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Frequent Importation of Enterovirus 71 from Surrounding Countries into the Local Community of Yamagata, Japan, between 1998 and 2003

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Phylogenetic analysis of 45 enterovirus 71 (EV71) isolates for 6 years in Yamagata, Japan, clarified that the annual outbreak of hand-foot-and-mouth disease was due to four genetically distinct subgenogroups, including a novel "B5." Our results suggest that the importation of EV71 from surrounding countries has had a major epidemiological impact on the local community used in our study.

Enterovirus 71 (EV71) is a member of the family *Picornaviridae*, and belongs to the genus *Enterovirus* (human enterovirus A) (14). EV71 is known to be a causative agent of hand-foot-and-mouth disease (HFMD), herpangina, aseptic meningitis, paralysis, and meningoencephalitis. The disease is common in young children, especially in the summer and early fall in temperate climates, as is typically seen with enterovirus-related diseases (14).

Because fatal cases have been reported in Asian countries, such as Malaysia (1997), Taiwan (1998), Singapore (2000), and Japan (2000), careful monitoring of the incidence of HFMD and a plan for dealing with severe outbreaks of HFMD are considered to be of great importance (3, 5, 16, 18, 20). To realize this, understanding the epidemiology and pathogenesis of EV71 is primary. However, there have been few longitudinal epidemiological analyses of EV71 in a local community except the report of T. Munemura et al., who reported that two distinct genogroups of EV71 had been circulating in Yokohama, Japan, between 1973 and 2000 (11). Furthermore, there is no uniform analysis protocol for the investigation of the genetic characteristics and the molecular epidemiology of EV71. M. J. Cardosa et al. noted that since recent studies of EV71 phylogeny by various groups had analyzed only 207 to 891 bp of different regions of the 5′ untranslated region, VP1, VP4, and VP2, a comparison of phylogenetic relationships was difficult (2). In reality, it was also impossible to determine the homology of EV71 sequences reported in Japan for the same reason (3, 11, 16). Therefore, it is now recommended that a molecular epidemiologic study of the complete sequence of the VP1 region, which is considered to play an important role in characterizing antigenicity and to be the most suitable region for the sequence analysis of genetic diversity, be performed (1, 2, 13, 17). We report here the molecular epidemiology of EV71 strains isolated from children in the local community of Yamagata, Japan, between 1998 and 2003, using sequence analysis of the VP1 region and genotyping.

We have a routine surveillance system for viral diseases in the Yamagata Prefecture. Between 1998 and 2003, a total of 9,805 throat or nasal swab specimens were taken from patients who visited pediatric clinics that are working in collaboration with the Yamagata Prefectural Institute of Public Health. Specimens were transported in a cooler box to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, for virus isolation. Virus isolation was carried out by means of a microplate method (12). We used HEF, HEp-2, Vero, and MDCK cell lines throughout the study period and added RD-18S and GMK cells in June 2001 (8). HEF cells, which were originally established by I. Ishikawa, were purchased from Riken Cell Bank, Tsukuba, Japan. Specimens were centrifuged at 3,000 rpm for 15 min, and 75 μl of the supernatant was inoculated onto cells of each cell line. Microplates were centrifuged for 20 min at 2,000 rpm and then incubated at 33°C in a 5% CO_2 incubator for 10 days. Among enteroviruses, only EV71 and coxsackievirus A16 (CoxA16) caused cell detachment and were isolated from both the HEF and GMK cell lines with this system as previously described (8). Then, these isolates were presumptively assumed to be EV71 or CoxA16 based on cell sensitivity and were finally typed by a microneutralization test. Specific antisera against EV71 (BrCr-CA-70 strain) and CoxA16 were provided from the National Institute of Infectious Diseases, Japan or the Virus Research Center, National Hospital Organization, Sendai Medical Center, Sendai, Japan.

To compare representative EV71 isolates and reference strains, we carried out sequence analysis on 45 isolates to determine the complete VP1 region (891 nucleotides) (Table 1). RNA extraction, reverse transcription-PCR, and sequence analysis were carried out as described previously (6, 9). Briefly, viral RNA was extracted from 200 μl of infected cell culture supernatant using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The viral RNA was then transcribed into cDNA with Maloney murine leukemia virus reverse transcriptase (Nippon Gene) and a random primer (Takara Bio Inc., Otsu, Japan). By using

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TABLE 1. Strains used in a molecular analysis of EV71

Strain	GenBank accession no.	Subgenogroup	Place	Yr	Clinical diagnosis or outcome[a]	Reference(s) or source
BrCr-CA-70	U22521	A	United States	1970	Encephalitis	1, 4, 6
2604-AUS-74	AF135883	B1	Australia	1974	Meningitis	1
2232-NY-77	AF135871	B1	United States	1977	NA	1
7633-PA-87	AF009534	B2	United States	1987	Gastroenteritis	1
7673-USA-87	AF009535	B2	United States	1987	NA	4
2222-USA-88	AF009540	B2	United States	1988	Fever	4
MY16/I/SAR/97	AF376073	B3	Malaysia	1997	HFMD	6
4350/SIN/98	AF376119	B3	Singapore	1998	HFMD	6
0899-MAA-97	AY207642	B3	Malaysia	1997	Encephalitis	4
5536/SIN/00	AF376122	B4	Singapore	2000	HFMD	6
S21082/SAR/00	AF376084	B4	Malaysia	2000	HFMD	6
2246-NY-87	AF009542	C1	United States	1987	Paralysis	1
S11051/SAR/98	AF376081	C1	Malaysia	1998	HFMD	6
1M-AUS-12-00	AF376098	C1	Australia	2000	HFMD	4
2M-AUS-3-99	AF376103	C2	Australia	1999	Myelitis	4
03750-MAA-97	AY207615	C2	Malaysia	1997	HFMD	4
001-KOR-00	AY125966	C3	Korea	2000	NA	4
013-KOR-00	AY125976	C3	Korea	2000	NA	4
SHZH-CHN-98	AF302996	C4	China	1998	NA	15
F2-CHN-00	AB115491	C4	China	2000	HFMD	15
H25-CHN-00	AB115492	C4	China	2000	HFMD	15
H26-CHN-00	AB115493	C4	China	2000	HFMD	15
671-Yamagata-98	AB213614	C2	Japan	1998	HFMD	This study
705-Yamagata-98	AB213615	C2	Japan	1998	HFMD	This study
932-Yamagata-99	AB213616	C2	Japan	1999	HFMD	This study
957-Yamagata-99	AB213617	C2	Japan	1999	HFMD	This study
983-Yamagata-99	AB213618	C2	Japan	1999	HFMD, encephalitis	This study
1002-Yamagata-99	AB213619	C2	Japan	1999	HFMD	This study
738-Yamagata-00	AB213620	B4	Japan	2000	HFMD	This study
739-Yamagata-00	AB213621	B4	Japan	2000	HFMD	This study
786-Yamagata-00	AB213622	B4	Japan	2000	HFMD	This study
934-Yamagata-00	AB177809	B4	Japan	2000	HFMD	This study
962-Yamagata-00	AB213623	B4	Japan	2000	HFMD	This study
980-Yamagata-00	AB213624	B4	Japan	2000	HFMD	This study
1067-Yamagata-00	AB213625	B4	Japan	2000	HFMD	This study
1084-Yamagata-00	AB213626	B4	Japan	2000	HFMD	This study
1116-Yamagata-00	AB177810	C2	Japan	2000	HFMD	This study
1141-Yamagata-00	AB177811	B4	Japan	2000	HFMD	This study
1585-Yamagata-01	AB177812	C2	Japan	2001	HFMD	This study
1695-Yamagata-01	AB213627	C2	Japan	2001	Herpangina	This study
1814-Yamagata-01	AB213628	C2	Japan	2001	HFMD	This study
2779-Yamagata-02	AB213629	C4	Japan	2002	Viral exanthem	This study
75-Yamagata-03	AB177813	C4	Japan	2003	HFMD	This study
100-Yamagata-03	AB177814	C4	Japan	2003	HFMD	This study
452-Yamagata-03	AB213630	C4	Japan	2003	HFMD	This study
638-Yamagata-03	AB213631	C4	Japan	2003	HFMD	This study
828-Yamagata-03	AB213632	C4	Japan	2003	Influenza-like illness	This study
944-Yamagata-03	AB213633	C4	Japan	2003	HFMD	This study
946-Yamagata-03	AB213634	C4	Japan	2003	HFMD	This study
1144-Yamagata-03	AB213635	C4	Japan	2003	HFMD	This study
1268-Yamagata-03	AB213636	C4	Japan	2003	HFMD	This study
1469-Yamagata-03	AB213637	C4	Japan	2003	HFMD	This study
1530-Yamagata-03	AB213638	C4	Japan	2003	HFMD	This study
1716-Yamagata-03	AB213639	C4	Japan	2003	HFMD	This study
1725-Yamagata-03	AB213640	C4	Japan	2003	HFMD	This study
1799-Yamagata-03	AB213641	C4	Japan	2003	HFMD	This study
1897-Yamagata-03	AB213642	C4	Japan	2003	HFMD	This study
2091-Yamagata-03	AB213643	C4	Japan	2003	HFMD	This study
2316-Yamagata-03	AB213644	C4	Japan	2003	HFMD	This study
2321-Yamagata-03	AB213645	C4	Japan	2003	HFMD	This study
2399-Yamagata-03	AB213646	C4	Japan	2003	HFMD	This study
2419-Yamagata-03	AB213647	B5[b]	Japan	2003	HFMD	This study
2542-Yamagata-03	AB177815	B5	Japan	2003	HFMD	This study
2716-Yamagata-03	AB177816	B5	Japan	2003	HFMD	This study
2933-Yamagata-03	AB213648	B5	Japan	2003	HFMD	This study
2934-Yamagata-03	AB213649	B5	Japan	2003	HFMD	This study
2972-Yamagata-03	AB213650	B5	Japan	2003	HFMD	This study

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TABLE 1—*Continued*

Strain	GenBank accession no.	Subgenogroup	Place	Yr	Clinical diagnosis or outcome[a]	Reference(s) or source
2916-TAI-98	AB286526	C2	Taiwan	1998	NA	GenBank
3254-TAI-98	AF286531	C4	Taiwan	1998	NA	GenBank
S110031-SAR-03	AY258307	B5	Malaysia	2003	NA	GenBank
S19741-SAR-03	AY258313	B5	Malaysia	2003	NA	GenBank

[a] NA, not available.
[b] Proposed subgenogroup in this study.

cDNA as a template, the VP1 region was amplified by PCR by means of 40 cycles of 94°C for 30 s, 50°C for 30 S, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced using a BigDye Terminator cycle sequencing FS ready-reaction kit on an ABI Prism 310 automatic sequencer (Applied Biosystems, Foster City, CA). For PCR and sequencing analysis, primers 159, 161, 162, 163, VP1F2, 011, and 16R were used as described previously (1, 13, 18, 19). Sequence data were analyzed with CLUSTAL W version 1.83, and a phylogenetic tree was constructed by the neighbor-joining method (15) using the same software.

There were 4, 4, 19, 3, 1, and 79 (a total of 110) EV71 isolates in each year between 1998 and 2003 in Yamagata, respectively (Fig. 1). Ninety-five strains were isolated from children with a clinical diagnosis of HFMD. Among them, a 1-year-old boy in 1999 and a 4-year-old girl in 2003 presented with encephalitis as a complication of HFMD. Other strains were isolated from patients with upper respiratory infection (four cases), influenza-like illness (three cases), herpangina (three cases), viral exanthema (three cases), and aseptic meningitis (two cases). Three cases might have been clinically diagnosed as influenza-like illness, as they did not show typical clinical manifestations of HFMD, such as vesicular lesions, but only a high fever. We isolated EV71 and influenza virus B from a single throat swab specimen from a patient with HFMD in March 2003 (dual infection), presumably because the HFMD outbreak started in the mid-influenza season.

The monthly distribution of EV71 isolates and the phylogenetic tree for the VP1 region are shown in Fig. 1 and 2, respectively. Between 1998 and 1999, six isolates were identified as subgenogroup C2. They had more than 95% nucleotide identity to the representative Malaysian C2 strain 03750-MAA-97. C2 had been reported in Malaysia, Taiwan, Australia, and Japan between 1997 and 1999 (2, 5, 7, 17). In 2000, all isolates analyzed were typed as B4 except for one strain, 1116-Yamagata-00, which was typed as C2. Their sequences were 97% identical to the representative B4 strains, 5536/SIN/00 from Singapore and S21082/SAR/00 from Malaysia. B4 was isolated in Singapore, Malaysia, Taiwan, and Japan between 1997 and 2002 (2, 5, 7). In 2001, all three isolates were identified as another C2 strain. Among C2 strains between 1998 and 1999, the sequence identity was 96 to 98%, whereas it was 99 to 100% among strains isolated between 2000 and 2001. However, between C2 strains isolated in 1998 to 1999 and in 2000 to 2001, the identity was only 93 to 95%. Therefore, it seems that evolutionary changes occurred between C2 strains in 1998 to 1999 and those in 2000 to 2001. It may be true that the latter strains were imported from surrounding countries; however, we could not find any strains with more than 95% identity to C2 strains in 2000 to 2001. In October 2002, only one EV71 strain was isolated; thereafter, a major outbreak of HFMD due to EV71 started in January in 2003. This was the winter season, when EV71 is rarely isolated. One strain in 2002, all analyzed strains between January and August 2003, and one strain in September 2003 had more than 95% identity to the representative Chinese C4 strains H25-CHN-00 and H26-CHN-00, and these isolates were identified as C4. A C4 isolate from Taiwan was also reported to GenBank in 1998 (Table 1 and Fig. 2), in addition to C4 isolates from China in 2000 (17). Unexpectedly, two isolates from September 2003

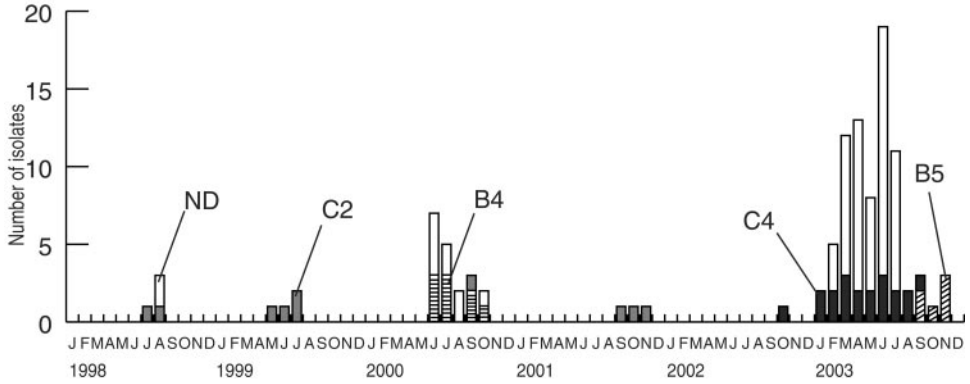

FIG. 1. Monthly distribution and subgenogroups of enterovirus 71 strains isolated in Yamagata, Japan, between 1998 and 2003. The subgenogroups, C2, B4, C4, and B5, were grouped according to the phylogenetic analysis shown in Fig. 2. ND, not done.

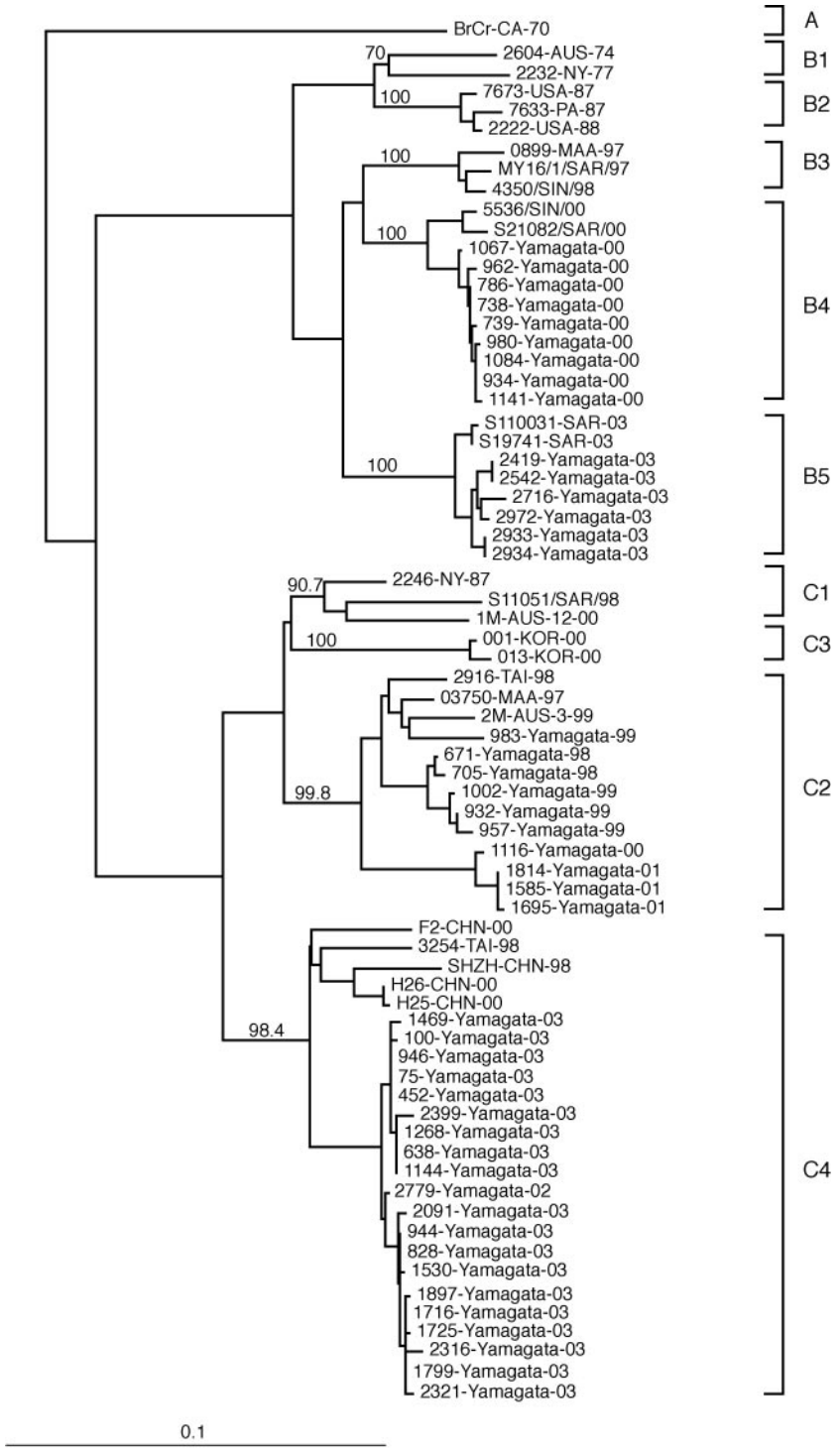

FIG. 2. Phylogenetic tree for the complete (891-nucleotide) VP1 region of representative EV71 strains isolated in Yamagata, Japan, between 1998 and 2003 and reference strains. Details of the EV71 strains, which belong to subgenogroups A, B1 to B5, and C1 to C4, are provided in Table 1. Branch lengths are proportional to the number of nucleotide differences. Numbers above the branches are the bootstrap probabilities (%). The marker denotes a measurement of relative phylogenetic distance.

and all isolates between October and November 2003 shared only 82 to 85% identity to the C4 strains that had been circulating immediately prior. Furthermore, they had only 88 to 94% identity with the representative B1 to B4 strains, while B1 to B4 showed 94 to 99% identity within each subgenogroup. Therefore, these isolates were suggested as belonging to a new subgenogroup, B5. Although we propose here the new subgenogroup B5 for the first time, we found similar strains, such as S110031-

SAR-03 and S19741-SAR-03 (98% nucleotide identity), which were submitted directly to GenBank. B5 appeared not only in Yamagata but also in Malaysia in 2003.

This is the first report of a longitudinal molecular epidemiological study analyzing the VP1 region of EV71 in a local community, such as Yamagata. This analysis supported the notion that EV71 circulates widely and actively in the Asia-Pacific region as one of the causative agents of HFMD, as previously reported (2, 7, 17, 19, 20). All of the four subgenogroups reported here had been circulating in the Asian-Pacific region throughout the study period described above. Therefore, cocirculation and the temporal change of several different subgenogroups are supposedly regular occurrences in HFMD outbreaks due to EV71, not only at the regional and national levels (5, 7, 20) but also locally. In this area, fatal encephalitis cases with HFMD have been reported: for example, there were cases in Taiwan in 1998 due to C2 and cases in Taiwan and in Singapore in 2000 due to B4 (2, 5, 7, 17, 18, 20). We had one encephalitis case of HFMD, which was due to a C2 strain in 1999 (983-Yamagata-99 in Table 1). The fact that EV71 strains belonging to identical subgenogroups causing fatal encephalitis in neighboring countries were prevalent in Yamagata during the same season shows the importance of the global surveillance of EV71 and a quick response from authorities (2, 5, 7, 17, 18, 20).

In particular, our study suggested that EV71 activity even in a local area is largely fuelled by frequent importations and maybe exportations from and to surrounding countries. During the 6-year study period, we found four subgenogroups, C2, B4, C4, and B5, and these subgenogroups replaced each other in circulation every 1 to 2 years. We do not have any clear reasons why these subgenogroups replace each other every year or so. As factors reflecting enterovirus evolution, interactions of the virus with its human host and the environment have been considered (10). Since VP1 capsid proteins are thought to be related to their involvement in antibody binding, population immunity probably acts to select new enterovirus strains (4, 10). In fact, our observation that B5 strains immediately replaced C4 strains in September 2003 indicates that subgenogroup replacement can occur within a very short time, and we found antigenic differences between C4 and B5 strains by a neutralization test with antiserum against the BrCr-CA-70 strain (data not shown). The replacement of subgenogroups in a local population is a novel finding of our study. The replacement of subgenogroups might occur in each local community, and this phenomenon could be observed totally as the cocirculation of several subgenogroups at the national and regional surveillance levels, as described by other papers (2, 5, 7, 20). Subunit VP1 vaccines are being developed as a preventive measure of severe neurological diseases of EV71 in children (21). Since EV71, possibly evolving and changing its antigenicity, circulates and causes HFMD and severe cases, antigenic analyses of different subgenogroups might contribute to the consideration of the effectiveness of a subunit vaccine as a next step.

Finally, a careful watch is needed on the epidemiology of EV71 locally, nationally, and regionally to survey the changes in the circulation of EV71 and to develop a better control strategy.

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