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Development of a sandwich ELISA to detect virus-like-particles in enterovirus A71 vaccines

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ABSTRACT

The goal of this paper was to develop a sandwich ELISA that can detect intact human enterovirus A71 (EV-A71) virus-like particles (VLPs) in vaccines. This assay specifically detected EV-A71 viruses from different sub-genogroups as well as EV-A71 VLPs, and treatment of VLPs with high heat and low pH reduced or completely abolished detection of the VLPs suggesting that the ELISA detected assembled particles. Using a purified VLP as a reference standard, a quantitative sandwich ELISA (Q-ELISA) was established which was used to monitor the yield and purity of the VLPs during manufacturing. Coupled with immunogenicity studies, the Q-ELISA was used to evaluate the performance of the VLPs and formalin-inactivated EV-A71 vaccine. This assay has the potential to play an important role in the development of an efficient process to produce and purify the VLPs and in examining the quality of EV-A71 vaccines.

1. Introduction

Human enterovirus 71 (EV-A71) is a non-enveloped, positive stranded RNA virus within the *Picornaviridae* family. During the lifecycle of a human enterovirus, viral structural proteins are translated from the viral RNA, cleaved and then assembled into protomers consisting of VP0, VP1, and VP3. Five copies of the protomers form a pentamer, and then twelve copies of the pentamers assemble into an empty capsid (procapsid) in the absence of viral RNA or into a provirion in the presence of viral RNA (reviewed in (Jiang et al., 2014)). Based on the variability in the VP1 coding sequence, EV-A71 is classified into 4 genotypes designated A, B, C, and D and the global distribution of these 4 genotypes is summarized by Yi and colleagues (Yi et al., 2017).

EV-A71 is a common cause of hand, foot and mouth disease (HFMD) in children. HFMD has become a serious public health issue worldwide due to its numerous and recurring outbreaks. Large outbreaks of EV-A71 are frequently detected in the Asia-Pacific region, including China, Taiwan, Australia, Singapore, and Malaysia (CDC, 1998; Chen et al., 2014; Horsley et al., 2014; Ooi et al., 2007; Wu et al., 2010; Zander et al., 2014; Zhang et al., 2009). Recently, EV-A71 infections were detected in Europe and Russia, further suggesting that EV-A71 has the potential of becoming a global infectious disease threat (Akhmadishina et al., 2015; Fischer et al., 2014; Mirand et al., 2015). The typical symptoms of HFMD are self-limiting and they include fever, oral lesions, and the development of papulovesicular exanthema on the hands, feet, and buttocks. However, EV-A71 infection can be fatal and has been associated with severe neurological disease including aseptic meningitis and fatal brain-stem encephalitis (McMinn et al., 2001b; Wang et al., 1999). The prevalence of EV-A71 and the severe complications resulting from infection warrant a need to develop a prophylactic vaccine against EV-A71.

Potential vaccine candidates for EV-A71 including DNA vaccines, synthetic peptides, recombinant viral proteins, live-attenuated virus, inactivated whole virus, and virus-like particles (VLPs) have been evaluated (reviewed by (Yi et al., 2017)). Of these candidates, three inactivated whole virus vaccines have recently been approved by the regulatory authorities in China and several others have entered clinical trials. Although the inactivated whole virus vaccines are safe and immunogenic, there is a potential risk of infection from incomplete inactivation of the virus during manufacturing. As a safer alternative, EV-A71 VLPs are empty viral particles and have been shown to be highly immunogenic (Chung et al., 2008; Ku et al., 2013; Li et al., 2013; Lim et al., 2015; Lin et al., 2012; Salmons et al., 2018; Zhang et al., 2015; Zhao et al., 2015). The absence of infectious viral genome in VLPs render them safer vaccine candidates.

A quantitative assay is required to monitor the production and purification of the EV-A71 VLPs throughout the manufacturing process. This assay is crucial in guiding the optimization of the upstream and downstream processes, specifically when VLPs are non-infectious and cannot be quantified using a plaque assay or tissue culture infectious

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dose 50 (TCID50) test. Additionally, accurately measuring the concentration of the viral particles is important for formulation of the vaccine. Several sandwich enzyme-linked immunosorbent assays (ELISA) have been developed to quantify EV-A71 viral antigens (Chung et al., 2010; Liang et al., 2011; Liu et al., 2011). These published assays do not distinguish between the assembled particles and the individual viral proteins due to the reagents used in the assays. This report describes a sandwich ELISA that detects assembled viral particle using a polyclonal antibody against VP1 as the capturing antibody and a monoclonal antibody E18 (Plevka et al., 2014) that recognizes a conformational epitope as the detecting antibody. This ELISA detected EV-A71 viruses from different subgenogroups as well as EV-A71 VLPs, but not CV-A16, another enterovirus. During the study, the assay was used to monitor the production and purification of the EV-A71 VLP vaccine and to assess the performance of the VLP and formalin-inactivated vaccines.

2. Method and materials

2.1. Cells

African green monkey kidney (Vero) cells were maintained in high glucose DMEM supplemented with GlutaMax™-I, penicillin-streptomycin, and 5% heat-inactivated fetal bovine serum (HI-FBS). Human rhabdomyosarcoma cells (RD) were cultured in Advanced RPMI supplemented with GlutaMax-I, penicillin-streptomycin, and 5% HI-FBS. All mammalian cells were maintained at 37 °C, 5% CO_2. Spodoptera Frugiperda cells (Sf9) (Gibco) were cultured in Sf900 II SFM supplemented with gentamicin, and maintained at 27 °C. All cell culture reagents were from Gibco, Thermo Fisher Scientific.

2.2. Viruses

All viruses were propagated in Vero or RD cells and the following EV-A71 viruses were used in this report: MY104/9/SAR/97 (accession no AF376072; B3 subgenogroup); MY821/3/SAR/97 (AF376077; B3); MY860/3/SAR/97 (AF376078; B3); SB0635/SAR/00 (AF376069; B4); SB1647/SAR/00; B4); SB18784 (B5); MR1958/2 (B5); 8M/AUS/6/99 (AF376109; C2); 9F/AUS/6/99 (AF376110; C2); VN5540 (C4); and VN5559 (C4) (McMinn et al., 2001a). Briefly, Vero or RD cell monolayers were inoculated with viruses, and viral supernatant was harvested and clarified by centrifugation at 3000xg for 10 min when > 90% cytopathogenic effect (CPE) was observed. The viral supernatant was stored in aliquots at −80 °C, and viral titers were determined by plaque assays as described below.

2.3. Production and purification of EV-A71 VLPs

EV-A71-B5 VLPs were produced using a recombinant baculovirus expression system as previously described (Lim et al., 2015). Briefly, Sf9 cells were infected with recombinant baculovirus containing the EV-A71-VLP cassette and the culture was harvested at day 4 or 5 post-inoculation (p.i.). VLPs were harvested by subjecting the cell lysates to centrifugation at 3000 × *g* for 30 min at 15 °C, and then the resulting pellet was resuspended in a hypotonic douncing buffer (1.5 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES) supplemented with 1% (v/v) Triton X. Alternatively, the culture was clarified by Sartopure® GF plus Midicap (Sartorius, Germany) to obtain a clarified supernatant. The supernatant was then subjected to diafiltration/ultrafiltration using a 750 kDa MWCO hollow fiber cartridge (GE Healthcare Life Sciences) to generate a retentate. The VLPs were further purified by size exclusion chromatography using HiPrep 26/60 Sephacryl S-500 HR (GE Healthcare Life Sciences).

2.4. Affinity purification of VLPs

E18 was purchased from (Mab Explorations Sdn Bhd, Malaysia). E18 was coupled onto HiTrap NHS-activated HP (GE Healthcare Life Sciences) according to the manufacturer's instructions. The clarified virus supernatant containing EV-A71 VLPs was exchanged into 20 mM Tris, 150 mM NaCl (pH7.5) and then concentrated 10 times using a 750 kDa MWCO hollow fiber cartridge. The concentrated virus supernatant was filtered and then applied onto the E18-conjugated column. The column was washed and then the bound VLPs were eluted with 50 mM Glycine HCl, pH 3.0. The eluted proteins were immediately neutralized with 1 M Tris-HCl (pH 8.8) and NaCl was added to make a final concentration of 150 mM.

2.5. Denaturation of VLPs

Affinity-purified VLPs were incubated at 100 °C or RT (control) for 10 min, and then stored in aliquots at −80 °C. To examine the effect of low pH treatment on the VLPs, purified VLPs were diluted 1/10 in 50 mM Glycine, 150 mM NaCl at various pH or PBS (control) at pH 7.4, and then incubated overnight at 4 °C. Samples from the above experiments were analysed by the sandwich ELISA or immunoblot analysis.

2.6. Determination of VLP concentrations by sandwich ELISA and Q-ELISA

NUNC Immuno plates (Thermo Scientific) were coated with purified polyclonal rabbit antibody against EV-A71 VP1 (10 μg/mL; 100 μL/well) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed once with PBS supplemented with 0.05% Tween 20 (PBST), and then blocked with PBS containing 1% casein hammerstein grade (MP Biomedicals) for 2 h at RT. After the wells were washed 3 times with PBST, samples (100 μL/well) prepared in diluent (PBS-0.2% casein) were added to the wells in duplicate for 1 h at RT, the wells were washed 6 times with PBST, and then E18 (0.1 μg/mL; 100 μL/well) was added to the wells for 1 h at RT. After washing the wells 6 times with PBST, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1/1000 dilution; 100 μL/well, Dako) was added to the wells for 1 h at RT. The wells were washed 6 times, and then SureBlue Reserve TMB microwell peroxidase substrate (100 μL/well, 1-component, KPL) was added to the wells for 5 min at RT. The stop solution (0.5 M HCl, 100 μL/well) was added to the wells, and the absorbance was immediately measured at 450 nm. The Q-ELISA was performed as above, except that the affinity-purified EV-A71-VLP with a known protein concentration was included on each 96-well plate in duplicate as a reference standard. The VLP concentrations of the unknown samples were determined using the standard curve.

2.7. Determination of protein content by SDS-PAGE and immunoblot analysis

Samples were mixed with 4X reducing sample buffer, incubated at 99 °C for 5 min, and then subjected to electrophoresis on a 12% SDS-PAGE gel. The gels were stained with Coomassie brilliant blue R-250 stain or processed for immunoblot analysis. For immunoblot analysis, proteins from the gels were transferred onto a nitrocellulose membrane, the membranes were blocked with PBS supplemented with 5% (w/v) skim milk for 2 h at RT, and then incubated with polyclonal antibody against VP1, VP0 or VP3 overnight at RT. The membranes were washed, and then incubated with HRP-conjugated secondary antibodies for 1 h at RT. After the membranes were washed, TMB membrane peroxidase substrate (KPL) was applied onto the membrane for 10 min at RT, and then the enzymatic reaction was stopped by washing the membranes with water.

2.8. Determination of protein concentration by microBCA

Protein concentrations were determined using Thermo Scientific micro BCA protein assay kit according to the manufacturer's instructions. Briefly, serial dilutions of the samples and standards were dispensed into the wells (150 µl/well). Micro BCA working reagent (150 µl/well) was added into the wells and the plates were incubated for 2 h at 37 °C. The OD of the wells were measured at 562 nm and the protein concentrations of the samples were determined using the standard curve.

2.9. Preparation of the formalin-inactivated EV-A71 virus

Vero cells were infected with MY104/9/SAR/97 and the culture was harvested by centrifugation at 3000 × *g* for 30 min at 10 °C when ≥ 90% CPE was observed. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline (DPBS, Gibco), frozen and thawed 3 times, and then homogenized with a tight douncer for 10 times. The homogenate was clarified by centrifugation at 3000 × *g* for 30 min at 10 °C, the resulting supernatant was loaded onto a 30% sucrose (w/v) cushion and then subjected to centrifugation at 100,000 × *g* for 2 h at 10 °C. The resulting pellet consisting of concentrated viral particles was resuspended in DPBS, clarified by centrifugation at 3000 × *g* for 30 min at 10 °C, and then loaded onto a 20–35% sucrose discontinuous gradient, with 5% intervals. After centrifugation at 100,000 × *g* for 3 h at 10 °C, 2 ml fractions were collected from the top of the gradient and were analyzed by plaque assay, SDS-PAGE, and immunoblot analysis. Fractions containing viral particles were pooled, concentrated, filtered, and then incubated with formalin (1/4000 dilution) for 3 days at 37 °C.

2.10. Mice immunogenicity studies

All animal care and use were conducted in accordance with the National Animal Welfare Standards and Guidelines of Malaysia under the Animals Act of 1953, incorporating all amendments up to 1 November 2006. Eight-to-ten week old female Balb/c mice were immunized intraperitoneally with 2 doses of antigens (200 µL/dose) and 0.1% (w/v) Brenntag alhydrogel 85 (Brenntag, Germany). Two weeks after the boost, the animals were euthanized and bled. The serum samples were heat-inactivated at 56 °C for 30 min. before they were analyzed.

2.11. Determination of virus titer by plaque assay and measurement of neutralizing antibody titer by plaque reduction neutralization test ($PRNT_{50}$)

Vero cells (1.5×10^5 cells/well) were seeded in 24-well plates overnight at 37 °C, 5% CO_2. For the plaque assay, medium was decanted from the plates, serial dilutions of the sample were added to the wells (200 µL/well) for 2 h at 37 °C, then overlay medium containing DMEM supplemented with Penicillin-Streptomycin, 2% HI-FBS, and 1.5% (w/v) Carboxymethyl cellulose was added into the wells. For $PRNT_{50}$, serial dilutions of the serum (250 µL) were mixed with MY104/9/SAR/97 (250 µL of 300 PFU/mL) for 1 h at 37 °C. The medium was decanted from the wells, the serum-virus mixture (200 µL/mL) was added to the wells for 2 h at 37 °C, and then the overlay medium was added to the wells. For both plaque assay and $PRNT_{50}$, cultures were incubated at 37 °C for 4–5 days for plaques to develop. Monolayers were then washed with PBS, and then fixed and stained with naphthalene black solution containing 0.1% (w/v) napthol blue black, 0.166 M sodium acetate, anhydrous, and 6% (v/v) acetic acid. The plaques were manually counted to obtain viral titers and $PRNT_{50}$ titers. The $PRNT_{50}$ titer was the highest dilution of serum that inhibited ≥ 50% of the plaques compared to the control.

2.12. Determination of the presence of antibodies against viral proteins by indirect ELISA

NUNC Immuno plates were coated with recombinant viral proteins, lysates containing EV-A71-infected RD cells that were incubated at 56 °C for 30 min, or purified EV-A71 VLP (100 µL/well) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. After the wells were washed once with PBST, PBS supplemented with 5% (w/v) skim milk was added for 2 h at RT, the wells were washed 3 times with PBST, and then 2-fold serial dilutions of the serum samples were added to the wells in duplicate. After 1 h incubation at RT, the wells were washed and HRP-conjugated anti-mouse IgG was added for 1 h at RT. SureBlue Reserve TMB microwell peroxidase substrate was added for 5 min at RT after the wells were washed. Stop solution was added to the wells and then the OD was measured at 450 nm. The P/N ratios from each sample were derived by dividing the average OD value of the samples by the average OD of the negative control from the same dilution. The negative controls were the pre-immune serum pooled from the animals and the dilution at which the P/N ≥ 2 was the titer of the sample.

3. Results

3.1. Sandwich ELISA detected assembled viral particles

To demonstrate that the sandwich ELISA recognize assembled particles, affinity-purified EV-A71 VLPs were denatured by incubating the VLPs at 100 °C or with low pH buffer, before they were analyzed by the sandwich ELISA. After incubation at 100 °C, the absorbance for the sample was at background level (Fig. 1A). Low pH treatment reduced the levels of detectable VLPs, specifically only 0.6% of the VLPs were detected after treatment with buffer at pH 2.5 (Fig. 1C). Higher level of VLPs were detected at pH 4.5 and 5.0, potentially because there is a

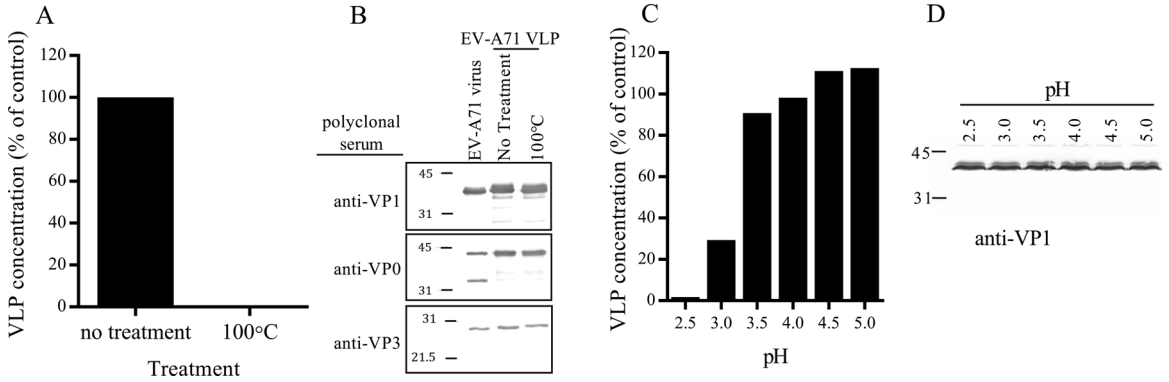

Fig. 1. Sandwich ELISA was unable to detect EV-A71 VLPs that were treated at 100 °C or with low pH buffer. EV-A71 VLPs were treated at (A and B) 100 °C for 10 min or (C and D) with low pH buffer at 4 °C overnight. All the samples were analyzed by the sandwich ELISA or immunoblot analysis. The absorbance from duplicate wells were normalized to no treatment control.

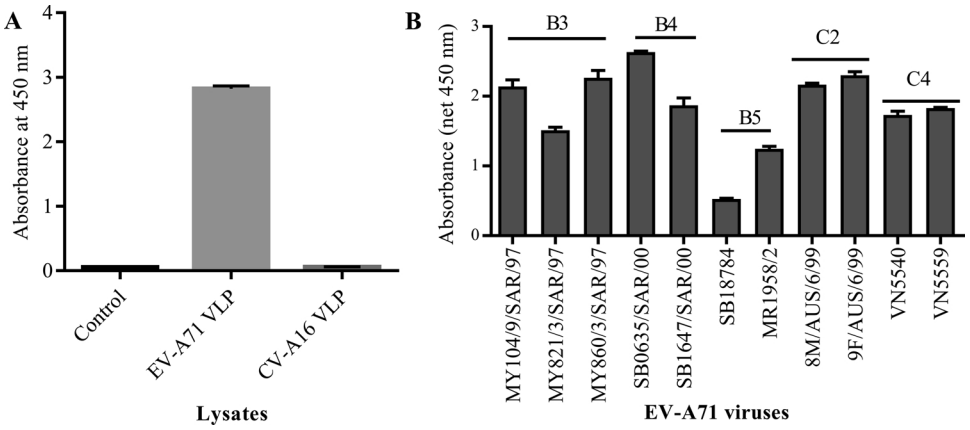

Fig. 2. Sandwich ELISA specifically detected EV-A71 viruses from different subgenogroups but not CV-A16 VLPs. Sandwich ELISA was performed using (A) lysates containing EV-A71 VLPs or CV-A16 VLPs or (B) supernatant containing various subgenogroups of EV-A71. Results from representative experiments are shown, the average and standard deviation of the net OD values from duplicates were plotted.

change in conformation allowing more E18 to bind to the particles or better binding of E18 to the particles. Similar levels of VP1 were detected in all samples regardless of treatment, suggesting that the VLPs were not lost but had disassembled (Fig. 1B and 1D). Collectively, these results showed that the sandwich ELISA detected assembled viral particles.

3.2. Sandwich ELISA specifically detected EV-A71 viruses from different subgenogroups, but not CV-A16

To demonstrate that the sandwich ELISA specifically detected EV-A71 VLPs, but not CV-A16 VLPs, lysates containing EV-A71 VLPs or CV-A16 VLPs were analyzed. The absorbance in wells containing CV-A16 VLPs was at background level demonstrating that the assay specifically detected EV-A71 (Fig. 2A). We then determined if the assay detects EV-A71 viruses from various subgenogroups. As shown in Fig. 2B, the assay detected EV-A71 virus from subgenogroups B3, B4, B5, C2, and C4. The OD values were different for all the samples possibly because the samples contained different amounts of EV-A71 virus particles or the antibodies had different binding affinity towards viruses from different subgenogroups (Fig. 2B). In conclusion, the sandwich ELISA specifically recognized EV-A71 virions from different subgenogroups, but not CV-A16.

3.3. Development of a quantitative sandwich ELISA (Q-ELISA) using a reference standard

Purified VLPs are required as a reference standard to develop a quantitative sandwich ELISA (Q-ELISA) and the most efficient way to obtain the cleanest VLP is by affinity chromatography as described previously (Plevka et al., 2014). By SDS-PAGE and immunoblot analysis, four major protein bands representing the molecular weight of VP1, VP0, VP3, and VP0-VP3 were detected in the reference sample. The presence of other minor bands suggest that further purification of the reference standard is required (Fig. 3A). The protein concentration of the purified VLPs was determined using microBCA, and the serial dilutions of the VLPs were analyzed using the sandwich ELISA. The net absorbance readings of the sample were plotted on the y-axis and the known protein concentrations on the x-axis (Fig. 3B). Using this reference standard, a Q-ELISA with a linear range from 0.2 to 1.22 µg/mL VLP was established.

3.4. Monitoring of VLP production and purification by Q-ELISA

Coupled with microBCA, Q-ELISA was used to monitor the production and purification of two different lots of VLPs during a manufacturing process. Culture supernatants containing EV-A71 VLP were harvested and clarified by depth filtration to generate clarified supernatant. The VLP concentrations and VLP-to-protein ratios for both WB1425 and WB1426 were ~32 µg VLP/mL and 0.004, respectively. The clarified supernatants were then subjected to ultrafiltration/diafiltration to produce retentates with 239.3 and 296.3 µg VLP/mL from lot WB1425 and WB1426, respectively. The process of ultrafiltration/diafiltration increased the VLP-to-protein ratios from 0.004 to > 0.2, improving the purity of the VLPs by 53 to 65-fold. Finally, the purity of the VLPs was further improved by size exclusion chromatography to VLP-to protein ratios of 0.972 and 2.381 (Table 1). The VLP-to-protein ratio of > 1 shows that further purification of the reference standard is required for future studies. In conclusion, the production and purification of the VLPs could be monitored using Q-ELISA and microBCA. The differences in VLP-to-protein ratios between the two different lots of VLPs demonstrated the variabilities between the different lots.

3.5. Using Q-ELISA to assess the performance of the EV-A71 vaccine

The Q-ELISA was also used to assess the performance of the EV-A71

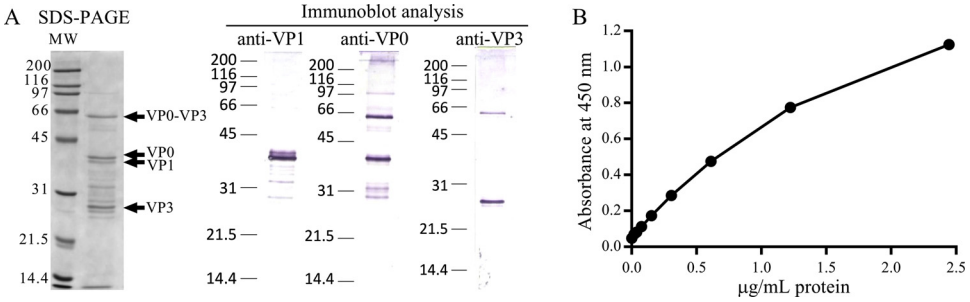

Fig. 3. Reference standard for Q-ELISA. (A) SDS-PAGE and immunoblot analysis. Affinity-purified EV-A71 VLP was subjected to electrophoresis on an SDS-PAGE gel. The gel was stained with coommassie brilliant blue or transferred onto nitrocellulose membrane. The membranes were probed with polyclonal sera against EV-A71 VP1, VP0, or VP3. (B) Sandwich ELISA was performed using the affinity-purified EV-A71 VLP. The protein concentration of the EV-A71 VLP was measured by microBCA. Serial dilutions of the VLPs were added into the wells in duplicates. The average OD values ± standard deviation and the protein concentrations of the samples were plotted on the y-axis and x-axis, respectively.

Table 1
Production and purification of EV-A71 VLP.

Lot #	Assay Sample	Q-ELISA μg VLP/mL	microBCA μg protein/mL	VLP to protein ratio
WB1425	Clarified supernatant	32.13	7,466.70	0.004
	Retentate	239.33	1,051.30	0.228
	SEC-purified VLP	57.15	24*	2.381
WB1426	Clarified supernatant	32.38	8,072.5	0.004
	Retentate	296.25	1,130.0	0.262
	SEC-purified VLP	40.55	41.7	0.972

* Protein concentration was < 25 μg/mL, below the limit of detection. The protein concentration was assigned 24 μg/mL to calculate the VLP to protein ratio.

VLP vaccine, ie. the ability for the vaccine to induce neutralizing antibodies against EV-A71 virus. Mice were immunized with various amounts of VLPs and euthanized two weeks after the boost. $PRNT_{50}$ was performed to determine the presence of neutralizing antibodies in the immunized mice and the reciprocal titers of the individual animals were plotted in Fig. 4A. VLPs induced the production of neutralizing antibodies in a dose-dependent manner and saturation occurred at 5 μg per dose VLP (Fig. 4B). Indirect ELISA was performed to assess the presence of antibodies against various viral components after immunization. VLPs induced the production of antibodies specific to EV-A71 virus, VLPs, VP1, and VP0 in a dose-dependent manner (Fig. 4C–F). Minimal amount of antibodies against VP3 (Fig. 4G) was detected in the immunized mice, regardless of the amount of VLPs immunized.

In addition to the VLP vaccine, the Q-ELISA was used to compare the performance of two different lots of formalin-inactivated EV-A71 vaccine: P1 and P2. The particle-to-protein ratios for P1 and P2 were 0.4 and 0.06, respectively, suggesting that P1 was a better quality vaccine lot. At 5 μg protein per dose of vaccine, P1 contained 6.6-fold higher amounts of viral particles than P2 (Fig. 5A). Immunogenicity studies show that mice immunized with P1 (1.97 μg particles per dose) produced 2.6-fold higher titer of neutralizing antibodies compared to mice immunized with P2 (0.3 μg particles per dose) (Fig. 5B). Consistent with the $PRNT_{50}$ titers, significantly higher levels of antibodies against EV-A71 virus, VLP, VP1, and VP0 were elicited in mice immunized with P1 particles compared to mice immunized with P2 particles (Fig. 5C–G). In conclusion, the Q-ELISA could be used to assess the performance of the VLPs and the formalin-inactivated vaccine.

4. Discussion

The yield, purity, stability and performance of the vaccine are important parameters in defining the upstream and downstream processes during EV-A71 vaccine production and the quality of the vaccine. Accurately measuring the vaccine concentration is crucial in formulation of the final vaccine product. Several assays have been used to examine the above parameters for EV-A71 vaccines. SDS-PAGE, immunoblot analysis, and high-performance liquid chromatography (HPLC) have been used to assess the purity of the VLPs (Zhao et al., 2015, 2017). Dynamic light scattering (DLS) have been used to measure the particle size and the stability of the VLPs (Lin et al., 2014; Zhao et al., 2015). In addition, several sandwich ELISAs have been established to measure the levels of EV-A71 antigen during EV-A71 vaccine production (Chung et al., 2010; Liang et al., 2011; Liu et al., 2011). Instead of using one assay to study one parameter, a Q-ELISA assay was developed to monitor the yield, purity, stability, and quality of the EV-A71 VLP vaccine.

The unique feature of the Q-ELISA was that it allows us to more specifically quantify the product of interest – assembled viral particles and to monitor the stability of the assembled viral particles. During the lifecycle of the virus, the viral proteins can exist as individual viral proteins, protomers, pentamers, or various forms of assembled viral particles. In all of the published sandwich ELISAs, polyclonal antibodies against EV-A71 have been used as the capturing antibody and polyclonal antibodies against EV-A71 or monoclonal antibodies were the detecting antibody (Chung et al., 2010; Liang et al., 2011; Liu et al., 2011). The major limitation of these published sandwich ELISAs is that

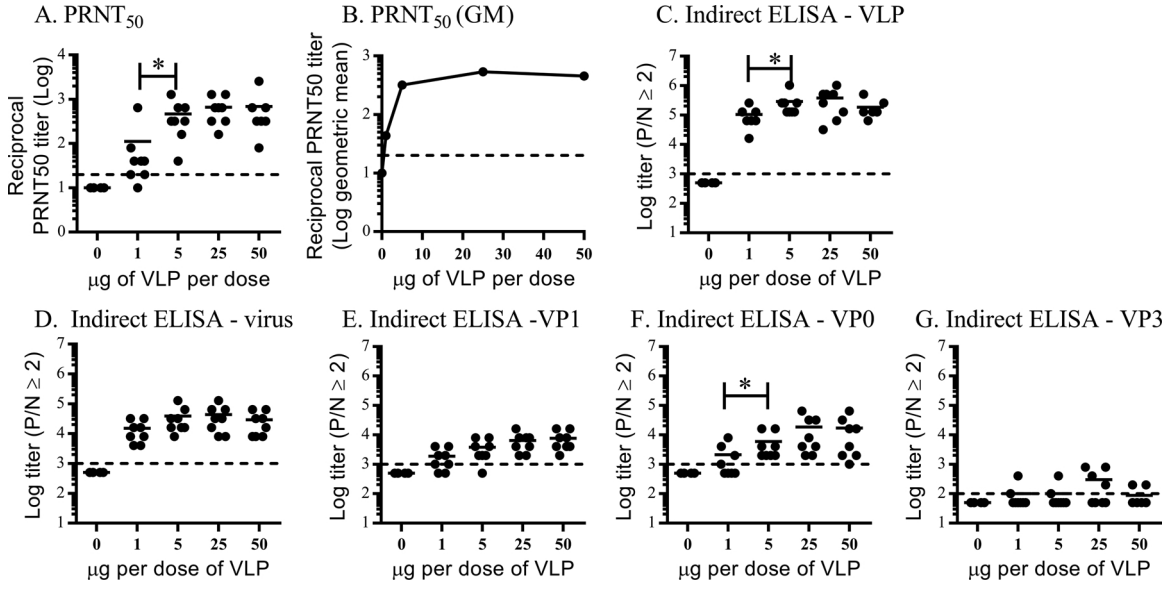

Fig. 4. Q-ELISA was used to evaluate the performance of the VLP vaccine. Mice (n = 4 to 8) were immunized with 0, 1, 5, 25, or 50 μg per dose of VLP, 2 doses at 2 weeks apart. Two weeks after the boost, the animals were euthanized and bled. (A and B) $PRNT_{50}$ was performed to examine the presence of neutralizing antibodies against EV-A71. (A) Each symbol represents the reciprocal $PRNT_{50}$ titer of the individual animal. (B) The geometric mean titer of the groups were plotted against the amount of VLP immunized. Antibody responses against (C) EV-A71 VLP; (D) lysates containing EV-A71 virus; (E) recombinant EV-A71 VP1; (F) EV-A71 VP0; or (G) EV-A71 VP3, following VLP immunization was examined by indirect ELISA. P/N was the ratio of the average OD of the sample divided by the average OD of the pooled pre-immune serum. The highest dilution with a P/N ≥ 2 is the reciprocal titer of the animals. Each symbol represents the antibody titer of the individual animal, the solid horizontal lines are the geometric mean of the group, and the dashed line is the limit of detection of the assay. Mann Whitney tests were performed to compare the treatment groups. *P* values are indicated as follows: *, $P \leq 0.05$.

A. Protein and particle content

Lot	P1	P2
Particle-to-protein ratio	0.4	0.06
Protein per dose (μg/dose)	5	5
Particle per dose (μg/dose)	1.97	0.3

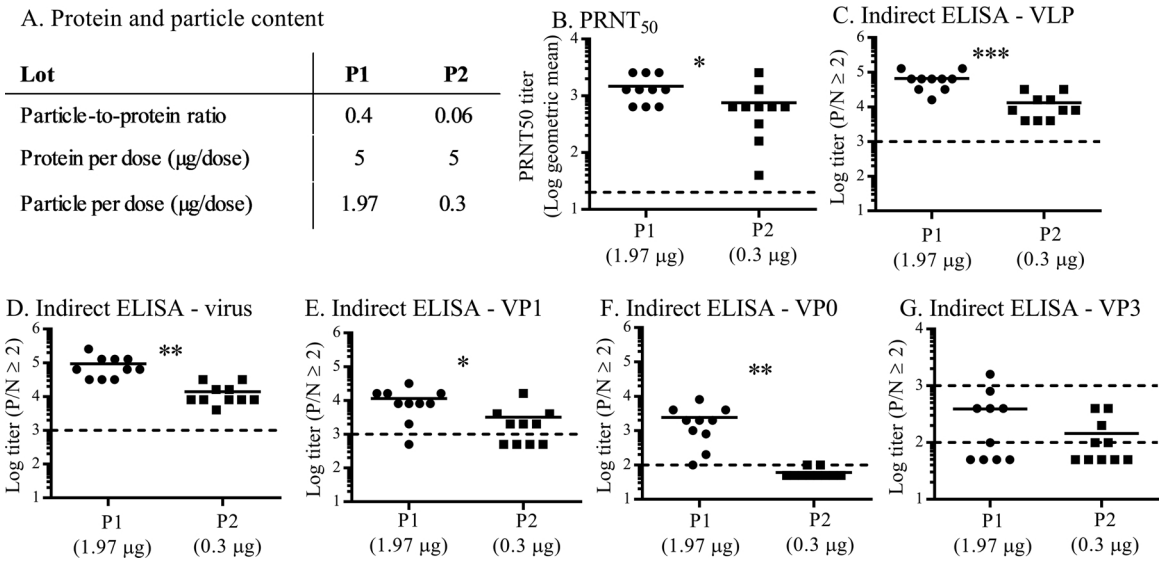

Fig. 5. Q-ELISA was used to evaluate the performance of two lots of formalin-inactivated vaccine. (A) Protein and particle concentrations of the vaccines were measured by microBCA and Q-ELISA. Mice were immunized with 5 μg protein of the formalin inactivated vaccine. (B) $PRNT_{50}$ was performed to examine the presence of neutralizing antibodies and indirect ELISA was performed to measure the antibodies response against (C) VLP; (D) virus; (E) recombinant EV-A71 VP1; (F) EV-A71 VP0; or (G) EV-A71 VP3.

they measure the different forms of viral proteins, including the unassembled viral proteins, protomers, pentamers, viral particles, and potentially denatured viral proteins. To overcome this limitation, E18, a monoclonal antibody that binds a conformational epitope, was used as the detecting antibody. Antibody footprinting experiment revealed that E18 binding sites were located between the VP0-VP3-VP1 protomers (Plevka et al., 2014), suggesting that E18 detected pentamers and/or assembled viral particles. Treatment of several picornaviruses with high temperature or low pH buffer resulted in the dissociation of viral particles (Curry et al., 1995; McGregor and Mayer, 1968; Tuthill et al., 2009). Denaturation of EV-A71 VLP at 100 °C and low pH treatment (Fig. 1) prevented the detection of the EV-A71 VLPs by the sandwich ELISA further confirmed that the assay specifically detected assembled viral particles. Based on the unique ability for this ELISA to detect assembled particles, we propose to use this assay to further study the stability of the EV-A71 particles under various storage conditions, such as buffer and storage temperature. Coupled with protein concentrations obtained from microBCA, the yield and purity of the VLP vaccine throughout the manufacturing procedure could be monitored (Table 1). Accurately measuring the concentration of the assembled viral particles in the crude material is crucial for optimization of the production and purification steps without over-estimating the yield. In conclusion, the sandwich ELISA specifically measured assembled particles allowing for the monitoring of the stability and quality of the VLP vaccine.

Another potential application of the Q-ELISA is to measure the potency of the EV-A71 vaccine. E18 recognizes important neutralizing epitopes on EV-A71 and inhibits EV-A71 infection in cell culture (Plevka et al., 2014). In addition, E18-purified EV-A71 VLPs induced the production of neutralizing antibodies in mice, nonhuman primates, and rabbits (Lim et al., 2015; Salmons et al., 2018). In this study, Q-ELISA was used to evaluate the performance and potency of two different types of vaccines: EV-A71 VLPs and formalin-inactivated EV-A71. The VLP concentration (as measured using the Q-ELISA) was correlated with the potency (as measured by PRNT50) of the vaccines. These results suggest that the Q-ELISA serve as an alternative to the time consuming and costly immunogenicity studies to evaluate the potency of different EV-A71 vaccines, including EV-A71 VLPs and inactivated whole virus.

The presence of multiple types of EV-A71 vaccines from different subgenogroups warrant the need for a standardize method to measure the concentrations and potency of the different vaccines. Although the assay was initially set up to evaluate EV-A71 VLPs, it also measured the concentration of the formalin-inactivated whole virus vaccine (Fig. 5), suggesting that formalin treatment did not alter the E18 epitope. Other than formalin, “binary” ethyleneimine and β-propiolactone inactivated vaccines elicited the production of neutralizing antibodies *in vivo* indicating that the E18 epitopes are present on the virus particles after chemical treatment (Cai et al., 2014; Hwa et al., 2013). These results further suggest that the Q-ELISA could be used to quantify and characterize all forms of inactivated EV-A71 vaccine. EV-A71 is classified into genogroups A, B, C, and D and the inactivated vaccines were produced from viruses from genogroups B and C (reviewed by (Reed and Cardosa, 2016)). Other VLP vaccines were also constructed using sequences from EV-A71 virus from C2, C4, B4, and B5 subgenogroups (Chung et al., 2008; Ku et al., 2013; Li et al., 2013; Lim et al., 2015). The Q-ELISA detected viral particles from different subgenogroups (Fig. 2B) suggesting that this assay is applicable to EV-A71 from different subgenogroups. Several groups have attempted to compare the potency of the inactivated and VLP vaccines and inconsistent results were obtained (Chou et al., 2012; Chung et al., 2008). This is potentially due to the lack of a standardize assay to measure the concentration and potency of the different vaccines. In general, this sandwich Q-ELISA is versatile and applicable to different types of EV-A71 vaccines from multiple subgenogroups.

During the manufacturing process of the EV-A71 vaccine, albeit VLPs or inactivated whole virus, assays are required to quantify and to characterize the vaccine. In this paper, we have demonstrated that the Q-ELISA could be used to measure the yield and to assess the purity, stability and quality of the EV-A71 vaccines. A higher purity reference standard and validation of the assay are essential before vaccine manufacturers can use it to monitor vaccine production. It is important to note that although this Q-ELISA is multi-functional, it will not completely replace assays such as SDS-PAGE, immunoblot analysis, and DLS because results from these assays will provide further insights into the characteristic of the vaccine.

Declaration of interest

The authors did this work while employed by Sentinext Therapeutics Sdn Bhd.

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