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REVIEW

Nucleic Acid Sequence-Based Amplification in Diagnosis of Disease Pathogens

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CONFLICTS OF INTEREST

THERE ARE NO CONFLICTS OF INTEREST FOR ANY OF THE AUTHORS.

ABSTRACT:

Nucleic acid sequence-based amplification (NASBA) is a simple technology which is used to amplify nucleic acid isothermally. The template is mainly RNA, can also be DNA. Combined with molecular beacon assay, NASBA is widely used to diagnose virus, bacteria, fungus, protozoa and other pathogens. NASBA has comparable sensitivity and specificity to PCR, but it's complicated to be applicable as it could not be integrated efficiently with relevant detection techniques. Here, we summarize the development of NASBA, especially its application in infectious disease diagnosis based on previous study, which provide reference for developing NASBA technology to detect trace amount of disease pathogens.

INTRODUCTION

There are many causes of diseases. Microorganism with small size, large quantity and a great variety is a pivotal pathogenic factor of diseases. Microorganism infects animals or plants and leads to contagion which is difficult to cure. Those infectious diseases have great negative influence on human life. Diagnosis is a series of means to judge the causes of diseases, which is the prerequisite for disease prevention, treatment, prognosis and monitoring. PCR is the most common method for microbial molecular diagnosis, with high specificity and sensitivity. At present, a series of technologies has been developed based on the principle of PCR. And one of them is NASBA, a simple technology which is used to amplify nucleic acid isothermally with short thermodynamic cycles, simple operation, high sensitivity and specificity. However, NASBA is limited in development due to high cost, complicated methods and incompatible with related detecting technologies. In this paper, we reviewed the principle, characteristic, development and the application of NASBA technology based on previous research, and discussed its publicity in diagnosis with great prospect.

1. Principle and Characteristic of NASBA

Nucleic acid sequence-based amplification (NASBA), which is also called self-sustained sequence republication assay (3SR)[3], is a kind of simple technology that depends on a pair of specific primers, and continuously amplifies nucleic acid at constant temperature. A NASBA reaction mixture

includes T7 RNA polymerase, RNase H, avain myeloblastosis virus (AMV) reverse transcriptase, primer P1 and P2, ribonucleotides, deoxyribonucleotides, template and buffer. The three kinds of enzymes participate in transcription, hydrolysis and reverse transcription, respectively. The 3' end of P1 is complementary to the template, and its 5' end contains promoters identified by the T7 RNA polymerase. P2 is complementary to the 5' end of template sequence. The NASBA reaction is similar to RT-PCR (reverse transcription-polymerase chain reaction), which is composed of cyclic and non-cyclic stages. In non-circular stage, with primer P1 complemented to single stranded RNA template, AMV reverse transcriptase catalyzes the synthesis of cDNA to form hybrid molecular RNA-DNA. RNase H targets and hydrolyzes phosphate diester bond of RNA coming from the RNA-DNA hybrid molecule. The remaining single stranded DNA anneals with primer P2, AMV reverse transcriptase then catalyzes the synthesis of the other single stranded DNA. Subsequently, the promoters of double stranded DNA are recognized by T7 RNA polymerase, transcription occurs and RNA forms at the ratio of 1:100. The transcriptional RNA serves as target, and cyclic stage begins.

NASBA amplifies RNA at the speed of 100^n, so targets can achieve an amplification of 10^9 fold in 1-2 hours[13]. Compared with PCR whose amplification speed is 2^n, NASBA greatly improves the amplification efficiency. The NASBA reaction is carried out at 41℃, under this circumstance, external double stranded DNA without T7 promoter does not undergo thermal denaturation nor transcription. Certain contaminants, such as DNA, heparin, EDTA, citrate, haemoglobin, albumin, and lipids, won't disturb the amplification process without thermal denaturation. So it is suitable to directly diagnose clinical samples, for instance, throat swab, blood, excrement, etc[13]. Besides the high specificity of NASBA, different scholars found that the high sensitivity of NASBA varied in different objects, but was similar to PCR. The isothermal process of NASBA does not require special instruments, which could fit conditions short of equipments[11]. Portable NASBA testing instrument makes it possible for on-site diagnosis[12]. It is not only suitable for detection of single stranded RNA, but also for double stranded nucleic acids (DNA and RNA) and sticky end nucleic acid. So it is widely used in the detection and sequencing of RNA.

On the other hand, limitations still exist in NASBA's application. Firstly, the extracted nucleotides are not single stranded nucleotides in most cases, and the secondary structure must be destroyed by incubating thermostatically at 65℃ for 2-5 minutes, then 41℃ for 2-5min in order to anneal to primers[5]and[13]. Then, the enzyme mixture is added, the reaction runs, and the process occupies 90min generally. The time is comparative with conventional PCR. Secondly, comparing with a new PCR invented in recent years, which accomplishes amplification within 15min, NASBA is time consuming and complicated to operate. Thirdly, three kinds of enzymes lead to high cost by NASBA even without molecular beacon or molecular hybridization. At the same time, although NASBA combined with molecular beacon assay is used most widely in quantification and qualification, but the whole experimental process is complex. Overall, NASBA is costly and complicated in operation to some extent.

2. Development of NASBA

J. C. Guatelli et al described the way in vitro amplifying nucleic acids isothermally depent on T7 RNA polymerase, RNase H and AMV reverse transcriptase, which was called 3SR in 1990. They also detected HIV-1 and preliminarily confirmed 3SR could be used to quantify and qualify special nucleic acid[1]. In 1991, Canadian scholar J. Compton named the assay with nucleic acid sequence-based amplification (NASBA). At the same time, his team optimized conditions, tested the amplification and evaluated sensitivity of NASBA for detecting HIV in plasma. They found NASBA could minimally detect 3 HIV particles in 100 microliter sample, verified the assay was specific to HIV[3]. The same year in December, Netherlandish Kievits T et al optimized the methods to extract nuclic acids from epidemical pathogens and turned NASBA to large-scale application[4]. In 1993, Gabrielle M E et al extended NASBA application

from virus to bacteria by detecting 16S rRNA of mycobacterium[5]. Organon Teknika, a company from Netherland, released NucliSens HIV-1 QT assay derived from NASBA in 1998[6]. After that, the company and BioMiSens launched a series of products including NucliSens Extractor kit[9] or NucliSens easyMAG[14]and[19] to extract nucleic acids, NucliSens Basic Kit for amplification[26]and[31], NucliSens EasyQ assay for molecular beacon analysis[11], and NucliSens miniMAG assay for electro chemiluminescent analysis[10]. The most widely used NASBA kit at present is NucliSens EasyQ assay which could detect Listeria, vibrio cholera, salmonella and so on[11]. At the same time, NABSA combined with a variety of detection technology to diagnose pathogens, including agarose gel electrophoresis[3], an enzyme-linked gel assay (ELGA)[5], electro chemiluminescent (ECL) labeled probes[20], biological fluorescence detection[7], Northern blot[8], enzyme-linked immunosorbent assay (ELISA)[1], molecular beacon, etc. Invention of kits including basic items for diagnosis simplified pathogen detection with NASBA. Targets range from HIV to all kinds of microorganism monitored by quantification or qualification of pathogens by real-time NASBA. Additionally, there are integrated microfluidic NASBA technology[34], immune NASBA[1], multiplex NASBA[18] and [32], etc.

During the period of 2001-2010, the research and application of NASBA reached a climax, such as detection of microbial species, integrated methods and the development of NASBA, such as multiplex NASBA. However, from 2011 to now, the research of NASBA has decreased with limited breakthrough in technology. It may due to the new invention and widely use of PCR, or the limitations of NASBA itself. Looking for cheap substitutes of the three kinds of enzymes to reduce the cost of NASBA, designing a easy-to-use kit, and weaving a NASBA integrated instrument are urgent problems to be solved in the development of NASBA.

3. Application of NASBA in Diagnosis

NASBA is widely used in pathogen diagnosis of virus, protozoa, fungus and bacteria. As HIV, Plasmodium, Aspergillus, Mycoplasma pneumoniae are common pathogenic microorganisms which are seriously threatened to human health, the application of NASBA provides reliable means to detect and prevent those diseases.

3.1. Immuno-deficiency Viral Disease Diagnosis, HIV-1

Human immunodeficiency virus (HIV), a single strand positive RNA virus, belonging to retroviruses family, lentivirus subfamily, was first diagnosed in 1981. With high mutation rate, high recombination rate and high replication rate, a large number of HIV variant strains could be produced rapidly. So far no effective cure has been found and there is no effective vaccine available for HIV prevention. HIV mainly attacks human T cells after infection, resulting in immune deficiency, which causes a series of infections, cancer, and eventually leads to death. HIV-1 is most common worldwide. There are four groups of HIV-1 (M, N, O, and P), and M group which has caused the global epidemic of HIV/AIDS was divided into many subtypes, named subtype A-D, E-H, J and K, and circulating recombinant form (CRFs), named CRF_A/B/C.

Nucleic acid was extracted from the blood of AIDS patients (whole blood, plasma, serum, blood, peripheral blood mononuclear cells) or reproductive tract samples. The substrate was added according to the NASBA reaction system, and isothermal amplification was run. Amplified products were analyzed by agarose gel electrophoresis or ECL or other methods. Internal or external standards could be used to detect different subtypes. For example, molecular beacon technique achieves real-time quantitative detection by establishing internal standards Qa, Qb, Qc combined with different ECL probes[20]. NucliSens EasyQ HIV-1 combined NucliSens HIV-1 QT with the molecular beacon assay, NucliSens EasyQ analyzer, for real-time detection of targets[21]. Nowadays, it's the main clinic method that extracting nucleic acid from dried blood spots (DBS) with NucliSENS EasyMAG and qualifying, quantifying with NucliSENS EasyQ HIV-1 V2.0[14] and [19]. LTR-based NASBA was modified based on NucliSens HIV-1 QT with only one internal standard, to diagnose M, N, O groups of HIV-1 specially[19]. Ultra-NucliSens suits

low concentration of pathogen samples with ultra-high sensitivity[22]. Multiplex NASBA which based on molecular beacon probes could detect HIV-1 and hepatitis B virus (HBV) simultaneously with sensitivity of 98% and specificity of 100%[18]. Overall, the application of NASBA greatly improves the diagnosis speed and accuracy of HIV-1.

3.2 Diagnosis of Plasmodium, a Parasitic Animal Disease

Female anopheles mosquito bite human body, inject parasite plasmodium into human bloodstream and lead to malaria. Four Plasmodium species, P. falciparum, P. vivax, P. ovale, and P. malariae, cause human malaria. Plasmodium grows in erythrocyte and breaks the cell, releasing merozoite and metabolic products. Morbidity and death rate of malaria are reduced rapidly at present, but it was one of the most dangerous infectious diseases in history. Malaria transmission depends on asexual parasites, and the quantitative density of gametocyte in peripheral blood cells is an essential risk assessment for malaria. A traditional way to qualify malaria and its gametophyte is blood smear microscopy. However, the sensitivity could not reach requirement for early infection patients or patients under treatment at the late stage. QT-NASBA and qRT-PCR are two methods developed to measure low concentration samples[23-25]. QT-NASBA is widely used in malaria diagnosis since 2000. The common template is 18S rRNA of ribosome small subunit. Pfs16 and Pfs25 mRNA is used specifically to diagnose faleiparuma mlaria, and Pvs25 mRNA is used specifically to diagnose Vivax malaria. To achieve realtime quantification, adding synthetic internal standard in the lysate of nucleic acid extraction, amplified wild type RNA and synthetic internal standards according to the NucliSens Basic Kit, then analyzed and obtained experimental data by NucliSens EasyQ analyzer. This method not only can diagnose 4 kinds of Plasmodium, but also can detect their gametophyte and analyze the parasite qualitatively[26].

Compared with microscope observation of blood smear microscopy and antigen detection, NASBA has higher sensitivity. While compared with semi-quantitative PCR, NASBA has better specificity, and is easier to operate. There are also some studies found that QT-NASBA reproducibility is worse than qRT-PCR[24], but still can effectively detect trace amount of Plasmodium in early diagnosis and late stage observation. The volume of parasite is big enough for microscopy observation which is easier and less time consuming. So NASBA is more suited for prognosis and monitoring the effective of drugs in parasitic diseases.

3.3 Diagnosis of Fungal Disease, Aspergillus

Aspergillus belonging to filamentous fungi can be parasitic on skin and upper respiratory tract. Aspergillus fumigatus and Aspergillus flavus are most common Aspergillus causing human diseases. Aspergillus is a kind of opportunistic fungus. Normal people have a certain resistance to Aspergillus, but immunocompromised patients are very sensitive to it. It is an important cause of morbidity and mortality in patients with immune dysfunction. Aspergillus mainly infects lung and respiratory tract, also infects skin and mucosa. A large number of hyphae can be found in the lesions. Those hyphae penetrate blood vessels can cause vasculitis, perivascular inflammation, thrombosis, and tissue ischemia, necrosis. Severe sepsis may occur, so that other tissues and systems involved. Some Aspergillus even causes cancer.

Early diagnosis plays a key role in the treatment of Aspergillosis, but it's not easy. Galactomannan (GM) is symbol of fungal infection. Galactomannan enzyme-linked immunosorbent assay（GM-ELISA）based on above-mentioned principle is short of specificity, and is prone to give false positive results. In recent years, NASBA-ELISA was used as diagnose assay in developed countries. Comparative studies proved that sensitivity of NASBA-ELISA is higher than qPCR and GM-ELISA, and its specificity is lower than qPCR with small difference[27-28]. Real-time-NASBA（RTi-NASBA）is widely used as well with 18S rRNA as target, and molecular beacons probe combining with primers makes real-time monitoring possible[29]. In 2011, fluorescence resonance energy transfer（FRET）combined with NASBA successfully achieved real-time detection[30]. However, molecular beacon is still the mostly used

method with relevant diagnostic kit, apparatus and analysis software.

3.4 The Diagnosis of Bacteroidal Disease, Mycoplasma Pneumoniae

Mycoplasma pneumonia is the smallest bacteria which can live independently, and causes mycoplasma pneumonia, a kind of primary atypical pneumonia. It is a common pathogen to induce pneumonia. Patients are most kids and teenagers. It spreads by oral and nasal secretions through air droplets at the acute stage, causing respiratory tract infection. In clinic, Mycoplasma pneumonia, Legionella pneumophila, Chlamydia pneumoniae and Rickettsia and other atypical pathogens cause primary atypical pneumonia. And mycoplasma pneumonia is the main pathogen of community-acquired pneumonia.

The NASBA assay for detecting mycoplasma pneumonia is similar to virus, fungus, and parasites. For example, extracting and purifying nucleic acid (16S rRNA) with NucliSENS easyMAG extraction system, amplifying with NucliSENS basic Kit, and analyzing with NucliSENS easyQ Director software[31]. In addition, there are large quantities of kits and analysis software to be used in diagnostic process of NASBA. Multiplex RTi-NASBA is applied successfully to diagnose mycoplasma pneumonia, chlamydophila pneumonia and legionella pneumophila causing pneumonia with designing different primers and choosing different molecular beacons according to the target bacteria[32]. But common NASBA is more sensitive than multiplex NASBA and PCR for mycoplasma pneumonia[33].

4. Perspective

Compared with PCR, NASBA has many advantages. First, the diagnosis speed of NASBA is much more quicker than PCR; Second, RNA is served as template so that external DNA has no interference, and DNA can also be template with another design; Third, external RNA could not be amplified with T7 RNA polymerase; Fourth, isothermal amplification technology leaves out thermodynamic cycles and could be achieved by cheap equipment. Compared with loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and rolling circle amplification (RCA), the primer of NASBA is short and easier to design, and the experiment can be carried out easily. By far, there is a series of NASBA diagnosis kits. QT-NASBA or RTi-NASBA combined with molecular beacon assay detecting online for disease diagnosis or monitoring and evaluation of treatment effect. There are ECL-NASBA, FRET-NASBA, and multiplex NASBA as well. Qualitative and quantitative analysis can be accomplished in accordance to the standard protocol of NucliSENS.

NASBA also has insurmountable defects. The use of three kinds of enzymes leads to high cost, so that NASBA is not suitable for long-term diagnosis. The operational process is complicated, and no instrument can be used to diagnose at one step. Unlike PCR whose reaction speed could be shortened easily, the complicated process of NASBA is very time-consuming. Integration between NASBA and means of diagnosis isn't very well, and the sensitivity and specificity is not outstanding in the detection of pathogens in trace amount.

In summary, NASBA is suitable for limited experiment conditions. External DNA and RNA should be clear away before NASBA detection. However, it will be the main method for HIV diagnosis. Looking for three cheap enzyme substitutes, design of a more convenient operation kit and the integration of NASBA with better detection instruments are the problems in NASBA development.

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