



## Asymptomatic circulation of HEV71 in Norway

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Received 16 May 2006; received in revised form 25 July 2006; accepted 26 July 2006

Available online 11 September 2006

### Abstract

Widespread circulation of human enterovirus 71 was discovered in a prospective study of fecal samples obtained from healthy Norwegian children. Molecular characterization of the virus determined that it belonged to genotype C1. Complete sequencing of this strain, HEV71 804/NO/03, revealed differences in the 5'UTR and polymerase with respect to more pathogenic genotypes that may explain its reduced neurovirulence.

Published by Elsevier B.V.

**Keywords:** Enterovirus 71; Epidemiology; Molecular typing

Human enterovirus 71 (HEV71) is associated with outbreaks of hand-foot-and-mouth disease (HFMD), aseptic meningitis and encephalitis. Like poliovirus, HEV71 has affinity for anterior horn cells (Chumakov et al., 1979) and is the most common non-polio enterovirus associated with poliomyelitis-like paralysis (Melnick, 1984). Since its initial isolation in California in 1969, HEV71 has caused epidemics in Australia, Europe, Asia, and the United States (Palacios and Oberste, 2005). More recently, HEV71 caused brain-stem encephalitis during HFMD outbreaks in Malaysia, in 1997 (Cardosa et al., 2003; Chan et al., 2000) and in Taiwan in 1998 (Ho et al., 1999; Lin et al., 2003). The molecular epidemiology of HEV71 has been widely studied (Brown et al., 1999; Cardosa et al., 2003; Chu et al., 2001; Herrero et al., 2003; McMinn et al., 2001; Shimizu et al., 2004). There are two major HEV71 genogroups (B and C) co-circulating worldwide (the HEV71 prototype strain BrCr, isolated in 1969, is the only known example of genogroup A). Genogroups B and C have been subdivided into genotypes: B1–B5 and C1–C4, respectively (Brown et al., 1999; Cardosa et al., 2003; McMinn et al., 2001; Mizuta et al., 2005). Here we

report asymptomatic circulation of HEV71 in Norway. Phylogenetic analysis of VP1 sequences revealed a single circulating strain of genotype C1.

Stool samples were obtained on a monthly basis from 113 healthy infants (60 males, 53 females) in a prospective cohort study focused on environmental triggers of type 1 diabetes. New-borns (6 weeks old) were recruited at their first visit to health care centres. Stool samples and clinical data were obtained monthly from September 2001 to November 2003 (Cinek et al., 2006), beginning at age 3 months and continuing up to 28 months. Total nucleic acids were extracted and analyzed for human enterovirus (HEV) RNA using real-time PCR; 11.3% were positive (Cinek et al., 2006).

The serotype of the HEV positive samples (145/1255) were determined by VP1 nucleotide sequencing. HEV71 was detected in 16.8% (19/113) of the children in the cohort (10 boys and 9 girls, median age 14.0 months, 75% <18 months old). Positive samples were detected from children residing in the following counties: Akershus (south-east), Nord-Trøndelag (central) and Hordaland (west coast). This finding suggests a wide geographical distribution. VP1-2A sequences of HEV71 Norwegian strains (200–630 nt in length) were deposited in the GenBank under accession numbers DQ317216, DQ317217, DQ317218, DQ317219, DQ317220, DQ317221, DQ317222, DQ317223, DQ317224, DQ317225, DQ317226, DQ317227,

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DQ317228, DQ317229, DQ317230, DQ317231, DQ317232, DQ317233, DQ317234, DQ317235, DQ317236, DQ317237, and DQ317238.

HEV71 was found to be circulating widely in a restricted period of time, from October 2002 until October 2003, with peak prevalence in July 2003 (seven cases) (Fig. 1). HEV71 infection was not associated with fever, symptoms of upper respiratory or gastrointestinal complaints as reported by parents (Cinek et al., 2006).

A representative isolate of the Norwegian strain (HEV71 804/NO/03) was recovered from a suspension stool sample, identified with HEV71-specific antibody and titrated in green monkey kidney cells (GMK-AH1) and in a human cervical adenocarcinoma cell line (HeLa). Viral RNA was extracted; reverse transcribed and amplified using primers

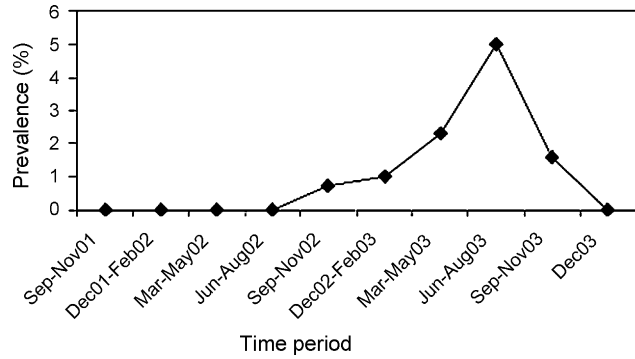


Fig. 1. Prevalence of HEV71 in stool samples from 113 asymptomatic children over the period September 2001 through December 2003.

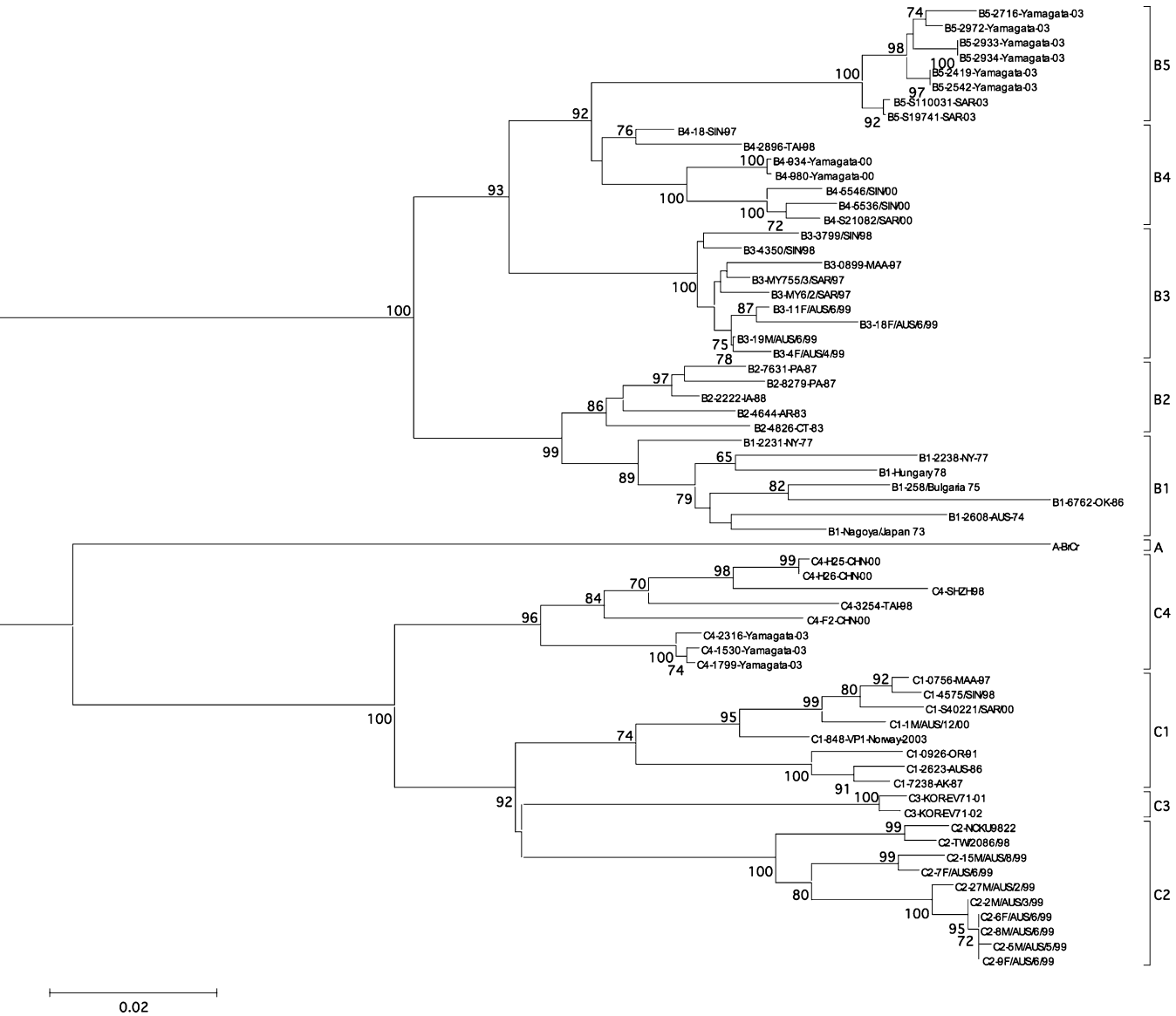


Fig. 2. Phylogenetic relationships of the sequence of the complete VP1 gene of HEV71 804/NO/03. The evolutionary distances were calculated using the Kimura 2-parameter model as a model of nucleotide substitution and the Neighbour-Joining method to reconstruct the phylogenetic tree (MEGA version 3.1 software package). Numbers above the branches are bootstrap values (percentage of 1000 pseudo-replicate datasets) supporting each cluster. Genogroups A, B and C and their respective sublineages are denoted in the figure. The scale bar represents the genetic distance (nucleotide substitutions per site).

Table 1  
Number of hospitalizations or outpatient visits in the age group 0–3 years old associated with a diagnosis of encephalitis, HFMD and herpangina in the period 1999–2004

Diagnosis	ICD-10 code <sup>a</sup>	1999	2000	2001	2002	2003	2004	Total
Enteroviral encephalitis	A85.0+	0	0	2	0	0	0	2
Other viral encephalitis, not elsewhere classified	A85	3	6	1	6	5	10	31
Unspecified viral encephalitis	A86	8	7	7	8	10	5	45
Hand, foot and mouth disease (HFMD)	B08.4	5	4	25	14	9	8	65
Herpangina	B08.5	0	1	2	4	8	5	19
Total	18	24	46	35	34	28 <sup>b</sup>	239	

<sup>a</sup> ICD, international classification of diseases.

<sup>b</sup> One case is counted twice because it was associated with two diagnoses (A85.0 + B08.5).

designed on the basis of previously published HEV71 strains (Appendix A). High Fidelity PCR Master Mix (Roche) reagents were utilized to minimize introduction of mutations during amplification. Products were cloned into pGEM-T-Easy vector (Promega). Sequencing was performed on both strands, using Big Dye terminator cycle sequencing reagents and the ABI 3730 XL Sequencer (Applied Biosystems, Foster City, CA). Raw sequence data was analyzed with Sequencher (version 4.2, Gene Codes Corporation, Ann Arbor, MI). Ambiguous nucleotides were resolved by re-sequencing. To avoid introduction of mutations by cell culture adaptation, new primers were designed based on the sequence obtained, and direct amplification and sequencing was performed to obtain the HEV71 sequence directly from the stool sample. The complete genome sequence of the Norwegian HEV71 strain was deposited in the GenBank under the accession number DQ452074.

The complete VP1 sequence of the isolate and 64 reference sequences collected from GenBank (Appendix B) were used to reconstruct the phylogenetic tree (Fig. 2) employing neighbor joining and the Kimura model of nucleotide substitution in the program MEGA (version 3.1) (Kumar et al., 2004). The statistical significance was evaluated by bootstrap re-sampling of the sequences 1000 times. The Norwegian strain was classified into genotype C1. Phylogenetic analysis of partial VP1 nucleotide sequences from HEV positive samples revealed that a single strain of HEV71 was circulating in Norway (data not shown).

We sought to investigate if the circulation of this strain was associated with an increase in the frequency of hospitalizations related to HEV71 infection. Clinical records of HFMD, herpangina and encephalitis were obtained from the Norwegian Surveillance System for Communicable Diseases and the Norwegian Patient Register. First, we focused in the analysis of cases

Table 2  
Comparative nucleotide sequence analysis of the HEV71 804/NO/03 and representative HEV71 strains of different lineages

Gene/region	Percentage of nucleotide identity between HEV71 804/NO/03 and					
	C2 Tainan/5746/98	C4 SHZH03	B2 MS/7423/87	B4 5865/SIN/00	A BrCr	CAV16-G10
5'UTR	91.2	85.8	83.5	83.5	81.8	79.9
P1 region						
VP4	85.0	86.5	82.6	83.6	83.1	65.7
VP2	89.2	87.0	84.4	82.5	81.5	68.2
VP3	88.7	88.8	81.3	81.8	82.4	71.6
VP1	90.2	89.9	83.8	83.2	82.8	64.2
P2 region						
2A	85.3	82.4	81.6	79.3	77.3	79.6
2B	86.9	75.1	74.7	74.7	76.1	79.8
2C	91.0	79.4	78.5	78.7	81.2	80.0
P3 region						
3A	89.9	74.0	75.2	75.6	79.1	75.6
3B	89.4	75.8	77.3	74.2	74.2	75.8
3C	90.2	76.5	75.8	75.0	74.7	77.4
3D	89.6	77.0	79.1	79.4	78.1	79.0
3'UTR	92.9	77.4	91.7	90.5	95.2	78.6
Overall	89.0	82.0	81.0	80.0	80.0	75.0

Nucleotide sequences of HEV71 reference strains were retrieved from GenBank (accession numbers AF304457, AY465356, U22522, AF316321, U22521 and U05876).

th EV71 diagnosis. Only one case of encephalitis was reported in children below the age of 3 years in 2003; no cases of herpangina or HFMD were reported. In other age groups, the virus was also barely detected: 1 case of HFMD in 2002, 3 in 2003. In previous years, there was one case with HFMD in 2001, none in 2000, and 3 or 4 cases with encephalitis along with 6 cases of HFMD in 1999. Since only a small proportion of HEV detected in clinical samples were serotyped, it is possible that some cases of symptomatic HEV71 infection were not detected. However, there was no increase in the numbers of hospitalizations of patients with encephalitis, HFMD or herpangina recorded during the period of this study in the same age range (Table 1). Thus, we conclude that the majority of HEV71 infections were either asymptomatic or associated with only mild disease.

The comparison of genomic sequences of HEV71 strains derived from fatal and non-fatal cases have been used to investigate the presence of neurovirulent determinants (AbuBakar et al., 1999; McMinn et al., 2001; Shih et al., 2000; Siafakas et al., 2005; Yan et al., 2001). Whereas genotype C1 epidemics in Malaysia, Singapore and Western Australia have been associated primarily with HFMD (McMinn et al., 2001), genotype C2 outbreaks in Malaysia and Taiwan have been associated with severe and fatal neurologic disease (Cardosa et al., 2003; Herrero et al., 2003; McMinn et al., 2001). The differences in neurovirulence between the C1 and C2 genotypes observed in these outbreaks may provide clues to EV71 pathogenicity determinants (McMinn, 2002). However, there is reason for caution in rendering this interpretation given a recent report wherein genotype C2 outbreaks in Japan were associated only with HFMD (Iizuta et al., 2005).

We compared the entire genome of HEV71 804/NO/03 with the HEV71 prototype BrCr (genotype A), HEV71 MS/73 (genotype B2), HEV71 5865/SIN/00 (genotype B4), HEV71 SHZ03 (genotype C4), Tainan/5746/98 (genotype C2) and CAV16 prototype G10. Overall, HEV71 804/NO/03 shared 89% nucleotide identity with the highly virulent C2 strain Tainan/5746/98 and 81% identity with other HEV71 genotypes, while the identity with CAV16 was 75% (Table 2). Non-structural genes are typically better conserved than structural genes. Indeed, enteroviruses are typed using the structural gene VP1. Interestingly, analysis of HEV71 804/NO/03 and other EV71 strains revealed higher homology in P1 region than in the non-structural genes P2 and P3 regions. The exception was C2-Tainan/5746/98 where close to 100% homology to HEV71 804/NO/03 was observed throughout the genome. Table 3 indicates amino acid differences between HEV71 804/NO/03 and the C2-consensus sequence. Interestingly, most of the changes are located at non-structural sites (30 out of 37, 81.1%; rate of amino acid substitution of structural genes:  $0.011 \pm 0.002$ ; non-structural genes:  $0.028 \pm 0.015$ ) and the higher number in the polymerase gene (11 out of 37, 29.8%). With the exception of the mutations in the polymerase described above, we could not find any relation between these changes and changes affecting pathogenesis or viral replication. However, only a reverse genetics based model of these changes will allow prediction of their function, provided that the hypothesis of differences in neurovirulence between C2 and C1 genotypes is correct.

Table 3

Amino acid differences between the genomes of HEV71 804/NO/03 and the C2-consensus sequence

Position	Coding region	C1-Norway-804-2003	C2-consensus <sup>a</sup>
195	VP2	I	V
293	VP2	F	Y
354	VP3	H	T
416	VP3	N	S
587	VP1	Q	R
596	VP1	N	D
854	VP1	T	A
928	2A	S	N
930	2A	R	M
944	2A	V	I
945	2A	Y	F
946	2A	I	V
1042	2B	H	S
1053	2B	R	K
1095	2B	I	V
1187	2C	L	M
1282	2C	V	I
1368	2C	D	E
1406	2C	S	R
1427	2C	S	N
1430	2C	A	V
1479	3A	E	D/V <sup>b</sup>
1487	3A	T	S
1496	3A	S	N
1581	3C	H	R
1603	3C	R	K
1806	3D	R	K
1828	3D	I	M
1859	3D	R	K
1928	3D	T	A
1936	3D	V	I
1992	3D	E	D
2077	3D	K	R
2127	3D	K	R
2141	3D	N	S
2167	3D	T	A
2182	3D	F	Y

<sup>a</sup> Variations observed between HEV71 804/NO/03 and majority (>66%) of C2 full genome sequences (AF304457, AF304458, AF304459, AF136379, AF176044, AF119796 and AF119795).

<sup>b</sup> Five strains had a E → D change. Two (AF119796 and AF119795) had a E → V mutation.

The potential secondary structure of the 5' untranslated region (5'UTR) was modeled in an attempt to elucidate the existence of structural elements and motifs associated with neurovirulence within the EV71 genome. Neurovirulent phenotype determinants have been localized to the enterovirus 5'UTR (De Jesus et al., 2005; Evans et al., 1985; Gromeier et al., 1996, 1999; Rinehart et al., 1997; Romero and Rotbart, 1995; Szendroi et al., 2000). The highly structured internal ribosome entry site (IRES) of the 5'UTR contains seven stem-loop structural domains (I–VII) which are essential for cap-independent initiation of translation and replication. Mutational studies of the loop sequence and the putative stem structure immediately 5' to the loop in domain V have indicated the importance of this region to virus transcription and initiation of translation (Borman et al., 1994; Nicholson et al., 1991). Mutations within domain V

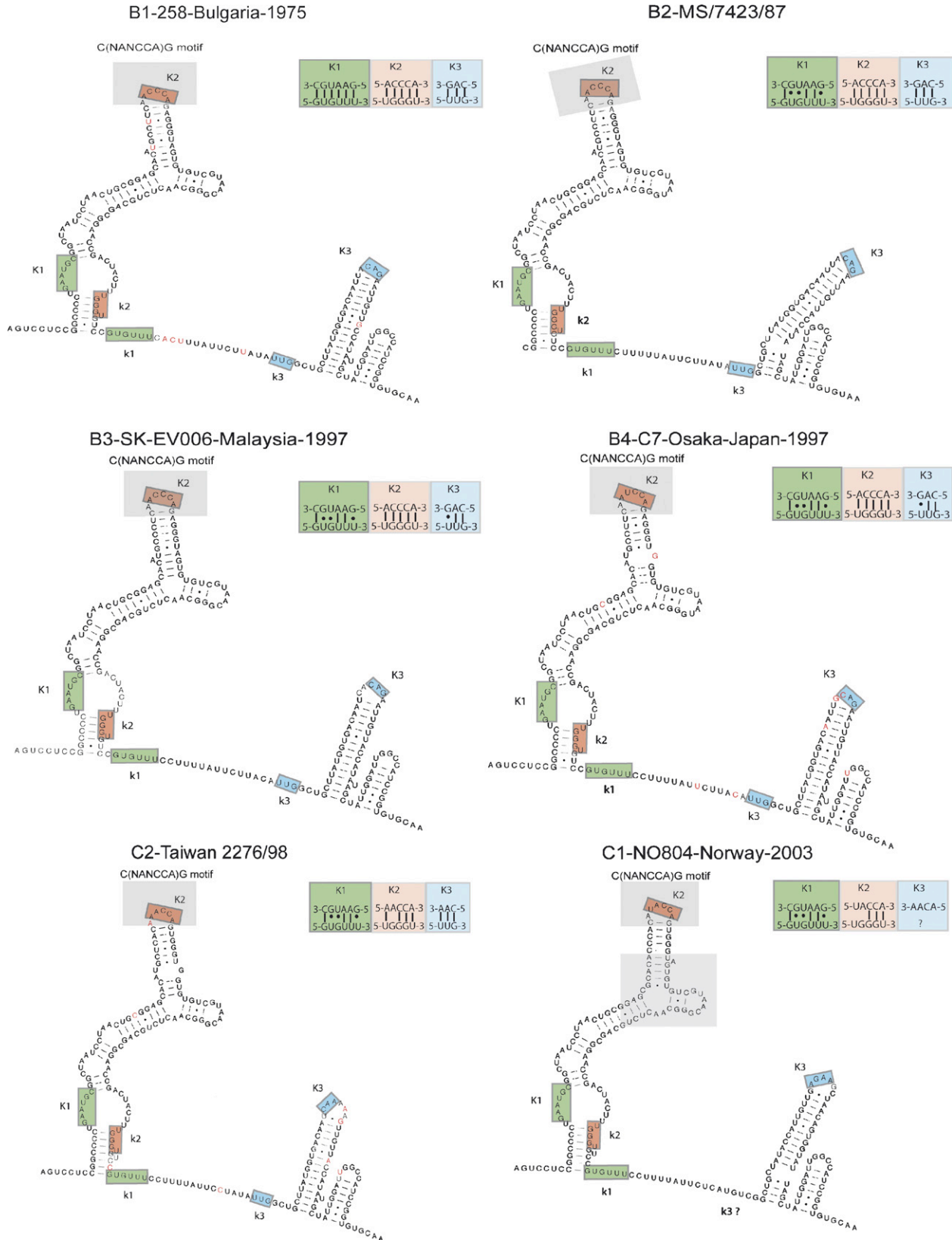


Fig. 3. Predicted RNA secondary structures of IRES domains V, VI and VII of B1-258-Bulgaria-1975 (AB059821), B2-MS/7423/87 (U22522), B3-SK-EV006-Malaysia-1997 (AB059826), B4-C7-Osaka-Japan-1997 (AB059825), C2-Taiwan 2276/98 (AF117634) and HEV71 804/NO/03. RNA structures were predicted based on the lowest free energy, using the Zuker algorithm as implemented in RNA structure (version 3.71). The three protrusions depicted for each viral RNA structure corresponds from the left to right to domains V, VI and VII. Conserved motifs are highlighted in color. Nucleotide positions found to be non-conserved in an alignment of all available sequences of the region are highlighted in red. Conserved tertiary “K” motifs are also denoted in the figure. K and k motifs paired by their numbers align together in the tertiary folding structure of the IRES.

the genome of poliovirus (PV) Sabin attenuated strains (at 480, 481 and 472 for Sabin 1, 2 and 3, respectively), act as temperature sensitive (ts) determinants and as major determinants of attenuation. The corresponding area containing those

mutations is the central loop (shaded in grey for genotype C1 in Fig. 3). In addition, a temperature sensitive mutant of HEV71-BrCr, including a U to C mutation at position 491, resulted in a marked reduction in virus growth (Arita et al., 2005). Interest-



Fig. 4. Phylogenetic relationships of the sequence of the domains V, VI and VII of HEV71 804/NO/03 and all available 5'UTR sequences. The evolutionary distances were calculated using the Kimura two-parameter model as a model of nucleotide substitution and the Neighbour-Joining method to reconstruct the phylogenetic tree (MEGA version 3.1 software package). Numbers above the branches are bootstrap values (percentage of 1000 pseudo-replicate datasets) supporting each cluster. Polished 5'UTR sequences were considered along their associated clinical outcome: (●) represent sequences associated with fatal outcome; (◐) central nervous system involvement; (○) herpangina or HFMD; (●) asymptomatic.

ingly, a series of conserved covariant mutations in this domain in the Norwegian C1 strain resulted in a slight shortening of the central loop that clearly differentiated this genotype from all other known genotypes (Fig. 3) (multiple 5'UTR sequences were obtained to confirm this finding). Other genotype C1 sequences also had this modification in the structure of the central loop (data not shown).

A 5'UTR secondary structure feature known to be highly conserved amongst the HEV is the C(NANCCA)G motif (loop in

parenthesis) in the secondary structure domain V (Siafakas et al., 2005). The first A in this motif is mutated to U in HEV71 804/NO/03. The changes observed in domain V might have direct impact on the tertiary folding structure of the IRES and its interaction with the 18S ribosomal RNA. Tertiary structure elements occur in the 3' end of a segment termed a “ribosome landing pad”. Those structures, identified as K1, K2 and K3 (Fig. 3), involve highly conserved sites in enteroviruses and rhinoviruses (Nicholson et al., 1991). K2 is suggested as a potential

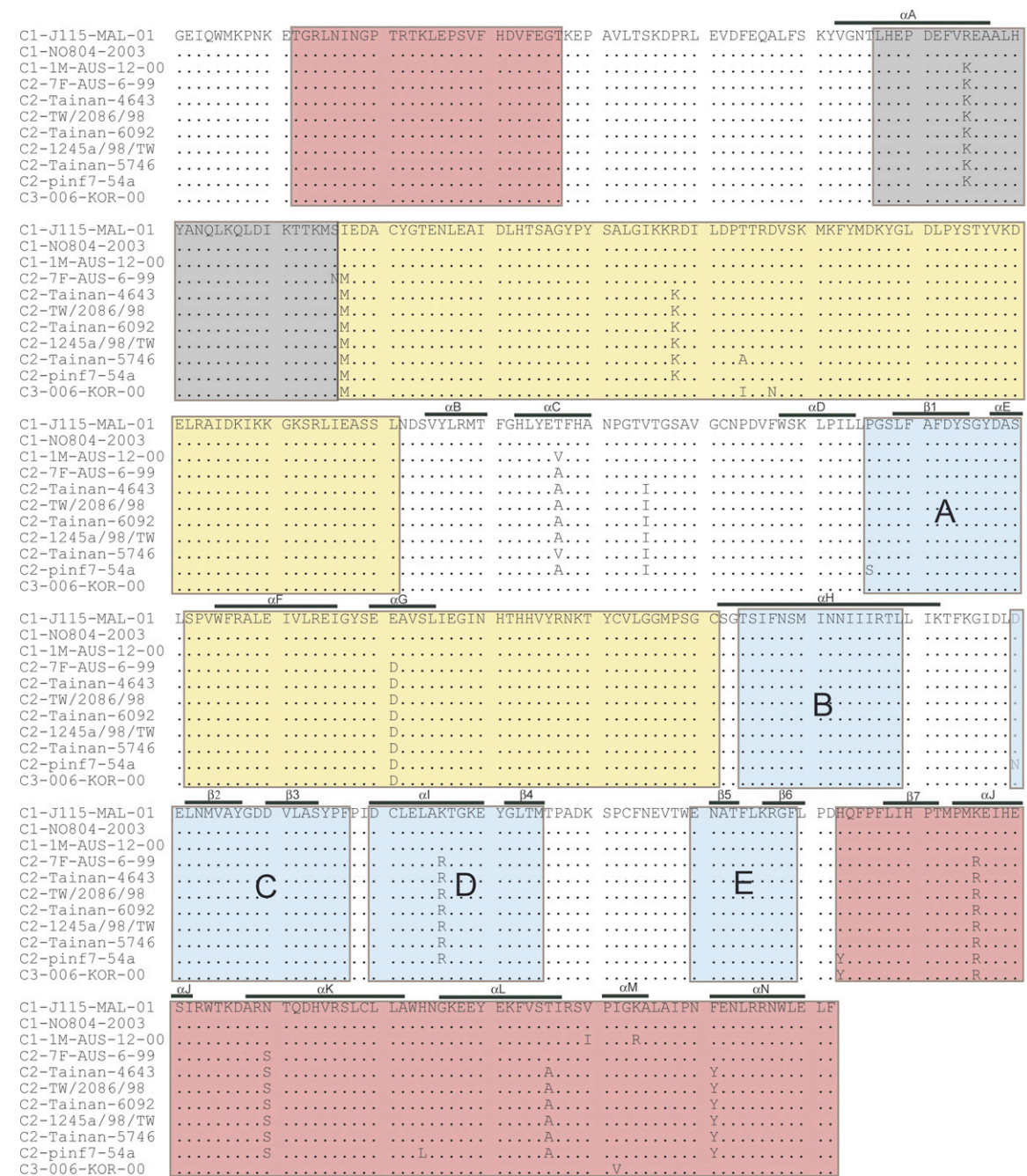


Fig. 5. Structural alignment of available HEV71 RNA-dependent RNA polymerase (3Dpol) sequences of genogroup C. Conserved residues are indicated (\*). The thumb subdomain is highlighted in grey and pink. The grey area appears beneath the fingers subdomain in the crystal structure of the polymerase. The finger subdomains are highlighted in yellow. The palm subdomains are highlighted in light blue. Black bars above the alignment represent alpha or beta helix structures.

binding site for the interaction between 40S ribosomes and/or transacting factor(s) and viral mRNAs. The tertiary folding structure K2 is theoretically destroyed by the A → U mutation of the C(NANCCA)G motif (Fig. 3) of HEV71 804/NO/03. The proposed K3 tertiary folding structure would also be theoretically destroyed by mutations of these conserved sites in HEV71 804/NO/03.

Interestingly, although the 5'UTRs of EV normally do not carry sufficient phylogenetic information to allow discrimination among serotypes (Casas et al., 2001), the analysis of all available HEV71 sequences for the domains V, VI and VII of the 5'UTR (Fig. 4) correlates with the genotype clustering of the VP1 gene (Fig. 2). Furthermore, the consensus predicted RNA secondary structure of the 5'UTR represented in Fig. 3 for each of the genotypes is conserved in all available sequences for each genotype (variable sites highlighted in red in the figure). The functional importance of this is evident at the RNA secondary structure of the 5'UTR, and especially the central loop modification in C1 (that clearly differentiates the C1 genotype from all other known genotypes) appears to correlate with clinical outcome. All 16 cases with C1 structure had a benign course; 26 of 57 cases with C2 structure presented with neurological disease; 17 of the 26 died. Three of 12 cases with B1–B2 structure had neurologic disease; 2 of 3 died. One of 4 cases with B3 structure had fatal neurologic disease. Seventeen of 41 cases with B4 structure had neurologic disease; 4 of the 17 had a fatal outcome (Fig. 4).

The crystal structure of the poliovirus RNA-dependent RNA polymerase (3Dpol) displays the characteristic “palm”, “finger”, and “thumb” subdomains, analogous to a right hand (Lanssen et al., 1997). Within the palm subdomain of the polymerase is the RNA recognition motif (RRM) characteristic of RNA-dependent RNA polymerases. The palm subdomain contains motifs A–E present in many polymerases (for review see O'Reilly and Kao, 1998). Eleven amino acid changes were observed when comparing the 3Dpol gene between HEV71 804/NO/03 and other C2 strains (see Table 3). While the palm-motifs A, B, C and E were conserved, a K → R substitution in C2 strains was observed in the alpha region of palm-motif D (Fig. 5). Two substitutions were also observed in the palm domain outside the palm-motifs. Four substitutions were observed in the thumb domain in positions involved in the alpha helix regions that are the core of the domain and three substitutions were observed in the finger domain. However, although structure prediction algorithms do not suggest that these changes modify the folding of the protein, we cannot exclude functional effects.

The finger and thumb subdomains of the polymerase play a role in modulating substrate recognition and proteolytic processing by the 3CD peptide. These subdomains have been implicated in oligomerization of the enzyme, a process that is critical to substrate binding and elongation of polypeptide chain. The crystal structure has revealed that 3Dpol molecules oligomerize along two interfaces: I and II. Interface-mediated dimerization (or oligomerization) has been pro-

posed to promote RNA binding. Interface II is formed largely by N-terminal peptide regions of the polymerase, of which only residues 13–37 (thumb) and 67–97 (beneath the finger domain) structural foldings are known. Given the unusual position of the 67–97 fragment in that interface of the crystal structure, it has been proposed that this N-terminal strand of the active polymerase is donated in trans after dimerization and is necessary for polymerase activity. A Y73H substitution of the Sabin strain of poliovirus 1 has been postulated as a neurovirulence determinant in poliovirus by impairing this dimerization (Paul et al., 2000). Indeed, substitutions in the amino acid position 73 (together with a change in position 363) of the HEV71-BrCr polymerase resulted in an attenuated neurological phenotype in a monkey model of infection (Arita et al., 2005). The HEV71 804/NO/03 polymerase presents an R to K substitution at position 75. Interestingly, the cynomolgous macaque model of HEV71 neurological infection demonstrates that the dual presence of 3Dpol substitutions and changes in the domain V of the 5'UTR increase the level of attenuation (Arita et al., 2005).

Our prospective study of HEV infection in Norwegian children over the period of September 2001 to November 2003 revealed the appearance of HEV71 in October 2002 and an incidence rate of HEV71 infection of 0.27 persons-years thereafter. Given a population at risk of approximately 180,000 (the number of infants below 3 years of age during the period of assessment), there may have been as many as 48,600 infants infected. The lack of any increase in neurological disease strongly suggests that the virus had low pathogenicity. It is striking therefore, that whereas all reports of C1 genotype infection in Asia are associated with HFMD with or without neurologic involvement, infection in Norway appears to be asymptomatic. The absence of disease in Norwegian children may reflect host factors such as genetic resistance, cross-reactive immunity, hygiene and nutritional status. However, viral factors are also plausible. There are no published sequences for the 5'UTR of C1 strains circulating in the US, Malaysia and Australia; nonetheless, direct comparison of sequences of C1 strains in Japan and Norway defines two clades (Fig. 4). Whether the absence of disease in Norwegian children reflects intrinsic viral properties or host factors remains to be determined.

## Acknowledgements

This study was supported by grants from the National Institutes of Health (AI55466, AI51292, UO1 NS047537), Norwegian Research Council (155300/320) and Ministry of Education of the Czech Republic (grant no. MSM0021620814). Environmental triggers of type 1 diabetes: the MIDIA study is supported by the Norwegian Research Council (135893/330, 156477/730). We are grateful to Lars Christian Stene at the Norwegian Institute of Public Health for guidance in statistical calculations and to Gabriel Ånestad for information on the clinical data registries.

**Appendix A. Oligonucleotide primers used for amplification of HEV71**

Primer (orientation)	5'–3' sequence	Region	Position <sup>a</sup>
ENV71-sp-1 (F)	TTAAACAGCTGTGGGTGCACCCAC	5'UTR	1–26
EV167A (F)	CAAGCACTTCTGTATCCCCG	5'UTR	168–187
EV580B (R)	ATTGTCACCATAAGCAGCCA	5'UTR	584–603
ENV71-1510 (R)	TTGTTGGTCCYCARATTAATCCACTGRTGTGGGCA	VP2	1485–1519
ENV71-sp-1345 (F)	GAGTATGTCATTGGGACATGGCAGG	VP2	1338–1363
ENV71-sp-1809 (R)	GGTATRTGRATACACGGGTGGGGTG	VP3	1800–1825
ENV71-sp-1809 (F)	CACCCACCCGTGTATYCAATACC	VP3	1800–1825
ENV71-sp-2250 (R)	TGCGCTCTGTAGTGAGTGTGCTGATCCATGG	VP3	2223–2254
ENV71-sp-2231 (F)	CCATGGATCAGYAACTCAYTACAG	VP3	2223–2248
ENV71-sp-2250 (F)	CCATGGATCAGCAACTCACTACAGAGCGCA	VP3	2223–2254
VP1-2S-ENV71 (F)	CARTAYATGTTTGTICCCSCCYGG	VP1	2895–2917
HEV71-sp-2921 (F)	CCAAGCCAGACTCCAGAGAA	VP1	2923–2942
ENV71SP (F)	GCACAGGTYTCIGTICCRITYATGTC	VP1	3003–3028
VP1-2A-ENV71 (R)	TCACAACCYTGRGCRGTGGTAGA	2A	3462–3484
ENV71-3497 (F)	TGTAATTGTCAGACAGGGGTGTAT	2A	3498–3521
ENV71-4430 (R)	CATACAGGTTCAATACGGTGTGCTCTTGAAGTGC	2C	4415–4450
ENV71-4405 (F)	TAATTACATGCAGTTCAAGAGCAA	2C	4407–4430
ENV71-4958 (R)	TGTATCTCACCTTGGACTSCTATC	2C	4959–4983
ENV71-4958 (F)	GATAGSAAGTCCAAGGTGAGATACA	2C	4959–4983
ENV71-4405 (R)	TTTGCTCTTGAAGTGCATGTAATTA	2C	4407–4430
ENV71-4409 (R)	TGCTCTTGAAGTGCATGTAATTATTCAT	2C	4401–4428
ENV71-5604 (R)	TTAGTATCAAGCGTTACCAGTGTGA	3C	5605–5629
ENV71-5604 (F)	TCACACTGGTAACTCTTGATACTAA	3C	5605–5629
ENV71-5790 (R)	GTTGGGAAATTGTACATCATAGTCC	3C	5791–5812
ENV71-5664 (F)	AAACAATTAGTCTGCTAGTGATGC	3C	5665–5689
ENV71-6513 (R)	CCAAATGTCATTCTCAAGTACTCTG	3D	6490–6514
ENV71-6655 (F)	TATGAYGCTAGYCTYAGYCCIGTGTGGTTCAG	3D	6648–6679
RPOL-1S (F)	YGARGCIWSIAGYYTIAAYGA	3D	6467–6487
RPOL-1A (R)	AWRTTRTRTATCATWGARTTRAAIAT	3D	6825–6850
EV7408B (R)	GCTATTCTGGTTATAACAAA	3'UTR	7392–7411

<sup>a</sup> Relative to the genome of HEV71/enterovirus 5865/sin/000009 (GenBank accession number AF316321).

**Appendix B. Clinical isolates used in phylogenetic analysis of the complete VP1 gene of HEV71**

Reference	Strain name	Year	Origin	Outcome <sup>a</sup>	Group	Nucleotide database accession no.
Brown and Pallansch (1995)	BrCr-CA-70	1970	USA	Encephalitis	A	U22521
Shimizu et al. (1999)	Nagoya/Japan 73	1973	Japan	NA	B1	AB059813
Brown et al. (1999)	2604-AUS-74	1974	Australia	Meningitis	B1	AF135883
Chumakov et al. (1979)	258/Bulgaria 75	1975	Bulgaria	Polio-like	B1	AB059814
Brown et al. (1999)	2238-NY-77	1977	USA	NA	B1	AF135876
Brown et al. (1999)	2231-NY-77	1977	USA	NA	B1	AF135870
Nagy et al. (1982)	Hungary 78	1978	Hungary	Polio-like	B1	AB059815
Brown et al. (1999)	4644-AR-83	1983	USA	NA	B2	AF135896
Brown et al. (1999)	4826-CT-83	1983	USA	NA	B2	AF135897
Brown et al. (1999)	6762-OK-86	1986	USA	NA	B1	AF135900
Brown et al. (1999)	2623-AUS-86	1986	Australia	HFMD	C1	AF135945
Brown et al. (1999)	7631-PA-87	1987	USA	Gastroenteritis	B2	AF009533
Brown et al. (1999)	8279-PA-87	1987	USA	NA	B2	AF009537
Brown et al. (1999)	7238-AK-87	1987	USA	Rash	C1	AF135952
Brown et al. (1999)	2222-IA-88	1988	USA	Fever	B2	AF009540
Brown et al. (1999)	0926-OR-91	1991	USA	NA	C1	AF009548
McMinn et al. (2001)	MY755/3/SAR/97	1997	Sarawak	HFMD	B3	AF376076
McMinn et al. (2001)	MY62/SAR/97	1997	Sarawak	ACS	B3	AF376075
Herrero et al. (2003)	0899-MAA-97	1997	Malaysia	Meningitis	B3	AY207642
Singh et al. (2000)	18-SIN-97	1997	Singapore	AFP	B4	AF251359
Brown et al. (1999)	0756-MAA-97	1997	Malaysia	NA	C1	AF135935
McMinn et al. (2001)	3799/SIN/98	1998	Singapore	HFMD	B3	AF376117
McMinn et al. (2001)	4350/SIN/98	1998	Singapore	HFMD	B3	AF376119
Li et al. (2005)	2896-TAI-98	1998	Taiwan	NA	B4	AF286516
McMinn et al. (2001)	4575/SIN/98	1998	Singapore	HFMD	C1	AF376120
Yan et al. (2000)	NCKU9822	1998	Taiwan	Fatal	C2	AF136379

## Appendix B (Continued)

Reference	Strain name	Year	Origin	Outcome <sup>a</sup>	Group	Nucleotide database accession no.
Chen et al. (2000)	TW/2086/98	1998	Taiwan	Benign	C2	AF119796
Chen et al. (2005)	3254-TAI-98	1998	Taiwan	NA	C4	AF286531
Cardosa et al. (2003)	SHZH98	1998	China	NA	C4	AF302996
McMinn et al. (2001)	11F/AUS/6/99	1999	Australia	Meningitis	B3	AF376089
McMinn et al. (2001)	18F/AUS/6/99	1999	Australia	HFMD	B3	AF376095
McMinn et al. (2001)	19M/AUS/6/99	1999	Australia	HFMD	B3	AF376096
McMinn et al. (2001)	4F/AUS/4/99	1999	Australia	GBS	B3	AF376105
McMinn et al. (2001)	7F/AUS/6/99	1999	Australia	Meningitis	C2	AF376108
McMinn et al. (2001)	15M/AUS/8/99	1999	Australia	HFMD	C2	AF376092
McMinn et al. (2001)	27M/AUS/2/99	1999	Australia	HFMD	C2	AF376102
McMinn et al. (2001)	2M/AUS/3/99	1999	Australia	Myelitis	C2	AF376103
McMinn et al. (2001)	6F/AUS/6/99	1999	Australia	Encephalitis	C2	AF376107
McMinn et al. (2001)	8M/AUS/6/99	1999	Australia	Myelitis	C2	AF376109
McMinn et al. (2001)	5M/AUS/5/99	1999	Australia	Meningitis	C2	AF376106
McMinn et al. (2001)	9F/AUS/6/99	1999	Australia	Ataxia	C2	AF376110
McMinn et al. (2001)	S21082/SAR/00	2000	Sarawak	HFMD	B4	AF376084
Ng et al. (2002)	5865/sin/000009	2000	Singapore	Fatal	B4	AF316321
Ng et al. (2002)	5666/sin/002209	2000	Singapore	Fatal	B4	AF352027
Yamagata et al. (2005)	934-Yamagata-00	2000	Japan	HFMD	B4	AB177809
Yamagata et al. (2005)	980-Yamagata-00	2000	Japan	HFMD	B4	AB213624
McMinn et al. (2001)	S40221/SAR/00	2000	Sarawak	HFMD	C1	AF376087
McMinn et al. (2001)	1M/AUS/12/00	2000	Australia	HFMD	C1	AF376098
Shimizu et al. (2004)	F2-CHN-00	2000	China	NA	C4	AB115491
Shimizu et al. (2004)	H25-CHN-00	2000	China	NA	C4	AB115492
Shimizu et al. (2004)	H26-CHN-00	2000	China	NA	C4	AB115493
Cardosa et al. (2003)	KOR-EV71-01	2001	Korea	NA	C3	AY125966
Cardosa et al. (2003)	KOR-EV71-02	2002	Korea	NA	C3	AY125967
Yamagata et al. (2005)	2542-Yamagata-03	2003	Japan	HFMD	B5	AB177815
Yamagata et al. (2005)	2716-Yamagata-03	2003	Japan	HFMD	B5	AB177816
Yamagata et al. (2005)	2419-Yamagata-03	2003	Japan	HFMD	B5	AB213647
Yamagata et al. (2005)	2933-Yamagata-03	2003	Japan	HFMD	B5	AB213648
Yamagata et al. (2005)	2934-Yamagata-03	2003	Japan	HFMD	B5	AB213649
Yamagata et al. (2005)	2972-Yamagata-03	2003	Japan	HFMD	B5	AB213650
Yamagata et al. (2005)	S110031-SAR-03	2003	Sarawak	NA	B5	AY258307
Yamagata et al. (2005)	S19741-SAR-03	2003	Sarawak	NA	B5	AY258313
Yamagata et al. (2005)	1530-Yamagata-03	2003	Japan	HFMD	C4	AB213638
Yamagata et al. (2005)	1799-Yamagata-03	2003	Japan	HFMD	C4	AB213641
Yamagata et al. (2005)	2316-Yamagata-03	2003	Japan	HFMD	C4	AB213644

NA, not available; CNS, central nervous system; AFP, acute flaccid paralysis; ACS, acute cardiogenic shock; GBS, Guillain-Barré syndrome.

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