International Journal of Life science & Pharma Research

NUCLEIC ACID SEQUENCE BASED AMPLIFICATION (NASBA)- PROSPECTS AND APPLICATIONS

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ABSTRACT

Nucleic acid sequence-based amplification (NASBA) is a sensitive, isothermal, transcription-based amplification system specifically designed for the detection of RNA targets. In some NASBA systems, DNA can also be amplified. This amplification system uses a battery of three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H and T7 RNA polymerase) leading to main amplification product of single-stranded RNA. Expensive equipments are not necessary to acquire a high level of precision. NASBA is an established diagnostic tool in clinical use, with a theoretically bigger analytical sensitivity than reverse transcription-polymerase chain reaction (RT-PCR) for pathogen detection. It has a potential for detection of viable cells through selective amplification of messenger RNA, even in a background of genomic DNA, which PCR does not possess. In the future, NASBA could be used to identify and subsequently quantify microorganisms (even those which cannot be readily cultured) and would be very efficient as routine diagnostic procedures.

Key words: NASBA, TMA, 3SR, TAS, Molecular beacon.

1.0 INTRODUCTION

Nucleic acid amplification is a valuable molecular tool not only in basic research but also in application oriented fields, such as clinical medicine development, infectious diseases diagnosis, gene cloning and industrial quality control *etc*. (Fakruddin, 2011). Several amplification methods have been developed already, such as polymerase chain reaction (PCR) (Saiki *et al.,* 1985), self-sustained sequence replication (3SR) (Guatelli *et al.,* 1990), loop mediated isothermal amplification (LAMP) (Notomi et al. 2000), strand displacement amplification (SDA) (Walker *et al.,* 1992) and rolling circle amplification (RCA) (Lizardi *et al.,* 1998) *etc*. None of these methods can directly amplify RNA with high sensitivity. NASBA is a novel nucleic acid amplification method developed by Compton (1991) which can amplify RNA. In this article, we overview the current status of NASBA and applications of the method.

2.0 WHAT IS NASBA

Nucleic acid sequence-based amplification" (NASBA) **(**Compton, 1991**)**, also known as "self-sustained sequence replication" (3SR) **(**Guatelli et al., 1990**)** and Transcription mediated amplification (TMA) (Gill and Ghaemi, 2008) is a sensitive transcription-based amplification system (TAS) for the specific replication of nucleic acids *in vitro*. The complete amplification reaction is performed at the predefined temperature of 41^0C. Three enzymes are involved in this homogeneous isothermal reaction: avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 DNA dependent RNA polymerase (DdRp). Because of the integration of RT into the amplification process, the method is especially suited for RNA analytes like mRNA, rRNA or genomic RNA (Deiman et al., 2002).

3.0 CHARATERISTICS OF NASBA

1. A single step isothermal amplification reaction at 41^0C. 2. Especially suited for RNA analytes because of the integration of RT into the amplification process. 3. The single-stranded RNA product is an ideal target for detection by various methods including solution probe hybridization. 4. The fidelity of NASBA is comparable to that of other amplification processes using DNA polymerases lacking the 3' exonuclease activity. 5. The use of a single temperature eliminates the need for special thermocycling equipment. 6. Efficient ongoing process resulting in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product. 7. Unlike amplification processes, such as PCR in which the initial primer level limits the maximum yield of product, the amount of RNA product obtained in NASBA exceeds the level of primers by at least one order of magnitude. 8. NASBA RNA product can be sequenced directly with a dideoxy method using RT and a labeled oligonucleotide primer. 9. The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned. 10. Three enzymes are required to be active at the same reaction conditions. 11. Low temperature can increase the nonspecific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO. 12. A single melting step is required to allow annealing of the primers to the target. 13. The NASBA enzymes are not thermostable and thus can only be added after the melting step. 14. The primers are not incorporated in the amplicon and thus labeled primers can not be used for detection. 15. The length of the target sequence to be amplified efficiently is limited to approx 100 to 250 nucleotides.

4.0 PRINCIPLES OF NASBA

Isothermal nucleic acid amplification of RNA is achieved by the simultaneous action of avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 RNA polymerase and RNase H (Guatelli et al., 1990). Nucleic acid sequence based amplification (NASBA) was developed in the early 90s to amplify nucleic acids without the help a thermal cycler (Compton, 1991). This technique mainly used to obtain many copies of RNA starting from a few RNA molecules. This approach make use of two specific primers flanking the sequence to be amplified. The first primer (P1) carries the binding sequence for the T7 RNA polymerase at its 5' end and is used to initiate the RNA reverse-transcription (RT) reaction, catalyzed by a reverse-transcriptase. RNA strand in the RNA– DNA hybrid molecules resulted from the RT reaction was then degraded by RNAse H. The remaining cDNA is then accessible to the second primer (P2) which initiates the synthesis of the complementary strand. A third enzyme, the T7 RNA Polymerase, docks the double strand DNA on the sequence at the 5' end of P1, transcribing many RNA copies of the gene. This process, i.e. the cycle of first strand synthesis/RNA hydrolysis/second strand synthesis and RNA transcription, is repeated indeterminately starting from the newly transcribed RNA (Fig. 1). RNA and double strand cDNA accumulate exponentially and can be detected by EtBr/agarose gel electrophoresis. Recently, fluorescently labelled probes and a fluorescence scanner are employed to follow the NASBA real-time amplification of a viral RNA genome (Leone et al., 1998). The reaction is performed at a single temperature, normally 41°C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification (Sergentet et al., 2008). The kinetics of the reaction are mainly determined

by the efficiency of primer binding, which is dependent on the sequence and structure of the target RNA, and the extent of nonspecific product synthesis due to mispriming events (Fahy et al., 1991).

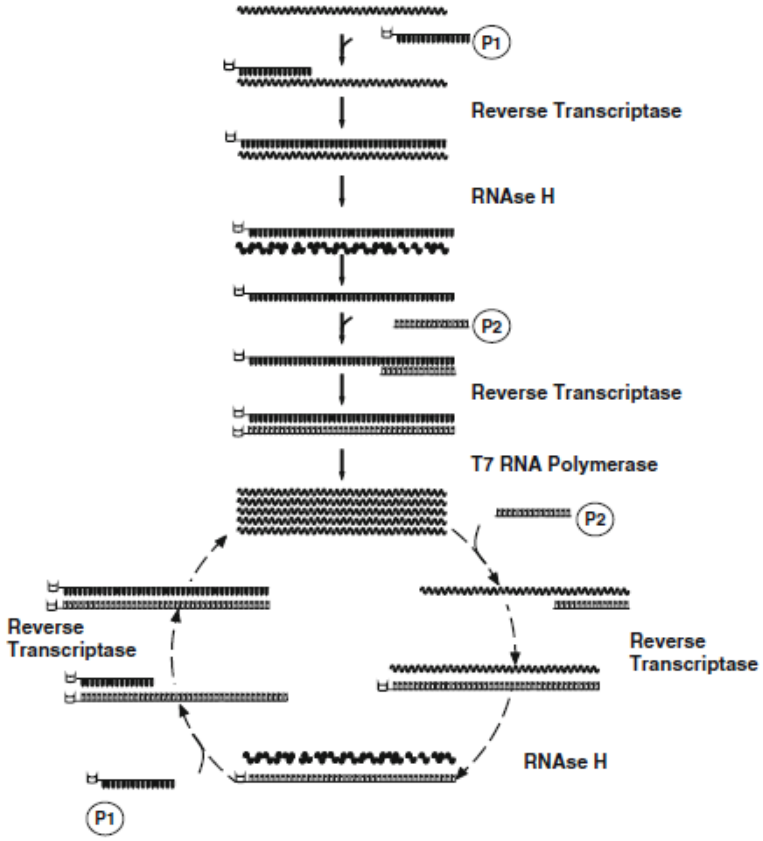

Figure 1: ***Principles of NASBA (Lauri & Mariani, 2009)***

5.0 PRIMER AND PROBE DESIGN FOR NASBA

The presence of a T7-promoter sequence at the 5' end of one of the primers, preferably at the 5' end of the "forward" primer is essential for NASBA. This sequence is used by the T7 DdRp at the end of the initiation phase and during the cyclic phase. The "reverse" primer may include a 5' nonspecific sequence that can be used for detection with a generic probe in ECL detection. Some rules have been published for the selection of the primer binding regions (Sooknanan et al., 1995). Primer and probe design rules have been summarized by Deiman et al. (2002). In general, the primer binding site should be conserved and specific for the target nucleic acid, the hybridizing part of the primer should be 20–30 bases, preferably 20, and the G/C composition should be 40–60%. The ability to form intramolecular and intermolecular structures should be avoided. The same rules apply to the hybridizing part of the probe sequence. The distance between the two primer binding sites is preferably 80 to 200 nucleotides.

6.0 OPTIMIZATION

NASBA assay must be optimized prior to application. First of all, the KCl concentration can be optimized in order to maximize primer specificity, sensitivity, and product yield. Generally, a high KCl concentration provides high specificity of primer set and target sequence (Malek et al., 1994). Too low concentrations may result in more primer-dimer

formation, as well as false positive results. Too high concentrations could inhibit the annealing of the primers to the target sequence. A 70 mM KCl concentration is suggested to be optimal for most primer sets. Uyttendaele *et al*. (1997) and Baeumner *et al*. (2003) used this concentration to detect respectively *Campylobacter jejuni* in food and *Escherichia coli* in drinking water. However, in other studies, authors utilized concentrations ranging from 50 to 90 mM (Blais et al., 1997; Min and Baeumner, 2002). The concentration of primer can also be optimized, but the majority of the studies used 0.2 μM (Min and Baeumner, 2002; Uyttendaele et al., 1999). Typical NASBA incubation times are 60-90 minutes to ensure optimal performance. In most of the studies, the time course of the amplification was 90 minutes (Blais et al., 1997).

7.0 DETECTION OF NASBA PRODUCTS

The product of NASBA reaction is mainly single-stranded RNA. Its detection was a labour-intensive procedure normally involving ethidium bromide (EtBr)-stained agarose gel electrophoresis. But to ensure product specificity, a confirmatory step, generally involving probe hybridization, was usually employed. However, the post-NASBA detection step has undergone significant improvements, such as the use of an enzyme-linked gel assay, enzymatic bead-based detection and electrochemiluminescent (ECL) detection and fluorescent correlation spectroscopy (Sergentet et al., 2008).

Agarose and polyacrylamide are the primary stabilizing media used in the electrophoresis of NASBA products. Because single-stranded RNA can form secondary structures, it must be electrophoresed in a denaturating system. The most frequently used denaturants for agarose gel electrophoresis of RNA are formaldehyde and glyoxal plus DMSO. The procedure for detecting RNA with ethidium bromide is similar to that for DNA, but Ethidium bromide does not stain RNA as efficiently as it does DNA (Jean et al., 2001; Jean et al., 2002).

Currently, the detection of NASBA products takes place at the end of amplification using electro-chemiluminescence (ECL) (Van Gemen et al., 1994). A specific capture probe, attached to magnetic beads via a streptavidin-biotin interaction, is used to immobilize the amplicon and a ruthenium-labeled detection probe hybridizes with the amplicon. A voltage induced oxidation-reduction reaction results in an ECL signal that is enhanced and detected by a photo-multiplier (Deiman et al., 2002).

Another detection method used for research and in-house qualitative NASBA reactions is the “enzyme linked gel assay” (ELGA) (Samuelson et al., 1998; Darke et al., 1998). In this technique, horseradish peroxidase (HRP)- labeled probes are used to detect the amplicon. The free and hybridized probes are separated by electrophoresis in a gel containing dextran sulfate. Incubation of the gel with a substrate for HRP results in visualization of the product.

Fluorescence correlation spectroscopy has also been used for detection in which a fluorescent labeled probe hybridizes to the amplicon and the increase in diffusion time is used to quantify the amplicon (Oehlenschlager et al., 1996).

The NASBA-like transcription mediated amplification procedure (TMA) uses acridinium ester-labeled DNA probes to detect the amplicon. Unbound probe is selectively inactivated by chemical means. A luminometer detects the light generated from the hybridized amplicons (Arnold et al., 1989).

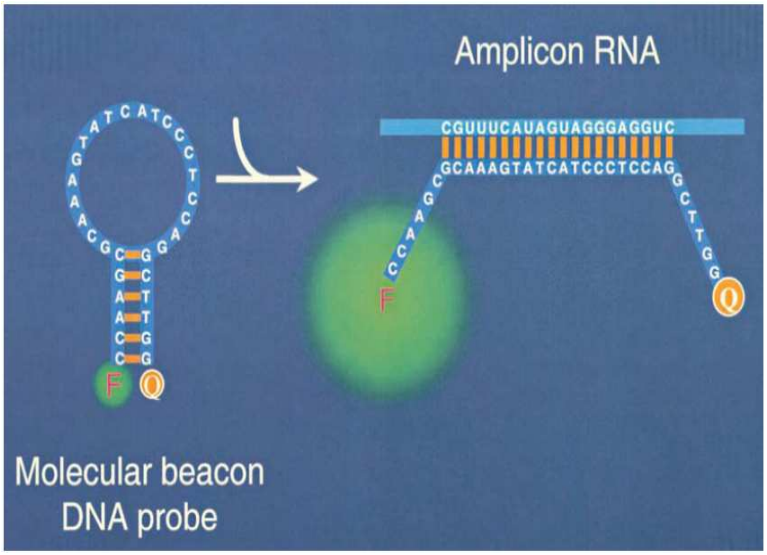

Figure 2. ***Molecular Beacon technology.***

A very elegant new method for the detection of NASBA products is the use of molecular beacons. Molecular beacons are DNA oligonucleotides labeled with a fluorophore at the 5' end and a quencher at the 3' end *(*Tyagi and Kramer, 1996*)*. The sequence of the very 3' end is complementary to that of the very 5' end and a hairpin stem is formed in such a way that the quencher absorbs the emitted light of the fluorophore (Fig. 2). The hairpin loop sequence is complementary to the target sequence of the amplicon. Because of the binding of the loop sequence to the target, the hairpin stem opens up and the quencher becomes separated from the fluorophore. The increase in light emitted can be detected by a fluorometer. Hybridization with the amplicon takes place during amplification enabling "real-time" detection to be performed. This not only shortens the total reaction time but also provides information about the kinetics of the reaction. As no extra detection step is required, the tubes remain closed and so carry-over contamination is prevented after amplification (Deiman et al., 2002).

8.0 ADVANTAGES AND DISADVANTAGES OF NASBA OVER OTHER AMPLIFICATION TECHNIQUES

NASBA is a technique with widespread applications in the area of RNA amplification and detection. The following are the advantages and disadvantages of NASBA.

(a) Advantages of NASBA

NASBA offers several advantages over other mRNA amplification methods. The amplification of nucleic acid sequence of more than 10^9 copies can be done in just 90 minutes by the three-enzyme action.1. NASBA is an isothermal reaction performed at 41°C, which obviates the need for a thermal cycler and may facilitate the production of point-of-test devices (Sergentet et al., 2008). 2. A major advantage of NASBA is the production of single-stranded RNA amplicons that can be used directly in another round of amplification or can be probed for detection without denaturation or strand separation (Deiman et al., 2002) 3. NASBA is specifically designed to detect RNA. NASBA can selectively amplified RNA in the presence of DNA background. 4. The amplification of DNA by NASBA has also been demonstrated using primers directed against easily accessible DNA regions such as plasmid DNA, low-melting point sequences or single-stranded regions (Voisset et al., 2000). 5. Several studies have reported that the amplification power of NASBA is comparable to or sometimes even better that of RT-PCR (Lunel et al., 1999). 6. The constant temperature maintained throughout the amplification reaction allows each step of the reaction to proceed as soon as an amplification intermediate becomes

available. Thus, the exponential kinetic of the NASBA process, which is caused by multiple transcription of RNA copies from a given DNA product, is intrinsically more efficient than DNA-amplification methods limited to binary increases per cycle (Sooknanan and Malek, 1995). 7. RNA being the genomic material of many RNA viruses, an RNA-based amplification technique in contrast to PCR avoids an additional reverse transcription (RT) step, thus reducing the risk of contamination and lowering hands-on time (Loens et al., 2005). 8. It helps in better RT-PCR reaction as it offers faster amplification kinetics. 9. Especially suitable for detection of Retroviruses. 10. It can measure replication of DNA viruses by detecting late mRNA expression. 11. It supports the detection of human mRNA sequences without the risk of DNA contamination. 12. Gene expression studies can be done without intron flanking primers or DNAses.

(b) Disadvantages of NASBA

There are also some disadvantages of NASBA. 1. RNA integrity is the main cause of concern for NASBA, as for RT-PCR and other RNA amplification procedures. (Loens et al., 2005) 2. Although the amplification reaction itself is isothermal at 41^0C, a single melting step prior to the amplification reaction is required to allow annealing of the primers to the target. (Deiman et al., 2002) 3. Furthermore, because the specificity of the reactions is dependent on thermolabile enzymes, the reaction temperature cannot exceed 42°C without compromising it. 4. Finally, the length of the amplified RNA target sequence should be in the range of 120–250 nucleotides, shorter or longer sequences being amplified less efficiently (Loens et al., 2005).

9.0 APPLICATIONS OF NASBA

Some examples are discussed below in detail.

9.1. Detection of Infectious Agents

The technique has been employed for the detection of many classes of infectious agents in different types of specimens: blood (Smits et al., 1997), serum (Griffith et al., 1997;,Hollingsworth, 1996), plasma (Griffith et al., 1997), seminal plasma (Dyer et al., 1996), semen (Christie et al., 1998), respiratory tract specimens (Loens et al., 2002), nasopharyngeal biopsies (Brink et al., 1998), genital tract specimens (Smits et al., 1995; Mahony et al., 2001), urine (Morre et al., 1996), skin biopsies (Smits et al., 1995), saliva (Wacharapluesadee and Hemachuda, 2001), cerebrospinal fluid (Heim and Schumann, 2002, Zhang et al., 2000), stools (Fox et al., 2002), breast milk (Shepard et al., 2000), cervical–vaginal lavage fluid (Shepard et al., 2000), amniotic fluid (Revello et al., 2003), sewage-treatment efflux (Jean et al., 2002), potato tubers (Leone et al., 1997; van beckhoven et al., 2002), liquid whole eggs (Cook et al., 2002) and poultry (Uyttendaele et al., 1996).

9.2. Blood borne Pathogens

9.2.1. Human Immunodeficiency Virus Genomic RNA

Since the first HIV-1 NASBA was described in 1991 by Kievits et al. (Kievtis et al., 1991**)**, the assay has been improved considerably. Since 1995, a quantitative NASBA test for HIV-1 viral load determination based on ECL detection is commercially available (NASBA HIV-1 RNA QT, bioMérieux). The primers and probes are targeted at the *gag* region of the viral genome **(**van Gemen et al., 1994**)**. The more sensitive second-generation assay, NucliSens HIV-1 QT (bioMérieux) **(**Ginocchio et al., 2003**)**, can detect a range of HIV-1 RNA between 25 and $>5\times10^6$ copies/mL and has a 95% detection rate of 176 copies/mL, using 1 mL of EDTA, citrate, or heparin plasma (Notermans et al., 2000). Another new assay for real-time detection of HIV-1 RNA with molecular beacons is under development. Molecular beacons with different fluorescent labels are directed against the amplicon of wild-type RNA and that of only one calibrator. Preliminary performance data show that this assay has sensitivity comparable to that of the ECL-based viral load assay. Moreover, application of real-time detection dramatically increases the throughput of the test and shortens hands-on time. With this assay the amplification and (real-time) detection of 48 samples requires only 90 min **(**Beuningen et al., 2001, Wiel et al., 2000**).**

9.2.2. Hepatitis C Virus Genomic RNA Hepatitis C virus

In 1994, a first NASBA assay, with ELGA detection, for the amplification of the HCV 5' noncoding region RNA (5'NCR) in serum was described **(**Sillekens et

al., 1994**)**. The conserved 5'NCR has been used as a target for the further development of qualitative and quantitative ECL-based NASBA assays for genotypes 1a, 1b, 2, 3, 4, and 5 **(**Damen et al., 1999; Lunel et al., 1999**)**. The qualitative detection limit was found to be 230 copies. Again, quantification was done in the presence of three calibrators. The HCV NASBA QT assay was over 10 times more sensitive than the bDNA assay, whereas the quantitative results of both assays were highly concordant. The HCV NASBA QT assay was comparable in sensitivity with the HCV MONITOR assay (Roche) **(**de Baar et al., 1999**)**. Besides a qualitative and quantitative HCV NASBA assay, a genotyping NASBA test has also been developed making use of the variable regions in the 5' NCR (Melsert et al., 1997). The primers are identical to those of the quantitative test but specific capture probes were developed that can only hybridize with the amplicon of the target and not with that of the calibrators if present. In this way, one amplification reaction can be performed and used to determine both the quantity and the genotype of the target.

9.2.3. Human Cytomegalovirus mRNA

Detection of CMV at an early stage of infection is a prerequisite for effective pre-emptive antiviral therapy. The first described NASBA, a qualitative assay, targeted the late pp67 (UL65) mRNA (Block et al., 1998); it was applied on mRNA isolated from whole blood from renal transplant patients. The sensitivity of NASBA proved to be higher than that of the antigenemia assay, whereas the sensitivities of cell culture and NASBA were comparable. NASBA detected the onset of CMV infection simultaneously with cell culture and the antigenemia assay. The NucliSens CMV pp67 test (bioMérieux, The Netherlands) successfully monitored CMV disease in HIV-infected individuals (Blank et al., 2002), solid-organ transplant recipients (Witt et al., 2000), and heart, lung, and bone marrow transplant recipients (Gerna et al., 1999) and the assay could be used as a virologic marker for initiation of pre-emptive antiviral CMV therapy (Gerna et al., 1999). Although the assay is not the most sensitive available, it specifically detects the clinical relevant stages of infection and has the best specificity and positive predictive value for disease development (Blank et al., 2002). Additionally, a multiplex real-time NASBA was described for the simultaneous detection and quantification of CMV encoded IE 1 and pp67 mRNA (Greijer et al., 2002).

9.2.4. Flavivirus Genomic RNA

NASBA has been shown to be a highly sensitive and specific method for the detection of West- Nile (WN) virus (Lacciotti et al., 2000). The primers are directed against the envelope gene of the virus. A 1000-fold improved sensitivity as compared to standard RT-PCR and equal sensitivity to *Taq*Man RT-PCR was demonstrated, resulting in a lower limit of detection of less than 1 pfu of WN virus. A qualitative NASBA test has also been developed for the detection of Dengue virus RNA. Primers and probe are derived from conserved regions in the 3'NCR. A sensitivity of 0.1 pfu was demonstrated. In addition, a subtyping NASBA assay has been developed making use of specific capture probes for subtypes 1 to 4. The sensitivity of this assay is dependent on the subtype, varying from 0.1 pfu for subtypes 2 and 3, 1 pfu for subtype 1 and 10 pfu for subtype 4 (Wu et al., 2000**).**

9.2.5. Enterovirus Genomic RNA

Very recently, a NASBA assay was developed for the detection of enteroviruses . The conserved 5'NCR of the viruses is used as target. The test can detect Coxsackie A viruses, Coxsackie B viruses, Polioviruses, Echoviruses and enteroviruses and the assay is therefore denomi nated as the pan-enterovirus test. Many sample types can be used including stool suspensions, CSF samples, pernasal swabs, and throat swabs. The clinical sensitivity of the test was shown to be equivalent or equal to 0.2 pfu poliovirus and 1pfu Coksackie B virus per input and comparable to RT-PCR (Fox et al., 2002). No crossreactivity was observed with Adenovirus or Rotavirus in stool samples or with a number of cultured Rhinoviruses (Ginocchio, 2000).

9.3. Respiratory Pathogens

Rapid diagnostic techniques for respiratory pathogens are not only important for clinical epidemiological reasons but are also useful to initiate appropriate treatment within the first 24 h or interrupt empirical treatment when the disease is

caused by a microbial species requiring a different treatment.

9.3.1. Mycoplasma pneumoniae

The specificity and sensitivity of the NASBA assay for detection of *Mycoplasma pneumoniae* were evaluated by Loens et al. (2002). *M. pneumoniae* RNA prepared from a plasmid construct was used to assess the sensitivity of the assay, and an internal control for the detection of inhibitors was constructed. The sensitivity of the NASBA assay was 10 molecules of in vitro-generated wildtype *M. pneumoniae* RNA and five color changing units (CCU) of *M. pneumoniae*. In protease-treated spiked throat swabs, nasopharyngeal aspirates, bronchoalveolar lavages, and sputum, the sensitivity of the NASBA assay, in the presence of the internal control, was 2×104 molecules of in vitro-generated RNA or 5 CCU of *M. pneumoniae*. The sensitivity of the NASBA assay was comparable to that of a PCR targeted at the P1 adhesin gene used in the study by Ieven et al. **(**1996**)**. A real-time NASBA for the detection of *M. pneumoniae* was developed by Ovyn et al. (Loens et al., 2003a). The authors concluded that real-time and conventional NASBA show high concordance in sensitivity and specificity, with a clear advantage for the real-time technology regarding handling, speed, and number of samples that can easily be tested in a single run.

9.3.2. Other Respiratory Pathogens

A conventional NASBA assay for the detection of the 16S rRNA of *Bordetella pertussis* (Loens et al., 2003b) and real-time NASBA assays for the detection of the 16S rRNA of *Chlamydia pneumoniae* **(**Coombes and Mahony, 2000**)** and *Legionella pneumophila* **(**Sillekens et al., 2002**)** in respiratory specimens have been developed. Finally, a multiplex real-time NASBA for the simultaneous detection of *M. pneumoniae, C. pneumoniae*, and *L. pneumophila* 16S rRNA was developed **(**van Deursen et al., 1999**)**.

9.3.3. Mycobacterium tuberculosis

Van der Vliet et al. designed a NASBA assay based on a highly conserved region of the 16S rRNA of *Mycobacteria* **(**van der Vliet et al., 1993**)**. A species-specific probe for the detection of *M. tuberculosis* amplicons was used in an ELGA-based detection system. Nucleic acids derived from 200 bacteria resulted in a positive signal. The assay correctly identified 32 *M. tuberculosis* strains isolated from different parts of the world.

9.3.4. Picornaviridae

Targeting the Human rhinoviruses (HRV) 5'NCR, two NASBAs for HRV typing were developed (Loens et al., 2003c). Another assay with the 5'NCR and viral protein 4 was developed for the detection of HRV in respiratory specimens. The assay proved to be specific and sensitive and was succesfully applied to analysis of HRV sequences in respiratory specimens **(**Samuelson et al., 1998**)**. Three groups applied NASBA for the detection of enteroviruses in respiratory samples, cerebrospinal fluid and stools **(**Fox et al., 2002**)**. Nucleic acid sequence-based amplification assays have also been developed for the detection of other respiratory viruses such as respiratory syncytial virus **(**Rahman et al., 2002a**)**, parainfluenzaviruses **(**Hibbitts et al., 2002**)**, and influenza viruses **(**Rahman et al., 2002b**)**.

9.4. Other NASBA Applications for Detection of Infectious Agents

Various other NASBA applications have been described: for malaria parasites (Smits et al., 1997), *Cryptosporidium parvum* (Baeumner et al., 2001), *Campylobacter* species (Uyttendaele et al., 1996), *Chlamydia trachomatis* (Morre et al., 1996), *Escherichia coli* (Min and Baeumner, 2002), *Listeria monocytogenes* (Uyttendaele et al., 1995), *Mycobacterium leprae* (van der Vliet et al., 1996), *Neisseria gonorrhoeae* (Mahony et al., 2001), *Salmonella enterica* (Cook et al., 2002), *Ralstonia solanacearum* (Bentsink et al., 2002), *Aspergillus* (loeffler et al., 2001), *Candida* species (Widjojoatmodjo et al., 1999), including a wide range of viruses such as hepatitis A virus (Jean et al., 2001), human papillomavirus 16 (Smits et al., 1995), varicella zoster virus (mainka et al., 1998), Epstein–Barr virus (Fox et al., 2002), dengue virus (Wu et al., 2001), West Nile virus **,** St Louis encephalitis virus (Lanciotti et al., 2001), simian immunodeficiency virus (Romano et al., 2000), feline

immunodeficiency virus (Jordan et al., 2002), avian influenza subtype H5 viruses (Collins et al., 2002), citrus tristeza virus (Lair et al., 1993), and apple stem pitting virus (Klerks et al., 2001).

9.5. Viability Studies

Van der Vliet et al. (1994) and Morré et al. (1998) applied NASBA to study the influence of antibiotics on bacterial viability. During the drug exposure, the viability of the *Mycobacteria*, expressed as the number of colonyforming units (CFUs) was compared with the presence of 16S rRNA as determined by NASBA and with the presence of DNA coding for the 16S rRNA as determined by PCR. Morré et al. (1998) investigated the value of RNA detection by NASBA for the monitoring of *Chlamydia trachomatis* infections after antibiotic treatment. Simpkins et al. found consistent and highly significant differences between the mRNA amplifications extracted from viable and heat-killed *Salmonella enterica* cells, whereas PCR amplification of both kind of samples was unaffected (Simpkins et al., 2000). On the other hand, when Birch et al. (2001) assessed three amplification techniques (mRNA NASBA, mRNA RT-PCR, and DNA PCR) for their ability to detect nucleic acid persistence in an *E. coli* strain following heat killing, NASBA offered the greatest sensitivity to detect a decrease in viability although residual DNA and mRNA could be detected by PCR and NASBA.

9.6. NASBA Application for the Detection of Noninfectious Targets

BCR-ABL mRNA in Ph+ chronic myeloid leukemia **(**Sooknanan et al., 1993**)**, factor V Leiden (Reitsma et al., 1996), circulating breast cancer cells (Lambrechts et al., 1999), macrophage-derived chemokine gene expression (Romano et al., 2001), cytokine production (Heim et al., 2000), in vivo kinetics of tissue factor messenger RNA (Franco et al., 2000), tumor necrosis factor (TNF)-α mRNA (Darke et al., 1998), and circulating tumor cells (Burchill et al., 2002) have all been more or less successfully detected by the NASBA procedure.

10.0 CHALLENGES OF NASBA

The inability of NASBA to selectively detect mRNA from *Mycobacterium avium* ssp. *paratuberculosis* (MAP) (Rodríguez-Lázaro *et al.* 2004a) was very disappointing. It would have been very advantageous to have a rapid NASBA-based viability assay. Other organisms for which a rapid viability assay would be useful include *Legionella*, *Listeria*, *Campylobacter*, etc. NASBA could also be used to test viable-but non-culturable (VBNC) state in several bacteria, e.g., *Campylobacter*. NASBA is now an established diagnostic tool in clinical use (Rodríguez-Lázaro et al. 2006), but it does not appear to be progressing toward implementation in food analysis. To be optimally applicable to the detection of viable microorganisms in foods, NASBA will need to be applied directly, i.e., with no prior enrichment of the samples in nutrient media to increase the number of target cells and to allow sampling of small volumes. The assay should be applied to nucleic acids extracted directly from the food sample. As NASBA is an isothermal process, it does not require an expensive thermocycling apparatus. However, detection of the amplified RNA signal can require complex equipment (Cook, 2003). Biosensor based assays (Baeumner et al., 2003) although sophisticated in their working principle, could appear relatively easy to use and could be developed into a format suitable for operation by nonskilled personnel. They may also mediate quantification of the target, through measurement of the signal intensity (Baeumner et al. 2003). Quantification may also be achieved by employing a NASBA assay in real-time format, with signal detection mediated by, e.g., molecular beacons (Rodríguez-Lázaro et al. 2004b). NASBA, as a more complex process than PCR, is unlikely to match the progress of PCR toward adoption as a routine diagnostic tool for food and environmental samples unless committed developmental effort is made to explore and capitalize on its potential to detect viable microbial cells, which PCR does not in itself possess.

11.0 FUTURE PROSPECTS OF NASBA

Considering the advantages of RNA amplification, rapid amplification, simple operation, and easy detection, NASBA has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel. The integration of isothermal

amplification and electrophoresis onto microchips could lead to NASBA on Chips for quick and accurate identification of disease producing genes at the patient's bed side. NASBA has all the characteristics required of real time assays (high sensitivity, quantitative) along with simple operation for easy adaptability to field conditions. If these characteristics of the NASBA method are used effectively, it should be possible to develop simple genetic testing devices that have not been realized yet despite a strong awareness of their necessity, in a wide range of fields, including infectious disease testing, food inspection and environmental testing. The continued development of features that can progress NASBA from the researcher's laboratory to a practical rapid analytical method, especially for at-line monitoring of foods for viable pathogen presence, is a worthy and exciting challenge. This method will be widely applied in clinical diagnostics, environmental monitoring, food safety, and health fields with a broader development prospects in the future.

12.0 CONCLUSION

NASBA method is a powerful innovative gene amplification technique emerging as a simple rapid diagnostic tool for early detection and identification of microbial diseases. The use of NASBA could contribute to the development of an affordable, portable and easy to use pathogen detection kit, suitable even for less well equipped laboratories.

13.0 ACKNOWLEDGEMENT

Authors are indebted to Bangladesh Council of Scientific and Industrial Research (BCSIR) for providing an excellent job environment for study and research.

14.0 REFERENCES

1. Arnold L. J., Hammond P. W., Wiese W. A. and Nelson N. C. Assay formats involving acridinium-ester-labeled DNA probes. *Clin. Chem.* 1989:**35:** 1588–1594.
2. Baeumner A.J., Cohen R.N., Miksic V. and Min J. RNA biosensor for the rapid detection of viable *Escherichia coli* in drinking water. *Biosensors Bioelectronics*. 2003: **18**: 405-413.
3. Baeumner A. J., Humiston M. C., Montagna R. A. and Durst R. A. Detection of viable oocysts of *Cryptosporidium parvum* following nucleic acid sequence-based amplification. *Anal. Chem.* 2001:**73**: 1176–1180.
4. Bentsink, L., Leone G. O. M., van Beckhoven J. R. C. M., Van Schijndel H. B., Van Gemen B. and Van der Wolf J. M. Amplification of RNA by NASBA allows direct detection of viable cells of *Ralstonia solanacearum* in potato. *J. Appl. Microbiol.* 2002: **93:** 647–655.
5. Beuningen, van R., Marras S. A., Kramer F. R., Oosterlaken T., Weusten J., Borst G. and van der Wiel P. Development of a high throughput detection system for HIV-1 using real-time NASBA based on molecular beacons. Genomics and Proteomics Technologies, Ramesh Raghavachari, Weihong Tan, eds., *Proceedings of SPIE.* 2001: **4264:** 66–72.
6. Birch L., Dawson C. E., Cornett J. H. and Keer J. T. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Lett. Appl. Microbiol.* 2001: **33:** 296–301.
7. Blais B.W., Turner G., Sooknanan R. and Malek L.T. A nucleic acid sequence based amplification system for detection of *Listeria monocytogenes hlyA* sequences. *Appl. Enviro.l Microbiol*. 1997: **63**: 310-313.
8. Blank B. S. N., Meenhorst P. L., Pauw W. et al. Detection of late pp67-mRNA by NASBA in peripheral blood for diagnosis of human cytomegalovirus disease in AIDS patients. *J. Clin. Virol.* 2002: **25**: 29–38.
9. Blok M. J., Goossens V. J., Vanherle S. J. V. et al. Diagnostic value of monitoring human cytomegalovirus late pp67 mRNA expression in renal-allograft recipients by nucleic acid sequence based amplification. *J. Clin. Microbiol*. 1998: **36**: 1341–1346.

10. Brink, A. A. T. P., Vervoort M. B. H. J., Middeldorp J. M., Meijer C. J. L. M. and Van den Brule A. J. C. Nucleic acid sequence-based amplification, a new method for analysis of spliced and unspliced Epstein–Barr virus latent transcripts, and its comparison with reverse transcriptase PCR. *J. Clin. Microbiol.* 1998: **36**: 3164–3169.
11. Burchill S. A., Perebolte L., Johnston C., Top B. and Selby P. Comparison of the RNAamplification based methods RT-PCR and NASBA for the detection of circulating timour cells. *Br. J. Cancer.* 2002: **86**: 102–109.
12. Christie I. L., Mullen J. E., Braude P. R. et al. Assisted conception in HIV discordant couples: evaluation of semen processing techniques in reducing HIV viral load. *J. Reprod. Immunol.* 1998: **41**: 301–306.
13. Collins, R. A., Ko L.S., So K.L., Ellis T., Lau L.T. and Yu A. C. H. Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. *J. Virol. Methods.* 2002: **103:** 213–225.
14. Compton J. Nucleic acid sequence-based amplification. Nature. 1991: **350**:91–92
15. Cook N. The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J. Microbiol. Methods.* 2003: **53:** 165–174.
16. Cook N., Ellison J., Kurdziel A. S., Simpkins S. and Hays J. P. A nucleic acid sequencebased amplification method to detect *Salmonella entereica* serotype *enteritidis* strain PT4 in liquid whole egg. *J. Food Protect.* 2002: **7**: 1177–1178.
17. Coombes B. K. and Mahony J. B. Nucleic acid sequence-based amplification (NASBA) of *Chlamydia pneumoniae* major outer membrane protein (ompA) mRNA with bioluminescent detection. *Comb. Chem. High Throughput Screen.* 2000: **3**: 315–327.
18. Damen M., Sillekens P., Cuypers H. T. M., Frantzen I. and Melsert R. Characterization of the quantitative HCV NASBA assay. *J. Virol. Methods.* 1999: **82**: 45–54.
19. Darke B. M., Jackson S. K., Hanna S. M. and Fox J. D. Detection of human TNF-α mRNA by NASBA. *J. Immunol. Methods.* 1998: **212**: 19–28.
20. de Baar, M. P., van der Schoot A. M., Goudsmit J. et al. Design and evaluation of a human immunodeficiency virus type 1 RNA assay using nucleic acid sequence-based amplification technology able to quantify both group M and O viruses by using the long terminal repeat as target. *J. Clin. Microbiol.* 1999: **37**: 1813–1818.
21. Deiman B., van Aarle P. and Sillekens P. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Molecular biotechnology*. 2002: **20(2)**:163-79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11876473 .
22. Dyer J. R., Gilliam B. L., Eron J. J. Jr., Grosso L., Cohen M. S. and Fiscus S. A. Quantitation of human immunodeficiency virus type 1 RNA in cell free seminal plasma: comparison of NASBA™ with Amplicor™ reverse transcription–PCR amplification and correlation with quantitative culture. *J. Virol. Methods* 1996: **60**: 161–170.
23. Fahy E., Kwoh D. Y. and Gingeras T. R. Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. *PCR Meth. Appl.* 1991: **1:** 25–33.
24. Fakruddin M. Loop mediated Isothermal Amplification (LAMP) - An Alternative to Polymerase Chain Reaction (PCR). Bangladesh Res. Pub. J. 2011: **5(4)**:425-439.
25. Fox, J. D., Han S., Samuelson A., Zhang Y., Neale M. L. and Westmoreland D. Development and evaluation of nucleic acid sequence-based amplification (NASBA) for diagnosis of enterovirus infections using the NucliSens Basic Kit. *J. Clin. Virol.* 2002: **24**: 117–130.
26. Franco R. F., De Jonge E., Dekkers P. E. P. et al. The in vivo kinetics of tissue factor messenger RNA expression during human endotoxemia: Relationship with activation of coagulation. *Blood.* 2000: **96**: 554–559.
27. Gerna G., Baldanti F., Middeldorp J. M. et al. Clinical significance of expression of human cytomegalovirus pp 67 late transcript in heart, lung and bone marrow transplant recipients, as

determined by nucleic acid sequence-based amplification. *J. Clin. Microbiol.* 1999: **37**: 902–911

28. Gill P. & Ghaemi A. Nucleic Acid Isothermal Amplification Technologies—A Review, Nucleosides, Nucleotides and Nucleic Acids, 2008: **27(3)**:224-243
29. Ginocchio C. C. Evaluations of NucliSens Basic Kit Applications:1) Detection of Enterovirus 2) CCR5 genotyping of HIV infected patients.16th Annual Clinical Virology Symposium and Annual Meeting Pan American Society for Clinical Virology, Clearwater, 2000: April 30–May 3.
30. Ginocchio C. C., Kemper M., Stellrecht K. A. and Witt D. J. Multicenter evaluation of the performance characteristics of the NucliSens HIV-1 QT assay used for quantitation of human immunodeficiency virus type 1 RNA. *J. Clin. Microbiol.* 2003: **41**: 164–173.
31. Greijer A. E., Adriaanse H. M. A., Dekkers C. A. J. and Middeldorp J. M. Multiplex realtime NASBA for monitoring expression dynamics of human cytomegalovirus encoded IE1 and pp67 RNA. *J. Clin. Virol.* 2002: **24**: 57–66.
32. Griffith B. P., Rigsby M. O., Garner R. B., Gordon M. M. and Chacko T. M. Comparison of the Amplicor HIV-1 monitor test and the nucleic acid sequence-based amplification assay for quantitation of human immunodeficiency virus RNA in plasma, serum and plasma subjected to freeze–thaw cycles. *J. Clin. Microbiol.* 1997: **35**: 3288–3291.
33. Guatelli J., Whitfield K., Kwoh D., Barringer K., Richman D. and Gingeras T. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retrovirus replication. *Proc. Natl. Acad. Sci. USA* 1990: **87**: 1874–1878.
34. Heim, A. and Schumann J. Development and evaluation of a nucleic acid sequence-based amplification (NASBA) protocol for the detection of enterovirus RNA in cerebrospinal fluid samples. *J. Virol. Methods.* 2002: **103**: 101–107.
35. Heim A., Zeuke S., Weiss S., Ruschewski W. and Grumbach I. M. Transient induction of cytokine production in human myocardial fibroblasts by Coxsackievirus B3. *Circ. Res.* 2000: **86**: 753–759.
36. Hibbitts S., Rahman A., Westmoreland D. and Fox J. D. 2002. Diagnosis of parainfluenza virus infections using NucliSens® Basic Kit NASBA with "end point" and "real-time" detection, 12th European Congress of Clinical Microbiology and Infectious Diseases.
37. Hollingsworth R. C., Sillekens P., van Deursen P., Neal K. R. and Irving W. L. on behalf of the Trent HCV Study Group (1996) Serum HCV RNA levels assessed by quantitative NASBA: stability of viral load over time and lack of correlation with liver disease. *J. Hepatol.* 1996: **25**: 301–306.
38. Ieven M., Ursi D., Van Bever H., Quint W., Niesters H. G. M. and Goossens H. Detection of *Mycoplasma pneumoniae* by two polymerase chain reactions and role of *M. pneumoniae* in acute respiratory tract infections in pediatric patients. *J. Infect. Dis.* 1996: **173**: 1445–1452.
39. Jean J., Blais B., Darveau A. and Fliss I. Detection of hepatitis A virus by the nucleic acid sequence based amplification technique and comparison with reverse trasncription PCR. *Appl. Environm. Microbiol.* 2001: **67**: 5593–5600.
40. Jean J., Blais B., Darveau A. and Fliss I. Rapid detection of human rotavirus using colorimetric nucleic acid sequence based amplification (NASBA) enzyme linked immunosorbent assay in sewage treatment effluent. *FEMS Microbiol. Lett.* 2002: **210**: 143–147.
41. Jordan H. J., Scappino L. A., Moscardini M. and Pistello M. Detection of feline immunodeficiency virus RNA by two nucleic acid sequence based amplification (NASBA) formats. *J. Virol. Methods* 2002: **103**: 1-13.
42. Keer J.T. and Birch L. Molecular methods for the assessment of bacterial viability. *J. Microbiol. Methods* . 2003: **53**: 175-183.
43. Kievits T., van Gemen B., van Strijp D. et al. NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Virol. Methods.* 1991: **35**: 273–286.
44. Klerks M. M., Leone G., Linder J. L., Schoen C. D. and van den Heuvel J. F. J. M. Rapid and

sensitive detection of apple stem pitting virus in apple trees through RNA amplification and probing with fluorescent molecular beacons. *Phytopathology* 2001: **91**: 1085–1091.

45. Lair S. V., Mirkow T. E., Dodds J. A. and Murphy M. F. A single temperature amplification technique applied to the detection of citrus tristeza viral RNA in plant nucleic acid extracts.*J. Virol. Methods* 1993: **47**:141–152.
46. Lambrechts, A. C., Bosma A. J., Klaver S. G. et al. Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence based amplification for the detection of circulating breast cancer cells. *Breast Cancer Res. Treat.* 1999: **56**: 219–231.
47. Lanciotti R. S. and Kerst A. J. Nucleic acid sequence based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. *J. Clin. Microbiol.* 2001: **39**: 4506–4513.
48. Lanciotti R. S., Kerst A. J., Allen B. C. and Velez J. O. Development of a NASBA based assay for the rapid detection of west nile virus. American Society of Tropical Medicine and Hygiene, Houston, 2000: Oct 29– Nov 2, poster presentation.
49. Lauri A. and Mariani P.O. Potentials and limitations of molecular diagnostic methods in food safety. *Genes & nutrition*. 2009: **4(1)**:1-12.
50. Leone G., van Schijndel H., van Gemen B., Kramer F.R. and Schoen C.D. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res*. 1998: **26**:2150–2155.
51. Leone G., van Schijndel H., van Gemen B., Kramer F.R. and Schoen C.D. Direct detection of potato leafroll virus in potato tubers by immunocapture and the isothermal nucleic acid sequence based amplification method NASBA. *J. Virol. Methods* 1997: **66**: 19–27.
52. Lizardi P.M., Huang X., Zhu Z. *et a1*. Mutation detection and single molecule counting using isothermal rolling circle amplification. *Nat Genet*. 1998: **19**: 225-232.
53. Loeffler J., Hebart H., Cox P., Flues N., Schumacher U. and Einsele H. Nucleic acid sequence based amplification of *Aspergillus* RNA in blood samples. *J. Clin. Microbiol.* 2001: **39**: 1626–1629.
54. Loens K., Ursi D., Goossens H. and Ieven M. Medical Biomethods Handbook. Edited by J.M. Walker and R. Repley. Humana press, Inc, Totowa, N.J. 2005.
55. Loens K., Beck T., Sillekens P. et al. Development of conventional NASBA for the detection of *Bordetella pertussis* in respiratory specimens, 13th European Congress of Clinical Microbiology and Infectious Diseases, 2003b: abstract P1369.
56. Loens K., Ieven M., Ursi D. et al. Detection of *Mycoplasma pneumoniae* by real-time NASBA. *J. Clin. Microbiol.* 2003a: **41**: 4448–4450.
57. Loens K., Ieven M., Ursi D. et al. Improved detection of rhinoviruses by NASBA® after nucleotide sequence determination of the 5'NCR of additional rhinovirus strains. *J. Clin. Microbiol.* 2003c: **41**: 1971–1976.
58. Loens K., Ursi D., Ieven M. et al. Detection of *Mycoplasma pneumoniae* in spiked clinical samples by nucleic acid sequence-based amplification. *J. Clin. Microbiol.* 2002: **40**: 1339–1345.
59. Mahony J. B., Song X., Chong S., Faught M., Salonga T. and Kapala J. Evaluation of the NucliSens Basic Kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence based amplification of 16S rRNA. *J. Clin. Microbiol.* 2001: **39**: 1429–1435.
60. Mainka C., Fuss B., Geiger H., Höfelmayr H. and Wolff M. H. Characterization of viremia at different stages of varicella zoster virus infection. *J. Med. Virol.* 1998: **56**: 91–98.
61. Malek L., Sooknanan R. and Compton J. Nucleic acid sequence-based amplification In Isaac PG. ed. Methods In Molecular Biology. **Vol28.** Protocols for Nucleic Acid Analysis by Nonradioactive Probes, Totowa, NJ: Humana Press, 1994: p***253***
62. Melsert R., Damen M., Cuypers T. et al. Combined quantitation and genotyping of hepatitis C virus RNA using NASBA. In Hepatitis C virus: Genetic Heterogeneity and

viral load, Paris, John Libbey Eurotext 1997: pp. 79–88.
63. Min J. and Baeumner A. J. Highly sensitive and specific detection of viable *Escherichia coli* in drinking water. *Anal. Biochem.* 2002: **303**: 186–193.
64. Morré S. A., Sillekens P. T. G., Jacobs M. V. et al. Monitoring of *Chlamydia trachomatis* infections after antibiotic treatment using RNA detection by nucleic acid sequence based amplification. *J. Clin. Pathol. Mol. Pathol.* 1998: **51**: 149–154.
65. Morré S. A., Sillekens P., Jacobs M. V. et al. RNA amplification by nucleic acid sequence based amplification with an internal standard enables reliable detection of *Chlamydia trachomatis* in cervical scrapings and urine samples. *J. Clin. Microbiol.* 1996: **34**: 3108–3114.
66. Notermans D. W., de Wolf F., Oudshoorn P. et al. Evaluation of a second-generation nucleic acid sequence-based amplification assay for quantification of HIV type 1 RNA and the use of ultra-sensitive protocol adaptations. *Aids Res. Hum. Retroviruses* 2000: **16**: 1507–1517.
67. Notomi T., Okayama H., Masubuchi H. *et al.* Loop-mediated isothermal amplification of DNA. Nucl Acid Res. 2000:**28**: e63.
68. Oehlenschlager F., Schwille P. and Eigen M. Detection of HIV-1 RNA by nucleic acid sequencebased amplification combined with fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. USA* 1996: **93 (**12): 811–12,816.
69. Rahman A., John R., Westmoreland D. and Fox J. D. Clinical evaluation of molecular amplification assays based on NASBA and RT-PCR for diagnosis of respiratory syncytial virus infection, Pan American Society for Clinical Virology, Florida. 2002a.
70. Rahman A., Westmoreland D. and Fox J. D. Evaluation of "end point" and "real time" molecular-based assays for amplification and detection of influenza viruses, 12th European Congress of Clinical Microbiology and Infectious Diseases. 2002b.
71. Reitsma P. H., van der Velden P. A., Vogels E. et al. Use of the direct RNA amplification technique NASBA to detect factor V Leiden, a point mutation associated with APC resistance. *Blood Coagul. Fibrin.* 1996: **7**: 659–663.
72. Revello M. G., Lilleri D., Zavattoni M., Furione M., Middeldorp J. and Gerna G. Prenatal diagnosis of congenital human cytomegalovirus infection in amniotic fluid by nucleic acid sequence- based amplification assay. *J. Clin. Microbiol.* 2003: **41**: 1772–1774.
73. Rodríguez-lázaro D., Hernández M., Agostino M.D. and Cook N. Application of Nucleic Acid Sequence Based Amplification For The Detection Of Viable Foodborne Pathogens :Progress And Challenges. *Journal of Rapid Methods & Automation in Microbiology*. 2006: **14**:218-236.
74. Rodríguez-Lázaro D., Lloyd J., Pla M., Ikonomopoulos J. and Cook N. Unexpected detection of DNA by NASBA in *Mycobacterium avium* subsp. *paratuberculosis*. Mol. Cell. Probes 2004a: **18**: 251–253.
75. Rodríguez-Lázaro D., Lloyd J., Herreweg A., Ikonomopoulos J., D'agostino M., Pla M. and Cook N. A molecular beacon-based real time NASBA assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. FEMS Microbiol. Lett. 2004b: **237**: 119–126
76. Romano J. W., Shurtliff R. N., Dobratz E. et al. Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology. *J. Virol. Methods* 2000: **86**: 61–70.
77. Romano J. W., Shurtliff R. N., Grace M. et al. Macrophage-derived chemokine gene expression in human and macaque cells: mRNA quantification using NASBA technology. *Cytokine* 2001: **13**: 325–333.
78. Saiki R. K., Scharff S., Faloona F. *et al.* Enzymatic amplification of β-globin genomic sequences and restriction site analysis diagnosis of sickle cell anemia. *Science.* 1985: **230**: 1350-1354.
79. Samuelson A., Westmoreland D., Eccles R. and Fox J. D. Development and application of a new method for amplification and detection of human rhinovirus RNA. *J. Virol. Methods* 1998: **71**: 197–209.

80. Sergentet-Thevenot D., M.P. Montet and C. Vernozy-Rozand. Challenges to developing nucleic acid sequence based amplification technology for the detection of microbial pathogens in food. *Revue*. Med. Vet. 2008: **159 (10)**:514-527.
81. Shepard R. N., Schock J., Robertson K. et al. Quantitation of human immunodeficiency virus type 1 RNA in different biological compartments. *J. Clin. Microbiol.* 2000: **38**: 1414–1418.
82. Sillekens P., Kok W., van Gemen B. et al. *Specific Detection of HCV RNA Using NASBA™ as a Diagnostic Tool. Hepatitis C Virus GEMHEP*, John Libbey Eurotext, Paris. 1994: pp. 71–82.
83. Sillekens P., Overdijk M., Roosmalen I. et al. Molecular diagnosis of *Legionella* infections using NASBA with real-time detection. *Clin. Microbiol. Infect.* 2002: **8 (Suppl. 1)**, 147.
84. Simpkins S. A., Chan A. B., Hays J., Pôpping B. and Cook N. An RNA transcription based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Lett. Appl. Microbiol.* 2000: **30**: 75–79.
85. Smits H. L., Gussenhoven G. C., Terpstra W., Schukkink R. A. F., van Gemen B. and van Gool T. Detection, identification and semi-quantification of malaria parasites by NASBA amplification of small subunit ribosomal RNA sequences. *J. Microbiol. Meth.* 1997: **28**: 65–75.
86. Smits H. L., van Gemen B., Schukkink R. et al. Application of the NASBA nucleic acid amplification method for the detection of human papillomavirus type 16 E6-E7 transcripts. *J. Virol. Methods* 1995: **54**: 75–81.
87. Sooknanan R. and Malek L. T. NASBA a detection and amplification system uniquely suited for RNA. *Bio/Technology* 1995: **13**: 563–564.
88. Sooknanan R., Malek L., Wang X.-H., Siebert T. and Keating A. Detection and direct sequence identification of BCR-ABL mRNA in Ph+ chronic myeloid leukemia. *Exp. Hematol.* 1993: **21**: 1719–1724.
89. Sooknanan R., van Gemen B. and Malek L. Nucleic acid sequence-based amplification. Molecular methods for virus detection, Academic press, London, UK , 1995: pp. 261–285.
90. Tyagi S. and Kramer F. R. Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotech.* 1996: **14**: 303–308.
91. Uyttendaele M., Bastiaansen A. and Debevere J. Evaluation of the NASBA nucleic acid amplification system for assessment of the viability of *Camplylobacter jejuni. Int. J. Food Microbiol*. 1997: **37**: 13-20.
92. Uyttendaele M., Debevere J. and Lindqvistr R. Evaluation of buoyant density centrifugation as a sample preparation method for NASBA-ELGA detection of *Campylobacter jejuni* in foods. *Food Microbiol*. 1999: **16**: 575-582.
93. Uyttendaele M., Schukkink R., Van Gemen B. and Debevere J. Comparison of the nucleic acid amplification system NASBA and agar isolation for detection of pathogenic *Campylobacters* in naturally conatminated poultry. *J. Food Protect.* 1996: **59**: 683–687.
94. Uyttendaele M., Schukkink R., Van Gemen B. and Debevere J. Development of NASBA, a nucleic acid amplification system, for identification of *Listeria monocytogenes* and comparison to ELISA and a modified FDA method. *Int. J. Food Microbiol.* 1995: **27**: 77–89.
95. van Beckhoven J. R. C. M., Stead D. E. and van der Wolf J. M. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by AmpliDet RNA, a new technology based on real-time monitoring of NASBA amplicons with a molecular beacon. *J. Appl. Microbiol.* 2002: **93**: 840–849.
96. van der Vliet G. M. E., Cho S.-N., Kampirapap K. et al. Use of NASBA RNA amplification for detection of *Mycobacterium leprae* in skin biopsies from untreated and treated leprosy patients. *Int. J. Leprosy* 1996: **64**: 396–403.
97. van der Vliet G. M. E., Schepers P., Schukkink R. A. F., van Gemen B. and Klatser P. R. Assessment of mycobacterial viability by RNA amplification. *Antimicrob. Agents Chemother.* 1994: **38**: 1959–1965.
98. van der Vliet G. M. E., Schukkink R. A. F., van Gemen B., Schepers P. and Klatser P. R. Nucleic acid sequence based amplification

(NASBA) for the identification of *Mycobacteria*. *J. Gen. Microbiol.* 1993: **139**: 2423–2429

99. van Deursen P., Gunther A. W., Spaargaren-van Riel C. C. et al. A novel quantitative multiplex NASBA method: application to measuring tissue factor and CD14 mRNA levels in human monocytes. *Nucleic Acids Res.* 1999: **27**: e15:i–e15:vi.
100. van Gemen B., Van Beuningen R., Nabbe A. et al. A one tube quantitative HIV-1 RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labelled probes. *J. Virol. Methods* 1994: **49**: 157–168.
101. Voisset C., Mandrand B. and Paranhos-Baccala G. RNA amplification technique, NASBA, also amplifies homologous plasmid DNA in non-denaturing conditions. *BioTechniques*. 2000: **3**: 236-240.
102. Wacharapluesadee S. and Hemachudha T. Nucleic acid sequence based amplification in the rapid diagnosis of rabies. *Lancet* 2001: **358**:892–893.
103. Walker G. T., Fraiser M. S., Schram J. L. *et al.* Strand displacement amplification—an isothermal, *in vitro* DNA amplification technique. Nucl Acid Res, 1992: **20**: 1691-1696.
104. Widjojoatmodjo, M. N., Borst A., Schukkink R. A. F. et al. Nucleic acid sequence based amplification (NASBA) detection of medically important *Candida* species. *J. Microbiol. Methods* 1999: **38**: 81–90.
105. Wiel van de P., Top B., Weusten J. and Oosterlaken T. A new, high throughput NucliSens assay for HIV-1 viral load measurement based on real-time detection with molecular beacons. XIII International AIDS Conference, Durban, poster presentation. 2000.
106. Witt D. J., Kemper M., Stead A. et al. Analytical performance and clinical utility of a nucleic acid sequence based amplification assay for detection of cytomegalovirus infection. *J. Clin. Microbiol*. 2000: **38**: 3994–3999.
107. Wu S. L., Putvatava R., Lee E. M. et al. Evaluation of nucleic acid sequence-based amplification (nasba) method for detection of dengue viral RNA. The 49th annual meeting of the American society of tropical medicine and hygiene. Houston, TX, USA. October 29–November 2-2000. Abstract. 2000.
108. Wu S.-J., Lee E. M., Putvatana R. et al. Detection of dengue viral RNA using a nucleic acid sequence based amplification assay. *J. Clin. Microbiol.* 2001: **39**: 2794–2798.
109. Zhang F., Tetali S., Wang X. P., Kaplan M. H., Cromme F. V. and Ginocchio C. C. Detection of human cytomegalovirus pp67 late gene transcripts in cerebrospinal fluid of human immunodeficiency virus type 1-infected patients by nucleic acid sequence based amplification. *J. Clin. Microbiol.* 2000: **38**: 1920–1925.