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An inactivated enterovirus 71 vaccine is safe and immunogenic in healthy adults: A phase I, double blind, randomized, placebo-controlled, study of two dosages

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ABSTRACT

Background: Hand, foot and mouth disease (HFMD), especially that caused by *enterovirus* 71 (EV71) infection, is a public health concern in the Asia-Pacific region. We report a phase I clinical trial of an EV71 candidate vaccine (INV21) based on a binary ethylenimine inactivated B2 sub-genotype formulated with aluminum hydroxide.

Methods: In this double-blind, placebo-controlled, randomized, dose escalation study adult volunteers received two vaccinations 28 days apart of low or high dose formulations of the candidate vaccine and were then monitored for safety and reactogenicity for four weeks after each dose, and for their immune responses up to 28 weeks.

Results: Of 36 adults enrolled, 35 completed the study as planned. Either no or mild adverse events were observed, mainly injection site pain and tiredness. Seroconversion was 100% after two vaccinations. High geometric mean neutralizing antibody titers (GMT) were observed 14 days post first dose, peaking 14 days post second dose (at Day 42) in both high and low dose groups; GMTs on days 14, 28, 42, and 56 were 128, 81, 323, 203 and 144, 100, 451, 351 in low- and high-dose groups, respectively. Titers for both doses declined gradually to Day 196 but remained higher than baseline and the placebo groups, which had low GMTs throughout the duration of the study. Cross-neutralizing antibody activity against heterologous sub-genotypes was demonstrated.

Conclusion: These data show that the EV71 candidate vaccine is safe and immunogenic in adults and supports further clinical development as a potential pediatric vaccine by initiating a dose-escalation study for determining the dose-dependent safety and immunogenicity of the vaccine in young naïve children.

1. Introduction

Enterovirus 71 (EV71) is one of the major causes of hand, foot and mouth disease (HFMD). It is a major public health concern in the Asia-Pacific region, but outbreaks have also been reported in Europe and North America, affecting mainly infants and young children [1,2]. The disease is usually manifested as lesions in the hands, feet and oral cavity. However, during outbreaks increasing numbers of severe cases have been recognized, with a range of neurologic symptoms including meningitis, encephalitis, myocarditis and polio-like paralysis, even leading to fatality [3,4]. There have been recent severe outbreaks in Southeast Asia [5] which have raised a great deal of concern in the region. In 1998, an outbreak in Taiwan claimed 78 lives [6]. The number of fatal cases per year in China has reached above 3000 since 2008 [7]. The overall fatality rate of HFMD has been estimated to be 229.9 fatalities per 100,000 symptomatic cases [8].

There is no specific licensed therapy for EV71 infection and treatment is currently restricted to management of the symptoms.

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[1] This paper is dedicated to our coauthor Dan Stinchcomb, who died on February 21, 2018.

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Prevention is reliant upon surveillance and isolation of infected individuals together with enhanced infection control. However, several strategies are currently being applied to develop vaccine candidates, some of which have resulted in commercialization [9].

EV71 is an RNA-virus, of the *Picornaviridae* family, with four capsid proteins (VP1-4) and seven nonstructural proteins. Variation in the VP1 protein has led to sub-classification into genogroups A, B and C, but the different VP1 genes share > 92% sequence homology [10], and animal studies have indicated that there is one serotype with respect to *in vivo* sero-neutralization [11]. EV71 has one single serotype as determined from hyperimmune animal sera. However, post-infection sera from children showed that different genotypes have antigenic variations among different EV71 strains [12,13]. The antigenic differences were determined through antigenic cartography since level of cross-reactivity varied within subgenotypes and genotypes to different levels. Several vaccine candidates are currently in development using different approaches [14], based on VP1 peptides [15], recombinant VP1 delivery [16,17], VP1 capsid-containing virus-like particles (VLP) [18], DNA vaccines [19], live attenuated EV-71 virus [20] or whole-virus vaccines using formaldehyde-inactivation [21]. The adjuvanted, inactivated, whole virus EV71 vaccines [14] are the most advanced in clinical development. In phase I and phase II clinical studies, those vaccines have been shown to induce high levels of neutralizing antibodies in adults and children [22–24]. Phase III clinical studies conducted in China have demonstrated that inactivated whole virus vaccines based on the C4 EV71 genogroup are efficacious, preventing severe, EV71 associated HFMD in children [23,25,26]. Presently, three inactivated EV71 vaccine products based on C4 sub-genotype have been licensed and are currently being marketed in China [9,27,28], while a genotype B based vaccine is under development in Taiwan [29]. Both B and C genotype viruses circulate frequently in many other countries, including Japan, Australia, Vietnam, Singapore and the US, highlighting the need for a vaccine which elicits cross-reactive immunity against strains from both genotypes [37,48,49].

In preclinical studies in mice and rabbits, we have demonstrated the safety and immunogenicity of a B2 genogroup EV71 virus particle candidate vaccine, inactivated with binary ethylenimine (BEI) and purified by ion-exchange and size-exclusion chromatography [30]. The rationale of selecting B2 subgenogroup is the severity of disease and neurovirulence caused by the virus and the capability of the virus to grow to high titers in cell culture. Moreover, there are not many EV71 vaccine candidates representing the B subgenotypes. BEI was used as the inactivating agent due to its superiority over beta-propiolactone (BPL) and formalin in preserving the antigenicity of the vaccine and faster inactivation kinetics [39,40]. This report describes the first phase I study of the safety and immunogenicity of two dose levels of the alum-adjuvanted EV71 vaccine candidate, termed INV21.

2. Methods

2.1. Study design

This was a single center, double blind, placebo controlled, randomized, dose escalation study to investigate the safety, tolerability and immunogenicity of two different doses of an investigational EV71 candidate vaccine, INV21, in healthy adults. The trial was performed from July 2011 to April 2012 at the Investigational Medicine Unit, National University Hospital, Singapore. The study protocol was approved by the site Domain Specific Review Board of the National Healthcare Group, which governs research in the institution (Reference number 2011/00249), and was registered as ClinicalTrials.gov NCT01376479. All subjects provided written, informed consent at enrolment, and the study was carried out according to current GCP and the Declaration of Helsinki.

2.2. Subjects

Eligible subjects were males or females aged 21–45 years, inclusive, in good health as determined by medical history and physical examination at the time of screening. At the screening visit potential subjects had a physical examination, ECG, laboratory testing for Na, K, Cl, Glucose, BUN, creatinine, phosphate, calcium, protein, albumin, alanine transaminase (ALT), aspartate transaminase (AST), bilirubin, white blood cell (WBC), hemoglobin, platelets and urinalysis. Normal ranges for laboratory tests were those used in the clinical laboratory; subjects with significant values outside of these ranges being excluded. All subjects had negative serology for HIV, Hepatitis C and Hepatitis B surface antigen. Other exclusion criteria included any chronic health condition, e.g. diabetes mellitus, history of hypersensitivity to any vaccine, prior vaccination within four weeks of the study, or any congenital condition or therapeutic treatment likely to lead to immunodeficiency. Female participants had to have a negative urine pregnancy test and were required to practice contraception by an approved method throughout the study period.

In each cohort 45 volunteers who met the above criteria were screened for their levels of EV71 antibodies, and the 18 subjects from each cohort with the lowest neutralizing antibody titers were included in the study.

2.3. Vaccine

The investigational product (INV21) consists of inactivated EV71 whole viral particles from a B2 sub genogroup EV71 strain closely related to the EV71/7423/MS/87 strain [28], formulated with 1 mg/mL aluminum hydroxide (Alhydrogel® 85, Brenntag) in phosphate buffered saline, pH 7.5 containing 0.002% (v/v) Tween 80. Two vaccine formulations with either 1.2 μg/mL (low dose) or 6.0 μg/mL (high dose) INV21 were supplied in sealed 3 mL vials. Placebo vials contained 1 mL phosphate buffered saline. The high and low dose were selected based on the literature for other EV71 vaccine candidates as well as a Japanese Encephalitis (JE) vaccine study. Preclinical studies in mice were also conducted to select the high and low dose for immunization of humans [30].

On Day 0, enrolled subjects received an intramuscular injection in the deltoid of 0.5 mL of the appropriate vaccine dose (n = 12) or placebo (n = 6) according to a sponsor-supplied randomization list. All injection syringes were prepared by independent unblinded study personnel and provided to the investigator for administration such that the investigator remained blind to the product administered. A second injection was similarly administered on Day 28. All laboratory and serology analyses were performed blind.

2.4. Safety and tolerability

Adverse events were assessed throughout the duration of the study, up to Day 196. In each cohort, there was a sentinel subject, who received the prime dose of INV21 vaccine before the rest of the cohort. The sentinel subject was observed for two days to assess safety prior to dosing the remaining volunteers in the cohort. Escalation to the higher dose was done based on review of the safety data through 14 days following the prime dose in the low dose cohort from a minimum of 12 subjects, including the sentinel. Similarly, there was review of 14 days of safety data following the prime dose in the high dose group from a minimum of 12 subjects including the sentinel to determine whether this cohort was to receive their booster dose. Subjects were given diaries to be completed for 14 days following each vaccination to

record solicited local reactions and systemic adverse events, and any other adverse events occurring before the subsequent clinical visit, up to Day 56. At the clinical visits, the investigators assessed the likely relationship of any adverse events to the immunizations.

Blood chemistry was monitored for toxicity grade change according to standard vaccine trial procedures (US Food and Drug Administration, FDA) and reported according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 4, 2009 [31,32].

2.5. Immunogenicity

Sera prepared on Days 0 (pre-dose 1), 14, 28 (pre-dose 2), 42, 56, 84 and 196 were used to assess the immune responses to the vaccine antigen, EV71 (sub-genotype B2). Neutralizing antibody titers were measured by standard methods as previously described [30] at a starting dilution of 1:8.

2.6. Cross-neutralization test

A standard ELISA-based microneutralization test (ELISA-MNT) was used with modification [33,34]. Sera were tested at a starting dilution of 1:32 and any reciprocal titer less than 32 was given a value of 8 for calculation of GMTs. Details of the procedure is described in the supplementary section.

2.7. Statistics

As this was an exploratory trial there was no specific hypothesis to test, and the number of subjects was chosen to provide data from a minimal dataset for safety and tolerability. The study was not powered to test for differences between the two doses of vaccine. Results are summarized with point estimates and 95% confidence intervals (CIs). Safety data were analyzed in terms of all subjects who received at least one study injection, and who provided safety data, according to their assigned treatment group. Chi square test was used to compare AE in groups when relevant. The neutralizing antibody titers (NT) were transformed into log10 titers for calculation of geometric mean titers (GMT) and 95% CI and back transformed to NT. Percentages of each group displaying seroconversion (defined as a four-fold or greater increase in titer compared with the baseline [Day 0] titer), were calculated for each group at each time point. ANOVA was used to compare immunogenicity between the dose groups and day. All statistical data analyses were done using SAS JMP (version 13.1.0).

3. Results

3.1. Demographics

A total of 90 eligible 21–45 year-old subjects were screened for possible inclusion, from which the 36 eventual participants with the lowest pre-existing EV71 titers, 23 men and 13 women, were selected (Fig. 1). All enrolled subjects were Asian, with similar demographic characteristics in each group (Table 1), however there was a greater proportion of males allocated to the two placebo groups by random chance. All subjects completed the study, except for one subject in the high-dose INV21 group who withdrew consent before receiving the second dose.

3.2. Safety and tolerability

Injections of investigational vaccine or placebo were generally well tolerated; there were no serious adverse events (SAE) or withdrawals due to adverse events during the study. A total of 42 unsolicited AEs were reported by 22 of the 36 subjects across all study groups up to Day 56; 11 subjects in the low-dose group reported 22 AEs, and 11 subjects in the high-dose group reported 20 AEs. All AEs were considered to be mild in severity, except for a case of hemorrhoids (of moderate severity) in a high-dose subject (starting on Day 8 after dose 1, and lasting two days). Only two unsolicited AEs were considered to be associated with study procedures, both in placebo recipients in the low-dose group: one case of musculoskeletal stiffness (stiff neck) lasting one day and one of headache lasting two days.

The most frequently reported solicited local reaction was pain at the injection site (Table 2), which was statistically significantly [$p < 0.05$] more frequent with either dose of INV21; 10 of 12 subjects (83.3%, 95% CI: 55.2–95.3) and 9 of 12 (75.0%, 95% CI: 46.8–91.1) for low- and high-dose recipients, respectively, than placebo 3 of 12 (25.0%, 95% CI: 8.9–53.2). The only apparent difference in local reactogenicity profiles between low- and high-doses of INV21 was the rate of swelling at the injection site ($p < 0.05$); no case was reported in the low-dose group compared with 4 out of 12 subjects (33.3%, 95% CI: 13.8–60.9)) in the high-dose group. However, compared with the placebo group, there was no significant differences ($p = 0.11$) in injection site swelling. One subject in each of the low- and high-dose groups reported erythema or pruritis. These local reactions were mild and transient, occurring within 1 to 2 days of vaccination, and all resolved spontaneously and promptly without sequelae.

The more common systemic reactions were muscle pain and tiredness. There were numerically more reports of muscle pain in the vaccinated groups; 6 of 12 subjects (50.0%, 95% CI: 25.4–74.6) and 5 of 12 (41.7%, 95% CI: 19.3–68.0), respectively, than in placebo; 2 of 12 (16.7%, 95% CI: 4.7–44.8). A similar trend was observed for tiredness; 5 of 12 (41.7%, 95% CI: 19.3–68.0) in both vaccinated groups vs. 3 of 12 (25%, 95% CI: 8.9–53.2) in placebo recipients. The only two reports of nausea were reported in high-dose recipients. However, all these systemic reactions did not show any statistical significance between vaccinated and placebo groups.

Observed vital signs (pulse rate, respiratory rate and blood pressure) remained constant during the post-vaccination period and did not show any differences between treatment groups. Temperature did slightly increase over 30 min post-vaccination in both vaccinated groups (by 0.24 °C and 0.55 °C in low and high dose groups, respectively) and in the placebo group (by 0.42 °C). All increases in temperature were transient and did not require treatment.

Blood chemistry tests did not indicate any clinically significant changes after either vaccine or placebo administration, with small transient changes in some parameters that did not show any particular pattern or dose dependency association (Tables 3A and 3B). One grade 4 event was noted, in a low-dose INV21 subject with low blood glucose (3.5 mmol/L) at baseline. Glucose returned to the normal range (4–7.8 mmol/L) between Days 7 and 35, but then dropped to 3.4 mmol/L before reaching 8.8 mmol/L at Day 56. This was graded as 4 due to the difference between baseline and Day 56 levels according to the protocol. However, this event was not reported as an adverse event as the patient remained well and asymptomatic, and it was not considered clinically significant.

3.3. Immunogenicity

At screening, most subjects in the two vaccine groups had some evidence of pre-existing EV71 antibodies (Table S1). Only 2 of the 36 subjects (5.6%, 95% CI: 1.5–18.1) had a titer < 8. However, antibody levels were generally low with 29 of 36 (80.6%, 95% CI: 65.0–90.2)) subjects having a titer ≤ 64, and a maximum titer in one subject was 256. GMTs on Day 0 were similar in all four groups, with a range from 20 to 32. GMTs in the placebo group showed no significant differences over the next 28 weeks (Fig. 2, Table S2).

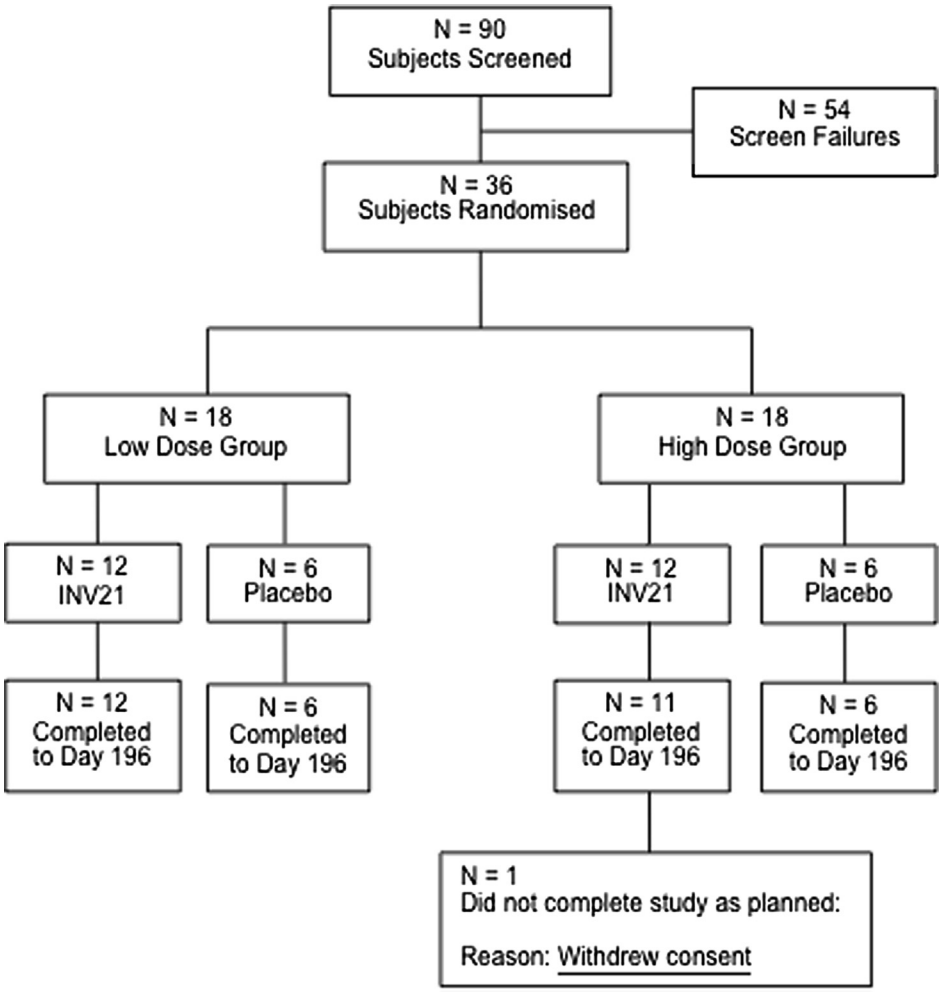

Fig. 1. Subject disposition.

Table 1
Summary of Demographics of the study population.

	Low dose		High dose	
	INV21	Placebo	INV21	Placebo
N	12	6	12	6
Gender, Male (%)	5 (42%)	5 (83%)	7 (58%)	6 (100%)
Age (years) Mean ± SD	33.7 ± 7.9	31.2 ± 5.5	30.9 ± 5.8	30.2 ± 7.2
Height (m) Mean ± SD	1.63 ± 0.11	1.70 ± 0.08	1.66 ± 0.09	1.71 ± 0.6
Weight (kg) Mean ± SD	61.1 ± 13.6	68.3 ± 10.9	62.1 ± 9.2	70.0 ± 10.1
BMI (kg/m^2) Mean ± SD	22.62 ± 2.52	23.62 ± 2.21	22.53 ± 2.83	23.83 ± 2.10

However, there were marked increases in GMTs in both vaccine groups by Day 14, increasing to 128 (95% CI: 32–509) and 144 (95% CI: 30–690) in low- and high-dose groups, respectively. GMTs had declined slightly in both groups by Day 28, immediately prior to the second dose, low-dose to 81 (95% CI: 23–290) and high-dose to 100 (95% CI: 21–481), but this was not statistically significant. The second dose boosted the GMTs to 323 (95% CI: 144–721) and 451 (95% CI: 163–1247) at Day 42. Although levels continued to decline over the following weeks, GMTs remained higher than baseline at 28 weeks: 81 (95% CI: 32–204) and 145 (95% CI: 54–393) for low- and high-dose, respectively. Both low- and high-dose showed no significant differences to their respective day.

When expressed as seroconversion, i.e. the percentage of subjects that achieved a four-fold or greater increase in titer over baseline, there was an evident benefit from the second dose (Fig. 3, Table S3). Two weeks after the first dose 58.3% (95% CI: 32.0–80.7) of subjects in both vaccine groups had seroconverted, these proportions declining slightly over the next two weeks before the second dose was administered on Day 28. At Day 42, two weeks after the second dose the seroconversion rate was 100% (95% CI; 75.8–100) in both groups. Over the following 22 weeks, there was an overall decrease in the level of GMT titer, but it remained >4 times higher than the pre-vaccination level. One subject in the placebo group seroconverted at Day 84.

3.4. Cross-neutralizing activity of antibodies elicited against EV71 vaccine with EV71 sub-genotypes B4, B5 and C4a

To examine if antibodies induced by the EV71 candidate vaccine (MS/87) based on the B2 sub-genotype could cross-neutralize

Table 2
Solicited local and systemic reactions after first and second doses combined.

Reaction	% (95% CI) of participants reporting adverse reaction					
	Low Dose		p-value	High Dose		p-value
	INV21 (n = 12)	Placebo (n = 6)		INV21 (n = 12[a])	Placebo (n = 6)	
Injection Site Reaction						
Pain	83.3 (55.2–95.3)	33.3 (9.7–70.0)	**0.03**	75.0 (46.8–91.1)	16.7 (3.0–56.4)	**0.02**
Edema	0.0 (0.0–24.3)	0.0 (0.0–24.3)		33.3 (13.8–60.9)	0.0 (0.0–24.3)	0.11
Erythema	8.3 (1.5–35.4)	0.0 (0.0–24.3)	0.47	8.3 (1.5–35.4)	0.0 (0.0–24.3)	0.47
Pruritis	8.3 (1.5–35.4)	0.0 (0.0–24.3)	0.47	8.3 (1.5–35.4)	0.0 (0.0–24.3)	0.47
Systemic Reaction						
Muscle Pain	50.0 (25.4–74.6)	33.3 (9.7–70.0)	0.50	41.7 (19.3–68.0)	0.0 (0.0–24.3)	0.06
Tiredness	41.7 (19.3–68.0)	33.3 (9.7–70.0)	0.73	41.7 (19.3–68.0)	16.7 (3.0–56.4)	0.29
Headache	8.3 (1.5–35.4)	16.7 (3.0–56.4)	0.60	33.3 (13.8–60.9)	16.7 (3.0–56.4)	0.46
Nausea	0.0 (0.0–24.3)	0.0 (0.0–24.3)		16.7 (4.7–44.8)	0.0 (0.0–24.3)	0.29
Rash on Body	25.0 (8.9–53.2)	0.0 (0.0–24.3)	0.18	8.3 (1.5–35.4)	0.0 (0.0–24.3)	0.47

[a] N = 11 for second dose, one subject withdrew 14 days after first dose.

Table 3A
Summary of frequently reported toxicity grade shifts from baseline in low dose.

	Low Dose							
	INV21 (n = 12) No. of Grades % (95% CI) of participants				Placebo (n = 6) No. of Grades % (95% CI) of participants			
	1	2	3	4	1	2	3	4
Serum chemistry								
Glucose (inc)	58.3 (32.0–80.7)	16.7 (4.7–44.8)		8.3 (1.5–35.4)	50.0 (18.8–81.2)	16.7 (3.0–56.4)		
Glucose (dec)	8.3 (1.5–35.4)	16.7 (4.7–44.8)			16.7 (3.0–56.4)	16.7 (3.0–56.4)		
Potassium (dec)	33.3 (13.8–60.9)				16.7 (3.0–56.4)			
Sodium (inc)	25.0 (8.9–53.2)				50.0 (18.8–81.2)			
Hematology[*]								
Haemoglobin (dec)	83.3 (55.2–95.3)		8.3 (1.5–35.4)		100.0 (61.0–100.0)			
Urinalysis[*]								
Urine Red Blood Cells	25.0 (8.9–53.2)					16.7 (3.0–56.4)		

Table 3B
Summary of frequently reported toxicity grade shifts from baseline in high dose.

	High dose							
	INV21 (n = 12) No. of Grades % (95% CI) of participants				Placebo (n = 6) No. of Grades % (95% CI) of participants			
	1	2	3	4	1	2	3	4
Serum chemistry								
Glucose (inc)	25.0 (8.9–53.2)	8.3 (1.5–35.4)			16.7 (3.0–56.4)	16.7 (3.0–56.4)		
Glucose (dec)	16.7 (4.7–44.8)	25.0 (8.9–53.2)			16.7 (3.0–56.4)	16.7 (3.0–56.4)		
Potassium (dec)		8.3 (1.5–35.4)						
Sodium (inc)	8.3 (1.5–35.4)				100.0 (61.0–100.0)			
Hematology[*]								
Haemoglobin (dec)	75.0 (46.8–91.1)	8.3 (1.5–35.4)			66.7 (30.0–90.3)			
Urinalysis[*]								
Urine Red Blood Cells	33.3 (13.8–60.9)		8.3 (1.5–35.4)		66.7 (30.0–90.3)			

[*] Laboratory results where toxicity was noted for greater than 20% of subjects in any dose group [a] N = 11 for second dose.

other EV71 sub-genotypes, we assessed the neutralizing activity in the ELISA-MNT assay of sera collected on day 42 post-vaccination against EV71 viruses belonging to sub-genotypes B4, B5 and C4a. Most individuals in the placebo groups did not show any detectable neutralizing titer (data not shown). Serum samples from both the low- and high-dose groups against homologous sub-genotype showed similar neutralizing activity (low-dose, 226 (95% CI: 94–544); high-dose, 362 (95% CI: 120–1090) with no statistical differences. In the vaccinated groups neutralizing titers against sub-genotype B4 were comparable to the homologous titers, although some individuals showed lower titers than other: low-dose, 99 (95% CI: 22–442); high dose, 97 (95% CI: 20–475) (Fig. 4, Table 4 and Table S4). The neutralizing activities against a B5 sub-genotype (7599) and two C4a sub-genotypes (4104 and 41) were all similar to the homologous titers. Similarly, the neutralizing titers against a third C4a sub-genotype (VN080) isolated from a different geographic location were slightly lower but within two-fold difference (low dose, 136 (95% CI: 50–374); high dose, 208 (95% CI: 62–701). Overall, these data indicate that antibodies induced by EV71 vaccine cross-neutralize EV71 viruses belonging to sub-genotypes B4, B5 and C4a. Both high and low dose showed no statistical differences respectively to each sub-genotype.

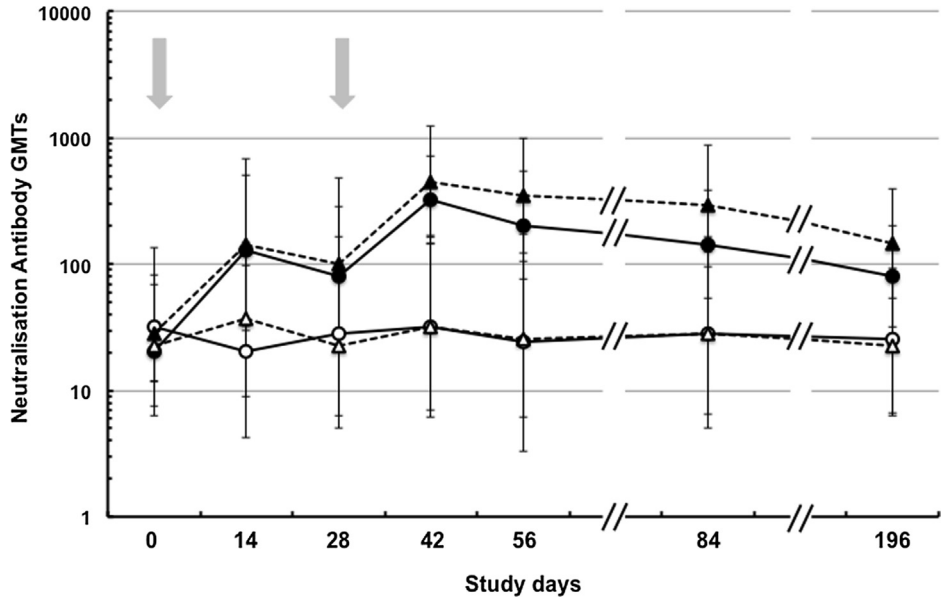

Fig. 2. Geometric mean titers (with 95% Confidence intervals bars) of neutralizing antibodies over the course of the study after two doses indicated by arrows of placebo (open symbols) or vaccine (closed symbols) in the groups receiving low (circles) or high (triangles) doses of INV21 (N = 12 for vaccine groups, N = 6 for placebo groups).

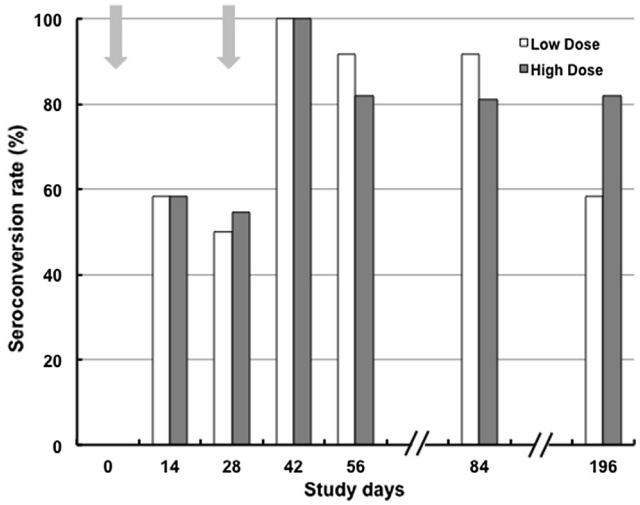

Fig. 3. Mean seroconversion rates (≥4-fold increase over baseline) as percentages of the two vaccine groups over the study period, following two doses of vaccine on Days 0 and 28 as (indicated by arrows) in low and high dose groups.

The higher dose vaccine did not result in higher titers than the lower dose for B4 and B5 subgenotypes, and although there was increase in titer against the homologous B2 subgenotype with the higher dose the difference did not achieve statistical significance. Since some individuals had lower titers than others due to preexisting antibodies, a clear-cut increase in titer was not observed. Additionally, the assay used for the cross-reactivity study and small number of samples could have played a role in this result. A titer of 8 was assigned to all titers those were <32 which resulted in low titers for B4 and B5. When those individuals are taken out of calculation, the titers slightly increased for B4 [(low dose, 256; high dose, 282.6) and B5 (low dose, 256; high dose, 322.5) (compare with Table 4)]. Although there was slight increase in titers, there was no significant statistical difference. Further investigation in a larger population of naïve children is required to see the clear effect of high dose.

We also tested the longevity and persistence of cross-neutralizing antibodies by comparing the titers of selected serum samples collected on day 196 post-vaccination with those from the same individual collected on day 42 post-vaccination against sub-genotypes B4, B5 and C4a. Although there was a small overall drop in titer against all genotypes by day 196 post-vaccination, this was not statistically significant and titers remained above the baseline indicating the persistence of neutralizing activity (Fig. 5).

4. Discussion

The EV71 enterovirus poses significant public health problem especially in Asia that warrants development of a safe and effective vaccine. Infection typically manifests as hand, foot and mouth disease similar to other enteroviruses, usually in young children, but more severe cases with serious complications in the central nervous system and fatalities have been reported in outbreak situations [1]. The present study explored the safety and immunogenicity of two doses of an inactivated EV71 whole viral candidate vaccine (INV21) in a single center, double blind, placebo controlled, randomized, dose escalation study in adult volunteers; the first use of this investigational vaccine in humans. EV71 has several genotypes, characterized by VP1 sequences, but has only one serotype in neutralization tests [10,11]. The INV21 candidate vaccine includes viral particles of a B2 sub genogroup EV71 strain closely related to the EV71/7423/MS/87 strain [30]. Previous animal studies have demonstrated the immunogenicity of INV21 in mice and rabbits, with no toxicology or safety signals [30]. In this Phase I clinical study in humans, the vaccine was well tolerated at both dose levels. The most frequent adverse event observed was transient pain at the injection site reported by 83% and 75% of low- and high-dose groups respectively, compared with 25% of the placebo recipients, which is typical of aluminum-adjuvanted vaccines. No subjects withdrew because of adverse events. No other safety signals were noted in terms of adverse events or the monitored clinical parameters.

This safety profile stands comparison with the safety profiles of other EV71 vaccines that have completed Phase I clinical trials. The phase I trial of NHRI B4-based vaccine conducted in 80 adult volunteers reported no severe adverse events, all being mild to moderate

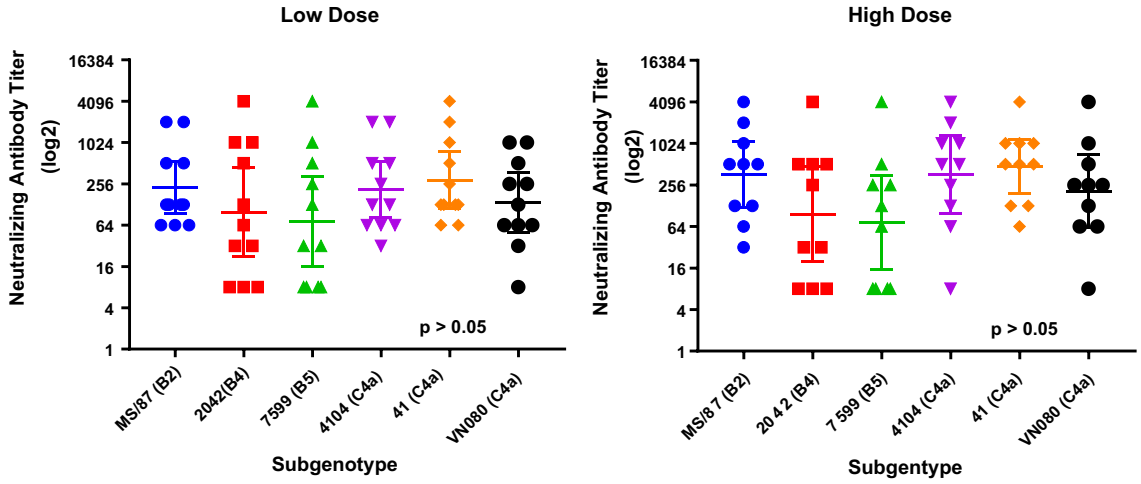

Fig. 4. Cross-neutralizing antibody titers of vaccinated volunteers against various EV71 sub-genotypes. Serum samples collected on day 42 post-vaccination were assessed for neutralization against EV71 sub-genotypes B4, B5 and C4a. Symbols indicate individual titers. Geometric mean titers (with 95% Confidence intervals bars) are presented (High dose n = 10, Low dose n = 11) are compared with those to the vaccine strain MS/87. Different EV71 isolates are represented as, 2042 (B4)-E2002042-TW, 7599 (B5)-E2007599-TW, 4104 (C4a)-E2004104-TW.

Table 4
Cross-neutralizing antibody titers against diverse sub-genotypes of EV71 at 42 days post-vaccination.

Sub-genotype	Low		High		p-value
	n	GMT (95% CI)	n	GMT (95% CI)	
MS/87 (B2)	11	226 (94–544)	10	362 (120–1090)	0.46
2042 (B4)	11	99 (22–442)	10	97 (20–475)	0.98
7599 (B5)	11	73 (16–325)	10	74 (15–351)	0.99
4104 (C4a)	11	212 (81–551)	10	362 (98–1337)	0.47
41 (C4a)	11	290 (112–751)	10	478 (196–1162)	0.40
VN080 (C4a)	11	136 (50–374)	10	208 (62–701)	0.56

Titers < 32 were given a value of 8 for the calculation of GMT values. The lower limit of detection of this assay is 32.
Different EV71 isolates are represented as, 2042 (B4)-E2002042-TW, 7599 (B5)-E2007599-TW, 4104 (C4a)-E2004104-TW.

intensity with myalgia being the most frequent systemic reaction and mild reaction at the local injection site [24]. In another phase I trial conducted severe adverse effects (high fever) were reported in 3.1% of the youngest subjects and mild fever for 22.2 of adult subjects [35]. Another phase I clinical trial reported mild adverse events with a third C4 vaccine candidate in [36]. In this study 37% of participants reported at least one injection-site or systemic adverse reaction. Fever was the most common systemic reaction (35–55%).

It was notable that the majority of subjects (>94%) already had antibodies (GMT > 8) against EV71, indicating some exposure to the virus, although generally low levels are felt to indicate that such exposure was not recent. This is expected to be typical of an adult population in a large Asian city, where exposure to EV71 is common during childhood [37]. The immune response to the INV21 candidate vaccine was evident even with this background (in the presence of pre-existing antibodies) with similar increases in GMTs in both low and high dose vaccine groups two weeks after the first dose. The second dose elicited 100% seroconversion and boosted antibody titers in both groups, and titers remained higher than baseline and the placebo group for a further 24 weeks. While these data in seropositive adults are promising, the immunogenicity of the vaccine needs to be confirmed in seronegative children. Interestingly, 75% of adults in Germany have been reported as having antibodies to EV71 [38]. Therefore, vaccine trials across the world and not just in Asia should be planned with preexposure in mind.

BEI is used as inactivating agent in the manufacturing process of INV21 candidate vaccine due to its advantage over formaldehyde or beta-propiolactone [39,40]. BEI-inactivation is faster, with defined kinetics, and residual BEI can be effectively neutralized by addition of sodium thiosulfate. It could potentially preserve the structure of viral epitopes. However, BEI inactivated materials have never been used in human vaccines, although it has shown to inactivate and induce immune response against foot and mouth disease virus (FMDV) [41], Ross River virus (RRV) [42], sheep pox [43], HIV [44], Nipah virus [45], porcine reproductive and respiratory syndrome virus (PRRSV) [46] and rabies virus [47]. The safety profile of INV21 is comparable to other EV71 vaccines that completed phase I trials [22,24,35,36], some of which have been commercialized. Both the low- and high dose formulations with BEI were tested in a pivotal safety study conducted in New Zealand White (NZW) rabbits at a GLP contract research organization (CRO) [30]. All clinical and histological findings were compiled by the CRO, who found no morbidity or mortality, or any skin reactions at the site of injection in any animals. Hematology and clinical chemistry parameters remained within the normal range and EV71 vaccine-treated animals exhibited no differences from the control groups in group averages of body mass, body mass change during the study period, or food consumption.

Various genotypes of EV71 are currently circulating in the world. For instance, currently EV71 sub-genotype C4a is reported to be circulating in Korea, B4, B5, C1 in Singapore and Malaysia, while B3, C2, C1 co-circulate in Australia and C2, C4a, B4 and B5 sub-genotypes were recently found to circulate in Japan [37,48,49]. In Taiwan, different serotypes of EV71 are in circulation; predominant genogroups occurred in 1998 (C2), 2000–2001 (B4), 2004–2005 (C4), 2008–2009 (B5) and 2010–2016 (B5, C4).

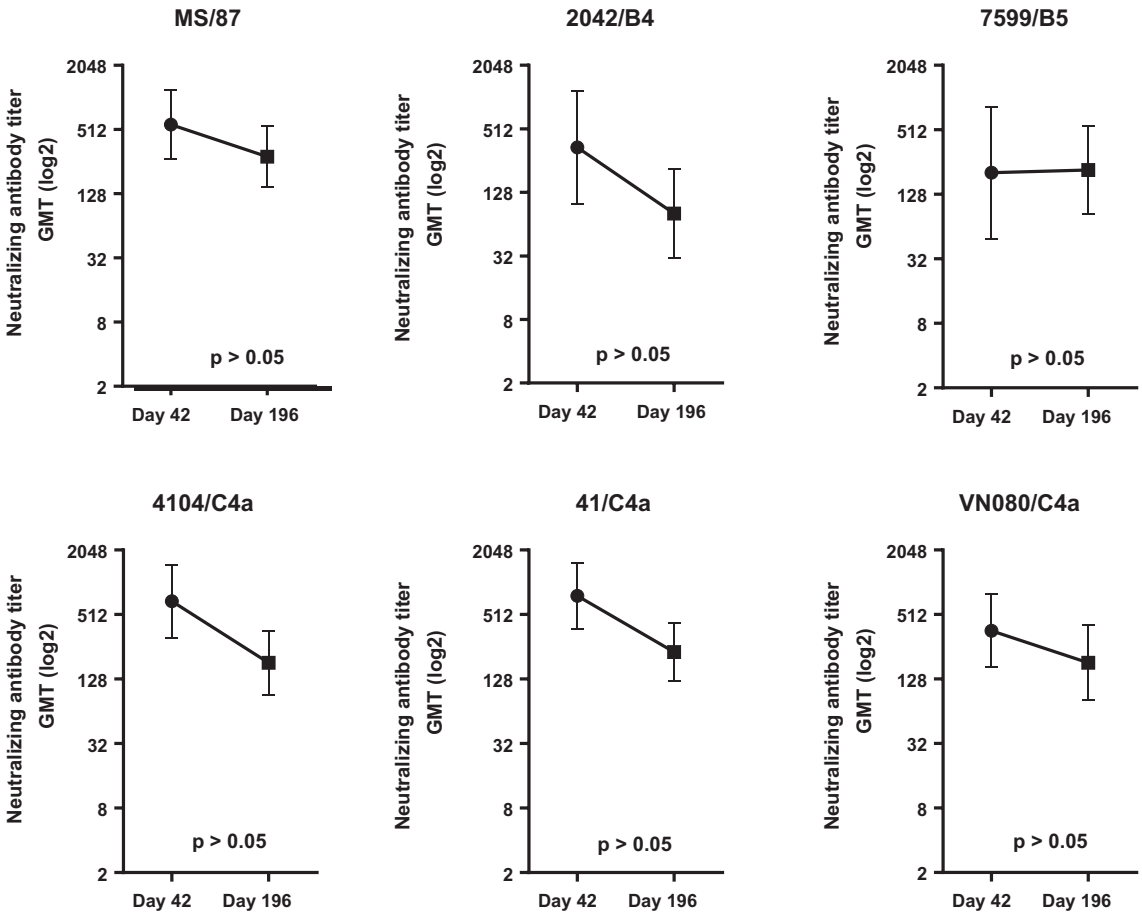

Fig. 5. Persistence of cross-neutralizing antibodies in the serum of vaccinated individuals. Paired sera samples collected on day 42 and 196 following immunization were tracked for their cross-neutralizing titers against B4, B5 and C4a EV71 sub-genotypes. Geometric mean titers (with 95% Confidence intervals bars) are presented (N-13). P values are shown in the inset.

In China C4a and in Vietnam C4a and B5 sub-genotypes are currently in circulation. Therefore, an EV71 vaccine that could provide protection against all sub-genotypes would be necessary.

In this Phase I clinical study, antibodies elicited by INV 21, a candidate EV71 vaccine based on B2 sub-genotype, cross-neutralized EV71 strains from sub-genotypes B4, B5 and C4a. While cross-neutralization of the B4 and B5 sub-genotypes was variable, cross-neutralizing titers against C4a sub-genotype were uniformly high. This result was unexpected, as sub-genotype C4a is genetically more divergent from B2 than are sub-genotypes B4 and B5. After further analysis it became clear that although the cross-reactivity against C4 was better than B4 and B5 at higher dose, it was within 4-fold difference. Since the VP1 protein is the major target of cross-neutralizing antibodies and B4 and B5 subgenotypes are closer to B2 than C4, the slight increase in cross-reactivity cannot be considered to be very significant indicating any major difference in antigenicity. Alternatively, the individuals might have been exposed to C4 subgenotypes prior to vaccination leading to higher cross-neutralizing titer which is unlikely. It is hard to draw definitive conclusions as this study was not designed to address the question of the response to specific genotypes. These exploratory results suggest that even though there are differences in genetic elements among various sub-genotypes of EV71, common antigenic epitopes present in the major immunogen (VP1) could generate neutralizing antibodies with strong cross-reactivity [50–52]. Serological cross-reactivity among sub-genotypes of EV71 has previously been investigated using hyperimmune serum raised in guinea pigs against B2 and C1 sub-genotypes and serum collected from 2-year-old children infected with B5 or C4 sub-genotypes of EV71 [52]. Similarly, antibodies raised in rabbits and collected from patients infected with one sub-genogroup of EV71 showed strong cross-neutralization with other sub-genogroups [53].

In addition, a challenge study conducted in new born mice vaccinated with synthetic peptide representing one of the VP1 epitopes of a B4 sub-genogroup demonstrated full protection against lethal challenge with EV71 strains belonging to B2, B4, B5, C2 and C4 sub-genogroups [54]. A report on an inactivated EV71 candidate vaccine based on a B4 sub-genotype (EV71/E59) showed that antibodies induced by the vaccine could cross-neutralize sub-genotypes B1, B4, B5, and C4A [55]. Antibodies induced by that vaccine however did not show good cross-reactivity with some isolates of C4B and C2 sub-genotypes [55]. Two C4 sub-genotype based EV71 vaccine candidates also induced cross-neutralizing antibodies in infants and children against B4, B5, C2, and C5 sub-genotypes [56]. Thus, the cross-neutralizing potential of EV71 vaccines based on either B2 (INV21 this study), B4 or C4 sub-genotypes could potentially confer broad protection against most of the currently circulating EV71 sub-genotypes, but this needs to be further confirmed [23].

A potential limitation of the study is the target population in which the study was conducted. Since most of the individuals had prior exposure to EV71, this study cannot predict a similar outcome of the vaccination in naïve population. Moreover, due to a small study population, a thorough and comprehensive analysis of safety of the vaccine with more statistical power could not be

performed. Therefore, future studies should be conducted in a larger population of naïve individuals. The selected demographic group posed another limitation. All subjects were Asian and the majority were males. The age selection was between 18 and 45 and no children or adults above 45 years of age were selected. The safety and immunogenicity profile may be different with other demographic groups.

In summary, the results of this phase I study showed that the inactivated EV71 candidate vaccine was safe, well tolerated and immunogenic in adults and induces cross-neutralizing antibodies against EV71 viruses. These observations support its further clinical development as a potential vaccine for use in children.

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Declaration of Competing Interest

The authors have no other conflicts of interest or funding to disclose.

Authors' contributions

PAT: Study design, data interpretation, writing; JO, RA, WK, SHH, LK, JF: data collection, data analysis; FV: data collection, data analysis, writing; JDS: Study design, data interpretation, writing; GSG: Study design, study monitoring, data interpretation, writing; CT, RR: Study design, data interpretation, writing; HD: data interpretation, writing; SCD: literature search, study design, data analysis, data interpretation, writing; DTS: Study design. All authors contributed to review and revision of the manuscript. All authors have seen and approved the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.023.

References

[1] Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis 2010;10:778–90.
[2] Wang Y-R, Sun L-L, Xiao W-L, Chen L-Y, Wang X-F, Pan D-M. Epidemiology and clinical characteristics of hand foot, and mouth disease in a Shenzhen sentinel hospital from 2009 to 2011. BMC Infect Dis 2013;13:539.
[3] Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, et al. Neurologic complications in children with enterovirus 71 infection. N Engl J Med 1999;341:936–42.
[4] Chang LY, Huang YC, Lin TY. Fulminant neurogenic pulmonary oedema with hand, foot, and mouth disease. Lancet 1998;352:367–8.
[5] Sabanathan S, Tan le V, Thwaites L, Wills B, Qui PT, Rogier van Doorn H. Enterovirus 71 related severe hand, foot and mouth disease outbreaks in South-East Asia: current situation and ongoing challenges. J Epidemiol Community Health 2014;68(6):500–2.
[6] Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71 infection in Taiwan. N Engl J Med 1999;341(13):929–35.
[7] Lyu K, Chen R. Achievements, challenges and prospects for the development of broadly protective multivalent vaccines and therapeutic antibodies against hand, foot and mouth disease. Sci Bull 2015;60:1305–15.
[8] Koh WM, Badaruddin H, La H, Chen MI, Cook AR. Severity and burden of hand, foot and mouth disease in Asia: a modelling study. BMJ Glob Health 2018;3(1):e000442.
[9] Hu Y, Zeng G, Chu K, Zhang J, Han W, Zhang Y, et al. Five-year immunity persistence following immunization with inactivated enterovirus 71 type (EV71) vaccine in healthy children: a further observation. Hum Vaccin Immunother 2018;14(6):1517–23.
[10] Chan YF, Sam IC, Abubakar S. Phylogenetic designation of enterovirus 71 genotypes and sub-genotypes using complete genome sequences. Infect Genet Evol 2009;10:404–12.
[11] Lee MS, Chang LY. Development of enterovirus 71 vaccines. Exp Rev Vacc 2010;9:149–56.
[12] Chia MY, Chung WY, Chiang PS, Chien YS, Ho MS, Lee MS. Monitoring antigenic variations of enterovirus 71: implications for virus surveillance and vaccine development. PLoS Negl Trop Dis 2014;8(7):e3044.
[13] Huang SW, Hsu YW, Smith DJ, Kiang D, Tsai HP, Lin KH, et al. Reemergence of enterovirus 71 in 2008 in Taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. J Clin Microbiol 2009 Nov;47(11):3653–62.
[14] Chong P, Hsieh SY, Liu CC, Chou AH, Chang JY, Wu SC, et al. Production of EV71 vaccine candidates. Hum Vaccin Immunother 2012;8:1775–83.
[15] Liu JN, Wang W, Duo JY, Hao Y, Ma CM, Li WB, et al. Combined peptides of human enterovirus 71 protect against virus infection in mice. Vaccine 2010;28:7444–51.
[16] Chen HF, Chang MH, Chiang BL, Jeng ST. Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. Vaccine 2006;24:2944–51.
[17] Meng T, Kolpe AB, Kiener TK, Chow VT, Kwang J. Display of VP1 on the surface of baculovirus and its immunogenicity against heterologous human enterovirus 71 strains in mice. PLoS ONE 2011;6:e21757.
[18] Chung YC, Ho MS, Wu JC, Chen WJ, Huang JH, Chou ST, et al. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. Vaccine 2008;26:1855–62.
[19] Tung WS, Bakar SA, Sekawi Z, Rosli R. DNA vaccine constructs against enterovirus 71 elicit immune response in mice. Genet Vaccines Ther 2007;5:6.
[20] Arita M, Nagata N, Iwata N, Ami Y, Suzaki Y, Mizuta K, et al. An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. J Virol 2007;81:9386–95.
[21] Ong KC, Devi S, Cardosa MJ, Wong KT. Formaldehyde-inactivated whole virus vaccine protects a murine model of Enterovirus 71 encephalomyelitis against disease. J Virol 2010;84:661–5.
[22] Li YP, Liang ZL, Gao Q, Huang LR, Mao QY, Wen SQ, et al. Safety and immunogenicity of a novel human Enterovirus 71 (EV71) vaccine: a randomized, placebo-controlled, double-blind, Phase I clinical trial. Vaccine 2012;30:3295–303.
[23] Zhu FC, Meng FY, Li JX, Li XL, Mao QY, Tao H, et al. Efficacy, safety, and immunology of an inactivated alum-adjuvant enterovirus 71 vaccine in children in China: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 2013;381:2024–32.
[24] Cheng A, Fung CP, Liu CC, Lin YT, Tsai HY, Chang SC, et al. A Phase I, randomized, open-label study to evaluate the safety and immunogenicity of an enterovirus 71 vaccine. Vaccine 2013;31:2471–6.
[25] Zhang W, Kong Y, Jiang Z, Li C, Wang L, Xia J. Comprehensive safety assessment of a human inactivated diploid enterovirus 71 vaccine based on a phase III clinical trial. Hum Vaccin Immunother 2016;12(4):922–30.
[26] Zhu F, Xu W, Xia J, Liang Z, Liu Y, Zhang X, et al. Efficacy, safety, and immunogenicity of an enterovirus 71 vaccine in China. N Engl J Med 2014;370(9):818–28.
[27] Mao Q, Wang Y, Bian L, Xu M, Liang Z. EV-A71 vaccine licensure: a first step for multivalent enterovirus vaccine to control HFMD and other severe diseases. Emerg Microbes Infect 2016;5(7):e75.
[28] Yee PTI, Laa Poh C. Impact of genetic changes, pathogenicity and antigenicity on enterovirus- A71 vaccine development. Virology 2017;506:121–9.
[29] Chou A-H, Liu C-C, Chang J-Y, Jiang R, Hsieh Y-C, Tsao A, et al. Formalin-inactivated EV71 vaccine candidate induced cross-neutralizing antibody against sub-genotypes B1, B4, B5 and C4A in adult volunteers. PLoS ONE 2013;8(11):e79783. https://doi.org/10.1371/journal.pone.0079783.
[30] Hwa SH, Lee YA, Brewoo JN, Partidos CD, Osorio JE, Santangelo JD. Preclinical evaluation of the immunogenicity and safety of an inactivated enterovirus 71 candidate vaccine. PLoS Negl Trop Dis 2013;7:e2538.
[31] http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074775.htm [Accessed March 13, 2014].
[32] http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf [Accessed March 13, 2014].
[33] Grund S, Adams O, Wählisch S, Schweiger B. Comparison of hemagglutination inhibition assay, an ELISA-based micro-neutralization assay and colorimetric microneutralization assay to detect antibody responses to vaccination against influenza A H1N1 2009 virus. J Virol Methods 2011;171(2):369–73.
[34] Cheng LW, Huang SW, Huang LM, Chang LY, Shao PL, Kiang D, et al. Comparison of neutralizing and hemagglutination-inhibiting antibody responses for evaluating the seasonal influenza vaccine. J Virol Methods 2012;182(1–2):43–9.
[35] Li YP, Liang ZL, Gao Q, Huang LR, Mao QY, Wen SQ, et al. Safety and immunogenicity of a novel human Enterovirus 71 (EV71) vaccine: a randomized, placebo-controlled, double-blind, phase I clinical trial. Vaccine 2012;30:3295–303.
[36] Meng FY, Li JX, Li XL, Chu K, Zhang YT, Ji H, et al. Tolerability and immunogenicity of an inactivated enterovirus 71 vaccine in Chinese healthy

adults and children: an open label, phase 1 clinical trial. Hum Vaccin Immunother 2012;8(5):668–74. https://doi.org/10.4161/hv.19521.
[37] Lin CL, Chen KH, Chen KT. Update on Enterovirus 71 infections: epidemiology, molecular epidemiology, and vaccine development. J Infect Dis Ther 2018;6:370. https://doi.org/10.4172/2332-0877.1000370.
[38] Diedrich S, Weinbrecht A, Schreier E. Seroprevalence and molecular epidemiology of enterovirus 71 in Germany. Arch Virol 2009;154(7):1139–42.
[39] Bahnemann HG. Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. Vaccine 1990;8:299–303.
[40] Fan C, Ye X, Ku Z, Kong L, Liu Q, Xu C, et al. Beta-propiolactone inactivation of Coxsackievirus A16 induces structural alteration and surface modification of viral capsids. J Virol 2017;91(8):e00038–e117.
[41] Bahnemann HG. Binary ethylenimine as an inactivant for foot-and-mouth disease virus and its application for vaccine production. Arch Virol 1975;47 (1):47–56.
[42] Aaskov J, Williams L, Yu S. A candidate Ross River virus vaccine: preclinical evaluation. Vaccine 1997;15(12–13):1396–404.
[43] Awad M, Michael A, Soliman SM, Samir SS, Daoud AM. Trials for preparation of inactivated sheep pox vaccine using binary ethyleneimine. Egypt J Immunol 2003;10(2):67–72.
[44] Race E, Frezza P, Stephens DM, Davis D, Polyanskaya N, Cranage M, et al. An experimental chemically inactivated HIV-1 vaccine induces antibodies that neutralize homologous and heterologous viruses. Vaccine 1995;13(1):54–60.
[45] Berhane Y, Berry JD, Ranadheera C, Marszal P, Nicolas B, Yuan X, et al. Production and characterization of monoclonal antibodies against binary ethylenimine inactivated Nipah virus. J Virol Methods 2006;132(1–2):59–68.
[46] Vanhee M, Delputte PL, Delrue I, Geldhof MF, Nauwynck HJ. Development of an experimental inactivated PRRSV vaccine that induces virus-neutralizing antibodies. Vet Res 2009;40(6):63.
[47] Larghi OP, Nebel AE. Rabies virus inactivation by binary ethylenimine: new method for inactivated vaccine production. J Clin Microbiol 1980;11(2):120–2.
[48] Koh WM, Bogich T, Siegel K, Jin J, Chong EY, Tan CY, et al. The epidemiology of hand, foot and mouth disease in Asia: a systematic review and analysis. Pediatr Infect Dis J 2016;35(10):e285–300.
[49] Chang PC, Chen SC, Chen KT. The current status of the disease caused by enterovirus 71 infections: epidemiology, pathogenesis, molecular epidemiology, and vaccine development. Int J Environ Res Public Health 2016;13(9). pii: E890.
[50] Liu CC, Chou AH, Lien SP, Lin HY, Liu SJ, Chang JY, et al. Identification and characterization of a cross-neutralization epitope of Enterovirus 71. Vaccine 2011;29(26):4362–72.
[51] Chang HW, Liu CC, Lin MH, Ho HM, Yang YT, Chow YH, et al. Generation of murine monoclonal antibodies which cross-neutralize human enterovirus genogroup B isolates. J Virol Methods 2011;173(2):189–95.
[52] Mizuta K, Aoki Y, Suto A, Ootani K, Katsushima N, Itagaki T, et al. Cross-antigenicity among EV71 strains from different genogroups isolated in Yamagata, Japan, between 1990 and 2007. Vaccine 2009;27(24):3153–8.
[53] Kung SH, Wang SF, Huang CW, Hsu CC, Liu HF, Yang JY. Genetic and antigenic analyses of enterovirus 71 isolates in Taiwan during 1998–2005. Clin Microbiol Infect 2007;13(8):782–7.
[54] Foo DG, Alonso S, Chow VT, Poh CL. Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. Microbes Infect 2007;9(11):1299–306.
[55] Chou AH, Liu CC, Chang JY, Lien SP, Guo MS, Tasi HP, et al. Immunological evaluation and comparison of different EV71 vaccine candidates. Clin Dev Immunol 2012;831282.
[56] Mao Q, Cheng T, Zhu F, Li J, Wang Y, Li Y, et al. The cross-neutralizing activity of enterovirus 71 sub-genotype c4 vaccines in healthy Chinese infants and children. PLoS ONE 2013;8(11):e79599.