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Review Article:
Genome-based Detection of Novel Coronavirus: An Overview Study

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ABSTRACT

The Coronavirus Disease 2019, named COVID-19 is a global problem. According to the declaration of the World Health Organization (WHO), it is a novel and extreme outbreak, spreading worldwide. Firstly, numerous patients reported exposure in Wuhan City, China at a large animals and seafood market. Accordingly, the first idea is suggesting the animal-to-human transmission of this infection pathogen. Next, since then, numerous patients have decelerated no exposure to animal shops, indicating that human-to-human transmission is occurring. The new coronavirus (2019-nCoV) is a positive RNA virus with a 29.8 kb genome and S, E, M, N, and Orf1 gene fragments. The most popular method for 2019-nCoV detection is genome-based approaches, like Polymerase Chain Reaction (PCR)-based tests that require expensive experimental equipment, a controlled working environment, and high-trained technicians; they are often lacking in massive viral outbreaks. Therefore, another rapid and simple genome-based assay was evaluated for this pathogen. In this review study, the RT-LAMP technique, as the main isothermal amplification assays with less time consumption and without the need for expensive equipment, compared to conventional PCR-based methods to 2019-nCoV identification, was discussed. Accordingly, the advantage or disadvantage of these techniques was compared. The obtained data indicated that this molecular and isothermal method could be used as a successful one-step process for portable screening and the rapid identification of 2019-nCoV.

1. Introduction

Coronaviruses (CoVs) are a large family from enveloped, positive-sense, single-stranded RNA viruses, including human HCoV-OC43, HCoV-HKU1, CoV-229E, and HCoV-NL63. They commonly cause mild respiratory illnesses. They also cause significant diseases in humans and animals. In this family, two species were prevalent in the last two decades, i.e., threatening and fatal; the Severe Acute Respiratory Syndrome (SARS) Coronavirus and Middle East Respiratory Syndrome (MERS) Coronavirus [1-3].

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Characterizing the novel coronavirus 2019

On December 30, 2019, the new case of cluster unidentified pneumonia was an outbreak. It was named the Coronavirus Disease 2019 (COVID-19) by the World Health Organization (WHO). This virus was first found in Wuhan City, China. Besides, it is very similar to the SARS coronavirus outbreak in 2003. Based on the spread of this virus, the WHO has categorized the COVID-19 outbreak as a public health emergency of international concern [4, 5]. This virus is a novel emerged coronavirus that has been never found in humans, i.e., named 2019-nCoV. According to the WHO notification in December 2019, Hubei Province (Wuhan City), has maintained the surveillance of influenza and similar diseases and detected multiple cases from viral pneumonia with a high rate of death. The genomic information of 2019-nCoV that isolates from Wuhan-Hu-1 indicated that this virus has RNA+ with 29.8 kb size and 5 fragment gene (Orf, S, E, M, N), i.e., critical for the viral detection (Figure 1) [6, 7].

The outbreak of COVID-19

After the emergence of SARS and MERS in 2003 and 2012, respectively, scientists believed that coronavirus outbreak always occurs in the world every 10 years. SARS outbreak lasted within a year of emergence; however, the MERS outbreak dispreads after 7 years. According to the WHO report (December 2018), MERS spread over 27 Middle Eastern countries. Furthermore, since the September of 2012, ≥2428 cases of MERS have been confirmed by 2278 laboratories with 838 deaths. However, SARS spread in 26 countries with 8098 reported cases and 774 deaths [8]. Currently, the recent outbreak of COVID-19 is a global health threat This is because of serious and dangerous concerns and its rapid spread in several countries, including Iran, and the possible fatal progression of the infection. At the beginning of the outbreak, numerous patients reported exposure at a large animal and seafood market in Wuhan City, China. Accordingly, the first idea was suggesting the animal-to-human transmission of this infection pathogen. Next, since then, numerous patients have decelerated no exposure to animal shops, indicating that human-to-human transmission was occurring. There is no confirmed vaccine and targeted therapy and treatment for COVID-19. The early detection of COVID-19 is difficult. This is because patients can remain asymptomatic or present non-specific flu-like clinical symptoms, including cough, fever, severe respiratory disease, shortness of breath, and pneumonia. These symptoms may appear in as few as 2 or up to 14 days after exposure and infection. Based on the obtained information, >113 million infected subjects had been confirmed worldwide with approximately 1.1 million infected cases from Iran. The 2019-nCoV is similar to SARS coronavirus; however, it has increased transmissibility, compared with the SARS. Thus, it often infected humans without any symptoms and the index could transfer the viral infection to others [7-9]. Therefore, developing rapid, correct, and one-step detection methods for the 2019-CoV identification in the early stages of the infection is necessary. Moreover, rapid treatment and the disease spread controlling are of significance.

The genome-based detection of 2019-nCoV

The most popular assay for viral RNA identification is based on quantitative RT-PCR [10]. This technique requires a run time of 3 hours. Besides, this approach requires expensive experimental equipment, a controlled working environment, and high-trained technicians. Accordingly, the genome-based detection of 2019-nCoV was presented.

qRT-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) includes two sequential steps. The first process is based on Reverse Transcriptase (RT) function; accordingly, cDNA was synthesized from an RNA template. Moreover, PCR is performed in the second step. RT-PCR has been evaluated as a common and conventional gene-based diagnostic method for the detection of infectious agents, including RNA. Currently, quantitative RT-PCR (qRT-PCR), for the detection of 2019-nCoV in blood or respiratory samples, is the main standard assay for molecular identification. This standard method is precise and sensitive; however, it requires expensive devices and trained personnel. For RNA viral agents, especially acute respiratory infection, a probe coupled with qRT-PCR from respiratory secretions are used to identify causative viruses, routinely. Currently, qRT-PCR based techniques are evaluated and applied worldwide by several researchers and disease control centers, and laboratories. Additionally, qRT-PCR has numerous limitations, especially the need for high purity targets and the access to expensive and especial laboratory equipment, as well as requiring a long time of about 3 h. Furthermore, qRT-PCR necessitates trained personnel and sophisticated device for RNA extraction and sample processing. These problems restrict the practical applicability of qRT-PCR in multiple cases. Therefore, these conditions can delay the required rapid interventions, with the prescription and administration of antiviral agents for infectious patients [11].

Figure 1. The genomic information of the novel coronavirus (2019-nCoV) [6, 7]

Real-Time RT-PCR

Real-Time RT-PCR assay is a common and precise technique, confirmed by the Centres for Disease Control (CDC) and used for the in vitro qualitative detection of 2019-nCoV in serum and respiratory specimens. The 2019-nCoV primer and probe sets are designed and synthesized for the conventional detection of SARS-like coronaviruses as well as the selectivity detection of N1 and N2 genes from 2019-nCoV. Due to the sensitivity of real-time RT-PCR, this technique should be performed using strict quality control and quality assurance process. This method helps to reducing the odds of false-positive amplification. Moreover, after finalizing the run, the relevant data are saved and analyzed per the manufacturers' instructions. By a manual threshold setting, data analyses should be separately performed for each target. Besides, thresholds should be calibrated to fall within the exponential step of the fluorescence curves and above any baseline. Moreover, the procedure selected for setting the threshold should be consistently used (Figure 2).

This method is a universal assay for 2019-nCoV detection; however, it has some limitations, as follows: Analysts should be trained with testing procedures and the interpretation of results prior to conducting PCR. If inadequate numbers of organisms are present in the specimen, false-negative data may occur, i.e., due to improper collection, handling, or transport. RNA viruses, in particular, have variability in the genome. Besides, conserved fragments into the viral genomes were selected; however, this variability resulted in mismatches between the designed primers and probes during performing the real-time RT-PCR technique. Therefore, the target sequences can result in decreased assay performance and possible false-negative data [12, 13].

RT-Loop-Mediated Isothermal Amplification (LAMP)

An alternative isothermal nucleic acid amplification assay is Loop-Mediated Isothermal Amplification (LAMP), i.e., evaluated in 2000 by Notomi. LAMP is a very simple method with a high rapid reaction and simple detection of the results. This method only requires one enzyme. Besides, all replication reaction was conducted under a constant temperature (60-65°C) in one hour. Therefore, a thermocycler device is not required and can be performed into Water Bath and Bain Marie. The LAMP products have the potential to offer an easy detection assay; the obtained results could be directly observed by the color visual change with the naked eye

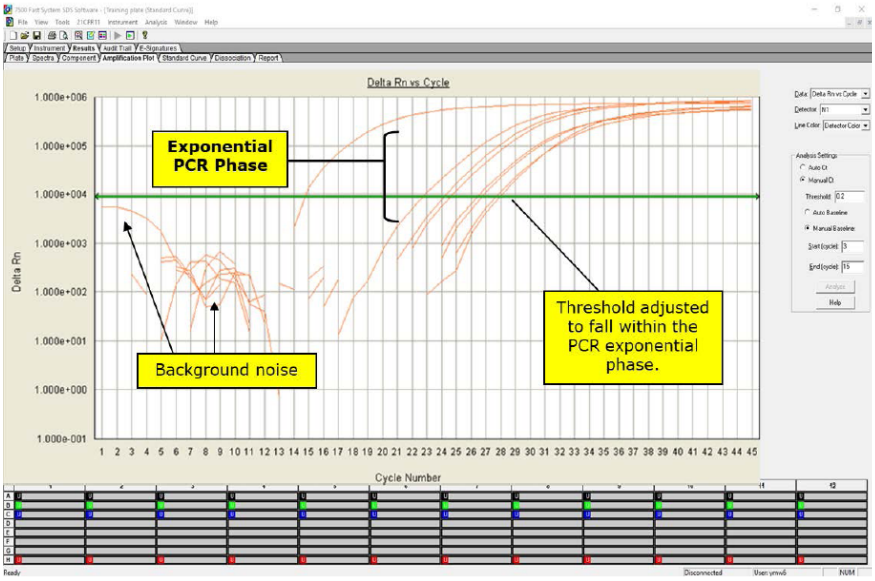

Figure 2. Amplification plot window from real-time RT-PCR and the determination of threshold [13]

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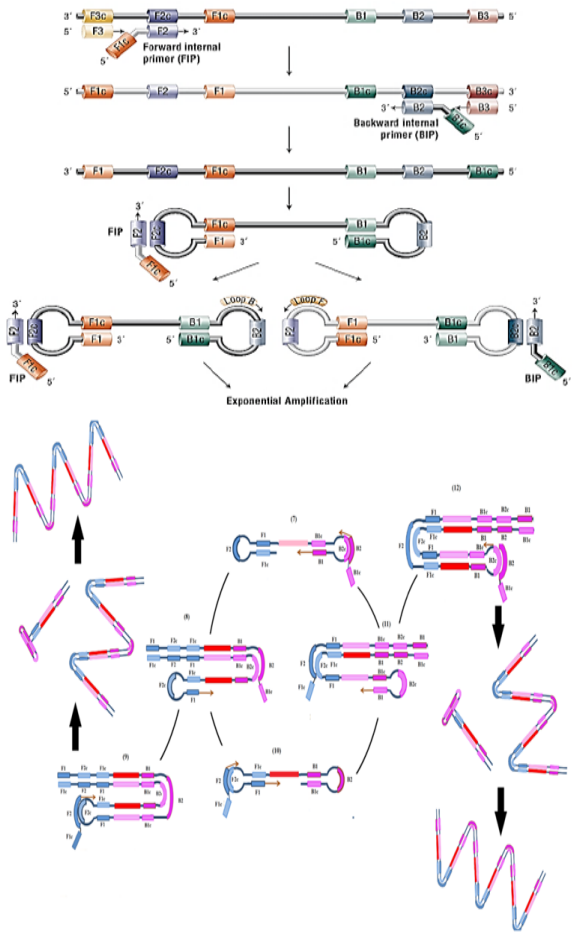

Figure 3. The schematic presentation of the LAMP reaction process and the development of dumbbell-like DNA strands [16, 17]

[14-17]. This assay can be used in microbiology and clinical laboratories without thermocycler and other molecular equipment. Furthermore, this technique can be implemented in the field and portable assay [14].

Bst polymerase plays an essential role in the LAMP reaction process. This enzyme is derived from Bacillus stearothermophilus, which lives in hot springs at a temperature of about 70°C and with 3/ to 5/ polymerase and exonuclease activity, also the ability to move the strand. At a suitable temperature, this polymerase with strand shifting function can remove non-patterned DNA strands without the heat cycle required for PCR reaction and synthesize new DNA strands from the target strand. In the first step of the reaction, the inner and outer primers can create dumbbell-like DNA strands from the target DNA template. Furthermore, these dumbbell-like DNA strands become new DNA patterns for the next step. The dumbbell-like DNA strand continues to amplify; eventually, it becomes a long strand of DNA (Figure 3) [16, 17].

Primer design, i.e., the main part of this method, is based on 6 regions in the target sequence. Two series of primers, called internal primers, including BIP and FIP as well as external primers, including B3 and F3 are designed for this method, which identify the 6 regions mentioned in the target sequence. Four key factors, including the melting temperature of the primers; the stability of the end of each primer; the GC value of each primer, and the lack of secondary structure in the primers must be considered in designing LAMP primers for a LAMP reaction to give an acceptable result. Overlooking these items can cause errors in the test result. Primers can be designed with two online software, LAMP designer and Primer explorer V5 [14-17]. In the design of LAMP primers, amplicons of 200-400 bp are considered. Therefore, primer design is easily possible for the conserved regions of 2019-nCoV with a gene structure of about 30 kb.

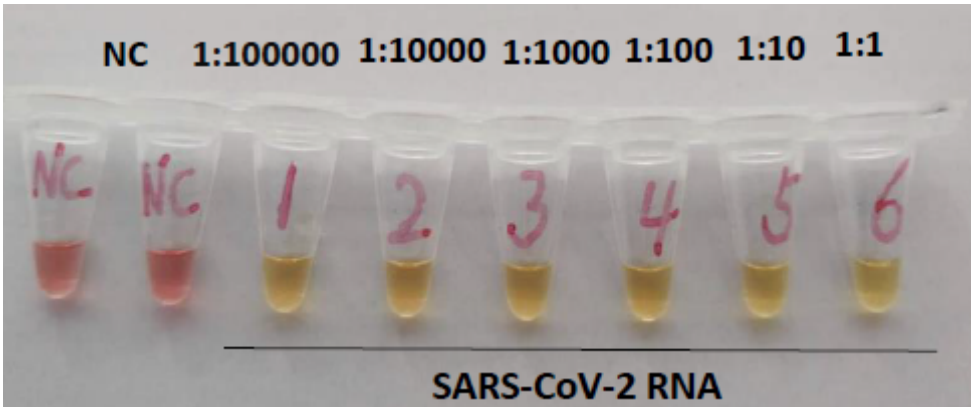

Figure 4. The colorimetric results of the LAMP products in different dilutions of RNA from 2019-nCoV [25]

Furthermore, the RT-LAMP assay was designed and developed for the rapid detection of pathogenic viral RNA; a two-step reaction (RT & LAMP) is conducted in one single tube [18-21]. This method can be combined with fluorescence reactors or pH change indicators (e.g. phenol red) within the LAMP reaction. Accordingly, after a visual specific color change into LAMP product, positive results are achieved [22]. Therefore, the LAMP product has the potential for a simple assay to visually and accessibly interpret the results. This method is also cost-effective and with very high specificity that can identify the desired gene target in less than one hour [15-17]. Additionally, less time consumption of RT-LAMP, compared with conventional PCR-based methods has been successfully used for quick identification and screening of pathogenic RNA viruses, like 2019-nCoV [23-27]. Researchers have used the RT-LAMP method to identify RNA viruses. They revealed that the RT-LAMP is very sensitive and selective; thus, the Limit of Detection (LOD) of this assay is approximately 10 copies per reaction, i.e., tenfold higher sensitivity than RT-PCR [19, 20]. In 2020, Lamb et al. developed a rapid method for 2019-nCoV detection using LAMP assay. These scholars believed qRT-PCR is a common and standard meth-

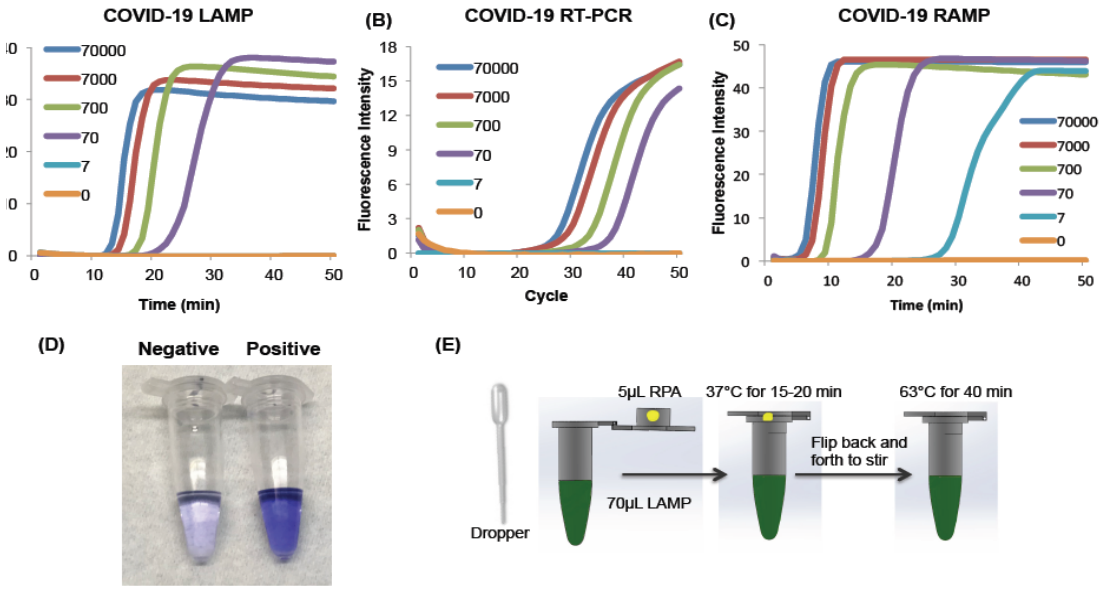

Figure 5. Comparing LAMP, RT-PCR, and closed-tube RAMP to 2019-nCoV detection [24]

Comparing LAMP (A), RT-PCR (B), and closed-tube RAMP (C) to 2019-nCoV detection at the concentrations of 70000, 7000, 700, 70, and 0 (without template) copy in reaction. D: Visible monitoring of LCV for the detection of 2019-nCoV with a RAMP tube; in this case, 100 copies (positive) and 0 copies (negative) from the newly synthesized DNA of 2019-nCoV were used. E: A sequential sample identification process was conducted at home; besides, the reaction can be performed by the samples incubating in a temperature-controlled incubator.

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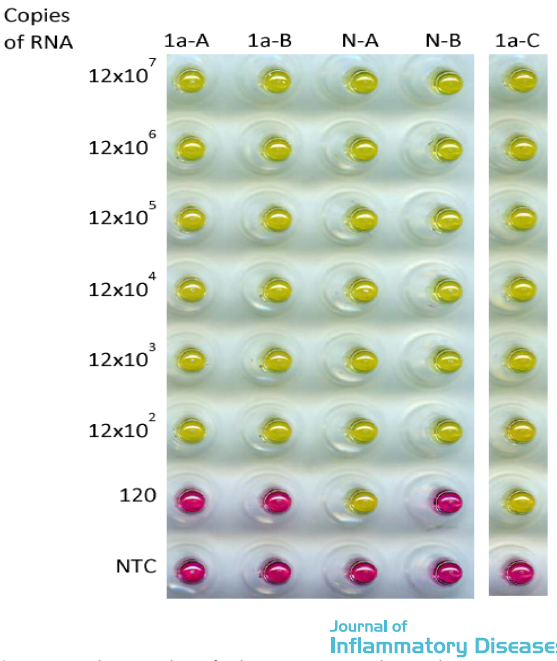

Figure 6. The results of Zhang›s research on the RNA synthetic amplicons of 2019-nCoV [26]

od to identifying 2019-nCoV, but RT-LAMP assay can be used for rapid and non-expensive in field experiments and hazardous conditions. In this RT-LAMP assay, specificity was evaluated by examining it against other related coronaviruses. The related data indicated that in immediate patient samples, RT-LAMP detected 2019-nCoV, specifically in <30 minutes. Immediate samples obtained from patients, including serum, urine, oral, and pulmonary swabs, and saliva can be used to identify the nucleic acid sequence of this virus. Besides, the specificity and sensitivity of this method can be evaluated as competing with qRT-PCR. Therefore, this method could be used for monitoring or detecting exposed humans; it could potentially help with screening efforts in the area and possible entrance points [23].

Yu et al. evaluated a colorimetric assay for the specific identification of 2019-nCoV by RT-LAMP, named iLACO (isothermal LAMP-based method for COVID-19) using 6 specific primers to amplify a segment of the conserve Orf1ab gene. In this study, comparing the sequences of 11 related viruses (including 9 coronaviruses & 2 influenza viruses) by BLAST software, the species-specificity of iLACO was confirmed. The sensitivity is proportional to Taq man-based real-time qPCR detection assay, identifying a synthesized and artificial RNA equivalent to 10 copies from 2019-nCoV. The time duration of this assay depended on the range and loading of the virus in the collected samples that varied from 15 to 40 minutes. The versatility, simplicity, and accuracy of this designed method indicated that iLACO process can be conveniently applied for monitoring and controlling COVID-19 threat, even in those cases where the specialized molecular device is not present and available [24]. In this assay, specific colorimetric material-based phenol red (WarmStart Colorimetric LAMP 2X Master Mix, NEB Company) was used; subsequently, the positive and negative results were determined with yellow and purple colors, respectively (Figure 4).

The negative and positive controls are presented according to purple and yellow colors, respectively.

Tholoth et al. also used the RT-LAMP method to screen 2019-nCoV at entry points and even homes, as a sensitive and accurate molecular method with minimal train-

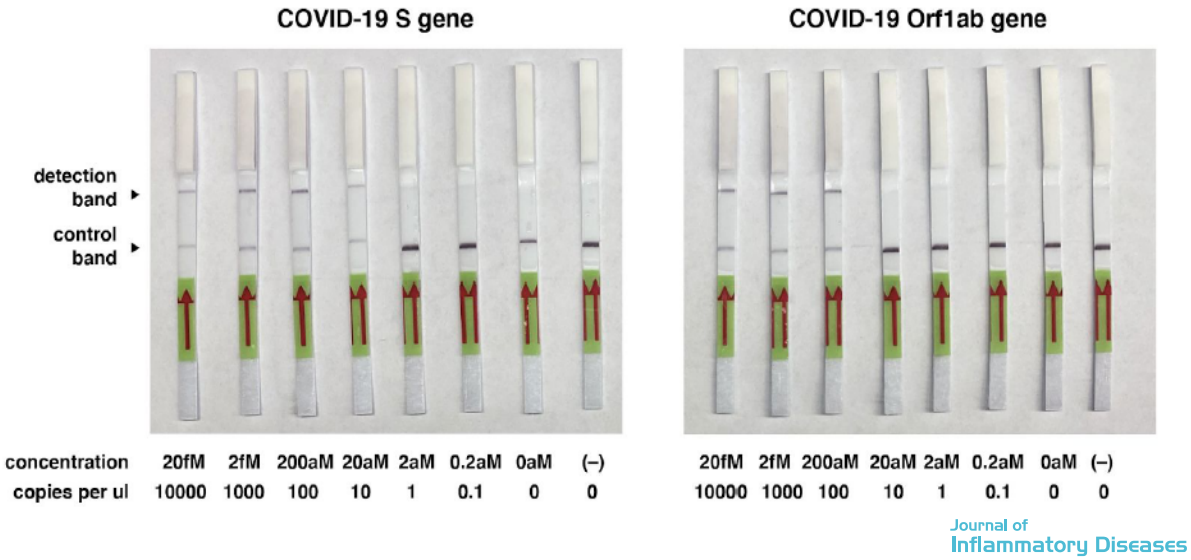

Figure 7. Identifying SHERLOCK COVID-19, using the RNA fragments of 2019-nCoV and available paper dipstick [27]

Table 1. Comparing 3 methods used for 2019-nCoV detection

Methods	Sensitivity	Specificity	Time (h)	Equipment	Simplicity of Work
RT-PCR	100 pg	Medium (a pair of primers)	3	Thermocycler	Complex, Hard
Real-Time RT-PCR	100 fg	High (a pair of primers and probes)	3	Thermocycler	Complex, Hard
RT-LAMP	100 fg	Very high (3 pair of primers)	1	Hot water bath	Simple, Easy

ing for each individual as well as the minimum device required. This method was named COVID-19 RAMP for increasing the sensitivity of the LAMP product, with two isothermal amplification reactions (LAMP & RPA, recombinase polymerase amplification), i.e., programmed using new primers and nested LAMP. Finally, it was compared with RT-PCR and LAMP. Initially, the RPA mix is prepared traditionally; however, in the second step, the LAMP reaction is performed using a Loopamp DNA replication kit; unlike the kit structure of other companies, the fluorescent dye is absent. The ratio of RPA to LAMP is 9:1; for the colorimetric assay of 2019-nCoV, Leuco Crystal Violet (LCV) solution, including crystal violet (0.5 mM), β-cyclodextrin (5 mM), and sodium sulfite (30 mM) is prepared and stored at -20°C until use. The LAMP reaction mixture also included the following: 1.6 µM of BIP and FIP primers, 0.8 µM of LB and LF primers, 1 µl of Bst polymerase enzyme, and 5.5 µl of LCV solution. For the RPA reaction, the mixture is placed at 38°C for 15-20 min. Moreover, for the LAMP reaction, the mixture is incubated at 63°C for 40 minutes. The color change of LCV is observed by the naked eye at the end of the incubation time that can be recorded using a smartphone, if necessary (Figure 5).

The obtained results indicated that the sensitivity of COVID-19 RAMP is 10-100 folds better than the COVID-19 LAMP and COVID-19 RT-PCR technique. Accordingly, Tholoth believed that this rapid method has the potential to significantly reduce false negatives while being capable to use minimal equipment and individual training [25].

The new method for RNA detection of 2019-nCoV according to pure form or cell lysis was developed by Zhang et al. using the LAMP method and based on colorimetric and ocular detection. Using RNA samples collected from purified respiratory swabs from infected patients in Wuhan, the procedure performed similarly to a commercial RT-qPCR kit, requiring only heating and ocular monitoring. This simple, fast, selective, and sensitive method provides the opportunity and ability to facilitate virus detection without the need for advanced diagnostic equipment. Zhang designed 5 pairs of complete LAMP primers (1a-A, 1aB, N-A, N-B, & 1aC) to identify the protected end regions of the 5/ ORF1ab and N genes. The sensitivity of the method was determined using synthetic RNA by serially-diluted consecutively from 120 million copies to 120 copies (in 25 µl of reaction) in the LAMP method. Therefore, the sensitivity of the method was determined by about 12 copies according to 4.8 copies per µl (Figure 6). The results of this work were completely consistent with real-time diagnostic data [26].

Five primer pairs (1a-A, 1aB, N-A, N-B, & 1aC) related to ORF1ab and N genes with a gene template between 120 million and 120 copies were examined. Yellow and pink indicate positive and negative responses, respectively.

In another work, an innovative method was used to detect the causative agent of COVID-19 in the form of genome identification using lateral flow assay kits. The relevant authors stated that this innovative method was proposed in the form of generating ideas for other researchers to continue their activities; thus, it lacks the aspect of usage in clinical laboratories and managing infected patients. This innovative diagnostic method helps with the development of the 2019-nCoV diagnosis. This guide uses the innovative CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) assay for viral identification. Using the new synthetic RNA fragments of 2019-nCoV, the virus can be specifically identified with a diagnostic limit of 20 to 200 atomoles (10 to 100 copies per µL). This method can be performed in the form of lateral flow assay kits in less than an hour without the need for special laboratory equipment. The SHERLOCK COVID-19 diagnostic guideline operates in 3 steps; they are performed in one hour using nucleic acid extraction similar to the qRT-PCR diagnostic method, as follows: Step 1: incubation for 25 min, the isothermal amplification of the extracted RNA sample using a commercial kit with available Recombinase Polymerase Amplification (RPA). RPA is an isothermal technique with 3 main enzyme axes, including recombinase, SSB or single-stranded DNA binding

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protein, and strand displacement polymerase. Step 2: time incubation for 30 min, the pre-amplified viral RNA sequence detection, using Cas13. Step 3: time incubation for 2 min, using a commercially available paper dipstick for the visual monitor of the detection data by naked eye according to Figure 7 [27]. Based on this approach, the S gene (left) and the ORF1ab gene (right) of the virus can be detected at the automol level.

5. Conclusion

In this review article, genome-based detection approaches were compared. Moreover, isothermal amplification, especially RT-LAMP was determined as a highly sensitive, accurate, and portable method for the 2019-nCoV detection. This method can evaluate a portable molecular diagnostic kit for use at points of entry and home by minimally-trained individuals and with minimal equipment to control and manage the prevalence of COVID-19. Comparing 3 gene-dependent methods for 2019-nCoV detection is presented in Table 1.

Despite the many advantages of the LAMP method, this assay has some disadvantages. One of the limitations and disadvantages of this method is the false-positive responses due to product cross-contamination. Therefore, according to the limitations of this method in interference reactions and cross-contamination, leading to false-positive results, it is always recommended that the closed tubes be used for the LAMP reaction. Even in the visual detection of the LAMP product, indicators and reporters were added within the initial LAMP reaction (before amplification). However, if it is necessary for the product analysis by electrophoresis, which in this case, the door tubes must be opened, this test should be performed under biosafety cabinet in a controlled manner for the prevention of the spread of possible contamination.

Currently, some companies are developing RT-LAMP-based diagnostic kits in the form of the closed tubes and providing them to specialized laboratories for screening 2019-nCoV infection [25]. Thus, using the RT-LAMP method, as an isothermal approach to identify 2019-nCoV, due to the lack of complex laboratory equipment, can provide suitable conditions for field and even home screening.

Ethical Considerations

Compliance with ethical guidelines

This work is a review study with no experiments on animal or human samples.

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Conflict of interest

The author declared no conflict of interest.

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