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Nanomolecular Magnetic Probe for Detection of Nucleic Acid Sequence-Based Amplification of Covid-19 RNA with the Naked Eye

Shokoofeh Moghadasi [1], Adele Rafati [2], Mohammad Reza Haghshenas [3], Pooria Gill [3,*]

[1] Department of Medical Nanotechnology, Faculty of Advanced Technologies in Medicine, Mazandaran University of Medical Sciences, Sari, Iran; shmoghadasi@yahoo.com (S.M.);
[2] Department of Medical Nanotechnology, Immunogenetics Research Center, Faculty of Advanced Technologies in Medicine, Mazandaran University of Medical Sciences, Sari, Iran; rafati.adele@gmail.com (A.R.);
[3] Department of Microbiology and Virology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran; haghshenas2001@yahoo.com (M.R.H.);
* Correspondence: p.gill@mazums.ac.ir, pooriagill@yahoo.com (P.G);

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Abstract: Nucleic acid amplification of Covid-19 RNA is the main subject for molecular detection of SARS-COV-2. However, the employment of target amplification methods such as PCR needs a converting step for Covid-19 RNA to DNA template to be amplified. In addition, Covid-19 RNA isolation needs some RNA extraction kits that their providing could increase the time and costs for the molecular detection of the virus. In this study, we introduced a magnetic nanoprobe that could be used to capture and amplify Covid-19 RNA through an isothermal amplification process, so-called nucleic acid sequence-based amplification, without needing to perform a separate step for the viral RNA converting to DNA template. By using engineered sequences appropriate to the target nucleic acid attached to the magnetic nanoparticles, identifying the target RNA from the virus could be possible by clumping the particles that could be seen with naked eyes. According to the isothermal amplification of the viral RNA via nucleic acid sequence-based amplification assisted with the magnetic nanoprobe, the nanomolecular method eliminated the need for special pieces of equipment and the time for detection of Covid-19 in specimens.

Keywords: magnetic nanoprobe; Covid-19 RNA; nanomolecular detection.

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1. Introduction

Nanoparticles have unique physicochemical properties that are very different from conventional materials, especially the electromagnetic properties of magnetic nanoparticles (MNP). These characteristics have attracted the attention of many researchers to investigate their potential biomedical applications, such as targeting and separating various molecules in analysis methods [1-7]. MNPs can be easily separated from the mixed components by a magnet. Due to the properties of MNPs, it is possible to collect MNPs exactly in the desired location by applying an external magnetic field [1].

MNPs can bind to ligands through protein or nucleic acid probes for specifically identifying the targets. As a result, the functional nanoparticles can be used to capture or purify microorganisms and then separate their compounds from other components under the influence of magnetic forces. Functional MNPs have been used for protein and DNA isolation, molecular

biological assays, and pathogen detection and isolation [7-10]. These nanoparticles can also be used in the development of biological nanosensors. For example, Perez *et al*. have been used MNPs coated with antibodies against virus surface proteins to detect viral particles in solution [11]. MNPs can also combine directly with DNA or RNA probes can be used for isolation and concentration of the genome materials [10]. RNA separation could be improved by magnetic separation technology, and hence the separation time and costs could be reduced [12].

Magnetic particles with immobilized affinity ligands (e.g., streptavidin, antibodies, glutathione, protein A, protein G, etc.) are commercially available for designing some magnetic affinity-based techniques for absorption and separation methods for biomolecules that those are usually quick and do not require expensive equipment (such as chromatography systems, centrifuges, filters, etc.) [12]. According to this idea, we described here a nanomolecular method for capturing Covid-19 RNA and then detecting the results through naked eyes (Figure 1). For amplifying the captured RNA, a magnetic oligonucleotide probe could be employed as one of the primers in nucleic acid sequence-based amplification (NASBA). In addition, the magnetic probe could be used for identifying the positive tests from the negative ones for Covid-19 RNAs.

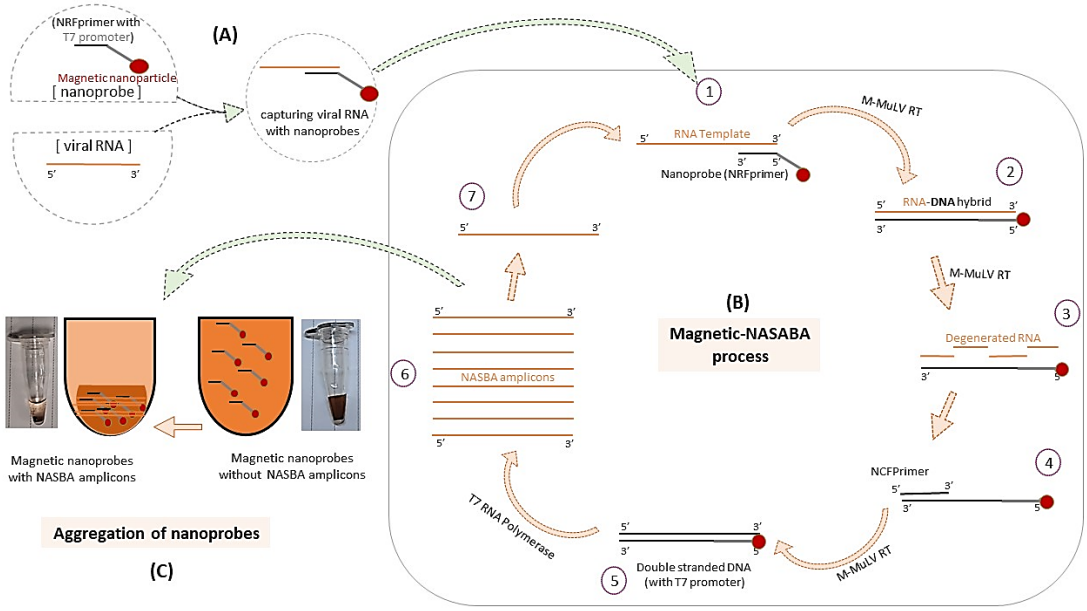

Figure 1. **(A)** capturing viral RNA with nanoprobes: Viral RNA is adsorbed to the nanoprobe when it is placed in the vicinity of prepared nanoprobes containing magnetic nanoparticles and NRF primer due to the presence of complementary sequences. **(B)** Magnetic-NASABA process: **1)** RNA template targeted with primer 1 (nanoprobe) participates in the process. **2)** The primer is annealed to a specific sequence, and complementary DNA is made by reverse transcriptase against early RNA. **3)** RNA-DNA hybrid is treated with M-MuLV reverse transcriptase (M-MuLV RT), and the primary RNA is degenerated**. 4)** Primer2 is annealed to a specific sequence of recently synthesized single-stranded DNA, and the complementary DNA strand is made by M-MuLV RT. **5)** Double-stranded DNA is created with a T7 promoter, which acts as a self-sustained pattern. Then, using this DNA as a template, T7 RNA polymerase synthesizes sense target RNAs. **6,7**) Each synthesized RNA can re-participate in these steps and produce more RNA amplicons.

2. Materials and Methods

2.1. Chemicals and instruments.

NTP Mix was purchased from Invitrogen by Life Technologies™ (USA). RiboRuler Low Range RNA Ladder and T7 RNA Polymerase were bought from Thermo Scientific (USA). Streptavidin-coated magnetic nanoparticles (fluidMAG-Streptavidin) with a diameter

of 100 nm were purchased from Chemicell (Germany). M-MuLV Reverse Transcriptase was bought from SinaClon Co Ltd (IRAN).

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 NASBA amplification, the oligo primers were synthesized by Bioneer Co. Ltd (South Korea) as follows:

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

Reverse 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'

2.3. Preparation of magnetic nanoprobe.

For preparing the magnetic probe,100 µl of streptavidin-coated magnetic nanoparticles was mixed with 4 µl of the reverse primer, and the mixture was shaken at room temperature for 20 min. Then the mixture was placed on a magnetic plate, and it was washed twice with free nuclease water.

2.4. Nucleic acid sequence-based amplification of viral RNA.

NASBA was performed in a final volume of 25 µl containing 2 mM NTPs, 13 % V/V DMSO (Sigma, Germany), 1 mM dNTPs, 9mM MgSO4, 0.5 µM reverse primer, 0.5 µM forward primer, 5 mM DTT (Thermo Scientific, USA), 1 µl RNA, 40 U T7 RNA polymerase, and 8 U reverse transcriptase. First, RNA and the enzyme-free reaction mixture were incubated for 5 minutes at 65 °C. After adding RNA and the enzymes to the mixture, the mixture was incubated for 90 minutes at 41 °C.

2.5. Nucleic acid sequence-based amplification of captured viral RNA on magnetic nanoprobe.

This reaction mixture was also prepared at 25 µl total volume contained 2 mM NTPs, 13 % V/V DMSO (Sigma, Germany), 1 mM dNTPs, 9mM MgSO4, 0.5 µM reverse primer, 0.5 µM forward primer, 5 mM DTT (Thermo Scientific, USA), 1 µl RNA, 40 U T7 RNA polymerase, and 8 U reverse transcriptase. First, RNA and enzyme-free reaction mixture were incubated for 5 minutes at 65 °C. After adding RNA, the enzymes, 1 µl of the prepared magnetic nanoprobe to the mixture, the mixture was incubated for 90 minutes at 41 °C.

2.6. Detection of nucleic acid sequence-based amplification of viral RNA via gel electrophoresis.

The NASBA products were analyzed by 2% agarose gel (containing GelStain-Green, Bioneer Company, South Korea) via electrophoresis in 1X TAE buffer.

2.7. Detection of nucleic acid sequence-based amplification of viral RNA via magnetic nanoprobes.

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

2.8. Limit of detection (LoD) of viral RNA using NASBA.

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 NASBA process was performed. Also, the serial dilution of the viral RNA was amplified with the NASBA assisted with magnetic probes.

3. Results

3.1. Visualization of RNA amplicons of NASBA process via gel electrophoresis.

For visualization of the NASBA amplicons, the electrophoretic behavior was checked via horizontal gel electrophoresis experiment Figure 2). NASBA amplicons from RNA with ~200 nt length were seen via the gel stained with gel stain green viewer fluorescent dye using UV-illuminator gel documentation system.

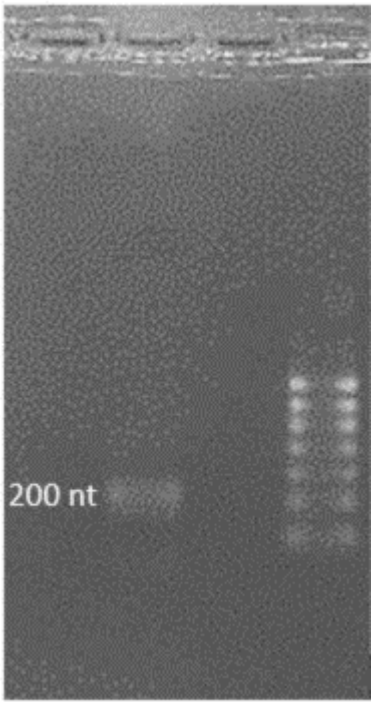

Figure 2. Gel electrophoresis of ~ 200 nt NASBA product (left band) and RiboRuler Low Range RNA ladder (right bands, 100, 200, 300, 400, 600, 800, 1000 nt).

3.2. RNA amplicons of NASBA assisted with magnetic nanoprobe.

RNA amplicons obtained from NASBA assisted with magnetic nanoprobe were visualized using gel electrophoresis experiment (Figure 3). The amplicons were seen with ~200 nt weight near to the equal band of RNA ladder in 2% agarose gel.

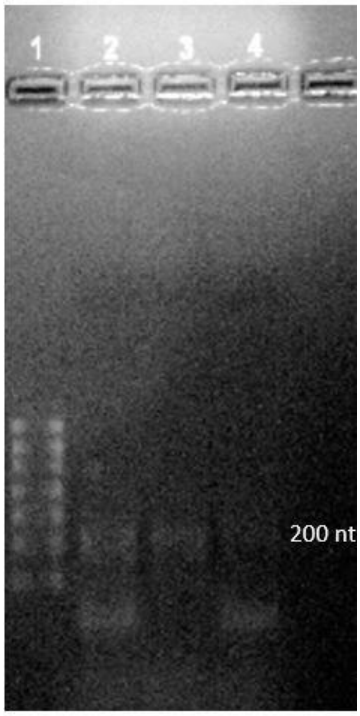

Figure 3. Gel electrophoresis of RNA amplicons amplified through NASBA (lane 3) and NASBA-assisted with magnetic nanoprobe (lane 2) near to RNA ladder (lane 1, 100, 200, 300, 400, 600, 800, 1000 nt)); Lane 4, as negative control.

3.3. Visualization of NASBA amplicons assisted with magnetic nanoprobes.

NASBA products of the reactions contained 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, and 0 ng viral RNA templates were checked using a gel electrophoresis experiment. The results showed positive bands up to NASBA reaction contained 10 ng RNA template (Figure 4). Also, no bands were seen in the NASBA reaction with no (or 0 ng) RNA template.

Figure 4. Results from the NASBA process using 100 ng (lane 2), 10 ng (lane 3), 1 ng (lane 4), 0.1 ng (lane 5), and 0 ng (lane 6) RNA templates; lane 1, RNA ladder.

NASBA reactions with magnetic nanoprobes contained 100 ng, 10 ng, 1 ng, 0.1 ng, and 0 ng viral RNA templates were checked with the naked eyes and photographed. The results showed positive bands up to NASBA reaction contained 0.1 ng RNA template (Figure 5). Also, no changes were seen between the reactions with no (or 0 ng) RNA template.

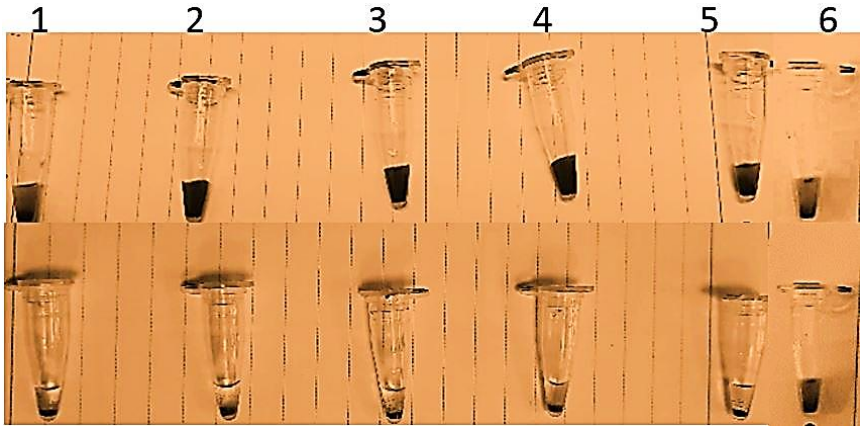

Figure 5. Visual colorimetric changes of the NASBA reactions before (top) and after (down) interactions with the nanoprobes with 100 ng, 10 ng, 1 ng, 0.1 ng, and 0 ng RNA templates (1st, 2nd, 3rd, 4th, 5th, and 6th microtubes).

4. Discussion

Nowadays, nucleic acid amplification is used as one of the molecular methods to increase the sensitivity and speed of detection [13]. Because these techniques detect specific sequences of nucleic acid, nucleic acid-based diagnostic tests can be performed to diagnose any infection that affects humans [14]. Nucleic acid amplification tests (NAATs) are the most common diagnostic tests used to diagnose pathogens, and many current SARS-CoV-2 detection techniques are primarily based on NAATs. The most common type of NAAT is polymerase chain reaction [15, 16]. PCR is a method for the detection of a wide range of pathogenic microorganisms and is considered the "Gold standard" for testing with high sensitivity and specificity [15, 17, 18]. PCR is used to amplify the target DNA sequence to detect deoxyribonucleic acid (DNA). Another type of experiment, called Reverse Transcriptase-PCR (RT-PCR), was developed to amplify RNA targets; In this technique, reverse transcriptase (RT) is used to convert the target viral RNA to complementary DNA (cDNA) and then amplify the resulting cDNA by conventional PCR. RT-PCR has been used since its development to detect human infection caused by RNA viruses [14, 17, 18]. Currently, the RT-PCR method is used to confirm COVID-19 infection. Although RT-PCR is the most widely used method for diagnosing SARS-CoV-2 infections, it requires expensive laboratory equipment and highly skilled laboratory personnel, relatively high costs associated with the purchase of equipment, maintenance and reagents, and a long lead time. The results as well as the existence of false positive and false negative results, are the limitations of this method. As a result, a number of companies and laboratories worldwide are working to improve further the efficiency of RT-PCR technologies and the development of various other techniques [15, 17-21]. In this regard, other techniques NAATs were developed as isothermal amplification methods and are being developed as a suitable alternative to PCR to improve COVID-19 molecular detection techniques [22, 23]. Isothermal amplification methods, unlike PCR, require only one temperature. So the need for thermal cycles is eliminated. These methods are generally fast and sensitive and can be easily implemented in service locations or in conditions where resources are limited).

NASBA is specifically designed to detect RNA [14] and used to detect other coronaviruses (SARS-CoVs) in previous outbreaks. NASBA generally uses three enzymes (Reverse Transcriptase, RNase H, and T7RNA polymerase) and two primers (reverse primer and forward primer) [15, 23, 24]. NASBA uses single-stranded RNA amplifiers. The generated

product is used directly in another round of amplification, which results in exponential amplification of the target nucleic acid and is a major advantage of the NASBA reaction over PCR, which increases the sensitivity because the whole enzyme Participants in this reaction are most active in the temperature range of 41 ° C. The NASBA reaction can produce more than one copy of the target RNA in 90 minutes at a constant temperature of 90 ° C without the need for thermal cycle steps [23, 25]. The use of the NASBA isothermal reaction allows It can be propagated in any portable device that can maintain a constant temperature of 41 °C [26]. Therefore, it has a high potential for use in POC. It is widely used to detect microorganisms in different types of samples, for example, clinical, environmental, and food samples, and the main product of the NASBA reaction, which is single-stranded RNA, can be detected by various methods, such as gel electrophoresis, ethidium bromide staining, enzyme-linked gel assay (ELGA), electrochemiluminescence detection (ECL) and also using molecular beacon [21, 24]. Also, real-time detection of duplication is possible by isothermal methods, and amplified products are detected by measuring turbidity or visual inspection for discoloration [24].

Conventional nucleic acid extraction techniques involve many steps that are long and sensitive to impurities. Magnetic nanoparticle-based (MNP) extraction techniques can be used to improve these conditions [13]. Magnetic separation techniques are usually rapid and do not require expensive equipment. Then, in the presence of magnetic fields and after the washing steps, most of the impurities in the supernatant are separated [13, 27]. MNPs can be combined directly with biological molecules. DNA or RNA can be isolated and concentrated using MNP-grafted selected oligonucleotides. RNA isolation functions are improved by magnetic separation technology, and separation time (and RNA degradation probability and cost are reduced. Recent advances in the use of nanoparticles with oligonucleotides and changes in their surface properties have paved the way for the development of new detection systems. It is possible to design nanoparticle probes used in molecular detection assays with numerous advantages compared to conventional assays [28]. In general, the primer and probe systems used can greatly increase the sensitivity of a molecular method Affect.

This study tried to introduce a cost-effective method with high sensitivity and specificity for coronavirus RNA detection. In the introduced Magnetic-NASABA method, a specific nanoprobe was used simultaneously to detect viral RNA and participate in the NASBA reaction to amplify viral RNA. To perform the NASBA reaction, the RNA calibrator used in the reaction was fabricated. In our study, according to the results, NASBA reaction using 2 enzymes Reverse Transcriptase and T7RNA polymerase instead of using 3 enzymes (Reverse Transcriptase, RNaseH, and T7 RNA polymerase) according to the common NASBA protocol, without any disturbance and quality reduction was performed. In the introduced method, M-MuLV Reverse Transcriptase enzyme was used, which acted as Reverse Transcriptase enzyme and showed the function of RNase H enzyme, thus simplifying the reaction. The results also showed that the NASBA reaction could be performed in the presence of nanoprobes. Nanoprobes prepared from magnetic nanoparticles and NRF primer, in addition to increasing the sensitivity and specificity of the target method by specifically identifying the target RNA, were able to participate directly in the NASBA reaction as one of the primers due to their primer content; therefore, the primer used in the nanoprobe retained its primer property to participate in the reaction, thus eliminating the need to use a separate NRF primer to perform the reaction.

In addition, by comparing the status of the nanoprobes in the reaction mix, before and after the reaction, it was observed that by tapping the desired microtubes, the nanoprobes were displaced in the form of clots in the microtubes where the reaction was performed; whereas in pre-reaction microtubes and non-reaction microtubes, the nanoprobe was dispersed in the reaction mix after hitting the microtube. As a result, the presence or absence of a reaction can be observed by observing the state of the nanoprobe. The detection limit of the Magnetic-NASBA method was evaluated using different concentrations of RNA for the reaction, and it was observed that the reaction was performed well using a minimum concentration of RNA per reaction. Also, by comparing the electrophoresis results of the NASBA and Magnetic-NASBA reactions, it was observed that the band formed for the Magnetic-NASBA reaction had better brightness and clarity; In addition, the possibility of eye detection in the Magnetic-NASBA method based on the observation of the nanoprobe status shows the superiority of the Magnetic-NASBA method over NASBA.

Various studies have been performed to diagnose COVID-19. For example, Anirudh Chakravarthy and colleagues developed a method called the PHAsed NASBA-Translation Optical Method (PHANTOM) for direct and hypersensitive diagnosis of coronavirus and its salient variants. In this method, RNA extracted from the virus was amplified isothermally using nucleic acid sequence amplification (NASBA) and detected by toehold-based biosensors in an in vitro transcription-translation assay (IVTT). NASBA's reaction with transcription-translational measurement results in producing a color that can be easily seen with the naked eye or with a cell phone camera or luminescence, which can be measured by luminometry. This diagnostic method is able to detect 100 copies of viral RNA by dyeing. The results showed that instead of spending 2 hours performing the NASBA reaction, it was possible to perform the reaction in 60 minutes while reaching the detection limit (i.e., 100 copies of viral RNA). Also, with faster luminescence detection, it was possible to reduce the assay time to 90 minutes or better [29].

In another study, Qianxin Wu and colleagues presented a two-stage diagnostic strategy for Coronavirus 2019 using a barcoded isothermal NASBA reaction called the Isothermal NASBA-Sequencing based high-throughput Test (INSIGHT). It combined point-of-care diagnosis with next-generation sequencing (NGS) to achieve population-scale testing. The first stage was the INSIGHT NASBA reaction, which can produce fast test results in 1 to 2 hours, and the second stage used NGS to improve test accuracy further and reduce user errors. The second stage can naturally serve as a confirmatory test for the patient's first stage results. The results showed that LoD-95 was defined as the amount of viral RNA input in which 95% of the samples were detectable. Also, in the experiments of stage 1 and stage 2, no false-positive result was observed [30].

Among the studies on viral RNA extraction using magnetic nanoparticles for the detection of SARS-CoV-2 is the study by Zhen Zhao and colleagues, which synthesized polyamine esters with group-coated magnetic nanoparticles. Reported carboxyl (PC) and the development of a viral RNA extraction method based on pcMNPs for the sensitive detection of COVID-19 by RT-PCR. They stated that the virus lysing and RNA binding steps could be performed in one step. The pcMNPs-RNA complexes could be introduced directly into subsequent RT-PCR reactions, which provides a fairly simple RNA extraction protocol. Also, pcMNPs have excellent viral RNA binding performance, resulting in a sensitivity of 10 copies and a line above 5 gradients in detecting SARS-CoV-2 viral RNA using RT-PCR [13].

Another study conducted by Sandeep B. Somvanshi and colleagues on viral RNA extraction using magnetic nanoparticles to detect SARS-CoV-2 provided the RNA extraction protocol using ferritic zinc nanoparticles whose surface by Silica and polymers containing carboxyl were functionalized for the detection of COVID-19. They stated that MNP's-RNA complexes could be used directly to amplify the RT-PCR process. Since this process does not involve any centrifugation process, the same RNA extraction protocol can be performed automatically [27].

Different studies have used coronavirus gene sequences to increase the specificity of the method [26]. The coronavirus-specific spike gene (S gene from Severe Acute Respiratory Syndrome Coronavirus 2) synthesized RNA calibrators and designed oligo primers (NCF and NRF) for the NASBA reaction. The design of a specific primer, in addition to having primer properties and the ability to participate in the NASBA reaction, made it possible to attach the primer to magnetic nanoparticles and create nanoprobes, which was effective in the specific detection of coronavirus nucleic acid and thus the need for acid extraction. The coronavirus nucleus was removed. The Magnetic-NASABA diagnostic method is reliable, but More research is needed on clinical specimens. The speed and accuracy of this technology can help diagnose faster and be effective in reducing complications and mortality from the disease.

According to the results obtained for our study, using the introduced Magnetic-NASBA method, simultaneous and specific propagation and detection of the target has become possible. Coronavirus RNA was accurately and reliably identified and isolated using a magnetic nanoprobe consisting of magnetic nanoparticles with a surface-modified sequence corresponding to the nucleic acid nucleus (NRF primer) in a short time without the need for a cycle. In thermal applications, by the NASBA isothermal reaction, the number of considered nucleic acid versions increased. Using this method, it is possible to amplify the desired nucleic acid with higher accuracy and sensitivity in less time compared to a process such as PCR. Also, the presence or absence of the reaction could be easily detected by visually observing the status of the nanoprobe after the reaction. In this way, coronavirus nucleic acid was identified and amplified simultaneously and completely; therefore, it is possible to save time and money, and the diagnosis of the disease will be faster and more accurate, which in turn will help to control and reduce the disease. Consequently, medical and pharmaceutical costs will be reduced. It should be noted that the simplicity and isothermal nature of this method offer great potential for the development of diagnostic devices that are used to diagnose pathogens point-of-care.

5. Conclusions

Recent advances in the use of nanoparticles with oligonucleotides and changing their surface properties have paved the way for the development of new detection systems. It is possible to design optically and chemically labeled nanoparticle probes used in molecular detection assays with numerous advantages compared to conventional assays. A new set of diagnostic applications was made possible by the advent of nucleic acid amplification technologies. One of these very suitable technologies for RNA amplification (NASBA) is Nucleic Acid Sequence-Based Amplification. The primer and probe systems used can greatly affect the sensitivity of a molecular method. In the method introduced, at the same time, a specific nanoprobe is used to detect viral RNA and also the NASBA process to amplify viral RNA. The possibility of identifying the virus by the NASBA process seems good, but more research is needed on the clinical specimen. Nevertheless, the speed and accuracy of this technology can help diagnose faster and thus reduce complications and mortality.

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Conflicts of Interest

The authors declare no conflict of interest.

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