UNIVERSITÉ DE LA MÉDITERRANÉE

FACULTE DE MÉDECINE DE MARSEILLE

ECOLE DOCTORALE DE SCIENCES DE LA VIE

Laboratory diagnosis, molecular identification and epidemiology of Human Enteroviruses in Marseille, 1985-2011

T H È S E

Présentée et publiquement soutenue devant

LA FACULTÉ DE MÉDECINE DE MARSEILLE

Le 16 decembre 2011

Par **Mlle TAN Yanqi Charlene**

Née le 29 mai 1984 à Singapour

Pour obtenir le grade de DOCTEUR de L'UNIVERSITÉ de la MÉDITERRANÉE

SPÉCIALITÉ : MALADIES INFECTIEUSES

Membres du Jury de la Thèse :

Professeur Valérie GIORDANENGO	Rapporteur
Professeur Bruno POZZETTO	Rapporteur
Professeur Jean-Louis MEGE	Examinateur
Professeur Xavier DE LAMBALLERIE	Directeur de thèse

Acknowledgements

It is humbling to see 3 years' worth of sweat, adrenaline, tears, and lack of sleep reduced to a mere stack of papers. This could not have been achieved without the help and support of many and I thank you all.

Aux Prof. Valerie Giordanengo and Prof. Bruno Pozzetto, merci d'avoir pris le temps et d'avoir fait le trajet pour évaluer ce travail. Votre patience et vos conseils ont été essentiels à l'aboutissement de ce manuscrit.

Au Prof. Xavier de Lamballerie, vos idées dépassent souvent la capacité de mes neurones et de mes mains. Merci d'avoir toujours été présent.

Au Prof. Jean-Louis Mège, je ne serais peut-être pas là aujourd'hui à présenter ma thèse devant vous si ce n'était pas pour tous vos encouragements lors de mon Master. Merci.

A tout l'équipe d'UMR190 d'hier et d'aujourd'hui, vous avez partagé vos protocoles, vos vannes, vos runs de séquençage, votre kloug roulé sous les aisselles, vos mots fléchés, vos manies, vos rires, et votre amitié. Merci surtout pour ce dernier. A Julie et Laurence, 'Tout ça pour ça'.

A Muriel, Olivier, Maxime, Celia et Vincent, merci à Vous, enfin, à Eux, puisqu'il n'y a pas Toi. Si je suis folle aujourd'hui, c'est entièrement votre faute.

A François, Katherine and Nicolas, vous m'avez accueilli avec tant de chaleur et de cœur. Votre gentillesse et votre soutien me redonnent le souffle, merci d'avoir été ma deuxième famille.

To Gwen, you are my rock, my shoulder to cry on, my someone to lean on, my dearest friend for whom I will exhaust all clichés. Despite the vast lands and many seas that separate us, you are always there with the right words to heal my wounds and lift my spirits. Thank you Hwee Lian.

To my dearest papa, mummy and boy, many years I've been away and this is all I have to show for it. You have been my pillars of support, through every decision and every hiccup. The sacrifices you were willing to make so I could pursue my adventure speak volumes of your love and trust. I could never thank you enough. I hope I am at least half the daughter and sister you hoped to raise.

A Mathias, la force que tu as pour me supporter chaque soir au retour du travail, que je sois fatiguée ou (surtout) fatigante. Tu m'as ouvert les yeux et m'a rempli le cœur, le monde s’offre à moi quand je suis avec toi. Merci d'avoir fait ce bout de route avec moi. Je t'aime.

CONTENTS

ABBREVIATIONS

5'NCR: 5'non-coding region
3'NCR: 3'non-coding region
AFP: Acute flaccid paralysis
AHC: Acute hemorrhagic conjunctivitis
BGM: Buffalo green monkey kidney cells
CAR: Coxsackievirus and adenovirus receptor
CDC: Centers for Disease Control and Prevention
CODEHOP: Consensus degenerate hybrid oligonucleotide primer
CRE: Cis-acting RNA element
CSF: Cerebrospinal fluid
Da, kDa: Dalton, kilodalton
DAF: Decay accelerating factor, CD55
DNA: Deoxyribonucleic acid
eIF: Eukaryotic initiation factor
dFBS: decomplemented foetal bovine serum
FMD: Foot-and-mouth disease
Gln: Glutamine
Hep2: Human laryngeal carcinoma cells
ICAM-1: Intercellular adhesion molecule-1, CD54
ICTV: International Committee on Taxonomy of Viruses
IFN: Interferon
Ig: Immunoglobulin
IPV: Salk formalin-inactivated vaccine
IRES: Internal ribosome entry site
KB: Human laryngeal carcinoma cells
LBM pools: Lim-Benyesh-Melnick antisera pools
LOS: Length-of-hospital stay
MHC: Major histocompatibility complex
MRC5: Human foetal lung fibroblasts
NEAA: Non-essential amino acids
NF-kB: Nuclear factor-kappa B
NSP: Nonstructural protein
NTP: Nucleoside triphosphate
nt: nucleotide
OPV: Sabin oral live-attenuated vaccine
ORF: Open reading frame
PCBP: Poly(rC) binding protein
POC: Point-of-care
PVR: Poliovirus receptor, CD155
RNA: Ribonucleic acid
RDRP: RNA-dependent RNA polymerase
RT-PCR: Reverse transcription-polymerase chain reaction
ssDNA: single stranded DNA
TBP: TATA-box binding protein
TLR: Toll-like receptor
VAPP: Vaccine-associated paralytic poliomyelitis
Vero: African green monkey kidney cells
VLP: Virus-like particle
WHO: World Health Organization

Abbreviations for viruses are in accordance with the recommendations of the International Committee on Taxonomy of Viruses

CV: Coxsackievirus
cVDPV: Circulating vaccine-derived poliovirus
E: Echovirus
EV: Enterovirus
FMDV: Foot-and-mouth disease virus
HAV: Hepatitis A virus
HEV: Human enterovirus
HRV: Human rhinovirus
iVDPV: Immunodeficient vaccine-derived poliovirus
PV: Poliovirus
SVV: Seneca Valley virus

INTRODUCTION

TO ENTEROVIRUSES

A/ PICORNAVIRIDAE

The *Picornaviridae* family derives its name from its small size (pico meaning small in Latin, unit measurement of 10^{-12}) and its RNA genome, literally meaning small RNA virus. Picornaviruses are small non-enveloped viruses with a single strand RNA genome of positive polarity, and include many notable human and animal pathogens that have played important roles in the development of modern virology. The International Committee on Taxonomy of Viruses (ICTV) formally recognizes 12 distinct genera and 28 species in the Picornaviridae family: *Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus, and Tremovirus*. **Table 1** shows the current genera in the Picornaviridae family.

The genus *Aphthovirus* comprises three species: *bovine rhinitis B virus, equine rhinitis A virus,* and *foot-and-mouth disease virus*. Foot-and-mouth disease virus (FMDV) was the first animal virus to be discovered in 1898. It causes foot-and-mouth disease (FMD), a highly contagious disease that infects cloven-hoofed animals such as domestic cattle, swine and sheep, as well as wild deer (1) FMD has debilitating effects that can cause drastic losses in meat and milk production, and is thus of great agricultural and economic interest. During the 2001 FMD epizootic in Great Britain, 6.5 million animals were slaughtered and losses were estimated to be about £3.1 billion for the agricultural and food industry (2-3). FMDV is considered absent in North and Central America, Australia, Japan, Chile and most of Europe but remains present in Africa, South America, and parts of Asia and Europe.

The genus *Kobuvirus* consists of two species: *Aichi virus* and *bovine kobuvirus*. Aichi virus has been isolated in children with non-bacterial gastroenteritis and from travellers to Southeast

Asian countries (4). Three other kobuviruses (porcine, murine and canine), as well as other human and bat kobu-like viruses have been identified.

The genus *Cardiovirus* contains two species: *Encephalomyocarditis virus* and *Theilovirus*. Serotypes of these species cause encephalitis and myocarditis in numerous host species including mammals, birds, and insects. In particular, Theiler's murine encephalomyelitis virus (TMEV) can cause acute polioencephalomyelitis or chronic persistent demyelinating infection of the white matter (5).

The genus *Parechovirus* consists of two species: *Human parechovirus* and *Ljungan virus*. Human parechoviruses causes mild gastronintestinal and respiratory infections. Ljungan virus has been associated with perinatal death in natural rodent reservoirs and laboratory murine models, as well as, with intrauterine fetal death and diabetes in humans (6).

The genus *Hepatovirus* has one species: *Hepatitis A virus* (HAV) which causes acute liver inflammation (7). It is often characterised by jaundice and is not associated with chronic disease. It is primarily transmitted via by contact with infected household members, foodborne and waterborne contaminations. Travel to countries with HAV endemicity and poor sanitary conditions is a notable source of imported infections, and the HAV vaccine is recommended to international travellers.

The genera *Avihepatovirus*, *Sapelovirus*, *Senecavirus*, and *Tremovirus* were officially recognized as members of the *Picornaviridae* in 2009, and contain *duck hepatitis A virus*, *avian sapelovirus*, *porcine sapelovirus*, *simian sapelovirus*, *Seneca Valley virus (SVV)*, and *avian encephalomyelitis virus* (8).

Human rhinoviruses (HRV, Enterovirus genus) are highly prevalent respiratory pathogens, and are the predominant cause of acute respiratory tract infections such as the common cold. HRVs are frequently encountered worldwide and throughout life, and thus a major cause of school and work absenteeism. More than 90% of children have experienced at least one HRV infection by the age of 2 (9). Rhinoviruses were originally classified under the original Rhinovirus genus, but have now been integrated into the Enterovirus genus on the basis of their sequence homology. Rhinoviruses share an identical genomic organization and have similar functional RNA secondary structures with Enteroviruses, but differ in their acid tolerance, receptor usage, and cell tropism (10). More than 100 different HRV serotypes have been described for the HRV-A and HRV-B species. A novel genetically distinct third genotype HRV-C, comprising 49 designated serotypes, has now been recently recognized (11).

A number of picornaviruses remained unassigned to a species or genus. They have been found to infect bats (bat picornavirus), bluegill fishes (bluegill picornavirus), eels (eel picornavirus), sheep (hungarovirus), mice (mosavirus, rosavirus), pigeons (pigeon picornavirus), seals (seal picornavirus), turkeys (turkey hepatitis virus) and wild birds (turdiviruses). Two human picornaviruses have also yet to be assigned: (i) human cosaviruses (HCoSV, named for human common stool associated picornaviruses) were first described in 2008 in stool samples of South Asian children with nonpolio acute flaccid paralysis (AFP) (12). HCoSV genetically resemble cardioviruses (32.7% and 46% aa identity in the P1 and 3D regions respectively) and SVV (28.8% and 48.7% aa identity in the P1 and 3D regions respectively). Five genetically divergent candidate species of cosaviruses (HCoSV-A to E) have been proposed (13); (ii) human salivirus/klassevirus was identified in 2009 in paediatric

diarrhoea patient samples tested negative for known diarrheal viruses (14-15). It shares the greatest identity with kobuviruses, sharing 52.3% nt identity with Aichi virus across the entire genome.

Genus	Species	Serotypes
Aphthovirus	Foot-and-mouth disease virus Equine rhinitis A virus Bovine rhinitis B virus	7 1 1
Avihepatovirus	Duck hepatitis A virus	1
Cardiovirus	Encephalomyocarditis virus Theilovirus	3 12
Erbovirus	Equine rhinitis B virus	3
Enterovirus	Human enterovirus A Human enterovirus B Human enterovirus C Human enterovirus D Simian enterovirus A Bovine enterovirus Porcine enterovirus Human rhinovirus A Human rhinovirus B Human rhinovirus C	22 60 21 4 1 2 2 77 25 49
Hepatovirus	Hepatitis A virus	1
Kobuvirus	Aichi virus Bovine kobuvirus	1 1
Parechovirus	Human parechovirus Ljungan virus	14 4
Sapelovirus	Porcine sapelovirus Simian sapelovirus Avian sapelovirus	1 3 1
Senecavirus	Seneca Valley virus	1
Teschovirus	Porcine teschovirus	11
Tremovirus	Avian encephamyelitis virus	1

Table 1 : Current genera and species of the *Picornaviridae* family.

B/ ENTEROVIRUSES

The genus *Enterovirus* contains 10 species: 4 human enteroviruses (HEV-A, HEV-B, HEV-C, HEV-D), 3 human rhinoviruses (HRV-A, HRV-B, HRV-C), bovine enterovirus, porcine enterovirus B and simian enterovirus A **(Figure 1)**.

Human enteroviruses are important human pathogens that have marked the history and development of modern virology. Poliovirus, in particular, has played a significant role since its identification as the etiological agent of poliomyelitis in 1909 by Landsteiner and Popper. Poliomyelitis is characterised by muscle weakness and loss of muscle control, and it is of particular public health interest because it can cause disabling paralysis. Notable sufferers of poliomyelitis include: Itzhak Perlman, Frida Kahlo, and Francis Ford Coppola. Franklin D. Roosevelt, 32nd President of the United States, was said to be the most famous polio survivor, but this diagnosis has since been challenged. At the turn of the 20th century, localized epidemics started being reported in developed countries, but by the 1940s and 1950s, paralytic poliovirus infections were hitting pandemic proportions (16). In 1948, the team of Enders, Weller and Robbins successfully cultivated poliovirus in nonneuronal tissue cultures, the first time it had been grown and manipulated outside the body (17). This significant breakthrough allowed scientists to grow cells and virus on a large scale, and thus provided the basis for the discovery of poliovirus vaccines. It was also the first animal virus to be analysed by X-ray crystallography (18).

The Salk formalin-inactivated vaccine (IPV) and the trivalent Sabin oral live-attenuated vaccine (OPV) were licensed in 1955 and 1963 respectively (19-20). The success of these two vaccines led the way for the organisation of poliovirus eradication campaigns. In 1988, the

Global Poliovirus Eradication Initiative was launched by the World Health Organisation (WHO), Rotary International, the Centers for Disease Control and Prevention (CDC) and UNICEF. The WHO Region of the Americas (36 countries) was declared polio-free in 1994, the WHO Western Pacific Region (37 countries) in 2000, and the WHO European Region (51 countries) in 2002 (21-23). In France, the last indigenous and imported cases were reported in 1989 and 1995 respectively (24). Today, only four countries (India, Nigeria, Pakistan and Afghanistan) are considered endemic (25). Nevertheless, minor outbreaks do occur in neighbouring countries: China reported its first cases of wild poliovirus in 2011, in the Xinjiang Uygur Autonomous Region which borders three of the endemic countries, including Pakistan from where it has been shown to be imported (26). In conflict zones, such as Democratic Republic of Congo, sporadic cases have also been reported.

The urgency of vaccine development propelled the poliovirus to become one of the best-studied viruses and the subsequent model system for studying other RNA viruses and their replication in eukaryotic cells. As such, poliovirus is the prototypic enterovirus and its study is the basis for our understanding of enteroviruses. Poliovirus remains today at the forefront of RNA virus research. In 2002, Cello *et al* demonstrated the chemical synthesis of poliovirus cDNA by ligation of in vitro-synthesized oligonucleotides, in the absence of both a natural template and live cells (27). Such an ability to design PV genomes allowed Coleman *et al* to study virus attenuation by altering the natural codon usage (28).

1/TAXONOMY

EV were originally classified according to their pathogenicity in man and in newborn mice: (i) the poliovirus group that caused paralysis in humans; (ii) the coxsackie A viruses that induced flaccid paralysis in mice; (iii) the coxsackie B viruses that provoked spastic paralysis in mice; and (iv) the enteric cytopathogenic human orphan (Echo) virus group which had no associated disease. It was quickly realised that this mode of classification was inadequate due to significant overlaps in the biological properties of viruses in these groups, possibly because mice were not natural hosts of enteroviruses (29). More recently identified EVs are named using consecutive numbers. Within these groups, 64 distinct serotypes were determined on the basis of their neutralisation by specific antisera in Lim Benyesh-Melnick pools. The development of molecular typing techniques paved the way for the correlation of enteroviral antigenic serotype and molecular genotype. In particular, nucleotide sequences of the VP1 region have been demonstrated to correlate best with neutralisation-based serotype, as it is the site of major epitopes associated with serotype-specific neutralisation. Based on sequence identity of the region coding for the VP1 capsid protein, HEV are now classified into four species: HEV-A, HEV-B, HEV-C including Poliovirus, and HEV-D. Members of a species share at least 75% nucleotide (85% amino acid) identity in VP1, thus presenting a more rapid method of distinguishing EV serotypes (30-31) **(Figure 1)**. Serotype identification on the basis of VP1 sequence has led to some modifications in enterovirus classification. CVA9, previously classified as a coxsackie A virus based on pathology, is genetically more similar to coxsackie B and other HEV-B viruses. E8 (strain Bryson) was initially typed in 1953 as a distinct serotype, but has since been reclassified as a strain of E1 serotype (32). The genetic similarity between CVA11 and CVA15 and between CVA13 and CVA18 in the VP1

region might account for the antigenic cross-reactivity observed for these two pairs, and has led to their reclassification as variants of CVA11 and CVA13 respectively. Furthermore, one classical HRV serotype, HRV-87, was shown to be a variant of Enterovirus 68 (EV68) (33).

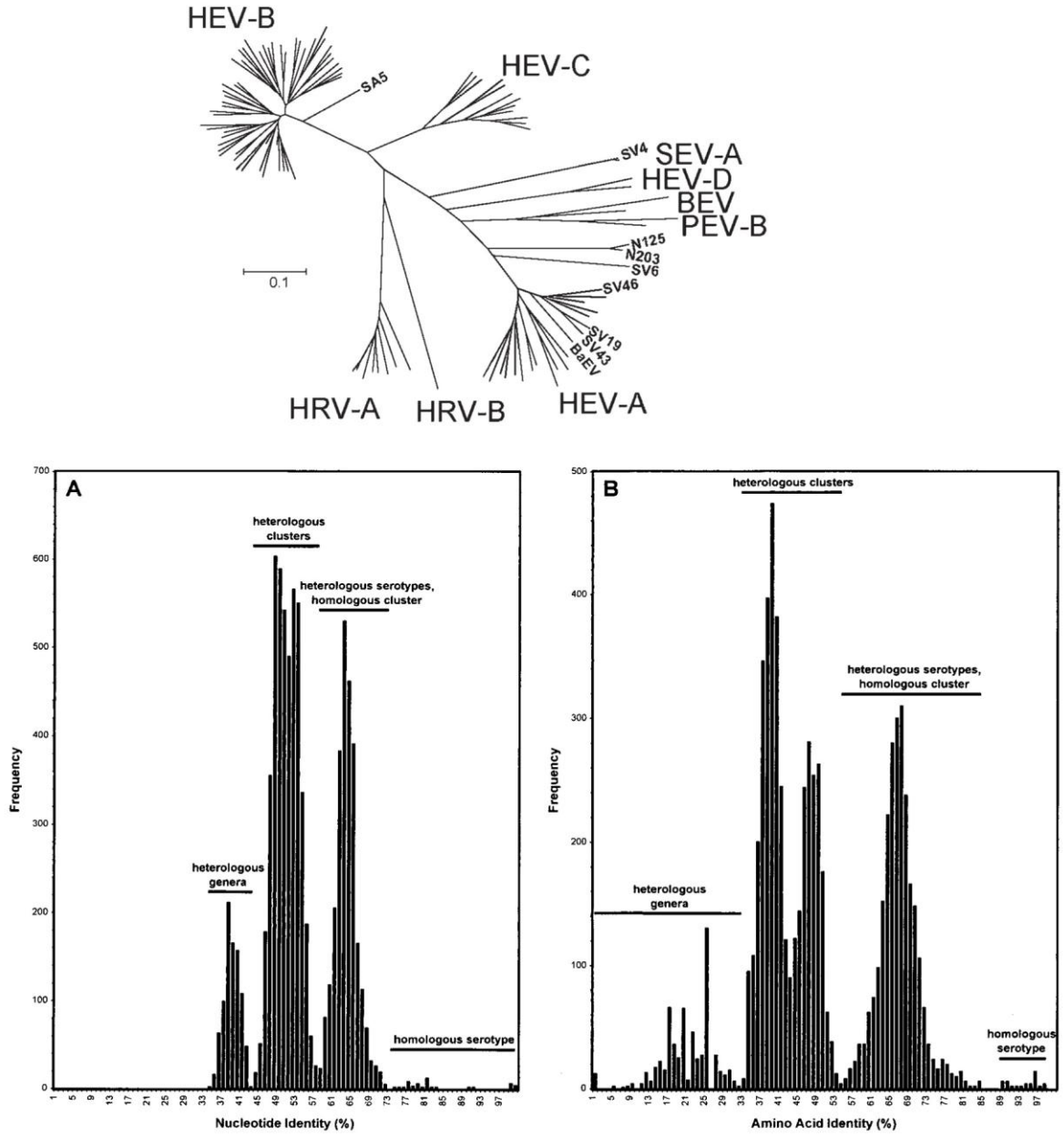


Figure 1 : Phylogenetic and genetic relationships of Enteroviruses based on VP1 gene sequence (31)

Frequency distribution of pairwise VP1 identity scores allow the distinction between sequences of the same serotype or species.

2/ EPIDEMIOLOGY

Human enteroviruses have no known extrahuman reservoirs or hosts.

2.1/ AGE

Age is an important factor in enterovirus transmission, severity of illness and outcome. Enteroviruses are the major cause of aseptic meningitis in both paediatric and adult populations in developed countries. Most primary infections occur during childhood due to the worldwide circulation of non-polio enteroviruses, and as such, higher rates of disease tend to be reported in children. Young children are the major source of enteroviral transmission in families (34). Encephalitis and aseptic meningitis associated with nonpolio enterovirus infection is most frequently reported in children 5 to 14 years old. In the particular case of poliovirus, the World Health Organisation guidelines for acute flaccid paralysis surveillance require coverage for children less than 15 years of age. Most diseases associated with enteroviruses tend to present more severely in adults than in children, leading to an overrepresentation of severe disease in adults.

2.2/ TRANSMISSION

Enteroviruses are primarily transmitted by faecal-oral and respiratory routes. They infect enteric and respiratory tract epithelial cells which facilitate direct excretion of viruses into the environment. They face the selection pressures of local secretory and cell-mediated gut immunity, and are acid stable, remaining infectious at pH values equal to or inferior to 3.0. Enteroviruses can survive for months in favourable environmental conditions (neutral pH, moisture, low temperature). At 20°C, poliovirus has been shown to be infectious at humidity levels greater than 50%, but decreases rapidly in infectivity if humidity falls below 40% (35).

The classic mode of EV transmission is the faecal-oral route with intermediate transmission via hands or fomites contaminated with faecal material. Direct contact with an EV-infected individual usually consists of hand contact with contaminated secretions and results in autoinoculation to the facial orifices. This is important considering young children are probably the most important hosts. Children regularly contaminate each other via soiled toys in day care centre, or even paediatric office waiting rooms (36-37).

Waterborne and foodborne transmission derive from the classic faecal-oral route, where water is the contaminated intermediate. It is considered minimal in developed countries where potable and used water are kept separate, but may predominate in areas with poor sanitary levels (38). EV can be transmitted by drinking contaminated water or eating food prepared with contaminated water or by contaminated persons. Transmission may also occur in contaminated recreational waters. Enteroviruses have been isolated in public swimming pools and wading pools in the presence of recommended chlorine levels (residual free chlorine 0.4ppm) and in the absence of coliform bacteria (39). Aseptic meningitis outbreaks have been associated with public swimming areas in Italy and in Germany (40-41). In a recent study by Begier *et al*, concurrent outbreaks of E30 and CVA1 infections in travellers returning from Mexico were associated with swimming in seawater (42).

Respiratory transmission may occur by direct of hand- or fomites-mediated inoculation of contaminated respiratory secretions into the mouth, nose or eye. This is the primary mode of transmission of the agents of acute hemorrhagic conjunctivitis (AHC), EV70 and CVA24 variant (43).

Severe presentations of EV infection in neonates have been associated with bloodborne vertical transmission from mother to foetus (44). Other forms of bloodborne transmission such as blood transfusion and mosquito bites do not seem to play a role in EV transmission.

2.3/ SEASONALITY

There is worldwide circulation of EV, with the exception of poliovirus. Enteroviral seasonality is influenced by geographical latitude: in temperate climates, EV activity has been noted to peak in summer and early autumn, whilst circulation is year-round in the tropical and semitropical regions. In France, a nationwide effort for enterovirus surveillance showed that between 2000 and 2004, the number of EV infections starts increasing each year in summer and early fall, with a peak in July before gradually petering out (45) **(Figure 2)**. In a 35-year surveillance study of enteroviruses in the United States, Khetsuriani *et al* detected a prominent summer-fall seasonality in EV cases, with 77.9% of all detections reported between June and October (46). Similar summer peaks have been described in Tunisia and Brazil (47-48).

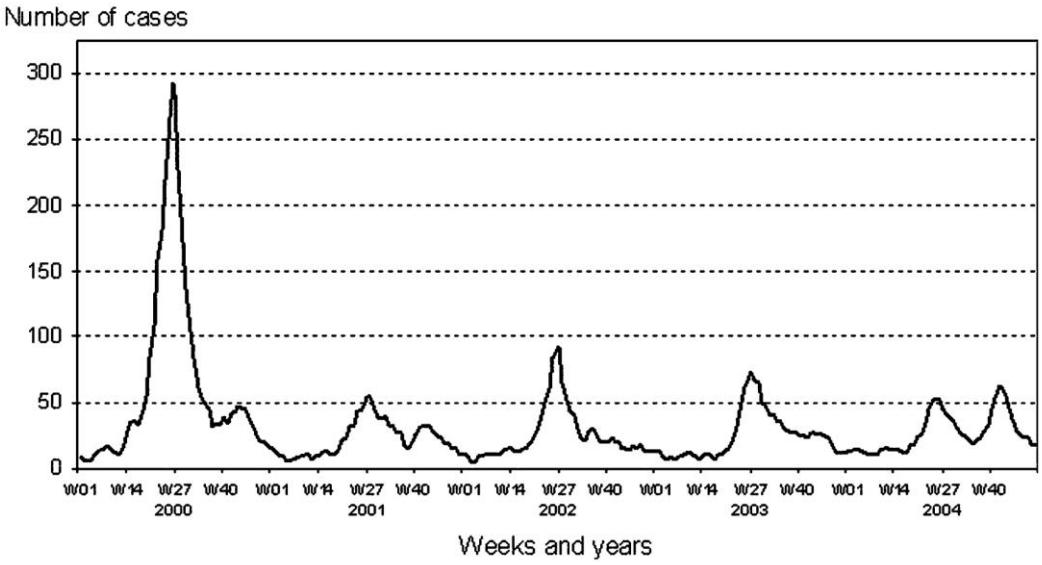


Figure 2 : Seasonal pattern of EV circulation, France 2000-2004 (45)

2.4/ IMPORTANT SEROTYPES

The prevalent serotype varies from year to year and it is not uncommon that several serotypes co-circulate. Recent surveillance studies describe a predominance of HEV-B serotypes, with E30 in particular, often one of the most frequent EV serotype isolated. This is reflected in the occurrence of E30-associated aseptic meningitis outbreaks, such as in Belgium in 2000, in Taiwan in 2001, in Brazil in 2001, in France in 2005, in Spain in 2006, and in Japan in 2007 (48-53).

Another HEV-B serotype, E13, has emerged as a global epidemic serotype in recent years. Before 2000, E13 had never been associated with outbreaks, only sporadically. In the United States, E13 accounted for 24% of all reported EV isolates in 2001, compared to 1.6% in 2000 (54). In Japan, 65 strains of E13 were isolated in 2001 and 2002, before which E13 has only been detected once (55). This was also the case described in Spain, where 135 E13 isolates were identified in 2000 alone (56). Furthermore, E13 has persisted since its re-emergence, and was the primary serotype detected during an outbreak of aseptic meningitis described in France in 2006 (52).

Enterovirus 71, a HEV-A serotype, has also emerged as a significant public health threat over the last two decades, but large-scale outbreaks have been confined to the Asia-Pacific region. EV71 is the main etiological agent of hand, foot and mouth disease (HFMD), which is usually considered a benign disease. However, it can cause serious complications of the central nervous system and result in fatal infections. In Taiwan in 1998, 129,106 cases of HFMD or herpangina were reported, including 405 cases with neurological disease and 78 deaths (57). Other outbreaks with neurological complications and/or fatal cases have been

described in Singapore in 1997, in Western Australia in 1999, in Japan in 2006 and in China in 2008 (58-61). No epidemic EV71 activity has been reported in Europe since the 1970s but sporadic cases have been increasingly isolated in France, Germany and the Netherlands (62-66).

In Finland in 2008, an untypical outbreak of HFMD (317 cases) was reported in an adult population. However, the main causative serotypes detected were CVA6 and CVA10 (67). CVA6 has previously been associated with an outbreak of herpangina and HFMD in Taiwan (68). These serotypes are considered rare, and may circulate endemically.

3/ DIAGNOSIS

Most EV infections are subclinical. Enteroviruses mainly infect the human gastrointestinal tract, but display few of the prominent gastrointestinal symptoms such as vomiting or diarrhoea. Many of the EV-associated diseases are likely to be secondary infections of nongastrointestinal cells (69). Furthermore, most symptoms are relatively generic. Nevertheless, EV testing can be prescribed if the patient is a young child, if infection occurs during the described EV seasonality, if an EV outbreak is currently underway, and if neurological symptoms suggest meningitis or encephalitis. The convenience of molecular diagnosis and the economy of sample use allow clinicians to prescribe EV testing with greater ease.

Laboratory detection of EV is performed today by the 'gold standard' method of a pan-EV quantitative RT-PCR (70). Amplification is targeted at the highly conserved domain V in the 5'NCR, thus capable of detecting all EV serotypes. The use of a fluorophore-coupled probe that anneals to another highly conserved motif in the target region allows accurate detections in real time, further reducing the time needed for diagnosis.

EV strains were previously identified by the classical method of seroneutralisation using Lim-Benyesh-Melnick (LBM) antiserum pools after virus isolation (71). However, this technique is time-consuming and incomplete method (given the identification of newer serotypes and the lack of the new antisera in LBM pools) has been replaced by molecular methods. Today, serotyping is performed by amplification of the VP1 capsid protein coding region (31).

4/ GENOME STRUCTURE

Enteroviruses are small, non-enveloped viruses that have a spherical shape of about 30nm in diameter. Enteroviruses share a same genome structure: a single-stranded RNA of positive polarity of approximately 7400 nucleotides **(Figure 3)**. It is covalently linked to a virus-encoded oligopeptide (VPg) at its 5' terminus which is implicated in the initiation of viral RNA replication. The enteroviral genome consists of a highly conserved 5' non-coding region (5'NCR), a coding region with a single open reading frame (ORF) which encodes a 250-kDa polyprotein, followed by a short 3' non-coding region (3'NCR) and a polyadenylated tail (72). The coding region contains a single open reading frame (ORF) which encodes a 250-kDa polyprotein, subsequently cleaved by virus-encoded proteinases into its structural and nonstructural proteins (73).

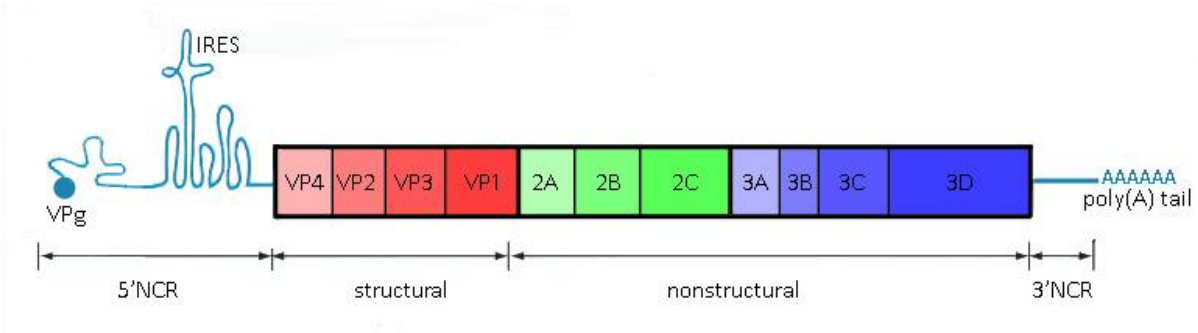


Figure 3 : Genome structure of human enteroviruses.

4.1/ NON-CODING REGIONS

The structural integrity of the 5' non-coding region (5'NCR) is essential for efficient viral replication of enteroviruses. The current model defines seven secondary structural domains **(Figure 4)** (74). Domain I is the characteristic cloverleaf element that is required for the initiation of negative-strand RNA synthesis. Domains II to VI contain the *cis*-acting internal

ribosome entry site (IRES), although studies have shown that only domains II, IV and V are required to act as a functional IRES. The IRES is responsible for recruiting ribosomes directly to a downstream AUG codon, in order to initiate the cap-independent translation mechanism. The 5'NCR also plays an important role in virulence, such that mutations in the 5'NCR decrease multiplication efficiency and virulence and alter cell tropism. This is best demonstrated in the Sabin vaccine, the three strains of which are attenuated by nucleotide substitutions in domain V of the 5'NCR (75-77). The sequence of domain II has also been shown to be a virulence determinant for CVB3 and PV. Domain IV contains a conserved hairpin loop with a 5'-ACCCC-3' motif that serves as an important recognition feature for poly(rC) binding protein (PCBP), as well as the GNRA tetraloop.

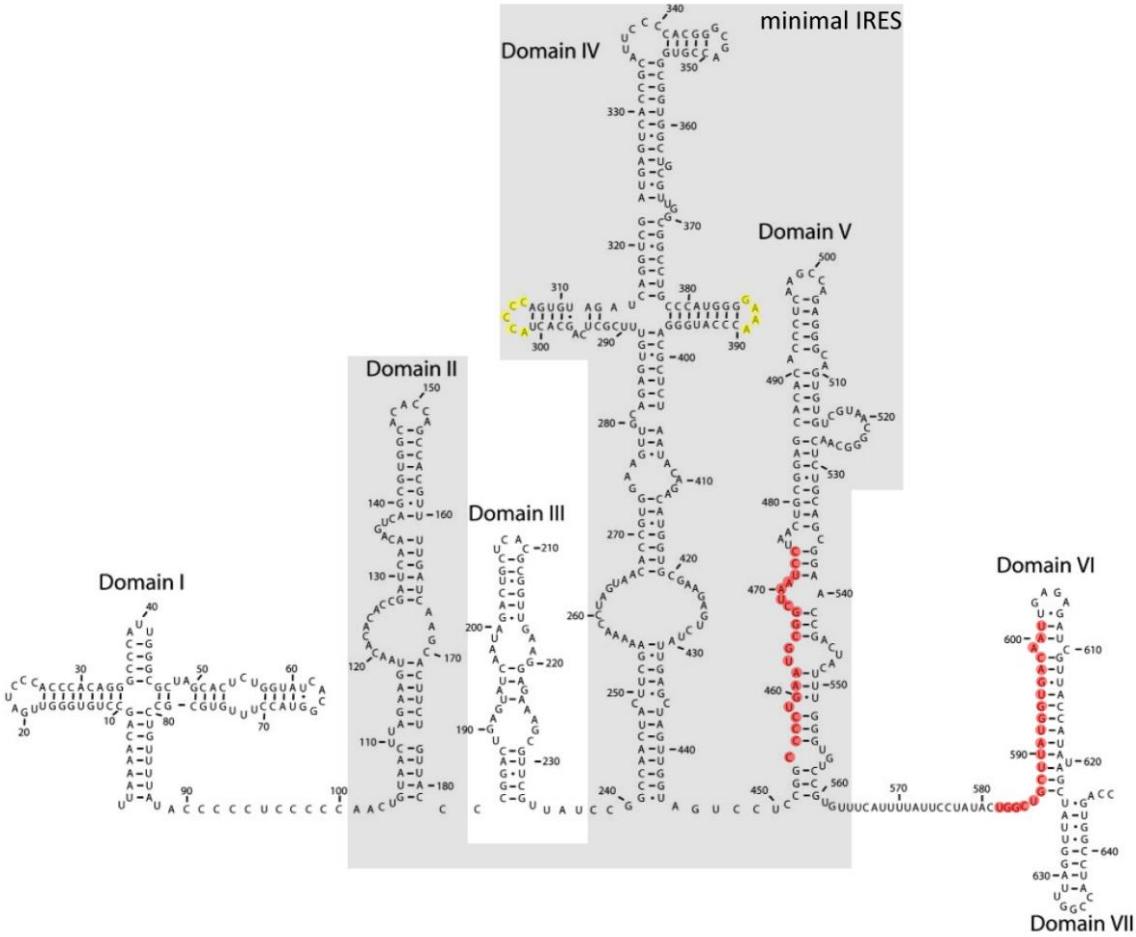


Figure 4 : Structural model of the CVB3 5'non-coding region (74)
Domain II, IV and V make up the minimal IRES. The PCBP recognition motif and GNRA tetraloop are shown in yellow. The annealing sites for diagnostic RT-PCR are shown in red.

The enteroviral 3'NCR is highly conserved among members of a species (70-99% identity) but highly divergent otherwise (<62% identity). It is necessary for efficient enterovirus replication and is the site of initiation of negative-strand RNA synthesis (78). Analysis of a mutant lacking the entire genome 3'NCR showed decreased viral fitness, which resulted from a defect in positive-strand RNA synthesis, suggesting that it is also involved in positive-strand synthesis. Predicted structures in the 3'NCR include three structural domains in the form of stem-loops (X, Y and Z) in HEV-A and HEV-B, and two (X and Y) in HEV-C and HEV-D. Enteroviruses cluster in the 3'NCR according to the number of structural domains, such that it mirrors the clustering observed in the 5'NCR.

The poly(A) tail is essential for the efficient replication of EV such that negative-strand synthesis increases with the length of the poly(A) tail (79). The team of Silvestri *et al* showed that the difference of a single adenosine residue in a poly(A)(13) tail increased negative-strand synthesis by tenfold compared to a poly(A)(12) tail. A poly(A) tail of 20 bases allows optimal efficiency of RNA synthesis.

4.2/ STRUCTURAL PROTEINS

The P1 region encodes the structural proteins VP4, VP2, VP3 and VP1 that form the enteroviral capsid of icosahedral symmetry.

VP4 is small and entirely internal in the mature virion. It is covalently linked to myristic acid (n-tetradecanoic acid) at a glycine residue of the amino terminus. The myristyl groups interact with amino acid side chains of VP4 and VP3 and plays a role in virus assembly and in the stability of the capsid (80). VP1, VP2 and VP3 are structurally similar **(Figure 5)**: each consists of 250 amino acids arranged as an eight-stranded antiparallel β-barrel with two

flanking α-helices (81). The β-barrels are joined at one end by four short loops such that it is wedge-shaped, allowing the capsid proteins to pack tightly around the symmetry axes. The uppermost three loops of the barrel are exposed, forming a peak at the fivefold axis. The exposed loops of VP1 constitute major neutralizing antibody sites (82). The peak at the fivefold axis is surrounded by a 15 Å deep canyon (24 Å deep and 30 Å wide) that is the putative site of binding. A hydrophobic pocket within the b-barrel of VP1 lies just beneath the floor of the canyon and is normally occupied by a lipid pocket factor. This factor must be displaced by interaction with the receptor for uncoating and release of RNA can occur. This pocket can also be filled by lipophilic antiviral compounds, which stabilize the capsid and prevent conformational changes associated with uncoating.

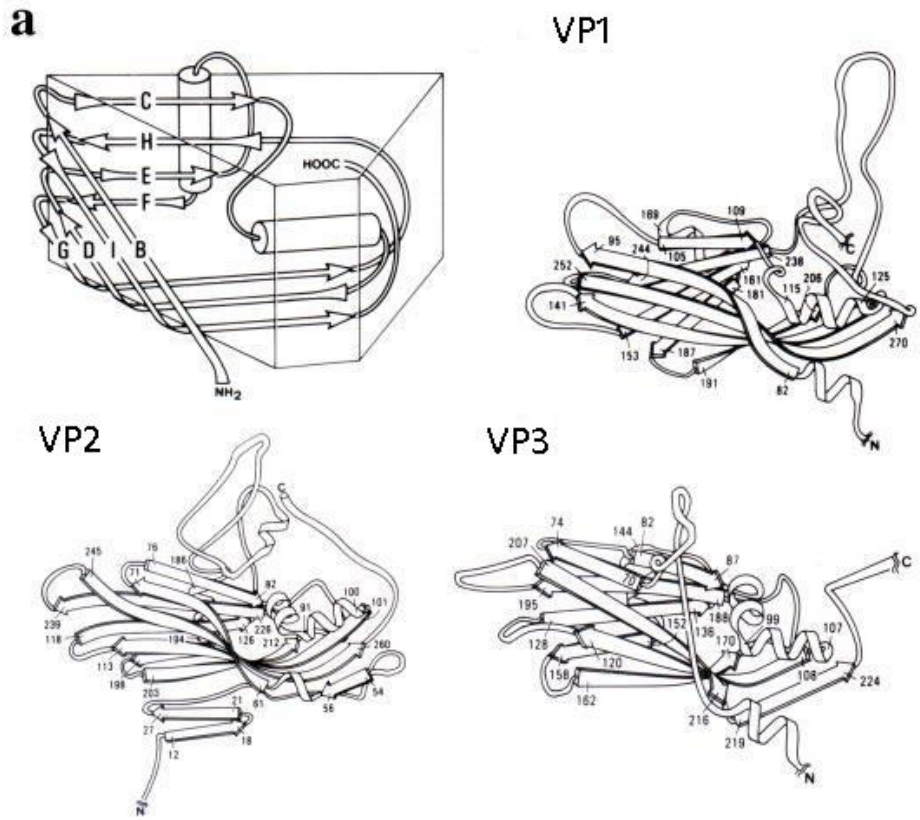


Figure 5 : Structure of capsid proteins VP1, VP2 and VP3 (102)
All three surface capsid proteins are structurally similar, and share a common eight-stranded β-barrel structure represented in (a).

4.3/ NONSTRUCTURAL PROTEINS

The P2 region encodes nonstructural proteins 2A, 2B and 2C. 2A is a small trypsin-like proteinase that cleaves at the VP1/2A junction, separating the structural P1 from the non-structural protein precursors. 2A protease is also responsible for the inhibition of host cell synthesis by cleaving eIF-4G (83-84). 2B and its precursor 2BC are responsible for enhancing membrane permeability in infected cells. 2B contains an cationic amphipathic helix domain that interacts with cellular membranes and forms the viroporin complex (85-86). 2C is the most strongly conserved protein in the enteroviral genome. It has well-documented nucleoside triphosphatase activity (ATPase and GTPase) and contains three known conserved motifs: an amino-terminal amphipathic helix, a NTP-binding site, and a putative zinc finger (87). These motifs are also often found in proteins of the helicase superfamily II, thus 2C is often attributed a putative helicase function. However, this helicase activity has yet to be demonstrated despite numerous studies.

The P3 region encodes the nonstructural proteins 3A, 3B, 3C and 3D. It is thought that protein 3A acts as the scaffold of the viral replication complex of unknown structure and composition (88). 3B is the precursor of VPg, whose uridylylation is essential for viral RNA synthesis (89). 3C is the catalytic core of the protease that releases the capsid proteins. It can fold and behave as a protease even while uncleaved in the polyprotein. It cleaves the host cell eIF5B initiation factor, as well as at its own N-terminus to generate 3AB and 3CD (90-91). 3CD processes the P1 region, cleaving between VP0-VP3 and VP3-VP1. 3D is the 53 kDa viral RNA-dependent RNA polymerase and requires a primer for viral protein synthesis. It contains the palm, thumb, and fingers domains commonly used to describe polymerases, with a characteristic core structure of RNA polymerases (92). It lacks a proofreading

mechanism such that it has a high error rate frequency in the order of 10-3 to 10-4 (93). This error rate is believed to contribute to the high genetic diversity observed in enteroviruses. However, a more recent study by Freistadt *et al* measuring the fidelity of 3D polymerase proposed that EV genetic variation is not due to intrinsically low polymerase fidelity (94).

5/ VIRAL REPLICATION CYCLE

Figure 6 provides an overview of the enteroviral replication cycle.

5.1/ CELL ATTACHMENT

Host range and cell tropism are dependent on receptor recognition. Viral attachment of EV is mediated by a variety of host cell receptors located in the canyons surrounding the fivefold axes of symmetry. The poliovirus receptor (PVR, or CD155) is recognized by all three poliovirus serotypes (95-96). Cellular binding sites for coxsackieviruses include the intercellular adhesion molecule-1 (ICAM-1, or CD54) which is expressed on respiratory epithelial cells, and the coxsackievirus and adenovirus receptor (CAR) which is strongly expressed in the developing central nervous system (97-98). CD155, CD54 and CAR are transmembrane glycoproteins belonging to the immunoglobulin superfamily, containing two, three and five extracellular immunoglobulin-like domains respectively. In all three receptors, the N-terminal domain D1 contains the virus recognition site, distal from the plasma membrane.

The cell attachment of echoviruses is mediated by integrin VLA-2 (CD49b/CD29), a cell surface glycoprotein that is also responsible for interaction with collagen and laminin (99). The capacity to bind echoviruses has been mapped to the a2 subunit, within the I domain (100). Decay accelerating factor (DAF, or CD55) is a glycoprotein that regulates the activity of the complement system, and protects host cells from attack by the immune system . It also functions as a cellular receptor for certain enteroviruses, including CVB3, CVB5, E7 and E12 (101). Different viruses can bind to different domains of the functional region: the binding site on CVB3 differs from that on echoviruses. The presence of DAF receptors are not

sufficient for cell entry and may require the presence of a co-receptor, such as in the case of CVA21.

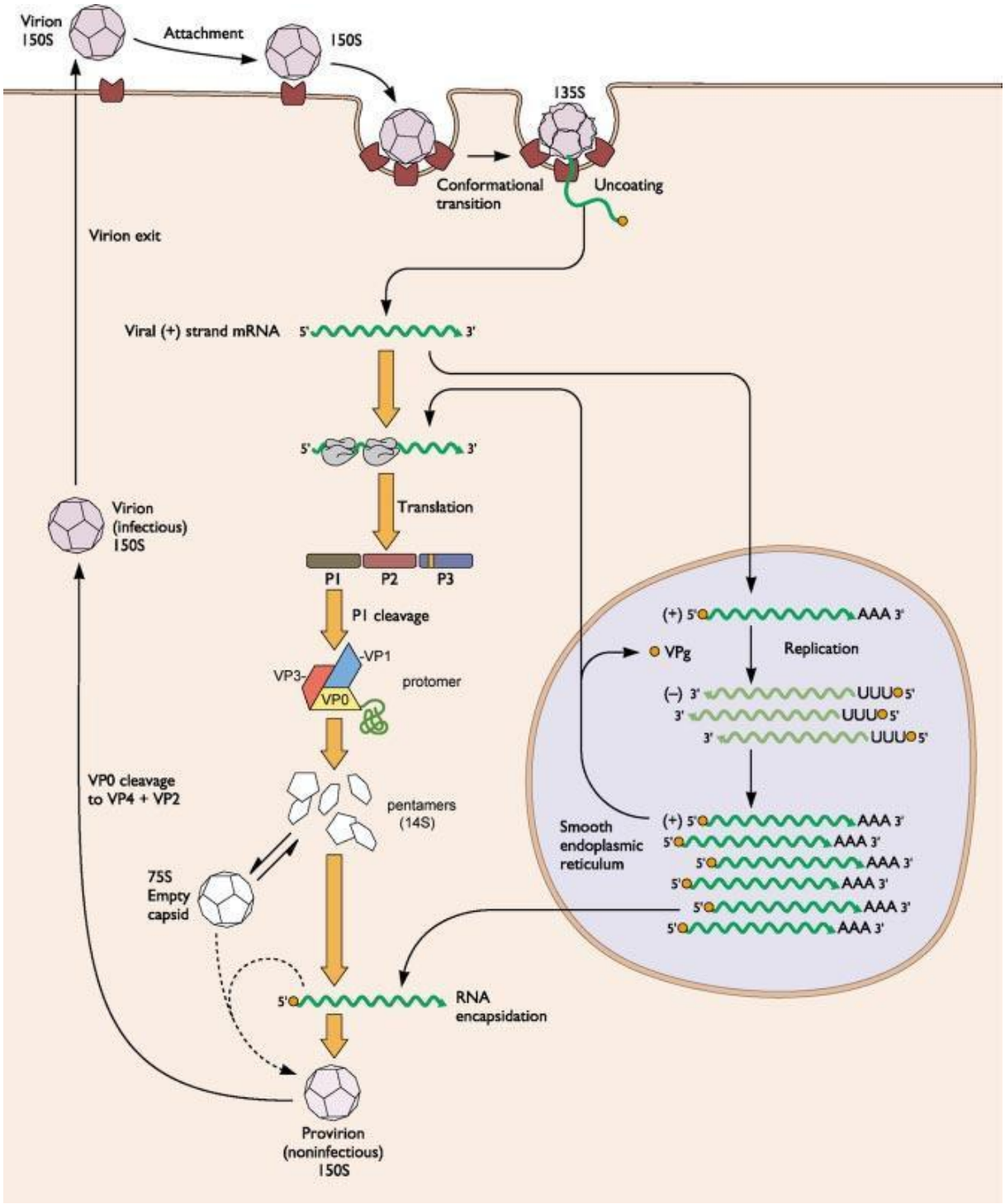


Figure 6 : Replication cycle of the enterovirus (102)
Enteroviral replication occurs entirely in the cytoplasm of the host cell.

5.2/ UNCOATING OF THE VIRAL GENOME

Enteroviruses replicate exclusively in the cytoplasm of the target cell. Purified genome RNA is infectious, indicating that the stage of viral RNA genome release into the cell cytoplasm is essential for the initiation of replication, but that viral structural proteins are not needed. Despite being the focus of many studies, the exact mechanism of uncoating remains unclear.

It is generally accepted that native 160S viruses sustain an irreversible conformational change upon binding to the cell receptor at physiological temperatures (102). The viral capsid is destabilised and the internal VP4 protein is released. The hydrophobic amino terminus of VP1 capable of binding to liposomes, is extruded to the surface. The resulting 135S virions with an altered structure are known as A particles. Upon longer incubation periods, 80S particles without genomic RNA are formed, but it has not yet been shown if this is part of the uncoating process. The team of Bostina *et al* have shown by cryo-electron tomography that RNA release occurs near the twofold symmetry axis (103).

The team of Brandenburg *et al* used fluorescent labels to trace the path of capsid proteins and RNA in live HeLa cells, and determined uncoating to take place within 20 minutes of cell receptor attachement and by a pathway independent of clarithrin- and caveolin-mediated endocytosis (104). However, the team of Coyne *et al* described the detection of virus in caveolin-containing vesicles of human brain microvascular endothelial cells, suggesting that poliovirus may use different entry mechanisms according to cell type. However, the poliovirus particle-to-pfu ration is relatively high and complicates our understanding of the uncoating process as it is unsure if the observed viral particles are infectious or defective.

5.3/ VIRAL PROTEIN TRANSLATION AND PROCESSING

The synthesis of viral proteins is preceded by the inhibition of host cell protein synthesis. Host cell mRNAs rely on cap-dependent initiation of translation, which is directed by the eIF-4F (eukaryotic Initiation Factor 4F) complex and comprises initiation factors eIF-4A, eIF-4E and eIF-4G. This complex recruits 40S ribosomal subunits to the 5' cap of cellular mRNAs, where it scans for the AUG start codon. Protein synthesis begins when the initiator tRNA charged with methionine associates with the complex and a 60S ribosomal subunit completes the 80S ribosome. When EV infection occurs, the 2A protease cleaves eIF-4G (formerly p220) such that host cell protein synthesis is inhibited. (105). However, it has been suggested that the cleavage of eIF-4G is necessary but insufficient for host cell shutoff (106). The team of Kuyumcu-Martinez *et al* demonstrated that the 3C protease expression in HeLa cells caused the partial cleavage of poly(A) binding protein (PABP) and the subsequent inhibition of host cell mRNAs. They proposed that host translation shutoff is mediated by both eIF-4G cleavage by 2A protease and PABP cleavage by 3C protease (107).

Cap-independent translation is initiated via the type I IRES located in the EV 5'NCR. A conserved polypyrimidine tract contains a *cis*-acting RNA element (CRE, UUUCC motif) that is essential for translation (108). *Trans*-acting host cell factors bind to the IRES and help recruit the 40S ribosomal unit that scans for the authentic AUG codon. The EV genome is translated as a single, large polyprotein that undergoes co- and post-translational processing into its viral protein components **(Figure 7)**. 2A protease hydrolyes a tyrosine-glycine bond at its own amino terminus to separate the structural P1 and nonstructural P2-P3 regions (91). This cleavage must occur for subsequent processing of the capsid proteins to proceed (109). The 3C protease precursor and the 3CD precursor then cleave the glutamine-glycine bonds

between the nonstructural proteins and between the structural proteins respectively (110-111). Myristoylation of the polyprotein is required for efficient processing by 3CD (80).

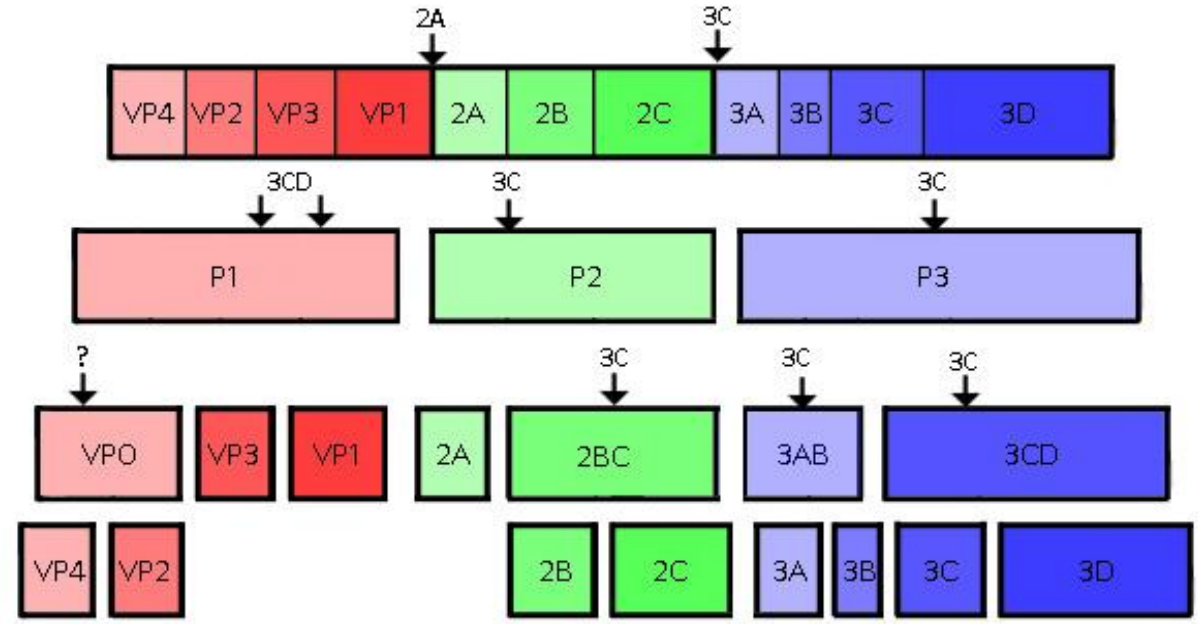


Figure 7: Polyprotein processing into viral proteins.
Viral proteases 2A, 3C and 3CD precursor cleave the translated polyprotein into its components. The cleavage sites of 2A and 3C are the tyrosin-glycine dipeptide and the glutamine-glycine dipeptide respectively.

5.4/ VIRAL RNA SYNTHESIS

The viral positive-strand genomic RNA is a template for both protein translation and RNA replication. The replication cycle occurs entirely in host cell cytoplasm and begins with the synthesis of negative-strand RNA intermediate. 5'NCR cloverleaf structure is necessary for the stability of ribonucleicprotein complexes with viral and cellular proteins. A CRE in the ORF acts as a template for the uridylylation of the virus-encoded VPg (112). In the poliovirus, this CRE is a stem-loop RNA structure located in the 2C region and is conserved in all human enteroviruses. VPg is covalently linked to two uridine residues to form $VPgpUpU_{OH}$ in a

reaction catalysed by the 3D polymerase. The VPg-linked poly(U) at the 5'terminus of negative-strand RNA complements the poly(A) tail at the 3'terminus of positive-strand RNA to form a poly(A)-poly(U) duplex, such that they are reciprocal templates and prime RNA synthesis (113). VpgpUpU$_{OH}$ is necessary for priming positive-strand replication and negative-strand RNA is used as a template for the synthesis of 40-70 copies of positive-strand progeny genomes.

5.5/ ASSEMBLY AND RELEASE OF VIRAL PROGENY

One molecule of each capsid protein, VP0, VP1 and VP3, comprise the immature protomer (114). Five protomers assemble into a 14S pentamer and twelve pentamers form the capsid **(Figure 8)**. The immature virion is formed by direct association of the capsid with the viral RNA, or by formation of an empty capsid into which viral RNA is injected (89). VP0 is cleaved into VP4 and VP2 to produce the mature infectious virion. The enteroviral capsid of icosahedral symmetry is composed of sixty copies of each structural protein, the structure of which has been illustrated by X-ray crystallography (115-117).

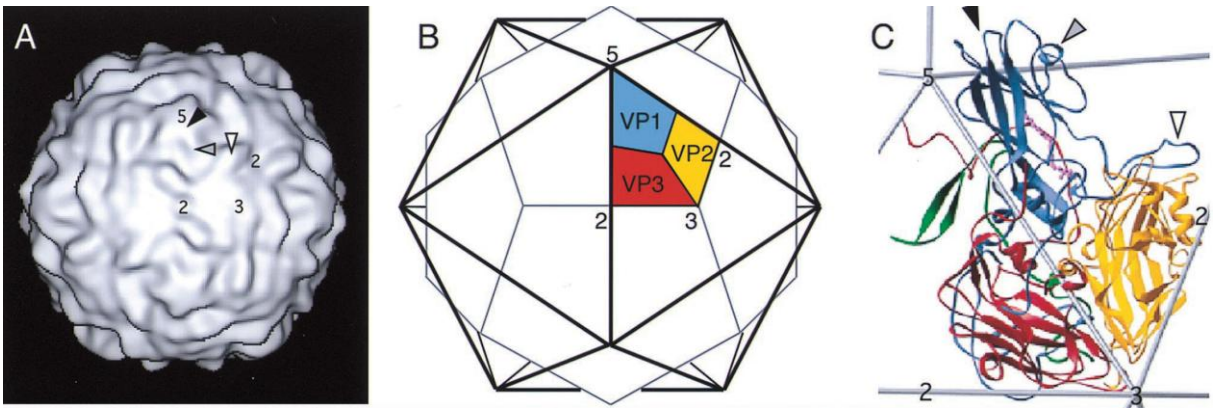


Figure 8 : Structure of the mature poliovirus particle (117)
A) X-ray crystallography visualization. B and C) The details of one protomer is shown, with capsid proteins VP1, VP2 and VP3 in blue, yellow and red respectively.

6/ CLINICAL ASPECTS OF ENTEROVIRUS INFECTIONS

6.1/ CLINICAL MANIFESTATIONS

Most enteroviral infections are asymptomatic or subclinical. When disease does result, EV can cause a wide spectrum of clinically distinct illnesses. This includes milder syndromes such as hand, foot and mouth disease, herpangina, and acute haemorrhagic conjunctivitis. On the other end of the spectrum, their neurotropism can cause serious, potentially life-threatening central nervous system complications such acute flaccid paralysis, aseptic meningitis and encephalitis. It has also been suggested that EV infection contribute to acute and chronic myocarditis, pleurodynia, and diabetes. Generally, there is no direct association between an individual serotype and a particular pathology. Exceptions whereby such an association exists, include CVA24 or EV70 and acute haemorrhagic conjunctivitis; PV or EV71 and acute flaccid paralysis; and EV71 and CVA16 and hand, foot and mouth disease.

Some EV-induced illnesses are mild and usually self-limiting. Herpangina is characterised by sudden fever and sore throat, caused by lesions on the soft palate and tonsils. Hand, foot and mouth disease is associated with the exanthema of the hand, feet and mouth (118). Acute haemorrhagic conjunctivitis involves periorbital swelling, excessive tearing and keratitis that can lead to eventual visual impairment (119).

However, EV can also cause serious neurological complications. Poliomyelitis is a paralytic disease and is classified depending on the involvement of spinal cord or brainstem (spinal or bulbar paralysis respectively) or both (bulbospinal paralysis) (120-121). Spinal polio is the most common form of paralysis and involves the destruction of motor neurons and spinal gray matter. The absence of nerve stimulation eventually leads to muscle atrophy and flaccid

paralysis. Bulbar polio affects motor cranial nerves and results in facial weakness and difficulties swallowing and talking.

EV infection has been identified as the primary cause of aseptic meningitis, the nonbacterial inflammation of the meninges, in both children and adults. Infected individuals often experience fever, headache, photophobia, rash and meningeal signs (122). Aseptic meningitis is usually self-limiting and has a more favourable prognosis than encephalitis. Encephalitis implicates infection of brain parenchyma and causes more severe neurological symptoms such as seizures. Meningoencephalitis, the association of both aseptic meningitis and encephalitis, is not uncommon.

There is also cause to believe that EV may be implicated in myocarditis, the inflammation of the myocardium. Acute myocarditis was present during the autopsies of newborns who died of overwhelming group B coxsackieviruses and EV negative-strand RNA has been detected in valvular tissue of chronic rheumatic heart disease patients (123-124). Furthermore, EV infection may play a part in insulin-dependent diabetes, since anti-coxsackie B virus IgM has been detected in a significant number of newly-diagnosed diabetes (125). The team of Yoon *et al* produced a transient form of diabetes in monkeys after inoculation with CVB4 (126).

6.2/ HOST IMMUNE RESPONSE

The efficacy of a functional immune response is important for the outcome of EV infections. This is particularly true in immunodeficient patients and neonates who are susceptible to severe forms of EV diseases (127-128).

In vitro studies showed that NF-kB (nuclear factor-kappa B) is activated early after EV infection. Host defense mechanisms such as NF-kB-mediated expression of cytokines and interferons are also triggered. However, the downstream effects of NF-kB are inhibited by enteroviral proteases 2A and 3C by cleavage of the p65-RelA component of NF-kB (129).

The B-cell humoral immune response is vital in neutralizing extracellular enterovirus (130). It consists of anti-enteroviral immunoglobulin (Ig) that is rapidly produced upon infection. IgM titers can persist for months, and years in the case of IgG and IgA. Since the majority of EV infections are asymptomatic, detectable levels of IgM can be found in healthy individuals. Sufficient levels of anti-enteroviral IgA can be transferred passively from mother to child via breast milk (131). Antigenic epitopes are found mainly on the surface capsid proteins, such as the BC loop in the VP1 protein. Cell surface Toll-like receptor 4 (TLR4) and intravesicular TLR3 are involved in the anti-enterovirus immune response (132). TLR3-knockout mice developed more severe myocarditis and suffered increased mortality when challenged with CVB3 (133).

The T-cell immune response plays a role in clearing intracellular enteroviral infection. CD4+ and CD8+ cytotoxic T-cells have been found in human individuals who have received PV vaccination. However, the expression of antigens by major histocompatibility complex (MHC) class I is inhibited in PV infected cells, which decreases T-cell activity (134).

6.3/ VACCINE

The only enterovirus for which a vaccine exists is the poliovirus. Two vaccines provide protection from poliovirus infection: The Salk formalin-inactivated vaccine (IPV) and the trivalent Sabin oral live-attenuated vaccine (OPV), licensed in 1955 and 1963 respectively

(19-20). Vaccine use culminated in the near-eradication of PV, which only persists endemically in four countries today.

The Sabin OPV was initially favoured for several reasons: (i) it replicates efficiently in the human gastrointestinal tract, the primary site of infection, but not in nervous system tissue; (ii) the possibility of oral administration of vaccine facilitated mass vaccination campaigns; and (iii) provides longer lasting immunity than the Salk vaccine, as well as providing herd immunity in non-vaccinated individuals (135). However, despite its many advantages, the use of OPV has one major drawback: given the potential of PV to mutate and to recombine in order to maintain fitness and viability, the live-attenuated vaccine can be considered genetically unstable. Its reversion to a more virulent neuropathogenic form has been detected in approximately 1 in 750 000 vaccinations, and causes vaccine-associated paralytic poliomyelitis (VAPP) (136). The Sabin vaccine is also the potential source of circulating vaccine-derived polioviruses (cVDPV), defined as OPV isolates with a VP1 nucleotide sequence divergence of more than 1% from that of the reference vaccine strain, and can be generated by recombination of vaccine strains and closely related HEV-C serotypes (137). Furthermore, immunodeficient vaccine recipients can become chronically infected and become long-term excretors of immunodeficient vaccine-derived polioviruses (iVDPV) but this remains extremely rare (138). The occurrence of cVDPV and iVDPV has created what is known as the 'OPV paradox': OPV use has prevented wild PV infections but is now responsible for the remaining forms of poliomyelitis. OPV use needs to be discontinued if complete eradication is to be achieved (139).

As of 2003, the public health benefit of OPV was still deemed by the WHO to outweigh the occasional risk of VAPP. Given the decline in wild poliovirus incidence and the relative

proportional increase in VAPP, the feasibility of using IPV as the primary polio vaccine has been further assessed (140-141). In contrast to OPV, IPV does not contain infectious virus and has no possibility of reversion. Intramuscular administration of IPV provides reduced intestinal immunity. IPV is also more expensive to produce, and the current production capacity is not sufficient for the 425 million doses estimated to replace OPV completely (142-143). IPV has been used in developed countries to replace OPV in the immunisation schedule, with virtually no evidence of VAPP. After such a switch in New Zealand in 2003, children found to excrete OPV strains before the change no longer did so a month after (144). The use of IPV remains unrecommended by the WHO in developing countries.

Since 1999, no wild PV2 strain has been detected (138). Wild type 1 and type 3 viruses still circulate but with a much reduced genetic diversity. Most wild PV lineages circulating in 1985 have been eradicated, with only one distinct PV1 lineage present in India today for example. Eradication efforts were first focused on the use of monovalent type 1 poliovirus vaccine, but this resulted in the re-emergence of PV3 in the population (145). Bivalent OPV is currently being used in India (146).

A new strategy has been implemented in the search for a better poliovirus vaccine with fewer associated risks. Due to the degenerative nature of the genetic code, adjacent amino acids can be encoded by many different pairs of codons. However, a bias in codon pair usage is such that certain codons are used more or less frequently than others. The team of Coleman *et al* synthesized PV genomes with rare codons while conserving the amino acid sequence of wild-type PV in a process called 'synthetic attenuated virus engineering' (28). They demonstrated that underrepresented synonymous codon pairs negatively affected translation and exhibited an attenuated phenotype. These attenuated polioviruses were

capable of providing immunity in mice. Such a technique could represent a new strategy for attenuating other viruses.

There is currently no vaccine available for nonpolio enteroviruses.

In the post-poliovirus eradication period, EV71 has emerged as an important neurotropic human enterovirus. While illnesses caused by other nonpolio enteroviruses tend to be mild and self-limiting, fatal EV71 infections have been described worldwide. There is thus a conscientious effort to look into developing an effective EV71 vaccine (147). The strategies that successfully generated the poliovirus vaccines have been implemented on an EV71 model. Inactivation by heat or formalin treatment of vaccine strains has been shown to confer protection against wild-type EV71 challenge in suckling mice after passive transfer of serum from adult mice inoculated with the inactivated vaccine strain (148-149). However, an increased dose of antigen and adjuvant is required to counterbalance the inactivation for viable levels of protection. The team of Arita *et al* also produced an EV71 strain (S1-3') attenuated by mutations based on those found in the attenuated poliovirus vaccine strain, in the 5'NCR and 3D regions. Monkeys inoculated with S1-3' strain survived a subsequent challenged with a lethal dose of the virulent parent strain, but did suffer from mild neurological symptoms (150-151). However, concerns regarding the conversion of a live attenuated vaccine strain to a neurovirulent revertant such as that witnessed for PV also surround EV71.

To avoid the risk of reversion, other teams have also considered vaccines consisting of either only a few 'subunit' viral proteins or empty virus-like particles (VLPs) (149, 152). This strategy is based on the stimulation of the immune responses to the virus upon interaction with viral

proteins, but does not run the risk of reversion since no intact copies of the viral genome are present.

6.4/ ANTIVIRAL TREATMENT

Despite the medical and socio-economic impact of enterovirus infections, there are currently no commercial antiviral treatments available. In theory, every step of the viral replication cycle is potentially a target for inhibiting enteroviruses. Antiviral drugs have been developed that target structural and non-structural proteins have shown to be interesting candidates for enterovirus treatment (153-155).

Assembly and release

Hydantoin prevents the encapsidation of viral RNA progeny. When tested at a concentration of 25 μg/mL, it inhibited PV1 and PV2 replication by at least 95% and PV3 and CVA21 by more than 99%. Resistance of poliovirus to hydantoin was mapped to the 2C region (156).

Capsid-binding proteins

One important feature of the viral capsid is the presence of a canyon with an underlying hydrophobic cavity. The canyon acts as a major site for virus-cellular receptor binding, and harbours the receptors for poliovirus, echovirus, and coxsackievirus. The hydrophobic pocket beneath it is the target binding-site for capsid-binding compounds, and is accessible through a pore in the canyon floor. This structure is located at the fivefold axis of symmetry, and occurs 60 times per capsid.

A wide range of synthetic compounds have exhibited in vitro antiviral activity by binding the capsid specifically in this hydrophobic pocket: rhodamine, flavonoids, chalcones, aralkylamino-pyridines, oxazolinyl isoxazole compounds from Sterling-Winthrop (known as WIN compounds). They block viral replication by inhibiting virus-cell attachment and/or uncoating of the genome (153). The exact mechanism of inhibition is unknown, but several hypotheses have been proposed: the binding of molecules in the hydrophobic pocket induces increased capsid structure rigidity that is unfavourable for uncoating **(Figure 9)**; It may also exert conformational perturbations in the canyon floor such that capsid attachment is suboptimal; the canyon and pocket act as ion flow channels and the capsid-binding compounds physically block ion flow and inhibits pH-dependent uncoating.

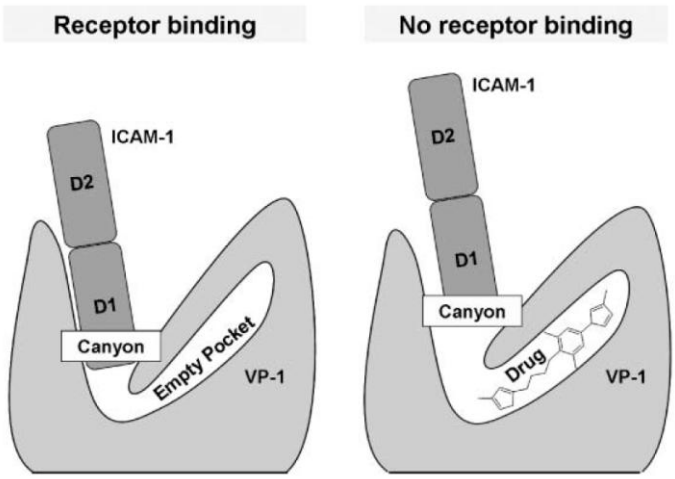


Figure 9 : Mechanism of action of WIN compounds (153)
Capsid-binding antiviral molecules inhibit viral replication by binding an internal hydrophobic pocket of VP1. This induces conformational changes that block virus-cell receptor interaction and increase structure rigidity to prevent uncoating.

The most extensively analysed series of molecules that target early events of viral replication are the WIN compounds, among which Pleconaril, an accidentally discovered WIN compound, showed particular promise as an anti-enteroviral drug. Pleconaril (3-{3,5-

demethyl-4-[[3-methyl-5-isoxazolyl}propyl]phenyl]-5-[trifluoromethyl]-1,2,4-oxadiazole) acts as an inhibitor of EV with an IC50 <0,03µM, and has been shown to inhibit not only prototype strains but also clinical isolates of commonly isolated EV serotypes (157-158). Rotbart *et al* reported on the preliminary outcomes of pleconaril use by compassionate administration for the treatment of potential life-threatening enterovirus infections, such as chronic enterovirus meningoencephalitis. 78% of patients had a favourable clinical response associated temporally with pleconaril treatment, with minimal adverse effects (159). However, concerns about its safety have delayed its approval for commercialisation by the American FDA.

Pleconaril is not effective against EV71. A group of pyridyl imidazolidinones such as BPR0Z-194, designed based on pleconaril and other WIN compounds, have demonstrated antiviral activity against EV, including EV71, CVA24, CVA9 and E9. BPR0Z-194 inhibits viral replication at the early stages of the cycle and a single amino acid mutation at position 192 in the VP1 region from a valine to a methionine residue has been found to confer resistance to the inhibitory effects of BPR0Z-194 in EV71 (160).

WIN 54954 has been tested against CVA21 in phase I trials, and shown to be effective in reducing the severity of symptoms and decreasing viral titers in nasal secretions (161).

V-073 is a capsid-binding molecular reported to have broad-spectrum anti-enteroviral activity developed primarily for nonpolio enterovirus infections. V-073 demonstrated to have potent *in vitro* anti-poliovirus activity against all PV strains tested to date, with an EC50 between 0.003 µM and 0.126 µM (162). Preliminary testing in mice showed that V-073 does

not have detrimental impact when used concurrently with primary or booster immunization with IPV (163).

Protease inhibitors

2A and 3C are enteroviral proteases that play a vital part in the maturation of viral progeny by co- and post-translational processing. Proteinase 2A is responsible for the cleavage of eIF-4G to inhibit host cell protein synthesis while 3C is the catalytic core of the proteinase that releases the capsid proteins.

Thiol alkylating agents such as iodoacetamide and N-ethylmaleimide, were shown to inhibit 79% and 84% of 2A protease activity respectively in a study by Konig *et al* (164). The anti-enteroviral effects of pan-caspase inhibitors, such as Z-VAD-FMK, on proteases 2A and 3C resulted in the decrease in both viral RNA and viral proteins (165).

Peptidic aldehydes that inhibit 3C protease activity were designed to mimic 3C substrates, based on the cleavage specificity of the enzyme for the P1-Gln-P1'-Gly bond contained in the compound (166). Rupintrivir (AG7088) has also been shown to specifically inhibit viral 3C function, while having no effect on cellular serine and cysteine proteases. Furthermore, rupintrivir is effective against representative members of all four human enterovirus species, with EC50 values ranging from 7 to 183 nM (153). Both single-dose and multiple-dose intranasal rupintrivir levels were safe and well-tolerated in healthy volunteers (167).

Targeting viral RNA replication

Guanidine hydrochloride is one of the most extensively studied anti-picornavirus molecules. Barton *et al* showed that guanidine inhibits a 2C function that is required for the initiation of

negative-strand synthesis, but not positive-strand RNA synthesis nor RNA elongation (168). Resistance and/or dependence to guanidine has been mapped in poliovirus to the 2C region in the form of a mutated asparagine and methionine at positions 179 and 187 respectively (169-170). Similarly, resistance of E9 to HBB (2-(a-hydroxybenzyl)-benzimidazole) has been mapped to mutations in the 2C region (171-172). However, the combined use of guanidine and HBB resulted in a synergistic effect, suggesting different inhibitory mechanisms for either drug. The thiazolobenzimidazole derivative TBZE-029 has been shown to effectively inhibit the replication of CVB3 (EC50 < 1.2 μg/mL). Resistance to TBZE-029 has also been mapped to the nonstructural 2C region. Curiously, the *in vitro* ATPase activity of 2C does not appear to be inhibited (173).

Nucleoside analogs that increase the error rate of 3D polymerase have also be tested. One such cytidine analog, 5-nitrocytidine, has been shown to inhibit RDRP in poliovirus, resulting in the loss of viral viability. The resistance phenotype to amiloride, a cellular ion transport blocker, has been attributed to mutations in the 3D region (153).

Some interferons (IFN) have been used in the treatment of many viral infections. Out of 17 type I IFNs tested by Yi *et al*, IFN-4, IFN-6, IFN-14, and IFN-16 displayed potent anti-EV71 activity (174). In particular, IFN-14 induced downstream antiviral effectors to provide superior antiviral effect. Most conventional IFNs were less effective or ineffective. However, bioinformatics analysis identified proteins in the IFN response pathway such as IRF9 that can be cleaved by enteroviral 3C protease, hindering thus the antiviral activity of IFN. It has been proposed that IFN can be combined with rupintrivir for a strong synergy effect in anti-EV71 treatment (175).

Arbidol is an antiviral molecule developed in Russia and used for the prevention and treatment of viral respiratory infections such as influenza infection, in both adult and paediatric populations (176). Arbidol (ethyl-6-bromo-4-[(dimethlyamino)-methyl-]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate) inhibits virus-mediated fusion of viral and cellular membranes, by stabilizing the viral envelope glycoprotein, haemagglutinin (HA) against the low pH transition to its fusogenic state (177-178). Arbidol has also been shown to be effective against Chikungunya virus, targeting in particular the E2 envelope protein (179). Curiously, the antiviral activity of Arbidol has been shown to be effective against non-enveloped viruses such as enteroviruses and rhinoviruses (180-181). Arbidol is a broad-spectrum antiviral agent that deserves more attention for the development of anti-enteroviral treatment.

However, drug resistance arises rapidly because of EV genetic diversity. By mirroring the strategy of using drug combinations with different mechanisms as in HIV antiviral therapy, it may be possible to delay or even prevent emergence of drug-resistant varieties.

7/ ENTEROVIRUS EVOLUTION

The molecular mechanism for enterovirus evolution couples mutation due to error rate of the viral polymerase and homologous recombination.

7.1/ REPLICATIVE RECOMBINATION

Homologous recombination involves the swapping of genetic elements between two enteroviral genomes during replication (182-183). The mechanism is commonly considered to be 'copy choice', in which the viral RNA-dependent RNA polymerase and the nascent strand switch templates during minus-strand RNA synthesis at highly homologous regions (184). Strand switching is precise such that there are no insertions or deletions at the crossover site. This precision has been verified by recombination analysis in non-coding regions, such that the conservation of reading frame does not supersede any imprecise recombination (185). Recombination can occur throughout the genome, but may give rise to recombinants with altered fitness. Viable recombination has been shown to occur principally in the non-structural region and strictly within a species, making naturally occurring recombination a reliable species criterion (186-188). No consensus sequence motifs for crossover sites have been determined. It has been suggested that the polymerase-template complex comes to a premature pause during minus-strand transcription, due to stable secondary structure elements. This unpairs a few bases of the nascent strand and the disassociated 3' terminus anneals to a different template strand. Strand elongation continues to produce the recombinant genome.

Phylogenetic analysis of the 5'NCR shows clustering into two groups, with group I 5'NCR found in HEV-C and HEV-D and group II 5'NCR found in HEV-A and HEV-B (189). It has been

proposed that the four distinct genetic lineages originally evolved by point mutations from a common enteroviral ancestor, but during the course of evolution, HEV-A viruses replaced their original 5'NCR by that of HEV-B viruses during a recombination event or vice versa **(Figure 10A)**.

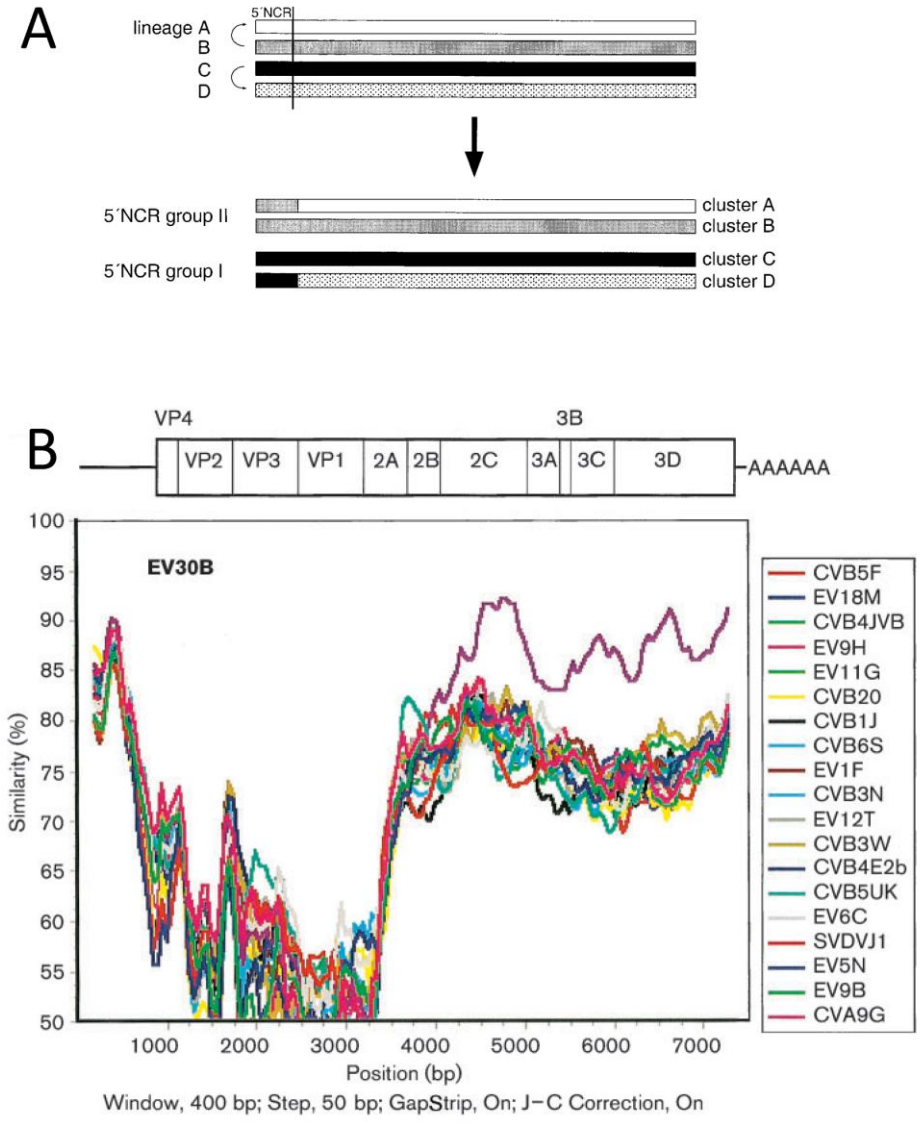


Figure 10 : Recombination in Enteroviruses (190-191)
(A) Proposed recombination event between the 5'NCR and the rest of the genome.
(B) Bootscanning analysis of complete E30 genome shows the diversity in the structural regions and the similarity in the nonstructural regions when compared to other serotypes.

Full-genome bootscanning analysis of circulating HEV-B strains with prototype EV strains showed that only the capsid-coding P1 region of modern strains displayed high similarity (bootstrap values > 70%) to the corresponding prototype virus, with calculated recombination points in or close to the VP4 and 2A regions (190-191) **(Figure 10B)**. Phylogenetic analysis for each genomic region in modern strains showed that they grouped reliably with prototype strains in the VP1 region, and to a lesser extent VP2. However, the modern strains studied share highly similar NSP regions: the sequences were less divergent and amino acid variability was the lowest in the 2C and 3D regions, demonstrating how common an occurrence recombination is among enteroviruses. The inconsistent topology of phylogenetic trees for the 2AB and VP4 regions underscore their roles as recombination hot spots.

The evolutionary overview of enteroviruses appears to be considered as genome fragments in a global reservoir, subjected to independent evolutionary forces and recombination events.

Identification of serotype only determines that the structural part of its genome is >70% similar to a prototype strain. There is currently still insufficient evidence to show that the shared NSP region is driven by a selection pressure due to the higher 'efficiency' of the encoded proteins. Should virulence determinants be shown to situate outside the structural genome region, standard VP1 typing would provide incomplete and misleading results.

7.2/ NON-REPLICATIVE RECOMBINATION

Viable recombinant viruses have also been produced from non-replicating and non-translatable regions of the EV genome (192-193). Using two parental poliovirus strains, one

containing lethal modifications in the 5'NCR and the other lacking the polyprotein and 3'NCR, such that infectious progeny can only be produced if recombination occurs, the team of Gmyl *et al* detected viable recombinants with different crossover points. Furthermore, these crossover points did not map to homologous regions in the parental strains. Further analysis using parental strains with interruptions within the 3D region such that the presence of the RNA-dependent RNA polymerase encoded by 3D is dependent on recombination events that preserved the correct reading frame, demonstrated the occurrence of precise nonreplicative recombination. This phenomenon implies the cleavage of phosphodiester bonds , possibly by endonuclease or cryptic ribosome-like activity, and the exposed termini are cross-ligated.

7.3/ THE CASE OF POLIOVIRUS RECOMBINATION

Poliovirus recombination is of particular clinical interest in the context of global poliovirus eradication. It has been widely reported that recombination occurs between the three live OPV strains, with recombinant type 2 and type 3 Sabin strains accounting for up to 50% and 67% of VAPP cases respectively in a study by Furione *et al (194)*. Recombination between vaccine strain polioviruses and wild poliovirus or other circulating HEV-C has also been increasingly studied, since outbreaks involving such recombinants have been reported worldwide (195). During an outbreak of pathogenic circulating vaccine-derived polioviruses in 2001-2002 in Madagascar, the team of Jegouic *et al* isolated type 2 strains with recombination sites in the 2A region, demarcating a 5' half derived from Sabin 2 and a 3' portion that greatly resembled CVA17 and CVA13 (196).

EXPERIMENTAL

CONTEXT AND STRATEGY

C/ LABORATORY DIAGNOSIS AND SURVEILLANCE

1/ ENTEROVIRUS DETECTION

1.1/ CELL CULTURE TECHNIQUES

The acid stability and absence of a lipid envelope contribute to the environmental stability of EV, which can thus, be isolated from a variety of clinical samples such as cerebrospinal fluid, stool and pharyngeal swabs. Before the advent of molecular diagnostic tools used today, the reference technique for EV laboratory detection was virus isolation in cell culture. No single cell line is sufficient for the isolation of all serotypes; a combination of at least four different cell lines has been recommended for sufficient serotype coverage (197-199). The characteristic cytopathic effect (CPE) of enteroviral infection is easily observed by light microscopy, in the form of rounded cells that detach from the culture dish and eventually undergo lysis. Subsequently, isolates were identified by the classical method of seroneutralisation using Lim-Benyesh-Melnick (LBM) antiserum pools. The cell culture technique is also relatively slow, often requiring 3 to 6 days for CPE to appear. Cell culture is also limited by low viral titers in some sample types such as CSF and by the difficulty of isolating certain serotypes that grow poorly or not at all in cell cultures. For example, CVA1, CVA19 and CVA22 have never been successfully grown in cell culture, requiring instead the intracranial inoculation of suckling mice (200). This is a difficult and impractical technique because it is time-consuming and requires animal maintenance. Nevertheless, cell culture remains a primordial step for the identification of EV serotype by seroneutralisation protocols.

1.2/ MOLECULAR TECHNIQUES

The limitations of cell culture lead to the development of molecular diagnostic techniques. PCR amplification techniques for the universal detection of EV allow clinicians to deliver a less time- and labour-intensive diagnosis. Primers are used to reverse transcribe and amplify domaine V of the 5'non-coding region, a highly conserved region among all enteroviruses, such that all serotypes are detected **(Figure 4)**. The amplified products could then be visualised in ethidium bromide-stained agarose gels. Diagnosis by RT-PCR has been demonstrated to be highly specific, and consistently more so than cell culture (201-202). Today, laboratory detection of EV is performed by the 'gold standard' method of a pan-enterovirus quantitative RT-PCR (70). In addition to the primers targeting 5'NCR, the use of a fluorophore-coupled probe provided accurate detections in real time, further reducing the time needed for diagnosis. However, EV detection remains genus-specific and does not allow the identification of their serotype.

1.3/ SEROLOGICAL TECHNIQUES

The use of serological tests, such as immunoassays, has been limited. However, the great diversity of EV serotypes and the lack of a shared antigen increase the likelihood of cross-reactions between different viral serotypes, often rendering such tests uninterpretable.

2/ ENTEROVIRUS SEROTYPE IDENTIFICATION

Serotype identification is vital for epidemiological purposes such as monitoring the emergence and spread of rare and highly pathogenic serotypes. This is especially pertinent in paediatric populations and in polio-free countries where there is a need to distinguish between vaccine strain polioviruses and nonpolio enteroviruses.

2.1/ CLASSIC SERONEUTRALIZATION

Conventional serotype identification is inherent in the detection assay by LBM mixed antiserum pools (71). Each isolate was screened against 24 antisera in seven intersecting pools, such that each serotype produced a specific pattern of neutralization **(Figure 11)**. However, this method of detection and identification is suboptimal: LBM pools available from the WHO were raised against prototype strains isolated in the 1950s and 1960s, and may fail because of antigenic drift, recombination or the presence of multiples serotypes in the tested sample (203). The time-consuming and labour-intensive seroneutralisation method using LBM pools has been shown to be insufficient for EV serotyping today.

TABLE I

Composition of combination serum pool

Combination Pool	Antisera* Present in Pools								Number of Sera Per Pool
A	A9	E18	E2	E10	B1	E1	B3	E12	8
B	A9	E11	E5	E13	E19	B4	E3		7
C	E11	E14	E2	E6″	B2	B3	E8		7
D	E14	E15	E5	E7	B1	B5	E3	E12	8
E	E15	E16	E6″	E9	E19	E1	B3	E8	8
F	E16	E17	E7	E10	B4	E3	E12		7
G	E17	E18	E9	E13	B5	B2	E8		7

* A = Coxsackie A; B = Coxsackie B; and E = ECHO.

TABLE II

Distribution of monkey typing sera in combination pools, and neutralization patterns obtained with prototype viruses

Type Serum*	Final Dilution of Serum	Serum Pools†							Prototype Virus Tested	Neutralization Pattern
		A	B	C	D	E	F	G		
A9	50	×	×						Grigg	AB
E11	50		×	×					Gregory	BC
E14	50			×	×				Tow	CD
E15	50				×	×			Charleston 96–51	DE
E16	50					×	×		Harrington	EF
E17	50						×	×	CHHE-29	FG
E18	50	×						×	Metcalf	AG
E2	100	×		×					Cornelis	AC
E5	100		×		×				Noyce	BD
E6″	32			×		×			D'Amori	CE
E7	100				×		×		Garnett	DF
E9	100					×		×	Bourne	EG
E10	100	×					×		Lang	AF
E13	100		×					×	Hamphill	BG
B1	32	×			×				Conn-5	AD
E19	50		×			×			Burke	BE
B5	200				×			×	Faulkner	DG
E1	200	×				×			Farouk	ABEG‡
B4	160		×				×		Texas-13	BF
B2	125			×				×	Ohio-1	CG
B3	320	×		×		×			Nancy	ACE
E3	500		×		×		×		Berardi	BDF
E8	500			×		×		×	Bryson	ACEG‡
E12	500	×			×		×		Travis	ADF
Number of sera in pool		8	7	7	8	8	7	7		
Serum concentration of pools		1:21	1:21	1:19	1:24	1:20	1:24	1:23		

* A = Coxsackie A; B = Coxsackie B; and E = ECHO.

† × indicates inclusion of serum in pool named at top of column.

Figure 11 : Serotype identification using Lim Benyesh-Melnick pools (71)

Table I show the distribution of 24 antisera into seven pools with which virus isolates are tested. Table II shows the neutralization pattern expected with serum containing different EV serotypes.

2.2/ MOLECULAR GENOTYPING TECHNIQUES

PCR-based methods have increasingly been solicited to provide information on EV species or serotype. Studies based on different genomic regions of enteroviruses have been undertaken to varying degrees of success (204).

Phylogenetic analysis of partial 5'NCR nucleotide sequences showed that human enteroviruses grouped into two distinct clusters with a minimum of 70% nucleotide identity within each: (i) group I cluster containing PV, CVA21, CVA24 and EV70 (HEV-C and HEV-D); and (ii) group II cluster containing coxsackie B viruses, echoviruses, CVA16 and EV71 (HEV-A and HEV-B) (189). Individual strains of the same serotype did not group together. This reflects the high level of conservation in the 5'NCR, but revealed the 5'NCR to be inadequate for serotype identification.

In a study by Ishiko *et al*, VP4 nucleotide sequences were studied to determine a phylogeny-based identification process (205). EV prototype strains clustered according to the four human enterovirus species, with nucleotide identity scores within each cluster ranging from 61.8% to 77.3% and poliovirus forming a stable subgroup within the HEV-C cluster. When this method was extended to clinical isolates, the team was able to distinguish between CVA24 and EV70 in patients with AHC.

Since EV serotype has been classically defined by neutralization, and VP1 contains major neutralization epitopes, VP1 sequence was theorised to correlate with serotype. In a study carried out by Oberste *et al*, partial or complete sequences of the VP1 structural gene from both prototype strains and clinical isolates were shown to correlate unambiguously with EV serotype. 37 out of 53 prototype serotypes tested were detected by this method (30).

Furthermore, pairwise nucleotide and amino acid identity comparison scores were distributed in three defined peaks, corresponding (from highest to lowest) to viruses of the same serotype, viruses of same species but different serotype and viruses of different species. This can be used as a computationally non-intensive method to assign serotype: sequences of at least 75% nucleotide or 85% amino acid identity can be considered to represent strains of the same serotype, if the next highest-scoring sequence of different serotype is 70%. This method was used to identify new serotypes from previously 'untypeable' specimens (206-207).

The team of Caro *et al* also studied a 1452 bp region encompassing the 3'end of VP1, the 2A and 2B regions, and the 5'end of 2C as a tool for genome variability analysis, with the 3'end of VP1 sufficient for providing serotype information (208). However; a comparative study carried out by Kottaridi *et al* using different RT-PCR protocols targeting different genomic regions showed that this method was suboptimal compared to the VP1 method previously described (209).

The central portion of VP2 coding region was also recently analysed by the team of Nasri *et al* (210). The region was chosen based on its sufficiently high genetic variability, but it is not as hypervariable as VP1 such that flanking primers could be designed with fewer degenerate positions. 55 field isolates 'untypeable' by seroneutralisation were amplified in the VP1 and VP2 regions for comparison: the serotype determined by both methods was concordant in 48 of them. Six strains could not be amplified in the VP1 region, four of which however were successfully typed in the VP2 region. This technique was further validated by a comparative study of 50 'untypeable' specimens in both the VP2 and VP1 regions: VP2 typing was

successful in 45% of the specimens, but VP1 typing yielded non-specific amplifications that rendered the assay inconclusive (211).

To circumvent the cell culture technique and its shortcomings as previously described, methods have been proposed for the direct amplification from clinical specimens. Achieving practical levels of sensitivity and specificity is particularly problematic, especially in CSF samples which typically contain very low viral titers. As a result, such methods frequently oblige the use of nested or semi-nested secondary amplifications. Furthermore, the use of highly degenerate, inosine-containing primers to counter the variability of VP1 sequence between serotypes can often yield nonspecific amplification host cell nucleic acids. Despite these obstacles, direct VP1 amplification from CSF samples has been tested, albeit with varying results ranging from 64-65% (49, 51) to 96% success (52). Tavakoli *et al* resorted to using four different protocols in order to amplify their clinical samples (212). Leitch et al also described a rapid PCR-based method using primers without inosine that specifically targeted the VP1 region of either HEV-A or HEV-B (213).

The most common sequencing technique used for serotyping purposes is based on Sanger's chain termination method, using didesoxynucleotides as DNA chain terminators. Pyrosequencing is a real-time ssDNA sequencing technique that involves the detection of pyrophosphate released during elongation by chemiluminescent enzymes, and has been used in fast sequencing assays of short DNA segments. Silva *et al* successfully identified EV serotype of 194 isolates and clinical samples from the National Reference Laboratory for Poliomyelitis and Enterovirues at the Robert Koch Institute by pyrosequencing, including samples with mixed viruses of different species and classically 'untypeable' isolates (214).

In order to directly amplify VP1 of all serotypes with sufficient sensitivity and specificity directly from clinical specimens, Nix et al used the consensus degenerate hybrid oligonucleotide primer (CODEHOP) approach (215-216). A CODEHOP primer consists of a nondegenerate consensus clamp in the 5' end and a degenerate consensus core in its 3' portion, such that the clamp increases stability of the primer-template duplex, allowing greater annealing temperatures for the minimization of nonspecific amplification, and the degenerate core targets all EV serotypes.

However, since EV-associated diseases are generally not serotype-specific, serotype identification has little influence on clinical management and is not systematically performed. Instead, species-specific amplification has been proposed as an interesting tool to rapidly screen clinical specimens and isolates for species of particular interest, in order to focus subsequent serotype identification. The team of Oberste *et al* successfully amplified 89.3% of previously uncharacterised nonpolio enterovirus isolates with species-specific primers in the 3'NCR, all of which the species identity was corroborated by partial VP1 sequencing (217).

3/ EV LABORATORY PROCESSING IN MARSEILLE, FRANCE

Figure 12 illustrates the processing of clinical samples registered in the Laboratory of Virology, University Hospital La Timone (Marseille, France) and for which the EV diagnostic test had been prescribed.

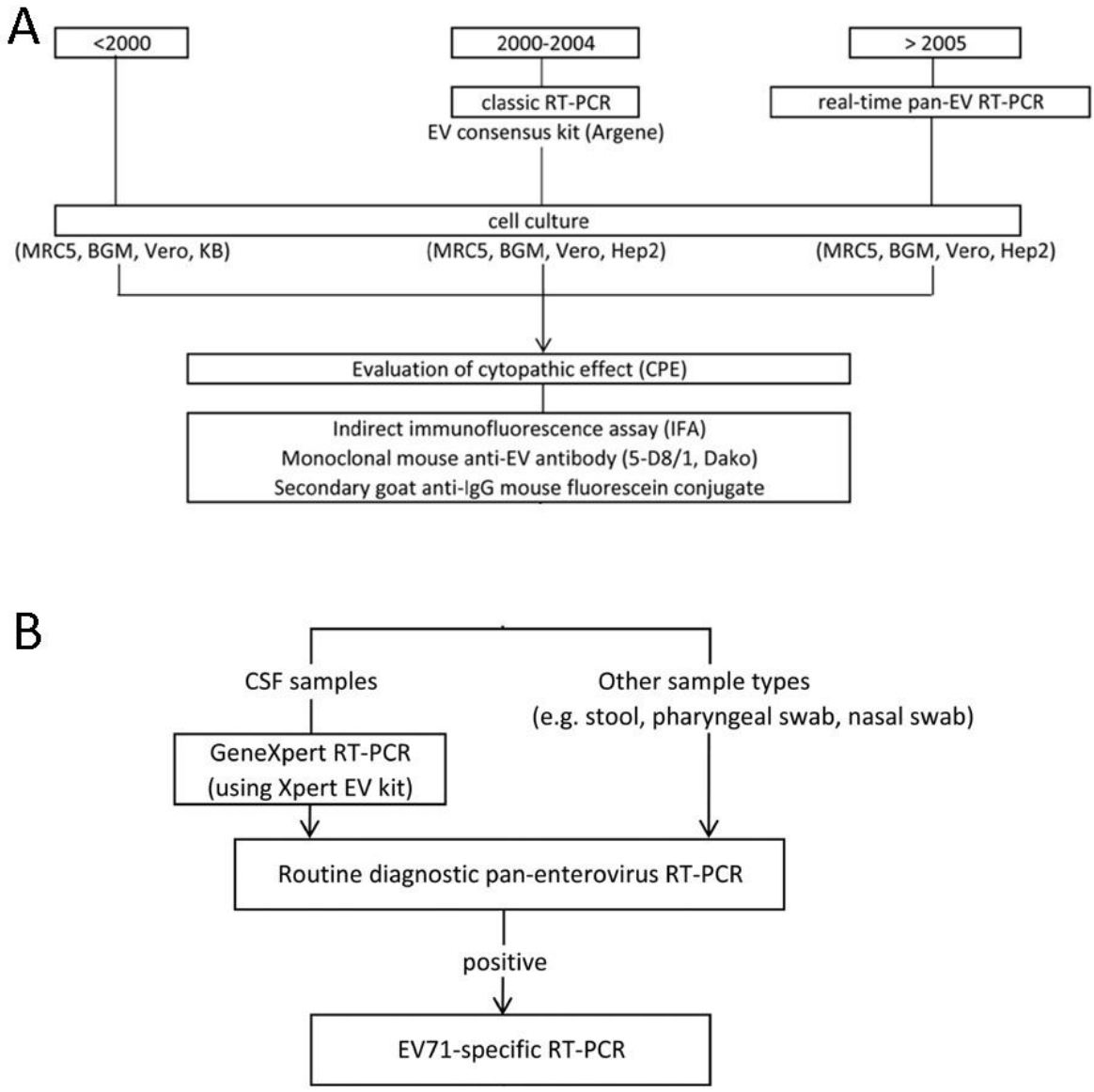


Figure 12 : Processing flowchart of clinical samples
(A) EV diagnostic processing, 1985-2005
(B) EV diagnostic processing, present

Prior to 2000, EV diagnosis consisted of growing samples in cell culture. Cell lines used for this purpose were MRC5 (human foetal lung fibroblasts), BGM (Buffalo green monkey kidney cells), Vero (African green monkey kidney cells) and KB (human laryngeal carcinoma cells). Once cytopathic effect was observed by light microscopy, the presence of EV antigen was verified by immunofluorescence assay using a monoclonal mouse anti-EV antibody (clone 5-D8/1, Dako) and a secondary goat anti-IgG mouse fluorescein conjugate.

From 2000 to 2004, EV diagnosis was achieved by classic RT-PCR using the Enterovirus Consensus kit (Argene). Samples were also inoculated on MRC5, BGM, and Vero cell lines as previously described, with the exception of KB cell line that was replaced by the similar Hep2 cell line (Human laryngeal carcinoma cells). Cell cultures were also evaluated by CPE and immunofluorescence assay.

From 2005 onwards, EV diagnosis was carried out using a real-time pan-enterovirus RT-PCR adapted by Watkins-Riedel *et al* to the Taqman format. Samples were also inoculated on MRC5, BGM, Vero and Hep2 cell lines, and cell cultures were evaluated by CPE and immunofluorescence assay.

The rapid detection of the presence of EV RNA in CSF samples is crucial for the provision of appropriate patient care. As such, the real-time pan-enterovirus RT-PCR diagnostic assay has since been implemented in two point-of-care (POC) laboratories connected with the core virology laboratory. Since 2008, CSF samples have been systematically tested at the POC

level for EV RNA using the Xpert EV kit on the GeneXpert integrated system (Cepheid) and verified with the routine real-time RT-PCR diagnostic assay (218) **(Figure 12B)**.

Since May 2010, clinical samples tested EV-positive by diagnostic RT-PCR are screened the following day using a EV71-specific real-time RT-PCR assay adapted from Tan *et al* with a modified hybridization probe (219).

Internal control

All nucleic acid extraction, reverse transcription and real-time RT-PCR were monitored by using a quantitated solution of bacteriophage MS2 (220). PCR detection of MS2 was performed using the same reagents on the same cycler with the same cycling programme as is used for EV detection. Diagnostic PCR results were validated if MS2 detection assay yielded a positive result with a Ct value not higher than one standard deviation as compared to the mean Ct value observed for the whole PCR plate

3.1/ EXPERIMENTAL STRATEGY

Given that the Laboratory of Virology of the Public Hospital of Marseille has a well-maintained and accessible virus strain bank spanning 1985 to 2005, the decision to create a viable sequence database came naturally.

Cell lines

We reproduced archived EV strains in the cell line in which they were originally isolated. MRC5 cells (Human foetal lung fibroblasts) were cultured in Basal Medium Eagle (BME), 10% decomplemented Foetal Bovine Serum (dFBS), 1% L-Glutamine (L-GLN), 1% Penicillin-Streptomycin (PS). Hep2 (Human laryngeal carcinoma cells), KB (Human laryngeal carcinoma cells), Vero (African green monkey kidney cells) and BGM (Buffalo green monkey kidney cells) cell lines were grown in Minimum Essential Medium Eagle (MEM), 5% dFBS, 1% L-GLN, 1% PS. All cell cultures were incubated at 37°C under 5% CO_2.

Genomic amplification

Reverse transcription was carried out with Reverse Transcriptase MultiScribe (Applied Biosystems) with random hexamers. Each viral cDNA was then amplified in the VP1, 2C and 3D regions using region-specific primers **(Table 2).** Amplification products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining, purified then sequenced on an ABI Prism 3130 DNA Sequencer (Applied Biosystems).

Primers/Probes		Nucleotide sequence	Position*		Gene	Study
DiagnosticE1	sense	CCCCTGAATGCGGCTAATCC	455-474			
DiagnosticE2	antisense	ATTGTCACCATAAGCAGCCA	601-582	EV	5'NCR	Watkins-Riedel *et al*
DiagnosticProbe	probe	FAM-CANGGACACCCAAAGTAGTCGGTTCC-TAMRA	559-537			
EV292	sense	MIGCIGYIGARACNGG	2547-2562			
EV222	antisense	CICCIGGIGGIAYRWACAT	2894-2876	EV	VP1	Oberste *et al*
EV040	sense	ATGTAYRTICCIMCIGGIGC	2876-2894			
EV011	antisense	GCICCIGAYTGITGICCRAA	3321-3302			
2C-F	sense	TTYGAYGGITAYAARCARCA	4532-4551	HEV-B	2C	Our design
2C-R	antisense	GGICCYTGRAAIARIGCYTC	5034-5015			
3D-2F	sense	TTYTGGWSIAARATHCCIGT	6560-6579	HEV-B	3D	Our design
3D-R	antisense	CKIACRTGRTCYTGIGTRTT	7158-7139			
EvVP1F	sense	GAGAGCTCTATAGGAGATAGTGTG	2463-2486†	EV71	VP1	Tan *et al*
EvVP1R	antisense	TGCCGTACTGTGTGAATTAAGAA	2644-2666†			
EvVP1-FL	probe	GATGACTGCTCACCTGTGTGTTTTGACC-FL	2550-2523†			Our design
EV71_Marseille	probe	FAM-ATTGGAGCATCATCAAATGCTAGTGA-TAMRA	2592-2617†			

Table 2: Nucleotide sequences of the primers and hybridization probes

* The position of all probes are those relative to CVB3 prototype strain Nancy (Accession No. JN048468)

† The position of all probes are those relative to EV71 prototype strain BrCr (Accession No. U22521)

With the exception of the work done on the Marseille VP1 database, a semi-nested PCR assay adapted by the French National Reference Centre for Enteroviruses from Nix *et al* was used when a virus could not be isolated from culture.

Phylogenetic analysis

All sequence chromatograms were analysed with Sequencher 4 software (Gene Codes Corporation). Multiple sequence alignments were realized with EBI ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) on default settings and manually edited with BioEdit (221). The nucleotide sequences were translated into and aligned as amino acids. Using the programme DAMBE (http://dambe.bio.uottawa.ca/dambe.asp), nucleotide sequences were aligned against the amino acid sequences. Phylogenetic trees were constructed with MEGA version 3.1 (Molecular Evolutionary Genetics Analysis)(222). For VP1, 2C and 3D, this was achieved using the Neighbor-Joining method on a Jukes-Cantor model. Partial VP1 sequences (<400 nucleotides) were omitted from phylogenetic analysis. Pairwise distance matrices were drawn to calculate p-distance, the proportion of nucleotide sites at which the two sequences differ for the totality of the sites compared. The consistency of tree topologies was tested by bootstrapping in 1000 pseudoreplicates.

Recombination analysis

Nucleotide alignments were used for bootscanning in SimPlot v.3.5.1. (http://sray.med.som.jhmi.edu/SCRoftware/simplot/), using a 500-nt sliding window in 20-nt steps (Neighbour-joining method, Kimura 2-parameter model) and 100 pseudoreplicates. Signals of >70% of the observed permuted trees were taken to indicate potential recombination events.

RESULTS AND DISCUSSION

ARTICLE #1

A Retrospective Overview of Enterovirus Infection Diagnosis and Molecular Epidemiology in the Public Hospitals of Marseille, France (1985-2005)

Foreword

Human Enteroviruses circulate worldwide, and are the major cause of aseptic meningitis outbreaks in both paediatric and adult populations. In the Northern Hemisphere, outbreaks associated with nonpolio enteroviruses have been described with a summer-early autumn seasonality. The prevalent serotype may vary from year to year, and there is often co-circulation of several serotypes. Recent epidemics of aseptic meningitis in France have been attributed primarily to Echovirus 30 (E30) (45, 223-224).

Enterovirus surveillance is carried out primarily in the context of poliovirus eradication, for the identification of imported cases in regions certified polio-free. In France, a sentinel laboratory network for the surveillance of Enteroviruses (Réseau de Surveillance des Enterovirus, RSE) was implemented to determine circulation patterns of different enterovirus serotypes, in addition to providing poliovirus surveillance. The ten most frequently isolated circulating serotypes belonged to the HEV-B species: (in decreasing order) E30, E13, E6, CVB5, E11, CVB4, E9, E7, CVB1 and CVB2 (45).

In Marseille, the need for enterovirus surveillance has led to the creation and compilation of a comprehensive database of human enteroviruses isolated in the Public Hospitals of Marseille (AP-HM). Clinical EV strains isolated between 1985 and 2005 were sequenced in

the VP1 coding region and analyzed systematically for epidemiological information as well as trends in laboratory diagnostic techniques. This study is likely to be the largest retrospective study including molecular data of circulating enteroviruses ever undertaken.

OPEN ACCESS Freely available online

PLoS one

A Retrospective Overview of Enterovirus Infection Diagnosis and Molecular Epidemiology in the Public Hospitals of Marseille, France (1985–2005)

Charlene Y. Q. Tan[1]*, Laetitia Ninove[1,2], Jean Gaudart[3], Antoine Nougairede[1,2], Christine Zandotti[2], Laurence Thirion-Perrier[1], Remi N. Charrel[1,2], Xavier de Lamballerie[1,2]

1 UMR190 Unite des Virus Emergents, Universite de la Méditerranee - Institut de Recherche pour le Développement, Marseille, France, **2** Federation de Microbiologie, Assistance Publique-Hopitaux de Marseille, Marseille, France, **3** LERTIM EA3283, Biostatistic Research Unit, Universite de la Mediterranee, Marseille, France

Abstract

Human enteroviruses (HEV) are frequent human pathogens and, associated in particular with large outbreaks of aseptic meningitis. Here, we have compiled a database of clinical HEV isolates from the Public Hospitals of Marseille, from 1985 to 2005. Amongst 654 isolates that could be characterized by complete sequencing of the VP1 gene, 98% belonged to species HEV-B; the most frequently isolated serotypes were Echovirus E30, E11, E7, E6 and E4. The high incidence of E30 and the recent emergence of E13 are consistent with reports worldwide and peak HEV isolation occurred mostly in the late spring and summer months. The proportion of echoviruses has decreased across the years, while that of coxsackieviruses has increased. Stool (the most frequent sample type) allowed detection of all identified serotypes. MRC5 (Human lung fibroblasts) cell line was the most conducive cell line for HEV isolation (84.9% of 10 most common serotype isolates, 96.3% in association with BGM (Buffalo green monkey kidney cells)). Previous seroneutralization-based serotype identification demonstrated 55.4% accuracy when compared with molecular VP1 analysis. Our analysis of a large number of clinical strains over 20 years reinforced the validity of VP1 serotyping and showed that comparative p-distance scores can be coupled with phylogenetic analysis to provide non-ambiguous serotype identification. Phylogenetic analysis in the VP1, 2C and 3D regions also provided evidence for recombination events amongst clinical isolates. In particular, it identified isolates with dissimilar VP1 but almost identical nonstructural regions.

Citation: Tan CYQ, Ninove L, Gaudart J, Nougairede A, Zandotti C, et al. (2011) A Retrospective Overview of Enterovirus Infection Diagnosis and Molecular Epidemiology in the Public Hospitals of Marseille, France (1985–2005). PLoS ONE 6(3): e18022. doi:10.1371/journal.pone.0018022

Editor: Darren Martin, Institute of Infectious Disease and Molecular Medicine, South Africa

Received October 21, 2010; **Accepted** February 22, 2011; **Published** March 18, 2011

Funding: This study was funded by Université de la Méditerranée - Institut de Recherche pour le Développement. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: charlene.tanyq@gmail.com

Introduction

Human enteroviruses (*HEV*, family *Picornaviridae*) are small non-enveloped viruses with a single-stranded RNA genome of positive polarity. The genome is approximately 7.4 kb long. Four structural proteins, VP1 to VP4, are assembled to form the virion capsid of icosahedral symmetry. The most conserved regions of the enteroviral genome are the 5′non-coding region (5′NCR) and the RNA-dependent RNA polymerase [1–2]. 64 distinct human serotypes were previously identified on the basis of their pathogenic potential and neutralization by specific antisera. They were then later reclassified into four species based on sequence identity of the region coding for the VP1 capsid protein. The four species are (i) *HEV-A*, (ii) *HEV-B*, (iii) *HEV-C* including *Poliovirus* (PV), and (iv) *HEV*-D [3–4].

Laboratory detection of enteroviruses is performed today by the 'gold standard' method of a pan-enterovirus real-time RT-PCR in the 5′NCR, which allows the detection of all enteroviruses but not the identification of their serotype [5]. Conventional serotyping consists of neutralization tests with Lim Benyesh-Melnick antiserum pools raised against prototype strains [6]. Modern day serotype identification is based on virus isolation in cell culture and the nucleotide sequence of the region coding for VP1 protein. VP1 sequences from prototype strains have been demonstrated to correlate best with neutralization-based serotype, as it is the site of major epitopes associated with serotype-specific neutralization [7–8]. More recently, molecular protocols aiming at identifying the HEV serotype directly from clinical samples have been proposed [9–12].

HEV cause a wide spectrum of illnesses ranging from mild (hand, foot and mouth disease, gastroenteritis, acute haemorrhagic conjunctivitis) to severe and potentially life-threatening (acute flaccid paralysis) [13–14]. Most enteroviral infections are asymptomatic or subclinical but their neurotropism can cause serious central nervous system complications such as aseptic meningitis and encephalitis. Enteroviruses are the major cause of aseptic meningitis in both pediatric and adult populations [15–16]. There is currently no antiviral treatment available for HEV infection [17].

There is worldwide circulation of enteroviruses, except for poliovirus which remains endemic in only four countries (Pakistan, India, Nigeria and Afghanistan) [18]. Seasonal aseptic meningitis outbreaks due to non-polio enteroviruses have been noted to peak in summer till early autumn in the Northern Hemisphere [19]. While the prevalent serotype varies from year to year, with co-circulation of several serotypes a common observation, recent

epidemics have been attributed primarily to Echovirus 30 (E30) [20–24]. The molecular mechanism for enterovirus evolution couples mutation due to viral polymerase error and homologous recombination by template switching [25]. The evolutionary overview of enteroviruses appears to be considered as genome fragments in a global reservoir, subjected to independent evolutionary forces and recombination events [26–28].

Here, we have compiled a comprehensive database of HEV isolated at the Public Hospitals of Marseille (AP-HM), France, spanning 1985 to 2005 with VP1 nucleotide sequences of clinical HEV strains. We systematically analyzed it for epidemiological information as well as trends in laboratory diagnostic techniques.

Results

Frequency of HEV isolation

Of 828 secondary cell cultures tested positive for HEV by immunofluorescence, 654 (79%) were successfully sequenced in the VP1 region and attributed their serotype. We identified 9 years with significantly high HEV isolation frequency during which the number of monthly isolates exceeded the upper control limit at 99% confidence level (UCL99 = 6.71, $p<0.01$) (**Figure 1**), 8 of which saw peak HEV isolation between May andAugust, in the late spring and summer months. The only exception was in 1987 when peak HEV isolation occurred in September and October, in the fall. Isolation levels in 2000 and 2005 were of great amplitude, and coincide with the occurrence of HEV epidemics in Marseille. HEV isolates in 2000 (n = 191) peaked in the summer months, with 93.2% (n = 178) occurring between May and August. 50.3% of the isolates in 2000 were attributed to the serotype E30 (n = 96), 15.7% to E13 (n = 30) and 7.9% to E11 (n = 15). In 2005, only 6.7% (n = 24) of all cases were isolated and typed as a result of a change in hospital diagnostic protocol. Nevertheless, the 2005 epidemic is evidenced by the number of cases diagnosed Enterovirus-positive with real time RT-PCR (n = 78, 151, 76 for May, June and July respectively). HEV isolates during the remaining peaks comprised numerous serotypes, including E30, E11, E7, E18 and CVB5, without clear predominance of any one serotype.

Serotype patterns

Overall, the 10 HEV serotypes most commonly isolated between 1985 and 2005 all belong to the HEV-B species: in decreasing frequency, E30, E11, E7, E6, E4, E13, CVB5, E14, CVB3 and E18 (**Figure 2**) and account for 77.1% of isolates with known serotype. The 5 most frequently encountered serotypes, E30, E11, E7, E6 and E4 account for 56.7% of all isolates, and remain the most prevalent serotypes even after factoring out the 2000 and 2005 epidemics. HEV-B accounted for 98% of all isolates.

Long-term circulation patterns varied for individual serotypes. Some serotypes have disappeared from Marseille: the last reported cases of CVA13, CVA17, E1, E2, E3, E12, E14, E31, E32, EV74 and PV all precede 1992. On the other hand, other serotypes have appeared with varying frequencies: CVA24 has been isolated with extremely low frequency (4 isolates since 1996), in contrast with E13 which reappeared in 2000 as an epidemic serotype. Only a single E13 infection was reported in Marseille prior to the 2000 outbreak. Serotypes such as E30, E7, E6 and E4 demonstrate more endemic patterns, with persistent isolation levels over 20 years. E30 in particular, has a strong propensity for epidemic eruptions and was the most commonly identified enterovirus

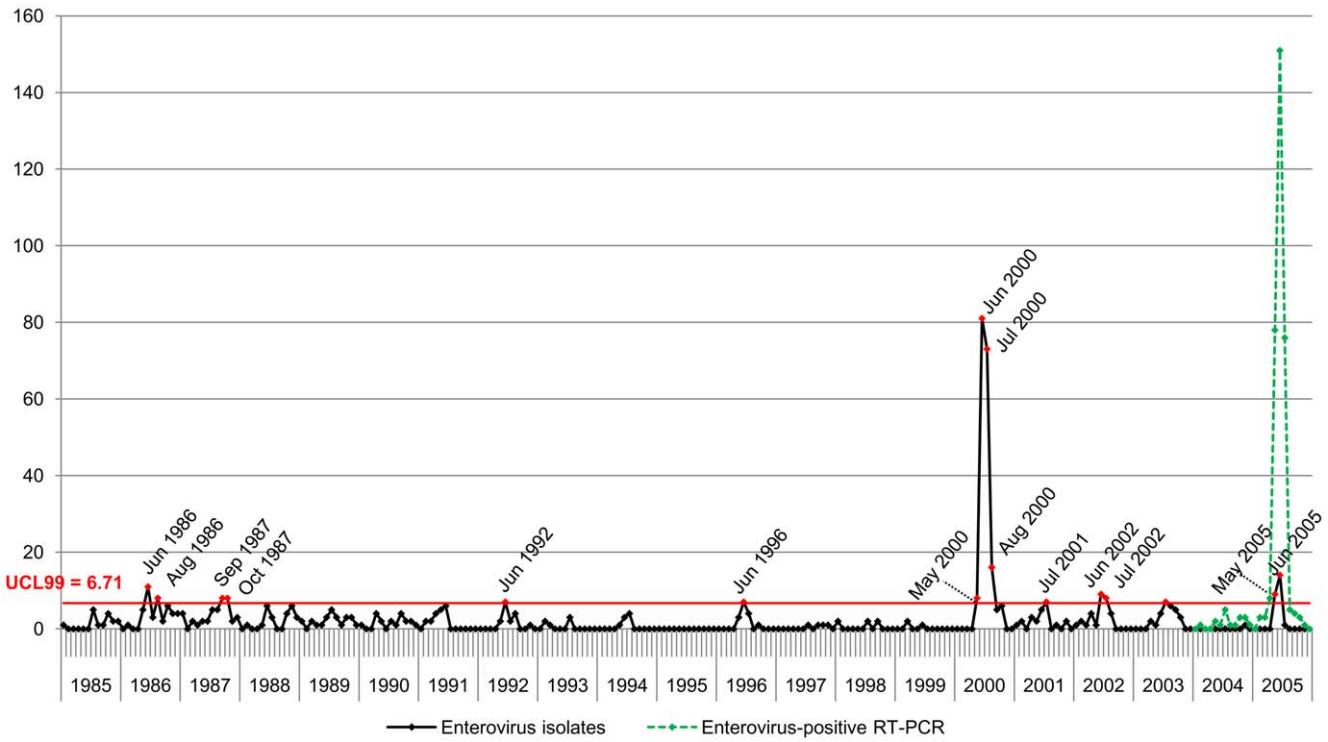


Figure 1. Monthly distribution of Enterovirus isolates (1985–2005), and Enterovirus-positive RT-PCR diagnostic cases (2004–2005). We identified 9 years of significantly high HEV isolation frequency during which the number of monthly isolates exceeded the upper control limit at 99% confidence level (UCL99 = 6.71, $p<0.01$). Peak isolation levels were mostly between May and August, except in 1987 in September and October. 2 epidemics were also recorded in 2000 and 2005.
doi:10.1371/journal.pone.0018022.g001

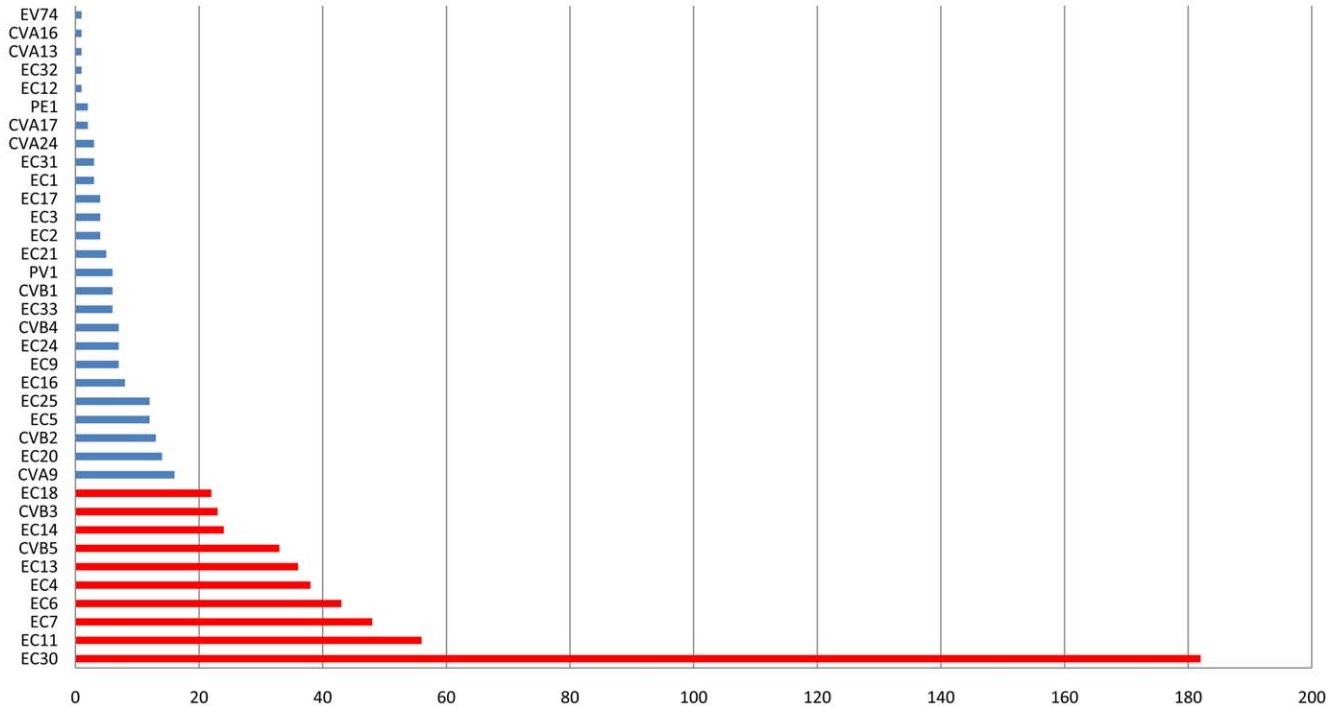


Figure 2. Distribution of Enterovirus serotypes isolated in Marseille, 1985–2005. The 10 most common serotypes isolated between 1985 and 2005 in Marseille account for 77.1% of all cases. Shown in red: (in decreasing order) E30, E11, E7, E6, E4, E13, CVB5, E14, CVB3 and E18. The top 5 most frequently encountered serotypes alone account for 56.7%.
doi:10.1371/journal.pone.0018022.g002

during seven years of the study period (1987, 1988, 1996, 2000, 2001, 2002 and 2005).

Classification and Regression Tree (CART)

The CART technique classified our data into groups through a series of splits that best differentiated observations of the data (**Figure 3**). The main discriminatory feature was the year of isolation which allowed the definition of 3 temporal periods: 1985–1987, 1988–2000 and 2001–2005. Paying attention to the change in proportion of each virus group across these 3 periods we observed that: (i) Poliovirus has gradually disappeared; (ii) Frequency of HEV-A and other HEV-C and remained consistently low (≤2%); (iii) The proportion of coxsackieviruses has increased from 4.9% to 24.8%, and (iv) The proportion of echoviruses have decreased from 89.2% to 73.5%.

EV isolation by sample type and cell line

Stool is used most frequently in suspected HEV infections over 20 years, save the 2000 and 2005 epidemics during which cerebrospinal fluid (CSF) was highly solicited. Stool samples allowed detection of all serotypes, including most of the non HEV-B serotypes and, notably, all the PV. Further examination of the most common HEV serotypes revealed that 84.9% of the clinical strains showed preferential growth in MRC5 cells, in particular the echoviruses. The additional use of BGM cells enhanced total recovery to 96.3% and allowed better detection of group B coxsackieviruses and Polioviruses (**Figure 4**).

Accuracy of serotype identification by seroneutralization

The serotypes of 204 clinical samples initially determined at the time of virus isolation by neutralization tests were challenged by VP1 nucleotide sequencing. Only 113 (55.4%) were corroborated by nucleotide sequence. Considering only serotypes with at least 4 strains, this technique was largely accurate (75–91%) for E20, E30, E24, CVB2 and E5. It was average (50–60%) for E14, CVB5, E7, E4 and E6 and poor (26.7–28.6%) for CVA9 and E11. No PV strain was detected using the neutralization technique. The 4 strains that were ultimately designated as PV1 by their VP1 sequences were initially typed as E20, E21, E24 and an adenovirus.

Phylogenetic analysis of clinical strains

Near full-length VP1 nucleotide sequences (777 nucleotides) of HEV clinical strains were analysed in a phylogenetic tree together with prototype reference sequences and VP1 homologues from NCBI GenBank This overall topology of four distinct clusters corresponding to the four HEV species is consistent with phylogenies previously described [8]. By visualizing the frequency of p-distance scores as a percentage of total scores, clinical VP1 scores fell into three established ranges: variants of the same serotype (≤0.25), sequences of different serotype but of the same species (>0.25 and <0.42) and finally, sequences of different species (≥0.42) (**Figure S1**). Overall, only 0.05%, 0.14% and 0.8% of the three categories respectively were exceptions to these definitions.

Molecular evolution of EC30 and EC13

The molecular evolution of E30 was studied in detail by phylogenetic analysis including 159 E30 VP1 sequences from the Marseille database (**Figure 5**). The phylogenetic tree presented 5 temporal clusters, with all Marseille strains clustering together in group 5 (bootstrap 74%) and the majority in a stable subgroup characterized by their period of isolation (2000–2005). Pairwise p-distance showed that the greatest nucleotide disparity between

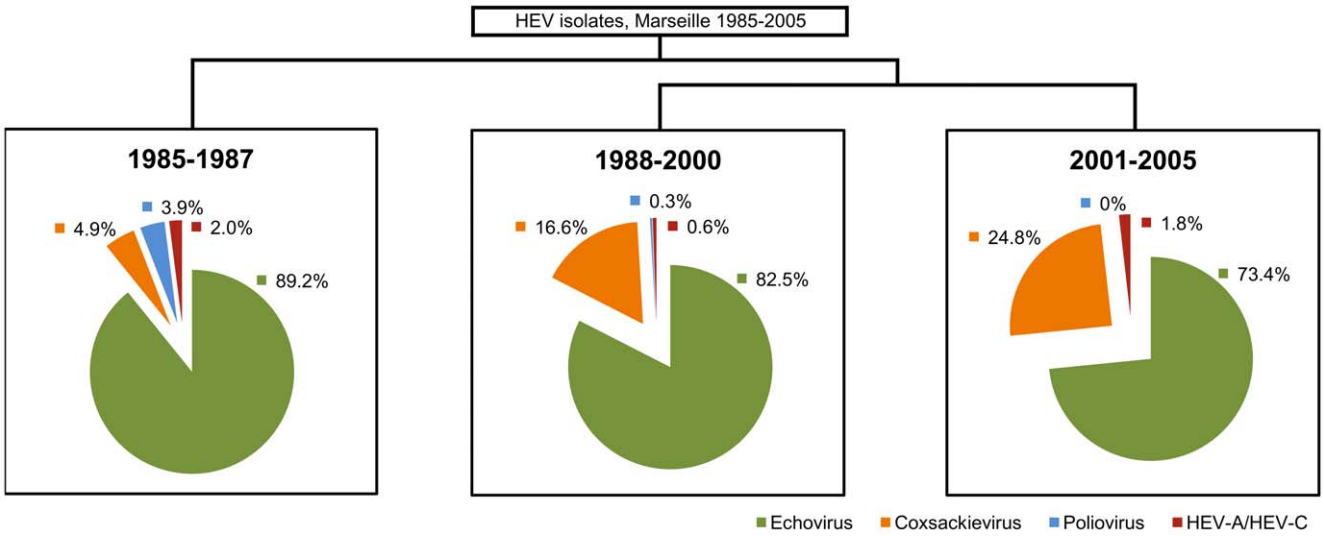


Figure 3. Classification by year of isolation. The Classification and Regression Tree (CART) split the data by year of isolation, describing 3 temporal periods: 1985–1987, 1988–2000 and 2001–2005. The change in proportion was observed for each virus group, with HEV-B further divided into Echoviruses and Coxsackieviruses, while Poliovirus was regarded as separate from HEV-C.
doi:10.1371/journal.pone.0018022.g003

clinical isolates was 0.157, between samples #553 and #497, both isolated in 2002 but which cluster differently in the phylogenetic tree. Notably, the clinical isolate #553 and the prototype strain Giles isolated in 1960 differed genetically by 0.255, which sits just beyond the intra-serotype threshold of 0.25. Its persistent circulation and the extent of its associated epidemics have generated a large genetic diversity within E30, and may go some way to account for this exceptional genetic distance.

The molecular evolution of E13 was also further studied by phylogenetic analysis including 36 E13 VP1 sequences from the Marseille database (**Figure 6**). All but two clinical E13 strains were isolated between 2000 and 2002, and clustered in one distinct group together with European and Asian strains from the same period. Within this group, clinical isolates differed in p-distance by no more than 0.036. In contrast, the greatest genetic distance observed between clinical isolates was 0.242, between #369 isolated in 1987, and #375 isolated in 2000.

Evidence for recombination

To examine the extent of intraspecies recombination, we designed primers that targeted portions of the 2C and 3D regions that distinguished HEV-B serotypes phylogenetically from other species. Of 65 HEV-B strains tested, 59 (90.8%) were successfully amplified and sequenced in the 2C region and 61 (93.8%) in the 3D region. Phylogenetic trees were constructed for the VP1, 2C and 3D genes (**Figure 7**). Incongruent tree topologies and inconsistent interserotype clustering show that the genetic relationship between different serotypes is not conserved throughout the genome. The maximum nucleotide distance in the VP1, 2C and 3D regions was 0.42, 0.262 and 0.271 respectively. To

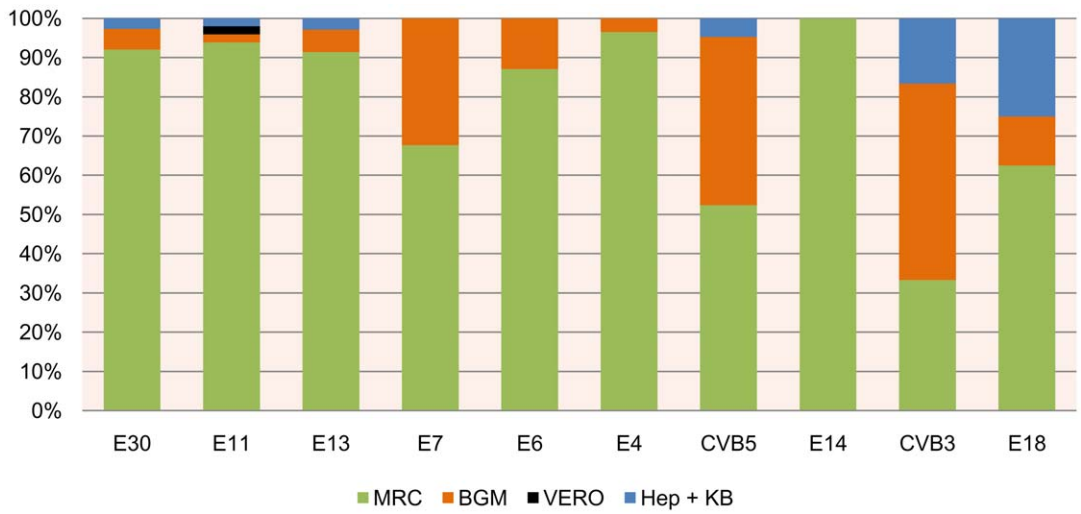


Figure 4. Isolation of the 10 most common Enterovirus serotypes by cell line. MRC5 cells were the most conducive culture line for 84.9% of the samples, with the addition of BGM cell lines enhancing total sample recovery up to 96.3% and covering all serotypes.
doi:10.1371/journal.pone.0018022.g004

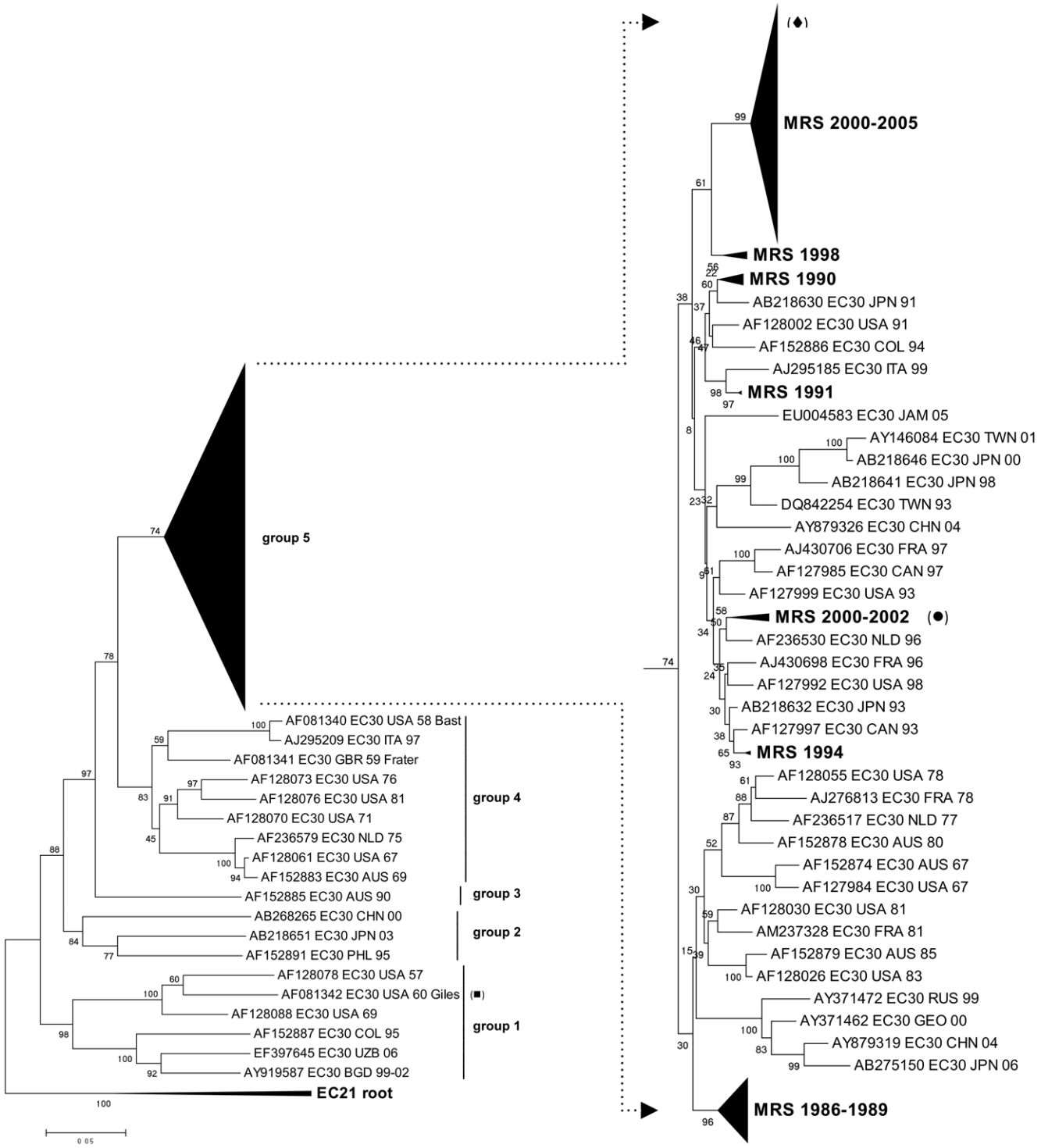


Figure 5. Phylogenetic tree of E30 isolates. 5 groups were observed, with all Marseille isolates clustering in group 5. Closer look at group 5 shows that the most Marseille isolates are genetically related, including all strains from the 2000 and 2005 epidemics. #553 (*diamond*) differs genetically from #497 (*circle*) and prototype Giles (*square*) by 0.157 and 0.312 respectively.
doi:10.1371/journal.pone.0018022.g005

reflect this higher level of conservation in the nonstructural region, all p-distance scores were normalized by expressing them as a percentage of the maximal p-distance in nucleotides for each region within HEV-B.

Three serotypes (E13, CVB3 and E30) with more than 4 antigenic variants in our study set were examined to evaluate intraserotype genetic relationships. Regardless of p-distance, all strains clustered according to their serotype in the VP1 region, as is expected since VP1 is the basis of serotype designation. E13 strains differed little genetically in all regions, by 0–8.3% in VP1, 0–14.9% in 2C and 0–7.7% in 3D. Phylogenetic trees also showed consistent grouping in all regions for these strains, all isolated in

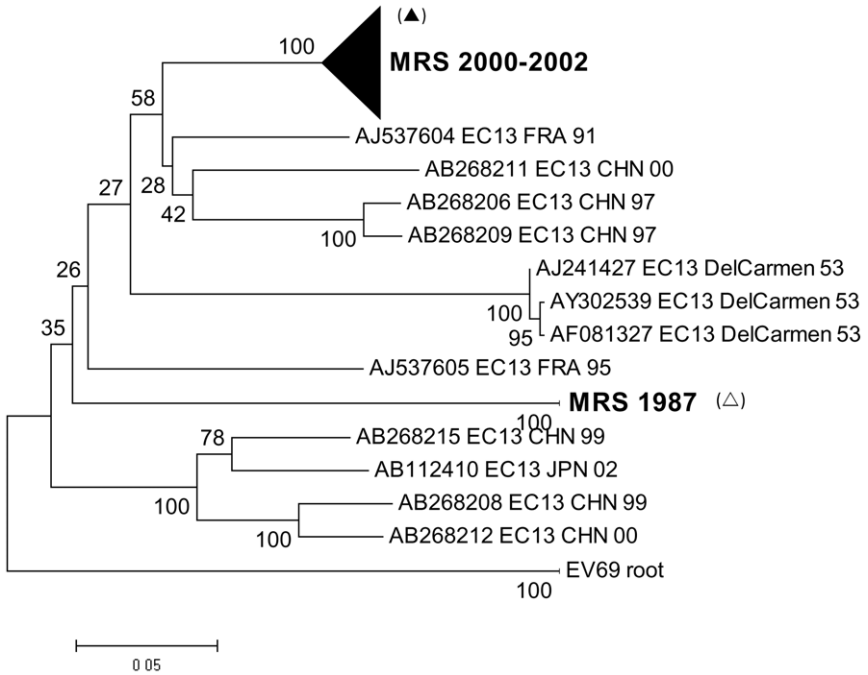


Figure 6. Phylogenetic tree of E13 isolates. All but two sequences were isolated between 2000 and 2002, and clustered in a distinct group. The greatest genetic distance was observed between #369 (*clear triangle*), isolated in 1987, and #375 (*filled triangle*), isolated in 2000.
doi:10.1371/journal.pone.0018022.g006

2000. There is thus no evidence for recombination among clinical E13 strains in Marseille. In contrast, CVB3 strains were more genetically distant, with 5.2–47.1% in VP1, 52.3–80.2% in 2C and 13.7–81.2% in 3D. Their greater diversity is reinforced by inconsistent clustering across the genome. It is thus highly possible that recombination events have occurred involving the nonstructural region of CVB3 strains. E30 on the other hand, is a more complex case: three strains isolated between 1987 and 1988 demonstrated little divergence with maximum p-distance of 4.3%, 5% and 8.5% in the VP1, 2C and 3D regions respectively and consistent clustering with one another in all regions. However, the remaining E30 strains presented greater genetic distance, by 0–20.7% in VP1, 64.1–75.2% in 2C and 62–88.6% in 3D. This disparity is also observed in their variable phylogenetic positions across the genome.

This pattern is exemplified by E30 strains #405559 and #404728, both isolated in 2000 and which are identical (p-distance = 0%) in their VP1 nucleotide sequence but differ by 73.3% and 74.2% in their 2C and 3D sequences respectively. Interestingly, the exact opposite clustering pattern was observed in 7 strains: 1 E30 (#406559), 1 CVA9 (#406891), 1 E25 (#406788) and 4 E13 (#406401, #406050, #406119 and #406241) strains differed by a maximum 92.6% in their VP1 region, but only by 14.9% in 2C and 8.5% in 3D. Phylogenetic analysis showed reliable grouping (bootstrap = 100%) in both the 2C and 3D regions for our cluster of interest.

Discussion

The present study describes the frequency of enteroviral serotypes isolated in Marseille between 1985 and 2005. Enteroviruses are known to circulate in the summer and autumn months in temperate regions [19,29–30]. In Marseille, we observed that HEV isolation peaked in the spring and summer months, notably during the 2000 and 2005 epidemics when unusual levels of HEV activity were detected as early as May. The last natural case of poliovirus in Marseille was described in 1988, in line with its complete elimination in France and the European continent in 1990 [31]. E30 was the most frequent enterovirus isolated in Marseille, in accordance with epidemiological data collected by the RSE, the sentinel laboratory network for the surveillance of Enteroviruses in France [30]. This high incidence also reflects similar circulation levels and the occurrence of E30-associated aseptic meningitis epidemics in 2000–2001 in North and South America, Europe and Asia [23–24,32]. The general distribution of E30 is temporally consistent with other European strains included in our analysis, and supports a microevolution as a continuous cline with rare re-emergence of more ancient strains. Unlike E30, E13 was considered a rare serotype with no outbreaks associated with this virus and had only ever been isolated once in Marseille prior to 2000. Its sudden emergence as a predominant serotype was also observed in other countries: in the United States, E13 accounted for 24% of all reported HEV isolates in 2001, compared to 1.6% in 2000 [33]. In Japan, E13 had only been isolated once before 2001, during which 65 strains were isolated [34]. E13 was first identified in Spain during an aseptic meningitis outbreak in 2000 [35].

Regarding the strategies and methodologies used during the study period for detection and characterization of enteroviruses, a number of observations could be made. Firstly, stool has been the most frequently used sample type for enterovirus isolation, and the most useful since it allowed the isolation of all serotypes. Secondly, MRC5 was the most conducive cell line for enterovirus isolation, and coupling with BGM cell line, resulted in a more extensive coverage for HEV-B strains. Thirdly, seroneutralization-based HEV typing showed 55.4% accuracy when compared to VP1 sequence analysis. This divergence in identification may be explained in part by technical insufficiency of the seroneutralization typing protocol (*e.g.* cross reacting activity, use of pools raised against strains prevalent more than 30 years ago). Another possible

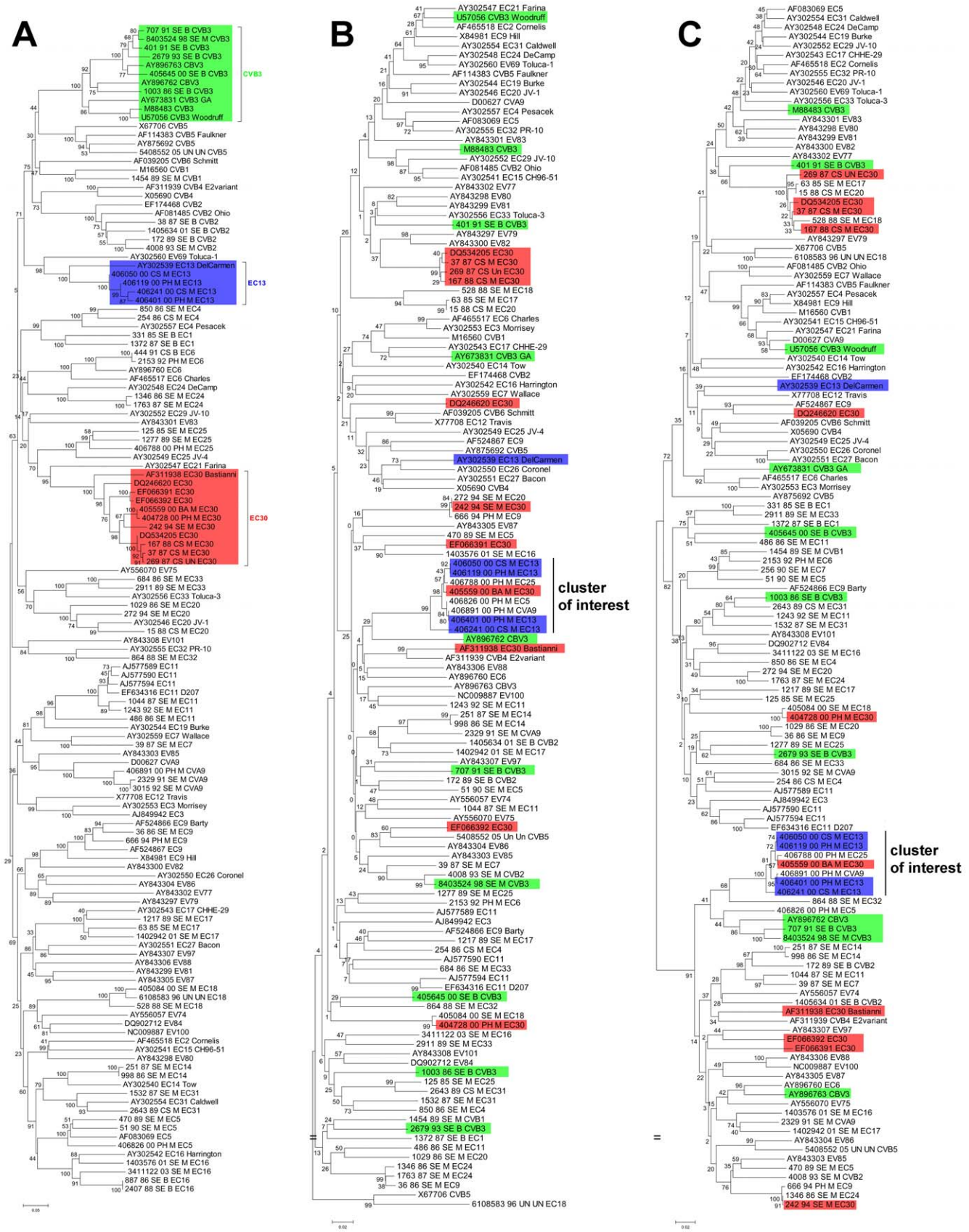


Figure 7. Phylogenetic trees of HEV-B in the VP1 (A), 2C (B) and 3D (C) regions. Inconsistent topologies suggest possible recombination events, especially in the case of E30 (*red*) and CVB3 (*green*). E13 clinical isolates (*blue*) however, differed little genetically across the genome, suggesting the absence of major recombination events. Our cluster of interest includes 7 strains (4 E13, 1 E30, 1 CVA9 and 1 E25) that group reliably (bootstrap 100%) in the 2C and 3D regions but not in the VP1 region.
doi:10.1371/journal.pone.0018022.g007

explanation could be the presence of more than 1 serotype in a patient sample, whereby the dominant serotype during its reproduction in culture for VP1 sequencing is different from the dominant serotype during initial culture for diagnostic seroneutralization. It has also been highlighted that poliovirus might be present in working stocks of other viruses, even when unambiguously identified and labeled [36].

The validity of VP1 serotyping protocol and pairwise genetic distance analysis has been primarily established with enterovirus prototype sequences or with clinical sequences spanning a short period of time [7]. Our analysis of a large number of clinical isolates over 20 years reinforces the pertinence of this technique that allows the identification of most HEV sequences using the simple computationally non-intensive genetic distance calculation. Furthermore, in the few instances whereby the genetic diversity within a serotype can be so significant as to exceed the 0.25 threshold, as observed in E30, genetic distance can be coupled with the phylogenetic analysis of VP1 to provide a non-ambiguous identification of HEV, a strategy previously validated with the delineation of Hepatitis C virus genotypes [37–38].

The serotypic identification of enteroviruses is challenged by the existence of recombination events [39–40]. Isolates sharing similar VP1 genes but differing in other parts of the genome may display different epidemiological or clinical properties. Phylogenetic topologies of different portions of the enteroviral genome describe HEV strains with genetically consistent VP1 regions and more interchangeable 2C and 3D regions, particularly demonstrated by E30 and CVB3 strains from Marseille. This suggests that closely related VP1 regions can be associated with divergent 2C and 3D regions. The mechanism of RNA recombination in enteroviruses is commonly accepted to involve template switching during RNA synthesis, with recombination points most frequently identified within the nonstructural region [28,41]. In contrast, we observed the opposite trend in an unusual cluster of 7 strains: #406891_CVA9, #406401_E13, #406050_E13, #406119_E13, #406241_E13, #406788_E25 and #406559_E30 showed little similarity in the VP1 region, but a marked resemblance in the nonstructural region. This suggests the circulation of highly similar HEV strains which differ primarily in the region by which they are attributed serotypes. Considering that all 7 strains were isolated during the 2000 Marseille epidemic during which the E30 and E13 serotypes were particularly prominent, this genetic similarity could explain the emergence of E13 as an epidemic serotype by a recombination between circulating E13 and epidemic E30 strains. This might also account for the lack of direct correlation between serotype and pathology, such as how several (VP1-defined) serotypes can provoke the same clinical manifestations. A new model of enteroviral genetics has been suggested, such that enteroviruses should be regarded as a pool of independently evolving genomic fragments [42]. We show that clinical strains of enteroviruses circulating over 20 years lend credence to this model by showing the inadequacies of the current model of demarcated serotypes.

In this work, serotypes from the HEV-B species account for 98% of all isolates in Marseille. This echoes HEV-B levels described in Spain (89.3%) [43], in the United States (89.5%) [19] and in Tunisia (92.2%) [44], studies which also used cell culture to first isolate the virus in the typing process. However, the use of cell culture techniques may distort any derived epidemiological data since some HEV serotypes (Coxsackievirus A and certain numbered HEVs) do not grow or grow poorly in cell culture, and suggests that the proportion of circulating non HEV-B serotypes has been underestimated. Such a study of clinical samples in a clinical virology laboratory is sure to encounter some limitations, in part by the bias associated with cell culture techniques, but also by other factors such as patient sample referral and enteroviral disease presentations that might not be actively investigated. This work should thus be more accurately regarded as a clinical profile of HEV-B serotypes in Marseille. As such, we feel the need to reinforce efforts for identifying HEV directly from clinical samples, bypassing the need for cell culture systems.

Materials and Methods

Marseille HEV strain collection and sequence database

Enterovirus samples. All samples taken for diagnostic purposes are accessible for research under French national regulations regarding biomedical research (Loi Huriet-Sérusclat (loi 881138)) without requiring neither specific written consent from the patient nor approval from an ethics committee. All clinical samples were obtained from the Laboratory of Virology, University Hospital La Timone (Marseille, France) from 1985 to 2005. Specimen types comprised of nasopharyngeal swabs, stool, cerebrospinal fluid, saliva and bronchoarterial specimens.

Cell lines. MRC5 cells (Human fetal lung fibroblasts) were cultured in Basal Medium Eagle (BME), 10% decomplemented Fetal Bovine Serum (dFBS), 1% L-Glutamine (L-GLN), 1% Penicillin-Streptomycin (PS). Hep2 (Human laryngeal carcinoma cells), KB (Human laryngeal carcinoma cells), Vero (African green monkey kidney cells) and BGM (Buffalo green monkey kidney cells) cell lines were grown in Minimum Essential Medium Eagle (MEM), 5% dFBS, 1% L-GLN, 1% PS. All cell cultures were incubated at 37°C under 5% CO_2.

Enterovirus diagnosis. (i) Prior to 2000, HEV diagnosis consisted of growing samples in cell culture in MRC5, BGM, Vero and KB cell lines. Once cytopathic effect (CPE) was observed, the presence of HEV antigen was tested by immunofluorescence with a monoclonal mouse anti-EV antibody (clone 5-D8/1, Dako) and a secondary goat anti-IgG mouse fluorescein conjugate [45]; (ii) From 2000 to 2004, diagnosis was achieved by classic RT-PCR using the Enterovirus Consensus Kit 5 (Argene) and inoculation of samples onto MRC5, BGM, Vero and Hep2 cell lines which were similarly evaluated by CPE and immunofluorescence; (iii) From 2005 onwards, a real-time pan-enterovirus RT-PCR was used (adapted from [5]) along with the inoculation of samples onto MRC5, BGM, Vero and Hep2 cell lines which were similarly evaluated by CPE and immunofluorescence. For all samples, the cell line in which CPE was most rapidly observed was recorded and the virus isolates stored in the Marseille Public Hospitals virus collection. Globally, from 1985 to 2005, the same cell culture detection and isolation protocol was performed based on the use of MRC5, Vero and BGM cells, which represent 96.5% of all isolates (cf Results section). The only change during the period was the replacement of KB with Hep2 cells from 2000 onwards, and which represent 0.5% and 3% of all isolates respectively).

Seroneutralization. A portion of HEV-positive samples (n = 204) processed between 1985 and 1994 were typed by seroneutralization with Lim-Benyesh-Melnick antiserum pools [6].

VP1 Sequencing. Strains recorded in the Marseille HEV collection were reproduced in the cell line in which they were originally isolated from culture. Supernatant was clarified by centrifugation and extracted using the EZ1 Virus Mini Kit (Virus Card 2.0) in an EZ1 BioRobot (QIAGEN) according to the manufacturer's protocol. Reverse transcription was carried out with Reverse Transcriptase MultiScribe (Applied Biosystems) with random hexamers. Each viral cDNA was then amplified by nested *Taq* DNA Polymerase PCR (Invitrogen) using 2 VP1-specific primer pairs (adapted from [7]). Amplification products were

visualized by 2% agarose gel electrophoresis and ethidium bromide staining, then purified with QIAquick PCR Extraction or Gel Extraction kits (QIAGEN). Sequencing was carried out using a Big Dye Terminator Cycle Sequencing Reaction kit and an ABI Prism 3130 DNA Sequencer (Applied Biosystems).

Marseille HEV sequence database. VP1 sequences were obtained and their serotype identified by phylogenetic analysis (n = 654). They were archived in the Marseille HEV VP1 database in the following format: #Reference number_Year_Sample type_Cell line_Serotype. No written patient consent was required as all strains were characterized for etiological purposes.

2C and 3D sequencing. HEV-B strains from the HEV database (n = 65) were chosen to be representative of serotypes and years for the period surveyed. 2C, coding region for the helicase/NTPase; and 3D, coding region for the RNA-dependent RNA polymerase, were chosen as representative of the P2 and P3 regions respectively. We designed primer pairs to specifically amplify HEV-B serotypes, targeting portion of the 2C and 3D regions that phylogenetically distinguished HEV-B from other HEV species. RT-PCR was performed as described above, using the specific primer pairs 2C-F (TTYGAYGGiTAYAARCARCA) and 2C-R (GGiCCYTGRAAiARiGCYTC) or 3D-2F (TTYTGGWSiAARATHCCiGT) and 3D-R (CKiACRTGRTCYTGiGTRTT). Amplification products were visualized, purified and sequenced as described above.

Sequence analysis

Phylogenetic analysis. All sequence chromatograms were analysed with Sequencher 4 software (Gene Codes Corporation). Multiple sequence alignments were realized with EBI ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [46] on default settings and manually edited with BioEdit [47]. The nucleotide sequences were translated into and aligned as amino acids. Using the programme DAMBE (http://dambe.bio.uottawa.ca/dambe.asp) [48], nucleotide sequences were aligned against the amino acid sequences. Phylogenetic trees were constructed with MEGA version 3.1 (Molecular Evolutionary Genetics Analysis) [49]. For VP1, 2C and 3D, this was achieved using the Neighbor-Joining method on a Jukes-Cantor model. Partial VP1 sequences (<400 nucleotides) were omitted from phylogenetic analysis. Pairwise distance matrices were drawn to calculate p-distance, the proportion of nucleotide sites at which the two sequences differ for the totality of the sites compared. The consistency of tree topologies was tested by bootstrapping in 1000 pseudoreplicates.

Statistical analysis of epidemiological data

A Classification and Regression Tree (CART) was established to determine characteristic features of the dataset as a series of if-then logical conditions [50]. The monthly frequency of Enterovirus isolations was plotted on a control chart for count data (Poisson distribution) estimating an upper control limit with $\mu \pm 3\sigma$ (99.73% confidence) [51–52]. Statistical analysis was carried out using the R.2.10.1 environment (http://www.r-project.org) and the qcc package [53].

Supporting Information

Figure S1 Pairwise p-distance scores of clinical Enterovirus VP1 sequences, 1985-2005. 20 years of clinical strains validated the three established ranges of genetic distance that indicate variants of the same serotype ($\leq$0.25), sequences of different serotypes but the same species (>0.25 and <0.42), or sequences of different species ($\geq$0.42).
(TIF)

Acknowledgments

We would like to thank Karine Barthelemy for her excellent technical assistance.

Author Contributions

Conceived and designed the experiments: CYQT LN AN CZ LT-P RNC XdL. Performed the experiments: CYQT LN AN. Analyzed the data: CYQT LN JG AN RNC XdL. Contributed reagents/materials/analysis tools: CYQT LN JG AN CZ LT-P RNC XdL. Wrote the paper: CYQT LN JG AN XdL.

References

1. Hewlett MJ, Florkiewicz RZ (1980) Sequence of picornavirus RNAs containing a radioiodinated 5′-linked peptide reveals a conserved 5′ sequence. Proc Natl Acad Sci 77: 303–307.
2. Diedrich S, Dreisel G, Schreier E (1995) Sequence comparison of echovirus type 30 isolates to other enteroviruses in the 5′noncoding region. J Med Virol 46: 148–152.
3. Pöyry T, Kinnunen L, Hyppiä T, Brown B, Horsnell C, et al. (1996) Genetic and phylogenetic clustering of enteroviruses. J Gen Virol 77: 1699–1717.
4. Hyypiä T, Hovi T, Knowles NJ, Stanway G (1997) Classification of Enteroviruses Based on Molecular and Biological Properties. J Gen Virol. pp 1–11.
5. Watkins-Riedel T, Woegerbauer M, Hollemann D, Hufnagl P (2002) Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. Diagn Microbiol Infect Dis 42: 99–105.
6. Lim KA, Benyesh-Melnick M (1960) Typing of Viruses by Combinations of Antiserum Pools. Application to Typing of Enteroviruses (Coxsackie and Echo). J Immunol 84: 309–318.
7. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, et al. (1999) Typing of Human Enteroviruses by Partial Sequencing of VP1. J Clin Microbiol 37: 1288–1293.
8. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA (1999) Molecular Evolution of the Human Enteroviruses: Correlation of Serotype with VP1 Sequence and Application to Picornavirus Classification. J Virol 73: 1941–1948.
9. Nix WA, Oberste MS (2006) Sensitive, seminested PCR Amplification of VP1 sequences for direct identification of all Enterovirus serotypes from original clinical specimens. J Clin Microbiol 44: 2698–2704.
10. Nasri D, Bouslama L, Omar S, Saoudin H, Bourlet T, et al. (2007) Typing of Human Enterovirus by Partial Sequencing of VP2. J Clin Microbiol 45: 2370–2379.
11. Mirand A, Henquell C, Archimbaud C, Chambon M, Charbonne F, et al. (2008) Prospective Identification of Enteroviruses involved in Meningitis in 2006 through Direct Genotyping in Cerebrospinal Fluid. J Clin Microbiol 46: 87–96.
12. Leitch ECM, Harvala H, Robertson I, Ubillos I, Templeton K, et al. (2009) Direct identification of human enterovirus serotypes in cerebrospinal fluid by amplfication and sequencing of the VP1 region. J Clin Virol 44: 119–124.
13. Melnick JL (1996) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, Channock RM, Melnick JL, et al. eds. Fields Virology. 3rd ed. Philadelphia, PA: Lippincott-Raven Publishers. pp 655–712.
14. Cherry JD (1992) Enteroviruses: Polioviruses (poliomyelitis), coxsackieviruses, echoviruses and enteroviruses. In: Feigin RD, Cherry JD, Demmler GJ, Kaplan SL, eds. Textbook of Pediatric Infectious Diseases. 3rd ed. PhiladelphiaPA: WB Saunders. pp 1705–1753.
15. Rotbart HA (2000) Viral meningitis. Semin Neurol 20: 277–292.
16. Tebruegge M, Curtis N (2009) Enterovirus infections in neonates. Sem Fetal Neonatal Med 14: 222–227.
17. Chen TC, Weng KF, Chang SC, Lin JY, Huang PN, et al. (2008) Development of antiviral agents for enteroviruses. J Antimicrob Chemother 62: 1169–1173.
18. Global Polio Eradication Initiative (2009) Strategic plan 2009-2013 framework document. Available: http://www.polioeradication.org/content/publications/PolioStrategicPlan09-13_Framework.pdf Accessed 06 October 2010.
19. Khetsuriani N, LaMonte-Fowlkes A, Oberste MS, Pallansch MA (2006) Enterovirus surveillance—United States, 1970-2005. MMWR Surveill Summ 55: 1–20.
20. Wang J-R, Tsai H-P, Huang S-W, Kuo P-H, Kiang D, et al. (2002) Laboratory Diagnosis and Genetic Analysis of an Echovirus 30-Associated Outbreak of Aseptic Meningitis in Taiwan in 2001. J Clin Microbiol 40: 4439–4444.

21. Ozkaya E, Hizel K, Uysal G, Akman S, Terzioglu S, et al. (2003) An Outbreak of Aseptic Meningitis due to Echovirus type 30 in two cities in Turkey. Eur J Epidemiol 18: 823–826.
22. Bernit E, de Lamballerie X, Zandotti C, Berger P, Veit V, et al. (2004) Prospective investigation of a large outbreak of meningitis due to Echovirus 30 during summer 2000 in Marseilles, France. Medicine 83: 245–253.
23. dos Santos GPL, Skraba I, Oliveira D, Lima AAF, de Melo MMM, et al. (2005) Enterovirus meningitis in Brazil, 1998-2003. J Med Virol 78: 98–104.
24. Cabrerizo M, Echevarria JE, Gonzalez I, de Miguel T, Trallero G (2008) Molecular epidemiological study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain during 2006. J Med Virol 80: 1018–1024.
25. Kirkegaard K, Baltimore D (1986) The mechanism of RNA recombination in poliovirus. Cell 47: 433–443.
26. Santti J, Hyypiä T, Kinnunen L, Salminen M (1999) Evidence of Recombination among Enteroviruses. J Virol 73: 8741–8749.
27. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, et al. (2003) Recombination in Circulating Enteroviruses. J Virol 77: 10423–10431.
28. Oberste MS, Maher K, Pallansch MA (2004) Evidence for Frequent Recombination within Species Human Enterovirus B Based on Complete Genomic Sequences of All Thirty-Seven Serotypes. J Virol 78: 855–867.
29. Pallansch MA, Roos R (2007) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Knipe DM, Howley PM, eds. Fields Virology. 5th ed. PhiladelphiaPA: Lippincott Williams & Wilkins. pp 840–893.
30. Antona D, Lévêque N, Chomel J-J, Dubrou S, Lévy-Bruhl D, et al. (2007) Surveillance of Enteroviruses in France, 2000-2004. Eur J Clin Microbiol Infect Dis 26: 403–412.
31. Malvy D, Drucker J (1993) Elimination of poliomyelitis in France: epidemiology and vaccine status. Public Health Reviews 21: 41–49.
32. MMWR (2006) Enterovirus surveillance - United States, 2002-2004. MMWR Morb Mortal Wkly Rep 55: 153–156.
33. Mullins JA, Khetsuriani N, Nix WA, Oberste MS, LaMonte A, et al. (2004) Emergence of Echovirus Type 13 as a Prominent Enterovirus. Clin Infect Dis 38: 70–77.
34. Mizuta K, Abiko C, Murata T, Itagaki T, Katsushima N, et al. (2003) Re-emergence of echovirus type 13 infections in 2002 in Yamagata, Japan. J Infect 47: 243–247.
35. Avellón A, Casas I, Trallero G, Pérez C, Tenorio A, et al. (2003) Molecular analysis of Echovirus 13 isolates and aseptic meningitis, Spain. Emerg Infect Dis 9: 934–941.
36. Davies M, Bruce C, Bewley K, Outlaw M, Mioulet V, et al. (2003) Poliovirus type 1 in working stocks of typed human rhinoviruses. Lancet 361: 1187–1188.
37. de Lamballerie X, Charrel RN, Attoui H, De Micco P (1997) Classification of hepatitis C virus variants in six major types based on analysis of the envelope 1 and nonstructural 5B genome regions and complete polyprotein sequences. J Gen Virol 78: 45–51.
38. Simmonds P, Holmes EC, Cha T-A, Chan S-W, McOmish F, et al. (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 74: 2391–2399.
39. Lindberg AM, Andersson P, Savolainen C, Mulders MN, Hovi T (2003) Evolution of the genome of Human enterovirus B: incongruence between phylogenies of the VP1 and 3CD regions indicates frequent recombination within the species. J Gen Virol 84: 1223–1235.
40. Simmonds P, Welch J (2006) Frequency and Dynamics of Recombination within Different Species of Human Enteroviruses. J Virol 80: 483–493.
41. Lukashev AN (2005) Role of recombination in evolution of enteroviruses. Rev Med Virol 15: 157–167.
42. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, et al. (2005) Recombination in circulating Human enterovirus B: independent evolution of structural and non-structural genome regions. J Gen Virol 86: 3281–3290.
43. Trallero G, Avellón A, Otero A, De Miguel T, Perez C, et al. (2010) Enteroviruses in Spain over the decade 1998-2007: Virological and Epidemiological Studies. J Clin Virol 47: 170–176.
44. Bahri O, Rezig D, Ben Nejma-Oueslati B, Ben Yahia A, Ben Sassi J, et al. (2005) Enteroviruses in Tunisia: Virological surveillance over 12 years (1992-2003). J Med Microbiol 54: 63–69.
45. Trabelsi A, Grattard F, Nejmeddine M, Aouni M, Bourlet T, et al. (1995) Evaluation of an Enterovirus Group-specific Anti-VP1 Monoclonal Antibody, 5-D8/1, in Comparison with Neutralization and PCR for Rapid Identification of Enteroviruses in Cell Culture. J Clin Microbiol 33: 2454–2457.
46. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.
47. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.
48. Xia X, Xie Z (2001) DAMBE: Software Package for Data Analysis in Molecular Biology and Evolution. J Hered 92: 371–373.
49. Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9: 299–306.
50. Breiman L, Friedman JH, Olshen RA, Stone CJ (1984) Classification and Regression Trees. BelmontCA: Wadsworth International. 368 p.
51. Winckel P, Zhang N (2007) Statistical Development of Quality in Medicine. Chicester: John Wiley & Sons. 280 p.
52. Montgomery DC (2000) Introduction to Statistical Quality Control. New York: John Wiley & Sons.
53. Scrucca L (2004) qcc: an R package for quality control charting and statistical process control. R News 4: 22–27.

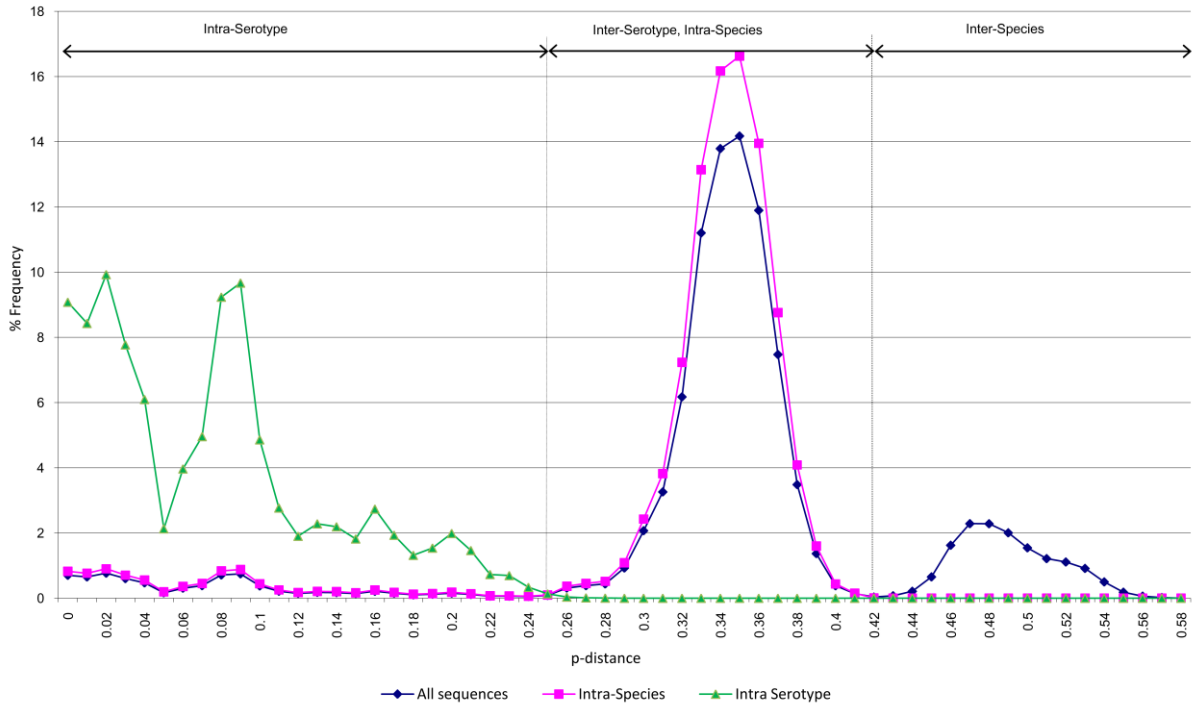


Figure S1 : Pairwise p-distance scores of clinical Enterovirus VP1 sequences, 1985-2005. 20 years of clinical strains validated the three established ranges of genetic distance that indicate variants of the same serotype (≤0.25), sequences of different serotypes but the same species (>0.25 and <0.42), or sequences of different species (≥0.42).

Discussion

Our systematic analysis of circulating EV serotypes in Marseille over 20 years has resulted in several major observations: Similar to RSE findings, the ten main serotypes in Marseille are members of the HEV-B species. The most frequently encountered serotype is E30, whose high incidence is reflected worldwide. Emergence of E13 considered a rare serotype prior to 2000 in Marseille. This epidemic serotype has also been increasingly observed in other countries: in the United States, E13 accounted for 24% of all reported EV isolates in 2001, a marked increase from 1.6% in 2000 (54).

Furthermore, the analysis of circulation patterns and laboratory strategy has allowed guidelines for streamlining diagnostic procedures in terms of time and manpower without sacrificing sensitivity. While no single cell line has been described as sufficient for the isolation of all serotypes, the use of MRC5 and BGM cell lines provides extensive coverage of enterovirus serotypes. Detection also begins earlier than described as well, since EV isolation peaked in the spring and summer months, even as early as May in Marseille.

The strategy of EV serotype identification based on genetic and phylogenetic analysis has previously been proposed by Oberste *et al* (31). However, this has been established primarily with prototype sequences or partial clinical sequences spanning a short period of time (1991-1998). Our analysis of over 600 clinical isolates over 20 years reinforces the validity of this technique.

The current overview of enterovirus evolution is modelled as a global reservoir of genomic fragments, in which genetically similar structural regions associate with more divergent nonstructural regions (183). However, the opposite trend was observed in an unusual cluster

of 7 strains (1 CVA9, 1 E25, 1 E30 and 4 E13) isolated during the epidemic in 2000, with different VP1 but highly similar 2C and 3D regions, suggesting that the emergence of epidemic E13 serotype could be due to recombination between circulating E13 and epidemic E30 strains.

ARTICLE #2

Impact of diagnostic procedures on patient management and hospitalization cost during the 2000 and 2005 enterovirus epidemics in Marseille, France

Foreword

Enteroviruses are responsible for a wide spectrum of human illnesses, the majority of which are mild and self-limiting. However, severe presentations such as meningoencephalitis, can be dangerous in neonates, immunocompromised patients or patients with pre-existing medical conditions. Two large outbreaks were reported in Marseille in 2000 and 2005 (223). In between these events, an important change in the laboratory diagnosis of EV infections was implemented: in 2000, laboratory diagnosis was based on cell culture and to a lesser extent, on classic RT-PCR amplification; in 2005, this was performed with a real-time RT-PCR assay.

Viral and epidemiological characteristics of both outbreaks were analysed and compared, especially in the period of time necessary for diagnostic result delivery and the impact on subsequent management of patients.

Impact of diagnostic procedures on patient management and hospitalization cost during the 2000 and 2005 enterovirus epidemics in Marseilles, France

L. Ninove[1,2], C. Tan[1], A. Nougairede[1,2], C. Zandotti[1,2], H. Richet[2], R. Charrel[1,2] and X. de Lamballerie[1,2]

1) *Unité des Virus Emergents, UMR190, IRD and Université de la Méditerranée, and* 2) *Fédération de Microbiologie, University Hospitals of Marseilles (AP-HM), France*

Abstract

Enteroviruses are frequent aetiological agents of central nervous system infections in humans. In 2000 and 2005, two large outbreaks of Echovirus 30 (a member of species *human enterovirus B*) were observed in the University Hospitals of Marseilles (France). Between the two epidemics, the diagnostic protocols for enterovirus infection were modified, moving from viral cultures and classic RT-PCR in 2000 to real-time RT-PCR in 2005. We compared some viral and epidemiological characteristics of the 2000 and 2005 outbreaks with special attention to diagnostic procedures and to the subsequent clinical management of patients. Despite similar virological and epidemiological characteristics during both outbreaks, our results show that real-time RT-PCR techniques used in 2005 noticeably shortened the period of time necessary to deliver diagnostic results and suggest that this was associated with a decrease in the duration of hospitalization for positive cases. In conclusion, this study suggests that the improvement of enterovirus diagnosis had a major financial impact on the management of the 2005 epidemic in Marseilles and may constitute an interesting example of how new diagnostic methods in microbiology can be self-financed through improvement in patient management.

Keywords: Diagnosis, enterovirus, meningitis
Original Submission: 20 January 2009; **Revised Submission:** 10 April 2009; **Accepted:** 15 April 2009
Editor: G. Greub
Article published online: 10 December 2009
Clin Microbiol Infect 2010; **16:** 651–656
10.1111/j.1469-0691.2009.02915.x

Corresponding author and reprint requests: L. Ninove, UMR190, Emergence des Pathologies Virales, Faculté de Médecine, 27 bd Jean Moulin, 13005 Marseille, France
E-mail: laetitia.ninove@univmed.fr

Introduction

The enteroviruses (EVs) are a genus of the family *Picornaviridae* which includes six species (representing over 70 serotypes) isolated from man: human enteroviruses A–E (HEV-A–HEV-E) and polioviruses [1]. During human infection, EVs which normally replicate in the intestinal tract may spread to other organs, and in particular to the central nervous system. Accordingly, EVs are the most frequent causes of aseptic meningitis in both paediatric [2] and adult [3] populations. In temperate regions, EVs circulate widely and cause seasonal epidemics of meningitis in the summer and autumn with sporadic cases during inter-epidemic periods [4]. Severe presentations (meningoencephalitis, encephalitis and polyradiculoneuritis) can be encountered, particularly in neonates, immunocompromised patients or patients with pre-existing medical conditions or underlying illnesses. However, in the vast majority of cases the disease is mild and self limiting, with a large number of asymptomatic or pauci-symptomatic infections [1]. No antiviral treatment is currently available and vaccination is not used against EVs other than polioviruses, because of the multiplicity of the antigenic types implicated in human infections and the lack of cross-reactive immunity.

During the past decades, echovirus 30 (EV-30), a member of the species *human enterovirus B* (HEV-B), has been responsible for meningitis outbreaks with increasing frequency. More specifically, large outbreaks were observed at the University Hospitals of Marseilles (France) in 2000 [5] and 2005. Interestingly, an important change in the management of hospitalized EV-infected patients occurred between the two epidemics: in 2000, the laboratory diagnosis of EV infections was mainly based on viral cultures and partially on classic RT-PCR amplification, while in 2005 virus detection was mainly achieved using a real-time RT-PCR technique. In the

present study, we compare some viral and epidemiological characteristics of the 2000 and 2005 outbreaks with special attention given to the period of time necessary to deliver diagnostic results and the subsequent clinical management of patients.

Patients and methods

Patients

Population studied. During the 2000 and 2005 outbreaks, 195 and 387 cerebrospinal fluid (CSF) samples from patients with clinical presentations suggesting EV infection were analysed. All patients were hospitalized in the University Hospitals of Marseilles, France.

Definition of confirmed cases. As previously proposed and discussed [5], confirmed cases of EV nervous system involvement were defined by the presence of (i) signs of meningeal irritation and/or other central nervous system involvement and/or febrile polyradiculoneuritis and (ii) positive culture or RT-PCR in CSF.

Laboratory diagnosis

Diagnosis of EV infection. Diagnosis was achieved using (i) inoculation of CSF samples onto human lung embryonic fibroblast MRC5 and continuous kidney Buffalo green monkey (BGM) cell lines (2000 and 2005 outbreak patients), (ii) classic RT-PCR using the Enterovirus Consensus kit (Argene, Verniolle, France) (2000 outbreak patients) and (iii) a real-time RT-PCR method adapted from Watkins-Riedel *et al.* [6] (2005 outbreak patients).

Genetic characterization of EVs. Genetic identification of serotypes was accomplished with cell culture isolates. During the 2000 outbreak, a first screening used restriction fragment length polymorphism (RFLP) analysis of PCR amplicons [7] for the identification of isolates closely related to the dominant EV-30 strain. A number of these EV-30 isolates and all isolates with different RFLP profiles were subsequently characterized by sequencing. A 785-bp PCR product located in the VP1 gene was synthesized and sequenced as reported previously [5], and then compared with a reference VP1 database for serotype determination [8]. In 2005, all isolates were sequenced using this method.

For the analysis of EV-30 isolates, sequences obtained from cell culture isolates were combined with a choice of relevant EV-30 VP1 sequences retrieved from the GenBank database and aligned using the CLUSTALW programme [9]. Phylogenetic analysis was performed with MEGA version 3.1 [10] using the Kimura 2-parameter algorithm for distance calculation and the Neighbor-Joining method for tree construction. An EV-21 sequence (China 1999, GenBank accession number AB268240) was used as an outgroup.

Results

Diagnosis of EV infection and viral characteristics of confirmed cases

In 2000, all samples (n = 195) were tested using cell culture and 106 were also tested using RT-PCR. A total of 139 confirmed cases was identified: 22 cases were detected using classic RT-PCR only, 70 using cell culture only and 47 using both methods. Among 117 isolates from confirmed cases, 95 were EV-30 (81%) and 22 belonged to other serotypes of HEV-B (19%). Thirty-one isolates of EV-30 were further characterized by sequencing of the VP1 gene sequence.

In 2005, 186 confirmed cases were identified, mainly using real-time RT-PCR and 24 isolates were obtained from these samples. The VP1 region of these isolates was sequenced and corresponded to 18 EV-30 (82%) and six isolates from other serotypes of HEV-B (18%). In all cases, viruses isolated in cell culture were also detected using real-time RT-PCR.

A total of 49 EV-30 VP1 sequences was obtained for analysis (31 strains from 2000 and 18 from 2005). A phylogenetic reconstruction is shown in Fig. 1. The tree indicates that all VP1 genes of 2000 and 2005 EV-30 strains were closely related and had a recent common ancestor. They belonged to a unique recent lineage identified by a 99% bootstrap resampling value. Genetic pairwise distances within this lineage were lower than 5%; in comparison, the genetic variability among all EV-30 isolates included in our tree was approximately 15%. Isolates from 2000 were organized as two distinct (but closely related) clusters (denoted 2000a and 2000b in Fig. 1). Isolates from 2005 belonged to a unique cluster which appeared as a sister group to the 2000b cluster.

Epidemiological characteristics of confirmed cases

The distribution of confirmed cases by age group was similar during the 2000 and 2005 outbreaks (Fig. 2). The median age was 11.2 years in 2000 and 10.0 in 2005, with a majority of confirmed cases occurring in patients younger than 15 years. The gender ratio (M/F) was 1.6 in 2000 and 1.4 in 2005.

Period of time necessary to deliver diagnostic results

In 2000, the mean time necessary for providing results to clinicians was 12.4 ± 4.3 days, i.e. 11.6 ± 3.9 days in the case of positive results (n = 139) and 14.3 ± 5.3 days in the case of negative results (n = 56). The average delay was

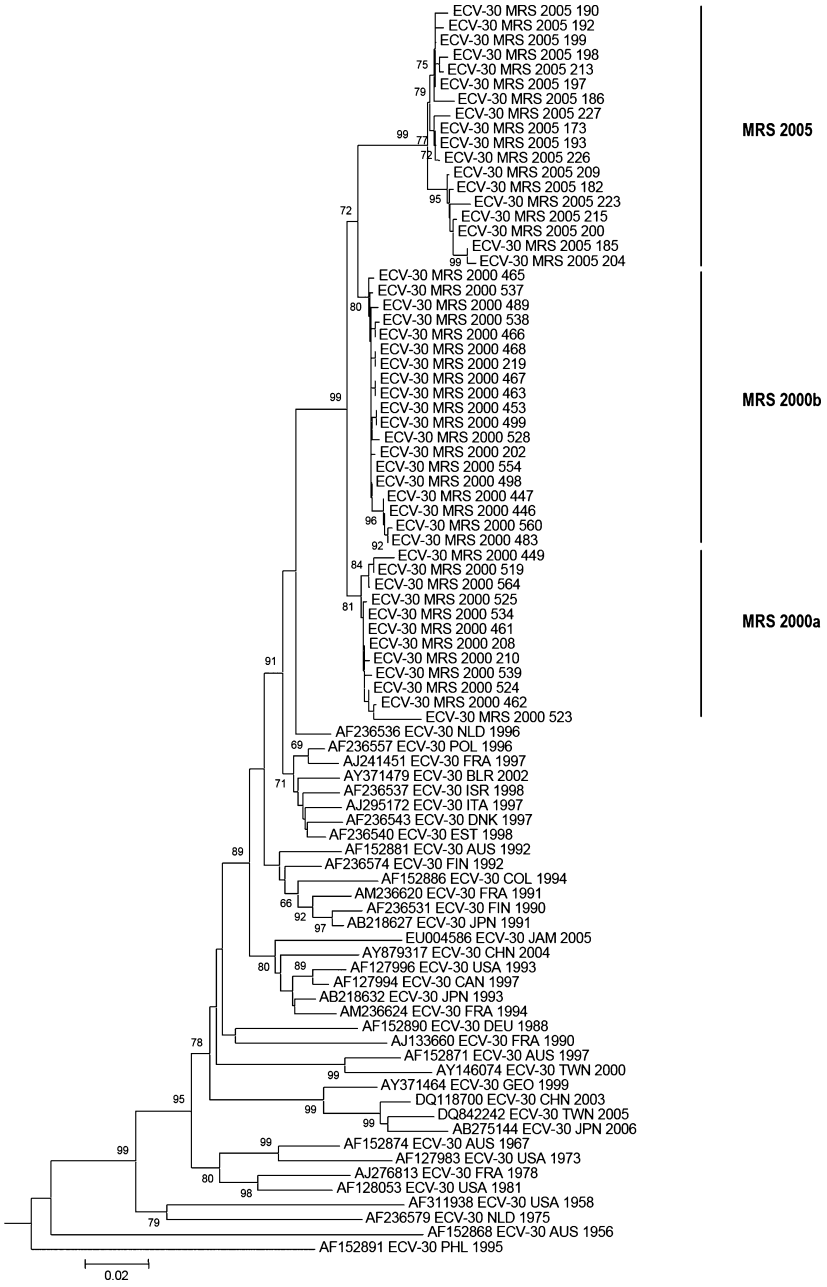


FIG. 1. Genetic relationship between 2000 and 2005 EV-30 isolates. The phylogenetic tree was built using Kimura 2-parameter algorithm and the Neighbor-Joining method. Bootstrap resampling values (500 replications) are indicated at main nodes. Abbreviations: AUS, Australia; BLR, Byelorussia; CAN, Canada; CHN, China; DEU, Germany; DNK, Denmark; EST, Estonia; FIN, Finland; FRA, France; GEO, Georgia; ISR, Israel; ITA, Italia; JAM, Jamaica; JPN, Japan; MRS, Marseilles (France); NLD, the Netherlands; PHL, Philippines; POL, Poland; TWN, Taiwan; USA, United States of America.

8.9 ± 4.2 days for classic RT-PCR results, i.e. 9.3 ± 4.5 days for positive results (n = 69) and 8.1 ± 3.6 days for negative results (n = 37). In 2005, all results were provided using the real-time RT-PCR assay. The mean time necessary for providing results to clinicians was 1.9 ± 0.8 days, similar for positive (n = 186) and negative results (n = 201).

Length-of-hospital stay

In 2000 and 2005, the mean lengths-of-hospital stay (LOS) in confirmed cases were 5.4 ± 4.8 days and 2.2± 1.8 days, respectively. This difference is significant (Student's test), i.e. hospitalizations were significantly shorter in 2005.

Discussion

Numerous previous studies have tried to model and document the clinical and economic impact of EV PCR diagnosis. Differentiating EV positive and negative patients is important [11–14] as the recommended treatments and the cost related to LOS or diagnostic and therapeutic interventions are different. A convergent line of arguments also suggests that results must be available early in the management of patients to impact significantly on patient care in the subgroup of EV-infected patients [12,15–17]. However, this

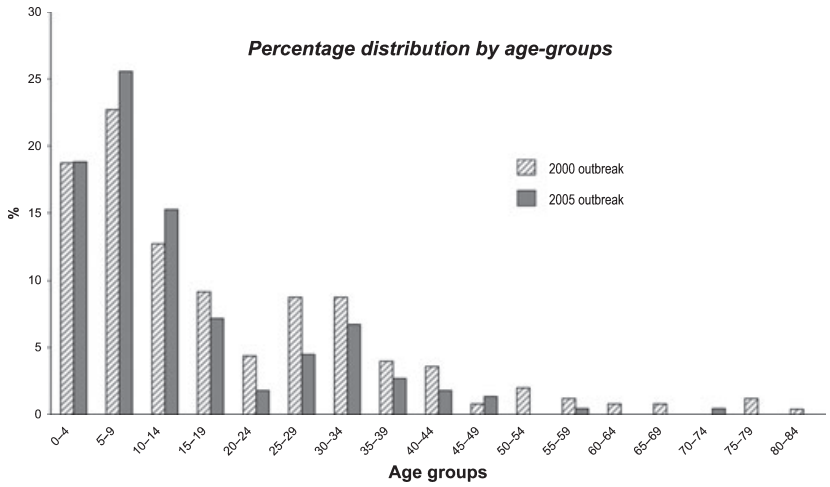


FIG. 2. Percentage distribution of confirmed cases by age groups during the 2000 and 2005 enterovirus outbreaks.

is intrinsically difficult to evaluate as, in any given study, it is not easy to establish that the groups of patients receiving either early or delayed diagnosis are comparable. Indeed, previously published studies have not provided precise information on the clinical, epidemiological and virological characterization of these groups.

The two successive enterovirus epidemics observed in Marseilles in 2000 and 2005 offered an interesting opportunity for a comparative study including a large number of patients. Analysis of the two outbreaks showed that the epidemiological characteristics of the patients were similar (age and gender ratio) and that, in both cases, a large majority of cases were a result of infection by EV-30 (over 80%). In addition, genetic analysis of the VP1 gene of several EV-30 isolates from 2000 and 2005 suggested that predominant strains that circulated during these two outbreaks were genetically closely related. Therefore, the characteristics of the patient management could have been expected to be very similar on both occasions.

However, we observed that the average duration of hospitalization of confirmed cases was significantly shorter in 2005 than in 2000. This had important direct financial implications. The overall direct cost of hospitalization at the University Hospitals of Marseilles during the 2000 EV outbreak has been estimated previously to be 541 EUR per patient per day [5]. Using this value for the calculation of costs, we could evaluate the cost of hospitalization of the 186 confirmed cases in 2005 at 221 377 EUR, using the actual average duration of hospitalization observed in 2005 (i.e. 2.2 days), and at 543 380 EUR, using the average duration of hospitalization observed in 2000 (i.e. 5.4 days). In other words, decreasing LOS from 5.4 to 2.2 days in the group of confirmed cases allowed us to save approximately 322 000 EUR.

A retrospective analysis of the characteristics of the 2000 and 2005 epidemics revealed that a major change in the management of virological diagnosis occurred between the two outbreaks. In 2000, diagnosis relied mainly on cell cultures inoculated with CSF samples. Using that technique, the mean time necessary for providing results to clinicians was 12.4 ± 4.8 days (in particular, negative results could not be delivered before 14–17 days). Only about half the samples received were tested using classic RT-PCR and, even in this case, the average delay was 8.9 days, reaching 9.3 days for positive results (which were systematically double checked to avoid false-positive results because of PCR contamination). As a result, the mean time for obtaining a positive result from the laboratory (approximately 10 days) was longer than the average time of hospitalization of confirmed cases. Consequently, the aetiological diagnosis was obtained most of the time after the patient's discharge and recovery. In contrast, during the 2005 epidemic, all positive diagnoses were delivered to the clinicians on the basis of the result of real-time RT-PCR. This technology allowed very rapid provision of results and, in particular, did not require the verification of positive results. As a result, the mean time for obtaining a diagnosis in 2005 dropped to 1.9 days and was similar for both negative and positive results. Importantly, it became shorter than the average duration of hospitalization of confirmed cases (2.2 ± 1.8 days). It can also be observed that the rapid availability of the diagnostic results was associated with an increase in demand: in 2000: 139 out of 195 samples (71%) were positive vs. 186 out of 387 (48%) in 2005, indicating that clinicians had spontaneously extended the indications for EV diagnosis.

It is not possible to infer from this observation a direct cause and effect relationship between the improvement of

the aetiological diagnosis and the shortening of the average time of hospitalization. Other factors, such as subtle differences in the virulence of the viruses or the effect of the experience acquired during the first outbreak, might be invoked partially to account for modifications in patient management. However, the hypothesis that obtaining an early diagnosis of EV infections has played a significant role in reducing LOS is highly plausible. This assumption was reinforced by interviews with clinicians in charge of hospitalized EV-infected patients, who confirmed that obtaining a rapid positive diagnosis of EV infection modified the management of patient care. Patients, or relatives of young patients, suffering from enterovirus meningitides could receive early reassuring information, unnecessary antibiotic or anti-herpetic treatments could be stopped earlier and, importantly, the absence of severe presentations associated with a biological diagnosis of EV infection allowed patients to be discharged earlier.

The authors are aware of some weaknesses in the present study. It was not possible to be certain of the comparability of the groups studied, although we can advance a number of epidemiological and virological arguments to support this. The retrospective evaluation of clinical cases explains the absence of several important parameters (e.g. the avoidance of antibiotic or antiviral treatments or reduction in the duration of administration, the reduction in radiological investigations, the reduction in nosocomial infections (1) and finally the reduction in global cost for hospitalized EV-infected patients). Similar methodological problems were encountered in previously published studies. However, a number of investigations have also tried to evaluate some of these parameters prospectively[13,17]. Overall the results have suggested that obtaining an early aetiological diagnosis by molecular methods permits healthcare services to shorten the duration of hospitalization, and also to decrease unnecessary diagnostic and therapeutic interventions, and consequently to reduce significantly the global cost of the clinical management of hospitalized EV-infected patients. However, it is clear that savings are more significant when the proportion of positive EV diagnoses is high, i.e. in the context of EV outbreaks or during the seasonal period of sustained EV circulation.

In conclusion, our study of two successive important EV outbreaks in southeast France supports previously published studies which suggested an important impact of enteroviral rapid molecular diagnosis on patient management and resultant health care savings. In the specific context of our study, the cost of the molecular diagnosis using real-time RT-PCR, despite an increase in requests for aetiological diagnosis, represented only a small percentage of the saving achieved by the implementation of rapid molecular testing. This constitutes a modest but interesting example of how new diagnostic methods in microbiology can be self-financed through the improvement of patient management.

Transparency Declaration

The authors have no conflict of interest in relation to this study.

References

1. The Universal database of the International Committee on Taxonomy of Viruses. http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm [accessed on 13 January 2009].
2. Cherry JD. Enteroviruses: Polioviruses (poliomyelitis), coxsackieviruses, echoviruses and enteroviruses. In: Feigin CJD, ed, *Textbook of pediatric infectious diseases*. Vol 1, 3rd edn. Philadelphia: WB Saunders, 1992; 12.
3. Helfand RF, Khan AS, Pallansch MA *et al.* Echovirus 30 infection and aseptic meningitis in parents of children attending a child care center. *J Infect Dis* 1994; 169: 1133–1137.
4. Rotbart HA. Enteroviral infections of the central nervous system. *Clin Infect Dis* 1995; 20: 971–981.
5. Bernit E, de Lamballerie X, Zandotti C *et al.* Prospective investigation of a large outbreak of meningitis due to echovirus 30 during summer 2000 in Marseilles, France. *Medicine* 2004; 83: 245–253.
6. Watkins-Riedel T, Woegerbauer M, Hollemann D, Hufnagl P. Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. *Diagn Microbiol Infect Dis* 2002; 42: 99–105.
7. Charrel RN, Bernit E, Zandotti C, de Lamballerie X. An approach based on RFLP assay to investigate outbreaks of enteroviral meningitis. *J Clin Virol* 2004; 29: 54–58.
8. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol* 1999; 37: 1288–1293.
9. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22: 4673–4680.
10. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004; 5: 150–163.
11. Marshall GS, Hauck MA, Buck G, Rabalais GP. Potential cost savings through rapid diagnosis of enteroviral meningitis. *Pediatr Infect Dis J* 1997; 16: 1086–1087.
12. Ramers C, Billman G, Hartin M, Ho S, Sawyer MH. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. *JAMA* 2000; 283: 2680–2685.
13. Nigrovic LE, Chiang VW. Cost analysis of enteroviral polymerase chain reaction in infants with fever and cerebrospinal fluid pleocytosis. *Arch Pediatr Adolesc Med* 2000; 154: 817–821.
14. Stellrecht KA, Harding I, Woron AM, Lepow ML, Venezia RA. The impact of an enteroviral RT-PCR assay on the diagnosis of aseptic meningitis and patient management. *J Clin Virol* 2002; 25: S19–S26.

15. Hamilton MS, Jackson MA, Abel D. Clinical utility of polymerase chain reaction testing for enteroviral meningitis. *Pediatr Infect Dis J* 1999; 18: 533–537.
16. Tattevin P, Minjolle S, Arvieux C *et al.* Benefits of management strategy adjustments during an outbreak of enterovirus meningitis in adults. *Scand J Infect Dis* 2002; 34: 359–361.
17. Robinson CC, Willis M, Meagher A, Gieseker KE, Rotbart H, Glodé MP. Impact of rapid polymerase chain reaction results on management of pediatric patients with enteroviral meningitis. *Pediatr Infect Dis J* 2002; 21: 283–286.

Discussion

Epidemiological characteristics, such as distribution by age group and ratio were similar during both outbreaks. The breakdown of isolated serotypes was also similar: HEV-B serotypes were identified in all isolates, 81-82% of which were attributed to E30 alone. Phylogenetic analysis indicated that all E30 strains from the 2000 and 2005 outbreaks are genetically similar and had a common ancestor.

However, we observed a marked decrease in the mean time necessary for providing diagnostic test results depending on the detection technique used: (i) in 2000, cell culture and classical RT-PCR techniques required 12.4 ± 4.3 days and 8.9 ± 4.2 days respectively; and (ii) in 2005, real-time RT-PCR results were available within 1.9 ± 0.8 days. Concordantly, the mean lengths-of-hospital stay (LOS) decreased significantly from 5.4 ± 4.8 days in 2000 to 2.2 ± 1.8 days in 2005. Furthermore, the difference in time needed to provide positive and negative results was negligible with real-time RT-PCR.

Diagnostic results should be delivered as early as possible in order to better manage patient care. This has shown to have an additional benefit of reducing the average duration of hospitalization, as well as the overall direct cost of hospitalization at the University Hospitals of Marseille. Decreasing LOS has reduced hospital spending by approximately 322 000 Eur.

While it is not possible to infer a direct causal relationship between the improvement of enterovirus diagnosis and the decrease in LOS, there is cause to believe that new diagnostic technologies contribute to obtaining an early diagnosis and plays a significant role in reducing hospital stay.

ARTICLE #3 (submitted)

Screening and detection of Human Enterovirus 71 infection by a real-time RT-PCR assay in Marseille, France, 2009-2011

Foreword

Human enterovirus 71 (EV71) has emerged as a public health threat over the last two decades (57, 225-226). It is recognized as the primary etiological agent of hand, foot and mouth disease (HFMD) and can cause severe neurological disease, especially in children under 5 years of age. While major epidemics associated with various genogroups have been reported in the Asia-Pacific region, only EV71 viruses of C1 and C2 genogroups have been sporadically isolated in Europe (64-66). Continuous enterovirus surveillance is of utmost importance, considering that the introduction of genetic variants distinct from the European lineages has the potential to provoke large-scale epidemics.

There is a thus a need to develop techniques capable of detecting EV71 rapidly and specifically, with the additional requirement of differentiating EV71 from the antigenically and clinically similar but less neurotropic CVA16. To this end, the team of Tan *et al* designed a real-time RT-PCR hybridization probe assay targeting the VP1 region for amplification that detected as few as 5 EV71 viral copies (219). The team of Wu *et al* developed a high-throughput multiplex bead suspension array that identified EV71 subgenogroups based on single nucleotide polymorphisms within the VP1 region (227).

In the context of enterovirus surveillance, we undertook the molecular screening of EV71 in clinical samples tested positive by routine pan-enterovirus diagnostic assay between January 2009 and September 2011.

Research Note

Screening and detection of Human Enterovirus 71 infection by a real-time RT-PCR assay in Marseille, France, 2009-2011

Charlene Y.Q. Tan[1], Géraldine Gonfrier[2], Laetitia Ninove[1,2], Christine Zandotti[2], Audrey Dubot-Pérès[1], Xavier de Lamballerie[1,2], Rémi N. Charrel[1,2]*

[1]UMR190 Emergence des Pathologies Virales, Aix-Marseille Université - Institut de Recherche pour le Développement - EHESP French School of Public Health, Marseille, France

[2]Fédération de Microbiologie, Assistance Publique-Hôpitaux de Marseille, Marseille, France

*Corresponding author: Rémi N. Charrel

UMR190 Emergence des Pathologies Virales

Faculté de Médecine, 27 bd Jean Moulin, 13005 Marseille, France.

Email: remi.charrel@univmed.fr

Tel : (33)-491-324420

Fax : (33)-491-324421

Abstract

Enterovirus-positive samples diagnosed in Marseille (January 2009-September 2011) were screened for EV71 by real-time RT-PCR. EV71 was detected in three children below the age of two with no history of overseas travel; two of these cases were associated with severe clinical presentation. Viruses demonstrated genetic similarity with other European genogroup C2 strains. Strain MRS/09/3663 complete sequencing revealed 97.6% identity across the entire genome with a 2008 Singapore isolate, without signs of possible recombination events. To our knowledge, this is the first detection of EV71 infection in Marseille, France, that confirms the current circulation of EV71 in France.

Keywords:

Picornaviridae; Enterovirus 71; Genogroup C2 viruses; Enterovirus; hand, foot and mouth disease

Enterovirus 71 (EV71, family *Picornaviridae,* species Human Enterovirus A, classified into genogroups based on the VP1 capsid sequence) is a frequent etiological agent of hand, foot and mouth disease (HFMD), along with Coxsackievirus A16 (CVA16). Unlike CVA16, EV71 can cause neurological complications (*e.g.*, brainstem rhombencephalitis, neurogenic pulmonary oedema), especially in children under 5 years of age. It is the most common nonpolio enterovirus associated with poliomyelitis-like paralysis (1). EV71 has emerged as a significant public health threat, with major epidemics reported in the Asia-Pacific region over the last two decades but none in Europe since the 1970s. Nevertheless, the possibility of genetic variants provoking epidemics in Europe underlies the continuous enterovirus surveillance. In this context, we describe the molecular screening for EV71 in EV-positive samples between 2009 and 2011 in the Public Hospitals of Marseille, France, and report the molecular characterisation of detected strains.

8041 samples registered in the Microbiology Department between January 2009 and September 2011 were processed as illustrated (Figure 1). Cerebrospinal fluids (CSF, 70.9% of samples) were tested for EV RNA with the Xpert® EV kit (GeneXpert®, Cepheid) and confirmed using the routine pan-enterovirus real-time RT-PCR test (2-3). Other samples types (*e.g.* stool, pharyngeal swabs…) were directly tested with the routine diagnostic assay. 682 (8.5%) samples tested EV-positive: 77.5% were sampled during the seasonal peaks of EV incidence (May-August); the male-to-female ratio was 1.73:1; 70% were obtained from patients below 10 years old (24% <1 yo, 46% 1-9 yo).

Prospective EV71 screening was carried out over two periods of peak EV activity (May-October 2010, May-September 2011): 174 patients with a positive pan-enterovirus assay were tested by a EV71-specific real-time RT-PCR assay adapted from Tan *et al*, with a modified hybridisation probe (FAM-attggagcatcatcaaatgctagtga-TAMRA) and a specific external control (4-5). Two samples tested EV71-positive (June 2010; April 2011). Retrospective screening was carried out on 182 EV-positive samples between 2009 and May 2010, yielding one additional EV71-positive sample (June 2009).

Case MRS/09/3663 was a 22-month-old female patient (10kg) hospitalised in June 2009. She was febrile and experienced loss of consciousness (*ca* 2 min), ocular revulsion, eye deviation to the right, and generalised tonic-clonic seizure (*ca* 15 sec). The post-critical phase included stiffness, hyperextension of the left arm, and persisting ocular revulsion. She was intubated, extubated after 12h. CSF WBC count was 7/mm^3 and RBC count was 20/mm^3. EV71 was detected in pharyngeal swab. The patient was discharged 3 days after admission and showed no sequelae.

Case MRS/10/8229 was a 1-month-old female patient (4.7kg) admitted in June 2010 with fever and appetite loss, without neck stiffness. CSF analysis revealed hyperproteinorrhachia (1.07g/L), glycorrhachia (2.9g/L), WBC count was 6/mm^3 and RBC count 2400/mm^3. EV71 was detected in stool and CSF samples. The patient remained febrile for two days, was discharged 3 days after admission and showed no sequelae.

Case MRS/11/8134 was a 3-month-old female patient admitted in April 2011 with persistent fever, diarrhoea, altered neurological status, convergent strabismus, and left eyelid ptosis. She was intubated, extubated two days later. CSF analysis revealed hyperproteinorrhachia, WBC count was 8/mm^3 and RBC count 2000/mm^3. EV71 was detected in pharyngeal swab. The patient was discharged 4 days after admission and showed no sequelae.

Two EV71-positive samples were isolated in BGM cells (strains MRS/09/3663 and MRS/11/8134), and their VP1 completely amplified for genogroup identification (297 aa). Sample MRS/10/8229 was partially amplified (118 aa) directly from CSF, using a semi-nested PCR assay. VP1 phylogenetic analysis showed that MRS/09/3663, MRS/10/8229 and MRS/11/8134 clustered with strains of the C2 subgenogroup (bootstrap 91%, Figure 2a), including strains isolated in Australia (1995-1999), USA (1995-1997), Japan (1997-2004, 2009-2010), Netherlands (1997-2010), Taiwan (1998, 2008), Great Britain (1999-2010), France (2000-2009), and Germany (2006-2007). MRS/09/3663 and MRS/11/8134 shared 90.2-98.7% identity with C2 strains, amongst which European strains from

2006-2008 were the most genetically similar. The closest non-European strains were isolated in Japan (2009-2010) and Singapore (2008). Notably, C2 subgenogroup includes a fatal EV71 infection reported in Brest, France (2007) sharing 97.9-98.2% identity with the Marseille strains.

The complete genome of strain MRS/09/3663 was amplified, gel-purified and sequenced: the genome is 7395 nt long. Phylogenetic analysis of the complete genome sequence showed that MRS/09/3663 grouped with a strain isolated in Singapore in 2008 (bootstrap 100%, 97.6% identity) (Figure 2b). Bootscanning analysis indicated that similarity to this strain is consistent across the entire genome, without evidence for recombination events.

The last EV71-associated epidemics in Europe were reported in Bulgaria (1975) and Hungary (1978) (6-7). In Marseille, a study of EV isolates did not identify EV71 between 1985 and 2005 (8). More recently, studies of sporadic cases in Europe identified mostly viruses from subgenogroup C1 from 1998 to 2006, and then, increasingly, C2 viruses since 2006 (9). The latter have been linked to severe neurological disease during the 1998 outbreaks in Taiwan and Australia (10). However, the VP1 A170V substitution suggested to be associated with increased neurovirulence was absent in the Marseille strains, in the fatal Brest strain, in other available European C2 strains and in fatal B3 and B4 strains from Malaysia and Singapore (11-12). There is consequently no firm evidence that virulence is associated with genogroup or with this specific substitution. C4 genogroup viruses, which have been increasingly identified in the Asia-Pacific region and associated with lethal infection and adult-onset encephalitis, are less frequent in Europe: in 2004, 2 cases were isolated in Austria, 1 in France, and 1 in Germany (13-17).

The current study reports the first detection of EV71 infection in Marseille, Southern France. None of the patients had a recent history of travel prior to disease onset, confirming that C2 genogroup viruses currently circulate in France and are, based on phylogenetic analyses, likely to have been

introduced from the Asia-Pacific region. In contrast with EV71 strains isolated in Asia, no evidence for recombination was identified following complete genome characterisation (18). Case reports indicate that EV71 currently circulating in Europe has the potential to cause severe presentations with neurological complications. A close epidemiological monitoring of EV71 infections, including follow-up of genogroup patterns is recommended.

Acknowledgements

We would like to thank Odile Py for her excellent technical assistance.

Transparency Declaration

The authors declare no conflict of interest and no particular funding in the conception of this work.

References

1. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infectious Diseases. 2010;10(11):778-90.
2. Ninove L, Nougairede A, Gazin C, Zandotti C, Drancourt M, de Lamballerie X, et al. Comparative detection of enterovirus RNA in cerebrospinal fluid: GeneXpert system vs. real-time RT-PCR assay. 2011.
3. Watkins-Riedel T, Woegerbauer M, Hollemann D, Hufnagl P. Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. Diagn Microbiol Infect Dis. 2002;42(2):99-105
4. Ninove L, Nougairede A, Gazin C, Thirion L, Delogu I, Zandotti C, et al. RNA and DNA bacteriophages as molecular diagnosis controls in clinical virology: a comprehensive study of more than 45,000 routine PCR tests. PLoS One. 2011;6(2):e16142.
5. Tan EL, Chow VTK, Kumarasinghe G, Lin RTP, Mackay IM, Sloots TP, et al. Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay. Molecular and Cellular Probes. 2006;20(2):135-40.
6. Chonmaitree T, Menequs M, Schervish-Swierkosz E, Schwalenstocker E. Enterovirus 71 Infection: Report of an outbreak with two cases of paralysis and a review of the literature. Pediatrics. 1981;67(4):489-93.
7. Chumakov M, Voroshilova M, Shindarov M, Lavrova I, Gracheva L, Koroleva G, et al. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol. 1979;60(3-4):329-40.
8. Tan CY, Ninove L, Gaudart J, Nougairede A, Zandotti C, Thirion-Perrier L, et al. A Retrospective Overview of Enterovirus Infection Diagnosis and Molecular Epidemiology in the Public Hospitals of Marseille, France (1985-2005). PLoS One. 2011;6(3).
9. Mirand A, Schuffenecker I, Henquell C, Billaud G, Jugie G, Falcon D, et al. Phylogenetic Evidence for a Recent Spread of 2 Populations of EV71 in European countries. J Gen Virol. 2010;91(9):2263-77.
10. McMinn P, Stratov I, Nagarajan L, Davis S. Neurological manifestations of Enterovirus 71 infection in children during an outbreak of Hand, Foot, and Mouth Disease in Western Australia. Clin Infect Dis. 2001;32(2):236-42.
11. Chan KP, Goh KT, Chong CY, Teo ES, Lau G, Ling AE. Epidemic Hand, Foot and Mouth Disease Caused by Human Enterovirus 71, Singapore. Emerg Infect Dis. 2003;9(1):78-85.
12. Vallet S, Legrand-Quillien M-C, Dailland T, Podeu G, Gouriou S, Schuffenecker I, et al. Fatal Case of Enterovirus 71 Infection, France, 2007. Emerg Infect Dis. 2009;15(11):1837-40.
13. Hamaguchi T, Fujisawa H, Sakai K, Okino S, Kurosaki N, Nishimura Y, et al. Acute Encephalitis Caused by Intrafamilial Transmission of Enterovirus 71 in Adult. Emerg Infect Dis. 2008;14(5):828-30.
14. Sun L-m, Zheng H-y, Zheng H-z, Guo X, He J-f, Guan D-w, et al. An Enterovirus 71 epidemic in Guangdong Province of China, 2008: epidemiological, clinical and virogenic manifestations. Japanese Journal of Infectious Diseases. 2011;64(1):13-8.
15. Diedrich S, Weinbrecht A, Schreier E. Seroprevalence and molecular epidemiology of enterovirus 71 in Germany. Arch Virol. 2009;154(7):1139-42.
16. Ortner B, Huang C-W, Schmid D, Mutz I, Wewalka G, Allerberger F, et al. Epidemiology of Enterovirus Types Causing Neurological Disease in Austria 1999-2007: Detection of Clusters of Echovirus 30 and Enterovirus 71 and Analysis of Prevalent Genotypes. J Med Virol. 2009;81:317-24.
17. Schuffenecker I, Mirand A, Antona D, Henquell C, Chomel J-J, Archimbaud C, et al. Epidemiology of Human Enterovirus 71 Infections in France, 2000-2009. J Clin Virol. 2011;50:50-6.
18. Huang Y-P, Lin T-L, Hsu L-C, Chen Y-J, Tseng Y-H, Hsu C-C, et al. Genetic Diversity and C2-like Subgenogroup Strains of Enterovirus 71, Taiwan, 2008. Virology Journal. 2010;7:277.

177 **Figure Legends**

178 **Figure 1: Flowchart for clinical specimens processing**

179 CSF: Cerebrospinal fluid; BGM: Buffalo Green Monkey renal cells; MRC5: Human foetal lung
180 fibroblasts; IFA: Immunofluorescence assay

181 **Figure 2: Phylogenetic and genetic analyses of the detected EV71 strains**

182 (a) In the VP1 coding region, MRS/09/3663, MRS/10/8229, and MRS/11/8134 clustered reliably
183 (bootstrap 91%) with sequences of the C2 subgenogroup, that included FJ824734, a fatal strain
184 isolated in France in 2007.

185 (b) Full-length MRS/09/3663 grouped reliably (bootstrap 100%) with FJ172159, isolated in 2008 in
186 Singapore.

187 (c) MRS/09/3663 was used as the query sequence with a 500-nt sliding window and steps of 20 nt.
188 Signals between 75% and 100% were observed across the entire genome with FJ172159 exclusively.
189 No other evidence of possible recombination events was observed.

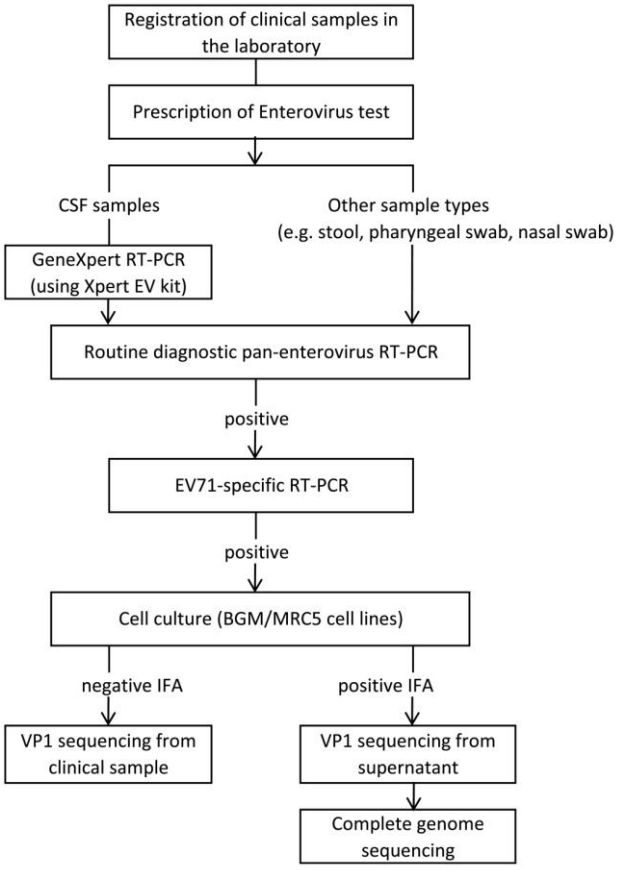
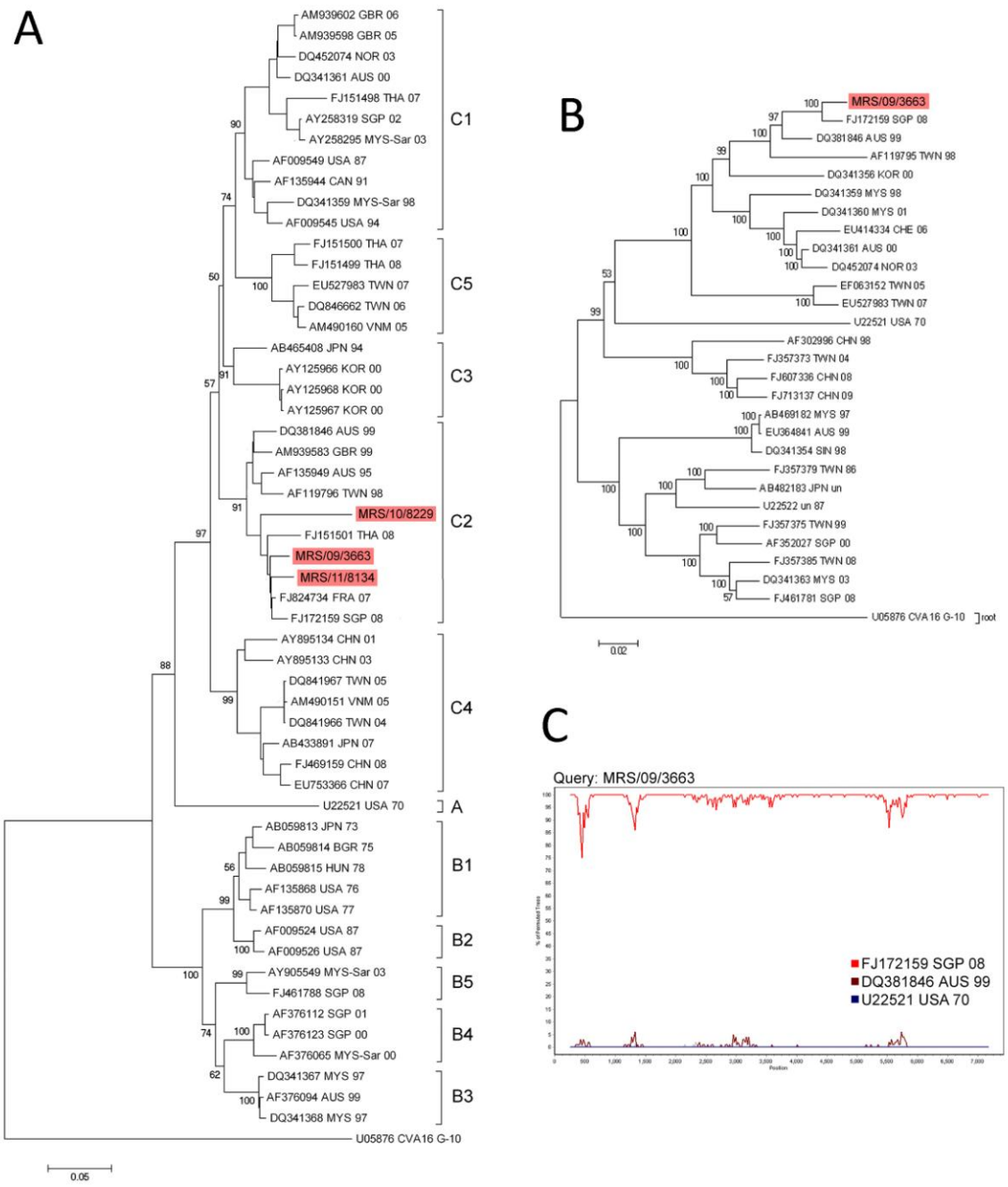


Figure 1: Flowchart for clinical specimens processing

CSF: Cerebrospinal fluid; BGM: Buffalo Green Monkey renal cells; MRC5: Human foetal lung fibroblasts; IFA: Immunofluorescence assay

Figure 2: Phylogenetic and genetic analyses of the detected EV71 strains

(a) In the VP1 coding region, MRS/09/3663, MRS/10/8229, and MRS/11/8134 clustered reliably (bootstrap 91%) with sequences of the C2 subgenogroup, that included FJ824734, a fatal strain isolated in France in 2007. (b) Full-length MRS/09/3663 grouped reliably (bootstrap 100%) with FJ172159, isolated in 2008 in Singapore.(c) MRS/09/3663 was used as the query sequence with a 500-nt sliding window and steps of 20 nt. Signals between 75% and 100% were observed across the entire genome with FJ172159 exclusively. No other evidence of possible recombination events was observed.


A
AM939602 GBR 06
AM939598 GBR 05
DQ452074 NOR 03
DQ341361 AUS 00
FJ151498 THA 07
AY258319 SGP 02
AY258295 MYS-Sar 03
AF009549 USA 87
AF135944 CAN 91
DQ341359 MYS-Sar 98
AF009545 USA 94
FJ151500 THA 07
FJ151499 THA 08
EU527983 TWN 07
DQ846662 TWN 06
AM490160 VNM 05
AB465408 JPN 94
AY125966 KOR 00
AY125968 KOR 00
AY125967 KOR 00
DQ381846 AUS 99
AM939583 GBR 99
AF135949 AUS 95
AF119796 TWN 98
MRS/10/8229
FJ151501 THA 08
MRS/09/3663
MRS/11/8134
FJ824734 FRA 07
FJ172159 SGP 08
AY895134 CHN 01
AY895133 CHN 03
DQ841967 TWN 05
AM490151 VNM 05
DQ841966 TWN 04
AB433891 JPN 07
FJ469159 CHN 08
EU753366 CHN 07
U22521 USA 70
AB059813 JPN 73
AB059814 BGR 75
AB059815 HUN 78
AF135868 USA 76
AF135870 USA 77
AF009524 USA 87
AF009526 USA 87
AY905549 MYS-Sar 03
FJ461788 SGP 08
AF376112 SGP 01
AF376123 SGP 00
AF376065 MYS-Sar 00
DQ341367 MYS 97
AF376094 AUS 99
DQ341368 MYS 97
U05876 CVA16 G-10
C1
C5
C3
C2
C4
A
B1
B2
B5
B4
B3
0.05
B
MRS/09/3663
FJ172159 SGP 08
DQ381846 AUS 99
AF119795 TWN 98
DQ341356 KOR 00
DQ341359 MYS 98
DQ341360 MYS 01
EU414334 CHE 06
DQ341361 AUS 00
DQ452074 NOR 03
EF063152 TWN 05
EU527983 TWN 07
U22521 USA 70
AF302996 CHN 98
FJ357373 TWN 04
FJ607336 CHN 08
FJ713137 CHN 09
AB469182 MYS 97
EU364841 AUS 99
DQ341354 SIN 98
FJ357379 TWN 86
AB482183 JPN un
U22522 un 87
FJ357375 TWN 99
AF352027 SGP 00
FJ357385 TWN 08
DQ341363 MYS 03
FJ461781 SGP 08
U05876 CVA16 G-10
root
0.02
C
Query: MRS/09/3663
FJ172159 SGP 08
DQ381846 AUS 99
U22521 USA 70

Discussion

Of EV-postive clinical samples screened both prospectively and retrospectively, three samples tested positive for EV71. All three viruses identified belong to the European C2 genogroup (up to 98.7% identity in VP1) and were detected in female patients between 1 and 22 months of age. Full-length genome analysis of strain MRS/09/3663 revealed great genetic similarity with a strain isolated in Singapore in 2008, with no evidence for genetic recombination across the entire genome. None of the patients had a recent history of international travel, and genetically similar C2 viruses have been described in other regions of France, suggesting that EV71 C2 genogroup viruses currently circulate in France and are likely to have been introduced from the Asia-Pacific region.

EV71 has the potential to provoke large-scale epidemics upon the introduction of genetic variants distinct from the European lineages. Viruses of the C4 genogroup are less common in Europe, but have been increasingly associated with outbreaks and fatal infections in the Asia-Pacific region (60, 228). Cross-immunity may develop for viruses of similar genogroups, and the alternation between predominant genogroups may favour a more efficient propagation in the population. In Taiwan, every major EV71 outbreak has been associated with a change between genogroup B and C viruses. Furthermore, recombination events may also give rise to recombinants that are distinct from viruses in circulation.

EV71 infections have been increasingly reported in France, including a case of fatal pulmonary oedema associated with acute severe respiratory distress reported in a 17-month-old boy (229). This underlines the importance of monitoring EV71 circulation and changes in genogroup patterns outside the Asia-Pacific region.

PERSPECTIVES (ongoing work)

Real-time RT-PCR detection of all EV71 genogroups

In Marseille, enterovirus testing proceeds as follows: upon registration of clinical specimens, routine pan-enterovirus diagnostic testing is carried out at the point-of care (POC) laboratory; EV-positive samples are then screened for EV71 the following day using our adapted real-time RT-PCR assay. In order to better monitor EV71 emergence, EV71 testing at the POC level is currently under evaluation.

However, the primers designed by Tan *et al* are highly specific, with neither degenerate positions nor inosine residues incorporated. Yet, our analysis of all available VP1 sequences highlighted the disparity in nucleotide sequence in the target annealing sites of their primers and probes. There is thus a possibility that more divergent EV71 strains may be overlooked by our current protocol.

We recovered and aligned all EV71 full-length VP1 nucleotide sequences available in GenBank (n=1956 as of 9th September 2011). Phylogenetic trees were constructed using the neighbor-joining method on the Jukes-Cantor substitution model and genogroup was attributed according to VP1 phylogeny with sequences described in Solomon *et al* (n=49).Based on this alignment, we degenerated the EV71 primers and probe previously used in our study. Next, we designed synthetic consensus DNA fragments that correspond with the amplified region of each EV71 subgenogroup (length = 204 bp).

Our objective is to test both the specific and the degenerate systems in a real-time RT-PCR assay, and compare the level the ability of each to detect all EV71 genogroups.

Analysis of 5'NCR RT-PCR diagnostic sequence for rapid EV species and PV identification

Clinical virologists have reinforced efforts to identify EV serotype directly from clinical samples by molecular techniques. In particular, it is important to develop tools that rapidly differentiate polioviruses from nonpolio enteroviruses. The team of Kilpatrick *et al* developed a real-time RT-PCR assay using degenerate poliovirus group-specific primers targeting the VP1 region, and detected all PV isolates tested (230).

There is generally no direct association between an individual serotype and a particular pathology. Serotyping has little influence on the clinical management of patients, and is not systematically performed in most laboratories. Furthermore, recombination events have challenged the validity of serotyping techniques based solely on capsid-encoding region (182). However, since recombination occurs in nature exclusively among members of the same species, it is plausible that species identity may hold some fundamental biological significance.

The highly sensitive diagnostic pan-EV assay detects all enteroviruses but does not allow the identification of serotypes. We attempt to evaluate the nucleotide sequence of the 5'NCR diagnostic end product as a tool for the rapid identification of EV species and in particular, PV.

We compiled all complete genome sequences for human enteroviruses available in GenBank (n=696 as of 13 September 2011) and aligned using ClustalW on default settings. VP1 nucleotide sequence was used to attribute EV serotype and species. 5'NCR sequence was analysed using an in-house programme based on the recognition of significant nucleotide motifs and on genetic distance-directed partition alignments.

We also included for analysis clinical samples diagnosed EV-positive in the Laboratory of Virology, University Hospital La Timone (Marseille, France) from January 2010 to July 2011. Specimen types comprised nasopharyngeal swabs, stool, CSF, saliva and bronchoarterial specimens. Archived strains from the Marseille EV database previously described were also included in the study set. Diagnostic 5'NCR amplification products were purified and sequenced. The VP1 region was then amplified directly from the corresponding original clinical specimen (or isolate in the case of strains from the Marseille EV database) using a semi-nested PCR protocol adapted from Nix *et al* by the French National Reference Centre for Enterovirus (Annex 1).

Currently, our programme is able to distinguish between group I(HEV-C, of which PV is a subgroup, and HEV-D) and group II (HEV-A and HEV-B) 5'NCRs, using a computationally less intensive method than phylogenetic analysis. Within group II, no clear distinction can be made between HEV-A and HEV-B. In group I, we attempt to distinguish between PV and HEV-C viruses. This is complicated by recombination events between PV and other members of the HEV-C species.

Our objective is to determine if 5'NCR could allow the rapid identification of PV group. Since the majority of EV identified in Europe belong to HEV-A and HEV-B, our test could help to assign priority and better allocate resources for serotyping. Analysis of 5'NCR diagnostic sequence could represent a rapid and hassle-free method streamlining EV surveillance efforts and rapidly detect potentially uncommon serotypes.

D/ CONCLUSIONS

Enteroviruses are responsible for a wide array of clinically distinct pathologies in both adult and paediatric populations, certain of which may result in fatal neurological complications. Today, EV surveillance is performed in the context of post-polio eradication vigilance, and provides important epidemiological data for nonpolio enteroviruses. It forms the basis for this work, which is focused on the aspects of laboratory diagnosis, molecular identification and epidemiology.

In Marseille, surveillance efforts culminated in the compilation and analysis of a database of 654 enterovirus strains isolated between 1985 and 2005. Data included the month and year of original isolation, the sample type from which and the cell line in which the strain was isolated, as well as VP1 nucleotide sequence and corresponding serotype. This is likely to be the largest retrospective study including molecular data ever undertaken. HEV-B species accounted for 98% of all isolates, including the most commonly isolated E30 and the emergent E13 serotypes. Our analysis of clinical isolates over 20 years also validates the VP1 serotyping strategy based on genetic and phylogenetic analysis previously proposed with prototype strains. Furthermore, phylogenetic analysis of different genomic regions identified strains of different serotypes which were genetically similar in the nonstructural regions despite distinct VP1 regions. This observation contradicts the current model of enterovirus recombination and could suggest the emergence of epidemic E13 as a result of an inter-serotypic recombination event.

Two large outbreaks of E30 were described in 2000 and 2005, in between which the EV diagnostic protocol used in the Public Hospitals of Marseille was modified. The transition

from cell culture and classic RT-PCR techniques in 2000 to real-time RT-PCR in 2005 was analysed, especially in terms of the time needed to deliver diagnostic results and subsequent management of patients. Our results showed that the outbreak in 2005 was characterised by a significant decrease in result delivery time, as well as in the duration of hospital stay, such that it contributed to a reduction in spending for patient management.

Analysis of clinical strains between 1985 and 2005 did not detect the circulation of EV71 in Marseille. EV71 is a neurotropic serotype that has caused major epidemics of hand, foot and mouth disease in the Asia-Pacific region, but not in Europe. We adapted a real-time molecular assay for EV71 screening in 356 samples which had been tested EV-positive. Three cases of EV71 infection were detected between 2009 and 2011, all of which involved C2 genogroup viruses in patients with no history of travel prior to disease onset. Genetically similar C2 viruses have also been isolated elsewhere in France, confirming that C2 genogroup EV71 viruses currently circulate in France, and are likely to have been introduced from the Asia-Pacific region with no evidence of recombination.

Enteroviruses have the potential to cause frequent epidemics, due to their great genetic diversity and their propensity for re-emergence. Less common serotypes such as CVA6 have been recently associated with disease in Europe. There is thus a need to maintain EV surveillance. By studying serotype circulation patterns and trends in laboratory strategy, it is possible to optimise surveillance in terms of time and manpower. Furthermore, the development of more sensitive and efficient detection techniques can contribute to subsequent reductions in spending on patient management, as well as increasing surveillance coverage.

REFERENCES

1. Grubman MJ. Foot-and-Mouth Disease. Clin Microbiol Rev. 2004;17(2):465-93.
2. Scudamore J, Trevelyan G, Tas M, Varley E, Hickman G. Carcass disposal: lessons from Great Britain following the foot and mouth disease outbreaks of 2001. Rev Sci Tech. 2002;21(3):775-87.
3. Thompson D, Muriel P, Russell D, Osborn P, Bromley A, Rowland M, et al. Economic costs of the foot and mouth disease outbreak in 2001. Rev Sci Tech. 2002;21(3):675-87.
4. Yamashita T, Sakae K, Kobayashi S, Ishihara Y, Miyake T, Mubina A, et al. Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from Southeast Asia. Microbiol Immunol. 1995;39(6):433-5.
5. Lipton H, Jelachich M. Molecular pathogenesis of Theiler's murine encephalomyelitis virus-induced demyelinating disease in mice. Intervirology. 1997;40(2-3):143-52.
6. Niklasson B, Almqvist PR, Hornfeldt B, Klitz W. Sudden infant death syndrome and Ljungan virus. Forensic Sci Med Pathol. 2009;5(4):274-9.
7. Brundage S, Fitzpatrick A. Hepatitis A. Am Fam Physician. 2006;73(12):2162-8.
8. Knowles NJ, Hovi T, Hyypiä T, King AMQ, Lindberg AM, Pallansch MA, et al. Picornaviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy: classification and nomenclature of viruses: Ninth report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier; 2011. p. 855-80.
9. Blomqvist S, Roivainen M, Puhakka T, Kleemola M, Hovi T. Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. J Med Virol. 2002;66(2):263-8.
10. Laine P, Savolainen C, Blomqvist S, Hovi T. Phylogenetic analysis of human rhinovirus capsid protein VP1 and 2A protease coding sequences confirms shared genus-like relationships with human enteroviruses. J Gen Virol. 2005;86(3):697-706.
11. Simmonds P, McIntyre C, Savolainen-Kopra C, Tapparel C, Mackay IM, Hovi T. Proposals for the classification of human rhinovirus species C into genetically assigned types. J Gen Virol. 2010;91(10):2409-19.
12. Kapoor A, Victoria J, Simmonds P, Silkas E, Chieochansin T, Naeem A, et al. A highly prevalent and genetically diversified Picornavirus genus in South Asian children. Proc Natl Acad Sci. 2008;105(51):20482-7.
13. Holtz LR, Finkbeiner SR, Kirkwood CD, Wang D. Identification of a novel picornavirus related to cosaviruses in a child with acute diarrhea. Virol J. 2008;5:159.
14. Greninger AL, Runckel C, Chiu CY, Haggerty T, Parsonnet J, Ganem D, et al. The complete genome of klassevirus - a novel picornavirus in pediatric stool. Virol J. 2009;6:82.
15. Holtz LR, Finkbeiner SR, Zhao G, Kirkwood CD, Girones R, Pipas JM, et al. Klassevirus I, a previously undescribed member of the family Picornaviridae, is globally widespread. Virol J. 2009;6:86.
16. Trevelyan B, Smallman-Raynor M, Cliff AD. The spatial dynamics of poliomyelitis in the United States: From epidemic emergence to vaccine-induced retreat, 1910-1971. Ann Assoc Am Geogr. 2005;95(2):269-93.
17. Enders JF, Weller TH, Robbins FC. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various embryonic tissues. Science. 1949;109(2822):85-7.
18. Steere R, Schaffer F. The structure of crystals of purified Mahoney poliovirus. Biochim Biophys Acta. 1958;28(2):241-6.
19. Salk J. Considerations in the preparation and use of poliomyelitis virus vaccine. JAMA. 1955;158(14):1239-48.
20. Sabin A. Oral, live poliovirus vaccine for elimination of poliomyelitis. Arch Intern Med. 1960;106:5-9.

21. Centers for Disease Control and Prevention. Certification of poliomyelitis eradication - the Americas, 1994. MMWR Morb Mortal Wkly Rep. 1994;43(39):720-2.
22. Centers for Disease Control and Prevention. Certification of poliomyelitis eradication - Western Pacific Region, October 2000. MMWR Morb Mortal Wkly Rep. 2001;50(1):1-3.
23. Centers for Disease Control and Prevention. Certification of poliomyelitis eradication - European Region, June 2002. MMWR Morb Mortal Wkly Rep. 2002;51(26):572-4.
24. Antona D, Guerin N. L'éradication de la poliomyélite: où en est-on en 2010? Bulletin Epidemiologique Hebdomadaire. 2010;48:489-93.
25. Centers for Disease Control and Prevention. Laboratory surveillance for wild and vaccine-derived poliovirus - Worldwide, January 2007- June 2008. MMWR Morb Mortal Wkly Rep. 2008;57(35):967-70.
26. Global Polio Eradication Initiative. Wild poliovirus confirmed in China. 2011.
27. Cello J, Paul AV, Wimmer E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science. 2002;297(5583):1016-8.
28. Coleman JR, Papamichail D, Skiena S, Futcher B, Wimmer E, Mueller S. Virus attenuation by genome-scale changes in codon pair bias. Science. 2008;320(5884):1784-7.
29. Hyypiä T, Hovi T, Knowles NJ, Stanway G. Classification of Enteroviruses Based on Molecular and Biological Properties. J Gen Virol. 1997(78):1-11.
30. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of Human Enteroviruses by Partial Sequencing of VP1. J Clin Microbiol. 1999;37(5):1288-93
31. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular Evolution of the Human Enteroviruses: Correlation of Serotype with VP1 Sequence and Application to Picornavirus Classification. J Virol. 1999;73(3):1941-8
32. Ohman T, King SL, Krithivas A, Cunningham J, Dickeson SK, Santoro SA, et al. Echoviruses 1 and 8 are closely related genetically, and bind to similar determinants within the VLA-2 I domain. Virus Res. 2001;1(1):1-8.
33. Blomqvist S, Savolainen C, Raman L, Roivainen M, Hovi T. Human Rhinovirus 87 and Enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. J Clin Microbiol. 2002;40(11):4218-23.
34. Hawkes MT, Vaudry W. Nonpolio enterovirus infection in the neonate and young infants. Paediatr Child Health. 2005;10(7):383-8.
35. Hemmes J, Winkler K, Kool S. Virus survival as a seasonal factor in influenza and poliomyelitis. Antonie van Leeuwenhoek. 1962;28:221-33.
36. Helfand RF, Khan AS, Pallansch MA, Alexander JP, Meyers HB, DeSantis RA, et al. Echovirus 30 infection and aseptic meningitis in parents of children attending a child care center. J Infect Dis. 1994;169(5):1133-7.
37. Pappas D, Hendley J, Schwartz R. Respiratory viral RNA on toys in pediatric office waiting rooms. Pediatr Infect Dis J. 2010;29(2):102-4.
38. Ramia S. Transmission of viral infections by the water route: implications for developing countries. Rev Infect Dis. 1985;7(2):180-8.
39. Keswick B, Gerba C, Goyal S. Occurrence of enteroviruses in community swimming pools. Am J Public Health. 1981;71(9):1026-30.
40. Faustini A, Fano V, Muscillo M, Zaniratti S, La Rosa G, Tribuzi L, et al. An outbreak of aseptic meningitis due to echovirus 30 associated with attending school and swimming in pools. Int J Infect Dis. 2006;10(4):291-7.
41. Hauri A, Schimmelpfennig M, Walter-Domes M, Letz A, Diedrich S, Lopez-Pila J, et al. An outbreak of viral meningitis associated with a public swimming pond. Epidemiol Infect. 2005;133(2):291-8.

42. Begier EM, Oberste MS, Landry ML, Brennan T, Mlynarski D, Mshar PA, et al. An outbreak of concurrent echovirus 30 and coxsackievirus A1 infections associated with sea swimming among a group of travelers to Mexico. Clin Infect Dis. 2008;47(5):616-23.
43. Lévêque N, Huguet P, Norder H, Chomel J-J. Enteroviruses responsible for acute hemorrhagic conjunctivitis. Med Mal Infect. 2010;40(4):212-8.
44. Cheng LL, Ng PC, Chan PKS, Wong HL, Cheng FW, Tang JW. Probable Intrafamilial Transmission of Coxsackievirus B3 With Vertical Transmission, Severe Early-Onset Neonatal Hepatitis, and Prolonged Viral RNA Shedding. Pediatrics. 2006;118(3):929-33.
45. Antona D, Lévêque N, Chomel J-J, Dubrou S, Lévy-Bruhl D, Lina B. Surveillance of Enteroviruses in France, 2000-2004. Eur J Clin Microbiol Infect Dis. 2007;26(6):403-12
46. Khetsuriani N, LaMonte-Fowlkes A, Oberste MS, Pallansch MA. Enterovirus surveillance--United States, 1970-2005. MMWR Surveill Summ. 2006;55(8):1-20
47. Bahri O, Rezig D, Ben Nejma-Oueslati B, Ben Yahia A, Ben Sassi J, Hogga N, et al. Enteroviruses in Tunisia: Virological surveillance over 12 years (1992-2003). J Med Microbiol. 2005;54(1):63-9
48. dos Santos GPL, Skraba I, Oliveira D, Lima AAF, de Melo MMM, Kmetzsch CI, et al. Enterovirus meningitis in Brazil, 1998-2003. J Med Virol. 2005;78(1):98-104
49. Thoelen I, Lemey P, Van der Donck I, Beuselinck K, Lindberg AM, Van Ranst M. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. J Med Virol. 2003;70(3):420-9.
50. Wang J-R, Tsai H-P, Huang S-W, Kuo P-H, Kiang D, Liu C-C. Laboratory Diagnosis and Genetic Analysis of an Echovirus 30-Associated Outbreak of Aseptic Meningitis in Taiwan in 2001. J Clin Microbiol. 2002;40(12):4438-44.
51. Cabrerizo M, Echevarria JE, Gonzalez I, de Miguel T, Trallero G. Molecular epidemiological study of HEV-B enteroviruses involved in the increase in meningitis cases occurred in Spain during 2006. J Med Virol. 2008;80(6):1018-24
52. Mirand A, Henquell C, Archimbaud C, Chambon M, Charbonne F, Peigue-Lafeuille H, et al. Prospective Identification of Enteroviruses involved in Meningitis in 2006 through Direct Genotyping in Cerebrospinal Fluid. J Clin Microbiol. 2008;46(1):87-96.
53. Akiyoshi K, Nakagawa N, Suga T. An outbreak of aseptic meningitis in a nursery school caused by echovirus type 30 in Kobe, Japan. Jpn J Infect Dis. 2007;60(1):66-8.
54. Mullins JA, Khetsuriani N, Nix WA, Oberste MS, LaMonte A, Kilpatrick DR, et al. Emergence of Echovirus Type 13 as a Prominent Enterovirus. Clin Infect Dis. 2004;38(1):70-7
55. Mizuta K, Abiko C, Murata T, Itagaki T, Katsushima N, Akiba T, et al. Re-emergence of echovirus type 13 infections in 2002 in Yamagata, Japan. J Infect. 2003;47(3):243-7
56. Avellón A, Casas I, Trallero G, Pérez C, Tenorio A, Palacios G. Molecular analysis of Echovirus 13 isolates and aseptic meningitis, Spain. Emerg Infect Dis. 2003;9(8):934-41
57. Ho M, Chen E-R, Hsu K-H, Twu S-J, Chen K-T, Tsai S-F, et al. An Epidemic of Enterovirus 71 Infection in Taiwan. N Engl J Med. 1999;341(13):929-35.
58. McMinn P, Stratov I, Nagarajan L, Davis S. Neurological manifestations of Enterovirus 71 infection in children during an outbreak of Hand, Foot, and Mouth Disease in Western Australia. Clin Infect Dis. 2001;32(2):236-42.
59. Chan KP, Goh KT, Chong CY, Teo ES, Lau G, Ling AE. Epidemic Hand, Foot and Mouth Disease Caused by Human Enterovirus 71, Singapore. Emerg Infect Dis. 2003;9(1):78-85.
60. Sun L-m, Zheng H-y, Zheng H-z, Guo X, He J-f, Guan D-w, et al. An Enterovirus 71 epidemic in Guangdong Province of China, 2008: epidemiological, clinical and virogenic manifestations. Jpn J Infect Dis. 2011;64(1):13-8.
61. Hamaguchi T, Fujisawa H, Sakai K, Okino S, Kurosaki N, Nishimura Y, et al. Acute Encephalitis Caused by Intrafamilial Transmission of Enterovirus 71 in Adult. Emerg Infect Dis. 2008;14(5):828-30.

62. Chonmaitree T, Menequs M, Schervish-Swierkosz E, Schwalenstocker E. Enterovirus 71 Infection: Report of an outbreak with two cases of paralysis and a review of the literature. Pediatrics. 1981;67(4):489-93.
63. Chumakov M, Voroshilova M, Shindarov M, Lavrova I, Gracheva L, Koroleva G, et al. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol. 1979;60(3-4):329-40.
64. Diedrich S, Weinbrecht A, Schreier E. Seroprevalence and molecular epidemiology of enterovirus 71 in Germany. Arch Virol. 2009;154(7):1139-42.
65. Schuffenecker I, Mirand A, Antona D, Henquell C, Chomel J-J, Archimbaud C, et al. Epidemiology of Human Enterovirus 71 Infections in France, 2000-2009. J Clin Virol. 2011;50:50-6.
66. van der Sanden S, Koopmans M, Uslu G, Van der Avoort H. Epidemiology of Enterovirus 71 in the Netherlands, 1963 to 2008. J Clin Microbiol. 2009;47(9):2826-33.
67. Blomqvist S, Klemola P, Kaijalainen S, Paananen A, Simonen M-L, Vuorinen T, et al. Co-circulation of coxsackieviruses A6 and A10 in hand, foot and mouth disease outbreak in Finland. J Clin Virol. 2010;48(1):49-54.
68. Lo S-H, Huang Y-C, Huang C-G, Tsao K-C, Li W-C, Hsieh Y-C, et al. Clinical and epidemiologic features of Coxsackievirus A6 infection in children in northern Taiwan between 2004 and 2009. J Microbiol Immunol Infec. 2011;44(4):252-7.
69. Morens DM, Pallansch MA. Epidemiology. In: Rotbart HA, editor. Human Enterovirus Infections. Washington D.C.: ASM Press; 1995. p. 3-23.
70. Watkins-Riedel T, Woegerbauer M, Hollemann D, Hufnagl P. Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. Diagn Microbiol Infect Dis. 2002;42(2):99-105
71. Lim KA, Benyesh-Melnick M. Typing of Viruses by Combinations of Antiserum Pools. Application to Typing of Enteroviruses (Coxsackie and Echo). J Immunol. 1960;84(3):309-18
72. Hewlett MJ, Florkiewicz RZ. Sequence of picornavirus RNAs containing a radioiodinated 5'-linked peptide reveals a conserved 5' sequence. Proc Natl Acad Sci. 1980;77(1):303-7.
73. Jacobson MF, Baltimore D. Polypeptide cleavages in the formation of poliovirus proteins. Proc Natl Acad Sci. 1968;61(1):77-84.
74. Bailey JM, Tapprich WE. Structure of the 5' Nontranslated Region of the Coxsackievirus B3 Genome: Chemical Modification and Comparative Sequence Analysis. J Virol. 2007;81(2):650-68.
75. Evans D, Dunn G, Minor PD, Schild G, Cann A, Stanway G, et al. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature. 1985;314(6011):548-50.
76. Kawamura N, Kohara M, Abe S, Komatsu T, Tago K, Arita M, et al. Determinants in the 5' noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. J Virol. 1989;63(3):1302-9.
77. Macadam A, Ferguson G, Arnold C, Minor PD. An assembly defect as a result of an attenuating mutation in the capsid proteins of the poliovirus type 3 vaccine strain. J Virol. 1991;65(10):5225-31.
78. Brown DM, Cornell CT, Tran GP, Nguyen JH, Semler BL. An Authentic 3′ Noncoding Region Is Necessary for Efficient Poliovirus Replication. J Virol. 2005;79(18):11962-73.
79. Silvestri LS, Parilla JM, Morasco J, Ogram SA, Flanegan JB. Relationship between poliovirus negative-strand RNA synthesis and the length of the 3′ poly(A) tail. Virology. 2006;345(2):509-19.
80. Krausslich H, Holscher C, Reuer Q, Harber J, Wimmer E. Myristoylation of the poliovirus polyprotein is required for proteolytic processing of the capsid and for viral infectivity. J Virol. 1990;64(5):2433-6.
81. Levy HC, Bostina M, Filman DJ, Hogle JM. Catching a Virus in the Act of RNA Release: a Novel Poliovirus Uncoating Intermediate Characterized by Cryo-Electron Microscopy. J Virol. 2010;84(9):4426-41.

82. Stirk H, Thornton J. The BC loop in poliovirus coat protein VP1: an ideal acceptor site for major insertions. Protein Eng. 1994;7(1):47-56.
83. Castello A, Alvarez E, Carrasco L. The Multifaceted Poliovirus 2A Protease: Regulation of Gene Expression by Picornavirus Proteases. J Biomed Biotechnol. 2011:369648.
84. Kempf BJ, Barton DJ. Poliovirus 2A(Pro) increases viral mRNA and polysome stability coordinately in time with cleavage of eIF4G. J Virol. 2008;82(12):5847-59.
85. Martinez-Gil L, Bano-Polo M, Redondo N, Sanchez-Martinez S, Nieva JL, Carrasco L, et al. Membrane Integration of Poliovirus 2B Viroporin. J Virol. 2011;85(21):11315-24.
86. van Kuppeveld F, Melchers WJ, Kirkegaard K, Doedens JR. Structure-function analysis of coxsackie B3 virus protein 2B. Virology. 1997;227(1):111-8.
87. Rodriguez PL, Carrasco L. Poliovirus protein 2C has ATPase and GTPase activities. J Biol Chem. 1993;268(11):8105-10.
88. Teterina N, Pinto Y, Weaver JD, Jensen KS, Ehrenfeld E. Analysis of Poliovirus Protein 3A Interactions with Viral and Cellular Proteins in Infected Cells. J Virol. 2011;85(9):4284-96.
89. Oh HS, Pathak HB, Goodfellow IG, Arnold JJ, Cameron CE. Insight into Poliovirus Genome Replication and Encapsidation Obtained from Studies of 3B-3C Cleavage Site Mutants. J Virol. 2009;83(18):9370-87.
90. de Breyne S, Bonderoff JM, Chumakov KM, Lloyd RE, Hellen CUT. Cleavage of eukaryotic initiation factor eIF5B by enterovirus 3C proteases. Virology. 2008;378(1):118-22.
91. Toyoda H, Nicklin MJH, Murray MG, Anderson CW, Dunn JJ, Studier FW, et al. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell. 1986;45(5):761-70.
92. Hansen J, Long A, Schultz S. Structure of the RNA-dependent RNA polymerase of poliovirus. Structure. 1997;5(8):1109-022.
93. Ward C, Stokes M, Flanegan JB. Direct measurement of the poliovirus RNA polymerase error frequency in vitro. J Virol. 1988;62(2): 558-62.
94. Freistadt MS, Vaccaro JA, Eberle KE. Biochemical characterization of the fidelity of poliovirus RNA-dependent RNA polymerase. Virol J. 2007;4:44.
95. Mendelsohn C, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell. 1989;56(5):855-65.
96. He Y, Bowman VD, Mueller S, Bator CM, Bella J, Peng X, et al. Interaction of the poliovirus receptor with poliovirus. Proc Natl Acad Sci. 2000;97(1):79-84.
97. Bergelson J, Cunningham J, Droguett G, Kurt-Jones E, Krithivas A, Hong J, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science. 1997;275(5304):1320-3.
98. Xiao C, Bator CM, Bowman VD, Rieder E, He Y, Hebert B, et al. Interaction of Coxsackievirus A21 with Its Cellular Receptor, ICAM-1. J Virol. 2001;75(5):2444-51.
99. Bergelson J, Shepley M, Chan B, Hemler M, Finberg R. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science. 1992;255(5052):1718-20.
100. King S, Cunningham J, Finberg R, Bergelson J. Echovirus 1 interaction with the isolated VLA-2 I domain. J Virol. 1995;69(5):3237-9.
101. Bergelson J, Chan M, Solomon K, St John N, Lin H, Finberg R. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc Natl Acad Sci. 1994;91(13):6245-8.
102. Hogle JM. Poliovirus Cell Entry: Common Structural Themes in Viral Cell Entry Pathways. Annu Rev Microbiol. 2002;56:677-702.
103. Bostina M, Levy HC, Filman DJ, Hogle JM. Poliovirus RNA Is Released from the Capsid near a Twofold Symmetry Axis. J Virol. 2011;85(2):776-83.
104. Brandenburg B, Lee LY, Lakadamyali M, Rust MJ, Zhuang X, Hogle JM. Imaging Poliovirus Entry in Live Cells. PLoS Biol. 2007;5(7):183.

105. Krausslich H, Nicklin MJH, Toyoda H, Etchison D, Wimmer E. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. J Virol. 1987;61(9):2711-8.
106. Bonneau A, Sonenberg N. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J Virol. 1987;61(4):986-91.
107. Kuyumcu-Martinez NM, Van Eden ME, Younan P, Lloyd RE. Cleavage of Poly(A)-Binding Protein by Poliovirus 3C Protease Inhibits Host Cell Translation: a Novel Mechanism for Host Translation Shutoff. Molecular and Cell Biology. 2004;24(4):1779-90.
108. Pestova T, Hellen CUT, Wimmer E. Translation of poliovirus RNA: role of an essential cis-acting oligopyrimidine element within the 5' nontranslated region and involvement of a cellular 57-kilodalton protein. J Virol. 1991;65(11):6194-204.
109. Nicklin MJH, Krausslich H, Toyoda H, Dunn J, Wimmer E. Poliovirus polypeptide precursors: expression in vitro and processing by exogenous 3C and 2A proteinases. Proc Natl Acad Sci. 1987;84(12):4002-6.
110. Lawson M, Dasmahapatra B, Semler BL. Species-specific substrate interaction of picornavirus 3C proteinase suballelic exchange mutants. J Biol Chem. 1990;265(26):15920-31.
111. Ypma-Wong M, Filman DJ, Hogle JM, Semler BL. Structural domains of the poliovirus polyprotein are major determinants for proteolytic cleavage at Gln-Gly pairs. J Biol Chem. 1988;263(33):17846-56.
112. Steil BP, Barton DJ. Conversion of VPg into VPgpUpUOH before and during Poliovirus Negative-Strand RNA Synthesis. J Virol. 2009;83(24):12660-70.
113. Steil BP, Kempf BJ, Barton DJ. Poly(A) at the 3′ End of Positive-Strand RNA and VPg-Linked Poly(U) at the 5′ End of Negative-Strand RNA Are Reciprocal Templates during Replication of Poliovirus RNA. J Virol. 2010;84(6):2843-58.
114. Putnak J, Phillips B. Differences between poliovirus empty capsids formed in vivo and those formed in vitro: a role for the morphopoietic factor. J Virol. 1981;40(1):173-83.
115. Muckelbauer JK, Kremer M, Minor I, Diana G, Dutko FJ, Groarke JM, et al. The structure of coxsackievirus B3 at 3.5 A resolution. Structure. 1995;3:653-67.
116. Hogle JM, Chow M, Filman DJ. Three-dimensional structure of poliovirus at 2.9 A resolution. Science. 1985;229(4720):1358-65.
117. Belnap DM, Filman DJ, Trus BL, Cheng N, Booy FP, Conway JF, et al. Molecular Tectonic Model of Virus Structural Transitions: the Putative Cell Entry States of Poliovirus. J Virol. 2000;74(3):1342-54.
118. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 2010;9(11):1097-105.
119. Wright P, Strauss G, Langford M. Acute hemorrhagic conjunctivitis. Am Fam Physician. 1992;45(1):173-8.
120. Sabin A. Paralytic poliomyelitis: Old dogmas and new perspectives. Rev Infect Dis. 1981;3(3):543-64.
121. Mueller S, Wimmer E, Cello J. Poliovirus and poliomyelitis: A tale of guts, brains, and an accidental event. Virus Res. 2005;111:175-93.
122. Rotbart HA. Meningitis and Encephalitis. In: Rotbart HA, editor. Human Enterovirus Infections. Washington D.C.: ASM Press; 1995.
123. Li Y, Pan Z, Ji Y, Peng T, Archard L, Zhang H. Enterovirus replication in valvular tissue from patients with chronic rheumatic heart disease. Eur Heart J. 2002;23(7):567-73.
124. Kaplan K, Klein S, McPhee J, Harper R. Group B coxsackievirus infections in infants younger than three months of age: a serious childhood illness. Rev Infect Dis. 1983;5(6):1019-32.
125. D'Alessio D. A case-control study of group B Coxsackievirus immunoglobulin M antibody prevalence and HLA-DR antigens in newly diagnosed cases of insulin-dependent diabetes mellitus. Am J Epidemiol. 1992;135(12):1331-8.

126. Yoon J, London W, Curfman B, Brown R, Notkins A. Coxsackie virus B4 produces transient diabetes in nonhuman primates. Diabetes. 1986;35(6):712-6.
127. Minor PD. Characteristics of poliovirus strains from long-term excretors with primary immunodeficiencies. Dev Biol (Basel). 2001;105:75-80.
128. Tebruegge M, Curtis N. Enterovirus infections in neonates. Sem Fetal Neonatal Med. 2009;14(4):222-7
129. Neznanov N, Chumakov KM, Neznanova L, Almansan A, Banerjee AK, Gudkov AV. Proteolytic cleavage of the p65-RelA subunit of NF-kappaB during poliovirus infection. J Biol Chem. 2005;280(25):24153-8.
130. Kemball CC, Alirezaei M, Whitton JL. Type B coxsackieviruses and their interactions with the innate and adaptive immune systems. Future Microbiol. 2010;5(9):1329-47.
131. Zaman S, Carlsson B, Morikawa A, Jeansson S, Narayanan I, Thiringer K, et al. Poliovirus antibody titres, relative affinity, and neutralising capacity in maternal milk. Arch Dis Child. 1993;68(2):198-201.
132. Triantafilou K, Triantafilou M. Coxsackievirus B4-induced cytokine production in pancreatic cells is mediated through toll-like receptor 4. J Virol. 2004;78(20):11313-20.
133. Negishi H, Osawa T, Ogami K, Ouyang X, Sakaguchi S, Koshiba R, et al. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc Natl Acad Sci. 2008;105(51):20446-51.
134. Deitz SB, Dodd DA, Cooper S, Parham P, Kirkegaard K. MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A. Proc Natl Acad Sci. 2000;97(25):13790-5.
135. Ehrenfeld E, Modlin JF, Chumakov KM. Future of polio vaccines. Expert Rev Vaccines. 2009;8(7):889-905.
136. Pliaka V, Achilleos C, Kyriakopoulou Z, Tsakogiannis D, Ruether IG, Gartzonica C, et al. Determination of antigenic properties of vaccine derived poliovirus strains. Vaccine. 2010;29(1):26-33.
137. Combelas N, Holmblat B, Joffret M-L, Colbere-Garapin F, Delpeyroux F. Recombination between Poliovirus and Coxsackie A Viruses of Species C: A Model of Viral Genetic Plasticity and Emergence. Viruses. 2011;3:1460-84.
138. Skern T. 100 years poliovirus: from discovery to eradication. A meeting report. Arch Virol. 2010;155:1371-81.
139. Dowdle WR, De Gourville E, Kew OM, Pallansch MA, Wood DJ. Polio eradication: the OPV paradox. Rev Med Virol. 2003;13(5):277-91.
140. Johns TL, Hutter GE. New combination vaccines: DTaP-IPV (Kinrix) and DTaP-IPV/Hib (Pentacel). Ann Pharmacother. 2010;44(3):515-23.
141. Verdijk P, Rots NY, Bakker WA. Clinical development of a novel inactivated poliomyelitis vaccine based on attenuated Sabin poliovirus strains. Expert Rev Vaccines. 2011;10(5):635-44.
142. Ellen H, Ruitenberg EJ. The global introduction of inactivated polio vaccine can circumvent the oral polio vaccine paradox. Vaccine. 2010;28(22):3778-83.
143. Bonnet M-C, Dutta A. World wide experience with inactivated poliovirus vaccine. Vaccine. 2008;26(39):4978-83.
144. Huang QS, Greening G, Baker MG, Grimwood K, Hewitt J, Hulston D, et al. Persistence of oral polio vaccine virus after its removal from the immunisation schedule in New Zealand. Lancet. 2005;366(9483):394-6.
145. John TJ, Jain H, Ravishankar K, Amaresh A, Verma H, Deshpande J, et al. Monovalent type 1 oral poliovirus vaccine among infants in India: report of two randomized double-blind controlled clinical trials. Vaccine. 2011;29(34):5793-801.
146. Sutter RW, John TJ, Jain H, Agarkhedkar S, Ramanan PV, Verma H, et al. Immunogenicity of bivalent types 1 and 3 oral poliovirus vaccine: a randomised, double-blind, controlled trial. Lancet. 2010;376(9753):1682-8.

147. Zhang D, Lu J, Lu J. Enterovirus 71 vaccine: close but still far. Int J Infect Dis. 2010;14(9):739-43.
148. Yu C, Chen C, Chen C, Wang J, Liu C, Yan J, et al. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci. 2000;7(6):523-8.
149. Wu C-N, Lin Y-C, Fann C, Liao N-S, Shih S-R, Ho M-S. Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine. 2001;20(5-6):895-904.
150. Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, et al. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. J Gen Virol. 2005;86(5):1391-401.
151. Arita M, Nagata N, Iwata N, Ami Y, Suzaki Y, Mizuta K, et al. An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. J Virol. 2007;81(17):9386-95.
152. Chung Y, Huang J, Lai C, Sheng H, Shih S-R, Ho M-S, et al. Expression, purification and characterization of enterovirus-71 virus-like particles. World J Gastroenterol. 2006;12(6):921-7.
153. De Palma AM, Vliegen I, de Clercq E, Neyts J. Selective Inhibitors of Picornavirus Replication. Med Res Rev. 2008;28(6):823-84.
154. Chen T-C, Weng K-F, Chang S-C, Lin J-Y, Huang P-N, Shih S-R. Development of antiviral agents for enteroviruses. J Antimicrob Chemother. 2008;62(6):1169-73.
155. Thibaut HJ, De Palma AM, Neyts J. Combating enterovirus replication: State-of-the-art on antiviral researc. Biochem Pharmacol. 2011;In Press.
156. Vance LM, Moscufo N, Chow M, Heinz BA. Poliovirus 2C Region Functions during Encapsidation of Viral RNA. J Virol. 1997;71(11):8759-65.
157. Florea N, Maglio D, Nicolau D. Pleconaril, a novel antipicornaviral agent. Pharmacotherapy. 2003;23(3):339-48.
158. Pevear DC, Tull TM, Seipel ME, Groarke JM. Activity of pleconaril against enteroviruses. Antimicrob Agents Chemother. 1999;43(9):2109-15.
159. Rotbart HA, Webster AD, Pleconaril Treatment Registry Group. Treatment of potentially life-threatening enterovirus infections with pleconaril. Clin Infect Dis. 2001;32(2):228-35.
160. Shih S-R, Tsai M-C, Tseng S-N, Won K-F, Shia K-S, Li W-T, et al. Mutation in enterovirus 71 capsid protein VP1 confers resistance to the inhibitory effects of pyridyl imidazolidinone. Antimicrob Agents Chemother. 2004;48(9):3523-9.
161. Turner R, Dutko F, Goldstein N, Lockwood G, Hayden F. Efficacy of oral WIN 54954 for prophylaxis of experimental rhinovirus infection. Antimicrob Agents Chemother. 1993;37(2):297-300.
162. Oberste MS, Moore D, Anderson B, Pallansch MA, Pevear DC, Collett MS. In vitro antiviral activity of V-073 against polioviruses. Antimicrob Agents Chemother. 2009;53(10):4501-3.
163. Kouiavskaia D, Dragunsky E, Liu H, Oberste MS, Collett MS, Chumakov KM. Immunological and pathogenic properties of poliovirus variants selected for resistance to antiviral drug V-073. Antivir Ther. 2011;16(7):999-1004.
164. Konig H, Rosenwirth B. Purification and partial characterization of poliovirus protease 2A by means of a functional assay. J Virol. 1988;62(4):1243-50.
165. Martin U, Jarasch N, Nestler M, Rassmann A, Munder T, Seitz S, et al. Antiviral effects of pan-caspase inhibitors on the replication of coxsackievirus B3. Apoptosis. 2007;12(3):525-33.
166. Cordingley MG, Callahan PL, Sardana VV, Garsky VM, Colonno RJ. Substrate requirements of human rhinovirus 3C protease for peptide cleavage in vitro. J Biol Chem. 1990;265(16):9062-5.
167. Hsyu P-H, Pithavala YK, Gersten M, Penning CA, Kerr BM. Pharmacokinetics and safety of an antirhinoviral agent, ruprintrivir, in healthy volunteers. Antimicrob Agents Chemother. 2002;46(2):392-7.
168. Barton DJ, Flanegan JB. Synchronous replication of poliovirus RNA: initiation of negative-strand RNA synthesis requires the guanidine-inhibited activity of protein 2C. J Virol. 1997;71(11):8482-9.

169. Pincus S, Diamond D, Emini E, Wimmer E. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J Virol. 1986;57(2):638-46.
170. Pfister T, Wimmer E. Characterization of the nucleoside triphosphatase activity of poliovirus protein 2C reveals a mechanism by which guanidine inhibits poliovirus replication. J Biol Chem. 1999;274(11):6992-7001.
171. Klein M, Hadaschik D, Zimmermann H, Eggers HJ, Nelsen-Salz B. The picornavirus replication inhibitors HBB and guanidine in the echovirus-9 system: the significance of viral protein 2C. J Gen Virol. 2000;81(4):895-901.
172. Hadaschik D, Klein M, Zimmermann H, Eggers HJ, Nelsen-Salz B. Dependence of echovirus 9 on the enterovirus RNA replication inhibitor 2-(alpha-Hydroxybenzyl)-benzimidazole maps to nonstructural protein 2C. J Virol. 1999;73(12):10536-9.
173. de Palma AM, Heggermont W, Lanke K, Coutard B, Bergmann M, Monforte A-M, et al. The thiazolobenzimidazole TBZE-029 inhibits enterovirus replication by targeting a short region immediately downstream from motif C in the nonstructural protein 2C. J Virol. 2008;82(10):4720-30.
174. Yi L, Chen Y, Kung H, He M. Potent inhibition of human enterovirus 71 replication by type I interferon subtypes. Antivir Ther. 2011;16(1):51-8.
175. Hung H-C, Wang H-C, Shih S-R, Teng I-F, Tseng C-P, Hsu JT-A. Synergistic inhibition of enterovirus 71 replication by interferon and rupintrivir. J Infect Dis. 2011;203(12):1784-90.
176. Boriskin YS, Leneva IA, Pecheur E-I, Polyak S. Arbidol: a broad-spectrum antiviral compound that blocks viral fusion. Curr Med Chem. 2008;15(10):997-1005.
177. Leneva IA, Russell RJ, Boriskin YS, Hay AJ. Characteristics of arbidol-resistant mutants of influenza virus: implications for the mechanism of anti-influenza action of arbidol. Antiviral Res. 2009;81(2):132-40.
178. Teissier E, Zandomeneghi G, Loquet A, Lavillette D, Lavergne J-P, Montserret R, et al. Mechanism of inhibition of enveloped virus membrane fusion by the antiviral drug arbidol. PLoS One. 2011;6(1):e15874.
179. Delogu I, Pastorino B, Baronti C, Nougairede A, Bonnet E, de Lamballerie X. In vitro antiviral activity of arbidol against Chikungunya virus and characteristics of a selected resistant mutant. Antiviral Res. 2011;90(3):99-107.
180. Shi L, Xiong H, He J, Deng H, Li Q, Zhong Q, et al. Antiviral activity of arbidol against influenza A virus, respiratory syncytial virus, rhinovirus, coxsackie virus and adenovirus in vitro and in vivo. Arch Virol. 2007;152(8):1447-55.
181. Zhong Q, Yang Z, Liu Y, Deng H, Xiao H, Shi L, et al. Antiviral activity of Arbidol against Coxsackie virus B5 in vitro and in vivo. Arch Virol. 2009;154(4):601-7.
182. Lukashev AN. Role of recombination in evolution of enteroviruses. Rev Med Virol. 2005;15(3):157-67
183. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, Ilonen J. Recombination in Circulating Enteroviruses. J Virol. 2003;77(19):10423-31
184. Kirkegaard K, Baltimore D. The mechanism of RNA recombination in poliovirus. Cell. 1986;47(3):433-43
185. Jarvis TC, Kirkegaard K. Poliovirus RNA recombination: mechanistic studies in the absence of selection. EMBO J. 1992;11(8):3135-45.
186. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, Ilonen J. Recombination in circulating Human enterovirus B: independent evolution of structural and non-structural genome regions. J Gen Virol. 2005;86(12):3281-90
187. Oberste MS, Maher K, Pallansch MA. Evidence for Frequent Recombination within Species Human Enterovirus B Based on Complete Genomic Sequences of All Thirty-Seven Serotypes. J Virol. 2004;78(2):855-67
188. Simmonds P, Welch J. Frequency and Dynamics of Recombination within Different Species of Human Enteroviruses. J Virol. 2006;80(1):483-93

189. Pöyry T, Kinnunen L, Hyppiä T, Brown B, Horsnell C, Hovi T, et al. Genetic and phylogenetic clustering of enteroviruses. J Gen Virol. 1996;77:1699-717
190. Santti J, Hyypiä T, Kinnunen L, Salminen M. Evidence of Recombination among Enteroviruses. J Virol. 1999;73(10):8741-9
191. Lindberg AM, Andersson P, Savolainen C, Mulders MN, Hovi T. Evolution of the genome of Human enterovirus B: incongruence between phylogenies of the VP1 and 3CD regions indicates frequent recombination within the species. J Gen Virol. 2003;84:1223-35
192. Gmyl AP, Korshenko SA, Belousov EV, Khitrina EV, Agol VI. Nonreplicative homologous RNA recombination: Promiscuous joining of RNA pieces? RNA. 2003;9:1221-31.
193. Gmyl AP, Belousov EV, Maslova SV, Khitrina EV, Chetverin AB, Agol VI. Nonreplicative RNA Recombination in Poliovirus. J Virol. 1999;73(11):8958-65
194. Furione M, Guillot S, Otelea D, Candrea A, Crainic R. Polioviruses with natural recombinant genomes isolated from vaccine-associated paralytic poliomyelitis. Virology. 1993;196(1):199-208.
195. Rakoto-Andrianarivelo M, Gumede N, Jegouic S, Balanant J, Andriamamonjy SN, Rabemanantsoa S, et al. Reemergence of recombinant vaccine-derived poliovirus outbreak in Madagascar. J Infect Dis. 2008;197(10):1427-35.
196. Jegouic S, Joffret M-L, Blanchard C, Riquet FB, Perret C, Pelletier I, et al. Recombination between polioviruses and co-circulating Coxsackie A viruses: role in the emergence of pathogenic vaccine-derived polioviruses. PLoS Pathog. 2009;5(5):e1000412.
197. Dagan R, Menegus M. A combination of four cell types for rapid detection of enteroviruses in clinical specimens. J Med Virol. 1986;19(3):219-28.
198. Kok TW, Pryor T, Payne L. Comparison of rhabdomyosarcoma, buffalo green monkey kidney epithelial, A549 (human lung epithelial) cells and human embryonic lung fibroblasts for isolation of enteroviruses from clinical samples. J Clin Virol. 1998;11(1):61-5.
199. Chonmaitree T, Ford C, Sanders C, Lucia H. Comparison of cell cultures for rapid isolation of enteroviruses. J Clin Microbiol. 1988;26(12):2576-80.
200. Lipson SM, Walderman R, Costello P, Szabo K. Sensitivity of rhabdomyosarcoma and guinea pig embryo cell cultures to field isolates of difficult-to-cultivate group A coxsackieviruses. J Clin Microbiol. 1988;26(7):1298-303
201. Landry ML, Garner R, Ferguson D. Comparison of the NucliSens Basic kit (Nucleic Acid Sequence-Based Amplification) and the Argene Biosoft Enterovirus Consensus Reverse Transcription-PCR assays for rapid detection of enterovirus RNA in clinical specimens. J Clin Microbiol. 2003;41(11):5006-10.
202. Rotbart HA. Nucleic acid detection systems for enteroviruses. Clin Microbiol Rev. 1991;4(2):156-68.
203. Bendig J, Earl P. The Lim Benyesh-Melnick antiserum pools for serotyping human enterovirus cell culture isolates--still useful, but may fail to identify current strains of echovirus 18. J Virol Methods. 2005;127(1):96-9.
204. Nasri D, Bouslama L, Pillet S, Bourlet T, Aouni M, Pozzetto B. Basic rationale, current methods and future directions for molecular typing of human enterovirus. Expert Rev Mol Diagn. 2007;7(4):419-34.
205. Ishiko H, Shimada Y, Yonaha M, Hashimoto O, Hayashi A, Sakae K, et al. Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. J Infect Dis. 2002;185(6):744-54.
206. Oberste MS, Maher K, Michele SM, Belliot G, Uddin M, Pallansch MA. Enteroviruses 76, 89, 90 and 91 represent a novel group within the species Human enterovirus A. J Gen Virol. 2005;86(2):445-51.
207. Norder H, Bjerregaard L, Magnius L, Lina B, Aymard M, Chomel J-J. Sequencing of 'untypable' enteroviruses reveals two new types, EV-77 and EV-78, within human enterovirus type B and substitutions in the BC loop of the VP1 protein for known types. J Gen Virol. 2003;84(4):827-36.

208. Caro V, Guillot S, Delpeyroux F, Crainic R. Molecular strategy for 'serotyping' of human enteroviruses. J Gen Virol. 2001;82(1):79-91
209. Kottaridi C, Bolanaki E, Markoulatos P. Amplification of Echoviruses genomic regions by different RT-PCR protocols—a comparative study. Mol Cell Probes. 2004;18(4):263-9.
210. Nasri D, Bouslama L, Omar S, Saoudin H, Bourlet T, Aouni M, et al. Typing of Human Enterovirus by Partial Sequencing of VP2. J Clin Microbiol. 2007;45(8):2370-9.
211. She RC, Hymas WC, Taggart EW, Petti CA, Hillyard DR. Performance of enterovirus genotyping targeting the VP1 and VP2 regions on non-typeable isolates and patient specimens. J Virol Methods. 2010;165(1):46-50.
212. Tavakoli NP, Wang H, Nattanmai S, Dupuis M, Fusco H, Hull R. Detection and typing of enteroviruses from CSF specimens from patients diagnosed with meningitis/encephalitis. J Clin Virol. 2008;43(2):207-11.
213. Leitch ECM, Harvala H, Robertson I, Ubillos I, Templeton K, Simmonds P. Direct identification of human enterovirus serotypes in cerebrospinal fluid by amplfication and sequencing of the VP1 region. J Clin Virol. 2009;44(2):119-24.
214. Silva PA, Diedrich S, de Paula Cardoso DD, Schreier E. Identification of enterovirus serotypes by pyrosequencing using multiple sequencing primers. J Virol Methods. 2008;148(1-2):260-4.
215. Nix WA, Oberste MS. Sensitive, seminested PCR Amplification of VP1 sequences for direct identification of all Enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44(8):2698-704.
216. Rose TM. CODEHOP-mediated PCR - a powerful technique for the identification and characterization of viral genomes. Virol J. 2005;2:20.
217. Oberste MS, Maher K, Williams AJ, Dybdahl-Sissoko N, Brown BA, Gookin MS, et al. Species-specific RT-PCR amplification of human enteroviruses: a tool for rapid species identification of uncharacterized enteroviruses. J Gen Virol. 2006;87(1):119-28.
218. Ninove L, Nougairede A, Gazin C, Zandotti C, Drancourt M, de Lamballerie X, et al. Comparative detection of enterovirus RNA in cerebrospinal fluid: GeneXpert system vs. real-time RT-PCR assay. Clin Microbiol Infect. 2011.
219. Tan EL, Chow VTK, Kumarasinghe G, Lin RTP, Mackay IM, Sloots TP, et al. Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay. Mol Cell Probes. 2006;20(2):135-40.
220. Ninove L, Nougairede A, Gazin C, Thirion L, Delogu I, Zandotti C, et al. RNA and DNA bacteriophages as molecular diagnosis controls in clinical virology: a comprehensive study of more than 45,000 routine PCR tests. PLoS One. 2011;6(2):e16142.
221. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95-8
222. Kumar S, Nei M, Dudley J, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 2008;9(4):299-306
223. Bernit E, de Lamballerie X, Zandotti C, Berger P, Veit V, Schleinitz N, et al. Prospective investigation of a large outbreak of meningitis due to Echovirus 30 during summer 2000 in Marseilles, France. Medicine. 2004;83(4):245-53.
224. Mirand A, Henquell C, Archimbaud C, Peigue-Lafeuille H, Bailly J-L. Emergence of recent echovirus 30 lineages is marked by serial genetic recombination events. J Gen Virol. 2007;88(Pt 1):166-76
225. Cordosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, et al. Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes. Emerg Infect Dis. 2003;9(4):461-8.
226. Wu Y, Yeo A, Phoon M, Tan EL, Poh CL, Quak S, et al. The largest outbreak of Hand, Foot and Mouth Disease in Singapore in 2008: the role of Enterovirus 71 and Coxsackievirus A strains. Int J Infect Dis. 2010;14(12):1076-81.

227. Wu Y, Tan EL, Yeo A, Chan K, Nishimura H, Cardosa MJ, et al. Novel Multiplex Oligonucleotide-Conjugated Bead Suspension Array for Rapid Identification of Enterovirus 71 Subgenogroups. J Clin Microbiol. 2011;49(1):419-22.
228. Huemer HP, Ortner B, Huang C-W, Schmid D, Mutz I, Wewalka G, et al. Isolating Asian Enterovirus 71 Subgenogroup C4 in two Austrian clinical samples in 2004. Euro Surveill. 2008 10 July 2008;13(28).
229. Vallet S, Legrand-Quillien M-C, Dailland T, Podeu G, Gouriou S, Schuffenecker I, et al. Fatal Case of Enterovirus 71 Infection, France, 2007. Emerg Infect Dis. 2009;15(11):1837-40.
230. Kilpatrick DR, Yang C-F, Ching K, Vincent A, Iber J, Campagnoli R, et al. Rapid Group-, Serotype-, and Vaccine Strain-Specific Identification of Poliovirus Isolates by Real-Time Reverse Transcription-PCR Using Degenerate Primers and Probes Containing Deoxyinosine Residues. J Clin Microbiol. 2009;47(6):1939-41.

Centre National de Référence des Enterovirus
Laboratoire de Virologie Est

Mode opératoire

Génotypage des Enterovirus

EV012 MOP Génotypage EV Nix/ 01

Version 01

31 Mai 2010

	Nom	Visa	Date
Rédacteur	Delphine FALCON		
Vérificateur	Isabelle SCHUFFENECKER		
Approbateur (RAQ)	Bruno LINA		

Modifications à intégrer dans la prochaine version :

Date	Modification	Page	Auteur

Localisation de la version imprimée :

Numéro de la pièce	Nb d'exemplaire	Nom du responsable	Visa	Date
A3-02-UTC-2-015 secrétariat	1	Bruno LINA		
A3-02-MIC-2-014A (CNR EV)	1 copie	Delphine FALCON		

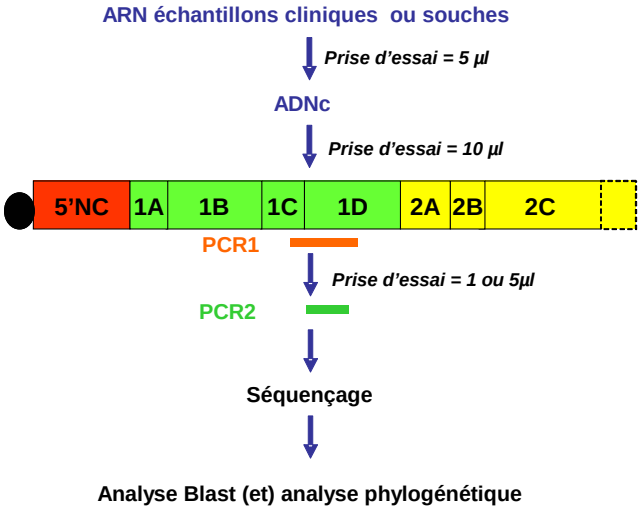
SOMMAIRE

1. BUT ET DOMAINE D'APPLICATION

Déterminer le sérotype et le génotype des Enterovirus (EV) associés aux infections à EV diagnostiquées par RT-PCR générique ou isolement de virus en culture. Cette technique est applicable aux prélèvements biologiques (LCRs, prélèvements respiratoires, selles, prélèvements cutanés…) et aux souches isolées en culture.

2. METHODOLOGIE GENERALE

Cette technique repose sur l'amplification par RT-PCR et le séquençage de la région 5' (300-350 pb) du gène 1D codant pour la protéine de capside VP1. Il s'agit d'une version optimisée au CNREV de la technique de génotypage générique du CDC (Nix et al., JCM, 2006, 44 :2698-2704) permettant en théorie l'amplification et le séquençage de tous les sérotypes d'EV. Le sérotype est déterminé par comparaison de la séquence obtenue pour un EV donné avec les séquences d'EV contenues dans la base de données GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). L'amplification des fragments à séquencer est réalisée au CNREV, tandis que le séquençage est confié à une société extérieure (Genoscreen, Lille ; Biofidal, Vaulx en Velin).



3. REACTIFS UTILISES POUR L'AMPLIFICATION DE LA REGION 5' DU GENE VP1

Remarque préliminaire :

A l'exception des enzymes et de la RNaseOut, tous les autres réactifs sont aliquotés dès la première utilisation de façon à **ne pas être décongelés plus de 2 fois**.

3.1 Amorces

Réaction	Nom des amorces	Séquence	Position	Gène
RT	AN32	GTY-TGC-CA	3009-3002	1D
	AN33	GAY-TGC-CA	3009-3002	
	AN34	CCR-TCR-TA	3111-3104	
	AN35	RCT-YTG-CCA	3009-3002	
PCR1	222	CIC-CIG-GIG-GIA-YRW-ACA-T	2969-2951	1D
	224	GCI-ATG-YTI-GGI-ACI-CAY-RT	1977-1996	1C
PCR2	AN88	TAC-TGG-ACC-ACC-TGG-NGG-NAY-RWA-CAT	2977-2951	1D
	AN89	CCA-GCA-CTG-ACA-GCA-GYN-GAR-AYN-GG	2602-2627	

Commentaires :

Les amorces sont synthétisées et purifiées par HPLC (Eurogentec). A réception, les amorces lyophilisées sont stockées à -20°C (congélateur CGL3 du secteur 1 (A3-2-LOG-011) en attendant leur reprise. Les solutions mère à 100 µM sont reconstituées avec de l'eau PPI (3706433, Aguettant), aliquotées sous 20µl et stockées à -20°. Les solutions de travail à 10 µM sont reconstituées avec de l'eau PPI, aliquotées sous un volume de 5 µl pour les amorces utilisées dans la RT, de 80 µl pour celles utilisées dans la PCR1, de 65 µl pour celles utilisées dans la PCR2 et stockées à -20°C. Les boîtes d'amorces sont annotées comme suit: nom de l'amorce, concentration, volume des aliquots, numéro de lot de l'amorce, date de reprise.

3.2 Réactifs utilisés pour la RT et les PCRs

Réactions	Réactifs	Fournisseurs	Références
RT	Superscript III	Invitrogen	18080-085
	DTT		
	dNTPs 5mM each	Eurogentec	NU-0010-50
	RNaseOut™ Recombinant Ribonuclease	Invitrogen	10777-019
PCR1	AmpliTaq® DNA polymerase	Applied Biosystems	N808-0166
	dNTPs 5mM each	Eurogentec	NU-0010-50
PCR2	FastStart DNA Polymerase	Roche Applied Science	12032937-001
	dNTPs 5mM each	Eurogentec	NU-0010-50

3.3 Réactifs pour électrophorèse en gel d'agarose

Agarose	Sigma	A9539-500G
TBE 5x	Sigma	T6400-4L
Syber®Safe DNA Gel Strain	Invitrogen	S33102
Marqueur poids moléculaire 1000 pb	Promega	G3161

4. AMPLIFICATION DE LA REGION 5' DU GENE VP1

4.1 Extraction des échantillons et souches

L'extraction s'effectue sur l'automate EasyMag de Biomérieux selon le mode opératoire **IN014 MOP EASYMAG 03**. La lyse des échantillons s'effectue en secteur 2 sous le PSM de la pièce A3-02-MIC-2-013F s'il s'agit de prélèvements biologiques (LCRs, prélèvements respiratoires, selles, biopsies) ou sous celui de la pièce A3-02-MIC-2-014A s'il s'agit d'isolats. Le programme d'extraction utilisé pour les prélèvements biologiques et les souches est le Specific B (qui remplace le protocole Specific A depuis juin 2010). Le volume extrait pour chaque échantillon ou souche est de 200µl. Le pré-traitement avant extraction se fait selon le mode opératoire **IN014 MOP EASYMAG 03.** L'élution de l'ARN s'effectue sous 70µl (cf tableau). L'ARN est aliquoté et stocké à -80°C (congélateur CGL 80/9).

Remarque : Pour les prélèvements biologiques qui ont une charge virale faible et pour lesquels le génotypage serait mis en défaut (absence d'amplification, amplification trop faible pour permettre le séquençage), une ré-extraction de l'échantillon peut-être envisagée avec le programme Generic en éluant sous 25 µl.

Type de prélèvement	Prétraitement	Volume extrait	Programme extraction	Volume silice	Volume élution
LCRs	Ajout matrice plasmatique	200µl	Specific B	50µl	70µl
Prélèvements respiratoires	Fluidification par PK	200µl	Specific B	50µl	70µl
Biopsies	Fluidification par PK après broyage mécanique	200µl	Specific B	140µl	70µl
Selles	Lyse déportée et centrifugation	200µl (surnageant)	Specific B	50µl	70µl
Souches	Dilution au 1/100°	200µl	Specific B	50µl	70µl

4.2 Synthèse d'ADNc (RT)

<u>En secteur 1</u> (pièce A3-02-LOG-2-011):

- Noter le nom des échantillons sur chaque tube de 0,2 ml
- Préparer le mix sur portoir réfrigéré, dans un tube de 0,6 ml, sous la hotte réservée aux CNR
- Vortexer brièvement le mix
- Distribuer 5 µl de mix dans chaque tube sur portoir réfrigéré

Réactifs	**Concentration initiale**	**Concentration finale (Qté finale*)**	**volume pour 1 échantillon**
Tampon	5x	1x	2 µl
DTT	0,1M	0,01 M	1 µl
H2O			0,4 µl
dNTP	5 mM	0,1 mM	0,2 µl
AN32	à 10 µM	1 pmol*	0,1 µl
AN33	à 10 µM	1 pmol*	0,1 µl
AN34	à 10 µM	1 pmol*	0,1 µl
AN35	à 10 µM	1 pmol*	0,1 µl
RNaseOut	40 U/µl	20 U*	0,5 µl
Superscript III	200 U/µl	100 U*	0,5 µl

<u>Rq</u> :Pr **n** échantillons et 2 témoins (positif et négatif), prévoir un volume pour **n+3** réactions

<u>En secteur 2</u> (pièce A3-02-MIC-2-014B) :

- Installer les tubes de 0.2 ml sur portoir réfrigéré sous le PSM dédié
- Ajouter 5 µl d'ARN extrait (échantillons, souches, témoin positif) ou 5µl d'eau (témoin négatif)
- Mélanger par aspiration – refoulement

<u>En secteur 3</u> (pièce A3-2-MIC-2-011) :

- Centrifuger rapidement les tubes
- Mettre les tubes dans le thermocycler (Mastercycler Personal, Ependorf)
- Lancer le programme de RT (RTNIX)
- En fin de RT, réaliser la PCR 1 ou stocker l'ADNc à -20°C (stockage à +4°C si la PCR1 est réalisée dans un délai de 24h après la RT)

RT Nix *(durée: 1h 05 min)*		
Etapes	Température	Durée
Hybridation amorces	22°C	10 min
Reverse transcription	50°C	50 min
	95°C	5 min

4.3 PCR 1

<u>En secteur 1</u> (pièce A3-02-LOG-2-011):

- Préparer le mix sur portoir réfrigéré, dans un tube de 1.5 ml, sous la hotte réservée aux CNR

Réactifs	Concentration initiale	Concentration finale (Qté finale*)	volume pr 1 éch
H2O			22,5 µl
Tampon	10 x	1x	5 µl
222	à 10 µM	50 pmol*	5 µl
224	à 10 µM	50 pmol*	5 µl
dNTP	5mM	0,2 mM	2 µl
Taq DNA pol	5U/µl	2,5U*	0,5 µl

Remarque : prévoir le même nombre de tubes que pour la RT.

<u>En secteur 2</u> (pièce A3-02-MIC-2-014B) :

- Vortexer brièvement le mix
- Distribuer 40 µl de mix dans chaque tube de RT sur portoir réfrigéré sous le PSM dédié
- Mélanger par aspiration-refoulement

<u>En secteur 3</u> (pièce A3-2-MIC-2-011) :

- Centrifuger rapidement les tubes
- Mettre les tubes dans le thermocycler (Mastercycler Personal, Ependorf)
- Lancer le programme de PCR1 (PCR1NIX)
- En fin de PCR1, réaliser la PCR2 ou stocker l'ADN à -20°C (stockage à +4°C si la PCR1 est réalisée dans un délai de 24h après la RT)

PCR 1 Nix *(durée : 2 h 37)*			
Etapes	Températures	Temps	Nombre de cycles
Dénaturation	95°C	5 min	1
Dénaturation	95°C	30 sec	40
Hybridation	42°C	50 sec	
Elongation	60°C	50 sec	
	15°C	∞	1

Taille attendue pour le fragment de PCR1 : 990pb

4.4 PCR 2

<u>En secteur 1</u> (pièce A3-02-LOG-2-011):

- Noter le nom des échantillons sur chaque tube de PCR2 de 0.2ml
- Préparer le mix sur portoir réfrigéré, dans un tube de 1.5 ml, sous la hotte réservée aux CNR
- Vortexer brièvement le mix
- Distribuer 49 µl de mix dans chaque tube

Réactifs	Concentration initiale	Concentration finale (Qté finale*)	volume pr 1 éch (CT<32)	volume pr 1 éch (CT>32)
H2O			33,5 µl	29,5 µl
Tampon	10 x	1x	5 µl	5 µl
AN88	à 10 µM	40 pmol*	4 µl	4 µl
AN89	à 10 µM	40 pmol*	4 µl	4 µl
dNTP	5mM	0,2 mM	2 µl	2 µl
Fast Start Taq	5U/µl	2,5U*	0,5 µl	0,5 µl

<u>Rq</u> : prévoir le même nombre de tubes que pour la RT ou la PCR1.

<u>En secteur 3</u> (pièce A3-02-MIC-2-014A)

- Ajouter **1 ou 5 µl** de PCR1 dans le tube de PCR2 sous la hotte réservée pour la distribution d'amplicons
- Mélanger par aspiration-refoulement

NB : <u>Lorsque la charge virale est faible (CT>32), la PCR2 est réalisée à partir de 5µl de PCR1</u>

<u>En secteur 3</u> (pièce A3-2-MIC-2-011) ;

- Centrifuger rapidement les tubes
- Mettre les tubes dans le thermocycler (Mastercycler Personal, Ependorf)
- Lancer le programme de PCR2 (PCR2NIX)
- En fin de PCR2, déposer les produits de PCR sur gel d'agarose ou stocker l'ADN à -20°

PCR 2 Nix *(durée : 2 h 02)*			
Etapes	Températures	Temps	Nombre de cycles
Dénaturation	95°C	5 min	1
Dénaturation	95°C	30 sec	40
Hybridation	60°C	50 sec	
Elongation	72°C	30 sec	
	15°C	∞	1

Taille attendue pour le fragment de PCR2 : 375 pb (350-400pb)

4.5 Contrôle PCR sur gel d'agarose à 2%

4.5.1 Préparation du gel à 2%

- Préparer le dispositif qui va contenir le gel (peignes),
- Dans un flacon de 250 ml, peser 1,6 g d'agarose
- Ajouter 80 ml de tampon TBE 1X et mélanger
- Mettre le mélange dans le four micro-ondes à puissance 600W durant 1 min et mélanger
- Remettre le mélange dans le four micro-ondes à puissance maximum durant 1 min
- Ajouter ensuite 8 µl de Syber®Safe DNA gel strain 10 000 x et mélanger
- Couler le gel
- Recouvrir le gel à l'aide de papier aluminium (le Syber®Safe DNA gel strain est sensible à la lumière)
- Attendre 30 min que le gel polymérise

4.5.2 Dépôt des produits de PCR et migration

- Déposer le gel d'agarose dans la cuve d'électrophorèse remplie de tampon TBE 1 X
- Sur un papier de parafilm, déposer 5 µl de tampon de charge 2X
- Ajouter 5 µl de marqueur de poids moléculaire
- Aspirer – refouler plusieurs fois avec le cône,
- Déposer 10 µl dans le premier puit
- Recommencer l'opération pour chaque échantillon et les témoins
- Brancher la cuve d'électrophorèse
- Faire migrer à 200 V pendant 30 min.
- Une fois la migration terminée, faire la photo du gel sur la table à UV.

4.5.3 Interprétation

- Si les bandes de PCR ont une intensité correcte, les produits de PCR 2 sont envoyés en séquençage.
- Si les bandes de PCR sont de faible intensité, refaire la PCR 2 avec 5µl de produit de PCR1.
- Si aucune bande n'apparaît sur le gel, prévoir de ré-extraire l'échantillon avec le protocole Generic (élution sous 25 µl) et refaire le protocole d'amplification en prenant 5µl de produit de PCR1 pour réaliser la PCR2.

5. SEQUENCAGE DE LA REGION 5' DU GENE VP1

La purification et le séquençage des produits de PCR sont sous traitées à la société Biofidal à Vaulx-en-Velin (69) ou à la société Genoscreen à Lille (59). Le séquençage est effectué avec les amorces AN232 et AN233.

Réaction	Nom des amorces	Séquence	Position	Gène
SEQ	AN232	CCA-GCA-CTG-ACA-GCA	2602-2616	1D
	AN233	TAC-TGG-ACC-ACC-TGG	2977-2963	

5.1 Envoi des échantillons chez Biofidal

- Contacter Mme MEBARKI au 04 37 45 02 96 ou par mail (fmebarki@biofidal.com) pour effectuer une demande de transport.
- Mettre les tubes de PCR 2 dans un flacon rigide.
- Glisser le flacon dans une enveloppe au nom de Biofidal.
- Un transporteur passe récupérer l'enveloppe au secrétariat du laboratoire.
- Remplir le formulaire de demande de séquençage et l'envoyer par mail à Mme MEBARKI.

5.2 Envoi des échantillons chez Genoscreen

- Transférer le produit de PCR 2 dans un tube eppendorf de 1,5 ml.
- Mettre les tubes de PCR 2 dans un flacon rigide.
- Remplir le formulaire de demande de séquençage et l'envoyer par mail à seq1@genoscreen.fr
- Mettre le flacon et une copie du formulaire dans l'enveloppe Chronopost pré-affranchie.
- Déposer l'enveloppe au secrétariat du laboratoire.

6. DETERMINATION DU SEROTYPE

6.1 Analyse Blast

6.1.1 Création des contigs

Pour chaque fragment génomique séquencé, un contig est constitué à partir des séquences forward et reverse grâce à l'application SeqMan (logiciel DNASTAR Lasergene v8)

- Ouvrir l'application Seq Man
- Sélectionner les séquences forward et reverse au format **.abi** par Add sequences, Add et Done
- Aligner les séquences par la fonction Assemble
- Ouvrir le document créé en double-cliquant sur contig 1
- Pour visualiser les chromatogrammes, sélectionner la séquence texte correspondante et faire CTRL-D

- Vérifier l'alignement créé par le logiciel : concordance des séquences forward et reverse sur les portions double brin et absence d'ambiguités sur les portions simple brin

- Enregistrer l'alignement sous 2 extensions : **.sqd** (File/Save as/Nom du fichier) et **.seq** (Contig/Save consensus/Single File/Nom du fichier) ; la version **.sqd** permet la visualisation des chromatogrammes et la version **.seq** est une sauvegarde de la séquence au format texte utilisée pour les alignements multiples.

6.1.2 Analyse Blast

Le sérotype est déterminé par comparaison de la séquence obtenue pour un EV donné avec les séquences d'EV contenues dans la base de données GenBank.

- Aller sur le site Blast du NIH (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
- Cliquer sur Nucleotide Blast
- Sélectionner (CTRL-A) et copier (CTRL-C) la séquence à comparer dans le fichier **.sqd**
- Coller (CTRL-V) la séquence copiée dans la fenêtre Enter Query
- Sélectionner le jeu de séquences GenBank : Choose search set : Others
- Stringence des critères d'alignement : utiliser par défaut « Highly similar sequences » ; si le % d'homologie est faible (<80%), il est prérable d'utiliser « More dissimilar sequences »
- Lancer la recherche d'homologie par Blast
- Enregistrer le document Blast au format **.html**

Critères d'interprétation :

% d'homologie > 80% avec séquences d'un sérotype donné : le virus séquencé est du même sérotype.

% d'homologie entre 70 et 80% avec séquences d'un ou de plusieurs sérotypes: une analyse phylogénétique est requise pour déterminer le sérotype du virus étudié.

% d'homologie <70% avec toutes les séquences GenBank: le virus appartient probablement à un sérotype non décrit et un séquençage plus complet devra être réalisé pour caractériser le virus.

6.2 Analyse phylogénétique

Réalisée selon la procédure **EV013 MOP Analyse phylogénétique EV - 01**