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Highlights

Vaccine xx (2011) xxx–xxx

Selection and characterization of vaccine strain for Enterovirus 71 vaccine development

Jui-Yuan Chang, Cheng-Peng Chang, Hutchinson Hau-Pong Tsai, Chen-Dou Lee, Wei-Cheng Lian, Ih-Jen-Su, I.-His Sai, Chia-Chyi Liu, Ai-Hsiang Chou, Ya-Jung Lu, Ching-Yao Chen, Pi-Hsiu Lee, Jen-Ron Chiang**, Pele Choi-Sing Chong*

- In this report a clinical isolate E59 was screened and selected from >100 clinical isolates.
- EV71 vaccine candidate was successfully produced from Vero cell grown in serum-containing medium.
- Sub-microgram of EV71 could elicit strong virus neutralizing antibody responses in animal models.

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Selection and characterization of vaccine strain for Enterovirus 71 vaccine development

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ABSTRACT

Enterovirus 71 (EV71) has recently emerged as an important neurotropic virus in Asia because effective medications and prophylactic vaccine against EV71 infection are not available. Based on the success of inactivated poliovirus vaccine, the Vero cell-based chemically inactivated EV71 vaccine candidate could be developed. Identification of EV71 vaccine strain which can grow to high titer in Vero cell and induce cross-genotype virus neutralizing antibody responses represents the first step in vaccine development. In this report we describe the characterization and validation of a clinical isolate E59 belonging to B4 sub-genotype based on VP1 genetic analysis. Before selected as the vaccine strain, the genetic stability of E59 in passage had been analyzed based on the nucleotide sequences obtained from the Master Virus Seed, Working Seed banks and the virus harvested from the production lots, and found to be identical to those found in the original isolate. These results indicate that E59 vaccine strain has strong genetic stability in passage. Using this vaccine strain the prototype EV71 vaccine candidate was produced from 20 L of Vero cell grown in serum-containing medium. The production processes were investigated, characterized and quantified to establish the potential vaccine manufacturing process including the time for virus harvest, the membrane for diafiltration and concentration, the gel-filtration chromatography for the down-stream virus purification, and the methods for viral inactivation. Finally, the inactivated virion vaccine candidate containing sub-microgram of viral proteins formulated with alum adjuvant was found to induce strong virus neutralizing antibody responses in mice and rabbits. Therefore, these results provide valuable information for cell-based EV71 vaccine development.

1. Introduction

Enterovirus 71 (EV71) infection has become an emerging infectious disease and presents serious public health problems in the Southeast Asia [1–5]. Since no effective anti-EV71 agent is available, developing an effective vaccine against EV71 infection is the best strategy to control the disease. EV71 virus was firstly isolated in 1969 in the United States [6]. EV71 is a non-enveloped RNA virus of the family Picornaviridae, has icosahedral symmetry with 25–30 nm in diameter, contains a single molecule of plus sense ssRNA (7.5–8.5 kb), and replicates in the cytoplasm. The icosahedral viral capsid is composed of 60 identical units that consist of four structural proteins in each unit: VP1, VP2, VP3 and VP4 [5,7,8]. The complete nucleotide sequence of the EV71 prototype strain BrCr has been determined [7].

The concern of potential virulent revertant virus as happened in polio and yellow fever attenuated vaccines [9,10], has made the inactivated EV71 vaccine a more favorable choice for vaccine development. In fact, preliminary studies have indicated that the heat-inactivated EV71 virion produced from Vero cell grown in various culture media elicited more effective immune response than those obtained from the recombinant VP1 protein or DNA vector vaccines in mice [3,8,11–15]. Since there is no certified EV71 vaccine available in the world, every party working on EV71 vaccine development collaborates with the regulatory authority in their own country. In Taiwan, Taiwan FDA suggests to us to adopt the guidelines for polio vaccine lot release in which there is no absolutely criteria for vaccine purity. In the present study, we

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describe the identification and characterization of a clinical isolate that was screened and selected from >100 clinical isolates. The manufacturing process for producing the prototype EV71 vaccine candidate was established includes the time for virus harvest, the membrane for diafiltration and concentration, the gel-filtration chromatography for the down-stream purification, and the methods for viral inactivation. The inactivated virion vaccine candidate formulated with alum adjuvant was found to elicit strong virus neutralizing antibody responses in mice and rabbits. Rabbit antibodies were also found to be capable of cross-neutralizing many B and C sub-genotypes of EV71. The up-stream and down-stream processes established in this report had been successfully used for the manufacture of EV71 vaccine candidates using either serum-containing or serum-free media. Therefore, these results provide valuable information for cell-based EV71 vaccine development.

2. Materials and methods

2.1. Ethics statement

All experiments were conducted in accordance with the guidelines of Laboratory Animal Center of NHRI. The animal use protocols have been reviewed and approved by the NHRI Institutional Animal Care and Use Committee.

2.2. Cells and virus

African green monkey kidney cell lines (Vero cell at passage #121) were purchased from the American Type Culture Collection (ATCC). Vero cells were grown in serum-containing medium (Medium-199 from Gibco/Invitrogen and 5% fetal bovine serum (FBS)), and cells were passaged twice weekly in T-flasks. FBS was purchased from Moregate Biotech, Australia and was certified by the manufacturer to be free of bovine spongiform encephalopathy (BSE). Vero cells were expanded and harvested at passage #125, and defined as the Master Cell Bank (MCB). The MCB was dispensed into ~400 vials (1 mL/vial), and stored in a liquid-nitrogen tank. One vial of the MCB was thawed and expanded as the Working Cell Bank (WCB) at passage #129. Like the MCB. WCB was dispensed into ~400 vials (1 mL/vial), and also stored in a liquid-nitrogen tank. The E59 strain (genotype B4), the clinical isolate of the EV71 virus, was obtained from the Taiwan CDC. E59 vaccine virus was adapted to grow in Vero cells supplemented with 2 μg/mL TPCK-trypsin (Sigma, USA) and 2 rounds of plaque purification in 6-well plates. EV71 E59 virus stocks were collected from the supernatants of infected Vero cells at 3 days post infection (DPI). The Master Virus Seed (MVS) and Working Virus Seed (WVS) were prepared at passage two and four, respectively. The titers of the virus stocks were determined by a plaque assay, and the stocks were stored at −80 °C. Furthermore, master and working cell and virus seed banks were established following the cGMP guidelines given by US FDA and characterized to fulfill the requirements for manufacture of biological products by Bioreliance (UK). EV71 virus RNA was extracted by a commercial kit produced by Geneaid (Taoyuan, Taiwan). The extracted viral RNA was amplified using the one-step RT-PCR (Promega, Madison, WI). Sequences of the oligonucleotide primers used in this study are available upon request. The amplified DNA was sequenced using the ABI 3730 XL DNA Analyzer (Applied Biosystem Inc., Foster City, CA). Nucleotide sequences of VP1 reported in this study have been submitted to public domain (Accession No. GQ150746.1), and the amino acid sequences of all four structural viral proteins reported in this study are available upon request.

2.3. Pilot production of EV71 virus using roller bottles

The production of EV71 virus was performed using E59 vaccine strain and roller bottle technology. In brief, Vero cells were grown in 850-cm^2 roller bottles (Corning Life Science, USA) in 400 mL of serum-containing medium (DMEM plus 5% fetal bovine serum), and bottles were rotated at 0.33 rpm at 37 °C on a 100-bottle roller rack. Each roller bottle culture was inoculated with 1.5–2 × 10^7 cells, and the cell density usually reached 1.5–2 × 10^8 cells after 6 days of cultivation. After culture medium replacement, Vero cells in each bottle were infected with EV71/E59 at an m.o.i. of 10^{-5}. Production batches were typically obtained from 50 × 400-mL roller bottles in each run. EV71 was collected from the culture supernatant of each bottle at the fifth DPI. Cell debris were removed by filtration through a 0.65-μm membrane (Sartorius Stedim Biotech, USA), and the crude virus bulk was 20- to 40-fold concentrated using a 300-kDa cut-off diafiltration membrane in a tangential flow filter (TFF) cassette (Sartorius Stedim Biotech).

2.4. Purification of EV71 virus using liquid chromatography

One liter of EV71 virus concentrate was purified using an AKTA Pilot liquid chromatography system (GE Healthcare, USA) equipped with Sepharose Fast Flow 6 gel. The column (100 mm × 900 mm) was packed with 7 L of gel. PBS was used as the eluting buffer and the flow-rate was set at 80 mL/min. Fractions (300 mL per fraction) were collected and analyzed by immunoblotting, and virus infectivity was measured using the $TCID_{50}$ assay. Fractions containing the virus were pooled, further concentrated and then inactivated with 0.2% formalin (v/v) at 4 °C for 6 weeks. The vaccine bulk was obtained after sterile filtration using a 0.22-μm filter, subjected to SDS-PAGE and Western blot analyses, and stored at 4 °C. The VP2 content was analyzed by Q-ELISA, and the total protein concentration of the vaccine bulk was also determined by the BCA protein assay.

2.5. Determination of viral titer

Virus titers were determined using the median endpoint of the tissue culture's infectious dose ($TCID_{50}$) as described previously by Liu et al. [8] The $TCID_{50}$ values were calculated using the Reed–Muench method [16].

2.6. SDS-PAGE analysis and Western blotting

SDS-PAGE and Western blot analyses of the purified EV71/E59 bulk were performed according the protocol reported previously by Liu et al. [8].

2.7. Animal immunogenicity studies

Immunogenicity studies were performed according to the protocol reported previously by Liu et al. [8]. Briefly, different concentrations of inactivated virus protein (5, 25 and 50 μg of the EV71/E59 vaccine candidate) were mixed with 5 mL of aluminum phosphate (containing 1.5 mg of aluminum) at room temperature for 3 h before immunization. Groups of six female BALB/c mice (6–8 weeks old) were immunized intramuscularly (i.m.) with 0.2 mL of the different dosage of alum-adsorbed inactivated EV71 immunogens, and the animals were boosted with the same dose at 2-week intervals after priming. Immunized mice were bled 1 week after the final boost, and the serum was collected and stored at −80 °C. In parallel, rabbits were immunized i.m. with 0.5–20 μg of EV71 protein formulated with 1.5 mg of alum per dose. Sera were collected 2 weeks after each immunization and used for immunological analysis. The specificity and anti-EV71 titer of the antisera were tested

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by Western blotting and virus neutralization assay based on the $TCID_{50}$ determination, respectively.

2.8. Virus neutralizing assay

Virus neutralization titer of each sample was determined using $TCID_{50}$ assay according to the protocol reported previously by Liu et al. [8].

2.9. VP2 epitope-specific enzyme-linked immunosorbent assay (VP2-ELISA)

The VP2-ELISA was performed according to the protocol reported previously by Liu et al. [17]. The VP2 content in the samples were determined from the standard curve that was established by an EV71 vaccine bulk reference standard (lot #RD01) which was standardized against the original working standard (VLP lot #2) reported previously by Liu et al. [17].

2.10. Peptide-ELISA

The reactivity of the antibody to synthetic peptide was analyzed by an enzyme-linked immunosorbent assay (ELISA) according to the protocol previously reported by Panezutti et al. [18]

3. Results and discussion

3.1. Selection and characterizations of EV71 virus vaccine strain

Based on US FDA and International Conference on Harmonization (ICH) guidelines, the virus vaccine strain should have full document of the passage history of the virus seed (http://www.fda.gove/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm and ICH documents Q5A and Q5D). Two potential EV71 vaccine strains, E59 and C4D, were firstly selected over other >100 clinical isolates based on historical background and medical information. E59 and C4D strains were then characterized for yield of virion and immunogenicity. One potential vaccine strain E59 was isolated in 2000 and identified to be B4 sub-genotype; the other virus isolate C4D was obtained in 2004 and determined to be C4 sub-genotype. These virus seeds were first adapted to replicate in the Vero cell grown in T-flask cell culture containing Medium-199 plus 5% fetal bovine serum. Based on $TCID_{50}$ assay, the virus titer of both viruses could reach to 10^7 by the 5th day of post infection.

To test whether these viruses produced in T-flask could elicit strong cross-neutralizing antibody responses, two groups of 3 rabbits were immunized intravenously (iv) twice in 1 month with 0.5 mL of live virus at 2×10^7 $TCID_{50}$/mL per dose. As shown in Table 1, the sera collected at 1 month after the second dose from rabbits immunized with C4D (C4 genotype) virus were found to be more effective cross-neutralizing different sub-genotypes of EV71 viruses collected by Taiwan CDC than those sera obtained from rabbits immunized with E59 live virus. It was a surprise that antisera obtained from rabbits immunized subcutaneously (sc) with 2×10^7 $TCID_{50}$/mL of formalin-inactivated C4D virion formulated with adjuvant alum were found to have poor neutralization titers against different EV71 isolates (Table 1). The highest neutralization titer of the sera was 64 and the sera had no neutralizing activity against A and B1 sub-genotype. It is about 200 times (13,308 vs. 64) less effective against itself (C4 isolate). In contrast, antisera obtained from rabbits immunized with the same amount of formalin-inactivated E59 virion formulated with alum were found to retain good neutralization titers against EV71 isolates (Table 1). The sera from rabbits immunized with live E59 virus and inactivated virion formulated with alum had varied neutralizing activity against other B4 isolates ranging from 3557 to 74,161 and 2512 to 21,831, respectively. These results are consistent with our previous report [8] in which the sera generated from rabbits immunized with inactivated virion formulated with alum have neutralization titer ranging from 3320 to 32,768. We observed similar antibody responses from mice immunized with formalin-inactivated C4D formulated with alum (data not shown). These results suggest that (i) the neutralization epitopes in C4D isolate are very sensitive to chemical modification and can be destroyed by formalin inactivation; (ii) C4D virions are aggregated and precipitated out because of extensive cross-linking due to formalin treatment; and (iii) formalin-inactivated C4D virions are poor immunogens. No obvious aggregated viral particles were observed in the solution containing formalin-treated C4D virion. C4D inactivated virion formulated with alum could elicit relative good IgG titer (1/6400) against VP2 peptide as determined by peptide-ELISA [8,18], so formalin-inactivated C4D virions were not poor immunogens.

Other inactivation methods were performed. These included as exposure to UV light (UV box equipped with a 3600 Å light source) for 10 min at room temperature, heat-treatment at 65 °C for 2 h, and cross-linking with 0.05% (v/v) glutaraldehyde for 6 weeks at 4 °C. All three methods (heat-treatment, UV exposure, and glutaraldehyde cross-linking) were found to be very efficient in inactivating EV71 virus in culture supernatant, but significant precipitations were observed in both the heat-treated and glutaraldehyde inactivated EV71 samples. Thus, rabbit immunogenicity studies were performed with UV inactivated EV71 samples. As shown in Table 2, the neutralizing antibody titer obtained from the UV inactivated EV71 vaccine formulations were found to be lower than those obtained from the formalin-inactivated vaccine formulation. The UV-inactivated vaccine formulation containing C4D virus did not improve the potency and still elicited weak neutralizing antibody responses (<1/32). Therefore, the neutralization epitopes in C4D were sensitive to chemical modification and could be destroyed by both UV and formalin inactivation. Taking all these results together, E59 isolate was selected as the vaccine strain for all future studies.

3.2. Qualification of cell substrates and virus seeds

The MCB certification was performed by Bioreliance (UK). The Vero cells at passage #125 was fully characterized according to the guidelines given by US FDA (http://www.fda.gove/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm), and found to contain no retroviruses, bovine viral contaminants or porcine viral contaminants and other adventitious agents. In addition, Vero cells from MCB did not produce palpable tumors during several weeks observation period following inoculation of athymic nude mice. The WCB was further tested and passed for sterility, mycoplasmas, and *in vivo* and *in intro* adventitious agents tests. The MVS certification was also performed by Bioreliance (UK) according to the guidelines given by US FDA, and found to contain no other viruses such as retroviruses, bovine viral contaminants or porcine viral contaminants and other adventitious agents. In addition, virus from MVS did not produce palpable tumors during several weeks observation period following inoculation of nude mice. The WVS was further tested and passed for sterility, mycoplasmas, and *in vivo* and *in intro* adventitious agents tests.

3.3. Genetic characterization of E59 strain

To be compliant with the Good Manufacturing Practice (GMP) guidelines and screen for the fast growth and high yield EV71 virus clone, E59 clinical isolate was first adapted to grow in Vero cell culture containing 5% of FBS and plaque purified. High-growth virus clones (PP2 and PP5) were selected and prepared for EV71

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Table 1
Summary of cross-neutralization titer of antisera obtained from rabbits immunized with EV71 viruses.

Clinical isolates (sub-genotypes)	Rabbit antisera (average titer)[a]			
	Live virus		Inactivated virion with alum	
	E59	C4D	E59	C4D
BrCr (A)	55	255	40	<10
b1 (B1)	569	3146	700	<10
C2A (C2)	3319	12,915	1783	16
C2B (C2)	7130	13,277	3320	32
C2C (C2)	28	67	15	<10
B4A (B4)	3557	4237	2512	16
B4B (B4)	23,940	40,286	12,915	16
B4C (B4)	22,986	19,953	4418	ND[b]
B4D (B4)	7130	10,071	3320	ND
E59 (B4)	14,260	14,260	21,831	32
B4F (B4)	28,452	30,560	8474	ND
B4G (B4)	74,161	26,553	15,280	ND
B4H (B4)	26,533	28,452	4529	32
C4A (C4)	<10	54	<10	<10
C4B (C4)	4635	14,260	2118	32
C4C (C4)	16	285	40	<10
C4D (C4)	1660	13,308	782	64
C4E (C4)	1496	25,830	2320	ND
C4F (C4)	1349	15,280	750	16
C4G (C4)	<10	85	15	<10
C4H (C4)	891	5036	447	16
C4I (C4)	17	466	201	32
C4J (C4)	20	36	15	<10

[a] Two to three rabbits were immunized with 2×10^7 $TCID_{50}$/mL of either live virus via intravenous route (IV) or formalin-inactivated virion formulated with alum by subcutaneous (SC) method.
[b] ND means not done.

Table 2
Rabbit immunogenicity studies of the chemically inactivated EV71 virions.[a]

Virus strain	Chemical treatment	VP2 Q-ELISA unit/dose	Adjuvant formulation ($AlPO_4$)	$TCID_{50}$ neutralization titer against E59 (GMT ± SE)
E59	Live	0.35	No	1457 (748)
E59	Formalin	0.29	Yes	5036 (346)
E59	UV	0.25	Yes	832 (273)
C4D	Live	0.21	No	1218 (430)
C4D	Formalin	0.25	Yes	31 (15)
C4D	UV	0.08	Yes	16 (8)

[a] Three rabbits were immunized with 2×10^6 $TCID_{50}$/mL of either live or chemically inactivated virion formulated with alum by subcutaneous (SC) method.

virus seed stocks for further evaluation, including genetic characterization and pilot production in roller-bottle cell cultures. The nucleotide sequences of clones PP2 and PP5 of E59 strain were found to be 100% similar. Compared with the published BrCr strain [7], the E59 strain had 25.1% nucleotide difference in P1 structural gene. As shown in Table 3, VP4, VP2, VP3 and VP1 have 26.8, 35.8, 18.5 and 35.7%, respectively nucleotide difference with those gene segments found in BrCr strain. In contrast, the amino acid sequences in viral proteins between the BrCr and E59 virus were found to be 0, 1.6 (4 amino acids different), 2.1 (5 amino acids different) and 6.7% (20 amino acids different) difference respectively. Although none of these amino acid changes was located in the identified virus neutralization epitopes of VP1 (residues 776–791) and VP2 (residues 205–220 based on BrCr numbering) [8], amino acids changed at position 163 of VP2 (Pro to Gln); position 557 of VP3 (Asp to His); and positions 587, 595, 663, 710, 739, 809 and 811 of VP1 (Pro to Gln, Pro to Gln, Lys to Glu, Arg to Gln, Pro-Gln, Glu to Lys and Ser to Pro, respectively) could significantly alter the global structure of viral capsid and cause the virus to switch from A to B genotype.

About 400 vials of Master Virus Seed (MVS) bank (1 mL/vial containing ~1×10^7 $TCID_{50}$) were prepared from clone PP2 at passage 2. To be used as a vaccine strain, the genetic stability of E59 in passage had been analyzed based on the nucleotide sequences obtained from the master (2 passages from clone PP2) virus seed, working seed banks and the virus harvested from the production lots, and found to be identical to those found in clone PP2. Therefore, these results indicate that E59 vaccine strain has strong genetic stability in passage.

Table 3
Genetic differences between the BrCr and E59 virus strains.

Structural gene segments	Differences	
	Nucleotides (%)	Amino acids
P1 (2586 bp/862 aa)	25.1	3.4%
VP4 (207 bp/69 aa)	26.8	0%
VP2 (762 bp/254aa)	35.8	1.6%, changes are: **N**114**D**; **P**163**Q**; **V**195**I**; **N**212**D**
VP3 (726 bp/242 aa)	18.5	2.1%, changes are: **L**329**P**; **V**517**I**; **D**557**H**; **E**560**Q**; **T**563**S**
VP1 (891 bp/297 aa)	35.7	6.7%, changes are: **D**579**N**; **K**583**R**; **P**587**Q**; **P**595**Q** **D**-596**N**; **V**599**A**; **K**608**E**; **A**623**T** **K**663**E**; **R**710**Q**; **D**729**E**; **D**732**E** **P**739**Q**; **S**748**L**; **S**749**T**; **S**806**L** **E**809**K**; **S**811**P**; **I**814**V**; **S**840**A**

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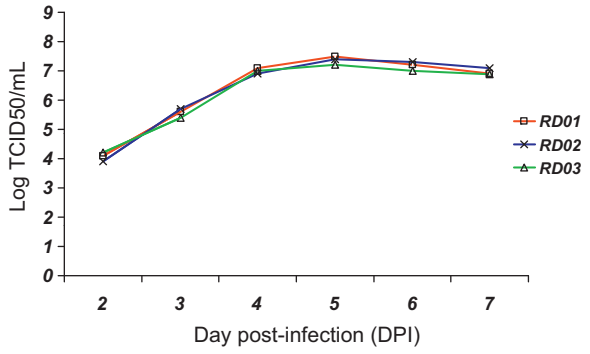

Fig. 1. The growth profiles of 3 research lots of EV71 virus produced from Vero cell grown in SC media.

3.4. Optimization of EV71 virus yield

Although many cell-based inactivated vaccines such as polio, influenza and hepatitis A are readily available on the open market, there is little information available on the manufacturing processes and the culturing systems. In addition, because of intellectual property rights and the proprietary technologies used in these vaccine products, the availability of comparison studies on virus and product yield influenced by the compositions of culture medium and systems used are very limited. Serum is required for optimal growth of mammalian cells as it is a source of nutrients, hormones and growth factors [19]. These serum factors also facilitate the attachment and spreading of cells, and provide protection against mechanical damage and shear forces [19,20]. Besides these advantages however, serum-containing (SC) medium may contain unwanted contaminants which are primary concerns in the safety of biological products [21]. To this end, the sources of bovine serum were selected from BSE-free cattle in Australia.

To evaluate the efficiency of E59 virus production in Vero cell grown in the SC medium, four different multiplicities of infection (MOI): 0.01, 0.001, 0.0001 and 0.00001 were first tested and compared for virus growth profile in the T-flask. The virus titers in general were found to have increased slightly on the 1st day and then increased steadily after 2 days of inoculation. The highest virus titer reached 3–5 $\times 10^7$ $TCID_{50}$/mL on the 5th day of post infection (DPI) for all MOI tested (data not shown). In addition, EV71 virus caused no significant cytopathic effects in Vero cell even by the 9th DPI. To test whether temperature could influence the virus growth and yield, the E59 virus was grown in various temperatures from 32 to 37 °C, no significant difference in virus growth profile and yield were observed (data no shown). Thus, the 10^{-5} MOI and 37 °C were selected and used in all later experiments.

3.5. Pilot scale up-stream process development

To fast track the production of a million doses of EV71 vaccine to immunize Taiwanese children, vaccine manufacturing was performed using the roller-bottle technology as described in Section 2. The virus growth profiles of three typical R&D batches (50 × 400-mL roller bottles in each run) are shown in Fig. 1. The virus titer could reach >10^7 $TCID_{50}$/mL by the 5th DPI and were consistent with the results obtained from T-flask. The production kinetic of the E59 virus was similar using 200 mL of the culture medium per bottle (data not shown).

To avoid large amounts of host cell debris which could increase difficulty in the downstream purification, EV71 virus was collected from the culture supernatant of each bottle at the 5th DPI before the significant cytopathic effects happening. Cell debris was then removed by filtration through a 0.65-μm membrane. To monitor and optimize the up-stream process, culture supernatants collected from each 20-L pilot-scale manufacturing runs were assayed for their titers of infectious virus particles using the $TCID_{50}$ assay and VP2 epitope contents by Q-ELISA that was developed previously [17]. Table 4 shows that the titers of infectious virus particles in pilot-scale crude supernatants harvested from four successful lots were found to be 3.6, 1.0, 2.5 and 0.4 × 10^7 $TCID_{50}$ units/mL in lot #RD01, 02, 03 and EV04, respectively. VP2 epitope contents in culture supernatants of these batches were measured by Q-ELISA and found to be 2.59, 2.39, 2.50 and 1.6 units/mL in lot #RD01, 02, 03 and EV04, respectively. Therefore, Q-ELISA units of the VP2 epitope content per 10^6 $TCID_{50}$ infective units were calculated to be 0.07 (2.59/36), 0.24 (2.39/10), 0.10 (2.5/25) and 0.40 (1.6/4.0) for lot

Table 4
Summary of in-process characterizations of EV71 vaccine bulk.

Lot #	Process step	Total volume (L)	$TCID_{50}$ [a] (× 10^7/mL)	Protein[b] (μg/mL)	VP2 epitope[c] (unit/mL)	Recovery[d] (%)	VP2 epitope/$TCID_{50}$ (unit/10^6)
RD01	Harvest	20.0	3.6	504.9 ± 10	2.59 ± 0.08	100	0.07
	Dif/Con	1.05	77.5	4654.6 ± 9.5	34.96 ± 4.5	70.9	0.04
	LC	0.54	37.2	40.4 ± 0.8	16.0 ± 1.7	16.7	0.04
	Formalin inactivated[e]	0.52	ND	36.9 ± 1.5	16.1 ± 2.3	16.2	ND
RD02	Harvest	20.0	1.0	411.0 ± 29.1	2.39 ± 0.23	100	0.24
	Dif/Con	0.96	76.6	4680.1 ± 53.6	32.59 ± 5.47	65.4	0.05
	LC	0.88	53.6	37.4 ± 0.39	15.6 ± 2.07	28.7	0.03
	Formalin inactivated[e]	0.80	ND	35.1 ± 1.67	17.3 ± 1.7	28.9	ND
RD03	Harvest	19.7	2.5	488.5 ± 27.6	2.50 ± 0.31	100	0.10
	Dif/Con	1.00	77.5	4600.5 ± 32.3	31.09 ± 7.5	63.1	0.04
	LC	0.88	37.3	41.7 ± 0.74	22.4 ± 1.43	34.1	0.06
	Formalin inactivated[e]	0.75	30.2 ± 2.82	15.4 ± 3.1	27.5	ND	
EV04	Harvest	20.0	0.4	313.1 ± 44.7	1.6 ± 0.2	100	0.40
	Dif/Con	1.00	76.2	1040.3 ± 49.81	30.9 ± 5.15	96.7	0.04
	LC	0.54	63.1	51.9 ± 0.14	44.18 ± 7.4	74.5	0.07
	Formalin inactivated[e]	0.50	ND	29.8 ± 1.32	23.06 ± 4.37	36.0	ND

ND is defined as not determined.
[a] $TCID_{50}$ is the median tissue culture infective dose of EV71 that produces pathological cytopathic effects (CPE) in 50% of inoculated cell cultures.
[b] Protein concentration was determined by the BCA protein assay.
[c] VP2-specific epitope content was determined by Q-ELISA.
[d] Recovery was calculated using the total VP2 epitope content at each step divided by the total VP2 epitope at the harvest stage.
[e] Vaccine bulk was obtained by formalin inactivation of virus bulk.
[f] Dif/Con is the diafiltration/concentration step.
[g] LC is the gel-filtration chromatography purification.

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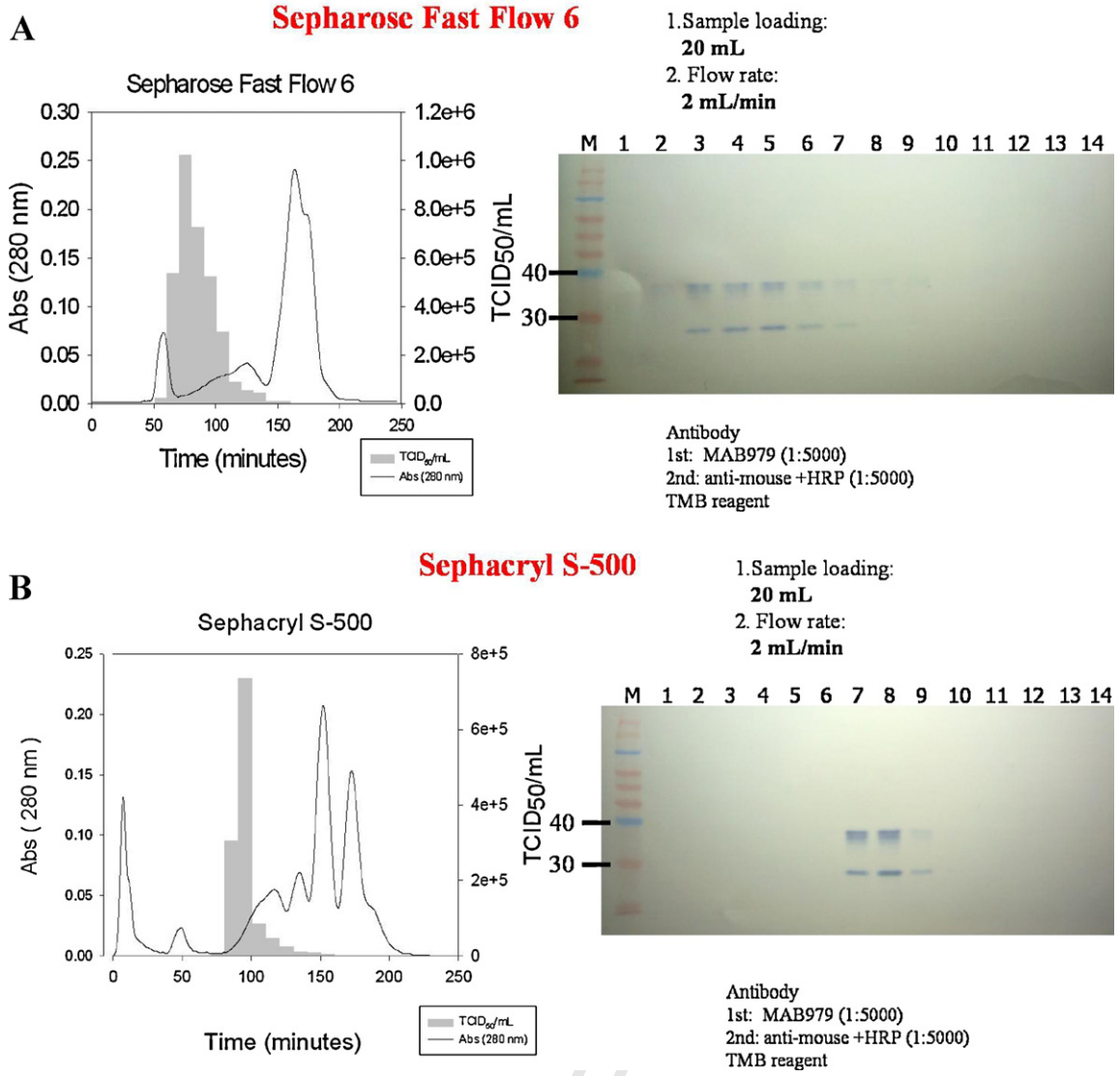

Fig. 2. Gel-filtration chromatography and Western blot analysis of EV71 virus obtained from the downstream purification process at research scale. (A) The gel-filtration chromatography was performed with Sepharose Fast Flow 6 column, and each fraction was analyzed by the Western blot using VP2-specific monoclonal antibody, MAB979. (B) The gel-filtration chromatography was performed with Sephacryl S-500 column, and each fraction was analyzed by the Western blot using VP2-specific monoclonal antibody, MAB979. The sample loading was 20 mL of the harvest concentrate, the flow-rate was 2 mL/min and the fraction was collected every 10 min.

#RD01, 02, 03 and EV04, respectively. These results show that the VP2 epitope content and the titer of infectious viral particles are not correlated. Similar preliminary results were observed with EV71 virus produced from Vero cell grown in the serum-free medium [17,22]. It suggests various amounts of defective EV71 virus particles produced in each production run. In contrast, the ratio between VP2 content and total protein in the harvest shown in Table 4 was found to be relatively consistent in all four lots (0.005, 0.006, 0.005 and 0.005 for lot #RD01, 02, 03 and EV04, respectively). Both results together suggest that EV71 viral antigens are consistently produced from Vero cell grown in the SC culture system, but various amounts of viral antigens assembled to be infectious virus particles.

To facilitate the downstream purification, the crude virus bulk was 20-fold concentrated using a 300-kDa cut-off diafiltration membrane in a tangential flow filter (TFF) cassettes. In research scale (5 L of crude virus bulk), a 1000-kDa cut-off diafiltration membrane had been tested in the TFF step and found to be less effective in the recovery of VP2 content as determined by Q-ELISA (data not shown). To monitor the efficiency and consistency of 300-kDa membrane in the TFF process, the protein content, VP2 epitope content and infectious viral particles in in-process materials were analyzed by BCA protein assay, Q-ELISA and $TCID_{50}$ virus titer assay, respectively. As shown in Table 4, the TFF process was found to have 60–90% efficiency and essentially did not recover all viral antigens based on Q-ELISA unit of VP2 (70.9%, 65.4%, 63.1% and 96.6% for lot #RD01, 02, 03 and EV04, respectively). The purpose of the TFF process was to concentrate the viral particles and to remove cell proteins. In most cases, more than 50% of total protein as shown in Table 4 was removed. Also, ELISA units of VP2 epitope per 10^6 $TCID_{50}$ infectious units were found to be consistent and correlated (0.04, 0.05, 0.04 and 0.04 for lots #RD01, 02, 03 and EV04, respectively). These results suggest that some of the EV71 viral fragments are removed from the virus bulk during the TFF step and infectious particles are concentrated.

3.6. Downstream process development and optimization

At the research stage, the concentrated EV71 viral stock (20 mL) was first loaded to either a Sepharose Fast Flow 6 gel column or a Sephacryl S-500, and subjected to liquid chromatography. The

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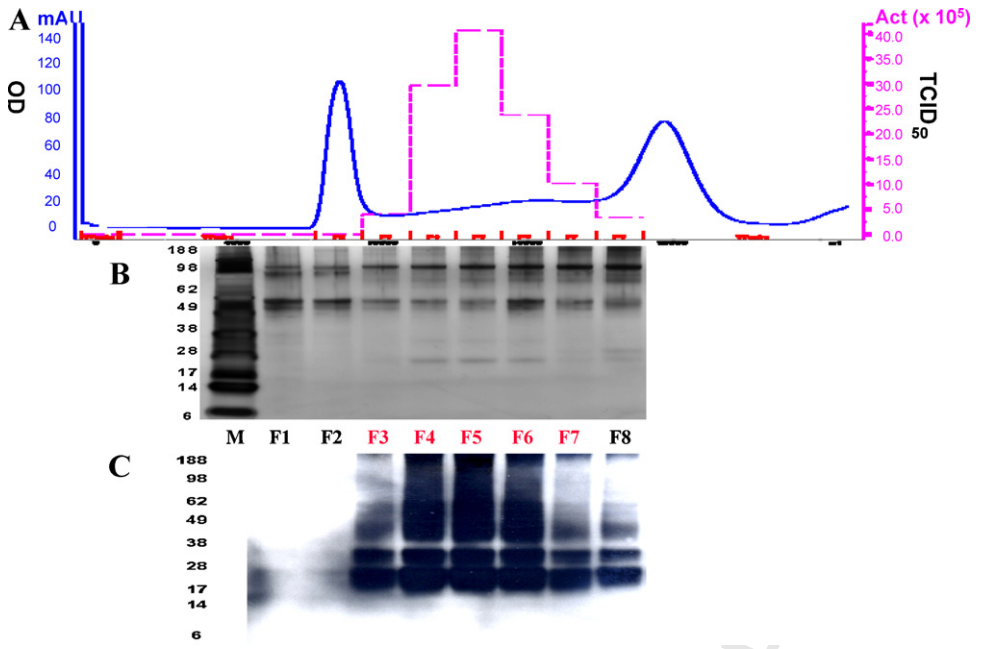

Fig. 3. Gel-filtration chromatography, SDS-PAGE and Western blot analyses of EV71 virus obtained from downstream purification process at pilot scale. The Panel A shows the elution profile of virus concentrate loaded and separated on the Sepharose Fast Flow 6 column using an AKTA Pilot chromatography system and monitored by UV absorption at 280 nm. The virus titer in each fraction determined by $TCID_{50}$ was shown in the histogram. The protein content in each collected fraction was separated by SDS-PAGE (Panel B) and analyzed by the Western blot using VP2-specific monoclonal antibody, MAB979 (Panel C).

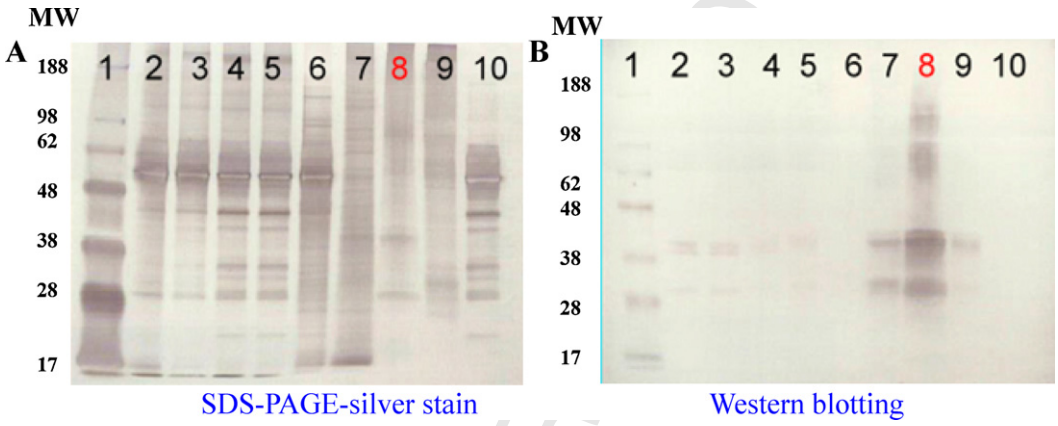

Fig. 4. The SDS-PAGE (Panel A) and Western blot (Panel B) analyses of EV71 virus products obtained from each production processes. Lane 1 is MW Markers; lane 2 is virus harvest from cell culture; lane 3 is the filtrate from 0.65 um micro-filtration; lane 4 is the virus concentrate obtained from the 300 K ultra-filtration step; lane 6 is the waste from the ultra-filtration step; lane 7 is the pooled fractions of Sepharose Fast Flow 6; lane 8 is the formalin-inactivated virus bulk from the pooled fractions; lane 9 is the pooled fractions of Sephacryl S-500 gel; and lane 10 is the SC culture medium.

Table 5
Mouse immunogenicity studies of formalin-inactivated EV71 virion produced from Vero cell grown in SC media.

Sample	Total protein (μg) per dose	VP2 ELISA unit/dose	Adjuvant		Neutralization titer based on $TCID_{50}$ after 2 immunizations (GMT ± SE)
			Type	μg	
RD03	1	0.5	PBS	0	105 (35)
RD03	5	2.5	PBS	0	214 (101)
RD03	1	0.5	$AlPO_4$	300	322 (132)
RD03	5	2.5	$AlPO_4$	300	624 (342)
EV04	1	0.8	$AlPO_4$	300	51 (78)
EV04	2	1.6	$AlPO_4$	300	416 (221)
EV04	1	0.8	$Al(OH)_3$	300	47 (45)
EV04	2	1.6	$Al(OH)_3$	300	62 (32)

Six mice per group were immunized with 2× chemically inactivated virion formulated with adjuvants by subcutaneous (SC) method.

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virus was identified mainly in fractions 3–7 based on Western blot analysis and $TCID_{50}$ virus titer (Fig. 2A). In contrast, the main virus fractions were found in fractions 7–9 when the Sephacryl S-500 gel column was used (Fig. 2B). Both gel-filtration chromatographies gave similar virus yield and purity, but the Sepharose Fast Flow 6 gel was selected because its GMP-grade material was commercially available.

Four pilot scale crude virus bulks (1 L) were purified using the AKTA Pilot liquid chromatography system equipped with a column (100 mm × 900 mm) packed with Sepharose Fast Flow 6 gel. A typical chromatographic run is shown in Fig. 3. The EV71 virus was identified and located in fractions 3–8 as determined by SDS-PAGE, Western blot, Q-ELISA and $TCID_{50}$. The EV71 virus fractions (4–7) were pooled and concentrated using a K100 TFF membrane. As shown in Table 4, ELISA units of VP2 epitope per 10^6 $TCID_{50}$ infectious units were again found to be consistent (0.04, 0.03, 0.06 and 0.07 for lots #RD01, 02, 03 and EV04, respectively). These results suggest that the virus bulk consistently pooled from the fractions contain both EV71 infectious and non-infectious particles. In addition, ELISA unit of VP2 epitope per μg/mL of protein was found to be around 0.45 and consistent for the first 3 lots (0.41, 0.42, and 0.53 for lots #RD01, 02, and 03, respectively). The ratio in lot #EV04 was found to be 0.85, possibly due to the pooling of fractions containing high VP2 content. The results indicate that the virus bulk still contaminated with residual cell and serum proteins even though the gel-filtration chromatography had essentially removed >99% (>4500 dropped to 40 μg/mL) of these proteins (Table 4). Furthermore, the recovery yield based on VP2 content determined by ELISA was not consistent (25, 44, 44, and 78% for lots #RD01, 02, 03 and EV04, respectively) for these four pilot runs. Overall, there is room for improvement in the chromatographic purification process.

3.7. Qualification of vaccine bulk

Since there is no certified EV71 vaccine available in the world, every party working on EV71 vaccine development collaborates with the regulatory authority in their own country. In Taiwan, Taiwan FDA suggests to us to adopting the guidelines for polio vaccine lot release in which there is no absolutely criteria for vaccine purity. To this end, the criteria used for assessment of vaccine bulk were defined (i) no residual infectivity; (ii) consistent ratio of VP2 epitope per total protein (>0.3); (iii) consistent high MW proteins found in vaccine bulk; and (iv) the residual DNA in the EV71 vaccine bulk should be less than 10 ng/dose based on the FDA guidelines. The chromatographically purified EV71 virus bulk was inactivated with formalin at 4°C for 6 weeks. Testing for residual infectivity was performed using the plaque assay in RD cell. After the formalin-inactivation no virus activity was found in all 4 lots tested and confirmed that EV71 vaccine bulks were fully inactivated. After 0.22-μm sterile filtration, the VP2 epitope content determined by ELISA and the total protein concentration measured by the BCA assay found both protein and VP2 epitope content losses during the inactivation and sterile filtration steps. Nevertheless, the Q-ELISA unit in all four lots was found to be close to the value found in the virus bulk before inactivation (Table 4). These results suggested that formalin inactivation did not modify the VP2 epitope content as determined by ELISA. The ratio of VP2 epitope per total protein was found to be consistently >0.3 (0.43, 0.49, 0.51 and 0.77 for lot #RD01, 02, 03 and EV04, respectively). In addition, the quality of EV71 vaccine bulk was analyzed by SDS-PAGE and Western blots, and shown in Fig. 4. The vaccine bulk produced in the presence of serum was found to contain fewer high MW proteins in the SDS-PAGE analysis, but these protein bands were not recognized by MAB979 (Fig. 4 lane 8) and could be residual proteins from cell and SC media. These high MW proteins were observed in all 4 lots of vaccine bulk (data not shown). This may also explain that the current vaccine bulk produced from Vero cell grown in SC medium has the ratio of ELISA unit of VP2 epitope per μg of total protein below 1. The residual DNA in the EV71 vaccine bulk was determined and found to be <50 pg/mL that would pass the criteria of acceptance for human vaccine (10 ng/dose) based on the FDA guidelines.

3.8. Mouse immunogenicity studies with different EV71/E59 vaccine candidates

The amount of VP2 epitopes in three different formalin-inactivated EV71 whole virions vaccine bulks determined by the ELISA were used for the dosage formulation and mouse immunogenicity studies. Mouse immunogenicity studies summarized in Table 5 revealed that EV71 vaccines were immunogenic and elicited good virus neutralization titer. In addition, EV71 vaccine formulated with $AlPO_4$ as adjuvant elicited better EV71 neutralizing antibody responses (geometric mean titer (GMT) = 624) than those obtained with the EV71 vaccine alone (GMT = 214). Furthermore, $Al(OH)_3$ was found to be less effective as adjuvant (Table 5). The number of VP2 epitope units and the magnitude of neutralizing titers were found to be dose-dependent in mice immunized with vaccine candidates produced in roller bottles.

4. Conclusion

An EV71 vaccine strain E59 was selected and characterized to be B4 sub-genotype based on the VP1 genetic analysis. The genetic stability of E59 in passage had been analyzed based on the nucleotide sequences and found to be identical to those found in the original isolate. These results indicate that E59 vaccine strain has strong genetic stability in passage. The Master and Working Virus Seed banks were prepared and validated to be free of contaminants according to US FDA guidelines. The manufacturing process for the prototype EV71 vaccine candidate based on E59 isolate produced from 20 L of Vero cell grown in serum-containing medium was established including the time for virus harvest, the membrane for diafiltration and concentration, the gel-filtration chromatography for the down-stream virus purification, and the methods for viral inactivation. Finally, the inactivated EV71 virion vaccine candidate containing sub-microgram of viral proteins formulated with alum adjuvant was found to induce strong virus neutralizing antibody responses in mice and rabbits. Therefore, these results provide valuable information for cell-based EV71 vaccine development.

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