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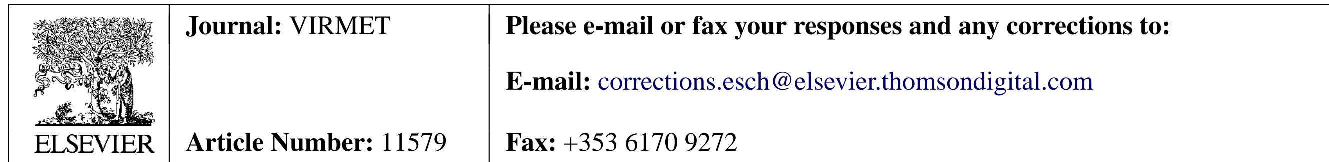

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Highlights

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Development of a quantitative enzyme linked immunosorbent assay for monitoring the Enterovirus 71 vaccine manufacturing process

Chia-Chyi Liu, Hsuen-Wen Chang, Grace Yang, Jen-Ron Chiang, Yen-Hung Chow, I-Hsi Sai, Jui-Yuan Chang, Sue-Chen Lin, Charles Sia, Chia-Hsin Hsiao, Ai-Hsiang Chou*, Pele Chong**

► A quantitative measurement of VP2 subunit throughout the vaccine production cycle was developed. ► The infectious particles determined by TCID50 assay was not directly correlated with VP2 content. ► The VP2 content and the magnitude of neutralizing titers were found to be dose-dependent.

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Development of a quantitative enzyme linked immunosorbent assay for monitoring the Enterovirus 71 vaccine manufacturing process

Chia-Chyi Liu[a,1], Hsuen-Wen Chang[a,1], Grace Yang[a], Jen-Ron Chiang[b], Yen-Hung Chow[a], I-Hsi Sai[a], Jui-Yuan Chang[a], Sue-Chen Lin[a], Charles Sia[a], Chia-Hsin Hsiao[a], Ai-Hsiang Chou[a,*], Pele Chong[a,c,**]

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A B S T R A C T

Enterovirus 71 (EV71), the etiologic agent causes outbreaks with significant mortality in young children in Asia and currently there is no vaccine available. In this study, we report a quantitative enzyme linked immunosorbent assay (Q-ELISA) to determine the concentration of the EV71 VP2 antigen. EV71 virus-like particles (VLPs) were produced in the baculovirus expression system and used as the EV71 antigen reference standard. Antisera from both EV71-immunized chickens and rabbits were very efficient and useful as capture antibodies to bind various forms of EV71 antigens, whereas a commercial VP2-specific virus neutralizing monoclonal antibody MAB979 was found to be suitable for quantifying the amount of VP2 antigen. This Q-ELISA was used successfully to determine VP2 content at each stage of EV71 vaccine manufacturing process, particularly during the upstream harvest, downstream purification and viral inactivation steps. The amount of VP2 antigen and the magnitude of neutralizing titers were found to be dose-dependent in mice immunized with vaccine candidates. These results indicate that Q-ELISA could provide off-line timely quantitative measurements of VP2 antigen throughout the production cycle to evaluate critical attributes and conditions that may affect virus yields in culture media, the quality of purification methods, the stability and potency of final vaccine formulations.

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1. Introduction

Since 1969 the first case of Enterovirus 71 (EV71) infection was recorded in CA, USA (Schmidt et al., 1974), EV71 has infected periodically young children throughout the world (for review, see Lee and Chang, 2010; Xu et al., 2010). Although the majority of EV71 infections in adults are asymptomatic, EV71 infection manifests most frequently as hand-foot-and-mouth disease (HFMD) or hyperangina in young children, who are potentially at risk for severe neurological complications, including aseptic meningitis, cerebella encephalitis and acute flaccid paralysis (AFP), that may lead to occasional deaths (Chumakov et al., 1979; Alexander et al., 1994; Gilbert et al., 1988; Chang et al., 1988; AbuBakar et al., 1999; Ho et al., 1999; McMinn, 2003). EV71 has now emerged as an important neurotropic virus, but there are no effective medications and a prophylactic vaccine (McMinn, 2002; Qiu, 2008). EV71 vaccine development efforts have been undertaken mainly in Asia, and promising preclinical immunogenicity results have been obtained using several prototype vaccine candidates. These include findings from studies with small animals immunized with chemically inactivated EV71 (Lee and Chang, 2010; Xu et al., 2010; Liu et al., 2011), a formulation of viral capsid protein peptides (Foo et al., 2007), a DNA plasmid carrying the major viral capsid protein VP1 (Tung et al., 2007), virus-like-particles (VLPs) formed by the physical association of the four EV71 capsid proteins VP1, VP2, VP3 and VP4 (Chung et al., 2008), and VP1-enriched milk produced by transgenic mice containing the VP1 gene (Chen et al., 2008). In light of the success of the Salk-inactivated poliovirus vaccine, the production of an inactivated whole virion EV71 vaccine is feasible, and the vaccine could be licensed if an animal potency assay and a method for quantifying viral antigens were available. Apart from vaccine formulation purposes, determining viral antigen yields in crude cell culture supernatants and purified virus preparations is a very important parameter to guide the scaling-up of the downstream process. Vaccine manufacturers are being challenged currently to modernize their scientific processes for optimizing the final product quality and improving process

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yields. In this report, we describe a quantitative enzyme-linked immunosorbent assay (Q-ELISA) to determine the concentration of EV71 antigen using different in-house EV71 virus preparations, EV71-specific antisera and a commercially-available EV71 VP2-specific virus neutralizing monoclonal antibody. This Q-ELISA has been used successfully to assess virus production yields and measure the amount of EV71 VP2 epitope in culture supernatants, the upstream harvest, downstream purification fractions and vaccine bulk.

2. Materials and methods

2.1. Ethics statement

All experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of NHRI. The animal use protocols have been reviewed and approved by the NHRI Institutional Animal Care and Use Committee (Approved protocol no. NHRI-095054-A).

2.2. Cells, media and virus

African green monkey kidney (Vero) cells were kindly provided by the Taiwan Centers of Disease Control (Taiwan CDC) which obtained the original Vero cell line (passage #125) from the American Type Culture Collection (ATCC, Rockville, MD, USA). The E59 strain (genotype B4), the clinical isolate of the EV71 virus, was obtained from the Taiwan CDC. EV71/E59 virus stocks were collected from the supernatants of infected Vero cells three days post infection (DPI). The titers of virus stocks were determined by a plaque assay, and these stocks were stored at −80 °C.

2.3. Production of EV71 virus-like particle (VLP) using the baculovirus expression system

EV71 VLP was produced using the recombinant baculovirus expression system described in Chung et al. (2008), with a small modification: the P1 gene was derived from the EV71 E59 virus isolate. Total protein concentration was determined by the BCA protein assay (Biorad, Hercules, CA, USA) and quantitative amino acid analysis that was performed by UBI-Asia, Taiwan.

2.4. Production of EV71 vaccine bulk using roller bottles

The production of EV71/E59 vaccine bulk was performed using the roller bottle technology. In brief, Vero cells were grown in 850 cm^2 roller bottles (Corning Life Science, Corning, NY, USA) in 200 mL of VP-SFM medium (GIBCO, Carlsband, CA, USA), 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 six 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 200 mL × 200 mL or 100 mL × 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, Haywood, CA, USA), and the crude virus bulk was 20- to 40-fold concentrated using a 100-kDa cut-off diafiltration membrane in a tangential flow filter (TFF) cassette (Sartorius Stedim Biotech). EV71 was purified using an AKTA Pilot liquid chromatography system (GE Healthcare, Salt Lake City, UT, USA) equipped with Sepharose Fast Flow 6 gel. The column (200 mm × 900 mm) was packed with 26 L of gel. PBS was used as the eluting buffer and the flow-rate was set at 80 mL per min. Fractions (160 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, concentrated further and then inactivated with 0.2% formalin (v/v). The vaccine bulk was obtained after sterile filtration using a 0.22-μm filter. The total protein concentration of the vaccine bulk was also determined by the BCA protein assay.

2.5. Production of EV71 virus in bioreactor

The production of EV71/E59 virus using serum-free VP-SFM medium in a BIOFLO 310 bioreactor (NBS, New Jersey, NJ, USA) was based on the microcarrier cell culture bioprocess previously reported by Chang et al. (2011). The total protein concentration of the purified virus bulk was determined by the BCA protein assay. Half of the purified EV71/E59 bulk (10 mL) was stored at −80 °C in 0.5-mL aliquots; the other half was inactivated by 0.2% (v/v) formalin at 37 °C for 3 days and stored at 4 °C.

2.6. SDS-PAGE and Western blot analyses

SDS-PAGE and Western blot analyses of the purified EV71/E59 bulk were performed according the protocol reported previously by Chang et al. (2011).

2.7. Densitometric analysis of Coomassie blue-stained EV71 proteins resolved by SDS-PAGE

A control calibration curve was established with increasing amounts of bovine albumin standard (Biorad) using SDS-PAGE and Coomassie blue staining. In the same gel, 5, 10 and 20 μL of either baculovirus-expressed EV71 VLP prepared according to Chung et al. (2008) or a sucrose gradient-purified EV71/E59 bulk were included to estimate the EV71 protein concentration. Coomassie brilliant blue R-250-stained albumin and EV71 protein bands were scanned individually at 150 dots per inch (DPI) setting using the Hewlett Packard ScanJet 4890 to obtain their respective integrated optical density (IOD) values. IOD recorded for each of the protein bands represented the total amount of protein molecules because Coomassie blue binds stoichiometrically to proteins (Hames, 1988). EV71 protein IODs obtained from the albumin calibration curve represented the estimated concentration of the viral protein.

2.8. Determination of virus titers

Virus titers were determined using the median endpoint of the tissue culture's infectious dose ($TCID_{50}$) as described previously by Liu et al. (2011) The $TCID_{50}$ values were calculated using the Reed–Muench method (Reed and Muench, 1938).

2.9. Preparation of inactivated EV71/E59 and animal immunogenicity studies

The EV71/E59 bulk chromatographically purified from roller bottle supernatants was inactivated with a 37% formaldehyde solution (Merck, West Point, NY, USA) at a ratio of 4000:1 (v/v) either at 4 °C for 45 days or 37 °C for 3 days. Different concentrations of inactivated virus protein were adsorbed on 5 mL of aluminum phosphate (1.5 mg of aluminum) at room temperature for 3 h before immunization. A group of six female BALB/c mice (18–25 g, 6–8 weeks old) was immunized intramuscularly (i.m.) with 0.2 mL of the alum-adsorbed inactivated EV71 immunogens, and they were boosted with the same dose every two weeks after priming. Immunized mice were bled two weeks after the boost, and the serum was collected for a virus neutralization assay based on the $TCID_{50}$ determination. In parallel, two rabbits were immunized i.m. three times with 0.5 μg of EV71 protein (determined by the BCA protein

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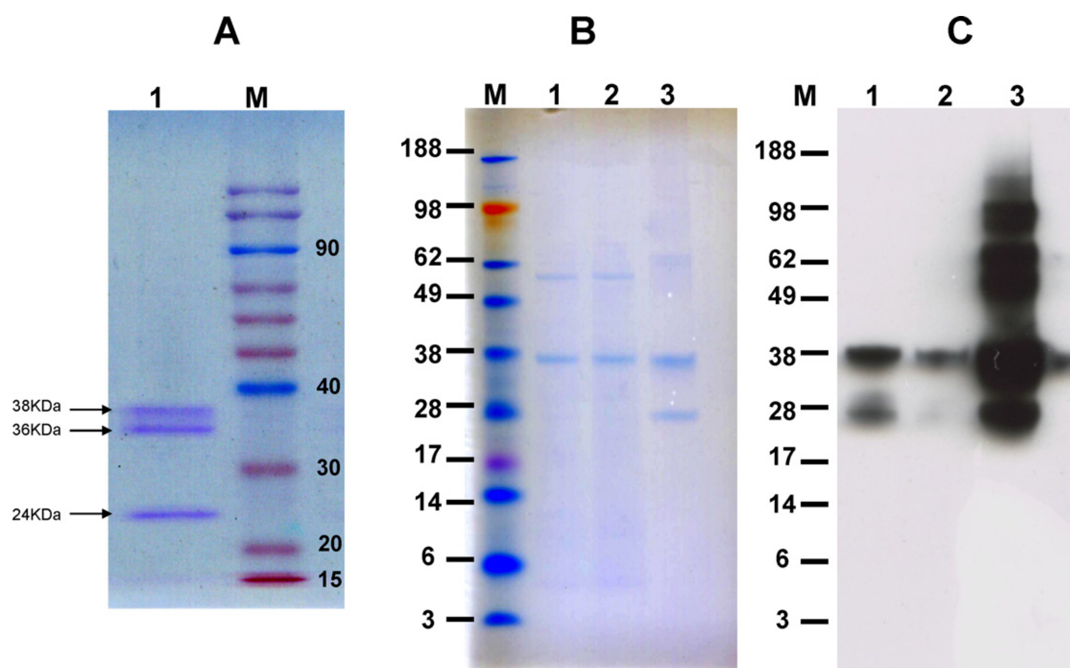

Fig. 1. Coomassie blue staining was used to visualize the EV71/E59 virus bulk separated on a SDS-PAGE gel (1A); two different lots of EV71 VLP proteins (1B, lanes 1 and 2) and formalin-inactivated EV71/E59 (1B, lane 3) were analyzed by SDS-PAGE; the viral proteins were analyzed by Western blot (1C) using the commercial EV71-specific monoclonal antibody MAB979.

assay) per dose. Rabbit sera were collected two weeks after each immunization and used for immunological analysis. Rabbits were bled out at four weeks after the final immunization, and a rabbit polyclonal IgG (RPAb) fraction was purified by Protein A affinity chromatography of pooled hyper-immune antisera and stored at −20 °C. The concentration of RPAb was determined by the BCA protein assay. The specificity and anti-EV71 neutralization titer of RPAb were tested using Western blot and virus neutralization assay, respectively.

2.10. Virus neutralization assay

Virus neutralization titer of each sample was determined using $TCID_{50}$ assay according to the protocol reported previously by Liu et al. (2011).

2.11. VP2 epitope-specific quantitative enzyme-linked immunosorbent assay (Q-ELISA)

The Q-ELISA was done by coating the wells of a 96-well Maxisorb ELISA plate (Nunc, Denmark) with 1 μg of either rabbit anti-EV71 polyclonal IgG antibodies (RPAb) or PY10267 in 100 μL of coating buffer (pH 9.6, Sigma) overnight at 4 °C. The wells were incubated with 250 μL of 5% skim milk to block non-specific binding of the conjugated secondary antibodies. The plate was left for 2 h at room temperature before it was washed three times, each time with 250 μL of assay buffer; then, 100 μL of twofold dilutions of either formalin-inactivated, chromatographically-purified EV71 bulk, or different virus preparations in PBS, pH7.4, containing 1% BSA (Biorad), was added to the wells. After incubation at room temperature for 2 h, the wells were washed four times with 250 μL of the wash buffer before 100 μL of assay buffer containing 0.1 μL of the MAB979 monoclonal antibody stock was added to each test well to detect the binding of native or denatured viral proteins to immobilized polyclonal capture antibodies. After the 1-h incubation at room temperature, wells were washed four times with 250 μL of wash buffer; 100 μL of an HRP-conjugated anti-mouse IgG antiserum (Jackson ImmunoResearch, West Grove, PA, USA) diluted at 1:30,000 in the assay buffer were then added to each test well. Binding was allowed during 30 min at room temperature before the plate was washed six times with assay buffer and blotted dry with a paper towel. Then, 50 μL of TMB peroxidase substrate (SureBlue^{TM}, KPL, Gaithersburg, MD, USA) was added, and the reaction occurred in the dark because the plate was covering with aluminum foil and left in a drawer for 30 min at room temperature. The reaction was stopped by adding 50 μL of 2N H_2SO_4 to each well, and absorbance at 450 nm was recorded with an ELISA reader (Spectra Max M2 model, Sunnydale, CA, USA).

3. Results

3.1. Reagents

Reagents and materials used in the Q-ELISA were firstly characterized. An EV71/E59 virus bulk was produced and purified using serum-free bioreactor as described previously (Chang et al., 2011) and its protein concentration was found to be 21.5 μg/mL by the BCA assay. The infectivity was measured by the $TCID_{50}$ assay and found to be 7.2×10^6 $TCID_{50}$ per mL. SDS-PAGE analysis of the purified EV71/E59 antigen revealed three major viral protein bands with estimated molecular weights of 28, 34–36 and 38 kDa (Fig. 1A). As shown in Table 1, the 34–36 kDa protein band matched the estimated molecular weights of the VP1 subunit of EV71 (Chung et al., 2008). The 28 kDa protein band might comprise two proteins with very close molecular weights (MW), such as VP2 and VP3, which were estimated to have MWs of 28 and 27 kDa, respectively (Table 1). As indicated in Table 1, the 38 kDa protein band corresponded to the incompletely processed procapsid protein VP0 (VP4–VP2). This is based on tryptic digestion and peptide mapping using mass spectrometry, both VP2 and VP4 protein sequences were identified in the 38 kDa protein band (Liu et al., 2011).

To characterize the specificity of the antibodies used in the Q-ELISA, immuno-dot blotting showed that both the chicken polyclonal antibody PY-10267 and the rabbit anti-EV71 antibody RPAb

Table 1
Summary of predicted and observed molecular weights (MW) of EV71/E59 viral proteins and incompletely processed viral polypeptides.

EV71 antigens	Amino acid sequences	Residues	Predicted MW[a] (kDa)	Observed MW[b] (kDa)
Viral antigen in virion				
VP4	1–69	69	7.49	8
VP2	70–323	254	27.78	28
VP3	324–565	242	26.52	27
VP1	566–862	297	32.73	34–36
Incompletely processed viral polypeptides (Fig. 1B, lanes 1 & 2)				
VP0 (VP4–VP2)	1–323	323	35.27	38
VP3-VP1	324–862	519	59.25	60
Viral antigens cross-linked by formalin inactivation based on VP2 recognized by MAB979 (Fig. 1C, lane 3)				
VP4–VP2	1–323	323	35.27	38
VP4–VP2–VP4			42.76	45–49
VP2–VP2			55.56	56–58
VP2–VP4–VP2			63.05	64–66
VP4–VP2–VP3–VP1	1–862	862	94.52	96–98

[a] The predicted MW is based on the corresponding amino acid sequence derived from the EV71/E59 strain genome.
[b] The observed MW is based on the SDS-PAGE and Western blot analyses of the EV71/E59 virus bulk.

reacted with purified EV71/E59 bulk. Both antisera had ELISA-reactive titers >50,000 (data not shown). A commercially available monoclonal antibody MAB979 was found to have a >1/64 neutralizing titer not only for EV71/E59 (genotype B4) but also for other EV71 isolates currently circulating in Taiwan, such as genotypes C4 and B5 (Liu et al., 2011). In addition, MAB979 recognized an epitope with the ***AGGTGTEDSHPPYKQ*** sequence that corresponded to residues 136–150 of VP2 (Liu et al., 2011). Another commercial monoclonal antibody M7064 (Dako, Denmark) that had no reactivity against EV71/E59 proteins, was served as a negative control in all immunological assays. These results suggested that a Q-ELISA could be established using a pair of selected antibodies, namely a polyclonal antibody as a capture reagent and the MAB979 monoclonal antibody as a reporting marker.

3.2. Preparation of purified EV71/E59 bulk and VLP as antigen reference standards

The quality of one potential reference standard (EV71/E59 bulk) derived from the 5 L bioreactor run has been described above and shown in Fig. 1A. EV71 virus-like particles (VLPs) as another potential reference standard was prepared from the baculovirus expression system according to Chung et al. (2008). As shown by SDS-PAGE (Fig. 1B, lanes 1 and 2), two major protein bands (60 and 38 kDa) were observed in VLP preparations. The 60 kDa protein band may correspond to the incompletely processed VP1–VP3 polypeptides that contained VP1 (34–36 kDa) and VP3 (27 kDa) (see Table 1). This 60 kDa protein band is being characterized currently. The 38 kDa protein was the VP0 procapsid protein described previously. The 38 kDa band (VP0) was recognized by MAB979 in the Western blot analyses (Fig. 1C, lanes 1 and 2). It was a surprise to observe a 28-kDa protein (VP2) band reacted with MAB979 in Fig. 1C, lane 1 since no protein band was detected in the SDS-PAGE analysis (Fig. 1B, lane 1).

The Coomassie blue-stained viral protein bands resolved in the SDS-PAGE could be scanned densitometrically to obtain integrated optical density (IOD) values that allowed for the determination of their respective concentrations from a known protein standard curve as shown in Fig. 2A. The viral antigens in two potential EV71 antigen reference standards (VLPs and EV71/E59 bulk) separated by SDS-PAGE as shown in Figs. 1B and 2A were scanned and their IOD values were recorded. The albumin protein standard curve obtained from the gel was found to be linear (R^2 = 0.9935, Fig. 2A). Based on the albumin calibration curve, the concentration of the 38 kDa

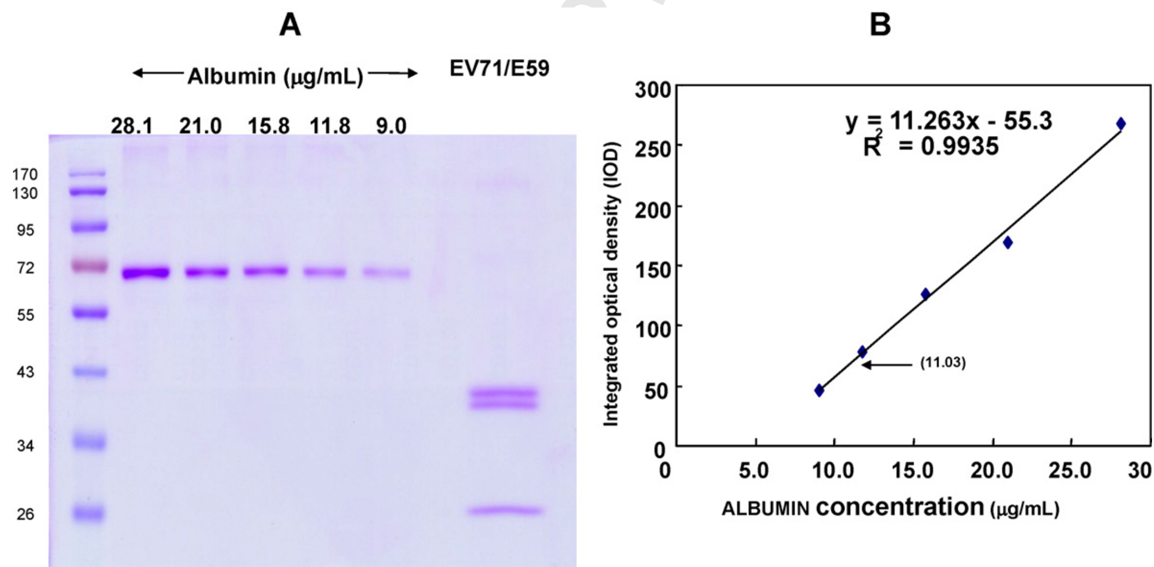

Fig. 2. Densitometric determination of EV71 viral antigen concentrations 40 μL of a purified EV71/E59 virus bulk, and various known concentrations of albumin standard (the numbers above the individual albumin bands) were separated on a 10.0% SDS-PAGE gel (A). The individual dye-bound albumin and 38-kDa bands were scanned with the Hewlett Packard ScanJet 4890 to obtain their respective IODs. The IOD values obtained for the individual albumin bands were plotted (B).

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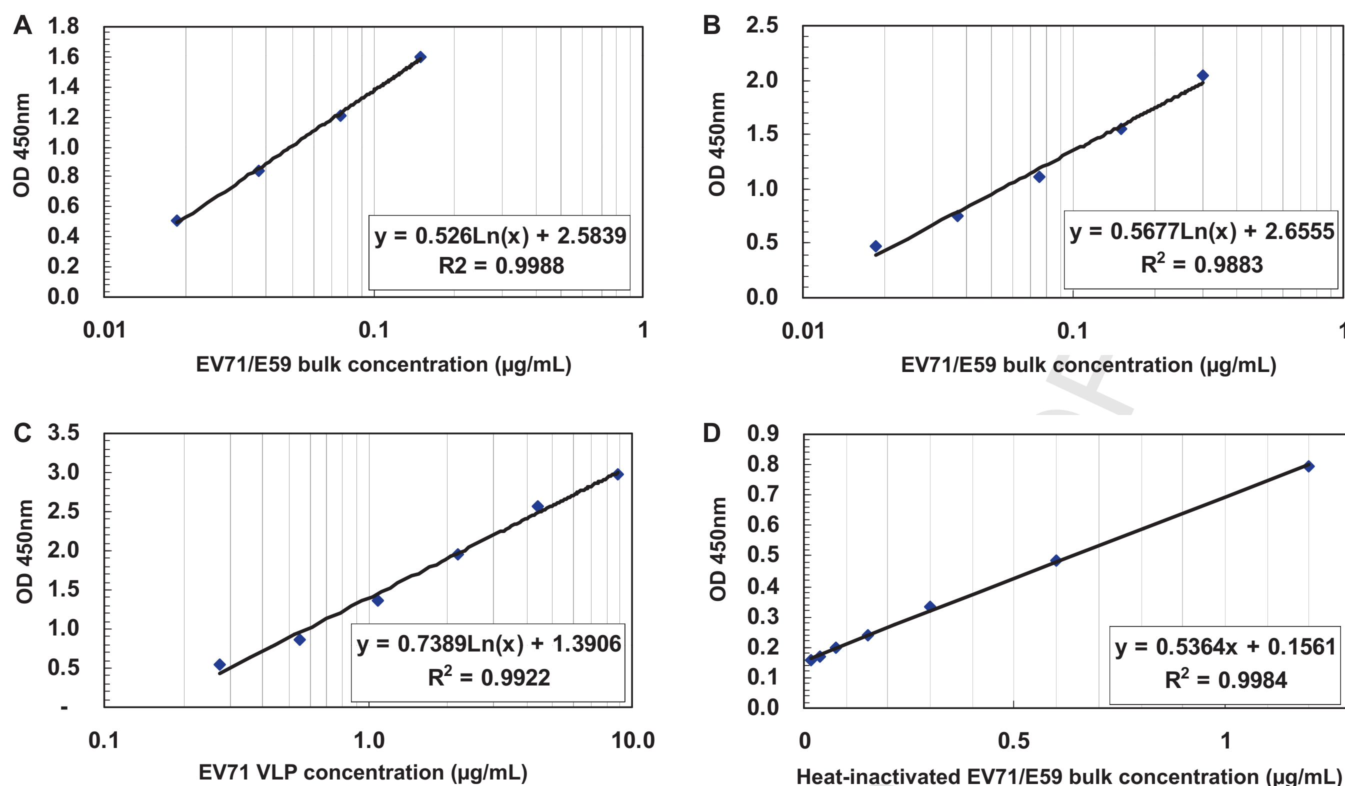

Fig. 3. Sensitivity and specificity of the PY-10267/MAB979 sandwich ELISA were calibrated with purified EV71/E59 bulk. The Q-ELISA was conducted by coating the wells with 0.1 µg of capture PY-10267 polyclonal antibodies. The secondary antibody MAB979 in (A) was added at 0.1 µL from the supplied stock per well. (B) and (C) show the sensitivity and specificity of the RPAb/MAB979 sandwich ELISA format calibrated with purified EV71/E59 virus bulk and the VLP Lot #2, respectively. (D) shows the sensitivity and specificity of the PY-10267/MAB979 sandwich ELISA format calibrated with purified, heat-inactivated EV71/E59 bulk.

(VP2–VP4), 36 kDa (VP1) and 27k-Da (VP3) proteins in EV71/E59 virus bulk (Fig. 2B) were calculated to be 5, 5.5 and 5 µg/mL, respectively. Therefore, the approximate concentration of EV71 viral antigens in the purified virus bulk (10 mL) was 15.5 µg/mL (the sum of VP0, VP1 and VP3), close to the value (21.5 µg/mL) obtained by the BCA protein assay. Using the same method, the arbitrary concentrations of the 60 kDa and 38 kDa protein bands in VLP Lot #2 were determined to be 50 and 75 µg/mL, respectively. The sum of these two concentrations (125 µg/mL) was consistent with the value of the total protein concentration determined by the BCA assay (135 µg/mL).

3.3. Q-ELISA development

Two assay formats were evaluated to establish the Q-ELISA. PY-10267 polyclonal antibodies were immobilized to capture varying amounts of viral antigens from the purified EV71/E59 bulk. The MAB979 monoclonal antibody was used to detect VP2 in purified virus bulk captured by PY-10267 antibodies. Results showed that this assay format could determine the VP2 epitope in EV71/E59 bulk in a dose-dependent manner (Fig. 3A). Similar results (Fig. 3B) were obtained from the second Q-ELISA format combining rabbit anti-EV71 polyclonal IgG antibody (RPAb, prepared in-house and found to have a >1000 EV71/E59 virus neutralization titer) and MAB979. Excellent linear fits (R^2 values 0.98–0.99) were obtained for each Q-ELISA format (Fig. 3A and B). Based on the current results, the best range of detection was found to be 0.05–0.16 µg/mL of the EV71/E59 bulk.

Similarly VLP Lot #2 (the second antigen reference standard) was tested by both Q-ELISA formats and the R^2 values were found to be consistent (0.99). A typical plot of absorbance vs. VLP antigen generated from the RPAb/MAB979 format is shown in Fig. 3C. Based on the current results, the Q-ELISA using VLP Lot #2 as an antigen working standard was found to be 10-fold less sensitive and had a detection range of 0.5–1.6 µg/mL of the VP0 antigen. Since the RPAb/MAB979 Q-ELISA format was established and characterized, it was selected for all future studies.

3.4. Stability of the potential antigen reference standards

Because the sensitivity of the assay obtained with VLP Lot #2 was 10-fold less than that obtained with purified EV71/E59 bulk, it was of interest to determine the stability of both VLP Lot #2 and purified EV71/E59 as long-term antigen reference standards for EV71 vaccine development. The stability profiles of these antigen reference standards stored at −80 °C for one and three months were evaluated by Q-ELISA. The VLP Lot #2 stored at −80 °C for one and three months was found to be relatively stable and yielded standard curves similar to those obtained before storage. In contrast, purified EV71/E59 required two to three times more frozen materials to generate standard curves similar to those obtained before storage. Other storage conditions for purified EV71/E59 bulk were studied. Unfortunately, five to ten times more material of the heat-treated EV71-E59 (56 °C for 3 h) or formalin-inactivated EV71/E59 bulk (3 days at 37 °C) was needed to generate a standard curve similar to that obtained with freshly prepared EV71/E59 bulk (Fig. 3D). The current results indicate that both the EV71/E59 bulk and VLP Lot #2 could be used as the antigen reference standard. But the stability profile and the concentration of antigen determined by SDS-PAGE and BCA assay showed VLP Lot #2 to be better and correlated (125 µg/mL vs. 135 µg/mL). For these reasons, VLP Lot #2 and the RPAb/MAB979 format were selected as reagents for subsequent Q-ELISA studies.

Table 2
Q-ELISA determination of VP2-specific epitope content in the serum-free culture supernatants, virus concentrates, chromatographically-purified virus bulks, and formalin-inactivated vaccine bulks produced at various key steps of the EV71 vaccine manufacturing process.

Lot #	Process step	Total volume (L)	$TCID_{50}$[a] ($\times 10^6$/mL)	Total protein[b] (μg/mL)	VP2 epitope[c] (Unit/mL)	Recovery[d] (%)	VP2 epitope/total protein (Unit/μg)
Lot #1	Harvest	39.0	5.4	1342 ± 46	7.1 ± 0.4	100	ND
	Dif/Con[e]	1.65	77.5	520 ± 10	166 ± 4.1	99.3	ND
	LC[f]	1.40	ND	28.6 ± 2.9	110 ± 14.9	55.8	3.8
Lot #2	Harvest	39.1	1.2	1534 ± 27	6.8 ± 0.5	100	ND
	Dif/Con	1.75	40	358 ± 9.7	228 ± 7.8	151	ND
	LC	1.50	ND	21.5 ± 2.5	113 ± 4.2	64.2	5.3
Lot #3	Harvest	41.0	2.3	1460 ± 5.5	11.9 ± 0.8	100	ND
	Dif/Con	1.10	57	430 ± 6.6	475 ± 21	107	ND
	LC	0.75	ND	66.7 ± 2.2	225 ± 16.7	34.6	3.4
	Formalin inactivated[g]	0.75		53.0 ± 1.5	198 ± 21.3	30.5	3.7

ND: 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] Total protein concentration was determined by the BCA protein assay.
[c] VP2-specific epitope content was determined by Q-ELISA using VLP Lot #2 as the antigen working standard and the RPAb/MAB979 ELISA format.
[d] Recovery was calculated using the total VP2 epitope content at each step divided by the total VP2 epitope at the harvest stage.
[e] Dif/Con is the diafiltration/concentration step.
[f] LC is the gel-filtration chromatography purification.
[g] Vaccine bulk was obtained by formalin inactivation of virus bulk.

3.5. The VP2 epitope content in a formalin-inactivated EV71-E59 vaccine bulk measured by the Q-ELISA

To determine whether the VP2 epitope content in a formalin-inactivated EV71-E59 vaccine bulk could be measured by the Q-ELISA, VLP Lot #2 was used as the antigen reference standard. The VP2 epitope content in the formalin-inactivated EV71/E59 vaccine bulk was found to be equal to 45.7 μg of VP0 per mL, which was greater than the value obtained by the BCA protein assay (18 μg/mL). It was interesting to know that the ratio between the VP2 epitope content determined by Q-ELISA and the total protein measured by the BCA assay was 2.53 (45.7/18). This discrepancy may be due to the fact that the VP2 epitope could exist in different forms and conformations (VP2 subunit, virion-associated VP2, incompletely processed VP0 and other VP2 associated antigens cross-linked by formalin). Therefore, arbitrary Q-ELISA units are preferable to describe the content of VP2 epitope in a given sample. To this end, one Q-ELISA VP2 epitope unit/mL was defined as 1 μg of VP0 (38-kDa protein band) per mL of VLP Lot #2 determined by the RPAb/MAB979 Q-ELISA format. In this example, 45 units of VP2 epitope per mL were in the formalin-inactivated EV71/E59 vaccine bulk.

3.6. Determination of VP2 epitope content in EV71/E59 vaccine produced from the roller bottle manufacturing process

Culture supernatants collected from each 40-L roller bottle manufacturing runs were assayed for their titers of infectious virus particles using the $TCID_{50}$ assay and VP2 epitope contents by Q-ELISA. Table 2 shows that the titers of infectious virus particles in pilot-scale crude supernatants harvested from three individual runs were 5.4, 1.2 and 2.3×10^6 $TCID_{50}$ units/mL in runs #1, 2 and 3, respectively. VP2 epitope contents in culture supernatants of these batches were measured by Q-ELISA and found to be 7.1, 6.7 and 11.9 units/mL in runs #1, 2 and 3, respectively. It was of interest to know whether the VP2 epitope content and the titer of infectious viral particles were correlated. Q-ELISA units of the VP2 epitope content per 10^6 $TCID_{50}$ infective units were found to be 1.3 (7.1/5.4), 5.6 (6.8/1.2) and 5.2 (11.9/2.3) for run #1, 2 and 3, respectively. Based on the current limited number of experiments, no direct correlation was established.

To monitor the consistency of the EV71 vaccine manufacturing process, the VP2 epitope content in in-process materials obtained from both the diafiltration/concentration and liquid chromatography purification steps were analyzed by Q-ELISA. After the diafiltration/concentration step, as shown in Table 2, Q-ELISA units of VP2 epitope per 10^6 $TCID_{50}$ infectious units were found to be 2.2 (166.5/77.5), 5.7 (228/40) and 8.3 (475/57) for runs #1, 2 and 3, respectively. Again, the VP2 epitope content measured by Q-ELISA did not correlate well with $TCID_{50}$ values. VP2 epitope/total protein ratios were calculated to be 3.9 (110/28.6), 5.2 (113/21.5) and 3.4 (225/66.7) for runs #1, 2 and 3, respectively (Table 2). These results suggest that there is room for improvement in the chromatography purification process, particularly in establishing criteria for pooling eluted fractions. In fact, based on the VP2 epitope determination by Q-ELISA the overall recovery yields of virus from the three runs were found to be 55.8%, 64.2% and 34.6% for runs #1, 2 and 3, respectively (Table 2).

The chromatographically-purified EV71/E59 bulk was inactivated with formalin at 37 °C for three days, and after 0.22-μm sterile filtration, the VP2 epitope content determined by Q-ELISA and the total protein concentration measured by the BCA assay were found to be 198.4 units/mL and 53.0 μg/mL, respectively (Table 2). There were protein (from 66.7 to 53.0 μg/mL) and VP2 epitope content (from 224.7 to 198.4 units/mL) losses during the inactivation and sterile filtration steps. Nevertheless, the Q-ELISA unit was found to be 3.7 VP2 epitopes per μg of total protein, a value close to that in virus bulk (3.4). A clinical lot (#23-04-0001) was recently manufactured using the same production process, and the number of VP2 Q-ELISA units per μg of total protein was found to be 3.9 (data not shown). These results suggested that formalin inactivation did not modify the VP2 epitope content as determined by Q-ELISA.

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

The amount of VP2 epitopes in three different formalin-inactivated EV71 whole virions vaccines determined by the Q-ELISA were used for the dosage formulation and mouse immunogenicity studies. The quality of EV71/E59 vaccine bulk produced from Vero cell grown in the Bioreactor with serum-free medium had been described above (Fig. 1). EV71/E59 vaccine bulk produced in Vero

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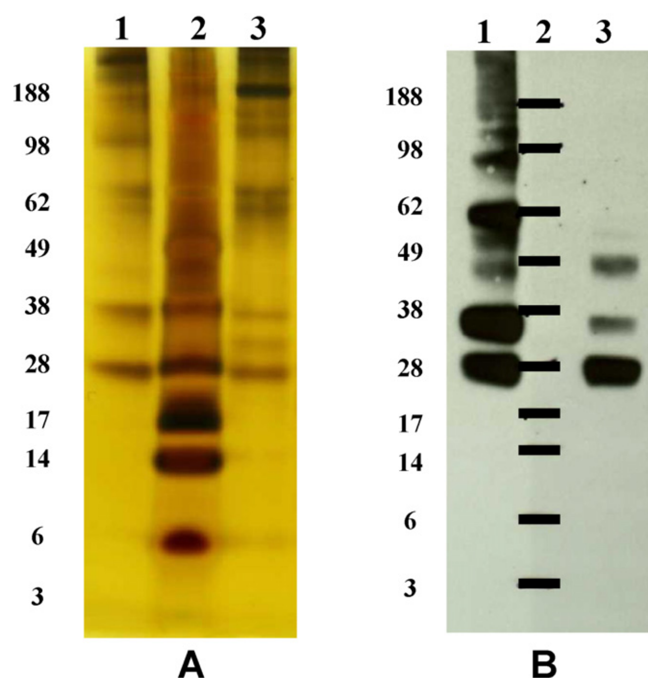

Fig. 4. The EV71 viral antigens in the vaccine bulk produced from serum-free culture medium (A, lane 1); and from serum-containing medium (lane 3) were separated by SDS-PAGE and sliver stained. The viral proteins recognized by the commercially available EV71-specific monoclonal antibody MAB979 in the Western blot (B).

417 cell grown in the presence of serum or serum-free medium was 418 analyzed by SDS-PAGE and Western blots, and shown in Fig. 4. The 419 vaccine bulk produced in the presence of serum was found to con- 420 tain fewer high MW proteins that were not recognized by MAB979 421 (Fig. 4B, lane 3). This may explain the vaccine bulk produced in 422 the presence of serum (Lot #07EV03 in Table 3) has lower Q-ELISA 423 unit of VP2 epitope per μg of total protein as compared to those 424 obtained from the serum-free.

Table 3
Mouse immunogenicity studies with three different EV71/E59 vaccine candidates.

Vaccine candidates[a]	Protein (μg)/dose[b]	Q-ELISA unit of VP2 epitope/dose[c]	GMT NT titers[d] (std. dev.)
1.4-L bioreactor (2.5 Q-ELISA units of VP2 epitope per μg of total protein)			
D16	0.5	0.75	1600 (499)
D16	2.5	6.25	2099 (1211)
Roller-bottle/serum containing medium (0.41 Q-ELISA unit of VP2 epitope per μg of total protein)			
07EV03	0.15	0.06	<20
07EV03	0.71	0.29	199 (70)
07EV03	3.5	1.45	447 (220)
07EV03	7.1	2.91	707 (511)
Roller bottle/serum-free medium (3.7 Q-ELISA unit of VP2 epitope per μg of total protein)			
Lot #3	0.01	0.05	28 (40)
Lot #3	0.07	0.26	100 (72)
Lot #3	0.34	1.29	854 (476)
Lot #3	0.68	2.58	911 (532)

[a] EV71/E59 vaccine candidates were prepared under various culture conditions using three different downstream purification methods. The immunization protocol was described in Section 2.
[b] The protein concentration of each vaccine candidate was measured by the BCA protein assay. Vaccines were formulated with alum as an adjuvant.
[c] VP2-specific epitope content was determined by the Q-ELISA described in Table 2.
[d] Virus neutralization titers were determined using the $TCID_{50}$ inhibition assay as described in Section 2 and are reported as geometric mean titers (GMT).

425 Mouse immunogenicity studies summarized in Table 3 revealed 426 that EV71 vaccines were highly potent in eliciting virus neutral- 427 ization titer. In addition EV71 vaccine produced in the serum-free 428 medium (one VP2 epitope unit formulated with alum as adju- 429 vant) elicited slightly better EV71 neutralizing antibody responses 430 (GMT = 854) than those obtained with the EV71 vaccine produced 431 in the presence of serum (GMT = 447). However, the difference was 432 found to be insignificant by the Student *t*-test (data not shown). 433 The number of VP2 epitope units and the magnitude of neutralizing 434 titers were found to be dose-dependent in mice immunized with 435 vaccine candidates produced in roller bottles either in the presence 436 or absence of serum in culture media. These results indicated that 437 the amount of VP2 Q-ELISA units present in the vaccine bulks could 438 be used to reflect the potency of the vaccine.

4. Discussion 439

440 This study aimed to develop an assay that measures the con- 441 centration of EV71 virus and/or viral antigens critical to vaccine 442 potency at each step of the vaccine production process. Therefore, 443 the assay should have excellent sensitivity and specificity, and be 444 suitable for application throughout the whole vaccine development 445 cycle during which viruses are cultured in live cells, harvested, puri- 446 fied, inactivated and sterile filtered before product formulation. 447 The plaque assay is used normally to estimate live virus titers (i.e., 448 the concentration of infectious virus particles) during the culture 449 process was not applicable or suitable for measuring and character- 450 izing the final inactivated product. Previous studies (Chung et al., 451 2008) had shown that the concentration of EV71 antigens could be 452 obtained by either the BCA protein assay or SDS-PAGE analysis if the 453 EV71 vaccine preparation was relatively pure. However, these anal- 454 yses are not suitable for determining the antigen contents in culture 455 supernatants and virus concentrates during the upstream vaccine 456 manufacturing process steps because of low antigen concentrations 457 in supernatants and host cell and/or serum protein contamination. 458 Considering all these factors, a Q-ELISA that could determine the 459 content of EV71 viral antigens was investigated and developed. The 460 final objective was to develop an assay much like the SRID assay 461 used for measuring the hemagglutinin (HA) content in the seasonal 462 flu vaccine QC potency test. To validate the usefulness and accuracy 463 of a Q-ELISA in EV71 vaccine development, we selected reagents for 464 their specificity, stability, efficiency and suitability. In addition to 465 the in-house-produced EV71-specific rabbit antisera, a commer- 466 cial chicken polyclonal EV71-specific antiserum PY-102676 was 467 evaluated and found to be useful as a capture antibody for dif- 468 ferent types of samples ranging from culture supernatants, virus 469 concentrates, chromatographically-purified virus and formalin- or 470 heat-inactivated virus. Using SDS-PAGE and gel-scanning analyses, 471 we estimated the protein concentrations of two potential EV71 472 viral antigen reference standards, namely purified VLPs and an 473 EV71/E59 bulk produced from Vero cells grown on microcarri- 474 ers in a bioreactor. The Q-ELISA was 10-fold more sensitive when 475 the EV71/E59 virus bulk (0.05–0.16 μg/mL) was used as a refer- 476 ence standard, while the biosafety issues and the instability of the 477 EV71/E59 virus bulk during storage at −80 °C made it unsuitable for 478 Q-ELISA development. The current results showed that the frozen 479 purified EV71/E59 required two to three times more materials to 480 generate standard curves similar to those obtained before stor- 481 age. To optimize the storage condition for purified EV71/E59 virus 482 bulk, we found that ten times more material of the heat-treated 483 EV71-E59 or formalin-inactivated EV71/E59 virion was needed to 484 generate a standard curve similar to that obtained with freshly 485 prepared EV71/E59 virus bulk. There are two possibilities: (1) the 486 heat-treatment and/or formalin-inactivation have altered the viral 487 structure and hindered the capture efficiency of rabbit anti-EV71

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antibodies; (2) the epitope of VP2 is modified by the heat-treatment or formalin, and has less binding affinity to MAB979. The direct ELISA experiments were performed to clarify this issue. Rabbit anti-EV71 antibodies had less binding titer to chemical-inactivated EV71 bulk as compared to those obtained from freshly prepared EV71 virus (data not shown). In addition, the chemical-inactivated EV71 vaccine bulk had the similar binding affinity to MAB979 as the freshly prepared EV71 bulk. Further study has shown the formalin-inactivated EV71 bulk to be stable at 4 °C, so it can be considered as a reference standard for Q-ELISA in future.

Based on the current stability and immunological studies, EV71 VLPs provided less sensitive range (0.2–1.6 μg/mL), but better and more consistent results than those obtained with the EV71/E59 virus bulk. VLPs consist of assembled viral proteins that together created a more authentic structure and conformation of viral antigens to mimic a native virus. Therefore, EV71 VLPs were used as an antigen reference standard for the Q-ELISA development. In addition, the current VLP standard only contains VP0 (VP4–VP2) as the viral antigen recognized by MAB979 (Fig. 1C, lane 2). Thus, the binding activity (VP0/MAB979) or antigenicity value of the VP2 antigen could be used directly as an index or arbitrary unit of VP2 epitope concentration. In fact, one Q-ELISA VP2 epitope unit/mL can now be defined as 1 μg of VP0 (38 kDa protein band) per mL of VLP Lot #2 determined by the RPAb/MAB979 Q-ELISA.

From a bioprocess development point of view, estimating the concentration of viruses present in sequential samples is essential to monitor the loss of product at each step of the manufacturing and purification process and to optimize the bioprocess conditions to increase product yields. The VP2 Q-ELISA was used to evaluate three 40-L batches of EV71/E59 produced in serum-free roller bottles culture system. The upstream bioprocess (culture supernatant harvest, diafiltration and concentration) steps were found to be very efficient because the VP2 epitope recovery throughout these steps was 100% (Table 2). Because of various amounts of non-infectious particles present in the culture supernatant harvest, the concentration of infectious particles determined by $TCID_{50}$ assay was not correlated directly with the VP2 epitope content measured by Q-ELISA. The downstream gel-filtration chromatography purification step, the yields for the first two batches were found to be between 56 and 64%. The third batch was found to have 2–3-fold more VP2 epitope content in the virus concentrates than those observed in the first 2 batches, but the chromatography yield decreased to 35%. The current results suggested that the conditions used in gel-filtration chromatography should be investigated and optimized, particularly in establishing criteria for pooling eluted fractions. Based on the VP2 Q-ELISA results, formalin-inactivation and sterile filtration steps did reduce product yields, less than 5% shown in Table 2 which is acceptable.

It was interesting to know that the ratio between the VP2 epitope content in the EV71 vaccine bulk determined by Q-ELISA and the total protein measured by the BCA assay was ranged 2.5–5.2. This discrepancy may be due to (i) the present of various amounts of host protein contaminants and (ii) the fact that VP2 epitope may exist in different forms and conformations (VP2 subunit, virion-associated VP2, incompletely processed VP0 and other VP2 associated antigens cross-linked by formalin). These various forms of VP2 were shown and identified in the Western blot analysis of formalin-inactivated EV71/E59 vaccine bulk (Fig. 1C, lane 3) but were not observed on the SDS-PAGE gel (Fig. 1B, lane 3). In addition, the binding affinity of MAB979 for its cognate VP2 epitope in VLP Lot #2 may be weaker than that in the EV71/B59 virion (native or formalin-inactivated). This hypothesis was supported by the fact that VLP Lot #2 required 10-fold more antigens (1.3 μg in Fig. 3C) to obtain an absorbance value (1.4 ELISA OD) similar to that observed with 0.13 μg of purified EV71/E59 bulk (Fig. 3A). The binding affinity of MAB979 to VP2 epitope may vary because the VP2 antigen could exist as (i) a subunit (28 kDa) in live EV71 particles, whereas the current VLP antigen standard could be mostly formed by incompletely processed VP1–VP3 and VP0 (Fig. 1B, lanes 1 and 2); (ii) a virion-associated component (98 kDa); (iii) a component of an incompletely processed VP0 (38 kDa); (iv) in other possible forms (45 and 64 kDa in Table 1); and (v) in various conformations. Therefore, this Q-ELISA could be used to evaluate critical factors influencing virus culture conditions, upstream harvest, down-stream purification processes and the stability of vaccine bulk.

When the Q-ELISA was used to analyze the quality of vaccine bulks produced in roller bottles in the presence of serum, the VP2 epitope units per μg of total protein ratio was found to be 10-fold lower than those obtained for the vaccine bulk produced in the absence of serum (Tables 2 and 3). The quality of the vaccine bulks produced in the presence of serum and analyzed by SDS-PAGE and Western blot using MAB979 was found to contain fewer high MW viral antigens (Fig. 4B, lane 3). Mouse immunogenicity studies summarized in Table 3 revealed that EV71 vaccines produced in serum-free medium (one VP2 epitope unit formulated with alum as adjuvant) elicited slightly better EV71 neutralizing antibody responses (GMT = 854) than those obtained with the EV71 vaccine produced in the presence of serum (GMT = 447). The dose–response immunogenicity studies also suggested that the number of VP2 epitope units in vaccine bulks produced in either serum-free media or serum-containing media could reflect the potency of the vaccine.

5. Conclusion

To comply with process development and validation guidelines enforced by regulatory agencies, vaccine manufacturers have an interest in developing assays that can monitor and characterize the purity and yield of viral preparations at sequential key stages of the manufacturing processes. In addition, vaccine manufacturers require assays to determine whether the concentration of viral antigen(s) correlates with vaccine potency. In this study, a Q-ELISA was successfully developed to provide off-line timely quantitative measurements of an important epitope of the EV71 VP2 subunit throughout the vaccine production cycle (cell culture supernatants, diafiltrates/concentrates, chromatographically-purified or sucrose-gradient centrifugation-purified virus particles, and formalin-inactivated virions). This Q-ELISA in future could serve as an assay to evaluate critical factors influencing virus culture conditions, upstream harvest and down-stream purification processes. The most important findings from the current analyses were the following: (i) the concentration of infectious particles determined by $TCID_{50}$ assay was not directly correlated with the cross-neutralization VP2 epitope content measured by Q-ELISA; and (ii) the amount of VP2 Q-ELISA units present in the EV71 vaccine bulks may reflect the potency of the vaccine because the number of VP2 epitope units and the magnitude of neutralizing titers were found to be dose-dependent in mouse immunogenicity studies.

Uncited reference Q1

Wu et al. (2004).

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