Mini-Review

RT-NASBA: An Ideal Approach for the Detection of Diarrhoea-Related Viruses

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Abstract | RT-NASBA is a rapid and sensitive diagnostic system, which combines nucleic acid sequence-based amplification (NASBA) and molecular beacon technique. Recently there have been two major progresses in the field, in which RT-NASBA technique was applied for the detection of diarrhoea related viruses. This mini review overviewed the basic principle of RT-NASBA and discussed the significance of these new findings.

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Diarrhoea is one of the most serious public health issues worldwide (Waters et al., 2000). The causative agents include parasites, bacteria and viruses, in which rotaviruses, astroviruses, noroviruses and sapoviruses are the most common pathogens and account for the majority of the viral-related diarrhoea (He et al., 2011; Le Guyader et al., 2008; Tran et al., 2010; van Maarseveen et al., 2010). According to previous reports, these viruses caused as high as 90% of the total diarrhoea epidemic in children (Farkas et al., 2000; Lindell et al., 2005; Parashar et al., 2006; Roman et al., 2002). Though spontaneous recovery occurs in approximately four days post-infection/symptom, some patients may develop irritable bowel syndrome if left untreated (Wahnschaffe et al., 2007).

Traditional diagnostic methods for these viruses are electron microscopy, northern blot, serological tests or antigen-detection assay, all of which were either low-sensitive, lab-intensive, or time-consuming (Atmar and Estes, 2001). In addition, in some cases, co-infection or multi-infection occurred, making the diagnostic complicated and cumbersome (Koh et al., 2008; Rohayem et al., 2004). Therefore, rapid and sensitive diagnostic assays are urgently needed to help physicians making faster and better treatment decision for these patients.

Nucleic acid sequence-based amplification (NASBA) (Compton, 1991), also known as self-sustained sequence replication (3SR) (Guatelli et al., 1990), is an isothermal nucleic acid amplification method which applied the combined action of reverse transcriptase, RNase H and T7 RNA polymerase. Since reverse transcriptase is already present in the reaction, NASBA is highly suitable for the detection of RNA targets (including viral RNA, ribosome RNA, messenger RNA) due to the fact that no separate reverse transcription reaction is needed (Compton, 1991). Since its development, NASBA has been successfully used for the detection of various kinds of pathogens including viruses (Costa et al., 2008; Forbi et al., 2010; Lau et al., 2010), bacteria (Churruca et al., 2007; Mollasalehi and Yazdanparast, 2013), parasites

(van der Meide et al., 2005) and fungi (Zhao et al., 2009). For diarrhoea disease, there are also reports on the application of NASBA for the detection of rotaviruses, noroviruses, and astroviruses. Jean et al. developed a colorimetric nucleic acid sequence-based amplification-enzyme-linked immunosorbent assay (NASBA-ELISA) for rapid detection and identification of human rotaviruses (Jean et al., 2002). After the NASBA process, biotinylated RNA amplicons were hybridized with a specific amino-linked oligonucleotide probe covalently immobilized on microtiter plates. The DNA-RNA hybrids were colorimetrically detected by the addition of streptavidin-peroxidase conjugate and tetramethylbenzidine substrate. Using the NASBA-ELISA system, as little as 0.2 PFU (4 x 10^1 PFU/ml) of rotavirus were detected within 6 h. Fukuda et al developed a two-step isothermal amplification assay system combining nucleic acid sequence-based amplification and reverse transcription-loop-mediated isothermal amplification assays, which achieved the detection of norovirus (NoV) genomes in oysters with a sensitivity similar to that of reverse transcription-seminested PCR (Fukuda et al., 2008). The time taken for the amplification of NoV genomes from RNA extracts was shortened to about 3 h. Tai et al. developed a rapid method to detect astroviruses in faecal specimens utilizing nucleic acid sequence-based amplification (NASBA) and several detection methodologies, including a sandwich hybridization assay based on DNA-tagged liposomes (liposome-strip detection assay) and electrochemiluminescence detection (ECL) (Tai et al., 2003).

As the development of molecular beacon technique, real-time NASBA (RT-NASBA) has been developed. The reaction is started with a 5-min incubation at 65 °C without enzymes which will denature all the secondary structures in the RNA molecular, and then 90-min incubation at 41°C with all the enzymes to complete the rest of the reaction. Molecular beacon, which hybridizes to the targeted region, is used to monitor the product in a real-time scenario. The basic principle of RT-NASBA is shown in Figure 1.

Firstly, cDNA is generated with the antisense primer P1, which contains a 5'-overhang T7 polymerase promoter sequence, by the activity of reverse transcriptase. RNA in the RNA:cDNA hybrid is hydrolyzed by RNase H to generate single strand cDNA. Secondly, sense primer P2 anneals to the cDNA and generates dsDNA with the help of reverse transcriptase. Thirdly, T7 RNA polymerase binds to the promoter site in the dsDNA and generates antisense RNA copies of the target RNA molecular to complete the non-cyclic phase. Subsequently during the cyclic phase, primer P2 binds to the antisense RNA molecular first and generates RNA-cDNA hybrid by reverse transcriptase. Then RNase H hydrolysis RNA and primer P1 binds to it and generates dsDNA. T7 RNA polymerase can now again generate new RNA copies, which can be used as templates for the next round of amplification. During the amplification, molecular beacon hybrids to and monitors the accumulation of RNA products in a real-time way.

There are several advantages of the RT-NASBA assay. The first one is direct amplification of RNA. No separate reverse transcription step is needed, which makes the assay highly suitable for the detection of RNA virus. The second one is high amplification efficiency. In conventional PCR or RT-PCR, amplification products were only duplicated after each cycle. However, 10-100 copies of RNA could be produced in each transcription step in RT-NASBA. Therefore, the positive signal of RT-NASBA could be obtained much earlier than PCR or RT-PCR. The third one is high specificity. As T7 promoter sequence is a prerequisite, other template without T7 promoter sequence could not be amplified, which guarantees the high specificity of the assay. The fourth one is the real time detection. No post-amplification steps, such as gel electrophoresis, hybridization, ELISA, are needed. The results are shown in a real time way. The last but maybe more important one is no contamination. The assay is conducted in a closed tube and the endpoint product is easier-to-degrade RNA, which makes the chance of contamination significantly lower than conventional PCR or RT-PCR.

RT-NASBA has been applied for the detection of several diarrhoea-related viruses. Patterson et al. developed molecular beacon based RT-NASBA to detect noroviruses GII from environmental samples (Patterson et al., 2006), which was the first example of a molecular beacon based NASBA assay for NV. The NASBA was performed using the Nuclisens Basic Kit (bioMerieux, Durham, NC, USA) and an EasyQ incubator and detection system (bioMerieux). Final concentrations of primers and molecular beacon were 400 nM and 100 nM per NASBA reaction, respectively. The NV beacon (NorBeacon) was labelled with 6-carboxy fluorescein (6-FAM) at its 5'-end and

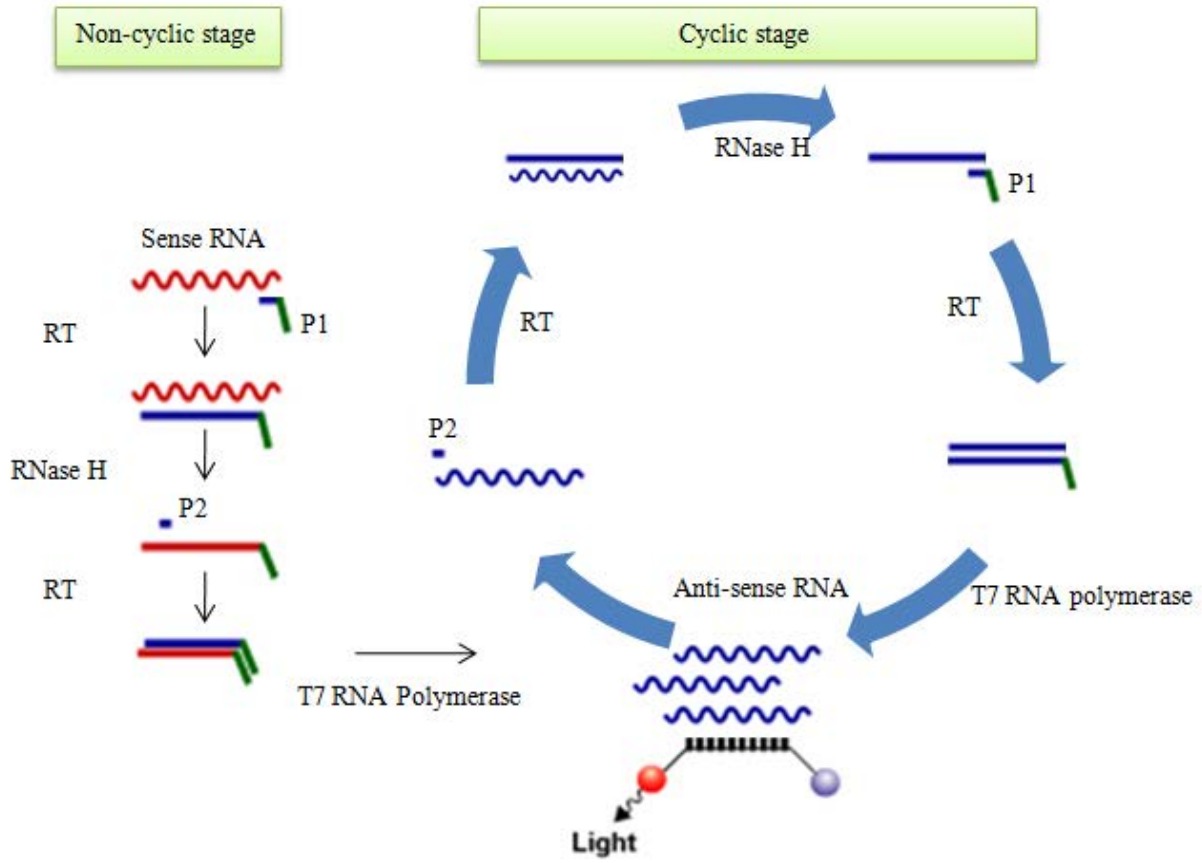

Figure 1: *Principle of RT-NASBA assay*

quencher Dabcyl at its 3'-end. The sensitivity of the assay was 100 copies of target RNA per reaction and intermittent amplification occurred even with as few as 10 copies. This method has the potential to be linked to a handheld NASBA device that would make this real-time assay a portable and inexpensive alternative to bench-top, lab-based assays. Lamhoujeb *et al.* improved the sensitivity and efficiency of the RT-NASBA assay by targeting the novel open reading frame 1-2 (ORF1-ORF2) junction of the noroviruses genome (Lamhoujeb et al., 2009). By testing 96 clinical faecal samples, the assay showed a limit of detection (LOD) of 0.01 particle detectable units, which were two logs lower than conventional RT-PCR. GII NoV was detected in 88.54% of the samples by real-time NASBA, in 86.46% by TaqMan RT-PCR, in 81.25% by conventional RT-PCR, and in 65.7% by ELISA. These results demonstrated that real-time NASBA with a molecular beacon probe was highly sensitive, accurate, and specific for NoV detection in clinical samples. Recently, our group conducted a comparison study on the detection of rotaviruses RNA by conventional RT-PCR, TaqMan RT-PCR and RT-NASBA (Mo et al., 2015b), which was the first direct comparison among three different assays for the detection of rotaviruses. The results showed that all three methods were able to detect and distinguish rotaviruses from other diarrhoea related viruses, with a 100% concordance rate during the course of an evaluation on 20 clinical stool samples. However, RT-NASBA was much quicker than the other two methods. More importantly, the LOD of RT-NASBA could reach as low as seven copies per reaction and was one to two logs lower than that of conventional RT-PCR and TaqMan RT-PCR, respectively.

As co-infection or multi-infection occurred in some cases (Koh et al., 2008; Rohayem et al., 2004), it is highly valuable to develop multiplex assays to detect several pathogens in one single tube. Though commercial multiplex real-time RT-PCR assays (such as AmpliSens Rotavirus/Norovirus/Astrovirus-FRT PCR kit (AmpliSens), RIDA GENE Viral Stool Panel I (R-Biopharm AG)) were developed for the detection of diarrhoea, no previous researches conducted on the development of multiplex RT-NASBA for that purpose. Therefore, we developed a multiplex RT-NASBA assay for the rapid and simultaneous detection of rotaviruses, astroviruses and noroviruses (Mo et al., 2015a). The LOD of the multiplex RT-NASBA assay was 7, 100, 200 copies per reaction for the rotavirus A, norovirus GII and astrovirus, respectively, which

were 10 to 100 times more sensitive than multiplex RT-PCR. Clinical evaluation indicated that the assay was 100% concordant to multiplex RT-PCR and was reliable for the detection of both single infection and multiple infections in real stool samples. To the best of our knowledge, this is the first multiplex RT-NASBA assay established for the detection of three major diarrhoea-causing viruses.

Now, we are developing RT-NASBA system for other diarrhoea related viruses, including sapoviruses, Coxsackievirus A16, and Enterovirus 71. We believe that more and more RT-NASBA-based diagnostic assays will be developed in the near future. And more importantly, multiplex RT-NASBA assays will also be developed for the purpose of simultaneous detection.

In summary, the rapid, specific and sensitive RT-NASBA assay would be highly suitable for the detection of these diarrhoea-causing viruses. When formatted in multiplex form, the assay could provide simultaneous detection of several viruses in a single run. The multiplex RT-NASBA will be useful for the high-throughput screening and diagnosis, especially in resource-limited countries where expensive thermocycling equipment is not available.

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