Evaluation of nucleic acid sequence based amplification (NASBA) and Reverse Transcription Polymerase Chain Reaction for detection of coxsackievirus B3 in cell culture and animal tissue samples

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Abstract
Enteroviruses are the causative agents of a number of diseases in humans. Group B coxsackieviruses are believed to be the most common viral agents responsible for human heart disease. Genomic data of enteroviruses has allowed developing new molecular approaches such as Nucleic Acid Sequence Based Amplification (NASBA) for detection of such viruses. In this study, coxsackievirus B3 (CVB3) was detected in virus-infected cell culture and specimens of artificially infected mice with specific primers using Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and NASBA techniques. According to the results, both techniques could be used for the detection of viruses in cell culture and artificially infected animals. NASBA reaction was simpler to perform than RT-PCR. The only variable factor that had to be optimized with NASBA is KCl concentration. The optimal concentration of KCl was determined as 90 mM. Serial dilutions of 1 μg of total RNA showed that both RT-PCR and NASBA could detect the virus at 10^{-5} dilution. Analyses of heart and spleen samples from infected animals were positive for presence of Coxsackievirus B3 with both RT-PCR and NASBA. In conclusion, NASBA offers some advantages over RT-PCR and is a suitable alternative technique for the sensitive detection of CVB3 in contaminated samples.

Keywords: Coxsackievirus B3; NASBA; RT-PCR

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INTRODUCTION

Human enteroviruses are causative agents of a number of diseases and are widespread throughout the world. Enteroviruses, particularly the group B coxsackieviruses, are believed to be responsible for the most common viral human myocarditis (Woodruff, 1980). It is speculated that they may play a role in development of idiopathic dilated cardiomyopathy (IDC) (Lerner and Wilson, 1987). Enteroviruses have a strong tropism for the myocardium (Lerner and Wilson, 1987). Inoculation of coxsackieviruses in experimental animals induces myocarditis, which can evolve into cardiomyopathy with associated virus persistence for several weeks after primary infection (Kandolf *et al*., 1987; Kawai *et al*., 1978). Evidence for involvement of enteroviruses in acute myocarditis was suggested by detection of neutralizing IgM and IgG antibodies specific to the group B coxsackieviruses (Baboonian *et al*., 1997). The presence of coxsackievirus B3 (CVB3) specific IgM in patient's sera after a phase of myocarditis indicated that enteroviruses could also establish chronic infection, which would result in IDC (Muir and Archard, 1994). At this stage, the detection of enteroviral particles or viral antigens in the myocardium by using conventional biological techniques was unsuccessful (Bowles and Towbin, 2000; Woodruff, 1980).

Genomic data of enteroviruses has allowed the development of new molecular approaches for detec-

tion of viruses (Rotbart, 1990). Hybridization studies, that use slot-blot or *in situ* hybridization, have demonstrated positive enterovirus detection in the myocardium of patients with acute myocarditis and IDC (Kandolf *et al*., 1987). The use of RT-PCR has allowed more specific and sensitive enterovirus detection in the human myocardium (Rey *et al*., 2001; Schwaiger *et al*., 1993; Severini *et al*., 1993; Grasso *et al*., 1992; Rotbart, 1990). Enteroviral RNA has been observed in heart tissue from approximately 25% of patients suffering from idiopathic acute myocarditis but also in 25% of patients undergoing transplantation due to end-stage chronic myocarditis. These findings suggest that molecular techniques could be valuable tools for clinical diagnosis of enterovirus endomyocardial infection (Baboonian *et al*., 1997).

Nucleic acid amplification techniques, such as PCR, are highly sensitive and specific tools for the amplification and detection of pathogen-specific sequences. As an alternative to PCR, the nucleic acid sequence-based amplification (NASBA) technique has been developed for detecting specific nucleic acids (Compton, 1991). NASBA is an isothermal nucleic acid amplification reaction, using two specific oligonucleotide primers (one containing a T7 promoter sequence) and three enzymes, AMV reverse transcriptase, RNase H and T7 RNA polymerase (Fig. 1) (Deiman *et al*., 2002; Jean *et al*., 2001; Compton, 1991; Kievits *et al*., 1991). NASBA can amplify DNA and RNA template sequences and its major reaction product is RNA complementary to the target sequence

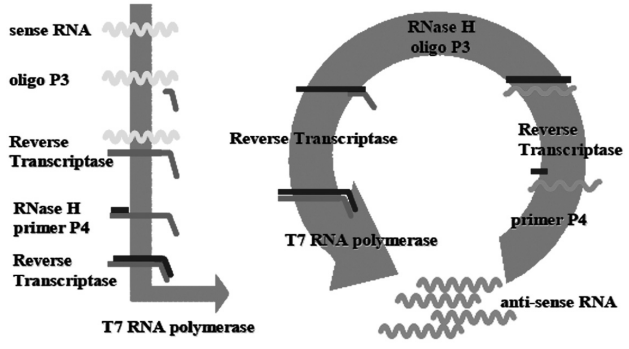

Figure 1. Schematic presentation of the NASBA method, the method is based on the simultaneous activity of AMV reverse transcriptase, RNase H and T7 RNA polymerase with two oligonucleotide primers (P3 and P4) to amplification of the desired fragment more than 10^{12} fold in duration of 90 to 120 minutes.

(Rodríguez-Làzaro *et al*., 2004). NASBA has proven to be a useful technique for the highly sensitive detection of viral nucleic acids in clinical samples (Loens *et al*., 2006; Yoo *et al*., 2005; Chan and Fox, 1999), microbial pathogens in food and environmental samples (Fykse *et al*., 2007; Jean *et al*., 2004; Cook, 2003). With some modifications, It has also been used in the quantization of viral nucleic acids (Schneider *et al*., 2005; Leone *et al*., 1998).

In the present study, NASBA and RT-PCR techniques were developed and evaluated for diagnosis of CVB3 in cell culture and artificially infected animals.

MATERIALS AND METHODS

Virus culture and RNA extraction: CVB3 Nancy strain, ATCC number VR-30 was grown on Vero cell culture and used as a positive control. Virus-infected cultures were collected 48 h after infection when all the cells showed the characteristic cytopathic effect. Uninfected cell culture was also used as a negative control. Aliquots of positive and negative cultures were collected and stored at -80ºC for the purpose of RNA extraction. Specimens were frozen and thawed three times and clarified by centrifugation at 13,000 $\times g$ for 10 min at 4ºC. Viral nucleic acid was extracted from the supernatant by the TriZol RNA extraction kit (Fermentas, Germany), according to the manufacturer's instructions.

Artificially infected animals: Eight Balb/c mice, aged 8 weeks, were divided into 2 same groups. Group 1, the control group, inoculated with medium, while group 2 inoculated with the cultured virus. A sample containing 10^5 plaque forming units (pfu) of the CVB3 was used to inoculate Balb/c mice via the intraperitoneal route (IP). After a week, a booster dose was used to inoculate again and 2 weeks later, samples were collected from heart, blood, feces and spleen, and then stored at -80ºC for RT-PCR and NASBA analyses.

Primer design: Primers were designed by the Oligo 6 software, (Molecular Biology Insights, Inc., Cascade, CO), according to the CVB3 genomic sequence in GenBank (Accession No: FJ000001). The P1 and P2

Table 1. Primers for detection of Coxsackievirus B3.

Primers	Sequence[a]	Position
P1	5´- CACACTACCGGTTTGTTGCTTCAG -3´	2258-2281
P2	5´- TCCCTCGGTCCAAAACACACTG -3´	2946-2967
P3	5´ -AATTCTAATACGACTCACTATAGGGAGAGACGTCATATTGCGGCATGGC-3´	1807-1827
P4	5´-TTGTACCGCTAGATTACTGC-3´	1631-1650

[a]Underlined sequence represents the T7 promoter sequence.

primers for the RT-PCR were designed on the basis of the VP1 and VP3 genes to amplify a 710 bp segment of CVB3 RNA. The NASBA primers, designated P3 and P4, were also directed against the VP3 and VP2 genes of CVB3, respectively. The P3 primer contains the T7 RNA polymerase promoter which is recognized by T7 RNA polymerase during the amplification step leading to the production of single stranded anti-sense RNA. Because the amplification of longer products is less efficient when using the NASBA reaction, the primers were selected in a manner to amplify a 225 bp product. The primers were checked for secondary structures and dimmers and primers' specificity were determined by the Blastn program. The primers were designed in a manner to specifically detect CVB3 (Table 1).

Reverse Transcription- Polymerase Chain Reaction (RT-PCR): RNA was amplified using a two step RT-PCR method. For reverse transcription, samples were denatured for 10 min at 65ºC followed by incubation at 37ºC during which reverse transcriptase was added. After incubation for 60 min, the reaction was stopped by heating at 95ºC for 5 min. PCR reaction was carried out followed by 35 cycles of at 94ºC for 45 s, 55ºC for 45 s, and 72ºC for 30 s. PCR products was analyzed by 1% agarose gel electrophoresis. RT-PCR products were confirmed by digestion with appropriate restriction enzymes.

Nucleic Acid Sequence Based Amplification (NASBA): The NASBA was carried out as described by Kievits *et al*. (1991) with some modifications. The reaction mixture was prepared in a total volume of 20 µl containing 5 µl of extracted RNA, 40 mM Tris (pH 8.5), 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiotritol (DTT), 1 mM of each deoxyribonucleoside triphosphate (dNTP), 2 mM each ribonucleoside triphosphate, 15% (v/v) dimethylsulphoxide (DMSO), and 5 pmol of each primer. The reaction mixture was incubated at 65ºC for 5 min and subsequently transferred to 41ºC. After 5 min, an enzyme mixture containing 2.1 µg of BSA, 0.08 U of RNase H (0.08 U/µl) of RNase H, 32 U of T7 RNA polymerase (32 U/µl) and 6.4 U of Avian Myeloblastosis Virus - Reverse Transcriptase (AMV-RT) (6.4 U/µl) was added to the mixture and the resulting reactions were performed at 41ºC for 90 min. NASBA products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS

In this study, the RT-PCR and NASBA assays were performed for the detection of CVB3 in virus-infected cultures and specimens of artificially infected mice. The results of NASBA were compared with those of RT-PCR.

Virus detection in cell culture: RNA was extracted from CVB3 infected cells and used to determine the appropriate reaction conditions and evaluate the sensitivities of the NASBA and RT-PCR assays. The size of NASBA and RT-PCR products were 225 and 710 bps, respectively. The NASBA and RT-PCR gave positive specific amplification signals when challenged with the RNA of CVB3 infected cells.

The NASBA assay of CVB3, was optimized for KCl concentration. The level of amplified product increased as the concentration of KCl was increased from 50 to 90 mM (Fig. 2). The sensitivity of NASBA and RT-PCR were determined by testing serial $\log_{10}$ dilutions of RNA obtained from CVB3-infected cells. The results showed that both NASBA and RT-PCR could detect CVB3 at the 10^{-5} dilution of a 1 µg RNA sample extracted from the virus-infected cells (Fig. 3).

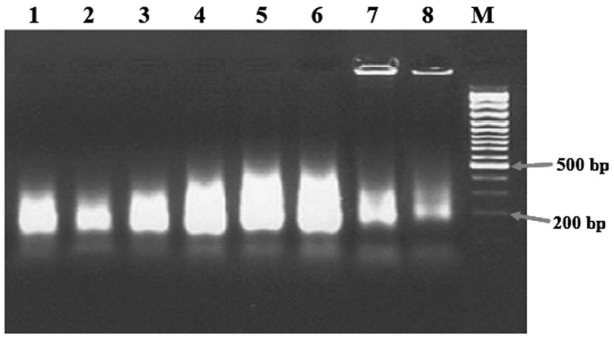

Figure 2. Optimization of KCl concentration for NASBA. NASBA was performed using a KCl concentration range of 50-120 mM. Lane 1, 50 mM; lane 2, 60 mM; lane 3, 70 mM; lane 4, 80 mM; lane 5, 90 mM; lane 6, 100 mM; lane 7, 110 mM and lane 8, 120 mM of KCl. Lane M, molecular weight marker (100 bp DNA Ladder, Fermentas, Germany).

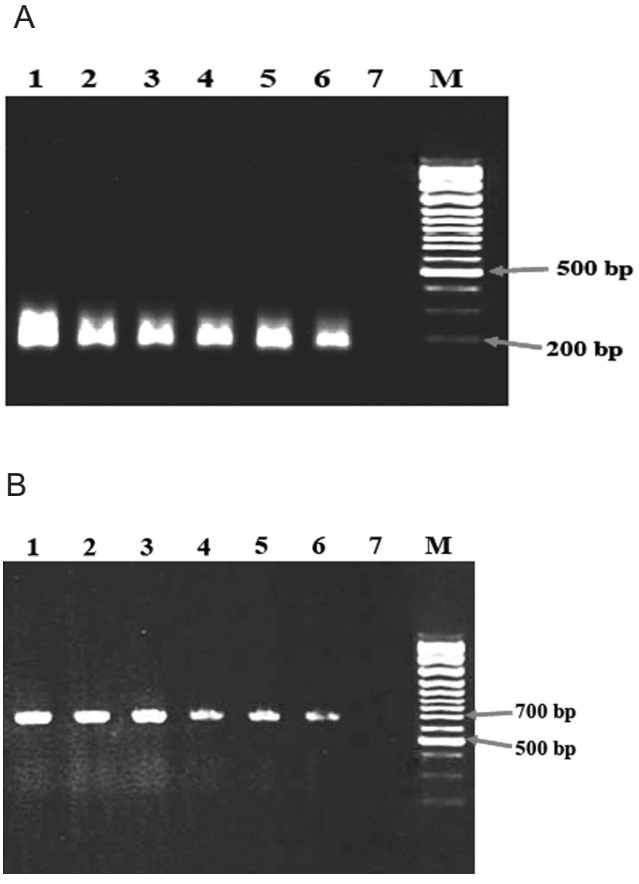

Figure 3. Sensitivity of NASBA and RT-PCR reactions. Sensitivity of reactions was determined by serial dilutions of total RNA from virus-infected cells. Lane 1, 1 µg; lane 2, 0.1 µg (10^{-1} dilution); lane 3, 0.01 µg (10^{-2} dilution); lane 4, 1 ng (10^{-3} dilution); lane 5, 0.1 ng (10^{-4} dilution); lane 6, 0.01 ng (10^{-5} dilution); lane 7, 1 pg (10^{-6} dilution) of RNA derived from infected Vero cells; Lane M, molecular weight marker (100 bp DNA Ladder, Fermentas, Germany). NASBA (A) and RT-PCR (B) can detect 10^{-5} dilution (lane 6) of initial RNA.

Assessment of specificity: Poliovirus vaccine was used for assessment of both NASBA and RT-PCR specificities. Neither NASBA nor RT-PCR could detect poliovirus extracted RNA under the conditions described for CVB3.

Analysis of artificially infected animals: The NASBA and RT-PCR were further evaluated by testing infected mice. Balb/C mice were inoculated by the IP route with 10^5 PFU of the virus. Samples were collected 2 weeks post-infection from heart, spleen, blood, and feces. The results of RT-PCR on heart and spleen samples were positive, but not on blood and feces samples. The NASBA also detected the virus in heart and spleen specimens (Fig. 4).

DISCUSSION

Enteroviruses are responsible for several diseases in humans. Among enteroviruses, CVB3 is the most

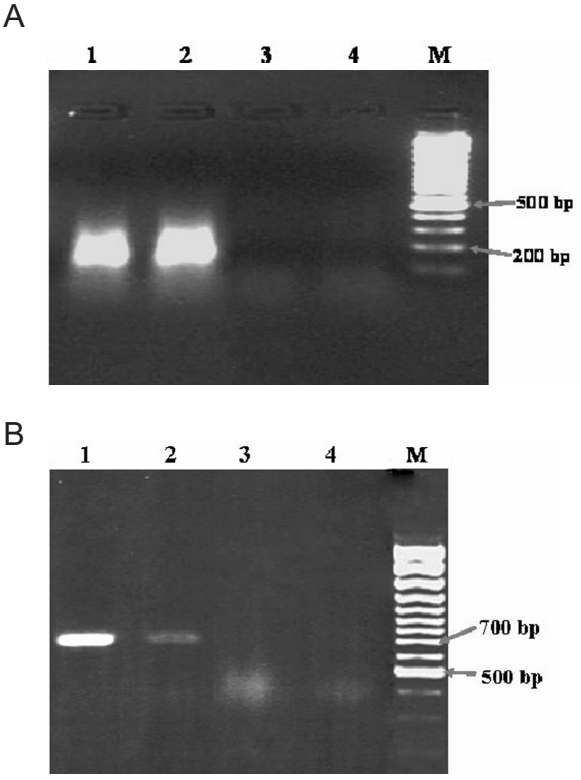

Figure 4. Analysis of infected mice tissues with NASBA (A) and RT-PCR (B). Lane 1, 1 µg RNA derived from heart; lane 2, 1 µg RNA derived from spleen; lane 3, 1 µg RNA derived from blood; lane 4, 1 µg RNA derived from feces; Lane M, molecular weight marker (100 bp DNA Ladder, Fermentas, Germany).

important viral agent of myocarditis and IDC (Lerner and Wilson, 1987; Woodruff, 1980). Detection of enteroviral RNA in myocardial tissue of patients with IDC has only been demonstrated using slot blot (Bowles, *et al*., 1986), *in situ* hybridization (Kandolf, 1988), and PCR (Shigekazu, *et al*., 2000; Jin, *et al*., 1990). Detection of enteroviral RNA in some diseases was demonstrated using the NASBA technique (Capaul and Gorgievski-Hrisoho, 2005; Ginocchio *et al*., 2005; Heim and Schumann, 2002; Fox *et al*., 2002).

In the present study, NASBA and RT-PCR techniques were used to detect CVB3 in cell culture and animal samples. The results revealed that RT-PCR and NASBA are suitable for detection of the virus in cell culture. The sensitivity of these techniques was determined by serial dilution of total RNA. Both the techniques could detect viral RNA at the 10^{-5} dilution of a 1 µg RNA sample derived from CVB3-infected Vero cells (Fig. 3). Experiments on animal models revealed that NASBA is a suitable alternative to RT-PCR for sensitive detection of the virus in heart and spleen samples. But there were no positive amplification signals in the case of feces and blood samples by both RT-PCR and NASBA. It may be due to the presence of inhibitors such as complex polysaccharides in feces which prevent amplification during RT-PCR and NASBA procedures. The negative results with blood samples may be related to low levels of the virus. The specificity test was only examined with the poliovirus. Of course, it is not enough for confirming the specificity of the techniques, but there was limited access to all entroviruses and the results showed an absence of amplification by both RT-PCR and NASBA techniques.

In a previous study, small amounts of viral genomes were detected in a latent state with focal distribution in the myocytes (Kandolf, 1988). Grasso *et al*. (1992) detected CVB3 at the 10^{-5}-10^{-6} dilutions of a 1 µg RNA sample derived from coxsackievirus B3-infected cells. Other studies have shown that NASBA and RT-PCR produce comparable results and are significantly more sensitive than the virus culture technique (Houde *et al*., 2006; Loens *et al*., 2006). Rutjes *et al*. (2005) have found that the rapid real-time NASBA assay is slightly less sensitive than the RT-PCR for the detection of entrovirus in water.

According to the present study, both NASBA and RT-PCR have the same sensitivity and specificity for detection of CVB3. NASBA offers some advantages over RT-PCR because it is an isothermal reaction, obviating the need for a thermal cycler and the optimal annealing temperature for primers does not have to be determined empirically. NASBA is a single step reaction, which decreases the risk of cross contamination in comparison to the two step RT-PCR. It is important to detect CVB3 in clinical laboratories in order to avoid false positive results. Because NASBA is performed in a single, closed tube format, it minimizes the risk of amplicon contamination due to fewer handling step. The amplification conditions for NASBA are generally constant, and optimization of conditions for each new assay can be simpler than RT-PCR. The concentrations of enzymes and primers used for NASBA are standardized and do not differ from assay to assay. The only variable factor that has to be optimized in NASBA is the KCl concentration. It is easily performed in a single experiment using a KCl concentration range of 50-120 mM. However, most targets will have optimal KCl concentrations between 70 and 90 mM. In this study, the optimal concentration of KCl was determined as 90 mM. NASBA is easier to perform and produces results more rapidly than RT-PCR.

In conclusion, NASBA is simpler and safer to perform than RT-PCR for detection of coxsackievirus B3. There is no advantage in sensitivity and specificity between the two techniques. Therefore, we recommend the NASBA as an alternative method for detection of this virus in cell culture. Performance of NASBA on samples obtained from cell cultures as well as tissue biopsies from infected animals is promising. However, its efficacy in detecting the CB3 virus in human samples awaits further investigations.

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