大韓바이러스學會誌 : 第 30 卷 第 1 號 2000
J. Korean. Soc. Virology, Vol. 30, No. 1, 2000

Detection and Characterization of Enteroviral RNA in Paraffin-embedded Heart Tissues from Patients with Dilated Cardiomyopathy

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=Abstract=

The aim of this study was to investigate viral etiology in dilated cardiomyopathy (DCM) by polymerase chain reaction (PCR) or nested reverse transcription PCR (RT-PCR), and characterize the enteroviral RNA presented in the clinical specimens. Twenty-eight paraffin-embedded heart tissue samples were assayed to detect cytomegalovirus, herpes simplex virus type 1, type 2, parvovirus, adenovirus, and enterovirus (EV) with each specific primer. Of these 28 patients (mean age: 27, M: 24, F: 4), 26 were histologically diagnosed as DCM and 2 as myocardial infarction (MI). Nested RT-PCR detected enteroviral RNA in 7 (26.9%) of 26 patients with DCM, and none of patients with MI. And none of DNA viruses tested were detected from the samples.

Amplified products were also genotyped by single-strand conformation polymorphism (SSCP). Three subtypes can be differentiated from 7 clinical specimens. Furthermore, direct sequence analysis was performed to determine whether genetic variation of EV is present in the explanted heart tissues from patients with DCM. Although most of the sequences among the wild isolates have the greatest similarity to those of coxsackievirus B3, there are specific regions of variable sequences (no 490 - no 510).

The data suggest that enterovirus may be a major viral pathogen for the DCM in Korea and nucleotide sequence data indicate that coxsackievirus B3 may be a leading etiologic agent of DCM.

Key Words: Dilated cardiomyopathy, Enterovirus, Genotype, 5'-nontranslating region, PCR

INTRODUCTION

Dilated cardiomyopathy (DCM) is defined as a heart muscle disease with characteristics of impairment of systolic function, dilatation of ventricles and myocardial fiber hypertrophy [1]. Histopathological study showed close association of myocarditis with DCM [21]. However, most cases of DCM are of unknown etiology although many evidences indicate that viral agents, especially enteroviruses (EVs), play an important role in the pathogenesis of myocarditis and DCM [16,31].

접수 : 2000년 3월 13일, 논문게재확정 : 2000년 4월 7일

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Since it has been very difficult to isolate etiologic agents from heart tissue, polymerase chain reaction (PCR) is, therefore, frequently used to detect viruses [3,7,8,10,11,15,19,23,24,29,30].

EVs are the most common cause of aseptic meningitis resulting in approximately 30,000-50,000 hospitalizations per year in the United States [18]. Other enteroviral diseases include mild illness such as common colds, hand-foot-and-mouth disease, as well as potentially life-threatening illnesses, including myocarditis and DCM. An association of enteroviral infection with heart diseases has been demonstrated previously by retrospective serology [9].

EV, a member of *Picornaviridae* family along with rhinovirus, cardiovirus, and aphthovirus is a single-stranded RNA virus. EVs include poliovirus (types 1-3), coxsackievirus A (types A1-A22, A24), coxsackievirus B (types B1-B6), echovirus (types 1-9, 11-27, 29-33), enterovirus (types 68-72). Different serotypes cause different infections [17]. However, the relationship of serotypes with enteroviral infections are not known. The reasons are partly due to classification of enteroviruses by phenotype, not genotype. Therefore, there is a limitation to study pathogenesis of enteroviral infection using serotypes. It is useful to differentiate enteroviruses according to genotypes and study pathogenesis. Site-directed mutagenesis study showed that 471-484 nucleotides sequences were important for the neurovirulence of poliovirus [27]. Therefore, nucleotide sequence analysis of 5'-nontranslating region (NTR) of wild strains of enterovirus may be helpful to study pathogenesis of enteroviral infections. In addition, the World Health Organization has recommended to conserve the use of the now limited stock of Lim and Benyesh-Melnick antiserum pools, which is essential for the determination of classic means of typing of an EV. Therefore, alternative typing methods such as PCR with restriction fragment length polymorphism analysis or PCR-single-strand conformation polymorphism (SSCP) are required for the epidemiolgical surveillance.

The purpose of this investigation was to determine the prevalence of viral infections in paraffin-embedded explanted heart tissue from patients with DCM undergoing heart transplantation in Korea. PCR and nested RT-PCR were used to screen for the presence of viral genomes in the heart tissues. Furthermore, we characterized the 5'-NTR of enteroviral RNA found from the paraffin-embedded heart tissues by SSCP and nucleotide sequence analysis.

MATERIALS AND METHODS

Patients population

We collected 28 paraffin-embedded myocardial tissue samples from patients who had undergone heart transplantation from April 1994 to March 1997. The patients ranged in age from 13 to 63 years (mean age: 27, M: 24, F: 4). Of these 28 patients, 26 were diagnosed as DCM and 2 as myocardial infarction (MI).

Template preparation

Viral nucleic acid was obtained by the isolation of total nucleic acids from paraffin-embedded heart tissue [25]. Briefly, five micrometer sections were cut from paraffin-embedded heart tissue and deparaffinized by immersing in 1 ml of xylene for 10 min at room temperature. The pellets were washed twice in 1 ml of 100% ethanol for 5 min and 95% ethanol once. After air-dried, the pellets were digested at 60℃ for 3 h (RT-PCR) or overnight (PCR) in 600 μl of TE (pH 8.0) buffer containing proteinase K (200 μg/ml) and 1% sodium dodecyl sulfate. To extract the RNA, the digested solution was spun for 5 sec and 200 μl of supernatants were put into new tube. The 150 μl of saturated phenol and 150 μl of chloroform-isoamylalcohol (24 : 1) were added and vortexed vigorously for 15 sec, and spun for 2 min at 12,000 xg. The 400 μl of remaining solution was saved for DNA extraction and the upper phase was removed. For RNA precipitation, 0.6 vol of isopropanol in the presence of 0.3 M sodium acetate (pH

5.3) and 1 μl of glycogen as a carrier was added and placed at -20℃ for 2 h before centrifuging at 5℃ at 12,000 xg for 30 min. The supernatant was removed and the pellet washed twice with 1 ml of 75% ethanol prior to centrifuge at 5℃ at 12,000 xg for 15 min. The supernatant was removed and the pellet was dried and resuspended in 10 μl of dH_2O. DNA purification was performed as the same method of RNA purification. For DNA precipitation, 2 vol of 100% ethanol and 1/10 vol of 3 M sodium acetate was added and placed at -20℃ for 2 h before centrifuging at 12,000 xg for 30 min. The supernatant was removed and the pellet was washed with 1 ml of 70% ethanol prior to centrifuge at 12,000 xg for 15 min twice. The supernatant was removed and the pellet was dried and resuspended in 30 μl of dH_2O and stored at -20℃.

RT-PCR for enterovirus

Enteroviral RNA was amplified by RT-nested PCR using primers of 5' nontranslated regions [20]. This region is highly conserved among the enterovirus serotypes and was selected in order to maximize the detection rate. First-strand cDNA, for use in the detection of enteroviral genome, was generated from 10 μl of extracted total RNA in the presence of 1 unit of RNase inhibitor, first strand buffer (50 mM Tris HCl, pH 8.3, 75 mM KCl), 10 mM of DTT, 0.2 mM of dNTP, 15 pmol of outer downstream primer, and 200 U of MMLV reverse transcriptase (Gibco BRL, Eggenstein, Germany). This mixture was heated to 37℃ for 1 h and inactivated at 90℃ for 5 min. The first amplification of nested PCR was carrried out by adding 1 μl of cDNA to 20 pmol U1 and D1 primer in a buffer containing 1.8 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), 0.2 mM dNTP, and 1 U of *Taq* polymerase (AmpliTaq, Perkin Elmer Cetus, Branchburg, NJ). This mixture reaction was performed on DNA thermal cycler (Perkin Elmer 9600, Norwalk, CT); 94℃ 5 min, 52℃ 15 sec, 72℃ 15 sec 1 cycle; 94℃ 15 sec, 52℃ 15 sec, 72℃ 15 sec 30 cycles; 94℃ 15 sec, 52℃ 15 sec, 72℃ 5 min 1 cycle. The second enzymatic amplification was performed by adding 3 μl of the first PCR product to 25 pmol U2 and D2 primer in 1x buffer, 0.2 mM dNTP, 1.25 U of *Taq* polymerase. This was heated to 94℃ for 4 min; 94℃ 1 min, 55℃ 1 min 30 secs, 72℃ 20 sec (+1 sec/each cycle); 35 cycles: 72℃ 5 min. The PCR product was visualized by staining with ethidium bromide after agarose gel electrophoresis.

Table 1. Oligonucleotide primers used and PCR product size

Virus	Target site	Primer sequence (5'-3')	PCR product size (bp)
Cytomegalovirus	Immediate early region	AAAGAGCCCGACGTCTACTACACGT (sense) CCAGGTACACCTTGACGTACTGGTC (antisense)	169
Adenovirus	Hexon region	AGCACGCCGCGGATGTCAAAG GCCGCAGTGGTCTTACATGC	308
Herpes simplex virus	DNA *pol* gene	GGAGGCGCCCAAGCGTCCGGCCG TGGGGTACAGGCTGGCAAGT	229 (HSV1) 241 (HSV2)
Parvovirus	VP1 gene	ATAAATCCATATACTCATT CTAAAGTATCCTGACCTTG	699
K-ras oncogene	12th codon	TATTATAAGGCCTGCTGAAAATGACTGAAT TTACCTCTATTGTTGGATCATATGTCCA	135
Enterovirus	5' conserved region	GGTGYGAAGAGYCTAYTGAG (U1) CACYGGRTGGCYAATCCA (D1) CCCCTGAATGCGGCTAAT (U2) ATTGTCACCATAAGCAGCCA (D2) Y=C or T, R=A or G	228 155

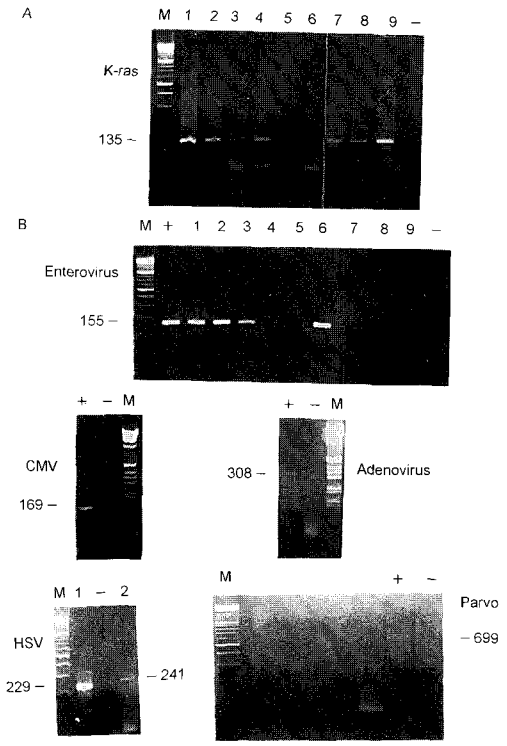

Figure 1. Ethidium bromide-stained agarose gel analysis of representative PCR and RT-PCR products. **(A)** The 135 bp *K-ras* product was amplified in all patient lanes. **(B)** RT-PCR amplification of enterovirus, PCR amplification products of positive and negative control of CMV, adenovirus, HSV1, 2, and parvovirus.

PCR for DNA virus

The sequences of the primers used for the DNA viruses and *K-ras* oncogene were identical to previously published sequences [25] and were listed in Table 1. PCR reaction mixture was performed on 1x buffer, 4 μl DNA, 0.2 mM dNTP, 25 pmol of each primer, and 1.25 U of *Taq* polymerase. The PCR reaction of each DNA virus was performed in different conditions. Cytomegalovirus (CMV): 94℃ 5 min, 94℃ 30 sec, 68℃ 30 sec, 72℃ 30 sec, 35 cycles; Adenovirus: 95℃ 5 min, 95℃ 1 min, 55℃ 1 min, 72℃ 1 min, 35 cycles; HSV1 and HSV2: 95℃ 5 min, 95℃ 1 min, 61℃ 40 sec, 72℃ 1 min, 35 cycles; Parvovirus: 94℃ 3 min, 94℃ 2 min, 37℃ 2 min, 72℃ 3 min, 40 cycles; *K-ras* 98℃ 6 min, 94℃ 2 min, 60℃ 1 min 30 sec, 72℃ 3 min, 40 cycles.

Nucleotide sequence analysis

Enteroviral-specific amplification product was analyzed by direct nucleotide sequencing method. This was performed using the T7 Sequenase v 2.0 Kit (Amersham Life Science, Cleveland, OH). The reaction products labeled with ^{35}S dATP were separated on a 8% denaturing polyacrylamide gel.

RESULTS

Detection of viral infection in paraffin-embedded tissue

Since the 5'-NTR is relatively conserved region we targeted this region for the detection of enteroviral genomes. Table 2 summarizes PCR and RT-PCR results obtained from explanted heart sample from patients with DCM or MI. *K-ras* primers were applied to confirm the presence of target nucleic acid in the sample preparations. PCR product of the 135 bps indicates the presence of nucleic acid in the paraffin-embedded heart tissues. Viral genomes of 169 bps, 308 bps, 229 bps, 241 bps, and 699 bps for cytomegalovirus (CMV), adenovirus, herpes simplex virus type 1 (HSV1), herpes simplex virus type 2 (HSV2), and parvovirus, respectively, were amplified by PCR (Figure 1). RT-nested PCR detected 155 bps enteroviral genome in 7 (26.9%) of 26 patients with DCM, and none of the patients with MI (Table 2). No DNA viral genomes tested were detected from the samples.

Genotyping of enteroviruses by single-strand conformation polymorphism

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) was performed to differentiate genomic information and results were compared with electrophoretic patterns of coxsackievirus B3 (CB3) and CB4 obtained from American Type Culture Collection

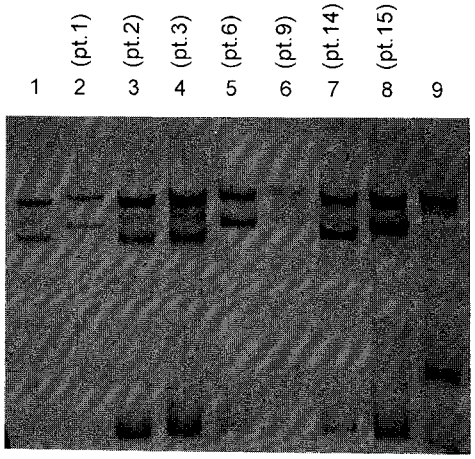

Figure 2. SSCP analysis of the enteroviral genotype. Enteroviral genomes were amplified from paraffin-embedded tissue using RT-PCR. The PCR products from 7 heart tissues migrated with different electrophoretic mobilities upon separation. Lane: 1, coxsackievirus B3; 2-8, heart tissues (patient numbers are shown in parenthesis); 9, coxsackievirus B4.

(Figure 2). Three different genotypes were recognized from seven EVs: genotype 1 (patients 1, 6, and 15), genotype 2 (patients 2, 3, and 14), and genotype 3 (patient 9).

Partial sequence analysis of 5'-nontranslating region

PCR products of 5'-NTR were also sequenced and compared with that of CB3 strain (Table 3). The nucleotide sequence data were obtained on the 155 bps PCR products. The region amplified in this study contained nucleotide position 448-602. Although most of the sequences among the wild isolates are highly conserved, there are specific regions of variable sequences (no 490 - no 510). Nucleotide sequences have the greatest homology of CB3: the 5'-NTR nucleotide sequences of wild strains showed 91.6-100% identity to those of CB3. Pairwise sequence comparisons among isolates showed that strains differed from one another by up to 5% in nucleotide sequence. The nucleotide at position 565 in all of the enteroviral RNA was an A, which is comparable with previous study. The results confirm the nucleotide at position 565 is important nulceotide in the pathogenesis of cardiovirulence. There were differences at 25 positions (16%) and there were 6 positions in which at least 50% of the enteroviral RNA differed from those of the CB3 at positions 491, 496, 497, 500, 504, and 505.

DISCUSSION

DCM is one of the most common cause of heart failure. Previous studies showed that myocarditis is closely linked with DCM [12,13] although etiology is not known. Animal model studies also strongly suggest close relationship between two disease entities [5]. Much progress has been made in elucidating the viral causes of DCM. The possible causative viruses of myocarditis include picornavirus, herpesvirus, adenovirus, and orthomyxovirus. The classical method for the detection of viruses is identification of cytopathic effects in cell culture. However, cell culture method is not suitable for detection of viruses in heart tissue.

The results obtained from *in situ* hybridization methods suggested that enteroviral genomes are present in the hearts of 12-46% of patients with DCM [4,28]. Recently, PCR technique is used to detect viral nucleic acid in myocardial tissues. The PCR allowed detection of viruses that had previously been very difficult to detect. However, the positive rate varied between reseachers [7,14,26] and the prevalence of enteroviral infection in DCM may vary between one population and another. We report the positive rate of enteroviral genomes in patients with DCM in Korea is 26.9%. The nucleotide sequence analysis data exclude the possibility of cross-contamination.

Although subtyping of viruses is not relevant to treatment, it is useful for epidemiologic study and for investigation of pathogenesis. In this study, in order to investigate whether there are specific genotypes to cause DCM and to check cross-contamination, the SSCP technique was applied. SSCP analysis was developed by Orita

Table 2. Summary of PCR and RT-PCR Results

Patient No.	Age/Sex	Disease	Entero	HSV1, 2	Parvo	Adeno	CMV	*K-ras*
1	26/M	DCM	+	−	−	−	−	+
2	40/M	DCM	+	−	−	−	−	+
3	45/M	DCM	+	−	−	−	−	+
4	28/M	DCM	−	−	−	−	−	+
5	22/M	DCM	−	−	−	−	−	+
6	35/M	DCM	+	−	−	−	−	+
7	33/M	DCM	−	−	−	−	−	+
8	43/M	DCM	−	−	−	−	−	+
9	34/M	DCM	+	−	−	−	−	+
10	59/F	DCM	−	−	−	−	−	+
11	41/M	MI	−	−	−	−	−	+
12	41/M	DCM	−	−	−	−	−	+
13	48/M	MI	−	−	−	−	−	+
14	63/M	DCM	+	−	−	−	−	+
15	13/F	DCM	+	−	−	−	−	+
16	47/F	DCM	−	−	−	−	−	+
17	41/M	DCM	−	−	−	−	−	+
18	35/M	DCM	−	−	−	−	−	+
19	13/F	DCM	−	−	−	−	−	+
20	60/M	DCM	−	−	−	−	−	+
21	16/M	DCM	−	−	−	−	−	+
22	47/M	DCM	−	−	−	−	−	+
23	30/M	DCM	−	−	−	−	−	+
24	29/M	DCM	−	−	−	−	−	+
25	43/M	DCM	−	−	−	−	−	+
26	18/M	DCM	−	−	−	−	−	+
27	15/M	DCM	−	−	−	−	−	+
28	56/M	DCM	−	−	−	−	−	+

et al. in 1989, and they demonstrated that a single nucleotide substitution was sufficient to cause a mobility shift of a fragment of single-stranded DNA in a gel [22]. Thereafter, the SSCP has been used as a rapid and inexpensive methods and yet provides valuable information with regard to strain typing. In this study, three different electrophoretic mobilities can be found from 7 clinical samples. Although a genotype resembling with coxsackievirus B3 was found from 3 patients with DCM, the presence of a specific genotype as a causative agent for the DCM was unlikely. However, it was very difficult to differentiate mobility shift of fragments. Therefore, standardization of method protocols is important for

Table 3. Alignment of the nucleotide sequence of a part of 5'-NTRs from RT-PCR products of enterovirus isolates from patients with DCM. The sequences used in the alignment are coxsackievirus B3. The sequence begins at position 448 and extends to position 602

No. of sample													
Cox B3	TC	CTCCGGCCCCC	TGAATGCGGC	TAATCCTAAC	TGCGGAGCAC	ACACCCTCAA	GCCAGAGGGC	AGTGTGTCGT					
1	**	**********	*********T	**********	**********	*T*****CT**	T***AG****	**********					
2, 3	**	**********	**********	**********	**********	**********	**********	**********					
6	**	**********	*********T	**********	**********	*T*****CT**	T***AG****	**********					
9	**	**********	**********	**********	**********	**GTT*G***	*****C*A*T	G*********					
14	**	**********	**********	**********	**T*******	*TG***CT**	T***AG***T	**********					
15	**	**********	*********T	**********	**********	*T*****CT**	T*T*AG****	**********					
Cox B3	AACGGGCAAC	TCTGCAGCGG	AACCGACTAC	TTTGGGTGTC	CGTGTTTCAT	TTTATTCCTA	TACTGGCTGC	TTATGGTGAC	AAT				
1	**********	**********	**********	**********	**********	**********	**********	**********	***				
2, 3	**********	**********	**********	**********	**********	**********	**********	**********	***				
6	**********	**********	**********	**********	**********	*********C	******TT**	**********	***				
9	**********	**********	**********	**********	**********	*********C	********T**	CCT********	***				
14	**T*A****T	**********	**********	**********	**********	**********	**********	**********	***				
15	**********	**C*******	**********	**********	**********	*********C	******T***	**********	***				

ensuring the reproducibilities of the mobility shift.

The nucleotide sequence of 5'-NTR was analyzed because it contains random mutations that may persist indefinitely in the complete absence of immunologic pressure. Furthermore, it is well known that the 5'-NTR is associated with secondary structure that is critical to ribosomal binding and other functions associated with replication.

The sequence variations indicated that no false positive due to cross-contamination had been amplified. Database comparison of the nucleotide sequences indicated in each case that the PCR products had highest homology with coxsackievirus B3 (CB3). Nucleotide sequence variation of the 5'-NTR in this study compared with the CB3 was 4.9% and was comparable with other study [2]. In addition, the 5'-NTR of enteroviruses isolated from Korean patients with DCM was relatively conserved except no 490 - no 510. It was also noted that nucleotide position 565 was an A, which confirms the hypothesis of the importance of no 565 as an A in the pathogenesis of cardiovirulence [6].

In conclusion, positive RT-PCR signals were found in 7 (26.9%) of 26 samples from patients with DCM and in 0% of control tissues, suggesting possible association of enteroviral infection with DCM. Furthermore, the nucleotide sequence data have great similarity with CB3, suggesting coxsackieviruses may be etiologic agents of DCM in Korea.

Acknowledgements

This study was supported by the academic research fund of Ministry of Education (997-021-F0013), Republic of Korea.

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