PCR (dPCR). Lateral flow immunoassay is rapid detection of antigen of COVID-19 or antibody (IgG or IgM) against COVID-19 [10]. Lateral flow immunoassay has advantage of fast result with low cost but suffer from poor sensitivity at earlier infection (5-6 days) [10]. Several rapid antigen assays has been developed and monoclonal antibodies against COVID-19 are under developed [5]. LAMP-technology is more or less similar to qRT-PCR in terms of sample collection for amplification of viral genetic material. But major disadvantage of this technology lies in limited studies [10]. Among them, qRT-PCR technique is highly specific and sensitive and gives results quickly i.e. within 2-3 hr [2]. Some qRT-PCR test kits are also available in "all in one kit" to reduce time, potential, laboratory handling and contamination. The major advantage associated with qRT PCR lies in its simultaneous amplification and analysis of samples to reduce false-positive result [2].
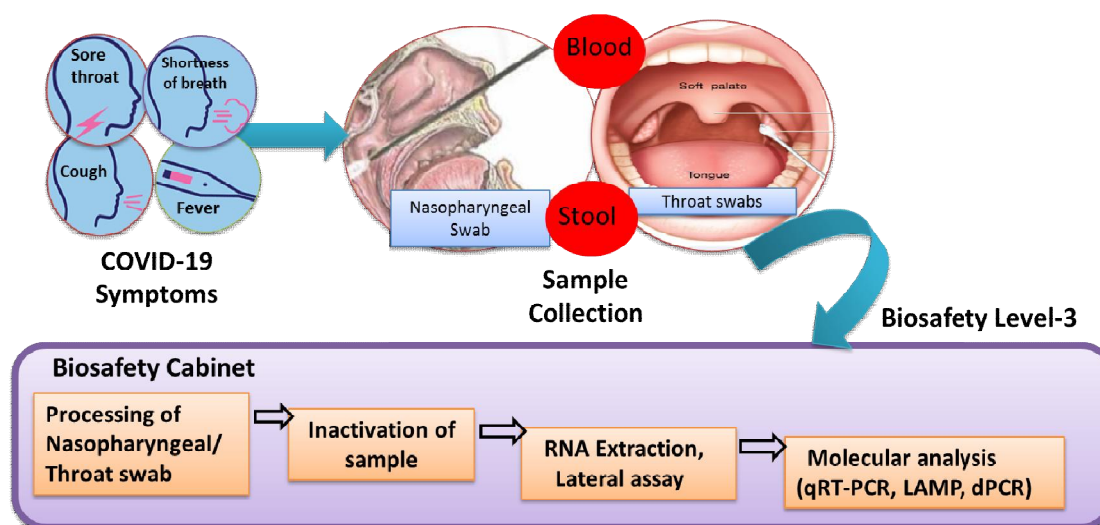
Fig. 1. Overview of COVID-19 working process
COVID-19 infected individual symptoms include sore throat, shortness of breath, fever, sneezing or cough.
Infected patient sample collected from nasopharyngeal aspirate/swabs, throat swabs, sputum, blood, and stool.
Remaining molecular analysis of sample has performed in Biosafety cabinet

2. SAMPLE COLLECTIONS

For COVID-19 detection, samples like nasopharyngeal aspirate/swabs, throat swabs, and/or sputum specimens are frequently used [2] while in early stage of infection nasopharyngeal aspirate/swabs and oropharyngeal (OP) swab are commonly recommended for screening [11] (Fig. 1). However, stool and blood samples can also be used [12] in severe COVID 19 infection. Recent studies showed that COVID-19 viral load was found to be more in throat compare to stool followed by blood [4]. Since viral load play a significant role in detection method, so nasopharyngeal aspirate/swabs has been routinely used for RNA extraction followed by qRT-PCR analysis. In early days of infection (within 5-6 days), infected patient demonstrate high COVID -19 load in upper and lower respiratory tract [13]. OP swabs were often tested than nasal swabs during outbreak in China [6]. However, RNA content in nasal swab was found to be significantly higher (63%) than OP swabs [14]. Nasopharyngeal aspirate/swabs have been properly collected by the inserting swab deeply as describe by WHO guideline 2019. For collection of nasopharyngeal sample a fresh long sterile swab is gently inserted into nostril parallel (1-2) to the palate until the discharge is met at turbinate and it should be kept for 10 sec, while time being target swirled 5 times (clockwise and anticlockwise) [15]. The collected samples must be immediately kept in viral transport medium under refrigerator condition for safe conveyance to COVID-19 detection center [15]. Recently, it was observed that samples of bronchoalveolar lavage (BAL) fluid yielded higher viral load in later stage of infection [16].

When handling with infectious virus we must use standard biosafety level 2 while some of laboratories argue with biosafety level-3 with enhance personal protective equipment for handling corona virus samples prior to viral inactivation [2,17].

3. PRIMERS AND PROBES

Specific primers were designed for COVID-19 diagnosis from ORF1a/b, ORF1bnsp14, RdRp, S, E, or N gene, nsp2 protein [18,19,20] (Table 1) (Fig. 2). For COVID-19 detection, WHO recommends first–line screening with an E gene followed by RdRp [21]. However, two nucleocapsid proteins target (N1 and N2) have been recommended in USA [22]. RdRp/Hel, S, and N genes of COVID-19 performance compared and among them RdRp/Hel assay gives efficient result with higher sensitivity and specificity at lower limit of detection [2]. Few studies recommended N gene based detection as it showed higher sensitivity [4]. Probe free COVID-19 testing was also developed using to nsp2 primer [19].
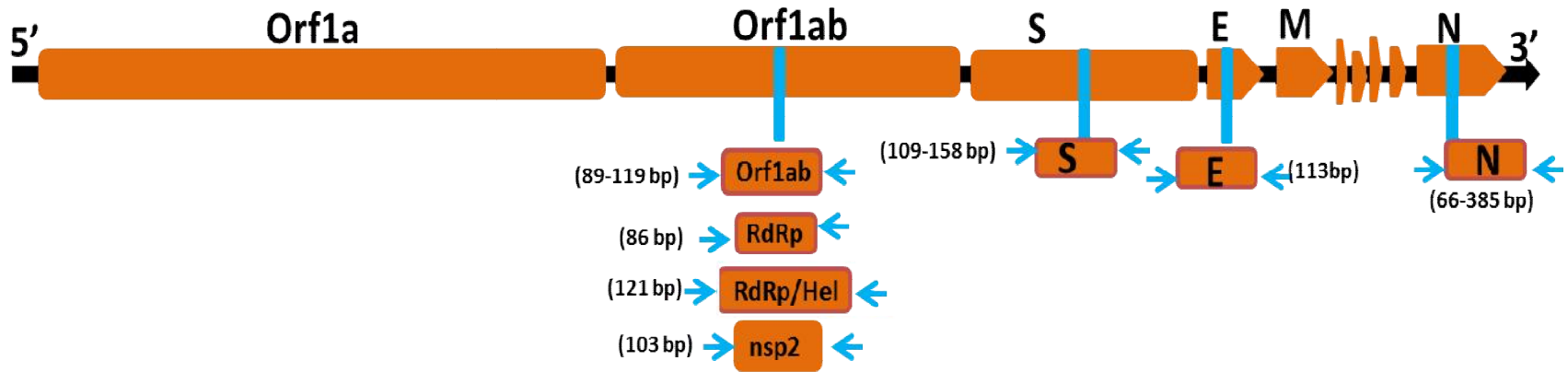
Fig. 2. COVID-19 structure

qRT PCR detect viral RNA (converted to cDNA) present in collected from infected patient sample. It detects by capturing and amplifying the viral genetic material (ssRNA) mainly envelope (E), spike (S), nucleocapsid (N), ORF1a/b, ORF1bnsp14, RdRp, nsp2 protein of COVID-19 and approximate product size range of each part mention in figure

Table 1. List of COVID-19 primer used in COVID 19 RT PCR study

Assay	Primer name	Primer sequence	Amplicon size (bp)	Primer size(bp)	References
COVID-19-nsp2	Forward	5'-ATGCATTTGCATCAGAGGCT-3'	103	20	[19]
	Reverse	5'-TTGTTATAGCGGCCTTCTGT-3'		20	
E gene (Sarbeco)	Forward	5´- ACAGGTACGTTAATAGTTAATAGCGT -3´	113	26	[22]
	Reverse	5´- ATATTGCAGCAGTACGCACACA -3´		22	
RdRP gene (SARSr)	Forward	5´- GTGARATGGTCATGTGTGGCGG -3´	86	22	[22]
	Reverse	5´- CARATGTTAAASACACTATTAGCATA-3´		26	
N gene (Sarbeco)	Forward	5´- CACATTGGCACCCGCAATC-3´	385	19	[22]
	Reverse	5´- GAGGAACGAGAAGAGGCTTG -3´		20	
S gene (RBD)	Forward	5'- CAATGGTTTAACAGGCACAGG -3'	121	21	[18]
	Reverse	5'- CTCAAGTGTCTGTGGATCACG -3'		21	
N gene	Forward	5'-CTCAGTCCAAGATGGTATTTCT-3'	66	22	[29]
	Reverse probe	5'-AGCACCATAGGGAAGTCC-3' 5'-FAM-ACCTAGGAACTGGCCCAGAAGCT-BHQ1-3'		18 23	
ORF1ab	Forward	5'-TCATTGTTAATGCCTATATTAACC-3'	89	24	[29]
	Reverse probe	5'-CACTTAATGTAAGGCTTTGTTAAG-3' 5'-FAM-AACTGCAGAGTCACATGTTGACA-BHQ1-3'		24 23	
Orf1ab (RPA)	Forward	5'-GAAATTAATACGACTCACTATAGGG CGAAGTTGTAGGAGACATTATACTTAAACC-3'	112	30	[28]
	Reverse	5'TAGTAAGACTAGAATTGTCTACATAAGCAGC-3'		30	
S gene (RPA)	Forward	5'-GAAATTAATACGACTCACTATAGGG AGGTTTCAAACTTTACTTGCTT TACATAGA-3'	109	30	[28]
	Reverse	5'TCCTAGGTTGAAGATAACCCACATAATAAG-3'		30	
ORF1 ab	Forward	5'-AGAAGATTGGTTAGATGATGATAGT-3'	118	25	[24]
	Reverse probe	5'-TTCCATCTCTAATTGAGGTTGAACC-3' 5'-FAM-TCCTCACTGCCGTCTTGTTGACCA- BHQ1-3'		25 24	
Covid-19 RdRp/Hel	Forward	5'-CGCATACAGTCTTRCAGGCT-3'	121	20bp	[2]
	Reverse Probe	5'-GTGTGATGTTGAWATGACATGGTC-3' FAM-TTAAGATGTGGTGCTTGCATACGTAGAC -IABkFQ		24bp 28	
COVID -19 - S	Forward	5'-CCTACTAAATTAAATGATCTCTGCTTTACT-3'	158	30	[2]
	Reverse	5'-CAAGCTATAACGCAGCCTGTA-3'		21	

Assay	Primer name	Primer sequence	Amplicon size (bp)	Primer size(bp)	References
	Probe	HEX-CGCTCCAGGGCAAACTGGAAAG -IABkFQ		22	
COVID -19 - N	Forward	5'-GCGTTCTTCGGAATGTCG-3'	97	18	[2]
	Reverse	5'-TTGGATCTTTGTCATCCAATTTG-3'		23	
	Probe	FAM -AACGTGGTTGACCTACACAGST -IABkFQ		22	
ORF1ab	Forward	5'-CCCTGTGGGTTTTACACTTAA-3'	119	21	[4]
	Reverse	5'-ACGATTGTGCATCAGCTGA-3		19	
	Probe	5'-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'		26	
N	Forward	5'-GGGGAACTTCTCCTGCTAGAAT-3'	99	22	[4]
	Reverse	5'-CAGACATTTTGCTCTCAAGCTG-3'		22	
	Probe	5'-HEX-TTGCTGCTGCTTGACAGATT-TAMRA-3'		20	
Internal references gene (RPP30),	Forward	5'-AGTGCATGCTTATCTCTGACA G-3'		21	[4]
	Reverse	5'-GCAGGGCTATAGACAAGTTCA-3'		21	
	probe	5'-Cy5-TTTCCTGTGAAGGCGATTGACCGA-BHQ-3'.		24	
RPP30	Forward	5'-GATTTGGACCTGCGAGCG-3'		18	[23]
	Reverse	5'-GCGGCTGTCTCCACAAGT-3'		18	
	Probe	VIC-CTGACCTGA-ZEN-AGGCTCT- 3IABkFQ		16	
RPP30 Shear	Forward	CCAATTTGCTGCTCCTTGGG		20	[23]
	Reverse	CATGCAAAGGAGGAAGCCG		19	
	Probe	FAM- AAGGAGCAA-ZENGGTTCTATTGTAG- 3IABkFQ		21	

4. RNA EXTRACTION

Good quality RNA extraction is prerequisite for various techniques employed in COVID-19 detection. Numerous commercially available kits were utilized in RNA extraction from collected samples [17]. Currently the COVID-19 testing commercial kits divided into two major group; the first one include detection of COVID-19 viral genome particle using RNA dependent DNA polymerase enzyme by qRT-PCR or hybridization based strategies in acute phase of infection. The second group includes lateral assay and ELISA assay are largely dependent on detection of antigen protein or antibody production from COVID-19 infected person. Lateral assay consequently identifies the person who recovering from an illness or medical treatment or developing antibodies to the contagious virus. This assay provides future ability to monitor immune assay of infected person [23].

5. QUANTITATIVE-REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (qRT-PCR)

qRT-PCR detection technique is based on amplification of viral genetic material and it considered to be standard method for detection of COVID-19 virus. Recently qRT-PCR test for COVID-19 virus isolation of sample from various parts that have been describe in above paragraph. qRT-PCR based detection were illustrated in Fig. 3, process start conversion of RNA genomic material into cDNA by RNA-dependent DNA polymerase (reverse transcriptase). This reaction includes small target specific forward and reverse primers (Orf1ab/RdRP/Hel/E/N/S/nsp2) designed to recognize complimentary sequence of RNA viral genome and the viral RNA first converted to cDNA in reverse transcription reaction. The amplification of DNA is monitor by fluorescent dye or a target specific fluorescent labeled probe (FAM/HEX/CY5/ROX). The automated setup of qRT-PCR machine amplify cDNA for approximately 40 cycle until the viral cDNA could be detected by fluorescent signal during extension phase [24]. The fluorescent intensity results collected at qRT PCR machine further data was analysed using various softwares. For qRT-PCR based COVID-19 detection, a large numbers of protocol are available online (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-

guidance). Most of available commercial kits recommends one step processing of RNA (http://www.labgenomics.co.kr). The one step method is preferred, as it is fast to set with limited sample handling, less pipetting error and reduces bench time. The component of qRT PCR include one-step real-time RT-PCR buffer, one step enzyme DNA polymerase and reverse transcriptase, Primers and probe mix (Orf1ab/RdRP/Hel/E/N/S/nsp2 and FAM/HEX/CY5/ROX labeled probe) and template RNA (Fig. 2) [2,22,25-29]. MS2 bacteriophage acts as an internal control, whereas cloned plasmid DNAs of RdRp and E gene acts as a positive control (http://www.labgenomics.co.kr). However, in some study human GAPDH gene was used as an internal control [4]. Different fluorescent dyes have been used in qRT-PCR analysis. Among different fluorescent dye FAM/HEX/CY5/ROX the FAM [2,22,25-29] and HEX [2] were most widely used for COVID-19 detection. Lu et al. [25] used multi-channel fluorescence dPCR system (FAM/HEX/CY5/ROX) to detect multiple target genes in a single multiplex system. Multi-channel fluorescent technique provide more efficient quantitative viral load in specimens, in respect to single fluorescent dye for monitoring COVID -19 [27]. Thermal cycling condition used for RdRp/Hel, Spike (S), Nucleocapsid (N) primers includes synthesis of cDNA by reverse transcription followed by PCR [2]. In COVID-19-nsp2 assay, positive sample shows unique peak with melting temp of around 80°C and reproducibility in terms of Ct was more satisfactory comparision to previously developed COVID-19-RdRp/Hel assay [19,2]. Previous study noticed that RdRp-P2 assay cross-reacted with SARSCoV *in vitro* but COVID-19-RdRp/Hel assay was highly selective and showed no cross-reactivity with other typical respiratory pathogens *in vitro* and in nasopharyngeal aspirates [22]. Primers based on genes like RdRP, E and N was used in cDNA synthesis followed by PCR analysis [22]. The determination of Ct (cycle threshold cycle) value is critical for analyzing results of qRT-PCR.

For positive control, the Ct value should be ≤40 and not detectable for negative control (Ref). The Ct value of internal control (MS2) gene should be at least 40 (Table 2) (http://www.labgenomics.co.kr). The interpretation of the result of patient specimens is calculated by reading the Ct value of each sample and determining if the value is below or above 40 (Table 3)

(http://www.labgenomics.co.kr). The Ct-value ≤35 was considered as positive for COVID-19, whereas Ct value >38 was described as negative test result [30].

The earliest COVID-19 RT-PCR diagnostic tests to come in scene included (1) LabCorp- COVID-19 RT-PCR diagnostic test [31] (2) 2019-nCoV Real-Time RT-PCR Diagnostic Panel (U.D. Centers for Disease Control and Prevention) [32] (3) TaqPath COVID-19 Multiplex Diagnostic Solution (Thermo Fisher-Applied Biosystems) [33] (4) Allplex 2019-nCoV Assay (Seegene) [34] (5) cobas SARS-CoV-2 (Roche) [35]. Additional information about test is mention in Table 4.

One of the improved qRT-PCR based detection using ePlex SARS-CoV-2 test was developed by GenMark Diagnostics, Inc. This was based on "The True Sample-to-Answer Solution" to detect nasopharyngeal swabs [36]. Each cartridge contains reagents for magnetic solid-phase viral RNA extraction, cDNA amplification and detection method includes eSensor technology and electrowetting. The target DNA mixed with ferrocene-labeled signal probe solution that complimentary to specific target site. If target DNA is present then it immediate hybridize with signal probe. The presence or absence of target DNA is determined voltammetry which generate specific electrical signal from ferrocene-labeled signal probe.

Table 2. Summary for the interpretation for control results

Control Type/Name	Used to monitor	RdRp gene (FAM)	E gene (Cy5)	MS2 phage (VIC/HEX)	Expected Ct values
Positive Control (PC)	Substantial reagent failure including primer and probe integrity	+	+	−	≤40
Negative Control (NC)	Reagent and/or environmental contamination	−	−	−	Not detected
MS2 RNA Internal Control (IC)	Failure in lysis and extraction procedure	−	−	+	≤40

Table 3. Summary of the interpretation for patient specimen results

| RdRp gene (FAM)	E gene (Cy5)	MS2 (VIC/HEX)	Result Interpretation	Action
+	+	±	SARS-CoV-2 detected	Report results to healthcare provider and appropriate public health authorities.
+	−	±		
−	+	±	Inconclusive result	Repeat testing using residual nucleic acid first. If the repeated test result is inconclusive, re-extract nucleic acid from the remaining sample and repeat qRT PCR. If the repeated result remains inconclusive, additional confirmatory testing should be conducted if clinically indicated.
−	−	+	SARS-CoV-2 not detected	Report results to healthcare provider.
−	−	−	Invalid result	Repeat extraction and qRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.
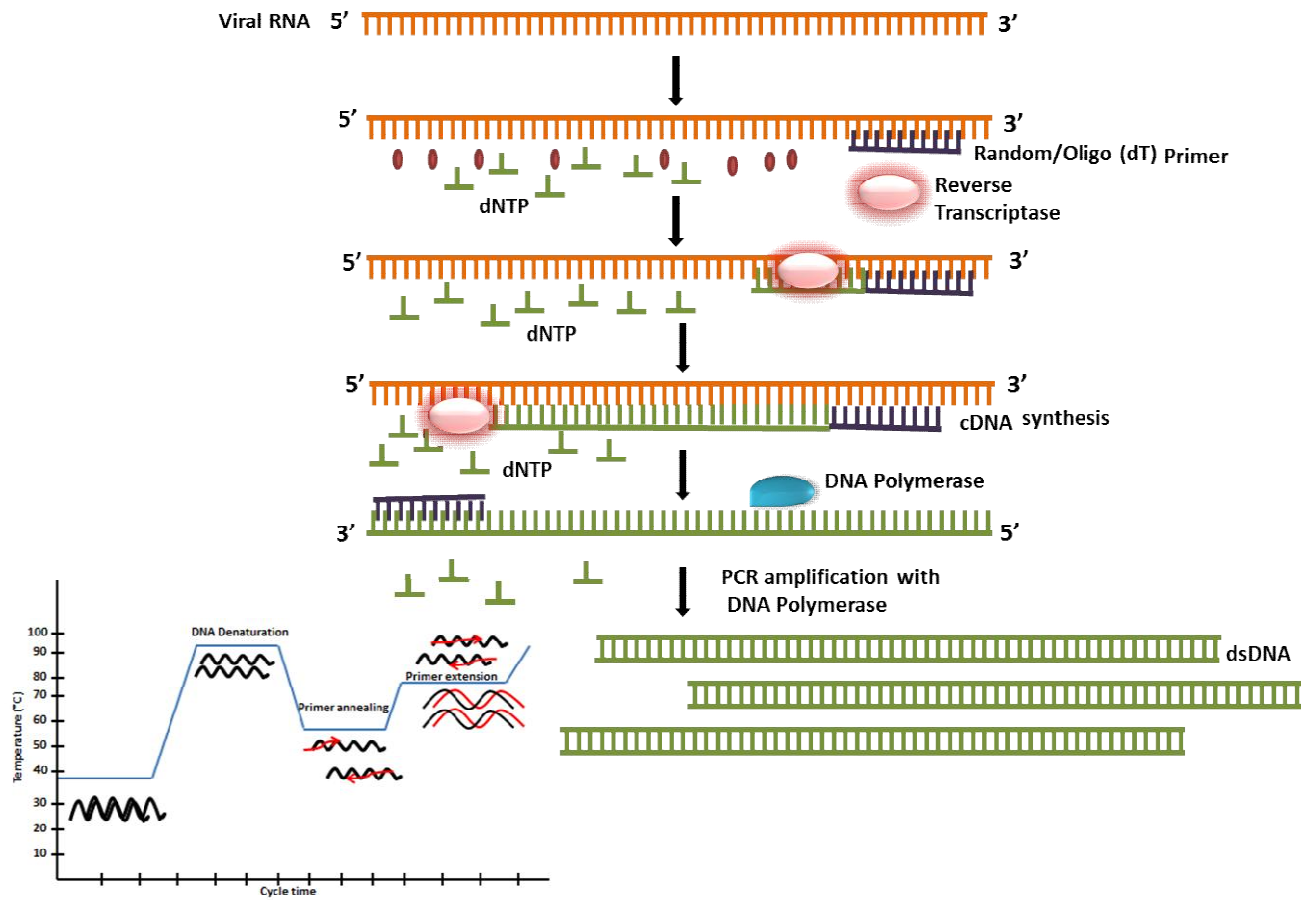
Fig. 3. Quantitative Reverse transcription-polymerase chain reaction (RT-PCR)
The RT-PCR amplify cDNA copy of a specific segment of genome of COVID-19

Table 4. List of molecular diagnostic test used in COVID-19 detection

Name of Test	Sample collection	Amplified region	Result time/additional information	Throughput information	Assay Name	Manufacturer/ organization name	EUA
Real-time RT-PCR	NS, OS, BLF, TS, Sputum	N gene	human RNase P gene used as control	264 samples/day	CDC 2019- Novel Coronavirus Real-Time RT-PCR Diagnostic Panel	CDC-US	US FDA
	NS, MS	N2 and E genes	46 min	High throughput[a]	Xpert Xpress SARS-CoV-2 test	Cepheid	US FDA
	NS, OS	viral RNA	results in ~3.5 h, instruments can process up to 384 results (cobas 6800 System) and 1056 results (cobas 8800 System) in 8 h	High throughput[a]	cobas SARS-CoV-2	Roche Molecular Systems, Inc.	Australia, CE mark, US FDA
	NS, OS, Serum, Blood , Stool	ORF1ab and N gene	30 min		Novel Coronavirus (2019-nCoV) Nucleic Acid diagnostic kit (PCR-fluorescence probing)	Sansure Biotech Inc.	China NMPA
	NS, OS	pp1ab	<75 min		LYRA SARS-CoV-2 assay	Diagnostic Hybrids, Inc. Quidel Corporation	US FDA
	NS, OS, MS, BLF	N1 and RdRP	results in <1 h without manual RNA extraction		SARS-CoV-2 assay	Diagnostic Molecular Laboratory −Northwestern Medicine	US FDA
	OS	E and RdRP genes	results within 90 min		STANDARD M nCoV RTdetection kit	SD BIOSENSOR	Korea MFDS
	NS	RNA	<2 min hands-on time and results in ~2 h		Simplexa COVID-19 Direct	DiaSorin Molecular LLC	US FDA
	NS, OS	ORF1ab regions 1 and 2	each Panther Fusion system can provide results in <3 h and process up to 1150 coronavirus tests in 24-h	1 sample/run	Panther Fusion SARS-CoV-2 assay (Panther Fusion System)	Hologic Inc.	Australia, US FDA

Name of Test	Sample collection	Amplified region	Result time/additional information	Throughput information	Assay Name	Manufacturer/ organization name	EUA
			period				
	NS, OS, MS, BLF sputum,	N gene	results in 2−4 days		COVID-19 RT-PCR test	LabCorp Laboratory Corporation of America	US FDA
	NS	ORF1ab and N gene	minimal hands-on time and an automated workflow delivers results in ~2 h	high throughput[a]	ARIES SARS-CoV-2 assay	Luminex Corporation	US FDA
	NS	viral RNA	results in 90 min, produces 100,000 test kits/wk	96 samples in 1 run	MiRXES FORTITUDE KIT 2.0	MiRXES Pte Ltd.	Singapore HSA
	NS, OS, MS, BLF	N gene	results the same day, 12−18 h from receipt of specimen		Viracor SARS-CoV-2 assay	Viracor Eurofins Clinical Diagnostics	US FDA
multiplex realtime RT-PCR	NS	ORF1ab and ORF8	results in ~45 min	94 samples/run	BioFire COVID-19 test	BioFire Defense, LLC	US FDA
	NS	E and RdRP genes	results in ~1 h, by differentiating novel coronavirus from 21 other bacterial and viral respiratory pathogens	1 sample/run	QIAstat-Dx Respiratory SARS-CoV-2 panel	Qiagen GmbH	US FDA
	NS, MS, anterior nasal, and saliva specimens	ORF1b and N and S genes			TaqPath COVID-19 combo kit	Rutgers Clinical Genomics Laboratory ThermoFisher-Applied Biosystems	US FDA
	NS, MS, OS, sputum	E, N, and RdRP genes	results in <2 h after extraction		Allplex 2019-nCoV assay	Seegene	CE mark, Korea MFDS, US FDA
PCR and lateral flow technologies	TS, NS	N gene	results in 30 min, the palm-sized device can be used in physician office or patients' home	144 tests/day	Accula SARS-CoV-2 test	Mesa Biotech Inc.	US FDA
isothermal nucleic	NS, OS	RdRP gene	positive results <5 min and	1 sample/run	ID NOW COVID-19	Abbott Diagnostics	US FDA

Name of Test	Sample collection	Amplified region	Result time/additional information	Throughput information	Assay Name	Manufacturer/ organization name	EUA
acid amplification technology			negative results in 13 min			Scarborough, Inc.	
real-time RT isothermal amplification test	NS, OS	ORF1ab and/ or N gene	results <1.5 h	high throughput[a]	iAMP COVID-19 detection kit	Atila BioSystems, Inc. D11	US FDA
CRISPR-based lateral flow assay isothermal amplification	respiratory samples	viral RNA	combines Sherlock's Cas12 and Cas13 enzymes for nucleic acid detection with Cepheid's GeneXpert test-processing instruments		CRISPR-based tests for SARS-CoV-2	Cepheid Sherlock Biosciences	US FDA
	respiratory samples	E and N genes	CRISPR Cas12a-based lateral flow assay results in 30−40 min	high throughput[a]	SARS-CoV-2 DETECTR	Mammoth Biosciences	US FDA

NS: Nasopharyngeal swabs; OS: Oropharyngeal swabs; TS: Throat swab; BLF: Bronchoalveolar lavage Fluid; MS: Midturbinate swabs; EUA: Emergency Use Authorization by U.S. FDA or different drug regulatory authorities; [a]Information from FDA https://www.fda.gov/media/136702/download

Even though qRT PCR is the most widely accepted technology for detection of COVID-19 but it has disadvantage of needed highly skilled personnel along with expensive instruments and can take more than one day to generate accurate results. Due to this reason, a number of research laboratories and companies is working to develop further more efficient and transient method for qRT-PCR technology and meanwhile working on various new technologies.

6. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

LAMP technology is a similar to RT-PCR, but uses single temp 60-65°C instead of series of temp. LAMP technology requires set of 4-6 specially designed primer to amplify nucleic acid in short time [37,38]. LAMP can be combined with reverse transcription (*i.e.* RT-LAMP) which combines reverse transcription and amplification allow for detection of RNA [39]. This technology and reagent can be detected due to formation of cloudy compound magnesium pyrophosphate' and can be seen by naked eye. The amplified product can be either visualize by fluorescence intercalating dyes or by measuring turbidity by PH indicator. Since RT-LAMP is quick technique in terms of time, sensitivity, simplicity makes it promising technology for COVID-19 detection [39]. Yu et al. developed isothermal LAMP based method-iLACO (*i*sothermal LAMP based method for COVID-19) (WarmStart master mix kit), which rapidly detect COVID-19 positive reactions results in color change of phenol red pH indicator from pink to yellow due to presence of extensive DNA polymerase activity meanwhile negative reaction remain pink [40]. Zhang et al. 2020 uses RT-LAMP technology for efficient conversion of RNA to cDNA using reverse transcription (WarmStart RTx) followed by amplification by DNA-dependent DNA polymerase (Bst 2.0 WarmStart) for quick calorimetric detection of amplified product with DNA binding dye (SYTO-9, Thermo Fisher S34854) [41].

7. CRISPR-BASED ASSAYS

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is RNA guided genome editing system found in bacteria recognizes nucleic acid sequence. These specific recognized sequences can be cut by family of enzyme Cas9, Cas12, Cas13 which produced by bacteria [42]. Cas13 and Cas12 family of enzyme can be used for CRISPR bases operation of COVID-19 to cut viral RNA sequence. The advance diagnosis of COVID-19 have been developed using the CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) and DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) technique. SHERLOCK technique were chosen S gene and Orf1ab gene target of COVID-19 genome. Recombinase polymerase amplification (RPA) and LwaCas13a CRISPR guide RNAs were used for specific detection. RPA converts viral RNA to dsDNA. The Cas13−tracrRNA complex binds to target sequence to activate Cas13 enzymatic activity and by the end it cleaves the target sequence and fluorescent RNA reporter [43]. DETECTR assay perform reverse transcription and RT-LAMP at the same time for RNA sample of infected person of COVID-19 followed by Cas12 detection of prior identified region of coronavirus genome region, after cleavage of reporter molecule admit the presence of virus. Like SHERLOCK technique, DETECTR technique were chosen E and N region for targeting COVID-19 [44].

CRISPR –based assay can be read through paper strip to detect presence or absence of COVID-19 and it don't require complex instrument. CRISPR test can be perform within one hour with highest specificity and sensitivity [45,46].

8. SEROLOGICAL AND IMMUNOLOGICAL ASSAYS

Serological test is working with analysis of plasma or blood serum, saliva, sputum to check the presence and absence of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies.

Serological test is urgently needed for vaccine development, epidemiological study and assessment of both short and long term antibody response. IgM can be detected in early stage of infection in serum while IgG in later stage of infection or prior to infection. In coming time, the immunological assay is getting fascinating due to monoclonal antibody production for detection of COVID-19. But due to some situation this assay can be impacted [47-51] viz. (1) a person could show positive result for COVID-19 from qRT-PCR assay but may show seronegative due to lag of production of antibody (2) a person can be negative for qRT-PCR result may be seropositive due to recovery from early stage infection (3) sensitivity and specificity limitation can mislead with false positive result of COVID-19 [52,53]. Serological testing of covid-19 includes ELISA,

Lateral flow assay, neutralization assay, Chemiluminescent immunoassay. Each of serological assays brings automation, easy, rapid detection of COVID-19.

8.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is plate based quantitative or qualitative technique. Plates are coated with COVID-19 viral genome particle of interest, viz. spike protein, antibodies andpeptides. ELISA test is directly use serum or plasma, blood sample and usually take 1-5 h for result. Patient of COVID-19 sample incubated with protein, antiviral antibody present will bind with coated protein then it form antibody-protein complex. This complex can be detected after washing with another tracer antibody that will produce color or fluorescent-based readout. ELISA test have ability to give high throughput efficiency with specific sensitivity. This test most frequently check IgG/IgM of infected patient [47,54].

8.2 Lateral Flow Immunoassay

This is qualitative (positive or negative) assay that is small potable and used at the point of care. This test is type of rapid diagnostic test (RDT), means result can be seen within 10-30 min using blood samples from a finger prick, saliva samples, or nasal swab fluids. Nitrocellulose membrane used in this assay, when sample load to band strip it moves via capillary action and bind to labeled antigen and continue to move until they are captured by immobilized antihuman antibody. The COVID-19 antibody (IgG/IgM)-antigen complex shows colored compound which shows positive result with respect to control band [47,54].

8.3 Neutralization Assay

This test is relies on ability of patient antibody to prevent viral infection of cultured cell in lab setting. For this test, whole blood, serum, or plasma samples from the patient are diluted and added at decreasing concentration to cell culture. When a cell and virus grow with decreasing concentration, the presence of neutralizing antibodies can prevent viral replication. The neutralization level and how many antibodies in patient serum block viral replication can be measured by determining the threshold level of inhibition. Time to result of this assay takes 3-5 days but recently reduces to hours [55,56].

8.4 Chemiluminescent Immunoassay

This test is typically quantitative, can use peptidebased magnetic chemiluminescence enzyme. The test relies on mixing of patient whole blood, plasma, or serum samples with known viral protein, and enzyme labeled antibodied that allow a light-based, luminescent read-out then secondary enzyme labeled antibody bind when any antibody in the patient react with viral protein. This binding illuminate light and amount of light can be used to calculate the number of antibodies present in the patient sample. Diazyme Laboratories, Inc. (San Diego, California) developed fully automated Diazyme DZ-lite 3000 Plus chemiluminescence Analyzer for COVID-19 serological test [57].

9. CONCLUSION

The ongoing pandemic disease or probably syndemic outbreak of COVID-19 has potent threat to global health.. There are several methods adopted for early detection of deadly COVID-19 but among them qRT PCR is widely accepted using set of specific primers ORF1a/b, ORF1bnsp14, RdRp, S, E, or N gene, nsp2 protein. The quick and reproducible nature of qRT PCR results makes its most adopted techniques for early detection of COVID-19.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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