Evolutionary trajectory of the VP1 gene of human enterovirus 71 genogroup B and C viruses

Sabine van der Sanden,1,2 Harrie van der Avoort,1 Philippe Lemey,3 Gökkhan Uslu1 and Marion Koopmans1,2

1National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2Erasmus Medical Center, Department of Virology, Rotterdam, The Netherlands
3Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium

From 1963 to 1986, human enterovirus 71 (HEV71) infections in the Netherlands were successively caused by viruses of subgenogroups B0, B1 and B2. A genogroup shift occurred in 1987, after which viruses of subgenogroups C1 and C2 were detected exclusively. This is in line with HEV71 typing in Australia, Europe and the USA, but is distinct from that in the Asian Pacific region, where HEV71 subgenogroups B3–B5 and C4–C5 have caused large outbreaks since 1997. To understand these observations in HEV71 epidemiology, the VP1-encoding regions of 199 HEV71 strains isolated in the Netherlands between 1963 and 2008 were used to study the detailed evolutionary trajectory and population dynamics of HEV71. Genogroup B viruses showed an epochal evolution, whereas genogroup C viruses evolved independently, which is in line with the co-circulation of C1 and C2 viruses in the Netherlands since 1997. Considering that strains from the Netherlands are interspersed phylogenetically with GenBank reference strains, the evolution of B1–B2, C1–C2 viruses has a global nature. Phylodynamic analysis confirmed that increased reporting of HEV71 infections in 1986 and 2007 reflected true epidemics of B2 and C2 viruses, respectively. Sequence analysis of the complete capsid region of a subset of isolates revealed several (sub)genogroup-specific residues. Subgenogroup B2-specific rabbit antiserum showed cross-neutralization of B0, B1 and B2 viruses, but not of subgenogroup C1 or C2 viruses, probably explaining the global shift to genogroup C in 1987 following a B2 epidemic. Anti-C1 rabbit serum neutralized both genogroup B and C viruses. Global herd immunity against C1 and C2 viruses possibly explains why epidemics with subgenogroups B4 and C4 are restricted to the Asian Pacific region.

INTRODUCTION

Human enterovirus 71 (HEV71) belongs to the species Human enterovirus A of the genus Enterovirus of the family Picornaviridae, and is the major causative agent of hand, foot and mouth disease (HFMD) (usually in children aged <5 years). In <1% of HEV71 infections, the virus is associated with neurological disease, including aseptic meningitis, poliomyelitis-like paralysis, brainstem encephalitis and neurogenic pulmonary oedema (Cardosa et al., 2003; Perez-Velez et al., 2007; Wang & Liu, 2009).

On the basis of VP1 nucleotide sequence comparisons, three genogroups, designated A, B and C, have been defined in previous studies; these show a genetic divergence of about 17% at the nucleotide level (Brown et al., 1999; McMinn et al., 2001). Genogroup A includes only one strain (BrCr-CA-70), which was isolated in California in 1970 (Brown et al., 1999). Genogroups B and C are reported more commonly and consist of subgenogroups B0–B5 and C1–C5, respectively (Brown et al., 1999; Chu et al., 2001; Li et al., 2005; McMinn et al., 2001; Mizuta et al., 2005; Tu et al., 2007; van der Sanden et al., 2009).

Strains of HEV71 isolated in the Netherlands from 1963 to 1986 successively belonged to subgenogroups B0, B1 and B2 (van der Sanden et al., 2009). Viruses belonging to the B2 subgenogroup were associated with increased reports of hospitalization in 1986, with meningitis and encephalitis as major clinical symptoms. In 1987, a switch of genogroup from B to C occurred and, from that time onward, all isolates of HEV71 belonged to subgenogroup C1 (1987–1997 and 2000–2007) and subsequently C2 (1997–2002 and...
2004–2007). The emergence of C2 was again associated with a peak in reporting of hospitalization in 2007. Subgenogroups B1, B2, C1 and C2 circulated more or less simultaneously in Europe, Australia and the USA (van der Sanden et al., 2009). An increase in the number of hospitalized cases due to B2 infection was also reported in the USA and Australia in the mid-1980s (Brown et al., 1999; Sanders et al., 2006). HEV71 strains belonging to subgenogroups B3–B5, C4 and C5 that have caused large outbreaks in the Asian Pacific region since 1997 have not been observed in the Netherlands or in other countries outside the Asian Pacific region (Brown et al., 1999; van der Sanden et al., 2009).

The complete VP1 regions of 199 HEV71 strains, isolated in the Netherlands in 1963–2008, have been sequenced previously and were used to assign the strains to specific subgenogroups on the basis of phylogenetic clustering with GenBank HEV71 reference strains (van der Sanden et al., 2009). Here, we describe the evolutionary trajectory of these genogroup B and C viruses in more detail in order to understand the previously described observations in HEV71 epidemiology. We studied virus population dynamics through time to assess whether the high number of hospitalized HEV71 cases in 1986 and 2007 was indeed the result of an increased incidence of HEV71 infection or resulted from an entrovirus-surveillance artefact. To determine a possible role of antigenic shift in the switch from genogroup B to C, differences in antigenicity among genogroup B and C viruses were studied by virus-neutralization assays using subgenogroup-specific rabbit antisera. The complete capsid-encoding regions of a selection of genogroup B and C strains were sequenced and used for amino acid sequence comparisons to find possible clues on antigenic differences.

RESULTS

VP1 nucleotide sequence comparison

The RIVM enterovirus collection contains 199 HEV71 strains isolated in distinct regions of the Netherlands between 1963 and 2008 as part of the national enterovirus surveillance. The complete VP1-encoding regions of these 199 HEV71 strains were sequenced successfully and were used to study the evolutionary trajectory of genogroup B and C viruses in more detail. Phylogenetic analysis based on the 199 Dutch isolates and reference strains (see Supplementary Tables S1 and S2, available in JGV Online) by means of a maximum-likelihood (ML) method showed evolution of genogroup B and C viruses as two monophyletic groups, with an intergenogroup divergence of 16–18% at the nucleotide level. Within genogroup B, Dutch isolates clustered within subgenogroups B0, B1 and B2. Clustering of subgenogroups B1 and B2, however, was supported by low bootstrap values (Fig. 1). The ML tree suggested a stepwise evolution of subgenogroup B1 to B2 via an intermediate group (B1*) containing isolates and reference strains of 1973–1979, previously assigned to group B1, explaining these low bootstrap values. Subgenogroups B3–B5, not observed in the Netherlands, also appear to be products of this epochal evolution, which is characterized by a continuous replacement of prevailing strains (Fig. 1). Within genogroup C, Dutch isolates clustered within subgenogroups C1 and C2, supported by high bootstrap values (Fig. 2). A different trajectory was observed for these viruses than for genogroup B viruses, as the ML tree showed independent evolution of subgenogroups C1 and C2 (Fig. 2). The C1 group showed two distinct branches with 93% sequence similarity: 1987–1997 and 2000–2007. Subgenogroups C3–C5, not observed outside the Asian Pacific region, also showed an independent evolution. Considering clustering of reference strains in the phylogenetic region, the evolution of B1, B2, C1 and C2 viruses in the USA and Australia seemed to have followed the same trajectory as observed in the Netherlands.

Phylodynamic analysis

Virus population dynamics were estimated over time by using Bayesian coalescent analysis (Drummond & Rambaut, 2007) of the VP1 sequence alignment of the 199 Dutch isolates. A measure of coalescence rate or relative genetic diversity through time was estimated by using a Bayesian skyline plot model (Fig. 3) (Drummond et al., 2005). The Bayesian skyline plot employs a piecewise-constant model to describe the change in effective population size through time. This highly parametric model can fit a wide range of demographic scenarios, thereby circumventing the need to select more restrictive demographic models a priori. Past population dynamics are reconstructed by jointly estimating genealogies and Bayesian skyline plot parameters, including group sizes and population sizes [parameterized as the product of generation time and effective population size (Ne)] for genealogical intervals, using Bayesian inference.

From 1963 to 1985, a constant diversity was observed, indicating endemic circulation of B0 and B1 strains. In 1986, the plot showed a sharp peak in Ne, indicating a fast exponential growth of the B2 virus population in this year. The B2 epidemic was followed by endemic circulation of C1 and C2 strains, as indicated by a constant Ne until 2007, when new evidence for an epidemic was observed by another rise in Ne.

Serum neutralization assays

To study cross-antigenicity among subgenogroups, rabbits were hyperimmunized with HEV71 isolates of subgenogroup B2 (11316; 1986) and C1 (480; 1991). Neutralization assays were performed to determine neutralizing-antibody titres against the immunization virus and to test cross-reactivity with another subgenogroup B2 (20557; 1985) and C1 (1416; 2001) isolate and isolates of B0 (10857...
Fig. 1. ML tree of the Dutch HEV71 isolates of genogroup B (85 in total; see Supplementary Table S1) and reference strains. The tree was generated on the basis of VP1 nucleotide sequence data (891 nt). Bootstrap analysis was performed using 1000 pseudoreplicates. Colours are used to elucidate virus clusters in the epochal evolution: light blue, B0 isolates; green, B1 isolates; blue, B1* isolates; red, B2 isolates. Arrows indicate isolates used in the virus-neutralization assays. Isolates of which the complete capsid-encoding region was sequenced are highlighted in grey.
Fig. 2. ML tree of the Dutch HEV71 isolates of genogroup C (114 in total; see Supplementary Table S2). Reference strains are indicated in bold. The tree was generated on the basis of VP1 nucleotide sequence data (891 nt). Bootstrap analysis was performed using 1000 pseudoreplicates. Arrows indicate isolates used in the virus-neutralization assays. Isolates of which the complete capsid-encoding region was sequenced are highlighted in grey.
and 10076; 1966), B1 (11977 and 15051; 1971), B1* (9443; 1974 and 16173; 1976), C2 (2485; 2007 and 1034; 2005) and A (BrCr; 1970) (indicated by arrows in Figs 1 and 2). A 10 dilution of the mock serum did not show toxicity to the Vero cell culture, excluding a significant influence of antibodies against non-viral cell components on the outcome of the assay. Samples collected prior to the first immunization showed no titres against HEV71, excluding aspecific neutralization of HEV71. The B2 antiserum neutralized the B0 viruses, B1 11977, B1* 16173 and B2 viruses (n=2; the test was repeated twice with the same serum) with titres ranging from 2560 to 46 080 (Table 1). The sera reacted poorly with B1 15051, B1* 9443 and the C1, C2 and A viruses. The mean titres (n=5) of the neutralization assays with the C1 antiserum indicated cross-reactivity of C1 antiserum with genogroup A, B and C viruses, except for B1 15051, which also could not be neutralized by C1 antiserum (Table 1).

Amino acid sequence comparison

To find possible clues for the antigenic differences observed in the neutralization assays, the complete capsid-encoding regions were determined for a subset of isolates (Figs 1 and 2) and used for amino acid sequence comparisons (Fig. 4). The capsid regions were highly conserved at the amino acid level. Strains belonging to genogroup B showed 97.7–98.8% amino acid similarity to strains belonging to genogroup C. Within the complete capsid region, 14 genogroup B- and C-specific residues were observed (Fig. 4). Considering residues that are located in previously predicted antigenic regions of the immunodominant VP1 capsid protein (Cello et al., 1993; Foo et al., 2007; Huang et al., 2009; Samuelson et al., 1994; Sivasamugham et al., 2006), B1–B5 viruses differed from genogroup C viruses at residue 164 and or 43. The B1* 9443 and B1 15051 isolates, which showed differences in antigenicity, differed from other genogroup B and C viruses,
including B1* and B1 isolates, at residue 60 of VP4 (9443 and 15051) and residue 149 of VP2 (15051) (Fig. 4). Genogroup- and subgenogroup-specific residues observed in the capsid regions of Dutch isolates were also conserved in HEV71 viruses isolated in the USA, Australia, the UK, Norway and Asia.

### DISCUSSION

Extensive enterovirus-surveillance activities throughout the years enabled us to perform a detailed study on the molecular epidemiology of HEV71 in the Netherlands over a period of 45 years (van der Sanden et al., 2009). From 1963 to 1986, infections were successively caused by viruses of subgenogroups B0, B1 and B2, followed by a shift to predominance of viruses belonging to subgenogroups C1 and C2. In the present study, we found interesting differences in the evolutionary trajectory between B and C viruses, explaining trends in HEV71 epidemiology observed previously; while phylogenetic analysis on the basis of the VP1-encoding region clearly demonstrated epochal evolution (or successive replacement) of subsequent subgenogroup B1–B5 viruses, genogroup C viruses showed an independent evolution, which is in line with co-circulation of C1 and C2 viruses in the Netherlands since 1997. Inclusion of reference strains from the USA and Australia in the phylogenetic analysis suggested that the evolution of B1, B2, C1 and C2 viruses has a global character. Multiple, successive clusters within subgenogroup C1 with an independent evolution suggest introduction of new lineages from abroad.

The transition between circulation of genogroup B and C strains is likely to result from antigenic shift: hyperimmune B2 antiserum neutralized B0, B1, B1* and B2 viruses, but cross-reacted poorly with C1 and C2 viruses. These results suggest that the B2 epidemic in the mid-1980s, the occurrence of which was supported by phylodynamic analysis, has probably resulted in herd immunity against viruses of genogroup B, but did not protect the population from genogroup C infections, explaining the observed shift from genogroup B to C. The amino acid sequences of the complete capsid regions showed 14 genogroup B- and C-specific residues. Residues 60 of VP4 and 149 of VP2 probably play a role in the antigenically distinct character of isolates B1 15051 and B1* 9443. Antibody-binding studies, however, will be necessary to determine the antigenic determinants (Cello et al., 1993; Foo et al., 2007; Samuelson et al., 1994).

On the basis of capsid amino acid sequence comparisons and the fact that the shift of genogroup following increased reporting of B2 infections was also observed in Europe, the USA and Australia in the mid-1980s, we expect that the scenario described above occurred globally. Remarkably, the type specificity of neutralization was unidirectional, as hyperimmune sera to a C1 virus did cross-neutralize B0, B1 and B2 viruses, as well as C1 and C2 viruses, which corresponds to the observation of solely genogroup C viruses in Europe and the USA since 1986. Global herd immunity against C1 and C2 viruses possibly explains why epidemics with subgenogroups B4 and C4 are restricted to the Asian Pacific region. This is supported by the results of a Taiwanese study, which showed neutralization of B4 and C4 viruses by C2-specific rabbit antiserum (titres around

<table>
<thead>
<tr>
<th>Subgenogroup/strain</th>
<th>Year</th>
<th>B2 antiserum</th>
<th>C1 antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10857</td>
<td>1966</td>
<td>15 360 (–)</td>
<td>17 920 (15 360–20 480)</td>
</tr>
<tr>
<td>10076</td>
<td>1966</td>
<td>5120 (–)</td>
<td>5120 (–)</td>
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<td>B1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11977</td>
<td>1971</td>
<td>46 080 (30 720–61 440)</td>
<td>15 360 (10 240–20 480)</td>
</tr>
<tr>
<td>15051</td>
<td>1971</td>
<td>20 (–)</td>
<td>20 (–)</td>
</tr>
<tr>
<td>B1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9443</td>
<td>1974</td>
<td>40 (20–60)</td>
<td>7552 (5120–10 240)</td>
</tr>
<tr>
<td>16173</td>
<td>1976</td>
<td>2560 (–)</td>
<td>5120 (–)</td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20557</td>
<td>1985</td>
<td>20 480 (–)</td>
<td>5120 (–)</td>
</tr>
<tr>
<td>11316</td>
<td>1986</td>
<td>7680 (–)</td>
<td>3840 (–)</td>
</tr>
<tr>
<td>C1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>480</td>
<td>1991</td>
<td>40 (20–60)</td>
<td>2320 (1280–5120)</td>
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<tr>
<td>1416</td>
<td>2001</td>
<td>10 (–)</td>
<td>5120 (–)</td>
</tr>
<tr>
<td>C2</td>
<td></td>
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<tr>
<td>1034</td>
<td>2005</td>
<td>10 (–)</td>
<td>5120 (–)</td>
</tr>
<tr>
<td>2485</td>
<td>2007</td>
<td>70 (60–80)</td>
<td>2304 (1280–5120)</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BrCr</td>
<td>1970</td>
<td>220 (120–320)</td>
<td>1920 (1280–2560)</td>
</tr>
</tbody>
</table>
Subgenogroup B5, however, has recently been described to be antigenically distinct from B1, B4, C2 and C4 viruses (Huang et al., 2009) and could pose a potential risk for epidemic spread outside the Asian region. Other epidemiological parameters, such as population density, lack of sanitation and climate, however, are also likely to play a role in the occurrence of outbreaks.

One should keep in mind that the serological assays in this study were performed using rabbit monospecific antisera.

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**Fig. 4.** Amino acid differences in the capsid region of HEV71 strains. Upper part, VP4/VP2/VP3 region; lower part, VP1 region.
to assure the presence of antibodies raised against only one subgenogroup. It is not certain whether the responses observed in rabbits are representative of human immune responses against live HEV71 and what effect enterovirus cross-reacting antibodies have on host susceptibility for HEV71 infection. Our results do not correspond to those of a recent Japanese study that describes cross-neutralization of genogroup B and C viruses by sera of guinea pigs immunized with B2 virus. However, these authors do report a slight difference in antigenicity between genogroups A and C and genogroup B (Mizuta et al., 2009). A Taiwanese study showed cross-neutralization of genogroup C viruses by sera of human patients infected with genogroup B (Kung et al., 2007), but this could also be explained by prior exposure rather than cross-neutralization. Our results are in line with another, more recent Taiwanese study, which showed antigenic differences between B1/B4 and C2/C4 on the basis of antigenic cartography, using human serum (Huang et al., 2009).

This study describes the genetic evolution of HEV71 genogroup B and C viruses in the Netherlands over a period of 45 years. Phylogenetic analysis showed evidence for the occurrence of epidemics in 1986 and 2007, for the first time demonstrating that these were true epidemics and not surveillance artefacts. These epidemics obviously led to increased virus sampling, but such higher sampling density is not necessarily associated with elevated population-size estimates. Importantly, the coalescence rate for the samples obtained during the epidemic was extremely rapid, with most lineages quickly coalescing to a single recent common ancestor. The sampling-size issue has been addressed elegantly by a comprehensive analysis of human influenza H3N2 epidemic dynamics (Rambaut et al., 2008). This study demonstrated that the population genetic history reflected the seasonal dynamics of the virus, but with a sampling density that was inevitably larger at the peak of the epidemic season. To demonstrate that the reconstruction reflects the underlying population dynamics, and not the heterogeneity in sampling density, Rambaut et al. (2008) performed extensive simulations under various demographic scenarios, but with the same seasonal sampling heterogeneity. Consistently, the true demographic dynamics were reconstructed independently of the sampling heterogeneity. Therefore, we also conclude epidemic outbreaks from the Bayesian skyline peaks, but we acknowledge that other epidemics may have gone unnoticed without appropriate sampling during these expansions.

Serological assays using hyperimmune rabbit sera showed the disability of subgenogroup B2 antisera to neutralize genogroup C viruses, possibly explaining the global shift from genogroup B to C following a B2 epidemic. Cross-neutralization of genogroup B and C viruses with C1 antiserum, on the other hand, probably explains why solely C1 and C2 viruses have been isolated in Europe and the USA after the shift from genogroup B to C in 1986. Extensive surveillance, however, should be performed to detect emerging subgenogroups, such as B3, that are antigenically distinct from other groups and do form a potential risk for causing outbreaks in our region.

**METHODS**

HEV71 isolates. The HEV71 strain collection used for this study has been described previously (van der Sanden et al., 2009). Briefly, 346 HEV71 isolates were submitted to the RIVM as part of enterovirus-surveillance activities from 1963 to 2008. Isolates were typed as HEV71 by the use of neutralization tests with monospecific antisera against HEV71. In total, 199 of these HEV71 isolates from the same number of patients from distinct regions in the Netherlands, at least two from non-epidemic years and five from epidemic years, were stored and used for the current study. This dataset forms a representative selection of viruses submitted to the RIVM for genotyping and reflects the diversity of HEV71 in the Netherlands.

RNA extraction. Viral RNA was extracted from HEV71 isolates freshly cultured on human rhabdomyosarcoma or human embryonic lung fibroblast (GABI) cell lines, using a MagNA Pure LC Total Nucleic Acid Isolation kit with a MagNA Pure LC instrument (Roche Diagnostics) (van der Sanden et al., 2009). Lysis was done by adding 100 μl cell culture to 400 μl lysis binding buffer (provided in the kit). Extraction was performed according to the manufacturer’s instructions. Viral RNA was eluted in 50 μl elution buffer provided in the kit.

HEV71 PCR assays. The VP1 capsid region was PCR-amplified in two overlapping regions as described previously (Bible et al., 2008; Brown et al., 1999; van der Sanden et al., 2009). The VP4/VP2/VP3-encoding region of a selection of isolates (Figs 1 and 2) was amplified as two overlapping regions, using the EV1FB (5'-'TAAAACAGCCTGTGGGTGTYACCC-3'), EV1RB (5'-'TGCAATGRTATGCAAACCTAC)-3), EV2FB (5'-'CTCTATGTTYGAC-WGACC-3') and EV2RB (5'-'ATCAGTCGCCCAYCTATC-3') primers for genogroup B isolates and the EV1FC (5'-'TAAAACAGCCTGTGG-TTGTYGACC-3'), EV1RC (5'-'TGCACRTGRATCARAACC-3'), EV2FC (5'-'CGAGATGTRYAAC-WGACC-3') and EV2RC (5'-'ATCAGTCGCCCAYCTATC-3') primers for genogroup C isolates. Reverse transcriptions were performed to convert the viral RNA to cDNA. For this reaction, 5.0 μl isolated viral RNA was incubated at 50 °C for 60 min together with 1.0 μl 50 μM antisense primer, 1 μl 10 mM dNTPs, 6.0 μl H2O. 4 μl 5 × First Strand Buffer (Invitrogen), 1.0 μl 0.1 M dithiothreitol, 1.0 μl 10 U RNase inhibitor ml⁻¹ (GE Healthcare) and 1.0 μl SuperScript III reverse transcriptase (Invitrogen). The reaction was terminated by incubation of the samples at 70 °C for 15 min.

In total, 2 μl cDNA was used for the PCR amplification, together with 12.5 μl HotStarTaq Mastermix (Qiagen), 2.5 μl 10 μM forward and reverse primers and 5.5 μl H2O. The PCR was carried out for one cycle of 15 min at 95 °C to activate the Taq polymerase, 35 cycles of 1 min at 95 °C, 1 min at 54 °C and 1 min at 72 °C, and a final cycle of 5 min at 72 °C. Amplicon-size analysis was performed using agarose gel electrophoresis. Purification of the PCR products was performed by using a QiAquick PCR Purification kit, according to the manufacturer’s protocol (Qiagen). Sequencing of the PCR products was carried out with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.2 on a model 3700 automated sequencer (both from Applied Biosystems).

Phylogenetic analysis. Editing of the sequence data was performed using the BioNumerics software (Applied Maths). ML trees were constructed with the PhyML software, using the general time-reversible (GTR) nucleotide-substitution model with discrete gamma-distrib-
uted rate variation among sites (Guindon & Gascuel, 2003). Reliability of the trees was tested by a bootstrap analysis using 1000 pseudoreplicates. The reference strains used in the phylogenetic analysis are presented in Supplementary Tables S1 and S2.

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDJB nucleotide sequence databases with the accession numbers AB524081–AB524279 for the VP1 regions (Supplementary Tables S1 and S2) and AB552972–AB552988 for the complete capsid regions (Fig. 4).

**Virus population dynamics.** On the basis of the VP1 nucleotide sequence data, virus population dynamics over time were estimated by using a Bayesian Markov chain Monte Carlo (MCMC) approach that incorporates the date of virus sampling. The best-fitting nucleotide-substitution model was determined by a model-selection procedure implemented in the HYPHY software (Kozakovsky Pond et al., 2005). The Bayesian analysis was subsequently performed with the BEAST software (Drummond & Rambaut, 2007) using the GTR nucleotide-substitution model with a discrete gamma distribution to accommodate rate variation among sites in the alignment. Furthermore, we partitioned alignment sites into codon positions, to allow different rates of substitution for the first plus second versus the third codon position. Lineage-specific rate heterogeneity (rate variation among the branches of the inferred phylogeny was significant) was taken into account by using the uncorrelated log-normal relaxed molecular clock model (Drummond et al., 2006).

Preliminary analysis on the increase of number of nucleotide mutations over time using Path-O-Gen software version 1.1 (http://tree.bio.ed.ac.uk/software/pathogen/) supported evolution of genogroups B and C as two monophyletic groups. The VP1 sequence data of genogroup B and C strains were therefore kept monophyletic during the MCMC analysis. To infer the dynamics of HEV71 genetic diversity through time, we employed a Bayesian skyline plot model (Drummond et al., 2005). We specified 20 groups of coalescent intervals to capture the past population dynamics in the piecewise-constant demographic function. The posterior distribution for the intervals to capture the past population dynamics in the piecewise-

**Preparation of virus for immunization.** Isolate 86-11316, from the B2 epidemic in 1986, and isolate C1 91-480, collected after the B2 epidemic, were selected for immunization of rabbits. Vero (African green monkey kidney) cells (ATCC) were maintained as monolayers for 50 000 000 generations; stationarity and mixing efficiency were examined by using Tracer (http://tree.bio.ed.ac.uk/software/tracer/).

**Neutralization assays.** Serum samples were inactivated at 56 °C for 30 min and were subsequently analysed in duplicate for neutralization of the B2 86-11316 and C1 91-480 immunization viruses by a virus CPE-reduction assay in Vero cells in a 96-well format (Lin et al., 2009; von Zeipel, 1979). Other isolates of subgenogroups B2 (20557; 1983) and C1 (1416; 2001) and isolates belonging to B0 (10857 and 10076; 1966), B1 (11977 and 15051; 1971, 9443; 1974 and 16173; 1976), C2 (1034; 2005 and 2485; 2007) and genogroup A (BrCr; 1970) were used to study cross-reactivity with the monospecific anti-B2 and anti-C1 sera (Figs 1 and 2). Selection of these viruses for the assay was based on the year of isolation and the virus titre being high enough for a neutralization assay. A twofold serial dilution of the serum samples was incubated with an equal volume of 100 TCID₅₀ chloroform-treated virus ml⁻¹. Vero cells in DMEM supplemented with 3 % FCS were added (400 000 cells ml⁻¹) and were checked for CPE after 5 days. The serum titre was defined as the highest dilution without visible CPE.

**Preparation of virus for immunization.** Isolate 86-11316, from the B2 epidemic in 1986, and isolate C1 91-480, collected after the B2 epidemic, were selected for immunization of rabbits. Vero (African green monkey kidney) cells (ATCC) were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) supplemented with 3 % fetal calf serum (FCS; PAA Laboratories) and were used for virus preparation and virus titration. Cytopathic effect (CPE)-positive cultures were freeze–thawed to release intracellular virus particles. Cell debris was removed by centrifugation at 800 g for 15 min. Virus particles were concentrated 150 times by ultracentrifugation of the supernatant at 55 793 g at 4 °C for 17.5 h. Virus titres were determined from the median end point of the tissue culture infectious dose (TCID₅₀) as described by Wu et al. (2004). The TCID₅₀ value was calculated according to the Spearman–Kärber method (Kärber, 1931). Prior to rabbit immunization, the virus particles were heat-inactivated by incubation of the virus suspension at 56 °C for 30 min.

**Immunization of rabbits.** Pathogen-free New Zealand White rabbits (Harlan Laboratories) were injected intramuscularly in each back leg with 0.5 ml virus solution containing 10⁻¹⁰ TCID₅₀ virus ml⁻¹ and 0.5 ml Freund's adjuvant (Freund's complete for the first immunization and Freund's incomplete for the boosts) (Harlan Laboratories). One rabbit was injected with processed uninfected cell culture (mock) to check for immune reaction to non-viral cell components. Rabbits were boosted with the same doses at 2, 4, 6, 8 and 10 weeks after the first immunization. Blood samples were collected prior to the first immunization and 1 week after boosts 2, 3, 4 and 5.

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