

REVIEW

EV-A71 vaccine licensure: a first step for multivalent enterovirus vaccine to control HFMD and other severe diseases

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Enteroviruses (EVs) are the most common viral agents in humans. Although most infections are mild or asymptomatic, there is a wide spectrum of clinical manifestations that may be caused by EV infections with varying degrees of severity. Among these viruses, EV-A71 and coxsackievirus (CV) CV-A16 from group A EVs attract the most attention because they are responsible for hand, foot and mouth disease (HFMD). Other EV-A viruses such as CV-A6 and CV-A10 were also reported to cause HFMD outbreaks in several countries or regions. Group B EVs such as CV-B3, CV-B5 and echovirus 30 were reported to be the main pathogens responsible for myocarditis and encephalitis epidemics and were also detected in HFMD patients. Vaccines are the best tools to control infectious diseases. In December 2015, China's Food and Drug Administration approved two inactivated EV-A71 vaccines for preventing severe HFMD. The CV-A16 vaccine and the EV-A71-CV-A16 bivalent vaccine showed substantial efficacy against HFMD in pre-clinical animal models. Previously, research on EV-B group vaccines was mainly focused on CV-B3 vaccine development. Because the HFMD pathogen spectrum has changed, and the threat from EV-B virus-associated severe diseases has gradually increased, it is necessary to develop multivalent HFMD vaccines. This study summarizes the clinical symptoms of diseases caused by EVs, such as HFMD, myocarditis and encephalitis, and the related EV vaccine development progress. In conclusion, developing multivalent EV vaccines should be strongly recommended to prevent HFMD, myocarditis, encephalitis and other severe diseases.

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INTRODUCTION

Enteroviruses (EVs) are a genus of positive-sense single-stranded RNA viruses associated with several human and mammalian diseases. The EV genus consists of 12 species: EV-A-H, EV-J and Rhinovirus A, B and C.¹ EVs cause a wide range of diseases, including hand, foot and mouth disease (HFMD), upper respiratory tract infection, diarrhea, viral myocarditis, encephalitis and aseptic meningitis (Table 1). The most common EV in Europe and the United States (US) is the echovirus (E), which is the main pathogen of aseptic meningitis, while HFMD caused by the EV-A group is prevalent in the western Pacific region. The EV-A group is responsible for more than 90% of HFMD cases.² EV-A71 and coxsackievirus (CV) CV-A16 from the EV-A group are the main pathogens responsible for worldwide HFMD outbreaks.^{3,4} In addition to EV-A71 and CV-A16, several other EV-A may also cause HFMD. In recent years, CV-A6 and CV-A10 have gradually replaced EV-A71 and CV-A16 as the main pathogens of HFMD outbreaks. For example, CV-A6 was the pathogen responsible for HFMD outbreaks in Taiwan in 2010, Japan in 2011, Thailand in 2012 and mainland China in 2013,^{5–8} whereas CV-A10 was responsible for the HFMD outbreaks in France in 2010 and Wuhan, China in 2013.^{2,9} In addition, EV-B may lead to sporadic HFMD cases,¹⁰ among which CV-B3 and CV-B5 have been the predominant

etiological agents. For example, CV-B3 was the main pathogen responsible for the 2012 HFMD epidemic in Shijiazhuang, China.¹¹ EV-B infections usually lead to serious illnesses. In the United States, 20 000–40 000 cases of acute myocarditis are caused by CV-B3 each year. Among patients with acute myocarditis, 3.5–8.5 of every 100 000 people (9000–20 000 in total) may develop dilated chronic myocarditis.¹² In fact, CV-B5 and E30 are the main causes of encephalitis and aseptic meningitis outbreaks.¹³

Because no effective treatments for HFMD and other diseases caused by EVs exist, vaccines have become the most effective solution in preventing EV-related diseases. In December 2015, two inactivated EV-A71 vaccines, which were the first HFMD vaccines, were approved in mainland China for preventing severe HFMD.^{14,15} In addition, a CV-A16 monovalent vaccine and an EV-A71-CV-A16 bivalent vaccine showed good efficacy in HFMD prevention according to the pre-clinical study results.^{16,17} Although research on CV-B3 vaccine has been conducted for years, no vaccine is available to protect children from viral myocarditis caused by CV-B3 infection.

Moreover, the results of epidemiological surveillance show that the EVs epidemic characteristics are changing. CV-A6 and CV-A10 are gradually replacing EV-A71 and CV-A16 as the major pathogens of HFMD, and the rising prevalence of CV-B3, CV-B5 and E30 is

Table 1 The clinical manifestation spectrum of enterovirus-associated diseases

Enterovirus group	Mild disease	Severe disease	Comment
EV-A	HFMD ^{#1}	NP-AFP ^{#4}	Associated with EV-A71 ^{#1,4-7} ; associated with CV-A16 ^{#1,2,5,6} ; associated with CV-A6 or CV-A10 ¹⁻³
	Herpangina ^{#2}	Aseptic meningitis ^{#5}	
	Onychomadesis ^{#3}	Encephalitis ^{#6}	
	Pharyngitis	Death ^{#7}	
	Diarrhea	Pulmonary edema	
	Respiratory symptoms	Neuromyelitis Optica	
EV-B	–	Infant sepsis	Associated with CV-B3 ^{#1,7-9} ; associated with CV-B5 or E30 ^{#1,4-7}
	HFMD ^{#1}	NP-AFP ^{#4}	
	Herpangina	Aseptic meningitis ^{#5}	
	Diarrhea	Encephalitis ^{#6}	
	Respiratory symptoms ^{#8}	Myocarditis ^{#9}	
	–	Death ^{#7}	
	–	Acute transverse myelitis	
	–	Acute pancreatitis	
EV-C	–	Papillitis	Associated with CV-A22 or CV-A24 ^{#1,2,5,10}
	–	Hepatitis	
	HFMD ^{#1}	NP-AFP ^{#4}	
	Herpangina ^{#2}	Aseptic meningitis ^{#5}	
	Acute hemorrhagic conjunctivitis ^{#10}	Hopkins syndrome	
Other EVs	Corneal endothelial dysfunction	–	Associated with rhinovirus ^{#1,8}
	HFMD ^{#1}	ND	
	Respiratory symptoms ^{#8}	–	

Abbreviations: not determined, ND; non-polio acute flaccid paralysis, NP-AFP.

[#]Typical clinical manifestation.

increasing the likelihood of consequent severe diseases to occur in infants.¹⁸ Therefore, multivalent EV vaccines should be researched and developed to prevent infants and children from being infected with severe diseases.

EV-RELATED DISEASE EPIDEMICS

EV-A group viruses

EV-A infections are the main causes of HFMD and herpangina (HA). It is reported that the EV-A group is responsible for more than 90% of HFMD cases,² among which EV-A71 and CV-A16 are the main pathogens of HFMD outbreaks worldwide.^{3,4} EV-A71 is responsible for most severe cases and deaths.¹⁹ CV-A6 and CV-A10 may also cause HFMD outbreaks, while other viruses in the EV-A group mainly cause HA and may also lead to sporadic HFMD cases.

Since the first EV-A71 strain was isolated in California in 1969, EV-A71-related HFMD outbreaks have occurred worldwide, particularly in the western Pacific region. For example, EV-A71 was the predominant pathogen responsible for HFMD outbreaks in Thailand in 2008, 2009 and 2011, with EV-A71 positive rates of 56%, 32% and 29%, respectively.²⁰ During the period 2008–2012, EV-A71 ranked as the primary pathogen responsible for HFMD outbreaks in several cities of mainland China. A total of 10 714 237 survivors and 3046 deaths were reported from 2008 to June 2014 in mainland China, with a case fatality rate of 0.03%.²¹ In another study based on enhanced HFMD surveillance, the risk of severe-case fatality was 3.0%, with >90% of deaths associated with EV-A71.²²

As another major HFMD pathogen, CV-A16, which co-circulates with EV-A71, also caused many HFMD outbreaks. A CV-A16-related HFMD outbreak was first reported in Toronto, Canada in 1957; subsequently, such outbreaks were also reported in Sydney, Australia in 1991, England and Wales in 1994, Taiwan during 2002–2003,

Singapore during 2002, 2005 and 2007, Vietnam in 2005, India in 2009, and Thailand in 2010.^{19,20,23–28} In mainland China, CV-A16 was the main pathogen responsible for HFMD epidemics. Although CV-A16 infection was considered only to cause mild symptoms, a few CV-A16 patients also developed aseptic meningitis and encephalitis.²⁹

Research groups in Taiwan and Japan found that other viruses in the EV-A group might also cause both HA and HFMD.⁵ Before 2007, CV-A6, CV-A4 and CV-A10 were the main HA pathogens, whereas EV-A71 and CV-A16 were the main HFMD pathogens. However, CV-A6 and CV-A10 attracted more attention recently because they replaced EV-A71 and CV-A16 as the main causes of HFMD outbreaks in many countries.^{7,8} CV-A2, CV-A4, CV-A5, CV-A6, CV-A8, CV-A10, CV-A12 and CV-A14 were also detected in HFMD clinical samples.^{7,19}

CV-A6 was responsible for the 2008 HFMD outbreak in Finland. Thereafter, CV-A6 was reported to cause HFMD outbreaks in Singapore in 2009, Taiwan during 2009–2010, Spain in 2011, Japan in 2011, Thailand in 2012, California in 2012 and Edinburgh, UK in 2013.^{5,6,30–34} In many cities of mainland China, CV-A6 emerged as the major etiologic agent of HFMD, replacing EV-A71 and CV-A16. CV-A6-related HFMDs were characterized by atypical HFMD symptoms such as nail loss, with high incidences in adults and during the winter.¹⁸

Among the 141 samples taken during the 2010 France HFMD/HA outbreak, 134 were EV-A, with 39.9% CV-A10 and 28% CV-A6.² Regarding the 2013 HFMD outbreak in the city of Wuhan, China, 463 viral strains were identified from 3208 HFMD samples, with 190 (41.0%) identified as CV-A10, 111 (21.2%) identified as EV-A71 and 52 (11.2%) identified as CV-A16.⁹ Therefore, after EV-A71 and CV-A16, CV-A6 and CV-A10 have emerged as the most significant HFMD pathogens.

EV-B group viruses

The EV-B group has a high infection rate in humans. In 2007, CV-B1 became the most commonly identified EV serotype in the United States, accounting for 25% of all EV infections with known serotypes. Reportedly, the HFMD-causing EV-B viruses included CV-B1-5, E7, E13 and E30. In addition to mild HFMD cases, the CV-B subgroup was also responsible for severe diseases such as myocarditis and encephalitis.³⁵

CV-B3 may cause severe diseases such as viral myocarditis, dilated chronic myocarditis and encephalitis in children, although most clinical symptoms of CV-B3-HFMD are mild. In the past, CV-B3 mainly caused sporadic HFMD cases, as was reported in Korea, Singapore, mainland China and Taiwan.^{8,11,36–39} Recently, in mainland China, CV-B3-related HFMD has been on the rise. In fact, during the HFMD outbreak in the Chinese city of Shijiazhuang in 2012, CV-B3 (3.02%, 26/861) was the second most predominant non-EV-A71/CV-A16 enterovirus, behind only CV-A10.¹¹ In the 2012 HFMD epidemic in Jiangsu, CV-B3 was the second major pathogen, behind CV-A16.⁴⁰ Therefore, with the rise of HFMD cases caused by CV-B3 infection, there is greater risk for HFMD patients to develop myocarditis, dilated chronic myocarditis and other severe diseases.

CV-B5 was the major pathogen of encephalitis outbreaks in South Korea and mainland China. CV-B5 was ranked the No.2 pathogen of the 2005 (26.58%, 21/79) and 2009 (19.63%, 21/107) HFMD epidemics in Korea.^{36–37} For the 2009 HFMD epidemic in Shandong China, CV-B5 was also the second major pathogen (12.7%, 14/110), with 11 of the 14 CV-B5 patients (78.6% (11/14)) developing neurological symptoms.⁴¹ The US Centers for Disease Control and Prevention statistics showed that CV-B group viruses (types 1–5) might cause more than 500 million infections each year.⁴² In addition to CV-B3, other EV-B viruses may also cause myocarditis and encephalitis. EV surveillance in Taiwan found that CV-B1 was the fourth predominant EV (3.4%) in 2008, with CV-B2 the fifth most common (7%) in 2006, CV-B4 the fifth most common in 2000 and 2001 (4.5% and 1.5%, respectively), the second most common in 2004 (14.9%), and the third most common in 2008 (12.0%). CV-B1 was reported to cause several viral myocarditis outbreaks.^{43,44}

The most commonly isolated EVs in Europe are E6, E7, E9, E11, E13 and E30. Over the past 35 years, US EV surveillance annual reports showed that E35, E6, E9, E11 and E30 were the four most prevalent EVs. Among them, E30 attracted the most attention because it caused several aseptic meningitis outbreaks.¹³ The prevalence of E30 among HFMD patients has also trended upward in recent years. In HFMD patients in Taiwan in 2008, E30 was the most predominant among all echoviruses.³⁹ From HFMD samples in Shandong, China 2010, 35 non-EV-A71/CV-A16 EVs were isolated, including eight identified as E30,⁴⁵ whereas E30 was also detected among patients with severe HFMD from that region in 2010–2011.⁴⁶

EV-C group viruses

CV-A21, CV-A24 and E96 from the EV-C group may cause HFMD. Among the 2,067 suspected HFMD cases in Guangzhou, China in 2011, CA21, CA24 and E96 each accounted for one case.⁴⁷ CV-A21 was reported to cause acute respiratory infection in adults, while CV-A24 was the main pathogen of acute hemorrhagic conjunctivitis, aseptic meningitis, corneal endothelitis, and acute flaccid paralysis (AFP). E96 was also associated with AFP.

EV INFECTION-RELATED CLINICAL MANIFESTATIONS

EVs may cause a wide spectrum of acute diseases with clinical manifestations ranging from non-specific febrile illness, HFMD, HA,

mild upper respiratory tract infection, and self-limiting gastroenteritis to more severe ones such as myocarditis, hepatitis, and encephalitis. However, different subgroups of EVs are associated with different clinical manifestations. For example, EV-A was associated with HFMD/HA,² EV-B with myocarditis/encephalitis, and CV-A24/E70 with hemorrhagic conjunctivitis.

EV-A group viruses

Mild diseases caused by EV-A viruses include HFMD/HA, upper respiratory tract infection, and diarrhea, while severe diseases include encephalitis and other neurological diseases. Severe HFMD symptoms are usually caused by EV-A71, with encephalitis and pulmonary edema being the most common. Although CV-A16 infections usually lead to mild symptoms, severe and fatal HFMD cases related to CV-A16 have been reported.²⁹ The clinical symptoms of CV-A6-related HFMD patients differed from those of patients with other HFMD types. CV-A6 HFMD patients usually have an atypical clinical rash, with nail loss being a prognostic sign. CV-A10 may cause HFMD as well as a number of severe diseases, such as aseptic meningitis.⁴⁸

EV-B group viruses

Among the illnesses caused by EV-B virus infections, the mild ones include HFMD, as well as respiratory and gastrointestinal symptoms, while the severe diseases include myocarditis, encephalitis and hepatitis. Each year, more than five million people are infected with CV-B1-5 virus in the United States. Up to 12% of EV-B-infected patients may have myocarditis, with CV-B3 being the main pathogen of viral myocarditis.⁴⁹ Of considerable importance because myocarditis commonly goes undiagnosed, studies have shown that patients with CV-B3 infection were also at risk of myocarditis, even in the absence of HFMD symptoms. CV-B5 is also associated with encephalitis.⁵⁰

THE DEVELOPMENT OF EV VACCINES

Because EV-A71 and CV-A16 are the major HFMD pathogens, their vaccine development has progressed the fastest (Table 2). The EV-A71 vaccine has already shown substantial protective effect against EV-A71-HFMD and EV-A71-related diseases in clinical trials. Advances in developing a CV-A16 vaccine animal model and good preclinical results on a CV-A16 monovalent vaccine and an EV-A71/CV-A16 bivalent vaccine also provide auspicious prospects for the prevention of EV-A71- and CV-A16-related diseases.^{16,17}

EV-A71 vaccines

EV-A71 vaccines include the whole virus-inactivated vaccine, virus-like particle (VLP) vaccine and peptide vaccine, among which the whole virus-inactivated vaccine has progressed the fastest. Inactivated EV-A71 vaccines developed by mainland China, Taiwan and Singapore used the C4, B4 and B3 genotype strains, respectively.⁵² Those virus strains were cultured in Vero cells or human diploid cells, before they were inactivated with formaldehyde. After purification, these inactivated viruses were mixed with aluminum salt adjuvants to obtain the final product. Preclinical research showed that these vaccines may induce animals to generate neutralizing antibodies (NTAbs) and exhibited good efficacy. The NTAbs level was positively correlated with the protective effect. In 2010, the National Taiwan Institute of Health (NHRI) began the first EV-A71 clinical trial in adults. The subjects were inoculated with 5 µg or 10 µg of EV-A71 vaccine, and the results showed that the EV-A71 vaccine had good safety and immunogenicity in adults.⁵² Currently, Singapore Inviragen also completed a phase I clinical trial of their EV-A71 vaccine (0.3 µg and 3 µg dosages), whereas three mainland China manufacturers have

Table 2 Research and development progresses regarding EV-A71 and CV-A16 vaccines

EV vaccine	Vaccine type	Vaccine strain	Subgenotype	Cell substrate	R&D status	Organization (country/region)
EV-A71 monovalent vaccine	Inactivated	FY-23	C4	KMB17	Production and registration approval	CAMS (China) ¹⁴
	Inactivated	H07	C4	Vero	Production and registration approval	Sinovac (China) ¹⁵
	Inactivated	FY7VP5	C4	Vero	Phase 3 clinical trial completed	Beijing Vigoo (China) ⁵¹
	Inactivated	E59	B4	Vero	Phase 1 clinical trial completed	NHRI (Taiwan) ⁵²
	Inactivated	–	B3	Vero	Phase 1 clinical trial completed	Inviragen (Singapore) ⁵³
	Inactivated	E59	B4	Vero	Pre-clinical	Adimmune Corporation (Taiwan) ⁵⁴
	Attenuated live virus	BrCr	A	Vero	Pre-clinical	NIID (Japan) ⁵⁵
	VLP	neu	C2	Sf9	Pre-clinical	National Taiwan University (Taiwan) ⁵⁶
	VLP	G082	C4	Sf9	Pre-clinical	IPS-CAS (China) ⁵⁷
CV-A16 monovalent vaccine	Inactivated	SZ05	B1b	Vero	Pre-clinical	IPS-CAS (China) ⁵⁸
	Inactivated	CC024	B	Vero	Pre-clinical	First Hospital of Jilin University (China) ⁵⁹
	Peptide	SZ05	B1b	Synthetic	Pre-clinical	IPS-CAS (China) ⁶⁰
EV-A71/CV-A16 bivalent vaccine	VLP	GD09/119	B	Saccharomyces cerevisiae	Pre-clinical	Beijing Institute of Microbiology and Epidemiology (China) ⁶¹
	Inactivated	FY573 & G08	C4 & B	Vero	Pre-clinical	IPS-CAS (China) ¹⁶
	VLP	FY & 09-7	C4 & B1	Sf9	Pre-clinical	IB-CAS (China) ⁶²
	VLP	SB12736-SAR-03 & SB3512/SAR/00	–	Sf9	Pre-clinical	The University of Queensland (Australia) ⁶³
	VLP	G082 & SZ05	C4 & B1b	Sf9	Pre-clinical	HuaLan (China) ¹⁷

Abbreviations: Chinese Academy of Medical Sciences, CAMS; Institute of Biophysics, Chinese Academy of Science, IB-CAS; Institute Pasteur of Shanghai, Chinese Academy of Sciences, IPS-CAS; National Health Research Institutes, NHRI; National Institute of Infectious Diseases, NIID; virus-like particle, VLP.

completed phase III clinical trials on their EV-A71 vaccines. In those phase III clinical trials, a total of 32 000 infants were vaccinated with EV-A71 vaccines. The clinical results showed that the protection rate against HFMD was >90% and that the protection rate against other EV-A71-related diseases was >80%. In addition, the proposed threshold level of NTAbs should be between 1:16 and 1:32 for EV-A71 vaccine protection effectiveness. Further research on the batch-to-batch consistency of those three EV-A71 vaccines in China substantiated high immunogenicity consistency among all batches.⁶⁴ The EV-A71 vaccine of Sinovac (Beijing, China) showed an efficacy rate of 95.1% (95% CI 63.6, 99.3) against EV-A71-associated HFMD during the extended follow-up and an overall efficacy rate of 94.7% (95% CI 87.8, 97.6) at two years, which indicated that the EV-A71 vaccine could provide a sustained high level of protection against EV-A71-associated HFMDs up to two years.⁶⁵ The phase III clinical trial for the EV-A71 vaccine developed by the Chinese Academy of Medical Sciences (CAMS; Kunming, Yunnan Province, China) using human diploid cells showed that the sero-conversion rate was 95% during the 2-year follow-up and that the protection rate was 97.4% and 100% against EV-A71-HFMD at the 1st and 2nd year, respectively.⁶⁶ Although the subgenotypes of the EV-A71 vaccine strains were different in mainland China, Taiwan and Singapore, serum samples obtained from children immunized against the C4 strain in mainland China or the B3 strain in Taiwan had good cross-neutralizing capacity against other EV-A71 subtypes. All studies have shown that children immunized with a single C4 strain vaccine gained cross-protection against other EV-A71 genotypes. Another study

showed that polio vaccine inoculation in infants, simultaneously or successively along with EV-A71 vaccine inoculation, would not interfere with the EV-A71 vaccine immunization effect.^{67,68} To ensure the accuracy and comparability for detecting NTAbs and antigen content in clinical trials, the National Institute for Food and Drug Control (NIFDC) established the first national standard on EV-A71 anti-serum and first national standard on EV-A71 antigen, and standardized the dosages of the EV-A71 vaccines from these three manufacturers.⁶⁹ On the basis of the studies of national standards, the first international standard for anti-EV-A71 serum was established by the National Institute for Biological Standards and Control (NIBSC) and NIFDC recently.⁷⁰ According to these results, two inactivated EV-A71 vaccines from CAMS and Sinovac Ltd were approved successively for preventing severe HFMD in mainland China in December 2015.

CV-A16 vaccine

With the successful experience regarding the inactivated EV-A71 vaccine, the research on a CV-A16 vaccine has also been focused on an inactivated vaccine. Two CV-A16 strains (CV-A16-SZ05 and CV-A16-G08) were inactivated by Cai *et al* with β -propiolactone, cultured on Vero cells and mixed with aluminum adjuvant. Mice were inoculated with this vaccine and the results showed that it could induce CV-A16-specific antibodies and an interferon- γ (IFN- γ) cellular immune response. The anti-CV-A16 serum could neutralize both homologous and heterologous CV-A16 strains and a mouse-adapted CV-A16 strain (MAV). CV-A16 mouse anti-serum could partially neutralize the virus attack on neonatal mice, while CV-A16-vaccinated

mice may be protected against CV-A16 MAV attacks.⁷¹ Qi An *et al* also prepared a CV-A16 vaccine by inactivating the virus with β -propiolactone, culturing the virus on Vero cells and formulating with aluminum salt. This vaccine also showed a good protective effect in the neonatal mouse model.⁷² Yang *et al* used a human diploid cell substrate in preparing inactivated CV-A16 vaccine. In the mouse and rhesus immune response research, this vaccine demonstrated a dose-dependent effect in inducing NTAb responses. In addition, the serum had good neutralizing effects against gene A and B subtypes of CV-A16, and this vaccine could induce IFN- γ cellular immune responses.⁷³

Recent studies have focused on CV-A16 VLP vaccine development and showed that VLP vaccines appear promising. A CV-A16 VLP vaccine was made from recombinant baculovirus-infected Sf9 cells. Serum from mice immunized with this vaccine could neutralize homologous (CV-A16/SZ05) and heterologous (CV-A16/GX08) strains of CV-A16 *in vitro*, and protect mice against attacks from lethal doses of CV-A16 virus challenge. Moreover, Zhao *et al*⁶¹ produced a CV-A16 VLP vaccine using *Saccharomyces cerevisiae* cells. This vaccine could also induce NTAbs and IgG antibodies, as well as cellular immune responses in mice. In the virus challenge test, this immune serum could protect neonatal mice against the lethal attack from a CV-A16 challenge.

EV-A71 and CV-A16 bivalent vaccine

Because EV-A71 and CV-A16 are the major pathogens of HFMD and other severe diseases, monovalent EV-A71 vaccine and monovalent CV-A16 vaccine were successfully developed and have shown some successful pre-clinical progress. Several institutes have recently begun to develop EV-A71-CV-A16 bivalent vaccines.^{62,74}

Inactivated bivalent vaccine. Recent studies have proved the feasibility and effectiveness of inactivated bivalent vaccines. Cai *et al* evaluated the immunogenicity and protective efficacy of the bivalent EV-A71/CV-A16 vaccine prepared on Vero cell substrates in the mouse model. Similar to the monovalent vaccine, serum from mice immunized with bivalent vaccine showed the same capacity in neutralizing EV-A71 or CV-A16 virus *in vitro*. Bivalent vaccine may protect animals against both EV-A71 and CV-A16 viruses, whereas monovalent vaccine may only protect against one virus. No interference was observed between EV-A71 and CV-A16 immunization.¹⁶ Sun *et al*⁷⁴ further demonstrated the protective effect of the inactivated bivalent EV-A71/CV-A16 vaccine in a mouse model *in vivo*.

VLP bivalent vaccine. Similar to inactivated monovalent vaccines, bivalent EV-A71/CV-A16 VLP vaccines may protect against both viruses. Gong *et al* used insect cells using baculovirus vectors to express EV-A71/CV-A16 VLPs. Immunogenicity was compared between monovalent EV-A71 VLPs, monovalent CV-A16 VLPs and bivalent VLPs vaccine. Serum from mice immunized with aluminum- or CpG-adjuvant VLPs bivalent vaccine could neutralize EV-A71 and CV-A16 *in vitro*.⁶² Ku *et al* also demonstrated that EV-A71/CV-A16-VLPs could induce high levels of antibodies, and protect mice from EV-A71 and CV-A16 infections.¹⁷ Sun *et al* investigated EV-A71/CV-A16 VLPs expressed on insect cells, and their research showed that the VLPs could protect neonatal mice from lethal EV-A71/CV-A16 challenge and demonstrated that NTAbs also had cross-protection against other sub-genotypes of EV-A71 and CV-A16.⁷⁴ Liu *et al*⁷⁵ recently found that CV-A10 VLPs efficiently induced antibodies capable of neutralizing CV-A10 infection *in vitro*.

EV-B group virus vaccines

Some studies have reported on CV-B1-CV-B6 joint inactivated vaccines and vaccines for CV-B3 and CV-B1.^{76,77} Because CV-B3 is the major cause of viral myocarditis and encephalitis, the study of EV-B enterovirus vaccines has been focused on CV-B3, particularly the attenuated CV-B3 vaccine. Virulence research on CV-B3 was mainly focused on the 5'-UTR region and the viral capsid protein region. The changes of some important nucleotide sites in the 5'-UTR region may affect virus binding with cardiomyocytes. In addition, the ribosome-binding sites of the 5'-UTR region (IRES) are the key sites for virus infection and translation.⁷⁸ With mutations of those IRES sites, the mutant virus cannot cause myocarditis or myocardial necrosis in mice.⁷⁹ With mutations of key VP1, VP2 and VP3 amino acids for CV-B3, the pathogenicity of the mutant virus also decreased significantly. However, the attenuated strain may still induce NTAbs in mice and may protect mice from lethal CV-B3 challenge. The decline in virulence after amino-acid mutations in the viral capsid protein may be due to the weak interaction between the virus and the receptor. More research is necessary to better understand the safety of the attenuated vaccine, notably the possible tissue damage caused by the attenuated virus as well as virulence recovery from the virus repair. Research on CV-B3 vaccine includes DNA vaccines, genetically engineered vaccines and the inactivated vaccine.⁸⁰ All three vaccines reported above showed protective effects in mice against myocarditis caused by CV-B3. Moreover, the protein vaccine showed superior protection compared with the DNA vaccine.

SUMMARY

EVs are among the most common disease-causing viruses in humans, particularly in infants and children. There is a wide spectrum of clinical manifestations caused by EV infections with varying degrees of severity. EV-A71 and CV-A16 were the main causes of HFMD epidemics in the western Pacific region. After 2008, CV-A6 and CV-A10 were the main pathogens of HFMD outbreaks. The prevalence of CV-A6 in mainland China and Southeast Asian countries after 2013 presented new challenges in HFMD prevention. Recently, the presence of CV-B3, CV-B5 and E30 among HFMD patients has trended upwards, which may lead to higher risk of more severe diseases for those patients.

Vaccine is the most effective and economical tool to control HFMD. In December 2015, two EV-A71 vaccines were approved for use in mainland China. The cost of immunization is ~\$30 per dose with two doses required for primary immunization,⁸¹ whereas the total costs per patient for severe HFMD and mild HFMD were \$2149.47 and \$513.22, respectively. Furthermore, the loss of disability-adjusted life years of the two disease forms were, respectively 3.47 and 1.76 person-years per 1000 persons in rural central China, from 2011 to 2013, and the cost of illness for EV-A71-associated disease in China was estimated as >\$450 million in 2013.⁸² Two reports forecasted that routine immunization with a 70% or 90% efficacious EV-A71 vaccine sold at \$25 or \$75 per dose, respectively, would be of great economic value.^{83,84}

The change in the HFMD pathogen spectrum and the threat of severe diseases caused by EV-B group viruses both indicate the necessity of developing a multivalent HFMD vaccine. Increasing numbers of investigators have recognized the importance of multivalent HFMD vaccines, which are mainly focused on EV-A71, CV-A16, CV-A6 and CV-A10.^{75,85,86} Recently, a trivalent-inactivated EV-A71/CV-A16/CV-A6 vaccine showed good protection from lethal challenge against each homologous virus in mice, which was similar to that of the corresponding monovalent vaccine groups.⁸⁷ Moreover,

a combination of formalin-inactivated EV-A71, CV-A6, CV-A10 and CV-A16 multivalent vaccine candidates could elicit serotype-specific neutralizing antibody responses in mice and rabbits, and no cross-neutralization efficacy was found among these viruses.⁸⁸ However, many related studies are limited, particularly the national surveillance systems for non-EV-A71 and non-CV-A16 EVs in endemic areas. Critically important is the decision regarding which EV serotypes should be incorporated in the development of a multivalent vaccine. How should the appropriate strain of each EV serotype be chosen? How should the immunogenicity be evaluated and how should the protective efficacy be standardized? How should the immune interaction between these incorporated EV antigens be addressed? Could these vaccines induce an antibody-dependent enhancement effect? Are multiple vaccines economically feasible? Other questions remain.

To control HFMD and other severe diseases caused by EV infections in the pediatric population, the enhanced surveillance of related diseases caused by EV-A71 and CV-A16, as well as CV-A6, CV-A10, CV-B3, CV-B5 and E30 has been suggested. Concurrently, the development of a multivalent vaccine based on both EV-A71 and CV-A16 is urgently needed.

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