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Genetic characterization of Enterovirus 71 strains circulating in Vietnam in 2012

Celeste Donato [a,1], Le Thi Hoi [b,1], Nguyen Thi Hoa [c], Tran Mai Hoa [b], Le Van Duyet [b], Ta Thi Dieu Ngan [b], Nguyen Van Kinh [b], Nguyen Vu Trung [b], Dhanasekaran Vijaykrishna [a,d,*]

[a] Duke-NUS Medical School, 8 College Rd, Singapore
[b] National Hospital of Tropical Diseases, 78 Giai Phong Street, Hanoi, Vietnam
[c] Department of Clinical Microbiology, Hanoi Medical University, Vietnam
[d] Department of Pathology, Singapore General Hospital, Singapore

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ABSTRACT

Background: Enterovirus 71 subgenogroup C4 caused the largest outbreak of Hand, Foot and Mouth Disease (HFMD) in Vietnam during 2011–2012, resulting in over 200,000 hospitalisations and 207 fatalities.
Methods: A total of 1917 samples with adequate volume for RT-PCR analysis were collected from patients hospitalised with HFMD throughout Vietnam and 637 were positive for EV71. VP1 gene (n=87) and complete genome (n=9) sequencing was performed. Maximum-likelihood phylogenetic analysis was performed to characterise the B5, C4 and C5 strains detected.
Results: Sequence analyses revealed that the dominant subgenogroup associated with the 2012 outbreak was C4, with B5 and C5 strains representing a small proportion of these cases.
Conclusions: Numerous countries in the region including Malaysia, Taiwan and China have a large influence on strain diversity in Vietnam and understanding the transmission of EV71 throughout Southeast Asia is vital to inform preventative public health measures and vaccine development efforts.

1. Introduction

Enterovirus 71 (EV71), a member of the virus family *Picornaviridae* (genus *Enterovirus),* is one of several aetiological agents associated with hand, foot and mouth disease (HFMD) in children (McMinn, 2002). Typically mild and self-limiting, HFMD is characterised by a range of symptoms including fever, pharyngitis, enanthem on the buccal muscoa, gums and palate, and papulovesicular exanthem on the hands, feet and buttocks (Grist et al., 1978). These clinical features are indistinguishable from HFMD caused by Coxsackie virus type A (CA), also a member of *Enterovirus* genus (McMinn, 2002). EV71 causes the majority of severe HFMD cases and in rare cases can be complicated by severe, potentially fatal neurological disease such as aseptic meningitis, cerebella ataxia, brain stem encephalitis, neurogenic pulmonary edema and acute flaccid paralysis (McMinn, 2002, 2014). Transmission of EV71 occurs person-to-person through direct contact with bodily fluids, with the virus shed in the saliva, faeces, vesicular fluid and respiratory droplets. The virus is also spread by indirect contact through contaminated surfaces and fomites (Solomon et al., 2010).

EV71 is classified based on the phylogenetic relationships of the VP1 gene into five genotypes (A–F) and subgenotypes B0–B5 and C0–C5 (McMinn, 2012; Rao et al., 2012). Since 1997, the burden of disease due to EV71 infection has been increasing in the Asia-Pacific region with numerous outbreaks reported in Malaysia (Chua et al., 2007), Brunei (AbuBakar et al., 2009), Australia (Sanders et al., 2006), Taiwan (Chen et al., 2007), Japan (Fujimoto et al., 2002), Singapore (Ang et al., 2009), South Korea (Cho et al., 2010), China (Tan et al., 2011) and Cambodia (Geoghegan et al., 2015).

In Vietnam, EV71 was first identified in 2003 associated with a large outbreak of HFMD and the high burden of disease has continued (Tu et al., 2007). EV71 infections have been reported in all provinces throughout Vietnam, although the highest disease burden has been previously reported in the Southern region (Nguyen et al., 2014; Tu et al., 2007). An outbreak in Southern Vietnam in 2005 was associated with 173 cases including 51 with neurological complications and 3 fatalities (Tu et al., 2007). The number of reported HFMD cases and deaths in Vietnam has been increasing, from 5719 and 23, respectively in 2007, 10,958 and 25 in 2008, and 10,632 and 23 in 2009 (Nguyen et al., 2014). Since 2011, HFMD has

* Corresponding author at: Duke-NUS Medical School Singapore, 8 College Road, Singapore, 169857, Singapore.
E-mail address: vijay.dhanasekaran@duke-nus.edu.sg (D. Vijaykrishna).
[1] Both authors equally contributed to this publication.

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been classified by the Vietnamese Ministry of Health as a severe infectious disease with outbreak potential and all hospitals report cases weekly through the national communicable disease surveillance system. In 2011–2012 there were over 200,000 HFMD hospitalisations in Vietnam and 207 fatalities (Khanh et al., 2012; Nguyen et al., 2014).

Our understanding of the molecular epidemiology of EV71 in Vietnam arises primarily from genetic analysis of sequences acquired from samples collected in Southern Vietnam (Geoghegan et al., 2015; Thoa le et al., 2013; Tu et al., 2007). The molecular epidemiology of EV71 in the cities and provinces of North Vietnam, as well as their regional role in dissemination of EV71 is less well understood. The aim of this study was to conduct genetic analysis of the VP1 gene from EV71 strains associated with severe disease that were isolated from paediatric patients throughout Vietnam in 2012, highlighting the complex temporal and geographic spread of the virus during an outbreak year.

2. Materials and methods

2.1. Specimen collection

Between September 2011 and January 2013, 1917 samples were collected from patients hospitalised with HFMD in Vietnam. Samples were collected as throat swabs and/or stool samples from patients hospitalised at the National Hospital for Tropical Diseases, Children's Hospital 1 and Children's Hospital 2, in Ho Chi Minh, and Vietnam National Hospital of Pediatrics in Hanoi.

A total of 1693/1917 samples had adequate volume for RT-PCR analysis as previously described (Perera et al., 2004) and 637 samples were found to be EV71 positive. Patient information including age, date of sample collection, gender, survival outcome and clinical disease severity score when the sample was taken (defined following the Vietnamese Ministry of Health clinical grading system) were recorded where possible (Khanh et al., 2012). Ethics approval was granted by the Institution Review Board of the National Hospital of Tropical Diseases prior to commencement of the study. Informed consent was obtained from the parent or guardian of the child prior to samples being included in the study.

2.2. Virus culture, RNA extraction, PCR and sequencing

Of the 637 EV71 positive samples a subset were selected for cell culture from 2012, based on location, clinical severity and sample volume. A total of 128/200 selected strains were able to be cultured and a subset of 100 was selected for sequencing. Viral RNA was extracted from cell culture supernatant using the QIAamp viral RNA extraction kit (QIAGEN). Amplification of the VP1 region were performed using primers described earlier (Yip et al., 2013), confirmed by 1% agarose gel electrophoresis and purified with a Wizard SV gel and PCR clean up System (Promega). The PCR products were directly sequenced using a BigDye Terminator v1.1 cycle sequencing kit, and analysed using the ABI 3130XL Genetic Analyzer (Applied Biosystem). A total of 78 samples had adequate quality sequence reads for analysis.

For direct NGS whole genome sequencing, extracted viral RNA from 20 samples was fragmented (approximately 400 base pairs) using the TruSeq Strand mRNA LT sample Prep kit (Illumina). Following first strand and second strand cDNA synthesis, and 3′ adenylation, adaptors were ligated to the ends of the ds-cDNA. Following enrichment by limited-cycle amplification PCR and normalization using the MiSeq Reagent kit 300 cycle V2 (Illumina), one nanogram of pooled DNA from individual samples (assigned a unique barcode sequence using the Nextera XT Index Kit (Illumina)) was then subjected to library preparation using the Nextera XT DNA sample preparation kit (Illumina). Sequencing was performed using the Miseq reagent kit v2 (300 cycles, Illumina) on an Illumina Miseq platform. Sequence assembly was performed using Geneious 7.1.3 utilizing a reference-based mapping tool. The reads obtained were processed to remove PCR primers using CLC Genomics Workbench (QIAGEN). Nine samples had adequate genome coverage to be used in analysis.

2.3. Phylogenetic analysis

Strains sequenced in this study were analysed along with EV71 strains retrieved from NCBI GenBank. Multiple sequence alignments were performed for VP1 sequences and complete genome sequences using MAFFT (Katoh and Standley, 2013). Maximum-likelihood phylogenetic trees were constructed for the B5, C4 and C5 strains using RAxML applying the nucleotide substitution models GTR+G+I with 1000 rapid bootstraps (Stamatakis, 2014; Stamatakis et al., 2008). Trees were visualised and annotated using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Recombination and selection analysis of complete genomes

Recombination detection was performed using the GARD and SBP algorithms available from the DataMonkey server (Delport et al., 2010; Kosakovsky Pond et al., 2006). Recombination was also investigated using the models RDP, GENECONV, MaxChi, Chimaera, SiScan and 3Seq, available in the Recombination Detection Program (RDP version 4.46) (Boni et al., 2007; Gibbs et al., 2000; Martin and Rybicki, 2000; Martin et al., 2015; Posada and Crandall, 2001; Smith, 1992). Estimates of the selection pressures acting at each codon site were investigated using the models SLAC, FEL, iFEL, MEME, FUBAR (p-value, < 0.01 or posterior threshold 0.9 to minimize false-positives) (Kosakovsky Pond and Frost, 2005; Murrell et al., 2013), available from the DataMonkey server using the nucleotide substitution model TrN as selected by the internal optimal model selection test (Delport et al., 2010). Sites were considered significant if they were detected by three or more models.

2.5. Accession numbers

The nucleotide sequences of samples from this study were deposited in GenBank under the accession numbers KU159434–KU159520.

3. Results and discussion

Since the emergence of EV71 in Asia in 1997, there have been several large epidemics associated with a high morbidity and mortality in numerous countries (AbuBakar et al., 2009; Ang et al., 2009; Chen et al., 2007; Cho et al., 2010; Chua et al., 2007; Fujimoto et al., 2002; Geoghegan et al., 2015; Sanders et al., 2006; Tan et al., 2011). Vietnam experiences an unusually high burden of disease, with the incidence of HFMD associated EV71 infection increasing since 2003 (Khanh et al., 2012; Tu et al., 2007). The biannual peaks and outbreaks of EV71 disease are often associated with changes between genogroup or subgenogroup strains. A subgenogroup switch from C5 to C4 strains was associated with a large outbreak in 2011–2012 (Khanh et al., 2012; Nguyen et al., 2014). Similarly, a switch from C4 to B5 occurred in 2012–2013 (Geoghegan et al., 2015). Based on strains sequenced in this study C4 strains were the dominant subgenogroup, with B5 strains detected at a lower frequency along with the sporadic detection of a C5 strain.

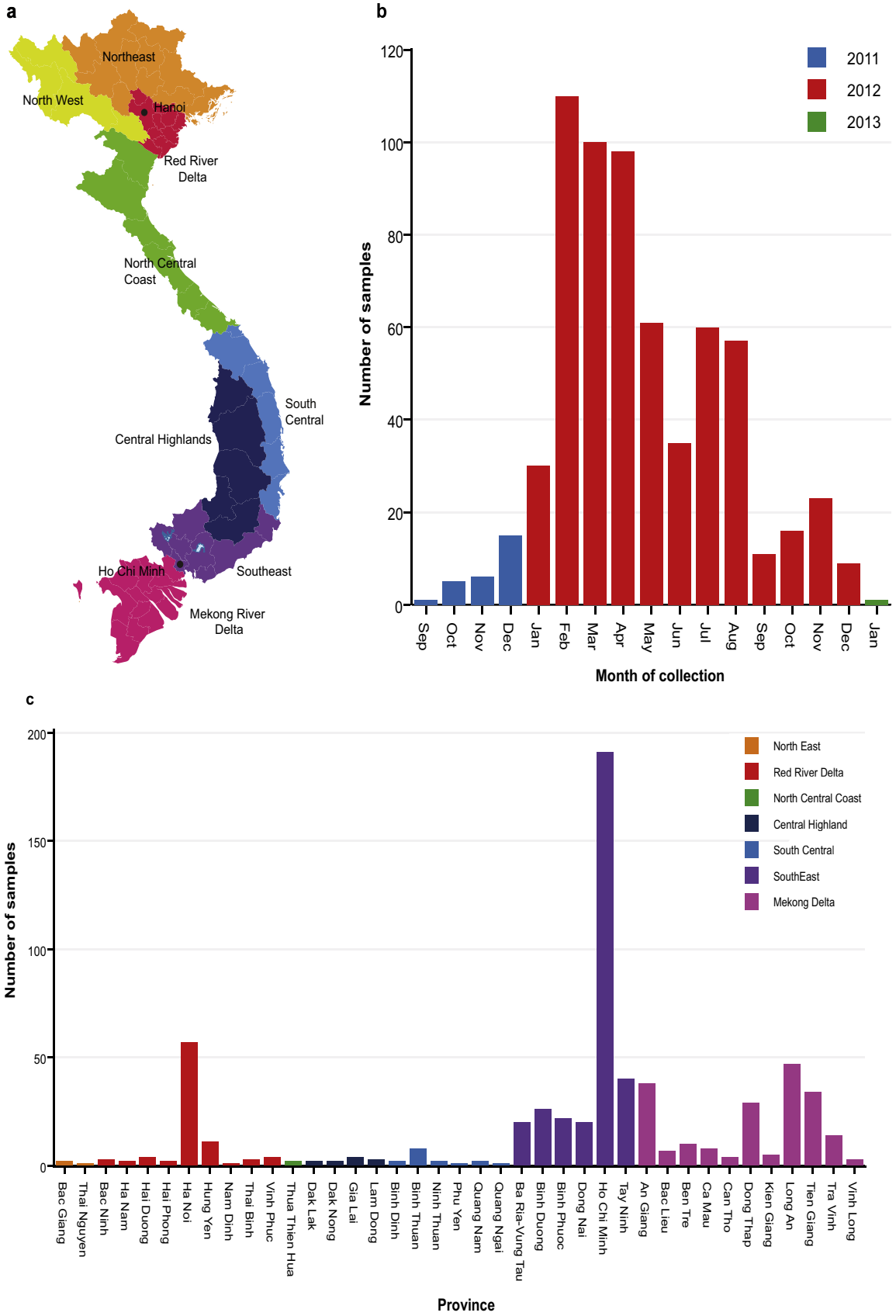

Fig. 1. (a) The major regions to designate province location within Vietnam. Temporal (b) and geographic (c) distribution of samples collected in this study. A total of 637 EV71 positive samples were collected between September 2011 and January 2013.

Table 1
Breakdown of age demographics for all 637 EV71 positive patients and genotype distribution of sequenced samples based on clinical severity score.

Severity score[a]		1 n=26/32	2a n=397/408	2b n=104/105	3 n=82/84	4 n=8/8
Age (years)	**Mean**	1.8	1.9	2.2	1.6	2.0
	Median	1.5	1.7	1.9	1.4	1.4
	Range	0.6–3.6	0.2–8.8	0.3–8.5	0.4–7.4	0.7–3.6
Genogroup[b]	**B5**	1	4	1	2	0
	C4	0	14	44	17	3
	C5	0	0	1	0	0

[a] Clinical severity score defined by Vietnamese Ministry of Health clinical grading system.
[b] Subset of 87 samples sequenced.

3.1. Patient demographics

Of the 1917 samples collected from patients with HFMD from 39 provinces throughout Vietnam, 637 were EV71 positive by RT-PCR. The majority of positive samples arose from provinces in the South East and Mekong River Delta regions (Fig. 1a and b). A subset of these samples was sequenced (VP1 only n=78 and complete genome n=9), from the Mekong Delta region (n=40), South East region (n=30), Red River Delta region (n=15) and North East region (n=2).

Several interesting demographic features of children with EV71 infection in Vietnam were revealed in this study, however these results have to be taken into consideration with a possible sampling bias associated with samples selected for sequencing. The age at hospital admission was known for 618/637 patients and when stratified by clinical severity, the median age of patients with disease category 1, 3 and 4 was younger than those with category 2a and 2b (Table 1). However these results should be interpreted with caution as the least number of samples were of disease category 1 (n=32) and 4 (n=8): the majority of our positive samples were of 2a (n=408), followed by 2b (n=105) and 3 (n=84). Previous studies have shown that younger age is a risk factor for severe infection, however studies in Europe reported considerably younger median ages of 4–5 months (Badran et al., 2011; van der Sanden et al., 2009). Other studies in Asia report higher ages of infection including mean age of 3.2 years in a study from Taiwan (Hsu et al., 2011) and median age of 3.5 years in a study from Hong Kong (Ma et al., 2010). This age-associated disease pattern may be implicated in the higher disease burden in Asia and requires further investigation.

The length of hospitalisation was known for 635/637 patients with an average stay of 4.3 days (range 1–32, median 4) and C4 strain infections were associated with longer hospitalisation (mean 5.6, median 5, range1–33) compared to B5 (mean 3.6, median 4, range 2–6). Gender was known for all 637 patients and gender ratio of female-to-male patients was 1:2.4. Several studies have revealed a higher severe infection rate in males, particularly outbreaks associated with C4a strains in Shanghai in 2012 (Wang et al., 2015). However, comparison to gender ratios in China must be considered in the context of the gender imbalance already present. There was uneven sampling between Northern and Southern provinces, however this reflects the imbalanced distribution of EV71 disease in Vietnam previously reported (Khanh et al., 2012). More extensive surveillance is required to understand the differing disease burden around the country.

3.2. Recombination detection, selection analysis and molecular characterization of virulence

No evidence of recombination was detected in the complete genome or VP1 sequences of EV71 strains characterised in this study. No sites were found to be under positive selection in the nine complete genomes or in the combined analysis with 154 Vietnam C4 genomes obtained from GenBank (2011–2012).

In this study, strains isolated from patients with varying clinical severity scores were analysed, however our analyses of the complete genomes and VP1 genes showed no association between any particular amino acid differences with disease severity, however adapting the strains to cell culture prior to sequencing may have confounded these results. Variations were observed at position 249 in the VP1 region and I1627T in the 2C region among these sequences with no specific association with disease severity. Amino acid substitutions L97R, E145G/Q and E164D/Q in the VP1 region have been previously associated with neurological symptoms, however these amino acids were conserved regardless of severity score in samples from this study (Cordey et al., 2012; Zhang et al., 2014). All C4 and C5 strains and all but two B5 strains from Vietnam exhibited a glutamic acid residue at position 145 in the VP1 region which has been suggested to be associated with increased virulence (Huang et al., 2009). However, numerous studies have reported no difference between strains isolated from complicated vs. uncomplicated infections (Wang et al., 2015). Discerning the viral genetic factors associated with virulence is difficult and the genetics of the patient is a significant factor in disease outcome. Understanding the proteomic markers in patients with differing severity of disease will provide meaningful insights into the pathogenesis of EV71 and reveal targets for specialized treatment.

3.3. Phylogenetic analysis

Based on available sequence data C4 strains (C4b) were first detected in Vietnam in 2005 with limited circulation and were not detected again until the subgenogroup re-emerged in 2011. C4 strains were the dominant subgenogroup circulating during this study with 78 strains sequenced in total, from the South Central (n=2), Mekong Delta region (n=39), South East region (n=26), Red River Delta region (n=10) and the North East region (n=1). There was no apparent geographic segregation of C4 strains around Vietnam, with closely related strains circulating in Northern and Southern provinces highlighting the rapid transmission around the country following introduction. The vast majority of strains circulating in 2011–2012 (from this study and GenBank) formed a single large lineage interspersed with strains isolated in Cambodia in 2012, highlighting the sustained transmission of strains from Vietnam into Cambodia (Fig. 2). There is strong phylogenetic support that this lineage was introduced into Vietnam from China, likely derived from strains circulating in 2010 in China, to cause the largest C4 epidemic in Vietnam, as previously described (Geoghegan et al., 2015). A small number of strains circulating in 2011–2012 did not cluster within the main lineage, including a single isolate from this study; ND-02_Vietnam:

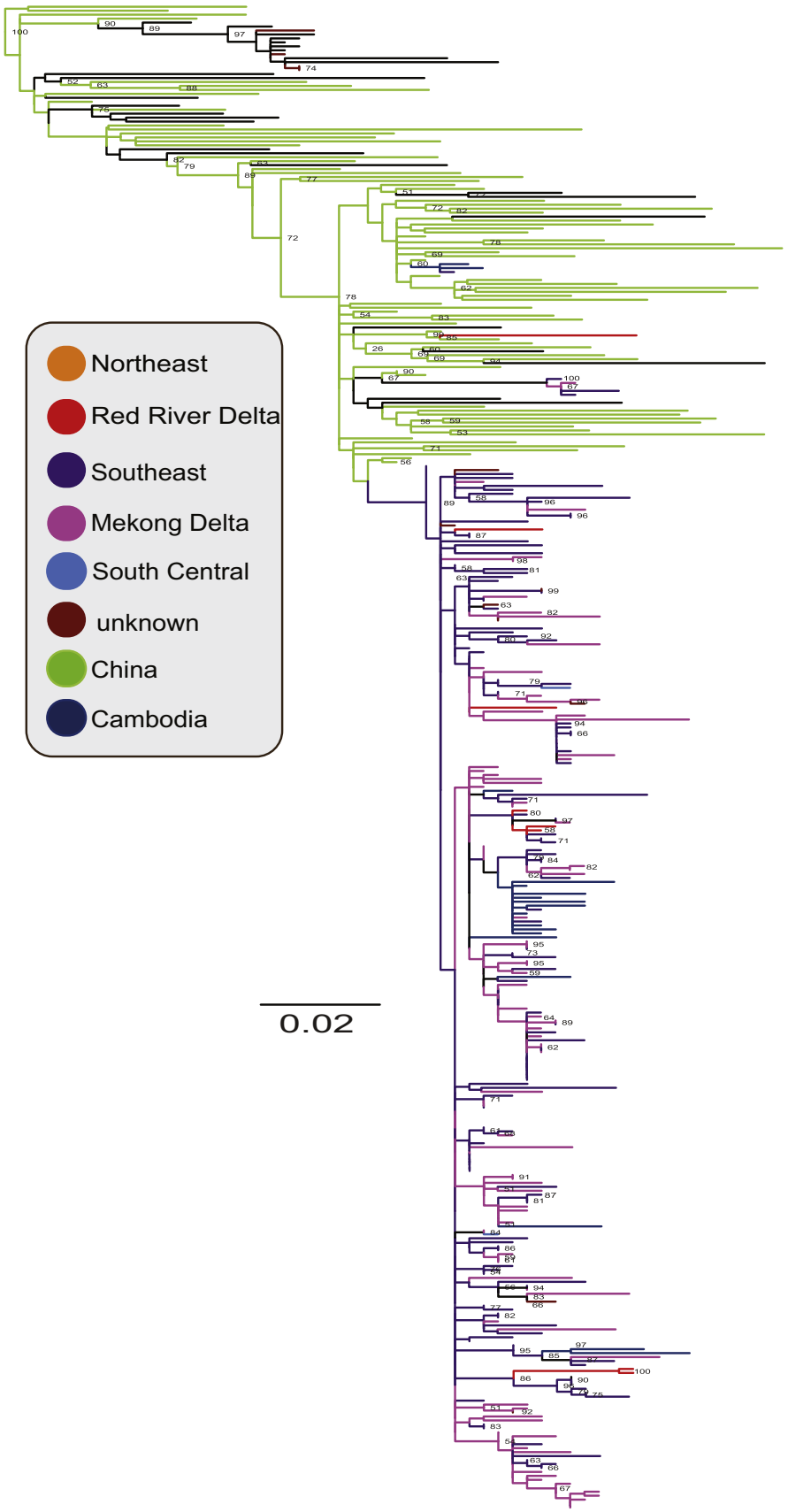

Fig. 2. The phylogenetic relationship of EV71 C4 subgenogroup viruses. The C4 tree was generated using the complete VP1 gene segment (891nts) using the maximum-likelihood method with GRT+G+I nucleotide substitution model and 1000 bootstrap replicates and nodes with values ≥ 50 are shown. The tree was midpoint rooted with the nodes ordered in a decreasing manner for clarity. Only the C4a lineage is shown and strain names removed for clarity.

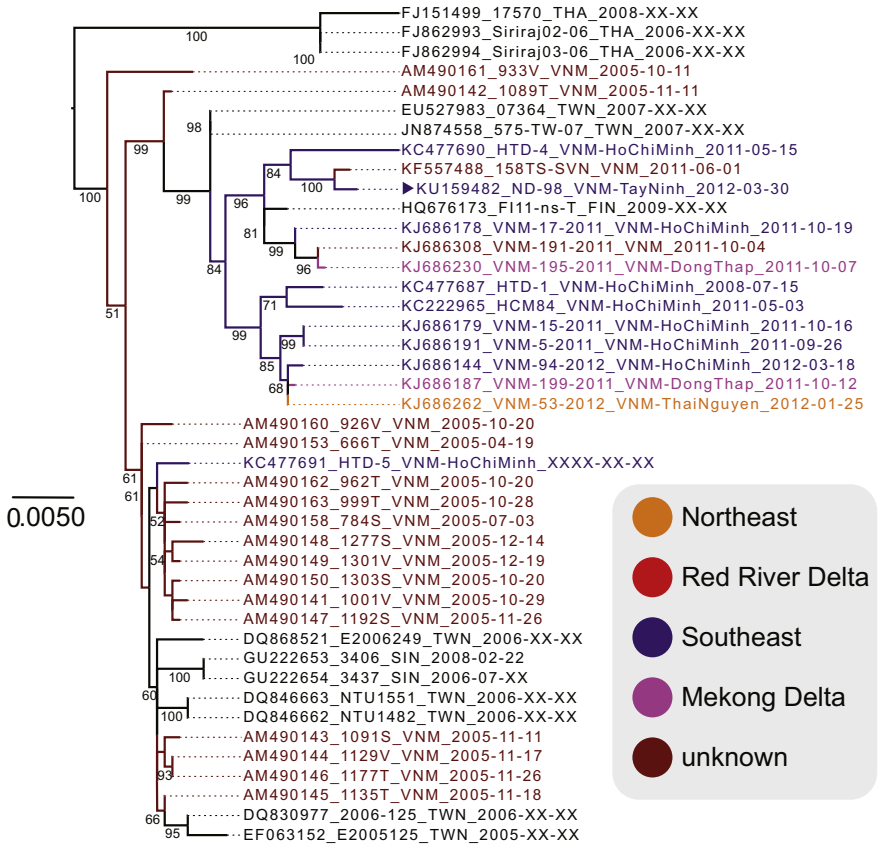

Fig. 3. The phylogenetic relationship of EV71 C5 subgenogroup viruses. The C5 tree was generated using the complete VP1 gene segment (891nts) using the maximum-likelihood method with GRT+G+I nucleotide substitution model and 1000 bootstrap replicates and nodes with values ≥50 are shown. The tree was midpoint rooted with the nodes ordered in a decreasing manner for clarity. Isolates are indicated by the GenBank Accession number_Strain_Country of isolation_Year of isolation. The country abbreviations are as follows: FIN-Finland, SGP-Singapore, THA-Thailand, TWN-Taiwan, VNM-Vietnam. Strains characterised in this are denoted by a ▸ symbol.

Hanoi_2012-01-2012. These strains formed small sub-clusters, closely related to several strains isolated in China in 2010 suggesting multiple lineages may co-circulate in Vietnam at varied frequencies. The reasons behind the epidemic dominance of a single large C4 sub-lineage are unknown, as viruses of the same lineage had caused repeated outbreaks in China since 2007/2008 and it is likely that C4 viruses were introduced repeatedly into Vietnam that did not cause outbreaks.

C5 strains have circulated in Vietnam since 2005 and in this study a single C5 strain ND-98_Vietnam: TayNinh_2012-03-30 was isolated from the South East region. Phylogenetic analysis of all available C5 strains revealed two lineages: one lineage comprised of three samples from Thailand circulating in 2006 and 2008 and another large lineage comprised of samples predominantly isolated from Vietnam since 2005, including strains from Singapore, Taiwan and one isolate from Finland (Fig. 3). The larger lineage was comprised of three sub-lineages; the one formed by a single isolate from the 2005 outbreak in Vietnam, and another containing strains from the 2005 outbreak in Vietnam and related strains from Singapore and Taiwan, isolated in the three years subsequent to the outbreak. A further sub-lineage was formed from viruses isolated in Vietnam from 2008 to 2012, Finland in 2009, and Taiwan in 2007. This sub-lineage appear to be derived from a minor population of strains circulating during the 2005 Vietnam outbreak (represented by AM490142_1089T_VNM_2005-11-11 in the tree) that spread to Taiwan, which may have been reintroduced into Vietnam suggesting possible sustained transmission of C5 strains between these geographically distinct countries, or these strains may have continued to circulate in Vietnam at a low prevalence, undetected over the intervening years (Geoghegan et al., 2015).

Based on available sequence data, B5 strains were first detected in Vietnam only in 2011 and increased in prevalence in 2012 to become the dominant subgenogroup characterised in 2013 replacing C4 (Geoghegan et al., 2015). In this study, eight B5 strains were sequenced from the Northern provinces in the North East region (n=1) and Red River region (n=5) with single strains also isolated from the South East and Mekong Delta regions. The majority of B5 strains sequenced in this study and the Vietnam strains available in GenBank formed a sub-lineage with strains circulating in Malaysia between 2010 and 2012 (Fig. 4). Within this sub-lineage, the B5 strains originating from the Northern provinces formed separate but closely related clusters to viruses isolated in the Southern provinces. The majority of B5 strains circulating in Vietnam were likely introduced from Malaysia, possibly through Thailand. The B5 strains in this cluster continued to circulate extensively in the Southern provinces into 2013. We detected the co-circulation of a small, separate cluster of strains from the South East and Mekong River Delta regions that clustered with strains isolated in Taiwan during 2011–2012. This suggests that there were multiple, separate introductions of B5 strains from Malaysia and Taiwan into Vietnam and these strains rapidly spread and co-circulated extensively around the country. Phylogenetic analysis reveals that Malaysia and Taiwan have extensive B5 strain diversity suggesting that distinct lineages of this subgenogroup are endemic in these countries and act as a source of strains circulating throughout South-East Asia, in particular Vietnam.

In conclusion, this study characterised EV71 strains isolated from children hospitalised in Vietnam during an outbreak of HFMD. The majority samples were collected in 2012 from Southern provinces where C4 strains were dominant, compared to the

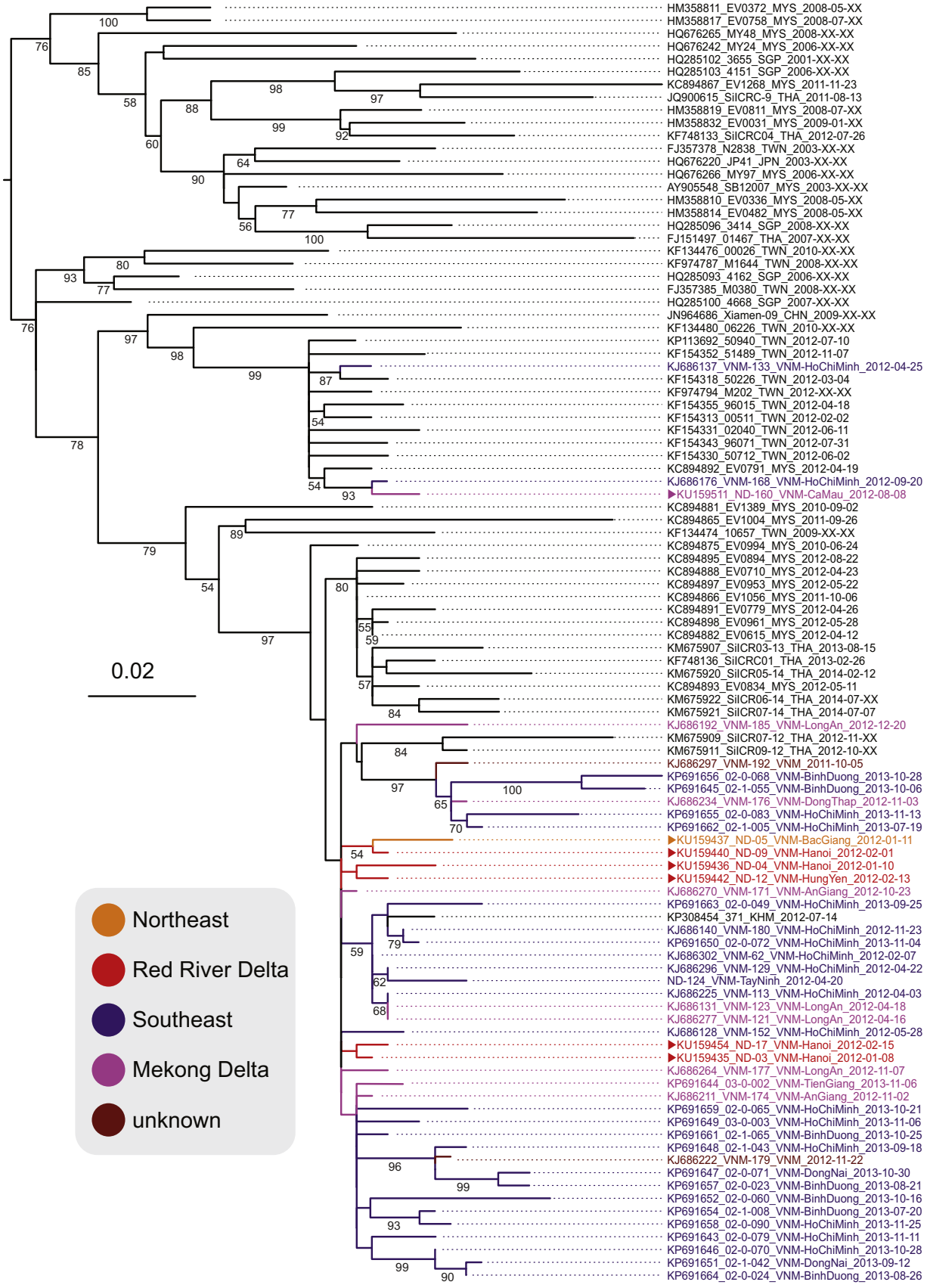

Fig. 4. The phylogenetic relationship of EV71 B5 subgenogroup viruses. The B5 tree was generated using the complete VP1 gene segment (891nts) using the maximum-likelihood method with GRT+G+I nucleotide substitution model and 1000 bootstrap replicates and nodes with values ≥50 are shown. The tree was midpoint rooted with the nodes ordered in a decreasing manner for clarity. Isolates are indicated by the GenBank Accession number_Strain_Country of isolation_Year of isolation. The country abbreviations are as follows: CHN-China, JPN-Japan, KHM-Cambodia, MYS-Malaysia, SGP-Singapore, THA-Thailand, TWN-Taiwan, VNM-Vietnam. Strains characterised in this are denoted by a ▸ symbol.

Northern provinces where C4 and B5 strains were circulating at similar frequencies. Our study also highlights the relative dominance of different lineages/sublineages in various Asian countries with frequent migration of EV71 strains between countries. For instance, China has acted as the epidemic source of C4 strains, whereas Malaysia and Taiwan were the source for B5 viruses. A greater understanding of the complex transmission of EV71 throughout South-East Asia and Vietnam in particular will help inform vaccination and pubic health strategies to decrease the burden of HFMD in the region.

Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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