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REVIEW

From Monovalent to Multivalent Vaccines, the Exploration for Potential Preventive Strategies Against Hand, Foot, and Mouth Disease (HFMD)

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Abstract
Hand, foot, and mouth disease (HFMD) recently emerged as a global public threat. The licensure of inactivated enterovirus A71 (EV-A71) vaccine was the first step in using a vaccine to control HFMD. New challenges arise from changes in the pathogen spectrum while vaccines directed against other common serotypes are in the preclinical stage. The mission of a broad-spectrum prevention strategy clearly favors multivalent vaccines. The development of multivalent vaccines was attempted via the simple combination of potent monovalent vaccines or the construction of chimeric vaccines comprised of epitopes derived from different virus serotypes. The present review summarizes recent advances in HFMD vaccine development and discusses the next steps toward a safe and effective HFMD vaccine that is capable of establishing a cross-protective antibody response.

Keywords Hand, foot, and mouth disease (HFMD) · Inactivated whole virus vaccine · Virus-like particles · Multivalent vaccines · Chimeric vaccines

Introduction

Human hand-foot-and-mouth disease (HFMD) caused several large outbreaks across the Asian-Pacific region, and it represents a global public health issue. Several viruses were identified as the primary HFMD-related pathogens, and this list includes enterovirus A71 (EV-A71), coxsackievirus A16 (CV-A16), CV-A6 and CV-A10, which all belong to the genus *Enterovirus* within the *Picornaviridae* family (Fang and Liu 2018). HFMD frequently occurs in children under five years old, and it is generally characterized by vesicular exanthema with self-limitation. There appears to be a link between the range of clinical manifestations and serotype differences, with some EV-A71 infections resulting in severe complications, including brainstem encephalitis, aseptic meningitis, acute flaccid paralysis, cardiopulmonary failure, or death, but other serotypes generally showing mild symptoms (Lin *et al.* 2019). Historically, EV-A71 and CV-A16 primarily accounted for the global HFMD outbreaks; however, other serotypes are gradually gaining dominance due to the broad inoculation of and protection by inactivated EV-A71 vaccines. Indeed, CV-A6 displaced EV-A71 and CV-A16 as the predominant serotype in 2013 in Shanghai, and CV-A10 has gradually become the dominating HFMD-related enterovirus (Song *et al.* 2017; Wang J *et al.* 2018; Bian *et al.* 2019).

Enteroviruses are positive-stranded RNA viruses with a genome size of approximately 7.4 kb, which encodes a single polyprotein of ~ 2100 amino acids. The polyprotein is divided into three subregions, namely, P1, P2, and P3. The P1 region encodes four structural proteins (VP4–VP2–VP3–VP1), and the P2 and P3 regions encode seven nonstructural proteins (P2–2A, 2B, 2C; P3–3A, 3B, 3C, 3D) (Fig. 1). The four structural proteins assemble to form the basic building block of the virion capsid, namely, a

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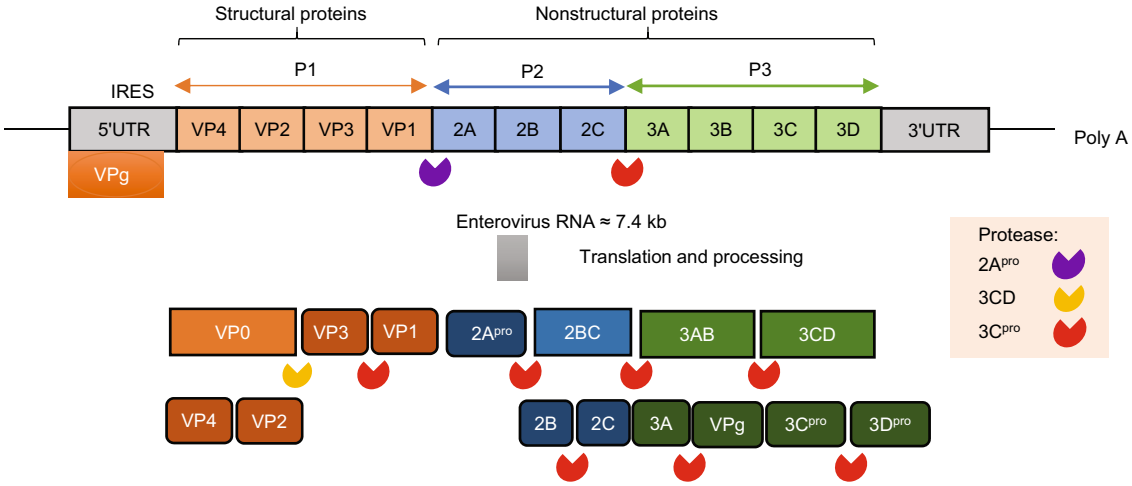

Fig. 1 The structure of enterovirus 71 genome and virion organization. The RNA genome of EV-A71 is approximately 7.4 kb, with an untranslated region (UTR) at the 5′ and 3′ ends of the genome. The 5′UTR contains an internal ribosomal entry site (IRES) for cap-independent translation. The 5′UTR is bound covalently to VPg (3B), and the 3′UTR includes a poly-A tail. The RNA is translated to a polyprotein that is sequentially cleaved by the viral 2A protease (2Apro), 3CD protease, and 3C protease (3Cpro).

protomer. Five protomers come together to form a pentamer, and 12 pentamers plus the viral genome form an icosahedral virion of ∼ 30 nm diameter (Yi *et al.* 2017).

Host Immune Responses to Natural Infection of HFMD-Related Viruses

Humoral immune responses against HFMD-related viruses produce virus-specific neutralizing antibodies, which are generally sufficient to curb virus spreading and makes HFMD a self-limiting disease. However, there were reported cases in infants and young children where severe complications developed despite normal or nearly normal antibody titers compared to patients with mild HFMD, which indicates that other factors contribute to the disease severity (Lim and Poh 2019). The following factors may influence severity: (1) Variation in the IgG composition. The different IgG subclasses that elicited by the viral infection behave differently in virus control. For instance, the IgG1 subclass, and to a lesser extent the IgG2 subclass, primarily mediate the virus-neutralizing activity, while the IgG3 subclass does not (Cao *et al.* 2013). (2) Variation in cellular immunity. The circulating virus-specific $CD8^+$ T cells and $CD4^+$ T cells must be effectively engaged for the timely clearance of virus-infected cells and helping the antibody production (Aw-Yong *et al.* 2019). (3) Genetic variations. There may be intrinsic differences between individuals in countering a viral invasion due to inherited variations in host factors that determine viral susceptibility at the cellular and organismal levels (Yee and Poh 2018). Among the three abovementioned factors, the cellular immune response is the most feasible target for vaccine-based prevention.

Critical Epitopes Recognized by Neutralizing Antibodies

As components of the virion capsid, VP1, VP2 and VP3 are the main targets of human neutralizing antibodies. VP1 contributes to the majority of neutralizing epitopes, and its binding may be used as a valuable assay to assess vaccine potency. VP2 and VP3 proteins harbor fewer neutralizing epitopes compared to VP1, despite structural similarity. Among the common HFMD-related enteroviruses, EV-A71 and CV-A16 show high conservation in capsid proteins, with approximately 80% sequence identity, and their neutralizing epitopes are largely overlapped (Anasir and Poh 2019).

The neutralizing epitopes are classified into linear epitopes and conformational epitopes. Most of the linear epitopes are located in the B–C, E–F, and G–H loops, and the C-terminus of VP1, the E–F loops of VP2, and N-terminal regions of VP3. CV-A16 also has linear epitopes in the canyon floor of VP1 and the G-H loop of VP3 (Xu *et al.* 2015; Fang and Liu 2018). Linear neutralizing antigenic sites were also reported in the E–F loop of CV-A10 VP2, in the G-H loop as well as the C-terminus of CV-A6 VP1 (Chen *et al.* 2018; Dai *et al.* 2019).The identification of conserved neutralizing linear epitopes provides important targets for the development of multivalent vaccines

(Xu *et al.* 2015). For example, MAB979 is an antibody raised by EV-A71 immunization, and it recognized residues 136–150 of VP2 on extensive synthetic peptide screening. It is cross-reactive with CV-A16, but with low neutralizing titers, which indicates a potential epitope for multivalent targeting (Liu C *et al.* 2011).

The conformational epitopes, which are comprised of amino acids that are discontinuous in sequence but are brought to proximity with three-dimensional protein folding, are more difficult to define compared to linear epitopes, and its ultimate validation may require structural analyses. A number of conformational neutralizing epitopes were identified, and most of these sites were derived from an EV-A71-related study (Fang and Liu 2018). A remarkable example is provided by mAb E18/19, which are two antibodies generated by immunization with an immature EV-A71 virus. Both antibodies neutralize EV-A71, but via different mechanisms. E18 binds to the virus and causes a conformational change of the bound virus that promotes the release of the viral genome and renders the virus inactivated E19 does not induce genome injection. Structural analyses subsequently revealed that E18 and E19 recognize conformation epitopes on the EV-A71 capsid, but their targets are different. The binding sites of E18 are located between the VP4–VP2–VP3–VP1 protomers, and the E19 sites are exclusively within a single promoter (Plevka *et al.* 2014). Additional conformational epitopes were identified in other structural features of the capsids of EV-A71, including the five-fold axis, "knob" and G-H loop, canyon northern rim, canyon floor, canyon southern rim, three-fold plateau, and two-fold plateau (Lee *et al.* 2013; Kiener *et al.* 2014; Jiang *et al.* 2015; Arthur Huang *et al.* 2017; Jia *et al.* 2017). Studies on CV-A6 indicated that there are also conformational epitopes located in the B–C, E–F, H–I loops of VP1 (Chen *et al.* 2018; Fang and Liu 2018).

Monovalent Candidates of HFMD Vaccines

Inactivated Whole Virus Vaccine

In comparison to other vaccine candidates, inactivated EV-A71 vaccines are the only vaccines entering the market. China's Food and Drug Administration (FDA) has issued drug certificates and production licenses for EV-A71 inactivated vaccines from 3 companies, namely, Sinovac, Vigoo, and the Chinese Academy of Medical Sciences (CAMS), all of which are based on C4 subgenotype—the most common genotype in China, although with different virus strain and variation in manufacturing process (Mao *et al.* 2016) (Table 1). Following successful phase I–III clinical trials, a recent large-scale cohort phase IV study of licensed inactivated EV-A71 vaccine revealed an overall protection effectiveness of 89.7% against EV-A71 infection along with a 4.58% rate of reported adverse reaction (Guan *et al.* 2019). However, both the Sinovac and CAMS EV-A71 vaccines were ineffective for CVA16-associated HFMD, unraveling their genotype specificity (Li *et al.* 2016; Li R *et al.* 2014). Besides Chinese mainland, inactivated EV-A71 vaccines were also developed in Taiwan region and Singapore, targeting B3 and B4 subgenotypes respectively. The EV-A71 developed in the Taiwan region has been evaluated in a phase II clinical trials involving a total of 365 infants or children aging from 2 months to 11 years, achieving a seroprotection (neutralization titer $\geq$ 1:32) lasting for 2 years in most participants without reported serious adverse events (SAEs) (ClinicalTrials.gov number, NCT02200237). In addition, a cross-reaction was observed against other EV-A71 strain genotypes, including B5, C4a, C4b, and C5 (Huang *et al.* 2019). A phase III clinical trial has initiated in 2019 and is expected to be completed in 2022 (ClinicalTrials.gov number, NCT03865238) (Lin *et al.* 2019). Only a small-scale phase I clinical trial has been conducted for EV-A71 vaccine developed in Singapore, and the study claimed that the vaccine induced a high immune response against HFMD caused by EV-A71, although the data has not been publicly disclosed (ClinicalTrials.gov number, NCT01376479). Immunizations with inactivated virions derived from CV-A16, CV-A10 and CV-A6 have been only studied in animal models, and the results provided immunological and functional evidence supporting their efficacy. The generated serum contained high levels of virus-specific neutralizing antibodies, and the serum from immunized mother mice afforded protection against lethal challenges with virulent HFMD-related viruses when it was passively transferred to neonatal mice (Qi An *et al.* 2014; Zhang *et al.* 2017a). Therefore, inactivated whole virus vaccine represents the most attainable monovalent HFMD-related vaccine.

Synthetic Peptide and Protein Vaccines

Synthetic peptide vaccines are usually related to the selected neutralizing epitopes, and the two peptides located in VP1, called SP55 (E–F loop; aa 163–177) and SP70 (G-H loop; aa 208–222) have been shown to elicit EV-A71 specific neutralizing antibodies. SP70 raised a relatively higher titer of neutralizing antibody against EV-A71 than that of SP55. However, the neutralizing antibody titer elicited from the peptide SP70 was just one-fourth of that observed in mice immunized with heat-inactivated EV-A71 (Foo *et al.* 2007). Thus, given that EV-A71 VP1 peptide or whole protein was only able to raise a neutralizing antibody response generally inferior to that of inactivated EV-A71 vaccines and

Table 1 Official licensed inactivated EV-A71 vaccines by the Chinese Food and Drug Administration.

Organizations	Sinovac Biotech Co., Ltd	Beijing Vigoo Biological Co., Ltd	Chinese Academy of Medical Sciences
EV-A71 Strain	H07 (C4)	FY (C4)	M01 (C4)
Inactivation technique	Formalin	Formalin	Formalin
Cell substrate	Vero cells	Vero cells	Human diploid KMB-17 cell
Dosages	400 U, two-dose	320 U, two-dose	100 U, two-dose
Adjuvant	Aluminum hydroxide	Aluminum hydroxide	Aluminum hydroxide
Population target	Children (6–35 month)	Children (6–35 month)	Children (6–71 month)
Enrollment	10,077	10,245	12,000
References	NCT01507857	NCT01508247	NCT01569581

consequently showed a protective effect in animal models limited to a low-dose virus challenge (Premanand *et al.* 2012), synthetic peptide and protein vaccines have only been tried in the research stage without progression to more commercial development.

Recombinant Subunit Vaccines

Virus-like particles (VLPs) are a special form of recombinant subunit vaccines for non-enveloped viruses, and can be generated by a number of biosystems. The principle of EV VLPs is to coexpress the genes encoding capsid protein precursor P1 and protease 3CD, which results in the cleavage of P1 into three capsid subunit proteins VP0, VP1, and VP3 through the action of the 3CD protease. VP0, VP1 and VP3 are subsequently self-assembled into VLPs, which adopt the natural structure of virus capsid and can serve as potential vaccine candidates after purification. VLPs derived from EV-A71, CV-A16, CV-A6 and CV-A10 species were reported to be successfully produced in baculovirus-insect cell (Somasundaram *et al.* 2016), *Pichia pastoris* yeast (Zhang *et al.* 2016) and *saccharomyces cerevisiae* yeast (Zhao *et al.* 2013; Zhou *et al.* 2016; Zhang W *et al.* 2018). Immunization study in mice showed that VLPs were able to elicit high titers of neutralizing antibodies and afford effective protection against lethal viral challenge (Wang X *et al.* 2018; Zhou *et al.* 2018). In addition, a recent study reported that VLP vaccines for HFMD induced a high antigen-specific B cell response that is comparable to inactivated vaccines (Yang *et al.* 2019) (Table 2). This study produced EV71-VLPs in *Pichia pastoris*, attaining a high expression level of EV71-VLPs greater than 250 mg/L (Yang *et al.* 2019). With the higher yield capacity to be more cost effective, EV71-VLPs produced in *Pichia pastoris* are in clinical trial (CXSL1900022), representing a good start toward future commercialization.

Recombinant Virus-Vector Vaccines

Researchers inserted *P1* and *3CD* genes of EV-A71 into one vesicular stomatitis virus (VSV) backbone to generate a recombinant VSV to produce VLPs, which protected neonatal mice against lethal viral challenge (Yan *et al.* 2016). A novel recombinant adenovirus vaccine, Ad-EVVLP, with *P1* and *3CD* genes of EV-A71 inserted into the adenoviral genome to express VLPs, induced EV-A71-specific neutralizing antibodies and Th1/Th2-balanced cellular responses in immunized mice, whereas inactivated EV-A71 vaccine activated only Th2-mediated neutralizing antibody responses to protect against virus challenge (Tsou *et al.* 2015) (Table 3). The immunogenicity of 71-6 epitope (aa 176–190 of VP3) was tested using the norovirus P particle as the vaccine carrier, and serum from mice immunized with the resulting chimeric P particle could protect suckling mice from a lethal dose of EV-A71 infection (Jiang *et al.* 2015).

Recent Development of Multivalent Vaccines

Patients with recurrent HFMD are a clear indicator of lack of efficient cross-reactivity among serotypes, informing the need for development of multivalent vaccine. Accordingly, several approaches have been attempted to develop vaccine covering multiple serotypes. The most straightforward approach is simply combining the existing monovalent vaccines into one formulation. There were studies showing that immunization of combined multiserotypic formulations, in the form of either activated virus or VLP, led to effective protection against corresponding viruses without interference, suggesting no cross-serotypic effect. Though simple "mixing" approach does show promise in providing a solution to the issue of multi-protection, it faces the problems of relatively high cost and reliability issue. Consequently, an alternative approach has been also explored utilizing chimeric vaccines generated by engineering vector to co-express viral proteins or peptides from multiple serotypes, generally achieved via partial antigenic substitution and insertion.

Table 2 The producing systems of enterovirus-related virus-like particle (VLP).

VLP-producing systems	Yield capacity	Properties	Status	Ref.
Baculovirus-insect cell	Moderate (64.3 mg/L)	Moderate-yield; Relatively high cost; Large stocks (cell & viruses); Contamination risk of virus	Lab	(Chung *et al.* 2010)
Saccharomyces cerevisiae yeast	Low (0.25 mg/L)	Low-yield; Low cost; Ease in manipulation	Lab	(Li *et al.* 2013)
Pichia pastoris yeast	High (270 mg/L)	High-yield; Low cost; Easy manipulation	Clinical trial (CXSL1900022)	(Yang *et al.* 2019)
Recombinant vesicular stomatitis virus (rVSV)	–	Attenuated(ΔM51); Replication-competent and may have adverse effects	Lab	(Yan *et al.* 2016)
Recombinant adenovirus 5 (Ad-EVVLP)	–	Replication-incompetent(ΔE1/ΔE3); 3C-specific cellular immunity Ad-EVVLPs from EV71 genes can protect against CVA16 infection	Lab	(Tsou *et al.* 2015)

Table 3 The characteristics of the primary experimental enterovirus vaccine formats.

Vaccine format	Conformation	Immunogenicity	mAb responses	Limitation	Advantages
Inactivated whole virus	Natural virion with genome	Strong (+++)	High; Cross-genotype protection	Low cross-serotypic protection	Mature technology
VLP	Natural virion without genome	Moderate (++)	High; Cross-genotype protection	Low cross-serotypic protection	Safe; Low cost; Explicit composition; Easy large-scale production and quality control
Synthetic peptide or recombinant subunit	Linear epitope or antigen	Relatively weak (+)	Low; Cross-genotype protection	Low cross-serotypic protection; Strong adjuvant requirement	Safe; Inexpensive; Explicit composition; Easy large-scale production and quality control
Novel chimeric vaccines	Natural virion without genome or linear epitopes of antigens	Relatively high (++/+++)	High; Cross-genotype protection	Required to know key neutralization domain and need to design the optimal chimeric strategy	May induce cross-protection of serotypes
Recombinant virus-vector vaccines	Natural virion without genome of target viruses but vectors	Relatively high (++/+++)	High; Cross genotype protection	Risk of vector replication	May induce cross-protection of serotypes; Comprehensive T-cell immune response

Inactivated Multivalent Vaccines

Bivalent vaccine approach was first tested on the two major causative agents, EV-A71 and CV-A16. A vaccine formulated by combining inactivated EV-A71 and CV-A16 viruses induced a balanced protective immunity in mice model against EV-A71 and CV-A16 infection without detectable immune interference (Cai *et al.* 2014). Furthermore, in rhesus macaques model, intradermal immunization of two doses of bivalent EV-A71/CV-A16 inactivated vaccine showed excellent virus containment and protection without immunopathological effect against a subsequent viral challenged with EV-A71 or CV-A16 (Fan *et al.* 2020). CV-A6 and CV-A10 of the inactivated whole-virus combination vaccines also induce antigen-specific systemic immune responses, which elicit active immunization to achieve a protection rate of $> 80\%$ in controlling homotypic and heterotypic CV-A6 and CV-A10 infections (Zhang Z *et al.* 2018). A trivalent vaccine candidate containing inactivated EV-A71, CV-A16, and CV-A6 delivered full protection from lethal challenge against EV-A71 and CV-A16, and protection from CV-A6

challenge was accomplished in a passive transfer study involving serum raised against the trivalent vaccine (Caine *et al.* 2015). Another inactivated-CV-A6, CV-A10, and CV-A16 trivalent vaccine induced sufficient neutralizing antibodies and cell-mediated immune responses, and there was no sufficient cross-protectivity against heterologous strains (Lim *et al.* 2018). Collectively, these results indicate that there is no immunological interference between the antigens in their ability to induce virus-specific immune responses, which provides proof-of-concept for multivalent vaccines for broad protection against HFMD.

Multivalent VLPs Vaccines

A bivalent EV-A71/CV-A16-VLPs vaccine induced a balanced neutralizing antibody response and passively protected mice against EV-A71 and CV-A16 infections (Ku *et al.* 2014). A tetravalent vaccine, including CV-A10-VLP, EV-A71-VLP, CV-A16-VLP, and CV-A6-VLP, elicited antigen-specific and long-lasting serum antibody responses and neutralization titers against EV-A71, CV-A16, CV-A10, and CV-A6 strains similar to the monovalent vaccines, which indicates good compatibility among the four antigens in the combination vaccine (Zhang W *et al.* 2018).

Novel Chimeric Vaccines

A chimeric EV-A71 virus, in which the VP1 (aa 210–225) epitope was replaced with the epitope of CV-A16, was constructed using a reverse genetics technique to produce an EV-A71/CV-A16 bivalent vaccine candidate (Yang *et al.* 2016). The other attempt was to replace the EV-A71-neutralizing epitope SP70 with the epitope of CV-A16 to form chimeric EV-A71 virus-like particles (ChiEV-A71 VLPs), and immunization with ChiEV-A71 VLPs in mice elicited robust Th1/Th2-dependent immune responses against EV-A71 and CV-A16. Passive immunization with sera raised against ChiEV-A71 VLPs conferred full protection against lethal challenge with EV-A71 and CV-A16 in neonatal mice (Zhao *et al.* 2015). Structural studies revealed that SP70 epitope replacement converted the surface charge potential of VLP, coupled with variations in amino acid sequences, which most likely accounted for the additional neutralization capability of the ChiEV-A71 VLP. A newly published patent showed that EV-A71 VLP displaying CV-A16 VP1 polypeptides maintained the important neutralizing antibody epitopes of EV-A71 itself, and a CV-A16 VLP displaying EV-A71 VP1 polypeptides elicited a protective neutralizing antibody response directed against EV-A71 and CV-A16 viruses (PCT/MY2017/050059-US2019/0224304 A1). Generally, the results above indicate that the substitution and incorporation of key peptides/proteins between serotypes into one construction will be a reasonable method to construct a multivalent HFMD vaccine.

Recombinant Virus-Vector Vaccines

Bivalent chimeric VLPs presenting SP70 of VP1 and VP2 E–F loop epitopes (aa 141–155) of EV-A71 used the hepatitis B virus core protein (HBc) as a carrier (HBc-E1/2) and induced higher IgG and neutralization titers against EV-A71 and CV-A16 than immunization with only one epitope incorporated into HBc. More importantly, passive immunization with recombinant HBc-E2 particles protected neonatal mice from lethal EV-A71 and CV-A16 infections, and therefore, the VP2 epitope is immunodominant between the two serotypes (Xu *et al.* 2015). Another bivalent chimeric VLP using the core carrier of a truncated hepatitis B virus (tHBc) displayed conserved epitopes of EV-A71 in SP90 (aa 208–222) of VP1, VP2 (aa 248–263) and CV-A16 in PEP91 (aa 271–285) of VP1, which induced humoral and cellular immune responses and protected neonatal mice born to dams from lethal EV-A71 and partially from CV-A16 infection (Huo *et al.* 2017). Researchers described a hexon-modified chimpanzee adenovirus serotype 68 (AdC68) bivalent vaccine that incorporated the neutralizing epitope of CV-A16, PEP71, and a shortened neutralizing epitope of EV-A71, sSP70, into the AdC68 hexon, and EV-A71-VP1 was cloned into the E1-region of the AdC68 vectors. The candidate elicited neutralizing antibodies against CV-A16 and EV-A71 and conferred protection to suckling mice against a lethal challenge of both viruses, which indicates a potential carrier and epitope-displaying platform (Zhang *et al.* 2015). Accordingly, the integration of chimeric VLPs into novel virus vectors induced the effect of multivalence, characterized by an enhanced broad systemic immune response.

Experimental Animal Model and Regimen

Murine and non-primate models are the two major animal models for evaluating HFMD-related vaccines. In mouse model, adult BALB/c or ICR mice of 6–8 weeks were routinely selected for immunogenicity assessment. Prime-boost represents the most common vaccination strategy with vaccine(s) applied two or three times with an interval of 2–4 weeks via intraperitoneal (i.p.) or intramuscular (i.m.) route (Wang and Yu 2014). Given the HFMD-related viruses only cause minor phenotypes in adult mice, the protective efficacy of experimental vaccines has to be indirectly examined using neonatal mice, which are more susceptible to virus infection than adult mice possibly due

to their immature immune systems (Yu *et al.* 2000; Fang and Liu 2018). One-day-old ICR mice infected with EV-A71 at a lethal dose of greater than 10^8 PFU can reach a mortality rate of 100% following i.p. inoculation (Yu *et al.* 2000). Neonatal mice infected with the highest dose of CV-A16 at $10^{6.5}$ CCID50 had a 100% mortality by day 6, and a 100% mortality by day 13 with the lowest dose at $10^{2.5}$ CCID50 (Li J *et al.* 2014). CV-A6 and CV-A10 infection murine model have been developed using 5-day-old neonatal mice with $10^{5.5}$ TCID50 viruses via i.m. inoculation (Zhang *et al.* 2017a, b). Moreover, researchers have generated a transgenic (Tg) mouse expressing hSCARB2, the cellular receptor of EV-A71, which can infect EV-A71 within 1- to 14-day-old at a dose of 3×10^4 to 10^6 PFU via subcutaneous (s.c.) injection and exhibited neurological disease and pathology very similar to that observed in humans (Yang *et al.* 2009; Fujii *et al.* 2013). The vaccine protectiveness is measured by transfusing sera from immunized adult mice to young mice and examining their effect on subsequent viral challenge. An alternative version of this two-step protocol was recently developed by first combining immunized serum with virus *in vitro* and then applying the neutralization mixture to suckling mice, whereby the time required for the process is greatly shortened (Wang *et al.* 2016). Gerbil has recently emerged as a new model animal for studying HFMD-related virus as research showed that gerbils up to 21-day-old were fully susceptible to CV-A16 of $10^{5.5}$ TCID50 and this susceptibility, marked by eventual death from neurological disorders, could be achieved on 60-day-old gerbils once the infection dose increased to 10^8 TCID50. Moreover, gerbils up to the age of 14-day-old were also susceptible to CV-A10 of $10^{8.5}$ TCID50, with all animals succumbed five days after infection (Sun *et al.* 2016; Yao *et al.* 2019; Chen *et al.* 2020).

The research exploring the non-human primate model of HFMD-related viruses is limited, but the results are promising. In one report, the neonatal rhesus monkeys were challenged with EV-A71 ($10^{4.5}$ CCID50/monkey) via intratracheal infection, and HFMD-liked vesicular lesions were found in the mouth and foot, demonstrating the suitability of neonatal non-human primate for dissecting the complete process of EV-A71 infection (Liu L *et al.* 2011). In another report, upon CV-A16 infection via nasal insufflation, rhesus macaques developed oral mucosa and limb vesicles, a major classical clinical manifestation of HFMD infection. Strikingly, the infected macaques did not elicit CV-A16-specific neutralizing antibodies and functional memory T-cells. Furthermore, transfusion of sera from macaques immunized with inactivated CV-A16 vaccine failed to mount protection against a viral challenge in young macaque recipient. These surprising revelations suggest that the immunological mechanism of CV-A16 infection need to be further investigated (Wang *et al.* 2017).

Conclusions

The inactivated EV-A71 vaccines show high efficacy, good immunogenicity persistence and acceptable safety profiles in the vaccination population and efficiently reduce the incidence of HFMD, especially severe cases. However, concerns have risen on changes in dominant HFMD-causing virus strains and emerging new disease-causing serotypes. Therefore, it is imperative to explore multivalent vaccine formulation with broad-spectrum protection and sufficient safety. This exploration would be facilitated by a combinatorial effort involving improved vaccine design and strategy, better utilization of old vaccine vector along with development of new vaccine platforms. Lastly, it will be also helpful to gain a better understanding of how immunological memories develop upon infection with different serotypes, which could serve as an instructive guide for vaccine development.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement This article does not contain any studies with human or animal subjects performed by any of the authors.

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