J Arch Mil Med. 2021 March; 9(1):e111603. doi: 10.5812/jamm.111603.

Published online 2021 May 16. **Review Article**

Reverse Transcription Polymerase Chain Reaction (RT-PCR) as a Tool for Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) Surveillance in the Military

Idris Zubairu Sadiq [1,*]

[1]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

[*]*Corresponding author*: Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria. Email: idrisubalarabe2010@gmail.com

Received 2020 November 25; **Accepted** 2021 April 18.

Abstract

Context: Military organizations like civilian communities are under considerable threat due to COVID-19 and other re-emerging infectious diseases, which may impair their operations and training. The aim of this review was to discuss the application of RT-PCR technology for detecting and surveillance of SARS-CoV-2 among armed forces to successfully halt COVID-19 transmission.
Evidence Acquisition: Relevant literature was collected from PubMed, Science Direct, and Google Scholar
Results: By combining both detection and quantification of pathogens, RT-PCR provides a reliable and sensitive method for detecting SARS-CoV-2 via targeting the viral ORF1ab, E, and N genes. Military surveillance plays an important role in the early detection and identification of positive cases, isolating infected patients, contact tracing, and quarantine to contain the spread of the disease among personnel. This technology emerges as a suitable tool for disease surveillance in the military and early detection and control of diseases.
Conclusions: The COVID-19 disease has spread into different parts of the world and may affect military training and operations. So, there is a need for active disease surveillance and preparedness plans in the military. Accordingly, RT-PCR, as a standard tool for SARS-CoV-2 detection, can be used for the surveillance and monitoring of military personnel to successfully curtail COVID-19.

Keywords: COVID-19, SAR-CoV-2, RT-PCR, Detection, Surveillance

1. Context

Polymerase chain reaction (PCR) involves the use of molecular procedures to amplify minute quantities and produce several copies of genetic materials (DNA and cDNA). It is a powerful method used for DNA amplification, and its utilization has been approved in detecting minute amounts of viral RNA in cells and tissues (1). At present, RT-PCR is one of the most sensitive procedures for detecting mRNA and quantifying SARS-CoV-2. It can precisely evaluate small quantities of genetic materials via producing cDNA from RNA by reverse transcription and subsequently amplifying cDNA by PCR (2). Among the diagnostic methods of COVID-19, RT-PCR (most reliable) and rapid Ag-Ab tests (with still questionable specificity and sensitivity) are most widely employed. Real-time reverse transcriptase–polymerase chain reaction using nasopharyngeal swabs has been proven to be highly effective for detecting the virus in clinical samples and confirming a diagnosis (3).

Towards the end of December 2019, a new coronavirus strain emerged in Wuhan, a city in China, with the ability to be transmitted from humans to humans. The disease is characterized by severe pneumonia symptoms resulting in the death of many people. Coronaviruses belong to the phylum incertae, order Nidovirales, family Coronaviridae, genus *Betacoronavirus*, and subgenus *Sarbecovirus* (4). These viruses are positive-sense and single-stranded RNA organisms (5) and have spikes, a membrane, an envelope, and nucleocapsid proteins (6). Coronaviruses can fall into one of the four subgenera: α-CoVs, β-CoVs, γ-CoVs, and Δ-CoVs, based on their genetic and antigenic materials, and their genome sizes vary from 26 to 32 Kbp, the largest genome for RNA viruses so far (7).

The SARS-CoV-2 virus was officially reported to the world health organization (WHO) office in China on 31 December 2019 as a pneumonia of unknown etiology (8). Previously known as the novel coronaviruses (2019 nCoV), it was later renamed the coronavirus disease 2019 (COVID-19). In severe cases, the virus causes complicated respiratory illnesses (5, 9). The SARS-CoV-2 virus differs from Middle

East respiratory syndrome coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) as it often exposes patients to severe respiratory pneumonia (5, 10). The disease has spread globally, mostly through human-to-human transmission.

Military organizations like civilian communities are also under considerable threat due to COVID-19 and other emerging infectious diseases transmitted through human-to-human interaction. Amid its global spread, COVID-19 may affect military training and operations, thereby necessitating military preparedness to counteract any form of the pandemic threatening military personnel. Armed forces are unique due to their functions in territorial defense of nations, and therefore, bioterrorism and bio-warfare often use pathogenic microorganisms to target the military and weaken its structure. For this reason, there is often a need to carry out disease surveillance in armed forces using molecular techniques that will guarantee the safety of all and prevent any form of outbreaks. The U.S. army has been at the forefront of the fight against the COVID-19 pandemic and has played a prominent part in mobilizing medical assistance and constructing emergency facilities to cope with the predicted outbreak (11). Germany has mobilized 15,000 troops to help city officials address the pandemic, while Poland, for example, has enabled thousands of soldiers to patrol locked streets, disinfect hospitals, and reinforce border control (12). Military operations have been curtailed or, in some cases, suspended in France, Italy, and Spain, the nations that were hardest hit by the outbreak (12). The aim of this review was to discuss the applications of RT-PCR for detecting and screening SARS-CoV-2 among armed forces and the successful halting of COVID-19 transmission.

2. Origin of SARS-CoV-2 and Symptoms of COVID-19

Several reasons demonstrated that SARS-CoV-2 was of zoonotic origin (10, 13). A report suggested that early-infected patients were exposed to the local animal market or seafood market in Wuhan, China (5), suggesting possible animal-to-human transmission. Earlier reports from the Middle East indicate the transmission of MERS-CoV from the camel to human (13, 14). With the global spread of the virus, it is now clear that human-to-human transmission (15, 16) remains the principal transmission route of the virus. Viral genome sequencing shows a high similarity between SARS-CoV-2 and the bat coronavirus (9, 17, 18). Upon the entry of the virus, the infected person may be normal, presenting no symptoms. The symptoms ranging from mild to severe, including fever, fatigue, shortness of breath, sore throat, runny nose, sneezing, and pneumonia, may manifest two to 14 days after exposure to the virus (17, 18).

3. Structural and Genomic Organization of SARS-CoV-2

Coronaviruses are large viruses with an approximately 30 kb plus-stranded RNA and about 125 nm diameter (19) that are usually capable of infecting a wide range of animals, including human beings (20). The SARS-CoV-2 virus consists of an enveloped, single, positive-stranded genomic RNA encoding nearly four (4) viral structural proteins, viz. spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Figure 1) 3 - 5 proteins, following the characteristic gene order [5′-replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3′] (21). The non-structural proteins are encoded by the *rep* gene making up about two-third (2/3) of the genome at the 5′ end. The S glycoprotein facilitates receptor binding and the subsequent entry of the virus into hosts' target cells and is thus a significant therapeutic target (22, 23). The proteins M and E play important roles in the assembly of viral particles, and the protein N is required for the synthesis of RNA (19).

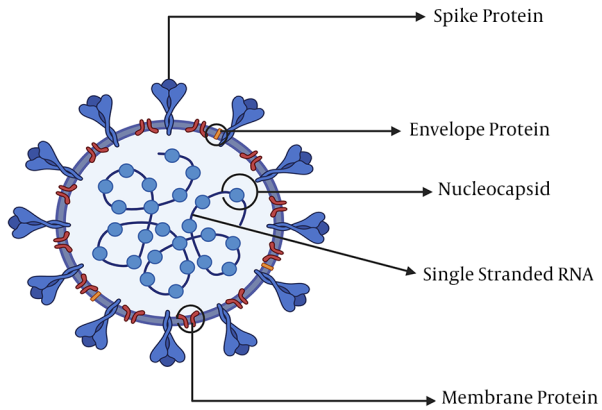

Figure 1. SARS-CoV-2 structure (created by Biorender, www.biorender.com)

The virion's most abundant structural protein is the M protein that is about ~ 25 - 30 kDa, consisting of three transmembrane domains (24) and maintaining the virion's shape. The N-terminal is a small glycosylated ectodomain, while the C-terminal is a much larger endodomain extending 6 - 8 nm into the viral particle (25). Though M proteins are co-translationally integrated into the ER membrane, almost all lack a signaling sequence. Reports indicate that the M protein exists in the virion as a dimer and may exhibit the configurations permitting it to foster the membrane curvature and bind to the nucleocapsid (26). Within

the virion, the E protein (proximately 8 - 12 kDa) exists in minute quantities and shows a highly divergent but comparable architecture (27). Both the N-terminal ectodomain and the C-terminal endodomain of the E protein are located in an ion channel. The recombinant viruses lacking the E protein remain not always lethal (28). The E-protein fosters the virus's assembly, release, fitness, and pathogenesis (29).

The N protein consisting of two domains, the C-terminal and N-terminal domains, is the only protein contained within the nucleocapsid. The protein's C- and N-terminal domains are able to bind to RNAs in vitro (19). Both the C- and N-terminal domains contribute to optimal RNA binding (30, 31). The frequent phosphorylation of the N protein (32) initiates a structural change that intensifies its affinity for viral versus non-viral RNAs (19). Specific RNA substrates for the N protein include the transcriptional regulatory sequences and packaging signals (33). The N protein binds RNA via a bead-on-a-string type interaction, whereas the packaging signal in the genome (34) binds the RNA binding domain (19). The N protein also binds to a key component of the replicase complex, the nonstructural protein-3 (31, 35), in addition to the M protein (36). Perhaps these protein-protein interactions will help the viral genome to bind to the replicase transcriptase complex and then bundle the encapsidated genome into viral particles (19).

4. Applications of RT-PCR for the Diagnosis of Acute Respiratory Infections

Real-time RT-PCR is commonly used for identifying causative viruses in the respiratory secretions of patients with acute respiratory infections (Figure 2) (37). Viral genetic materials are initially isolated from respiratory specimens and then subjected to RT-PCR via the synthesis of cDNA. Earlier reports have confirmed the use of RT-PCR-based technologies in public health laboratories for managing international outbreaks (38-41). The emergence of COVID-19 has highlighted the dominant application of real-time RT-PCR for diagnostic purposes, and the method remains the principal tool for detecting the virus's various strains among many existing diagnostic methods (42). It has become instrumental not only in the diagnosis but also in the treatment of COVID-19 (43). Although RT-PCR has a high sensitivity and reliability, the procedure may disrupt the viral genetic material, causing false-negative results in some cases (43). The outbreaks of acute respiratory illnesses are often considered health priorities during military postings, deployments, and operations (44). Influenza and acute respiratory infections can significantly impair military operations (44) and can be used as a pathogen(s) or bio-warfare to suppress enemies during war confrontations. The value of RT-PCR lies in its ability to detect RNA viruses in a given specimen, amplify target genes, and measure viral loads or monitor responses to antiviral drugs (45). By merging both quantification and detection of RNAs from pathogens within the shortest possible time, RT-PCR protocols become a dominant technique in molecular medicine and diagnostic laboratories (46).

5. COVID-19 Surveillance in the Military

Infectious disease surveillance in the military aims to monitor, reduce, control, and prevent outbreaks. Specifically, COVID-19 surveillance may involve the finding of active cases, isolating infected patients, contact tracing, and quarantine. The symptoms of the COVID-19 diseases, including fever, fatigue, shortness of breath, sore throat, runny nose, sneezing, and pneumonia-like symptoms, should be monitored among military personnel. This has been proven to be very effective for identifying COVID-19 cases in many countries. Observing good personal hygiene (washing hands and covering nose when sneezing and coughing), sanitizing public utilities such as door handles, stair rails, as well as providing health education to armed forces may help to contain such diseases in military organizations (47). Laboratory-based surveillance often plays a key role in disease prevention via detecting sources of pathogens, contributing to a more reliable risk assessment of the infectious disease (44, 48). Such knowledge can also be useful in formulating successful prevention methods and providing care to deployed military personnel. Military laboratories should be able to detect pathogens early enough to prevent unnecessary outbreaks and issue timely warnings. Multi-drug resistant pathogens should also be detected in military hospitals and communities.

6. The Specimens Used for COVID-19 Detection

Sample(s) can be obtained from the suspected patient(s) or those who have had close contact with confirmed patient(s). The specimens to be collected, according to the WHO, include upper respiratory specimens (pharyngeal/nasopharyngeal swabs) and/or lower respiratory specimens (bronchoalveolar lavage sputum/endotracheal aspirate) (49) (Figure 2). An investigation on the types of clinical specimens from 1070 COVID-19 patients for the detection of SARS-CoV-2 revealed that bronchoalveolar lavage fluid specimens rendered the highest positive rates (14 out of 15 patients, 93%), followed by sputum (72/104, 72%), nasal swabs (5/8, 63%), fibrobronchoscope brush biopsy (6/13, 46%), pharyngeal swabs (126/398, 32%), feces (44/153, 29%),

and blood (3/307, 1%). None of 72 urine specimens tested positive (50). Figure 2 shows the possible specimens that can be used for detecting COVID-19.

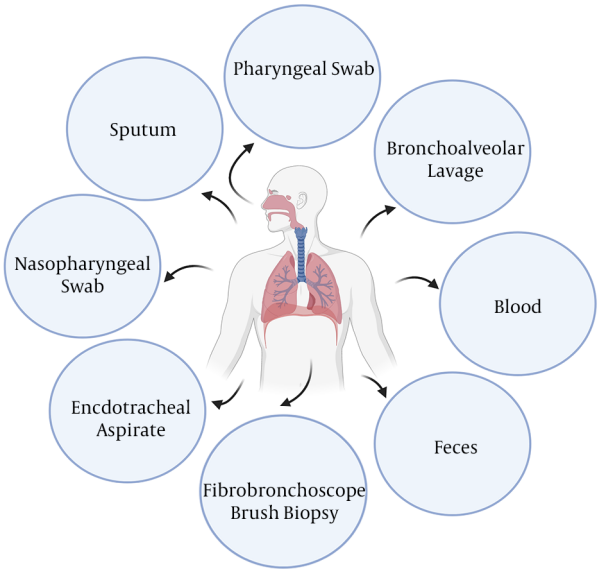

Figure 2. Specimens for detecting COVID-19 (created by Biorender, www.biorender.com)

7. Principle of RT-PCR Procedure

Polymerase chain reaction consists of three stages; denaturation, annealing, and extension. In the denaturation phase, the reaction temperature is raised up to 95°C, which melts double-stranded DNA into a single-stranded molecule via disrupting hydrogen bonds between complementary bases. In the annealing phase, the temperature is decreased to around 45 - 60°C that is far below the melting temperature (Tm) of primers to allow them to bind to the template. Finally, in the extension process, the temperature is raised to 72°C, which is optimal for DNA polymerase activity to allow hybridized primers to expand.

The polymerase chain reaction is an advanced technique providing a fast and accurate method to amplify DNA and allowing gene cloning and manipulation in biomedical research. This procedure has facilitated the diagnosis of infectious diseases and cancers based on genetic features. The use of reverse transcriptase to determine RNA levels and real-time DNA amplification and quantification are among advanced applications of PCR (51). In this technique, specific probes/primers, which are used to detect target viral genes, are key elements. For SARS-CoV-2 detection, primers and probes should be chosen from the virus's E, RdRP, and N genes. For example, the primers and probes distributed by TIB MOLBIOL are used in most countries to detect the presence of SARS-CoV-2 via amplifying E, RdRP, and N genes. These can be used as controls for RNA extraction or PCR. An example of an internal control RNA includes the Equine Arthritis Virus genomic fragment, β actin. The genetic material of the virus (RNA) is initially extracted from the sample and reverse transcribed to cDNA, which is then amplified using a real-time PCR machine. As the reaction progresses, the probe anneals to a viral target sequence for which the two primers (forward and reverse) are specific. In the RT-PCR phase, Taq polymerase, a highly thermo-stable enzyme, eliminates the probe by its nuclease activity, separating the quencher dye from the reporter dye, thereby producing a fluorescent signal (52), which is then detected at each cycle.

8. Specific Primers for Detecting SARS-CoV-2

Primers are short-stranded pieces of nucleic acid, annealing to a region of the genetic material to be amplified. One of the most important factors influencing the success and quality of quantitative real-time PCR is the design of primers and probes. In fact, precise and consistent quantification depends on the use of effective primers and probes (53). For longer PCR products, selecting efficient primers becomes more difficult. To design effective primers, it should be kept in mind to avoid the formation of primer-dimers, minimize self-complementarity, and not using primers with too low (melting temperature) Tm and/or internal stability (54). Several different primers have been used to detect SARS-CoV-2 in different respiratory samples (37, 55-60). These primers are very specific for viral genes, annealing to target genes and allowing SARS-CoV-2 detection. The common primers used for detecting SARS-CoV-2 are presented in Table 1.

9. Detection of SAR-CoV-2 RNA Using RT-PCR

Currently, the identification of viral RNA by RT-PCR is regarded as the gold standard for COVID-19 diagnosis and treatment follow-up (61). The detection of this pathogenic virus in respiratory specimens requires isolating the genetic material of the virus (RNA) followed by its conversion to cDNA and finally cDNA amplification. In this process, specific primers (forward and reverse) are used to amplify the target gene(s). The two most common targets are the ORF1ab and "N" genes, both of which are positive reference genes (61, 62) (Figure 3). The amplification condition can be set as follows: 50°C for 15 min, 95°C for 3 min, and then 45 cycles of 95°C for 15 s and 60°C for 30 seconds (59, 62).

Table 1. The Common Primers Used for the Detection of SARS-CoV-2

Target Genes	Intended Purposes	Country	Forward Primer	Reverse Primer	Probe	Reference
N gene	COVID-19	Thailand	CGT TTG GTG GAC CCT CAG AT	CCC CAC TGC GTT CTC CAT T	CAA CTG GCA GTA ACC A	(36)
N gene	COVID19	Japan	AAA TTT TGG GGA CCA GGA AC	TGG CAG CTG TGT AGG TCA AC	ATG TCG CGC ATT GGC ATG GA	(37)
N gene	COVID-19	China	GGG GAA CTT CTC CTG CTA GAA T	CAG ACA TTT TGC TCT CAA GCT G	TTG CTG CTG CTT GAC AGA TT	(38)
N1 gene	COVID-19	USA	GAC CCC AAA ATC AGC GAA AT	TCT GGT TAC TGC CAG TTG AAT CTG	ACC CCG CAT TAC GTT TGG TGG ACC	(39)
N2 gene	COVID-19	USA	TTACAA ACA TTG GCC GCA AA	GCG CGA CAT TCC GAA GAA	ACA ATT TGC CCC CAG CGC TTC AG	
N3 gene	COVID-19	USA	GGG AGC CTT GAA TAC ACC AAA A	TGT AGC ACG ATT GCA GCA TTG	AYC ACA TTG GCA CCC GCA ATC CTG	
RdRp /Orf1b-nsp14	COVID-19	Hong Kong	TGG GGY TTT ACR GGT AAC CT	AAC RCG CTT AAC AAA GCA CTC	TAG TTG TGA TGC WAT CAT GAC TAG	(40)
RdRp/Orf1	COVID-19	Germany	GTG ARA TGG TCA TGT GTG GCG G	CAR ATG TTA AAS ACA CTA TTA GCA TA	CAG GTG GAA CCT CAT CAG GAG ATG C	(19)
RdRp/Orf1	COVID-19	China	CCC TGT GGG TTT TAC ACT TAA	ACG ATT GTG CAT CAG CTG A	CCG TCT GCG GTA TGT GGA AAG GTT ATG G	(41)
orf1a	COVID-19	China	AGAAGATTGGTTAGATGATGA1	TTCCATCTCTAATTGAGGTTGA.	TCCTCACTGCCGTCTTGTTGAC	(42)

Usually, fluorescence acquisition in qPCR with dsDNA binding dyes occurs during the melting process at a temperature between the melting points of primers and the amplicon (63). The fluorophore-quencher probe is cleaved during thermocycler senses the fluorescent signal and tracks amplification progression in real-time (64). The diagnostic algorithm used to detect SARS-CoV-2, the causative agent of COVID-9, uses an initial screening to detect the presence of SARS-CoV-1, SARS-CoV-2, and other viruses belonging to the *Sarbecovirus* subgenus. For confirming the diagnosis, probes, and primers unique to the RdRP gene (RNA-dependent polymerase RNA) are used. Many labs in the world use the diagnostic algorithm and reagents employed by Corman et al. (37) in Cuba, as well as their interpretation to detect SARS-CoV-2 (37). For the "E" gene, a sample is considered positive if Ct is $\leq$ 36. In these cases, the diagnosis is confirmed using the RdRP gene, whose positivity is warranted at a Ct of $\leq$ 40. According to a report from a hospital in Wuhan, China, positive results were obtained for a patient only when both target genes (ORF1ab and N) were assessed (62). Accordingly, the result was considered positive when the Ct value was $<$ 37 and negative when Ct was $\geq$ 40. A moderate viral load, defined as a Ct value from 37 to $<$ 40, needed re-testing for confirmation (61). For widespread use of these molecular technologies in armed forces, military scientists must focus on designing a portable RT-PCR-based technology that is capable of integrating all procedures in one device and detecting infectious agents at affordable costs.

Figure 3 shows the target genes (Orf1ab, "E", and "N") used for the diagnosis of COVID-19 applying specific forward and reverse primers. These genes are then amplified via RT-PCR to produce a million copies of them. The presence of the genes generates signals that are detected by the RT-PCR device, confirming the diagnosis of COVID-19.

10. Conclusions

The achievements acquired by scientists in the fields of diagnosis and treatment of COVID-19 since the beginning of the outbreak encompass the sequencing of the viral genome, development of effective and reliable methods for detecting the virus, drug repurposing, and producing vaccines, which are currently on trials. The global spread of COVID-19 has exposed military personnel to all sorts of threats, and therefore, there is a need to secure military organizations by implementing disease surveillance programs and the effective diagnosis and rapid management of the disease using molecular tools such RT-PCR. Currently, RT-PCR is regarded as the gold standard for detecting COVID-19 in respiratory samples, including bronchoalveolar lavage fluid, sputum, nasal and pharyngeal swabs, fibrobronchoscope brush biopsy, as well as in feces, and blood. Reverse-transcriptase-PCR procedures ensure the early detection and rapid and effective control of the disease. The method is sensitive and reliable as it detects the genetic materials of pathogens. In some diseases where antibodies are initially detected using other methods, for example, in HIV, RT-PCR is employed to confirm the presence of the viral genetic material, making the diagnostic procedure more efficient. In conclusion, RT-PCR will continue to remain a reliable diagnostic test for detecting

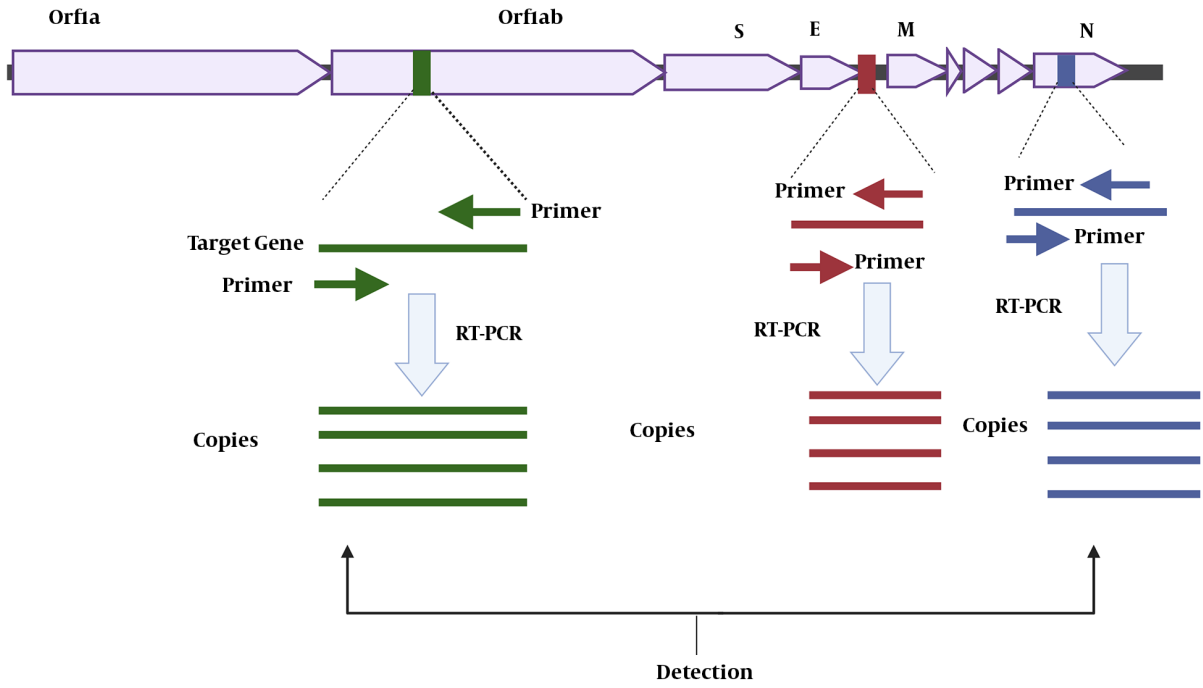

Figure 3. Detection of SARS-CoV-2 Using Target Genes (Created by Biorender, www.biorender.com)

SARS-CoV-2 and monitoring patients in the course of treatment.

Footnotes

Authors' Contribution: The sole author designed, analyzed, and interpreted data and prepared the manuscript.

Conflict of Interests: No competing interests have been declared.

Funding/Support: There is no funding/support.

References

1. Bartlett JM, Stirling D. *PCR protocols*. **226**. Totowa, New Jersey: Humana Press; 2003.
2. Gubler U. Methods in molecular biology. In: O'Connell J, editor. *RT-PCR protocols*. Totowa, New Jersey: Humana Press; 2002.
3. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected Pneumonia in Wuhan, China. *JAMA*. 2020;**323**(11):1061–9. doi: 10.1001/jama.2020.1585. [PubMed: 32031570]. [PubMed Central: PMC7042881].
4. International Committee on Taxonomy of Viruses. *Taxonomic information*. 2020. Available from: https://web.archive.org/web/20180304035352/https://talk.ictvonline.org/taxonomy/.
5. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;**395**(10223):497–506. doi: 10.1016/S0140-6736(20)30183-5. [PubMed: 31986264]. [PubMed Central: PMC7159299].
6. Yu IM, Oldham ML, Zhang J, Chen J. Crystal structure of the severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein dimerization domain reveals evolutionary linkage between corona- and arteriviridae. *J Biol Chem*. 2006;**281**(25):17134–9. doi: 10.1074/jbc.M602107200. [PubMed: 16627473]. [PubMed Central: PMC7946579].
7. Schoeman D, Fielding BC. Coronavirus envelope protein: current knowledge. *Virol J*. 2019;**16**(1):69. doi: 10.1186/s12985-019-1182-0. [PubMed: 31133031]. [PubMed Central: PMC6537279].
8. WHO. *Novel coronavirus (2019-nCoV) situation report*. World Health Organisation; 2020. Available from: https://www.who.int/docs/default-source/coronaviruse/situation415reports/20200121-sitrep-1-2019-ncov.pdf..
9. Jiang S, Xia S, Ying T, Lu L. A novel coronavirus (2019-nCoV) causing pneumonia-associated respiratory syndrome. *Cell Mol Immunol*. 2020;**17**(5):554. doi: 10.1038/s41423-020-0372-4. [PubMed: 32024976]. [PubMed Central: PMC7091741].
10. Perlman S. Another decade, another coronavirus. *N Engl J Med*. 2020;**382**(8):760–2. doi: 10.1056/NEJMe2001126. [PubMed: 31978944]. [PubMed Central: PMC7121143].
11. Micallef JV. *The national security implications of COVID-19*. 2020. Available from: https://www.military.com/daily-news/2020/04/27/national-security-implications-covid-19.html.
12. Salaün T, Siebold S, Baker L. *Europe's armed forces face a war against coronavirus as military infections rise*. 2020. Available from: https://www.weforum.org/agenda/2020/04/coronavirus-european-armed-forces/.
13. Hui DS, Azhar EI, Kim YJ, Memish ZA, Oh MD, Zumla A. Middle East respiratory syndrome coronavirus: risk factors and determinants

of primary, household, and nosocomial transmission. *Lancet Infect Dis*. 2018;**18**(8):e217–27. doi: 10.1016/S1473-3099(18)30127-0. [PubMed: 29680581]. [PubMed Central: PMC7164784].
14. Sabir JS, Lam TT, Ahmed MM, Li L, Shen Y, Abo-Aba SE, et al. Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. *Science*. 2016;**351**(6268):81–4. doi: 10.1126/science.aac8608. [PubMed: 26678874].
15. Majumder MS, Mandl KD. Early transmissibility assessment of a novel coronavirus in Wuhan, China. *SSRN*. 2020:3524675. doi: 10.2139/ssrn.3524675. [PubMed: 32714102]. [PubMed Central: PMC7366781].
16. CDC. *Transmission of novel coronavirus (2019-nCoV)*. Center for Diseases Control; 2020. Available from: https://www.cdc.gov/coronavirus/2019-ncov/about/transmission.html.
17. CDC. *Symptoms of novel coronavirus (2019nCoV)*. Center for Diseases Control; 2020. Available from: https://www.cdc.gov/coronavirus/2019-ncov/about/symptoms.html.
18. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. 2020;**382**(18):1708–20. doi: 10.1056/NEJMoa2002032. [PubMed: 32109013]. [PubMed Central: PMC7092819].
19. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol*. 2015;**1282**:1–23. doi: 10.1007/978-1-4939-2438-7_1. [PubMed: 25720466]. [PubMed Central: PMC4369385].
20. Barcena M, Oostergetel GT, Bartelink W, Faas FG, Verkleij A, Rottier PJ, et al. Cryo-electron tomography of mouse hepatitis virus: Insights into the structure of the coronavirion. *Proc Natl Acad Sci U S A*. 2009;**106**(2):582–7. doi: 10.1073/pnas.0805270106. [PubMed: 19124777]. [PubMed Central: PMC2613939].
21. Song Z, Xu Y, Bao L, Zhang L, Yu P, Qu Y, et al. From SARS to MERS, thrusting coronaviruses into the spotlight. *Viruses*. 2019;**11**(1):59. doi: 10.3390/v11010059. [PubMed: 30646565]. [PubMed Central: PMC6357155].
22. Du L, Yang Y, Zhou Y, Lu L, Li F, Jiang S. MERS-CoV spike protein: a key target for antivirals. *Expert Opin Ther Targets*. 2017;**21**(2):131–43. doi: 10.1080/14728222.2017.1271415. [PubMed: 27936982]. [PubMed Central: PMC5457961].
23. Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S. The spike protein of SARS-CoV–a target for vaccine and therapeutic development. *Nat Rev Microbiol*. 2009;**7**(3):226–36. doi: 10.1038/nrmicro2090. [PubMed: 19198616]. [PubMed Central: PMC2750777].
24. Armstrong J, Niemann H, Smeekens S, Rottier P, Warren G. Sequence and topology of a model intracellular membrane protein, E1 glycoprotein, from a coronavirus. *Nature*. 1984;**308**(5961):751–2. doi: 10.1038/308751a0. [PubMed: 6325918]. [PubMed Central: PMC7095125].
25. Nal B, Chan C, Kien F, Siu L, Tse J, Chu K, et al. Differential maturation and subcellular localization of severe acute respiratory syndrome coronavirus surface proteins S, M and E. *J Gen Virol*. 2005;**86**(Pt 5):1423–34. doi: 10.1099/vir.0.80671-0. [PubMed: 15831954].
26. Neuman BW, Kiss G, Kunding AH, Bhella D, Baksh MF, Connelly S, et al. A structural analysis of M protein in coronavirus assembly and morphology. *J Struct Biol*. 2011;**174**(1):11–22. doi: 10.1016/j.jsb.2010.11.021. [PubMed: 21130884]. [PubMed Central: PMC4486061].
27. Godet M, L'Haridon R, Vautherot JF, Laude H. TGEV corona virus ORF4 encodes a membrane protein that is incorporated into virions. *Virology*. 1992;**188**(2):666–75. doi: 10.1016/0042-6822(92)90521-p. [PubMed: 1316677]. [PubMed Central: PMC7131960].
28. DeDiego ML, Alvarez E, Almazan F, Rejas MT, Lamirande E, Roberts A, et al. A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *J Virol*. 2007;**81**(4):1701–13. doi: 10.1128/JVI.01467-06. [PubMed: 17108030]. [PubMed Central: PMC1797558].
29. Nieto-Torres JL, DeDiego ML, Verdia-Baguena C, Jimenez-Guardeno JM, Regla-Nava JA, Fernandez-Delgado R, et al. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. *PLoS Pathog*. 2014;**10**(5). e1004077. doi: 10.1371/journal.ppat.1004077. [PubMed: 24788150]. [PubMed Central: PMC4006877].
30. Chang CK, Sue SC, Yu TH, Hsieh CM, Tsai CK, Chiang YC, et al. Modular organization of SARS coronavirus nucleocapsid protein. *J Biomed Sci*. 2006;**13**(1):59–72. doi: 10.1007/s11373-005-9035-9. [PubMed: 16228284]. [PubMed Central: PMC7089556].
31. Hurst KR, Koetzner CA, Masters PS. Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein. *J Virol*. 2009;**83**(14):7221–34. doi: 10.1128/JVI.00440-09. [PubMed: 19420077]. [PubMed Central: PMC2704785].
32. Stohlman SA, Lai MM. Phosphoproteins of murine hepatitis viruses. *J Virol*. 1979;**32**(2):672–5. doi: 10.1128/JVI.32.2.672-675.1979. [PubMed: 228084]. [PubMed Central: PMC353599].
33. Molenkamp R, Spaan WJ. Identification of a specific interaction between the coronavirus mouse hepatitis virus A59 nucleocapsid protein and packaging signal. *Virology*. 1997;**239**(1):78–86. doi: 10.1006/viro.1997.8867. [PubMed: 9426448]. [PubMed Central: PMC7130520].
34. Kuo L, Masters PS. Functional analysis of the murine coronavirus genomic RNA packaging signal. *J Virol*. 2013;**87**(9):5182–92. doi: 10.1128/JVI.00100-13. [PubMed: 23449786]. [PubMed Central: PMC3624306].
35. Hurst KR, Koetzner CA, Masters PS. Characterization of a critical interaction between the coronavirus nucleocapsid protein and nonstructural protein 3 of the viral replicase-transcriptase complex. *J Virol*. 2013;**87**(16):9159–72. doi: 10.1128/JVI.01275-13. [PubMed: 23760243]. [PubMed Central: PMC3754073].
36. Sturman LS, Holmes KV, Behnke J. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J Virol*. 1980;**33**(1):449–62. doi: 10.1128/JVI.33.1.449-462.1980. [PubMed: 6245243]. [PubMed Central: PMC288560].
37. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. 2020;**25**(3). doi: 10.2807/1560-7917.ES.2020.25.3.2000045. [PubMed: 31992387]. [PubMed Central: PMC6988269].
38. Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, et al. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. *Euro Surveill*. 2012;**17**(49). doi: 10.2807/ese.17.49.20334-en. [PubMed: 23231891].
39. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. 2003;**348**(20):1967–76. doi: 10.1056/NEJMoa030747. [PubMed: 12690091].
40. Corman VM, Eickmann M, Landt O, Bleicker T, Brunink S, Eschbach-Bludau M, et al. Specific detection by real-time reverse-transcription PCR assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China. *Euro Surveill*. 2013;**18**(16):20461. [PubMed: 23611031].
41. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Euro Surveill*. 2012;**17**(39). doi: 10.2807/ese.17.39.20285-en. [PubMed: 23041020].
42. Pang J, Wang MX, Ang IYH, Tan SHX, Lewis RF, Chen JI, et al. Potential rapid diagnostics, vaccine and therapeutics for 2019 novel coronavirus (2019-nCoV): A systematic review. *J Clin Med*. 2020;**9**(3). doi: 10.3390/jcm9030623. [PubMed: 32110875]. [PubMed Central: PMC7141113].
43. Pan Y, Long L, Zhang D, Yuan T, Cui S, Yang P, et al. Potential false-negative nucleic acid testing results for severe acute respiratory syndrome coronavirus 2 from thermal inactivation of samples with low viral loads. *Clin Chem*. 2020;**66**(6):794–801. doi: 10.1093/clinchem/hvaa091. [PubMed: 32246822]. [PubMed Central: PMC7184485].
44. Velasco JM, Yoon IK, Mason CJ, Jarman RG, Bodhidatta L, Klungthong C, et al. Applications of PCR (real-time and MassTag) and enzyme-

linked immunosorbent assay in diagnosis of respiratory infections and diarrheal illness among deployed U.S. military personnel during exercise Balikatan 2009, Philippines. *Mil Med*. 2011;**176**(10):1096–100. doi: 10.7205/milmed-d-11-00027. [PubMed: 22128641]. [PubMed Central: PMC7107564].
45. Menon PK, Kapila K, Ohri VC. Polymerase chain reaction and advances in infectious disease diagnosis. *Med J Armed Forces India*. 1999;**55**(3):229–31. doi: 10.1016/S0377-1237(17)30450-1. [PubMed: 28775636]. [PubMed Central: PMC5531883].
46. Kleines M, Schellenberg K, Ritter K. Efficient extraction of viral DNA and viral RNA by the Chemagic viral DNA/RNA kit allows sensitive detection of cytomegalovirus, hepatitis B virus, and hepatitis G virus by PCR. *J Clin Microbiol*. 2003;**41**(11):5273–6. doi: 10.1128/jcm.41.11.5273-5276.2003. [PubMed: 14605182]. [PubMed Central: PMC262516].
47. Ho ZJ, Hwang YF, Lee JM. Emerging and re-emerging infectious diseases: challenges and opportunities for militaries. *Mil Med Res*. 2014;**1**:21. doi: 10.1186/2054-9369-1-21. [PubMed: 25722877]. [PubMed Central: PMC4341224].
48. Sanchez JL, Cooper MJ, Myers CA, Cummings JF, Vest KG, Russell KL, et al. Respiratory infections in the U.S. military: Recent experience and control. *Clin Microbiol Rev*. 2015;**28**(3):743–800. doi: 10.1128/CMR.00039-14. [PubMed: 26085551]. [PubMed Central: PMC4475643].
49. WHO. *Laboratory testing for coronavirus disease (COVID-19) in suspected human cases*. World Health Organization; 2020. Available from: https://apps.who.int/iris/bitstream/handle/10665/331329/WHO-COVID-19-laboratory-2020.4eng.pdf?sequence=1&isAllowed=y.
50. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. 2020;**323**(18):1843–4. doi: 10.1001/jama.2020.3786. [PubMed: 32159775]. [PubMed Central: PMC7066521].
51. Ishmael FT, Stellato C. Principles and applications of polymerase chain reaction: basic science for the practicing physician. *Ann Allergy Asthma Immunol*. 2008;**101**(4):437–43. doi: 10.1016/S1081-1206(10)60323-7. [PubMed: 18939735].
52. CDC. *2019-novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel*. Center for Diseases Control; 2020. Available from: https://www.fda.gov/media/134922/download.
53. Rodriguez A, Rodriguez M, Cordoba JJ, Andrade MJ. Design of primers and probes for quantitative real-time PCR methods. *Methods Mol Biol*. 2015;**1275**:31–56. doi: 10.1007/978-1-4939-2365-6_3. [PubMed: 25697650].
54. Rychlik W. Selection of primers for polymerase chain reaction. *Mol Biotechnol*. 1995;**3**(2):129–34. doi: 10.1007/BF02789108. [PubMed: 7620973].
55. Department of Medical Sciences; Ministry of Public Health. *Diagnostic detection of novel coronavirus 2019 by real time RT-PCR*. Thailand: World Health Organization; 2020. Available from: https://www.who.int/docs/default-source/coronaviruse/conventional-rt-pcr-followed-by-sequencing-for-detection-of-ncov-rirl-nat-inst-health-t.pdf.
56. Nao N, Shirato K, Katano H, Matsuyama S, Takeda M. *Detection of second case of 2019-nCoV infection in Japan (corrected version)*. Japan: Department of Virology III, National Institute of Infectious Diseases; 2020.
57. National Institute for Viral Diseases Control and Pervention. *China-CDC specific primers and probes for detection 2019 novel coronavirus*. China; 2020. Available from: http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html.
58. WHO. *US-CDC 2019-novel coronavirus (2019-nCoV) real-time rRT-PCR panel: Primers and probes*. 2020. Available from: https://www.who.int/docs/default-source/coronaviruse/uscdcrt-pcr-panel-primerprobes.pdf?sfvrsn=fa29cb4b_2.
59. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of Pneumonia. *Clin Chem*. 2020;**66**(4):549–55. doi: 10.1093/clinchem/hvaa029. [PubMed: 32031583]. [PubMed Central: PMC7108203].
60. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*. 2020;**395**(10224):565–74. doi: 10.1016/S0140-6736(20)30251-8. [PubMed: 32007145]. [PubMed Central: PMC7159086].
61. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, et al. Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients. *Clin Infect Dis*. 2020;**71**(15):793–8. doi: 10.1093/cid/ciaa345. [PubMed: 32221523]. [PubMed Central: PMC7184442].
62. Liu R, Han H, Liu F, Lv Z, Wu K, Liu Y, et al. Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin Chim Acta*. 2020;**505**:172–5. doi: 10.1016/j.cca.2020.03.009. [PubMed: 32156607]. [PubMed Central: PMC7094385].
63. Mehndiratta M, Palanichamy JK, Ramalingam P, Pal A, Das P, Sinha S, et al. Fluorescence acquisition during hybridization phase in quantitative real-time PCR improves specificity and signal-to-noise ratio. *Biotechniques*. 2008;**45**(6):625-6–630 passim. doi: 10.2144/000112994. [PubMed: 19238793].
64. Udugama B, Kadhiresan P, Kozlowski HN, Malekjahani A, Osborne M, Li VYC, et al. Diagnosing COVID-19: The disease and tools for detection. *ACS Nano*. 2020;**14**(4):3822–35. doi: 10.1021/acsnano.0c02624. [PubMed: 32223179]. [PubMed Central: PMC7144809].