Review of Enterovirus 71 Vaccines

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Enterovirus 71 (EV71) and coxsackieviruses are the major causative agents of hand, foot, and mouth disease (HFMD) outbreaks worldwide and have a significant socioeconomic impact, particularly in Asia. Formalin-inactivated (FI) EV71 vaccines evaluated in human clinical trials in China, Taiwan, and Singapore were found to be safe and to elicit strong neutralizing antibody responses against EV71 currently circulating in Asia. The results from 3 different phase 3 clinical trials performed in young children (6–60 months) indicate that the efficacy of FI-EV71 vaccines is >90% against EV71-related HFMDs and >80% against EV71-associated serious diseases, but the vaccines did not protect against coxsackievirus A16 infections. Here we discuss the critical factors affecting EV71 vaccine product registration, including clinical epidemiology, antigenic shift issues in cross-protection and vaccine strain selection, standardized animal models for potency testing, and cost-effective manufacturing processes for potential incorporation of FI-EV71 vaccine into Expanded Programme on Immunization vaccines.

Keywords. human enterovirus A (HEV-A); hand, foot and mouth disease; enterovirus 71; inactivated whole virion vaccine; waning immunity.

Coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) are the predominant causes of hand, foot, and mouth disease (HFMD) and herpangina. EV71 has emerged as a serious threat to public health across the Asia-Pacific region [1–3]. EV71 infects infants and young children, and 71% of them are asymptomatic [4]. HFMD is a febrile illness characterized by a maculopapular rash or blisters on the hands, soles, and buttocks and is associated with painful ulcerative lesions of the mouth. It is usually a self-limiting infection, but is highly contagious and efficiently propagated to household contacts by oropharyngeal secretions or fecal–oral transmission [5]. EV71 is a neurotropic virus responsible for severe central nervous system (CNS) complications including aseptic meningitis, cerebellar ataxia, poliomyelitis-like paralysis, acute brainstem encephalitis, and fulminant neurogenic pulmonary edema associated with high mortality [6]. Children who recover from brainstem encephalitis are left with significant neurologic sequelae [1–3, 6]. Therefore, large-scale EV71 outbreaks have a major impact on healthcare and daycare systems, and may cause widespread reactions in the population during epidemics. In the absence of effective treatment, the development of efficacious vaccines to prevent EV71 outbreaks has been a national priority in some Asian countries [3, 7, 8].

EV71 is an RNA virus that belongs to the human enterovirus A (HEV-A) species of the Picornaviridae family. Its single open reading frame codes for a polyprotein that contains 3 regions (P1, P2, and P3). P1 encodes 4 viral structural proteins (VP1–VP4) released by proteolytic cleavage. The VP1, VP2, and VP3 proteins are exposed on the virion surface in its crystal structure [9] and are responsible for immune responses and host–receptor binding. VP1 contains the major neutralization epitopes and is used in viral identification and evolutionary analyses. EV71 uses the human scavenger receptor class B, member 2 (hSCARB2) and the P-selectin glycoprotein ligand 1 (PSGL-1) among other minor cellular receptors (annexin-2, sialylated glycans, and heparin sulfate) to infect host cells, and infected cells produce both infectious particles and noninfectious defective particles.
Yamayoshi et al. [12] have recently reported that hSCARB2 is capable of viral binding, uncoating, and internalization, resulting in high EV71 infectivity in hSCARB2 cells, whereas the low infection efficiency of leukocyte-PSGL1-expressing cells is due to the inability of PSGL1 to induce viral uncoating.

CLINICAL AND MOLECULAR EPIDEMIOLOGY

EV71 was first isolated in California in 1969. Subsequently, endemic and large epidemic infections have occurred in different regions of the world [2, 3]. There is only 1 EV71 serotype, but based on VP1 gene phylogenetic studies, EV71 has been classified into 3 genotypes (A, B, and C). Genotype A contains only the prototype strain (BrCr). Genotypes B and C each have 5 subgenotypes (B1–B5 and C1–C5, respectively). A B0 subgenotype was retrospectively identified in the Netherlands [13]. The C4 genotype has been further classified into C4a and C4b lineages [3]. More recently, 3 additional genotypes including the Indian D genotype and 2 African genotypes (E and F) were identified, illustrating the wide genetic diversity of EV71 [14]. No association could be established between genotype and disease severity [15]. EV71 epidemics occur throughout the year but usually peak in summer months. However, the seasonal distribution and cyclical patterns (every 2–4 years) of outbreaks vary depending on the year and the country [15]. Epidemics (Figure 1) have occurred in the United States of America, Europe, Australia, and Asia [2, 3, 15]. Most countries use different case definitions, sample collections, data analysis, and laboratory testing procedures to report HFMD cases; therefore, disease burdens are likely underestimated.

Genotype A transiently reemerged in China in 2008 [16]. In contrast, genotypes B and C have continued to circulate and coexist around the world since the 1970s, causing outbreaks with CNS complications [15]. High mortality rates were observed during large epidemics of polio-like disease in Bulgaria in 1975 and of acute meningoencephalitis in Hungary in 1978, respectively [1–3, 6–8, 17]. Since the 1990s, recurrent HFMD epidemics with neurological complications and deaths have been reported in Austria, Australia, France, Germany, Greece, Hungary, Norway, the United Kingdom, and the United States [1–3, 6–8, 13, 17]. The most severe EV71 epidemics spread through the Asia-Pacific region including Australia, China, Hong Kong, Japan,
Malaysia, Singapore, Taiwan, and Vietnam (Figure 1) [2–3, 15, 17]. The first major EV71 outbreak occurred in 1997 in Malaysia where co-circulating neurovirulent B3, B4, C1, and C2 genotypes were responsible for 41 deaths among young children [18]. In Singapore, 30% of HFMD that affected thousands of children over a period of 7 years was caused by EV71 [11], and subgenotypes B5 and C2 caused the largest epidemic in 2008 [19]. Outbreaks of HFMD with severe neurological complications and herpangina that occurred in Japan between the late 1990s and 2013 were attributed to 4 co-circulating genotypes: B4, B5, C2, and C4 [3,20]. Since 2000, the Taiwan Centers for Disease Control has reported between 93 000 and 140 000 cases of HFMD and herpangina annually. EV71 was detected in 21% of all HEV-A isolates between 2000 and 2009 and was associated with 82% of severe cases. A major epidemic of EV71 subgenotypes C2 and B4 that caused 1.5 million infections and 78 deaths in 1998 was followed by smaller outbreaks of switching genotypes (Figure 1) [3, 21]. More than 7 million HFMD cases were reported in China between 2008 and 2012, of which 2457 were fatal [22]. The largest ever recorded epidemic occurred in 2009 with 1.1 million cases and 353 deaths and continued to escalate in 2010 and 2011 with >1.5 million cases, 27 000 neurological complications, and 905 deaths [22]. Severe pandemics associated with high mortality rates spread recently through Vietnam in 2011 and Cambodia in 2012 [15, 17].

**SWITCHING GENOTYPES AND GENETIC RECOMBINATION**

EV71 epidemics that occur cyclically every 2–4 years could be caused by a single genotype/subgenotype; however, the co-circulation of divergent isolates and unpredictable switching of genotypes and subgenotypes are frequently observed (Figure 1). Genotype C4 has persisted with progressive drift through time in China [22]. Intra–genotype B shifts from B3 to B4 (1997–2000) and B4 to B5 (2000–2003) have occurred in Malaysia. Sequential intergenotype shifts, from C2 to B4 then from C4 to B5, were observed in Taiwan [15, 21, 23]. The co-circulation of several EV71 genotypes and CVA16 during epidemics has been responsible for intratypic genetic recombination between the B and C genotypes in Taiwan and intertypic recombination between genotype C2 and CVA16 in China [2, 15, 23]. Another interserotypic recombination happened between EV71 genotype C2 and coxsackievirus A8 to create genotype B4, responsible for outbreaks in Japan and Taiwan in 1998 [15, 23]. The predominant C4a genotype may be a double recombinant virus among EV71 genotypes B, C, and CVA16 [15–16, 23]. Almost each major HFMD outbreak was correlated to genetic variations caused by EV71 switches [3, 15–16, 21, 23]. Thus, a continuous monitoring of antigenic variation and genetic evolution is critical for epidemic control and vaccine design.

**SUCCESSFUL DEVELOPMENT OF INACTIVATED EV71 VACCINES**

We [24] and Kung et al [17] recently reviewed the merits of experimental vaccines evaluated in animal models. Recombinant VP1 subunits expressed either in *Escherichia coli* or in the baculovirus expression system and formulated with complete Freud adjuvant/incomplete Freud adjuvant elicited neutralizing antibody responses in mice. Vaccination with DNA plasmid constructs encoding VP1 resulted in low neutralizing responses. Antisera raised by oral immunization with VP1 produced either by *Salmonella typhimurium* or *Bifidobacterium longum* were able to protect newborn mice against lethal EV71 challenge. Mice fed with VP1 produced in transgenic tomatoes developed low neutralization titers. Synthetic immunogens based on conserved, immunodominant neutralization epitopes of EV71 are safe and cost-effective, but they require strong adjuvant such as complete Freud adjuvant, which is not acceptable for human use to elicit neutralizing antibody titers. Vaccination of neonatal mice with an adenovirus vector expressing a conserved neutralization epitope conferred protection against lethal EV71 challenge. Immunization with virus-like particles produced either in the baculovirus expression system or *Saccharomyces cerevisiae* have elicited potent and cross-neutralizing immune responses in mice and monkeys, and conferred protection in neonatal mouse challenge models. Live-attenuated EV71 vaccines are still in an early development phase.

Only formalin-inactivated (FI) EV71 virion formulated in alum elicited satisfactory cross-neutralizing antibodies responses in experimental animal models. For regulatory, economic, and market acceptability reasons, FI-EV71 vaccines were selected for clinical development. Five inactivated EV71 vaccines have been rapidly developed in the past few years (Table 1). The Vaccine Research and Development (R&D) Center of the National Health Research Institutes (NHRI) of Taiwan produced a B4-based FI-EV71 vaccine (EV71vac) and launched the first human phase 1 clinical trial in adults in 2010. A single vaccine dose of 5 µg or 10 µg was safe and highly immunogenic [25]. It elicited 100% seroconversion in naive volunteers and strong virus neutralizing antibody (VNA) responses (geometric mean titer [GMT] = 210) against the vaccine strain as well as against the B1, B5, and C4a strains in 85% of the vaccinees [26]. In contrast, neutralizing responses against C4b and CVA16 were weak in 20% of the subjects, and 90% of the vaccinees did not develop any VNA against an atypical C2 strain. Inviragen (Takeda Pharmaceuticals Co Ltd) reports the results of a phase 1 trial in adults with an inactivated EV71 B2 vaccine (Table 1). All subjects who received 0.6 µg or 3 µg of vaccine at days 0 and 28 seroconverted and developed VNA GMTs of 323 and 452, respectively [17].

Inactivated EV71 vaccines based on different C4 isolates were independently developed and evaluated by 3 different Chinese

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### Table 1. Formalin-Inactivated Enterovirus 71 Candidate Vaccines Tested in Human Clinical Trials

<table>
<thead>
<tr>
<th>Organizations</th>
<th>Manufacturing Processes</th>
<th>Dosage of EV71 Antigen, µg</th>
<th>Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHRI (Taiwan)</td>
<td>Vero cell and EV71 B4 subgenotype (GMP-certified)</td>
<td>5 and 10</td>
<td>Adults 20–43 y (60) Phase 1 completed</td>
</tr>
<tr>
<td>Sinovac Biotech Co Ltd (China)</td>
<td>Vero cell and EV71 C4a subgenotype FY7VP5 strain</td>
<td>0.25, 0.5, and 1</td>
<td>Adults, children (&gt;5 y) Phase 1 completed</td>
</tr>
<tr>
<td></td>
<td>Cell factory/Bioreactor (SFM) Gel-filtration chromatography</td>
<td>1</td>
<td>Children (18–60 mo) Phase 2 completed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25, 0.5, and 1</td>
<td>Children (6–35 mo) (10 245) Phase 3 completed</td>
</tr>
<tr>
<td>Beijing Vigoo Biological Co Ltd (China)</td>
<td>Vero cell and EV71 C4a subgenotype H07 strain</td>
<td>0.4, 0.8, and 1.6</td>
<td>Adults, children (&gt;5 y) Phase 1 completed</td>
</tr>
<tr>
<td></td>
<td>Cell factory (SFM) Gel-filtration chromatography</td>
<td>0.8</td>
<td>Children (18–60 mo) Phase 2 completed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.8</td>
<td>Children (6–35 mo) (10 077) Phase 3 completed</td>
</tr>
<tr>
<td>CAMS (China)</td>
<td>Human diploid cell KMB-17 and EV71 C4a subgenotype H07 strain</td>
<td>0.25</td>
<td>Adults, children (&gt;5 y) Phase 1 completed</td>
</tr>
<tr>
<td></td>
<td>Cell factory (medium + serum) Gel-filtration chromatography</td>
<td></td>
<td>Children (18–60 mo) Phase 2 completed</td>
</tr>
<tr>
<td>Inviragen (Singapore)</td>
<td>Vero cell and EV71 B2 subgenotype</td>
<td>0.6 and 3</td>
<td>Adults (36) Phase 1 completed</td>
</tr>
</tbody>
</table>

Abbreviations: CAMS, Chinese Academy of Medical Sciences; EV71, enterovirus 71; GMP, Good Manufacturing Practices; NHRI, National Health Research Institutes, Taiwan; SFM, serum-free medium.

* The antigen dosage is calculated based on the report by Liang et al [8] that the specific activity of the EV71 antigen reference standard established in China is 421.1 U/µg.

### Table 2. Clinical Data From Enterovirus 71 Vaccine Phase 3 Trials

<table>
<thead>
<tr>
<th>Organization</th>
<th>No. of Sites</th>
<th>Cohort Size</th>
<th>Target Population (Age)</th>
<th>Dosage*</th>
<th>SAE in Vaccinees (Controls), %</th>
<th>Seroconversionb,</th>
<th>VNA, GMT</th>
<th>HFMD</th>
<th>EV71-Related Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinovac Biological Co Ltd</td>
<td>Jiangsu Province: 3 Beijing: 1</td>
<td>10 245</td>
<td>Children (6–35 mo)</td>
<td>400</td>
<td>1.2 (1.5)</td>
<td>88.1 (day 56)</td>
<td>191 (1 y)</td>
<td>94.8</td>
<td>88.0</td>
</tr>
<tr>
<td>Beijing Vigoo Biological Co Ltd</td>
<td>Jiangsu Province: 3</td>
<td>10 077</td>
<td>Children (6–35 mo)</td>
<td>320</td>
<td>2.2 (2.6)</td>
<td>91.7 (day 56)</td>
<td>92 (1 y)</td>
<td>90</td>
<td>80.4</td>
</tr>
<tr>
<td>CAMS</td>
<td>Guangxi Province: 2</td>
<td>12 000</td>
<td>Children (6–71 mo)</td>
<td>100</td>
<td>1.1 (2.1)</td>
<td>100 (day 56)</td>
<td>170 (4 wk)</td>
<td>97.4</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CAMS, Chinese Academy of Medical Sciences; EV71, enterovirus 71; GMT, geometric mean titer; HFMD, hand, foot, and mouth disease; SAE, serious adverse event; VNA: virus neutralizing antibody.

* The antigen dosage is expressed in units per 0.5 mL. The Chinese reference standard is 421.1 U/µg.

* Seroconversion is based on a 4-fold increase in baseline neutralizing antibody titer.
within 24 hours and a grade 3 fever. The rate of seldom- reported serious adverse events (SAEs) in vaccinees was not different from that observed in the control groups and SAEs were not causally related to vaccination. The Vigoo vaccine [27, 28], was >90% efficacious against EV71-related HFMD and >80% protective against EV71-associated serious diseases including herpangina. In the Sinovac trial, the incidence rate of EV71-associated disease was 0.3% vs 2.1% in the control group, corresponding to an 89.3% efficacy [27–29]. In the Chinese Academy of Medical Sciences study [30], the seroconversion rate was 100% after 2 vaccinations, with a VNA GMT of 170.6. The vaccine was 97.4% efficacious against EV71-related diseases. All C4-based vaccines prevented herpangina and EV71-associated hospitalizations. Immune sera from subjects immunized with the Vigoo and Sinovac vaccines cross-neutralized the circulating EV71 genotypes and subgenotypes (B4, B5, C2, C5) associated with epidemics in recent years [31]. Furthermore, preexisting antibodies due to stealth infections of young children did not interfere with vaccine efficacy against different EV71 genotypes [31]. However, the vaccines did not protect against CAV16 [26, 27]. Interestingly, the VNA titers decreased by half after 6 months, but this waning did not affect vaccine efficacy [27]. Most important, phase 3 results suggest that a VNA titer of 1/16 can serve as a correlate of protection against EV71-related HFMD [17, 27, 31]. Despite differences in seed strains and manufacturing processes, C4-based vaccines have shown batch consistency and efficacy [32], which should facilitate their licensure and market entry in China if there were no issues regarding vaccine stability, manufacturing capacity, and production cost, for which information is not available.

CHALLENGES FOR EV71 VACCINE REGISTRATION

Both C4-based and B4-based antibodies cross-neutralized the current circulating EV71 isolates [26–31], but the B4 vaccine poorly neutralized an atypical C2 strain [26]. In addition, no FI-EV71 vaccines protected against CVA16, which is predominantly responsible for annual HFMD outbreaks [26, 29, 30]. It is likely that FI-EV71 vaccinations may not significantly reduce the number of clinical cases of HFMD during outbreaks. In this regard, promising experimental bivalent FI-EV71/FI-CVA16 vaccines are being developed and have elicited balanced protective responses against both viruses [33, 34]. Furthermore, because of the risk of intertypic and intratypic recombination and the possible emergence of new strains, only results from multinational efficacy trials will reveal if a monovalent vaccine can elicit broad protection against divergent epidemic viruses. Asian countries have not yet harmonized their HEV-A surveillance systems. A global surveillance network for enterovirus outbreaks similar to the World Health Organization global influenza surveillance and response system is urgently needed to monitor immune responses to EV71 vaccines in the future.

The phase 3 trials have clearly shown that humoral immunity correlates with protection but wanes after the first 6 months, raising the issue of persistence of cross-protective antibody levels. The fast waning of VNA responses during natural infection may explain the outbreak of epidemics caused by new EV71 genotypes or subgenotypes emerging every 2–4 years. Because a booster injection 1 year after vaccination elicited a >10-fold increase in neutralizing antibody titer [26], a third immunization at 18–24 months is highly recommended for long-lasting protection. The development of mucosal vaccines that are attractive may not be necessary, as parenteral immunization confers protection. Prospective studies should be conducted during EV71 and coxsackievirus epidemics to assess the role of cellular immunity in long-term cross-protection and viral pathogenesis. In addition, longitudinal studies are necessary to evaluate the role of efficacious EV71 vaccines in controlling antigenic shift, virus fitness, and the emergence of new viruses.

In addition to a global surveillance network, standardized animal models, which are necessary to understand EV71 pathogenesis and evaluate the potency and consistency of vaccine batches, are not yet available [35, 36]. Macaques develop antibody responses to EV71 vaccines, similar to those observed in human; however, they are not suitable to study neurovirulence and pulmonary edema complications, and their use is limited by ethical and economic considerations [35]. Neonatal suckling mice and immunodeficient animals have been widely used in evaluating the protective efficacy of EV71 vaccine candidates, but they do not mimic human infections [35]. The NHRI [36] and Fujii et al [37] have successfully developed transgenic mice carrying the human receptor hSCARB2. HFMD-like skin rashes were observed in transgenic animals infected with B4 and B5 clinical EV71 isolates, and severe limb paralysis and death occurred in animals inoculated with a C2 strain [36]. The presence of EV71 in tissues and CNS was accompanied by the upregulation of proinflammatory mediators (CXCL10, CCL3, tumor necrosis factor–α, and interleukin 6) and correlated with the recruitment of T lymphocytes and disease severity [36]. In addition, passive administration of the monoclonal anti-EV71 VP1 neutralizing antibody N3 [38] reduced symptoms induced by EV71 B5 infection and protected the transgenic mice against EV71 C2-induced severe limb paralysis and death. The transgenic mouse model [36, 37], once standardized, will be useful...
to assess the cross-protective ability of vaccines against EV71 using hSCARB-2 as receptor and to evaluate immunotherapeutic strategies.

ECONOMIC CONSIDERATIONS

An ideal EV71 vaccine should be inexpensive, safe, compatible with large-scale production, easy to administer, and acceptable to parents. Companies in the Asia-Pacific region have little capability to take a new vaccine from research to product launch, so cooperation between R&D institutes and both Asian and global companies are urgently needed to improve and scale up the current manufacturing processes for broad approval by regulatory authorities. Due to intellectual property rights and proprietary technologies, information on the influence of culture medium and production systems on vaccine yields is totally missing. Both the roller-bottle and cell factory technologies used in producing current clinical lots are easy to implement and operate, although labor intensive. Developing countries could start implementing these technologies first and subsequently optimize the manufacturing processes for large-scale vaccine production.

Clinical trials have revealed that administration of 2 doses of 400 units (1-μg dose) of EV71 vaccine achieved efficacy [27–29]. We have recently reported [24] that a 40-L pilot-scale production batch could yield 50 000 1-μg doses of FI-EV71 at a cost of US$0.4/dose, which translates into 200 000 doses of the lowest C4-based protective dose (0.25 μg) of vaccine tested in a phase 3 trial [30] at US$0.1/dose. However, the large-scale production of EV71 vaccines will require an improvement of the current manufacturing processes. The use of bioreactors, microcarriers, and perfusion technology could increase cell growth and virus yield by 1 order of magnitude. To lower the production cost of FI-EV71 vaccines, a simple and efficient downstream chromatographic purification step could be optimized to copurify immunogenic defective and infectious virus particles [24]. Lee et al [39] forecasted that routine immunization with a 70% efficacious EV71 vaccine sold at US$25 per dose would be of great economic value. With this profit margin and the new emerging vaccine markets in the Asia-Pacific region, the global vaccine companies should become interested in manufacturing EV71 and future multivalent HFMD vaccines.

CONCLUSIONS

Based on the current phase 3 results, national EV71 immunization programs should be implemented in China to build strong herd immunity. A 2-dose regimen with FI-EV71 vaccines should be given to children at 6–7 months of age, with a third dose at 18–24 months. Multinational efficacy trials should be conducted to evaluate the degree of cross-immunity against circulating genotypes and subgenotypes. A global HFMD surveillance network should be established, and continuous epidemiological surveillance is critical to identify and detect the potential emergence of new EV71 variants. Harmonization and standardization of vaccine strain, quality control reagents and immunoassays, and animal models at the international level is urgently required to evaluate the potency of vaccine candidates and determine which manufacturing process yields the most potent and most affordable products. Although the coadministration of FI-EV71 with the commercial pediatric pentavalent vaccine Pediacel (Sanofi Pasteur) did not affect antibody responses against its individual components, clinical trials should be conducted to determine whether EV71 vaccines should ultimately be combined with Expanded Programme on Immunization vaccines [40].

Notes

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