Molecular epidemiology of enterovirus 71 strains isolated from children in Yamagata, Japan, between 1990 and 2013

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Enterovirus 71 infections have become a major public issue in the Asia-Pacific region due to the large number of fatal cases. To clarify the longitudinal molecular epidemiology of enterovirus 71 (EV71) in a community, we isolated 240 strains from children, mainly with hand-foot-and-mouth diseases, between 1990 and 2013 in Yamagata, Japan. We carried out a sequence analysis of the VP1 region (891 bp) using 223 isolates and identified six subgenogroups (B2, B4, B5, C1, C2 and C4) during the study period. Subgenogroups C1 and B2 were found only between 1990 and 1993 and have not reappeared since. In contrast, strains in subgenogroups C2, C4 and B5 appeared repeatedly with genomic variations. Recent reports from several local communities in Japan have suggested that identical predominant subgenogroup strains, which have also been found in the Asia-Pacific region, have been circulating in a wide area in Japan. However, it is likely that there is a discrepancy between the major subgenogroups circulating in the Asia-Pacific region and those in Europe. It is necessary to continue the analysis of the longitudinal epidemiology of EV71 in local communities, as well as on regional and global levels, to develop strategies against severe EV71 infections.

INTRODUCTION

Enterovirus 71 (EV71) is a small, single-stranded, positive-sense non-enveloped RNA virus belonging to the genus Enterovirus within the family Picornaviridae (McMinn, 2002; Solomon et al., 2010). Together with coxsackievirus A16 (CVA16), CVA10 and other CVA viruses, EV71 is known to be a causative agent of hand-foot-and-mouth disease (HFMD) (McMinn, 2002; Solomon et al., 2010). Although HFMD is typically a self-limiting illness in children, EV71 infection sometimes causes neurological manifestations such as brainstem encephalitis, and fatal cases in infants and children have been reported in the 20th–21st century, mainly across the Asia-Pacific region (McMinn, 2002; Solomon et al., 2010). For example, in an outbreak of HFMD in Shangdong Province, China, in 2007, 1149 cases were reported, among which 11 children (0.96%) presented with neurological complications and three died (mortality rate 0.26%) (Zhang et al., 2009). EV71 infection has since become a major public issue in this area and there has been notable progress in studies of its epidemiology, identification of receptors, development of antiviral agents and vaccine development, all of which are necessary to control and ameliorate the severity of the disease (Liang et al., 2013; McMinn, 2002; Solomon et al., 2010; Wu et al., 2001; Zhu et al., 2013).

Several epidemiological studies have clarified that EV71 is an active circulating agent capable of rapid evolution into several different genogroups and that EV71 consists of 14 subgenogroups (A, B1–5, C1–5, D, E and F), although no relationship between severity and genogroup has yet been confirmed (Liang et al., 2013; McMinn, 2002; Singh et al., 2002a; Solomon et al., 2010; Bessaud et al., 2014). The

Abbreviations: CVA, coxsackievirus A; EV71, enterovirus 71; HFMD, hand-foot-and-mouth disease.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AB936432–AB936561.
Development of a vaccine against EV71 has been pursued as a preventive measure and the main target region for vaccine development has been the VP1 region, which was defined as the neutralization determinant (McMinn, 2002; Oberste et al., 1999; Wu et al., 2001). As the number of studies involving antigenic analysis and measurement of neutralizing antibody against different subgenogroups targeting the VP1 region has been limited, we carried out a molecular epidemiological study of EV71 strains isolated in Yamagata, Japan, between 1990 and 2007 (Mizuta et al., 2009). Our results from a sequence analysis of the VP1 region, genotyping, a seroepidemiological survey and an antigenic analysis suggest that there is cross-antigenicity among the different EV71 subgenogroups circulating in the community and that it might be possible to prevent severe illnesses due to EV71 infections through the development of a vaccine that effectively induces neutralizing antibodies against EV71, as has been shown with the measles virus vaccine (Mizuta et al., 2009). In reality, studies related to vaccine development have suggested cross-protection among different EV71 subgenogroups, and it is recommended that future research focuses on strengthening the monitoring of prevalent strains and strain variations to check the cross-protective effects of vaccines, particularly if genogroup replacement has occurred (Arita et al., 2007; Liang et al., 2013; Solomon et al., 2010). Furthermore, good surveillance programmes are needed in many different geographical regions to provide accurate and relevant information about EV71 transmission and evolution (Solomon et al., 2010). We have further analysed the EV71 Yamagata isolates, and here describe the longitudinal molecular epidemiology of EV71 between 1990 and 2013.

**METHODS**

Virus isolation and identification were carried out by means of a microplate method using nasopharyngeal samples from children with HFMD, viral exanthematous disease or respiratory illnesses, as described previously (Mizuta et al., 2005, 2008, 2009). EV71 strains were isolated using human embryonic lung fibroblast (HEF), Vero, Vero E6, human rhabdomyosarcoma (RD)-18S and green monkey kidney (GMK) cell lines at the Virus Research Center, National Hospital Organization, Sendai Medical Center, Sendai, Japan, between 1990 and 1997, and thereafter at the Department of Microbiology, Yamagata Prefectural Institute of Public Health. The isolates were identified as EV71 based on a neutralization method. Sequence analysis for the complete VP1 region (891 nt) of the EV71 isolates was carried out as described previously (Mizuta et al., 2005, 2009), except that we also used primers 222 and 224 (Oberste et al., 2000; Singh et al., 2002b). Sequence data for the isolates from Yamagata had the following GenBank accession numbers: [GenBank accession numbers].

**Fig. 1.** Monthly distribution (odd months numbered) and subgenogroups of EV71 strains isolated in Yamagata, Japan, between 1990 and 2013. The subgenogroups, C1, C2, B2, B4, C4 and B5, were grouped according to the phylogenetic analysis shown in Fig. 2. ND, Not done.
numbers: AB177809–AB177816, AB213614–AB213650 and AB433862–AB433892. Sequence data were analysed with CLUSTAL W (version 1.83), and a phylogenetic tree was reconstructed by the neighbour-joining method using the same software (Saitou & Nei, 1987).

**RESULTS**

A total of 240 EV71 strains were isolated in Yamagata between 1990 and 2013. The number of EV71 strains isolated in Yamagata by month is shown in Fig. 1. Although we did not measure the neutralizing titres for EV71 isolates after 2007, these isolates were neutralized with the identical antisera used in the previous study (Mizuta et al., 2009). A total of 223 of the 240 isolates were used for sequence analysis, and representative Yamagata strains were used for further phylogenetic analysis. Available EV71 strains that had not yet been typed in previous studies (Mizuta et al., 2005, 2009) were also sequenced in this study.

The phylogenetic tree for the VP1 region and the monthly distribution of subgenogroups are shown in Figs 1 and 2, respectively. In 1990, three isolates were identified as belonging to subgenogroup C1 and one isolate was identified as belonging to B2. In 1993, one strain was identified as B2. Between 1997 and 1999, all analysed strains were typed as C2. In 2000, all analysed strains, except for one strain isolated in September and identified as C2, were typed as B4. All four isolates in 2001 were again typed as C2. Isolates between October 2002 and August 2003 were typed as C4, except for one strain isolated in June 2003 and identified as B5. C4 and B5 strains co-circulated in September, with only B5 observed thereafter until November 2003. All isolates between 2006 and 2007 were again typed as C4. All isolates, except for one strain (2950-Yamagata-2009 typed as B5), between 2009 and 2010 were typed as C2. The strain 2950-Yamagata-2009 was the only isolate from the Shonai area, which is located on the Japan Sea in Yamagata, whereas the other strains isolated between 2009 and 2010 were from inland areas. In 2012, all isolates were typed as B5, and B5 and C2 strains co-circulated in 2013. C2, C4 and B5 strains appeared repeatedly in Yamagata. C2 strains with 93–100% nucleotide similarity to each other

![Fig. 2. Phylogenetic tree for the complete (891 nt) VP1 region of representative enterovirus 71 (EV71) strains isolated in Yamagata, Japan, between 1990 and 2013 and reference strains. Subgenogroups B5, C2 and C4 strains repeatedly appeared in Yamagata with genomic variations with several years’ interval as shown by the different levels of grey shading. Yamagata and European strains are indicated by ● and ■, respectively. Branch lengths are proportional to the number of nucleotide differences. Numbers above the branches are the bootstrap probabilities (%). Bar, measurement of relative phylogenetic distance (0.02 nucleotide substitutions per site).](http://jmm.sgmjournals.org)

![Fig. 3. Summary of EV71 genotypes circulating in the Asia-Pacific region (grey shading) and Europe since 1980 based on data from the following references: Bible et al. (2008); Cardosa et al. (2003); Diedrich et al. (2009); Herrero et al. (2003); Huang et al. (2008a, b, 2010); Kung et al. (2007); Ortner et al. (2009); Podin et al. (2006); Sanders et al. (2006); Schuffenecker et al. (2011); Wu et al. (2010); Van der Sanden et al. (2009); Zhang et al. (2009).](http://jmm.sgmjournals.org)
circulated every year between 1997 and 2001, and again appeared between 2009 and 2010 with 94–100 % similarity. The nucleotide similarity between C2 strains from 1997 to 2001 and those from 2009 and 2010 was 90–95 %. C4 strains circulated between 2002 and 2003 with 98–100 % similarity, and then disappeared and reappeared between 2006 and 2007 with 97–100 % similarity. The nucleotide similarity between C4 strains from 2002 to 2003 and those from 2006 to 2007 was 95–96 %. B5 strains replaced C4 strains in September 2003 and circulated for 3 months with 98–100 % similarities and then disappeared. Only one B5 strain was isolated in November 2009 and there was another outbreak of B5 strains between 2012 and 2013 with 95–100 % similarity. The nucleotide similarity was 96, 94–95 and 94–95 % between the B5 strains from 2003 and the one from 2009, the one from 2009 and those from 2012–2013, and those from 2003 and those from 2012–2013, respectively.

DISCUSSION

We showed the temporal distribution of EV71 subgenogroups over a 24-year period from 1990 to 2013, in Yamagata, Japan. Apart from a study undertaken in Sydney, Australia, between 1983 and 2011, to the best of our knowledge no such longitudinal analysis of the VP1 region of EV71 strains circulating in a local community has been reported (Sanders et al., 2006). Our longitudinal studies revealed several interesting epidemiological features, as reported previously based on the data obtained between 1990 and 2007 (Mizuta et al., 2005, 2009), and we further found several new findings in the last 6-year observation period.

We found that six subgenogroups, B2, B4, B5, C1, C2 and C4, appeared one after another or concurrently during the study period. C1 and B2 disappeared by 1993 in Yamagata and these subgenogroup strains have not yet reappeared. However, the three other subgenogroups, excluding B4, have repeatedly appeared in Yamagata during the study period.

Based on the data between 1990 and 2007 (Figs 1 and 2) (Mizuta et al., 2009), we previously reported that genogroup C strains circulated for longer periods with genomic variations, whereas genogroups B strains appeared for only one season. We observed genogroups B strains, B4 and B5, for only 5 and 6 months in 2000 and 2003, respectively, whereas C2 and C4 strains circulated for 5–6 years despite breaks (Mizuta et al., 2009). However, in this study, after the disappearance of B5 strains in 2003, we subsequently observed one B5 strain in 2009 and another outbreak of B5 strains between 2012 and 2013. Thus, these results suggested that not only genogroup C strains but also genogroup B strains have the potential to circulate repeatedly with active genomic variations.

In Japan, the genotyping of EV71 strains has been reported recently based on partial VP1 and/or VP4-2 sequence analysis. Momoki et al. (2009) reported that one and three isolates between September and December in 2008 belonged to C2 and B5, respectively, and they suggested that, in 2008, C2 replaced C4, which had been in circulation between 2005 and 2007 in Yokohama City. Hosomi et al. (2010) reported that four strains in Kochi in 2010 belonged to C2 and Nakata et al. (2012) found that there was an outbreak of HFMD due to C2 strains in Osaka in 2010. Two isolates (OC091494 and OC091495) from Osaka City in 2010 showed 99 % sequence similarity with Yamagata isolates in 2010, apart from a 96 % similarity between the Osaka isolates and 2008-Yamagata-2010 (Fig. 2). Katsumi et al. (2013) reported the isolation of seven B5 strains and one C2 strain in 2012 in Sendai City. Kodama et al. (2013) reported that seven strains isolated in Ishikawa Prefecture in 2010 belonged to C2, and 14 strains isolated between 2012 and 2013 belonged to B5, although the investigation was carried out based on VP4–VP2 analysis. Taken together, these observations and our data from Yamagata suggest that the predominant subgenogroup changed from C4 to C2 across a wide area of Japan between 2007 and 2009. B5 strains probably caused a nationwide epidemic between 2012 and 2013, as reported from Sendai and Ishikawa (Katsumi et al., 2013; Kodama et al., 2013). Recent reports have also suggested that identical predominant subgenogroup strains have been in simultaneous circulation all over Japan.

As we reported previously, the fact that the detected subgenogroups in Yamagata (B2, B4, B5, C1, C2 and C4) were also found in the Asia-Pacific region suggests the active circulation of EV71 in this area (Bible et al., 2008; Cardosa et al., 2003; Diedrich et al., 2009; Herrero et al., 2003; Huang et al., 2008a, b, 2010; Kung et al., 2007; Mizuta et al., 2005, 2009; Ortnner et al., 2009; Podin et al., 2006; Sanders et al., 2006; Schuffenecker et al., 2011; Wu et al., 2010; van der Sanden et al., 2009; Zhang et al., 2009) (Fig. 3). On the other hand, several European countries, such as France, the Netherlands, Germany and the UK, recently reported the molecular epidemiology of EV71 (Bible et al., 2008; Diedrich et al., 2009; Schuffenecker et al., 2011; van der Sanden et al., 2009). When we compare the subgenogroups circulating in Asia-Pacific region and those in Europe, we can find several differences: (i) there was a co-circulation of genogroup B and C strains in the Asia-Pacific region throughout almost the entire study period, whereas only genogroup C strains were in circulation in Europe (except for subgenogroup B2 strains found in Germany between 1997 and 1998); (ii) B3, B4 and B5 strains have been reported only in the Asia-Pacific region; (iii) genogroup C, C1, C2 and C4 strains circulated as major subgenogroups in the Asia-Pacific region, whereas C1 and C2 strains were predominant in Europe between 1990 and 2009; and (iv) subgenogroup C5 strains are only observed in the Asia-Pacific region (Fig. 3). These observations suggest that EV71 circulates mainly in the Asia-Pacific region and in Europe, although this idea does not completely exclude the possible introduction of EV71 from Europe to Asia-Pacific region and vice versa. It is conceivable that the reappearance
of C2 in Yamagata in 2009 might be the result of the introduction of EV71 from Europe or Singapore (Figs 1–3).

Several capsid residues of EV71 that alter infectivity in the mouse model have been identified. For example, a novel lysine-to-glutamic acid substitution at position 244 in VP1 of B5 strain is critical for mouse adaptation and virulence, and a single mutation from glutamine to glutamic acid at residue 145 in VP1 of C4 strain generates a mouse-virulent phenotype (Zaini & McMinn, 2012; Zaini et al., 2012a). In Yamagata, all B5 strains had lysine at position 244, whereas all C4 strains had glutamic acid at residue 145 during the study period. It will also be necessary to clarify mechanisms of severity and pathogenicity in EV71 infections.

Finally, to resolve the public health issue of EV71, we have to continue to analyse the longitudinal epidemiology of EV71 in local communities such as Yamagata, as well as on regional and global levels.

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REFERENCES


