

Diagnosis of group A coxsackieviral infection using polymerase chain reaction

M Hosoya, H Ishiko, Y Shimada, K Honzumi, S Suzuki, K Kato, H Suzuki

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See end of article for authors' affiliations

Correspondence to:
Dr M Hosoya, Dept of Pediatrics, Fukushima Medical University School of Medicine, Hikarigaoka 1, Fukushima 960-1295, Japan; mhosoya@fmu.ac.jp

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Aims: To examine the relation between enteroviral infection, especially group A coxsackieviral infection, and acute febrile illness over two summers using tissue culture and polymerase chain reaction (PCR).

Methods: Throat swabs were collected from 246 children from June to August 1997 and 1998.

Results: Enteroviruses were isolated from 33/246 samples and 35 other viruses were isolated. Enteroviral genomes were detected in 54/178 samples from which no virus was isolated. Of 41 enteroviral genotypes identified by sequence analysis of PCR products, 38 were group A coxsackieviruses, which are usually difficult to isolate using tissue culture.

Conclusion: Results indicate that viral detection and identification based on PCR is useful in the diagnosis of group A coxsackieviral infection.

Human enteroviruses include polioviruses, echoviruses, group A and B coxsackieviruses, and enteroviruses (types 68–71). These subgroups were originally differentiated from each other by their different effects in tissue culture and animal inoculation. Polioviruses, echoviruses, and group B coxsackieviruses are easy to isolate using tissue culture but, in general, group A coxsackieviruses are difficult. Suckling mice inoculation is therefore used instead.¹ Virologic studies in the early 1950s using this technique clearly indicated that several group A coxsackieviruses were the cause of epidemic herpangina,^{2,3} but the full range of clinical manifestations of coxsackievirus group A infections remains undefined. Diagnostic studies utilising suckling mice inoculation are seldom performed today. Recently polymerase chain reaction (PCR) methods have been developed for the detection of most enterovirus serotypes.^{4–6} Phylogenetic analysis of enteroviral genome sequences allows classification of enteroviral genotypes.^{7,8} We have previously reported enteroviruses associated with febrile seizures in the summer months using PCR and subsequent phylogenetic analysis of amplified genome sequences.⁹ In the present study, we investigated the association of enteroviruses, especially group A coxsackieviruses, with a broader range of acute febrile illnesses including herpangina, febrile seizures, and pharyngitis/tonsillitis in the summer months, using both tissue culture and PCR.

MATERIALS AND METHODS

Samples

Between June and August 1997 and 1998 in the Fukushima Prefecture, Japan, throat swabs were collected for viral surveillance from 246 children who had a body temperature of more than 38.5°C and clinical evidence of pharyngeal and/or tonsillar infection. Specimens were collected into transport medium containing 2 ml of Eagle's minimum essential medium with gelatin (5 mg/l), penicillin (400 U/l), streptomycin (400 µg/l), and amphotericin B (1.25 µg/l), divided into two parts and stored at –70°C until analysis. One part was used for viral isolation by cell culture and the other for viral detection by PCR.

Twenty four children (aged 0.25–6.50 years, mean 2.21) were given a clinical diagnosis of herpangina based on sudden onset of fever and characteristic vesicular and/or ulcerated lesions on the oropharyngeal mucosa. Twenty one children (aged 0.67–4.50 years, mean 1.97) were given a clinical diagnosis of febrile

seizures if they fulfilled the following five criteria: age 6 months to 5 years; fever >38°C; seizure duration <15 minutes; absence of CNS infection as determined using standard techniques, such as bacterial culture and viral isolation; and absence of persistent neurological abnormalities after the attack. Details of the 21 patients have been reported previously.⁹ The other 201 children studied (aged 0.16–15.9 years, mean 3.87 years) had neither a characteristic enanthema in the oral cavity nor neurological manifestations. They were given a diagnosis of pharyngitis/tonsillitis. Of these 201 patients, eight had conjunctivitis, five exanthematous manifestations, one stomatitis, one parotitis, and one cervical lymphadenitis.

Virus isolation

Hep-2, Vero, RD-18S (a human rhabdomyoma cell line), HMV-II (a human malignant melanoma cell line), and MDCK cells were used for viral isolation. Confluent cell cultures were seeded in microplate wells and inoculated with 100 µl of maintenance medium and 50 µl of throat swab samples. Cell cultures were incubated at 34°C in 5% CO₂ and observed for seven days for the cytopathic effect. Blind passage was then performed once if cytopathic effect was not observed. Virus isolates were identified by neutralisation tests using sera from Denka Seiken Co. Ltd (Tokyo, Japan) as originally reported by Schmidt and colleagues.¹⁰

PCR for detection of enteroviral genome

Throat swab samples negative for viruses by culture were screened for enteroviral genome by PCR. One nested PCR method (PCR-FMU) has been described previously.⁶ In brief, RNA was extracted from 250 µl of each sample using Isogen-LS (NipponGene, Tokyo, Japan) (an acid guanidinium thiocyanate kit). Nucleic acid was pelleted by centrifugation, washed once with 70% ethanol, dried, and dissolved in 10 µl sterile water. cDNA synthesis was performed using 2.5 U Moloney murine leukaemia virus reverse transcriptase (Toyobo, Osaka, Japan) from 3 µl of resuspended RNA. A 10 µl aliquot of cDNA product was used as template for amplification using Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Primers targeted the 5' non-coded region of coxsackievirus B1 sequence,¹¹ as reported by Zoll and colleagues.⁵ F1 (5'-CAAGCACTTCTGTTCCCGG) complementary to genomic sense RNA at positions 160–180, and R1 (5'-ATTGTACCATAAGCAGCCA) antisense RNA 580–599, were

Table 1 Detection and identification of viruses from throat swab using cell culture

	Herpangina (n=24)	Pharyngitis/ tonsillitis (n=201)
<i>Enteroviruses</i>		
<i>Coxsackievirus</i>		
A16	1	0
B1	0	2
B2	0	3
B5	0	3
<i>Echovirus</i>		
9	0	8
18	0	13
30	0	2
<i>Enterovirus</i>		
71	0	1
<i>Others</i>		
<i>Adenovirus</i>		
1	0	5
2	0	8
3	0	13
5	0	4
7	0	1
<i>Parinfluenza virus</i>		
1	0	1
3	0	1
<i>HSV</i>		
1	1	1

No virus was isolated from 21 samples taken from patients with febrile seizures.

used for first round amplification; F2 (5'-TCCTCCGGCCCC TGAATGCG) sense positions 445–464 and R1 were used for second round amplification. The products were run on 2% agarose ethidium bromide gels and photographed under ultraviolet light. A positive PCR reaction was expected to produce a 155 base pair (bp) band. The PCR using these general primers could detect 60 of 66 different enterovirus serotypes; no amplification product was observed from coxsackievirus types A11, A17, and A24 and echovirus types 16, 22, and 23.⁵ The sensitivity of PCR-FMU, calculated from extractions of serial dilutions of titrated echovirus type 7, corresponded to 10^{-3} TCID₅₀/ml.⁶ Negative controls were included for each step of the assay.

PCR for enterovirus typing

Samples positive by PCR-FMU were tested for enteroviral genome sequences using PCR-MBCL at Mitsubishi Kagaku Bio-Clinical Laboratory (MBCL). Primers were set in the 5'

non-coded region and the VP2 region: OL68-1 (5'-GGTAAATTCACCACCANCC)⁷ complementary to genomic sense RNA at positions 1178–1197, and MD91 (5'-CCTCCGGCCCCCTGAATGCGGCTAAT)⁴ antisense RNA at 444–468 of attenuated poliovirus Sabin 1 strain,¹² were used for first round amplification; OL68-1 and EVP4 (5'-CTACTTTGGGTGTCCGTGTT)⁴ antisense RNA at 541–560 were used for second round amplification. PCR-MBCL amplified an approximately 650 bp product including part of the 5' non-coding region, the complete VP4 region, and the 5' end of the VP2 region. This method detected all 64 prototype enterovirus strains tested.¹³ The sensitivity of PCR-MBCL corresponded to approximately 10^{-1} – 10^{-2} TCID₅₀/ml.⁹

The PCR products were gel isolated, purified, and sequenced on an automated DNA sequencer using a fluorescent dideoxy chain terminator. The MBCL database for the complete VP4 region of all 64 human enteroviruses was constructed from nucleotide sequencing data of the VP4 region of 49 prototype enterovirus strains and 15 previously published enteroviral sequences obtained from GenBank.¹³ The VP4 sequences from each PCR product were phylogenetically analysed along with those of prototypes in the MBCL database using the SINCA program (Fujitsu Limited, Tokyo, Japan). Genetic distances were estimated using the Kimura two parameter method, and unrooted phylogenetic trees were constructed using the neighbour joining method. Statistical significance of phylogenies using neighbour joining was estimated by bootstrap analysis with 1000 resamplings of data sets.

Diagnosis of enteroviral infection

Diagnosis of enteroviral infection was made when enterovirus was detected in throat swab samples using cell culture and/or PCR. Enteroviral detection from any site except lower intestinal tract is commonly considered to be causally related to a specific presenting illness.

RESULTS

Viral isolation

Coxsackievirus A16 and herpes simplex virus type 1 were isolated from one each of 24 throat swab samples taken from patients diagnosed clinically with herpangina (see table 1). No virus was isolated from 21 samples taken from patients with febrile seizures. Sixty six viruses (32 enteroviruses, 31 adenoviruses, two parainfluenza viruses, and one herpes simplex virus) were isolated from 66 (32.8%) of 201 patients with other febrile illnesses during summer months.

Table 2 Detection and identification of viruses from throat swabs using polymerase chain reaction

	Herpangina (n=22)	Febrile seizures (n=21)	Pharyngitis/tonsillitis (n=135)
<i>Virus detected by PCR-FMU</i>	17	11	26
<i>Virus identified by PCR-MBCL</i>	14	11	16
<i>Enteroviruses</i>			
<i>Coxsackievirus</i>			
A2	0	0	4
A4	3	4	2
A5	3	1	7
A6	4	1	1
A8	1	1	0
A10	3	1	2
B5	0	1	0
<i>Echovirus</i>			
18	0	1	0
30	0	1	0

FMU, Fukushima Medical University; MBCL, Mitsubishi Kagaku Bio-Clinical Laboratories.

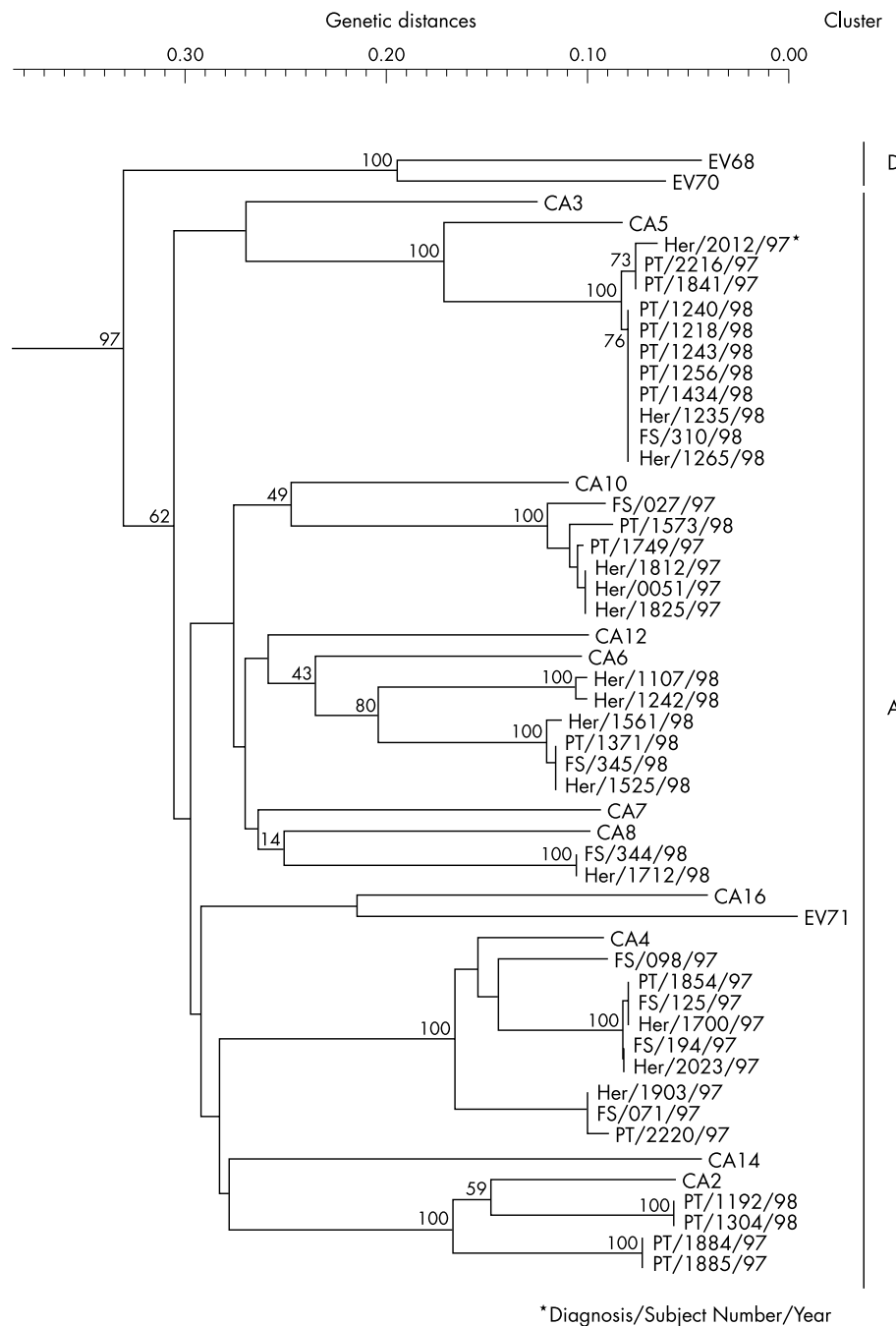


Figure 1 Phylogenetic tree of 64 prototype enteroviruses and 38 enteroviruses detected from patients with febrile illness during two summers. The phylogenetic tree was constructed based on the nucleotide sequences of the VP4 protein coding region. Sixty four prototype enterovirus strains were classified into four genetic clusters (A, B, C, and D). All 14 enteroviruses detected from patients with herpangina (Her), 8/11 enteroviruses detected from patients with febrile seizures (FS), and all 16 enteroviruses detected from patients with pharyngitis/tonsillitis (PT) were within the A cluster of enteroviruses (coxsackievirus group A-like genotype). Coxsackievirus group A-like genotypes detected from herpangina, febrile seizures, and pharyngitis/tonsillitis in two summers were distinctly clustered depending on the year when the samples were taken (1997 or 1998), rather than on the type of illness.

Screening for the presence of the enterovirus using PCR

The presence of the enteroviral genome sequence in throat swab samples from which no virus was isolated by tissue cultures was identified using PCR-FMU in 17/22 samples taken from patients with herpangina, 11/21 samples taken from patients with febrile seizures, and 26/135 samples taken from patients with pharyngitis/tonsillitis during summer months (see table 2). Enteroviral genome was detected from samples from one patient with pharyngitis/tonsillitis associated with exanthema and one patient with stomatitis.

Identification of enterovirus using phylogenetic analysis

Fifty four original samples that had positive results using PCR-FMU were reexamined using PCR-MBCL (see table 2). Positive results were obtained from 14/17 samples from patients with herpangina, all 11 samples from patients with febrile seizures, and 16/26 samples from patients with pharyngitis/tonsillitis. Thus, the presence of enteroviral genome was confirmed in 41 (75.9%) of 54 tested samples. Phylogenetic analysis using the VP4 protein coding region (207 bp) of 64 prototype enterovirus strains from the MBCL

database produced four major clusters. Analysing the sequences of the PCR products allowed us to identify the genotypes of enteroviruses. Genotypes of all 14 enteroviruses detected from samples of patients with herpangina, 8/11 enteroviruses detected from samples of patients with febrile seizures, and all 16 enteroviruses detected from samples of patients with pharyngitis/tonsillitis were within the A cluster of enteroviruses (coxsackievirus group A-like genotype).

Enteroviruses belonging to the coxsackievirus A5-like genotype, which were identified from samples of patients with herpangina, febrile seizures, and pharyngitis/tonsillitis during two summers formed two distinct clusters (fig 1). The clusters related to the year when the samples were taken rather than the type of illness. Other genotypes had similar clusters.

When baseline characteristics (age, degree of fever, or days after onset when the samples were taken) were compared among all patients with group A coxsackieviral infection, those with herpangina (2.34 (1.45) years) and febrile seizures (1.94 (1.05) years) were younger than those with pharyngitis/tonsillitis (4.39 (3.38) years). Among those with pharyngitis/tonsillitis, there were no significant differences in baseline characteristics while distinguished groups infected with group A coxsackieviruses, other enteroviruses, or adenoviruses.

DISCUSSION

In general, group A coxsackieviruses (except for some serotypes such as A9, A10, and A16) are rarely isolated using standard tissue culture methods. In the present study, only one (A16) of 33 isolated enteroviruses was a group A coxsackievirus. Recently developed PCR techniques are very sensitive for detecting most enteroviral genomes from a variety of samples.⁴⁻⁶ A PCR method for specific detection of group A coxsackieviruses has also been reported,¹⁴ but could not identify the enterovirus serotype (genotype). Phylogenetic analysis of VP-1 sequences of enteroviral genome has allowed classification of enteroviral genotypes.⁸ Alignment studies of the sequenced enteroviruses revealed highly conserved sequences within the 5' non-coding region and the VP2 region. When the primers were set in these conserved regions, PCR methods could detect most enterovirus serotypes.⁵⁻⁷ Subsequent phylogenetic analysis of complete VP4 sequences, between the 5' non-coding region and the VP2 region, allowed us to identify enterovirus genotypes.⁹⁻¹³ As viral isolation is not required for identification of the virus, a combination of PCR based viral detection and phylogenetic analysis based viral identification is expected to be useful for the detection and identification of group A coxsackieviruses. Thirty eight (92.7%) of 41 enteroviruses identified using these methods from samples in which no virus was isolated by tissue culture were group A-like coxsackieviruses by genotyping.

Group A coxsackieviruses are thought to be major herpangina pathogens. PCR-FMU detected enteroviruses in 17 of 22 patients with culture negative herpangina, of whom 14 (82.4%) had enteroviral genome detected by PCR-MBCL. This indicates that the two methods have different sensitivity. All 15 enteroviruses identified by both cell culture and PCR-MBCL were group A coxsackieviruses. Our results confirm that enteroviruses, and especially group A coxsackieviruses, are important causative agents of febrile illness associated with febrile seizures in the summer. The pathogens causing acute

pharyngitis/tonsillitis were detected in 92 (45.8%) of 201 patients. Common pathogens were enteroviruses (58/92), followed by adenoviruses (35/92). Of 48 enteroviruses identified both by cell culture and PCR-MBCL, 16 (33.3%) were group A coxsackieviruses. There were no significant differences in the baseline characteristics (age, degree of fever, and clinical features) of groups variously infected with group A coxsackieviruses, other enteroviruses, or adenoviruses.

Clustering by year shown by phylogenetic analysis of group A coxsackievirus isolates rather than by type of illness, indicates that the viruses prevalent each year cause a variety of summer time febrile illnesses, including herpangina, febrile seizures, and pharyngitis/tonsillitis.

In conclusion, PCR and subsequent phylogenetic analysis of amplified sequences is very useful for the detection and identification of enteroviruses and thus for the diagnosis of group A coxsackieviral infections. The methods will also be useful for the study of the molecular epidemiology of enteroviral infections.

Authors' affiliations

M Hosoya, K Honzumi, H Suzuki, Department of Pediatrics, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan
H Ishiko, Y Shimada, Infectious Disease Laboratory, Mitsubishi Kagaku Iriko, Ltd., Tokyo 174-8555, Japan
S Suzuki, K Kato, Fukushima Institute for Public Health and Environmental Science, Fukushima 960-8560, Japan

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