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Review

From research lab to standard environmental analysis tool: Will NASBA make the leap?

Birgitte K. Hønsvall [a, b, *], Lucy J. Robertson [c]

[a] University College of Southeast Norway, Raveien 205, 3184 Borre, Norway
[b] Trilobite Microsystems AS, Raveien 205, 3184 Borre, Norway
[c] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Adamstuen Campus, Oslo, Norway

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ABSTRACT

Nucleic acid sequence-based amplification (NASBA) is a sensitive and efficient molecular tool for amplification of RNA and has been widely adopted in clinical diagnostics. Monitoring of water and other environmental samples demands sensitive techniques, as potential pathogens may be in low concentrations and require only a few infectious units to infect their host. NASBA has qualities that should be advantageous for analysis of environmental samples, such as short reaction times, high sensitivity, and not readily affected by inhibitory substances that are often abundant in environmental samples. NASBA is well suited for incorporation into lab-on-a-chip (LOC) devices, as part of analysis systems that can be taken into the field for on-site screening.

In this review, we explore advantages and drawbacks of NASBA as a tool for environmental analyses, and try to answer the question of whether it will be a recognised technique in the same manner as in clinical diagnostics.

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1. Background

In the investigation of samples for microorganisms, molecular techniques based on analysis of nucleic acids provide approaches that are often considered to be more specific and of higher sensitivity than can be achieved by traditional detection techniques based on culturing. Potential harmful organisms are widespread

* Corresponding author. University College of Southeast Norway, Raveien 205, 3184 Borre, Norway.
E-mail addresses: birgitte.honsvall@trilobite.no, birgitte.honsvall@hbv.no (B.K. Hønsvall), lucy.robertson@nmbu.no (L.J. Robertson).

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throughout the environment, many of these are waterborne, and often the infective dose is low, such that very few infective agents are needed to cause infection and illness. Surface waters and recreational waters may be potential sources for pathogens, which can be inadvertently swallowed by people when they swim or during other recreational activities, or enter bloodstreams through wounds and scratches. One part of the many approaches used in order to know where it is safe to take a swim, where the water we drink is safe, or the food we consume will not make us sick, is screening for potentially harmful organisms. For safe drinking water, monitoring methods with low limits of detection (LOD) are required, and therefore molecular approaches are considered advantageous compared with traditional culturing techniques. Culture-based methods are only applied to bacteria and a few viruses, and, in addition to being time-consuming and labour intensive, have the risk of yielding false negative results, as not all viable organisms can be cultivated in the laboratory. Viruses are commonly detected using PCR or RT-PCR (Mattison and Bidawid, 2009), and larger pathogens such as *Cryptosporidium* and *Giardia* are normally detected and enumerated by immunofluorescent antibody test (IFAT) and examination by microscopy (e.g., ISO method 15553; US EPA 1623).

Most organisms are only infectious when they are viable, and therefore detection is not always sufficient - we also want to assess their state of viability. Other organisms produce toxins, and therefore may still have a toxic effect, even when they are dead. In those cases, an optimal assay would detect the toxin directly. Some toxin-producing algae are only harmful at some stages of their lifecycles. By monitoring seawater to detect these toxic algal blooms at an early stage, we can lower the risk of illness, for instance, from harvesting shellfish in these waters at the time of algal blooms.

Molecular techniques are more sensitive, quicker to perform, more objective, and less labour-intensive than traditional culture based methods. They may also provide additional information that cannot be obtained by culturing methods, regarding such factors as virulence, toxicity, or state of viability.

Several different techniques for nucleic acid amplification and detection have been developed over the last 20–30 years (Monis and Giglio, 2006), of which polymerase chain reaction (PCR) is by far the most commonly used, and has become a standard tool in a range of applications from clinical diagnostics to environmental analyses, from studies of evolution to forensic medicine. An alternative method, which is particularly applicable to RNA amplification, is nucleic acid sequence-based amplification (NASBA) (Compton, 1991). This sensitive molecular detection tool has been used for detection of viruses (Dyer et al., 1996; Kievits et al., 1991; Mo et al., 2015b; Starkey et al., 2006), bacteria (Heijnen and Medema, 2009), toxic algae (Delaney et al., 2011), fungi (Brenier-Pinchart et al., 2014) and pathogens (Baeumner et al., 2001; Cook, 2003), among others.

In the clinical lab, NASBA has relatively rapidly been adopted as a rapid and reliable diagnostic tool, being first used for the detection and quantification of HIV-1 in sera (Kievits et al., 1991), but also now used routinely for diagnosis of other viral diseases (e.g. hepatitis, dengue, human papilloma virus etc.). In addition, other infections are diagnosed in the clinical lab by NASBA, particularly blood-borne and respiratory tract bacterial and fungal infections (e.g. *Mycoplasma pneumoniae* and *Chlamydia pneumonia* (Bessede et al., 2010; Loens et al., 2002)) and parasitic diseases such as malaria, sleeping sickness, and leishmaniasis (Basiye et al., 2011; Gonçalves et al., 2016; van der Meide et al., 2008). The use of NASBA in clinical diagnostics has advanced to such an extent that chip and cassettes have even been developed for real-time assays that are compatible with mobile telephones; these provide a readily available and relatively cheap platform for analysis when laboratory infrastructure is not available (Mauk et al., 2015).

Although NASBA is acknowledged as being a valuable clinical diagnostic tool, for environmental analyses NASBA still seems to be largely confined to the research lab, and does not yet seem to be widely accepted as a useful analytical tool. In this review we consider the pros and cons of using NASBA for environmental analyses, and try to answer the question of whether NASBA will become an accepted technique in environmental analyses, as it has been in clinical diagnostics.

2. The NASBA reaction

The NASBA reaction consists of two phases, a non-cyclic phase and a cyclic phase (Fig. 1). The mRNA template is converted to double-stranded DNA (dsDNA) by reverse transcription in the non-cyclic phase. These dsDNA products are subsequently used as the template for mRNA transcription in the cyclic phase; 10–100 copies of RNA can be produced from each template (Compton, 1991). Three enzymes are involved in the NASBA reaction: avian myeloblastosis virus reverse transcriptase (AMV RT), RNase H, and T7 RNA polymerase. dNTPs, appropriate buffer components, and primers are also required. The forward primer consists of a nucleotide sequence complementary to the RNA target, and a promoter sequence recognized by T7 RNA polymerase at its 5' end. A 65 °C denaturation step can be included prior to the reaction in order to break up secondary structures of the RNA. The NASBA reaction is isothermal, and the temperature is kept at 41 °C. Hence, there is no need for a thermocycler, a simple heating device is sufficient to run the reaction.

The main target molecule for NASBA is RNA, but DNA may also be amplified if they are first converted to single-stranded molecules (Compton, 1991). An advantage of NASBA over reverse transcriptase-PCR (RT-PCR) is that the reverse transcriptase step is included in the reaction set-up. Thus, there is no need to prepare complementary DNA (cDNA) prior to running the NASBA reaction, as is necessary before RT-PCR, thus saving time, labour and decreasing the risk of contamination. Another benefit of NASBA, over RT-PCR, is that DNA does not interfere with the reaction, as the reaction temperature is kept below the DNA melting temperature, and dsDNA strands are not separated (Simpkins et al., 2000).

NASBA has been reported to be equally sensitive or more sensitive than RT-PCR, has a shorter reaction time, thus speeding up time to results, and is less labour-intensive (Delaney et al., 2011; Mo et al., 2015a; Starkey et al., 2006).

2.1. Real-time detection with NASBA

Real-time detection using NASBA can be achieved by including fluorescent markers that bind to the amplicon. In this way of detection, the reaction tube can remain closed, and the contamination risk decreases. Leone et al. (1998) developed a real-time NASBA assay by incorporating molecular beacons (Tyagi and Kramer, 1996), and this technique is the most used. Molecular beacons consist of two complementary stem sequences framing a probe sequence. One end is conjugated to a fluorophore, the other end to a quencher. When the probe sequence of a molecular beacon binds to its complementary target-sequence, the fluorophore and the quencher are physically separated, and the fluorophore will emit light when excited (Fig. 2A). If the probe has no target to bind, the molecular beacon folds into a stem-and-loop structure (Fig. 2B). In this conformation, the quencher is adjacent to the fluorophore, and any energy absorbed by the fluorophore is given off as heat by the quencher instead of detectable light. Detection of different targets in the same solution can also be achieved by using