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REVIEW: A Review on the Efficiency of NASBA Molecular Technique for Diagnosis of Acute Toxoplasmosis in Pregnant Mothers

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ABSTRACT

Introduction: Toxoplasmosis is a worldwide disease caused by an intracellular protozoan called Toxoplasma gondii and in mothers who become infected during pregnancy, it can cause serious damage to the fetus by passing through the placenta. The aim of this study was to review the effectiveness of the NASBA molecular technique in diagnosing the acute form of Toxoplasmosis in pregnant mothers.

Material and Methods: In this study, the websites of PubMed, Google Scholar, SID, Magiran, Web of Science, IranDoc were searched and articles related to the title have been reviewed from 1990 to 2020.

Results: Nucleic Acid Sequence-Based Amplification (NASBA) is an isothermal method that has the processes of nucleic acid extraction, amplification, and identification of amplified products. This technique is based on transcription and is specifically used for RNA amplification, so it is highly specific in identifying living and active microorganisms. All steps in this amplification method are performed at 41 °C and the amplified products can be identified by appropriate detection methods such as electrochemical luminescence (ECL).

Conclusion: Since all steps of amplification are performed by NASBA at the same temperature of 41°C, unlike molecular PCR technique, a thermocycler is not required, so setting it up will not cost much for laboratories and it can be useful in providing a suitable solution for toxoplasmosis screening in pregnant mothers.

Introduction

Toxoplasmosis is caused by an intracellular and forced protozoan called Toxoplasma gondii, which has a worldwide spread and is one of the most common causes of infection in humans (1-3). Seroepidemiological studies show anti-Toxoplasma antibodies in the blood serum of one third of the world's adult population, indicating its spread and contagious potential for humans (3, 4). Based on the geographical climates of Iran, Toxoplasmosis is most prevalent in the coastal areas of the Caspian Sea, so that its highest prevalence in Iran is in the northern temperate regions. However, it has been recorded to a lesser extent in cold mountainous as well as in warm regions,

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which indicates the importance of sufficient humidity and temperature for oocyte survival and parasitic infectivity (5, 6). Congenital toxoplasmosis infection is caused by the transmission of the parasite from mother to the fetus, which can potentially lead to neurological defects in the baby, choriotinitis and even miscarriage. They do so to identify infected mothers and provide early treatment (7). A pregnant mother is able to transmit the infection to the fetus throughout the entire pregnancy period, however in late pregnancy there is greater possibility of transmitting the parasite through the placenta but less likely to develop complications in the fetus (8). Transmission of the infection to the fetus occurs in mothers who become infected for their first time during pregnancy (9, 10). Thus, acute and primary infections during pregnancy pose a major diagnostic challenge (2). Because the clinical signs of toxoplasmosis are varied, they may be confused with other diseases, so the use of laboratory diagnostic methods is essential to confirm clinical diagnoses (11). Prenatal diagnosis of congenital toxoplasmosis provides the basis for its treatment and leads to an informed decision about whether to continue or terminate the pregnancy (12). Over the past decades, the development of nucleic acid experiments has led to significant advances in the diagnosis and evaluation of the effect of treatment against the pathogen (13).

The NASBA method, introduced by Compton in 1991, is used specifically for RNA amplification. This technique involves three enzymes called Reverse Transcriptase, RNaseH and T7RNA Polymerase, as well as two types of primers, one of which contains the T7 promoter at its 5' end (14). NASBA is a single-temperature technique (41°C) that provides 109 copies of amplified RNA in 1-2 hours. This method of amplification, unlike the PCR technique, does not require a thermocycler; therefore, it can be done in laboratories with limited facilities. Because using a simple heat block or incubator, the required conditions for nucleic acid amplification are satisfied (10, 15). It also requires fewer cycles for amplification compared with PCR. When we use PCR, the amplified fragments are doubled at each stage, and it takes at least 20 cycles to amplify about one million fragments, but at NASBA, at each transcription stage, 100-1000 copies of RNA are produced. So with 4-5 reproductive cycles around one million RNAs are amplified (16). The technical knowledge of manufacturing NASBA technique identification kits is the exclusive property of the French company Biomeriux, and for the first time in 2004 with a new approach by the Department of Medical Biotechnology of Tarbiat Modares University, this invention was recognized and formed in the Islamic Republic of Iran (17). Considering the mentioned advantages, it can be said that NASBA method is a cost-effective molecular technique and can help in quick and easy detection of pathogens, even in laboratories that are limited in terms of facilities (18).

The aim of this study was to introduce the NASBA technique and evaluate its effectiveness in identifying pregnant mothers as soon as possible who have infected with toxoplasmosis during their pregnancy. So that with early detection, better and more informed decisions can be made about whether to continue or terminate the pregnancy.

Methods

In this unsystematic review article, data were collected using the keywords NASBA (nucleic acid sequence-based amplification), diagnosis, toxoPlasmosis, Congenital, Acute, toxoplasmosis, congenital, acute and a combination of them from databases. PubMed data, SID and Google scholar search engine were collected. In this study, published studies from 1990 to 2020 that were related to the title of the article were reviewed.

Results

Introduction to NASBA technique

NASBA Molecular Technique is a method

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that has the processes of nucleic acid extraction, amplification and identification of amplified products. This technique is based on transcription (18, 19). In this method, the first primer is attached to its target sequence on the template RNA. The primer has about 20 bases at its 3' end, which complements the target sequence, and at its 5' end has a T7 promoter sequence. After binding of the first primer, the reverse transcriptase (RT) enzyme forms a DNA strand from the RNA strand. The product of this reaction is a DNA-RNA hybrid which is sensitive to the enzyme RNaseH and this enzyme destroys DNA-bound RNA results a single strand DNA. Then the second primer binds to this single stranded DNA. The reverse transcriptase enzyme in this condition, with its DNA Dependent DNA-Polymerase activity, converts the single stranded DNA (ssDNA) to double stranded DNA (dsDNA). Because this double-stranded DNA has a T7 promoter sequence in one end, the T7RNAPolymerase enzyme starts the transcription process by identifying this promoter region and produces a large number of RNAs (about 1000-100 copies of each strand). Each of these amplified RNAs can now be patterned and this cycle repeated (18-22). Since all the enzymes participating in this technique are most active in the temperature range of 41 °C, all steps of this reaction are performed in this exact temperature range and thus NASBA is classified as a single temperature technique (***Figure 1***) (18).

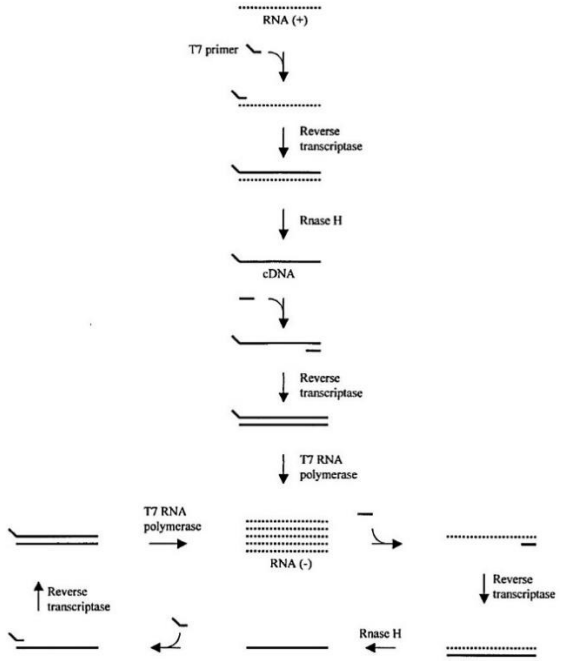

Figure 1. Schematic representation of the replication process in the NASBA technique

RNA extraction

The method of Boom et al. (1990) can be used to extract RNA. In this method, cells are first destroyed by a lubricating buffer containing guanidium isothiocyanate (GuSCN) to release RNA. Then the silica suspension is added. Released RNAs under high-salt conditions adhere to existing silica particles, which act as solid phases. Then, DNA removes from the solution by DNase, and the silica particles washed twice with buffer to remove proteins and inhibitors (twice with 70% alcohol, and once with acetone, respectively). After centrifugation, the resulting silica precipitate to which the RNAs are attached is dried at 56 °C and finally the RNAs are extracted using column chromatography and a suitable buffer (22, 23).

The reason for using RNA amplification in NASBA technique

The NASBA technique has the ability to identify living cells through mRNA replication. When living cells are to be evaluated, RNA amplification is more appropriate than DNA because DNA is more stable, is double-stranded and can be separated from dead cells for a long time. While mRNA molecules are semi-stable so they have a shorter lifespan (about a few minutes) and decompose immediately after cell destruction. So, mRNA detection can be used to indicate cell viability (14, 24). One of the problems that researchers always face in RNA replication is the contamination of the sample with DNA. Because DNA is more stable than RNA, it stays in the solution of the microorganism for a long time, so if the RNA sample contains DNA contamination, in the PCR technique, this DNA can be amplified instead of or at the same time as RNA. And lead to a false positive answer. In addition to

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exons, the genome of eukaryotic cells also contains introns, whereas in transcriptional RNA, introns are removed during the processing and exons are sequentially linked together. Accordingly, if the primer is designed to complement a region of two consecutive exons, that is, one part is paired on one exon and the other part is paired with an adjacent exon sequence, then this primer can never be inserted into DNA having an intron sequence between exons. And therefore, the presence of DNA in solutions containing purified RNA does not lead to a false positive because DNA is not able to amplify under these conditions (25). On the other hand, due to the use of low heat (41 ° C) in NASBA technique, this technique is not affected by DNA contamination of the sample because at 41 ° C it is not possible to separate the two strands of DNA, so under these conditions the primer does not bind to DNA and reproduction never take place (15).

Primer Design

In NASBA, RNA transcription and replication are performed by the enzyme T7RNAPolymerase, so it is necessary to add a specific promoter sequence for this enzyme to one of the primers. The presence of the T7 promoter sequence at the 5' end of one of the primers (preferably the first or forward primer) is essential because the relevant enzyme for RNA transcription and replication identifies and binds to this promoter sequence. In general, the binding site of two primers in nucleic acid should be protected and specific, and the distance between these two sites should preferably be between 80-200 nucleotides (18). The forward primer sequence has three sections: the RNA template hybrid section, the section containing the T7 promoter sequence, and the section between the hybrid region and the T7 promoter sequence. The presence of several purine bases (adenosine and guanosine) in the intermediate section of the first primer is essential because it increases the efficiency of the enzyme for RNA transcription. The hybridization part of the primer should be about 20-30 bases (preferably 20 bases) and 40-40% of these bases should include the G/C base composition (18, 26).

Disadvantages of NASBA

Since it is not possible to increase the reaction temperature to more than 41 °C to stabilize the enzymes participating in the NASBA technique and prevent their denaturation, nonspecific reactions are likely to occur in NASBA. In most studies, this problem has been solved by using different types of detection methods such as electrochemical luminescence (ECL) methods, enzyme hybridization labeled in ELISA and labeled probes in real-time NASBA, so that by using these methods, possible non-specific products are eliminated in the identification stage and the diagnosis of the pathogen is not perturbed (27-30).

Discussion

Congenital toxoplasmosis usually occurs when the mother comes in contact with the parasite for the first time during her pregnancy, that is, in the acute form of toxoplasmosis, which, usually does not cause problems for her if the immune system is healthy but can be dangerous to the fetus. Congenital toxoplasmosis is one of the most important fetal diseases and its serum prevalence in general populations and pregnant women in Iran has been reported 39.2% and 41%, respectively. Infection of the fetus is through the parasite crossing the placental barrier, so the parasite must be in the active form of tachyzoite to be able to cross the barrier (7, 10, 31). The thicker the placental barrier, the lower the probability of parasite passage, so the probability of parasite passage in the first trimester of pregnancy is the lowest (10-25%) and the highest (90-60%) in the third trimester of pregnancy due to thinning of the placenta. The risk of fetal infection increases with increasing the duration of pregnancy, but the likelihood of seeing complications in the fetus is reversed (10). Currently, the most common way to diagnose the acute form of toxoplasmosis is to detect IgM antibodies using serological

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tests, but there are two problems with using serological tests: one is that many false positive results can occur in these tests, and the other is that IgM antibodies can persist for months or even years, complicating the determination of whether or not the infection is acute, leading to misinterpretation of test results and thus abortion (3, 32, 33).

NASBA amplification, which is a single temperature and a sensitive method, was initially used to detect infection with RNA viruses. *Kievits et al.* first used this method to replicate RNA of HIV-1 in 1991 (21, 34). Since 1995, the technique has been combined with the Electrochemical Luminescence Detection (ECL) method into commercial kits for HIV-1 virus detection (18).

Since this technique is specifically designed for RNA replication, it has only the ability to identify living and active microorganisms by mRNA replication. Because mRNAs have a very short half-life and are destroyed immediately after cell death. Therefore, identification of mRNAs indicates the viability and activity of the microorganism and confirms the severity of the infection (14, 15, 18, 24, 35, 36).

The results of a study by Nowruzi et al. in 2011 with the aim of detecting a live parasite of Toxoplasma gondii by NASBA method in rats showed that this technique was effective for amplifying RNA purified from the tachyzoite form of the parasite, so it can be used for rapid diagnosis of toxoplasmosis in infants (16). Recently, the NASBA technique has been used for parasites such as *Plasmodium falciparum*, *Trypanosome sp.*, and *Leishmania sp*. (22, 29, 37-40). A 2005 study by *Schneider et al.* compared the real-time QT-NASBA technique with the real-time QT-PCR in determining the amount of Plasmodium falciparum parasite showed that this technique reduce analysis time and risk of contamination while keeping the sensitivity and specificity (29). In another study, Niazi et al. examined the sensitivity and specificity of the NASBA method for molecular diagnosis of cutaneous leishmaniasis. In this study, NASBA method and real-time PCR method for detecting Leishmania parasite in skin wound samples suspected of leishmaniasis were compared. NASBA sensitivity was 81% and Real Time PCR sensitivity was 51%, but both methods were 100%specific. They concluded that the NASBA technique with high sensitivity and specificity can be used to detect cutaneous leishmaniasis (41).

Conclusion

In general, since all steps of the NASBA technique are performed at the same temperature of 41 °C, unlike molecular PCR, does not require a thermocycler, so setting it up will not cost much for laboratories. Finding complete information about this technique can be useful in future studies and provide appropriate strategies for screening toxoplasmosis in pregnant mothers.

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Conflicts of interest

Authors declare that there is no conflict of interests.

Authors' contributions

All authors have intellectually committed to the study design and process. The final manuscript was revised and accepted by all authors.

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