Prevalence and Genetic Diversity of Human Enteroviruses in the Context of Poliovirus Eradication

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Prevalentie en genetische diversiteit van humane enterovirussen in de context van poliovirus eradicatie

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The Enterovirus Poem

Human enteroviruses, clusters A to D?
They're challenging you
They're intriguing me!

Polio's the worst; but the battle's begun
And we'll strike at it with vaccines
Until we see we've won.

It spreads so fast in droplets and faeces
It breeds in the intestinal tract
It plagues our kids - it's the worst of diseases.

And EV71? It gets on our nerves
Regally sweeping the Asian Pacific
Sharply raising morbidity curves.

Diarrhoea, meningitis, a rash on the skin
Otitis, paralysis and pharyngitis
See those vesicles on that child's chin?

There's always more, much more to learn-
Epidemiology. Pathogenesis. Therapies.
But the time will come. It will be our turn!

Sabine

Contents

Chapter 1

General Introduction

The family of *Picornaviridae*

Viruses belonging to the family *Picornaviridae* are small, non-enveloped viruses with a single-stranded, positive-sense RNA genome. Up to present, thirteen genera within this large family have been designated by the International Committee on Taxonomy of Viruses: *Aphtovirus, Erbovirus, Teschovirus, Sapelovirus, Senecavirus, Tremovirus, Avihepatovirus, Cardiovirus, Hepatovirus, Cosavirus, Parechovirus, Kobuvirus* and *Enterovirus* (Fig. 1). Members within the latter six genera have been reported to cause human disease. Enteroviruses are, next to viruses of the *Herpesviridae* family, the major viral cause of neurologic disease with a known etiology in humans, including meningitis, encephalitis and acute flaccid paralysis (24, 38, 47). By that they form a serious threat for human health. A well known representative of these is poliovirus (species *Human enterovirus C*), which has inextricably been associated with large outbreaks of neurologic disease in children. This thesis will focus on prevalence and genetic diversity of human enteroviruses (belonging to species A to D) in the context of poliovirus eradication.

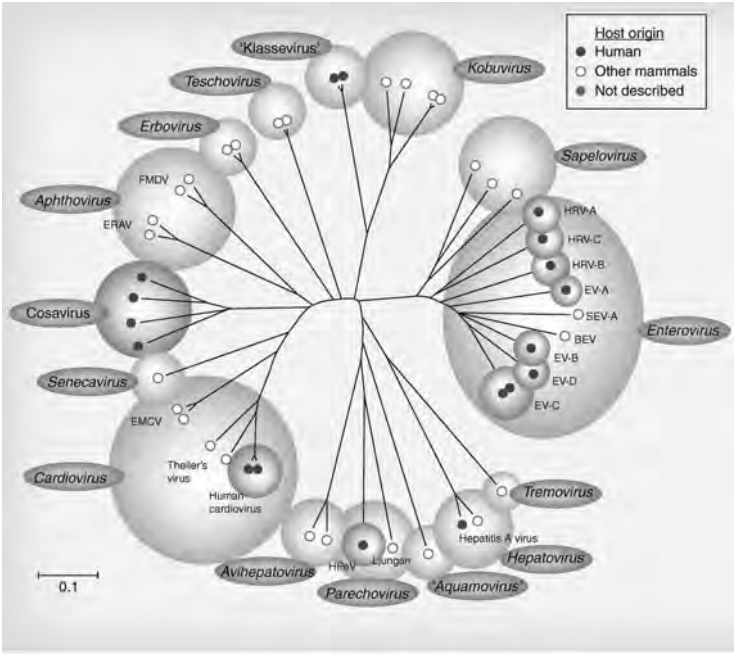

Fig. 1: Genera and their species within the *Picornaviridae* family. Phylogenetic tree was constructed by comparison of 3Dpolymerase amino acid sequences of representative members of each of the currently classified genera and two proposed genera; *Aquamovirus* and *Klassevirus* (positions 5711–7252 in the prototype HPeV1 Harris genome (accession number L02971)). Abbreviations: HPeV: human parechovirus; EV: enterovirus; SEV: simian enterovirus; BEV: bovine enterovirus; HRV: human rhinovirus; ERAV: equine rhinitis virus; FMDV: foot-and-mouth disease virus; EMCV: encephalomyocarditis virus. Source: Wildenbeest *et al.* (2010) (56).

Genome structure of human enteroviruses

As for other picornaviruses, the genome of human enteroviruses consists of a single-stranded, positive-sense RNA molecule of ~7400 nucleotides (nts) (Fig. 2) (26, 50). The first ~750 nts form the 5'nontranslated region (5'NTR), which contains the internal ribosome entry site necessary for cap-independent viral RNA translation. In addition, parts of the 5'NTR are necessary for genome replication. The next ~6600 nt encode a single polyprotein which is autocatalytically processed in the VP0, VP3 and VP1 capsid proteins, forming a protective shield around the naked RNA molecule, and the non-structural proteins 2A-C and 3A-D, involved in viral replication and protein processing. As for most picornaviruses, the enterovirus VP0 capsid protein is cleaved into VP4 and VP2, bringing the number of distinct capsid proteins to 4. The capsid proteins each form an eight-stranded, anti-parallel β-barrel, which is quite conserved in amino acid composition among human enteroviruses. The surface exposed loops connecting the individual β-sheets, on the other hand, are highly variable and form the major parts of the virus that are exposed to the host immune system and its neutralizing antibodies (26). These sites thus accommodate the major serotype determining factors. The non-structural proteins show less diversity in amino acid composition than the structural proteins, as evolutionary restrictions are necessary to preserve enzyme functionality. In most picornaviruses 2A and 3C are proteases processing the viral polyprotein. The 2B protein is likely involved in the intracellular rearrangement to enable viral replication, during which 2C appears to act as a helicase. The 3A protein is suggested to be involved in the transport of VPg (3B) to the replication complexes. Here, VPg binds to the 3'end of the viral RNA and primes the viral RNA synthesis conducted by the viral polymerase ($3D^{pol.}$). The polyprotein encoding region is flanked by a 3'NTR of ~70 to 80 nucleotides and a poly(A) tail, involved in viral replication as well (26).

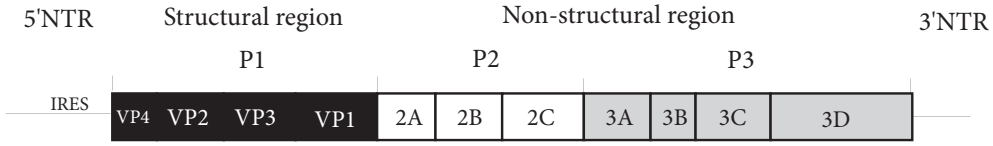

Fig. 2: Genome organization of human enteroviruses. Abbreviations: NTR: Non-Translated Region; IRES: Internal Ribosome Entry Site.

Classification of human enteroviruses

Human enteroviruses were traditionally classified into the species *Coxsackievirus A* (CA), *Coxsackievirus B* (CB) and *Poliovirus* on the basis of pathogenicity in humans and experimental animals. The introduction of enterovirus sensitive cell culture techniques enabled isolation of enteroviruses that did not replicate in experimental

animals. Enteroviruses discovered by these techniques and of which the association with human disease was unknown, were called Enteric Cytopathogenic Human Orphan (ECHO) viruses (33). Serotypes within the species were recognized in neutralization assays using serotype specific neutralizing antibodies.
Infections with human enteroviruses cause a wide spectrum of clinical syndromes, from relatively mild (like gastroenteritis and exanthema) to severe (like meningitis and acute flaccid paralysis), making classification on the basis of pathogenicity complicated. Since the early 1960s, it therefore was decided to no longer classify newly discovered enteroviruses within the traditional species, but to label them with numeric names, like human enterovirus 68 and 71.

The development of molecular techniques enabled characterization of enteroviruses at the genetic level. Comparisons of the amino acid sequences of enterovirus capsid proteins showed that certain serotypes, previously assigned to the same species on the basis of pathogenicity, were actually genetically distantly related. On the basis of these genetic distances human enterovirus serotypes were therefore reclassified into *human enterovirus species A* to *D*, keeping the traditional names of the enteroviruses (Table 1) (7, 33, 43-45, 57). As part of the genetic analysis, it became also evident that human ECHO virus 22 and 23 showed less than 30% amino acid identity in the polyprotein region to other picornaviruses (32). Together with the observation that the host cell translation machinery is not offset during infection with ECHO virus 22 and 23, something that does occur during infection with other enteroviruses, and presence of 3 capsid proteins (VP0-3-1) instead of 4, these genetic distances justified the classification of ECHO virus 22 and 23 into a new genus of the *Picornaviridae*, *Parechovirus*, and renaming of the viruses as *Human Parechovirus* (HPeV) 1 and 2 (32). Up to present, 14 types belonging to this genus have been identified (3-5, 22, 34, 36, 53) (and unpublished data Oberste, M.S.).

Table 1: Classification of human enterovirus serotypes to human enterovirus species A-D.

	Cluster A	Cluster B	Cluster C	Cluster D
Polioviruses			1-3	
Coxsackie A viruses	2-8, 10, 12, 14, 16	9	1, 11, 13, 15, 17-22, 24	
Coxsackie B viruses		1-6		
Echoviruses		1-9, 11-21, 24-27, 29-34		
Enteroviruses	71, 76, 89-92	69, 73-75, 77-88, 93, 97-98, 101, 106-107	95-96, 99, 102, 104-105, 109	68, 70, 94

The global poliovirus eradication initiative

As mentioned before, poliovirus (species *Human enterovirus C*) has been associated with large outbreaks of neurologic disease in children. In the 1980s, the virus circulated in >125 countries, each year causing hundred thousands of cases of acute flaccid paralysis (<1% of all poliovirus infections). The paralysis, which is a consequence of infection and destruction of motor neurons, is temporarily in most of the cases, but can lead to irreversible skeletal deformities and tightening of the joints. Respiratory failure as a result of impaired signalling of the muscles of the axial skeleton has formed an important cause of death among poliovirus infected children (40, 58).

Up to present, poliovirus is the only enterovirus for which an effective vaccine is available. The first vaccine against poliovirus, developed by Jonas Salk, was licensed in 1955. This vaccine contains inactivated poliovirus particles (IPV) of the three different poliovirus serotypes and is injected intramuscularly, inducing a strong systemic humoral immunity protective against poliovirus induced disease (40). Several years later the oral polio vaccine (OPV) of Albert Sabin, containing live, attenuated polioviruses of the three different serotypes, was licensed. These strains have been selected to replicate successfully in the human intestinal tract, but not in the cells of the central nervous system. In addition to a systemic humoral immunity, these strains (in contrast to IPV) generate a strong intestinal immunity that, upon natural exposure, protects against replication and transmission of poliovirus. Implementation of national poliovirus immunization programmes in several industrialized countries, including the Netherlands, resulted in elimination of endemic poliovirus circulation. The disease, however, remained wide spread in developing countries. In 1988, the World Health Assembly passed a resolution to eradicate wild type poliovirus globally which is feasible because a) humans are the only reservoir of the virus, b) the virus

causes only acute, non-persistent infections, c) the virus is only transmitted by infected humans and their stools, d) survival of the virus in the environment is finite and e) immunization interrupts transmission (21). A worldwide vaccination campaign was launched by the World Health Organization (WHO), using trivalent (t)OPV, not only because of the generation of both a mucosal and systemic humoral protection by this vaccine but also because of its simple way of administration (by ingestion) and relatively low production costs/dose. In addition, secondary transmission of the live vaccine viruses would indirectly immunize children that do not participate in routine immunization programmes. The tOPV vaccination campaign has been very successful as the number of countries with endemic wild poliovirus circulation decreased from >125 in 1988 to 4 in 2010 (Fig. 3). Wild type 2 has likely been eradicated since 1999 (55). By that, the global number of cases of poliomyelitis reduced from 350 000 in 1988 to 1291 in 2010. Supplementary vaccination campaigns with monovalent OPV 1 and 3, having a superior immunogenicity to these types compared to tOPV, have recently been introduced in the remaining endemic regions to tackle the last circulating P1 and P3 viruses (9, 25). Bivalent vaccine (containing both type 1 and 3 serotypes) has recently been shown to be non-inferior to monovalent vaccines regarding its protective efficacy and has been introduced to keep population immunity high for both types 1 and 3 (51).

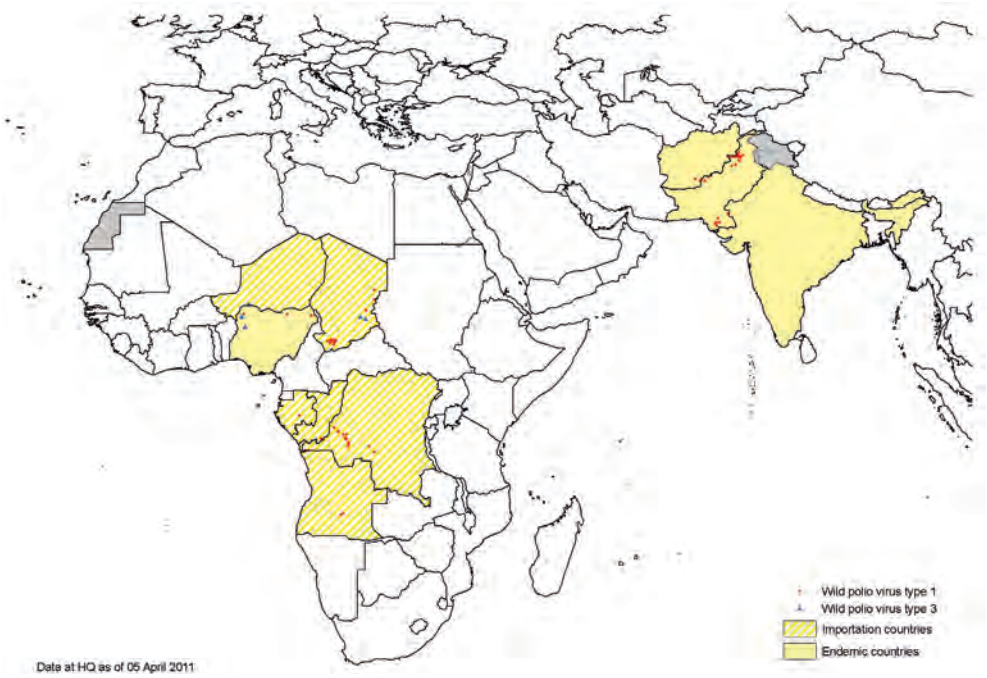

Fig. 3: Polio cases world wide as of April 5, 2011. Source: www.polioeradication.org.

The use of live attenuated strains for vaccination, however, is not without risks. Upon replication in the human intestinal tract the sites of attenuation can mutate, which may result in reversion of the OPV strains toward a neurovirulent phenotype able to cause vaccine associated paralytic poliomyelitis (19, 23). Prolonged circulation of vaccine-related strains in areas with a low vaccination coverage or prolonged replication of vaccine strains in patients with a B-cell immunodeficiency, increases the risk for emergence of such viruses (called circulating (c) or immunodeficiency-associated (i) Vaccine Derived Polioviruses (VDPVs) when having a VP1 nucleotide difference of >1% compared to prototype vaccine viruses) (2). In the past, cVDPVs have caused polio outbreaks in regions with low immunization coverage (11). High rates of OPV vaccination will prevent spread of all types of VDPVs, but to overcome the risk of evolution of new VDPVs, IPV will be introduced where possible in the final stages of poliovirus eradication. Three years after eradication of poliomyelitis, vaccination with OPV will be stopped everywhere in the world.

Human enterovirus 71: the new "poliovirus"?

Human enterovirus 71 (EV71) (species *human enterovirus A*) is considered to be the major threat for poliomyelitis like disease in children after eradication of poliovirus. Together with Coxsackievirus A16 (CA16), EV71 is the major causative agent of the generally mild and self limiting Hand Foot and Mouth Disease (HFMD) in children with fever, vesicles on the hands and feet and oral ulcers. Infections with EV71, however, can progress to severe neurological disease, in particular in very young children (< 3 years), including brainstem encephalitis and acute flaccid paralysis. EV71 infections have specifically been linked to long-term cognitive and motor deficits, and cardiopulmonary failure resulting from brainstem encephalitis forms a major cause of death among EV71 infected children (16, 46, 49).

Since the first isolation of EV71 in 1970 (California, USA), EV71-associated neurologic disease has been observed in outbreaks throughout the world: i.e. Bulgaria (1975), Hungary (1978), and the USA (1986) (6, 8, 20, 28). The incidence of EV71 infection, however, seems to be particularly high in the Asian Pacific region since 1997. Countries within this region, including Malaysia, Singapore, Japan, China and Taiwan, have been affected by one or more massive outbreaks of EV71 with ten to hundred thousands of cases of HFMD and several hundreds of fatal cases as a consequence of neurologic disease (1, 10, 12-15, 17, 18, 27, 29-31, 48). Genotyping of EV71 strains isolated from these outbreaks revealed that these strains belonged to distinct, recently emerged subgenogroups (defined on the basis of diversity within the VP1 nucleotide sequence) (8, 17, 35, 37, 39, 52). In response to the massive outbreak of

EV71 in China, which started in 2008 and which has already taken >500 child lives up to present, HFMD has been classified as a notifiable disease in the Asian Pacific region.

Enterovirus infections in the Netherlands

Primary diagnosis for all enterovirus infections, including those of poliovirus and enterovirus 71, has been performed by the virological laboratories of the Dutch Working Group on Clinical Virology (covering the whole country) since the early sixties. Faecal samples, throat swabs and cerebrospinal fluid (CSF) samples have been collected through the years from patients with systemic viral infection with disease symptoms varying from diarrhoea and skin rash to neurological disease like meningitis and paralysis. Detection of enteroviruses in clinical samples and their characterization has been performed locally by the labs using enterovirus sensitive cell culture techniques, serum neutralisation assays and molecular techniques (41, 42, 54). Non-typed or non-typable isolates (both ~13% of samples with an enterovirus characteristic cytopathogenic effect in cell culture each year) have routinely been sent to the National Institute for Public Health and the Environment (RIVM, Bilthoven), where they are cultured on a transgenic mouse cell line with the human poliovirus receptor (L20B) to exclude presence of poliovirus. On a yearly basis, the members of the surveillance system report the number of samples tested, the number of enterovirus positive samples, the results of the enterovirus typing experiments and the age distribution of the patients (> or < 15 years) to the RIVM. On the basis of these typing data, a sudden increase in the number of hospitalized cases of EV71 infection after a long period of low incidence was noticed in the Netherlands in 2007 (Fig. 4). At that time, it was not clear whether this increase reflected a real epidemic and whether it was related to the increased incidence of EV71 infection in the Asian Pacific region.

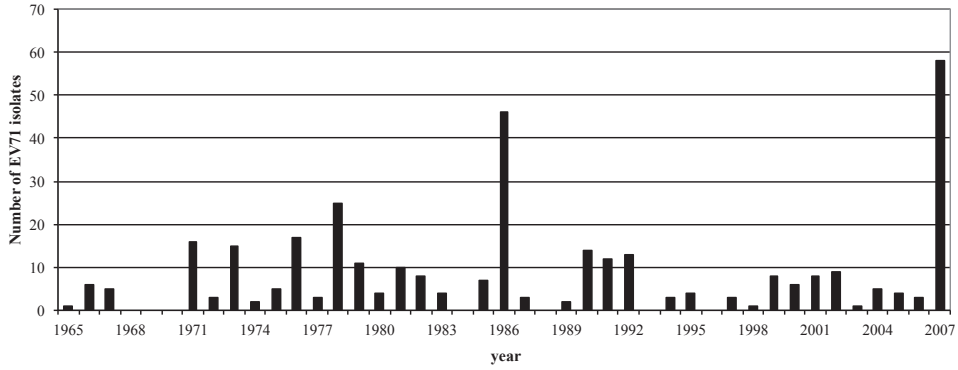

Fig. 4: Number of EV71 infections reported in the Netherlands in 1965 to 2007.

Thesis outline

The aim of this thesis was to gain insight in the prevalence and genetic diversity of human enteroviruses in the context of poliovirus eradication. Firstly, this was done by studying genetic and antigenic diversity of OPV strains collected during a WHO study on the efficacy of mOPV1 vaccination in newborns in eradicating poliovirus 1 (**chapter 2**). Secondly, we studied the evolutionary trajectory of EV71, considered to be the major threat for poliomyelitis like disease in children following eradication of poliovirus, to find explanations for its epidemiological trends. **Chapter 3** describes the genetic diversity of EV71 and infection associated disease symptoms in the Netherlands between 1963 and 2010 in relation to the diversity observed in Asia. Viral population dynamics were analyzed to study whether the increase in EV71 reporting in the Netherlands in 2007 reflected a real epidemic or was the result of changes in detection methods or increased awareness (**chapter 4**). In depth genetic and serological analyses were performed to find clues on the role of antigenic drift and genome recombination in the evolutionary trajectory of EV71 (**chapters 4 and 5**). In the third part of this thesis (**chapter 6)** we evaluated 50 years of enterovirus typing data, collected as part of routine enterovirus diagnostics in the Netherlands, to find clues on the potential consequences of poliovirus eradication and the associated decrease in poliovirus mucosal immunity on prevalence of other enteroviruses. To serve this purpose we explained the majority of the diagnostic deficit observed in the enterovirus surveillance in the Netherlands in 2000 to 2007 by retrospective testing of unexplained samples for presence of human parechovirus (**chapter 7**). In **chapter 8** results are discussed in relation to the current knowledge in the field.

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Chapter 2

Shedding of Vaccine Viruses with Increased Antigenic and Genetic Divergence after Vaccination of Newborns with Monovalent Type 1 Oral Poliovirus Vaccine

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Abstract

For the final stages in the eradication of poliovirus type 1 (P1), the World Health Organization advocates the selective use of monovalent type 1 oral poliovirus vaccine (mOPV1). To compare the immunogenicity of mOPV1 with that of trivalent OPV (tOPV) in infants, a study was performed in Egypt in 2005. Newborns were vaccinated with mOPV1 or tOPV immediately after birth and were challenged with mOPV1 after 1 month. Vaccination with mOPV1 at birth resulted in significantly higher seroconversion against P1 viruses and lower excretion of P1 viruses than vaccination with tOPV. Intratypic differentiation of the viruses shed by the newborns revealed the presence of remarkably high numbers of antigenically divergent (AD) P1 isolates, especially in the mOPV1 study group. The majority of these AD P1 isolates (71%) were mOPV1 challenge derived and were shed by newborns who did not seroconvert to P1 after the birth dose. Genetic characterization of the viruses revealed that amino acid 60 of the VP3 region was mutated in all AD P1 isolates. Isolates with substitution of residue 99 of the VP1 region had significantly higher numbers of nonsynonymous mutations in the VP1 region than isolates without this substitution and were preferentially shed in the mOPV1 study group. The widespread use of mOPV1 has proven to be a powerful tool for fighting poliovirus circulation in the remaining areas of endemicity. This study provides another justification for the need to achieve high vaccination coverage in order to prevent the circulation of AD strains.

Introduction

Polioviruses are the causative agents of human poliomyelitis and belong to the genus *Enterovirus* in the family *Picornaviridae*. The virus is transmitted primarily by the fecal-oral route and replicates in the human intestinal tract. The virus may also be transmitted through respiratory droplets and may replicate for a short period in the upper respiratory tract and tonsillar tissue. From either site of primary replication, the virus may invade the central nervous system and cause paralysis following infection and destruction of motor neurons. Three serologically different types of poliovirus can be distinguished (poliovirus type 1 [P1], P2, and P3), and only limited cross-protection exists between serotypes (35).

In 1988, the World Health Assembly passed a resolution to eradicate wild poliovirus globally. A worldwide vaccination campaign with the trivalent oral poliovirus vaccine (tOPV) was launched by the World Health Organization (WHO). This vaccine contains the three attenuated poliovirus vaccine strains developed by Albert Sabin in the proportion of 10:1:6 for P1, P2, and P3, respectively. These OPV strains have been selected to replicate successfully in the human intestinal tract but not in the cells of the central nervous system. In addition to a strong systemic humoral response, these strains generate strong intestinal immunity (12). Sabin type 1 is considered to be the most stable of the three attenuated poliovirus serotypes (19). This strain has 54 mutations compared to the parental Mahoney strain, of which 6 are primarily responsible for attenuation. Sabin type 2 has two major determinants of attenuation, and Sabin type 3 has three determinants of attenuation (11, 32). Upon replication in the human intestinal tract, the sites of attenuation can mutate, which results in reversion of the Sabin strains toward a parental neurovirulent phenotype. Also as a consequence of replication in the host, antibodies are produced that recognize the antigenic sites of the Sabin strains (42). This immunogenic pressure could favor the selection of antigenically divergent (AD) viruses with substituted residues in parts of these antigenic sites. AD Sabin viruses might circulate among a population for a long period and evolve into vaccine-derived polioviruses (VDPVs; with differences of >1% from the prototype Sabin viruses in the VP1 region) capable of causing outbreaks. These viruses might escape current diagnostic screening methods, and the risk for generation of these viruses should be reduced as much as possible (1, 9, 16).

The tOPV vaccination campaigns have been very successful, since the number of countries with endemic wild poliovirus circulation decreased from >125 in 1988 to 4 in 2006, and wild type 2 poliovirus has likely been eradicated since 1999 (5). The tOPV vaccine, however, is known to be less immunogenic against type 1 and 3 polioviruses. After tOPV administration, the superior replicative capacity of the P2 vaccine strain interferes with effective replication of the other two serotype viruses in

the human intestine (30). To eradicate wild P1 as well, vaccination with monovalent type 1 oral poliovirus vaccine (mOPV1) was introduced in the remaining countries where poliovirus is endemic, since this vaccine is more immunogenic for type 1 than the tOPV (4, 20).

In 2005/2006, a clinical study was conducted in Egypt to compare the immunogenicity of mOPV1 with that of the tOPV in newborns (15). Newborns were vaccinated with mOPV1 or tOPV as soon as possible after birth and were challenged with mOPV1 4 weeks later. Vaccination with mOPV1 at birth resulted in a higher humoral and mucosal protection against P1 at day 28 than vaccination with tOPV at birth.

In line with the recommendations of the WHO Polio Laboratory Network, we determined the antigenic characters of all the viruses shed by the newborns of the Egyptian study by using an intratypic differentiation (ITD) enzyme-linked immunosorbent assay (ELISA). The outcome of this analysis, an unexpectedly high percentage of AD isolates, prompted further investigation. To determine the possible presence of VDPVs and to gain insight into the genetic and antigenic evolution of the mOPV1 and tOPV isolates shed by the newborns in this study, we determined the sequences of the capsid regions of these isolates. We looked for correlates with antigenic change and rates of mutagenesis in the viruses and compared the evolution rates of the viruses shed by vaccinees of both study groups. We also linked the serological data collected during the study to the excretion of Sabin 1 isolates.

Materials and Methods

Study design

The design of the study of the comparative immunogenicity of mOPV1 versus tOPV has been described previously (15). Briefly, 530 newborns from three sites in Egypt located in Greater Cairo and Alexandria were randomly distributed into two groups (A and B). Group A received mOPV1 with a titer of $10^{6.8}$ 50% cell culture infective doses per dose as soon as possible after birth and before receiving breast milk (median interval, 60 min). Group B received tOPV (titers of $10^{6.9}$ for type 1, $10^{5.5}$ for type 2, and $10^{6.5}$ for type 3) as soon as possible after birth and before receiving breast milk. The newborns of both group A and group B were challenged with mOPV1 (titer, $10^{6.8}$) 28 days after the birth dose. Three blood samples were collected per child: one at birth (cord blood), one at day 28 just before the challenge with mOPV1, and one at day 56. Five stool samples per child were collected: at day 28 just before the challenge dose was administered and at days 35, 42, 49, and 56. The blood samples were shipped to the Enterovirus Diagnostic Laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, where the antibody titers were determined

by using a modified neutralization assay for antibodies to poliovirus types 1, 2, and 3 (15). Serological results were reported as reciprocal titers of serum dilutions that exhibited 50% neutralization. Seroconversion was defined as a ≥4-fold increase over the expected decline in the maternal antibody titer. The half-life of maternal antibody decay was assumed to be 28 days, consistent with direct measurements from previous studies. Change from a seronegative immune status to a reciprocal titer of ≥8 was also considered seroconversion. The stool samples were shipped to the National Institute for Public Health and the Environment (RIVM, The Netherlands) for virological analysis.

Randomization and inoculation of stool samples

A total of 2,459 fecal samples were received at the RIVM in four shipments and were labeled with blinded code numbers that could not be used to deduce the identities of individual vaccinees or vaccination groups by the testing laboratory. For each fecal sample, a 20% (wt/vol) suspension was prepared in phosphate-buffered saline containing 20% chloroform. For each sample, two tubes with monolayers of L20B cells, a transgenic murine cell line expressing the human poliovirus receptor, were inoculated with 0.1 ml stool suspension and observed for cytopathogenic effect (CPE) for 10 days. Cytopathogenic effect-positive cultures were passed once more in L20B cells (41).

Sabin-specific PCR assay and ITD

The presence and identities of poliovirus serotypes in the viral cultures were determined by a Sabin-specific PCR assay (43). Viral RNA was isolated from the viral culture, eluted in a final volume of 50 µl using the MagnaPure LC method (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and amplified in a two-step reverse transcriptase PCR (RT-PCR) assay according to standard procedures recommended by the WHO (41). Virus harvests containing a mixture of serotypes were passed in the presence of polyclonal antisera against two of the three serotypes in order to separate the virus mixture (40).

The antigenic characters of the isolates were determined by the ITD-ELISA described in the WHO Polio Laboratory Manual (40). With this assay the isolates were characterized as Sabin-like (SL), non-Sabin-like (NSL), double-reactive (DR), or nonreactive (NR), based on their reactivities with Sabin-like and wild-type-specific cross-absorbed polyclonal antibodies (39).

Capsid sequencing

The VP1 regions of all isolates were amplified in a two-step RT-PCR assay using the Y7 forward (5'-GGTTTTGTGTCAGCGTGTAATGA-3') and Q8 reverse (5'-AAG AGGTCTCTATTCCACAT-3') primers (33). Viral RNA was converted to cDNA by

incubation of 3.0 µl of the isolated RNA at 42°C for 60 min with 4.0 µl 50 µM Q8 primer (Isogen), 3.0 µl 10x PCR buffer (670 mM Tris-HCl [pH 8.8], 20 mM $MgCl_2$, 170 mM ammonium sulfate, 0.06 mM EDTA [pH 8], 0.12 mM (β-mercaptoethanol), 16 µl 2.5 mM deoxynucleoside triphosphates (Roche), 1.25 µl 10-U/µl avian myeloblastosis virus RT (Promega), 0.16 µl 125-U/µl RNase inhibitor (Amersham, Life Science), and 2.59 µl water. The reverse transcription reaction was terminated by incubation of the samples at 94°C for 3 min and subsequent chilling on ice. In total, 15 µl of the cDNA was used for the PCR amplification, together with 3.5 µl 10x PCR buffer, 2 µl 50 µM Y7 primer, 0.5 µl 5-U/µl Taq polymerase (Roche), and 29 µl water. PCR was carried out for 25 cycles of 30 s at 94°C, 45 s at 42°C, and 1 min at 60°C, with a final cycle of 7 min at 60°C. The PCR products were purified according to the manufacturer's protocol for the QIAquick PCR purification kit (Qiagen, 2002) and were sequenced using a fluorescence-labeled dideoxynucleotide technology from Applied Biosystems (Foster City, CA) with forward primer Y7 and reverse primer Q8. The VP2 and VP3 regions of selected P1 isolates were amplified by RT-PCR and sequenced by the procedures described above using primers 3F2 (5'-GAGCCCATCAAGGATGTCC-3') and n5R (5'-CCTAGGATCTGAAGCTGG-3') for VP2 and primers 5F (5'-ATATCT-YACTGCAGACAA-3'), n6R (5'-TGACCTAACCCCTGTGCT-3'), 6F (5'-CTCA-TGTACTATGGTAGT-3'), and 7R (5'-CACTTGATTTAAGGCATG-3') for VP3 (3).

Sequence data analysis

The nucleotide sequences were assembled using Seqman software (version 3.61; DNAStar). Sequence data were obtained by using both the sense and antisense primers. The P1, P2, and P3 sequences were aligned with the Sabin reference strains obtained from mOPV1 and tOPV by using Clustal W (38) from BioEdit software, version 7.0.0 (21). Synonymous and nonsynonymous mutations were determined with DNA Sequence Polymorphism, version 4.10 (37). Nucleotide mutation rates were estimated by linear regression. At ambiguous sites, only the base found in the higher molar proportion was scored.

Statistical methods

Data were analyzed with the statistical software package R (36). Synonymous and nonsynonymous mutation rates were estimated by linear regression. The serological data of the Egyptian study were plotted in boxplots using Excel.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB467375

to AB467543 and AB467720 to AB467724 for the P1 isolates of the mOPV1 study group, accession numbers AB467544 to AB467719 for the P1 isolates of the tOPV study group, accession numbers AB467725 to AB467814 for the P2 isolates, and accession numbers AB467815 to AB467867 for the P3 isolates.

Results

Mucosal immunity following the birth dose of mOPV1 or tOPV

Of the 530 children initially enrolled, 421 newborns participated throughout the study: 231 in group A (mOPV1 at birth) and 190 in group B (tOPV at birth) (Table 1). These children represented the group used for the final analyses, and the sample sizes were sufficient for analyzing differences in the responses to mOPV1 and tOPV and for some subset comparisons of the groups and subgroups. For the primary outcome measure, seroconversion to P1, mOPV1 demonstrated approximately twofold greater seroconversion to the birth dose than tOPV1, as reported previously (15). Mucosal immunity as measured by virus excretion following challenge was the secondary outcome measure of the study. Significantly more newborns who received mOPV1 at birth shed P1 at 28 days before administration of the challenge dose than newborns who received tOPV at birth (P = 0.02; odds ratio [OR], 2.34; 95% confidence interval [CI_{95}], 1.09 to 5.33) (Table 1), consistent with the previously reported higher seroconversion in group A (15). After the mOPV1 challenge at day 28, however, a higher proportion of newborns in group B than in group A shed P1 virus at all four sampling times, consistent with higher mucosal immunity in newborns of group A. Newborns never shedding P1 virus on any of the sampling days were significantly more common in the mOPV1 group (136/231) than in the tOPV group (79/190) (P = 0.000; OR, 2.01; CI_{95}, 1.34 to 3.03).

Table 1: Shedding of Sabin 1 virus before and after challenge*

P1 virus shedding	% of newborns (no./total tested) in group**		P***
	A (mOPV1)	B (tOPV)	
None	58.9 (136/231)	41.6 (79/190)	0,000
Before challenge	12.6 (29/231)	5.8 (11/190)	0,020
7 days after challenge	25.9 (59/228)	41.5 (78/188)	0,001
14 days after challenge	22.3 (50/224)	29.3 (54/184)	0,111
21 days after challenge	12.6 (28/222)	15.3 (28/183)	0,471
28 days after the challenge	8.2 (18/220)	13.2 (24/182)	0,140

* Data are from reference 15.
** In group A, 294 newborns were enrolled for the study and 231 completed the study requirements. In group B, 236 newborns were enrolled for the study and 190 completed the study requirements.
*** Calculated by Fisher's two-tailed test.

Virus isolation

The Sabin-specific PCR detected the presence of 186 P1 isolates, 35 P2 isolates, and 19 P3 isolates in group A (231 newborns). In group B (190 newborns), 202 P1, 67 P2, and 44 P3 isolates were detected. All L20B-positive cultures contained only polioviruses that reacted as Sabin-like viruses in the Sabin-specific PCR.

Antigenic characterization

In addition to screening for wild poliovirus by PCR, all these isolates were antigenically characterized by the ITD-ELISA (40) using cross-absorbed polyclonal Sabin and wild-type-specific antibodies (Table 2). In total, 44.6% of the P1 isolates in the mOPV1 study group (83/186 isolates from 53/95 children) and 21.8% of the P1 isolates in the tOPV study group (44/202 isolates from 34/111 children) had an AD character (NR, NSL, or DR). These percentages were much higher than the percentages of AD P2 and P3 isolates in both study groups and also much higher than the percentages of isolates or children observed in routine screening of Sabin-related isolates in the global surveillance network (<5% for P1) (27). The elevated percentage of AD P3 isolates in the mOPV1 study group is not significant and is most likely due to the low number of P3 isolates shed by this group (n = 3). The difference in the percentage of AD P1 isolates shed by newborns between group A and group B was significant (P = 0.001; OR, 2.89; CI_{95}, 1.82 to 4.62).

Table 2: Antigenic characterization by ITD-ELISA

Antigenic characterization	% of isolates					
	Group A (mOPV1)			Group B (tOPV)		
	P1	P2	P3	P1	P2	P3
	(n=186)	(n=35)	(n=19)	(n=202)	(n=67)	(n=44)
SL	55,4	94,3	84,2	78,2	91	97,7
NR	22,6	5,7	15,8	12,4	4,5	2,3
NSL	16,1	0	0	6,4	0	0
DR	5,9	0	0	3,0	4,5	0

VP1 capsid sequence analysis

Because the ITD-ELISA revealed remarkably high numbers of AD P1 isolates, it was necessary to characterize these isolates further in consistency with WHO-recommended procedures (40). All known P1 VDPVs and most, but not all, of the known P2 and P3 VDPVs react as AD in the ITD-ELISA (9). The VP1 capsid region was sequenced to determine whether any isolate could be defined as a VDPV and to understand the antigenic character and evolution of these isolates. The VP1 regions of 345 P1 isolates (involving 81 newborns of group A and 94 newborns of group B), 90 P2 isolates, and 53 P3 isolates were sequenced successfully. Sequences could not be obtained from the remaining 43 P1 isolates, 12 P2 isolates, and 10 P3 isolates because of poor sequencing quality, possibly caused by the presence of multiple strains of the same serotype. These isolates without definitive sequences were distributed randomly among newborns of both groups A and B; all of these isolates had a Sabin-like character by ITD-ELISA and therefore most likely contained only a small number of nucleotide changes and had no amino acid changes associated with AD isolates.

No VDPVs (defined as 10 or more changes in the VP1 region) of type 1 or type 3 were found in the study groups (Fig. 1A and 1C). The number of nucleotide mutations in the VP1 regions of P1 isolates ranged from none to eight. The maximum number of nucleotide mutations found in the VP1 regions of the P3 isolates was six. One newborn of group B shed a type 2 VDPV with a VP1 divergence of 1.22% (11 mutations in VP1) at 56 days after tOPV vaccination (Fig. 1B). Two earlier stool specimens collected from the same vaccinee at days 42 and 49 contained viruses with 5 and 7 mutations, respectively, all of which were also present in the isolate collected at day 56, demonstrating the accumulation of 6 mutations in a 2-week period in this child and implying the accumulation of 11 mutations within 8 weeks of receipt of the birth dose. The child had already seroconverted to P2 by day 28, also consistent with the response to the tOPV birth dose this child received. In addition, one newborn who had received an extra dose of mOPV1 during the routine vaccination activities, and was therefore excluded from the study, shed a type 1 VDPV with a VP1 divergence of

1.3% at 49 days after the birth dose. No evidence of immunodeficiency or disease was found during clinical follow-up of these two newborns with VDPVs.

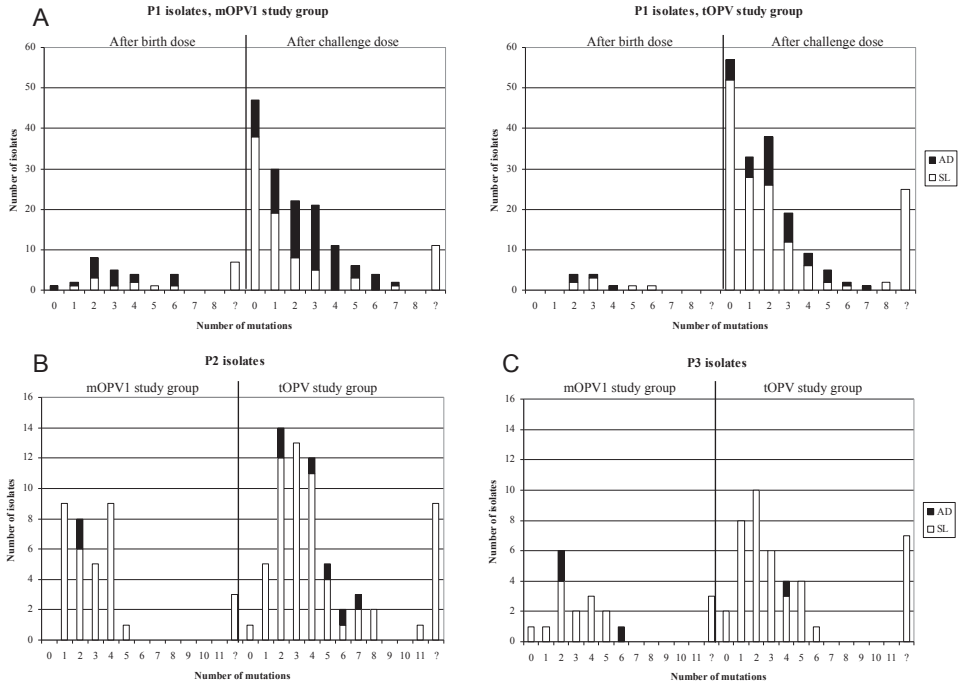

Fig. 1: Numbers of nucleotide mutations in the VP1 regions of isolates. (A) Birth dose- and challenge dose-derived P1 isolates; (B) P2 isolates; (C) P3 isolates.

VP2 and VP3 capsid sequencing

In the VP1 regions of the P1 isolates, no substitutions were found that distinguished the Sabin-like from the AD isolates (Table 3). The VP2 and VP3 capsid regions of 133 P1 isolates (91 Sabin-like isolates and 42 isolates with an AD character) were sequenced to identify the determinants that correlated with the observation of antigenic divergence. In the VP2 region, no amino acid substitutions were found that distinguished the Sabin-like P1 isolates from the AD P1 isolates. In the VP3 region, Ala59 and Lys60 were preferred sites of nonsynonymous mutation (Table 3). Both of these amino acids are associated with neutralization antigenic site III (N-AgIII). Isolates with a mutation of Ala59 to a glutamic acid kept the Sabin-like antigenic character. However, replacement of Lys60 with a threonine, asparagine, or glutamine resulted in an AD character by the ITD-ELISA (Table 3). It therefore appears that the mutation of Lys60 is the major determinant of the AD characteristics observed among the P1 isolates in this study. One isolate (E334) had mutations of Ala59 to valine and Lys60 to glutamine but was characterized as Sabin like by the ITD-ELISA, indicating the possibility of a more complex antigenic structure in this region.

Linkage of P1 shedding and serological data

Using the serological data and VP1 sequence data, it was possible to determine whether observed P1 isolates were most likely derived from the birth dose or the challenge dose. Seroconversion to P1 following the birth dose and/or shedding of P1 virus before administration of the challenge dose at day 28 (observed for 37 newborns) (Table 4) was interpreted as evidence of replication of the birth dose vaccine viruses. Isolates shed after the challenge dose (170 newborns) were assumed to be derived from the challenge dose unless the isolates included mutations in the VP1 region identical to those of the isolate detected at day 28 (6 newborns). The absence of seroconversion to P1 after the birth dose and the absence of shedding of P1 virus at day 28, in combination with seroconversion to P1 after administration of the challenge dose and/or shedding of P1 virus any time after the challenge dose, were interpreted as evidence of replication of the challenge virus (Table 4). It could not be determined whether 11 children (8 in group A and 3 in group B) had undergone seroconversion. Two of these indeterminate children (both in group A) never shed P1 virus; two (both group A) shed birth dose virus; and seven (four in group A and three in group B) shed virus following the challenge dose.

In total, P1 virus shedding was detected at any time in 95 newborns of group A and 111 newborns of group B. Birth dose P1 virus was shed from 26 newborns (27%) of the mOPV1 study group (group A) and 11 newborns (10%) of the tOPV study group (group B), of whom the majority (21/26 in group A and 7/11 in group B) also demonstrated seroconversion to P1 following the birth dose (Table 4). The majority of the newborns who ever shed P1 virus shed challenge-derived P1 viruses (73% [69/95] in group A and 91% [101/111] in group B). In total, 71% of these newborns in group A (49/69) and 77% of these newborns in group B (78/101) seroconverted only after administration of the challenge dose virus. AD P1 isolates were shed mainly by the newborns belonging to this category, especially in the mOPV1 study group, and were thus mOPV1 (challenge dose) derived.

Table 3: Amino Acid mutations in the VP1 and VP3 regions of P1 isolates*

Group	ITD-ELISA	Isolate	AA position in VP1 (N-AgI)													AA position in VP1									VP1 (N-AgII)				AA position in VP3 (N-AgIII)										
			90	91	92	93	94	95	96	97	98	99	100	101	102	103**	104**	**106****	**134****	142**	145**	147**	149**	150**	223	224	249**	290**	58	59	60	70	71	72	73	76	77	79	**225****
		OPV1	I	T	V	D	N	S	A	S	T	K	N	K	D	K	L	T	F	F	T	N	H	A	A	L	N	T	S	A	K	V	R	L	S	P	H	T	M
		WT	M					P				T						A	L												T								L
A	NR	E1226										R						A									S				Q								
A	NR	E1940										M						A													Q								
A	NR	E1884										T						A													Q								
A	NR	E1735										N		M																	N								
A	NR	E1573										E								S				V	V		S				N								
A	NR	E1940																A			A										Q								
A	NR	E201																A												E	Q								
B	NR	E2104	M															A													N								
B	NR	E2063							T									A												E	Q								
B	NR	E1259																				S									N								
B	NR	E1198																A				S									N								
B	NR	E2030																A									D				N								
B	NR	E1125																A												E	N								
B	NR	E2063							T									A													R								
A	NSL	E532										E						S				S									T								
A	NSL	E2342										E						A								I					T								
A	NSL	E2442										E						A													T								
A	NSL	E1410										E						A													T								
A	NSL	E1074																A													T								
A	NSL	E1736																A													T								
A	NSL	E784										N						A									S				T								
A	NSL	E1190										N						A													T								
A	NSL	E1244																A									D				T								
B	NSL	E1894										N						S													T								
B	NSL	E771										M						A													T								
B	NSL	E1501										E																			T								
A	NSL	E1011	L												***	***								V							T								
B	NSL	E1894																													T								
A	DR	E710																													N								

A	SL	E2196				N			A								E		
A	SL	E1665																	
A	SL	E2052																	
A	SL	E1057																	L
A	SL	E708																	
A	SL	E1440							S								E		
A	SL	E2196				N			A								E		
A	SL	E433				T													
A	SL	E572				N	N												
A	SL	E505	M														E		
A	SL	E2352		S		N			A										
A	SL	E333			T					S							E		
A	SL	E1685			T	E		R	A							M			
A	SL	E78									S						E		
A	SL	E334							A						S		V	Q	
A	SL	E1559															N		
B	SL	E1529	M														E		
B	SL	E542	L																L
B	SL	E1629			T				A								E		
B	SL	E2316			T				A								E		
B	SL	E496				N													
B	SL	E358				T			A										
B	SL	E495				E													
B	SL	E108					E		A										
B	SL	E496										Y							
B	SL	E350							S						S		E		
B	SL	E232							A										
B	SL	E635																	L
B	SL	E2176				R			A										
B	SL	E817								A									
B	SL	E372							A				V						
B	SL	E888												I					
B	SL	E1315							A						D				

* The VP3 regions of E2176, E817, E372, E888 and E1315 were not sequenced. The isolates in this table are presented to show that in the VP1 region, no substitutions that distinguish SL isolates from AD (NSL, NR, or DR) isolates were present. Major determinants of attenuation are boldfaced. **, the amino acid (AA) is not located in an antigenic site; ***, the amino acid was deleted.

Table 4: Overview of shedding of P1 isolates and seroconversion against P1

Shedding of P1 after birth dose or challenge dose	Group*	No. of newborns:		Seroconversion to P1**		Avg (range) log 2 titer of antibody against P1			No. of newborns with sero-conversion to:	
		Total	Shedding at least 1 AD P1 isolate	Before dose	After dose	Day 0	Day 28	Day 56	P2	P3
No shedding	A	26	0	-	-	8.65 (5.17-10.5)	7.07 (3.5-10.5)	5.99 (2.83-10.17)	5	4
	B	19	0			8.96 (6.17-10.5)	7.39 (4.5-9.83)	5.94 (3.5-8.5)	10	7
	A	11	0	-	+	8.38 (7.5-9.5)	7.65 (4.83-9.83)	10.17 (9.5-10.5)	1	4
	B	12	0			7.44 (5.17-9.17)	5.64 (3.83-7.83)	10.06 (9.17-10.5)	9	5
	A	97	0	+		6.14 (2.5-10.17)	10.14 (7.17-10.5)	10.00 (6.5-10.5)	13	5
	B	48	0			5.60 (2.5-9.83)	9.74 (6.83-10.5)	9.25 (4.5-10.5)	34	14
	A	2	0	?	?	10,5	10,5	10,5	0	0
	B	0	0						0	0
Subtotal	A	136	0						19	13
	B	79	0						53	26
Shedding after birth dose	A	2	0	-	-	9.5 (9.17-9.83)	9.5 (8.83-10.17)	8.84 (8.5-9.17)	1	2
	B	1	0			7,83	5,17	4,83	0	0
	A	1	0	-	+	7,5	4,83	10,5	0	1
	B	3	0			9.61 (8.83-10.17)	9.61 (9.17-10.17)	10,5	3	1
	A	21	12	+		7.47 (5.17-9.5)	10.13 (7.5-10.5)	10.39 (9.83-10.5)	2	0
	B	7	4			6.17 (3.5-8.17)	10.02 (9.5-10.5)	10.07 (8.5-10.5)	7	2

	A	2	2	?	?	10,5	10,5	10,5	0	0
	B	0	0						0	0
Subtotal	A	26	14						3	3
	B	11	4						10	3
Shedding after challenge dose	A	6	0	-	-	10.06 (8.83-10.5)	9.17 (6.5-10.17)	8.17 (4.83-10.17)	1	1
	B	9	0			9.43 (6.5-10.5)	8.54 (5.5-9.83)	8.02 (6.5-9.83)	5	3
	A	49	30	-	+	7.5 (2.83-10.5)	5.95 (2.5-10.17)	10.3 (7.17-10.5)	6	5
	B	78	28			7.35 (3.17-10.5)	5.6 (2.5-10.17)	9.98 (5.17-10.5)	49	19
	A	10	6	+		5.43 (2.5-10.17)	9.9 (7.83-10.5)	10,5	1	0
	B	11	2			6.83 (3.5-10.17)	9.23 (5.5-10.5)	9.65 (5.5-10.5)	11	2
	A	4	3	?	?	10,5	10,5	10,5	0	0
	B	3	0			10,5	10,5	10,5	2	0
Subtotal	A	69	39						8	6
	B	101	30						67	24
Total	A	231	53						30	22
	B	191***	34						130	53

* **Group** A, mOPV1 study group; group B, tOPV study group.
** -, no seroconversion; +, seroconversion; ?, indeterminable.
*** One newborn who was already shedding the birth dose virus started shedding the challenge dose virus as well.

The difference between groups A and B in the number of newborns shedding challenge dose P1 virus with an AD character who seroconverted against P1 only after the challenge dose could not be ascribed to a difference in the neutralizing antibody titers against P1 at day 28 (mean titers, 5.95 log 2 for group A and 5.6 log 2 for group B; P = 0.34 by a one-way analysis of variance [ANOVA] test) (Fig. 2). In addition, these titers did not differ significantly between newborns shedding only Sabin-like isolates (mean titer for group A, 5.39 log 2 [range, 2.83 to 8.5 log 2]; mean titer for group B, 5.5 log 2 [range, 2.5 to 10.17 log 2]) and newborns shedding at least one AD isolate (mean titer for group A, 5.93 log 2 [range, 2.5 to 10.17 log 2]; mean titer for group B, 5.46 log 2 [range, 2.83 to 9.17 log 2]) (P by one-way ANOVA, 0.396 for group A and 0.927 for group B).

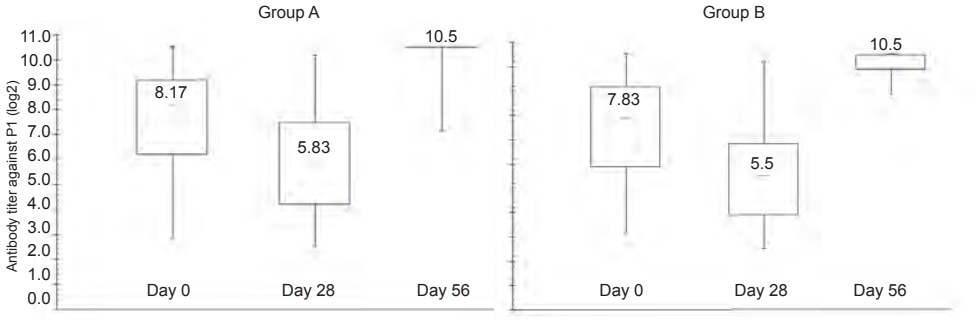

Fig. 2: Serological data for newborns with seroconversion against P1 after the challenge dose and shedding of challenge dose P1 virus (49 newborns in group A and 78 in group B). Values given in the boxplots are median titers.

In total, 56% of the newborns shedding P1 virus in group A (53/95) shed at least one AD P1 isolate. In group B, this percentage was 31.6% (34/111). In group A, 26% of the newborns who shed AD P1 virus (14/53) were shedding virus from the birth dose (Table 4). In group B, this percentage was 12% (4/34). The majority of the newborns shedding AD P1 virus (74% [39/53] in group A and 88% [30/34] in group B), however, shed challenge dose virus. These shedding newborns mainly seroconverted to P1 after administration of the challenge dose. In this category, 30 of the 49 newborns in group A (61%) shed AD P1 virus. In group B, this number was significantly lower: 28 of the 78 newborns (36%). The proportion of AD P1 isolates (birth dose and challenge derived) increased with time of shedding, particularly in the mOPV1 study group (Fig. 3). There were 21 infants who shed virus after the challenge dose but seroconverted after the birth dose, and it is therefore uncertain which dose was the source of the virus observed after challenge. If these children are excluded from the analysis, then the percentage of newborns shedding birth dose-derived AD P1 isolates was 56% in the mOPV1 group (20/36) versus 27% in the

tOPV group (6/22). The percentage of newborns shedding challenge dose-derived AD P1 isolates was 56% in the mOPV1 group (33/59) versus 31% in the tOPV group (28/90). These results are not significantly different from those with the 21 infants included.

A significant proportion of children who did not shed virus following vaccination nevertheless seroconverted during the study. Among newborns in group A, 42.0% (97/231) seroconverted to the mOPV1 birth dose although shedding of virus was never detected in any sample, whereas 80% (49/61) of children in group A who seroconverted to the challenge dose shed virus at some point after challenge. Because of the study design, the first time point where shedding of birth dose-derived virus is possible is 28 days following vaccination. In contrast, shedding as a result of the challenge dose can be measured at 7, 14, 21, and 28 days following vaccination. This indicates the likelihood that the measurement of birth dose shedding is underestimated; therefore, interpretation of the comparison of shedding rates from the birth dose and the challenge dose is not possible.

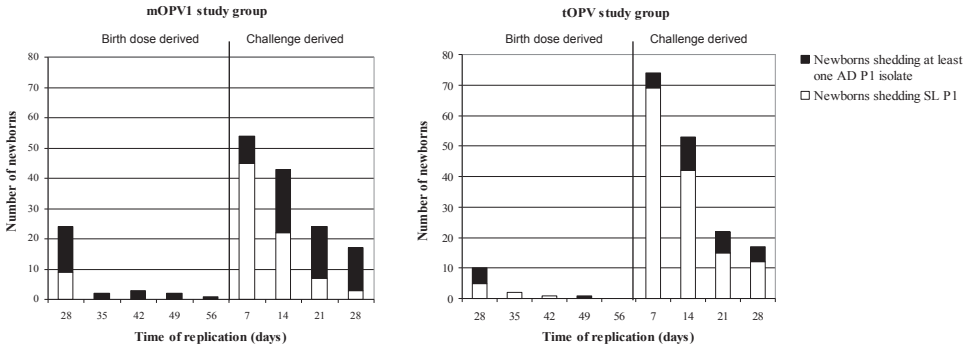

Fig. 3: Shedding of antigenically divergent P1 isolates over time.

Sites of mutations

The average number of nucleotide mutations in isolates of both study groups increased with time of shedding following vaccination (Table 5). Nonsynonymous/synonymous mutation ratios were calculated from the total numbers of nonsynonymous and synonymous mutations for all isolates at the same sampling instance and were similar among isolates of both study groups. For the challenge dose isolates, the first phase of evolution (14 to 28 days of replication in group A and 7 to 28 days of replication in group B) was characterized primarily by the accumulation of nonsynonymous mutations (nonsynonymous/synonymous mutation ratio, >1) (Table 5). Reversion of Thr106, one of the major determinants of attenuation, occurred relatively quickly after vaccination. At 14 days, 49% of the P1 isolates of the mOPV1 study group and

60% of the P1 isolates of the tOPV study group had replaced this residue. After 28 days, however, the ratio of nonsynonymous to synonymous mutations decreased in group B. This resulted from the accumulation and fixation of silent mutations and the decline in the rate of nonsynonymous mutations.

Table 5: Average percentage of VP1 difference and nonsynonymous/synonymous mutation ratio

Group, virus, and day	VP1 difference (%)	No. of mutations		Nonsynonymous/synonymous mutation ratio	No. of isolates
		Synonymous	Nonsynonymous		
Group A (mOPV1)					
Birth dose-derived virus					
Day 28	0.35 (±0.20)	24	39	1,63	23
Day 35	0.39 (±0.04)				2
Day 42	0.41 (±0.10)				3
Day 49	0.55 (±0.16)				2
Day 56	0,66				1
Challenge dose-derived virus					
Day 7	0.08 (±0.15)	19	17	0,89	50
Day 14	0.18 (±0.11)	23	48	2,09	41
Day 21	0.36 (±0.18)	23	46	2,00	22
Day 28	0.32 (±0.15)	16	33	2,06	16
Group B (tOPV)					
Birth dose-derived virus					
Day 28	0.34 (±0.15)	14	16	1,14	10
Day 35	0.41 (±0.12)				2
Day 42	0,33				1
Day 49	0,39				1
Day 56					0
Challenge dose-derived virus					
Day 7	0.08 (±0.13)	18	24	1,33	64
Day 14	0.24 (±0.19)	32	66	2,06	45
Day 21	0.26 (±0.17)	11	34	3,09	19
Day 28	0.33 (±0.17)	19	30	1,58	15

In addition to Thr106, Lys99 was a preferred site for mutation (Table 3). Significantly more newborns in group A (28.4% [23/81]) shed at least one isolate with a mutation of Lys99 than newborns in group B (11.7% [11/94]) (P = 0.007; OR, 2.99; CI_{95}, 1.28 to 7.32). Up to 4 weeks of replication, isolates with a mutation of residue 99 had

a significantly higher number of nonsynonymous nucleotide mutations in the VP1 region than isolates without this amino acid mutation (P = 0.000 by a mixed-effect Poisson regression model) (see the supplementary material). In group A, 89.7% (26/29) of the isolates with a Lys99 mutation were AD, compared to 41.7% (5/12) of the isolates with a Lys99 mutation in group B.

Sequence comparison of the P1 isolates obtained from different newborns showed that each newborn whose isolates had more than three mutations had a unique mutation pattern. The synonymous- and nonsynonymous-mutation rates in the VP1 region were estimated from the slope of the regression lines using the numbers of mutations of all isolates shed by an individual (Table 6). In both groups A and B, the average nonsynonymous-mutation rate was higher than the average synonymous-mutation rate (Table 6, "Total"). For group A, however, this difference was not significant (P = 0.107). When these rates were observed separately for newborns shedding P1 isolates without (-Δ99) and with (+Δ99) replacement of lysine 99, the isolates from both groups A and B with replacement of residue 99 had a ~3-fold higher nonsynonymous-mutation rate than isolates without this replacement (P = 0.000) (Table 6, -Δ99 and +Δ99; see also supplementary material). The synonymous-mutation rates, on the other hand, did not differ significantly between isolates with and without replacement of residue 99 in the mOPV1 study group. In the tOPV study group this difference was significant (P = 0.005), but the number of isolates with replacement of this residue was small in this study group.

The titers of neutralizing antibody to P1 at day 28 did not differ significantly between newborns shedding Sabin-like and AD P1 isolates without replacement of lysine 99 and newborns shedding P1 isolates with replacement of residue 99 after administration of the challenge dose (mean titer, 6.76 log 2 [range, 2.5 to 10.17 log 2]) (P by a one-way ANOVA test, 0.081 for group A and 0.114 for group B).

Table 6: Substitution rates for VP1 capsid sequences of isolates without and with replacement of residue 99*

	Group and type of mutation	Rate (10^-2 mutations/site/yr) (variation)**			P***
		Total	-Δ99	+Δ99	
A					
	Synonymous	2.4 (0.20)	2.4 (0.16)	2.5 (0.28)	0,949
	Nonsynonymous	3.9 (0.27)	2.2 (0.04)	7.6 (0.64)	0,000
B					
	Synonymous	1.9 (0.09)	1.6 (0.07)	4.7 (0.22)	0,005
	Nonsynonymous	3.3 (0.14)	2.6 (0.09)	8.8 (0.16)	0,000

* -Δ99 and +Δ99, without and with replacement of residue 99, respectively.
** For group A, the *P* values for the significance of differences between rates of synonymous and nonsynonymous mutations were 0.107 for all isolates, 0.078 for -Δ99 isolates, and 0.028 for +Δ99 isolates. For group B, the corresponding *P* values were 0.013, 0.033, and 0.085, respectively.
*** Calculated for -Δ99 and +Δ99 isolates using the one-way ANOVA test (alpha = 0.05)

Shedding of P2 and P3 isolates

In the tOPV study group, 130 newborns seroconverted to P2 and 53 seroconverted to P3 (Table 4); of these, 47 newborns shed P2 virus (36%) and 26 shed P3 virus (49%) at any time, while 39 shed P2 virus (30%) and 24 shed P3 virus (45%) at the time of the challenge dose. This is consistent with the preferential replication of P2 following the first dose of tOPV. In the VP1 regions of the P2 isolates, four preferred sites of mutation were observed: Ile143 (a major determinant of attenuation), Lys169 (antigenic site II), Asn171, and Arg103 (Table 7). VP1 sequencing of the P3 isolates revealed the presence of two preferred sites of mutation: Ala54 and Thr6 (an important determinant of attenuation) (Table 8).

Although newborns of group A were vaccinated solely with mOPV1, 30 newborns seroconverted against P2 (13%) and 22 against P3 (9.5%) (Table 4). In addition, 21 of these newborns also shed a P2 isolate (2 of which were AD) and 6 newborns shed a P3 isolate (3 of which were AD). Seroconversion of these newborns to P2 and/or P3 is consistent with the isolation of P2 and P3 viruses and makes contamination of these fecal samples highly unlikely. This finding strongly implies that the study population was exposed secondarily to vaccine-related viruses from the families or communities outside the vaccine doses administered as part of the study protocol. The effect is to overestimate the rate of seroconversion and shedding that can be attributed to the vaccines administered.

Table 7: Amino acid mutations in the VP1 regions of P2 isolates

Group	Antigenic characterization by ITD-ELISA	Isolate	Amino acid mutation at the following position in VP1:											
			101	**102**	**103**	**104**	**105**	**142**	**143***	**144**	**168**	**169**	**170**	**171**
		Sabin 2	A	S	R	L	F	Y	I	D	G	K	W	N
		WT			K								R	
A	DR/NR	E382							T					
B	NR	E2135							T			E		
B	NR	E1815			K				T					
B	DR	E2214							N					
B	NR	E2400	D	R										
B	DR	E808												
B	NR	E1630							T					
B	DR	E514			K				T					
A	SL	E216												
A	SL	E1560												D
A	SL	E561							N					
A	SL	E137							S					
B	SL	E1104							N					D
B	SL	E2363							T					D
B	SL	E958							T					
B	SL	E983							T			E		
B	SL	E700										E		
B	SL	E196												D
B	SL	E74								E				
B	SL	E13			K				T					
B	SL	E972							V					
B	SL	E483							N	E				
B	SL	E2148							T					D
B	SL	E694					S							D

* A determinant of attenuation

Table 8: Amino acid mutations in the VP1 regions of P3 isolates

Group	Antigenic characterization by ITD-ELISA	Isolate	Amino acid mutation at the following position in VP1:						
			4	**5**	**6***	**7**	**53**	**54**	**55**
		Sabin 3	D	L	T	S	L	A	P
		WT			I				
A	NR	E1305	G						
B	NR	E229			I			V	
A	SL	E2352			I			V	
A	SL	E127	G						
A	SL	E428						T	
A	SL	E28	G		I				
A	SL	E21							
B	SL	E1771			I				
B	SL	E643			I			T	
B	SL	E299							

* A determinant of attenuation.

Discussion

This study describes the antigenic and genetic variety of the vaccine viruses shed by newborns participating in a clinical trial in Egypt in 2005 and 2006 to determine the relative efficacy of mOPV1 versus tOPV in newborns (15). The participants were enrolled in the study immediately after birth and did not participate in routine vaccination activities. Therefore, their initial immunity was provided solely by maternal antibody that could be directly measured, which made possible the comparative evaluation of the immunogenicity of mOPV1 versus tOPV. The average titers of maternal antibodies against P1, P2, and P3 were similar among the newborns of the mOPV1 and tOPV study groups (15). The superiority of mOPV1 in humoral response was also observed in mucosal response. Priming of newborns with mOPV1 resulted in reduced shedding of vaccine virus compared to priming with tOPV.

Unexpectedly, another finding was that the proportion of AD vaccine viruses shed by the newborns was much higher than that previously observed, especially in the mOPV1 study group. Although it is likely that no strictly comparable study, with a birth dose of mOPV1 in an Egyptian study population and using these methods of virus characterization, has been done, from which an expectation could be derived, it is clear that these rates have not been observed elsewhere in unselected healthy individuals or through the global AFP (acute flaccid paralysis) surveillance activities (27). The majority of these AD isolates appeared to be derived from the mOPV1 challenge dose provided at the age of 28 days. Replacement of lysine 60 in the VP3 capsid region, as described by previous studies, explains the AD character observed

in this study (3, 42). This residue is located in the loop of antigenic site III and is also involved in the interaction between the virus and the cellular receptor CD155 (2, 22). This effect was compensated for in one strain by an additional mutation of Ala59 to valine as shown previously (42).

Excretion of more AD P1 isolates, coupled with an overall lower rate of shedding, suggests high immunogenic pressure at some of the antigenic sites in the mOPV1 study group. This, however, could not be specifically correlated with antibody titers, and the lack of correlation implies the presence of other parameters that may be more predictive for the shedding of AD P1 isolates.

In both study groups, the majority of the AD P1 isolates were shed by newborns who did not seroconvert to P1 after the birth dose (mOPV1 or tOPV). The majority of these newborns in the tOPV study group (88%), however, did seroconvert to P2 and/or P3 after the birth dose, indicating replication of P2 and P3 in the gut prior to the mOPV1 challenge dose (Table 4). Interference between these replicating P2 and P3 strains and the mOPV1 challenge strain might result in a more limited and less widespread replication of P1 within the tOPV study group and possibly in a lower likelihood of AD P1 isolates than within the mOPV1 study group (3). The amounts of P1 virus in the stool samples could be quantified in order to study the replication of P1 viruses in both study groups.

VDPVs are presently defined as viruses with more than 1% sequence difference in the VP1 gene, compared to the corresponding OPV strain. This degree of sequence variation has been equated to approximately 1 year of replication after infection and, by inference, also after the administration of an OPV dose (28). This period is considerably longer than the 4- to 6-week excretion period of the immunocompetent vaccinees of this study (8). In the present study, the mean overall VP1 synonymous- and nonsynonymous-mutation rates are several fold higher than those reported previously from circulating viruses or those in immunodeficient patients (18, 25, 26, 28, 31, 42, 43). It has been reported previously that rapid reversion of attenuating amino acids in the capsid region can occur in primary vaccinees (14); however, the overall rate of mutations in a large study using molecular characterization by genomic sequencing of a large number of isolates has only rarely been determined.

Isolates with mutations at residue 99 had nonsynonymous-mutation rates ~3 times higher than those of isolates without this mutation and were inclined to have an AD character, whereas P1 isolates with Thr106 did not have significantly more nonsynonymous changes than isolates without this mutation. Previous studies have shown that residue 99, which forms a trypsin cleavage site in antigenic site I of the Sabin virus, is a hot spot for change in the VP1 region of VDPVs (10, 13, 17,

19, 23, 24, 28, 29, 34, 42, 43). It is not clear what role mutation of residue 99 could play in the evolution of VDPVs. The observation of a much faster accumulation of nonsynonymous mutations shortly after vaccination in this study should be considered in estimating the duration of replication or circulation of Sabin viruses and VDPVs. The difference in the age and dose of origin for P1 virus shedding complicates the comparison of mutation rates between the different groups. The limited number of children shedding at each time point also limits the determination of difference between intervals and rates of mutation. These study design differences and observed rates may influence differences in the nonsynonymous/synonymous mutation ratios at the different time points and should be kept in mind when these ratios are compared. Regardless, these observations imply that VDPVs analyzed in the past might not be as old as expected.

It should be noted that the P2 VDPV observed in this study was not detected by the current WHO-recommended screening algorithm for VDPVs and was detected only by sequencing of the complete VP1 gene. This finding reiterates the need for new assays for the rapid screening of P2 (and P3) OPV isolates for VDPV detection. The present WHO-recommended VDPV screening methods still detect all known VDPVs of serotype 1.

There are some limitations in this study. The degree to which the study participants would be exposed to poliovirus through family or community contacts during the 8-week study period was unknown at the start of the study. This would have the effect of reducing the specificity of the measured outcomes that could be attributed to the study vaccines. Somewhat surprisingly, 52 newborns vaccinated with mOPV1 at birth seroconverted to P2 and/or P3 during their first month, indicating significant exposure of the study participants to P2 and P3 vaccine strains through their environments. Alternatively, some of these children may represent false-positive seroconversions because of the method of determining seroconversion relative to a projected decay of maternal antibody (15). As noted in Table 4, there were also 11 children whose dose-specific seroconversion could not be determined. On the basis of previous studies, the proportion of misclassification due to these factors is expected to be small but could not be verified within this study design. Because of these observations, we cannot entirely rule out the possibility that some of the AD viruses isolated from the mOPV1 group were secondary infections, but the rates of AD isolates were much higher than the numbers of P2/P3 isolates observed. The significant difference in the proportion of AD P1 isolates shed between the mOPV1 and tOPV study groups, however, cannot be easily ascribed to the circulation of P1 in the environment, because newborns of both groups should have been equally likely

to come into contact with environmentally derived P1 isolates, and the proportion of AD P1 viruses would be expected to be no higher than the ~5% observed in AFP surveillance.
Another limitation in the study design is the limited number of stool specimens following the birth dose compared to the more frequent sampling following the challenge dose. As noted previously, this almost ensures that the rate of shedding from the birth dose is underestimated compared to that from the challenge dose. In addition, it makes the two groups of children inherently noncomparable for a variety of factors. Even though many of these can be addressed directly, by subdividing groups as presented, absolute specificity cannot be ensured. These limitations, however, do not seem to negate the primary conclusions of the study, even if the explanation of the causation is ambiguous.

In summary, this study shows the reduced shedding of mOPV1 challenge virus associated with seroconversion to a birth dose of mOPV1 compared to a comparable dose of tOPV. Unexpectedly, however, vaccination with mOPV1 at birth results in the excretion of a significantly higher proportion of AD P1 viruses, possibly due to antigenic pressure and a longer and more widespread replication of P1 viruses than occurs in the presence of heterologous strains in the case of vaccination with tOPV. In the mOPV1 study group, more isolates with mutations of VP1 amino acid residue 99 occurred, which resulted in a significant increase in additional nonsynonymous mutations. It is uncertain what role this could play in the evolution of VDPVs, but it does suggest the possibility of additional studies of this population. Results from this study, however, also showed that newborns vaccinated with mOPV1 at birth were better protected against P1 and had a clearly lower excretion rate of Sabin viruses after the challenge dose than newborns vaccinated with tOPV at birth. In turn, a high mucosal protection against P1 reduces shedding of vaccine-derived P1 and therefore reduces the risk for transmission and evolution of VDPVs. In areas with good vaccination coverage and thus a high level of mucosal protection, transmission of vaccine virus is expected to be minimal and vaccination with mOPV1 could be very effective in eradicating the last polioviruses of type 1. In areas with low vaccination coverage and a high number of susceptible individuals, vaccination with mOPV1 could potentially lead to transmission of AD P1 isolates like those observed in this study and could possibly increase the risk for the development of VDPVs. However, until now, regular surveillance activities (based on AFP and environmental surveillance) have not provided evidence for a contribution of vaccination with mOPV1 to the evolution and circulation of VDPVs in Egypt after this study or in countries where mOPV1 has been used for several years already (Nigeria, India) (6,

7). This study describes accelerated genetic and antigenic changes observed following vaccination of newborns and may provide some insight into the earliest steps in the process of the generation of VDPVs.

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Supplementary Material Chapter 2

Description of the Poisson regression model designed to analyse the correlation between substitution of lysine 99 of the VP1 region and the number of silent and non-silent mutations in the VP1 region.

Data were analysed using the statistical software package R (R Development Core Team, 2008). After initial observation of the VP1 sequence data, we studied the effect of time of replication and substitution of lysine 99 (VP1) on the number of silent and non-silent nucleotide mutations in the VP1 region of P1 isolates, using a mixed effect Poisson regression model (Molenberghs, G., and G. Verbeke. 2006). The cumulative number of mutations y_{ij}, where index ij is the jth observation of individual i, were assumed to be Poisson distributed with accumulated intensity parameter μ_{ij}:

$$y_{ij} \sim \text{Poisson}(\mu_{ij})$$

The log accumulated intensity is a linear combination of the explanatory variables (here time of replication, substitution or no substitution of lysine 99 and silent or non-silent character of mutations) and a random effect-term for each individual. This term accounts for correlated measurements within each individual. Using the serological data and VP1 sequence data it was possible to determine whether observed P1 isolates were most likely derived from the birth dose or the challenge dose. Time of replication of these viruses was defined as the number of weeks on which the virus was excreted after administration of the vaccine. For birth dose vaccine viruses the day of birth was set as t=0. For challenge dose viruses day 28 (week 4) was set as t=0. A model selection procedure showed that the relation between log accumulated intensity and time appeared not to be linear, but quadratic instead. The effect of substitution of lysine 99 appeared not to change in time (no time x substitution of lysine 99 interaction). In addition, there appeared to be no interaction of time x non-silent character of mutations, but only an interaction of substitution of lysine 99 x non-silent character of mutations. The final model is given by:

$$\log(\mu_{ij}) = \beta_0 + \beta_1 t_{ij} + \beta_2 t^2_{ij} + \beta_3 L_{ij} + \beta_4 S_{ij} + \beta_5 L_{ij} S_{ij} + b_i$$

In the above equation β_0 is the intercept, which is interpreted as the log accumulated intensity of silent mutations of isolates without substitution of lysine 99 ($L_{ij} = 0$ and $S_{ij} = 0$) at time = 0. β_1 and β_2 represent the change in log accumulated intensity for these isolates per week. β_3 represents an additional change in log accumulated intensity in

case of substitution of lysine 99 (L_{ij} = 1). For non-silent mutations, β_4 represents an additional change for the log accumulated intensity (S_{ij} = 1). Finally, β_5 represents an additional change in log accumulated intensity for non-silent mutations in case of substitution of lysine 99 (L_{ij} = 1 and S_{ij} = 1). The random intercept b_i is assumed to be normally distributed with expectation zero and standard deviation σ_b. The interpretation of this term is that each individual has its own specific log accumulated intensity level which is constant over time and that we wish to take into account.

Results

Table S1 shows a summary of the parameter estimates. The model confirmed that time is an important factor for accumulation of mutations in the VP1 region (p-value = 0.000 for time and time^2). For silent mutations, substitution of lysine 99 has a small positive, but not significant, effect on the accumulation of mutations (relative rate RR = 1.27 [0.86, 1.85], p-value = 0.228). However, for non-silent mutations, substitution of lysine 99 has a large positive significant effect on the accumulation of mutations (RR = 2.12 [1.58, 2.84], p-value = 0.000).

The results are graphically shown in Figures S1 and S2. Accumulated mutation intensities are plotted against time of replication. These values are obtained by plugging the concerning parameters from Table S1 into the above model expression. 95% confidence intervals are calculated using the covariance between the parameters. The accumulated mutation intensity increases over time and reaches a maximum at about 3.5 weeks. After that it shows a small decrease, which may be due to a model artifact. For silent mutations, the accumulated mutation intensity is a factor 1.27 higher if lysine 99 is substituted. For non-silent mutations, the accumulated mutation intensity becomes a factor 2.12 higher if lysine 99 is substituted.

Table S1: Parameter estimates, standard errors and p-values of the Poisson regression model.

		Estimate	Std. Error	p-value
Intercept	β_0	-2.919	0.315	0.000
time	β_1	1.675	0.263	0.000
time^2	β_2	-0.235	0.051	0.000
sub lysine 99	β_3	0.235	0.195	0.228
non silent mutations	β_4	0.417	0.122	0.001
sub lysine 99 × non silent	β_5	0.516	0.211	0.014

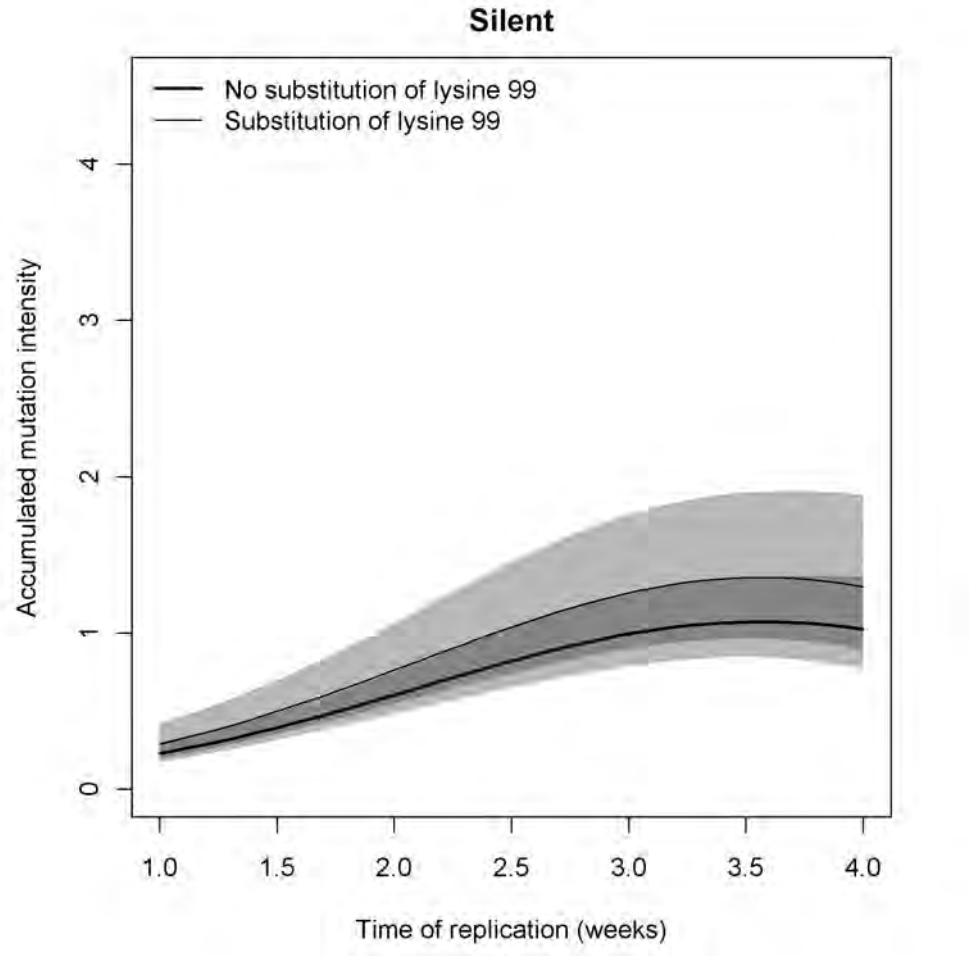

Fig. S1: Accumulated mutation intensity for silent mutations of isolates with and without substitution of lysine 99 (VP1) versus time. The shaded areas represent 95% confidence intervals.

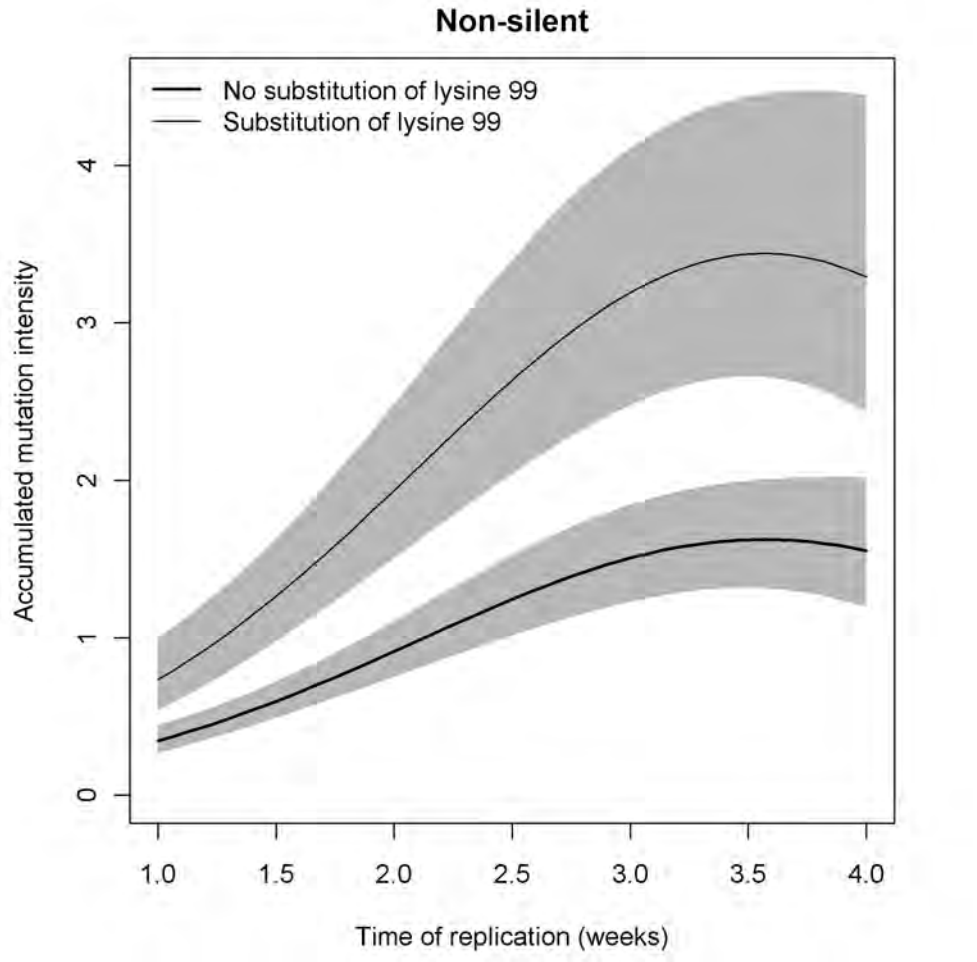

Fig. S2: Accumulated mutation intensity for non-silent mutations of isolates with and without substitution of lysine 99 (VP1) versus time. The shaded areas represent 95% confidence intervals.

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Chapter 3

Epidemiology of Enterovirus 71 in The Netherlands, 1963 to 2008

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Abstract

The incidence of enterovirus 71 (EV71) infection has greatly increased in the Asian Pacific region since 1997. Several large outbreaks, caused by different subgenogroups of EV71, occurred with high rates of morbidity and a substantial number of deaths. In 2007, 58 cases of EV71 infection requiring hospitalization were reported in The Netherlands after a period of low endemicity of 21 years. These events triggered a study on the epidemiology of EV71 in The Netherlands. Genetic analysis of the VP1 capsid region of 199 EV71 isolates collected from 1963 to 2008 as part of enterovirus surveillance activities revealed a change in the prevailing subgenogroups over time. From 1963 to 1986 infections were caused by three different and successive lineages belonging to subgenogroup B (the novel lineage designated B0, as well as B1 and B2). In 1987, following a major epidemic the previous year, the B genogroup was replaced by genogroup C strains of lineages C1 and, later, C2. Analyses of the clinical data suggested that there were differences between infection with genogroup B and with genogroup C strains in terms of the age groups affected and the severity of illness. From comparative analysis with genomic data available in the public domain, we concluded that EV71 strain evolution shows a global pattern, which leads to the question of whether the recently emerged C4 lineage strains will also spread outside of Asia.

Introduction

The genus *Enterovirus* of the family *Picornaviridae* consists of nine species of which five infect humans: poliovirus and human enterovirus A, B, C, and D. Together with 11 serotypes of coxsackievirus A, human enterovirus 71 (EV71) belongs to the human enterovirus A species. Human EV71 was first isolated in 1969, and from that time onward outbreaks of this virus have been described globally (38). On the basis of VP1 nucleotide sequence comparisons, three genogroups have been distinguished: A, B, and C (4, 29). Genogroup A includes only one strain (BrCr-CA-70) isolated in California in 1970 (4). Genogroup B is more common and consists of the previously defined subgenogroups B1 to B5, and genogroup C consists of subgenogroups C1 to C5 (4). Together with its close relative coxsackievirus A16, EV71 is the major causative agent of hand, foot, and mouth disease (HFMD) (usually in children of <5 years of age). EV71, however, is also associated with neurologic disease, including aseptic meningitis, poliomyelitis-like paralysis, brainstem encephalitis, and neurogenic pulmonary edema (6, 36). The incidence of EV71 appears to have increased in the Asian Pacific region since 1997. Several large outbreaks of HFMD have occurred with high rates of morbidity and substantial numbers of deaths (5, 6, 8, 9, 11, 21, 25, 27–29, 36, 40, 42). An association between genogroup and severity of disease has not yet been reported (4, 6).

The increasing numbers of EV71 cases reported in Asia and the observation of an EV71 outbreak in The Netherlands in 2007 after a period of low endemicity of 21 years raised the question of whether the epidemiology of EV71 is changing. Therefore, we did a detailed analysis of the epidemiological and genetic data on EV71 circulation in The Netherlands over a 45-year period (1963 to 2008).

Materials and Methods

Isolation of enteroviruses in The Netherlands

In The Netherlands only the severe, hospitalized cases of EV71 infection are diagnosed and reported as part of the national enterovirus surveillance system. Mild cases of EV71 infection are hardly reported since HFMD is not classified as a notifiable disease. Primary diagnosis for all enterovirus infections in The Netherlands is performed by virological laboratories that participate in the Weekly Sentinel Surveillance system of the Dutch Working Group on Clinical Virology. Fecal samples, throat swabs, and cerebrospinal fluid (CSF) samples are collected from children with systemic viral infection, varying from meningitis to gastrointestinal disorders. The clinical samples are cultured on combinations of enterovirus-sensitive cell lines: RD, tertiary monkey kidney, LLC-MK2, Vero, HEp-2, and various human fibroblast cell lines. Viral isolates

with an enterovirus-characteristic cytopathic effect are confirmed as enteroviruses by an immunofluorescence test with broadly reactive monoclonal antibodies (Dako, CA) (41, 47) or by a specific PCR assay (32, 33). Typing of enterovirus-positive isolates is locally performed by the virological laboratories by the use of serum neutralization tests with polyclonal typing pools (provided by the National Institute for Public Health and the Environment, RIVM, Bilthoven, The Netherlands) (46). Cell cultures of nontyped and nontypeable isolates are routinely sent to the RIVM for typing and characterization and form the basis for the historical RIVM collection of EV71.

Review of historic collection at RIVM for representativeness

In order to determine if differences in the surveillance setup influenced the data and EV71 isolates collected, a review was done of the protocols and technical developments from the beginning of the surveillance data collection.

RNA extraction

Viral RNA was extracted from cell cultures using a MagNA Pure LC Total Nucleic Acid Isolation Kit with a MagNA Pure LC instrument (Roche Diagnostics, Almere, The Netherlands). Lysis was done by adding 100 µl of cell culture to 400 µl of lysis binding buffer (provided in the kit). Extraction was performed according to manufacturer's instructions. Viral RNA was eluted in 50 µl of elution buffer.

EV71 PCR assay

The VP1 gene was amplified as two overlapping regions using the 159F (5'-ACYATGAAAYTGTGCAAGG-3'), 162R (5'-CCRGTAGGKGTRCACGCRAC-3'), 161F (5'-CTGGGACATAGAYATAACWGG-3'), and NP1Anew (5'-CCACYCTGA–AGTTGCCCACG-3') primers, which, except for NP1Anew, have been published previously (4). The previously published NP1A primer did not align optimally with our EV71 reference strains.

Reverse transcriptions were performed to convert the viral RNA to cDNA. For this reaction, 5.0 µl of the isolated viral RNA was incubated at 42°C for 60 min together with 1.0 µl of 25 µM antisense primer, 3.0 µl of 10X PCR buffer (pH 8.3) (100 mM Tris-HCl, 500 mM KCl), 1.8 µl of 25 mM $MgCl_2$, 2.0 µl of 10 mM concentrations of the deoxynucleoside triphosphates, 1.2 µl of 10 U/µl avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands), 0.4 µl of RNase inhibitor (Amersham, Life Science), and 15.6 µl of H_2O. The reaction was finished by incubation of the samples at 95°C for 5 min, followed by incubation on ice.

In total, 15 µl of the cDNA was used for the PCR amplification, together with 3.5 µl of 10X PCR buffer, 1.8 µl of 25 mM $MgCl_2$, 1 µl of 25 µM forward primer, 0.4 µl of

5 U/µl Taq polymerase (Roche), and 28.3 µl of water. The PCR was carried out for 1 cycle of 3 min at 94°C and 30 cycles of 45 s at 94°C, 45 s at 42°C, and 1 min at 68°C, with a final cycle of 7 min at 68°C. Amplicon size analysis was performed using gel electrophoresis. Purification of the PCR products was performed according to the manufacturer's protocol (QIAquick PCR Purification Kit; Qiagen).
Sequencing of the PCR products was carried out with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.2 (Applied Biosystems, Foster City, CA) on an automated sequencer (Applied Biosystems model 3700) using the 159F, 162seq (5'-GTTATRTCTATRTCCCAGTT-3'), 161F, and NP1Anew primers.

Phylogenetic analysis

Editing of the sequence data was performed using Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Isolates were assigned to specific genogroups and subgenogroups according to previously defined nomenclature (4). Phylogenetic dendrograms were generated by the neighbor-joining method (bootstrap analysis with 1,000 pseudoreplicate data sets) using TREECON software, version 1.3b (43). The reference strains used in the phylogenetic analysis are presented in Table 1.

Comparison with globally available data from systematic literature review

Data on the global presence of EV71 subgenogroups were derived by an extensive literature review using the Pubmed and Scopus literature databases. During the first search, abstracts and/or their titles were screened for the terms "enterovirus 71," "enterovirus type 71," "hev71," "hev-71," "ev71," or "ev 71." During the second search articles were screened on the basis of their main keywords: "outbreaks," "genotype," or "phenotype." Alternatively, articles were screened on basis of their scope: "epidemiology," "classification," "genetics," "pathogenicity." A third search was performed during which titles were screened for the terms "outbreak*," "epidem*," "case*," "fatal," "evolution," "genotyp*" (title/abstract), "genogroup*" (title/abstract), "subgenogroup*" (title/abstract), "phenotyp*," "phylogen*," "typing," "types," "serotyp*," "identificat*," "characteri*," "genetic," "circulation," or "new". Articles that were selected on the basis of the first search in combination with the second search or third search were used for reviewing. Year and country of detection and subgenogroup were recorded and used for a comparative analysis with the epidemiology in The Netherlands.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB491212 to AB491220.

Table 1: Reference strains used for phylogenetic analysis

Reference strain	Year of isolation	Place of isolation	Genogroup	Accession no.
BrCr	1970	USA	A	AB433863
2608-AUS-74	1974	Australia	B1	AF135885
2609-AUS-74	1974	Australia	B1	AF135886
2230-NY-76	1976	USA	B1	AF135869
2258-CA-USA-79	1979	USA	B1	AF135880
2952-SD-81	1981	USA	B2	AF135888
3663-MA-82	1982	USA	B2	AF135889
6887-SYD-86	1986	Australia	C1	AY722887
2219-IA-87	1987	USA	B2	AF009539
7238-AK-USA-87	1987	USA	C1	AF135952
7628-PA-USA-89	1987	USA	B2	AF009530
2222-IA-USA-88	1988	USA	B2	AF009540
Y90-3205	1990	Japan	B2	AB433863
2640-AUS-95	1995	Australia	C1	AF135946
2641-AUS-95	1995	Australia	C2	AF135947
2355-OK-97	1997	USA	C2	AF135942
MY821-3-SAR-97	1997	Sarawak	B3	AY125997
2896-TAI-98	1998	Taiwan	B4	AF286516
4350-SIN-98	1998	Singapore	B3	AF376119
TW-2086-98	1998	Taiwan	C2	AF116819
SHZH-CHN-98	1998	China	C4	AY465356
26M-AUS-2-99	1999	Australia	B3	AF376101
F1-CHN-00	2000	China	C4	AB115490
H25-CHN-00	2000	China	C4	AB115492
IM/AUS/12/00	2000	Australia	C1	AF376098
S2861-SAR-00	2000	Sarawak	B4	AF376085
2027-SIN-01	2001	Singapore	B4	AF376111
01-KOR-00	2003	Korea	C3	AY125966
03-KOR-00	2003	Korea	C3	AY125968
TW-2004-104	2004	Taiwan	C4	DQ666684
1301V/VNM/05	2005	Vietnam	C5	AM490149
2928-Yamagata-06	2006	Japan	C4	AB433878
H0/6364/255/2006	2006	United Kingdom	C2	AM939607
EV71/BRU/2006/33930	2006	Brunei	B5	FM201328
1961-Yamagata-07	2007	Japan	C4	AB433892

Results

Isolation of EV71

A review of the protocols and technical developments showed that diagnosis of EV71 has consistently been performed since 1963. Cell lines susceptible to EV71 and standardized serological typing reagents have been used throughout the years (24). EV71 cannot be neutralized by these reagents and formed part of the untypeable cell cultures that were routinely submitted to the RIVM for typing during the entire study period. Monospecific typing sera against EV71 became available at the RIVM in 1977, and isolates that could not be typed in the years 1963 to 1976 were tested retrospectively. A representative selection of the isolates was included in the RIVM biobank.

Trends in EV71 reporting

Between 1965 and 2008, 346 EV71 isolates were identified (Fig. 1). In total, 198 of these isolates (57%) from the same number of patients and 1 EV71 isolate from 1963 were stored and used in this study. Data on the origin of clinical samples, date of isolation, clinical presentation, and age of patient were obtained from the submission forms sent by the virological labs. In total, 55.3% of the EV71 isolates were obtained from feces; 15.1% were from throat swabs; 0.5% were from nose swabs; 3% were from fluid from vesicles on hands, feet, and mouth; and 0.5% were from CSF. For 25.6% of the isolates collected between 1982 and 2007, the sample origin was not known. In 1986 and in 2007 an increase in the number of cases of EV71 infection requiring hospitalization was reported. A mean EV71 isolation rate of 4.2% in 1986 and 6.3% in 2007 versus 0.5% in the preceding and following years indicates that these peaks are real.

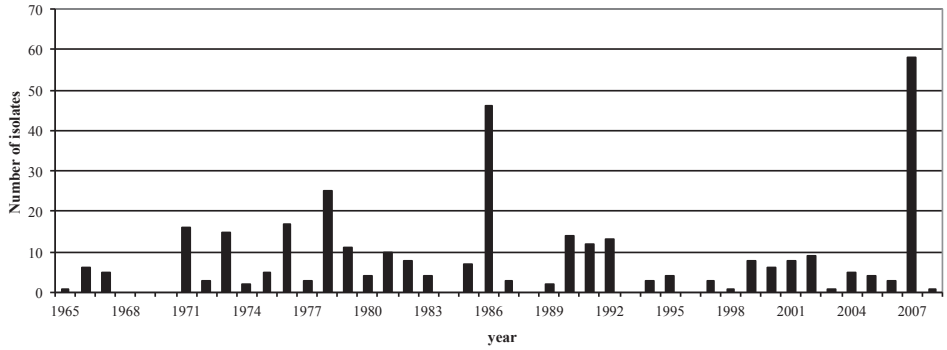

Fig 1: Number of EV71 cases reported by members of the Weekly Sentinel Surveillance system of the Dutch Working group on Clinical Virology (1965 to 2008).

Change of subgenogroup over time

The complete VP1 regions of 199 EV71 strains isolated in The Netherlands in the years 1963 to 2008 were sequenced successfully and were assigned to specific genogroups and subgenogroups on the basis of robust phylogenetic clustering with EV71 reference strains from the GenBank (4). Figure 2 shows the genetic relationships of Dutch isolates representative for the collection studied and the reference strains obtained from GenBank.

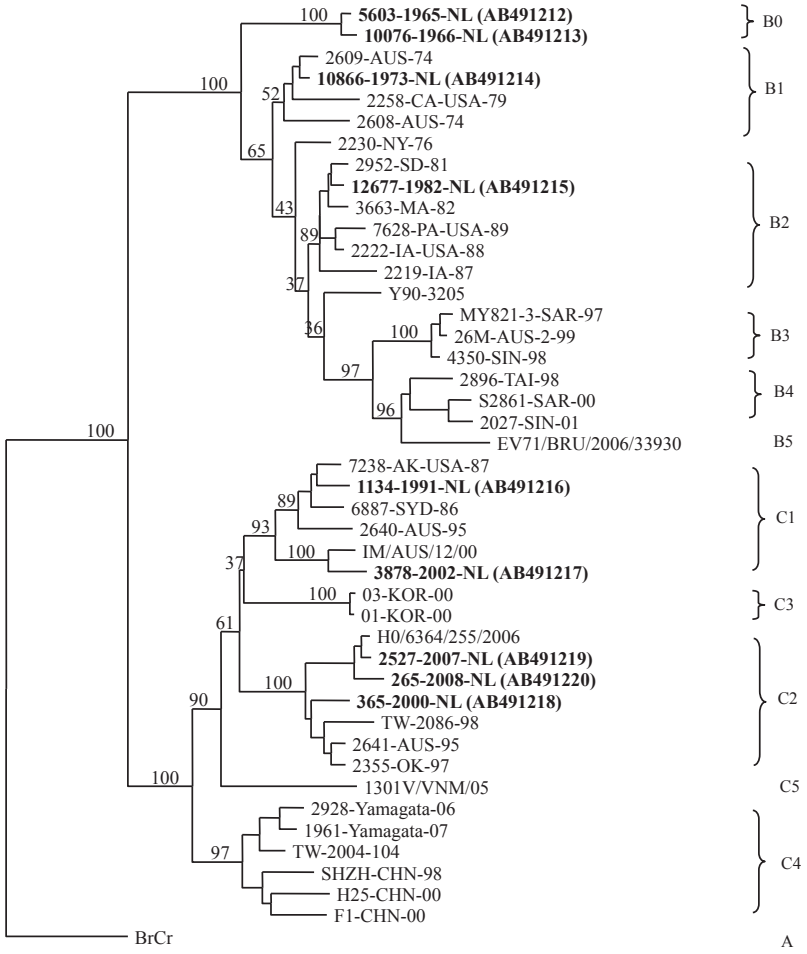

Fig. 2: Phylogenetic analysis of EV71 isolates from the Netherlands (bold) and reference strains. The dendrogram was generated by the neighbor-joining method (bootstrap analysis with 1,000 pseudoreplicate data sets) on the basis of a multiple alignment of the nucleotide sequences of the VP1 region (891 nucleotides).

In total, 85 isolates clustered with reference strains of genogroup B, of which 28 isolates belonged to subgenogroup B1, and 47 isolates belonged to subgenogroup B2. The remaining 10 isolates from 1963 to 1967 formed a distinct cluster within genogroup B, with 90.51% homology to subgenogroups B1 and B2. This difference justifies the classification of these isolates in a new subgenogroup, B0. A clear change in the dominating subgenotype over time could be observed when the genotyping results were added to the surveillance data (Fig. 3). Isolates of subgenogroup B0 were observed in 1963 to 1967, followed by a predominance of strains belonging to subgenogroup B1 from 1971 to 1979. In 1977, strains of subgenotype B2 were observed for the first time in The Netherlands at a low frequency (2 in total), and B2 was the only subgenogroup observed in The Netherlands from 1981 to 1986, with a peak of hospitalized cases in 1986. No isolates were found in this study that clustered within subgenogroups B3 and B4.

After 1986, no strains of genogroup B were observed. All Dutch EV71 strains (114 in total) isolated after 1986 were assigned to genogroup C. Fifty isolates belonged to subgenogroup C1 (isolated in 1987 to 1997 and 2000 to 2007), and 64 belonged to subgenogroup C2 (dominating in 1997 to 2000 and 2002 to 2008). Only one EV71 strain (subgenogroup C2) was isolated in 2008. In this study no strains were observed that clustered within the C3, C4, or C5 subgenogroups.

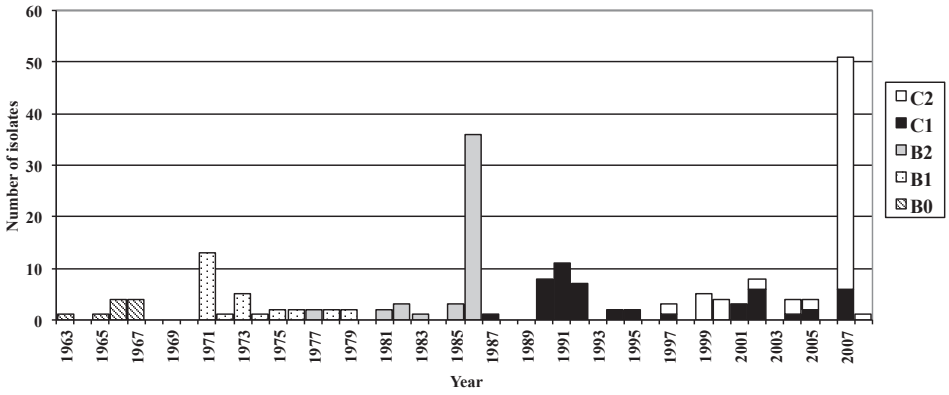

Fig. 3: Successive appearance of EV71 subgenogroups in The Netherlands from 1963 to 2008.

Seasonal distribution of EV71

The month of sampling was known for 168 EV71 isolates (82%). Cases of EV71 infection were reported throughout the year, with a peak in the summer months of June and July. A second, but smaller, peak was observed in September and October (Fig. 4), including subgenogroups of both genogroup B and C. No difference in

seasonal distribution could be observed between isolates belonging to genogroups B and C.

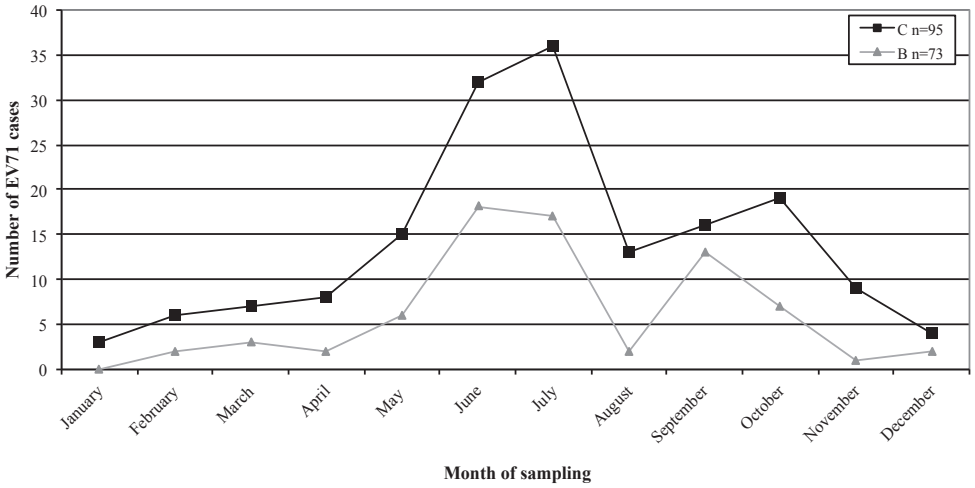

Fig. 4: Seasonal distribution of Dutch EV71 isolates, 1963 to 2008.

Clinical data

Clinical data were known for 78 children infected with EV71 of genogroup B (92%) and 62 children infected with EV71 of genogroup C (54%) (Table 2). In both genogroups the major reported clinical manifestations were fever, meningitis or encephalitis, and gastrointestinal symptoms (diarrhea and vomiting). The number of cases of typical HFMD (vesicles on hand, feet, and in mouth) was low, as expected, since only hospitalized cases of EV71 infection are usually diagnosed and reported in The Netherlands. There was no indication of EV71-related fatalities for the patients of this study.

The majority of the children showed more than one clinical symptom. For statistical analysis, the children were therefore categorized to one of the following groups: children with neurological complications (like meningitis, encephalitis, or convulsion) or children with other, milder complications only (i.e., gastrointestinal symptoms, rash, inflammation of the upper respiratory tract, or HFMD) (Table 2). Neurological complications were significantly more often reported among children infected with EV71 of genogroup B than among children infected with genogroup C ($P = 0.000$; odds ratio, 9.57; 95% confidence interval, 4.13 to 22.63). Gastrointestinal disease appeared to be more associated with genogroup C infection ($P = 0.008$; odds ratio, 3.12; 95% confidence interval, 1.27 to 7.89).

Table 2: Clinical symptoms of children infected with EV71*.

Symptom	No. of instances (%) by EV71 genogroup		P value**
	B	C	
Fever	31 (40)	31 (50)	0,236
Gastrointestinal symptoms	11 (14)	21 (34)	0,008
Meningitis/encephalitis	50 (64)	11 (18)	0,000
Paresis	2	0	0,503
Convulsions	3	2	1,000
Headache	2	1	1,000
Erythema	1	0	1,000
Vesicles hand, feet, mouth	7	6	1,000
Exanthema	2	4	0,406
Laryngitis	0	1	0,406
Stomatitis	3	2	1,000
Inflam. upper resp. tract/otitis	9	3	0,227
Parotitis	2	0	0,503
Pneumonia	0	1	0,443
Bronchitis	4	2	0,963

* The total number of children infected was 78 for genogroup B and 62 for genogroup C. The number of children with neurological complications was 55 (71%) and 13 (21%), respectively; the number with other complications was 23 (29%) and 49 (79%) for infection with genogroup B and C viruses, respectively.

** *P* values were calculated with a two-tailed Fisher's test on the basis of number of children for whom clinical data were available.

The age of children with EV71 infection was known for 166 children (genogroup B, n = 72; genogroup C, n = 94) (Fig. 5). The age of children infected with EV71 of genogroup B (median of 24 months) was overall significantly higher than the age of children infected with EV71 of genogroup C (median of 5 months) (P = 0.000, one-way analysis of variance). Comparison of the ages of children with neurological complications (n = 62; median of 24 months) and children with other, milder symptoms (n = 62; median of 13 months) showed that there was no significant difference in age (P = 0.539, one-way analysis of variance), excluding a correlation between the high number of neurological cases among children infected with genogroup B and the significantly higher ages of these children.

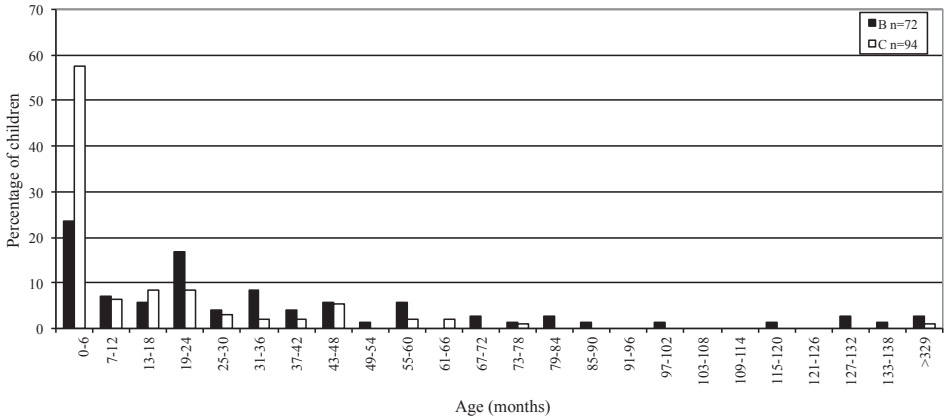

Fig. 5: Age of Dutch children infected with EV71, 1963 to2008.

Comparison with globally available data from systematic literature review

Data on the global presence of EV71 subgenogroups were derived by an extensive literature review. In total, 326 articles were selected on the basis of our search strategy, and of these 30 papers contained data meeting our criteria. These were finally used to record place and time of isolation and subgenogroup (Fig. 6).

Except for some cases of genogroup B2 infection in Japan and Taiwan, no data were reported on EV71 infections in the Asian region before 1997. Reports on EV71 epidemiology outside of this region showed circulation of B1 and B2 strains in Europe, the United States, and Australia in the 1970s up to 1986/1987. In Japan subgenogroup B2 was observed until 1989. The shift of genogroup B to genogroup C infection observed in our country in 1987, followed by the prevalence of genogroup C1 and, later, C2, also occurred in the United States and Australia in the same period of time. Recent reports on EV71 circulation in the United Kingdom, Austria, and Norway confirmed analogous circulation of C1 and C2 strains. Strains of subgenogroups B3, B4, C3, C4, and C5 have caused large outbreaks in the Asian Pacific region since 1997. Except for two infections with subgenogroup C4 in Austria in 2004, these subgenogroups have not been observed outside the Asian Pacific region.

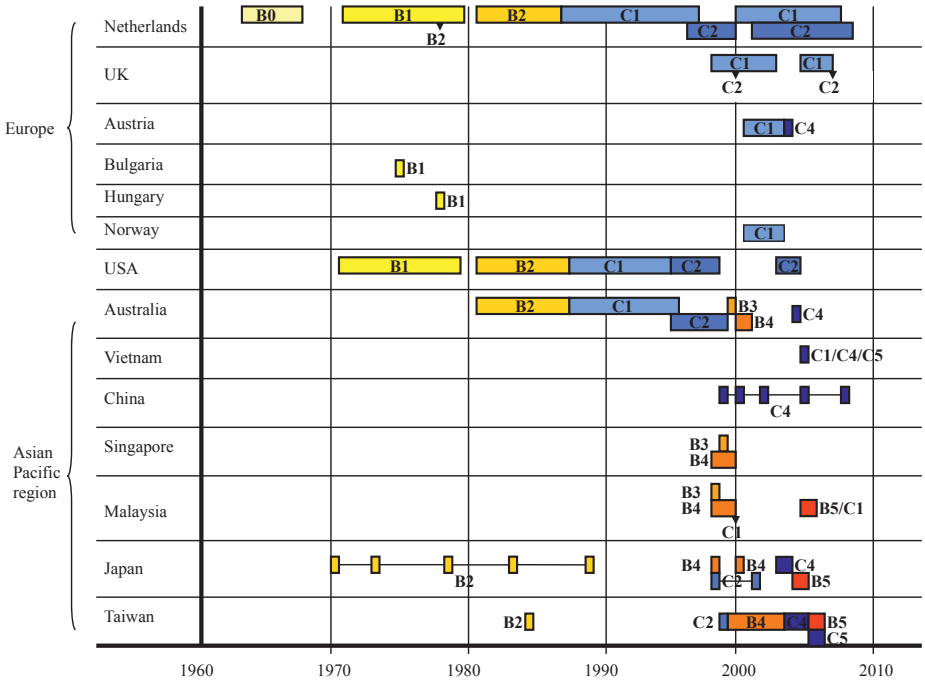

Fig. 6: Global presence of subgenogroups of EV71. Overview was made on the basis of an extensive literature review for the United Kingdom (2), Austria (35), Bulgaria (14), Hungary (30), Norway (45), the United States (3, 4, 36), Australia (4, 37, 39), Vietnam (42), China (11, 17, 18, 26), Singapore (6, 8, 29, 30, 40), Malaysia (1, 3, 5, 6, 13, 19, 29, 34, 36), Japan (3, 12, 31), and Taiwan (12, 22, 23, 25, 44). Black triangles represent single cases of subgenogroups other than the dominating subgenogroup in the period of time concerned.

Discussion

This study describes the epidemiology of EV71 in The Netherlands from 1963 to 2008. Consistency in typing of EV71 since 1977 and extensive retrospective testing of untypeable isolates from 1963 onwards made it possible to generate a detailed and realistic overview of a period of 45 years. This enabled observation of a clear change of prevailing subgenogroups over time in The Netherlands. The new subgenogroup B0, which was detected during the 1960s, has not been described before as retrospective typing of EV71 strains isolated before the first description of the EV71 serotype in 1974 has not been done previously. The seasonal distribution of EV71- a peak in the summer months of June and July and a second, but smaller, peak in September and October - does not differ from that of all other enterovirus infections diagnosed in The Netherlands (24).

The majority of the EV71 strains included in this study were isolated from feces and throat swabs. Only one isolate (0.5%) was obtained from CSF. This is in line with

the results of a previous study in which specimens from the gastrointestinal and respiratory tracts were shown to have higher diagnostic yields for EV71 than CSF (36). Primary diagnosis of enteroviruses in CSF only from patients with neurologic disease results in an underestimation of the real number of EV71 infections.

Comparison of data on EV71 epidemiology in the public domain showed that the change in the prevailing subgenogroups (B1, B2, C1, and C2) and the shift of genogroup B to genogroup C infection occurred more or less simultaneously in Europe, the United States, and Australia. Since the first publications on EV71 in Asia in 1997, in addition to the strains found elsewhere, additional subgenogroups (B3, B4, C4, and C5) have been reported to cause large outbreaks with high rates of severe morbidity and mortality in this region only. The global nature of the epidemiology of some subgenogroups, however, raises the question of whether strains of the C4 and C5 lineages will also spread outside Asia. The epidemic of subgenogroup B2 infection in our country in 1986 was followed by a switch to a new genogroup (genogroup C), possibly driven by herd immunity against genogroup B. Recurrence of the same scenario for viruses of genogroup C (global epidemiology of C1 and C2) might imply that epidemics of subgenogroup C4 infections in other parts of the world will be unlikely and that there might be a switch to a new genogroup.

A change in the clinical presentation of EV71 infection over time has been suggested previously (3, 34). This is the first time, however, that a clinical difference between EV71 lineages has been described showing more severe disease associated with the genogroup B strains. These results, however, should be interpreted with caution. Mild cases of infection with genogroup B might have been reported less frequently during the 1980s than infections with genogroup C in recent years, and the higher average age of people infected may have contributed to more severe illness. In our data set, however, there was no significant correlation between the high number of neurological cases among children infected with genogroup B and the significantly higher ages of these children. In addition, fatal and severe cases of EV71 infection have been reported to occur especially among children of <4 years of age, with an increase in the number of cases with decreasing age (7, 10, 16, 20). During the outbreak of subgenogroup C4 infection in China in 2008, 63.6% of the Chinese children infected were <2 years of age, and 39% were <1 year of age (11). More data on the age of children infected with EV71 of different subgenogroups of B and C should be analyzed to verify differences in the age groups affected.

It is not clear why the Asian Pacific region has experienced an increased incidence of EV71 infection since 1997. A change in clinical presentation could coincide with a change in the viral transmission pathway. A shift of neurological complications to gastrointestinal disease with vomiting and diarrhea, in combination with other epidemiological parameters like crowding, lack of sanitation, and climate, could enhance virus transmission and explain the high rates of morbidity during epidemics in the Asian Pacific region. An increased susceptibility of the population to EV71 infection could be another explanation for the increased incidence. A study in Taiwan showed that EV71 seroconversion rates between 1994 and 1997 (a year before the large outbreak in 1998) were significantly lower (3% to 4%) than the rates before 1994 (7% to 11%) (27). As a consequence, the size of the susceptible population increased, potentially passing a critical threshold. It is of interest to study host susceptibility among the Dutch population before and after the large outbreaks in 1986 and 2007.

To our knowledge this is the first extensive description of EV71 epidemiology in Europe. This study suggests that the change in the circulating virus from genogroup B to genogroup C coincided with a change in clinical presentation and in age group affected. The epidemiology of EV71 subgenogroups B1, B2, C1, and C2 appeared to have a global nature, which implies that a subgenogroup C4 outbreak, as was observed in China in 2008, can be expected to occur in other parts of the world as well. Repeat of the 1986 scenario in The Netherlands would imply that new cases of severe EV71 infection will occur only after the introduction somewhere in the world of a new genotype virus, e.g., subgenogroup D (15). In line with this scenario is the isolation of only one EV71 strain in The Netherlands in 2008 under surveillance regimen identical to that of the preceding years.

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Chapter 4

Evolutionary Trajectory of the VP1 Gene of Human Enterovirus 71 Genogroup B and C Viruses

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Abstract

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 (3, 19, 27). 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 (2, 16). Genogroup A includes only one strain (BrCr-CA-70), which was isolated in California in 1970 (2). Genogroups B and C are reported more commonly and consist of subgenogroups B0–B5 and C1–C5, respectively (2, 5, 14, 16, 17, 24, 25). Strains of HEV71 isolated in the Netherlands from 1963 to 1986 successively belonged to subgenogroups B0, B1 and B2 (25). 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 (25). An increase in the number of hospitalized cases due to B2 infection was also reported in the USA and Australia in the mid-1980s (2, 22). 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 (2, 25). 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 (25). 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 asses 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 enterovirus-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.

Materials and Methods

HEV71 isolates

The HEV71 strain collection used for this study has been described previously (25). 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) (25). 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 (1, 2, 25). 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'-TAAAACAGCCTGTGGGTTGYACC-3'), EV1RB (5'-TGCACATGRATGCAAAACC-3'), EV2FB (5'-CCTGATGTWYTGACWGAAACC-3') and EV2RB (5'-ATCACATCTGCCACYCTATC-3') primers for genogroup B isolates and the EV1FC (5'-TAAAACAGCCTGTGGGTTGYACC-3'), EV1RC (5'-TGCACRTGRATGCARAACC-3'), EV2FC (5'-CCGGATGTRYTAACWGAAACC-3') and EV2RC (5'-ATCACATCTGCCACCCTATC-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 H_2O, 4 µl 5x First Strand Buffer (Invitrogen), 1.0 µl 0.1 M dithiothreitol, 1.0 µl 10 U RNase inhibitor μl^{-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 H_2O. 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-distributed rate variation among sites (9). 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/DDBJ 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 (12). The Bayesian analysis was subsequently performed with the BEAST software (7) 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 lognormal relaxed molecular clock model (6). 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 (8). We specified 20 groups of coalescent intervals to capture the past population dynamics in the piecewise constant demographic function. The posterior distribution for the Bayesian skyline plot parameters yields the most plausible piecewise constant expectations for the coalescence rates through time in the genealogies, which, in turn, represent the most plausible evolutionary histories for the sequence data. The MCMC analysis was run for 50 000 000 generations; stationarity and mixing efficiency were examined by using Tracer (http://tree.bio.ed.ac.uk/software/tracer/).

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 ($TCID_{50}$) as described by Wu et al. (2004) (29). The $TCID_{50}$ value was calculated according to the Spearman–Kärber method (11). 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 10^7–10^8 $TCID_{50}$ 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 supernatant of 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.

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 (15, 26). Other isolates of subgenogroups B2 (20557; 1985) 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 $TCID_{50}$ 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.

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) 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 analysis, 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.

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. →

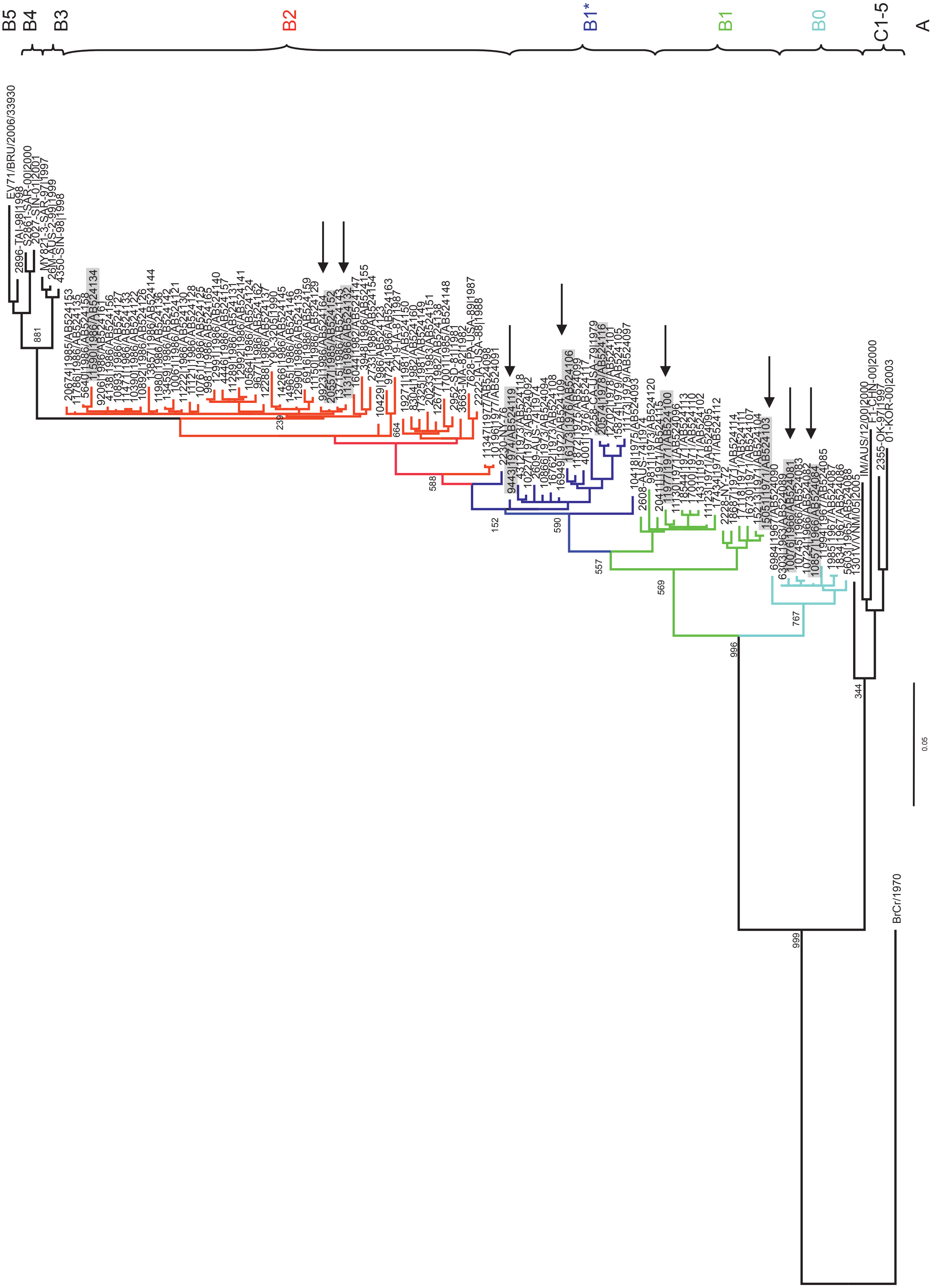
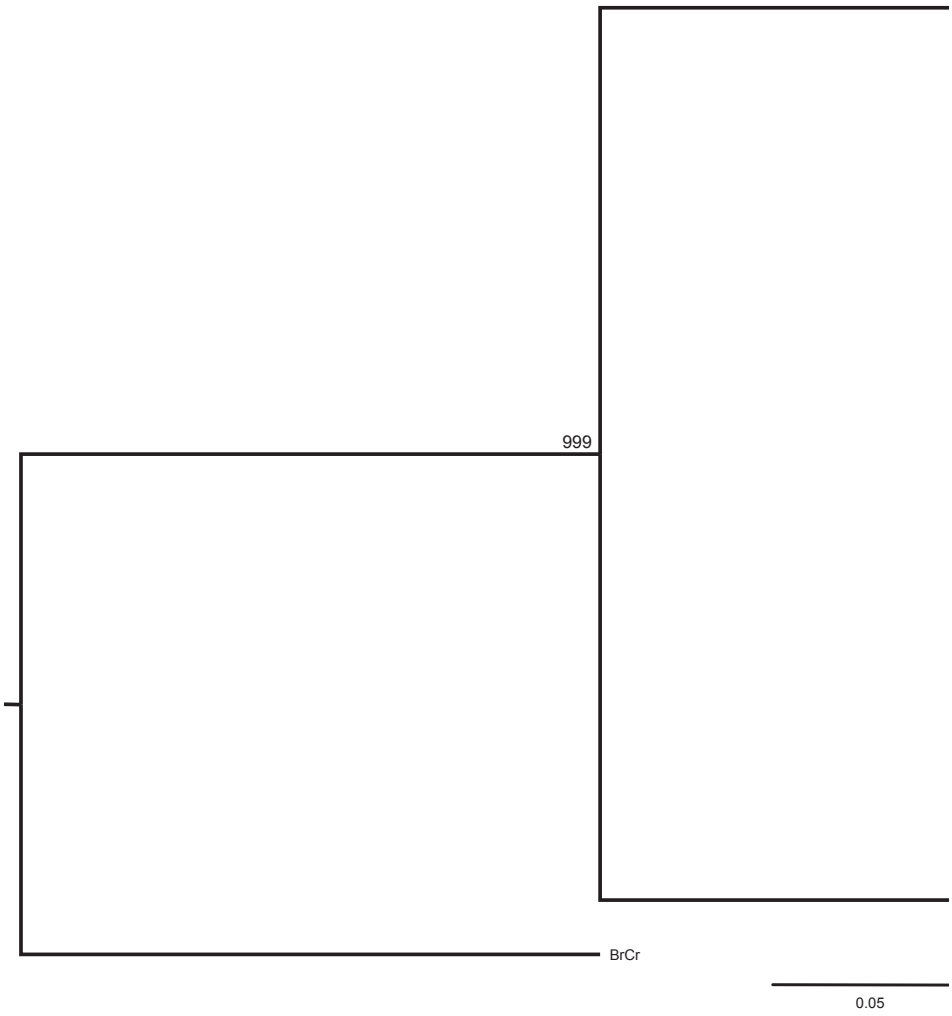
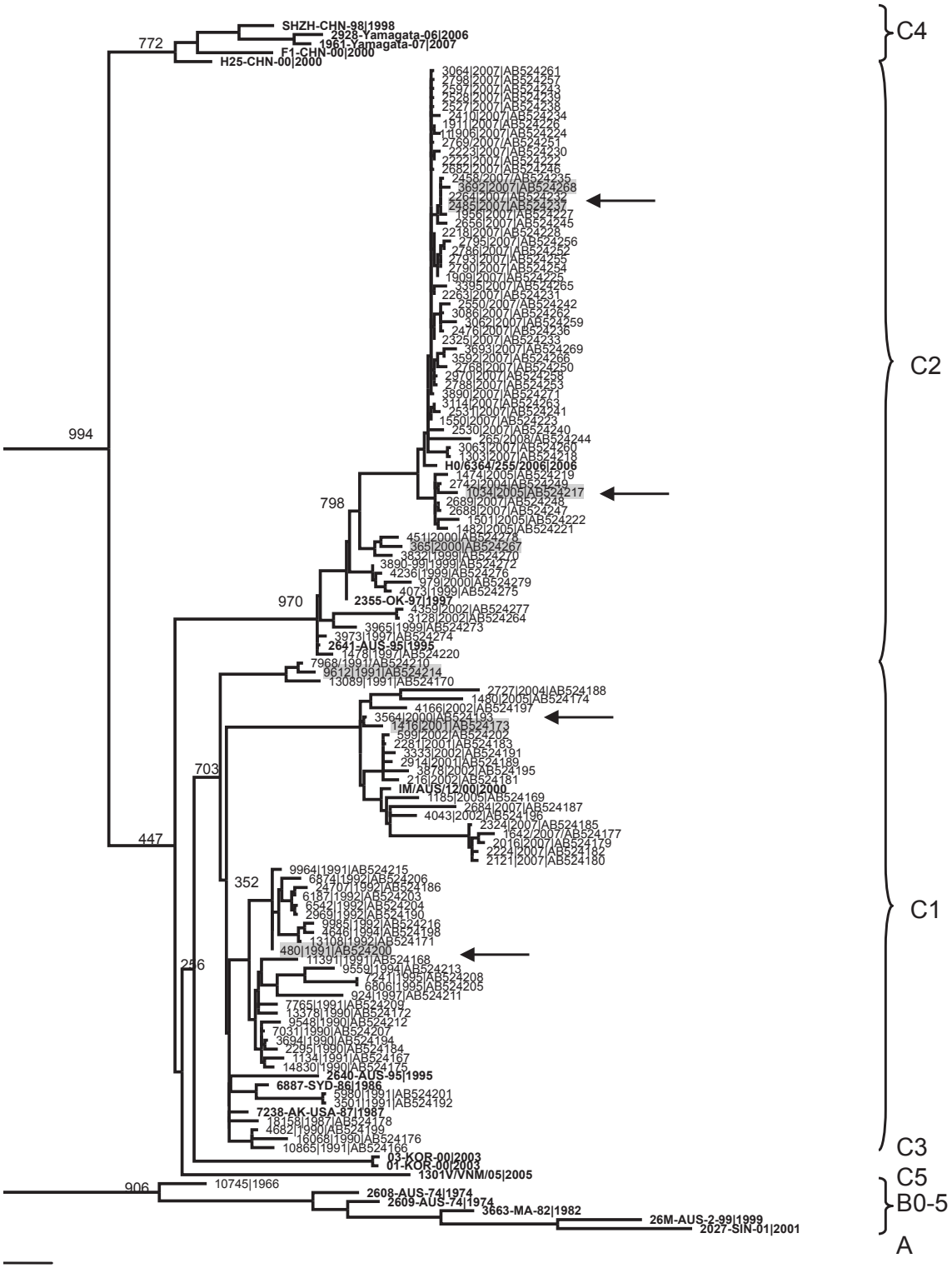

B5
B4
B3
B2
B1*
B1
B0
C1-5
A
0.05

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.

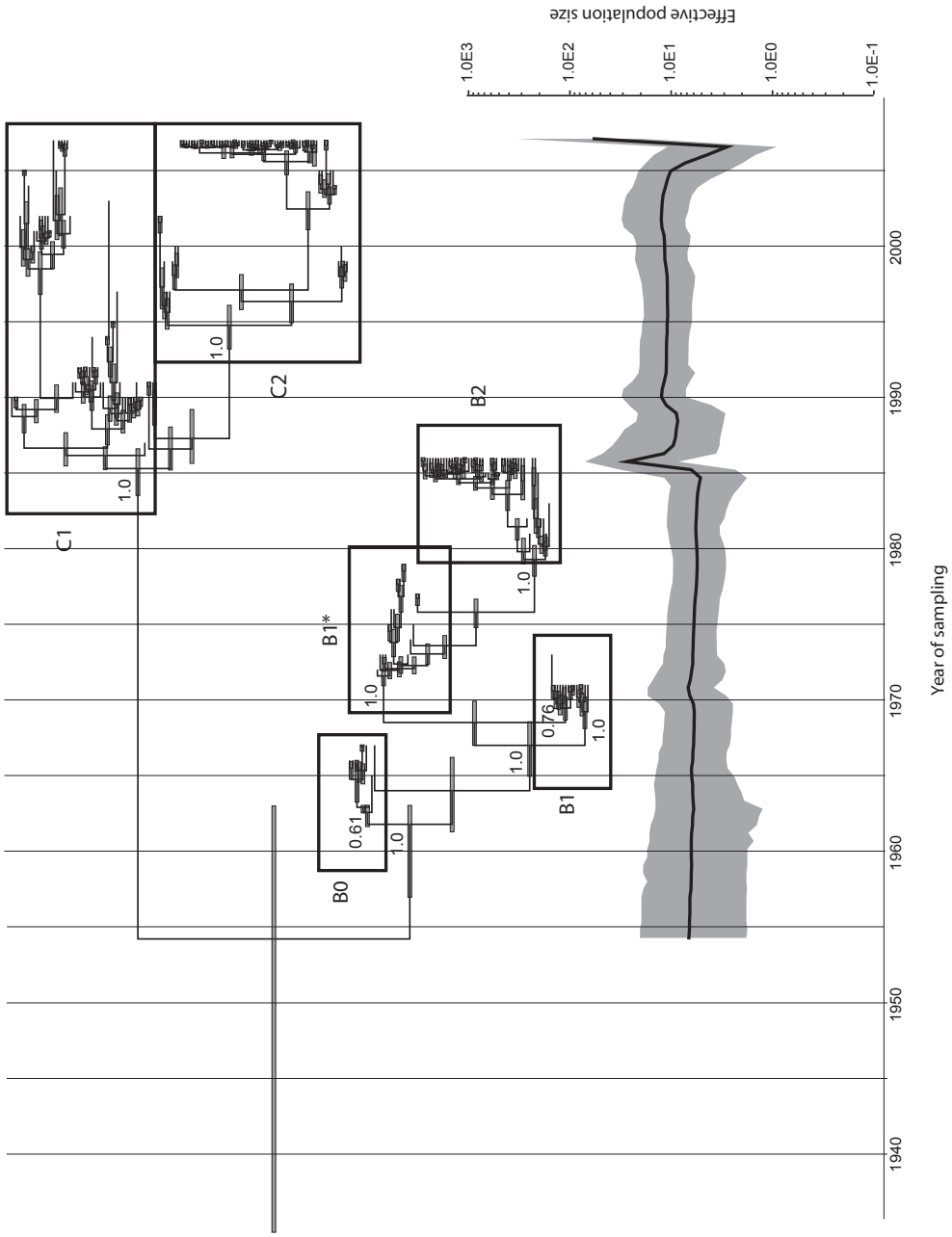




Phylodynamic analysis

Virus population dynamics were estimated over time by using Bayesian coalescent analysis (7) 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) (8). 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.

Fig. 3: Maximum Clade Credibility tree with 95% highest posterior density (HPD) intervals for the node times generated by the MCMC method in BEAST, based on a multiple alignment of VP1 nucleotide sequences of Dutch HEV71 isolates. Below the tree, the Bayesian Skyline with 95% HPD intervals shows the relative measure for genetic diversity through time (values plotted on y-axis). →



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 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 x10 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 46080 (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 antisera indicated cross-reactivity of C1 antisera with genogroup A, B and C viruses, except for B1 15051, which also could not be neutralized by C1 antisera (Table 1).

Table 1: Mean neutralizing-antibody titers (with range) of rabbit B2 and C1 antisera against HEV71 subgenogroups

Subgenogroup	Strain (year)	B2 antiserum	C1 antiserum
B0	10857 (1966)	15360 (-)	17920 (15360-20480)
	10076 (1966)	5120 (-)	5120 (-)
B1	11977 (1971)	46080 (30720-61440)	15360 (10240-20480)
	15051 (1971)	20 (-)	20 (-)
B1*	9443 (1974)	40 (20-60)	7552 (5120-10240)
	16173 (1976)	2560 (-)	5120 (-)
B2	20557 (1985)	20480 (-)	5120 (-)
	11316 (1986)	7680 (-)	3840 (-)
C1	480 (1991)	40 (20-60)	2320 (1280-5120)
	1416 (2001)	10 (-)	5120 (-)
C2	1034 (2005)	10 (-)	5120 (-)
	2485 (2007)	70 (60-80)	2304 (1280-5120)
A	BrCr (1970)	220 (120-320)	1920 (1280-2560)

Amino acid sequence comparison

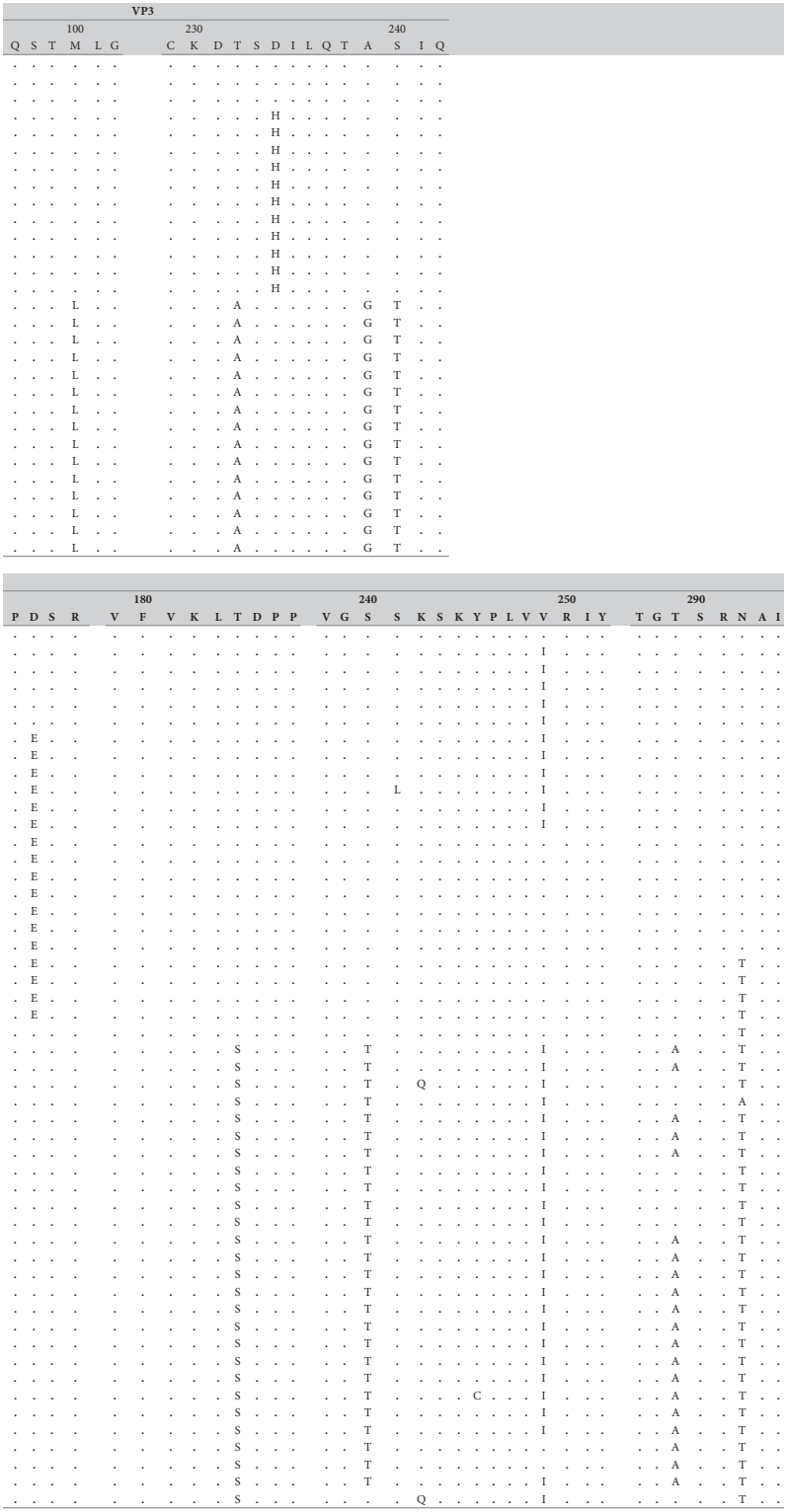
To find possible clues for the antigenic differences observed in the neutralization assays, the complete capsid-encoding regions were determined for a subset of isolates (Figs 1 and 2) and used for amino acid sequence comparisons (Fig. 4). The capsid

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 (4, 10, 21, 23, 28), 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.

Fig. 4: Amino acid differences in the capsid region of HEV71 strains. Upper part, VP4/VP2/VP3 region; lower part, VP1 region. →

	VP4			VP2																							
		60		40											150					200		90					
	D	I	F	P	S	Y	C	S	D	A	D	A	Y	K	Q	T	Y	M	N	T	L	P	G	R	D	G	P	W
B0 10857 (1966)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B0 10076 (1966)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1 15051 (1971)	.	V	.	.	.	.	.	.	.	D	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1 11977 (1971)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1* 9443 (1974)	.	V	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1* 16173 (1976)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1* 20574 (1978)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1* Nagoya, Japan (1973)	.	.	.	.	.	.	.	.	.	D	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B2 20557 (1985)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B2 11316 (1986)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B2 11590 (1986)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B2 7423-MS-87, USA (1987)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B3 MY821-3-SAR-97, Sarawak (1997)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B4 PP37-MAL-01, Malaysia (2001)	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 480 (1991)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C1 9612 (1991)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C1 1416 (2001)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C1 S10862-SAR-98, Sarawak (1998)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C1 1M-AUS-12-00, Australia (2000)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C1 804/NO/03, Norway (2003)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C1 S40221-SAR-00, Sarawak (2000)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.
C2 365 (2000)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C2 1034 (2005)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C2 2485 (2007)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C2 3692 (2007)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C2 7F-AUS-6-99, Australia (1999)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C2 pinf7-54A TW, Taiwan	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C3 03-KOR-00, Korea (2000)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	S	.	.	.
C4 Fuyang.Anhui.P.R.C/17.08/3, China (2008)	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	A	.	.	.	.	N	.	.	.

	VP1																											
							20				40											60					160		
	D	**S**	**V**	**S**	**R**	**A**	**L**	**T**	**Q**	**A**	**D**	**T**	**G**	**K**	**V**	**P**	**A**	**S**	**N**	**A**	**S**	**D**	**E**	**P**	**G**	**A**	**P**	**K**
B0 10857 (1966)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B0 10076 (1966)	.	.	.	.	.	.	.	.	.		.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B1 15051 (1971)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1 11977 (1971)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1 2228-NY-72, USA (1972)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1 2608-AUS-74, Australia (1974)	.	.	.	.	.	.	.	.	H		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* 9443 (1974)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* 16173 (1976)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* 20574 (1978)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* Nagoya, Japan (1973)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* 2609-AUS-74, Australia (1974)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B1* 2258-CA-USA-79, USA (1979)	.	.	M	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 19271 (1981)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 20557 (1985)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 11316 (1986)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 11590 (1986)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 2952-SD-81, USA (1981)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 7423-MS-87, USA (1987)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B2 Y90-3205, Japan (1990)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B3 MY821-3-SAR-97, Sarawak (1997)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B3 26M-AUS-2-99, Australia (1999)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B4 PP37-MAL-01, Malaysia (2001)	.	.	.	G	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B4 2896-TAI-98, Taiwan (1998)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
B5 EV71/BRU/2006/33930, Brunei (2006)	.	.	.	.	.	.	.	.	.		.	.	.	E	.	.	.	.	.	T	.	.	.	.	.	.	.	.
C1 480 (1991)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 9612 (1991)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 1416 (2001)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 2121 (2007)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 7238-AK-USA-87, USA (1987)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 2640-AUS-95, Australia (1995)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 S10862-SAR-98, Sarawak (1998)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 1M-AUS-12-00, Australia (2000)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 S40221-SAR-00, Sarawak (2000)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 804/NO/03, Norway (2003)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C1 H0/5440/288/2005, UK (2005)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 1478 (1997)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 365 (2000)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 3128 (2002)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 1034 (2005)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 2485 (2007)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 3692 (2007)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 2641-AUS-95, Australia (1995)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 2355-OK-97, USA (1997)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 7F-AUS-6-99, Australia (1999)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 pinf7-54A TW, Taiwan	.	.	.	.	.	.	.	.	R		.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
C2 H0/6364/255/2006, UK (2006)	.	.	.	.	.	.	.	.	R		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C3 03-KOR-00, Korea (2000)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C4 Fuyang.Anhui.P.R.C/17.08/3, China (2008)	.	.	.	.	.	.	.	.	H		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C4 H25-CHN-00, China (2000)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C5 1301V/VNM/05, Vietnam (2005)	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A BrCr, USA (1970)	.	.	.	.	K	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.



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 (25). 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 cocirculation 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 (4, 21, 28). 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 1: 10 000) (13). Subgenogroup B5, however, has recently been described to be antigenically distinct from B1, B4, C2 and C4 viruses

(10) 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 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 (18). A Taiwanese study showed cross-neutralization of genogroup C viruses by sera of human patients infected with genogroup B (13), 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 (10).

This study describes the genetic evolution of HEV71 genogroup B and C viruses in the Netherlands over a period of 45 years. Phylodynamic 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 (20). 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 B5, that are antigenically distinct from other groups and do form a potential risk for causing outbreaks in our region.

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Supplementary Material Chapter 4

Table S1: Genogroup B strains isolated in the Netherlands and reference strains used for phylogenetic analysis (VP1 encoding region).

Genogroup	Strain	Year	Place	Accession nr.
B0	6303	1963	NL	AB524089
	5603	1965	NL	AB524088
	10076	1966	NL	AB524081
	10724	1966	NL	AB524082
	10745	1966	NL	AB524083
	10857	1966	NL	AB524084
	11994	1967	NL	AB524085
	1834	1967	NL	AB524086
	1985	1967	NL	AB524087
	6984	1967	NL	AB524090
B1	11123	1971	NL	AB524095
	11130	1971	NL	AB524096
	11977	1971	NL	AB524100
	14311	1971	NL	AB524102
	15051	1971	NL	AB524103
	15213	1971	NL	AB524104
	16730	1971	NL	AB524107
	17000	1971	NL	AB524110
	17118	1971	NL	AB524111
	17434	1971	NL	AB524112
	18544	1971	NL	AB524113
	18687	1971	NL	AB524114
	20411	1971	NL	AB524115
	9811	1973	NL	AB524120
	10196	1977	NL	AB524091
	2608-AUS-74	1974	Australia	AF135885
B1*	16949	1972	NL	AB524109
	10227	1973	NL	AB524092
	10866	1973	NL	AB524094
	16762	1973	NL	AB524108
	4312	1973	NL	AB524118
	9443	1974	NL	AB524119
	10418	1975	NL	AB524093
	11872	1975	NL	AB524099
	16173	1976	NL	AB524106
	4001	1976	NL	AB524117
	11347	1977	NL	AB524098
	12702	1978	NL	AB524101
	20574	1978	NL	AB524116
	11173	1979	NL	AB524097
	15874	1979	NL	AB524105
	2609-AUS-74	1974	Australia	AF135886
	2230-NY-76	1976	USA	AF135869
	2258-CA-USA-79	1979	USA	AF135880
B2	17325	1981	NL	AB524149
	19271	1981	NL	AB524150
	8304	1982	NL	AB524160
	12677	1982	NL	AB524138
	16044	1982	NL	AB524147
	20233	1983	NL	AB524151
	17001	1985	NL	AB524148
	20557	1985	NL	AB524152
	20674	1985	NL	AB524153
	2733	1986	NL	AB524154

	3948	1986	NL	AB524155
	4138	1986	NL	AB524156
	4446	1986	NL	AB524157
	5640	1986	NL	AB524158
	6916	1986	NL	AB524159
	9206	1986	NL	AB524161
	9677	1986	NL	AB524162
	9724	1986	NL	AB524163
	9923	1986	NL	AB524164
	9981	1986	NL	AB524165
	10061	1986	NL	AB524121
	10390	1986	NL	AB524122
	10429	1986	NL	AB524123
	10564	1986	NL	AB524124
	10761	1986	NL	AB524125
	10892	1986	NL	AB524126
	10893	1986	NL	AB524127
	11121	1986	NL	AB524128
	11150	1986	NL	AB524129
	11222	1986	NL	AB524130
	11289	1986	NL	AB524131
	11316	1986	NL	AB524132
	11471	1986	NL	AB524133
	11590	1986	NL	AB524134
	11786	1986	NL	AB524135
	11980	1986	NL	AB524136
	12288	1986	NL	AB524137
	12990	1986	NL	AB524139
	12991	1986	NL	AB524140
	12992	1986	NL	AB524141
	13459	1986	NL	AB524142
	13715	1986	NL	AB524143
	13857	1986	NL	AB524144
	14266	1986	NL	AB524145
	14965	1986	NL	AB524146
	2952-SD-81	1981	USA	AF135888
	3663-MA-82	1982	USA	AF135889
	2219-IA-87	1987	USA	AF009539
	2222-IA-USA-88	1988	USA	AF009540
	7628-PA-USA-89	1989	USA	AF009530
	Y90-3205	1990	Japan	AB433863
B3	MY821-3-SAR-97	1997	Sarawak	AY125997
	4350-SIN-98	1998	Singapore	AF376119
	26M-AUS-2-99	1999	Australia	AF376101
B4	2896-TAI-98	1998	Taiwan	AF286516
	S2861-SAR-00	2000	Sarawak	AF376085
	2027-SIN-01	2001	Singapore	AF376111
B5	EV71/BRU/2006/33930	2006	Brunei	FM201328

Table S2: Genogroup C strains isolated in the Netherlands and reference strains used for phylogenetic analysis (VP1 encoding region).

Genogroup	Strain	Year	Place	Accession nr.
C1 '87-'97	18158	1987	NL	AB524178
	2295	1990	NL	AB524184
	3694	1990	NL	AB524194
	4682	1990	NL	AB524199
	7031	1990	NL	AB524207
	9548	1990	NL	AB524212
	13378	1990	NL	AB524172
	14830	1990	NL	AB524175
	16068	1990	NL	AB524176
	480	1991	NL	AB524200
	1134	1991	NL	AB524167
	3501	1991	NL	AB524192
	5980	1991	NL	AB524201
	7765	1991	NL	AB524209
	7968	1991	NL	AB524210
	9612	1991	NL	AB524214
	9964	1991	NL	AB524215
	10865	1991	NL	AB524166
	11391	1991	NL	AB524168
	13089	1991	NL	AB524170
	2969	1992	NL	AB524190
	6187	1992	NL	AB524203
	6542	1992	NL	AB524204
	6874	1992	NL	AB524206
	9985	1992	NL	AB524216
	13108	1992	NL	AB524171
	24707	1992	NL	AB524186
	4646	1994	NL	AB524198
	9559	1994	NL	AB524213
	6806	1995	NL	AB524205
	7241	1995	NL	AB524208
	924	1997	NL	AB524211
	1185	2005	NL	AB524169
	6887-SYD-86	1986	Australia	AY722887
	7238-AK-USA-87	1987	USA	AF135952
	2640-AUS-95	1995	Australia	AF135946
C1 '00-'07	3564	2000	NL	AB524193
	1416	2001	NL	AB524173
	2281	2001	NL	AB524183
	2914	2001	NL	AB524189
	216	2002	NL	AB524181
	599	2002	NL	AB524202
	3333	2002	NL	AB524191
	3878	2002	NL	AB524195
	4043	2002	NL	AB524196
	4166	2002	NL	AB524197
	2727	2004	NL	AB524188
	1480	2005	NL	AB524174
	1642	2007	NL	AB524177
	2016	2007	NL	AB524179
	2121	2007	NL	AB524180
	2224	2007	NL	AB524182
	2324	2007	NL	AB524185
	2684	2007	NL	AB524187
	IM/AUS/12/00	2000	Australia	AF376098
C2 '97-'02	1478	1997	NL	AB524220
	3973	1997	NL	AB524274

	3832	1999	NL	AB524270
	3890	1999	NL	AB524272
	3965	1999	NL	AB524273
	4073	1999	NL	AB524275
	4236	1999	NL	AB524276
	365	2000	NL	AB524267
	451	2000	NL	AB524278
	979	2000	NL	AB524279
	3128	2002	NL	AB524264
	4359	2002	NL	AB524277
	TW-2086-98	1998	Taiwan	AF116819
	2355-OK-97	1997	USA	AF135942
	2641-AUS-95	1995	Australia	AF135947
C2 '04-'07	2742	2004	NL	AB524249
	1034	2005	NL	AB524217
	1474	2005	NL	AB524219
	1482	2005	NL	AB524221
	1501	2005	NL	AB524222
	1303	2007	NL	AB524218
	1550	2007	NL	AB524223
	1906	2007	NL	AB524224
	1909	2007	NL	AB524225
	1911	2007	NL	AB524226
	1956	2007	NL	AB524227
	2218	2007	NL	AB524228
	2222	2007	NL	AB524229
	2223	2007	NL	AB524230
	2263	2007	NL	AB524231
	2264	2007	NL	AB524232
	2325	2007	NL	AB524233
	2410	2007	NL	AB524234
	2458	2007	NL	AB524235
	2476	2007	NL	AB524236
	2485	2007	NL	AB524237
	2527	2007	NL	AB524238
	2528	2007	NL	AB524239
	2530	2007	NL	AB524240
	2531	2007	NL	AB524241
	2550	2007	NL	AB524242
	2597	2007	NL	AB524243
	2656	2007	NL	AB524245
	2682	2007	NL	AB524246
	2688	2007	NL	AB524247
	2689	2007	NL	AB524248
	2768	2007	NL	AB524250
	2769	2007	NL	AB524251
	2786	2007	NL	AB524252
	2788	2007	NL	AB524253
	2790	2007	NL	AB524254
	2793	2007	NL	AB524255
	2795	2007	NL	AB524256
	2798	2007	NL	AB524257
	2970	2007	NL	AB524258
	3062	2007	NL	AB524259
	3063	2007	NL	AB524260
	3064	2007	NL	AB524261
	3086	2007	NL	AB524262
	3114	2007	NL	AB524263
	3395	2007	NL	AB524265
	3592	2007	NL	AB524266
	3692	2007	NL	AB524268

	3693	2007	NL	AB524269
	3890	2007	NL	AB524271
	265	2008	NL	AB524244
	H0/6364/255/2006	2006	UK	AM939607
C3	01-KOR-00	2003	Korea	AY125966
	03-KOR-00	2003	Korea	AY125968
C4	SHZH-CHN-98	1998	China	AY465356
	F1-CHN-00	2000	China	AB115490
	H25-CHN-00	2000	China	AB115492
	TW-2004-104	2004	Taiwan	DQ666684
	2928-Yamagata-06	2006	Japan	AB433878
	1961-Yamagata-07	2007	Japan	AB433892
C5	1301V/VNM/05	2005	Vietnam	AM490149
A	BrCr	1970	USA	AB433863

Chapter 5

Detection of Recombination Breakpoints in the Genomes of Human Enterovirus 71 Strains isolated in the Netherlands in Epidemic and Non-epidemic Years, 1963-2010

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Abstract

Evolutionary history of human enterovirus 71 (EV71) in the Netherlands shows displacement of virus subgenogroups, that only partly can be explained by antigenic changes. Additionally, occasional epidemics have occurred that remain to be explained. Previous studies have shown subgenogroup specific recombination events in the genome of Asian EV71 strains. To find clues on the role of genome recombination in evolution of the EV71 subgenogroups found in Europe and in the evolution of strains capable of causing outbreaks, we analyzed the genomes of 19 strains representing the genetic diversity of EV71 in the Netherlands between 1963 and 2010. We selected viruses from EV71 endemic and epidemic years (1986 and 2007). Subgenogroup specific genome recombination events were detected for subgenogroup B0, B1 and B2 viruses, in line with observed genome recombination events in Asian subgenogroup B3 and B4 viruses. Considering recombination events distinguishing strains from epidemic years from those of non-epidemic years, breakpoints for recombination were detected in the 5'UTR of B2 viruses from the outbreak in 1986, with highest similarity of the 5'UTR to B4 and B3 strains isolated during outbreaks in the Asian Pacific region. No indications for recombination were found in genogroup C isolates. Except for the '86 B2 isolates, Dutch isolates phylogenetically interspersed with international reference strains of the same subgenogroup, indicating a global dissemination of (recombinant) EV71 viruses. The difference observed in the 5'UTR of EV71 strains isolated in endemic versus epidemic years suggests that changes in the 5'UTR cause evolution of strains capable of causing outbreaks.

Introduction

Human enterovirus 71 (EV71) is a member of the *Human Enterovirus A* species of the genus *Enterovirus* of the *Picornaviridae* family. Around twenty percent of EV71 infections are symptomatic and usually manifest as the mild, self limiting Hand, Foot and Mouth Disease with skin rash and fever (HFMD) (42). Around 1% of infected people, however, develop severe neurological complications like meningitis and poliomyelitis-like paralysis. The EV71 genome consists of a positive sense RNA molecule of around 7400 nucleotides (nt). The first 750 nucleotides form the 5' untranslated region (5'UTR), which contains the internal ribosome entry site necessary for viral RNA translation. The region from nt 750 to ~7330 encodes a single polyprotein which is processed to the viral capsid proteins VP4–2–3–1 (P1), and the non-structural proteins 2A–C (P2) and 3A–D (P3). The 3'UTR region of ~84 nucleotides is involved in viral RNA synthesis (5, 16).On the basis of the nucleotide sequences of the VP1 encoding region, EV71 strains have been divided into genogroups A–C, respectively, showing a genetic divergence of about 17% at the nucleotide level (4, 27). Genogroup A includes only 1 strain (BrCr-CA-70), which was isolated in California in 1970 (4). Genogroups B and C are more commonly reported, and consist of sub-genogroups B0–B5 and C1–C5, respectively (4, 12, 23, 27, 29, 36, 38).

Since 1997, the incidence of EV71 infections appears to be increased in the Asian Pacific region. Several large outbreaks of HFMD caused by EV71 have occurred in this region, with substantial numbers of deaths as a result of neurological complications (1, 6-10, 12, 13, 17-19, 21, 33, 38). Genotyping of EV71 strains revealed that these outbreaks were caused by viruses belonging to different, recently emerged subgenogroups: B3–B5, C4 and C5. Except for some single isolated cases of C4 infection, viruses of these subgenogroups have not been observed outside the Asian Pacific region. Our previous studies on EV71 epidemiology in the Netherlands showed that reported EV71 infections in the Netherlands in 1963–1986 were successively caused by viruses of subgenogroups B0, B1, B1* (an intermediate group of viruses in the stepwise evolution of subgenogroup B1 to B2) and B2. In 1986, a B2 epidemic occurred followed by a switch to circulation of genogroup C viruses with an antigenic character distinct from that of the previously circulating genogroup B viruses. Since then, only C1 and C2 viruses have been observed (38, 39). In 2007, another epidemic occurred, this time caused by subgenogroup C2 viruses. Cross neutralization experiments analyzed through antigenic cartography showed that viruses of subgenogroups B and C are antigenically distinct (20, 30, 39). Within genogroups, however, cross-neutralization has been observed, suggesting that the evolution of subgenogroups within the

genogroups is not primarily immune driven. This is in line with the observation that diversity within the capsid encoding regions of viruses within the same genogroup is mainly based on silent transitions (28, 39). These observations leave the question what drives the apparent evolution of subgenogroups. In addition, it remains unclear why B2 and C2 viruses were able to cause outbreaks in the Netherlands in 1986 and 2007, respectively, while viruses belonging to these subgenogroups had been circulating for years already. A possible driving force could be selection of recombinant viruses with increased fitness. Previous studies have shown evidence for recombination in the non-structural genes of EV71 subgenogroup B3, C2 and C4 strains isolated during outbreaks in the Asian Pacific region (28, 44, 45). To find clues on the role of genome recombination in the evolution of EV71 subgenogroups observed in Europe and in the evolution of strains capable of causing outbreaks, we analyzed the near complete genome sequences of a selection of EV71 viruses isolated in the Netherlands in endemic and epidemic years between 1963 and 2010 (38, 39).

Materials and methods

EV71 isolates

The EV71 strain collection of the RIVM has been described previously (38, 39). Briefly, this collection contains 199 EV71 strains isolated from different parts of the Netherlands as part of enterovirus surveillance activities from 1963 to 2008, and has recently been supplemented with 13 EV71 isolates of 2010. These EV71 isolates have previously been divided into subgenogroups B0 (isolates of 1963–1967), B1 (1971–1973), B1* (1973–1979), B2 (1977–1986), C1 (1987–1997 and 2001–2007, 2010) and C2 (1997–2008, 2010) on the basis of nucleotide sequence comparisons of the VP1 encoding region (38, 39). On the basis of the year of isolation, at least two strains of each subgenogroup were selected for complete genome sequencing (Table S1 in supplementary material). Strains were considered to be from endemic or epidemic periods based on the results of BEAST analyses that revealed not only periods of stable equilibrium but also episodes of expanding virus diversity reflecting epidemics (39). For validation of initial findings, additional B2 isolates and C2 isolates were included for partial genome sequencing (nucleotides 1–1260).

RNA extraction and genome amplification

Viral RNA was extracted from EV71 isolates cultivated in human rhabdomyosarcoma or human embryonic lung fibroblast (GaBi) cell lines using the MagNA Pure LC Total Nucleic Acid Isolation Kit with a MagNA Pure LC instrument (Roche Diagnostics, Almere, the Netherlands) as described previously (39). Viral RNA was eluted in a

total volume of 50 µl. The genomes were PCR amplified in eight overlapping regions, using genogroup B and genogroup C specific primers (Table S2 in supplementary material). These primers were designed on the basis of published EV71 sequences and primers (44). Details on the PCR amplification protocol have been described previously (39). Amplicon size analysis was performed using gel electrophoresis. Purification of the PCR products was performed according to the manufacturer's protocol of the QIAquick® PCR Purification Kit (Qiagen, 2002). Sequencing of the PCR products was carried out with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.2 (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (Applied Biosystems model 3700).

Sequence analysis

Editing of the sequence data and generation of consensus sequences of the complete genomes by assembling overlapping DNA sequences determined from both strands was performed using Bionumerics (version 6.1; Applied Maths BVBA, Sint-Martens Latem, Belgium). The newly generated genome sequences were aligned together with the complete genome sequences of EV71 reference strains available in GenBank (Table S1) using the Clustal W method implemented in BioEdit (7.0.9.0; (14)). Neighbor joining trees were constructed of the 5'UTR, the structural P1 and the non-structural P2 and P3 regions using Treecon version 1.3b (37). One thousand bootstrap replicates were used to test the support for branches within these trees. Nucleotide BLAST analysis on genome regions possibly exchanged by recombination were performed to find potential recombination parents (2). To determine potential recombination breakpoints in the EV71 genomes, sequence alignments including genome sequences of virus strains detected by the BLAST analyses (Table S1) were analyzed using both RDP3 (26) and Simplot version 3.5.1 (25). For the Simplot analyses a sliding window of 200 nucleotides was moved along the complete genome alignment with steps of 20 nucleotides. A sliding window of 80 nucleotides was used for similarity plots of the 5'UTR region. Isolates of corresponding subgenogroups, sharing high percentages of similarity over their complete genomes, were grouped during the comparisons. RDP3 was used with default settings. Only recombination events detectable with three or more of the seven recombination detection methods implemented in RDP3 were taken as significant evidence of recombination. Recombinant sequences were identified using the VisRD (22), PHYLPRO (41) and subtree prune and regraft methods (3, 15) implemented in RDP3.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/Genbank nucleotide sequence databases with the accession numbers AB575911–AB575948 (Table S1).

Results

Phylogenetic analyses

The structural and non-structural protein encoding regions of the genomes of 19 EV71 strains, representing all EV71 subgenogroups observed in the Netherlands from 1963 to 2010, were sequenced successfully in eight overlapping fragments. Sequence data of the first fragment (nt 1–1260) of 13 additional B2 isolates and 6 C2 isolates was obtained in order to extend the study on genetic diversity in the 5'UTR. Sequence data of the first 40–70 nucleotides of the 5'UTR (748 nt in total) and the last 10–60 nt of the 3'UTR (84 nt in total) could not be determined, because of the position of the EV1F and EV8R primers, and were thus not included in the genome analyses.

Separate neighbor joining trees were constructed of the 5'UTR, P1, P2 and P3 regions of Dutch EV71 isolates and EV71 reference strains to find indications for subgenogroup specific recombination events among Dutch isolates and among strains isolated during epidemic and non-epidemic years. Strains belonging to the same subgenogroup consistently clustered together along the genome, except for Dutch B2 strains from the outbreak in 1986. In the 5'UTR, these viruses formed a separate cluster with the Asian B3 and B4 reference strains (92.6–96.5% similarity, Fig. 1), whereas the Dutch B2 isolates of preceding, non-epidemic years clustered with the B2 reference strain and Dutch B1 and B0 isolates, indicating a possible recombination event in the 5'UTR of the B2 outbreak strains (91–93% similarity between two B2 clusters).

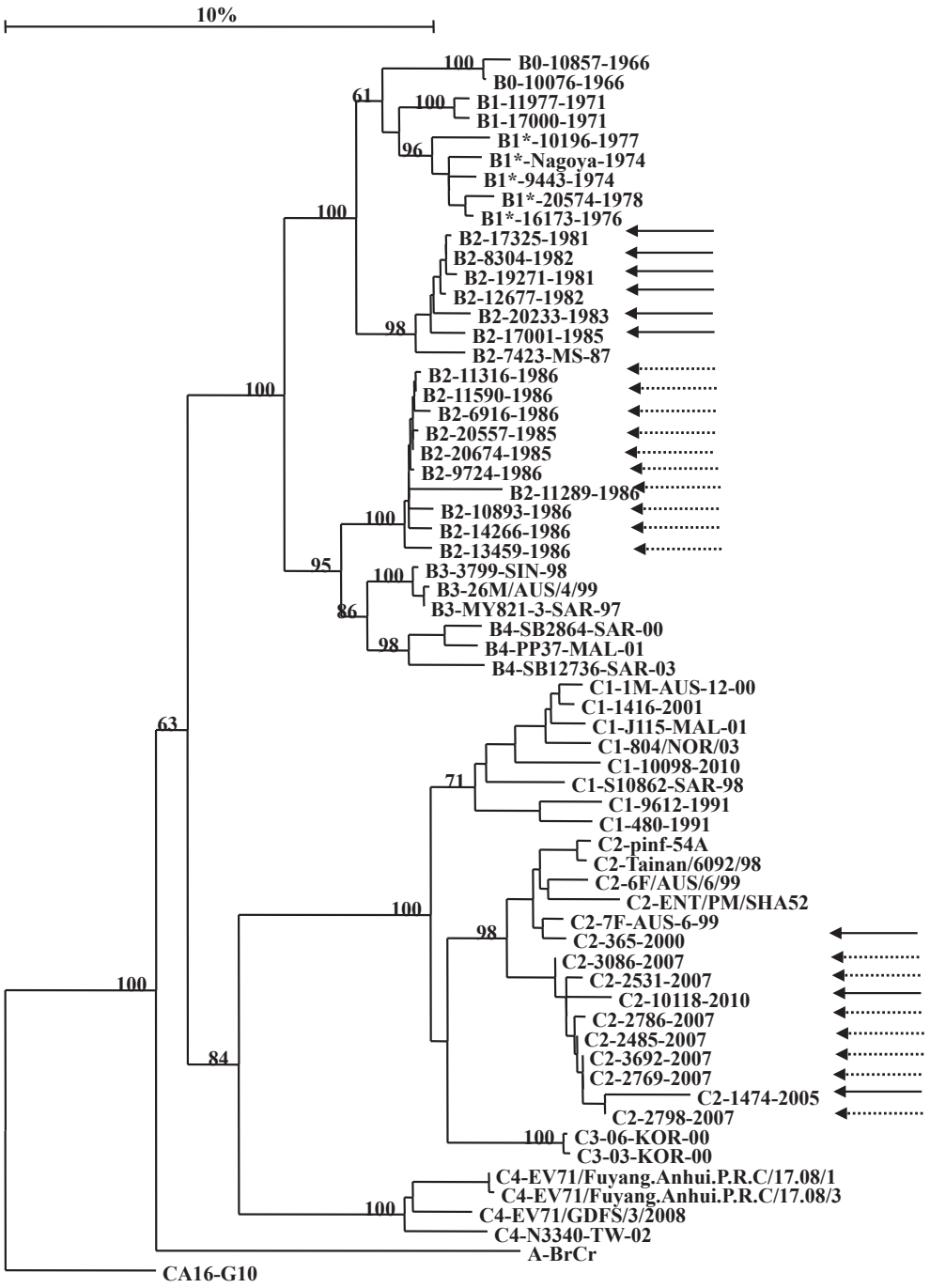

Fig. 1: Neighbor joining tree of the 5'UTR (748 nt) of Dutch EV71 isolates and reference strains. Bootstrap analysis was performed using 1000 pseudoreplicates. Dashed arrows indicate B2 and C2 strains from outbreaks in 1986 and 2007, respectively. Solid arrows indicate B2 and C2 strains isolated in non-epidemic years.

Among the C2 strains isolated in The Netherlands in epidemic and non-epidemic years no indications for recombination were observed. Considering indications for subgenogroup specific recombination events among strains isolated in the Netherlands, comparison of the phylogenetic trees for the different genome regions showed an increased distance between the Dutch B0 isolates and other EV71 subgenogroups in the P2 and P3 encoding regions (B0 versus B1/2/3/4 83.7–88.6% similarity in P2 and 80.3–82% in P3, Figs. 2–4).

Recombination analyses

Nucleotide BLAST analysis on genome regions possibly exchanged by recombination indicated that EV71 and cluster A coxsackie A (CA) reference strains showed the highest percentage of similarity to the EV71 strains analyzed. Recombination analysis within complete genome sequence alignments using RDP3 revealed several recombination breakpoints in our EV71 isolates and the EV71 and CA reference strains included in the analysis (Table S3). Focusing on strains isolated in epidemic versus non-epidemic years, evidence for recombination was detected in the 5'UTR of Dutch B2 isolates sampled in the 1986 outbreak ($p = 1.4 \times 10^{-9}$; Recombination event 14 in Table 1). The first 316 nucleotides of the 5'UTR of these viruses apparently derived from a B4 like parent. Simplot analysis of the 5'UTR sequence alignments confirmed that these B2 strains (8 included in this study) and two B2 strains from 1985 (20557, 20674) shared >90% similarity with Asian B3 and B4 strains in this region, but up to 230 nt only 62.5–77% similarity to Dutch B2 isolates of non-epidemic years (1981–1985), B0 and B1 isolates (Fig. S1 in supplementary material, Coxsackie A strains with the highest percentage of similarity to the query are presented in the simplots). No evidence for recombination was detected among the Dutch C2 strains of the epidemic year 2007 and those isolated in non-epidemic years.

Considering subgenogroup specific recombination events, recombination breakpoints were detected in Dutch subgenogroup B0 genomes just after the structural encoding region and just before the 3D encoding region (nt 5702) ($p = 1.15 \times 10^{-67}$ and $p = 0.040$, respectively; Recombination events 4 and 12 in Table 1). In the capsid region B0 isolates were most closely related to B1 and B2 isolates (88–97%), whereas the 2A–3C and 3D encoding regions of B0 viruses are likely derived from CA4/CA16 and CA7 like viruses, respectively (Fig. S2). Although the neighbor joining trees showed no indications for recombination for Dutch B1 and B2 viruses in the P2 and P3 regions, simplots showed that the similarity of these subgenogroups against each other and against other subgenogroups dropped to <87% in the 3D encoding region (Figs. S3 and S4). The RDP3 analysis revealed

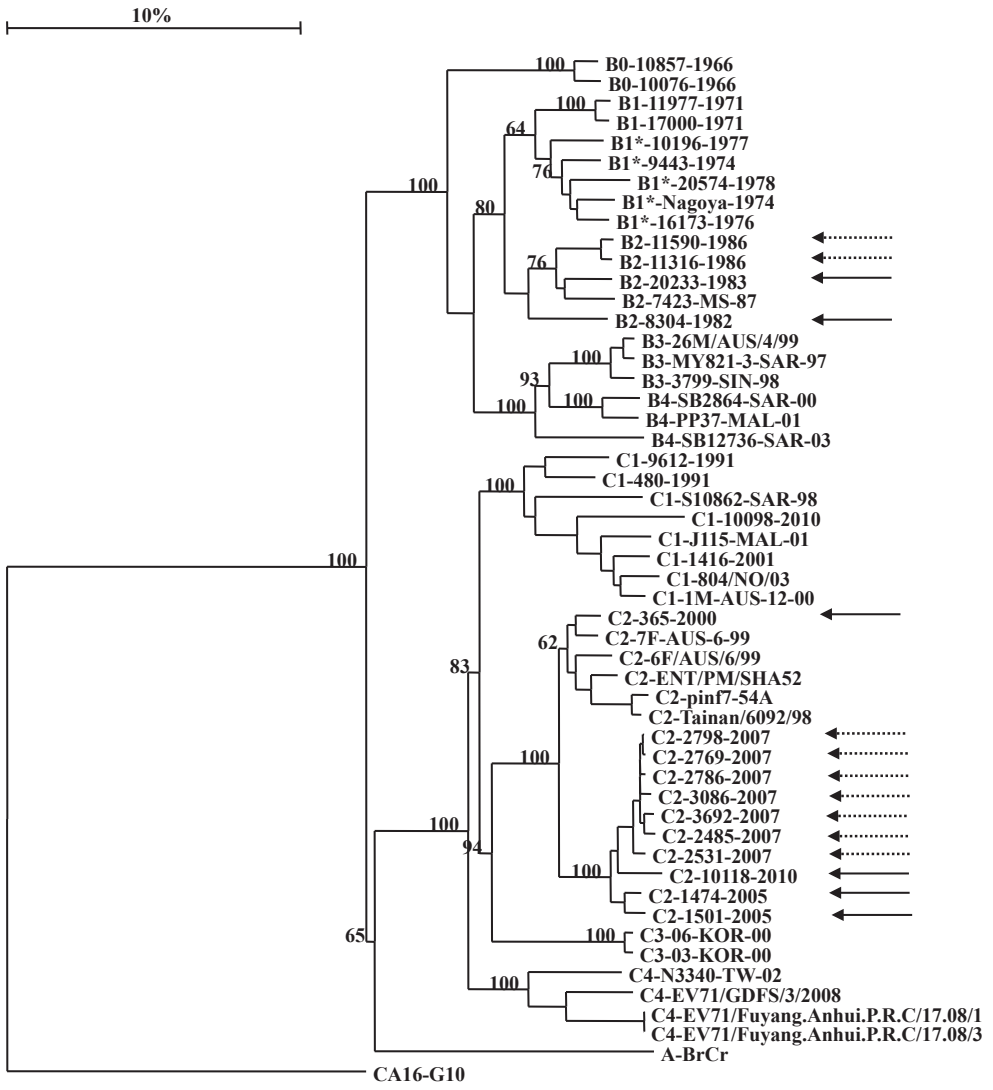

Fig. 2: Neighbor joining tree of the P1 encoding region (2586 nt) of Dutch EV71 isolates and reference strains. Bootstrap analysis was performed using 1000 pseudoreplicates. Dashed arrows indicate B2 and C2 strains from outbreaks in 1986 and 2007, respectively. Solid arrows indicate B2 and C2 strains isolated in non-epidemic years.

clear evidence that these patterns of sequence similarity were most likely caused by two distinct recombination events, with breakpoints just after the structural region and just before the 3D region, within the common ancestor of the B1, B2 and B4 viruses (events 9 and 13 in Table 1; $p = 5.5 \times 10^{-38}$ and 3.06×10^{-3}, respectively).

No evidence for recombination was detected among the Dutch C1 and C2 strains. The pattern of similarity shared between these strains and the CA8 reference strain and Asian subgenogroup C3 and C4 strains (Figs. S5 and S6) can be explained by recombination events in genomes of the CA8, C3 and C4 viruses (recombination event 2, 21 and 3 in Table 1, respectively).

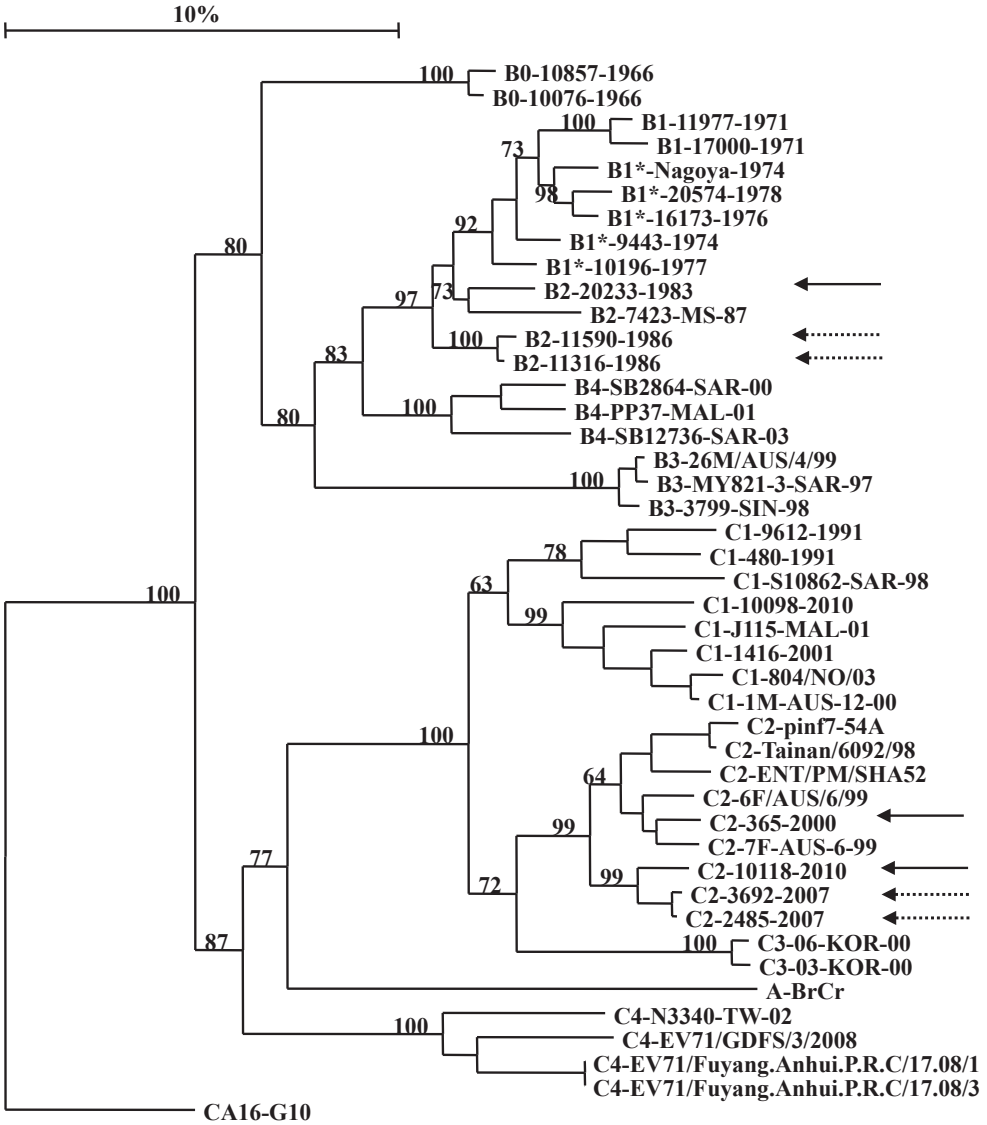

Fig. 3: Neighbor joining tree of the P2 encoding region (1734 nt) of Dutch EV71 isolates and reference strains. Bootstrap analysis was performed using 1000 pseudoreplicates. Dashed arrows indicate B2 and C2 strains from outbreaks in 1986 and 2007, respectively. Solid arrows indicate B2 and C2 strains isolated in non-epidemic years.

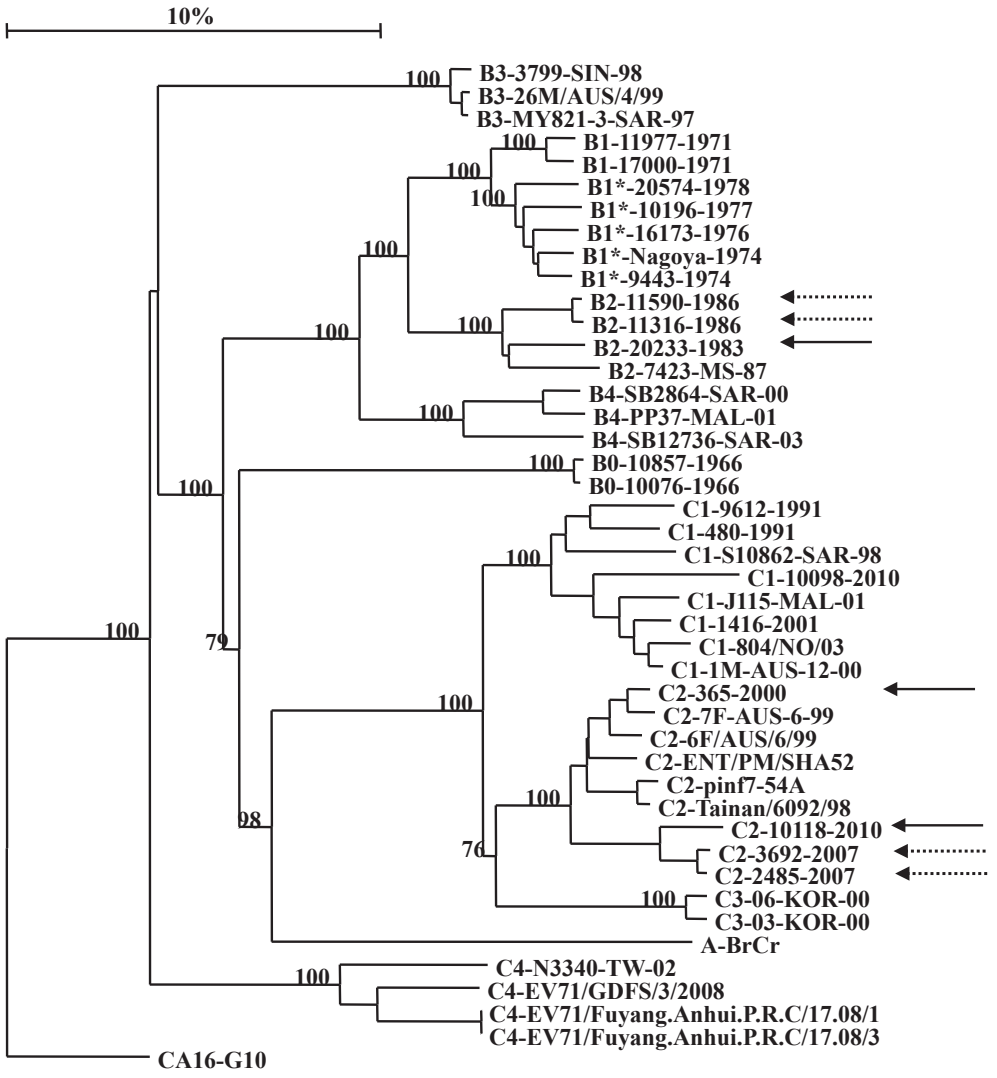

Fig. 4: Neighbor joining tree of the P3 encoding region (2259 nt) of Dutch EV71 isolates and reference strains. Bootstrap analysis was performed using 1000 pseudoreplicates. Dashed arrows indicate B2 and C2 strains from outbreaks in 1986 and 2007, respectively. Solid arrows indicate B2 and C2 strains isolated in non-epidemic years.

Table 1: Summary of enterovirus A recombination events detected by RDP3. The analysis was performed using a complete genome sequence alignment. The recombination event numbers as they appear in the supplementary Table S3 (complete overview of the recombination events detected by RDP) and the breakpoint positions in the recombinant sequence(s) are indicated in the first and second columns, respectively.

Recomb. event nr.	Breakpoint pos. in recomb. sequence Begin	End	Recombinant Sequence(s)	Minor Parent **	Major Parent ***	Detection Methods and p-Values RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
1	0*	3643	B3-reference strains	B4-reference strains, Dutch B2 strains of '86 (11316, 11590)	CA14-G-14, CA4-High_ Point	3,12E-82	1,18E-77	1,89E-79	1,54E-40	3,56E-33	6,03E-84	3,12E-32
2	420*	3614	CA8-Donovan	Unkown	Dutch C1 and C2 strains C1-2-3-reference strains	3,77E-81	6,20E-77	4,22E-79	3,82E-36	2,47E-39	1,30E-69	2,24E-112
3	513*	3574	C4-reference strains	Dutch C1 and C2 strains C1-2-3- reference strains	CA14-G-14	9,18E-78	1,28E-63	1,40E-71	3,43E-43	3,52E-36	2,25E-63	7,08E-78
4	12*	3768	Dutch B0 strains	Dutch B1 and B2 strains B1-2- reference strains	Unknown	1,15E-67	1,67E-65	3,04E-62	3,13E-20	4,87E-27	3,12E-83	3,26E-4
9	402*	3514	Dutch B0, B1 and B2 strains B1-2-3-4 reference strains	Dutch C1 and C2 strains C1-2-3-reference strains	CA5-Swartz CA12-Texas-12	5,53E-38	2,79E-9	1,50E-28	4,21E-28	1,35E-23	5,90E-46	4,26E-30
12	5702	7333*	Dutch B0 strains	Dutch C1 and C2 strains C1-2-3-reference strains	CA4-High_ Point C4-reference strains CA16-G10	0,040	NS	NS	3,79E-11	1,93E-9	3,08E-13	NS

13	3454*	5420	Dutch B1 and B2 strains B1-2-4 reference strains	CA14-G-14 B3-reference strains C4-reference strains CA4-High_ Point	Unknown	3,06E-3	NS	4,79E-4	1,57E-8	5,13E-7	1,48E-41	NS
14	0*	316*	Dutch B2 strains of '86 (11590, 11316)	B4-reference strains	Dutch B2 strain 20233 Dutch B1 strains 17000, 10196 B2-reference strain	1,36E-9	2,55E-2	2,61E-9	7,17E-3	2,80E-5	5,08E-5	9,24E-7
21	6651*	7380*	C3-reference strains	Unknown	C2-reference strains Dutch C2 strain 365	5,79E-5	NS	6,39E-5	1,48E-2	NS	1,76E-7	NS

*:The actual breakpoint position is undetermined (it was most likely either at the 3' or 5' ends of the sequence or overprinted by a subsequent recombination event).
**:Sequence(s) within the dataset that most resemble the parent contributing the smaller fraction of sequence.
***Sequence(s) within the dataset that most resemble the parent contributing the larger fraction of sequence.
NS: No significant P-value was recorded for this recombination event using this method.

Discussion

The RIVM EV71 collection covers a period of 47 years of EV71 circulation in the Netherlands (1963 up to present), enabling a study on recombination events not only among different subgenogroups observed in the Netherlands, but also among viruses of endemic and epidemic years. Recombination has previously been shown to play a significant role in the evolution of enterovirus genomes and the recombination breakpoints detected in this study (5'UTR/VP4, VP1/2A, 3C/3D) are known hot-spots of enterovirus recombination (31, 32). Regarding EV71, breakpoints at the interface of VP1/ 2A, 2A/2B and 3C/3D have been detected in the genomes of subgenogroup B3 and C4 strains isolated in the Asian Pacific region (11, 43, 44). To our knowledge, however, this is the first study that describes indications for recombination in the 5'UTR region of EV71. This observation is remarkable because it was unique to strains involved in increased spread, as was evidenced by phylodynamic analyses (39). Dutch B2 viruses of the epidemic year 1986 were closely related in the 5'UTR to B3 and B4 strains isolated during large outbreaks in the Asian Pacific region and to the first 230 nt this region was only distantly related to Dutch B0, B1 and B2 viruses isolated in preceding, non-epidemic years. Based on previously characterized EV71 RNA secondary structures, the first 230 nt of the 5'end of the genome contains both a cloverleaf structure that is involved in the initiation of positive strand synthesis, and two of the five stem-loop structures of the internal ribosomal entry site that is involved in genome translation (24, 35, 40). Previous studies on attenuated poliovirus strains have shown that the 5'UTR contains determinants of tissue tropism (34). Thus, considering the major role of the 5'UTR in viral replication, translation and tissue tropism, the question is whether exchange of the 5'UTR could have resulted in evolution of B2, B3 and B4 strains with increased viral fitness capable of causing outbreaks. This same question could be asked of the C4 strains isolated during a large outbreak in China in 2008; these viruses too were only distantly related to other subgenogroup B and C viruses in the 5'UTR (this study). Extended *in vitro* studies on replication kinetics and translation efficiency, using recombinant reporter viruses, are the next steps in investigating this potential correlation. Genome analysis of the C2 strains isolated in the Netherlands in 1997–2010 revealed no indications for recombination among C2 isolates from the epidemic in 2007. Other epidemiological parameters, like host susceptibility are likely to play a role in this outbreak. Analysis of clinical data from Dutch children infected with EV71, showed that genogroup B infections were significantly more often associated with neurologic disease than genogroup C infections (38). This difference, however, is likely not correlated to the recombination event in the 5'UTR of B2 outbreak strains, as no differences in disease severity were observed among infections with these strains and genogroup B strains isolated in preceding, non epidemic years.

Subgenogroup specific recombination events were detected in the non-structural encoding region (P2 and P3) of subgenogroup B0, B1 and B2 viruses observed in the Netherlands in 1963–1986. In line with this is the detection of subgenogroup specific genome recombination events among B3 and B4 strains isolated in the Asian Pacific region (this study and (44)). These observations, and the observed cross-reactivity between type B subgenogroups, suggest that genome recombination plays a role in the evolution of type B subgenogroups and not primarily antigenic drift (20, 39). Among Dutch C1 and C2 isolates, however, no evidence for recombination was found which is in line with the results of a recent French study on C1 and C2 isolates from France, Germany and Austria (1994–2009) (28). Except for B2 isolates of the epidemic in 1986, Dutch B1, B2, C1 and C2 isolates showed a high percentage of similarity over their entire genomes to international reference strains of their respective subgenogroups. Besides indicating the global dissemination of these EV71 subgenogroups, it suggests that recombination events detected in the Dutch genogroup B viruses had occurred before they were introduced into the Netherlands. Genetic factors increasing host susceptibility for EV71 infection, in combination with a high prevalence of EV71 strains and other EV strains, could explain the quick emergence of new EV71 subgenogroups in the Asian Pacific region.

This study showed that genome recombination likely plays a role in the evolution of type B subgenogroups. No evidence for recombination, however, was found for subgenogroup C1 and C2 viruses isolated in the Netherlands since 1987. The fact that the Dutch B2 isolates of the epidemic in 1986 were closely related in the 5'UTR to B3 and B4 viruses from large outbreaks in the Asian Pacific region, raises the question whether exchange of the 5'UTR plays a role in the evolution of viruses capable of causing outbreaks. This observation emphasizes the need to analyze (near) complete genomes instead of only the capsid encoding region during studies on EV71 epidemiology.

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Supplementary Material Chapter 5

Table S1: Dutch isolates and reference strains used for complete genome analyses. Partial genome sequences were available for strains indicated in italics.

Strain	Subgenotype	Year	Place	Acc. number
10076	B0	1966	NL	AB575911
10857	B0	1966	NL	AB575912
11977	B1	1971	NL	AB575913
17000	B1	1971	NL	AB575914
9443	B1*	1974	NL	AB575915
16173	B1*	1976	NL	AB575916
10196	B1*	1977	NL	AB575917
20574	B1*	1978	NL	AB575918
Nagoya	B1*	1974	Japan	AB482183
19271	B2	1981	NL	AB575919
17325	B2	1981	NL	AB575920
8304	B2	1982	NL	AB575921
12677	B2	1982	NL	AB575922
20233	B2	1983	NL	AB575923
17001	B2	1985	NL	AB575924
20674	B2	1985	NL	AB575925
20557	B2	1985	NL	AB575926
11316	B2	1986	NL	AB575927
11590	B2	1986	NL	AB575928
6916	B2	1986	NL	AB575929
9724	B2	1986	NL	AB575930
10893	B2	1986	NL	AB575931
11289	B2	1986	NL	AB575932
13459	B2	1986	NL	AB575933
14266	B2	1986	NL	AB575934
7423-MS-87	B2	1987	USA	U22522
MY821-3-SAR-97	B3	1997	Sarawak	DQ341367
3799-SIN-98	B3	1998	Singapore	DQ341354
26M/AUS/4/99	B3	1999	Australia	EU364841
SB2864-SAR-00	B4	2000	Sarawak	DQ341366
PP37-MAL-01	B4	2001	Malaysia	DQ341365
SB12736-SAR-03	B4	2003	Malaysia	DQ341362
480	C1	1991	NL	AB575935
9612	C1	1991	NL	AB575936
1416	C1	2001	NL	AB575937
10098	C1	2010	NL	AB575938
S10862-SAR-98	C1	1998	Malaysia	DQ341359
1M-AUS-12-00	C1	2000	Malaysia	AY794031
J115-MAL-01	C1	2001	Malaysia	AY794028
804/NO/03	C1	2003	Norway	DQ452074
365	C2	2000	NL	AB575939
1474	C2	2005	NL	AB575940
2485	C2	2007	NL	AB575941
3692	C2	2007	NL	AB575942
2531	C2	2007	NL	AB575943
2769	C2	2007	NL	AB575944
2786	C2	2007	NL	AB575945
2798	C2	2007	NL	AB575946
3086	C2	2007	NL	AB575947
10118	C2	2010	NL	AB575948

ENT/PM/SHA52	C2	1997	Malaysia	AM396584
Tainan/6092/98	C2	1998	Taiwan	AF304459
6F/AUS/6/99	C2	1999	Australia	DQ381846
7F-AUS-6-99	C2	1999	Australia	AF376108
pinf7-54A	C2	2005	Taiwan	DQ060149
03-KOR-00	C3	2000	Korea	DQ341356
06-KOR-00	C3	2000	Korea	DQ341355
N3340-TW-02	C4	2002	Taiwan	EU131776
EV71/Fuyang.Anhui.P.R.C/17.08/1	C4	2008	China	EU703812
EV71/Fuyang.Anhui.P.R.C/17.08/3	C4	2008	China	EU703814
EV71/GDFS/3/2008	C4	2008	China	FJ194964
BrCr	A	1970	USA	U22521
CA2-Fleetwood	CA2	1947	USA	AY421760
CA2-Kanagawa	CA2	2003	Japan	AB162738.1
CA3-Olson	CA3	1948	USA	AY421761
CA4-High Point	CA4	1948	USA	AY421762
CA5-Swartz	CA5	1950	USA	AY421763
CA6-Gdula	CA6	1949	USA	AY421764
CA7-Parker	CA7	1949	USA	AY421765
CA8-Donovan	CA8	1949	USA	AY421766
CA10-Kowalik	CA10	1950	USA	AY421767
CA12-Texas-12	CA12	1948	USA	AY421768
CA14-G-14	CA14	1950	South Africa	AY421769
CA16-G10	CA16	1951	South Africa	U05876

Table S2: Primer sets used for PCR amplification and sequencing of complete EV71 genomes.

Primer	Sequence (5'-3')	Genome position (nt) *	PCR/Seq
EV1FB	TAAAACAGCCTGTGGGTTGYACC	2-23	PCR/Seq
EV1RB	TGCACATGRATGCAAAACC	1286-1268	PCR/Seq
VP4VP2B	CAGCGATCCGGCTCCCACGAG	750-768	Seq
EV2FB	CCTGATGTWYTGACWGAAACC	1200-1221	PCR/Seq
EV2RB	ATCACATCTGCCACYCTATC	2462-2443	PCR/Seq
1826B	GAAGTTAGGAACCTGCTTGAG	1833-1853	Seq
EV3FB	GGGGCRCCCAAYACAGCTTACATWWTRGC	2333-2361	PCR/Seq
EV3RB	CCTGGGCRGTGGTAGACGACACTART	3476-3451	PCR/Seq
EV4FB	CAGGTYTCAGTYCCATTYATGTCACC	3005-3030	PCR/Seq
EV4RB	GCTGTYTTKGCTTTRATCCAWGC	4044-4022	PCR/Seq
EV5FB	YAACTCYAAGAGAAAGCACTATCC	3526-3559	PCR/Seq
EV5RB	GTRGATGCAATRACAAACTTAGATG	4743-4719	PCR/Seq
EV6FB	CCKGAYCAYTTTGAYGGRTACAAAC	4559-4585	PCR/Seq
EV6RB	CCRTACTGCACRACRTCYCCC	5760-5740	PCR/Seq
EV7FB	GTTGAGCGRCACCTYAAYAGAGC	5216-5238	PCR/Seq
EV7RB	GYAGRTCYAACCCATACTTRTCC	6395-6373	PCR/Seq
EV8FB	CATYAAGAARAGRGACATYCTYG	6310-6332	PCR/Seq
EV8R**	TTGCTATTCTGGTTATAACAAATTTACCC	7413-7385	PCR/Seq
EV1FC	TAAAACAGCCTGTGGGTTGYACC	2-23	PCR/Seq
EV1RC	TGCACRTGRATGCARAACC	1286-1268	PCR/Seq

VP4VP2C	ATGGGBTCACARGTRTCHA	750-768	Seq
EV2FC	CCGGATGTRYTAACWGAAACC	1200-1221	PCR/Seq
EV2RC	ATCACATCTGCCACCCTATC	2462-2443	PCR/Seq
1826C	GAAGTTAGGAACTTGCTAGAG	1833-1853	Seq
EV3FC	GGRGCACCCAAYACAGCCTATATAATAGC	2333-2361	PCR/Seq
EV3RC	CYTGRGCRGTGGTRGATGAYACGAGC	3476-3451	PCR/Seq
EV4FC	CAGGTYTCAGTTCCRTTYATGTCACC	3005-3030	PCR/Seq
EV4RC	GCTGTYTTCGAYTTRAYCCAGGC	4044-4022	PCR/Seq
EV5FC	AACTCAAKGAGAAARCACTAYCC	3526-3559	PCR/Seq
EV5RC	GTRGAGGCAATCACAAACTTGGAGG	4743-4719	PCR/Seq
EV6FC	CCAGAYCAYTTTGAYGGRTACAAAC	4559-4585	PCR/Seq
EV6RC	AYCCATACTGGACCACATCKCC	5760-5740	PCR/Seq
EV7FC	GTTGAGCGCCACTTRAAYAGAGC	5216-5238	PCR/Seq
EV7RC	GKAGRTCCAACCCATACTTRTCC	6395-6373	PCR/Seq
EV8FC	CATCAAGAARARGGATATYTTGG	6310-6332	PCR/Seq

* Position on genome of EV71 subgenogroup B2 reference strain 7423-MS-87
** EV8R has been used for both genogroup B and C viruses

Table S3: The characteristics of detectable Enterovirus A recombination events.

Recombination Event Number	Number In Supplementary .RDP File	Breakpoint Positions						Recombinant Sequence(s)	Minor Parent	Major Parent	Detection Methods and p-Values						
		In Alignment		In Recombinant Sequence		Relative to 10076|B0|1966											
		Begin	End	Begin	End	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
1	1	1*	3766	0*	3643	0*	3577	B3-26M/AUS/4/99 B3-MY821-3-SAR-97 B3-3799-SIN-98	B4-SB2864-SAR-00 11316|B2|1986 11590|B2|1986 B4-PP37-MAL-01 B4-DQ341365-MA-2001 B4-SB12736-SAR-03	CA14-G-14 CA4-High_Point	3,12E-82	1,18E-77	1,89E-79	1,54E-40	3,56E-33	6,03E-84	3,12E-32
2	2	434*	3753	420*	3614	357*	3564	CA8-Donovan	Unknown (C4-N3340-TW-02) Unknown (C4-EV71/GDFS/3/2008) Unknown (C4-EV71/FUYANG.ANHUI.P.R.C/17.08/3) Unknown (C4-EV71/FUYANG.ANHUI.P.R.C/17.08/1)	480|C1|1991 9612|C1|1991 1416|C1|2001 10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 C1-S10862-SAR-98 C2-7F-AUS-6-99 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52	3,77E-81	6,20E-77	4,22E-79	3,82E-36	2,47E-39	1,30E-69	2,24E-112
3	3	530*	3702	513*	3574	452*	3513	C4-N3340-TW-02 C4-EV71/GDFS/3/2008 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/3 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/1	C2-7F-AUS-6-99 480|C1|1991 9612|C1|1991 1416|C1|2001 10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 C1-S10862-SAR-98 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52	CA14-G-14	9,18E-78	1,28E-63	1,40E-71	3,43E-43	3,52E-36	2,25E-63	7,08E-78

4	4	67*	3943	12*	3768	0*	3754	10857|B0|1966 10076|B0|1966	11977|B1|1971 17000|B1|1971 9443|B1|1974 16173|B1|1976 10196|B1|1977 20574|B1|1978 20233|B2|1983 11316|B2|1986 11590|B2|1986 B2-7423-MS-87 B1-_Nagoya	Unknown (CA5-Swartz)	1,15E-67	1,67E-65	3,04E-62	3,13E-20	4,87E-27	3,12E-83	3,26E-4
5	5	761*	3462	739*	3361	673*	3273	CA6-Gdula	Unknown (480|C1|1991) Unknown (11977|B1|1971) Unknown (17000|B1|1971) Unknown (9443|B1|1974) Unknown (16173|B1|1976) Unknown (10196|B1|1977) Unknown (20574|B1|1978) Unknown (20233|B2|1983) Unknown (11316|B2|1986) Unknown (11590|B2|1986) Unknown (9612|C1|1991) Unknown (1416|C1|2001) Unknown (10098|C1|2010) Unknown (365|C2|2000) Unknown (2485|C2|2007) Unknown (3692|C2|2007) Unknown (10118|C2|2010) Unknown (B2-7423-MS-87) Unknown (C1-S10862-SAR-98) Unknown (C2-7F-AUS-6-99)	BrCr-A	5,78E-47	8,13E-11	2,50E-33	1,53E-4	1,86E-17	4,16E-42	1,69E-59

Recombination Event Number	Number In Supplementary .RDP File	Breakpoint Positions						Recombinant Sequence(s)	Minor Parent	Major Parent	Detection Methods and p-Values						
		In Alignment		In Recombinant Sequence		Relative to 10076|B0|1966											
		Begin	End	Begin	End	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
									Unknown (B4-PP37-MAL-01) Unknown (C3-03-KOR-00) Unknown (C3-06-KOR-00) Unknown (C2-6F/AUS/6/99) Unknown (C1-1M-AUS-12-00) Unknown (C1-J115-MAL-01) Unknown (C1-804/NO/03) Unknown (B1-_Nagoya) Unknown (B4-DQ341365-MA-2001) Unknown (B4-SB2864-SAR-00) Unknown (B4-SB12736-SAR-03) Unknown (C2-Tainan/6092/98) Unknown (C2-pinf7-54A) Unknown (C2-ENT/PM/SHA52)								
6	6	618*	3535	603*	3408	539*	3346	BrCr-A	C3-03-KOR-00 11977|B1|1971 17000|B1|1971 9443|B1|1974 16173|B1|1976 10196|B1|1977 20574|B1|1978 20233|B2|1983 11316|B2|1986 11590|B2|1986 480|C1|1991 9612|C1|1991 1416|C1|2001	CA12-Texas-12 CA3-Olson CA10-Kowalik	1,06E-42	5,00E-30	1,47E-27	3,10E-26	1,95E-32	8,23E-41	1,27E-57

									10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 B2-7423-MS-87 C1-S10862-SAR-98 C2-7F-AUS-6-99 B4-PP37-MAL-01 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03 B1-_Nagoya B4-DQ341365-MA-2001 B4-SB2864-SAR-00 B4-SB12736-SAR-03 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52							
7	7	797*	3485	769*	3354	708*	3296	CA7-Parker	CA14-G-14 CA4-High_Point	Unknown (CA4-High_Point) Unknown (CA14-G-14) Unknown (CA16-G10)	2,24E-24	NS	1,79E-27	2,46E-20	1,29E-15	8,30E-39	2,44E-7
8	8	572*	3526	560*	3403	494*	3337	CA14-G-14	Unknown (CA3-Olson) Unknown (BrCr-A) Unknown (CA2-Fleetwood) Unknown (CA10-Kowalik) Unknown (CA12-Texas-12)	CA4-High_Point	2,11E-23	NS	2,96E-26	1,59E-10	3,72E-13	8,20E-67	3,80E-12
9	9	414*	3637	402*	3514	337*	3448	B2-7423-MS-87 10076|B0|1966 10857|B0|1966 11977|B1|1971 17000|B1|1971 9443|B1|1974 16173|B1|1976 10196|B1|1977 20574|B1|1978 20233|B2|1983 11316|B2|1986 11590|B2|1986 B3-MY821-3-SAR-97 B3-3799-SIN-98	C1-S10862-SAR-98 480|C1|1991 9612|C1|1991 1416|C1|2001 10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 C2-7F-AUS-6-99 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00	CA5-Swartz CA12-Texas-12	5,53E-38	2,79E-9	1,50E-28	4,21E-28	1,35E-23	5,90E-46	4,26E-30

Recombination Event Number	Number In Supplementary .RDP File	Breakpoint Positions						Recombinant Sequence(s)	Minor Parent	Major Parent	Detection Methods and p-Values						
		In Alignment		In Recombinant Sequence		Relative to 10076|B0|1966											
		Begin	End	Begin	End	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
								B4-PP37-MAL-01 B1-_Nagoya B3-26M/AUS/4/99 B4-DQ341365-MA-2001 B4-SB2864-SAR-00 B4-SB12736-SAR-03	C1-J115-MAL-01 C1-804/NO/03 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52								
10	10	3598	7537*	3460	7394*	3409	7333*	CA2-Fleetwood	CA10-Kowalik	CA4-High_Point	3,74E-19	0,000	6,77E-25	3,54E-2	1,26E-13	1,73E-42	NS
11	11	637	3641	623	3538	557	3452	CA4-High_Point	C2-Tainan/6092/98 480|C1|1991 9612|C1|1991 1416|C1|2001 10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 C1-S10862-SAR-98 C2-7F-AUS-6-99 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03 C2-pinf7-54A C2-ENT/PM/SHA52	CA16-G10	1,72E-14	NS	2,12E-18	1,38E-20	1,01E-15	2,48E-32	2,33E-7
12	12	5891	7536*	5702	7333*	5702	7333*	10076|B0|1966 10857|B0|1966	C1-804/NO/03 480|C1|1991 9612|C1|1991 1416|C1|2001 10098|C1|2010 365|C2|2000 2485|C2|2007 3692|C2|2007 C2-7F-AUS-6-99 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99	CA4-High_Point C4-EV71/GDFS/3/2008 C4-N3340-TW-02 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/3 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/1 CA16-G10	0,040	NS	NS	3,79E-11	1,93E-9	3,08E-13	NS

									C1-1M-AUS-12-00 C1-J115-MAL-01 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52								
13	13	3641*	5607	3454*	5420	3452*	5418	20574|B1|1978 11977|B1|1971 17000|B1|1971 9443|B1|1974 16173|B1|1976 10196|B1|1977 20233|B2|1983 11316|B2|1986 11590|B2|1986 B2-7423-MS-87 B4-PP37-MAL-01 B1-_Nagoya B4-DQ341365-MA-2001 B4-SB2864-SAR-00 B4-SB12736-SAR-03	CA14-G-14 B3-3799-SIN-98 B3-26M/AUS/4/99 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/3 C4-EV71/FUYANG.ANHUI.P.R.C/17.08/1 CA4-High_Point CA16-G10	Unknown (C2-pinf7-54A) Unknown (480|C1|1991) Unknown (9612|C1|1991) Unknown (1416|C1|2001) Unknown (10098|C1|2010) Unknown (365|C2|2000) Unknown (2485|C2|2007) Unknown (3692|C2|2007) Unknown (10118|C2|2010) Unknown (C1-S10862-SAR-98) Unknown (C2-7F-AUS-6-99) Unknown (C3-03-KOR-00) Unknown (C3-06-KOR-00) Unknown (C2-6F/AUS/6/99) Unknown (C1-1M-AUS-12-00) Unknown (C1-J115-MAL-01) Unknown (C2-Tainan/6092/98) Unknown (C2-ENT/PM/SHA52) Unknown (CA8-Donovan)	3,06E-3	NS	4,79E-4	1,57E-8	5,13E-7	1,48E-41	NS
14	14	1*	392*	0*	316*	0*	315*	11590|B2|1986 11316|B2|1986[P]	B4-SB2864-SAR-00 B4-PP37-MAL-01 B4-DQ341365-MA-2001 B4-SB12736-SAR-03	20233|B2|1983 17000|B1|1971 10196|B1|1977 B2-7423-MS-87	1,36E-9	2,55E-2	2,61E-9	7,17E-3	2,80E-5	5,08E-5	9,24E-7

Recombination Event Number	Number In Supplementary .RDP File	Breakpoint Positions						Recombinant Sequence(s)	Minor Parent	Major Parent	Detection Methods and p-Values						
		In Alignment		In Recombinant Sequence		Relative to 10076|B0|1966											
		Begin	End	Begin	End	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
15	15	2*	3841	1*	3710	0*	3652	CA12-Texas-12	CA10-Kowalik	10098|C1|2010 9612|C1|1991 1416|C1|2001 365|C2|2000 2485|C2|2007 3692|C2|2007 10118|C2|2010 C1-S10862-SAR-98 C2-7F-AUS-6-99 C3-03-KOR-00 C3-06-KOR-00 C2-6F/AUS/6/99 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03 C2-Tainan/6092/98 C2-pinf7-54A C2-ENT/PM/SHA52	4,51E-8	NS	3,60E-13	4,59E-15	8,27E-3	2,19E-60	NS
16	16	5252	5347	5125	5220	5063	5158	BrCr-A	B2-7423-MS-87 11977|B1|1971 17000|B1|1971 9443|B1|1974 16173|B1|1976 10196|B1|1977 20574|B1|1978 20233|B2|1983 11316|B2|1986 11590|B2|1986 B1-_Nagoya	CA12-Texas-12 CA2-Fleetwood CA3-Olson CA6-Gdula CA10-Kowalik	2,09E-4	2,70E-7	1,02E-8	2,19E-2	NS	2,42E-7	NS
17	17	6942*	7400*	6807*	7265*	6753*	7211*	CA5-Swartz	C1-S10862-SAR-98 9612|C1|1991 1416|C1|2001 10098|C1|2010 C1-1M-AUS-12-00 C1-J115-MAL-01 C1-804/NO/03	Unknown (C3-06-KOR-00) Unknown (C3-03-KOR-00)	7,05E-7	NS	4,06E-4	0,030	3,38E-2	NS	NS
18	18	6204	7226	6103	7125	6015	7037	CA6-Gdula	CA12-Texas-12	Unknown (CA2-Fleetwood)	4,89E-6	NS	4,75E-6	1,79E-6	6,92E-3	3,95E-14	NS

19	19	4466	5682	4340	5556	4277	5493	CA10-Kowalik	CA6-Gdula	CA2-Fleetwood	3,16E-4	NS	1,10E-4	1,39E-6	1,79E-4	5,06E-11	4,18E-2
20	20	3833	5543	3698	5408	3644	5354	CA5-Swartz	CA16-G10	Unknown (365|C2|2000) Unknown (480|C1|1991) Unknown (1416|C1|2001) Unknown (10098|C1|2010) Unknown (2485|C2|2007) Unknown (3692|C2|2007) Unknown (10118|C2|2010) Unknown (C1-S10862-SAR-98) Unknown (C2-7F-AUS-6-99) Unknown (C3-03-KOR-00) Unknown (C3-06-KOR-00) Unknown (C2-6F/AUS/6/99) Unknown (C1-1M-AUS-12-00) Unknown (C1-J115-MAL-01) Unknown (C1-804/NO/03) Unknown (C2-Tainan/6092/98) Unknown (C2-pinf7-54A) Unknown (C2-ENT/PM/SHA52)	3,05E-5	NS	4,93E-6	8,39E-5	1,83E-3	1,07E-11	NS
21	21	6779*	7510*	6651*	7380*	6590*	7317*	C3-06-KOR-00 C3-03-KOR-00	Unknown (C1-S10862-SAR-98) Unknown (9612|C1|1991) Unknown (C1-804/NO/03)	C2-ENT/PM/SHA52 365|C2|2000 C2-7F-AUS-6-99 C2-6F/AUS/6/99	5,79E-5	NS	6,39E-5	1,48E-2	NS	1,76E-7	NS

Recombination Event Number	Number In Supplementary .RDP File	Breakpoint Positions						Recombinant Sequence(s)	Minor Parent	Major Parent	Detection Methods and p-Values						
		In Alignment		In Recombinant Sequence		Relative to 10076|B0|1966											
		Begin	End	Begin	End	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
22	22	5608*	5982*	5473*	5847*	5419*	5793*	CA5-Swartz	Unknown (10196|B1|1977) Unknown (20233|B2|1983) Unknown (11316|B2|1986) Unknown (11590|B2|1986) Unknown (B2-7423-MS-87)	B4-DQ341365-MA-2001 B4-PP37-MAL-01 B4-SB2864-SAR-00	5,17E-4	NS	2,57E-2	3,48E-2	9,96E-5	1,66E-7	NS
23	24	5916*	6511*	5744*	6337*	5727*	6322*	10196|B1|1977	20574|B1|1978	B1-_Nagoya 9443|B1|1974	1,19E-2	NS	1,05E-2	NS	NS	NS	4,12E-2
24	32	2751	3537	2673	3397	2618	3348	CA3-Olson	CA8-Donovan	Unknown (CA5-Swartz)	4,01E-2	NS	NS	9,62E-3	3,98E-3	1,97E-24	NS

*: The actual breakpoint position is undetermined (it was most likely either at the 3' or 5' ends of the sequence or overprinted by a subsequent recombination event).
Minor Parent: Sequence(s) within the dataset that most resemble the parent contributing the smaller fraction of sequence.
Major Parent: Sequence(s) within the dataset that most resemble the parent contributing the larger fraction of sequence.
Unknown: Only one parent and a recombinant need be in the alignment for a recombination event to be detectable. The sequence listed as unknown was used to infer the existance of a missing parental sequence.
NS: No significant P-value was recorded for this recombination event using this method.

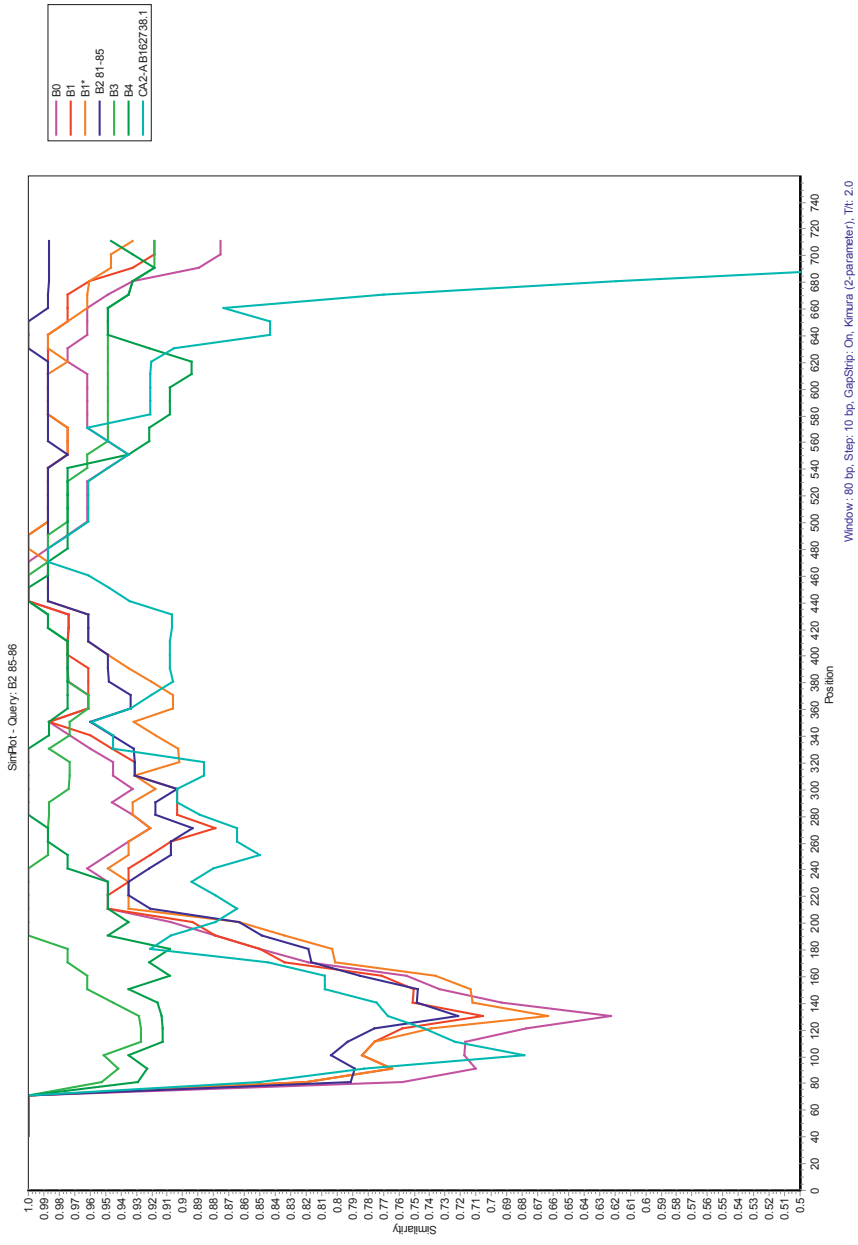

Fig. S1: Simplot analysis of the 5'UTR of EV71 genogroup B viruses and Coxsackie A2 reference strain. Dutch B2 isolates of 1986 and two B2 isolates of 1985 (20557, 20674) taken as query.

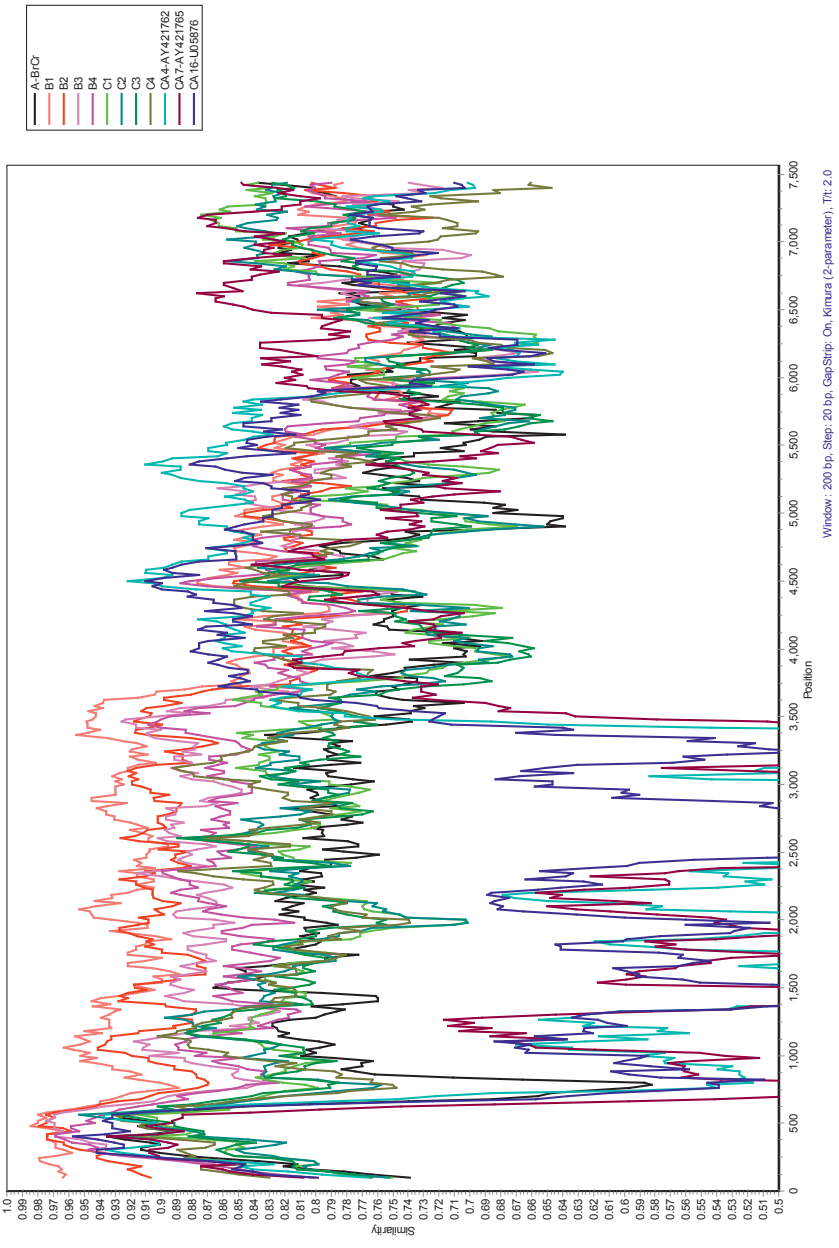

Fig. S2: Simplot analysis of the complete genomes of EV71 genogroup A, B and C viruses and Coxsackie A4, A7 and A16 reference strains. Sub-genogroup B0 taken as query.

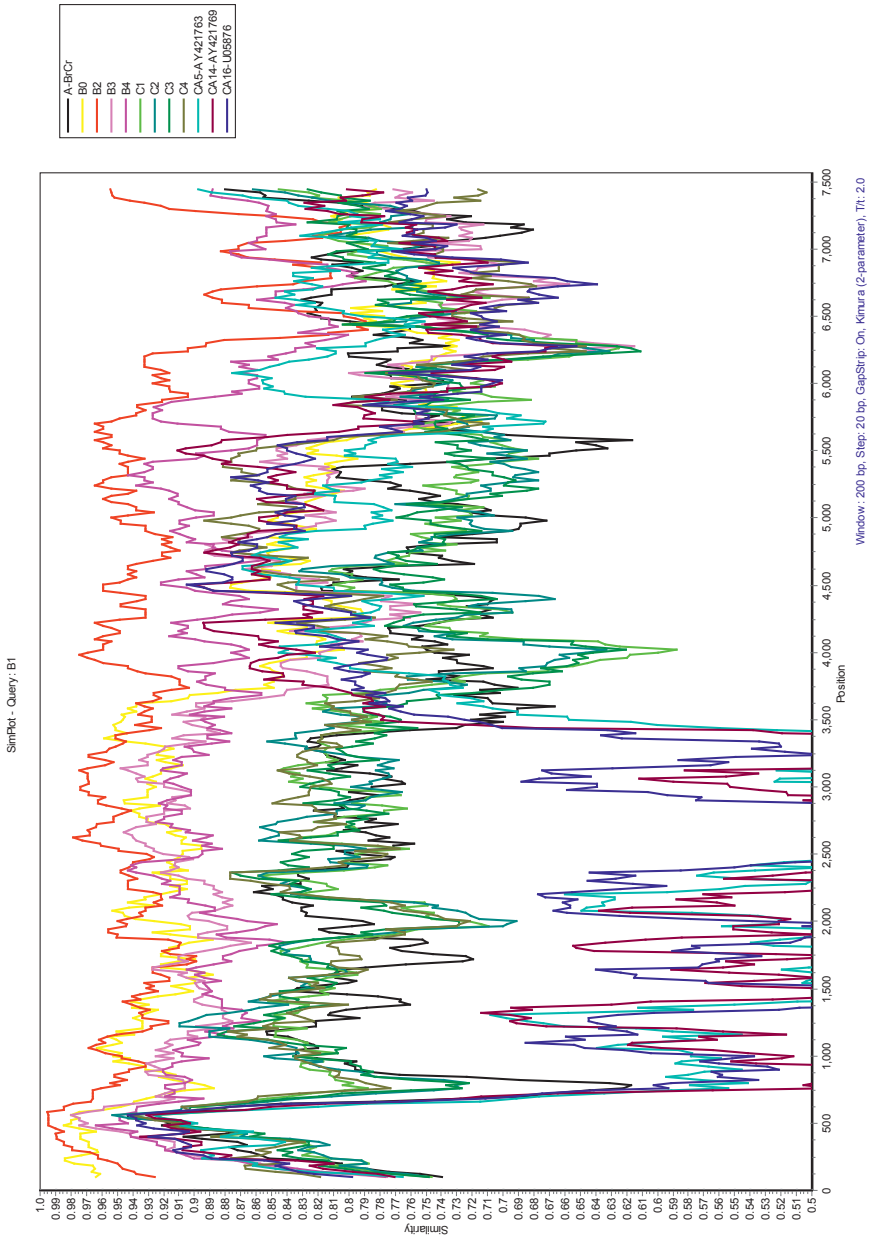

Fig. S3: Simplot analysis of the complete genomes of EV71 genogroup A, B and C viruses and Coxsackie A5, A14 and A16 reference strains. Subgenogroup B1 taken as query.

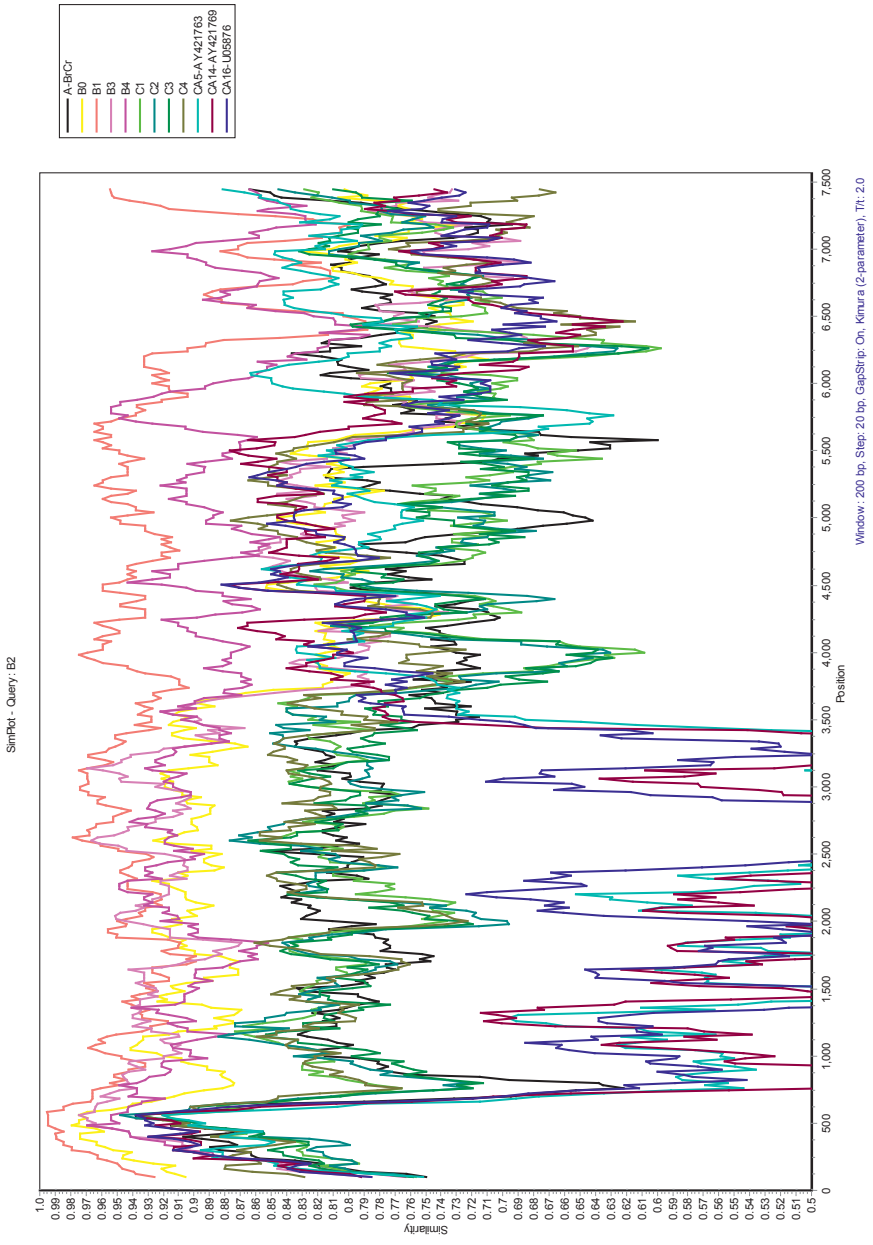

Fig. S4: Simplot analysis of the complete genomes of EV71 genogroup A, B and C viruses and Coxsackie A5, A14 and A16 reference strains. Subgenogroup B2 taken as query.

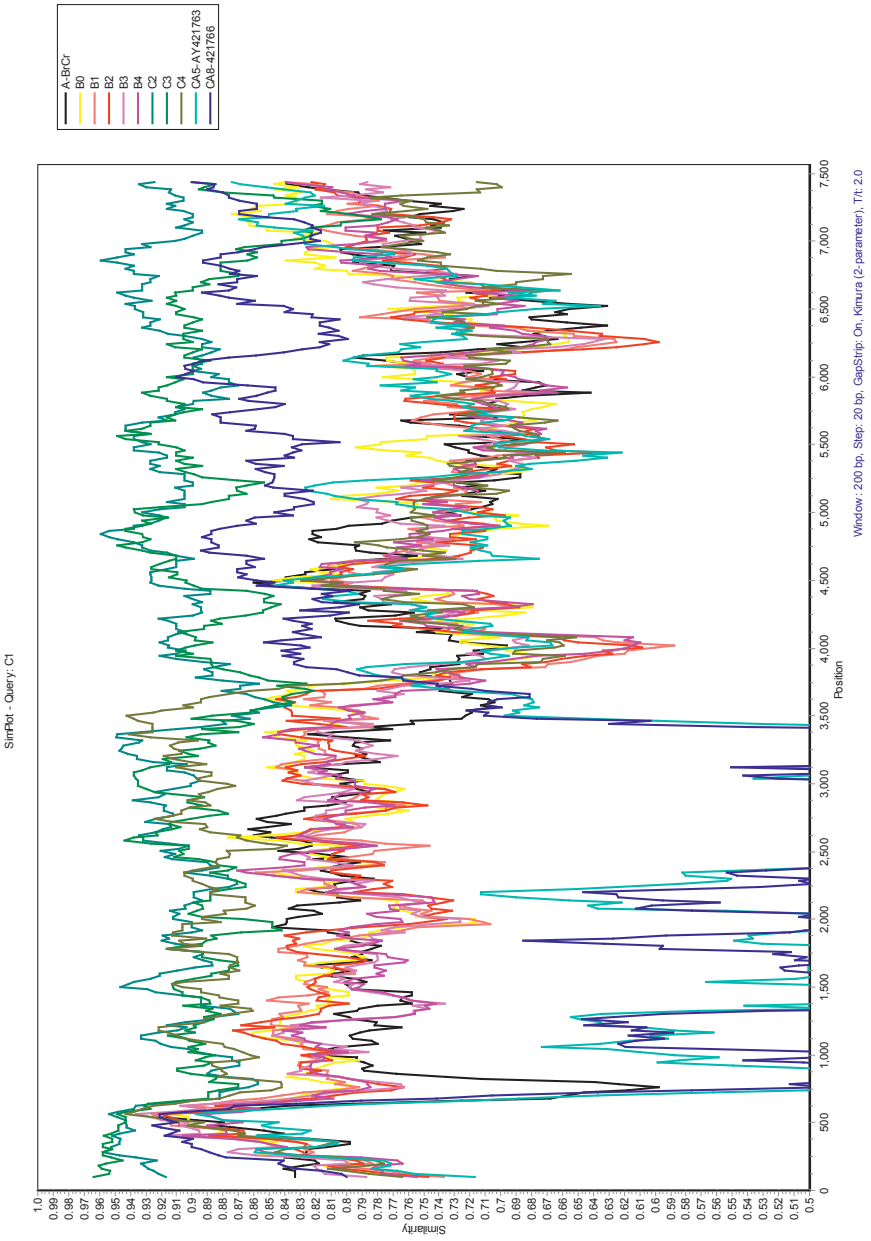

Fig. S5: Simplot analysis of the complete genomes of EV71 genogroup A, B and C viruses and Coxsackie A5 and A8 reference strains. Subgenogroup C1 taken as query.

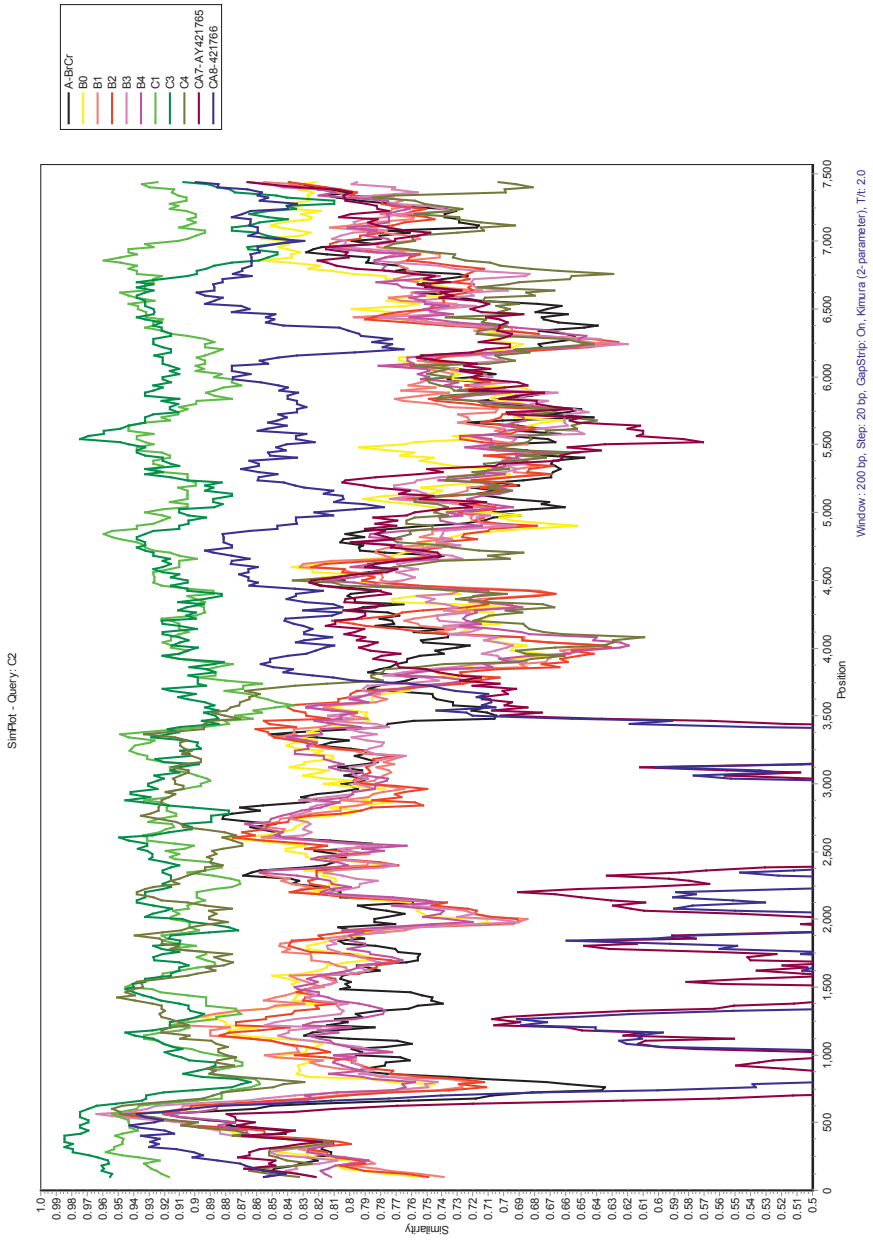

Fig. S6: Simplot analysis of the complete genomes of EV71 genogroup A, B and C viruses and Coxsackie A7 and A8 reference strains. Subgenogroup C2 taken as query.

Chapter 6

Evaluation of 50 Years of Enterovirus Surveillance Data to assess Changes possibly associated with Poliovirus Eradication

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Abstract

Laboratories of the Dutch Working Group on Clinical Virology have routinely performed enterovirus diagnostics since implementation of the national immunization programme in the Netherlands in 1957. Enterovirus positive samples have routinely been typed as part of this surveillance to document absence of poliovirus circulation. We analyzed trends in the surveillance data, and tried to study possible consequences of eradicating poliovirus and the associated decrease in poliovirus mucosal immunity on prevalence of enteroviruses that are most closely related to poliovirus. To define enterovirus relatedness we performed an extensive literature search on enterovirus heterotypic immune responses and studied homology in published (cross-reactive) B- and T-cell epitopes. Considering changes in isolation frequency of viruses defined to be closely related to poliovirus that could not be ascribed to documented changes in detection and typing methods, several cluster B serotypes were isolated at a relatively lower frequency during the first years of the study period (1959-1966) than in later years. Cluster C viruses, most closely related to poliovirus, were isolated at low rates throughout the complete study period (1959 up to 2009). This is in line with low prevalence of these viruses in the environment. On the basis of the current knowledge on the role of heterotypic immunity in human enterovirus infections a correlation between poliovirus immunity and observed trends in prevalence of cluster B and C viruses cannot be excluded.

Introduction

Human enteroviruses were traditionally classified into the species *Coxsackievirus A* (CA), *Coxsackievirus B* (CB), *Poliovirus* and *ECHO virus* on the basis of pathogenicity in humans and experimental animals. Serotypes within the species were recognized in neutralization assays using serotype specific antibodies. Enteroviruses cause a wide spectrum of disease symptoms, from relatively mild symptoms like diarrhoea and skin rash to severe disease with meningitis or acute flaccid paralysis, making classification on the basis of pathogenicity complicated. Since the early 1960s newly discovered enteroviruses were therefore no longer classified on the basis of pathogenicity, but were labeled with a numeric name, like human enterovirus (EV) 68 and 71. The development of molecular techniques enabled characterization of enteroviruses on the genetic level. Amino acid sequence comparisons of the structural regions showed that certain serotypes, previously assigned to the same species, were actually genetically distantly related. On the basis of these genetic distances human enterovirus serotypes were therefore reclassified into four species (cluster A-D), keeping the traditional names of the serotypes (Table 1) [1-4].

Table 1: Classification of enterovirus serotypes to human enterovirus species A-D.

	Cluster A	Cluster B	Cluster C	Cluster D
Polioviruses			1-3	
Coxsackie A viruses	2-8, 10, 12, 14, 16	9	1, 11, 13, 15, 17-22, 24	
Coxsackie B viruses		1-6		
Echoviruses		1-9, 11-21, 24-27, 29-34		
Enteroviruses	71, 76, 89-92	69, 73-75, 77-88, 93, 97-98, 101, 106-107	95-96, 99, 102, 104-105, 109	68, 70, 94

The introduction of the inactivated poliovirus vaccine (IPV) in the national immunization programme in the Netherlands in 1957 resulted in elimination of endemic poliovirus circulation in the Netherlands [5]. There are, however, subpopulations that refuse vaccination for religious reasons and are less protected against poliovirus by herd immunity because of their social and geographic clustering (the Bible Belt; southwest to northeast of the Netherlands). This was demonstrated by the occurrence of small outbreaks of polio in single villages within this risk area in 1961, '62, '63, '66, '69 and '71 (all type 1 poliovirus) and outbreaks affecting the whole Bible Belt in 1978 (type 1) and 1992/93 (type 3) [5]. During all these outbreaks, no indications of poliovirus circulation among people living outside the risk area, either

unvaccinated or IPV immunized, were found [5-8]. As IPV induces only systemic humoral protection, mucosal immunity against poliovirus is thus waning in the biggest part of the general Dutch population since the last nation wide poliovirus outbreak in 1956 (pre vaccination period) [9].

Laboratories of the Dutch Working Group on Clinical Virology, covering the whole country, have performed primary diagnosis for all enterovirus infections, including those of poliovirus, since introduction of IPV in 1957. In this study, we provide a detailed overview of the enterovirus typing data collected as part of these diagnostics at the National Institute for Public Health and the Environment (RIVM) between 1959 and 1989, and from national surveillance activities in the Netherlands from 1996 to 2009. We analyzed trends in surveillance data and studied possible consequences of absence of poliovirus circulation and the associated decrease in (mucosal) immunity on prevalence of other enteroviruses related to poliovirus. Knowledge on enterovirus heterotypic immune responses, B- and T-cell epitopes was extracted from the literature and used to define enteroviruses that are immunologically closely related to poliovirus. To rule out that differences in the detection and typing methods for enterovirus diagnostics influenced the observed serotype diversity a review was done of the protocols and technical developments from the onset of the surveillance data collection.

Materials and Methods

Definition of enterovirus strain relatedness

Enteroviruses immunologically most closely related to poliovirus were defined on the basis of a) data on enterovirus heterotypic immune responses drawn from the Pubmed and Scopus literature databases (search strategy in supplementary Table S1), b) homology to poliovirus in published enterovirus T-cell epitopes, as determined by T-cell proliferation assays using synthetic peptides, and c) homology to poliovirus in published, linear B-cell epitopes, as determined by peptide-antibody binding studies and neutralization assays using antigenic variants. For this, B- and T-cell epitopes present on structural proteins were included, as these epitopes are expected to elicit an effective humoral immune response important for preventing disease outcome. T-cell epitopes of non-structural proteins were not included as responses triggered by these antigens are assumed to play a less discriminative role in disease outcome, and therefore their level of protection is likely not reflected by the surveillance data based on number of symptomatic infections (severe and mild). Polyprotein amino acid sequences of serotypes that could be identified with the standard typing

methods used for surveillance were obtained from GenBank (Table S2), and used to study the percentage of similarity to poliovirus in B- and T-cell epitopes. For this, the Unweighted PairGroup Method with Arithmetic Mean (UPGMA) implemented in Bionumerics software version 6.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) was used.

Enterovirus typing data

The RIVM has performed primary diagnosis for enterovirus infections from 1959 to 1989. Faecal samples, throat swabs and cerebrospinal fluid (CSF) samples were collected through the years from patients with systemic viral infection with disease symptoms varying from diarrhoea and skin rash to neurological symptoms like acute flaccid paralysis. For this period the total number of samples analysed by the RIVM, the number of enterovirus positive samples and typing results have been documented. In 1989, all primary diagnostic virological activities were transferred from RIVM to existing medical microbiology laboratories. In response to the 1992/3 poliovirus type 3 outbreak in the Netherlands a new surveillance system was implemented to document the absence of poliovirus circulation. Within this sytem, 22 virological laboratories since 1996 yearly report the number of stool samples cultivated on poliovirus-sensitive cell lines, the number of EV-positive cultures and results of all EV typing experiments to the RIVM. The combined data sets were used as a basis for this study.

Review of enterovirus detection and typing methods in 1959 to 2009

In order to review if differences in the enterovirus detection and typing methods could bias the data, a review was done of the protocols and technical developments from the onset of the surveillance data collection and their possible effects on results of virus isolation and typing.

Results

Definition of enteroviruses related to poliovirus

Using the selection criteria listed in the methods section, 5 enterovirus T cell and 4 B cell epitopes, described in the literature, were included for further evaluation (Table 2). The highest percentages of similarity in the T-cell epitopes were found among viruses within the same enterovirus cluster (shown for cluster C, including poliovirus, in Fig. 1a). Among cluster C viruses, CA11, CA13, CA17 and CA20 showed a high percentage of similarity to poliovirus (relative to other cluster C serotypes) in at least 4 out of 5 epitopes, suggesting that these viruses are antigenically most closely

related to poliovirus (Fig. 1b). Although more distantly related to poliovirus in most epitopes, CA1 showed 100% similarity to poliovirus in epitope T3. Considering diversity in T-cell epitopes among the different clusters, cluster C viruses seem to be most closely related to cluster B viruses (Fig. 1a). This close relation is supported by *in vitro* cross-reactive T cell responses among poliovirus and cluster B viruses (described in [10, 11]).

Regarding B-cell epitopes, epitope 91-107 (B3) has been described to elicit a serotype specific immune response [12-15]: even within cluster C, the percentages of similarity between different serotypes are low in this epitope (Fig.2b). The same goes for epitope B4, but for this epitope no data on serotype specificity have been published. Considering data available on heterotypic binding of antibodies to peptides covering B-cell epitope VP1 42-50 (B1) (Table 2) and the percentages of similarity in this region, cluster C viruses again are more closely related to cluster B viruses than to cluster A and D viruses (Fig. 2a). Among viruses within cluster C this epitope is completely conserved (100% similarity). Based on similarity in the B2 epitope, CA1, CA11 and CA20 are most closely related to poliovirus (Fig. 2b).

Table 2: Enterovirus B- and T-cell epitopes extracted from the literature. Template virus: Serotypes used in experiments described in the literature. Available information on cross-reactivity is included in column 6 and 7. Abbreviations: AA, amino acid sequence; P, poliovirus; CB, Coxsackie B virus; EV, enterovirus; CA, Coxsackie A virus. Sources: [11, 13-15, 31, 40-49].

Epitope Nr.	Type	Location (AA)*	Sequence in Polio 1*	Template virus	Cross-reactivity among	No/low cross-reactivity against	Reference
T1	T-cell	VP1 99-116	TNKDKLFAVWKITYKDTV	P3	-	-	Graham et al. 1993
T2	T-cell	VP4 21-40	GGSTINYTTINYYRDSASNA	CB4	ECHO11	CB3, P1, CA16	Martilla et al. 2002
T3	T-cell	VP2 12-31	RVLQLTLGNSTITTQEAANS	CB4	CB3, ECHO11	P1, CA16	Martilla et al. 2002; Cello et al. 1996
T4	T-cell	VP2 200-219	LRTNNCATLVLPYVNSLSID	CB4	CB3, ECHO11, CA16	P1	Martilla et al. 2002; Cello et al. 1996
T5	T-cell	VP3 41-60	KNMMELAEIDTMIPFDLSAT	CB4	-	-	Martilla et al. 2002
B1	B-cell	VP1 42-50	PALTAVETG	P1, CB1, CB3, Cluster B conserved peptides	CB, ECHO, CA9, P1	EV71, EV70	Samuelson et al. 1994/95; Cello et al. 1993; Miao et al. 2009; Haarman et al. 1994
B2	B-cell	VP1 73-80	SESSIESF	Cluster B conserved peptides	ECHO, CB	-	Lewis et al. 1992, Cello et al. 1993
B3	B-cell	VP1 91-107	TVDNPASTTNKDKLFAV	EV71, CB3, CB4, P1	no other serotypes	-	Foo et al. 2008; Reimann et al. 1991, Wychowski et al. 1983
B4	B-cell	VP1 212-229	FSKVPLKDQSAALGDSLY	EV71, P1	-	-	Page et al. 1988; Foo et al. 2007a, b

*P1 Mahoney reference strain (V01148.1)
- not tested

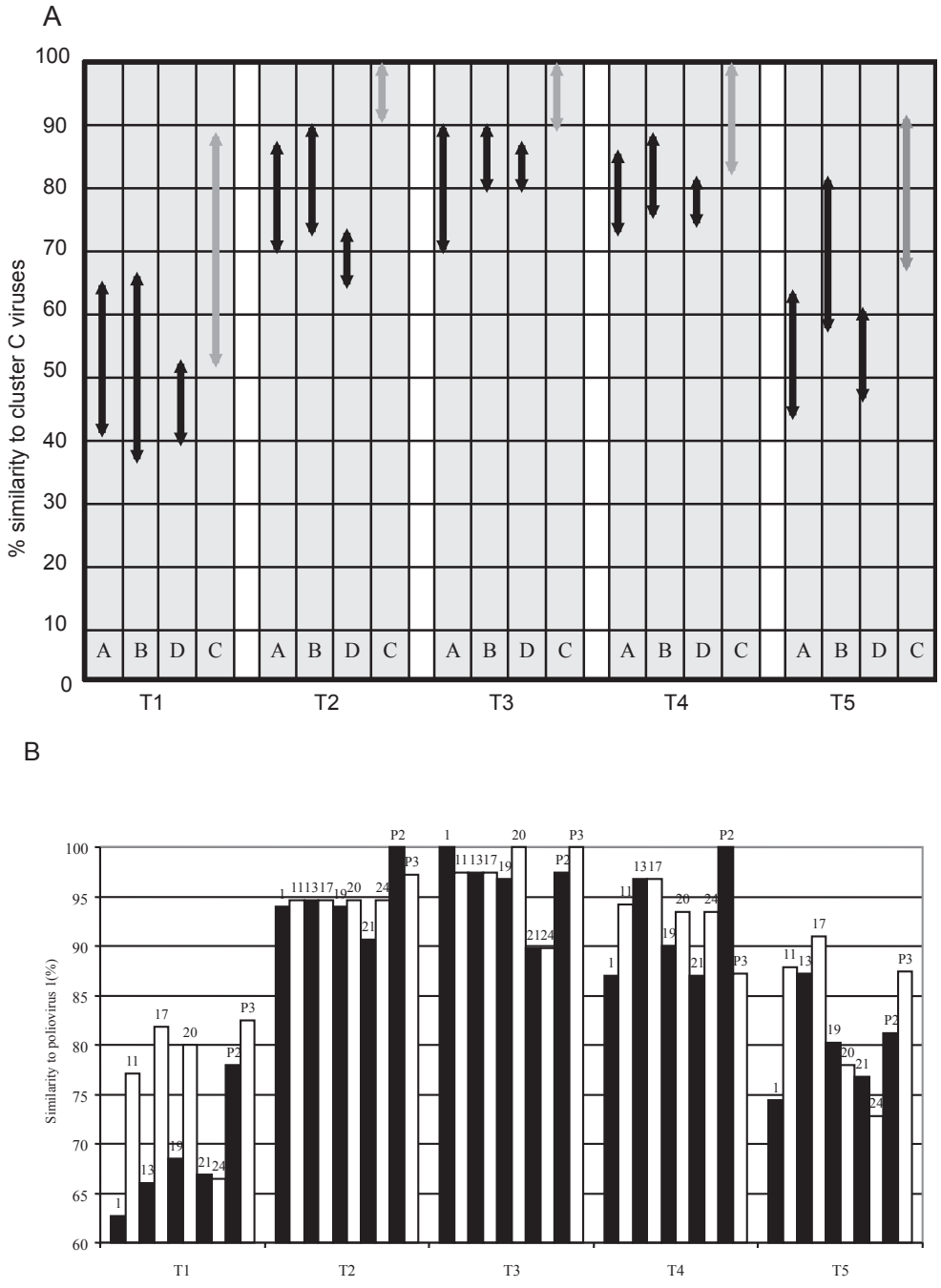

Fig. 1: Percentages of similarity in the T-cell epitopes listed in Table 2 between viruses belonging to different enterovirus clusters (A, B, D) and cluster C enteroviruses. A: Cluster A, B and D viruses versus cluster C viruses, including poliovirus. Grey arrows indicate genetic diversity within cluster C. B: Cluster C viruses versus poliovirus type 1 (Mahoney, V01148.1). Coxsackievirus A serotypes and poliovirus (P) serotypes are presented above bars.

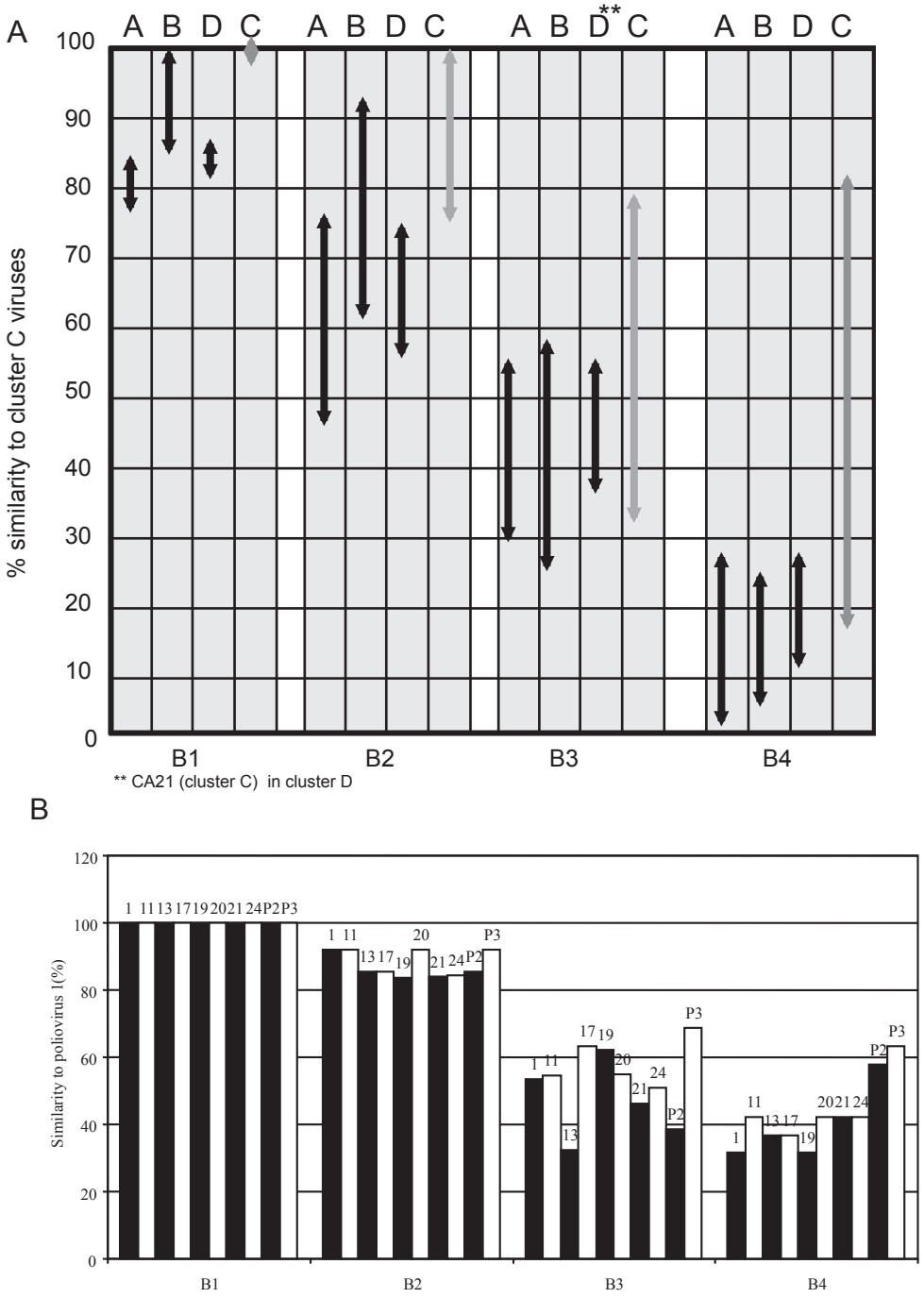

Fig. 2: Percentages of similarity in the B-cell epitopes listed in Table 2 between viruses belonging to different enterovirus clusters (A, B, D) and cluster C enteroviruses. A: Cluster A, B and D viruses versus cluster C viruses, including poliovirus. Grey arrows indicate genetic diversity within cluster C. B: Cluster C viruses versus poliovirus type 1 (Mahoney, V01148.1). Coxsackievirus A serotypes and poliovirus (P) serotypes are presented above bars.

Isolation and typing of enteroviruses in the Netherlands

On average 1870 samples were tested annually for presence of enterovirus as part of primary diagnostics at the RIVM (1959-1989) and 9800 samples as part of national surveillance activities (1996-2009). Of these ~13% (1959-1989) and 7.8% (1996-2009) tested positive. Looking at the overall picture of typing data (Fig. 3, poliovirus not included), the majority of the non-polio enterovirus infections reported in both study periods were caused by cluster B viruses. Low isolation rates were observed for cluster A, cluster C and in particular for cluster D viruses (only 3 EV70 isolates during the complete study period). Isolation rates could not be calculated for the years 1962, '63, '64 and '78 as the total numbers of tested samples were unkown or enterovirus positive samples were not further typed for practical reasons.

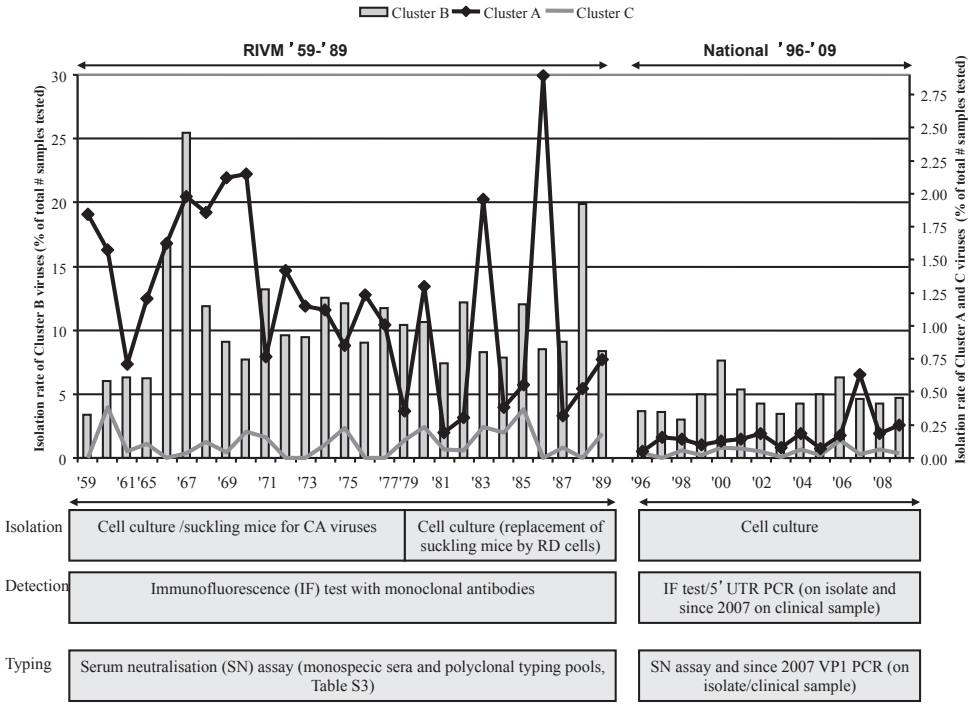

Fig. 3: Isolation of cluster A, B and C viruses as part of enterovirus diagnostics at the RIVM ('59-'89) and national surveillance activities ('96-'09). Isolation frequencies (percentage of total number of samples tested) of cluster B viruses (bars) are plotted on left y-axis. Isolation frequencies of cluster A and C viruses (lines), excluding poliovirus, are plotted on right y-axis. Detection and typing methods used throughout the study period are depicted beneath the graph. Abbreviations: CA, Coxsackie A virus; RD, Rhabdomyosarcoma.

Considering changes in isolation frequency of serotypes defined to be closely related to poliovirus (previous section) that could not be ascribed to changes in detection and typing methods (depicted in Fig. 3), cluster B viruses were isolated at relatively lower

frequencies in the first years ('59 to '65/'66) of the study period than in following years (Fig. 3). Serotype specific isolation data showed that this was the case for several CB and ECHO virus serotypes (presented in Fig 4 a and b and supplementary Fig. S2-3). Of these viruses, ECHO25 showed a discrepancy in ratio of the average annual isolation rate in both study periods (national/RIVM≥1, Fig. S1-4, calculated as rates in both periods cannot be directly compared), suggesting that the isolation frequency of this virus has increased in the second study period compared to the first period.

Focusing on cluster C viruses expected to be antigenically most closely related to poliovirus, CA11, CA17 and CA20 have not been observed at all or sporadically in the complete study period despite availability of typing sera (Fig. S4 and Table S3). CA1 has only been observed up to the mid 1970s when suckling mice for isolation of CA viruses were replaced by the for CA1 insensitive RD cell line (Fig. 3) [16]. CA13 has been observed more frequently, but at very low rates (1 or 3 cases per positive year), since 1974 while the typing serum used for identification of these isolates was available since 1959 (Fig. S4). For CA21 and CA24, assumed to be less closely related to poliovirus, a sudden increase in isolation frequency was observed since 1980 and 1983 respectively. For CA24 this coincided with the availability of typing serum against this virus in 1982 (monospecific) and 1984 (HR typing pool). For CA21 the increase was identified using monospecific typing serum available since the 1960s and in later years by the HR typing pool (1984). Looking at the total isolation frequency of cluster C viruses through time (Fig. 3), years with increased reporting of infections (3 to 8) were followed by years with only 1 or no positive cases. Up to 1979, this pattern was shaped by isolation of CA1 and in the following years in particular of CA21 and CA24.

For cluster A viruses, assumed to be less closely related to poliovirus than cluster B and C viruses, periodicity in increased reporting of symptomatic infections was observed as well, but only in the first study period (1959-1989). Up to 1979, when suckling mice were used for isolation of CA viruses, this pattern was shaped by (co-) isolation of CA2, 4-7, 10 and CA16, whereas in the following years peaks in reporting could only be attributed to increased isolation rates of CA16 and in 1986 also of EV71 (EV71 typing sera since 1977). In the second study period, CA16 showed a more endemic pattern of circulation, resolving the pattern of periodical increases.

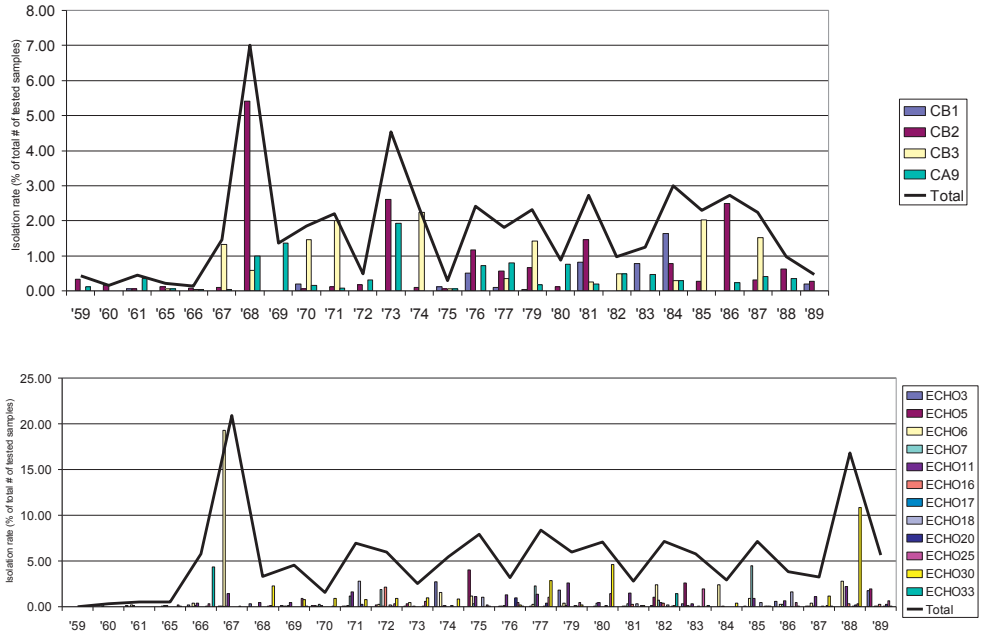

Fig. 4: Isolation of Coxsackie B and ECHO viruses as part of enterovirus diagnostics at the RIVM (1959-1989). Figure presents a selection of serotypes of which isolation rates (percentage of total number of samples tested) were lower in the first years of the study period than in following years. Abbreviations: CB, Coxsackie B virus.

Discussion

This study provides an extensive overview of enterovirus typing data collected as part of routine enterovirus diagnostics in the Netherlands from the late 1950s until recent years. As the Netherlands have consistently used IPV for routine vaccination since 1957, we used this dataset to find clues on the potential consequences of eradicating poliovirus and the associated waning mucosal immunity on prevalence of other enteroviruses.

In this study, cluster C Coxsackie A viruses were defined to be immunologically most closely related to poliovirus and, if cross-protection does exist, are thus expected to be most susceptible for poliovirus immunity. Remarkably, it are also these viruses that have been observed at very low frequencies not only in hospital based settings (this study and [17-20]), but also in the environment of IPV and (previously) OPV countries [21-26]. Additionally, surveillance data of the current study suggested an increase in prevalence through time for some of these viruses (CA13 and CA21). The

observation of periodical increases in number of cluster C infections (this study), suggesting that a new cohort of susceptible individuals is needed for a new elevation, may be an indication for existence of cross-protective immunity among cluster C viruses. This observation, however, is based on very small numbers of isolates and replacement of suckling mice by the RD cell line for isolation of CA viruses (1979) influenced collection of CA serotypes. In the literature databases no indications for cross-reactivity among cluster C viruses other than a high prevalence of cluster C CA viruses in stools from healthy children living in Madagascar, a country with a relatively low poliovirus immunization coverage [27], can currently be found. Studies on heterotypic immune responses among cluster C viruses will be needed to verify or exclude a possible correlation.

Regarding viruses belonging to other enterovirus clusters, cluster B viruses are expected to be immunologically closely related to poliovirus. The increase in isolation frequency of several cluster B serotypes, nine years after the last nationwide polio outbreak, could well be a consequence of waning mucosal immunity against poliovirus. This is not supported by the common reporting of symptomatic CB and ECHO infections, including serotypes for which we observed changes, as part of surveillance activities in (previously) OPV countries and thus in presence of mucosal immunity [18-20]. It is unkown, however, whether the relative isolation frequencies of cluster B viruses in OPV countries (expressed as percentage of total number of samples tested) are similar to or differ from that in IPV countries. This information would be of great value in the search for clues on consequences of poliovirus eradication.

Cluster A and D viruses are expected to be less closely related to poliovirus than cluster B and C viruses. In line with this, cluster A viruses have been reported to be the most prevalent enteroviruses (> cluster B) in the environment of OPV and IPV countries in recent years but also in years just before or after introduction of poliovirus vaccination (1950s/60s) [21-26]. The drop in isolation frequency of certain cluster A CA viruses in the Netherlands since 1979, following replacement of suckling mice by the RD cell line, suggests that prevalence of these viruses in the hospital based setting has been underestimated for years. In line with this is the slight increase in isolation rates of these viruses in recent years following introduction of a detection and typing PCR directly on clinical samples (Fig. S1).
Enteroviruses of cluster D were rarely reported [18, 19]. An influence of poliovirus immunity on prevalence of these viruses is not obvious regarding the percentage of similarity to poliovirus in B-cell and T-cell epitopes and data on heterotypic binding of antibodies (Table 2).

The approach used in this study to define enteroviruses closely related to poliovirus has some limitations. We studied homology in complete T-cell epitopes, whereas T-cell receptors recognize only 2 or 3 amino acids of the epitopes that project out of the MHC groove [28]. Serotypes that are immunologically closely related to poliovirus but that have a low percentage of similarity to poliovirus in the entire T-cell epitope, might therefore have been wrongly classified as distantly related. Except for cluster B and C viruses, however, the surveillance data showed no increases in prevalence of specific serotypes that could not be ascribed to documented changes in detection and typing methods. Missing knowledge on immunodominance and the cross-reactive nature of B- and T-cell epitopes among poliovirus and viruses of cluster A, C and D formed another difficulty in the selection of viruses immunologically most closely related to poliovirus.

Published data on heterotypic immune responses originated from *in vitro* studies on heterotypic binding of antibodies among enteroviruses and *in vitro* T-cell proliferation assays [29-31]. It is not clear, however, to what extend these data represent cross-protection in natural infection. Regarding humoral immunity, of which the importance in protection is demonstrated by chronic and life-threatening enterovirus infections in agammaglobulinemic patients, Yagi et al (1992) and Jeon et al (2007) demonstrated that antibodies with cross-binding capacities could not neutralize viruses of other serotypes. This is in line with what is observed in standard neutralisation assays used for typing of enteroviruses (low heterotypic titers: 10-40) and most of the studies on heterologous protection of systemic humoral immunity in humans and animal models: systemic humoral protection appears to be serotype specific [29, 32, 33]. Wu and colleagues (2007) and Schmidt et al. (1967) do report on heterotypic antibody reponses in mice and rhesus monkeys experimentally infected with enteroviruses (CA16 vs EV71 and CB vs ECHO) [34, 35]. These responses, however, were very weak (titer of 1:4) and cross-neutralizing antibodies persisted only shortly, making a cross-protective role of these antibodies in natural infection disputable.

Heterotypic T-cell protection, on the other hand, has been demonstrated in several mice experiments (for instance for Influenza, vaccinia virus, CA16 and EV71) [28, 32, 36, 37]. This type of immunity seemed not to prevent infection and associated disease outcome, but aided in recovery by accelerating virus clearance. There is no direct evidence for existence of this kind of protection in humans, but the observation that severity of illness in individuals infected with H3N2 and H1N1 Influenza viruses during sequential or concurrent epidemics was less during the second infection might be a clue for this [38, 39]. An accelerated clearance of cluster C Coxsackie A viruses by poliovirus vaccine induced heterotypic immunity and a potentially associated reduction in transmission could explain why these viruses circulate at very low rates in the environment.

Using 50 years of enterovirus surveillance data collected in the Netherlands, we studied possible consequences of eradicating poliovirus on prevalence of non-polio enteroviruses. Cluster B and cluster C viruses were defined to be antigenically most closely related to poliovirus. Interestingly, it are also these viruses with a persistently low prevalence (cluster C viruses) and/or for which an increase in prevalence was observed (cluster C and B viruses) that could not be attributed to changes in isolation and typing methods. Extended studies on the role of heterotypic immune-protection in human enterovirus infections and disease outcome will be needed to find out whether these trends are somehow related to poliovirus immunity and whether we thus can expect global changes in prevalence of these viruses or in severity of infection-associated disease in the post-OPV era.

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Supplementary Material Chapter 6

Table S1: Search strategy used for literature search on enterovirus (heterotypic) immune-responses, B- and T-cell epitopes.

Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process			
#	**Searches**	**Results**	**Search Type**
1	(enterovirus* or enteroviral).ti. or *enterovirus A, human/ or exp *enterovirus B, human/ or *enterovirus C, human/ or *enterovirus D, human/	5522	Advanced
2	(enterovirus, bovine or enterovirus, porcine).sh.	23	Advanced
3	1 not 2	5506	Advanced
4	(epitop* or ctl* or t cell* or t lymphocyte* or th1 or th2 or b cell* or b-lymphocyt* or immune response* or immunological response* or immunity or immunodominant* or proliferative response).ti.	218305	Advanced
5	(antigenic site* or antigenicity or antigen or antigens or antisera or peripheral blood mononuclear cells or pbmc or neutralization or cross-reactivity or cross reaction*).ti.	148706	Advanced
6	(antibodies or antibody or immunoglobulin* or peptide* or seroepidemiolog*).ti.	316691	Advanced
7	3 and (4 or 5 or 6)	592	Advanced
8	*antigens, viral/ or *antigens, cd4/ or *antigens, cd8/ or *immunity, cellular/ or *lymphocyte activation/ or (exp *histocompatibility antigens/ or exp *t-lymphocytes/)	226392	Advanced
9	*antibodies, viral/ or *antibodies, monoclonal/ or *antibody specificity/ or exp *b-lymphocytes/	128630	Advanced
10	exp *epitopes/ or *epitope mapping/ or *neutralization tests/ or *cross reactions/ or *peptide fragments/	70040	Advanced
11	3 and (8 or 9 or 10)	531	Advanced
12	7 or 11	753	Advanced
13	(swine or bovine or porcine).ti. or exp poliovirus vaccines/	107560	Advanced
14	12 not 13	689	Advanced
15	14 and (english or german or dutch).lg.	599	Advanced
16	15 not (letter or editorial or news).pt.	578	Advanced

Table S2: Genbank reference strains used for clustal analysis

Serotype	Acc. number	Serotype	Acc. number
CA2	AY421760	ECHO15	AAQ73078.1
CA3	AY421761	ECHO16	AAQ73079.1
CA4	AY421762	ECHO17	AAQ73080.1
CA5	AY421763	ECHO18	AAL37163.1
CA6	AY421764	ECHO19	AAQ73081.1
CA7	AY421765	ECHO20	AAQ73083.1
CA8	AY421766	ECHO21	AAQ73084.1
CA10	AY421767	ECHO24	AAQ73085.1
CA12	AY421768	ECHO25	CAA62259.2
CA14	AY421769	ECHO25	AAQ73086.1
CA16	U05876	ECHO26	AAQ73087.1
EV71	AAR32993.1	ECHO27	AAQ73088.1
EV71	AF176044_1	ECHO29	AAQ73089.1
CB1	AAC00531.1	ECHO30	AAL37155.1
CB2	Q9YLG5.3	ECHO31	AAQ73091.1
CB3	P03313.4	ECHO32	AAQ73092.1
CB4	P08292.4e	ECHO33	AAQ73093.1
CB4	AAB33885.1	P1	CAA24461.1
CB5	Q03053.3	P2	AAN85443.1
CB6	Q9QL88.4	P3	AAN85444.1
CA9	P21404	CA1	AAQ02675.1
ECHO1	O91734.4	CA11	ABM21503.1
ECHO2	AAQ04841.1	CA13	AAQ04834.1
ECHO3	AAQ73090.1	CA17	CAV31568.1
ECHO4	AAQ73094.1	CA19	AAQ02681.1
ECHO6	AAA65044.1	CA20	ABC84488.1
ECHO7	AAK85711.1	CA21	AAQ02684.1
ECHO9	AAO48739.1	CA22	ABM21517.1
ECHO11	AAL39108.2	CA24	ABM54551.1
ECHO12	S44251	EV70	D00820
ECHO13	AAQ73076.1	EV68	AY426531
ECHO14	AAQ73077.1		

Table S3: Antisera (monospecific or in pools) used for typing of common enteroviruses in neutralisation assays. Years in which sera became available are indicated.

Serotype	Mono-specific	AG pool ('68)	HR pool ('84)	Serotype	Mono-specific	AG pool ('68)	HR pool ('84)
CA1	>1959			ECHO2	>1958	x	
CA2	>1959		x	ECHO3	>1958	x	
CA3	>1959		x	ECHO4	>1960	x	
CA4	>1959		x	ECHO5	>1958	x	
CA5	>1959		x	ECHO6	>1961	x	
CA6	>1959		x	ECHO7	>1959	x	
CA7	>1959		x	ECHO8	>1959		
CA8	>1959			ECHO9	>1960	x	
CA9	>1959	x		ECHO11	>1959	x	
CA10	>1959		x	ECHO12	>1958	x	
CA11	>1959		x	ECHO13	>1958	x	
CA13	>1959		x	ECHO14	>1960	x	
CA14	>1959		x	ECHO15	>1961		x
CA16	>1959		x	ECHO16	>1959		x
CA17	>1959		x	ECHO17	>1959		x
CA19	>1959			ECHO18	>1960		x
CA20	>1979			ECHO19	>1960		x
CA21	>1962		x	ECHO20	>1959	x	
CA22				ECHO21	>1963	x	x
CA24	>1982		x	ECHO24	>1961		x
EV71	>1977		x	ECHO25	>1961	x	
EV 86				ECHO26	>1961		x
CB1	>1960	x		ECHO27	>1961	x	
CB2	>1959	x		ECHO29	>1964	x	
CB3	>1959	x		ECHO30	>1965	x	
CB4	>1959	x		ECHO31	>1965		x
CB5	>1959	x		ECHO32	>1966		x
CB6	>1961	x		ECHO33	>1966	x	x
EV70	>1974		x	ECHO34	>1969		
ECHO 1	> 1961	x					

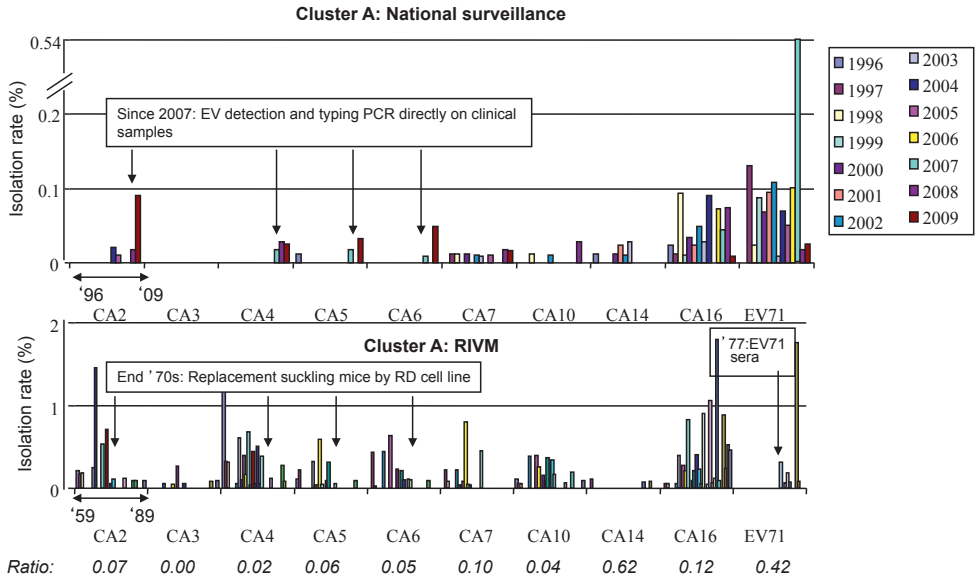

Fig. S1: Annual isolation rates (% of total number of tested samples) of cluster A viruses in 1959-1989 (RIVM, lower panel) and in 1996-2009 (national surveillance, upper panel). For each serotype the average annual isolation rate was calculated for both study periods. The ratios of these rates (1996-2009 : 1959-1989) are presented below the figure. Abbreviations: CA, Coxsackie A; EV, Enterovirus; RD, Rhabdomyosarcoma.

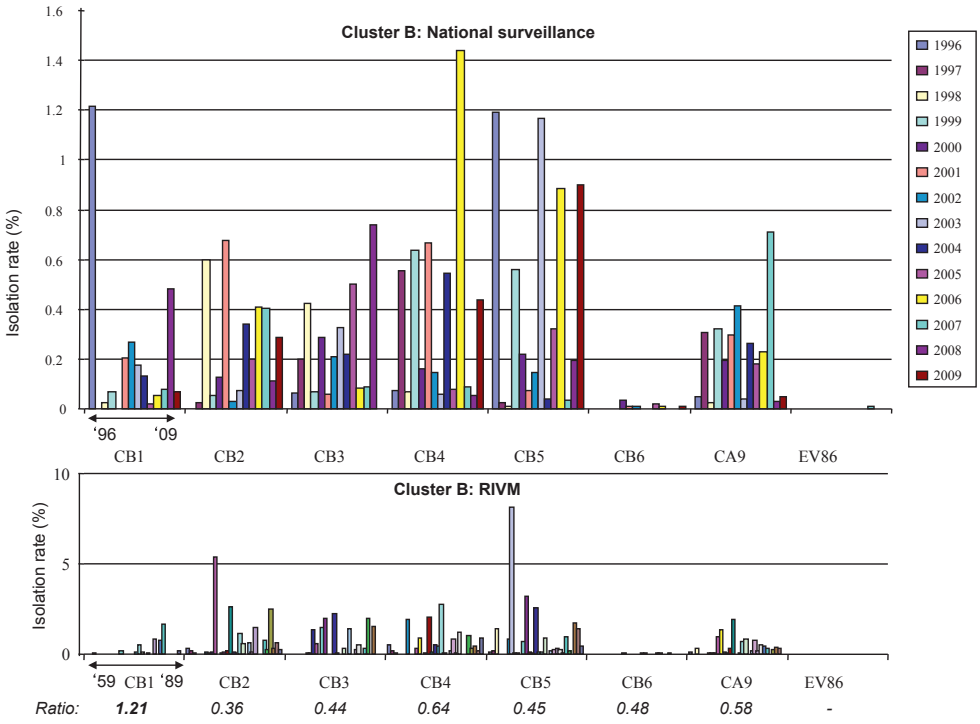

Fig. S2: Annual isolation rates (% of total number of tested samples) of cluster B viruses (CB1-6, CA9 and EV86) in 1959-1989 (RIVM, lower panel) and in 1996-2009 (national surveillance, upper panel). For each serotype the average annual isolation rate was calculated for both study periods. The ratios of these rates (1996-2009 : 1959-1989) are presented below the figure. Abbreviations: CB, Coxsackie B; CA, Coxsackie A; EV, enterovirus.

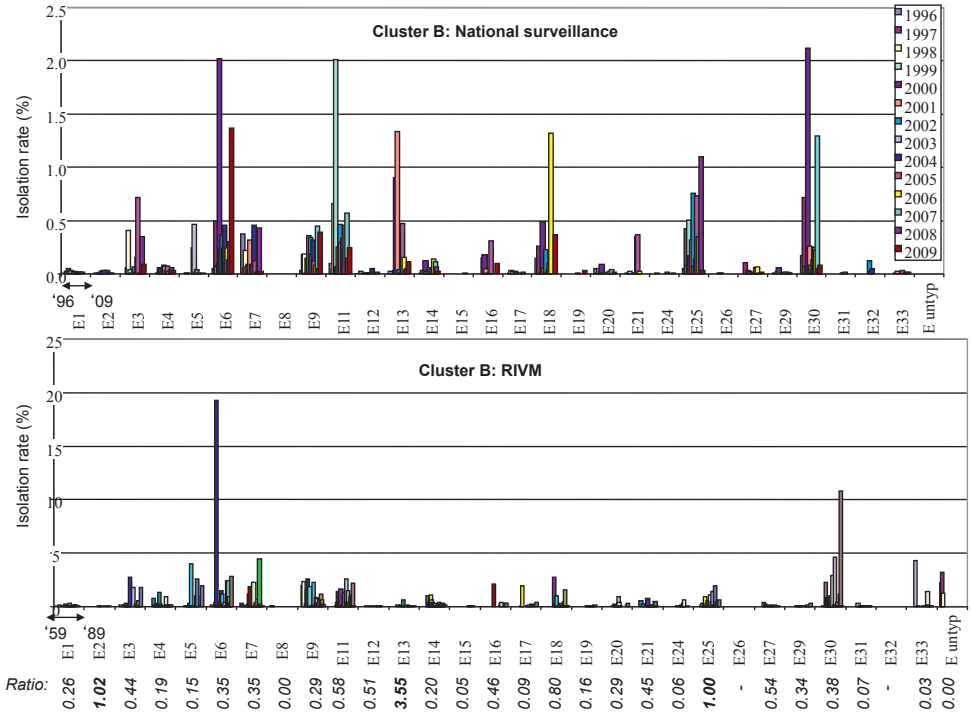

Fig. S3: Annual isolation rates (% of total number of tested samples) of cluster B viruses (ECHO) in 1959-1989 (RIVM, lower panel) and in 1996-2009 (national surveillance, upper panel). For each serotype the average annual isolation rate was calculated for both study periods. The ratios of these rates (1996-2009 : 1959-1989) are presented below the figure. Abbreviations: E, ECHO virus; Untyp, untyped ECHO virus isolates.

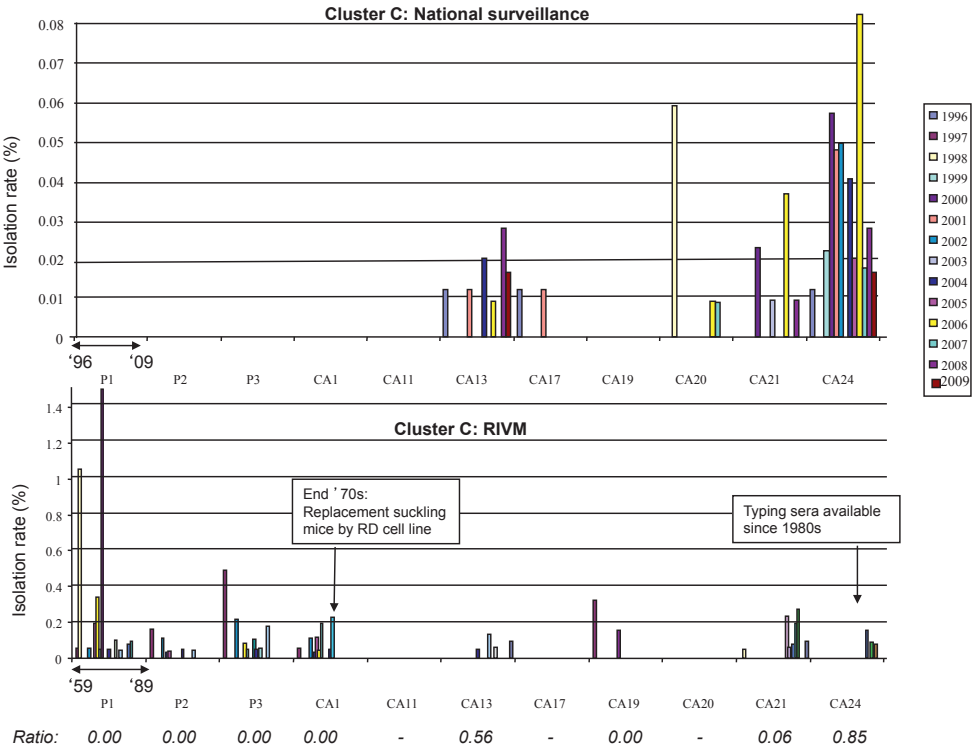

Fig. S4: Annual isolation rates (% of total number of tested samples) of cluster C viruses in 1959-1989 (RIVM, lower panel) and in 1996-2009 (national surveillance, upper panel). For each serotype the average annual isolation rate was calculated for both study periods. The ratios of these rates (1996-2009 : 1959-1989) are presented below the figure. Abbreviations: CA, Coxsackie A; P, Poliovirus; RD, Rhabdomyosarcoma.

Chapter 7

Prevalence of Human Parechovirus in The Netherlands in 2000 to 2007

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Abstract

Infection with human parechovirus 3 (HPeV3) was described for the first time in Japan in 2004 and reportedly is more often associated with severe disease than infection with HPeV1 or HPeV2. In 2004, infections with HPeV3 were observed for the first time in The Netherlands. Genetic analysis showed several different lineages, suggesting endemic circulation. We analyzed 163 cell culture isolates from the same number of patients tested in routine virological laboratories as part of the national enterovirus surveillance program. Isolates were collected between 2000 and 2007 and could not be characterized by routine methods. In total, 155 isolates (95%) were found positive for HPeV by a reverse transcription-PCR assay targeting the 5' untranslated region, explaining the majority of the diagnostic deficit in enterovirus surveillance for these years. Typing of the isolates by use of partial genome sequencing of the VP1/2A region revealed the presence of 55 HPeV1, 2 HPeV2, 89 HPeV3, 1 HPeV4, and 8 HPeV5 isolates. We compared isolation dates, age groups affected, and clinical pictures, which were reported as part of the routine surveillance. Clear differences in epidemiology were observed, with HPeV3 occurring at intervals of 2 years and in the spring-summer season, whereas HPeV1 was observed in small numbers throughout each year, with a low in the summer months. HPeV3 infection affected younger children than HPeV1 infection and was significantly more often associated with fever, meningitis, and viremia.

Introduction

Human parechovirus 1 (HPeV1) and HPeV2, initially known as echovirus 22 (EV22) and EV23, were first isolated in the summer of 1956 in Japan from stool specimens of children suffering from diarrhea (9). From that time on, HPeV infections have been detected all over the world. Common symptoms caused by these viruses are gastroenteritis, respiratory infections, and, in a smaller proportion of cases (usually in children <1 year old), encephalitis and flaccid paralysis (12, 20). Based on these disease symptoms and viral physicochemical properties (stability at a pH of <3 and in organic solvents), EV22 and -23 were initially grouped within the genus *Enterovirus* of the family *Picornaviridae* (18, 19). Subsequent molecular characterization showed that the viruses were quite distant from the enteroviruses: EV22 and EV23 showed no more than 30% amino acid identity with other picornaviruses, and in contrast to most other human viruses in this family, they do not shut down the host cell protein translation machinery (9, 18). For these reasons, EV22 and -23 were grouped into a new genus of the *Picornaviridae*, *Parechovirus*, and were renamed HPeV1 and HPeV2. With this new genus, nine picornavirus genera are known at present: *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, and *Parechovirus* (19).

In 2004 a new serotype of parechovirus (HPeV3), which reportedly was associated with more-severe disease than HPeV serotype 1 and 2 viruses (4, 5, 10), was described in Japan. In 2006 a fourth HPeV serotype was isolated from a neonate with fever (3). This isolate could not be neutralized by antibodies against serotypes 1 to 3. Together with a recently reported close relative (T92-15), HPeV2 CT86-6760 has been reclassified as a fifth genotype (HPeV5), since it is genetically distinct from the HPeV2 isolates (1). In 2007 a sixth serotype was reported, which could not be neutralized by antibodies against serotypes 1, 2, and 3 and which had an amino acid sequence distinct from those of types 1 to 5 (22). Recently a second HPeV6 isolate was recovered, which seemed to be a recombinant of HPeV6, -5, and -1 (2). Infection with HPeV5 and HPeV6 in The Netherlands was described for the first time in December 2007 (7).

In the summer of 2004, HPeV3 was observed for the first time in The Netherlands (Arnhem). An outbreak was suspected when 14 children (aged 2 to 8 weeks) were admitted to the same hospital within a short time with clinical manifestations ranging from aseptic meningitis to a sepsis-like syndrome (21). Genetic analysis of the viral isolates, however, revealed that the infections were caused by several different lineages of HPeV3, which suggested endemic circulation of HPeV3 in The Netherlands (see Fig. 3). This observation triggered the present study on the prevalence and genetic diversity of parechoviruses in The Netherlands.

Materials and Methods

Clinical samples

Primary diagnosis of enterovirus infections in The Netherlands is performed by 21 virological laboratories, which participate in the Weekly Sentinel Surveillance System of the Dutch Working Group on Clinical Virology. Fecal samples and throat swabs from children with systemic viral infections, with manifestations ranging from meningitis to gastrointestinal disorders, are cultured on combinations of enterovirus-sensitive cell lines: RD, tertiary monkey kidney (tMK), LLC-MK2, Vero, HEp-2, and various human fibroblast cell lines. Viral isolates with a cytopathic effect (CPE) characteristic of enteroviruses are confirmed as enteroviruses by a specific PCR assay (15, 17) or by an immunofluorescence test with broadly reactive monoclonal antibodies (Dako, CA). Cerebrospinal fluid samples are usually tested directly (without cell culture) by an enterovirus PCR assay for the presence of enteroviruses. Enterovirus PCR-positive isolates are typed by 15 of 21 virological laboratories by use of serum neutralization tests with polyclonal typing pools (provided by the National Institute for Public Health and the Environment [RIVM], Bilthoven, The Netherlands) (23). The RIVM receives a subset of all cell culture isolates from various types of specimens and from patients with different types of enterovirus infection. Isolates submitted to the RIVM either were negative by the locally performed enterovirus PCR or immunofluorescence test or were not typed by the local laboratory. Isolates that are typed at the local level are not sent to the RIVM. Positive diagnostic results are reported through the Weekly Sentinel Surveillance System of the Dutch Working Group on Clinical Virology.

Characterization of isolates for enterovirus surveillance

Isolates submitted to our institute (RIVM) are routinely cultured on transgenic cells with the human poliovirus receptor (L20B) to exclude the presence of poliovirus in the isolates (24). In addition, the isolates are cultured on a cell line most similar to the cell line used by the supplying laboratory and are tested by an enterovirus PCR assay (15, 17). From 2000 to 2007, a total of 1,344 cell culture isolates were submitted to the RIVM, of which 1,023 isolates (76%) were confirmed as enterovirus positive by an enterovirus PCR assay and 248 isolates (19%) were either nontypeable or not typed. The remaining 73 isolates either were negative by cell culture at the RIVM or were proven to be positive for adenovirus, reovirus, or rhinovirus. Background data on the age of each patient, the year and month of isolation, and clinical symptoms were extracted from the enterovirus surveillance database at the RIVM.

Criteria for inclusion in the study

Human parechoviruses are reported to grow selectively on cell cultures of simian origin (Vero, tMK, or LLC-MK2), with a CPE resembling that of enteroviruses, but to test negative by the enterovirus PCR assay (3, 7). By using these criteria for screening of the biobank of the 248 nontyped and nontypeable isolates, 163 cell culture isolates from individual patients were selected. These isolates were screened for the presence of HPeV by an in-house HPeV confirmatory PCR assay.

RNA extraction

Viral RNA was extracted from cell cultures using the MagNA Pure LC total-nucleic-acid isolation kit with a MagNA Pure LC instrument (Roche Diagnostics, Almere, The Netherlands). External lysis was done by adding 200 μl cell culture to 300 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.

HPeV confirmatory PCR assay

Primers were designed on the basis of a genome alignment of the reference strains HPeV1 Harris, HPeV2 Gregory, HPeV3 A308/99, HPeV3 Can82853-01, and HPeV5 CT86-6760 by using the BioEdit sequence alignment editor, version 7.0.0 (8). For the detection of HPeV, forward primer PE5F (5'-CCACGCTYGTGGAYCTTATG-3') and reverse primer PE5R (5'-GGCCTTACAACTAGTGTTTGC-3') were designed, targeting the 5' untranslated region (5'UTR) (nucleotides 292 to 553). For serotype classification, a PCR assay was designed using forward primer PEVF (5'-CAGCIG-GTGARCAGATGAC-3') and reverse primer PEVR (5'-ATCTAATTCACACTCTT-CYTC-3'), targeting the VP1/2A region (nucleotides 2790 to 3238). In case PCR amplification with this primer set failed, primers Sab5F (5'-CTTCAGCTCAAGAT-GATGG-3') and Sab5BR (5'-GAAACCTCTATCTAAATAWG-3') were used. Reverse transcriptions were performed to convert the viral RNA to cDNA. For reverse transcription, 2.5 μl of the isolated viral RNA was incubated at 94°C for 2 min together with 1.0 μl of 50 μM antisense primer and 5.5 μl H_2O. The samples were subsequently chilled on ice for 2 min and incubated at 42°C for 60 min together with 1.5 μl of 10x PCR buffer (pH 8.3) (100 mM Tris-HCl, 500 mM KCl), 1.8 μl of 25 mM $MgCl_2$, 1.5 μl of 10 mM deoxynucleoside triphosphates, 0.5 μl of 10-U/μl avian myeloblastosis virus reverse transcriptase (Promega, Leiden, The Netherlands), and 0.7 μl of H_2O. The reaction was finished by incubation of the samples at 94°C for 2 min, followed by incubation on ice.

PCR amplification of the 5' UTR and VP1/2A region was performed on a LightCycler instrument. The 20-μl PCR mixture, containing 2 μl of cDNA from the reverse

transcription (RT) reaction, 10 pmol of each primer, 4.5 mM $MgCl_2$, 0.16 μl TaqStart antibody solution (BD Biosciences, Breda, The Netherlands), and 2 μl LightCycler DNA Master Sybr green I (Roche Diagnostics, Almere, The Netherlands), was added to a capillary tube and loaded into the LightCycler instrument. The 5' UTR was amplified in the presence of 2 μl of 5x Q-solution (Qiagen, Venlo, The Netherlands). Samples were denatured for 1 min at 95°C and subjected to 45 cycles at 96°C for 3 s, 40°C for 4 s, and 72°C for 15 s. Amplicon size analysis was performed using gel electrophoresis. The PCR products were purified according to the manufacturer's protocol for the QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands). Positive samples were typed by sequencing of the VP1/2A PCR product. The RT-PCR products were sequenced with the ABI Prism BigDye terminator cycle sequencing ready reaction kit, version 3.2 (Applied Biosystems, Foster City, CA) on an automated sequencer (model 3700; Applied Biosystems) using primer PEVF. Sequence data were edited using SeqMan software (DNAStar Inc., Madison, WI). The strains were genetically classified by using Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

Validation of the HPeV confirmatory PCR assay

To evaluate the specificity of the HPeV confirmatory PCR assay, 86 cell culture isolates that tested positive by the enterovirus PCR assay were included in this test. To determine the detection limit of the test, RNA was extracted from 10^0 to 10^{-8} dilutions of HPeV1 Harris-positive cell culture supernatants ($10^{6,6}$ 50% tissue culture infective doses [$TCID_{50}$]/ml) and HPeV2 Gregory-positive cell culture supernatants ($10^{6,2}$ $TCID_{50}$/ml) by using the MagNA Pure LC total-nucleic-acid isolation kit (Roche Diagnostics, Almere, The Netherlands) and was amplified by the RT-PCR assay. HPeV-positive isolates were typed by sequencing of the VP1/2A region. To test the sensitivity of this PCR assay, 15 HPeV1 isolates cultured between 1960 and 1967 were included in the test. These isolates had previously been characterized as HPeV1 by a serum neutralization assay.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB443710 to AB443864.

Results

Validation of the HPeV confirmatory PCR assay

To evaluate the specificity of the HPeV confirmatory PCR assay, 86 enterovirus-positive isolates were included in this test. In total, 84 of these 86 isolates (98%) did

not react in our HPeV PCR assay. Two isolates had a positive result. The presence of HPeV in these cell culture isolates was confirmed by sequencing of the VP1/2A region, indicating mixed infections of enterovirus (EV18 and coxsackievirus A20) and HPeV. To determine the detection limit of the confirmation test RNA was extracted from 10^{0} to 10^{-8} dilutions of HPeV1 Harris cell supernatants ($10^{6,6}$ $TCID_{50}$/ml) and HPeV2 Gregory cell supernatants ($10^{6,2}$ $TCID_{50}$/ml) and was amplified by the RT PCR assay. For HPeV1 Harris, PCR-positive signals could be observed for dilutions from 10^{0} to 10^{-2}, representing ≥53 infectious virus particles in the reaction mixture. For HPeV2 Gregory, dilutions of 10^{0} to 10^{-1} gave PCR-positive results, representing ≥210 infectious virus particles per reaction mixture.

Identification of HpeV

In total, 163 unique patient isolates collected during enterovirus surveillance from 2000 to 2007 fit the selection criteria for the study in that they showed CPE only on cells of simian origin (Vero, tMK, and LLc-MK2) and tested negative for enteroviruses by PCR. The 5' UTR PCR assay found 155 isolates (95%) to be HPeV, while 8 isolates (5%) were negative by both the enterovirus PCR assay and the HPeV PCR assay.
The 155 HPeV-positive isolates and the 2 isolates positive by both the enterovirus PCR assay and the HPeV PCR assay were further characterized on the basis of the VP1/2A sequence data. For reference, 15 HPeV1 isolates from our historic collection (1960 to 1967) were tested; all of them gave positive reactions. On the basis of similarity comparisons, 55 of the 155 isolates (35%) were characterized as HPeV1-like, 2 (1.3%) as HPeV2-like, 89 (57%) as HPeV3-like, 1 (0.6%) as HPeV4-like, and 8 (5%) as HPeV5-like. The HPeV4 isolate was the isolate that has been reported by Benschop et al. in 2006 (3). The two samples from cases of mixed infections with HPeV and enterovirus contained HPeV3-like isolates.

Seasonality of HpeV

The month of sampling was known for 54 HPeV1 and 84 HPeV3 isolates. HPeV3 was isolated mainly during the spring and summer seasons, with a peak in July and August. This is in contrast with the seasonal distribution of HPeV1. A low level of HPeV1 circulation was observed throughout the year, with a low in the summer months (Fig. 1). For HPeV2, HPeV4, and HPeV5 isolates, no seasonal distribution could be described, possibly because of the low numbers.

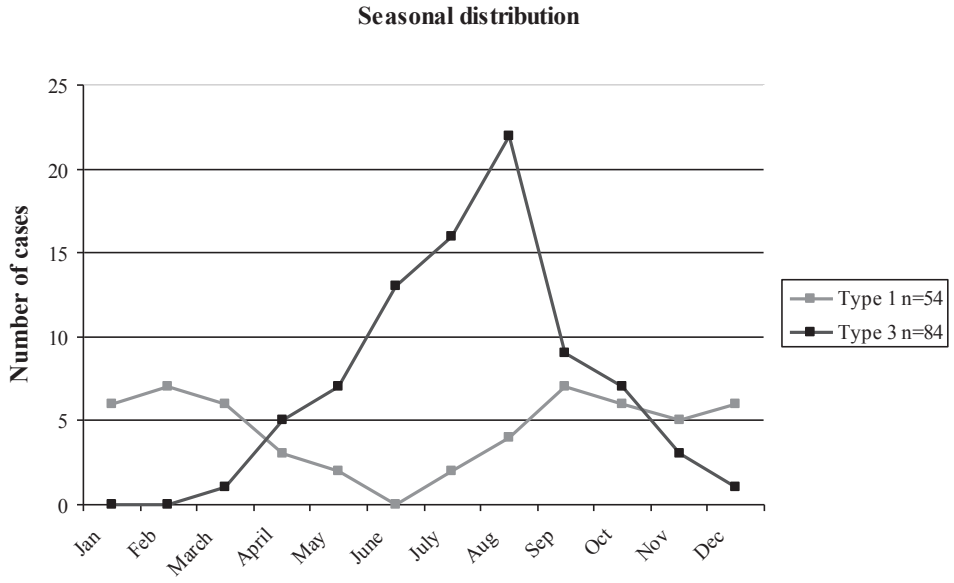


Fig. 1: Seasonal distribution of HPeV1 and HPeV3 isolated by routine virological laboratories in The Netherlands, 2000–2007.

A periodicity could be seen in the occurrence of HPeV3 during the years 2000 to 2007. HPeV3 infections with more than 20 cases occurred at intervals of 2 years (Fig. 2A). In 2006 a decrease in the number of HPeV3 isolates (n = 11) in the RIVM collection was observed as a result of the implementation of HPeV3 diagnostics in routine laboratories (Fig. 2B). The 14 cases in 2004 (Fig. 2B) represent the cases in Arnhem where HPeV3 infections were observed for the first time in The Netherlands.

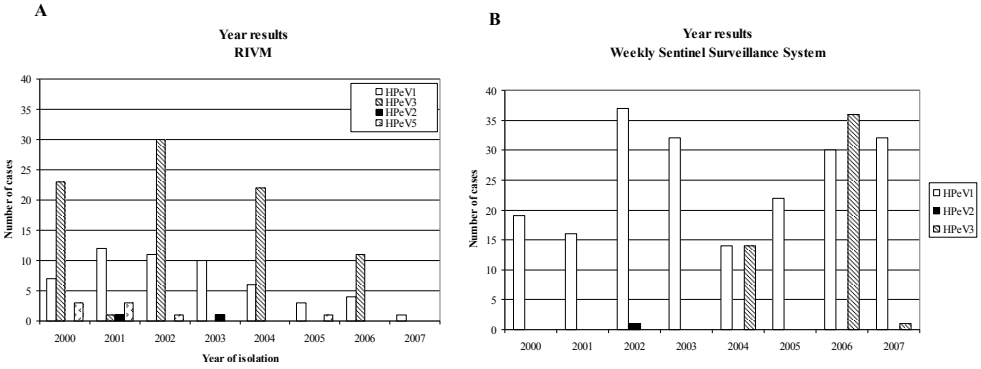


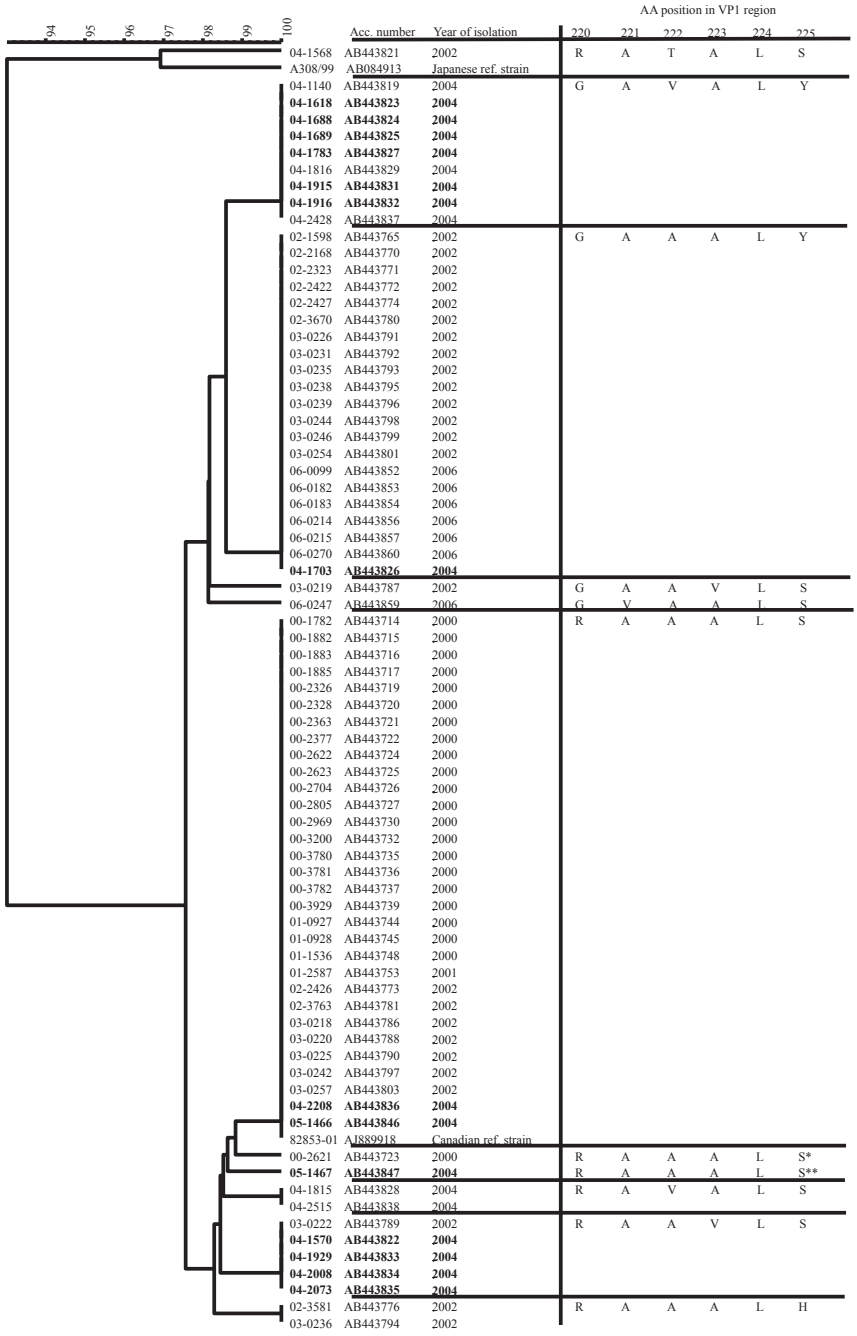
Fig. 2: Distribution of HPeV during 2000 to 2007. (A) HPeV isolated at the RIVM; (B) HPeV reported by 21 diagnostic laboratories participating in the Weekly Sentinel System of the Dutch Working Group for Clinical Virology.

Clustering of the viral isolates on the basis of the VP1/2A sequence data showed that the seasonal peaks (at 2-year intervals) were caused by viruses belonging to more than one lineage (two to six lineages per season), although a major variant was found in each season (Fig. 3). The viruses isolated from the patients in 2004 belonged to four lineages. In 2006 the majority of the cases were caused by an HPeV3 lineage that also was common in 2002. All HPeV3 isolates, except for one in 2002, were most closely related to the Canadian HPeV3 reference strain CAN82853-01. Variability between these different lineages was observed in the C-terminal region of VP1 in the surface-exposed loop (amino acid positions 219 to 225 in VP1). Only two isolates (00-2621 and 05-1467) showed an additional substitution in the VP1/2A region (residues 184 and 191 of VP1, respectively). Only one isolate was most closely related to the Japanese HPeV3 reference strain A308/99.

Typing of HPeV1 and HPeV2 by serum neutralization tests with pooled or monotypic antisera has been performed routinely by 15 of 21 laboratories since the early 1990s. The HPeV1 and -2 isolates detected in this study were isolates that were not typed by other laboratories and were sent to the RIVM to exclude poliovirus infection. The total number of HPeV1 cases in The Netherlands reported by all members of the Weekly Sentinel Surveillance System of the Dutch Working Group on Clinical Virology confirms the annual occurrence of HPeV1 (Fig. 2B). The data available for 2007 indicate isolation of a mean of 3 HPeV isolates per week (32 were typed as HPeV1 and only 1 as HPeV3, confirming the biannual periodicity).

Age

HPeV3 is reported to infect significantly younger children than HPeV1 (4). For 53 cases of HPeV1 infection and 84 cases of HPeV3 infection, the age of the patient was known (Fig. 4). HPeV1 was isolated from children with a mean age of 9.2 months (range, 0 to 35 months). HPeV3 infected children with a mean age of 1.9 months (range, 0 to 15 months). The ratio of male to female patients was 1.12 for HPeV1 and 1.26 for HPeV3.



* Substitution of arginine for lysine at aa position 184 (VP1)

** Substitution of threonine for alanine at aa position 191 (VP1)

Fig. 3: Clustering of the Dutch HPeV3 isolates from 2000 to 2007 and reference strains. The tree is based on a multiple alignment of the amino acid sequences of the VP1/2A region (48 amino acids) by use of cluster analysis with the unweighted-pair group method using average linkages. The isolates associated with the 14 HPeV3 cases in Arnhem in 2004 are boldfaced.

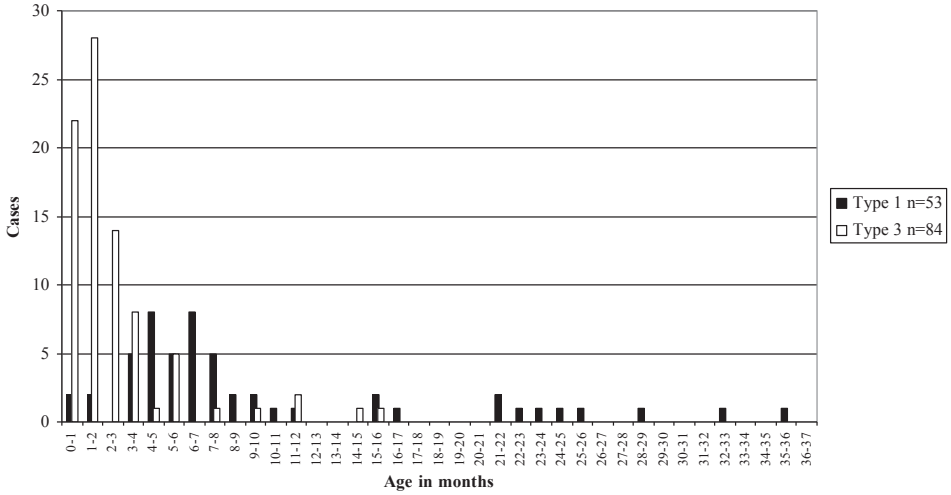


Fig. 4: Ages of patients with HPeV1 or HPeV3 infections.

Clinical symptoms

Clinical symptoms were known for 33 children infected with HPeV1 and 34 children infected with HPeV3 (Table 1). HPeV3 infection was significantly more often associated with fever (P = 0.013), meningitis, and viremia (P = 0.001) than HPeV1 infection. Gastrointestinal symptoms such as diarrhea and vomiting were more often associated with HPeV1 infection (P = 0.006). Respiratory or other symptoms could not be associated with a specific serotype. Gastrointestinal symptoms, fevers, joint aches, and rashes were reported for children infected with HPeV5 (n = 3). No symptoms were documented for the three patients from whom HPeV2 (n = 2) and HPeV4 (n = 1) were isolated.

Table 1: Clinical symptoms of children infected with HPeV1 and HPeV3

Symptom type	No. of children infected with:		P*
	HPeV1 (n=33)	HPeV3 (n=34)	
Gastrointestinal	19	8	0,006
Respiratory	6	2	0,15
Meningitis/viremia	3	16	0,001
Fever	8	19	0,013
Other	6	8	0,765

* Calculated by Fisher's two-tailed test.

Discussion

This study clarifies a large number of cell cultures with unexplained CPE collected during enterovirus surveillance from 2000 to 2007. In total, 163 of these isolates showed CPE only on cells of simian origin and were negative by the enterovirus PCR assay. Testing of these 163 isolates by an HpeV-specific PCR assay revealed the presence of 155 HPeV-positive isolates, explaining the majority of the diagnostic deficit in enterovirus surveillance for these years. Our HPeV confirmatory-PCR assay had a detection limit sufficient for use as a confirmatory test of cell culture isolation. For direct detection of HPeV in clinical samples, it remains to be seen if a more sensitive diagnostic PCR assay is needed, because the concentration of virus in these samples is unknown.

Each year approximately 3.5% of all isolates reported by the 21 diagnostic laboratories as part of the enterovirus surveillance is represented by HPeV1. HPeV3 infections occurred at intervals of 2 years and appeared to represent 3.5% of all infections diagnosed for enterovirus surveillance in these years. These numbers emphasize the prevalence of HPeV3 and HPeV1 in The Netherlands, which thus has been underestimated for many years.

This study clearly shows the difference in seasonal distribution between HPeV1 and HPeV3 that was indicated by previous studies based on lower numbers of HPeV1 and HPeV3 isolates (2, 4). In addition, HPeV3 showed a clear periodicity in causing infections, with seasonal peaks in 2000, 2002, 2004, and 2006. In each of these years, the outbreaks were caused by viruses belonging to more than one lineage, indicating endemic circulation of HPeV3. Analysis of the VP1/2A sequence data showed that divergence between the different lineages occurred in the C-terminal variable loop of VP1, which is expected to be highly immunogenic (5). This suggests antigenic drift. Occurrence of variability in this region, however, can be expected. Variability among picornaviruses is reported to occur mainly in the surface-exposed, flexible loops of the capsid proteins, which connect the highly conserved β-sheets (11). It is not clear why HPeV3 infections occur at intervals of 2 years. The periodicity might be linked to a periodical occurrence of the source of HPeV3, to the minimal size of the susceptible population required for a seasonal epidemic, or to antigenic drift. Given the very young age of patients, antigenic variation may be necessary to escape protection from maternal antibodies. Periodical occurrence seems not to be a feature unique to HPeV3 infections. Serotypes 3, 4, and 5 of coxsackievirus B, diagnosis of which forms part of the national enterovirus surveillance system, are other examples of viruses that appear to show independent periodicity in causing infections (data not shown). It might be of interest to analyze the capsid regions of these viruses to determine whether antigenic drift occurs or not.

The HPeV3 isolates detected during our study were isolated from patients with a mean age of 1.9 months and were significantly more associated with fever, meningitis, and viremia than HPeV1 infection. The children infected with HPeV1 were clearly older, with a mean age of 9.2 months. Gastrointestinal symptoms such as diarrhea and vomiting were more often associated with HPeV1 infection. These findings and the size of our study group strengthen the suggestions of Benschop et al. (2006) that children infected with HPeV3 are significantly younger and more often show sepsis-like illness than children infected with HPeV1 (4). The difference in behavior between HPeV1 and HPeV3 might be explained by the difference in receptor usage between HPeV1 and HPeV3. An RGD (arginine-glycine-aspartic acid) motif is present in the C-terminal loop of VP1 of HPeV1, -2, -4, -5, and -6 and is responsible for the attachment of the parechovirus to the αvβ1and αvβ3 (fibronectin) receptors present at the cell surface (6, 19). HPeV3 does not have an RGD motif (5, 10) and therefore uses another, RGD-independent route of cell entrance, which could be associated with a difference in tissue tropism and a different pathogenesis from that of HPeV1 infection.

The results of a previous study suggested that HPeV3 infections were especially but not only found in boys (male/female ratio, 9:1) (4). Our study, however, showed that the distribution of HPeV3 infections (male/female ratio, 1.26:1) does not clearly differ from that of enterovirus infections. Reports of enterovirus isolations are consistently more common from males than from females (13, 14).

This study shows that HPeV has been endemic in The Netherlands for the past 8 years and explains the majority of the diagnostic deficit in the enterovirus surveillance of 2000 to 2007. Routine implementation of an HPeV diagnostic PCR test in enterovirus surveillance is therefore recommended and is actually already in place in The Netherlands, since 2006 (16). This is the first time that HPeV3 infections have been reported to occur at intervals of 2 years. This study includes a large number of HPeV isolates, strengthening our observations and the suggestions from previous studies, such as the suggestion of Benschop et al. (2006) that HPeV3 is more often associated with sepsis-like illness in very young children (especially 0 to 4 months) (4).

Acknowledgements

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Chapter 8

General Discussion

Our study on genetic and antigenic diversity of vaccine-related polioviruses, shed by mOPV1 primed newborns (chapter 2), has resulted in new insights that will be of value for the final fight against the remaining polioviruses. When the goal of poliovirus eradication has been achieved, other enteroviruses, whether or not as a consequence of waning poliovirus immunity, may form, amongst others, the major viral threats of neurologic disease in children. A potential candidate for this is human enterovirus 71 (EV71), for which we gained important insights in its evolutionary trajectory. In this chapter, the value of the work described in this thesis will be discussed in relation to the current knowledge in the field.

The polio endgame: we are not there yet

The world wide vaccination campaign with trivalent OPV has significantly reduced the number of countries with endemic poliovirus circulation and succeeded in eradicating wild type 2 poliovirus. There are, however, still 4 countries left with endemic circulation of wild type 1 and 3 polioviruses (Nigeria, Afghanistan, India and Pakistan). As a consequence, explosive outbreaks of poliomyelitis caused by viruses imported from these endemic regions affect regions that were previously poliovirus free, but where present vaccination coverage is apparently lower than reported (i.e. Tajikistan and the Republic of Congo, 2010). Monovalent OPV1 and 3 have been shown to be superior in inducing systemic and mucosal humoral immune protection against the respective poliovirus serotypes (chapter 2 and (8, 18, 48)) and are currently used as supplementary tools in fighting the last circulating wild type polioviruses.

Our study in chapter 2 is the first one to describe the genetic and antigenic characteristics of isolates shed by mOPV1 primed newborns and by that feeds the discussion on the OPV-paradox: on one hand priming of newborns with mOPV1 resulted in a better systemic and mucosal protection against P1 infection than tOPV. From this point of view administration of mOPV1 directly after birth would be a powerful tool to tackle the last wild type polioviruses. Shedding of a remarkable high proportion of genetically and antigenically divergent (AD) viruses by these newborns, on the other hand, forms the reverse side of the medal and emphasizes the need to achieve high vaccination coverage (>90%) when applying this tool. The use of mOPV thus will have to go hand in hand with efforts to increase acceptance of poliovirus vaccination, interaction with (migrating) children in conflict areas and improvement of monitoring of vaccination coverage. In addition, the observation argues against the suggestion done by Caceres et al. (2001) that mOPV can be an excellent tool in controlling new polio outbreaks in the post-polio eradication era as presence of non-immunized populations will enhance evolution of cVDPVs (8). Considering the

immaturity of the immune system of neonates and shedding of a high proportion of genetically and antigenically divergent OPV strains, the discussion on the OPV-paradox gets an additional dimension: Are neonates at risk for vaccine associated paralytic poliomyelitis and should we thus administer mOPV at birth?

As fluctuations in vaccination coverage are inevitable, the risk for evolution of VDPVs capable of causing poliomyelitis will remain as long as OPV is used. The possibility to recode viral RNA genomes through chemical synthesis may offer future solutions for this. Without changing the amino acid sequence, Wimmer and colleagues (2009) succeeded in synthesizing polioviruses with a marked reduction in replicative fitness by introducing underrepresented codons or codon pairs in the P1 encoding region (in line with studies of Burns et al. (2006, 2009)) (6, 7, 58). Considering the fact that these vaccine strains harbor hundreds of mutations compared to the wild type poliovirus, reversion to a wild type phenotype is unlikely making these viruses promising vaccine candidates.
This field of research, however, is still in its infancy and introduction of IPV in all countries in the final stages of poliovirus eradication is therefore a more obvious solution to reduce the risk for evolution of new VDPVs. Vaccination with IPV, however, is expensive and may not be affordable for low and middle-low-income countries. To find solutions for this, a WHO study was conducted in Oman in 2007/2008 in which the immunogenicity of fractional doses of IPV delivered intradermally with a needle free devise was evaluated. Although the mean IgG titers against poliovirus were lower after intradermal administration of the fractional doses of IPV than after routine administration of full doses intramuscularly, this vaccination strategy resulted in high seroconversion rates and seems to be a promising tool for application in low income countries (38). IPV vaccination, however, does not induce sufficient mucosal immunity to protect against poliovirus replication and subsequent transmission when there has been no previous contact with live poliovirus (OPV or natural infection) (25). Design of new tools, like antiviral drugs and possibly the previously mentioned synthetic vaccine viruses, are therefore clearly needed to control new outbreaks in the final stages of eradication and thereafter. In addition, antiviral drugs will be needed to treat B-cell immunodeficient patients with prolonged shedding of iVDPVs, not only because of the high risk for paralysis in these patients but also for the risk of transmission of iVDPVs to the community at the time OPV vaccination is stopped (9, 34). Only a small number of these patients have been reported to the WHO (44 since 1961), but according to the WHO these numbers likely represent only the tip of the ice berg. Regarding the needs for antiviral drugs, the Task Force for Child Survival and Development, with the support of the RIVM, Atlanta Centers

for Disease Control and Prevention (CDC) and WHO, has established an initiative to assist the development and evaluation of compounds with suspected or demonstrated anti-poliovirus activity.

When the world is eventually polio free it is of great importance to monitor eradication of poliovirus. Currently this relies on cell culture isolation of poliovirus from stool samples on poliovirus sensitive cell lines. This method, however, is directed only at the tip of the surveillance pyramid, namely diseased individuals, and therefore relatively insensitive. The switch of OPV to IPV with an associated decrease in mucosal immunity against poliovirus, however, will enable monitoring by periodic screening of sera for presence of poliovirus specific IgA. This is a less laborious and less time consuming approach. The applicability of this test was demonstrated by a study in the Netherlands which showed that IgA positive responses of a population born before and after introduction of IPV vaccination in the Netherlands could largely be explained by the occurrence of episodes of proven poliovirus circulation (24, 25, 39). In collaboration with Atlanta CDC, the RIVM currently evaluates the application of a poliovirus IgA and IgM detection ELISA for fast diagnosis of poliovirus infection and the monitoring of poliovirus circulation. In addition, we currently explore possibilies to develop portable minilabs with serological microarrays for detection of poliovirus specific IgA or IgM in finger stick blood. This technology could be done by field workers in resource poor regions, increasing acceptance and coverage of monitoring.

Human enterovirus 71: really getting on our nerves

Human enterovirus 71 (EV71), considered to be the major threat for poliomyelitis like disease in children following eradication of poliovirus, appears to circulate persistently at low levels in Europe and the USA, sometimes causing relatively small outbreaks (chapters 3 and 4). Reports on fatal cases in Europe and the USA are rare (51). In Asia, on the other hand, EV71 outbreaks occur with ten thousands of cases of Hand Foot and Mouth Disease (HFMD) and hundreds of fatal cases as a result of neurologic disease. Why is this picture so different from the one in the western world?

Reconstruction of past EV71 population dynamics on the basis of EV71 VP1 sequences collected globally in 1970-2008, showed sharp increases in relative genetic diversity of genogroup B and C viruses in the late 1990s indicating a true increase in viral spread (53). This increase seemed to coincide with the emergence of new subgenogroups (B3-5, C2-5) of which circulation, except for C2, has only been observed in the Asian Pacific region (chapter 3, (53)). Until now, the driving

force behind the fast evolution of new subgenogroups is not well understood. Trends observed in EV71 epidemiology, for instance in Taiwan (Fig. 1), suggest that the emergence of new subgenogroups is antigenically driven to escape population immunity. The antigenic difference between genogroup B and C viruses, observed in our serological study (chapter 4) and in a recent Taiwanese study based on antigenic cartography using human sera (27), seems to fit the alternating pattern of genogroup B and C outbreaks in Taiwan. This picture, however, is not in line with our suggestion that genogroup C immunity might cross-protect against genogroup B infection (chapter 4, based on Dutch strains). Extended serological studies using Asian strains and respective rabbit antisera (kindly provided by Dr. Shimizu and Dr. Mizuta from Japan, and Dr. Tu and Dr. Thao from Vietnam), showed that sera against a Japanse C2 outbreak strain (1997, Fig. 1) could not neutralize a B4 strain (Japan, 1997). The B4 sera on its turn could not neutralize a C4 strain isolated in Japan in 2003 (Van der Sanden et al., unpublished data). These observations and the observation done by Huang et al. (2009) that B5 is antigenically distinct from other genogroup B and C viruses, fit trends not only observed in Taiwan but also in other Asian countries (overview in chapter 3), suggesting that antigenic drift plays a role in emergence of new subgenogroups to escape herd immunity (27). Like for seasonal Influenza A (H3N2) viruses, Asia seems to form the cradle for emergence of these new EV71 variants (46).

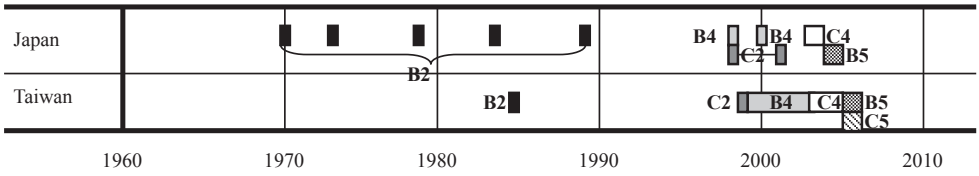

Fig.1: Enterovirus 71 subgenogroups observed in Japan and Taiwan (source: chapter 3).

In mainland China a picture distinct from that in other Asian countries is seen: all viruses isolated from the massive outbreak in 2008 to 2010 belong to the same subgenogroup; C4. How is C4 able to persist? The persistence could be explained by a circulation network in which EV71 continuously hops from one epidemic to the other. Such a network has previously been shown for circulation of seasonal Influenza A (H3N2) viruses in east and Southeast Asia (46). Another explanation could be found among study results published by Tee et al. (2010), who demonstrated toggling of VP1 amino acid residues through time on the core trunk branches of C1 phylogenies, suggesting that these mutations are necessary for virus persistence, possibly by generating antigenic novelty (53). In our sequence dataset of Dutch C1 isolates we saw toggling of two of these residues through time as well, but this did

not result in antigenic changes according to our serological assays. In collaboration with Guangdong CDC (China), we currently study genetic diversity and population dynamics of EV71 C4 strains to gain insight in its epidemiological behaviour in China.

The relatively high number of severe and fatal cases reported during outbreaks in the Asian region leaves the question whether recently emerged subgenogroups have an increased neurovirulence. Up to present, however, severity of disease could not be related to specific subgenogroups and sequencing of strains isolated from cases of mild disease and from fatal cases revealed no markers for increased neurovirulence (49, 50). A significant difference in viral load between mild and severe cases was not observed either (61). Nishimura et al. (2009), however, made an interesting observation: they showed a difference in binding capability to the EV71 receptor PSGL-1, present on human leukocytes, among strains belonging to the same, but also belonging to different subgenogroups. Subgenogroup C1, C2, C4, B3 and B4 strains, except for C1 all known to be associated with large outbreaks in Asia, replicated in a PSGL-1 dependent manner, whereas BrCr (genogroup A, USA), B1 (Japan) and C1 (Thailand) strains, belonging to "older" subgenogroups, replicated in a PSGL-1 independent manner (40). Regarding the observed depletion of T-cells and changes in cytokine levels in severe cases of EV71 infection, testing of the hypothesis that EV71 induced apoptosis in PSGL expressing leukocytes is correlated to a severe disease outcome would be of great interest. Additional categorization of PSGL binding and non-binding strains in a more extended study including strains isolated from severe and non-severe cases and strains of old and the more recently emerged subgenogroups could reveal insight in potential increased neurovirulence of new subgenogroups.

The high number of severe cases observed during outbreaks in Asia could also just be a consequence of an increase in total number of infections. Regarding the total number of EV71 infections reported for the C4 outbreak in China (990 000) the percentage of fatal cases (537 or 0.05%) seems not to exceed the normal percentage of severe and fatal infections reported for other enteroviruses. But how can the increased viral spread, as was evidenced by studies on population dynamics (Tee et al, 2010) be explained? Aside from the potential introduction of antigenically divergent EV71 strains in a population with low/no immunity against these new types, an overall drop in seroprevalence rate against EV71 in the population could explain the increased incidence of EV71 infection as well. This is in line with study results of Lu et al. (2002), who showed that the number of EV71 seropositive children decreased several fold in the years preceding the Taiwan outbreak in 1998 (33). Other factors

increasing virus fitness and transmission could play a role as well. Interestingly from this point of view, our detection of recombination breakpoints in the 5'UTR of EV71 was unique to strains isolated during outbreaks (chapter 5). In addition, this study implied that recombination in the non-structural encoding genes of EV71 plays a significant role in evolution of type B subgenogroups. Leitch et al. (2009), observed the same phenomenon for ECHO 30: recombinant forms of this virus emerged, dominated and disappeared over a period of 3 to 5 years (36). Untill now, however, it is not clear whether these recombination events represent just accidental events or events that drive selection of viruses with increased fitness.

But why is the complete situation restricted to the Asian region? Aside from epidemiological factors like crowding and differences in climate, it could be possible that Asian people are more susceptible for EV71 infection due to genetic host factors. The class I HLA-A33 genotype and specific polymorphisms in the cytotoxic T lymphocyte antigen-4 (CTLA-4) gene, involved in T-cell anergy and apotosis, were shown to be correlated to increased susceptibility for (severe) EV71 infection (10, 62). Both the HLA-A33 genotype and CTLA-4 polymorphisms were observed more frequently among Asian children than among white subjects, suggesting a role for genetic components in the susceptibility for EV71 infection. Regarding the human EV71 receptors PSGL-1 and SCARB2, it would be of interest to study polymorphism in genes expressing these receptors as well, to find out whether there is a race dependency in EV71 binding capabilities and associated susceptibility for infection (31).

Apart from possible host genetic factors, immunity of the European population against circulating C viruses, might cross-protect against infection with subgenogroups observed in Asia. This is implied by the results of our extended serum neutralization assays, which showed cross-neutralization of Asian B3, B4, C4 and to a lesser extend of C5 viruses by rabbit sera against the Dutch C1 isolate (Van der Sanden et al., data unpublished). Extended *in vitro* and *in vivo* studies will be necessary to verify the cross-protective efficacy of C1 serum. Regarding the clinical impact of EV71 outbreaks, introduction of new subgenogroups in Europe, such as that of subgenogroup B5 reported to be antigenically distinct from other genogroup B and C viruses, should be monitored carefully. For that reason the Dutch enterovirus typing tool, used by virology labs to type enterovirus isolates as part of routine diagnostics, has been supplemented with the possibility to type EV71 isolates on the level of subgenogroup (http://www.rivm.nl/mpf/enterovirus/typingtool#/).

Human Parechoviruses: the hide-and-seek game is over

The majority of the diagnostic deficit observed in national enterovirus surveillance for years could be explained by human parechovirus, in particular of types 1 and 3 (chapter 7). Although prevalence of HPeV is expected not to be related to poliovirus immunity (on the basis of phylogenetic distances (29)), it is important to note that it has been underestimated in the Netherlands for years.
Our study was based on cell culture isolation of HPeV strains and could therefore not give insight in prevalence of types not growing well in cell culture. Benschop et al. (2008) and Pham et al. (2011), however, genotyped HPeV variants directly from stool samples, collected from Dutch and Japanese children for diagnostic purposes, and confirmed that HPeV1 and 3 are the most prevalent types (2, 43). HPeV 4, 5 and 6 were found in only small proportions (2).

HPeV infections appear to be as prevalent as enterovirus infections: HPeV1 and 3 viruses are isolated at average annual isolation rates (percentage of total number of tested samples) of 0,28 and 0,24% (for HPeV3 positive years) respectively, which is in the range of isolation rates of commonly reported Coxsackie B (serotypes 1 to 5) and ECHO viruses (E9, E13 and E18) (chapter 6, 7, and (2)). But what is the clinical relevance of HPeV compared to that of enteroviruses? HPeV1 infections as well as the less frequently reported HPeV4, 5 and 6 infections are reported to be most often associated with relatively mild disease like gastroenteritis and respiratory infections (1-3, 16, 32, 42, 56). Infections with HPeV3 on the other hand, as was shown by previous studies and in chapter 7, are reportedly more often associated with CNS involvement in very young children (4, 5, 22, 30). Wolthers et al. (2008) considered HPeV as a second major cause of CNS-associated disease as HPeV and enteroviruses were found in 4.6% and 14% of CSF samples from Dutch children (< 5 years of age), respectively (samples collected as part of routine virologic diagnostics, 2004-2006) (59). One should keep in mind, however, that all enterovirus serotypes were taken together, making a direct comparison complicated. This issue was addressed by the study of Harvala et al. (2009), who showed that HPeV strains found in CSF samples, collected in Scotland in 2006 to 2008 for diagnostic purposes, all belonged to type 3 and that its isolation frequency in 2008 exceeded that of the enteroviruses (total of 8 serotypes), emphasizing their clinical relevance (21). Disease symptoms reported for HPeV infection by both studies and by a study of Verboon-Maciolek et al. (2008), closely resembled those of enterovirus infection (21, 54, 59).

Being aware of the clinical impact of HPeV infection, the majority of the labs of the Dutch Working Group on Clinical Virology have implemented a HPeV diagnostic

PCR since 2006 (41). As a consequence we can see a clear decrease in the number of non-typable isolates and an increase in number of detected HPeV strains in recent years (Fig. 2). Untill now, HPeV of types 1 to 6 have been reported as part of the surveillance system. The newly identified types 7 to 14 have not been observed, suggesting restricted circulation, but they might be present among the relatively large collection of non-typed HPeV isolates. Typing of these HPeV strains will substantially increase knowledge on HPeV epidemiology.

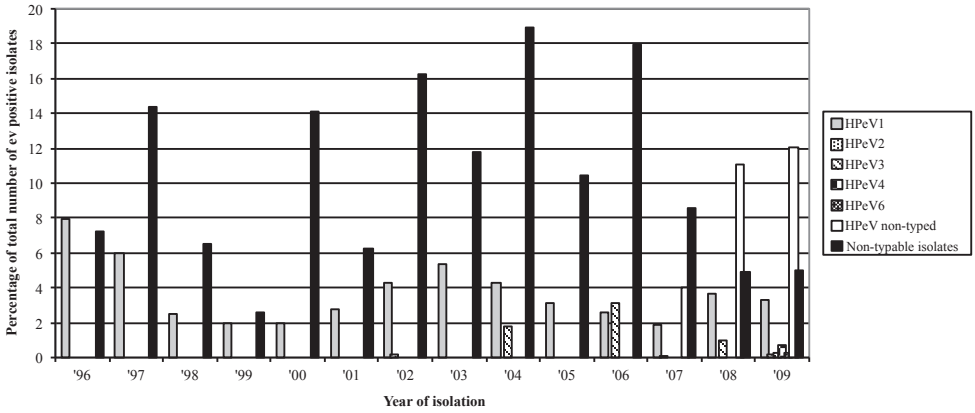

Fig. 2: Proportion of HPeV strains isolated by the Dutch Working Group on Clinical Virology in 1996 to 2009. Results of chapter 3 are not included.

Future Challenges

As mentioned before, in the final stages of poliovirus eradication and thereafter, tools like antiviral drugs will be needed to control new polio outbreaks and to treat patients with long term shedding of iVDPVs. Design of anti-polio drugs, however, has never been seriously considered by pharmaceutical industry due to the overwhelming success of poliovirus vaccination resulting in a small market. Several compounds known to block infection of picornaviruses, in particular human rhinovirus, have been described (reviewed in (15)), and some of these, like the 3C protease inhibitor Ruprintivir and its analog, the 3A inhibitor Enviroxime or the capsid binding agent R78206 (a pirodavir analog), showed broad reactivity against poliovirus type 1, 2 and 3 as well (13). None of these or other anti-picornavirus compounds, however, have ever been licensed for treatment because of poor pharmacokinetics, poor therapeutic efficacy in natural infection, side effects or interaction with other drugs. Screening for analogs or new compounds is therefore of utmost importance.

Non-polio enteroviruses, in particular Coxsackie B- and ECHO viruses, are the leading cause of aseptic meningitis: they account for 80 to 90% of all cases for which

an etiologic agent is identified (37, 47, 52, 65). Although disease is usually self-limiting, these viruses can cause life threatening infections (encephalitis, hepatitis or myocarditis) in neonates and agammaglobulenic patients (17, 55). Administration of intravenous immunoglobulin (IVIg) is currently the only option to treat such infections, but its therapeutic efficacy has not been proven. Increased awareness of the clinical relevance of non-polio enteroviruses, in particular of the emerging EV71, Coxsackie B- and ECHO viruses, has triggered research groups to develop antiviral drugs, but just as for poliovirus, these are in early stage of research (11, 12, 14, 45, 60). Although Coxsackie A (CA) viruses are reported to be less often associated with serious disease (63, 65), inclusion of these viruses in initial screenings for anti-enterovirus components should be considered, regarding the time-consuming trajectory for development and approval of drugs, the potential underestimation of the number of severe clinical cases of CA infection (chapter 6) and the potential increase in prevalence of these viruses in the post-polio eradication era.

A drawback of the use of antivirals against a highly prevalent and neurotrophic virus like EV71, is that a large number of children present already severe, CNS-associated disease before administration to the hospital. One could suggest that prophylactic use of anti-EV71 drugs in children with primary disease symptoms (HFDM) would overcome this problem. Regarding the large number of cases of HFMD during outbreaks of EV71 (ten to hundred thousands), a major drawback of this strategy is the increased risk for evolution of viruses resistant to the drugs. From these points of view, research needs to focus on prevention of EV71 infection as well. Currently, personal hygiene, good sanitation, and disinfection of surfaces are the only control measures during EV71 outbreaks. Considering the high population density in the Asian regions, and the period of time EV71 can be shed in faeces (for up to 6 weeks, (20)), more effective tools, like vaccines, will be needed to reduce transmission and disease burden. Vaccines based on formaldehyde-inactivated whole virus preparations, VP1 capsid proteins, or virus like particles have been shown to elicit a protective humoral antibody response against EV71 in animals (reviewed in (45)). One of the barriers to develop an EV71 vaccine, however, is the lack of a suitable animal model for large-scale testing of vaccine efficacy: Laboratory mice are only susceptible to human EV71 infection in the first 4 days of life and large-scale studies with Cynomolgus monkeys, that are susceptible for EV71 infection and develop CNS associated disease upon subcutaneous or intraspinal inoculation with EV71, are not feasible regarding ethical and financial aspects (19, 23, 35, 64). The recent identification of the human EV71 receptors PSGL-1 and SCARB, however, will greatly enhance vaccine research as it allows development of transgenic mice expressing human EV71 receptors, a method

which has successfully been applied for poliovirus and echovirus 1 (26, 28, 44).
Aside from a suitable animal model, development of vaccines, but also of antiviral drugs, will remain a challenge as long as genetic diversity and antigenic evolution of EV71 are keys of uncertainty. Monitoring diversity as part of HFMD surveillance and national enterovirus surveillance activities is therefore of utmost importance. If EV71 indeed appears to show antigenic drift, this would greatly complicate vaccine development as subgenogroups emerge quickly and can cocirculate during outbreaks.

Although infections with human parechoviruses of type 3 are often associated with severe neonatal disease, no data on treatment options are currently available. Wildenbeest et al. (2010) suggested that administration of IVIg and monoclonal antibodies might be the most feasible options for treatment of HPeV infection on short term. In the meantime, HPeV should be included in screening for compounds with activity against picornaviruses as well (57).

Concluding remarks

The world wide poliovirus vaccination campaign has greatly reduced the number of cases of poliomyelitis and has undoubtedly saved a lot of child lives. Total elimination of all polioviruses (wild type and VDPV) will require achievement of high immunization coverage, using tOPV, mOPV, bOPV and in a later stage IPV, active monitoring of poliovirus circulation and development of anti-polio drugs.

On the basis of the current knowledge, it is not possible to predict the consequences of poliovirus eradication on prevalence and pathogenesis of non-polio enteroviruses. This question, however, should not be ignored, regarding the clinical relevance of enteroviruses (they are the main causative agents of aseptic meningitis) and the potential risk for evolution of new polio-like viruses. Studies on the role of heterotypic immune protection in enterovirus infection and disease outcome and active monitoring of enterovirus prevalence in the pre- and post-polio era will be important.

EV71 has formed a major public health concern in the Asian Pacific region since 1997. Regarding the massive outbreaks with high rates of morbidity and substantial numbers of deaths, it is not unreasonable to consider this virus as one of the major virologic threats for neurologic disease in children. The public health measures currently used to control outbreaks, however, are not clearly effective. Development of more powerful tools like anti-EV71 drugs and vaccines is needed and its progress will greatly depend on knowledge on the pathogenesis and the evolutionary

trajectory of EV71. We believe that the work presented in this thesis contributes to the understanding of the epidemiologic behaviour of EV71 and by that, together with the work published by other research groups, forms the first steps towards effective intervention strategies.

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Summary

In 1988 the World Health Assembly passed a resolution to eradicate poliovirus globally. The World Health Organisation (WHO) launched a worldwide vaccination campaign, using the trivalent oral poliovirus vaccine (tOPV) that contains live, attenuated polioviruses of the three different serotypes. The vaccination campaign has been very successful as the number of countries with wild type poliovirus circulation decreased from >125 in 1988 to 4 in 2010 and poliovirus type 2 has likely been eradicated. Supplementary vaccination campaigns with monovalent (m) and bivalent (b) OPV of types 1 and 3, having a superior protective efficacy to these types compared to tOPV, have recently been introduced in the remaining endemic regions to eradicate the last circulating type 1 and 3 polioviruses.

As part of a WHO study on the protective efficacy of mOPV1 in newborns, birth dose administration of mOPV1 was shown to generate a better systemic and intestinal immune protection against poliovirus 1 infection than tOPV at birth (chapter 2). These results suggest that vaccination with mOPV1 at birth is a powerful tool in eradicating the last type 1 polioviruses. Shedding of a high proportion of genetically and antigenically divergent vaccine viruses, especially by mOPV1 primed newborns, however, warns against the use of this tool in areas in which the population is at risk for polio outbreaks caused by circulating vaccine derived polioviruses (areas with low vaccination coverage) (chapter 2). In addition, an accelerated accumulation of mutations in the genomes of vaccine strains just after vaccination, whether or not resulting in a phenotypic change of the virus, suggests that circulating vaccine derived polioviruses are less old than estimated previously.

In the second part of this thesis, we studied prevalence and genetic diversity of human enterovirus 71 (EV71), a virus considered to be the major threat for poliomyelitis like disease in children following poliovirus eradication. Together with the since 1997 increased incidence of EV71-associated Hand Foot and Mouth Disease and neurologic disease in the Asian Pacific region, the sudden increase in reporting of EV71 infections in the Netherlands in 2007, triggered us to study the molecular epidemiology of EV71 in the Netherlands. Phylodynamic analyses on the basis of VP1 nucleotide sequences of EV71 strains collected in the Netherlands in 1963 to 2008, showed a pattern of endemic circulation with occasional occurrence of relatively small outbreaks (chapter 4). The in Asia recently emerged subgenogroups, associated with large outbreaks, were not observed in the Netherlands (chapter 3). Isolates collected in the Netherlands from 1963 to 1986 belonged to EV71 genogroup B of which the molecular epidemiology seemed to be characterized by successive emergence, dominance and disappearance of recombinant but not antigenically distinct forms of EV71 (chapter 3, 4 and 5). An outbreak of genogroup B viruses occurred in 1986, caused by recombinant viruses with a 5'untranslated region

highly similar to that of viruses isolated in the Asian Pacific region (chapter 5). Following this outbreak, circulation of genogroup B viruses was replaced by circulation of genogroup C strains with an antigenic character different from that of B strains circulating in previous years. This shift seemed to coincide with a change in clinical presentation and age group affected (chapters 3 and 4). In contrast to the evolutionary trajectory of genogroup B viruses, recombination seemed to play no role in the (independent) evolution of type C subgenogroups. The occurrence of a type C outbreak in 2007 could neither be explained by emergence of recombinant or antigenically distinct variants. Trends observed in EV71 epidemiology in the Netherlands were in line with those observed in other European countries and the USA, indicating global circulation of the respective subgenogroups.

To find clues on the potential consequences of eradicating poliovirus and the associated decrease in (intestinal) immune protection on prevalence of other enteroviruses, we studied trends in enterovirus isolation data collected as part of routine enterovirus diagnostics in the Netherlands in 1959 to 2009. Regarding changes in isolation frequency of enteroviruses expected to be immunologicaly closely related to poliovirus (chapter 6), several cluster B serotypes were isolated at a lower frequency during the first years of the study period (up to 1966) than in following years. These changes could not be ascribed to documented changes in enterovirus detection and typing methods. Cluster C viruses, most closely related to poliovirus, were isolated at low rates throughout the complete study period (1959 up to 2009). This is in line with low prevalence of these viruses in the environment. Extended studies on the role of heterotypic immune-protection in human enterovirus infections and disease outcome will be needed to find out whether the trends observed for cluster B and C viruses are somehow related to poliovirus immunity and whether we thus can expect global changes in prevalence of these viruses in the post-OPV era.

As part of our study on trends in enterovirus prevalence, we explained the majority of the diagnostic deficit observed in the national enterovirus surveillance by testing unexplained samples, collected in 2000 to 2007, for presence of human parechovirus (HPeV) (chapter 7). Prevalence of HPeV, especially of types 1 and 3 appeared to have been underestimated for years in the Netherlands. HPeV1 and HPeV3 show some striking differences in their epidemiology: infections with HPeV3 were reported at intervals of two years and in the spring-summer season, whereas HPeV1 was observed in small numbers throughout each year with a low in the summer months. Children infected by HPeV3 were significantly younger and showed significantly more often severe disease symptoms than children infected with HPeV1. Outbreaks of HPeV3 were caused by several genetic variants, indicating endemic circulation of HPeV3 in the Netherlands.

Samenvatting

In 1988 stelde de World Health Assembly zich ten doel poliovirus wereldwijd uit te roeien. De Wereldgezondheidsorganisatie (WHO) startte daartoe een wereldwijde vaccinatie campagne met het trivalente orale poliovirus vaccin (tOPV). Dit vaccin bevat levende maar afgezwakte poliovirussen van de drie voorkomende serotypen. De campagne is tot nu toe erg succesvol geweest: het aantal landen met circulatie van wild type poliovirus is afgenomen van >125 in 1988 naar 4 in 2010. Bovendien is wild poliovirus type 2 zeer waarschijnlijk uitgeroeid (sinds 1999). Monovalent (m) en bivalent (b) OPV, die een aantoonbaar betere bescherming bieden tegen infectie met type 1 en 3 dan tOPV, worden momenteel gebruikt in aanvullende vaccinatie campagnes in de laatste endemische landen om poliovirus daadwerkelijk uit te roeien.

Een recente WHO studie naar de beschermende werking van mOPV1 in neonaten, toonde aan dat vaccinatie met mOPV1 vlak na de geboorte resulteerde in een betere systemische en mucosale bescherming tegen infectie met poliovirus 1 dan vaccinatie met tOPV vlak na de geboorte (hoofdstuk 2). Vaccinatie van pasgeborenen met mOPV1 wordt daartoe verondersteld een effectief middel te zijn voor het uitroeien van de laatste poliovirussen. Uitscheiding van relatief veel genetisch en antigeen divergente vaccin virussen, vooral in de mOPV1 studie groep, waarschuwt echter tegen het gebruik van dit middel in gebieden met een verhoogd risico voor polio uitbraken veroorzaakt door verwilderde vaccin virussen (gebieden met een lage vaccinatie graad) (hoofdstuk 2). De versnelde accumulatie van mutaties in het genoom van vaccin stammen vlak na vaccineren, al dan niet leidend tot fenotypische veranderingen van het virus, suggereert bovendien dat circulerende vaccin virussen minder oud zijn dan eerder geschat.

Hoofdstukken 3 tot 5 van dit proefschrift staan in het teken van de prevalentie en genetische diversiteit van humaan enterovirus 71 (EV71). Dit virus wordt verondersteld de belangrijkste bedreiging voor poliomyelitisachtige aandoeningen in kinderen te vormen na uitroeiing van poliovirus. In 2007 nam het aantal gerapporteerde EV71 infecties in Nederland plotseling toe. Samen met de sinds 1997 verhoogde incidentie van EV71-geassocieerde Hand- Voet- en Mondziekte en neurologische aandoeningen in Azië-Pacific, stimuleerde deze observatie ons om de moleculaire epidemiologie van EV71 in Nederland in kaart te brengen. Fylodynamische studies op basis van VP1 nucleotide sequenties van Nederlandse EV71 stammen uit 1963 tot 2008, toonden een endemische circulatie aan met het occasioneel voorkomen van relatief kleine uitbraken (hoofdstuk 4). De in Azië recent ontstane subgenogroepen, geassocieerd met grote uitbraken, zijn niet in Nederland gevonden (hoofdstuk 3). Nederlandse stammen uit 1963 tot 1986 behoorden tot EV71

genogroup B, waarvan de moleculaire epidemiologie gekarakteriseerd leek te worden door het opkomen, domineren en wederom verdwijnen van recombinante maar niet antigeen divergente varianten (hoofdstukken 4 en 5). In 1986 vond in Nederland een uitbraak plaats, veroorzaakt door recombinante genogroup B virussen waarvan het 5'niet-coderende-regio van het genoom bijzonder veel gelijkenis vertoonde met die van genogroup B virussen uit Azië (hoofdstuk 5). Deze uitbraak werd gevolgd door een verschuiving in de circulatie van genogroup B naar genogroup C virussen, die naast het hebben van een ander antigeen karakter ook met een ander ziektebeeld geassocieerd leken te zijn dan de voorheen circulerende genogroup B virussen (hoofdstukken 3 en 4). Anders dan voor het evolutionare traject van genogroep B virussen, lijkt recombinatie geen rol te spelen in het (onafhankelijk) ontstaan van type C subgenogroepen. De type C uitbraak in 2007 kon eveneens niet gerelateerd worden aan het ontstaan van recombinante of antigeen divergente stammen. De trends in de EV71 epidemiologie in Nederland komen overeen met die in andere Europese landen en de Verenigde Staten. Dit impliceert een globale circulatie van de desbetreffende EV71 subgenogroepen.

In de zoektocht naar antwoorden op de vraag of uitroeiing van poliovirus en de geassocieerde afname in (mucosale) immuniteit zal leiden tot verhoogde prevalentie van andere enterovirussen, analyseerden we in hoofdstuk 6 trends in enterovirus isolatie data verzameld als onderdeel van routine enterovirus diagnostiek in Nederland in 1959 tot 2009. Wanneer we kijken naar veranderingen in isolatie frequentie van virussen die vermoedelijk immunologisch aan poliovirus verwant zijn (hoofdstuk 6), valt het op dat verschillende cluster B serotypen relatief minder vaak geïsoleerd zijn in de eerste jaren van de studieperiode (1959 tot 1966) dan in de volgende jaren. Deze verschuiving kon niet gerelateerd worden aan veranderingen in diagnostische methoden. De aan poliovirus meest verwante cluster C virussen zijn gedurende de gehele studieperiode slechts sporadisch geïsoleerd. Deze observatie komt overeen met de lage prevalentie van deze virussen in de omgeving. Bredere studies over de rol van heterogene immuun protectie in humane enterovirus infectie en geassocieerde ziektebeeld zullen nodig zijn om te achterhalen of de waargenomen trends voor cluster B en C virussen gerelateerd zijn aan poliovirus immuniteit en of we een verandering in prevalentie van deze virussen kunnen verwachten in het post-OPV tijdperk.

Als onderdeel van bovenstaande studie naar trends in enterovirus prevalentie, hebben we het overgrote deel van het diagnostische deficit in de nationale enterovirus surveillance verklaard door samples van onverklaarde infecties, verzameld in 2000

tot 2007, te testen voor aanwezigheid van humaan parechovirus (HPeV) (chapter 7). Prevalentie van HPeV in Nederland, vooral die van serotypen 1 en 3, bleek al jaren onderschat te zijn. HPeV1 en HPeV3 laten opmerkelijke verschillen zien in hun epidemiologie: infecties met HPeV3 lijken tweejaarlijks voor te komen en vinden voornamelijk plaats in het lente-zomer seizoen, terwijl infecties met HPeV1 jaarlijks worden gerapporteerd met een minimum aantal infecties in de zomermaanden. Kinderen geïnfecteerd met HPeV3 bleken significant jonger te zijn en toonden significant vaker een ernstig ziektebeeld dan kinderen met een HPeV1 infectie. Uitbraken met HPeV3 werden veroorzaakt door verschillende genetische varianten wat een endemische circulatie van HPeV3 in Nederland impliceert.

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Curriculum Vitae

Sabine van der Sanden werd geboren op 20 november 1982 te Eindhoven. Na het behalen van haar VWO diploma aan het Rythovius College in Eersel in 2001, startte zij met de opleiding Biotechnologie aan de Universiteit van Wageningen (WUR). Na het *cum laude* afronden van de propedeutische fase, koos Sabine voor de cellulaire/moleculaire richting met als specialisatie virologie. In 2004/2005 deed zij een Bachelor afstudeerstage op het Laboratorium voor Virologie van de WUR, met als doel het voor vaccin doeleinden tot expressie brengen van tekeneiwitten in het Baculovirus expressie system. Als onderdeel van haar Master opleiding, startte Sabine in 2005 een onderzoeksstage op het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in Bilthoven. Hier bestudeerde zij de genetische diversiteit van het destijds pas ontdekte Humaan Parechovirus type 3. In 2006 sloot zij haar Master opleiding af met een afstudeerstage op de afdeling Humane Retrovirologie op het Academisch Medisch Centrum in Amsterdam. Hier bestudeerde zij de mogelijkheden om replicatie van het humaan immuundeficiëntievirus (HIV) te remmen door expressie van de bij de replicatie betrokken gastheer-eiwitten te remmen door middel van RNAi silencing. Voor dit werk ontving Sabine de Professor Van der Want prijs 2006/2007 (prijs voor beste afstudeermanuscript van het Laboratorium voor Virologie van WUR).

In 2006, startte Sabine haar promotieonderzoek op de afdeling Virologie van het RIVM onder leiding van Prof.dr. M.P.G. Koopmans en Dr. H. van der Avoort. Hierbij stond het verkrijgen van inzicht in de prevalentie en genetische diversiteit van humane enterovirussen in het licht van poliovirus eradicatie centraal. Om moderne enterovirus detectie- en typeringsmethoden eigen te maken, deed Sabine in 2007 een werkstage op het Centers for Disease Control and Prevention (CDC) in Atlanta (de Verenigde Staten). Met de tijdens haar promotietraject ontwikkelde expertise rondom enterovirus 71, bezocht Sabine in 2010 het CDC in Guangzhou (China) waar zij een training voor studies naar genetische diversiteit verzorgde. De resultaten die het promotieonderzoek heeft opgeleverd, staan gepresenteerd in dit proefschrift. Met dit werk heeft Sabine ook enkele prijzen gewonnen, zoals een posterprijs op de Europic meeting in Barcelona in 2008 en de prijs voor beste jonge onderzoeker van het Centrum voor Infectieziektebestrijding (RIVM) in 2010.

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In preparation:

Sabine van der Sanden†, Da-Wei Guan†, Han-Ri Zeng, Wei Li, Huan-Ying Zheng, Cong Ma, Juan Su, Zheng Liu, Xue Guo, Xin Zhang, Leng Liu, Chang-Wen Ke, Marion Koopmans. "Molecular Epidemiology of Human Enterovirus 71 in Mainland China, 1998-2010". †Contributed equally

Travel scholarships:

Sept 2011: Travel grant for the 14th annual meeting of the European Society for Clinical Virology, Madeira.
Sept 2010: Travel grant for Europic Meeting on Picornaviruses, Scotland.
Apr 2010: Travel grant for European Congress of Virology, Italy.
July 2009: Travel grant for meeting of the American Society for Virology, Canada.
May 2008: Travel grant for Europic Meeting on Picornaviruses, Spain.

Erasmus MC
University Medical Center Rotterdam

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student: Sabine M.G. van der Sanden
Erasmus MC Department: Virology
Research School: Molecular Medicine
Postgraduate School

PhD period: Oct 2006 – Sept 2011
Promotor: Prof.dr. M.P.G. Koopmans
Copromotor: Dr. H.G.A.M. van der Avoort

1. PhD training

	Year	Workload in Hours
General courses		
- Biomedical English Writing (Erasmus MC)	2007	80
- Endnote 9.0.1. (RIVM)	2007	7
- Oral presentations: design and performance (RIVM)	2008	20
- Design of scientific posters (RIVM)	2008	8
- Basic Introduction Course on SPSS (Erasmus MC)	2009	16
Specific courses		
- Virology course (Erasmus MC)	2008	40
- 15th international bioinformatics course on virus evolution and molecular epidemiology (Erasmus MC)	2009	40
- Advanced course Immunology (UMC)	2011	42
Seminars and workshops		
- Symposium Post-infectious diseases: molecular mimicry and beyond (Erasmus MC)	2007	16
- Workshop on Phylogeny and Genetics in microbiology and virology (Erasmus MC)	2008	8
- Mini Symposium Norovirus (RIVM)	2008	8
- Symposium "Zoönosen, bestrijd ze samen" (RIVM)	2009	8
- Workshop Bioinformatic Analysis, Tools and Services (Erasmus MC)	2009	8
- Minisymposium on Picornaviruses "Clinical relevance and antiviral therapy" (UMC St. Radboud)	2010	4
- Meeting Dutch Working group on Clinical Virology (RIVM)	2010	8
- Symposium Future Virology (Radboud University)	2011	6
- Dutch Annual Virology Symposium (KNAW)	2008-2011	28
- Workshops Proneri (PhD network RIVM, NVI, MNP): - How to improve your PhD? (2007) - Carreer options after having finished your PhD (2008) - How to get it published? (2009) - Software and management skills (2010)	2007-2010	24

Presentations (oral/poster)

- Europic Meeting Picornaviruses, Barcelona (oral + poster)	2008	16
- Wintermeeting European Society for Clinical Virology, Amsterdam (oral)	2009	8
- CIb PhD meeting, Zandvoort (oral)	2009	8
- Master class CDC Atlanta, Bilthoven (oral)	2009	8
- Spring Meeting Nederlandse Vereniging voor Medische Microbiologie, NVMM, Papendal (oral)	2009	8
- Meeting American Society for Virology, Vancouver (oral)	2009	8
- DAPS (Dutch Annual Picornavirus Society) meeting, Amsterdam (oral)	2010	8
- European Congres of Virology, Cernobbio (poster)	2010	8
- Europic meeting on Picornaviruses, St. Andrews (poster)	2010	8
- Meeting Dutch Working group on Clinical Virology, Bilthoven (oral)	2010	8
- Labmeeting surveillance of infectious diseases and emergence response, Guangzhou (oral)	2010	8
- 100th anniversary NVMM, Scientific Spring meeting, Papendal (oral)	2011	8
- Meeting on human enteroviruses, Edinburgh (oral).	2011	8
- Annual Meeting of the European Society for Clinical Virology, Funchal (poster)	2011	8
- Oral presentations (5) for department LIS-VIR	2006-2011	20

(Inter)national conferences

- Europic Meeting Picornaviruses, Barcelona, Spain	2008	40
- Wintermeeting European Society for Clinical Virology, Amsterdam, The Netherlands	2009	32
- Spring Meeting NVMM, Papendal, The Netherlands	2009	16
- Meeting American Society for Virology, Vancouver, Canada	2009	40
- European Congres of Virology, Cernobbio, Italy	2010	40
- Europic meeting on Picornaviruses, St. Andrews, Scotland	2010	40
- MEEGID X Conference, Amsterdam, The Netherlands	2010	24
- Labmeeting surveillance of infectious diseases and emergence response, Guangzhou, China.	2010	16
- 100th anniversary NVMM, Scientific Spring meeting, Papendal, The Netherlands	2011	20
- Annual meeting European Society for Clinical Virology, Funchal, Madeira.	2011	24

Other		
- Working Visit to CDC Atlanta, USA	2007	160
- Meeting World Health Organisation (WHO) on poliovirus eradication, Cairo, Egypt	2007	16
- CIb PhD meeting, Zandvoort, The Netherlands	2009	24
- WHO meeting on poliovirus eradication, Bilthoven, The Netherlands	2009	20
- Master class CDC Atlanta, Bilthoven, The Netherlands	2009	8
- DAPS meeting, Amsterdam, The Netherlands	2010	6
- Masterclass Prof. R. Rappuoli (Novartis, Siena), Amsterdam, The Netherlands.	2010	8
- Meeting on human enteroviruses, University of Edinburgh, Scotland	2011	8
- PhD meetings, department LIS-VIR, RIVM (6)	2011	9
- Coach personal development (RIVM)	2009/2010	12

2. Teaching activities

	Year	**Workload in hours**
Lecturing		
- Workshop on viral population dynamics, Guangdong CDC, China	2010	80
Supervising Bachelor and Master's theses		
- Supervising MSc Biotechnology graduate student	2009	90
- Supervising BSc graduate student	2009/2010	90
Other		
- Organisation Virology Introduction Day for high school students (RIVM)	2010	24
- Board of Proneri: organization of workshops, lectures and PhD meetings for PhD students of the RIVM, NVI and MNP.	2006-2010	160
- Organisation of retreat for Virology department, RIVM.	2009	15
Total Workload in hours (2006-2011)		1533