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Development of a high-growth enterovirus 71 vaccine candidate inducing cross-reactive neutralizing antibody responses

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ABSTRACT

Although Enterovirus 71 (EV71) has only one serotype based on serum neutralization tests using hyper-immune animal antisera, three major genogroups (A, B and C) including eleven genotypes (A, B1-B2, and C1-C5) can be well classified based on phylogenetic analysis. Since 1997, large-scale EV71 epidemics occurred cyclically with different genotypes in the Asia-Pacific region. Therefore, development of EV71 vaccines is a national priority in several Asian countries. Currently, five vaccine candidates have been evaluated in clinical trials in China (three C4 candidates), Singapore (one B2 candidate), and Taiwan (one B4 candidate). Overall, the peak viral titers of these 5 vaccine candidates could only reach about 10^7 $TCID_{50}$/mL. Moreover, genotypes of these 5 candidates are different from the current predominant genotype B5 in Taiwan and South-Eastern Asia. We adapted a high-growth EV71 genotype B5 (HG-B5) virus after multiple passages and plaque selections in Vero cells and the HG-B5 virus could reach high titers (>10^8 $TCID_{50}$/mL) in a microcarrier-based cell culture system. The viral particles were further purified and formulated with alum adjuvant. After two doses of intramuscular immunization in rabbits, the HG-B5 vaccine candidate could induce cross-reactive neutralizing antibodies against the three major EV71 genogroups. In conclusion, a high-growth EV71 virus was successfully adapted in Vero cells and could induce broad spectrum neutralizing antibody titers against three (A, B5, and C4) genotypes in rabbits.

1. Introduction

Human enteroviruses consist of more than 100 serotypes and usually cause self-limited infections in children except EV71 and polioviruses. EV71 is a non-enveloped, icosahedral positive-sense single strand RNA virus in the genus *Enterovirus* within the family *Picornaviridae*. EV71 RNA genome is around 7.4 kb and includes a single open reading frame (ORF) that can be divided into structural P1 region, and non-structural P2 and P3 regions [1,2]. The P2 and P3 genes encode seven accessory proteins 2A-2C and 3A-3D, respectively, and are related to viral replication and virulence [1]. The P1 protein can be processed by viral protease 3CD to produce capsid subunit proteins VP0, VP1, and VP3; all three of which self-assemble to form non-infectious viral capsid. The protein VP0 can be further cleaved to yield VP2 and VP4, which are associated with the formation infectious EV71 virions [3,4].

Most EV71 infections manifest as mild cases of hand-foot-mouth disease (HFMD) or herpangina in young children under 5 years of age, who are potentially at risk for severe neurological and cardiopulmonary complications [5]. The prototype EV71 strain, A (BrCr), was first isolated in California in 1970 [6]. Since 1997, EV71 has become the causative agent responsible for the epidemics of central nervous system diseases in the densely populated Asia-Pacific countries such as Taiwan, China, Malaysia, Singapore, Cambodia and Vietnam [5,7,8]. According to the molecular epidemiology and phylogenetic analysis of VP1 sequence, EV71 was divided into three major genogroups (A, B, and C) including eleven genotypes (A, B1-B5, and C1-C5) [2,5]. Recently, several minor strains were isolated and clustered into four additional genogroups, including genogroup D and G in India and genogroup E and F in African [9–11].

In Taiwan, phylogenetic analyses revealed that nationwide epidemic with different predominant genotypes occurred in 1998 (C2), 2000–2001 (B4), 2005 (C4), 2008 (B5) and 2012 (B5) and this genotype replacement has also been observed in other EV71 epidemic countries [8,12–16]. Meanwhile, the Asian pandemics have

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been associated with co-circulation of different genotypes and increase the potential of genetic recombination resulted in the emergence of novel strains [17–19]. Although EV71 has only one serotype, several human studies using post-infection sera obtained from children to measure cross-neutralizing antibody titers against different genotypes have detected antigenic variations among different EV71 genotypes [20–23]. Genogroup A viruses were only identified in 1970 in United States and disappeared afterward. In 2008, genogroup A re-emerged in Anhui China and gradually spread to multiple Provinces in China. The reason for the reemergence of genogroup A in China is not clear [24]. Based on serological studies, EV71 genogroup A is apparent antigenic differences from genogroup B and C [3,22]. Therefore, an ideal EV71 vaccine should induced cross-neutralizing antibody responses against three EV71 major genogroups.

Based on historical experiences in development of polio vaccines, the inactivated EV71 vaccine is more likely to pass regulatory hurdles [25]. Moreover, formalin inactivated EV71 virus formulated with alum has been confirmed to be immunogenic in animal and human studies [26,27]. Currently, five formalin inactivated whole virus EV71 vaccine candidates, using different viral genotypes, mammalian cell lines and manufacturing technologies, are evaluated in clinical trials (Supplementary Data 1). Four of these five vaccine candidates were produced using Vero cells (the popular cell line for human vaccine production) but their peak virus titers could not grow very well ($\sim 10^7$ PFU/mL). Genotypes of the current 5 vaccine candidates include one B2, one B4, and three C4 strains (Supplementary Data 1), which are different from the current predominant genotype B5 in Taiwan and South-Eastern Asia [7,13]. In the present study, we adapted high-growth ($>10^8$ $TCID_{50}$/mL) EV71 genotype B5 (HG-B5) virus in Vero cells. The HG-B5 viral particles were purified by sucrose gradient ultracentrifugation and then the purified vaccine candidate was formulated with alum to immunize rabbits. The results showed that the HG-B5 vaccine candidate could elicit cross-reactive neutralizing antibody titers against three (A, B5, and C4) EV71 genotypes in rabbits and is a promising approach for further development.

2. Materials and methods

2.1. Ethics statement

The animal protocol has been approved by the NHRI Institutional Animal Care and Use Committee (approval no. NHRI-IACUC-101,038-A) following the Institutional Animal Care and Use Committee Guidebook published by the US Office of Laboratory Animal Welfare (http://grants.nih.gov/grants/olaw/guidebook.pdf). All animal experiments in this study were performed in accordance with approved guidelines of the NHRI Ethics Committee.

2.2. Virus and cells

An EV71 genotype B5 strain, NHRI-B5-141-TW-08 (B5-141; GenBank accession number JN874552), was isolated from a 20-month-old herpangina patient in 2008 and had been used to generate rabbit antisera that could neutralize multiple EV71 genogroups.[3] Human rhabdomyosarcoma (RD; ATCC CCL-136) cell and African green monkey kidney (Vero; ATCC CCL-81) cells were used to grow enterovirus 71 following the standard procedures [28]. Vero cells are commonly used for producing human vaccines [29]. In this study, Vero cells were grown in VP-SFM medium (Thermo Scientific) supplemented with 200 mM L-glutamine (Thermo Scientific) and a HG-B5 viral strain was obtained by serial passages and multiple plaque selections.

2.3. Determination of virus growth curves in cell cultures

Confluent Vero cells in 24-well plates (Corning) were infected with EV71 viruses with 10^{-3} and 10^{-4} multiplicity of infection (MOI) and the supernatant was harvested at 3–8 days post infection for measuring viral titers. Potentials of the Vero cell-adapted EV71 virus strain as a vaccine seed virus were evaluated in a microcarrier-based spinner flask cell culture system. Cytodex 1 microcarriers (GE Healthcare) were hydrated, autoclaved, and preconditioned according to methods described by Tseng et al. [30].

2.4. Virological analysis

Viral titers were measured using the 50% tissue culture infectious doses ($TCID_{50}$) assay based on observing cytopathic effect (CPE) in Vero cells. In addition, the plaque and immuno-plaque assays were also employed to measure plaque forming unit (PFU) of infectious virus titers in Vero cells. A positive control with pre-specified acceptable range is included for conducting $TCID_{50}$ and plaque assays. In the $TCID_{50}$ assay, monolayer of Vero cells ($3 * 10^4$ cells/well) in 96-well plates (Corning) were inoculated with serial dilution of virus and determined by Reed-Muench method [31].

In the plaque assay, monolayer of Vero cells ($5 * 10^5$ cells/well) in 6-well plates inoculated with 500 μL serial dilution of virus and stained by 0.5% crystal violet for plaque assay. In the immune-plaque assay, monolayer of Vero cells ($3 * 10^5$ cells/well) in 12-well plates were inoculated with 200 μL serial dilution of virus and virus antigens were detected with EV71-specific monoclonal antibody MAB979 (Chemicon International). The plate was then incubated with a horseradish peroxidase (HRP) – conjugated goat anti-mouse secondary antibody (AbD Serotec) and the immune-plaques were revealed by adding TMB substrate solution (KPL).

2.5. RT-PCR and sequencing

Viral RNA was extracted from 140 μL of culture supernatant using QIAamp Viral RNA Mini Kit according to the manufactory protocol (Geneaid Biotech). cDNA of EV71 was synthesis by Super-Script II Reverse Transcriptase (Invitrogen). As showed in Supplementary Data 2, full-length polymerase chain reaction (PCR) and sequencing of EV71 was performed using specific primers. The sequences were aligned and analyzed by using the *Clustal W* in *MEGA 5*.

2.6. Viral purification

Two forms of EV71 particles, infectious (full) and non-infectious (empty) particles, had been documented in previous studies [3,30,32]. In this study, we purified these two forms of EV71 particles using sucrose gradient. The EV71 culture supernatant was inactivated by 0.01% (v/v) formaldehyde at 37 °C for overnight, filtered by 0.65 μm membrane, and concentrated with a Sartocon Silice 200 Hydrosart MWCO 100 K Cassette (Sartorius). The crude virus concentrate was loaded onto a 10–40% continuous sucrose gradient and centrifuged at 35,000 rpm for 4 h using SW41 rotor (Beckman). Fractions (0.5 mL per fraction) were collected from bottom to top of ultracentrifuge tube and analyzed using Western blotting. Fractions with full and empty particles were merged, respectively, and concentrated by diafiltration using Amicon 100 K centrifugal filter. The protein concentration of purified EV71 full and empty particles was determined using BCA assay (Thermo Scientific).

2.7. Transmission electron microscopy analysis

Purified EV71 particles were deposited on a carbon-coated 200 mesh copper grid for 1 min at room temperature. The excess sample was removed by filter paper and then the copper grid was stained with 2% uranyl acetate solution for 3 min, which was then removed by filter paper. The stained grid was dried for 1 day at room temperature and observed under a JEM 1200EX transmission electron microscopy [3].

2.8. Western blotting

For immunoblotting, proteins were directly transferred onto the NC membrane. The membrane was subsequently soaked overnight at 4 °C in 2.0% skim milk in PBS. Membrane was incubated with PBS buffer containing EV71-specific monoclonal antibody MAB979 for 1 h at room temperature. The membrane was then incubated with PBS buffer containing a horseradish peroxidase (HRP) – conjugated goat anti-mouse secondary antibody. The protein bands were revealed by adding TMB substrate solution.

2.9. Animal immunization

Eight two-month-old New Zealand White rabbits were confirmed to be EV71 seronegative and assigned into four treatment groups (2 rabbits/group). Different concentrations of purified HG-B5 full and empty particles (0.05 μg full, 0.25 μg full, 0.05 μg empty, and 0.25 μg empty) were adjuvanted with 300 μg alum phosphate ($AlPO_4$), respectively. Rabbits were immunized intramuscularly with two doses at a 2-week interval. The sera from immunized rabbits were collected and stored at −80°Cfor measuring neutralizing antibody titers against EV71 viruses.

2.10. Determination of neutralizing antibody titers

Neutralizing antibody titer of each sample was determined according to the previous protocol reported by Chia et al. [3]. Briefly, sera were heat-inactivated at 56 °C for 30 min and diluted serially twofold from 1:4. The 50 μL of twofold serially diluted sera were mixed with an equal volume of virus working solution containing 100 $TCID_{50}$/well of EV71 at 37 °C for an hour in 96-well microtiter plates. Human rhabdomyosarcoma (RD) cells at 3×10^4/well were added and incubated at 37 °C in 5% CO_2 for 4 days and the CPE was observed in an inverted microscope. Each serum dilution includes three replicates and the neutralization titers were read as the highest dilution that could result in a reduction of the CPE in at least two of three replicate wells. Each test sample was run simultaneously with cell control, positive serum control, and virus back titration. The cutoff level of seropositivity was set at 1:8.

3. Results

3.1. Adaption of the HG-B5 in Vero cells

The parental B5-141 virus isolated from throat swab could not cause clear CPE or plaque in Vero cells and viral titer was only about $\sim 10^7$ $TCID_{50}$/mL (Fig. 1 and Supplementary Data 3). For selection of high-growth B5-141, serial passages and plaque selections in Vero cells were conducted in 6-well plates at 37 °C. The culture supernatants were directly collected for measuring viral titers using $TCID_{50}$ assay. After 23 passages in Vero cells, the adapted B5-141 could form clear plaque and two clones, B5-141-4 and B5-141-6, were selected in the 24th and 25th passages, respectively (Supplementary Data 3). Generally, highly virulent clones could cause clear plaques by efficient lysis of monolayer Vero cells and have ability to induced high viral tiers during amplification [31]. After another plaque selection and three passages, two high viral strains, B5-141-4-2 and B5-141-6-5, were further selected to generate viral stocks.

The virus titers of the B5-141-4-2 and B5-141-6-5 were measured by plaque assay in 6-well plates and immuno-plaque assays in 12-well plates. Comparing with the parental B5-141 virus, the B5-141-4-2 and B5-141-6-5 viruses could cause very clear CPE and form plaques in plaque and immuno-plaque assays and their titers could reach to $>10^8$ PFU/mL (Fig. 1). In addition, the B5-141-4-2 and B5-141-6-5 viruses could produce more viral proteins than the parental B5-141 virus based on western blot assay (data not shown).

3.2. Growth kinetics of the HG-B5 viruses

The growth properties of HG-B5 strains (B5-141-4-2 and B5-141-6-5) were evaluated in 24-well plates with confluent Vero cells. The Vero cells were infected by two different MOI (10^{-3}, and 10^{-4}) and the supernatant was harvested at 3–8 days post infection (DPI) for measuring viral titers by $TCID_{50}$ assay. Generally, the viral titers increased steadily during 3–5 DPI and decreased slightly during 7–8 DPI (Table 1). The peak viral titers of the B5-141-6-5 virus could reach over 10^8 $TCID_{50}$/mL at 5 DPI under the 10^{-3} MOI and 7 DPI under the 10^{-4} MOI. Therefore, four pilot scale-up production of B5-141-6-5 strain were conducted using microcarrier-based cell cultures in serum-free VP-SFM medium. As shown in Table 2, Vero cells were grown on microcarriers at concentration 5 g/L in spinner flasks and infected with B5-141-6-5 (MOI = 10^{-3}) following the cell density reached to $\sim 1.5 * 10^6$ cells/mL. When the 90% CPE were observed on microcarrier-grown Vero cells, the culture supernatants were collected for measuring titers. Overall, viral titers of microcarrier-based serum-free culture system could reach around 10^8 $TCID_{50}$/mL in four runs, which confirm the commercial potential of the B5-141-6-5 strain.

3.3. Sequence variations of the HG-B5 viruses

To identify genetic mutations occurring during Vero cell adaptation, complete genomes of the B5-141, B5-141-4-2, and B5-141-6-5 were sequenced and analyzed. Comparing with the parental B5-141 virus, five and four nucleotide changes were detected in the B5-141-4-2 and B5-141-6-5 viruses, respectively (Table 3). To further determine the amino acid differences, the deduced sequence of B5-141 strain were aligned with B5-141-4-2 and B5-141-6-5 strains to reveal that one signature (T7A) in VP4 and two signatures (T802N and P811A) in VP1 were specific for B5-141-4-2 and B5-141-6-5 strains and may be related to increase viral growth in the present study.

3.4. Purification of the HG-B5 viral particles

The harvested culture medium were separated from microcarriers and cell debris by 3000 rpm centrifugation and 0.65 μm filtration and were concentrated by 100 K cassette as described in Materials and Methods. Subsequently, the concentrated and crude viruses were purified using sucrose gradient ultracentrifugation and then all fractions were analyzed using Western blotting and electron microscopy methods. The full (infectious) particles contain viral genome and were usually located at fractions 6–8 (F6-8) with about 32–36% sucrose concentrations (Fig. 2). The empty (non-infectious) particles lack viral genome and usually located at fractions 11–13 (F11-13) with about 26–30% sucrose concentrations. Based on Western blotting analysis by using EV71 VP2-specific monoclonal antibody MAB979, full particles mainly contain VP2 (28 KDa) and empty particles mainly contain VP0 (VP2 + VP4; 36 KDa) (Fig. 2A). The results indicate that purified viral particles in F6-8 and F11-13 contained different protein conformations [3,33].

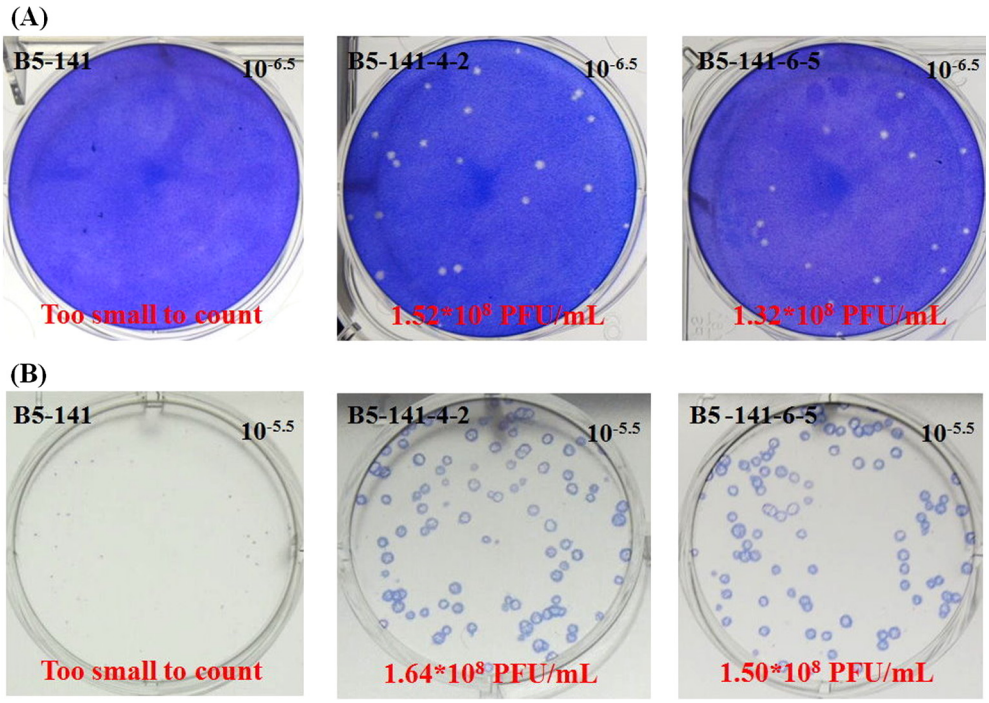

Fig. 1. The plaque morphology and plaque forming unit (PFU) of Vero cell-adapted EV71 B5-141, B5-141-4-2, and B5-141-6-5 vaccine candidates. (A) The results show plaque assay in 6-well plate of EV71 viruses with $10^{6.5}$ dilution factors in Vero cells. (B) The results show immune-plaque assay in 12-well plates of EV71 viruses with $10^{5.5}$ dilution factor.

Table 1
Daily virus titers of the Vero cell-adapted B5-141 viruses amplified in Vero cells using 24-well plates under different multiplicity of infection (MOI).

Days post infection	Virus titers (log_{10} $TCID_{50}$/mL) of B5-141-4-2		Virus titers (log_{10} $TCID_{50}$/mL) of B5-141-6-5	
	MOI = 0.001	MOI = 0.0001	MOI = 0.001	MOI = 0.0001
3	4.88	3.18	5.29	4.40
4	6.60	6.40	7.25	6.75
5	7.00	7.00	8.16	7.40
6	7.50	7.50	7.60	7.50
7	7.60	7.50	7.50	8.00
8	7.00	7.60	7.50	7.60

Table 2
Four pilot scale-up production of the B5-141-6-5 strain using microcarrier-based serum-free Vero cell culture systems in spinner flasks.

	Run 1	Run 2	Run3	Run 4
Harvest volume	370 mL	400 mL	380 mL	380 mL
Microcarrier density	5 g/L	5 g/L	5 g/L	5 g/L
Cell density (cells/mL) before infection	$1.60 * 10^6$	$2.74 * 10^6$	$1.92 * 10^6$	$1.88 * 10^6$
MOI	0.001	0.001	0.001	0.001
Harvest time (days post infection)	5	5	6	5
Virus titers (Log 10)	7.9	8.2	8.3	8.3

Table 3
Genetic variations among the parental B5-141 and Vero cell-adapted viruses.

	Nucleotide changes				Amino acid changes			
Gene	Position[a]	B5-141	B5-141-4-2	B5-141-6-5	Position[a]	B5-141	B5-141-4-2	B5-141-6-5
P1-VP4	766	A	G	G	7	T	A	A
P1-VP1	3152	C	A	A	802	T	N	N
P1-VP1	3178	C	G	G	811	P	A	A
P3-3A	5097	C	T	T	1450	I	I	I
P3-3D	7092	A	G	A	2115	Q	Q	Q

[a] Based on numbering of 5511-SIN-00 (accession no. DQ341364).

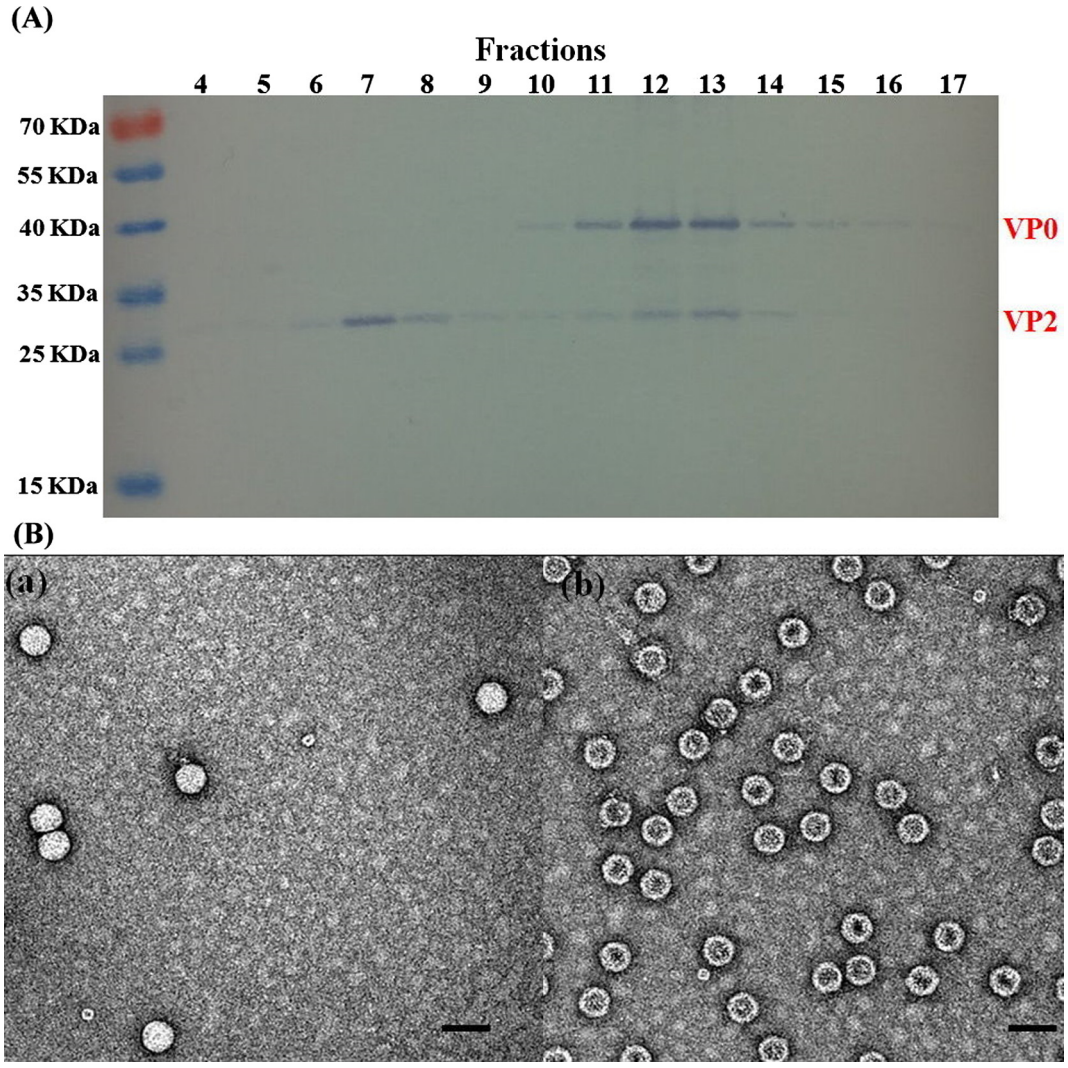

Fig. 2. Characterization of B5-141-65 viruses analyzed by Western blotting and electron microscopy. (A) The EV71 viruses were purified by sucrose gradient and fractions were detected by EV71 VP2 specific monoclonal antibody MAB 979. Full particles located at fractions 6–8 mainly contain VP2 (28 KDa) protein and empty particles located at fractions 11–13 mainly contain VP0 (36 KDa) protein in Western blotting assay. (B) EV71 particles were stained with 2% uranyl acetate solution and analyzed by transmission electron microscopy. (a) Full particles from fraction 7 had a solid structure and (b) empty particles from fraction 12 revealed a defective structure.

3.5. Immunogenicity of the HG-B5 viruses in rabbits

To determine the immunogenicity of purified full and empty particles, rabbits were immunized with different dosages (0.05 μg and 0.25 μg) of full or empty antigens formulated with 300 μg alum and boosted with the same dose at a 2-week interval. The antisera from all groups were collected for evaluating cross-neutralizing antibody titers against EV71 genotype A, B5 and C4 viruses (Table 4). After two doses of vaccination, the rabbits immunized with full particles could induce 2-fold higher neutralizing antibody titers than empty particles. Comparing neutralizing antibody responses against genotype B5 virus (homologous strain), the high dosage (0.25 μg) groups elicited almost 4-fold higher (362/92 in full particles and 181/45 in empty particles) antibody titers than the low dosage (0.05 μg) groups. However, the high dosage groups displayed almost 1.4-fold higher (64/45 in full particles and 32/23 in empty particles) antibody titers than the low dose groups against genotype C4 virus. For the genotype A virus, only high dosage F particle group could induce detectable neutralizing antibody titers (1:8). Overall, the HG-B5 vaccine candidates can induce neutralizing antibody titers against the three (A, B5, and C4) genotypes in rabbits.

4. Discussion

Generally, the isolation and identification of viral strains that can grow to a high viral titer in host cell lines represent the first step in vaccine development. The parental B5-141 virus had been used to generate rabbit antisera that could neutralize multiple EV71 genotypes [3]. In the present study, the HG-B5 viruses were adapted from the parental B5-141 and could reach over 10^8 $TCID_{50}$/mL in a microcarrier -based serum-free Vero cell culture system, which is commonly used for commercial production of human vaccines [34]. Meanwhile, propagation of viruses in serum-free culture medium is a desirable feature in vaccine development to eliminate contamination with zoonotic diseases and avoid induction of antibody against fetal calf serum [35].

Comparing with the parental B5-141 virus, three amino acid changes, one signature (T7A) in VP4 and two signatures (T802N and P811A) in VP1, were detected in the HG-B5 viruses. The individual significance of these three mutations needs to be verified using reverse genetics to generate mutant viruses. In addition, structural studies elucidating interaction between EV71 capsid proteins and Vero cell receptors will help understand the mechanism of virus replication.

Table 4
Cross-reactive neutralizing antibody responses in rabbits immunized with EV71 empty or full particles adjuvanted with alum phosphate.

EV71 genotypes	B5-141-6-5 empty particles		B5-141-6-5 full particles[a]	
	0.05 μg	0.25 μg	0.05 μg	0.25 μg
A (U22521-BrCr)[b]	<8	<8	<8	8
B5 (NHRI-141-TW-08)[c]	45	181	91	362
C4 (70516TW-08)[d]	23	32	45	64

Accession numbers:
[a] MG674288.
[b] JN874547.
[c] JN874552.
[d] JN874557.

Three major particles have been identified during enteroviruses replication in cells including putative procapsid, mature virus, and empty capsid [36,37]. During the beginning of assembly, putative procapsids are found in infected cells and contain 60 copies of the structural proteins VP0, VP1, and VP3, but are devoid of genome [38]. When the mature virus is formed, the RNA genome cause the catalytic cleavage of VP0 into VP2 and VP4, and the mature virus is released from infected cell [39]. After the mature virus interacted with a receptor on the surface of host cell, the uncoating process is activation and then the empty capsid is formed. In the present study, F and E particles of B5-141-6-5 were produced using microcarrier-based serum-free culture system and separated using sucrose gradient ultracentrifugation. In the electron microscopy analysis, F particles in F6-8 were similar with mature viruses which contained viral genome and had full particle structure. However, all two types of putative procapsid and empty capsid particles were devoid of viral genome and might not be separated by sucrose gradient ultracentrifugation in this study. Hence, E particles in the F11-13 could contain putative procapsid and empty capsid particles and all displayed a defective structure in negative staining. The EV71 VP2-specific monoclonal antibody MAB979 can recognize VP0 (VP2 + VP4) and VP2 structural proteins and was used in the Western blotting assay [3]. The VP0 protein of F particles had been cleaved into VP2 and VP4 proteins, therefore F6-8 displayed one major VP2 protein band in Western blotting assay. In contrast, the E particles in F11-13 displayed one major VP0 (could come from putative procapsids) and one minor VP2 (could come from empty capsid) protein bands in Western blotting assay. Overall, two-types of non-infectious particles, putative procapsid and empty capsid, existed in the F11-13 and can't be distinguished by electron microscopy and Western blotting assay in this study.

A previous study had demonstrated approximate 10,000-fold higher RNA content in the F particles than in the E particle by real-time RT-PCR analysis [4]. The low RNA content of the E particle presented empty physical appearance in electron microscopy analysis and low infectivity in $TCID_{50}$ analysis [4]. To investigate the immunogenicity of purified F and E particles, rabbits were immunized with different amount antigens in the present study. After two doses of vaccination in rabbits, the full particles could induce 2-fold higher neutralizing antibody titers than the empty particles. The results indicate that both particles were immunogenic but the F particles were more immunogenic than the E particles in rabbits, which were similar with the immunogenic evaluation of formalin-inactivated EV71 full and empty particles in mouse model in previous studies [4,40]. Moreover, the B5-141-6-5F particles could induce neutralizing antibody titers against all three major genogroups and is desirable for further development. Interestingly, the EV71 E particles could also induce neutralizing antibody titers, which is different from IPV [40,41]. Two types of non-infectious particles were produced during the EV71 replication cycle and they could not be differentiated using electron microscopy and Western blotting assay. In development of IPV, *in vitro* potency assays for quantifying D antigens were developed for predicting vaccine immunogenicity [32,42]. Therefore, *in vitro* potency assays should be also developed for EV71 vaccines.

In summary, we successfully adapted and selected a high-growth EV71 genotype B5 strain which is the current predominant genotype in Taiwan and South-Eastern Asia. Four pilot scale-up production of the HG-B5 strain were performed by using spinner flask system containing 5 g/L microcarrier in serum-free VP-SFM medium and the peak virus titers of all four runs could reach around 10^8 $TCID_{50}$/mL. As shown in the Table 4, the purified HG-B5 vaccine candidate could induce significant higher cross-neutralizing antibody responses against B5 (TW-08) strain than those in A (BrCr) and C4 (TW-08) strains. The results were consistent with antigenic analysis of cross-reactive neutralizing antibody profiles in our previous study [3]. The rabbits immunized with B5 strain could induce higher neutralizing antibody titers against homologous B5 (1:4096) strain than those against heterologous A (1:32) and C4 (1:512) strains [3]. Therefore, it is important to monitor vaccine-induced cross-reactive neutralizing antibody titers against recent circulating viruses. For the A (BrCr) virus, only high dosage F particle group could induce detectable neutralizing antibody titers (1:8) in the present study. Basically, serology data in previous EV71 rabbit and human studies showed that antisera generated using genogroup B and C viruses consistently have a lower neutralizing antibody titers against genogroup A viruses [3,22]. International guidance regarding protective level of EV71 serum neutralizing antibody has not been established. Based on poliovirus vaccine studies, serum neutralizing antibody titers $\geqq 1:8$ is suggested to be protective. Moreover, a recent study had demonstrated that mice receiving the passive antisera with an *in vitro* neutralizing titer of 1:8 could obtain 58% survival rate [43]. Therefore, serum neutralizing antibody titers $\geqq 1:8$ could also be suggested to be protective for EV71. Overall, the purified HG-B5 vaccine candidate had a potential to induce broad spectrum of cross-reactive neutralizing antibody titers against three (A, B5, and C4) EV71 genotypes in rabbits. Therefore, the high-growth EV71 B5-141-6-5 vaccine virus appears to be a promising candidate for future development.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.01.041.

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