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Human Picornaviruses Associated with Neurological Diseases and Their Neutralization by Antibodies

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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...
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.,
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-Maciele 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 agammaglobulinemia (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 α- 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 demyelination (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 immunoglobulin 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 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
universal for them. In addition, VP1 encompasses a strain-specific discontinuous neutralization epitope at the 5-fold symmetry axis with residue 145 critically contributing to antibody interaction (Lee et al., 2013). Two other neutralizing epitopes were localized to residues 136–150 of VP2 (known as VP2-28) (Liu et al., 2011), and to VP3 residues 55–69 that form a “knob” and are 100% conserved across EV71 subgenogroups A-C4 (Kiener et al., 2014). Much less is known about neutralizing epitopes in CVA16. Most of the experimentally proven neutralizing epitopes of CVA16 were localized to VP1 (GH, EF, C-terminal loops and B and C β-sheets) (Ren et al., 2015; Shi et al., 2013); one more continuous epitope was found in GH loop of VP3 (Chong et al., 2012) (Fig 3 (b)). Additional antigenic sites of CVA16 were predicted in silico in EF and HI loops of VP2 (Ren et al., 2015). Although peptide vaccines are usually less immunogenic compared to the viral particles, this can be mitigated using adjuvants or fusing viral epitopes with highly immunogenic antigens. Using such an approach, a tandem of three well described EV71 epitopes—SP55, SP70 and VP2-28—separated by Gly-Ser linker and fused to thioredoxin was expressed in E. coli. The recombinant protein induced EV71-specific neutralizing responses in immunized mice, serving as a proof-of-concept for peptide vaccines against HFMD (Li et al., 2014b). In another study, a hepatitis B virus-like particle displaying EV71 SP55 and VP2-28 epitopes induced neutralizing responses in immunized mice, and could also be cross-protective against CVA16 (Xu et al., 2015b). In terms of immunogenicity EV71 is one of the best-studied picornaviruses with two EV71 vaccines approved by CFDA and further work will be driven by the necessity of multivalent HFMD vaccines.
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,
which is just being developed for EV68 and not available for HPeV3, hampering investigation of these viruses. Therefore, their antigenicity should be studied directly from human sera using novel methods, such as peptide arrays (Hansen et al., 2013), classical phage display or phage display enhanced with next generation sequencing (NGS) (Christiansen et al., 2015). Successful use of custom phage display library and NGS was reported by Xu et al. who analysed antibody-peptide interactions in sera of over 550 donors and identified numerous previously undescribed viral epitopes, proving the utility of NGS-enhanced phage display for epitope identification (Xu et al., 2015a).

Another future direction is the search for EV68 and HPeV3 neutralizing antibodies. In the absence of antivirals (Linden et al., 2015; Wildenbeest et al., 2010), such antibodies could be valuable therapeutics, especially for HPeV3 that infects infants for whom vaccination is not a suitable option. A useful approach for that is identification of individual’s immune response to a given virus followed by respective B-cell cloning (Kwakkenbos et al., 2014). This approach was utilized to generate two broadly neutralizing antibodies against HPeV (Shakeel et al., 2015; Westerhuis et al., 2015). Unfortunately, these antibodies did not neutralize HPeV3. High throughput approaches, such as sequencing of antibody repertoire (Georgiou et al., 2014) and screening of large antibody fragments libraries using ribosomal, bacterial, yeast or phage display (Bradbury et al., 2011) are also utilized for antibody discovery. For instance, phage display technology has already yielded monoclonal neutralizing antibodies with therapeutic potential for EV71 (Zhang et al., 2015). Over 50 phage-display derived antibodies have been approved by the U.S. Food and Drug Administration (FDA) or European Medicines Agency (EMA) as of May, 2016. 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.
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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.
Genus | Species | Genotype
--- | --- | ---
Enterovirus | Enterovirus A | Enterovirus A71
 |  | Coxsackievirus A6
 |  | Coxsackievirus A16
 | Enterovirus B | Coxsackie virus B3
 |  | Coxsackievirus B4
 |  | Echovirus 9
 |  | Echovirus 11
 | Enterovirus C | Poliovirus 1, 2 and 3
 | Enterovirus D | Enterovirus D68
 | Rhinovirus A | Rhinovirus A2
 | Rhinovirus B | Rhinovirus B4
 | Rhinovirus C | Rhinovirus C15

Parechovirus | Parechovirus A | Human parechovirus 3
 | Parechovirus B

Hepatovirus | Hepatovirus A | Hepatitis A virus

Cardiovirus | Cardiovirus

Cosavirus | Cosavirus A

Kobuvirus | Aichivirus A

Salivirus | Salivirus A
Figure 2

Blood vessel
Brain parenchyma

Picornavirus
Impaired tight junction
Intact tight junction
Migrating cell

(a) (b) (c) (d) (e)