Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 3, 2023, 292
https://doi.org/10.33263/BRIAC133.292

Nanomolecular Magnetic Probe for Colorimetric RT-LAMP of Viral RNA

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Scopus Author ID 56718702000
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Received: 21.05.2022; Accepted: 26.06.2022; Published: 12.07.2022

Abstract: Amplification of the RNA from the Covid-19 virus is considered the main objective of the molecular diagnosis of SARS-Cov-2. However, the use of target-based amplification methods such as polymerase chain reaction requires a step to convert the RNA of the Covid-19 virus into a DNA template to lead to amplification. In addition, isolating the RNA of the Covid-19 virus requires RNA purification kits, which will increase the time and costs of molecular detection of this virus. In this study, the magnetic nanoprobe is introduced that it could capture and amplify Covid-19 RNA through an isothermal amplification process called loop-mediated isothermal amplification without requiring a step to convert the viral RNA into a DNA template. By using the engineered sequences corresponding to the target nucleic acid attached to magnetic nanoparticles, it becomes possible to identify the target RNA of this virus through color changes due to pH changes that can be seen with the naked eye due to the presence of pH indicators in the reaction mix. According to the isothermal amplification of the viral RNA via LAMP assisted with the magnetic nanoprobe, the nanomolecular method eliminated the need for special equipment and the time for detecting Covid-19 in specimens.

Keywords: magnetic nanoparticles; RT-LAMP; colorimetric detection; SARS-Cov2.

1. Introduction

The occurrence of pandemics threatens global health every few years, which is inevitable [1]. Major pandemics such as cholera, plague, influenza, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East Respiratory Syndrome (MERS-CoV) have affected human health and life [1]. Recently the outbreak of COVID-19 has been a major problem that global public health has struggled with. COVID-19 is created by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the family of Betacoronavirus [2]. Covid-19 symptoms are diverse, but it is often associated with a fever, cough, headache, fatigue, breathing problems, and reduced senses (such as tasting and smelling). These symptoms may occur one to 14 days after exposure to the virus. At least one-third of people who become infected do not show significant symptoms. The virus can be transmitted quickly from an infected person to a healthy person [3] when people breathe in air contaminated by droplets containing viral particles. the risk that threatens such people is at the

highest level in closed spaces, in sound, which is in covered spaces with longer distances; this risk is lower. [4]. This transmission can also happen when fluids contaminated with the virus are splashed or spread inside the eyes, mouth, or nose or through contaminated surfaces. People exposed to such contamination should be quarantined for 20 days, even if they have not shown clinical symptoms, causing various conditions, from a mild fever to life-threatening [5]. The most important point in controlling the spread of COVID-19 is identifying asymptomatic carriers because the symptomatic carriers are easily identified. Still, the main cause of disease transmission and its continuous chain are asymptomatic carriers [5, 6]. Hence, screening tests for asymptomatic diseased individuals are essential [7]. The most valuable point of care tests are visual detection assays; they can be analyzed eye naked, without needing instruments and an expert person.

The diagnostic methods of COVID-19 can be classified in two ways, such as protein-based- and nucleic acid-based-detections [8-10]. Protein-based methods detect virus spike antigen or body humoral antibodies [8, 9], and the visual detection occurs using gold nanoparticles or other nanomaterials. DNA-based methods usually are based on DNA and RNA amplification reactions, identification, and screening. However, the accuracy of protein-based methods is not enough. They are not used for detecting SARS-CoV-2 because the people who are carriers have low levels of antibodies or antigens of the virus that need to be detected result can be false negative [11-13]. Therefore, it is better to use visual detection systems based on nucleic acid (DNA or RNA) because they are more accurate and will detect the presence of viral genomes. So nucleic acid-based visual detection has a priority for us.

In nucleic acid-based amplification, the methods are usually divided into two groups, i.e., isothermal or non-isothermal amplification. For point-of-care diagnosis, the isothermal methods are usually preferred because they eliminate equipment, trained person, and laboratory and require only a heating block or water bath to maintain a constant temperature. Non-isothermal methods such as real-time PCR, which is already used as a gold standard detection method for coronavirus, have some limitations such as the demand for pure samples, lab instruments, time consumed reaction (about 2 h), trained person, and complex analyzing facilities [14-16].

Among the isothermal methods, loop-mediated isothermal amplification (LAMP) and reverse transcriptase (RT)-LAMP methods are the most important because the amplification reaction occurs fast and has high sensitivity and specificity. Loop-mediated isothermal amplification (LAMP) is rapid, has high sensitivity and specificity, and does not need expensive reagents or instruments [17, 18]. RT-LAMP allows the direct detection of RNA [14]. In these methods, we have four pairs of primers instead of two that can detect even a single viral genome, greatly increase specificity, reduce the possibilities of false positives and false negatives, and increase sensitivity. The reaction does not require any instrument for being occurred, and the result can also be evaluated based on turbidity, fluorescent emission, and colorimetric [19]. However, it is preferable to design a point-of-care detection method for diagnosing viral RNA of Covid-19 without needing a pretreatment process [20]. The use of magnetic iron nanoparticles with the surface probes in the presence of a magnetic field can lead to aggregation, so isolation of the genetic content that is especially attached to the probes has occurred [21]. In this study, we designed a magnetic nanoparticle with a probe on its surface. The probe is the specific primer for the LAMP reaction. If there is a viral genetic content in a clinical sample, the nanoparticle magnetically identifies the genome through its probe, and then the RNA could be captured (Figure 1).

With the process of washing and aggregation affected by the magnetic field, the additional contents could be removed. Then, the magnetic nanoprobes attached to LAMP amplicons change the pH that would be visualized using a pH indicator dye [22].

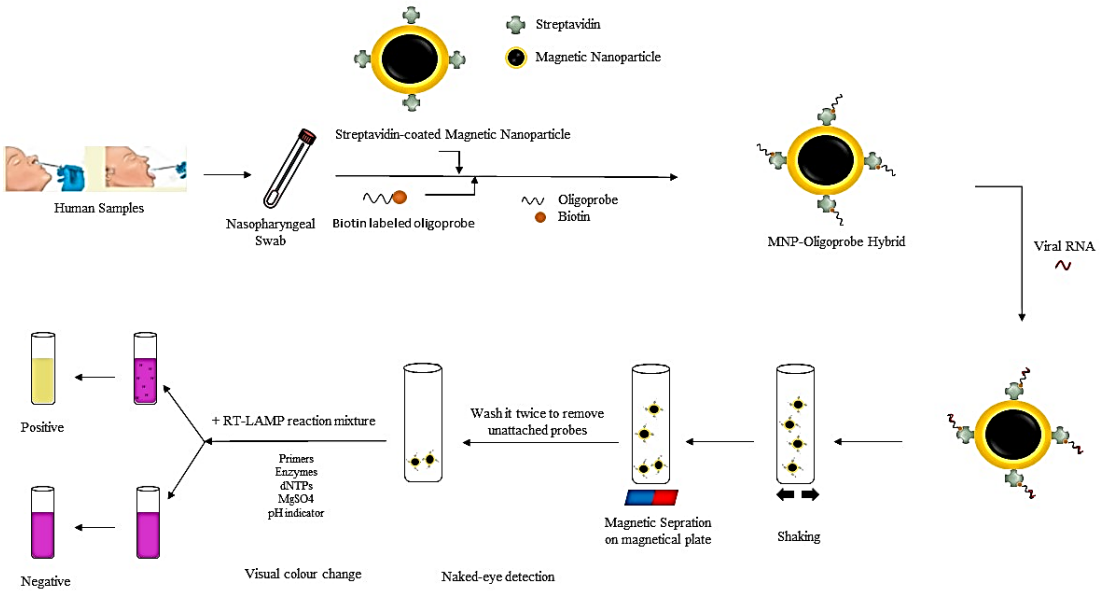


Figure 1. Schematic illustrated Nanomolecular magnetic probe for Loop-mediated Isothermal amplification of viral RNA. Capturing viral RNA with nanoprobes: Viral RNA is adsorbed to the nanoprobe when it is placed in the vicinity of prepared nanoprobes containing magnetic nanoparticles and B3 primer due to the presence of complementary sequences. Magnetic-RT-LAMP process: 1) RNA template targeted with primer 1 (nanoprobe) participates in the process. 2) The primer is annealed to a specific sequence, complementary DNA is made by reverse transcriptase against early RNA, and LAMP process is started. Each synthesized RNA can re-participate in these steps and produce more RNA amplicons. PH changes due to mass production of amplicon produced during LAMP reaction lead to a change in the color of the positive reaction to a negative reaction (without color change), which can be seen and examined with the naked eye qualify.

2. Materials and Methods

2.1. Chemicals and instruments.

Streptavidin-coated magnetic nanoparticles (fluidMAG-Streptavidin) with a diameter of 100 nm were purchased from Chemicell (Germany). Specific Primers (FIP, BIP, LF, LB, CF3, and biotin-labeled-CB3) were synthesized by Bioneer (United States). T7 RNA polymerase was purchased from Thermo Fisher Scientific (United States). NTPs (ATP, GTP, CTP, and UTP) were purchased from Invitrogen (United States). DNTPs (ATP, GTP, CTP, and TTP) were purchased from Invitrogen (United States). $MgSO_4$ was purchased from Sigma-Aldrich (United States). Bsm DNA polymerase was purchased from Thermo Fisher Scientific (United States). RevertAid reverse transcriptase was purchased from Thermo Fisher Scientific (United States). Phenol Red powder was purchased from Sigma-Aldrich (United States). 1000 bp DNA ladder was purchased from GeneDireX (China). RNA ladder was purchased from Thermo Fisher Scientific (United States). EmeraldAmp PCR master mix was purchased from TAKARA (Japan). The thermal condition for performing the reactions is provided by a master cycler gradient (Eppendorf, Germany). For electrophoresis, we used the electrophoresis chamber model OA-78 (Fargen, Tehran, Iran). To supply the required voltage for electrophoresis, a mobility power supply (Paya Pajohesh) was utilized. To form the hybrid of MNP-Probe, we need shaking conditions provided by a shaker-incubator (Behdad-Tehran,

Iran). To observe and analyze the electrophoresis behavior, Gel-Doc Multi Gene LED/UV (Fargen, Tehran, Iran) and ToupView software (ToupTek Photonics, China) were utilized.

2.2. Oligonucleotides and sequences.

For synthesis RNA calibrator from the "S" gene of NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, a 209-bp DNA sequence inserted to pUC57 was purchased from GeneScript Co. Ltd (China). For RT-LAMP amplification, the oligoprimers were synthesized by Bioneer Co. Ltd (South Korea) as follows:

Forward bumper primer: 5' CTG ACA AAG TTT TCA GAT CCT CAG 3'

Backward bumper primer with T7 handle: 5' (Biotin) AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GAG TAC CAA AAA TCC AGC CTC TT 3'

Forward inner primer: 5′TCCCAGAGACATGTATAGCATGGAATCAACTCAGGACTTGTTCTTACC3′

Backward inner primer:5′TGGTACTAAGAGGTTTGATAACCCTGTTAGACTTCTCAGTGGAAGCA3′

Loop forward primer: 5′CCAAGTAACATTGGAAAAGAAA3′

Loop backward primer: 5′GTCCTACCATTTAATGATGGTGTTT3′. To identify the conserved region of the coronavirus genome, six primers CF3, CB3, CFIP, CBIP, CLF, and CLB were used in the mix of Rt-LAMP reactions.

2.3. Preparation of magnetic nanoprobe.

One hundred microliters of the streptavidin-coated magnetic nanoparticles were mixed with 4 µL of backward bumper primer. Then the obtained mixture was shaken at room temperature for 20 minutes. The obtained mixture was placed on a magnetic plate and then washed twice with nuclease-free water [23].

2.4. RNA extraction.

RNA was extracted from biological samples by biotin-labeled CB3 probe (biotin-AGT ACC AAA AAT CCA GCC TCT T) were synthesized by Bioneer, Korea hybridized with Streptavidin (fluidMAG-Streptavidin) magnetic nanoparticle (MNP) of 100nm diameter size was purchased from Chemicell (Germany). COVID-19 viral RNA was reverse transcribed using 5' (Biotin) AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GAG TAC CAA AAA TCC AGC CTC TT 3'.

2.5. Fabrication of RNA target sequences using artificial DNA construct.

14 µL of DNA construct was added to 11 µL of reaction mixture, comprising T7 RNA polymerase transcription buffer 1X [200 mM Tris-HCl pH 7.9 at 25°C, 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl and 10 mM spermidine], 2 mM each NTPs [ATP,GTP,CTP,UTP] and 0.6 $\frac{U}{\mu L}$ T7 RNA polymerase. Reactions were incubated at 37°C for 2 hours. The reaction was evaluated using electrophoresis on gel agarose 2%.

2.6. cDNA synthesis and PCR reaction.

For cDNA Synthesis, 20 µL of mixture, RevertAid reverse transcriptase 1X reaction buffer [250 mM Tris-HCl pH 8.3 at 25°C, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT], 0.2 µM each CF3/CB3 primer, 1 mM each dNTPs [ATP, GTP, CTP, TTP], 1 µL RNA template (which was received a temperature of 65°C for 5 minutes and then transferred into ice) and 200 $\frac{U}{\mu L}$ RevertAid reverse transcriptase was made. Then we put this mix in the thermocycler at 42° C for 1 hour.

For PCR reaction mix, 20 µL of the mixture, comprising 1X EmeraldAmp PCR master mix, 0.2 µM CF3 forward primer, 0.2 µM CB3 backward primer, and 1 µL cDNA was prepared. The thermocycler temperature program for this PCR reaction is as follows (Table 1). The reaction was evaluated using electrophoresis on gel agarose 1.5%.

Table 1. PCR thermal cycling program.

Denaturation	10 s	98°C
Annealing	30 s	55°C
Extension	20 s	72°C
Go to 1, repeat 30 cycle		
Hold 4°C, 20s		

2.7. RNA synthesis from PCR product.

1 µL of DNA PCR product (which was received at a temperature of 95°C for 5 minutes and then transferred into ice) was added to 24 µL of the reaction mixture, comprising T7 RNA polymerase transcription buffer 1X [200 mM Tris-HCl pH 7.9 at 25°C, 30 mM MgCl2, 50 mM DTT, 50 mM NaCl and 10 mM spermidine], 2mM each NTPs [ATP, GTP, CTP, UTP], 30 $\frac{U}{\mu L}$ T7 RNA polymerase. Reactions were incubated at 37°C for 2 hours. The reaction was evaluated using electrophoresis on gel agarose 2%.

2.8. Reverse transcriptase loop-mediated isothermal amplification of viral RNA.

RT-LAMP reaction was performed with reaction mixture at 25 µl total volume contained: 1.6 µM forward inner primer/backward inner primer, 0.2 µM F3/B3 bumper primers, 0.4 µM loop forward/loop backward primers, 1.4 mM each dNTP, 6 mM MgSO4, 0.32 u/(µL) Bsm DNA polymerase, 8 u/(µL) RevertAid reverse transcriptase, 1µL RNA template. The thermocycler carried out the reverse transcription reaction at 50°C for 90 minutes [24].

2.9. Optimization of pH indicator for visual detection of RT-LAMP.

To develop this RT-LAMP technology, we used phenol red as a pH-sensitive indicator dye to distinguish positive samples from negative ones. Positive reactions resulted in the color change of phenol red from pink to yellow due to producing H+ during the reaction. However, the negative results still kept pink. Positive and negative reactions also were investigated by gel electrophoresis. From this point of view, the indicator must detect the minimum amount of protons produced during the reaction that causes reducing in the pH; the change in pH must be noticeable to the indicator. So the initial pH of 2 mM phenol red was adjusted with 100 mM KOH to ~5 [22].

2.10. RT-LAMP of captured viral RNA on magnetic nanoprobe.

RT-LAMP reaction was performed with reaction mixture at 25 µl total volume contained: 1.6 µM forward inner primer/backward inner primer, 0.2 µM F3/B3 bumper primers, 0.4 µM loop forward/loop backward primers, 1.4 mM each dNTP, 6 mM MgSO4, 0.32 u/ (µL) Bsm DNA polymerase, 8 u/ (µL) RevertAid reverse transcriptase. After adding 1µL RNA template, 1 µl of the prepared magnetic nanoprobe and phenol red was added to the mixture. The thermocycler carried out the reverse transcription reaction at 50°C for 90 minutes.

2.11. RT-LAMP-based Detection of viral RNA via gel electrophoresis.

The RT-LAMP products were analyzed by 1.5% agarose gel (containing GelStain-Green, Bioneer Company, South Korea) via electrophoresis in 1X TAE.

2.12. RT-LAMP BASED Detection of viral RNA via magnetic nanoprobes.

The LAMP product of the captured viral RNA on magnetic nanoprobes was evaluated using electrophoresis with 2% agarose gel (containing GelStain-Green, Bioneer Company, South Korea) in 1X TAE buffer.

2.13. Limit of detection (LoD) of viral RNA using LAMP.

The 1:10 dilutions of the viral RNA (100 ng, 10 ng, 1 ng, and 0.1 ng) in nuclease-free water were prepared, and the RT-LAMP process was performed. Also, the serial dilution of the viral RNA was amplified with the RT-LAMP assisted with magnetic probes.

3. Results and Discussion

3.1. Results.

3.1.1. Fabrication of RNA target sequences using artificial DNA construct.

RNAs were synthesized according to the given protocol. RNAs made from synthetic DNA construct have different lengths, so they are not seen as a single band on gel electrophoresis but as bands containing sequences of different lengths. The following image was obtained on 2% agarose gel (Figure 2).

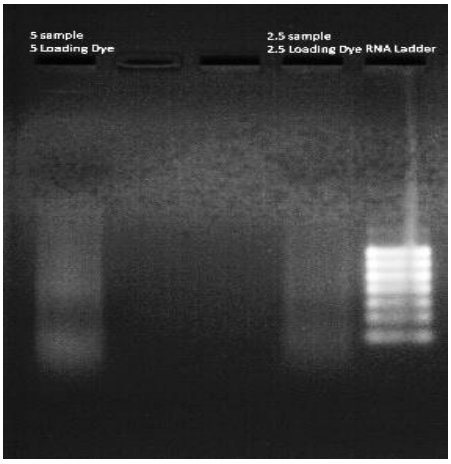


Figure 2. Gel electrophoresis of synthesized RNA (smear behavior) and RiboRuler Low Range RNA ladder (right bands).

3.1.2. cDNA synthesis and PCR reaction.

The next step after RNA synthesis is the synthesis of cDNA and RNA. Electrophoresis cannot determine whether cDNA synthesis has occurred or not, and we can only confirm the occurrence of cDNA synthesis when the following reaction occurs. The Synthesized cDNA was inserted into the PCR amplification reaction, and the product of the reaction was seen as a single band in the following image obtained on 1.5% agarose gel. By synthesizing RNA using cDNA produced during the PCR reaction, a single band is expected to be seen on 2% agarose gel electrophoresis. The weight of the PCR product using a DNA ladder was determined to be 200 bp (Figure 3).

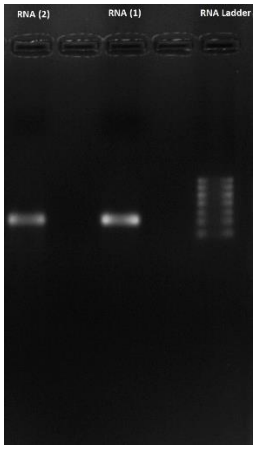


Figure 3. Gel electrophoresis of ~ 200 nt RNA products as artificial COV-2 RNA-target (left bands) and RiboRuler Low Range RNA ladder (right bands).

3.1.3. Reverse transcriptase loop-mediated isothermal amplification of viral RNA.

1.5 µl of MgSO4 (1:10), 3.5 µl of dNTP mix, 2 µl of FIP primer (diluted 1: 5), 2 µl of BIP primer (diluted 1: 5), 1 µl of F3 primer diluted (1:20), 1 µl of B3 primer diluted (1: 20), 1 µl of LF primer (diluted 1:10), 1 µl of LB primer (diluted 1:10). Then 1 µl of Bsm DNA Polymerase enzyme and 1 µl of M-MuLV Reverse Transcriptase enzyme add 1 µl of an RNA virus and 1.25 µl of red phenol reagent. Place this 25 µl volume of the reaction mixture in a thermocycler for 50 minutes at 50 ° C. Occurrence of RT-LAMP reaction was shown as ladder-shaped behavior on 1.5% agarose gel (Figure 4).

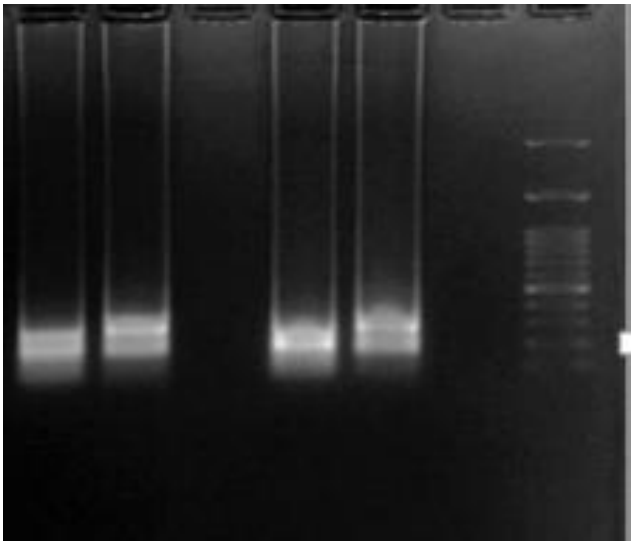


Figure 4. Ladder-shaped behavior of RT-LAMP products on 1.5% agarose gel.

3.1.4. Optimization of pH indicator for visual detection of RT-LAMP.

Indicator optimization was performed using the slope of the buffer volume from the highest volume (Bsm DNA polymerase reaction buffer = 2.5 µL) to the lowest volume (Bsm DNA polymerase reaction buffer = 0 µL). It was observed that when we do not use the reaction buffer, the color change before the reaction and after the reaction is quite obvious. Electrophoresis of the samples showed that the reaction occurred well with all buffer volumes used in reactions. Even when the buffer was not used, the ladder-shaped result of electrophoresis on 1.5% agarose gel confirms it (Figures 5 and 6).

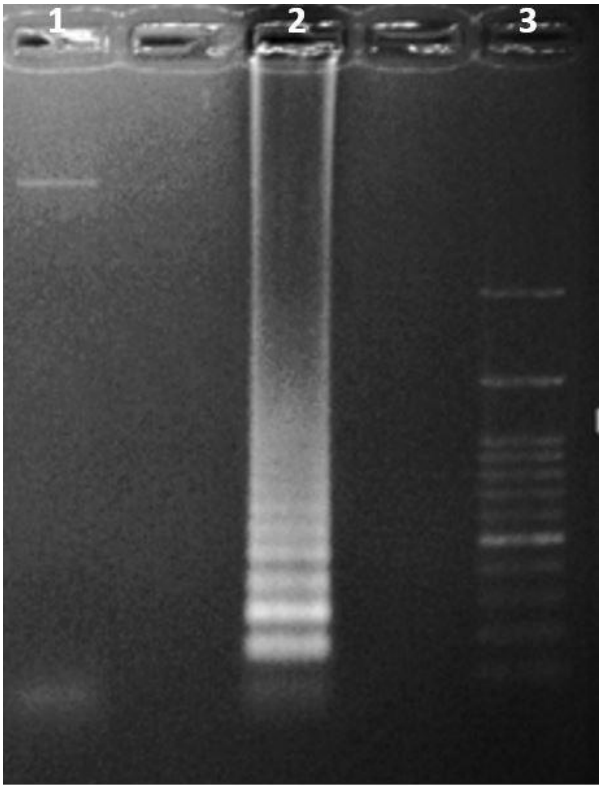


Figure 5. Ladder-shaped behavior of RT-LAMP products on 1.5% agarose gel (lane 2); Lane 1, Negative result of RT-LAMP; Lane 3, Gene ruller.

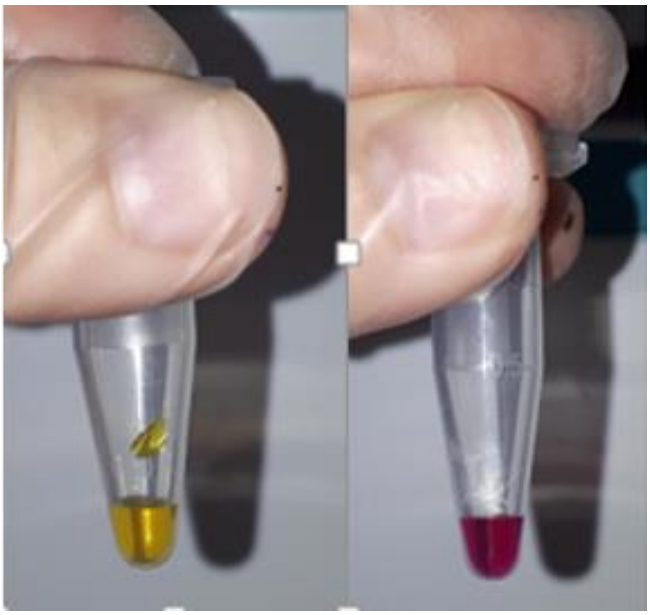


Figure 6. Preparation of pH indicator (left, acidic pH; right, alkaline pH).

3.1.5. RT-LAMP of captured viral RNA on magnetic-nanoprobe.

Conventional RT-LAMP, RT-LAMP with added MNP, and RT-LAMP with added MNP, and additional CB3 primer took place. As can be seen, the reaction is well performed in all three modes, and the ladder-shaped result of electrophoresis on 1.5% agarose gel confirms it (Figure 7).

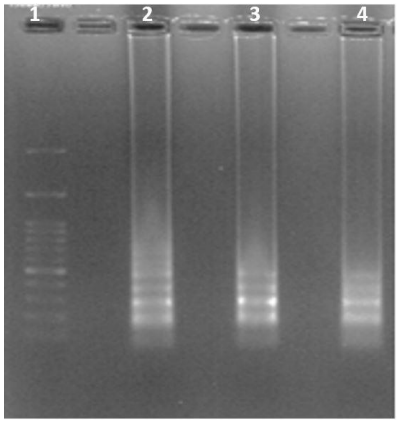
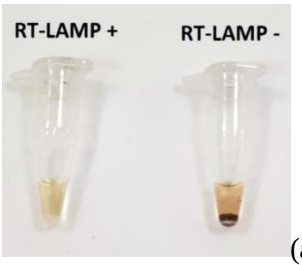


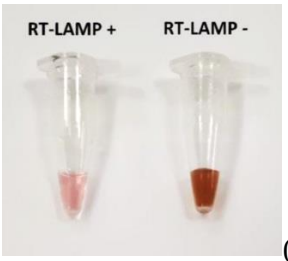
Figure 7. Electrophoresis of RT-LAMP products (lane 2), RT-LAMP products with magnetic nanoprobes (lane 3), RT-LAMP products with magnetic nanoprobes, and additional CB3 primer (lane 4); lane 1, gene ruler.

3.1.6. RT-LAMP-based Detection of viral RNA via magnetic nanoprobes with the naked eye.

The reaction is well performed in the presence of phenol red and magnetic nanoprobes with the concentration in the previously mentioned protocol. The color change of the reaction from pink to yellow, a sign of a positive reaction, is quite evident (Figure 8). The ladder-shaped electrophoresis result on 1.5% agarose gel confirms it (Figure 9).



(**a**)



(**b**)

Figure 8. Visual detection of RT-LAMP reaction result. (**a**) Before performing the RT-LAMP reaction; (**b**) After performing the RT-LAMP reaction.

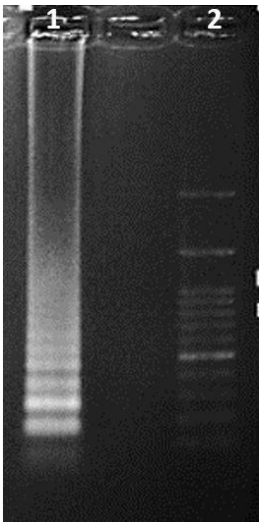


Figure 9. RT-LAMP electrophoresis result on 1.5% agarose gel (lane 1); Lane 2, gene ruller.

3.1.7. Limit of detection (LoD) of viral RNA using LAMP.

The reaction is performed in all dilutions prepared from the RNA; electrophoresis results confirm this.

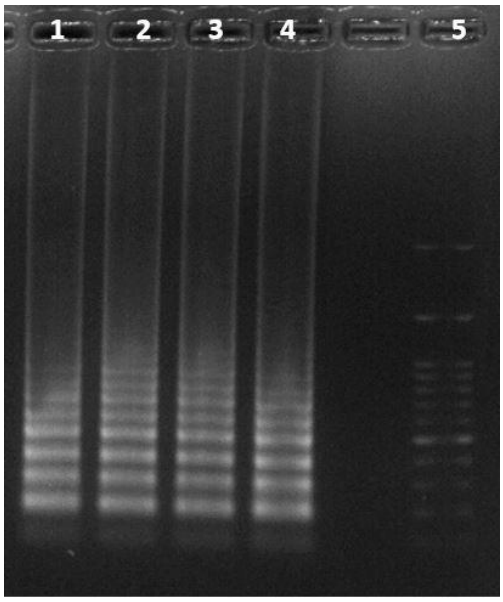
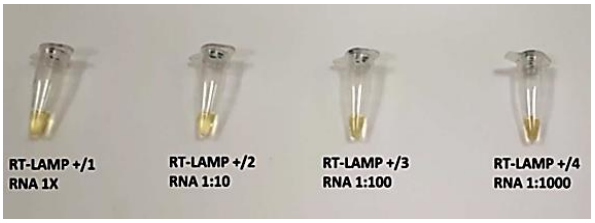


Figure 10. Electrophoresis results of RT-LAMPs (limit of detection tests) using RNA dilutions (lane 1, 1:1000 dilution, lane 2, 1:100 dilution, lane 3, 1:10 dilution, and lane 4, 1:1 dilution); Lane 5, gene ruller.

(**a**)



(**b**)

Figure 11. Checking out the limit of detection using visual analysis. (**a**) Before reaction: microtubes containing 1 X, 1:10, 1: 100 and 1: 1000 of RNA; (**b**) After reaction: Microtubes containing 1 X, 1:10, 1: 100 and 1: 1000 of RNA.

3.2. Discussion.

The concern for corona diagnosis has been to move towards rapid diagnosis with high sensitivity and accuracy. In all methods, nucleic acid-based methods for corona detection are usually preferred to serological tests because serological tests require at least one to two weeks of infection for antibodies to be produced and detected [25]. Based on nucleic acid tests, the disease can be diagnosed in the first week of infection. So nucleic acid-based methods are preferred [26]. In nucleic acid-based methods, the gold standard is the RT-PCR method because it is easy, accessible, and user-friendly. However, we still have two major concerns about RT-PCR in corona detection: 1. low Sensitivity and False Negative 2. Accuracy and false-positive [27-29], although this is the gold standard, they endeavored to get FDA-approved methods to enter the field of corona diagnosis. In the meantime, RT-LAMP has already been one of the methods that can be tested in diagnosing other viral diseases such as influenza, MERS, Ebola, and Zika [30, 31]. RT-LAMP has been one of the methods that can compete with the gold standard, the RT-PCR method [18, 32-33]. Due to the occurrence of the Corona pandemic, many scientists in the field of nanomolecular diagnostics turned to the RT-LAMP method for diagnosing COVID-19. DNA production in RT-LAMP reaction was detected by measuring

the turbidity or fluorescent dyes, etc. [6, 9] until the use of pH indicator dyes to detect the presence of the virus's genetic material became available [22]. pH indicators have a priority in rapid detection because they create the property of visual detection [34], so their methods went towards visual detection. Still, their work had shortcomings, for example, the need for an RNA or DNA purification kit, Lack of accuracy, etc. In this study, we used a nanoprobe to shorten the RNA or DNA purification part of the process, which can specifically detect the S gene region of the viral RNA, then enter directly into the RT-LAMP reaction and perform the reaction, so no RNA purification kits are required, so several elution steps are eliminated. To identify the positive or negative result of the RT-LAMP reaction without the need for instruments and a trained person, we used a visual detection method based on pH indicators such as phenol red, where the color change indicates a positive reaction and no color change indicates a negative reaction [22, 35].

Available methods for nucleic acid detection include non-isothermal methods and isothermal methods. The use of the PCR method has limitations due to the need for thermocyclers and specialists and the relatively long time to achieve test results, reminding us of the need for other methods. The isothermal amplification method can be used to save time because it eliminates the need for a thermal phase, and we can achieve more copies of the target nucleic acid in less time. The simplicity of these methods offers excellent potential for the development of manual DNA diagnostic devices that can be used to detect pathogens at a point of care. Using designed sequences attached to the magnetic nanoparticles also makes it possible to identify and isolate the target nucleic acid from the sample with high sensitivity and specificity. Considering the current state of the world caused by coronavirus in societies, rapid and accurate diagnosis of the disease will help to control and reduce it. The limitations of PCR, such as the relatively long time it takes to get a test result, as well as the possibility of false results [36], prevent many patients from being diagnosed or mistreated by healthy people, which in turn impairs the healing process and causes resistance to the virus. Achieving a way to detect the coronavirus specifically and in a short period is very important. In this way, after taking a sample by identifying the nucleic acid of the virus and the appearance of color marks and observing them with the naked eye, we can easily find out the presence or absence of the virus in a short time. Thus a lot of time and money will be saved, and patients will be identified faster. While COVID-19 diagnostic methods, which are based on the detection of virus nucleic acid, have attracted a great deal of attention, the cumbersome and time-consuming process of sample processing has made it difficult to use these methods because, in these methods, extraction of high purity virus nucleic acids is a prerequisite for the continuation of assay steps and low extraction efficiency can act as an inhibitor of amplification reactions and also lead to false negatives [37, 38].

Currently, in the control and detection of SARS-CoV-2, RNA extraction methods use rotating silica columns that utilize a silica membrane or fiberglass to bind nucleic acids. In these traditional methods, one step is required to lyse the viral particle in a suitable buffer to release nucleic acids from the virus before attaching to the column membrane. Several centrifugation steps are performed to bind, wash and separate the extracted nucleic acids. In addition, due to the use of toxic salts and organic solvents in the lysis and extraction steps, several wash steps are required to ensure the removal of substances that can have an inhibitory effect on the amplification reaction. The purification column can also become clogged or contaminated. In general, the rotating column approach is laborious and time-consuming due to several centrifuges and column transfer; it is not done automatically, and many specialized

operators are needed to work with it. Finally, due to their low diagnostic efficiency, they are unsuitable for monitoring and controlling sudden outbreaks such as COVID-19.

Rapid, convenient, and automatic nucleic acid extraction methods are desirable in the molecular diagnosis of SARS-CoV-2 and the monitoring and prevention of other infectious diseases. As an alternative, magnetic nanoparticle-based extraction methods (MNPs) have been introduced that do not require centrifugation, are easy to work with, and perform the separation process automatically and with high efficiency [39-41]. In conventional methods based on magnetic nanoparticles, the nucleic acids in the lysed samples can be adsorbed specifically on the magnetic nanoparticles due to the functional groups located on the nanoparticle surface. They are separated in the supernatant. After rapid washing steps to remove impurities, purified nucleic acids can be released from the surface of magnetic nanoparticles by washing buffer. Although magnetic nanoparticle-based extraction strategies are much simpler and faster than rotary column-based methods, they still involve several processing steps such as lysis, bonding, and rinsing, which pose challenges in clinical diagnosis [42].

4. Conclusions

In the recent method, the nucleic acid is separated using magnetic nanoparticles whose surface is functionalized. This separated genetic content enters the amplification reaction as a magnetic nanoparticle-nucleic acid complex. This method reduces the time, risk of contamination, and the possibility of false negatives. In this study, we isolated RNA from the sample using magnetic nanoparticles attached to a probe that can detect viral RNA and perform the RT-LAMP amplification isothermal reaction. By using the indicator, we see the presence of RNA virus in the sample as a change in color. Due to the ease and no need to have special skills, this protocol can significantly reduce the complex steps before molecular detection of COVID-19 at the molecular level. Previous protocols include steps such as lysis and binding buffer, adsorption, removal of supernatants, desorption, and RNA sample collection for RT-PCR. While in this research protocol, the process of purification of genetic content from the sample is performed, and MNP's-RNA complexes can be used directly for amplification in the LAMP process. This protocol does not involve any centrifugation process. Since this proposed protocol does not involve centrifugation, the RNA extraction protocol can be performed automatically. Because of the simple and cost-effective nature of this protocol, it could be a viable alternative to conventional techniques in the future instead of dealing with time-consuming methods that need trained personnel and complex devices. Thus, it demonstrates its application in the active detection of COVID-19. In addition, performing a single-step RT-LAMP reaction and identifying the reaction product through colorimetric and visual analysis makes this method a good candidate for the miniaturization methods of molecular detection reactions on microfluidic substrates. This study aimed to obtain a simple, fast, sensitive, and cost-effective molecular detection method for diagnosing the SARS-CoV-2 virus. The results of this study demonstrated that the LAMP amplification method showed a faster rate of amplification reaction than the RT-PCR method. This method eliminates the need for complex equipment such as a thermocycler. Due to the addition of magnetic-probe nanoparticles to the main composition of the RT-LAMP reaction, the time required to perform the complex steps of gene separation has been eliminated, the detection speed has been increased, and the purification and separation efficiencies have been boosted. The LAMP reaction was detected by adding phenol red as a pH indicator in the main composition of the RT-LAMP reaction, and color change was analyzed. Also, the accuracy of this diagnosis was confirmed using gel

electrophoresis, which indicates that there are no complex devices or operators for detection and that it is complex and time-consuming to analyze. Due to the use of primers designed to identify specific parts of the target sequence, this method has such high specificity. Using a concentration gradient of RNA in the RT-LAMP reaction and analyzing the results by electrophoresis showed that the sensitivity of this method is very high because, at the lowest concentration (1: 1000), the bands obtained in the gel-electrophoresis did not decrease in intensity. The reaction RT-LAMP is well performed at this RNA concentration, which is confirmed by the change in color of the reaction medium at all concentrations prepared.

Funding

This research was funded by the Vice-Chancellor of Research and Technology, Mazandaran University of Medical Sciences, grant number 7250.

Acknowledgments

The authors thank the personnel of the central Covid-19 Laboratory for their collaborations with the Vice-Chancellor of Health Affairs, Mazandaran University of Medical Sciences.

Conflicts of Interest

The authors declare no conflict of interest.

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