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 infamous picornavirus is poliovirus, for which significant epidemics of poliomyelitis were reported from the end of the nineteenth century. A successful vaccination campaign has brought poliovirus close to eradication, but neurological diseases caused by other picornaviruses have increasingly been reported since the late 1990s. In this review we focus on enterovirus 71, coxsackievirus A16, enterovirus 68 and human parechovirus 3, which have recently drawn attention because of their links to severe neurological diseases. We discuss the clinical relevance of these viruses and the primary role of humoral immunity in controlling them, and summarize current knowledge on the 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 [1]. They infect diverse hosts, from lower vertebrates to mammals. Members of the genera Cardiovirus, Cosavirus, Enterovirus, Hepatovirus, Kobuvirus, Parechovirus and Salivirus infect humans (Fig. 1) [2].

Hepatovirus A, Parechovirus A and multiple enterovirus species can cause symptomatic infections in humans. They typically result in mild disease of the gastrointestinal or respiratory tracts, but are sometimes associated with severe conditions. For instance, coxsackievirus B (CV-B) type 3 has an established role in viral myocarditis [3], and CV-B4 is well-documented as a viral trigger for the onset of type 1 diabetes [4].

Several human picornaviruses can target the central nervous system (CNS) and cause severe neurological diseases. The most well known of these is poliovirus (PV). It caused epidemics of poliomyelitis and was a recognized health care burden from the end of the nineteenth century [5] until the development of vaccines and a worldwide vaccination campaign brought it close to eradication [6]. However, other neurotropic picornaviruses still have the potential to cause outbreaks of neurological diseases, such as severe and life-threatening meningoencephalitis, encephalitis or acute flaccid paralysis (AFP). They are mostly representatives of the genera Enterovirus and Parechovirus [2, 7]. A recent metagenomic study also identified members of the genera Cosavirus, Cardiovirus and Kobuvirus in clinical samples from AFP children [8]; however, whether they are aetiologic agents of CNS infections or innocuous commensals remains to be investigated. The connections between representatives of the genera Enterovirus and Parechovirus and neurological diseases in humans are described below in detail.

PICORNAVIRUS CNS TARGETING

Picornaviruses spread via the faecal–oral or respiratory routes, and the primary sites for their replication are the gastrointestinal or respiratory tracts. Nevertheless, at least some enteroviruses (EVs) and human parechoviruses (HPeVs) can target the CNS [9–14].

For successful infection to occur, the target cell must be both susceptible to and permissive for virus replication [15]. Whereas susceptibility requires the presence of suitable viral receptor(s) on the cell surface, permissiveness relies on intracellular components that allow the viral replication to occur.
Picornaviruses utilize a variety of widely expressed molecules as their entry receptors [16]. Such receptors are often present on the surface of cells within the CNS, making them susceptible to infection. For example, poliovirus receptor (PvR) is expressed in the motor neurons of the spinal cord anterior horns, which are affected during poliomyelitis [17]. Human scavenger receptor class B member 2 (hSCARB2), which is utilized by EV-A71 and coxsackievirus A16 (CV-A16), is expressed on a variety of cells, including neurons and glial cells [18]. Intracellular adhesion molecule 5 (ICAM-5) – a protein receptor for EV-D68 – is expressed in telencephalic grey matter [19]. Neurons are indeed also permissive for picornaviruses [20, 21] and the reduced immune surveillance and weaker IFN responses in nervous tissue make it a plausible replication site for IFN-sensitive picornaviruses [22].

There is molecular evidence suggesting that picornaviruses can invade the CNS by three possible mechanisms: peripheral nerve infection, blood–brain barrier (BBB) 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. 2a, b). The evidence for this came from tissue culture studies and in vivo experimental models for PV and also from EV-A71 patient material [10, 20, 23, 24].

The second mechanism proposes that during viraemia, viruses cross the BBB and infect neural cells. Indeed, high levels of viraemia and inflammation can decrease tight-junction protein expression, disrupt BBB integrity and facilitate viral invasion (Fig. 2c) [25, 26]. Although inflammation-induced BBB breakdown has not been shown directly for picornaviruses, their prolonged viraemia may correlate with severe CNS infections. Cheng et al. observed significantly more severe CNS complications in EV-A71 patients with viraemia detected at days 4–6 compared to...
days 1–3 of illness, suggesting an association between prolonged viraemia and disease severity [27]. However, further investigations are needed to confirm that this observation is indeed due to prolonged viraemia and not its delayed onset. Picornaviruses can also cross the BBB in an active manner: PV can move through the BBB at a rate comparable to that of a BBB-crossing antibody (Fig. 2d) [28]. Such trafficking happens independently of the PvR and appears to rely on transferrin receptor 1 [29].

The third mechanism of neurotropism involves the migration of infected cells, such as dendritic cells, monocytes, macrophages, T- and B-cells, and myeloid nestin+ cells to the CNS, and is called a Trojan-horse invasion (Fig. 2e) [30]. 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 [33]. Infection of neurons with subsequent inflammation of brain parenchyma results in encephalitis, which can have long-term sequelae or be fatal [34]. Inflammation of the spinal cord grey matter results in myelitis and can lead to limb paralysis [33]. All of these conditions can be caused by different picornaviruses and their incidence is highest in children [35].

NEUROTROPIC PICORNAVIRUSES IN FOCUS

Confirmed neurotropic picornaviruses are members of the genera Enterovirus and Parechovirus. The genus Enterovirus includes many recognized pathogens, such as PV, coxsackieviruses A (CV-A), CV-B, rhinoviruses and other EVs, whereas the genus Parechovirus is smaller and includes one human pathogenic species – Parechovirus A. The infections are common: according to the Division of Viral Diseases of the Centers for Disease Control and Prevention (CDC) EVs cause 10–15 million infections and tens of thousands hospitalizations annually in the USA alone (www.cdc.gov/non-polio-enterovirus/about/overview.html, updated on 19 September 2014). Human EV and HPeV species are responsible for about 80% of aseptic meningitis cases [36] and 11% of reported encephalitis cases [37]. Several types of EV can trigger myelitis with limb paralysis [38].

Not all isolates of EVs and HPeVs cause CNS diseases. Enteroviruses associated with CNS infections include (but may not be limited to) PV types 1, 2 and 3, echovirus types 1–7, 9, 11–21, 24, 25, 27, 29, 30–33, CV types A1–A11, A13, A16, A17, A19–A22, A24, B1–B6, and EV types A71, A76, B9, A90, A91, B75, B77, B81, B85, B86, B87, B88, B93, B97, B100, C96, C109, D68, D70 and D94 (reviewed by Tapparel et al. [2]). Parechovirus CNS infections are almost exclusively caused by HPeV-3 [7]. In this review we will discuss EV-A71, CV-A16, EV-D68 and HPeV-3, which have gained attention due to their recent emergence and connection with CNS infections.

Enterovirus 71 and coxsackievirus A16

EV-A71 was initially discovered as a CNS-targeting picornavirus: the first isolates came from two children with neurological symptoms in 1969 in California, USA [39]. In 1973 it was identified as an aetiological 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 have also been observed [40]. In the middle of the 1970s it caused a few small outbreaks of aseptic meningitis in the USA, Europe and Australia [41–44], and two rather large outbreaks of polio-like disease in Bulgaria and Hungary that predominantly affected infants, with up to 21% showing symptoms of paralysis, of which over a quarter were lethal [45–47].

EV-A71 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 (URTIs) and non-specific rashes were occasionally observed. A proportion of EV-A71 infections had neurological and systemic manifestations, but, unlike earlier outbreaks, when aseptic meningitis was the most frequent neurological manifestation, more recent outbreaks were characterized by the increased incidence of much more severe brainstem encephalitis and a high mortality rate [48, 49]. 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 a mortality rate of almost 20% [50]. In total, that outbreak affected about 1.5 million people [51]. In 1999 an outbreak in Western Australia resulted in 6000 cases of HFMD and 29 cases of CNS disease, at least 9 of which were severe, with 4 developing long-term neurological sequelae [52]. EV-A71 outbreaks, most of which had lethal cases, continued in Korea, Singapore, Japan, Malaysia, Vietnam and Thailand [51]. In 2008 there was a large EV-A71 outbreak in China, with almost half a million reported HFMD cases and 122 fatal cases [53]. The virus kept on circulating in China and contributed to HFMD cases with CNS complications and fatalities until at least 2014 [54]. By then, over 7.5 million cases of HFMD had been reported in China alone, of which over 80 000 had neurological involvement, and the Chinese government announced that the development of measures to control EV-A71 was a national priority [55].

EV-A71 infections have been detected in Europe, including in Denmark, France, Spain and the Netherlands, and although the incidence of EV-A71 infections is low, occasional lethal cases have already been reported [56–59]. Interestingly, German researchers recently described a case of paediatic encephalitis caused by a novel EV-A71 genotype that likely arose from a recombination event [60]. Although no epidemic activity of EV-A71 has so far been reported in Europe, the European Centre for Disease Prevention and Control (ECDC) risk assessment reported increased detections of EV-A71 in the first half of 2016 compared to previous years, necessitating preparedness to control its spread in Europe [61].

Importantly, EV-A71 often alternates or co-circulates with other enterovirus A genotypes, mostly CV-A16, which is
another recognized HFMD agent. CV-A16 also predominantly infects children but, unlike EV-A71, typically causes milder symptoms and has a much lower morbidity and mortality rate. In a comparative study of 177 EV-A71 and 64 CV-A16 patients in Taiwan, only 6.3% of CV-A16 infections developed into aseptic meningitis, but 32% of EVA-71 cases resulted in aseptic meningitis, encephalitis, polio-like syndrome, encephalomyelitis and fatal pulmonary oedema; 7.9% of EV-A71 infections were lethal [62]. Occasional severe and fatal CV-A16 cases have been reported in the USA [63], Taiwan [64], France [65], Japan [66] and China [67]. Outbreaks of CV-A16 were documented in Canada (1957) [68], Australia (1991) [69], England and Wales (1994) [70], and India (2009) [71], and were linked to HFMD. It co-circulated with EV-A71 in 1998 during the unprecedented HFMD epidemic in Taiwan [50] and continued circulating in Taiwan, becoming dominant in 2002 and 2003 [72]. The co-circulation of CV-A16 and EV-A71 was documented during HFMD outbreaks in Vietnam in 2005 [73] and China in 2008 onwards [74]. In Singapore CV-A16 was the major cause of HFMD epidemics in 2002, 2005 and 2007, whereas in 2006 it was EV-A71 [75].

Co-circulation of EV-A71 and CV-A16 allows viral co-infections [76] and recombination, which happened during the HFMD outbreak in China [77]. Recombination events between different genotypes can yield new virus variants that can further expand and emerge globally [78], contributing to EV-A71 and CV-A16 spread. Thus, both EV-A71 and CV-A16 require attention as clinically important pathogens.

**Enterovirus 68**

EV-68 belongs to the D species of the genus *Enterovirus*. Unlike enteric viruses, it replicates at lower temperature (33 °C), is sensitive to acidic conditions and causes respiratory infections [79]. It was first isolated in 1962, but only 26 cases were reported until 2005, receiving little attention from the clinical and scientific communities [80]. This changed in 2008–2010, when several outbreaks of acute respiratory illness caused by EV-D68 were reported by the Centers for Disease Control and Prevention (CDC) in the Philippines, Japan, the Netherlands and the USA [81]. The affected individuals typically presented with URTI symptoms – cough, fever, rhinorrhea, difficulties in breathing and hypoxia – although severe lower-respiratory-tract infections (LRTIs) were also detected [80]. Individuals infected with EV-D68 often required hospitalization: for example, a prospective study in the Netherlands reported that out of 24 EV-D68-positive subjects, 23 were hospitalized [81]. Deaths were reported in the Philippines and in Japan, but not in the USA and the Netherlands [81]. In all studies, EV-D68 was reported as a paediatric pathogen, with the exception of the work by Meijer et al., which reported a significant number of patients over 50 years old [82].

Following the initial outbreaks, EV-D68 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 [83–86]. Further concern was raised after the EV-D68 outbreak in the USA in 2014 [87], when over 1000 patients across the country were diagnosed with acute respiratory illness (ARI) caused by EV-D68. 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 the intensive care unit (ICU) and 15 cases were fatal. Simultaneously, an increased incidence of EV-D68 ARI was reported in Canada, where over 200 cases were identified, resulting in 140 hospitalizations and one death [87]. In 2016 EV-D68 was detected in patients with neurological manifestations in the Netherlands, France, the UK, Italy, Portugal and Germany [61].

Intriguingly, the 2014 EV-D68 outbreak in the USA overlapped with an outbreak of AFP with brain stem and spinal cord grey matter lesions. Infection with EV-D68 was confirmed in 5 out of 11 (45%) of the AFP patients [88]. Furthermore, EV-D68 has also been detected in four cases of AFP in Canada, two in Norway and one in France [87, 89, 90]. A recent retrospective study identified EV-D68 in respiratory secretions from 12 of 25 (48%) patients with sporadic paralysis, strengthening the EV-D68 link to CNS disease [91]. Interestingly, all the EV-D68 strains identified in association with paralytic disease formed a distinct genetic cluster, suggesting the emergence of a novel clade and ongoing evolution of this virus [92]. Direct linkage of EV-D68 to neurological disease has been complicated by difficulties in detecting the virus or its RNA in patients’ cerebrospinal fluid (CSF), with only two successful studies so far [13, 80]. However, we should note that other neurological picornaviruses – PV and EV-A71 – are also rarely recovered from CSF, and therefore neurological involvement of EV-D68 cannot be ruled out on the basis of negative CSF samples.

The apparent incidence of EV-D68 has increased over the last decade. Although this observation could be related to significant improvements in the detection techniques, the accumulating clinical data suggest that EV-D68 should be considered to be an emerging pathogen. The concerns raised by respiratory EV-D68 infections, and especially by their possible link to AFP, necessitate careful surveillance of the virus spread, detailed studies on its pathogenesis and evolution, and the development of preventive and/or treatment options.

**Human parechovirus 3**

HPeV-3 belongs to species A within genus *Parechovirus* and is the second most commonly detected human parechovirus after HPeV-1 [93]. HPeV-3 was first isolated in 1999 from an infant with severe CNS disease [94], and since then different studies have recognized it as the most or second most prevalent virus causing CNS infections in infants under three months old [7, 95–98]. Outbreaks of HPeV-3 usually occur in the summer/autumn seasons and have a distinct biennial pattern [96, 98]. They have been documented in Europe [99], North America [100], Asia [101] and Australia
[102], and are regularly associated with a variety of clinical presentations, from mild gastrointestinal or respiratory illness to life-threatening conditions in neonates [36, 96, 103]. It can cause systemic infections with possible neurological involvement in infants, which are collectively described as ‘sepsis-like illnesses’ [98, 99, 104]. Such illnesses typically present with fever, seizures, irritability, respiratory and gastrointestinal problems, and occasional rash, and are indistinguishable from severe EV infections [34, 105]. The fraction of symptomatic HPeV-3-infected infants that develop sepsis-like illness can exceed 80%; most such patients require hospitalization and up to one-third of them are admitted to the ICU [104–106]. The CNS symptoms of HPeV-3 infection can include meningitis, meningoencephalitis, encephalitis or cerebral haemorrhage, with occasional white-matter alterations [107, 108]. Whereas HPeV-3 meningitis typically has a good prognosis, meningoencephalitis entailing white-matter alterations may have long-term sequelae, such as cerebral palsy, learning disabilities, epilepsy or visual impairment [34]. In addition, HPeV-3 is occasionally associated with haemophagocytic lymphohistiocytosis [109] and sudden death syndrome in infants [106]. Furthermore, in Japan it was linked to myositis in children and epidemic myalgia in adults [110, 111], HPeV-3 encephalitis in the absence of an immune response can also be fatal [112, 113].

Overall, HPeV-3 represents a significant threat to neonatal health care. The incidence of HPeV-3 infections and the increasing number of CNS disease cases [96] with no treatment options available necessitate a search for antivirals and better understanding of HPeV neutralization by antibodies [114].

**NEUTRALIZING ANTIBODIES IN PICORNAVIRUS INFECTIONS**

As with other viruses, the severity and outcome of picornavirus infections depends on both viral and host factors. The 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, which establish a complex signalling network, triggering the expression of antiviral genes in infected cells and the activation of specific adaptive responses [115]. The adaptive responses rely on 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 B-cells is of the greatest importance for the control of picornaviral infections.

Immunocompromised individuals, 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 EV infections [116]. A study of 201 such patients in the USA identified EVs as the most common cause of meningitis and encephalitis in them [117]. Moreover, patients with a- or hypogammaglobulinemia can develop chronic infection with HPeV-1, which is very uncommon in healthy individuals [118]. They can also develop HPeV-3 myocarditis and encephalitis, which are usually limited to neonates [119]. Supporting these observations, individuals undergoing immunosuppressive therapy, for instance, cancer treatment with rituximab, that leads to prolonged B-cell deficiency and hypogammaglobulinemia are susceptible to severe and even lethal enterovirus infections [120].

Controlled infections in mice directly confirm the role of antibodies in picornavirus infections. Experiments on mice with different immunodeficiencies showed that whereas up to 70% of mice deficient in innate immune responses survived EV-A71 infection, mice with severe combined immunodeficiency developed limb paralysis and died in almost 100% of cases [121]. In mice infected with a neurotropic coronavirus – Theiler’s encephalomyelitis virus – immune-suppression with anti-IgM antibodies led to virus-induced demyelination [122].

At the moment, there are no antivirals for treatment of severe picornavirus infections [114, 123] and the only therapeutic option is intravenous immunoglobulin (IVIG). However, because of the BBB’s low permeability to antibodies, IVIG is rarely effective in CNS infections, although intraventricular immunoglobulin administration may be beneficial [124]. Antibodies can also be effective at mucosal sites and prevent picornavirus viraemia and CNS invasion [125]. Successful management of severe picornavirus infections using intravenous immunoglobulins (IVIGs) [126] indicates the potential efficacy of passive immunization. However, the presence of specific neutralizing antibodies and their titres cannot be controlled in IVIG preparations, and the reliable option 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, which are beneficial in time-critical point-of-care setups. In addition to passive immunization, the success of the polio vaccine is encouraging for the development of vaccines against other picornaviruses. Controlling picornavirus infections with vaccines is not a feasible approach for the entire family Picornaviridae, but could be realistic for some viruses. Both vaccine and antibody development require thorough understanding of viral neutralization, and below we summarize current knowledge regarding the neutralization of CNS-invading picornaviruses.

**NEUTRALIZATION OF PICORNAVIRUSES**

**Neutralization of EV-A71 and CV-A16**

Since its initial isolation, EV-A71 has undergone extensive evolution and now 14 different genotypes have been identified based on viral protein 1 (VP1) coding sequences. They are referred to as A, B1–B5, C1–C5, D, E and F [127, 128].
C4 is further classified into two lineages, C4a and C4b [77]. Genotypes B3, B4, B5, C1, C2, C4 and C5 contributed to recent outbreaks [129]. Interestingly, cross-neutralization of different genotypes by immune sera from animals and humans is limited, indicating that EV-A71 became antigenically diverse [130–132]. Furthermore, using monoclonal neutralizing antibodies, Chen et al. even observed antigenic variation between isolates classified to the same genotype [133]. Thus, whereas some neutralizing epitopes on EV-A71 are conserved and confer cross-neutralization within and between genotypes, others can be variable between or even within genotypes. Since EV-A71 genotyping is based merely on VP1 coding sequences, it does not necessarily reflect viral antigenicity, as neutralizing epitopes can also be present on other viral proteins. This discrepancy should be taken into consideration when choosing vaccine strains and designing neutralizing antibodies. CV-A16 is less genetically diverse, showing a relatively slow evolution rate, and has three genotypes: A, B1 (B1a, B1b, B1c) and B2 [134]. EV-A71 can undergo intra- and intergenotype shifts that occur due to recombination events during co-circulation with different EV-A71 or CV-A16 genotypes [135]. The resultant new viruses can cause outbreaks, as happened in China in 2008 when a large HFMD outbreak was caused by a recombinant EV-A71 [77]. An effective vaccine should neutralize multiple genotypes of EV-A71 and also CV-A16. This necessity underpins difficulties in HFMD vaccine development.

The first live-attenuated EV-A71 vaccine strain was reported by Arita et al. in 2007. It induced broadly neutralizing responses in immunized monkeys, but was neurotropic when inoculated intravenously and its further development was halted [136]. 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 EV-A71-induced HFMD in humans [137]. 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 [138]. Importantly, EV-A71 infection also produces empty particles that are antigenically similar to infectious virions [139], giving grounds to develop virus-like particle (VLP) vaccine candidates. Such vaccine candidates were generated in baculovirus or Saccharomyces cerevisiae systems by co-expression of viral protein precursor P1 with viral protease 3 CD [140, 141]. The structure of baculovirus-expressed EV-A71 VLPs was studied to near-atomic detail. Its conformation could be localized on the VLP, indicating that it retains the antigenic properties of the virus [142]. Thus, recombinant VLP vaccines are expected to induce functional neutralizing responses against the virus. Indeed, the VLP vaccine candidate produced in the baculovirus system showed promise in in vivo studies: 89 % of immunized mouse pups survived a challenge with 1000 median lethal doses (LD$_{50}$) of EV-A71. By comparison, in the same experiment the survival of pups immunized with inactivated EV-A71 was 58 % [140]. The same vaccine candidate also induced protective responses in monkeys [143], although whether it could also protect humans is not yet known.

In terms of protection against different EV-A71 genotypes, the approved C4-based inactivated vaccines cross-protected humans against A, B3–B5 and C1–C5 (CAMS vaccine) [144], and B4, B5, C2, C4 and C5 (Sinovac vaccine; cross-neutralization of other genotypes was not tested) [145], while the VLP C2-based vaccine protected monkeys against B4, B5, C3, C4 and C5 (other genotypes were not tested) [143].

Multivalent vaccines may offer protection against EV-A71 and co-circulating CV-A. Bivalent EV-A71/CV-A16 vaccines based on inactivated viruses or VLPs can elicit high titres of neutralizing antibodies in immunized mice and protect from EV-A71 and CV-A16 infections [146]. Yet broader protection is desired to mitigate other HFMD contributors, such as coxsackievirus A6 (CV-A6) [147], and one trivalent EV-A71/CV-A16/CV-A6 inactivated vaccine candidate protecting mice from lethal challenge with these viruses was reported [148].

Several heterologous viruses can be targeted using peptide vaccines or chimeric VLPs presenting well-defined neutralizing epitopes from several viruses. Neutralizing epitopes on EV-A71 are localized on the viral structural proteins VP1, VP2 and VP3 (Fig. 3a, 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 [149–151]. SP55 has 85–100 % sequence identity within genotypes A, B1–B5 and C1–C4 and SP70 is 100 % conserved across these genotypes and thus is universal for them [150]. In addition, VP1 encompasses a strain-specific discontinuous neutralization epitope at the five fold symmetry axis with residue 145 contributing critically to antibody interaction [152]. Two other neutralizing epitopes were localized to VP2 residues 136–150 (known as VP2-28) [153] and VP3 residues 55–69, which form a ‘knob’ and are 100 % conserved across EV-A71 genotypes A, B1–B5 and C1–C4 [154]. One more epitope was localized to the I $\beta$-strand of VP1, two others were localized to the N-terminal loop of VP3 [155] and another one was localized within the N-terminal 20 amino acids of VP4 [156]. Mice immunized with corresponding peptides or with the epitope exposed on chimeric VLPs developed neutralizing responses to EV-A71. However, these four epitopes are not exposed on the virion surface [157] and whether they could elicit neutralizing responses during infection is unclear. Much less is known about neutralizing epitopes in CV-A16. Most of the experimentally proven neutralizing epitopes of CV-A16 were localized to VP1 (GH, EF, C-terminal loops, B and C $\beta$-sheets) [158, 159], while two more continuous epitopes were found in the GH loop of VP3 [160] (Fig. 3b). Zhang et al. reported an epitope within the N-terminal 20 amino acids of VP4, which appeared to be shared with EV-A71 [161]. Additional
antigenic sites of CV-A16 were predicted in silico in the EF and HI loops of VP2 [158].

A peptide vaccine candidate was made using a tandem of three well-described EV-A71 epitopes – SP55, SP70 and VP2-28 – separated by Gly-Ser linkers, fused to thioredoxin and expressed in E. coli. The recombinant protein induced EV-A71-specific neutralizing responses in immunized mice and served as a proof-of-concept for HFMD peptide vaccines; however, whether it could also be protective in humans was not studied [162]. Peptide vaccines are easier to produce compared to inactivated virus vaccines, do not require live virus to be handled and allow immunization with a lower protein load [163]. Adjuvants or fusion with highly immunogenic epitopes can improve their immunogenicity, although they are usually still less effective compared to the viral particles. Chimeric VLPs expressing heterologous neutralizing epitopes could be a more effective yet safe approach for multivalent vaccine design. No significant impact on capsid structure and function is observed when heterologous peptides are inserted in the VP1 BC loop on EV-A71 VLP, indicating that this is a plausible insertion site [164]. Another suitable site could be the VP1 GH loop: a chimeric EV-A71 VLP expressing CV-A16 VP1 GH loop epitope induced robust immune responses against both viruses in mice [165]. It is important to study whether it could elicit protective responses in humans. Overall, in terms of immunogenicity, EV-A71 is one of the best-studied picornaviruses, with two EV-A71 vaccines approved by CFDA, and further work will be driven by the necessity for multivalent HFMD vaccines.

Neutralization of enterovirus 68

EV-D68 is an emerging virus and little is known about its antigenicity. Imamura et al. studied the immunogenic properties of 12 EV-D68 types belonging to all three lineages of EV-D68 – A, B and C [85]. Immunized guinea pigs generated high titres of neutralizing antibodies for the original virus and viruses of the same lineage, but very little cross-neutralization between genetic lineages was found [166]. Importantly, the majority of sequence variation between EV-D68 lineages is localized to the VP1 BC and DE surface loops [55, 85, 166], which are the most variable and are probably 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 EV-D68 detections [167], 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. 3b) are undergoing positive selection and might be associated with antigenic differences between EV-D68 genetic lineages [166]. Nevertheless, to date the exact immunogenic epitopes of EV-D68 are only predictive and have not been mapped.

Not much is known about the distribution of EV-D68 neutralizing antibodies in the human population. One study undertaken in Finland addressed this question, reporting high titres of neutralizing responses to EV-D68 in 80% of the studied individuals [168]. However, neutralization responses in this study were addressed against the prototype EV-D68 Fermon strain, which is antigenically very different from the currently circulating EV-D68 strains [91, 167]. Therefore, neither EV-D68 antigenicity, nor the population protection levels are understood well at the moment.

Neutralization of HPeV-3

Almost nothing is known about HPeV-3 antigenicity. For the parechoviruses, only the antigenicity of HPeV-1 has been studied [169]. There are three HPeV structural...
proteins: VP0, VP1 and VP3. In parechoviruses VP0 is not processed to VP2 and VP4 [170, 171]. Three neutralizing epitopes are known on HPeV-1. One is localized to residues 83–97 of VP0 [172], another is found on VP1 and encompasses the receptor-recognizing arginine-glycine-aspartic acid (RGD) motif [173], and the third one is formed by VP0 and VP3 [169, 174]. Whereas antisera generated against the HPeV-1 VP0 peptide were not tested for HPeV-3 neutralization, two other monoclonal antibodies did not cross-react with HPeV-3 [169, 174]. Only a non-neutralizing epitope has been described for HPeV-3 [170].

The data on HPeV-3 seroprevalence in the population are also sparse. A study of sera from different populations in Finland and the Netherlands only revealed neutralizing responses to HPeV-3 in about 10% of the samples and very low titres of neutralizing antibodies post-infection [175]. By contrast, researchers in Japan detected neutralizing responses to HPeV-3 in 67% of individuals between 7 months and 40 years old [94], and reported high titres of neutralizing antibodies after 3 months post-infection in all studied individuals [176]. The virus strains used in the European and Japanese studies were different, suggesting that there is antigenic variation in HPeV-3. 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 HPeV-3.

The treatment option for human parechovirus infections could be IVIG [126], but titres of neutralizing antibodies against HPeV-3 in European IVIG preparations are very low [177]. Developing vaccines does not seem feasible because the subjects of severe HPeV-3 infections are infants. Passive immunization protects against HPeV-3 [176] and thus the development of therapeutic antibodies is a necessity for which studies of HPeV-3 antigenicity are required.

CONCLUSIONS AND FUTURE PERSPECTIVES

We understand the antigenicity of the picornaviruses discussed above poorly and our knowledge is largely limited to studies of EV-A71. Although studies of EV-A71 have already resulted in two CFDA-approved HFMD vaccines, multivalent HFMD vaccines to control different EV-A71 genotypes and also co-circulating CV-A16 are the next goal. The proof-of-concept studies for such vaccines are promising [148, 178], but further work is needed to identify the optimal combination of antigens for balanced, broadly protective immunity. Targeting multiple viruses with a single vaccine also requires delivery systems for the effective presentation of multiple epitopes. In this regard, VLP and peptide vaccines may be preferable to inactivated ones, offering tailored solutions in terms of presented antigens, together with comparable immunogenicity, high safety, less tedious production and economic feasibility [163].

We know almost nothing about EV-D68 and HPeV-3 antigenicity. The classical approach to studying virus antigenicity and developing vaccines relies on animal models, which are just being developed for EV-D68 and are not available for HPeV-3, hampering investigation of these viruses. Therefore, their antigenicity should be studied directly from human sera using novel methods, such as peptide arrays [179] or high-throughput phage display enhanced with next-generation sequencing (NGS), e.g. mimotope variation analysis (MVA) (patent priority number US 14/079,626, approved by USPTO on 1 February 2017 [180]). Successful use of a custom phage display library and NGS-enhanced phage display was reported by Xu et al., who analysed antibody–peptide interactions in the sera of 569 individuals and identified numerous previously undescibed viral epitopes, proving the utility of such an approach for epitope identification [181].

Another future direction is the search for EV-D68 and HPeV-3 therapeutic antibodies. In the absence of antivirals [114, 123], such antibodies could be valuable therapeutics, especially for HPeV-3, which infects infants for whom vaccination is not a suitable option. A useful approach for this is identification of an individual’s immune response to a given virus followed by respective B-cell cloning [182]. This approach was utilized to generate two broadly neutralizing antibodies against HPeV [169, 174]. Unfortunately, these antibodies did not neutralize HPeV-3 in a conventional microneutralization test. It is worth noting that antibodies that do not neutralize the virus in vitro may still be protective in the context of an organism, where they can mediate virus opsonization and clearance [183, 184]. On the other hand, non-neutralizing antibodies may also be devastating due to antibody-dependent enhancement of infection [185, 186] and such a possibility should be excluded using experiments in vivo. Overall, antibodies that neutralize the virus in vitro are more desirable for further development as therapeutics.

High-throughput approaches, such as sequencing of the antibody repertoire [187] and screening of antibody fragment libraries using ribosomal, bacterial, yeast or phage display [188], are also utilized for antibody discovery. For instance, phage display has been utilized successfully to search for therapeutic antibodies. As of May 2016 it yielded over 50 therapeutic antibodies approved by the USA Food and Drug Administration or European Medicines Agency for different indications, while over 500 are currently in clinical trials. With regard to picornavirus therapy, phage display technology has yielded an anti-EV-A71 VP4 monoclonal antibody that cross-neutralized at least the A and C4 types of EV-A71, as well as CV-A16 in cell culture [161]. Further in vivo studies on the therapeutic potential of this antibody are needed.

Overall, picornavirus antigenicity and neutralization studies have already brought encouraging results; however, more challenges lie ahead. Obtaining a 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, the USA 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 is necessary for effective virus control. In addition, unlike PV and EV-A71, which affected millions of people and killed tens of thousands, other human picornaviruses have not been recognized as causing large epidemics and at the moment the market the therapies against them seems relatively small. This may limit public- and private-sector interest towards these viruses and delay the development of therapeutic options against them. Hence, drawing public attention to the health-care threats that picornaviruses offer is another important area of activity for the medical and scientific communities.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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