대한임상미생물학회지 : 제3권 제2호 2000
Korean J Clin Microbiol 2000 ; 3 : 121-131

무균성 수막염 환자에서 분리한 enterovirus의 혈청형 및 계통발생학적 분석

이정희[1,2], 안병윤[2], 반성환[3], 김상현[3], 김의종

서울대학교병원 임상의학연구소 바이러스연구소[1] 고려대학교 자연자원대학원 유전공학과
순천향대학교 의과대학 구미순천향병원 소아과[3] 서울대학교 의과대학 임상병리학교실[4]

Serotyping and Phylogenetic Analysis of Enteroviruses Isolated from Patients with Aspetic Meningitis

Jung-Hee Lee, M.S.[1,2], Byoung-Yoon Ahn, Ph.D.[2], Sung-Hwan Ban, M.D.[3], Sang-Hyun Kim, M.D.[3], and Eui-Chong Kim, M.D.[1,4]

Virus Research Center, Clinical Research Institute, Seoul National University Hospital[1]; Graduate School of Natural Resources, Korea University[2]; Department of Pediatrics, Kumi Hospital, Soonchunhyang University College of Medicine, Kumi[3]; Department of Clinical Pathology, Seoul National University College of Medicine[4], Seoul, Korea

Background : The determination of serotype of enteroviruses is useful for the discrimination between sporadic and epidemic infections. The conventional serotyping method is time-consuming and labor-intensive. Recently, molecular method was introduced for the serotyping of enteroviruses. The aim of this study was to establish a method to isolate and analyze enteroviruses from various specimens utilizing molecular biological techniques and to determine which strains were phylogenetically related to clinical samples.

Methods : Clinical samples in this study included 164 cerebrospinal fluid (CSF), 136 stool, 15 sera, 6 throat swab, 5 urine, and 4 sputa, which were obtained from hospitalized patients, primarily infants or children presenting symptoms of aseptic meningitis in 1998. RD cells were used for enterovirus isolation. RT-PCR was performed with RD cell lysate showing CPE. The primers 011 and 012 were used for the VP1 region, and the primers EN1 and EN2 for 5' -UTR. The nucleotide sequences of VP1 region were determined and analyzed with BLAST program.

Results : Among 333 samples, only 23 samples produced CPE: 17 samples at first and six samples at the second blind passage. Fifteen isolates were related to coxsackievirus B2 , two to echovirus 4, three to echovirus 6, and three to echovirus 18. All 23 viral isolates displayed a nucleotide sequence identity of 80-95%, compared with the reference serotypes. However, the identity was increased up to 93-100% when the VP1 region was translated into amino acids

Conclusions : Since CB2 type was 55% among enteroviral isolates, the CB2 was determined as the major causative serotype of enteroviral meningitis in 1998. CB2 type was emerged between June and July, EC4 and EC6 was limited to July, and EC18 was in August. ***(Korean J Clin Microbiol 2000;3:121-131)***

Key words : Enterovirus, Aseptic meningitis, Nucleic acid sequence, Coxsackievirus

INTRODUCTION

The enteroviruses belong to the family *Picornaviridae* and comprise 67 distinct serotypes, including polioviruses 1 to 3, coxsackieviruses A1 to A24, coxsackieviruses B1 to B6, echoviruses 1 to 34, and enteroviruses 68 to 72.

접수번호 : CM 3-2-7
교 신 저 자 : 김 의 종
(110-744) 서울시 종로구 연건동 28
서울대학병원 임상병리과
Tel : 02) 760-3500 Fax : 02) 764-3698
E-mail : euichong@plaza.snu.ac.kr

Coxsackievirus A23 has been reclassified as echovirus 9, and echovirus 10 as reovirus 1, and echovirus 28 as rhinovirus 1A, echovirus 34 as a variant of coxsackievirus A24, and enterovirus 72 as hepatitis virus A [1]. The enteroviruses historically have been divided into 4 subgroups (i.e., poliovirus, coxsackievirus A, coxsackievirus B, and echovirus). Serotypes within a specific subgroup maintain RNA sequence homology of greater than 65 percent within region coding for capsid protein, but newly recognized enteroviruses could not be categorized unambiguously. They have therefore been sequentially numbered from type 68 upward and classified simply as enteroviruses.

Enteroviruses are among the most common and the most important viral pathogens in humans. The consequences of infection are either asymptomatic virus shedding or a broad spectrum of acute diseases ranging from a mild cold to myocarditis and encephalitis [2]. Additionally, enteroviruses have been implicated in the etiology of diabetes, chronic cardiomyopathy, and fetal malformation [3].

The determination of serotype of enteroviruses is useful for the discrimination between sporadic and epidemic infections. The conventional serotyping method is time-consuming and labor-intensive. Recently, molecular method was introduced for the serotyping of enteroviruses. The aim of this study was to establish a method to isolate and analyze enteroviruses from various specimens utilizing molecular biological techniques and to determine which strains were phylogenetically related to clinical samples.

MATERIALS AND METHODS

Specimens

Clinical samples in this study were 164 cerebrospinal fluid specimens, 136 stool specimens, 15 serum specimens, 6 throat swabs, 5 urine specimens, and 4 sputa. All specimens were obtained from hospitalized patients, primarily infants or children presenting symptoms of aseptic meningitis. Specimens were stored frozen at -70℃ until the inoculation on cultured cells. One gram of stool was placed in 5 ml of phosphate buffered saline (PBS), and suspended by vortex. A clear supernatant of stool was obtained by centrifugation at 3,000 X g for 15 min and filtration with 0.45-μm-pore-size membrane. CSF and serum were inoculated directly onto cultured cells without treatment.

Cell culture and virus infection

RD cells were used for enterovirus isolation. RD cell line, derived from a human rhabdomyosarcoma[4], was donated by Dr. Oberste at the Centers for Disease Control and Prevention at 27th passage. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) at 37℃ in 5% CO_2. A monolayer of cells was washed with 0.01 M PBS at room temperature. The cells were treated with 0.4 ml of 0.2% pre-warmed trypsin for 1 min. Trypsin solution was discarded and the cells were resuspended in fresh 10% DMEM by several pipetting and transferred into a fresh tube. Cell concentration was adjusted to 1 X 10^4 cell/ml by a counting chamber method, and 1 ml of cell suspension was cultured in 15-ml glass tube. After monolayer was formed, media was changed to DMEM with 2% FBS and 0.1 ml of specimen was inoculated, and incubated at 37℃ with 5% CO_2. The cytopathic effect (CPE) was examined under a microscope daily for 10 days. Culture tubes with CPE-positive were frozen at 20℃. If any CPE was not detected, three or four blind passages were performed weekly.

Preparation of viral RNA

When CPE was observed in 75-100% of the cells, cells were harvested. After freezing and thawing, cell debris was removed by centrifugation at 20,000 X g. RNA was extracted from the supernatant using the QIAamp Viral RNA kit™ (QIAGEN, USA). RNA for RT-PCR was obtained from 140μl of cell culture supernatant.

Reverse transcription and polymerase chain reaction

For reverse transcription , viral RNA was added in a 20-μl reaction mixture containing 20 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of deoxynucleotide triphosphates (Boehringer Mannheim), 1.25μM random hexamer primer, 25 U of RNase inhibitor (Boehringer Mannheim), and 40U of Avian Myeloblastosis Virus (AMV) reverse transcriptase (Boehringer Mannheim), and preheated at 65℃ for 2 min and then cooled to room temperature. Subsequently, they were briefly centrifuged to bring the contents to the bottom of the tube. The remaining reagents were added and incubated at 42℃ for 1 hr. One microliter of the RT reaction was used as a template for the PCR reaction. PCR was performed in a mixture containing 20 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphate, 1 U of Taq DNA polymerase (Boehringer Mannheim) with 0.4 pmol each primers. The primers 011 and 012 were used for the VP1

Coxsackievirus B2

	10	20	30	40	50	60	70	80
Ohio-1	CCTGAAACCG	TGGACGATTA	CAACTGGCAA	ACATCTACAA	ATCCCAGCCT	TTTTTGGACT	GAAGGGAATG	CACCTCCACG
SNUH003	.....G..T.	.....C..	.....	..G..A....	.C.....T.	...C.....C	.....A..C.	.G..C.....
SNUH009	.....G..T.	.....C..	.....	..G..A....	.C.....	C	.....A..C.	.G..C.....
SNUH018	..C.....T.	.T..T.....	.....	..T..A....	.C..T..T..	C..C.....C	.....C.	.....
SNUH024	..G.....T.	.A..T.....	.....	..T..A....	.C..T..T..	C.....C.C	..G.....C.	.....
SNUH046	.....G..T.	.....C..	.....	..G..A....	.C.....	...C.....C	.....A....	.G..C.....
SNUH064	.....G..T.	....T..C..	.....	..G..A....	.C.....	C	.....A....	.G..C.....
SNUH065	..G.....T.	.A..T.....	.....	..T..A....	.C..T..T..	C	..G.....C.	.....
SNUH075	..G.....T.	.A..T.....	.....	..T..A....	.C..T..T..	C	..G.....C.	.....
SNUH076	..G.....	.A..T.....	...A.....	..T..A..T.	.C..T..T..	C.....C	..G.....C.	.....
SNUH077	..G.....T.	.A..T.....	.....	..T..A....	.C..T..T..	C.....C	..G.....C.	.....
SNUH078	.....G..T.	.....C..	.....	..G..A....	.C.....	...C.....C	.....A....	.G..C.....
SNUH084	.....G..T.	.....	.....	..G..A....	.C.....	...C.....C	.....A....	.G..C.....
SNUH109	.....G..G.	...GAT.C..	.....	..G..A....	.C.....	...C.....C	.....A....	.G..C.....
SNUH132	.....G..T.	....T..C..	.....	..G..A....	.C.....	...C.....C	.....A....	....C.....
SNUH151	.....G..T.	.....C..	.....	..G..A....	.C.....	...C.....C	.....A....	.G..C.....

	90	100	110	120	130	140	150	160
Ohio-1	CATGTCAATT	CCATTCATGA	GCATAGGCAA	TGCCTATAGT	ATGTTCTATG	ATGGTTGGTC	CGAGTTTAGG	CATGACGGTG
SNUH003	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH009	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH018	T.....C	.....	....T..T..	...T..C..C	.....	....G.....	T..A..C...	.....T....
SNUH024	T.....C	.....	....T.....	.CG...C..C	.....T....	....C.....	T..A.....	.....T..C.
SNUH046	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH064	C	..G.....	.....T..	C..A..C..C	.....G..C.	.C..G.....	.....C...	.....T....
SNUH065	T.....C	.....T....	.....	...T..C..C	.....T....	....C.....	T..A.....	.....T..C.
SNUH075	T.....	.....	....T.....	...T..C..C	.....T....	....C.....	T..A.....	.....T..C.
SNUH076	T.....C	.....	....T.....	...T..C..C	.....T....	....C.....	T..A.....	.....T..C.
SNUH077	T.....	.....	....T.....	...T..C..C	...C.T....	....C.....	T..A.....	.....T..C.
SNUH078	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH084	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH109	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....
SNUH132	C	..G.....	.....T..	C..A..C..C	.....	.C..G.....	.....C...	.....T....
SNUH151	C	..G.....	.....T..	C..A..C..C	.....C.	.C..G.....	.....C...	.....T....

	170	180	190	200	210	220	230	240
Ohio-1	TGTACGGCCT	GAATACCCTT	AACAATATGG	GCACAATATA	TGCTAGGCAC	GTCAACGCTG	ACAACCCAGG	TAGCATCACC
SNUH003	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH009	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH018	....T..A..	.....T..C	.....C....	.T.....	C..C..A..T	..T.....C.	.....	...T.....T
SNUH024	....T..A..	.....T..C	.....C....	.T..C.....	C..C..A..T	..T.....C.	.....	C..T..T..T
SNUH046	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH064	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH065	....T..A..	.....T..C	.....C....	.T.....	C..C..A..T	..T.....C.	.....	C..T..T..T
SNUH075	....T..A..	.....T..C	.....C....	.T.....	C..C..A..T	..T.....C.	.....	C..T..T..T
SNUH076	....T..A..	.....T..G	.....C....	.....	C..C..A..T	..T.....C.	.....	C..T..T.AT
SNUH077	....T..A..	.....T..C	.....C....	.T.....	C..C..A..T	..T.....C.	.....	C..T..T..T
SNUH078	.T..T..AT.	A..C.....A	.....C....	.T..T.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH084	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH109	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH132	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A
SNUH151	.T..T..AT.	A..C.....A	.....	.T..C.....	...C..A...	.....T....	.T..T..G..	...T.....A

	250	260	270	280	290	300	310	320
Ohio-1	AGCACAGTGA	GAATATACTT	CAAACCCAAA	CATGTCAAGG	CATGGATTCC	TCGCCCGCCT	CGTTTGGCAC	AGTATCTTAA
SNUH003	....G....	.G..T..T..	...G..T..G	.....T....	.....G....	G..A..A..C	.....C.	....C.....
SNUH009	.....G....	.G..T..T..	...G..T..G	.....T....	.....G....	G..A..A..C	.....C.	....C.....
SNUH018	.....	.....T..	.....	.....T....	.T...G.C..	A..T..C...	...C.T..T.	.A.....C..

SNUH024			.....T.		.G.....		.C..T..		.T	G.C.	A..G	C..G		.C.A..T.	A	.C
SNUH046	.G.		G..T..T.		.G..T..G		.C..T..			G...	G..A	A..C		.C....C.		C.
SNUH064	.G.		G..T..T.		.G..T..G		.C..TC.			G...	G..A	A..C		.C....C.		CG
SNUH065			.....T.				.C..T..		.T	G.C.	A..T	C..G		.C.A..T.	A	..C.
SNUH075			.....T.		G		.C..T..		.T	G.C.	A..G	C..G		.C.A..T.	A	..C.
SNUH076			.....T.				.C..TC.		AT	G.C	A..T	C...		.C.A..T.	A	G.C.
SNUH077			.....T.				.C..T.		.T	G.C	A..T	C...		.C.A..T.	A	..C.
SNUH078	G.		G..T..T		G..T..G		....T.			G..	G..A	A..C		.C....C		C.
SNUH084	G.		G..T..T		G..T..G		....T.			G..	G..A	A..C		.C....C		C.
SNUH109	G.		G..T..T		G..T..G		....T.			G..	G..A	A..C		.C....C		C.
SNUH132	G.		G..T..T		G..T..G		....T.			G..	G..A	A..C		.C....C		CG
SNUH151	G.		G..T..T		G..T..G		....T.			G..	G..A	A..C		.C....C		C.

	330	340	350	360	370	380	390	400
Ohio-1	AGCCAATAAT	GTGAATTTTG	AGATCACCGA	TGTGACAGAA	AAGAGAGATA	GTCTCACGAC	CACGGGGGCC	TTTGGACAAC
SNUH003	...T..C..C	.....C....	.T.....T..	....	.....G..GC	TCACG..C..	TGT.GGN...	....
SNUH009	...T..C..C	.....C....	.T.....T..	....	.....G..G.	.CTCACGAC.	ACT.TG....	....
SNUH018	....	..T.......	.T.....T..	....	..AGAG.GAC	AG........	....	....
SNUH024	....	....	....A..T..	....	.....G..C.	.C........	....	....
SNUH046	...T..C..C	.....C....	.T.....T..	....	.....G..G.	CCTCACGAC.	ACT.GTGCG.	....
SNUH064	...T..C..C	.....C....	.T.....T..	....	G....G..G.	.C........	....	....
SNUH065	....	....	....A..T..	....	.....GACAG	....	....	....
SNUH075	....	....	....A..T..	....	.....GACA.	C.TAT.AC.A	.GG.ACCTCT	.A.AACA.CG
SNUH076	....	....	....A..T..	....	.....GACAT	C.TAT.AC.A	.GG.ACC...	....
SNUH077	....	....	....A..T..	....	.....G..C.	C.TAT.AC.A	.GG.AACT..	....
SNUH078	...T..C..C	.....C....	.T.....T..	....	.....G..G.	.CTCACGAC.	A.T.T.....	....
SNUH084	...T..C..C	.....C....	.T.....T..	....	.....G..G.	.C........	...TGGT...	....
SNUH109	...T..C..C	.....C....	.T.....T..	....	.....G..G.	.C..ACGAC.	....	....
SNUH132	...T..C..C	....	.T.....T..	....	.....G..CT	CAGC..T.GG	....	....
SNUH151	...T..C..C	.....C....	.T.....T..	....	.....G..G.	CCTCACGAC.	A.T.TGCG..	....

Echovirus 4

	10	20	30	40	50	60	70	80
WA93-1821	CCAGCAAAGG	TCGATGATTA	CAGTTGGCAA	ACATCCACTA	ACCCCAGTGT	GTTCTGGACA	GAGGGGAACG	CGCCTGCCCG
SNUH087	....	.T.....C..	...C.....G	.....A..A.	....	...T.....G	..A..A..T.	.A........
SNUH123	....	.T.....C..	...C.....G	.....A..A.	....	...T.....G	..A..A..T.	.A........
	90	100	110	120	130	140	150	160
WA93-1821	CATATCCATC	CCGTTCATTA	GCGTTGGGAA	TGCTTATAGC	AGTTTCTACG	ATGGATGGTC	AAACTTCTCA	CAGAATGGCC
SNUH087	.........T	........C.	.T........	....	....	.C..G.....	G........C	....
SNUH123	.........T	..A.....C.	.T........	....	....	.C..G.....	GC.......C	....
	170	180	190	200	210	220	230	240
WA93-18	GATATGGGTA	CAACACTTTA	AATAACATGG	GACAGTTGTT	CTTTAGACAT	GTGAATAAGC	CCAGCCCCAA	CACTTACACG
SNUH087	....C..T..	......C...	..C.......	.G..A..A..	....	.....C..A.	....T.....	.........A
SNUH123	....C..T..	......C...	..C.......	.G..A..A..	C	.....C..A.	....T.....	.........A
	250	260	270	280	290	300	310	320
WA93-18	AGCGTGGCTC	GCATTTACTT	CAAGCCAAAA	CATGTGAGAG	CGTGGGTCCC	GCGACCACCA	CGATTGTGCC	CGTACATAAA
SNUH087	..T..C..C.	....A.....	...A......	.......AG.	.....A....	......G..G	....	.T.....C..
SNUH123	..T..C..C.	....A.....	...A......	.......AG.	.....A....	......G..G	....	.T.....C..
	330	340	350	360	370	380	390	400
WA93-1821	TGCAGGGGAC	GTTAATTTCA	AACCAACACC	TGTGACAGAA	AAGAGGGCAA	GCTTAATCAC	CACT......	....
SNUH087	C..G......	.....C....	.G........	C.....T..C	..A...AA..	..C....T..	...AGGCC..	....
SNUH123	C..G.....T	.....C....	.G........	C.....T..C	..A....A..	....	....	....

Echovirus 6

	10	20	30	40	50	60	70	80
NM95-2070	CCACAAGCCG	TGGACGATTA	TAACTGGCAA	ACTTCTACAA	ACCCAAGTGT	CTTTTGGACT	GAGGGAAATG	CTCCACCTAG
SNUH030	....GG..T.	....T.....	C.........	.....C..T.				
SNUH095	.....G..T.	....T.....	C.........	........T.	.C			
SNUH117	..C..G..T.	....T.....	C.........	........T.				

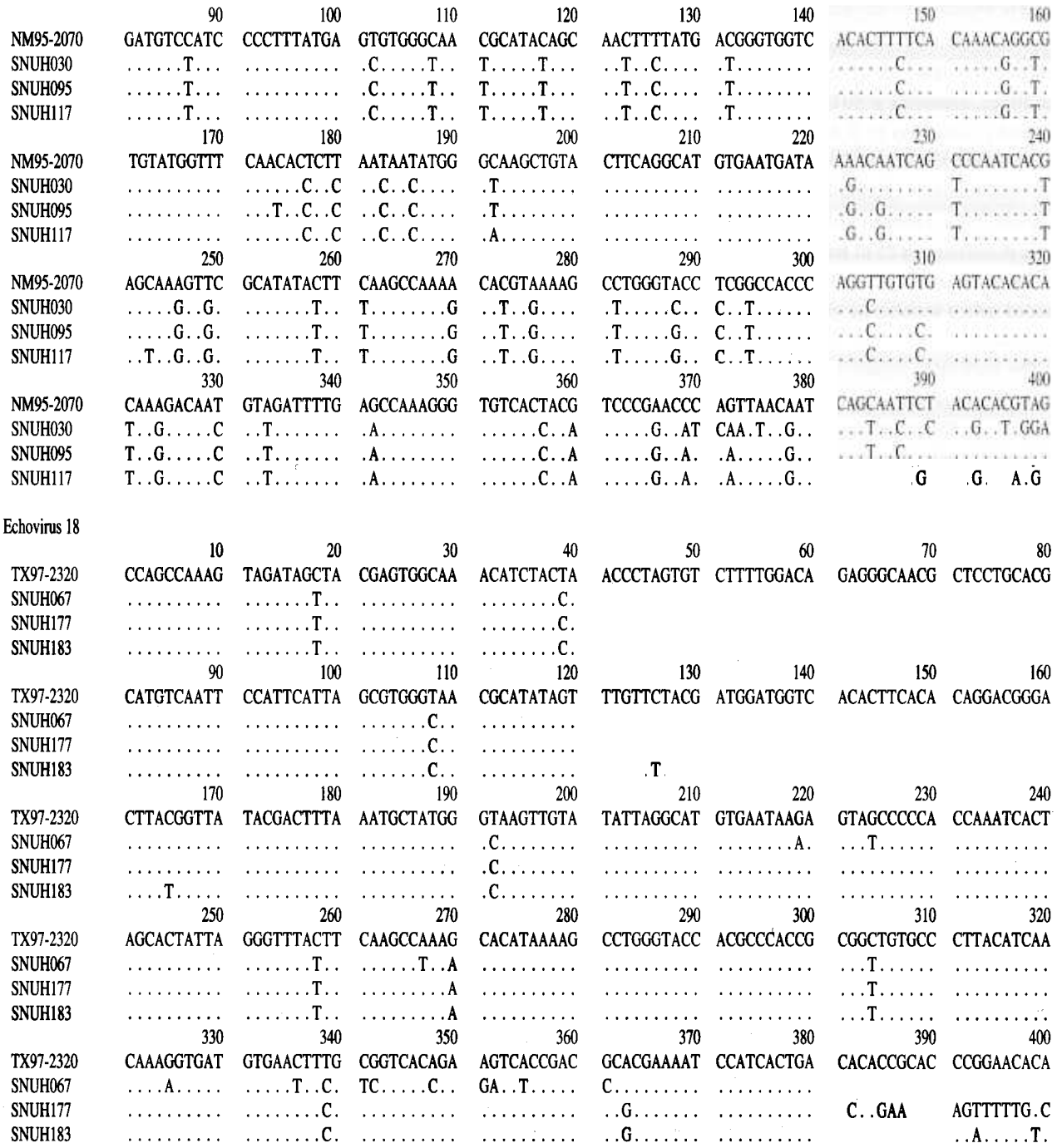

Fig. 1. Nucleotide sequence alignment of VP1 sequences.

region, and the primers EN1 and EN2 for 5' -UTR [5, 6].

Amplification of VP1 region was accomplished in 35 cycles consisting of denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min, and elongation at 72℃ for 1 min. For 5' -UTR, PCR was performed by denaturation at 94 ℃ for 45 sec, annealing at 50℃ for 45 sec and elongation at 72℃ for 45 sec. The PCR products were analyzed by electrophoresis in 2% agarose gels.

Analysis of nucleotide sequences

PCR products were purified from agarose gels by elution with QIAEXII™ (QIAGEN, USA). The method of cycle sequencing using dye-labeled terminators of ABI PRISM™

(Perkin-Elmer) was employed to determine the nucleotide sequences of VP1 and the 5′-UTR region. A sequencing reaction was done for 25 cycles, with denaturation at 96℃ for 10 sec, annealing at 50℃ for 5 sec, elongation at 60 C for 4 min by GeneAmp PCR System 2400 (Perkin-Elmer). The terminated reaction product was purified by using a Centri-Sep spin column™ (Perkin-Elmer) and concentrated utilizing a speed vac apparatus (Savant Instruments, USA). Samples were dissolved in a 25 μl loading buffer containing deionized formamide and EDTA (pH 8.0) and were subsequently loaded onto a sequencing gel following denaturation at 90℃ for 2 min.

The nucleotide sequence analysis was first done using the BLAST program in a search of the database of the National Centers for Biotechnology Information (NCBI). All regions of homologous sequences were aligned and the nucleotide identities and amino acid similarities were calculated with the DNASIS program (HITACH, Japan). The selected data sets were used as an input file to produce phylogenetic trees with the MEGA program. Dendrograms were constructed to visualize the overall relationship of 23 viral isolates and were performed subsequent to manual adjustment of the sequence into a standard MEGA format [7].

RESULTS

Virus isolation in RD cell

Among 333 samples, only 23 samples produced CPE: 17 samples at first and six samples at the second blind passage. Specimens showing no CPE at the second passage were also negative for CPE at the third and fourth passages. These 23 positive samples were derived from 19 stool and 4 CSF samples. The age of the patients was between 2 days and 13 years with 83% of the patients younger than 5 years old.

RT-PCR

The RNA, extracted from culture lysates, was used as a template to synthesize cDNA using random hexamer primer

Table 1. Comparative analysis of nucleotide sequences of VP1

Sample No.	Maximum homology with reference strain (%)				Serotype
	CB2	EC4	EC6	EC18	
SNUH003		69	65	64	CB2
SNUH009		68	65	64	CB2
SNUH018		69	64	62	CB2
SNUH024		68	66	63	CB2
SNUH030		68	84	67	EC6
SNUH046		68	65	65	CB2
SNUH064		67	64	62	CB2
SNUH065		68	65	64	CB2
SNUH067		69	66	91	EC18
SNUH075		68	67	66	CB2
SNUH076		67	66	65	CB2
SNUH077		68	67	64	CB2
SNUH078		68	65	65	CB2
SNUH084		68	65	63	CB2
SNUH087		85	67	66	EC4
SNUH095		70	83	68	EC6
SNUH109		68	64	63	CB2
SNUH117		68	86	68	EC6
SNUH123		85	65	65	EC4
SNUH132		67	65	63	CB2
SNUH151		68	65	65	CB2
SNUH177		70	67	94	EC18
SNUH183		69	68	95	EC18

Comparative homology of VP1 sequence of tested samples that regarded as positive using program DNASIS. Reference sequences used for comparison are: CB2, AF081312; EC4, AF081643; EC6, AF081625; EC18, AF081638.

and PCR was done using primer pairs, 011/012 and EN1/EN2. Products of 500 bp and 153 bp fragments were obtained with 011/012 and EN1/EN2, respectively.

Sequence analysis of VP1 and 5′-UTR region

The 400 bases of VP1 and 130 bases of 5′-UTR were sequenced utilizing the DNA automatic sequencer (ABI, model 373, version 1.2.1). Nucleotide sequence alignments of the VP1 and 5′-UTR sequences are shown in Figures 1 and 2, respectively. Fifteen isolates were related to coxsackievirus B2 type (CB2), two to echovirus 4 type (EC4), three to echovirus 6 type (EC6), and three to echovirus 18 (EC18) (Table 1). The maximum homology of VP1 sequences was discriminate between different serotypes. All 23 isolates in this study showed high nucleotide sequence homology in 5′-UTR, even between disparate members such as CB2 and EC18 (Fig. 2).

All 23 viral isolates displayed a nucleotide sequence identity of 80-95%, compared with the reference serotypes. However, the identity was increased up to 93-100% when the VP1 region was translated into amino acids (Fig. 3). The reference strains that used in analysis were obtained from GenBank: Ohio-1 (AF081312) for CB2, WA93-1821 (AF081643) for EC4, NM95-2070 (AF081625) for EC6, TX97-2320 (AF081638) for EC18.

DISCUSSION

The various methods, including neutralization, hemagglutination inhibition, complement fixation, immunofluorescence, counterimmunoelectrophoresis, enzyme-linked immunoassay, and virus agglutination, have been used for virus detection and identification. However, these methods are time-consuming, labor-intensive work and can be somewhat subjective [8]. Especially, neutralization test requires more than 60 serotypes in combined cell culture-mouse systems to screen completely for increase in antibody titer. So, in presented study, RD cell and PCR methods were chosen for isolation and identification of enteroviruses. The RD cell line, derived from a human rhabdomyosarcoma, supports the replication of most of the prototype strains of enterovirus. The CPE of infected RD cells develops quickly and often destroys the monolayer within 2 days after inoculation. In this study, some clinical specimens, later proved to contain EC4 and 18, produced CPE the day after inoculation. CB2, on the other hand, do not replicate well in RD cells [9]. CPE of RD cell infected with CB2 occasionally did not appear within 10 days after inoculation. Cultures that were regarded as negative by the first 10-day-incubation were tested again with blind passage system about 3-4 times. Indeed, 6 samples were positive for enteroviruses in sub-passages. From this results, RD cell seems to be unsuitable in detecting primarily all of enteroviruses, particularly, coxsackieviruses. And it would be desirable to use another cells simultaneously in addition to RD cell culture. In the previous study, RD cells were superior to cynomolgus monkey kidney (CMK) cells for the isolation of echoviruses 3, 6, 11, 12, 13, 19, 21, 22, 27, 30, and 31, while coxsackie B1-B5 viruses were recovered only in CMK cells [4].

The products that were synthesized with these degenerate primers were analyzed in detail by cycle sequencing using dye-labeled terminators, followed by analyzing of these sequences using program BLAST and DNASIS for seeking serotype showing the highest similarity with the nucleotide sequences of isolates. The causative enteroviral agents that were determined by BLAST program of NCBI were CB2, EC4, EC6, and EC18.

All of 23 viral isolates showed nucleotide similarity in VP1 region of 80-85% with the homologous sequence. However, when nucleotides of VP1 region were translated into amino acids, the identity was increased up to 93-100%. In particular, the 96th amino acid of ohio-1 reference strain in CB2 group was isoleucine (I) but that of all 15 clinical isolates was valine (V). Moreover, the 114th amino acid of ohio-1 was glutamic acid (E) but that was aspartic acid (D) in 9 out of 15 isolates.

The coxsackieviruses are believed to be the most common viral agent for myocarditis in humans [10]. Clinical illness is most frequently seen in infants and young children. In this study, one infant showed symptoms of myocarditis and thrombocytopenia. He was born by preterm delivery at 35th week gestation. Petechia was developed at 2 days after birth and hemoglobin, WBC, and platelet count were 16.9 g/dl, 22,800/μl, and 23,000/μl, respectively. At 10 days after birth, tachycardia (200/min) was developed, and creatine kinase and lactate dehydrogenase of serum were 225 U/L and 871 U/L, respectively. CB2 was isolated from stool collected at 13 days after birth.

In the analysis of VP1 capsid protein, diverse variations were exhibited in this region but the length of the areas seems to be fairly constant in all isolates. This suggests that antigenic heterogeneity occurs primarily as point mutations against outer circumstances that has strong influence of host immune system and thus generates new serotypes of the same ancestor virus during evolution [11].

Dendrograms generated from the VP1 and 5′-UTR were

Coxsackievirus B2

	10	20	30	40	50	60	70	80
CB2-5' UTR	CCTAACTGCG	GAGCGTGCGC	TTGCAACCCA	GTGAGTAGCA	CGTCGTAATG	GGTAACTCTG	CAGCGGAACC	GACTACTTTG
SNUH003	....	..C......A	C.T.......	.C..C.G..G			......C...	....
SNUH009	.C........	.....C....	..T.......	....				
SNUH018		.C						
SNUH024		.C						
SNUH046								
SNUH064	A		.T.	.C. C			.C.	
SNUH065				C				
SNUH075				C				
SNUH076			.A..	C	T.			
SNUH077		A..	C.C.					
SNUH078		AC.	C.T.	.G				
SNUH084		AC.	C.T.					
SNUH109		.C.	T.					
SNUH132		A.						
SNUH151		.C.	A	C				

	90	100	110	120	130
CB2-5' UTR	GGTGTCCGTG	TTTCCTTTAT	TCCTTGTCTG	GCTGCTTATG	GTGACAATTG
SNUH003	....	C	CT...CA...	....	....A...AA
SNUH009		C			........AA
SNUH018			C.		.........A
SNUH024			A		........AA
SNUH046		C	.C		........AA
SNUH064		C	A		........AA
SNUH065					........AT
SNUH075			..A.		........AA
SNUH076		.C.	.T.A.		........AA
SNUH077		.C.	.T...		........AT
SNUH078		.C	CT .CA..		C..A.
SNUH084		..	C. ...A.		AA.
SNUH109		.C.	.. C.		AA.
SNUH132			.. C.	.G	.AA
SNUH151		C .C			.AA

Echovirus 4

	10	20	30	40	50	60	70	80
EC4-5' UTR	CCTAACTGCG	GAGCACACGC	TCACAAGCCA	GTGAGTGGTG	TGTCGTAATG	GGTAACTCCG	CAGCGGAACC	GACTACTTTG
SNUH087	....	....	..T.......	....	....	........T.	....	....
SNUH123			A					

	90	100	110	120	130
EC4-5' UTR	GGTGTCCGTG	TTTCCTTTTA	ACTTCATTTT	GGCTGCTTAT	GGTGACAATT
SNUH087	....	....T.....	.T.....A..	....	.....A...A
SNUH123					.........A

Echovirus 6

	10	20	30	40	50	60	70	80
EC6-5' UTR	CTTAACTGCG	GAGCAGGTGC	TCACAATCCA	GTGGGTGGCC	TGTCGTAACG	GGCAACTCTG	CAGCGGAACC	GACTACTTTG
SNUH030								
SNUH095		C						
SNUH117	....	.C........	C....C...T	....				C

	90	100	110	120	130
EC6-5' UTR	GGTGTCCGTG	TTTCCTTTTA	TCTCTTTGTT	GGCTGCTTAT	GGTGACAATT
SNUH030	....	A.........	.......A..	....	
SNUH095			.A.A..		
SNUH117			T.A.A.		

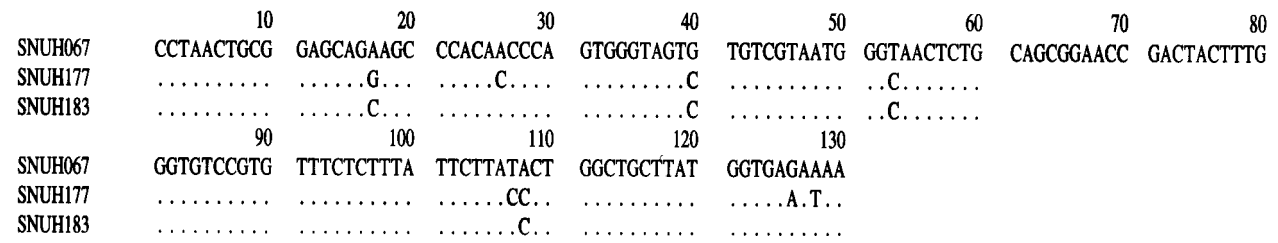

Fig. 2. Nucleotide sequence alignment of 5(-UTR sequences. Comparison of 5(-UTR sequences that obtained from 23 isolates showed considerably less variation than those of VP1.

constructed with the program MEGA as explained in Materials and Methods. Enteroviruses isolated in this study formed main two clusters in the VP1 phylogenetic tree (data not shown). Ohio-1 reference strain of CB2 was together with SNUH-018, 024, 065, 075, 076, 077, and SNUH-018 was closest related with Ohio-1. CB2 cluster was subdivided into two groups regarding their central axis, Ohio-1. The sequences other than CB2 were divided into three groups in accordance with each reference strain of three serotypes: EC4, EC6, and EC18. SNUH-087 and 123 were included in EC4 cluster; SNUH-030, 095 and 117 were in EC6; SNUH-067, 177 and 183 were in EC18. The strains of EC4 group were more related to EC18 than EC6. In the 5′-UTR dendrogram, division of the tree was not distinctive and branching order differed from the tree of VP1. Reference sequence of CB2 was closest related with SNUH-067 in which included EC6. Reciprocal comparative analysis of all strains in 5′-UTR region showed considerably less variation than the VP1.

In the phylogenetic analysis, tree of the VP1 sequences was well divided in line with reference strains but comparative tree based on the 5′-UTR sequences result in segregation into several clusters indistinctly. There were a few differences in the region. One explanation for this could be that evolution of the 5′-UTR may be more slow than the VP1 genome [5].

In conclusion, since CB2 type was 55% among enteroviral isolates, the CB2 was determined as the major causative serotype of enteroviral meningitis in 1998. CB2 type was emerged between June and July, EC4 and EC6 was limited to July, and EC18 was in August.

Acknowledgments

This work was supported by grants HMP-99-M-04-0002 from the Korean Ministry of Health and Welfare, and No. 04-99-065 from the Seoul National University Hospital Research Fund.

We thank Mark Pallansch and Steve Oberste for help with technical advises of enteroviral culture and phylogenetic analysis.

REFERENCES

1. Miller MJ. *Summary of current nomenclature, taxonomy, and classification of various microbial agents. Viral taxonomy. Clin Infect Dis 1993; 16(5):612-3.*
2. Muir P, Kammerer U, Korn K, Mulders MN, Poyry T, Weissbrich B, et al. *Molecular typing of enteroviruses: current status and future requirements. The European Union Concerted Action on Virus Meningitis and encephalitis. Clin Microbiol Rev 1998;11(1):202-27.*
3. Tam PE, Messner RP. *Coxsackievirus-induced chronic inflammatory myopathy: virus variants distinguish between acute cytopathic effects and pathogenesis of chronic disease. Virology 1997;233(1):199-209.*
4. Bell EJ, Cosgrove BP. *Routine enterovirus diagnosis in a human rhabdomyosarcoma cell line. Bull World Health Organ 1980;58(3):423-8.*
5. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. *Typing of human enteroviruses by partial sequencing of VP1. J. Clin Microbiol 1999;37(5):1288-93.*
6. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. *Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 1999;73(3):1941-8.*
7. Oberste MS, Maher K, Pallansch MA. *Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 5′ end of the region encoding VP2. Virus Res 1998;58:35-43.*
8. Kim EC, JH Lee, HJ Chung, YJ Lee. *Coxsackievirus A16 isolated from patients with hand-foot-mouth disease in Cheju Province in the spring of 1998.*

```
Coxsackievirus B2
 Ohio-1     PETVDDYNWQTSTNPSLFWTEGNAPPRMSIPFMSIGNAYSMFYDGWSEFRHDGVYGLNTLNNMGTIYARHVNADNPGSITSTVRIYFKPKHV
 SNUH003    ..............F.....................................................................................
 SNUH009
 SNUH018
 SNUH024                    P                  R
 SNUH046
 SNUH064                                       L
 SNUH075
 SNUH076          K
 SNUH077
 SNUH084
 SNUH109        GY
 SNUH132
 SNUH151

Echovirus 4
 WA93-821   AK  S  V  A  I.V  S  N.SQN.R..Y  QLFF  KPS.NTY..VA
 SNUH087    AK  S  V  A  I.V  S  N.SQN.R..Y  QLFF  KPS.NTY..VA
 SNUH123    AK  S  V  A  I.V  S  H.SQN.R..Y  QLFF  KPS.NTY..VA

Echovirus 6
 NM95-070   QA..  V  V  N  H.SQT  F  KL.F  DKTISP
 SNUH030    RAC S V  V  N  H.SQT  F  KL.F  DKTISP K
 SNUH095    QA. T A  P V  N  H.SQT  F  K..F  DKTISP K
 SNUH117    QA.  V  V  N  H.SQT  F  KL.F  DKTISP K

Echovirus 18
 TX97-2320  AK..S.E  V  A  I.V  L  H.TQ..''..YT  A..KL  KSS.HQ  I.V
 SNUH067    AK..S.E  V  A  I.V  L  H.TQ..''..YT  A..KL  KSI.HQ  I.V
 SNUH177    AK..S.E  V  A  I.V  L  H.TQ..''..YT  A..KL  KSS.HQ  I.V
 SNUH183    AK..S.E  V  A  I.V  L  H.TQ..''..YT  A..KL  KSS.HQ  I.V

Coxsakievirus B2
 Ohio-1     KAWIPRPPRLAQYLKANNVNFEITDVTEKRDSLT
 SNUH003    ...V.............D.....LTT.
 SNUH009    ...V.............D.....E.S
 SNUH018    ...V.............D.....EGQ
 SNUH024    ...V.........................
 SNUH046    ...V.............D.....ETSR
 SNUH064    Q..V.............D.....E.E...
 SNUH075    ...V.........................TTTN
 SNUH076    QD.V.........................T.NY
 SNUH077    ...V.........................TYN
 SNUH084    ...V.............D.....E...
 SNUH109    ...V.............D.....E..A
 SNUH132    ...V.............D.....SAM
 SNUH151    ...V.............D.....ETSR

Echovirus 4
 WA93-1821  R..V  CP.IN.GD  KP.P.....A..I
 SNUH087       CP.IN.GD  KP.P..D..K..I
 SNUH123       CP.IN.GD  KP.P..D..E..I

Echovirus 6
 NM95-2070     V  CE.THKD..D..PKG..TS.TQ
 SNUH030       V  CE.THKD..D..PKG..TS.T.
 SNUH095       V  CE.THKD..D..PKG..TS.TQ
 SNUH117       V  CE.THKD..D.VPKG..TS.TQ
```

```
Echovirus 18
 TX97-2320      ...V......CP.INKGD...AV.E..DA.K.I.
 SNUH067        ...V......CP.INKSD...VV.EISD
 SNUH177        ...V......CP.INKGD...AV.E..DA.K.I.
 SNUH183        ...V......CP.INKGD...AV.E..DA.K.I.
```

Fig. 3. Amino acid sequence alignment of VP1 protein. The figure showed the important properties that each serotype exhibits different amino acids. The first line of each serotype is the reference sequence obtained from GenBank.

Korean J Clin Microbiol 1999;2(2):172-6.

9. Wilfert CM, Lauer BA, Cohen M, Costenbader ML, Myers E. *An epidemic of echovirus 18 meningitis. J Infect Dis 1975;131(1):75-8.*
10. Fujioka S, Koide H, Kitaura Y, Deguchi H, Kawamura K, Hirai K. *Molecular detection and differentiation of enteroviruses in endomyocardial biopsies and pericardial effusions from dilated cardiomyopathy and myocarditis. Am Heart J 1996;131(4):760-5.*
11. Koonin EV, Dolja VV. *Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol 1993;28(5):375-430.*

국문초록

무균성 수막염 환자에서 분리한 enterovirus의 혈청형 및 계통발생학적 분석

이정희[1,2], 안병윤[2], 반성환[3], 김상현[3], 김의종[1,4]

서울대학교병원 임상의학연구소 바이러스연구소[1], 고려대학교 자연자원대학원 유전공학과[2],
순천향대학교 의과대학 구미순천향병원 소아과[3], 서울대학교 의과대학 임상병리학교실[4]

배경 : Enterovirus의 혈청형을 파악하면, 산발적 발생과 집단발생을 감별할 수 있다. 전통적인 혈청형 검사법은 시간과 노동력이 많이 소요된다. 최근 분자생물학적 기법을 통하여 혈청형을 알아내는 방법이 소개되었다. 본 연구의 목적은 enterovirus를 배양하고, 분자생물학적 방법으로 분석하여 혈청형을 파악하는 것이다.

방법 : 1998년 무균성 수막염이 의심되어 입원한 환자로부터 채취한, 척수액 164 검체, 대변 136개, 혈청 15개, 인후도말 6개, 소변 5개, 객담 4개를 대상으로 하였다. RD cell에서 세포병변효과(CPE)가 관찰되면, RNA를 추출하여 RT-PCR로 VP1과 5' UTR 부위를 증폭하였다. VP1의 염기서열은 BLAST 프로그램으로 분석하였다.

결과 : 총 333개의 검체 중 23개의 검체에서 CPE가 관찰되었다. 17개는 처음부터 CPE가 보였으며, 6 검체는 두번째 계대배양에서 CPE를 보였다. 15 개는 coxsackievirus B2 (CB2)이었으며, echovirus 4가 2개, echovirus 6가 3 개, echovirus 18이 3개이었다. 같은 혈청형끼리 핵산의 상동성은 80-95%이었으며, 아미노산의 상동성은 93-100%이었다.

결론 : 분리된 enterovirus 중에서 55%가 CB2이었으므로, 1998년에 유행한 무균성 수막염의 주요 원인은 CB2이었을 것으로 생각한다. 이는 6월과 7월에 발생하였고, EC4와 EC6는 7월에, EC18은 8월에 발견되었다.