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Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: prevalence, intrafamilial spread, and risk factors for infection

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SUMMARY

Studies of non-polio enterovirus prevalence and transmissibility in developing countries are limited and few studies have investigated specific risk factors for infection. An epidemiological survey of non-polio enterovirus among families in Mongolia was conducted in the late summer of 2003. Stools of 122 healthy persons were collected weekly for 5 weeks. Eight serotypes of non-polio enteroviruses (echovirus 30, 33, 12, 25, coxsackievirus A10, A2, A4, A24) were isolated from 62 persons, with an overall isolation rate of 51%, and 64% and 35% among children under 10 years and adults over age 21 years. Fifty-four per cent of isolations were due to intrafamilial infection. Analysis of risk factors for infection suggested contamination of indoor kitchen, bathroom, toilet, and waste disposal area. Hand washing after defecation was protective against infection. Our study findings stress the importance of hand washing and cleaning hygienic facilities to prevent infection by enteric viruses in the home environment.

INTRODUCTION

Though most enterovirus infections are asymptomatic, enterovirus can become a serious public health concern with a capability of causing a spectrum of clinical illnesses such as paralysis, aseptic meningitis, encephalitis, herpangina, hand, foot, and mouth disease, upper respiratory disease, cardiac disease, and acute haemorrhagic conjunctivitis [1–3]. Enterovirus prevalence and transmissibility vary by factors such as climate, geography, crowding, and socio-economic status; previous studies among healthy individuals in different countries have shown diversity in isolation rate [4–13]. However, studies of enterovirus, especially non-polio enterovirus, in developing countries are limited and few studies have investigated specific risk factors for infection.

In Mongolia, enteric diseases are widespread, imposing a heavy burden on the population's health. Hepatitis A is considered highly endemic: 8250 cases were clinically diagnosed as hepatitis A in 2000 among the national population of 2·7 million [14]. Diarrhoea is a significant problem resulting in a high infant mortality rate which was 51·97/1000 births as of 2002 [15]. Despite our knowledge that enteric diseases are endemic, no study has been done on non-polio enterovirus prevalence and transmissibility in Mongolia.

Therefore, this study aims to determine non-polio enterovirus prevalence in urban areas of Mongolia, to attempt isolation of enterovirus from hand, to assess

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the degree of intrafamilial spread of non-polio enterovirus as a measure of its transmissibility, and to investigate risk factors for non-polio enterovirus infection such as living environment and hygiene practices.

METHODS

Study site

This study was conducted from July to August 2003 in Ulaanbaatar and Tov province in Mongolia. Four districts were chosen from Ulaanbaatar: Chingeltei, Bayangol, Bayanzurkh, and Songinokhairkhan. From Tov province, Zuum mod (provincial centre) and Bayanchandmani (provincial district centre) were selected. Areas with different living environments such as type of residence and water supply were considered for site selection.

Areas within the six districts can be classified into three distinct categories: slum area in Ulaanbaatar, apartment area in Ulaanbaatar, and remote but accessible area from Ulaanbaatar (Zuum mod and Bayanchandmani). In Ulaanbaatar, *gers* (traditional Mongolian dwelling consisting of tent-like wooden structure covered with felt, no inside hygienic facilities) and houses (structure with one or more rooms, some with piped water and/or inside hygienic facilities) co-exist on dirt roads in slum areas, whereas apartment (provided with piped water and hygienic facilities) areas have paved roads and often have local shopping centres. Zuum mod and Bayanchandmani are located 45 and 80 km from Ulaanbaatar and are surrounded by grassland and gentle hills.

Study population and oral poliovirus vaccine (OPV) administration

Four to five infants who were scheduled for OPV immunization during July 2003 and their families were consecutively sampled in each of the six areas. An infant (study child), mother, father, and one youngest sibling were included in the study from each household. For some cases, a relative living in the same household as the study child, or a contact of a sibling of the study child who lived in a different household were sampled for investigation. OPV (SB Biologicals, Rixensart, Belgium) was administered to study children under routine immunization on the first week.

Sample collection

Stool specimens were collected from all participants at weekly intervals for 5 weeks. Swab samples were taken from palms and fingers of 46 available subjects who took care of study children on the second week. Swabs soaked in 1 ml PBS (Nissui, Tokyo, Japan) were used. All samples were carried at 4 °C and stored at −20 °C. Interviews to determine living environment and hygiene practices were conducted using a semi-structured questionnaire. Questions on living environment of each household were asked of the mother or father, and questions on hygiene behaviour were asked of all subjects except study children. For children too young to answer questions, the mothers answered for them. This did not seem inappropriate as in the case of very young children the mothers instructed them in hygienic activities or they did those activities together. The questionnaire was reviewed and revised by health workers participating in the study.

Ethical approval was obtained from the University of Tokyo Ethical Committee, and approval was given by the Ministry of Health in Mongolia. Written informed consent was collected from all participants or their guardians.

Isolation and identification of enterovirus in stools

Virus isolation was done according to the WHO recommended method [16] with the following modifications. Stool extracts were treated with PBS containing 10% chloroform and stored at −20 °C until inoculation onto rhabdomyosarcoma (RD), HEp-2 and L20B cell lines. Isolation was conducted using 24-well plates. Two sequential passages of 7 days were performed in the three cell lines before recording as negative. Samples which showed a cytopathic effect (CPE) in RD and/or HEp-2 cells after 14 days of observation were passaged to L20B cells and observed for another week. For samples which showed CPE in L20B cells, identification for poliovirus was conducted. For samples which showed CPE in RD and/or HEp-2 cells but not in L20B cells, identification for non-polioviruses was performed using enterovirus antisera (RIVM, Bilthoven, The Netherlands). For samples which were poliovirus positive in L20B cells and also showed CPE in RD and/or HEp-2 cells, samples were analysed for concurrent non-polio enterovirus infection. A total of 50 μl of diluted tissue culture fluid from RD and/or HEp-2 cells was mixed with 50 μl of antiserum to poliovirus types 1+2+3 in RD and/or HEp-2 cells respectively, and observed for 1 week. For CPE-positive samples, virus fluid was re-inoculated onto

L20B cells to confirm the absence of poliovirus and observed for another week.

RNA extraction and RT–PCR

For non-poliovirus samples for which a virus serotype could not be identified by neutralization using the RIVM kit, viral RNA was extracted from 140 μl of infected cell culture fluid using QIAamp Viral RNA kit (Qiagen, Hilden, Germany). Extracted RNA (5 μl) was used for genomic amplification by RT–PCR using Access RT–PCR kit (Promega, Madison, WI, USA). RT–PCR was conducted using 50 pmol of primers OL68-1 (5′-GGTAAYTTCCACCACCA-NCC-3′, antisense) and EVP4 (5′-CTACTTTGGG-TGTCCGTGTT-3′, sense) in the 5′NTR-VP4-VP2 region [17]. The condition for RT–PCR was 45 min at 48 °C, 2 min at 94 °C, 35 cycles at 94 °C for 10 s, 50 °C for 10 s, 65 °C for 1 min, and 5 min at 65 °C.

Sequencing analysis

Amplified products were purified using QIAquick PCR purification kit (Qiagen). Nucleotide sequencing reaction was performed using ABI PRISM BigDye Termination Cycle Sequencing kit with 3·2 pmol of each primer (Applied Biosystems, Foster City, CA, USA). Sequence data were collected using ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems).

For non-polio enteroviruses, clusters of each virus type were made by Clustal W software after 1000 times bootstrapping [18], using their nucleotide sequences in the 5′NTR-VP4-VP2 region identified using OL68-1 and EVP4 primers. (The same method was used for the analysis of intrafamilial spread of non-polio enteroviruses.) One sample in each virus type was chosen and its virus type was determined by RT–PCR using 187 (5′-ACIGCIGYIGARACIGG-NCA-3′, sense), 188 (5′-ACIGCIGTIGARACIGG-NG-3′, sense), 189 (5′-CARGCIGCIGARACIGG-NGC-3′, sense), 222 (5′-CICCIGGIGGIAYRWA-CAT-3′, antisense), 012 (5′-ATGTAYGTICCICCI-GGIGG-3′, sense), 040 (5′-ATGTAYRTICCIMCI-GGIGC-3′, sense), and 011 (5′-GCICCIGAYTGI-TGICCRAA-3′, antisense) in the VP1 region [19]. A total of 20 pmol of each antisense primer and 40 pmol of sense primer were used for the RT–PCR reaction with the same cycle condition as above.

Previously reported sequences used in the comparison were obtained from GenBank. Molecular-based enterovirus typing method described by Oberste et al. [20] was used with nucleotide sequence data of VP1 region.

Restriction fragment length polymorphism (RFLP) analysis

For samples positive for poliovirus, RFLP analysis was conducted for intra-typic differentiation to distinguish between vaccine and wild polioviruses [21]. The purified PCR products were digested with *Dde*I, *Hpa*II, and *Hae*III (Takara, Kyoto, Japan) at 37 °C for 2 h. The digested fragments were electrophoresed on 2% agarose gel.

Detection of enterovirus on hands

RNA was extracted from 980 μl PBS in which the swab was soaked. RT–PCR was conducted using 19 μl RNA and 80 pmol of EVP4-OL68-1 primers and UG1-UC1 (5′-GAATTCCATGTCAAATCT-AGA-3′, sense) primers [21] with the same condition as with stool samples. Subsequently, nested PCR was conducted using puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) with 1 μl PCR product of EVP4 and OL68-1 and 20 pmol of OL68-1 and OL24 (5′-CTACTTTGGGTGTCCG-3′, sense) primers [22] in a 25 μl reaction. PCR products were electrophoresed in 2% agarose gels.

Statistics

Univariate analysis was conducted to screen statistically significant ($P<0·05$) variables: χ^2 tests for categorical data and Student's t test for continuous data were adopted. Fisher's exact test was applied for data with small frequencies and Yates' correction was used with 2×2 categorical data. Logistic regression was performed for each significant variable and odds ratios were calculated for each risk factor. Analysis of variance (ANOVA) was conducted for analysis of factors associated with degree of intrafamilial spread, and $P<0·1$ was considered significant due to the small number of households analysed. The degree of intrafamilial spread was calculated by dividing the number of family member(s) infected by the total number of family members in the household. In the analysis of association between hygiene practices and non-polio enterovirus isolation, infants were excluded from analysis due to the difficulty in acquiring accurate data on their behaviour. For the analysis of factors associated with the degree of intrafamilial

Table 1. *Enterovirus isolated from stool specimens collected from families in Ulaanbaatar and Tov province, Mongolia, late summer 2003, by age*

		Age group (years)													
		0–1		2–10		11–20		21–30		31–40		41+		Total	
Virus		30 tested		23 tested		12 tested		37 tested		12 tested		8 tested		122 tested	
Group	Type	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Polio	1	9	(30)	1	(4·3)	1	(8·3)	1	(2·7)	0	(0)	0	(0)	12	(9·8)
	2	5	(16·7)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	5	(4·1)
	3	6	(20)	0	(0)	0	(0)	0	(0)	1	(8·3)	0	(0)	7	(5·7)
Total persons shedding poliovirus*		15	(50)	1	(4·3)	1	(8·3)	1	(2·7)	1	(8·3)	0	(0)	19	(15·6)
Concurrent infections†		1 P1+P2‡ 3 P1+P3‡ 1 P2+P3													
Echo	30	7	(23·3)	9	(39·1)	0	(0)	5	(13·5)	4	(33·3)	1	(12·5)	26	(21·3)
	33	7	(23·3)	6	(26·1)	6	(50)	9	(24·3)	1	(8·3)	0	(0)	29	(23·8)
	12	1	(3·3)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	1	(0·8)
	25	1	(3·3)	1	(4·3)	0	(0)	0	(0)	0	(0)	0	(0)	2	(1·6)
	Total*	16	(53·3)	16	(69·6)	6	(50)	14	(37·8)	5	(41·7)	1	(12·5)	58	(47·5)
Coxsackie	A10	4	(13·3)	3	(13)	1	(8·3)	0	(0)	0	(0)	0	(0)	8	(6·6)
	A2	0	(0)	1	(4·3)	0	(0)	0	(0)	0	(0)	0	(0)	1	(0·8)
	A4	0	(0)	1	(4·3)	0	(0)	0	(0)	0	(0)	0	(0)	1	(0·8)
	A24	0	(0)	0	(0)	1	(8·3)	0	(0)	0	(0)	0	(0)	1	(0·8)
	Total*	4	(13·3)	5	(21·7)	2	(16·7)	0	(0)	0	(0)	0	(0)	11	(9)
Total persons shedding non-polio enterovirus*		17	(56·7)	17	(73·9)	8	(66·7)	14	(37·8)	5	(41·7)	1	(12·5)	62	(50·8)
Concurrent infections†		1 1 E33+CA10		2 E33+CA10 1 E33+CA4‡ 1 E30+CA10											

* There are individuals infected with multiple virus types during the course of 5 weeks; such individuals are counted as one.
† Number of persons concurrently yielding multiple virus types during the course of 5 weeks.
‡ P1, poliovirus type 1; P2, poliovirus type 2; P3, poliovirus type 3; E30, echovirus 30; E33, echovirus 33; CA10, coxsackie A10; CA4, coxsackie A4.

spread, one study child who lived in an orphanage was excluded. Statistic analysis was performed using SPSS 11.0.1J (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Demographic characteristics

A total of 29 households involving 122 subjects participated in the study. The mean age of the study children was 3·9 months [± standard deviation (S.D.) 1·0 month], siblings 5·9 ± 4·1 years, mothers 26·6 ± 5·4 years, fathers 28·9 ± 5·9 years, relatives 30·1 ± 17·1 years, and contacts of siblings 29·7 ± 23·2 years. A total of 70/122 (57%) of subjects were female. From Chingeltei, Bayangol, Songinokhairkhan, Bayanzurkh, Zuum mod, and Bayanchandmani, 25, 17, 23, 20, 23, and 14 subjects respectively, participated.

Poliovirus and non-polio enterovirus isolation

Poliovirus isolation status is shown in Table 1. Fifteen study children (50%) excreted poliovirus, and polioviruses were isolated from four familial contacts: one sibling (age 2 years), two mothers (ages 21 and 34 years), and one uncle (age 16 years). Intra-typic differentiation revealed all polioviruses to be Sabin-like (vaccine type).

Table 2. *Non-polio enterovirus isolated from stool specimens collected from families in Ulaanbaatar and Tov province, Mongolia, late summer 2003, by sex and age*

Age group (years)	Female		Male	
	%	(No. positive /no. tested)	%	(No. positive /no. tested)
0–1	43	(6/14)	69	(11/16)
2–10	64	(7/11)	83	(10/12)
11–20	70	(7/10)	50	(1/2)
21–30	39	(9/23)	36	(5/14)
31–40	50	(3/6)	33	(2/6)
41+	17	(1/6)	0	(0/2)
Total	47	(33/70)	56	(29/52)

In the 5 weeks of observation of 122 subjects, 69 non-polio enteroviruses were isolated (Table 1). Eight serotypes of non-polio enteroviruses (echovirus types 30, 33, 12, 25, coxsackie A10, A2, A4, and A24 variant) were isolated from 62 persons with an overall isolation rate of 51% (62/122). By neutralization, only echovirus 30 was identified; therefore, molecular typing was performed for the samples for which a virus serotype could not be identified by neutralization. By analysis of the VP1 region, molecular typing identified the serotypes of all samples, which included echovirus 12, 25, 33, coxsackie A2, A4, A10, and A24. Among the serotypes, echovirus 30 and 33 predominated: 21% (26/122) and 24% (29/122) of the subjects were shedding the respective viruses. In the 5-week period, six individuals yielded multiple virus types: one study child (age 5 months) excreted echovirus 30, 33, and coxsackie A10, one sibling (age 4 years) excreted echovirus 33 and coxsackie A4, one sibling (age 2 years) excreted echovirus 30 and coxsackie A10, and one study child (age 5 months) and two siblings (ages 2 and 4 years) excreted echovirus 33 and coxsackie A10. Overall the non-polio enterovirus isolation rate was highest among the 2–10 years age group (74%) (Table 1). 57% (17/30), 69% (18/26), 45% (13/29), 36% (8/22), 12·5% (1/8), and 71% (5/7) of study children, siblings, mothers, fathers, relatives, and contacts excreted non-polio enteroviruses.

Overall, 47% (33/70) of females and 56% (29/52) of males excreted non-polio enteroviruses (Table 2). Comparison between the two sexes indicated that there was a tendency for higher isolation rates among males in younger age groups and females in older age groups.

Table 3. *Number of households which showed intrafamilial spread of non-polio enterovirus, Ulaanbaatar and Tov province, Mongolia, late summer 2003*

Virus		No. of household(s) with intrafamilial spread/No. of household(s) with at least one infected individual
Group	Type	
Echo	30	8/10
	33	8/10
	12	0/1
	25	1/1
	Total different households*	16/20
Coxsackie	A10	1/7
	A2	0/1
	A4	0/1
	A24	0/1
	Total different households*	1/10
Total different households*		16/23

* There are households with multiple virus types; such households are counted as one.

Detection of enterovirus on hands

No enterovirus was detected from any of the hand swabs of 46 subjects.

Familial spread of non-polio enterovirus

We determined intrafamilial spread through isolation of the same virus type with high sequence homology in the 5′NTR-VP4-VP2 region from two or more members in the same household. The number of households tested and households which showed intrafamilial spread of non-polio enterovirus are shown in Table 3. All isolates from members living in the same household had 100% sequence homology except two households from which sequences differed by one nucleotide. Intrafamilial spread was responsible for 54% (37/69) of non-polio enterovirus infections.

Out of 16 households in which intrafamilial spread was confirmed, mode of transmission within the household could be observed in nine households by the difference in timing of virus isolation among family members. It supports the view that siblings

Table 4. *Order of transmission of non-polio enterovirus within household determined by difference in timing of virus isolation, Ulaanbaatar and Tov province, Mongolia, late summer, 2003. Modes of transmission for the 16 households which showed intrafamilial spread are shown*

Household	Virus	Week 1		Week 2		Week 3	Week 4		Week 5
1	E30	S	→	M					
2	E30	V S M C	→	F					
3	E30			S	→	V M			
4	E30			S	→	V M F			
5	E30						V S	→	M
6	E30	V M F							
7	E30	S C							
8	E33	V S M F	→	C					
9	E33	V S M	→	F					
10	E33			S	→	V			
11	E33						V S	→	M F C
12	E33	V S M F							
13	E33	V S F							
14	E33			S M					
15	E25					V S			
	E30					V S			
16	E33								V M
	CA 10	V S							

V, study child; S, sibling; M, mother, F, father; C, contact.

are chiefly responsible for introducing virus into the family (Table 4). Fifty-nine per cent (32/54) of intrafamilial contacts excreted virus when the sibling was excreting virus, whereas only 9% (2/23) excreted virus when the sibling was not excreting virus ($\chi^2_{DF=1}=14{\cdot}74$, $P<0{\cdot}001$ with Yates' correction; odds ratio 15·27, 95% confidence interval 3·25–71·86). Echoviruses were isolated from 62% (36/58) of people in contact with siblings excreting echovirus: 72% (13/18), 61% (11/18), 47% (7/15), 33% (1/3), and 100% (4/4) of study children, mothers, fathers, relatives, and contacts of siblings respectively excreted virus when the sibling excreted virus. Coxsackie A virus seemed to be self-limiting, among 15 people who came into contact with siblings shedding virus, only one infant (7%) excreted virus.

Factors associated with non-polio enterovirus infection

Factors associated with non-polio enterovirus isolation are shown in Tables 5 and 6. Factors associated with intrafamilial spread of non-polio enterovirus are shown in Table 7. Overall, crowding, water storage, cover on stored water, years of maternal and paternal education, type of water supply (well/water distribution service/piped water), and location of toilet (inside/outside/none) showed no influence on enterovirus isolation. However, for people living in houses, households with an inside toilet showed a significantly higher degree of intrafamilial spread compared to those with outside toilets (Table 7).

DISCUSSION

Limitation

Generalization should be made carefully for the following reasons: our results reflect non-polio enterovirus isolation under routine immunization of OPV, and interference may have occurred between polio and non-polio enterovirus, serological analysis on each serotype was not conducted, therefore, prior infection was not considered, enterovirus prevalence varies by year and by season, therefore the period of 5 weeks only provides a snapshot of the situation in Mongolia, a larger study population is required to assure generalizability, and a lack of throat swabs may have decreased the isolation rate as well as the detection of certain serotypes.

Impact of molecular typing

For non-poliovirus isolates, only echovirus 30 was identified through neutralization. The use of

Table 5. *Associations between demographic characteristics and non-polio enterovirus isolation, Ulaanbaatar and Tov province, Mongolia, late summer 2003*

Variables	No.	OR	95% CI
Age group (years)			
0–1	30	9·1	1·0–83·8
2–10	23	19·8	2·0–195·9
11–20	12	14·0	1·3–156·3
21–30	37	4·6	0·5–38·3
31–40	12	5·0	0·5–54·4
41+	8	1·0	
Family status			
Study child	30	9·1	1·0–83·8
Sibling	26	15·7	1·7–149·8
Mother	29	5·7	0·6–52·2
Father	22	4·0	0·4–38·6
Relative	8	1·0	
Area			
Ulaanbaatar slum area	49	7·9	2·9–21·6
Ulaanbaatar apartment area	32	1·6	0·6–4·3
Tov province	37	1·0	
Sex (age $\leqslant$10 years)			
Male	28	2·8	0·9–8·8
Female	25	1·0	
Sex (age $\geqslant$10 years)			
Male	24	0·6	0·2–1·8
Female	45	1·0	

OR, Odds ratio (calculated by univariate logistic regression); CI, confidence interval.

molecular typing in addition to neutralization allowed the identification of the serotypes of all samples including the coxsackie A group which is generally difficult to identify through neutralization. The effectiveness of the use of molecular typing to identify non-polio enteroviruses was confirmed in our study.

Isolation rate of non-polio enterovirus

Although there is difficulty in comparing data due to differences in specimens sampled, specimen sampling frequency, selection of subjects, and virus isolation and identification methods, the observed overall isolation rate of 51 and 64% among children under 10 years of age is by far the highest isolation rate reported. Previous studies conducted among young children in the United States [8] and Hungary [12] reported an isolation rate of 21 and 26% respectively, and an isolation rate of 61% was reported among children under 10 months of age in Ghana [13].

Enterovirus infections peak in the warm months and are least frequent in the cold months [23, 24]. The warm season in Mongolia is very short, and after the severe winter season, enterovirus infections may become extremely prevalent in the summer. Additionally, the sharp increase of tourists during the summer season, as well as the holding of Naadam, a national sports festival to which Mongolians gather from all over the country, in the beginning of July in Ulaanbaatar, may have created an environment which facilitated virus introduction as well as person-to-person transmission, therefore influencing the incidence of infection.

Age, sex and isolation rate of non-polio enterovirus

In our study, the 21–40 years age group showed a high isolation rate of 39%. Data on the isolation rate of non-polio enteroviruses among healthy adults is limited, and to our knowledge, this is the highest isolation rate reported among adults. Our study demonstrates that infection usually occurs in childhood, but asymptomatic infection can be remarkably high among adults, depending on previous infection history with a given serotype in a given locale.

Overall, more males were shedding non-polio enteroviruses than females. This supports the findings that enteroviral diseases occur more frequently in males [8, 25, 26] and indicates that the higher morbidity among males is due to a higher infection rate among males. However, although the sample size in the age groups was small, there appeared to be higher infection rate among females above age 11 years. This is consistent with a finding that there was predominance of female patients due to enteroviral disease in a group over the age of 10 years [8]. It has been suggested that biological reasons are responsible for the higher infection rate among males such as longer duration of virus excretion and higher virus titre in stools of males [27]. However, our finding of mother > father > relative infected within the same household suggests that higher infection rate among adult females may reflect higher contact frequency and intimacy with infected children.

OPV interference by non-polio enterovirus

A review of immunity among children in developing countries has indicated low rates of seroconversion

Table 6. *Associations between hygiene behaviours and non-polio enterovirus isolation, Ulaanbaatar and Tov province, Mongolia, late summer 2003*

Variables	No.	OR	95% CI
Hand washing after defecation			
Always	55	1·0	
Not always	37	2·5	1·1–5·8
Hand washing before eating			
Always	46	1·0	
Not always	46	1·3	0·6–3·0
Hand washing before cooking			
Always	57	1·0	
Not always	13	0·8	0·2–2·8
Bathing frequency			
More than 2 times a week	41	1·0	
Weekly or less frequent	51	0·6	0·3–1·4
How bottom is cleaned			
Toilet paper	83	1·0	
Other (cotton, newspaper)	6	0·5	0·1–2·7
How many times hands washed*		Mean (S.D.)	*P* value
Negative	47	4·1 (0·3)	$P=0·394$
Positive	45	3·7 (0·3)	

OR, Odds ratio (calculated by univariate logistic regression); CI, confidence interval; S.D., standard deviation.
* *t* test. Question was asked for subjects to recall how many times they washed their hands the day before the questionnaire survey was conducted.

to poliovirus types 1 and 3 after administration of three doses of OPV [28]. Factors such as sub-optimal vaccine potency, breaks in the cold chain, sub-optimal vaccine schedule, and concurrent enteric infections have been postulated as reasons for the poor seroconversion rate. The effect of concurrent enteroviral infections in reducing seroconversion rate has been repeatedly suggested [29–31], and low rates of seroconversion have been reported more frequently during summer than in winter [32, 33].

Our study showed that there was high transmission by multiple types of non-polio enteroviruses in the summer season in Mongolia. Mongolia has reduced its OPV schedule to birth, 2, 3, and 4 months of age from 2003. To avoid interference by non-polio enteroviruses during OPV immunization, attention must be paid to children who will be receiving most of their OPV in the summer season. We discovered infants concurrently yielding multiple non-polio enteroviruses, and the efficacy of OPV administered to such infants is doubtful. A study of immunity to poliovirus in children receiving OPV in the summer season may be required. This is considered important to maintain high herd immunity and to avoid the risk of vaccine-derived poliovirus infection.

Detection of enterovirus on hands

The finding that persons who do not always wash their hands after defecation are 2·5 times more likely to be infected compared to those who answered that they always washed their hands highlights the importance of hand contact in the transmission pathway. This was consistent with studies of echovirus 30 which showed that hand washing was protective against infection [34, 35]. However, despite the high isolation rate of non-polio enteroviruses from stools, no enterovirus was detected from any of the hand swabs. The lack of detection may have been associated with the hands being rubbed onto many other objects or body parts, therefore, the chance of isolating virus from hands at random times in a real-life setting was very small. The PCR method was adopted due to the limited amount of samples and due to its high sensitivity for virus detection; however, the detection limit of the method may have overlooked the presence of virus due to a small virus load.

Table 7. *Associations of living environment and demographic character with degree of intrafamilial spread of non-polio enterovirus in Ulaanbaatar and Tov province, Mongolia, late summer 2003*

Variables	No. of households	Mean (S.D.)†	95% CI	*P* value
Residence				
Ger	5	0·73 (0·25)	0·42–1·05	$P=0·097$*
House	14	0·55 (0·42)	0·31–0·80	
Apartment	10	0·30 (0·35)	0·05–0·55	
Kitchen				
Sole use	17	0·40 (0·42)	0·18–0·62	$P=0·095$*
Shared	3	0·92 (0·14)	0·56–1·28	
None	9	0·55 (0·30)	0·32–0·77	
Bathroom				
Sole use	10	0·38 (0·41)	0·08–0·67	$P=0·041$**
Shared	4	0·94 (0·13)	0·74–1·14	
None	15	0·46 (0·36)	0·26–0·66	
Waste				
Tube	10	0·68 (0·43)	0·37–0·98	$P=0·053$*
Special hollow outside	12	0·51 (0·37)	0·28–0·75	
Outside, no special place	7	0·21 (0·23)	0·01–0·42	
Toilet‡				
Inside	5	0·95 (0·11)	0·81–1·09	$P=0·003$***
Outside	9	0·33 (0·35)	0·06–0·61	
Income (Togrog)§				
1–50 000	7	0·71 (0·39)	0·35–1·08	$P=0·136$
50 001–100 000	17	0·48 (0·39)	0·28–0·68	
100 001–150 000	2	0·50 (0·35)	−2·68–3·68	
150 001–200 000	3	0·08 (0·14)	−0·28–0·44	

CI, confidence interval.

* $P<0·1$, ** $P<0·05$, *** $P<0·01$.

† ANOVA. Degree of intrafamilial spread was calculated by number of family member(s) infected divided by total number of family members in the household. S.D., standard deviation.

‡ Only households residing in house are included.

§ \$1·00 = 1140 Togrog as of July 2003.

Intrafamilial spread and transmissibility of non-polio enterovirus

Examination of the order of virus excretion among family members revealed that siblings or young children aged 2–10 years were chiefly responsible for introducing virus into the household. Our study provides direct evidence that young children are the most important transmitters of enteroviruses compared to studies in which statistics were used to associate number of young children and number of infected family members.

Transmissibility of echovirus was high, while coxsackie A virus infection was self-limiting. The low transmissibility of coxsackie A virus may be explained by multiple reasons: herd immunity may have been established due to previous infection, supported by an indication that coxsackievirus infections peaked in early summer whereas echovirus tended to be restricted to late summer [8]; transmissibility of coxsackie A virus may be lower than coxsackie B virus; the dry climate of Mongolia may have limited the transmissibility of coxsackie A virus; relatively low sensitivity of the used cell lines towards coxsackie A virus [1] may have limited its detection; and coxsackie A virus may spread mainly through the respiratory route rather than the faecal–oral route, therefore lack of throat swabs may have decreased

our detection rate. Additionally, it is indicated that coxsackie A24 variant spreads primarily by direct or indirect contact with eye secretions [36], thereby limiting its detection. There were limitations in comparing transmissibility of echovirus and coxsackie A virus, however, echoviruses showed considerably higher transmissibility than coxsackie A viruses when stool specimens were examined using the L20B, Hep-2, and RD-A cell lines.

Risk factors of non-polio enterovirus infection

We observed difference in the degree of intrafamilial spread by type of residence. *Ger* dwellers had the highest risk of intrafamilial infection. Although not statistically evident, low accessibility to water and high density and intimacy among family members due to small housing space [4·3 m^2 (1·10 m^2 per person)] appeared to be the major reasons for the high infection rate.

For those living in a house or apartment, multiple families sharing a kitchen and/or bathroom was a significant risk factor for promoting intrafamilial spread of non-polio enterovirus. Additionally, for those living in a house, a higher risk of intrafamilial infection was associated with an inside toilet rather than an outside toilet. A virological study of families whose infants were recently vaccinated with OPV showed that 15 and 10% of swab samples taken from bathroom/toilet and kitchen respectively were positive for poliovirus [37]. The absence of such facilities inside the residence may have decreased the chance of contracting virus, and on the other hand, having and sharing such facilities increased the introduction of virus into the household as well as the chance of infection. Cleaning of kitchens, bathrooms, toilets, and waste disposal areas as well as hand washing, especially after defecation, are probably the most important factors to prevent infection in the home environment.

Our findings contradict previous studies of pathogens which are transmitted by the faecal–oral route. In a study of *Helicobacter pylori*, infection among those who used outdoor toilets was significantly higher than those who used indoor toilets [38]. In a study of hepatitis A, the absence of a toilet [39] and a kitchen [40] were associated with a higher prevalence of anti-hepatitis A virus antibodies. The difference in study finding may reflect differences in hygiene practices and knowledge of hygiene among the different populations. Urban areas of Mongolia have gone through a transition of housing during the 70 years of Soviet dominance. Soviet-style apartments providing a toilet, bathroom, kitchen, and piped water were built, and the lifestyle changed drastically for those who moved into apartments from a *ger*, lacking such facilities. The reasons for contradiction with previous studies may be because Mongolians living in urban areas are going through a transition in lifestyle; however, the knowledge of hygiene and hygiene practices has not yet reached the standards required for the new lifestyle.

Access to improved sanitation is one of the millennium goals targeted by the United Nations. Our study findings show that the mere presence of hygienic facilities is not sufficient to decrease infection with enteric viruses. Hygiene education, stressing the importance of cleaning hygienic facilities, and hand washing after defecation is required. A comprehensive sanitation programme encompassing provision of sanitary facilities as well as education in hygiene is indispensable for improved health.

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Intrafamilial Transmission of a Sabin 1-Related Poliovirus in Shizuoka Prefecture, Japan

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On June 6, 2005, a 41-year-old male presented with fever (39.0°C) and malaise, and was admitted to a clinic in Shizuoka Prefecture, Japan. Immediately before the onset, the patient had taken care of his 9-month-old daughter, who had diarrhea and who had previously received her first dose of oral poliovirus vaccine (OPV) on May 30, 2005.

To address the possibility of vaccine-associated paralytic poliomyelitis (VAPP) due to intrafamilial poliovirus transmission, clinical samples were collected from the patient and his vaccinated daughter on June 10 for virus isolation and identification. Polioviruses were isolated from a throat swab of the patient and from a stool sample of the daughter in Vero cells (1). Both isolates were identified as a type 1 poliovirus by the microneutralization test (Table 1). Two different methods, which were based on genetic and antigenic approaches and which used PCR-restriction enzyme fragment length polymorphism and Sabin-specific monoclonal antibodies, respectively, were employed for intratypic differentiation (ITD) of polioviruses to distinguish between the vaccine and wild polioviruses (2,3). As shown in Table 1, the ITD assays identified both type 1 poliovirus isolates as Sabin 1-like polioviruses, which were commonly found in the stool samples from healthy OPV recipients and their close contacts (4,5).

During clinical follow-up, the patient and his daughter completely recovered without any sequelae, although intrafamilial transmission of the type 1 OPV strain was epidemiologically suspected. However, another possible VAPP case, that of a 36-year-old male who had a contact infection with type 3 OPV strain from his son, was recently reported in Ehime Prefecture, Japan (6). These incidents revealed the existence of susceptible populations and the risk for VAPP as long as OPV is still used in Japan (7), and provide a strong argument for the introduction of an inactivated poliovirus vaccine instead of OPV to reduce the risk of VAPP, concomitant with routine OPV immunization (8,9). In the meantime, highly qualified disease and laboratory surveillance activities for VAPP are also needed.

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Table 1. Virus isolation and characterization of polioviruses

Virus ID	Sample			Poliovirus isolate			
				Serotype	ITD		Final result
	Source	Sample	Date of sampling		PCR-RFLP[1)]	MoAb[2)]	
0517	Patient	Throat swab	June 10, 2005	Type 1	Sabin 1	Sabin-like	Sabin 1
0518	Daughter	Stool	June 10, 2005	Type 1	Sabin 1	Sabin-like	Sabin 1

[1)]: PCR-restriction enzyme fragment length polymorphism test as a genetic intratypic differentiation (ITD) assay.
[2)]: Neutralization test using Sabin-specific monoclonal antibodies as a antigenic ITD assay.

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Molecular Epidemiology of Echoviruses 11 and 13, Based on an Environmental Surveillance Conducted in Toyama Prefecture, 2002-2003†

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Nineteen echovirus 11 (E11) and 12 E13 isolates were isolated from three rivers in Toyama Prefecture, Japan, during an environmental surveillance conducted from April 2002 to March 2003. The nucleotide sequences of E13 isolates were closely related to those from patients with aseptic meningitis, with less than 1.3% divergence in the VP1 region of the viral capsid gene, and belonged to the same clade responsible for a worldwide outbreak that started in 2000. In contrast, E11 isolates were clustered into three genomic groups and were not closely related to echovirus strains isolated from patients. These results suggest that the combination of both virus isolation from environmental sources and phylogenetic analysis could be complementary assessment approaches to trace prevalent and minor circulating enteroviruses in the human population.

Environmental surveillance has been conducted in Toyama Prefecture, Japan, four times since 1979 in order to study enteric virus water pollution (18–20). Assessment of enteric viruses found in the environment also plays a role in understanding virus circulation in the community (26, 27). In particular, environmental surveillance for human poliovirus (genus *Enterovirus*) under an eradication program is reported to be the most sensitive method to detect wild or vaccine-derived polioviruses circulating in the human population (7, 32, 33).

Large outbreaks of aseptic meningitis caused by human echovirus 13 (E13) (genus *Enterovirus*) have been reported in various areas in the world since 2000 (3, 4, 22, 23, 30). In addition, a nationwide outbreak of aseptic meningitis caused by E13 occurred in Japan during the summer of 2002 (19) after a small outbreak in a limited area in 2001. According to an Infectious Agents Surveillance Report of Japan, E13 was the most common causative agent (67.2%) isolated from aseptic meningitis patients in 2002, followed by human echovirus 11 (E11) (genus *Enterovirus*) (12.3%) (10). However, E13 had not been isolated clinically during the previous 20 years in Japan (9), and its isolation was rare until 2000. Thus, the outbreaks of aseptic meningitis caused by E13 seem to be a case of reemerging enterovirus infection (21, 29).

Human enterovirus infection is known to be generally asymptomatic, and thus, environmental surveillance has been reported to be a sensitive method to detect silently circulating viruses (26, 27). We show here several enteroviruses isolated during an environmental surveillance conducted in Toyama, and we especially analyzed E13 and E11 phylogenetically and compared their genetic sequences to available clinical isolates.

Virus isolation from rivers. Water samples (800 ml each) were collected from fixed points of three rivers (Oyabe, Itachi, and Sembo) (Fig. 1) twice a month from April 2002 to March 2003, as described previously (18). On the day of collection, samples were concentrated using a negatively charged membrane filter (mixed cellulose ester membrane filter; Advantec Co. Ltd., Tokyo, Japan) as described previously (18). After the initial collection, water samples were centrifuged at 3,000 rpm for 30 min, $MgCl_2$ was added to the supernatant to a final concentration of 0.05 M, and the pH was adjusted to 3.5. The samples were then absorbed to the filter under positive pressure. Absorbents on the filter were next eluted with 3% beef extract solution by sonication for 5 min and then centrifuged at 12,000 rpm for 30 min. The supernatants were collected and stored at 4°C until virus isolation.

A total of 0.2 ml of the supernatant was inoculated onto Vero, MA104, RD-18S, and Hep2 cell lines. Eight tubes for each cell line were used for virus isolation (18). Isolates were identified by specific antisera against each enterovirus (Denka Co. Ltd., Tokyo, Japan). Reoviruses (mammalian orthoreovirus) were characterized using neutralization and hemagglutination inhibition tests with type-specific antisera (19).

A total of 171 viruses were isolated at fixed points of three rivers in Toyama twice a month from April 2002 to March 2003 (Table 1). Reoviruses were the most common virus isolates, followed by E11 and E13. Three isolates could not be typed. Cell lines in which viruses were isolated are shown in Table S2 of the supplemental material.

There were two periods when viruses were frequently isolated: one was from July to September 2002, and the other was from December 2002 to February 2003 (Fig. 2). Twelve E13

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† Supplemental material for this article may be found at http://aem.asm.org/.

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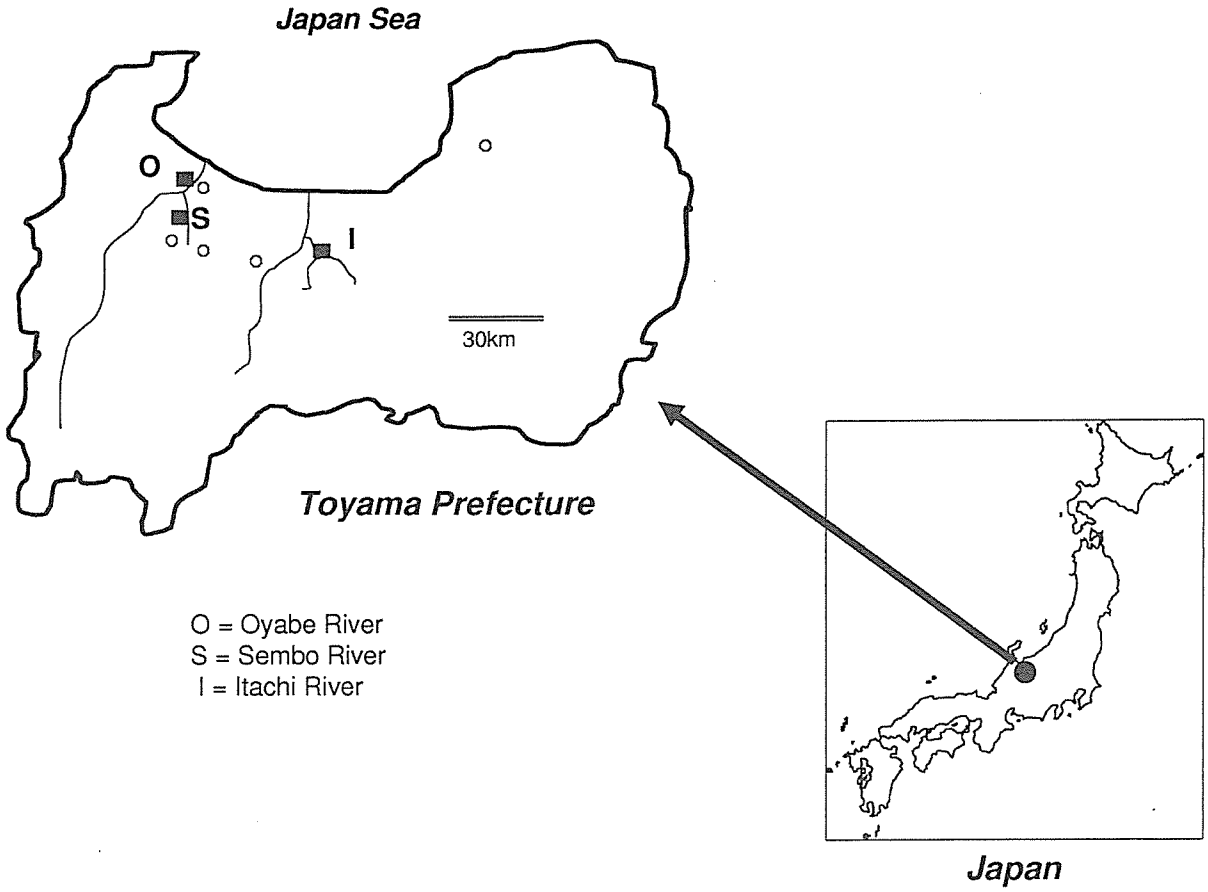

FIG. 1. Locations of the three rivers (Itachi [I], Oyabe [O], and Sembo [S]) in Toyama Prefecture. Squares (■) indicate the fixed points for water sampling. Circles (○) indicate the locations of hospitals where aseptic meningitis patients were admitted. Double lines indicate distance.

strains were isolated from May to December 2002. Nineteen E11 strains were isolated from September 2002 to January 2003. Most reoviruses were isolated in the latter periods, although they were isolated throughout the season.

Type 2 poliovirus was isolated in November 2002 after a routine immunization scheduled during the previous month. Differentiation of poliovirus isolates was performed by PCR-restriction fragment length polymorphism and sequencing methods as described previously (34), and isolates were characterized as vaccine type (data not shown).

TABLE 1. Virus isolation from three rivers in the Toyama Prefecture, Japan, between April 2002 and March 2003

Virus[a]	No. of virus isolates			
	Itachi River	Sembo River	Oyabe River	Total
E7		3		3
E11	4	14	1	19
E13	1	8	3	12
CB2	1	1		2
CB3		4	1	5
CB4		2		2
Poliovirus type 2		1[b]		1
Reovirus type 1	3		1	4
Reovirus type 2	33	48	38	119
Reovirus type 3	1			1
Not typed				3
Total	43	84	44	171

[a] E, echovirus; CB, coxsackievirus type B.
[b] A result of intratypic differentiation was Sabin 2.

Virus isolation from patients with aseptic meningitis. Clinical specimens (stool, cerebral spinal fluid, and throat swab) from seven aseptic meningitis patients diagnosed in Toyama in 2002 were used for virus isolation as described previously (13, 31). Five E13 viruses were also isolated from one stool specimen, two cerebral spinal fluid specimens, and two throat swabs from seven patients with aseptic meningitis in June and July 2002. Eight E11 isolates from aseptic meningitis patients collected between 1993 and 1998 in Hyogo Prefecture, Japan, were also used for analysis.

RT-PCR and nucleotide sequence analysis. E13 and E11 isolates were used for sequencing analysis. The viral RNA was extracted from virus fluid using a QIAamp Viral RNA Mini kit (QIAGEN, MD) and was then used for reverse transcription-PCR (RT-PCR) (Access RT-PCR system; Promega, WI). For amplification of the partial VP1 region of the viral capsid protein, two sets of panenterovirus degenerate primers described previously by Oberste et al. were used (24). Briefly, for amplification of the region upstream of VP1, sense primer 187

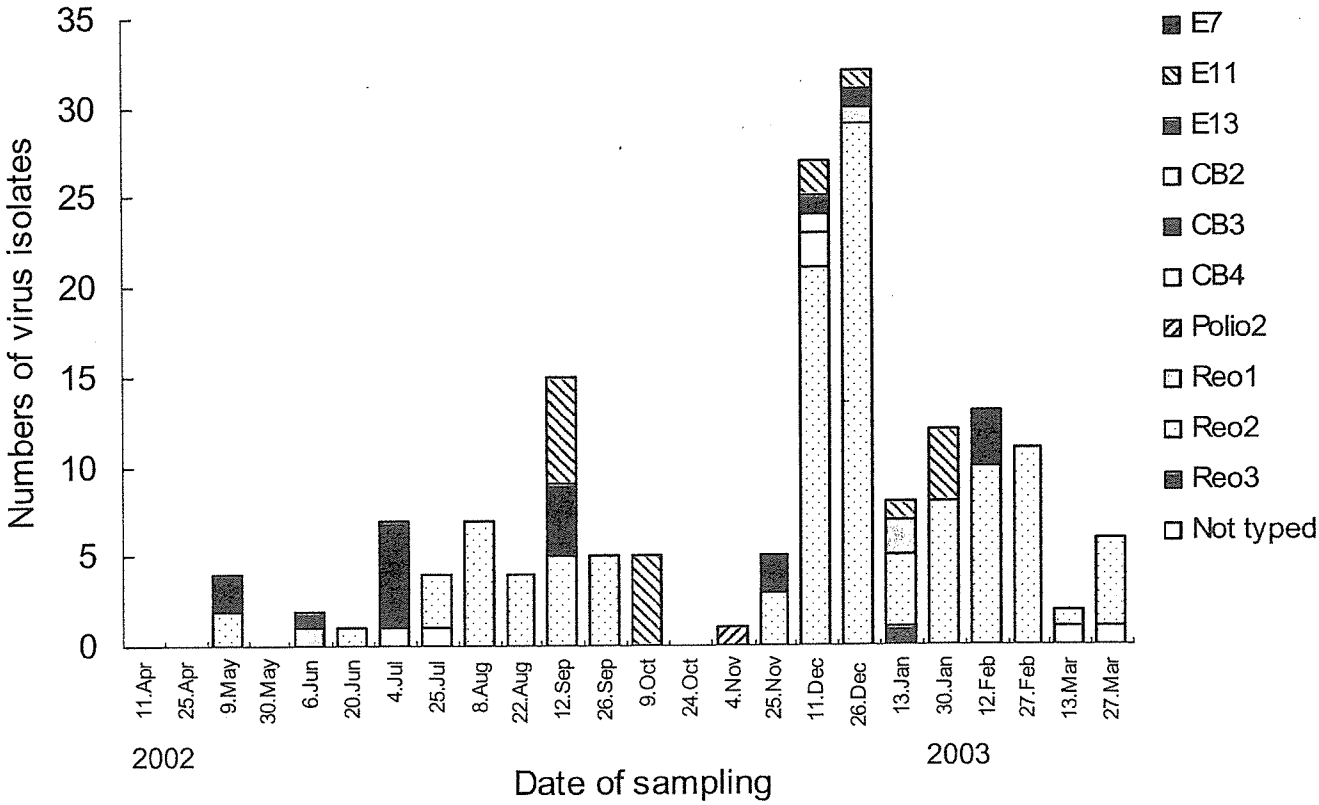

FIG. 2. Total numbers of virus isolates from three rivers from April 2002 (Apr) to March 2003 (Mar). Waters from fixed points of three rivers were collected on the indicated dates. CB2, coxsackievirus type B2; Polio2, poliovirus type 2; Reo1, reovirus type 1.

and antisense primer 222 were used, and for amplification of the downstream region, sense primer 012 and antisense primer 011 were used (24). RT-PCR was carried out under the following conditions: reverse transcription at 48°C for 45 min, inactivation at 94°C for 2 min, and 35 cycles of annealing at 50°C for 10 s, polymerization at 65°C for 1 min, and denaturation at 94°C for 10 s. After 35 cycles, an additional elongation step of 65°C for 1 min was done. The PCR product was purified by using a QIAquick PCR purification kit (QIAGEN) and directly sequenced using a PRISM Big-Dye Terminator cycle sequencing reaction kit on an automated DNA sequencer (Perkin-Elmer Applied Biosystems) as described previously (31).

Genetic relationships among E13 isolates or E11 isolates were analyzed by MEGA 3.1 software (16) using the partial VP1 region of E13 (703 bp; positions 2579 to 3281 on strain Del Carmen) and of E11 (561 bp; positions 2765 to 3325 on strain Gregory). Phylogenetic trees were constructed by neighbor-joining methods after estimation of genetic distance using the Kimura two-parameter method (14). The transition/transversion rate was set at 2.0, and a bootstrapping test was performed 1,000 times (8).

Sixty-one- and 51-nucleotide sequences of E13 and E11, respectively, were available in GenBank. The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank (1, 2, 5, 12, 15, 25).

The nucleotide sequences of E11 and E13 isolates were phylogenetically compared. For E13, the nucleotide divergence was less than 1.3% among isolates from the three rivers in the partial VP1 region (703 bp). The nucleotide sequence divergence was 0.9 to 1.3% between 12 environmental isolates and 5 clinical isolates, and at most, one amino acid substitution was found. Therefore, environmental isolates were closely related to clinical isolates in Toyama Prefecture (Fig. 3a). Compared to 19 other isolates from Japan during 2001 to 2002 found in GenBank, the divergence was 1.2 to 1.6%, with, at most, one amino acid substitution. Phylogenetic analysis showed that all E13 isolates in Japan belonged to the same cluster (Fig. 3a).

Moreover, isolates in Toyama from both environmental sources and patients were compared with the other E13 sequences available from GenBank, including the above-mentioned 19 strains from Japan. Phylogenetic analysis showed that the Toyama isolates belonged to the major genomic group labeled as the 2000–2002 cluster in Fig. 3b, which was described previously by other studies (1, 2, 5, 12, 15). Divergence of nucleotide sequences between Toyama isolates and others in this group was 1.9 to 2.5% (amino acid divergence, 0.3 to 0.7%), indicating that these Toyama isolates belonged to the major genomic group circulating worldwide.

On the other hand, nucleotide sequences of E11 were phylogenetically analyzed and antigenically categorized into two major strains: strain Gregory as the prototype and strain Silva as prime type (Fig. 4). There were several kinds of subgenotypes within these two major strains (6, 25). Although E11 was not isolated from patients in Toyama during the period of this

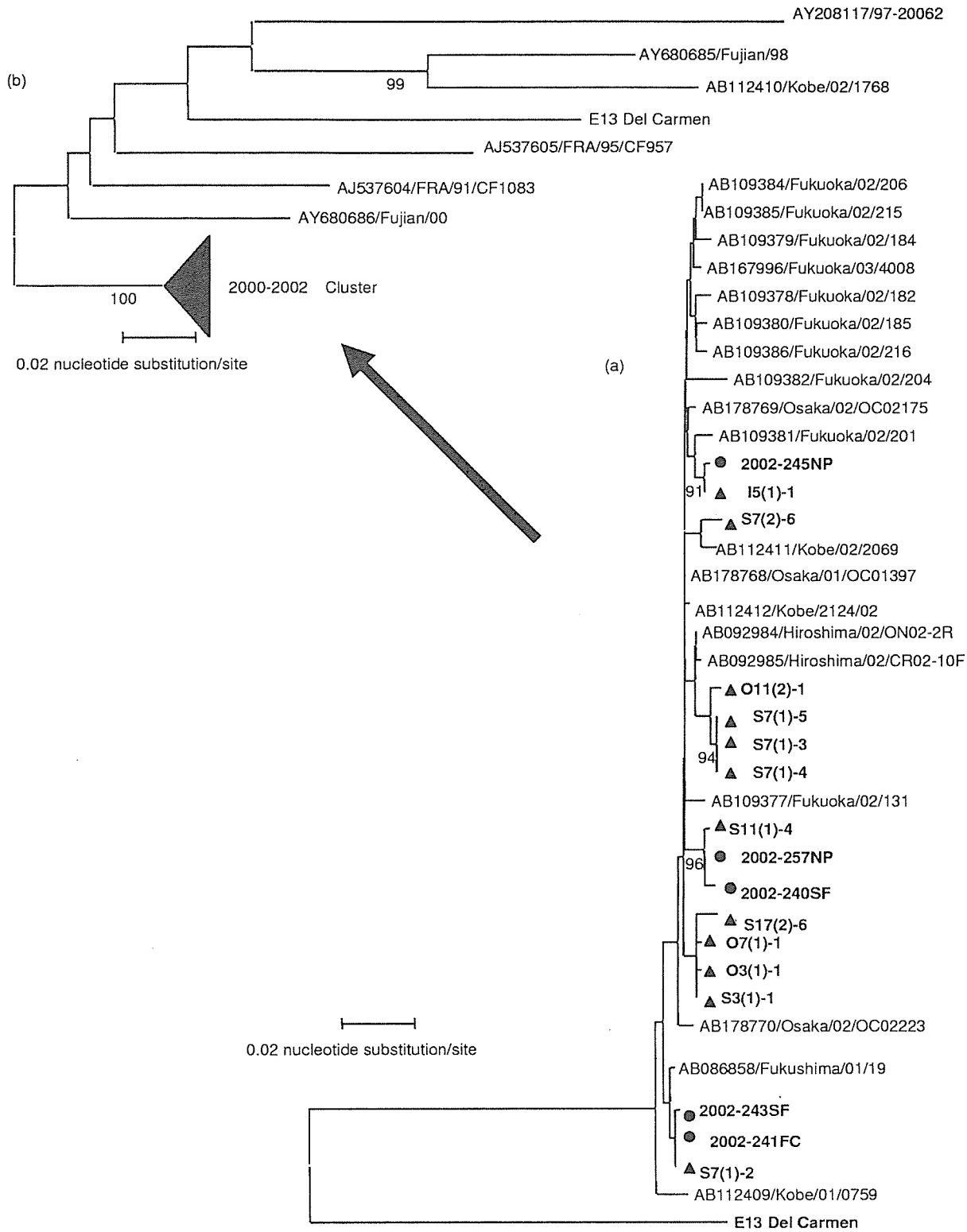

FIG. 3. (a) Phylogenetic relationships between the Japanese isolates. Phylogenetic trees for E13 using the partial VP1 region (703 bp) were generated by the neighbor-joining method with 36 strains: 17 Toyama isolates (12 from river samples and 5 from patients) and 19 other Japanese isolates (GenBank accession no. AB086858, AB092984, AB092985, AB109377 to AB109382, AB109384 to AB109386, AB112409, AB112411, AB112412, AB167996, and AB178768 to AB178770). Bootstrap values (in percentages) for 1,000 replicated trees are indicated. Circles and triangles specify patient isolates and environmental isolates, respectively. S, O, and I indicate Sembo, Oyabe, and Itachi, respectively. (b) Phylogenetic trees were reconstructed using 61 strains (accession no. AB112410, AJ537604 to AJ537609, AY227334 to AY227347, AY268561, AY268563 to AY268569, AY268571 to AY268580, AY680685, AY680686, and AJ241427 from GenBank and including the 19 other Japanese isolates). Bootstrap values (in percentages) for 1,000 replicated trees are indicated. The major genomic group isolated during 2000 to 2002 was compressed within the same cluster (2000–2002 cluster). Arrows from tree a to tree b indicate that Japanese isolates in tree a are included in the compressed cluster. The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank.

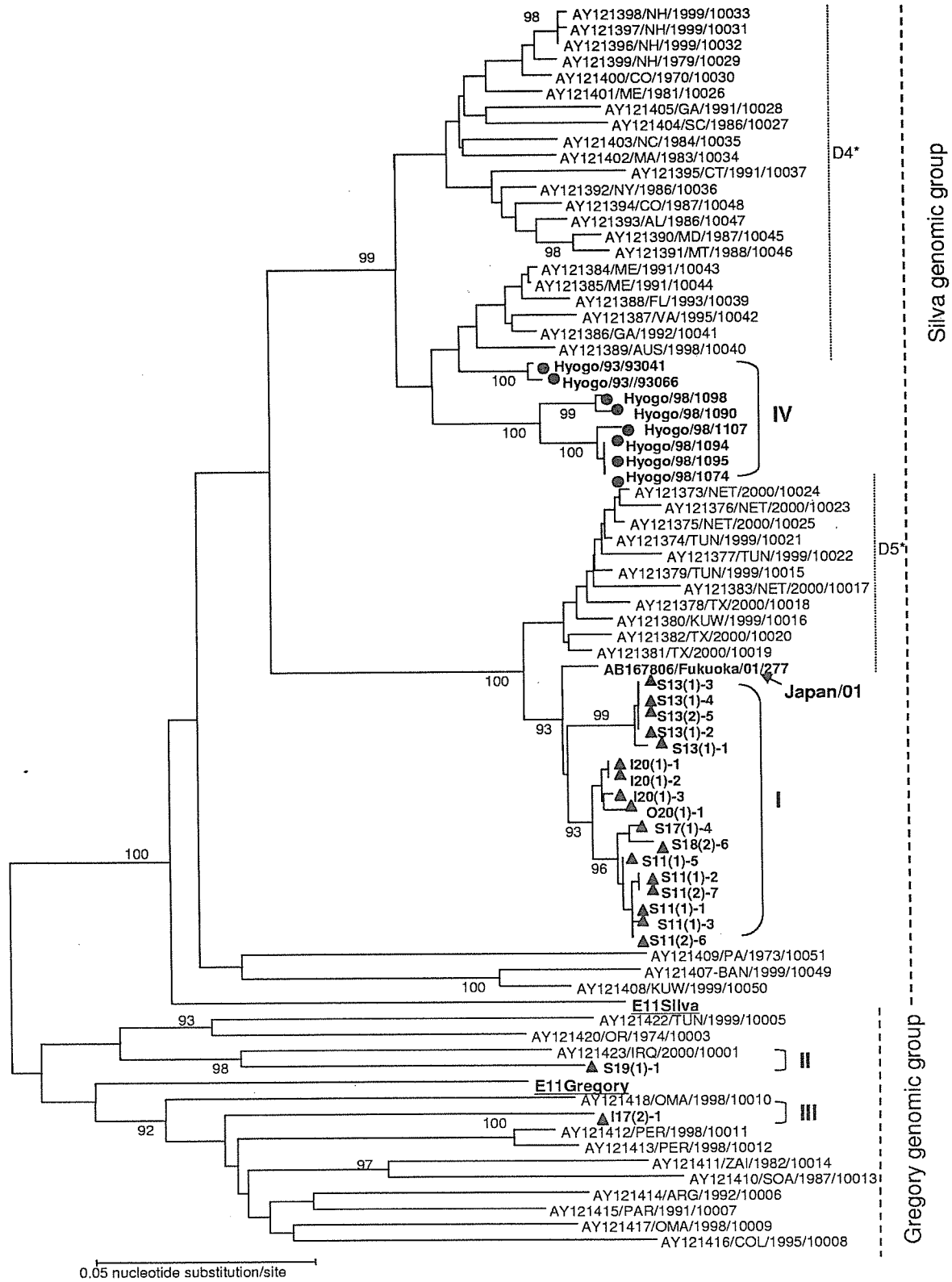

FIG. 4. Phylogenetic tree for E11 using the partial VP1 region (561 bp) generated by the neighbor-joining method with 78 strains: 19 Toyama River isolates, 8 patient isolates from Hyogo, and 51 other isolates (accession no. AY121373 to AY121405, AY121407 to AY121418, AY121420, AY121422, AY121423, AB167806, AF295498, and AF081326 from GenBank). Circles and triangles indicate viruses isolated from patients and the environment, respectively. Bootstrap values (in percentages) for 1,000 replicated trees are indicated. Subgenomic groups I to IV in this study are shown, with D4 and D5 clusters being named as suggested previously by Oberste et al. (25). The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank. Isolate identifiers consist of a three-letter country abbreviation (ARG, Argentina; AUS, Australia; BAN, Bangladesh; COL, Colombia; DOR, Dominican Republic; ZAI, Democratic Republic of Congo; IRQ, Iraq; KUW, Kuwait; NET, The Netherlands; OMA, Oman; PAR, Paraguay; PER, Peru; SOA, South Africa; TUN, Tunisia; Tur, Turkey; or USA, United States), with two-letter abbreviations for U.S. states. The viral isolate from Fukuoka City (accession no. AB167806) is indicated by arrows (Japan/01).

study, 19 E11 samples were isolated from the three rivers. Eight clinical isolates from Hyogo Prefecture between 1993 and 1998 were also used in the analysis together with the 51 E11 sequences available in GenBank.

Environmental isolates were divided into three genomic groups, groups I, II, and III (Fig. 4). Seventeen isolates fell into major genomic group I, with 1.5 to 2.1% nucleotide divergence within the partial VP1 region (561 bp). Oberste et al. previously described that the genomic group of Silva consisted of five subgroups, subgroups D1 to D5 (25). Toyama isolates had a mean of 18.3% divergence in nucleotides compared to the E11 Silva strain and only 3.9 to 5.1% divergence compared to the 1999–2000 D5 subgroup. Therefore, we classified Toyama isolates as belonging to subgroup D5. Moreover, nucleotide sequences of these isolates were very similar to aseptic meningitis isolates from Fukoaka City in 2001 (nucleotide divergence, 1.8 to 2.3%). Clinical isolates from Hyogo in 1993 and 1998 (group IV) were categorized as belonging to subgenomic group D4 from 1970 to 2001 (Fig. 4). I17(2)-1 (group III) and S19(1)-1 (group II) isolates were classified as belonging the Gregory genomic group and had nucleotide divergences from the genomic group of 21.2 to 24.0% and 18.9 to 21.5%, respectively.

Concluding remarks. There was a large outbreak of aseptic meningitis caused by E13 in the summer of 2002 in Japan, which coincided with a small outbreak caused by E11 (9). The aim of this study was to assess the performance of an environmental surveillance of river water isolates compared with isolates from clinical samples. These viruses were also isolated from the rivers in Toyama Prefecture by environmental surveillance. E13 was detected not only in Toyama Prefecture but also in other places in Japan simultaneously. The phylogenetic analysis of E13 showed that the isolates from both river waters and patients belonged to the same genomic cluster, one of the major genotypes circulating worldwide since 2000.

E11 was recently detected during the autumn/winter from river water in Toyama and was compared to isolates from elsewhere in Japan. Although there was no outbreak of aseptic meningitis in Toyama, E11 might have been silently circulating in the human population. Phylogenetic analysis showed that the isolates were divided into three genomic groups: isolates in group I belonging to the Silva genomic group, which seemed to be circulating mainly in Toyama, and minor E11 isolates of I17(2)-1 or S19(1)-1 belonging to the Gregory genomic group, which also seemed to be circulating in other areas of Japan. Thus, this environmental survey was capable of detecting enteroviruses of different genomic groups with high sensitivity and was capable of tracing minor circulating viruses.

In addition, many reoviruses were isolated during the surveillance, most frequently between December 2002 and February 2003. Matsuura et al. suggested in a previous study (19) that stool from not only humans but also animals might have contaminated the waters.

Since there were only a few reports of E11 and E13 isolation in Japan between 2003 and 2005 (11) according to the Infectious Agents Surveillance Report, these viruses appeared to temporarily cease circulation or to circulate silently. This report shows that the combination of conventional virus isolation from environmental sources together with phylogenetic analysis of clinical isolates is a useful approach in understanding enterovirus circulation and transmission. Therefore, environmental surveillance should be considered a complementary assessment tool to trace prevalent and minor enteroviruses circulating in the human population.

Nucleotide sequence accession numbers. The sequence data in this study were deposited in GenBank under accession no. AB239081 to AB239124.

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