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Human Picornaviruses Associated with Neurological Diseases and Their Neutralization by Antibodies

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ABSTRACT

Picornaviruses are the most commonly encountered infectious agents in mankind. They typically cause mild infections of the gastrointestinal or respiratory tract, but sometimes also invade the central nervous system. There, they can cause severe diseases with long-term sequelae and even be lethal. The most famous picornavirus is polio that was a huge burden for mankind for a long time. A successful vaccination campaign brought polio close to eradication, but neurological diseases caused by other picornaviruses have been increasingly reported since the late 1990s. In this review we focus on enterovirus 71, coxsackievirus A16, enterovirus 68 and human parechovirus 3 that have recently drawn attention because of their links to severe neurological diseases. We discuss the clinical relevance of these viruses, the primary role of humoral immunity to control them and summarize current knowledge on neutralization of such viruses by antibodies.

INTRODUCTION

Picornaviridae is one of the largest viral families. According to the International Committee on Taxonomy of Viruses (ICTV) it contains 31 genera that together enclose 54 viral species (Adams *et al.*, 2015). They infect diverse hosts, from lower vertebrates to mammals. The genera *Kobuvirus, Salivirus, Cosavirus, Cardiovirus, Hepatovirus, Parechovirus*, and *Enterovirus* infect humans (Fig. 1) (Tapparel *et al.*, 2013).

Hepatovirus A, Parechovirus A and multiple enterovirus genera can cause symptomatic infections in humans. They typically result in mild disease of gastrointestinal or respiratory tract, but sometimes are associated with severe conditions. For instance, coxsackievirus B (CVB) type 3 has an established role in viral myocarditis (Fairweather *et al.*, 2012), and CVB4 is well-documented as a viral trigger of type 1 diabetes onset (Yeung *et al.*, 2011).

Several human picornaviruses can target the central nervous system (CNS) and cause severe neurological diseases. The most well known of them is polio (PV). It caused outbreaks of flaccid paralytic disease in children and was a health care burden for a long time, until development of vaccines and a worldwide vaccination campaign brought it close to eradication (Morales *et al.*, 2016). However, other neurotropic picornaviruses still have potential to cause outbreaks of neurological diseases, such as severe and life-threatening meningoencephalitis, encephalitis or acute flaccid paralysis (AFP). A recent metagenomic study identified members of *Cosavirus*, *Cardiovirus*, *Kobuvirus*, *Enterovirus* and *Parechovirus* genera in clinical samples from AFP children (Victoria *et al.*, 2009). This study corroborated epidemiological and experimental work that has already established firm connections between

Enterovirus and *Parechovirus* and neurological diseases in humans and is described below in detail.

PICORNAVIRUS CNS TARGETING

Picornaviruses spread via the fecal-oral or respiratory routes, and the primary sites of their replication are the gastrointestinal or respiratory tracts. Nevertheless, at least some enteroviruses (EV) and human parechoviruses (HPeV) are routinely neurotropic (Rhoades *et al.*, 2011; Wiley *et al.*, 2015).

Picornaviruses utilize a variety of widely expressed molecules as their entry receptors (Evans & Almond, 1998). Such receptors are often present on the surface of cells within the CNS. For example, a receptor for PV—CD155—is expressed in the motor neurons of the spinal cord anterior horns, which are affected during poliomyelitis (Gromeier *et al.*, 2000). Human scavenger receptor class B member 2 (hSCARB2) that is utilized by EV71 and CVA16, is expressed on a variety of cells, including neurons and glial cells (Jiao *et al.*, 2014). Thus, the CNS cells are susceptible for infection. In addition, the nervous tissue has reduced immune surveillance and weaker interferon (IFN) responses, and is a plausible site for replication of IFN-sensitive picornaviruses (Ida-hosonuma *et al.*, 2005). Hence, the CNS cells are also permissive for viral replication.

There is molecular evidence suggesting that picornaviruses can invade the CNS by three possible mechanisms: peripheral nerve infection, blood-brain barrier crossing and “Trojan horse” invasion.

The first mechanism is peripheral nerve infection followed by retrograde axonal transport and trans-synaptic spread in nervous tissue (Fig. 2 (a) and (b)). The evidence for this came from tissue culture studies and *in vivo* experimental models for

PV and also from EV71 patient material (Chen *et al.*, 2007; Daley *et al.*, 2005; Ren & Racaniello, 1992; Wong *et al.*, 2008).

The second mechanism proposes that during viremia viruses cross the blood-brain barrier (BBB) and infect neural cells. Indeed, high levels of viremia and inflammation can decrease tight junction protein expression, disrupt BBB integrity and facilitate viral invasion (Fig. 2 (c)) (Chai *et al.*, 2014; Daniels *et al.*, 2014). Although the inflammation-induced BBB breakdown has not been directly shown for picornaviruses, their prolonged viremia correlates with severe CNS infections and supports this possibility (Cheng *et al.*, 2014). Picornaviruses can also cross the BBB in an active manner: PV can move through the BBB at a rate comparable to a BBB-crossing antibody (Fig. 2 (d)) (Yang *et al.*, 1997). Such trafficking happens independently of the PvR and appears to rely on transferrin receptor 1 (Mizutani *et al.*, 2016).

The third mechanism of neurotropism involves migration of infected cells, such as dendritic cells, monocytes, macrophages, T- and B-cells and nestin^{+} myeloid cells to the CNS, and is called a “Trojan horse” invasion (Fig. 2 (e)) (Tabor-Godwin *et al.*, 2010; Vuorinen *et al.*, 1996; Wahid *et al.*, 2005).

Neurotropic picornaviruses often target different regions of the CNS, and hence vary in their clinical manifestations. Infection of meningeal cells or cells of the ventricular lining results in aseptic meningitis—a non-bacterial inflammation of tissues lining the brain (Irani, 2008). Infection of neurons with subsequent inflammation of brain parenchyma results in encephalitis that can have long-term sequelae or be fatal (Verboon-Maciolek *et al.*, 2008). Inflammation of the spinal cord grey matter results in myelitis and can lead to limb paralysis (Irani, 2008). All these

conditions can be caused by different picornaviruses and their incidences are highest in children (Nicolosi *et al.*, 1986).

NEUROTROPIC PICORNAVIRUSES IN FOCUS

Confirmed neurotropic picornaviruses are members of *Enterovirus* and *Parechovirus* genera. The genus *Enterovirus* includes many recognized pathogens, such as PV, CVA, coxsackieviruses B (CVB), rhinoviruses and EV, whereas genus *Parechovirus* is smaller and includes one human pathogenic species—Parechovirus A. The infections are common, and in the US alone over 10 million symptomatic EV cases are reported annually (Strikas *et al.*, 1986). Human EV and HPeV can be responsible for about 80% of aseptic meningitis cases (Esposito *et al.*, 2014) and 11% of reported encephalitis cases (Koskiniemi *et al.*, 2001). Several types of EV can trigger myelitis with limb paralysis (Kincaid & Lipton, 2006).

Not all serotypes of EV and HPeV cause CNS diseases. Enteroviruses associated with CNS infections include PV types 1, 2 and 3, echovirus types 9, 11, 30 and 33, CVA type 16, CVB types 3 and 5 (Mistchenko *et al.*, 2006), EV types 68 (Messacar *et al.*, 2015) and 71 (McMinn *et al.*, 2001). Parechovirus CNS infections are almost exclusively caused by HPeV3 (Piralla *et al.*, 2014). In this review we will discuss EV71, CVA16, EV68 and HPeV3 that have gained attention due to their recent emergence and connection with CNS infections.

Enterovirus 71 and Coxsackievirus A16

EV71 was initially discovered as a CNS-targeting picornavirus: the first isolates came from two children with neurological symptoms in 1969 in California (Schmidt *et al.*, 1974). In 1973 it was identified as an etiological agent for hand-foot-and-mouth

disease (HFMD), a childhood exanthema characterized by rashes on the palms and soles, oral ulcers and brief febrile illness, but cases of aseptic meningitis were also observed (Hagiwara *et al.*, 1978). In the middle of 1970s it caused a few small outbreaks of aseptic meningitis in the USA, Europe and Australia (Alexander *et al.*, 1994; Blomberg *et al.*, 1974; Ishimaru *et al.*, 1980; Kennett *et al.*, 1974) and two rather large outbreaks of polio-like disease in Bulgaria and Hungary that affected predominantly infants with up to 21% of paralytic manifestations of which over a quarter were lethal (Chumakov *et al.*, 1979; Nagy *et al.*, 1982; Shindarov *et al.*, 1979).

EV71 became a major health care threat in the late 1990s after a series of large outbreaks across the Asia-Pacific region. Most of the affected individuals were children; they often developed HFMD or herpangina, but upper respiratory tract infections (URTI) and non-specific rashes were occasionally observed. A fraction of EV71 infections had neurological and systemic manifestations, but, unlike earlier outbreaks when aseptic meningitis was the most frequent neurological manifestation, more recent outbreaks were characterized with the increased incidence of much more severe brainstem encephalitis and high mortality rate (Chan *et al.*, 2000; Huang *et al.*, 1999). The first and largest outbreak in this series occurred in 1998 in Taiwan. Sentinel physicians reported almost 130,000 cases of HFMD, and over 400 cases of severe neurological involvement with almost 20% mortality rate (Ho *et al.*, 1999b) and in total that outbreak affected about 1.5 million people (Solomon *et al.*, 2010). In 1999 an outbreak in Western Australia resulted in 6000 cases of HFMD and 29 cases of CNS disease, at least nine of which were severe and four developed long-term neurological sequelae (McMinn *et al.*, 2001). EV71 outbreaks, most of which had lethal cases, continued in Korea, Singapore, Japan, Malaysia, Vietnam and Thailand

(Solomon *et al.*, 2010). In 2008 there was a large EV71 outbreak in China with almost half a million reported HFMD cases and 122 fatal cases (Yang *et al.*, 2009). The virus kept on circulating in China and contributed to HFMD cases with CNS complications and fatalities at least until 2014 (Liu *et al.*, 2015a). By then, over 7.5 million cases of HFMD were reported in China alone, of which over 80,000 had neurological involvement and Chinese government declared the development of measures to control EV71 spread as a national priority (Liu *et al.*, 2015b).

Enterovirus 71 infections have been lately detected in Europe, including Denmark, France, Spain and the Netherlands, and although the incidence of EV71 infections there is low, occasional lethal cases have already been reported (Cabrerizo *et al.*, 2014; Fischer *et al.*, 2014; van der Sanden *et al.*, 2011; Schuffenecker *et al.*, 2011). Interestingly, German researchers have recently described a case of pediatric encephalitis caused by a novel EV71 genotype that likely arose from a recombination event (Karrasch *et al.*, 2016). Although no epidemic activity of EV71 has so far been reported in Europe, the European Centre for Disease Prevention and Control (ECDC) risk assessment reported increased detections of EV71 in the first half of 2016 as compared to previous years, necessitating preparedness to control EV71 spread in Europe (ECDC, 2016).

Importantly, EV71 often alternates or co-circulates with other *Enterovirus A* genotypes, mostly with CVA16, another recognized HFMD agent. Coxsackievirus A16 also predominantly infects children but, unlike EV71, typically causes milder symptoms and has much lower morbidity and mortality rate. In a comparative study of 177 EV71 and 64 CVA16 patients in Taiwan, only 6.3% of CVA16 infections developed aseptic meningitis, whereas 32% of EV71 cases resulted in aseptic meningitis, encephalitis, polio-like syndrome, encephalomyelitis and fatal pulmonary

edema of which 7.9% were lethal (Chang *et al.*, 1999). However, occasional severe and fatal CVA16 cases have been reported in USA (Wright *et al.*, 1963), Taiwan (Wang *et al.*, 2004), France (Legay *et al.*, 2007), Japan (Goto *et al.*, 2009), and China (Chen *et al.*, 2015).

The outbreaks of CVA16 were documented in Canada (1957) (Robinson *et al.*, 1958), Australia (1991) (Ferson & Bell, 1991), England and Wales (1994) (Bendig & Fleming, 1996) and India (2009) (Kar *et al.*, 2013) and were linked to HFMD. It co-circulated with EV71 in 1998 during the unprecedented HFMD epidemic in Taiwan (Ho *et al.*, 1999a) and continued circulating in Taiwan becoming dominant in years 2002 and 2003 (Chang, 2008). The co-circulation of CVA16 and EV71 was documented during HFMD outbreaks in Vietnam in 2005 (Tu *et al.*, 2007) and in China in 2008 onwards (Liu, 2014). In Singapore CVA16 was the major cause of HFMD epidemics in years 2002, 2005 and 2007, whereas in 2006 it was EV71 (Ang *et al.*, 2009).

Co-circulation of EV71 and CVA16 allows viral co-infections (He *et al.*, 2013) and recombination, which happened during the HFMD outbreak in China (Zhang *et al.*, 2010a). Recombination between EV71 and CVA16 and accumulation of point mutations can give rise to viruses with altered antigenicity, thus limiting population protection and complicating control of viral spread. Both EV71 and CVA16 require attention as clinically important pathogens.

Enterovirus 68

Enterovirus 68 belongs to the D species of the *Enterovirus* genus. Although genetically it is an enterovirus, EV68 shares properties of both entero- and rhinoviruses and in most cases causes respiratory infections (Oberste *et al.*, 2004). It

was first isolated in 1962, but only 26 cases were reported until 2005 and EV68 received little attention from clinical and scientific communities (Khetsuriani *et al.*, 2006). This changed in 2008–2010 when several outbreaks of acute respiratory illness caused by EV68 were reported by the Centers for Disease Control and Prevention (CDC) in the Philippines, Japan, the Netherlands and the USA (Imamura *et al.*, 2011). The affected individuals typically presented with URTI symptoms—cough, fever, rhinnorea, difficulties in breathing and hypoxia—although severe lower respiratory tract infections (LRTI) were also detected (Khetsuriani *et al.*, 2006). Individuals infected with EV68 often required hospitalization: for example, prospective study in the Netherlands reported that out of 24 EV68-positive subjects, 23 were hospitalized (Imamura *et al.*, 2011). Deaths were reported in the Philippines and in Japan, but not in the US and the Netherlands (Imamura *et al.*, 2011). In all studies EV68 was reported as a paediatric pathogen, except for the work done by Meijer et al. who reported a significant number of patients over 50 years old (Meijer *et al.*, 2014).

Following the initial outbreaks, EV68 continued its seasonal circulation, and was occasionally detected in respiratory samples from paediatric patients with URTI and severe LRTI in different countries, further supporting its clinical relevance and place as a concern for medical society (Esposito *et al.*, 2015; Gimferrer *et al.*, 2015; Imamura *et al.*, 2013; Lu *et al.*, 2014). The concern has been raised further after the EV68 outbreak in the USA in 2014 (Khan, 2015) when over one thousand patients from 47 states have been diagnosed with acute respiratory illness (ARI) caused by EV68. The outbreak resulted in a significant increase in hospital admissions: in Kansas City alone over 300 patients were hospitalized, of which 15% were admitted to ICU and 15 cases were fatal. Simultaneously, an increased incidence of EV68 ARI was reported in Canada, where over 200 cases have been identified, resulting in 140

hospitalizations and one death (Khan, 2015). In 2016 EV68 was detected in patients with neurological manifestations in the Netherlands, France, UK, Italy, Portugal and Germany (ECDC, 2016).

Intriguingly, the 2014 EV68 outbreak in North America overlapped with an outbreak of a polio-like disease with the brain stem and the spinal cord grey matter lesions and AFP. Infection with EV68 was confirmed in 5 out of 11 (45%) of the American AFP patients (Messacar *et al.*, 2015). Furthermore, EV68 has been also detected in four cases of AFP in Canada, two in Norway and one in France (Khan, 2015; Lang *et al.*, 2014; Pfeiffer *et al.*, 2015). A recent retrospective study identified EV68 in respiratory secretions from 12 of 25 (48%) patients with sporadic paralysis, strengthening the EV68 link to CNS disease (Greninger *et al.*, 2015). Interestingly, all the EV68 strains identified in association with paralytic disease formed a distinct genetic cluster suggesting ongoing emergence and adaptation of this virus (Du *et al.*, 2015). Direct linkage of EV68 to neurological disease has been complicated by difficulties to detect EV68 or its RNA in patients' CSF and so far only two studies succeeded to detect EV68 in patients' CSF (Khetsuriani *et al.*, 2006; Kreuter *et al.*, 2011). However, we should note that other neurological picornaviruses—PV and EV71—are also rarely recovered from the CSF, and therefore neurological involvement of EV68 cannot be ruled out on the basis of negative CSF samples.

The incidence of EV68 has clearly increased over the last decade. Although this observation could be related to significant improvements in the detection techniques, the accumulating clinical data suggests that EV68 should be considered an emerging pathogen. The concerns raised by respiratory EV68 infections and especially by their possible link to AFP necessitate careful surveillance of the virus

spread, detailed studies of its pathogenesis and evolution and development of preventative and/or treatment options.

Human parechovirus 3

Human parechovirus 3 belongs to species A within genus *Parechovirus* and is the second most common human parechovirus (Wolthers *et al.*, 2008). HPeV3 was isolated in 1999 from an infant with severe CNS disease (Ito *et al.*, 2004), and since then it has been recognized as the most or second most prevalent virus causing CNS infections in infants under 3 months old (Harvala *et al.*, 2009, 2011; Piralla *et al.*, 2014; van der Sanden *et al.*, 2008). Outbreaks of HPeV3 usually occur in summer-autumn seasons and have a distinct biennial pattern (Harvala *et al.*, 2011; Wolthers *et al.*, 2008). They have been documented in Europe (Benschop *et al.*, 2006), North America (Boivin *et al.*, 2005), Asia (Yamamoto *et al.*, 2009), and Australia (Cumming *et al.*, 2015) and are regularly associated with a variety of clinical presentations, from mild gastrointestinal or respiratory illness to life-threatening conditions in neonates (Esposito *et al.*, 2014; Harvala *et al.*, 2011; Tapia *et al.*, 2008). It can cause systemic infections with possible neurological involvement in infants that are collectively described as "sepsis-like illnesses" (Benschop *et al.*, 2006; Selvarangan *et al.*, 2011; Wolthers *et al.*, 2008). Such illnesses typically present with fever, seizures, irritability, respiratory and gastrointestinal problems and occasional rash being indistinguishable from severe EV infections (Shoji *et al.*, 2013; Verboon-Maciolek *et al.*, 2008). The fraction of symptomatic HPeV3-infected infants that develop sepsis-like illness can exceed 80%; most of such patients require hospitalization and up to one third of them are admitted to the ICU (Schuffenecker *et al.*, 2012; Selvarangan *et al.*, 2011; Shoji *et al.*, 2013). The CNS symptoms of

HPeV3 infection can include meningitis, meningoencephalitis, encephalitis or cerebral hemorrhage with occasional white matter alterations (Khatami *et al.*, 2015; Kurz *et al.*, 2015). Whereas HPeV3 meningitis typically has good prognosis, meningoencephalitis entailing white matter alterations may have long-term sequelae such as cerebral palsy, learning disabilities, epilepsy or visual impairment (Verboon-Maciolek *et al.*, 2008). In addition, HPeV3 is occasionally associated with hemophagocytic lymphohistiocytosis (Aviner *et al.*, 2014) and sudden death syndrome in infants (Schuffenecker *et al.*, 2012). Furthermore, in Japan it was linked to myositis in children and epidemic myalgia in adults (Mizuta *et al.*, 2013; Yamamoto *et al.*, 2015). Fatal cases of HPeV3 infections are known: they resulted from encephalitis in the absence of an immune response and sometimes also involved white matter necrosis (Bissel *et al.*, 2015; van Zwol *et al.*, 2009).

Overall, HPeV3 represents a significant threat to neonatal health care. The incidence of HPeV3 infections and number of CNS disease cases increases (Harvala *et al.*, 2011) with no treatment options available necessitating search for antivirals and understanding of HPeV neutralization by antibodies (Wildenbeest *et al.*, 2010).

NEUTRALIZING ANTIBODIES IN PICORNAVIRUS INFECTIONS

Like with other viruses, the severity and outcome of picornavirus infections depends both on the viral and host factors. Host immune status is a key regulator of infection, and failure to mount an appropriate response inevitably leads to severe disease. The viral infection is detected by the specific pattern recognition receptors of the innate immunity that establish a complex signalling network, triggering the expression of antiviral genes in infected cells and the activation of specific adaptive responses (Dotzauer & Kraemer, 2012). The adaptive responses rely on the specific populations

of T-cells and antibody-producing B-cells. Although both innate and cellular adaptive immunity are essential, a large body of evidence indicates that efficient production of specific antibodies by the B-cells is primary for the control of picornaviral infections.

Picornaviruses typically infect children, likely due to their naive immune system. Severe picornavirus infections in healthy adults are uncommon. However, immunocompromised adults, in particular those with impaired B-cell responses, are susceptible to prolonged and/or severe picornavirus infections. For example, patients with X-linked agammaglobulinemina (XLA) have markedly reduced levels of B-cells and serum antibodies and are susceptible to enterovirus infections (Halliday *et al.*, 2003) with severe neurological manifestations (Quartier *et al.*, 2000). Moreover, patients with a- or hypogammaglobulinemia can also develop chronic HPeV1 infection (van de Ven *et al.*, 2011), as well as HPeV3 myocarditis and encephalitis that are extremely uncommon in adults (Mardekian *et al.*, 2015). Individuals undergoing immunosuppressive therapy further support the critical role of antibody responses in the control of picornaviruses. For instance, cancer treatment with rituximab leads to prolonged B-cell deficiency and hypogammaglobulinemia and patients receiving such therapy are susceptible to severe and even lethal enterovirus infections (Servais *et al.*, 2010).

Direct confirmation for the role of antibodies in picornavirus infections comes from controlled infections in animal models. Experimental infections in mice with different immunodeficiencies proved the significance of adaptive responses: whereas up to 70% of mice deficient in innate immune responses survived EV71 infection, mice with severe combined immunodeficiency developed limb paralysis and died in almost 100% of cases (Liao *et al.*, 2014). In Theiler's encephalomyelitis virus (TMEV)-infected mice—a common model for neurotropic picornaviruses—

immunosuppression with anti-IgM antibodies led to virus-induced demyelinization (Rodriguez *et al.*, 1990).

At the moment there are no antivirals for treatment of severe picornavirus infections (Linden *et al.*, 2015; Wildenbeest *et al.*, 2010) and the only therapeutic option is intravenous immunoglobulin (IVIG). However, because of the low BBB permeability to antibodies, IVIG is rarely effective in CNS infections although intraventricular immmunoglobulin administration may be beneficial (Quartier *et al.*, 2000). Antibodies can also be effective at mucosal sites and prevent picornavirus viremia and CNS invasion (Nathanson & Bodian, 1962). Successful management of severe picornavirus infections using IVIG (Wildenbeest *et al.*, 2013) indicates the potential efficacy of passive immunization to control picornavirus infections. However, the presence of specific neutralizing antibodies and their titres cannot be controlled in IVIG preparation, and the reliable options of passive immunization against picornaviruses should be based on the production of specific neutralizing or broadly neutralizing antibodies that target known viral epitopes. Production of specific antibodies could also contribute to rapid and specific serology-based diagnostics of picornavirus infections that are beneficial at time-critical point-of-care setups. In addition to passive immunization, the success of the polio vaccine encourages development of vaccines against other picornaviruses. Controlling picornavirus infections with vaccines is not a feasible approach for the entire *Picornaviridae* family, but can be realistic for some virus types. Both vaccine and antibody development require thorough understanding of viral neutralization, and below we summarize the current knowledge of the neutralization of CNS-invading picornaviruses.

NEUTRALIZATION OF PICORNAVIRUSES

Neutralization of EV71 and CVA16

EV71 is genetically diverse and contains 14 genotypes: A, B1–B5, C1–C5, D, E, and F (Bessaud *et al.*, 2014); C4 is further classified into two lineages C4a and C4b (Zhang *et al.*, 2010a). Genotypes B3, B4, B5, C1, C2, C4 and C5 contributed to recent outbreaks (Chong *et al.*, 2015). CVA16 is less genetically diverse showing relatively slower evolutionary rate and has 3 genotypes: A, B1 (B1a, B1b, B1c) and B2 (Zhang *et al.*, 2010b). EV71 can undergo intra- and intergenotype shifts that occur due to recombination events during co-circulation with CVA16 or different EV71 genotypes and correlate with most outbreaks (Bible *et al.*, 2007). An effective vaccine should neutralize multiple serotypes of EV71 and also CVA16. This necessity underpins difficulties in EV71 vaccine development.

The first live-attenuated vaccine strain was reported by Arita *et al.* in 2005. It induced broadly-neutralizing responses in immunized monkeys, but was neurotropic when inoculated intravenously and its further development was halted (Arita *et al.*, 2007). Development of inactivated vaccines was more successful: five such vaccines developed by different organizations entered clinical trials and three of them have already completed Phase III showing 80.4–97.4% efficacy against EV71-induced HFMD in humans (Liu *et al.*, 2015a). Two C4-based vaccines developed by the Chinese Academy for Medical Sciences (CAMS) and Sinovac Biotech Co Ltd were approved by the Chinese Food and Drug Administration (CFDA) as of January 2016 (Mao *et al.*, 2016). In addition to live-attenuated and inactivated vaccines, virus-like particle (VLP) vaccine candidates were generated in baculovirus or *Saccharomyces cerevisiae* systems using co-expression of viral protein precursor P1 with viral protease 3CD (Chung *et al.*, 2008; Li *et al.*, 2013). The VLP vaccine candidate

produced in baculovirus system showed promise in *in vivo* studies: the survival rate of VLP-immunized mouse pups after lethal EV71 challenge was superior to those immunized with inactivated virus (Chung *et al.*, 2008) and it also induced protective responses in monkeys (Lin *et al.*, 2012). The approved inactivated vaccines cross-protected against B1, B5 and C4a (CAMS vaccine) (Chou *et al.*, 2013) and B4, B5, C2 and C5 (Sinovac vaccine) (Mao *et al.*, 2013); VLP vaccines protected monkeys against B4, B5, C3, C4 and C5 (Lin *et al.*, 2012), but none of them neutralized CVA16.

Multivalent vaccines may offer protection against EV71 and co-circulating CVA. Bivalent EV71/CVA16 vaccines based on inactivated viruses or VLP can elicit high titres of neutralizing antibodies in immunized mice and protect from EV71 and CVA16 infections (Ku *et al.*, 2014). Yet broader protection is desired to mitigate other HFMD contributors, such as coxsackievirus A6 (CVA6) (Liu *et al.*, 2014), and one trivalent EV71/CVA16/CVA6 inactivated vaccine candidate protecting mice from lethal challenge with these viruses was reported (Caine *et al.*, 2015).

Peptide vaccines consisting of well-defined neutralizing epitopes represent a promising approach to target against several heterologous viruses (Li *et al.*, 2014a). They are easier to produce compared to inactivated virus vaccines, do not require handling live virus and allow immunization with lower protein load. Neutralizing epitopes on EV71 are localized on viral structural proteins VP1, VP2 and VP3 (Fig. 3 (a) and (b)). Three continuous neutralizing epitopes have been localized to VP1 residues 163–177 (known as SP55) and 215–219 (part of SP70 which localizes to residues 208–222) and to region 240–260 (Chang *et al.*, 2010; Foo *et al.*, 2007; Lim *et al.*, 2012). SP55 has 85–100% sequence identity within A–C4 groups (Foo *et al.*, 2007) and SP70 is 100% conserved across EV71 genotypes A–C4 and thus is

403 universal for them. In addition, VP1 encompasses a strain-specific discontinuous
404 neutralization epitope at the 5-fold symmetry axis with residue 145 critically
405 contributing to antibody interaction (Lee *et al.*, 2013). Two other neutralizing
406 epitopes were localized to residues 136–150 of VP2 (known as VP2-28) (Liu *et al.*,
407 2011), and to VP3 residues 55–69 that form a "knob" and are 100% conserved across
408 EV71 subgenogroups A-C4 (Kiener *et al.*, 2014). Much less is known about
409 neutralizing epitopes in CVA16. Most of the experimentally proven neutralizing
410 epitopes of CVA16 were localized to VP1 (GH, EF, C-terminal loops and B and C β-
411 sheets) (Ren *et al.*, 2015; Shi *et al.*, 2013); one more continuous epitope was found in
412 GH loop of VP3 (Chong *et al.*, 2012) (Fig 3 (b)). Additional antigenic sites of CVA16
413 were predicted *in silico* in EF and HI loops of VP2 (Ren *et al.*, 2015). Although
414 peptide vaccines are usually less immunogenic compared to the viral particles, this
415 can be mitigated using adjuvants or fusing viral epitopes with highly immunogenic
416 antigens. Using such an approach, a tandem of three well described EV71 epitopes—
417 SP55, SP70 and VP2-28—separated by Gly-Ser linker and fused to thioredoxin was
418 expressed in *E. coli*. The recombinant protein induced EV71-specific neutralizing
419 responses in immunized mice, serving as a proof-of-concept for peptide vaccines
420 against HFMD (Li *et al.*, 2014b). In another study, a hepatitis B virus-like particle
421 displaying EV71 SP55 and VP2-28 epitopes induced neutralizing responses in
422 immunized mice, and could also be cross-protective against CVA16 (Xu *et al.*,
423 2015b). In terms of immunogenicity EV71 is one of the best-studied picornaviruses
424 with two EV71 vaccines approved by CFDA and further work will be driven by the
425 necessity of multivalent HFMD vaccines.
426

Neutralization of enterovirus 68

EV68 is an emerging virus and little is known about its antigenicity. Imamura *et al.* studied the immunogenic properties of twelve EV68 genotypes belonging to all three genetic lineages of EV68—A, B and C (Imamura *et al.*, 2013). Immunized guinea pigs generated high titres of neutralizing antibodies to the original virus and to viruses of the same lineage, but very little cross-neutralization between genetic lineages was found (Imamura *et al.*, 2014). Importantly, the majority of sequence variation between EV68 lineages is localized to the VP1 BC and DE surface loops (Imamura *et al.*, 2013, 2014; Liu *et al.*, 2015b), which are the most variable and likely are epitope-containing in enteroviruses. Indeed, VP1 likely contains antigenic determinants as the increase in its gene diversity correlates with an increase in the number of EV68 detections (Meijer *et al.*, 2012), reflecting the appearance of antigenically new viruses in the population. Substitution dynamics within the viral genome also suggests the localization of antigenic epitopes to VP1: several positions in VP1 BC and DE loops (Fig. 3 (b)) are undergoing positive selection and might be associated with antigenic differences between EV68 genetic lineages (Imamura *et al.*, 2014). Nevertheless, to date the exact immunogenic epitopes of EV68 are only predictive and have not been mapped.

Not much is also known about the distribution of EV68 neutralizing antibodies in human population. One study done in Finland addressed this question reporting high titres of neutralizing responses to EV68 in 80% of the studied individuals (Smura *et al.*, 2010). However, neutralization responses in this study were addressed against the prototype EV68 Fermon strain, which is antigenically very different from the currently circulating EV68 strains (Greninger *et al.*, 2015; Meijer *et al.*, 2012).

Therefore, neither EV68 antigenicity, nor the population protection levels are understood well at the moment.

Neutralization of HPeV3

Almost nothing is known about HPeV3 antigenicity. Of the parechoviruses, only antigenicity of HPeV1 has been studied (Shakeel *et al.*, 2015) and so far three neutralizing epitopes on HPeV1 structural proteins VP0, VP1 and VP3 are described. One is localized to residues 83-97 of VP0 (Joki-Korpela *et al.*, 2000), another is found on VP1 and encompasses receptor recognizing arginine-glycine-aspartic acid (RGD) motif (Alho *et al.*, 2003) and the third one is formed by VP0 and VP3 (Shakeel *et al.*, 2015; Westerhuis *et al.*, 2015). Whereas antisera generated against HPeV1 VP0 peptide was not tested for HPeV3 neutralization, two other monoclonal antibodies did not cross-react with HPeV3 (Shakeel *et al.*, 2015; Westerhuis *et al.*, 2015). For HPeV3 only non-neutralizing epitope has been described (Shakeel *et al.*, 2016)

The data on HPeV3 seroprevalence in population is also sparse. A study of sera from populations in Finland and Netherlands revealed neutralizing responses to HPeV3 only in about 10% of the samples and very low titres of neutralizing antibodies post infection (Westerhuis *et al.*, 2013). On the contrary, researchers in Japan detected neutralizing responses to HPeV3 in 67% of individuals between 7 months and 40 years old (Ito *et al.*, 2004), and reported high titres of neutralizing antibodies after 3 months post infection in all studied individuals (Aizawa *et al.*, 2015). The virus strains used in European and Japanese studies were different, suggesting that some strains of HPeV3 are strongly immunogenic, whereas others are not. The determinants of immunogenicity within this virus are currently unknown and

investigation of the Japanese A308/99 strain immunogenicity may shed light on the neutralization of HPeV3.

The treatment option for human parechovirus infections could be IVIG (Wildenbeest *et al.*, 2013), but titres of neutralizing antibodies against HPeV3 in European IVIG preparations are very low (Westerhuis *et al.*, 2012). Developing vaccines does not seem feasible because subjects of severe HPeV3 infections are infants. Passive immunization protects against HPeV3 (Aizawa *et al.*, 2015) thus developing therapeutic antibodies is a necessity for which studies of HPeV3 antigenicity are required.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our understanding of antigenicity of picornaviruses that target CNS is very poor and is largely limited to studies of EV71. Although studies of EV71 have already resulted in two CFDA-approved HFMD vaccines, multivalent HFMD vaccines to control different EV71 genotypes and also co-circulating CVA16 are the next goal. Proof-of-concept studies of such vaccines are promising (Caine *et al.*, 2015; Sun *et al.*, 2014), but further work is needed to identify the optimal combination of antigens for balanced, broadly protective immunity. Targeting multiple viruses with a single shot also requires delivery systems for effective presentation of multiple epitopes. In this regard, VLP and peptide vaccines may be preferable over inactivated vaccines, offering tailored solutions in terms of presented antigens together with comparable immunogenicity, high safety and less tedious production, and economical feasibility (Li *et al.*, 2014a).

We know almost nothing about EV68 and HPeV3 antigenicity. Classical approach to study virus antigenicity and develop vaccines relies on animal models,

501 which is just being developed for EV68 and not available for HPeV3, hampering
502 investigation of these viruses. Therefore, their antigenicity should be studied directly
503 from human sera using novel methods, such as peptide arrays (Hansen *et al.*, 2013),
504 classical phage display or phage display enhanced with next generation sequencing
505 (NGS) (Christiansen *et al.*, 2015). Successful use of custom phage display library and
506 NGS was reported by Xu *et al.* who analysed antibody-peptide interactions in sera of
507 over 550 donors and identified numerous previously undescribed viral epitopes,
508 proving the utility of NGS-enhanced phage display for epitope identification (Xu *et*
509 *al.*, 2015a).

510 Another future direction is the search for EV68 and HPeV3 neutralizing
511 antibodies. In the absence of antivirals (Linden *et al.*, 2015; Wildenbeest *et al.*, 2010),
512 such antibodies could be valuable therapeutics, especially for HPeV3 that infects
513 infants for whom vaccination is not a suitable option. A useful approach for that is
514 identification of individual's immune response to a given virus followed by respective
515 B-cell cloning (Kwakkenbos *et al.*, 2014). This approach was utilized to generate two
516 broadly neutralizing antibodies against HPeV (Shakeel *et al.*, 2015; Westerhuis *et al.*,
517 2015). Unfortunately, these antibodies did not neutralize HPeV3. High throughput
518 approaches, such as sequencing of antibody repertoire (Georgiou *et al.*, 2014) and
519 screening of large antibody fragments libraries using ribosomal, bacterial, yeast or
520 phage display (Bradbury *et al.*, 2011) are also utilized for antibody discovery. For
521 instance, phage display technology has already yielded monoclonal neutralizing
522 antibodies with therapeutic potential for EV71 (Zhang *et al.*, 2015). Over 50 phage-
523 display derived antibodies have been approved by the U.S. Food and Drug
524 Administration (FDA) or European Medicines Agency (EMA) as of May, 2016.
525 About 500 antibodies are undergoing clinical trials. These include antibodies targeting

infectious agents, for example Rabies virus, which is now in Phase II (Frenzel *et al.*, 2016).

Overall, picornaviruses antigenicity and neutralization studies have already brought encouraging results, however more challenges are ahead. The detailed understanding of viral immunogenicity is clearly an important task to focus on, but it should be carried out in parallel with broader studies of viral biology and spread. Although many research groups in Europe, US and Asia are very active in the field of picornavirus research, we are still far from a thorough understanding of viral epidemiology, pathogenesis, evolution and inhibition, which are necessary for effective virus control. Apart from scientific challenges, development protective measures against picornaviruses may face additional financial and regulatory difficulties due to the endemic nature of diseases that they cause. Hence, drawing the public attention to the health care threats that picornaviruses impose is another important activity area for the medical and scientific communities.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

The authors declare that there are no ethical issues.

REFERENCES

Adams, M. J., Lefkowitz, E. J., King, A. M. Q., Bamford, D. H., Breitbart, M., Davison, A. J., Ghabrial, S. A., Gorbalenya, A. E., Knowles, N. J. & other authors. **(2015, July).** Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2015). *Arch Virol*.

Aizawa, Y., Watanabe, K., Oishi, T., Hirano, H., Hasegawa, I. & Saitoh, A. (2015). Role of Maternal Antibodies in Infants with Severe Diseases Related to Human. *Emerg Infect Dis* **21**.

Alexander, J. P. J., Baden, L., Pallansch, M. A. & Anderson, L. J. (1994). Enterovirus 71 infections and neurologic disease--United States, 1977-1991. *J Infect Dis* **169**, 905–908.

Alho, A., Marttila, J., Ilonen, J., Hyypiä, T. & Hyypia, T. (2003). Diagnostic Potential of Parechovirus Capsid Proteins Diagnostic Potential of Parechovirus Capsid Proteins. *J Clin Microbiol* **41**, 2294–2299.

Ang, L. W., Koh, B. K., Chan, K. P., Chua, L. T., James, L. & Goh, K. T. (2009). Epidemiology and control of hand, foot and mouth disease in Singapore, 2001-2007. *Ann Acad Med Singapore*.

Arita, M., Nagata, N., Iwata, N., Ami, Y., Suzaki, Y., Mizuta, K., Iwasaki, T., Sata, T., Wakita, T. & Shimizu, H. (2007). An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. *J Virol* **81**, 9386–9395.

Aviner, S., Sofer, D., Shulman, L. M., Bibi, H. & Weitzman, S. (2014). Hemophagocytic lymphohistiocytosis associated with parechovirus 3 infection. *J Pediatr Hematol Oncol* **36**, e251-3.

Bendig, J. W. & Fleming, D. M. (1996). Epidemiological, virological, and clinical features of an epidemic of hand, foot, and mouth disease in England and Wales. *Commun Dis Rep* **6**, R81-6.

Benschop, K., Schinkel, J., Minnaar, R. P., Pajkrt, D., Spanjerberg, L., Kraakman, H. C., Berkhout, B., Zaaijer, H. L., Beld, M. G. H. M. & Wolthers, K. C. (2006). Human parechovirus infections in Dutch children and the association between serotype and disease severity. *Clin Infect Dis* **42**, 204–10.

Bessaud, M., Razafindratsimandresy, R., Nougairede, A., Joffret, M.-L., Deshpande, J. M., Dubot-Peres, A., Heraud, J.-M., de Lamballerie, X., Delpeyroux, F. & Bailly, J.-L. (2014). Molecular comparison and evolutionary analyses of VP1 nucleotide sequences of new African human enterovirus 71 isolates reveal a wide genetic diversity. *PLoS One* **9**, e90624.

Bible, J. M., Pantelidis, P., Chan, P. K. & Tong, C. Y. (2007). Genetic evolution of enterovirus 71: epidemiological and pathological implications. *Rev Med Virol* **17**.

Bissel, S. J., Auer, R. N., Chiang, C.-H., Kofler, J., Murdoch, G. H., Nix, W. A., Painter, M., Richer, M., Sartelet, H. & other authors. **(2015).** Human Parechovirus 3 Meningitis and Fatal Leukoencephalopathy. *J Neuropathol Exp Neurol* **74**, 767–777.

Blomberg, J., Lycke, E., Ahlfors, K., Johnsson, T., Wolontis, S. & von Zeipel, G. (1974). Letter: New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease. *Lancet* **2**, 112.

Boivin, G., Abed, Y. & Boucher, F. D. (2005). Human parechovirus 3 and neonatal

infections. *Emerg Infect Dis* **11**, 103–105.

Bradbury, A. R. M., Sidhu, S., Dübel, S. & McCafferty, J. (2011). Beyond natural antibodies: the power of in vitro display technologies. *Nat Biotechnol* **29**, 245–254. Nature Publishing Group.

Cabrerizo, M., Tarrago, D., Munoz-Almagro, C., Del Amo, E., Dominguez-Gil, M., Eiros, J. M., Lopez-Miragaya, I., Perez, C., Reina, J. & other authors. (2014). Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot and mouth disease in Spain. *Clin Microbiol Infect* **20**, O150-6.

Caine, E. A., Fuchs, J., Das, S. C., Partidos, C. D. & Osorio, J. E. (2015). Efficacy of a Trivalent Hand, Foot, and Mouth Disease Vaccine against Enterovirus 71 and Coxsackieviruses A16 and A6 in Mice. *Viruses* **7**, 5919–5932.

Chai, Q., He, W. Q., Zhou, M., Lu, H. & Fu, Z. F. (2014). Enhancement of blood-brain barrier permeability and reduction of tight junction protein expression are modulated by chemokines/cytokines induced by rabies virus infection. *J Virol* **88**, 4698–4710.

Chan, L. G., Parashar, U. D., Lye, M. S., Ong, F. G., Zaki, S. R., Alexander, J. P., Ho, K. K., Han, L. L., Pallansch, M. A. & other authors. (2000). Deaths of children during an outbreak of hand, foot, and mouth disease in sarawak, malaysia: clinical and pathological characteristics of the disease. For the Outbreak Study Group. *Clin Infect Dis* **31**, 678–683.

Chang, G., Luo, Y., Wu, X., Si, B., Lin, L. & Zhu, Q. (2010). Monoclonal antibody induced with inactived EV71-Hn2 virus protects mice against lethal EV71-Hn2 virus infection. *Virol J* **7**, 106. BioMed Central.

Chang, L. Y. (2008). Enterovirus 71 in Taiwan. *Pediatr Neonatol* **49**, 244–252.

Chang, L. Y., Lin, T. Y., Huang, Y. C., Tsao, K. C., Shih, S. R., Kuo, M. L., Ning, H. C., Chung, P. W. & Kang, C. M. (1999). Comparison of enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. *Pediatr Infect Dis J* **18**, 1092–1096.

Chen, C.-S., Yao, Y.-C., Lin, S.-C., Lee, Y.-P., Wang, Y.-F., Wang, J.-R., Liu, C.-C., Lei, H.-Y. & Yu, C.-K. (2007). Retrograde axonal transport: a major transmission route of enterovirus 71 in mice. *J Virol* **81**, 8996–9003.

Chen, W.-J., Arnold, J. C., Fairchok, M. P., Danaher, P. J., McDonough, E. a., Blair, P. J., Garcia, J., Halsey, E. S., Schofield, C. & other authors. (2015). Epidemiologic, clinical, and virologic characteristics of human rhinovirus infection among otherwise healthy children and adults. *J Clin Virol* **64**, 74–82. Elsevier B.V.

Cheng, H.-Y., Huang, Y.-C., Yen, T.-Y., Hsia, S.-H., Hsieh, Y.-C., Li, C.-C., Chang, L.-Y. & Huang, L.-M. (2014). The correlation between the presence of viremia and clinical severity in patients with enterovirus 71 infection: a multi-center cohort study. *BMC Infect Dis* **14**, 417.

Chong, P., Guo, M.-S., Lin, F. H.-Y., Hsiao, K.-N., Weng, S.-Y., Chou, A.-H., Wang, J.-R., Hsieh, S.-Y., Su, I.-J. & Liu, C.-C. (2012). Immunological and biochemical characterization of coxsackie virus A16 viral particles. *PLoS One* **7**, e49973.

Chong, P., Liu, C. C., Chow, Y. H., Chou, A. H. & Klein, M. (2015). Review of enterovirus 71 vaccines. *Clin Infect Dis* **60**, 797–803.

Chou, A.-H., Liu, C.-C., Chang, J.-Y., Jiang, R., Hsieh, Y.-C., Tsao, A., Wu, C.-L., Huang, J.-L., Fung, C.-P. & other authors. (2013). Formalin-Inactivated EV71 Vaccine Candidate Induced Cross-Neutralizing Antibody against

Subgenotypes B1, B4, B5 and C4A in Adult Volunteers. *PLoS One* **8**, e79783. Public Library of Science.

Christiansen, A., Kringelum, J. V., Hansen, C. S., Bøgh, K. L., Sullivan, E., Patel, J., Rigby, N. M., Eiwegger, T., Szépfalusi, Z. & other authors. **(2015).** High-throughput sequencing enhanced phage display enables the identification of patient-specific epitope motifs in serum. *Sci Rep* **5**, 12913. Nature Publishing Group.

Chumakov, M., Voroshilova, M., Shindarov, L., Lavrova, I., Gracheva, L., Koroleva, G., Vasilenko, S., Brodvarova, I., Nikolova, M. & other authors. **(1979).** Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Arch Virol* **60**, 329–340.

Chung, Y.-C., Ho, M.-S., Wu, J.-C., Chen, W.-J., Huang, J.-H., Chou, S.-T. & Hu, Y.-C. (2008). Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. *Vaccine* **26**, 1855–1862.

Cumming, G., Khatami, A., McMullan, B. J., Musto, J., Leung, K., Nguyen, O., Ferson, M. J., Papadakis, G. & Sheppeard, V. (2015). Parechovirus Genotype 3 Outbreak among Infants, New South Wales, Australia, 2013-2014. *Emerg Infect Dis* **21**, 1144–1152.

Daley, J. K., Gechman, L. A., Skipworth, J. & Rall, G. F. (2005). Poliovirus replication and spread in primary neuron cultures. *Virology* **340**, 10–20.

Daniels, B. P., Holman, D. W., Cruz-Orengo, L., Jujjavarapu, H., Durrant, D. M. & Klein, R. S. (2014). Viral pathogen-associated molecular patterns regulate blood-brain barrier integrity via competing innate cytokine signals. *MBio* **5**, e01476-14.

Dotzauer, A. & Kraemer, L. (2012). Innate and adaptive immune responses against picornaviruses and their counteractions: An overview. *World J Virol* **1**, 91–107. Baishideng Publishing Group Co., Limited.

Du, J., Zheng, B., Zheng, W., Li, P., Kang, J., Hou, J., Markham, R., Zhao, K. & Yu, X.-F. (2015). Analysis of Enterovirus 68 Strains from the 2014 North American Outbreak Reveals a New Clade, Indicating Viral Evolution. *PLoS One* **10**, e0144208.

ECDC. **(2016).** *Enterovirus detections associated with severe neurological symptoms in children and adults in European countries*.

Esposito, S., Rahamat-Langendoen, J., Ascolese, B., Senatore, L., Castellazzi, L. & Niesters, H. G. M. (2014). Pediatric parechovirus infections. *J Clin Virol* **60**, 84–89. Elsevier B.V.

Esposito, S., Zampiero, A., Ruggiero, L., Madini, B., Niesters, H. & Principi, N. (2015). Enterovirus D68-associated community-acquired pneumonia in children living in Milan, Italy. *J Clin Virol* **68**, 94–96.

Evans, D. J. & Almond, J. W. (1998). Cell receptors for picornaviruses as determinants of cell tropism and pathogenesis. *Trends Microbiol* **6**, 198–200.

Fairweather, D., Stafford, K. a. & Sung, Y. K. (2012). Update on coxsackievirus B3 myocarditis. *Curr Opin Rheumatol* **24**, 401–407.

Ferson, M. J. & Bell, S. M. (1991, December). Outbreak of Coxsackievirus A16 hand, foot, and mouth disease in a child day-care center. *Am J Public Health*.

Fischer, T. K., Nielsen, A. Y., Sydenham, T. V, Andersen, P. H., Andersen, B. & Midgley, S. E. (2014). Emergence of enterovirus 71 C4a in Denmark, 2009 to 2013. *Eurosurveillance* **19**.

Foo, D. G. W., Alonso, S., Phoon, M. C., Ramachandran, N. P., Chow, V. T. K. &

Poh, C. L. (2007). Identification of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides. *Virus Res* **125**, 61–68.

Frenzel, A., Schirrmann, T. & Hust, M. (2016). Phage display-derived human antibodies in clinical development and therapy. *MAbs* 1–18.

Georgiou, G., Ippolito, G. C., Beausang, J., Busse, C. E., Wardemann, H. & Quake, S. R. (2014). The promise and challenge of high-throughput sequencing of the antibody repertoire. *Nat Biotechnol* **32**, 158–68. Nature Publishing Group.

Gimferrer, L., Campins, M., Codina, M. G., Esperalba, J., Martin, M. D. C., Fuentes, F., Pumarola, T. & Anton, A. (2015). First Enterovirus D68 (EV-D68) cases detected in hospitalised patients in a tertiary care university hospital in Spain, October 2014. *Enferm Infecc Microbiol Clin*.

Goto, K., Sanefuji, M., Kusuhara, K., Nishimura, Y., Shimizu, H., Kira, R., Torisu, H. & Hara, T. (2009, October). Rhombencephalitis and coxsackievirus A16. *Emerg Infect Dis*.

Greninger, A. L., Naccache, S. N., Messacar, K., Clayton, A., Yu, G., Somasekar, S., Federman, S., Stryke, D., Anderson, C. & other authors. **(2015).** A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012–14): a retrospective cohort study. *Lancet Infect Dis* **15**, 671–682. Elsevier Ltd.

Gromeier, M., Solecki, D., Patel, D. D. & Wimmer, E. (2000). Expression of the human poliovirus receptor/CD155 gene during development of the central nervous system: implications for the pathogenesis of poliomyelitis. *Virology* **273**, 248–257.

Hagiwara, A., Tagaya, I. & Yoneyama, T. (1978). Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. *Intervirology* **9**, 60–63.

Halliday, E., Winkelstein, J. & Webster, a. D. B. (2003). Enteroviral infections in primary immunodeficiency (PID): A survey of morbidity and mortality. *J Infect* **46**, 1–8.

Hansen, L. B., Buus, S. & Schafer-Nielsen, C. (2013). Identification and Mapping of Linear Antibody Epitopes in Human Serum Albumin Using High-Density Peptide Arrays. *PLoS One* **8**, e68902. Public Library of Science.

Harvala, H., Robertson, I., Chieochansin, T., McWilliam Leitch, E. C., Templeton, K. & Simmonds, P. (2009). Specific association of human parechovirus type 3 with sepsis and fever in young infants, as identified by direct typing of cerebrospinal fluid samples. *J Infect Dis* **199**, 1753–1760.

Harvala, H., McLeish, N., Kondracka, J., McIntyre, C. L., McWilliam Leitch, E. C., Templeton, K. & Simmonds, P. (2011). Comparison of human parechovirus and enterovirus detection frequencies in cerebrospinal fluid samples collected over a 5-year period in edinburgh: HPeV type 3 identified as the most common picornavirus type. *J Med Virol* **83**, 889–896.

He, S.-J., Han, J.-F., Ding, X.-X., Wang, Y.-D. & Qin, C.-F. (2013). Characterization of enterovirus 71 and coxsackievirus A16 isolated in hand, foot, and mouth disease patients in Guangdong, 2010. *Int J Infect Dis* **17**, e1025–e1030.

Ho, M., Chen, E. R., Hsu, K. H., Twu, S. J., Chen, K. T., Tsai, S. F., Wang, J. R. & Shih, S. R. (1999a). An epidemic of enterovirus 71 infection in Taiwan: Taiwan enterovirus epidemic working group. *N Engl J Med* **341**.

Ho, M., Chen, E.-R., Hsu, K.-H., Twu, S.-J., Chen, K.-T., Tsai, S.-F., Wang, J.-R. & Shih, S.-R. (1999b). An Epidemic of Enterovirus 71 infection in Taiwan. *N Engl J Med* **341**, 929–935.

Huang, C. C., Liu, C. C., Chang, Y. C., Chen, C. Y., Wang, S. T. & Yeh, T. F. (1999). Neurologic complications in children with enterovirus 71 infection. *N Engl J Med* **341**, 936–942.

Ida-hosonuma, M., Iwasaki, T., Yoshikawa, T., Nagata, N., Sato, Y., Sata, T., Fujita, T., Taya, C., Yonekawa, H. & other authors. **(2005).** The Alpha / Beta Interferon Response Controls Tissue Tropism and Pathogenicity of Poliovirus The Alpha / Beta Interferon Response Controls Tissue Tropism and Pathogenicity of Poliovirus. *J Virol* **79**, 4460–4469.

Imamura, T., Suzuki, A., Meijer, A., Niesters, H. G. M., Lojo, J. C., Jose, R.-L., Hodinka, R. L., Coffin, S., Ostroff, S. M. & other authors. **(2011).** Clusters of acute respiratory illness associated with human enterovirus 68—Asia, Europe, and United States, 2008-2010. *MMWR Morb Mortal Wkly Rep* **60**, 1301–4.

Imamura, T., Suzuki, A., Lupisan, S., Okamoto, M., Aniceto, R., Egos, R. J., Daya, E. E., Tamaki, R., Saito, M. & other authors. **(2013).** Molecular evolution of enterovirus 68 detected in the Philippines. *PLoS One* **8**, e74221.

Imamura, T., Okamoto, M., Nakakita, S., Suzuki, A., Saito, M., Tamaki, R., Lupisan, S., Roy, C. N., Hiramatsu, H. & other authors. **(2014).** Antigenic and receptor binding properties of enterovirus 68. *J Virol* **88**, 2374–2384.

Irani, D. N. (2008). Aseptic Meningitis and Viral Myelitis. *Neurol Clin* **26**, 635–viii.

Ishimaru, Y., Nakano, S., Yamaoka, K. & Takami, S. (1980). Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. *Arch Dis Child* **55**, 583–588.

Ito, M., Yamashita, T., Tsuzuki, H., Takeda, N. & Krnji, S. (2004). Isolation and identification of a novel human parechovirus. *J Gen Virol* **85**, 391–398.

Jiao, X.-Y., Guo, L., Huang, D.-Y., Chang, X.-L. & Qiu, Q.-C. (2014). Distribution of EV71 receptors SCARB2 and PSGL-1 in human tissues. *Virus Res* **190**, 40–52.

Joki-Korpela, P., Roivainen, M., Lankinen, H., Pöyry, T. & Hyypiä, T. (2000). Antigenic properties of human parechovirus 1. *J Gen Virol* **81**, 1709–1718.

Kar, B. R., Dwibedi, B. & Kar, S. K. (2013). An outbreak of hand, foot and mouth disease in Bhubaneswar, Odisha. *Indian Pediatr* **50**, 139–142.

Karrasch, M., Fischer, E., Scholten, M., Sauerbrei, A., Henke, A., Renz, D. M., Mentzel, H.-J., Boer, K., Bottcher, S. & other authors. **(2016).** A severe pediatric infection with a novel enterovirus A71 strain, Thuringia, Germany. *J Clin Virol* **84**, 90–95.

Kennett, M. L., Birch, C. J., Lewis, F. A., Yung, A. P., Locarnini, S. A. & Gust, I. D. (1974). Enterovirus type 71 infection in Melbourne. *Bull World Health Organ* **51**, 609–615.

Khan, F. (2015). Enterovirus D68: Acute Respiratory Illness and the 2014 Outbreak. *Emerg Med Clin North Am* **33**, e19–e32.

Khatami, A., McMullan, B. J., Webber, M., Stewart, P., Francis, S., Timmers, K. J., Rodas, E., Druce, J., Mehta, B. & other authors. **(2015).** Sepsis-like disease in infants due to human parechovirus type 3 during an outbreak in Australia. *Clin Infect Dis* **60**, 228–236.

Khetsuriani, N., LaMonte-Fowlkes, A., M, O. S. & Mark, P. (2006). Enterovirus Surveillance — United States, 1970-2005. *MMWR Surveill Summ* **55**, 1–20.

Kiener, T. K., Jia, Q., Meng, T., Chow, V. T. K. & Kwang, J. (2014). A novel universal neutralizing monoclonal antibody against enterovirus 71 that targets the highly conserved 'knob' region of VP3 protein. *PLoS Negl Trop Dis* **8**, e2895.

Kincaid, O. & Lipton, H. L. (2006). Viral myelitis: an update. *Curr Neurol Neurosci Rep* **6**, 469–474.

Koskiniemi, M., Rantalaiho, T., Piiparinen, H., von Bonsdorff, C. H., Farkkila, M., Jarvinen, A., Kinnunen, E., Koskiniemi, S., Mannonen, L. & other authors. **(2001).** Infections of the central nervous system of suspected viral origin: a collaborative study from Finland. *J Neurovirol* **7**, 400–408.

Kreuter, J. D., Barnes, A., McCarthy, J. E., Schwartzman, J. D., Oberste, M. S., Rhodes, C. H., Modlin, J. F. & Wright, P. F. (2011). A Fatal Central Nervous System Enterovirus 68 Infection. *Arch Pathol Lab Med* **135**, 793–796. College of American Pathologists.

Ku, Z., Liu, Q., Ye, X., Cai, Y., Wang, X., Shi, J., Li, D., Jin, X., An, W. & Huang, Z. (2014). A virus-like particle based bivalent vaccine confers dual protection against enterovirus 71 and coxsackievirus A16 infections in mice. *Vaccine* **32**, 4296–4303.

Kurz, H., Prammer, R., Bock, W., Ollerieth, R., Bernert, G., Zwiauer, K., Aberle, J. H., Aberle, S. W., Fazekas, T. & Holter, W. (2015). Intracranial hemorrhage and other symptoms in infants associated with human parechovirus in Vienna, Austria. *Eur J Pediatr*.

Kwakkenbos, M. J., Bakker, A. Q., van Helden, P. M., Wagner, K., Yasuda, E., Spits, H. & Beaumont, T. (2014). Genetic manipulation of B cells for the isolation of rare therapeutic antibodies from the human repertoire. *Methods* **65**, 38–43.

Lang, M., Mirand, A., Savy, N., Henquell, C., Maridet, S., Perignon, R., Labbe, A. & Peigue-Lafeuille, H. (2014). Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014. *Eurosurveillance* **19**.

Lee, H., Cifuente, J. O., Ashley, R. E., Conway, J. F., Makhov, A. M., Tano, Y., Shimizu, H., Nishimura, Y. & Hafenstein, S. (2013). A Strain-Specific Epitope of Enterovirus 71 Identified by Cryo-Electron Microscopy of the Complex with Fab from Neutralizing Antibody. *J Virol* **87**, 11363–11370. American Society for Microbiology.

Legay, F., Leveque, N., Gacouin, A., Tattevin, P., Bouet, J., Thomas, R. & Chomelt, J.-J. (2007). Fatal coxsackievirus A-16 pneumonitis in adult. *Emerg Infect Dis* **13**, 1084–1086.

Li, H.-Y., Han, J.-F., Qin, C.-F. & Chen, R. (2013). Virus-like particles for enterovirus 71 produced from Saccharomyces cerevisiae potently elicits protective immune responses in mice. *Vaccine* **31**, 3281–3287.

Li, W., Joshi, M. D., Singhania, S., Ramsey, K. H. & Murthy, A. K. (2014a). Peptide Vaccine: Progress and Challenges. *Vaccines* **2**, 515–536.

Li, Y.-X., Zhao, H., Cao, R.-Y., Deng, Y.-Q., Han, J.-F., Zhu, S.-Y., Ma, J., Liu, L., Qin, E.-D. & Qin, C.-F. (2014b). Recombinant tandem multi-linear neutralizing epitopes of human enterovirus 71 elicited protective immunity in mice. *Virol J* **11**, 79.

Liao, C.-C., Liou, A.-T., Chang, Y.-S., Wu, S.-Y., Chang, C.-S., Lee, C.-K., Kung, J. T., Tu, P.-H., Yu, Y.-Y. & other authors. **(2014).** Immunodeficient mouse models with different disease profiles by in vivo infection with the same clinical isolate of enterovirus 71. *J Virol* **88**, 12485–12499.

Lim, X. F., Jia, Q., Khong, W. X., Yan, B., Premanand, B., Alonso, S., Chow, V. T. K. & Kwang, J. (2012). Characterization of an isotype-dependent monoclonal antibody against linear neutralizing epitope effective for prophylaxis of enterovirus 71 infection. *PLoS One* **7**, e29751.

Lin, Y. L., Yu, C. I., Hu, Y. C., Tsai, T. J., Kuo, Y. C., Chi, W. K., Lin, A. N. & Chiang, B. L. (2012). Enterovirus type 71 neutralizing antibodies in the serum of macaque monkeys immunized with EV71 virus-like particles. *Vaccine* **30**, 1305–1312. Elsevier Ltd.

Linden, L., Wolthers, K. & van Kuppeveld, F. (2015). Replication and Inhibitors of Enteroviruses and Parechoviruses. *Viruses* **7**, 4529–4562.

Liu, C.-C., Chou, A.-H., Lien, S.-P., Lin, H.-Y., Liu, S.-J., Chang, J.-Y., Guo, M.-S., Chow, Y.-H., Yang, W.-S. & other authors. **(2011).** Identification and characterization of a cross-neutralization epitope of Enterovirus 71. *Vaccine* **29**, 4362–72.

Liu, C.-C., Chow, Y.-H., Chong, P. & Klein, M. (2014). Prospect and challenges for the development of multivalent vaccines against hand, foot and mouth diseases. *Vaccine* **32**, 6177–6182. Elsevier Ltd.

Liu, J. K. H. (2014). The history of monoclonal antibody development - Progress, remaining challenges and future innovations. *Ann Med Surg* **3**, 113–116. Elsevier Ltd.

Liu, S.-L., Pan, H., Liu, P., Amer, S., Chan, T.-C., Zhan, J., Huo, X., Liu, Y., Teng, Z. & other authors. **(2015a).** Comparative epidemiology and virology of fatal and nonfatal cases of hand, foot and mouth disease in mainland China from 2008 to 2014. *Rev Med Virol* **25**, 115–128.

Liu, Y., Sheng, J., Fokine, A., Meng, G., Shin, W., Long, F., Kuhn, R. J., Kihara, D. & Rossmann, M. G. (2015b). Structure and inhibition of EV-D68, a virus that causes respiratory illness in children. *Science (80-)* **347**.

Lu, Q.-B., Wo, Y., Wang, H.-Y., Wei, M.-T., Zhang, L., Yang, H., Liu, E.-M., Li, T.-Y., Zhao, Z.-T. & other authors. **(2014).** Detection of enterovirus 68 as one of the commonest types of enterovirus found in patients with acute respiratory tract infection in China. *J Med Microbiol* **63**, 408–414.

Mao, Q.-Y., Wang, Y., Bian, L., Xu, M. & Liang, Z. (2016). EV71 vaccine, a new tool to control outbreaks of hand, foot and mouth disease (HFMD). *Expert Rev Vaccines* **15**, 599–606.

Mao, Q., Cheng, T., Zhu, F., Li, J., Wang, Y., Li, Y., Gao, F., Yang, L., Yao, X. & other authors. **(2013).** The Cross-Neutralizing Activity of Enterovirus 71 Subgenotype C4 Vaccines in Healthy Chinese Infants and Children. *PLoS One* **8**, e79599. Public Library of Science.

Mardekian, S. K., Fortuna, D., Nix, A., Bhatti, T., Wiley, C. A., Flanders, A., Urtecho, J., Sloane, J., Ahmad, J. & Curtis, M. T. (2015). Severe Human Parechovirus Type 3 Myocarditis and Encephalitis in an Adolescent with Hypogammaglobulinemia. *Int J Infect Dis*.

McMinn, P., Stratov, I., Nagarajan, L. & Davis, S. (2001). Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. *Clin Infect Dis* **32**, 236–242.

Meijer, A., Benschop, K. S., Donker, G. A. & van der Avoort, H. G. (2014). Continued seasonal circulation of enterovirus D68 in the Netherlands, 2011-2014. *Eurosurveillance* **19**.

Meijer, A., van der Sanden, S., Snijders, B. E. P., Jaramillo-Gutierrez, G., Bont, L., van der Ent, C. K., Overduin, P., Jenny, S. L., Jusic, E. & other authors. **(2012).** Emergence and epidemic occurrence of enterovirus 68 respiratory infections in The Netherlands in 2010. *Virology* **423**, 49–57.

Messacar, K., Schreiner, T. L., Maloney, J. A., Wallace, A., Ludke, J., Oberste, S., Nix, A., Robinson, C. C., Glodé, M. P. & other authors. **(2015).** A cluster

of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. *Lancet* **385**, 1662–1671.

Mistchenko, A. S., Viegas, M., Latta, M. P. Della & Barrero, P. R. (2006). Molecular and epidemiologic analysis of enterovirus B neurological infection in Argentine children. *J Clin Virol* **37**, 293–299.

Mizuta, K., Yamakawa, T., Nagasawa, H., Itagaki, T., Katsushima, F., Katsushima, Y., Shimizu, Y., Ito, S., Aoki, Y. & other authors. **(2013).** Epidemic myalgia associated with human parechovirus type 3 infection among adults occurs during an outbreak among children: findings from Yamagata, Japan, in 2011. *J Clin Virol* **58**, 188–193.

Mizutani, T., Ishizaka, A. & Nihei, C.-I. (2016). Transferrin Receptor 1 Facilitates Poliovirus Permeation of Mouse Brain Capillary Endothelial Cells. *J Biol Chem* **291**, 2829–2836.

Morales, M., Tangermann, R. H. & Wassilak, S. G. F. (2016). Progress Toward Polio Eradication - Worldwide, 2015-2016. *MMWR Morb Mortal Wkly Rep* **65**, 470–473.

Nagy, G., Takátsy, S., Kukán, E., Mihály, I. & Dömök, I. (1982). Virological diagnosis of enterovirus type 71 infections: Experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. *Arch Virol* **71**, 217–227.

Nathanson, N. & Bodian, D. (1962). Experimental poliomyelitis following intramuscular virus injection. III. The effect of passive antibody on paralysis and viremia. *Bull Johns Hopkins Hosp* **111**, 198–220.

Nicolosi, A., Hauser, W. A., Beghi, E. & Kurland, L. T. (1986). Epidemiology of central nervous system infections in Olmsted County, Minnesota, 1950-1981. *J Infect Dis* **154**, 399–408.

Oberste, M. S., Maher, K., Schnurr, D., Flemister, M. R., Lovchik, J. C., Peters, H., Sessions, W., Kirk, C., Chatterjee, N. & other authors. **(2004).** Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. *J Gen Virol* **85**, 2577–2584.

Pfeiffer, H. C., Bragstad, K., Skram, M. K., Dahl, H., Knudsen, P. K., Chawla, M. S., Holberg-Petersen, M., Vainio, K., Dudman, S. G. & other authors. **(2015).** Two cases of acute severe flaccid myelitis associated with enterovirus D68 infection in children, Norway, autumn 2014. *Eurosurveillance* **20**, 21062.

Piralla, A., Mariani, B., Stronati, M., Marone, P. & Baldanti, F. (2014). Human enterovirus and parechovirus infections in newborns with sepsis-like illness and neurological disorders. *Early Hum Dev* **90**, S75–S77. Elsevier Ireland Ltd.

Quartier, P., Foray, S., Casanova, J. L., Hau-Rainsard, I., Blanche, S. & Fischer, A. (2000). Enteroviral meningoencephalitis in X-linked agammaglobulinemia: intensive immunoglobulin therapy and sequential viral detection in cerebrospinal fluid by polymerase chain reaction. *Pediatr Infect Dis J* **19**, 1106–1108.

Ren, J., Wang, X., Zhu, L., Hu, Z., Gao, Q., Yang, P., Li, X., Wang, J., Shen, X. & other authors. **(2015).** Structures of coxsackievirus A16 capsids with native antigenicity, implications for particle expansion, receptor binding and immunogenicity. *J Virol*.

Ren, R. & Racaniello, V. R. (1992). Poliovirus Spreads from Muscle to the Central Nervous System by Neural Pathways. *J Infect Dis* **166**, 747–752.

Rhoades, R. E., Tabor-Godwin, J. M., Tsueng, G. & Feuer, R. (2011). Enterovirus infections of the central nervous system. *Virology* **411**, 288–305. Elsevier Inc.

Robinson, C. R., Doane, F. W. & Rhodes, A. J. (1958). Report of an outbreak of febrile illness with pharyngeal lesions and exanthem: Toronto, summer 1957; isolation of group A Coxsackie virus. *Can Med Assoc J* **79**, 615–621.

Rodriguez, M., Kenny, J. J., Thiemann, R. L. & Woloschak, G. E. (1990). Theiler's virus-induced demyelination in mice immunosuppressed with anti-IgM and in mice expressing the xid gene. *Microb Pathog* **8**, 23–35.

van der Sanden, S., de Bruin, E., Vennema, H., Swanink, C., Koopmans, M. & van der Avoort, H. (2008). Prevalence of Human Parechovirus in The Netherlands in 2000 to 2007. *J Clin Microbiol* **46**, 2884–2889.

van der Sanden, S., van Eek, J., Martin, D. P., van der Avoort, H., Vennema, H. & Koopmans, M. (2011). Detection of recombination breakpoints in the genomes of human enterovirus 71 strains isolated in the Netherlands in epidemic and non-epidemic years, 1963-2010. *Infect Genet Evol* **11**, 886–894.

Schmidt, N. J., Lennette, E. H. & Ho, H. H. (1974). An Apparently New Enterovirus Isolated from Patients with Disease of the Central Nervous System. *J Infect Dis* **129**, 304–309.

Schuffenecker, I., Mirand, A., Antona, D., Henquell, C., Chomel, J.-J., Archimbaud, C., Billaud, G., Peigue-Lafeuille, H., Lina, B. & Bailly, J.-L. (2011). Epidemiology of human enterovirus 71 infections in France, 2000-2009. *J Clin Virol* **50**, 50–6.

Schuffenecker, I., Javouhey, E., Gillet, Y., Kugener, B., Billaud, G., Floret, D., Lina, B. & Morfin, F. (2012). Human parechovirus infections, Lyon, France, 2008-10: evidence for severe cases. *J Clin Virol* **54**, 337–341.

Selvarangan, R., Nzabi, M., Selvaraju, S. B., Ketter, P., Carpenter, C. & Harrison, C. J. (2011). Human parechovirus 3 causing sepsis-like illness in children from midwestern United States. *Pediatr Infect Dis J* **30**, 238–242.

Servais, S., Caers, J., Warling, O., Frusch, N., Baron, F., De Prijck, B. & Beguin, Y. (2010). Enteroviral meningoencephalitis as complication of Rituximab therapy in a patient treated for diffuse large B-cell lymphoma. *Br J Haematol* **150**, 379–381. Blackwell Publishing Ltd.

Shakeel, S., Westerhuis, B. M., Ora, A., Koen, G., Bakker, A. Q., Claassen, Y., Wagner, K., Beaumont, T., Wolthers, K. C. & Butcher, S. J. (2015). Structural Basis of Human Parechovirus Neutralization by Human Monoclonal Antibodies. *J Virol* **89**, 9571–9580.

Shakeel, S., Westerhuis, B. M., Domanska, A., Koning, R. I., Matadeen, R., Koster, A. J., Bakker, A. Q., Beaumont, T., Wolthers, K. C. & Butcher, S. J. (2016). Multiple capsid-stabilizing interactions revealed in a high-resolution structure of an emerging picornavirus causing neonatal sepsis. *Nat Commun* **7**, 11387.

Shi, J., Huang, X., Liu, Q. & Huang, Z. (2013). Identification of conserved neutralizing linear epitopes within the VP1 protein of coxsackievirus A16. *Vaccine* **31**, 2130–2136.

Shindarov, L. M., Chumakov, M. P., Voroshilova, M. K., Bojinov, S., Vasilenko, S. M., Iordanov, I., Kirov, I. D., Kamenov, E., Leshchinskaya, E. V & other authors. **(1979).** Epidemiological, clinical, and pathomorphological characteristics of epidemic poliomyelitis-like disease caused by enterovirus 71. *J Hyg Epidemiol Microbiol Immunol* **23**, 284–295.

Shoji, K., Komuro, H., Miyata, I., Miyairi, I. & Saitoh, A. (2013). Dermatologic manifestations of human parechovirus type 3 infection in neonates and infants. *Pediatr Infect Dis J* **32**, 233–236.

Smura, T., Ylipaasto, P., Klemola, P., Kaijalainen, S., Kyllönen, L., Sordi, V., Piemonti, L. & Roivainen, M. (2010). Cellular tropism of human enterovirus D species serotypes EV-94, EV-70, and EV-68 in vitro: Implications for pathogenesis. *J Med Virol* **82**, 1940–1949. Wiley Subscription Services, Inc., A Wiley Company.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M. J., McMinn, P. & Ooi, M. H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis* **10**, 778–790. Elsevier Ltd.

Strikas, R. A., Anderson, L. J. & Parker, R. A. (1986). Temporal and geographic patterns of isolates of nonpolio enterovirus in the United States, 1970-1983. *J Infect Dis* **153**, 346–351.

Sun, S., Jiang, L., Liang, Z., Mao, Q., Su, W., Zhang, H., Li, X., Jin, J., Xu, L. & other authors. **(2014).** Evaluation of monovalent and bivalent vaccines against lethal Enterovirus 71 and Coxsackievirus A16 infection in newborn mice. *Hum Vaccines Immunother* **10**, 2885–2895.

Tabor-Godwin, J. M., Ruller, C. M., Bagalso, N., An, N., Pagarigan, R. R., Harkins, S., Gilbert, P. E., Kiosses, W. B., Gude, N. A. & other authors. **(2010).** A novel population of myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS. *J Neurosci* **30**, 8676–8691.

Tapia, G., Cinek, O., Witso, E., Kulich, M., Rasmussen, T., Grinde, B. & Ronningen, K. S. (2008). Longitudinal observation of parechovirus in stool samples from Norwegian infants. *J Med Virol* **80**, 1835–1842.

Tapparel, C., Siegrist, F., Petty, T. J. & Kaiser, L. (2013). Picornavirus and enterovirus diversity with associated human diseases. *Infect Genet Evol* **14**, 282–293.

Tu, P. Van, Thao, N. T. T., Perera, D., Huu, T. K., Tien, N. T. K., Thuong, T. C., How, O. M., Cardosa, M. J. & McMinn, P. C. (2007). Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. *Emerg Infect Dis* **13**, 1733–1741.

van de Ven, A. A. J. M., Douma, J. W., Rademaker, C., van Loon, A. M., Wensing, A. M. J., Boelens, J.-J., Sanders, E. A. M. & van Montfrans, J. M. (2011). Pleconaril-resistant chronic parechovirus-associated enteropathy in agammaglobulinaemia. *Antivir Ther* **16**, 611–614.

Verboon-Maciolek, M. A., Krediet, T. G., Gerards, L. J., de Vries, L. S., Groenendaal, F. & van Loon, A. M. (2008). Severe neonatal parechovirus infection and similarity with enterovirus infection. *Pediatr Infect Dis J* **27**, 241–245.

Victoria, J. G., Kapoor, a., Li, L., Blinkova, O., Slikas, B., Wang, C., Naeem, a., Zaidi, S. & Delwart, E. (2009). Metagenomic Analyses of Viruses in Stool Samples from Children with Acute Flaccid Paralysis. *J Virol* **83**, 4642–4651.

Vuorinen, T., Vainionpaa, R., Vanharanta, R. & Hyypia, T. (1996). Susceptibility of human bone marrow cells and hematopoietic cell lines to coxsackievirus B3 infection. *J Virol* **70**, 9018–9023.

Wahid, R., Cannon, M. J. & Chow, M. (2005). Dendritic cells and macrophages are productively infected by poliovirus. *J Virol* **79**, 401–409.

Wang, C.-Y., Li Lu, F., Wu, M.-H., Lee, C.-Y. & Huang, L.-M. (2004). Fatal coxsackievirus A16 infection. *Pediatr Infect Dis J* **23**, 275–276.

Westerhuis, B., Koen, G., Wildenbeest, J. G., Pajkrt, D., de Jong, M. D., Benschop, K. S. M. & Wolthers, K. C. (2012). Specific cell tropism and

neutralization of human parechovirus types 1 and 3: Implications for pathogenesis and therapy development. *J Gen Virol* **93**, 2363–2370.

Westerhuis, B., Kolehmainen, P., Benschop, K., Nurminen, N., Koen, G., Koskiniemi, M., Simell, O., Knip, M., Hyöty, H. & other authors. **(2013).** Human parechovirus seroprevalence in Finland and the Netherlands. *J Clin Virol* **58**, 211–215. Elsevier B.V.

Westerhuis, B., Benschop, K., Koen, G., Claassen, Y. B., Wagner, K., Bakker, A. Q., Wolthers, K. C. & Beaumont, T. (2015). Human Memory B Cells Producing Potent Cross-Neutralizing Antibodies against Human Parechovirus: Implications for Prevalence, Treatment, and Diagnosis. *J Virol* **89**, 7457–7464.

Wildenbeest, J. G., Harvala, H., Pajkrt, D. & Wolthers, K. C. (2010). The need for treatment against human parechoviruses: how, why and when? *Expert Rev Anti Infect Ther* **8**, 1417–1429.

Wildenbeest, J. G., Wolthers, K. C., Straver, B. & Pajkrt, D. (2013). Successful IVIG treatment of human parechovirus-associated dilated cardiomyopathy in an infant. *Pediatrics* **132**, e243-7.

Wiley, C. A., Bhardwaj, N., Ross, T. M. & Bissel, S. J. (2015). Emerging Infections of CNS: Avian Influenza A Virus, Rift Valley Fever Virus and Human Parechovirus. *Brain Pathol* **25**, 634–650.

Wolthers, K. C., Benschop, K. S. M., Schinkel, J., Molenkamp, R., Bergevoet, R. M., Spijkerman, I. J. B., Kraakman, H. C. & Pajkrt, D. (2008). Human parechoviruses as an important viral cause of sepsislike illness and meningitis in young children. *Clin Infect Dis* **47**, 358–363.

Wong, K. T., Munisamy, B., Ong, K. C., Kojima, H., Noriyo, N., Chua, K. B., Ong, B. B. & Nagashima, K. (2008). The Distribution of Inflammation and Virus in Human Enterovirus 71 Encephalomyelitis Suggests Possible Viral Spread by Neural Pathways. *J Neuropathol & Exp Neurol* **67**, 162 LP-169.

Wright, H. T. J., Lanfding, B. H., Lenette, E. H. & McAllister, R. M. (1963). Fatal infection in an infant associated with Coxsackie virus group A, type 16. *N Engl J Med* **268**, 1041–1044.

Xu, G. J., Kula, T., Xu, Q., Li, M. Z., Vernon, S. D., Ndung'u, T., Ruxrungtham, K., Sanchez, J., Brander, C. & other authors. **(2015a).** Comprehensive serological profiling of human populations using a synthetic human virome. *Science (80-)* **348**, aaa0698-aaa0698.

Xu, L., He, D., Yang, L., Li, Z., Ye, X., Yu, H., zhao, H., Li, S., Yuan, L. & other authors. **(2015b).** A Broadly Cross-protective Vaccine Presenting the Neighboring Epitopes within the VP1 GH Loop and VP2 EF Loop of Enterovirus 71. *Sci Rep* **5**, 12973. Nature Publishing Group.

Yamamoto, M., Abe, K., Kuniyori, K., Kunii, E., Ito, F., Kasama, Y., Yoshioka, Y. & Noda, M. (2009). Epidemic of human parechovirus type 3 in Hiroshima city, Japan in 2008. *Jpn J Infect Dis* **62**, 244–245.

Yamamoto, S. P., Kaida, A., Naito, T., Hosaka, T., Miyazato, Y., Sumimoto, S.-I., Kohdera, U., Ono, A., Kubo, H. & Iritani, N. (2015). Human parechovirus infections and child myositis cases associated with genotype 3 in Osaka City, Japan, 2014. *J Med Microbiol*.

Yang, F., Ren, L., Xiong, Z., Li, J., Xiao, Y., Zhao, R., He, Y., Bu, G., Zhou, S. & other authors. **(2009).** Enterovirus 71 outbreak in the People's Republic of China in 2008. *J Clin Microbiol* **47**, 2351–2352.

Yang, W.-X., Terasaki, T., Shiroki, K., Ohka, S., Aoki, J., Tanabe, S., Nomura,

T., Terada, E., Sugiyama, Y. & Nomoto, A. (1997). Efficient Delivery of Circulating Poliovirus to the Central Nervous System Independently of Poliovirus Receptor. *Virology* **229**, 421–428.

Yeung, W.-C. G., Rawlinson, W. D. & Craig, M. E. (2011). Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. *BMJ* **342**, d35.

Zhang, X., Sun, C., Xiao, X., Pang, L., Shen, S., Zhang, J., Cen, S., Yang, B. B., Huang, Y. & other authors. **(2015).** Phage display-derived cross-reactive neutralizing antibody against enterovirus 71 and coxsackievirus A16. *Jpn J Infect Dis*.

Zhang, Y., Zhu, Z., Yang, W., Ren, J., Tan, X., Wang, Y., Mao, N., Xu, S., Zhu, S. & other authors. **(2010a).** An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. *Virol J* **7**, 94.

Zhang, Y., Wang, D., Yan, D., Zhu, S., Liu, J., Wang, H., Zhao, S., Yu, D., Nan, L. & other authors. **(2010b).** Molecular evidence of persistent epidemic and evolution of subgenotype B1 coxsackievirus A16-associated hand, foot, and mouth disease in China. *J Clin Microbiol* **48**, 619–622.

van Zwol, A. L., Lequin, M., Aarts-Tesselaar, C., van der Eijk, A. A., Driessen, G. A., de Hoog, M. & Govaert, P. (2009). Fatal neonatal parechovirus encephalitis. *BMJ Case Rep* **2009**.

FIGURE LEGENDS

Fig. 1. Classification of picornaviruses. The scheme shows picornavirus genera and species that infect humans. Clinically important genotypes are also shown. The genotypes associated with neurological infections are highlighted with heavy font.

Fig. 2. Routes of picornavirus entry to the CNS. (a) Picornaviruses can infect peripheral nerve and invade CNS via retrograde axonal transport. (b) They can and spread further in CNS in a trans-synaptic manner. (c) During viremia picornaviruses can enter the CNS via hematogenous route through a disintegrated blood-brain barrier. (d) They can also cross the blood-brain-barrier in an active manner, possibly relying on cellular transferrin receptor 1. (e) Picornaviruses can infect migrating cells and invade CNS in a "Trojan horse" manner.

Fig. 3. Enterovirus structure and localization of immunogenic epitopes on viral surface. (a) Icosahedral picornavirus capsid, shown for EV71 (PDB ID: 3VBS), consists of 60 identical structural units (asymmetric units). Each asymmetric unit is composed of four viral structural proteins: VP1 (dark green), VP2 (grey), VP3 (light green) and VP4 (attached to the inner surface of the capsid and not seen in the cartoon). (b) Localization of known immunogenic epitopes on EV71 (PDB ID: 3VBS) (upper panel), CVA16 (PDB ID: 5C4W) (middle panel) and predicted epitope-containing loops on EV68 (PDB ID: 4WM8) (lower panel). For simplicity, only one structural unit is mapped on each virus. Structural proteins VP1 (dark green), VP2 (grey) and VP3 (light green) are shown. The epitopes are marked in orange and are pointed with arrows on a zoomed image. Overlapping epitopes are marked in red.

Figure 1

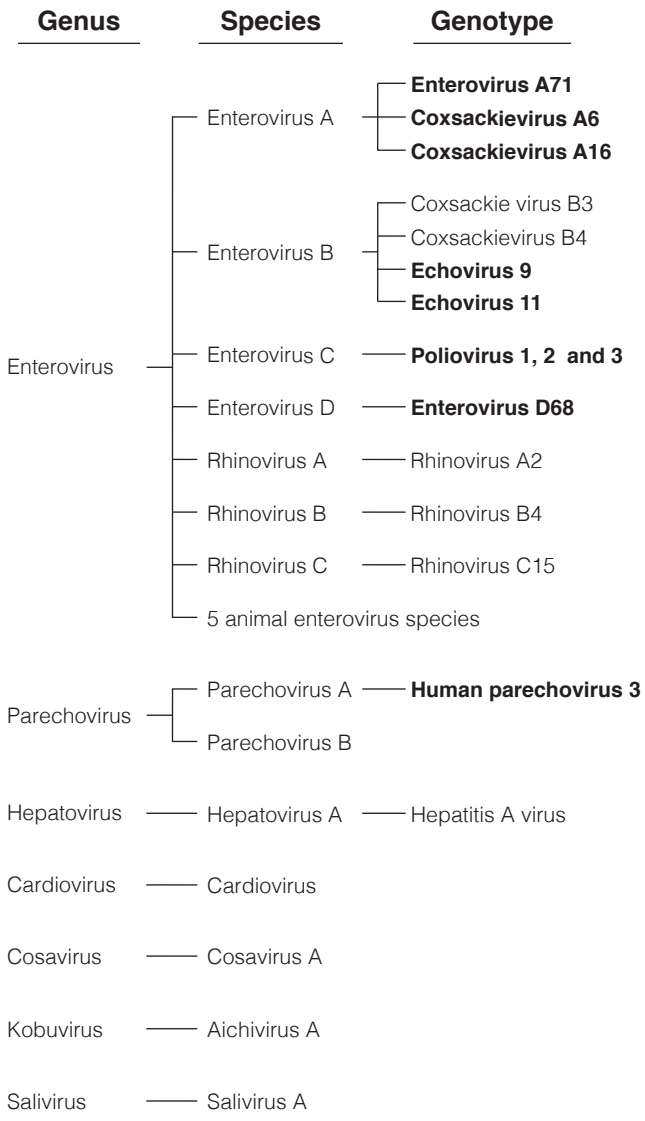

Figure 2

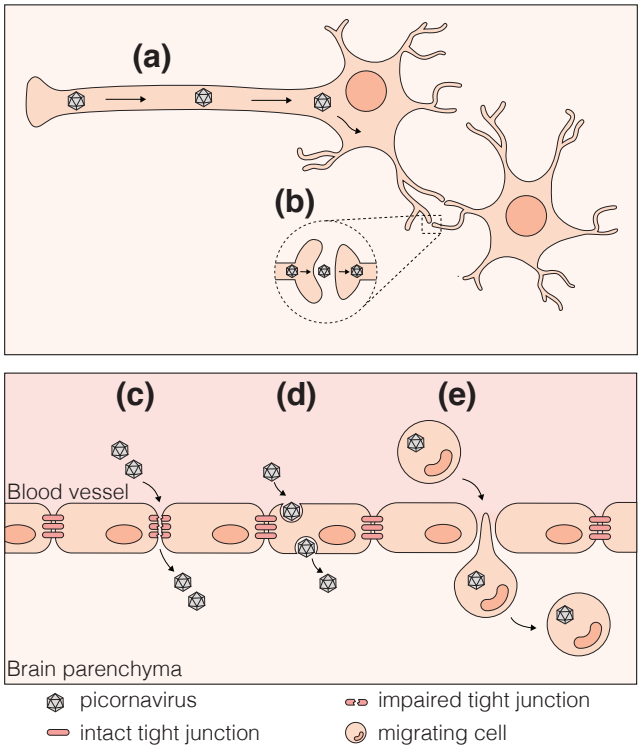

Figure 3