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RT-PCR Detection of Coxsackievirus B3: A Viral Myocarditis

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Backgrounds and Aims: Coxsakievirus B3 (CVB3), one of the six Coxsakievirus B serotypes, is a member of the Enterovirus genus within the Picornaviridae family. CVB3 is an important pathogen of viral myocarditis, which accounts for more than 50% of viral myocarditis cases. The genome of CVB3, like that of other Entroviruses, is a single-stranded, sense, polyadenylated RNA molecule with 7400 nucleotides in length and a single open reading frame (ORF), flanked by 5′ and 3′ non-translated regions. The capsids of coxcakieviruses are composed of the four structural proteins: viral protein-1 (VP1), VP2, VP3, and VP4. In the present study, a new set of primers were designed based on the VP1 for RT-PCR detection of CVB3.

Materials and Methods: Total RNA was extracted from CVB3-infected HeLa cell line and cDNA was synthesized using random primers. Then, PCR was carried out by specific primers and the PCR product analysis was performed using 1% agarose gel electrophoresis. Moreover, the sensitivity and specificity of this method were determined using serial dilution of CVB3 cDNA and three genuses of entroviruses, respectively.

Results: RT-PCR assay revealed a 234 bp specific amplified fragment. The sensitivity of this test was determined 5.72 fg/µl cDNA. On the other hand, the specificity was successful in comparison with coxsackievirus A16, Echovirus 36 and Rhinovirus.

Conclusions: The RT-PCR is a highly sensitive and rapid technique for detecting CVB3 infection. Moreover, this method can be used as an easy diagnostic test in regard with CVB3 detection in the clinical laboratories.

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Introduction

Myocarditis is a non-familial form of heart muscle disease [1, 2], defined as an inflammation of the heart muscle, and identified by immunohistological, clinical or histopathologic criteria [2, 3, 4]. Previous studies have demonstrated that viral myocarditis and the affiliate inflammatory response can be stated as basic determinants of the degree of virus-associated myocardial damage [5]. Viral myocarditis is regarded as a moderate disease, though it sometimes causes irreversible myocardopathy and progressive cardiac disorder [6]. Many different viruses have been considered as the agent of viral myocarditis, including enteroviruses, cytomegalovirus, adenovirus, parvovirus B19 and hepatitis C virus [7]. Among the normal infectious agents, the group B coxsackie viruses are important pathogens of viral myocarditis, which accounts for more than 50% of viral myocarditis cases [6]. The group B coxsackieviruses consists of six serotypes, which are members of the Enterovirus genus within the Picornaviridae family [8-11] associated with a wide spectrum of human illness ranging in severity from subclinical infection to rapidly fatal disease, with symptoms as diverse as acute myocarditis, aseptic meningitis, encephalitis, Bornholm disease (epidemic pleurodynia), nonspecific rashes and febrile illness [9]. This disease is composed of three distinct stages including viremic injury, immune infiltration, and reclamation [12]. Earlier studies have suggested that mechanisms of viral myocarditis involve direct myocyte injury by Coxsakievirus B3 (CVB3) and subsequent immune-mediated damage of the heart [13]. The primary route of enteroviral infection is fecal-oral. Oral lymph nodes and gut are assumed to be the initial sites of enteroviruses replication. Then, viremia occurs rapidly and these spread viruses infect the secondary sites such as central nervous system, pancreas, skeletal muscles or heart [14].

The CVB3 genome is 7,400 nucleotides in length consisting of a positive single stranded RNA flanked by non-translated regions that encodes 11 proteins within a single open reading frame [15-18]. The 5'-terminal region is linked to a virus-encoded protein, and the 3' terminus is completed with a polyadenosine tail [19, 20]. The viral genome is packaged in a naked capsid approximately 29 nm in diameter [14]. The CVB3 capsid is composed of four viral polypeptides known as VP1 to VP4 [21- 23]. The VP1 capsid gene has the conserved sequences to design specific primers for preformation of the species-specific polymerase chain reaction [24, 25]. Reverse transcription polymerase chain reaction (RT-PCR) assay is used as a highly sensitive, specific and common diagnostic method for identification of the infectious agents [26, 27]. The comparative study is one of the key advantages of RT-PCR assay and diagnosis by RT-PCR can increase the enteroviruses detection yield in tissue culture [27].

In the current study, a new set of specific primers was designed based on the VP1 gene for CVB3 detection in RT-PCR assay.

Materials and Methods

Viral culture and RNA extraction

Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS) was used for growth and maintenance of HeLa cell cultures. The cells were infected at 90% confluency with CVB3 (Nancy strain) in a medium containing 1% FCS. After 24 hours that cytopathic effect was thoroughly observed, the infected cells were harvested and centrifuged at 5000 rpm for 5 min. The cell sediment was used for the total RNA extraction. Hence, after two cycles of freezing and thawing, total RNA was extracted using RNX reagent according to the manufacturers' instruction. The integrity of the extracted RNA was assessed by resolution on a 1% agarose gel and ethidium bromide staining. Each RNA sample was stored at -80°C until two step RT-PCR experiment.

Primer design

For the RT-PCR method, primers were designed according to the CVB3 genomic sequence (VP1 gene) in Gen Bank (Accession No: JX312064). The specific primers (Forward primer: 5′-GGGTCACACGTCACAAGTAGTG-3′ and reverse primer: 5′-GTCAGCTCCAGGTCGAACC-3′) were designed in regard with amplification of a 234 bp segment from CVB3 RNA.

Reverse transcription (cDNA synthesis)

cDNA was synthesized in a 20 µl reaction mixture. First, 4 µl of total RNA(542 ng/ µl) and 1 µl of random primer (10 mM) were added into a microtube and heated at 65°C for 5 min. and then chilled on ice. After addition of 1 µl of dNTP (10 mM), 2 µl of DTT (0.1 M), 1 µl of RevertAid™ Reverse Transcriptase (200 U/µl) (Fermentas) and 4 µl of RT buffer (5X), the reaction mixture was incubated at 42°C for 90 min. The reaction was terminated by incubation at 95°C for 5 min. The synthesized cDNA was stored at -20°C until PCR reaction.

PCR reaction

PCR was performed using specific primers. The amplification reaction was prepared in a volume of 12 µl containing 170 ng of cDNA, 10 pmol of each primer, and master mix of Taq DNA polymerase (Ampliqon PCR Kit, Denmark). The PCR was performed by initial denaturation at 94°C for 4 min., 35 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 45 sec and a final extension at 72°C for 5 min. PCR procedure was carried out using an automated thermal cycler (Techgene, Germany) and all PCR products were analyzed by 1% agarose gel electrophoresis.

Specificity and sensitivity of primers in RT-PCR detection

The specificity of primers was determined by RT-PCR detection for some serotypes of enteroviruses genus including coxsackievirus A16, Echovirus 36 and Rhinovirus. Furthermore, in order to evaluate the sensitivity of RT-PCR primers for detection of CVB3, after preparation of cDNA, 5 dilution of cDNA (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}), from 5.72 µg/ml to 5.72 fg/µl, were prepared and used as a template for PCR. Then, the amplified

DNA was analyzed using 1% agarose gel electrophoresis.

Results

RT-PCR Detection

Total RNA was extracted from CVB3-infected HeLa cells according to the above procedure. Observation of 28S, 18S rRNA and tRNA bands confirmed the integrity of extracted total RNA (Fig. 1-B). Moreover, in order to confirm the presence of CBV3 genome in total RNA, reverse transcription was performed and cDNA was synthesized in a 20 µl reaction mixture. At last, cDNA was used for PCR method. After two-step RT-PCR amplification, one fragment of 234 bp was amplified, which confirmed the RNA genome of virus in total RNA extracted from the infected cells (Fig. 1-A).

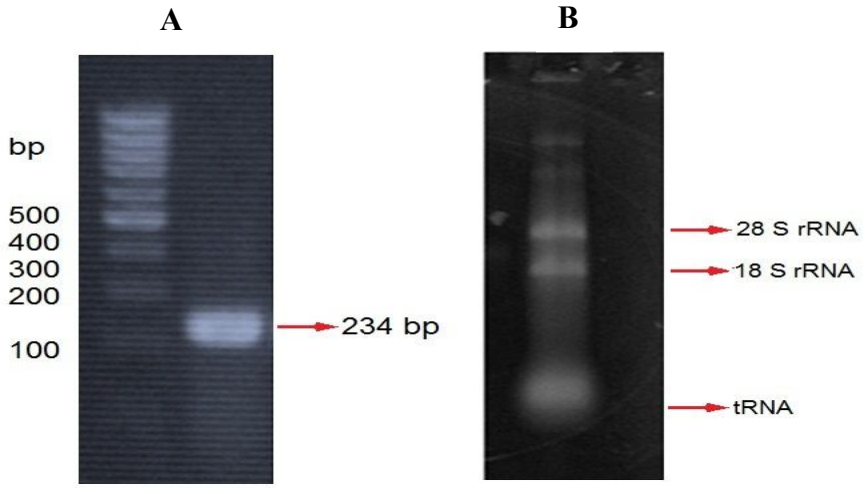

Fig. 1. (A) Analysis of RT-PCR product using 1% agarose gel electrophoresis. The amplified 234 bp fragment confirmed the RNA genome of CVB3 in the extracted total RNA. (B) Agarose gel electrophoresis of extracted total RNA.

RT-PCR selectivity and sensitivity

The selectivity of CVB3 RT-PCR detection was examined by total RNA extracted from cells infected with CVB3 and 3 different enterovirus strains including Coxsackieviruse A, Echovirus, and Rhinovirus. According to Fig. 2-A, the selectivity of RT-PCR assay was confirmed. On the other hand, 5-fold serial dilutions of the cDNA (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}) were prepared from 5.72 µg/µl to 5.72 fg/µl. Analysis of PCR products by 1% agarose gel electrophoresis showed amplification of a 234 bp specific band after RT-PCR assay until 10^{-8} dilution (10^{-2} to 10^{-8}) (Fig. 2-B).

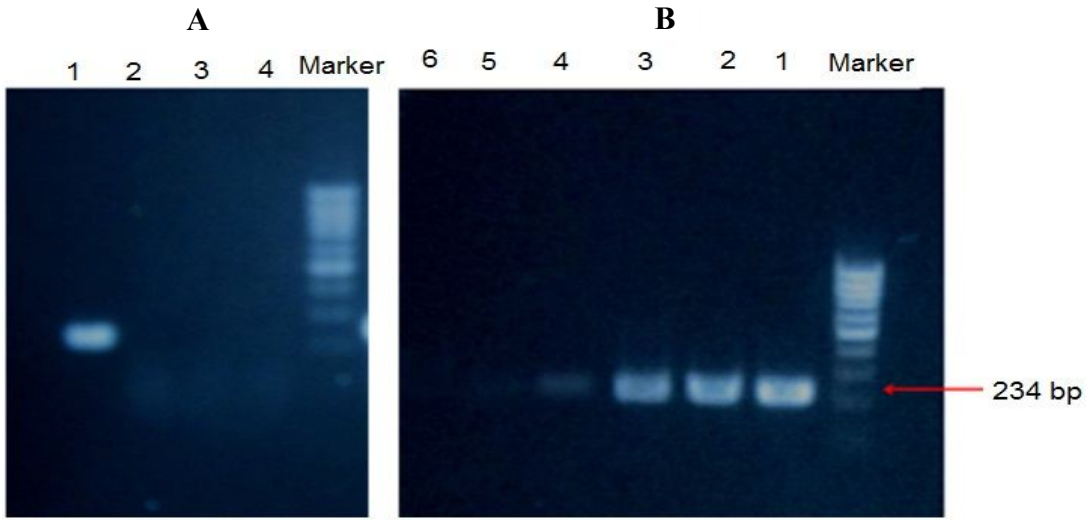

Fig. 2. (A) Specificity of RT-PCR was determined of cDNA that synthesis from total RNA extraction of CVB3 (1), CVA16 (2), Echovirus (3) and Rhinovirus (4), 1 kb DNA ladder (Marker). (B) Sensitivity of RT-PCR was determined by serial dilutions of cDNA that synthesis from total RNA extraction of CVB3 infected HeLa cells. 10^{-2} (1), 10^{-4} (2), 10^{-6} (3), 10^{-8} (4) and 10^{-10} (5) concentration of cDNA. Lane 6 is negative control and 100 bp DNA ladder (Marker).

Discussion

CVB3 is a RNA virus which belongs to the category of non-enveloped, linear, positive sense ssRNA viruses from *Picornavirus* family and the *Enterovirus* genus. This virus is regarded as a human pathogen, which is causative agents for at least 50% of acute myocarditis, and 25% of dilated cardiomyopathy cases [8-11, 28]. Detection and identification of CVB3 RNA in heart patients has used various serologic and molecular methods such as in situ hybridization [28], nucleic acid sequence based amplification (NASBA) [29], and RT-PCR [6, 9, 30, 31]. VP1 is a major capsid protein, playing an important role in directing viruses towards the permissive host for infection [32] and consequently inducing cell damage by cytopathic effect or apoptosis [33], which is necessary for virus lytic cycle. Therefore, VP1 could be used as a critical factor for detection of CVB3.

Pauschinger et al. (1999) used two step RT-PCR assay to examine the myocardial biopsies of 45 patients with myocarditis and 26 samples of the negative control for detecting enteroviral RNA. In their study, in patients with myocarditis as positive controls, enteroviral RNA was detected in 40% cases and 234 bp specific bands were produced after amplification of RT-PCR assay. In addition, no positive results were observed for the negative controls [34]. Bourlet et al. (1997) studied detection of coxsackievirus B3 in intestinal tissue of orally-infected mice by a standardized RT-PCR assay. In their study, intestinal tissue fragments were digested by trypsin to cell inoculation from day 1 to day 4 of mice post-infection. When a cytopathic effect was detected, the Total RNA was

extracted, and then CBV3 genome in intestinal tissue was detected using the RT- PCR assay. Total RNA extracts of other enteroviruses (negative controls) , were demonstrated to be negative by the RT-PCR assay. Sensitivity of the RT-PCR assays was 1 to 10^{-2} TCID50/100 µl in various experiments [30]. Weiss et al.'s (1991) study was conducted on the detection of coxsackievirus B3 RNA in animal models, viral infected-HeLa cells, and myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy via the RT-PCR. The sensitivity of the technique was established at approximately 1 to 100 PFU of virus per gram of tissue, and the specificity was established as limited to the coxsackievirus B3 serotype among nine viruses tested in patients with myocarditis [26]. Saeedinia et al. (2008) evaluated NASBA and RT-PCR in order to detect coxsackievirus B3 in cell culture and animal tissue samples. In their 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 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 [29]. Dierssen et al. (2008) provided a one-step transcription/real-time (TaqMan probe) PCR assay (TM-PCR) with a new consensus primer and probe sequences for generic detection of human pathogenic enteroviruses species. In this study, 95% detection limit was found to be 100 copies per run using in vitro transcribed coxsackievirus B3 RNA. TM-PCR was compared to an in house nested-PCR assay implemented in detecting enterovirus RNA from CSF samples of patients suffering from meningitis and encephalitis. Concordant results were obtained in all the samples (11 positive, 101 negative) [35].

In the present research, initial setup of RT-PCR method was applied in order to detect coxsackievirus B3 in the infected-HeLa cell. In addition, the sensitivity and specificity of this test was determined. The results of the present study revealed that, the sensitivity of this test is determined 5.72 fg/µl cDNA, and the specificity was successful compared with coxsackievirus A16, Echovirus 36 and Rhinovirus.

Conclusion

In this study, a new set of specific primers were based on the VP1 gene in regard with CVB3 detection in the cell culture. The results revealed that RT-PCR is a highly sensitive and suitable technique for detection of CVB3 infection. Moreover, this method can be used in the clinical laboratories as a diagnostic and easy test for detection of CVB3.

Conflict of Interests

The authors declare no conflict of interest**.**

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