

# Neutralizing Antibodies of Inactivated Thai Enterovirus A71 Strain in Mice for Development of Enterovirus A71 Vaccine

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## Abstract

Since enterovirus A71 is known as a pathogen which may cause severe complications as critical neurological manifestations, pulmonary edema, cardio respiratory failure and even death to infected children, therefore, the vaccine against EV-A71 infection has been expected to prevent such serious problems even in Thailand. In this study, we developed a vaccine candidate from a sub genotype C4 EV-A71 strain collected from a Thai fatal case. The target virus was firstly compared VP1 nucleotide and amino acid identities with other 13 Thai strains of one C4, three C5 and nine B5 sub genotypes. For nucleotide homologies, the virus shared 96.3%, 91.5%, and 90.3%, respectively, while it contained amino acid identities as 99.9%, 100% and 97.2%, with C4, C5 and B5 strains, respectively. Before vaccine development, the target virus was initially confirmed to be a single strain by inoculation of a single plaque serially from first cell culture to another, and the passage 9 still showed positive to the monoclonal antibody against EV-A71 by IFA. For production of the virus, EV-A71 could be cultured very well in Vero cells using roller bottles which the yield was 4.4 - 4.5 x 10<sup>9</sup> pfu/ml at day 3 - 4 post infection. By purification, total proteins left were monitored after 100 kDa tangential flow filtration and 10% - 50% sucrose density gradient centrifugation as 46.2% and 1.0% mean, respectively. Immunogenicity of the inactivated EV-A71 produced was tested in mice. After a single injection of 1 or 2.5 µg purified total proteins, it induced neutralizing antibodies against the homologous virus especially when with alum, as compared to placebo groups. After 2nd immunization, both 1 and 2.5 µg with alum induced many antibodies than those without alum and the groups of a single immunization. The 3rd immunization of the vaccines gave very much titer in all immunized groups even without alum; however, highest was 24,525 TCID<sub>50</sub>/ml after 4 weeks by 1 µg with alum. All titers seemed to maintain after 6 weeks studied. This study confirmed that an inactivated EV-A71 vaccine with triple injections is a good choice for further development.

**Keywords:** Enterovirus A71; Enterovirus A71 vaccine; Inactivated enterovirus A71 vaccine; EV-A71, EV-A71 vaccine;

## Introduction

Hand, foot and mouth disease (HFMD) is a common illness caused by enteroviruses worldwide and it is commonly contagious among infants and children, especially under the age of five. In general, most cases have symptoms as fever, throat and mouth sores (herpangina), rash and blisters on palms and foot soles, other body areas, and then recover. The virus is spread through saliva, nasopharyngeal secretions and stool. It can survive on environmental surfaces for a long period of time, making the disease prone to outbreaks in day care centers and schools. However, large outbreaks of HFMD have been mainly caused by Coxsackie A16 (CA16) and Enterovirus A71 (EV-A71) [1, 2]. Although the illness is usually self-limiting, there are some severe complications which are likely to be caused by EV-A71 infection that more serious manifestations may progress as aseptic meningitis to acute flaccid paralysis, respiratory distress, pulmonary edema, and cardio respiratory failure which can often be fatal. Even if the patient recovers, there remains a high likelihood of chronic neurological sequel. There were reports of complications and deaths in many countries caused by EV-A71. In Taiwan during 1998, 129,101 cases of HFMD were reported, where some among 405 serious cases of neurological complications such as meningitis and encephalitis, were led to 78 deaths, while in 2008, 387 severe cases and 14 fatalities were proved to be by EV-A71 [3, 4]. Between 2008 and 2012, 7,200,092 cases of HFMD and 2,457 deaths were reported from China [5]. Including in other Asia-Pacific countries, during 2011-2012 in Vietnam, over 200,000 HFMD cases were hospitalized and 207 fatalities [6]. Also in South Korea, Singapore, Malaysia and Thailand, HFMD seems to occur endemically and those with severe complications and fatalities were proved mostly of EV-A71 infection [7-10]. EV-A71 genotypes were usually identified

during endemics and outbreaks worldwide, which is classified into three genotypes A, B, and C and then further divided into 11 sub-genotypes. Genotype A has the prototype strain BrCr only, whereas genotypes B and C have five sub-genotypes including B1 to B5, and C1 to C5, respectively, which is dependent on sequence variations in the structural protein VP1 [11]. During 2012 and 2014, sub-genotype C4 was found predominantly in China and Viet Nam, B5 was most common in Taiwan, Malaysia and Japan, while in Thailand both C4 and B5 could be found most equally [12]. The VP1, VP2, and VP3 proteins of EV-A71 which are exposed on the virion surface are responsible for immune responses and host-receptor binding. VP1 contains major neutralization epitopes and is used in viral identification and molecular evolutionary analyses.

Since EV-A71 tends to be a neurotropic virus and is more likely to be associated with complications including neurological symptoms and heart disease such as myocarditis that can even be fatal, preventive interventions such as vaccines targeted EV-A71 would benefit infants and young children who are most susceptible to neurological symptoms to prevent them from HFMD disease complications [13-15]. There have been several types of EV-A71 vaccine candidates, including live-attenuated virus, inactivated whole-virus, and virus-like particles, recombinant proteins, recombinant vectors, and peptide vaccines, of which each type has different advantages. Several research groups in China, Taiwan and Singapore have been pursuing clinical trials of inactivated whole EV-A71 vaccines and some has been approved in China since 2015. The results from three different phase 3 clinical trials in China (Sinovac Biotech Co., Beijing Vigoo Biological Co., and Chinese Academy of Medical Sciences /CAMS, China) performed in young children (6–60 months) indicated that the efficacy of EV-A71 vaccines is >90% against EV-A71-related HFMD and >80% against EV-A71-associated serious diseases, and they applied for licensure approval in China in the end of 2014, but anyway, the vaccines showed no protection against coxsackievirus A16 infections [16]. These vaccines were based on the formalin-inactivated whole virus, where the phase 3 trials in young children either 6–35 or 6–71 month age groups of more than 10,000 volunteers have shown similar safety and efficacy profiles [17]. Moreover, some candidates have been evaluated greater neutralizing antibody, specific T-cell responses, and good safety in children as well [18 - 20].

Although, no subtype-specific sequences related to increased virulence have not yet been identified and since sub-genotypes C4 and B5 were found circulating predominantly in Thailand since 2012, therefore, in this study, formalin-inactivated whole EV-A71 vaccine candidate derived from the C4 sub-genotype strain isolated from a patient who had clinical signs of severe HFMD and fatality was developed, and the immunogenicity of the candidate was evaluated in mice [12]. Moreover, preliminary small scale virus production and purification processes were also developed. Our results provided a foundation for the development of inactivated EV-A71 whole virus vaccine in Thailand.

## Methods

### Ethics statement

Animal studies were performed at the Department of Medical Sciences, Thailand and approved by the respective institutional animal care and use committees (Approval No. 53-011).

### Determination of molecular epidemiological identity of the isolated EV-A71 VP1

The isolated EV-A71 THA-08-29961 strain of sub-genotype C4 was subjected to determine the identities of VP1 nucleotide and amino acid sequences with those of other 13 Thai EV-A71 of C4 strain and B5 strains by alignment and analysis of the sequences using the Alibee-Multiple Alignment. The nucleotide sequences of samples in this study were retrieved from NCBI GenBank under the accession numbers FJ556874 (THA-08-29961 strain), FJ151494, FJ151497, FJ151499, FJ862993, FJ862994, KM675907, KM675909, KM675911, KM675920, KM675921, KM675922, KF748133 and KF748136.

### Virus

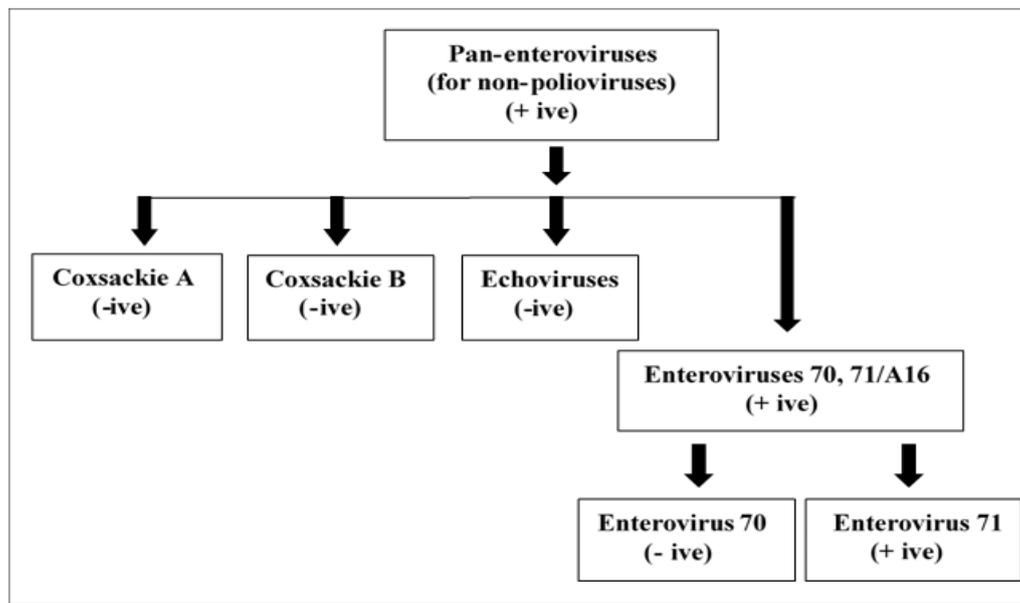
The sub-genogroup C4 EV-A71, strain THA-08-29961 isolated from a fatal case with severe hand-foot and mouth disease in Thailand in 2008 (Kindly provided by Guntapong R, et al, National Institute of Health, Department of Medical Sciences, Thailand) was used to prepare the virus stock by propagation in 90% Rhabdomyosarcoma (RD, ATCC: CCL-136) confluent cell monolayer in modified Eagle's medium (MEM) with 2% fetal bovine serum (FBS) as described elsewhere to prepare as the target EV-A71 [1].

### Virus isolation and plaque purification of the target EV-A71

To confirm that the target virus strain THA-08-29961 was a single EV-A71 isolate without contamination of other enteroviruses, prior to vaccine development, the virus was cultured in RD cells in many passages, every time a single plaque from one passage was picked up and inoculated onto other cell monolayers. The virus-infected cell cultures were step-wise identified for EV-A71 by indirect immunofluorescence assay (IFA) as described by the manufacturer (Light Diagnostics, Millipore). In brief, the cell culture was firstly typed with Pan-enteroviruses antibodies to identify for non-polioviruses, if positive, further infected cells were typed individually with each monoclonal antibody recognizing coxsackie A, coxsackie B, echoviruses, and enteroviruses 70, 71, A16, if it was positive with the latter, the culture was then typed with two monoclonal antibodies specific individually to enterovirus 70 and enterovirus 71 as the scheme shown in Figure 1.

### Determination of EV-A71 titer

Viral titration was performed by the plaque assay based on the method described in [2]. Confluent monolayers of RD cells were prepared in 24-well plates ( $2 \times 10^5$  cells/well). The cells were infected with serial dilutions of viral suspensions, overlaid with



**Figure 1:** This diagram shows all polyclonal antibodies and monoclonal antibodies specific to respective various enteroviruses taken to test the collected virus for a single strain of enterovirus 71

1.5% agarose gel in the culture medium DMEM + 2% FBS, and incubated at 37°C for 3 days. To visualize the plaques, stain the gel with crystal violet, and the viral titer was estimated in pfu/ml by plaque assay [21].

### Production and purification of EV-A71

2.0 x 10<sup>7</sup> Vero cells (CCL-81, ATCC, VA, USA) were seeded in a 850 cm<sup>2</sup> roller bottle containing the culture medium EMEM + 10% FBS until cell monolayer was performed. EV-A71 was produced by inoculating the confirmed EV-A71 isolate onto Vero cell monolayer at a multiplicity of infection (MOI) of 10–5 with EMEM + 2% FBS. EV-A71 was collected from the culture supernatant of each bottle at day 4 post infection by centrifugation at 2,000 RPM at 4°C for 20 min. Cell debris were removed by filtration through a 0.65 µm membrane (Sartorius Stadium Biotech, USA). Before virus purification, the crude virus was subjected to inactivation by freeze-thawing at 37°C 3 times. The lysate was collected by centrifugation at 2,000 RPM at 4°C for 20 min and then treated with 0.025% formalin at 37°C for 24 hours. The inactivated EV-A71 was then concentrated 20- to 40- fold, and to remove some unwanted proteins, nucleic acids and salts by using a 100 kDa cut-off tangential flow filtration membrane cassette (Sartorius Stadium Biotech, USA). The virus was then purified further by ultracentrifugation through 10% - 50% sucrose density gradient at 36,000 RPM at 4°C for 3 hrs. Fractions were collected to check for the virus antigen VP1 by quantitative ELISA as described further, and total protein amount was also determined by the Bradford assay as described by a manufacturer (Bio-Rad). Remaining active virus was monitored infectivity as estimated in pfu/ml assay [21]. The inactivated virus bulk was obtained after sterile filtration using a 0.22 µm filter, and subjected to SDS-PAGE and Western blot analyses, then stored at -20°C.

### SDS-PAGE and Western Blot Analyses

SDS-PAGE and Western blot analyses of the purified EV-A71 antigens from Vero cell cultures were performed according to the protocols reported previously by Liu CC, et al [22]. and molecular weight markers (PageRuler Prestained Protein Ladder, Thermo Scientific) were also run simultaneously. For immunoblotting, the proteins were directly electro transferred onto the PVDF membrane. Each membrane was incubated with PBS buffer containing diluted (1:1000) either EV-A71-specific monoclonal antibodies against VP1 or VP2 antigen. Antibodies were bound for 2 hr at room temperature. Binding of the respective antibodies to the viral proteins was detected by adding 2 mL PBS buffer containing a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Thermo Fischer Scientific) at a dilution of 1:10,000. After 1-hr incubation at room temperature, the membrane was washed 6 times with the assay buffer and blotted dry. The protein bands were revealed by adding TMB substrate solution (KPL).

### Determination of EVA71 VP1 amount by indirect ELISA

Micro titer plates were coated with anti-EV71 VP1 mouse polyclonal antibody (GeneTex, USA) at 4°C overnight. EV-A71 samples were added onto the coated plates, and anti-EV71 VP1 mouse monoclonal antibody (Abnova, USA) was then added into the wells. To determine the reaction, anti-mouse IgG antibody-horse radish peroxidase conjugate (KPL, USA), and 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (KPL, USA) were added. In this assay, diluting the reagents, blocking, washing the reaction mixtures, and removing non-specific binding agents from each step were done by using the solutions and buffers from KPL, USA, which the procedures were as described by the manufacturer. To estimate the amount of VP1 in the samples, the standard curve of

VP1 protein was established by replacing the sample with various concentrations of VP1 (EV71) protein (Immune Technology Corp., USA) between 12.5 and 500 ng/ml.

### Mouse immunization

To determine the immunogenicity induced by inactivated EV-A71, female BALB/c mice (6 weeks of age) were randomly divided into six groups (8 mice per group). Each group of mice was intramuscularly administered purified inactivated EV-A71 virions with varying antigen amounts of 1 or 2.5 µg total purified protein, with or without 0.73 mg alum (Alhydrogel, Sigma Aldrich) per dose, or phosphate-buffered saline (PBS) as a control. Two weeks after the first immunization, all mice were given a booster dose (2nd immunization) using the same vaccines. Four weeks after the first immunization (2 weeks after the first booster dose), 3 mice from each group were injected a second booster dose (3rd immunization) with the same vaccines. Serum samples were collected at week 2, 4, 8, 12 and 16 after primary immunization for assessment of the humoral immune responses, while for the groups who received a second booster, the serum was collected at week 2 after the administration.

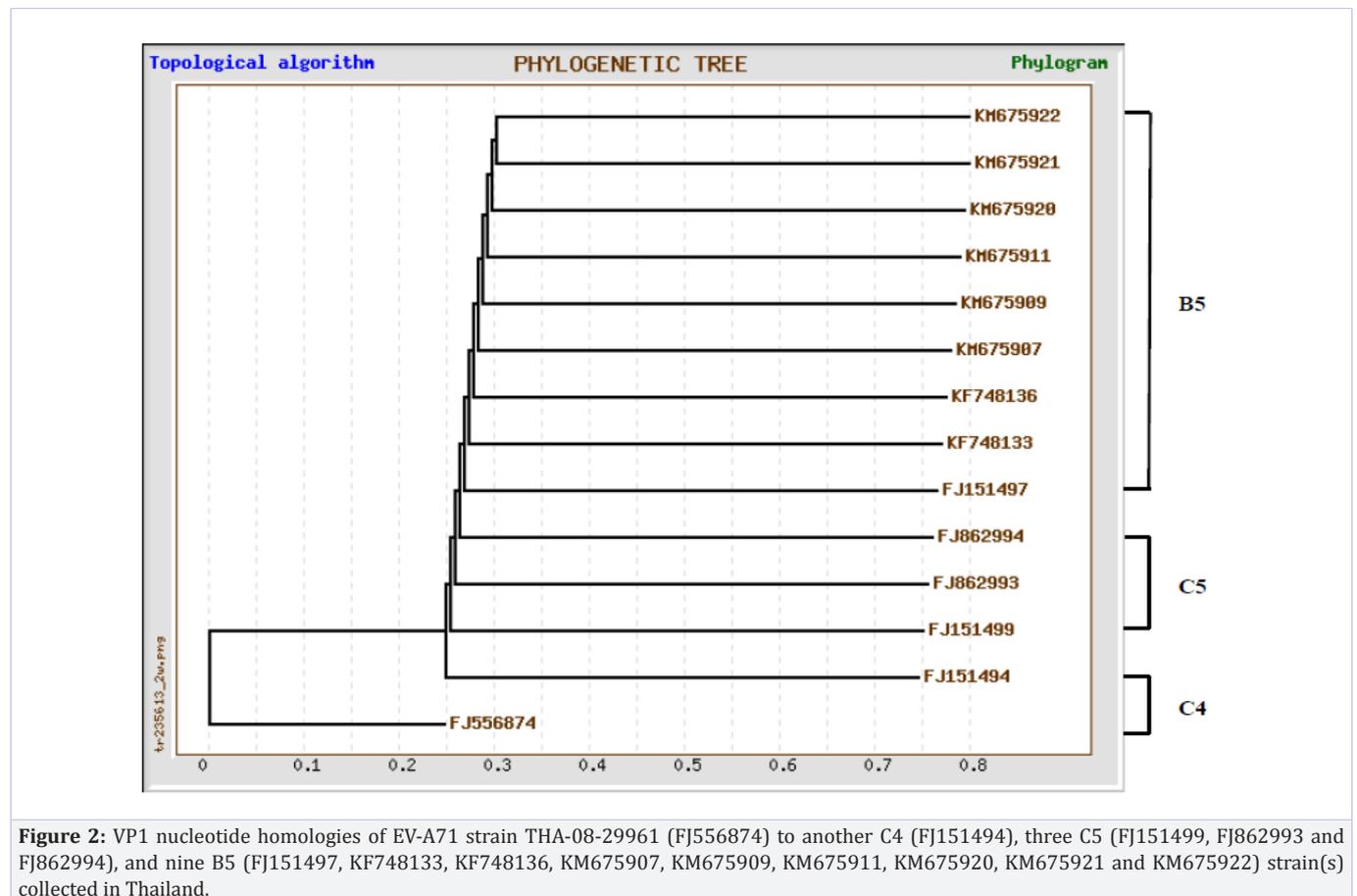
### Determination of neutralization of mouse antisera

Individual mouse serum samples were heat-inactivated at 56°C for 30 min, after that the sera were made two-fold serial dilutions with DMEM in 96-well plates. Fifty µl of each two-fold

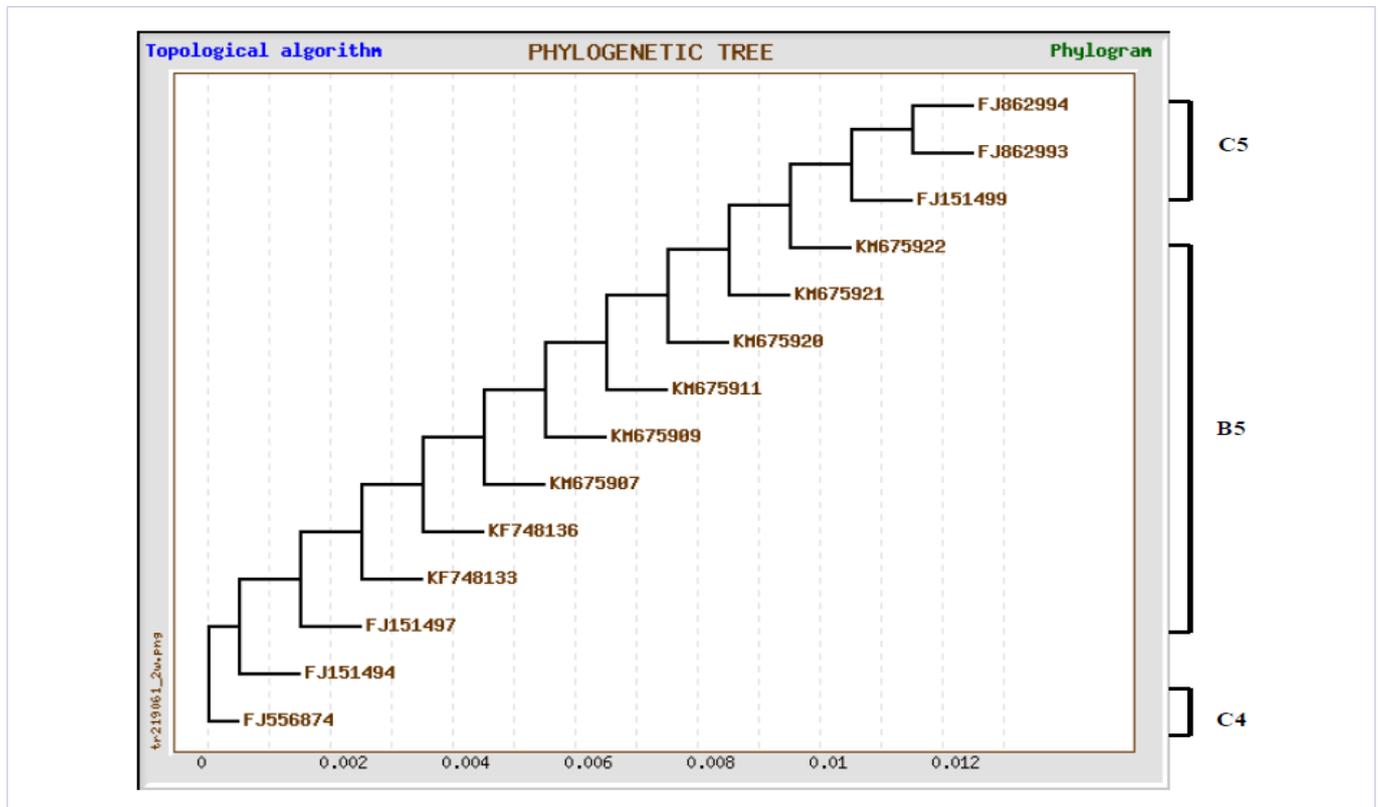
serial dilution was mixed with an equal volume of an EV-A71 suspension containing 100 TCID<sub>50</sub> and incubated at 37°C for one hr. Triplicate reactions were made for each serum dilution. One hundred µl of 10<sup>5</sup> cells/ml Vero cells were added into each well in the medium of DMEM + 2% FBS, and incubated at 37°C for 7 days. The end-point neutralizing titer was defined as the highest serum dilution in which at least two of the three replicates were negative for CPE. Score of neutralization antibody titer was defined using the Reed and Muench formula [23].

### Results

Molecular epidemiological identities of the isolated EV-A71 VP1: All sequences studied were of EV-A71 strains collected from EV-A71-infected patients in Thailand. Nucleotide sequence of VP1 of the isolated strain THA-08-29961 (FJ556874) which was sub-genotype C4 shared nucleotide and amino acid identities as 96.3% and 99.9%, respectively, with the other C4 strain (FJ151494), while shared 91.5% and 100.0%, respectively, with the three C5 strains studied (FJ151499, FJ862993 and FJ862994), and were as 90.3% and 97.2%, respectively, to those of nine B5 strains studied (FJ151497, KF748133, KF748136, KM675907, KM675909, KM675911, KM675920, KM675921 and KM675922). When the C4 THA-08-29961 strain was compared to all sequences taken, the homologies of all fourteen nucleotides and amino acid sequences were 85.1% and 97.8%, respectively, as shown in Figures 2 and 3.



**Figure 2:** VP1 nucleotide homologies of EV-A71 strain THA-08-29961 (FJ556874) to another C4 (FJ151494), three C5 (FJ151499, FJ862993 and FJ862994), and nine B5 (FJ151497, KF748133, KF748136, KM675907, KM675909, KM675911, KM675920, KM675921 and KM675922) strain(s) collected in Thailand.

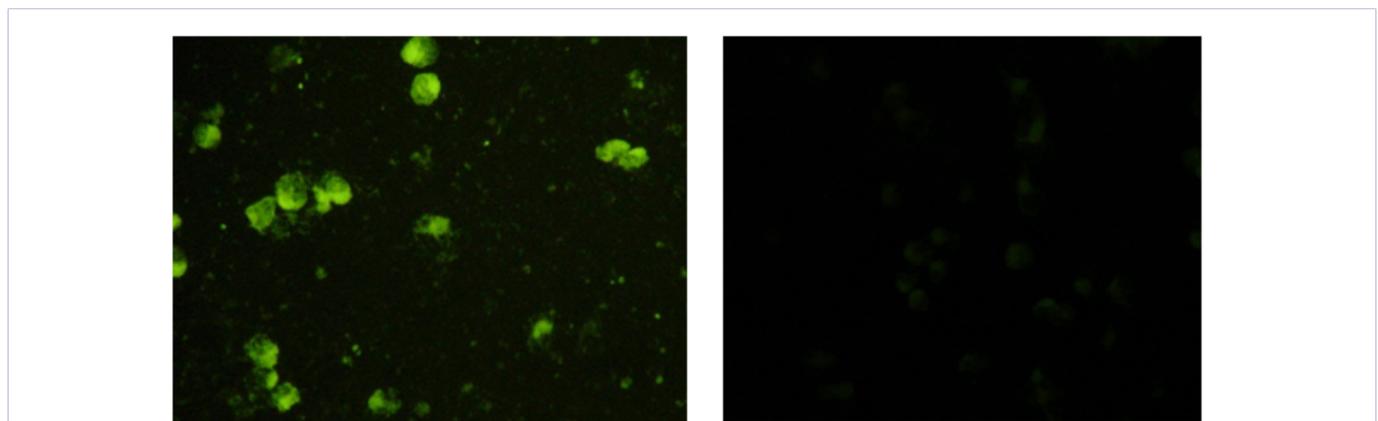


**Figure 3:** VP1 amino acid homologies of EV-A71 strain THA-08-29961 (FJ556874) to the other C4 (FJ151494), three C5 (FJ151499, FJ862993 and FJ862994), and nine B5 (FJ151497, KF748133, KF748136, KM675907, KM675909, KM675911, KM675920, KM675921 and KM675922) strain(s) collected in Thailand.

### Virus isolation and plaque purification of EV-A71

The sample virus strain THA-08-29961, after purification by inoculating a single plaque from one cell passage to another passages in RD cells until passage 9, was confirmed to be a single EV-A71 strain by typing with various polyclonal or monoclonal antibodies by IFA. From typing, the final culture showed primarily positive with the Pan-enterovirus antibodies, indicating that it

was in the group of non-polioviruses, but negative to monoclonal antibodies against coxsackie A, coxsackie B, and echoviruses, while positive to antibodies against the group of enteroviruses 70, 71 and coxsackie A16, and lastly individually negative or positive with the antibodies against merely enterovirus 70 or 71, respectively. These meant that the target virus was a single strain of EV-A71 as the positive signal of IFA with the anti-enterovirus 71 monoclonal antibody shown in Figure 4.

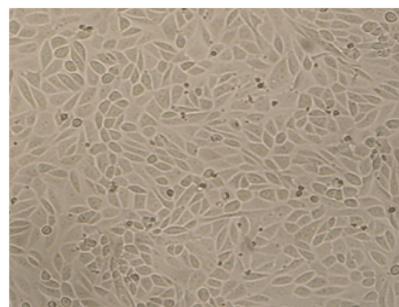


**Figure 4:** Immunofluorescence assay of EV-A71 strain THA-08-29961 infected-RD Cells and normal RD cells after staining with monoclonal antibody specific to EV-A71.

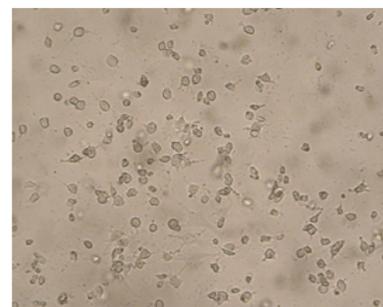
### Production and purification of EV-A71

When the isolated EV-A71 THA-08-29961 was cultured in Vero cell monolayer attached on the inner surface of an 850 cm<sup>2</sup> roller bottle, it infected and grew well, as cytopathic effect could be observed more day by day as some were shown in Figure 5. Maximum amount of EV-A71 was achieved on day 3 and 4 as the mean titers were  $4.4 - 4.5 \times 10^9$  pfu/ml, and the virus amount started to decline after day 4 as shown in Figure 6. After inactivation by the method used, no infectivity of the virus was left as determined by the plaque assay. For purification, unwanted culture components were tried to remove by procedures mentioned in Materials and Methods. After virus inactivation,

total proteins left were monitored after each purification step as some from three different purification runs were shown in Table 1. By some key steps shown, total proteins were left after the 100 kDa tangential flow filtration and 10% - 50% sucrose density gradient centrifugation as 46.2% and 1.0%, respectively. In addition, by sucrose density gradient centrifugation that fractions were collected, all fractions were monitored the target inactivated virus by determination of EV-A71 VP1 antigen by the method described earlier. There was no significant amount of VP1 antigen in almost all fractions except in the sediment of the last fraction (data not shown) that was collected for animal immunogenicity studies.



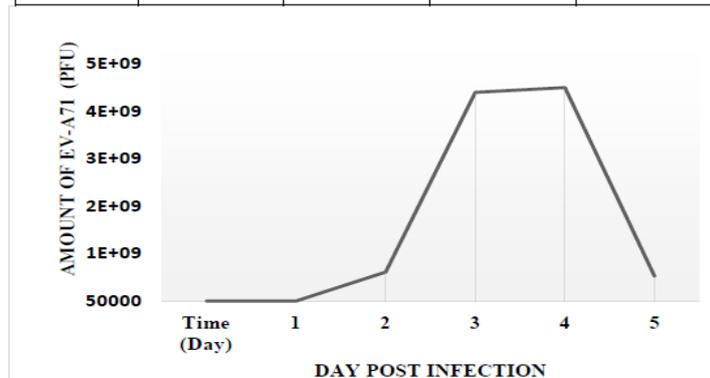
Normal Vero cell monolayer



EV-A71 infected-Vero cell monolayer at day 3 post infection

**Figure 5:** Cytopathic effect of EV-A71 strain THA-08-29961-infected Vero cell culture at day 3 post infection in a roller bottle compared to normal Vero cells.

| Day post infection | EV-A71 titer (pfu) in 850 cm <sup>2</sup> roller bottles |                   |                   |                   |
|--------------------|--|-------------------|-------------------|-------------------|
|                    | 1  | 2                 | 3                 | Mean              |
| 1                  | n.d.   | $1.4 \times 10^5$ | $1.4 \times 10^5$ | $1.4 \times 10^5$ |
| 2                  | $3.9 \times 10^8$  | $7.2 \times 10^8$ | $7.2 \times 10^8$ | $6.1 \times 10^8$ |
| 3                  | $9.5 \times 10^9$  | $2.2 \times 10^9$ | $1.4 \times 10^9$ | $4.4 \times 10^9$ |
| 4                  | $8.4 \times 10^9$  | $2.4 \times 10^9$ | $2.6 \times 10^9$ | $4.5 \times 10^9$ |
| 5                  | n.d.   | $8.8 \times 10^8$ | $1.5 \times 10^8$ | $5.2 \times 10^8$ |



**Figure 6:** Amounts of EV-A71 strain THA-08-29961 grown in Vero cells in roller bottles of three different cultures at day 1 to 5 post infections, and the mean amounts were plotted

**Table 1:** Amounts of inactivated EV-A71 strain THA-08-29961 produced from Vero cell cultures in the form of total protein in µg left after each purification step from three different purification runs. Percentages are shown in parentheses.

| Purification step                         | µg Total protein (%) |                   |                  |       |
|---|----------------------|-------------------|------------------|-------|
|   | Experiment 1         | Experiment 2      | Experiment 3     | Mean% |
| Inactivation                              | 37,380.6<br>(100)    | 3,340.5<br>(100)  | 1,336.1<br>(100) | 100   |
| 100K Tangential flow filtration           | 17,086.3<br>(45.2)   | 1,571.7<br>(47.1) | 620.3<br>(46.4)  | 46.2  |
| 10 – 50 % Sucrose gradient centrifugation | 449.75<br>(1.1)      | 33.7<br>(1.0)     | 14.4<br>(1.0)    | 1.0   |

### SDS-PAGE and Western Blot Analyses

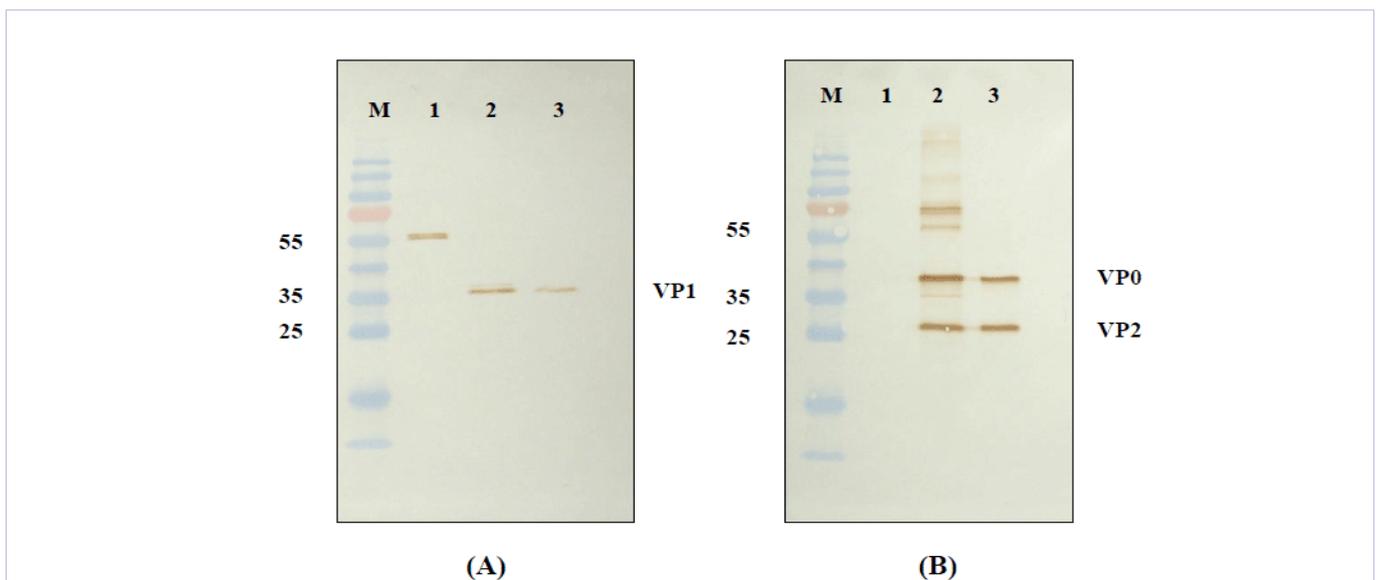
To determine the antigens of EV-A71 THA-08-29961 particles produced from Vero cell culture, Western blot analysis was performed. When the purified proteins from EV-A71 cultures were stained either with VP1-specific or VP2-specific monoclonal antibodies, protein bands were seen positive and the sizes of VP1 was estimated around 36 kDa as shown in Figure 7 A, while there were two bands against VP2-specific monoclonal antibody of VP0 and VP2 found at around 38 kDa and 28 kDa, respectively, as shown in Figure 7 B. Moreover, in B some aggregated proteins of VP2 or VP0 were also found larger than 60 kDa or more which might be the particle of VP4 + VP2 +VP3 [22].

### Immunogenicity of the inactivated EV-A71 vaccines in mice

As shown in Table 2 which all titers were mean titers of each group of mice, before immunization with the inactivated EV-A71, all mice had neutralizing antibodies as minimal as

482 - 565 TCID50/ml. By immunization of inactivated EV-A71 THA-08-29961, after the 1st injection of whether 1 or 2.5 µg purified total protein for 2 weeks neutralizing antibodies against the homologous virus (EV-A71 THA-08-29961) started to be observed much higher (Column B: Group 1 – 4) than those of before immunization (Column A) except the groups 5 – 6 (injected with PBS and PBS with alum, respectively) which still maintained at the background levels. Remarkably, when the antigens were with alum (Column B: Group 2 and 4), the antibodies were higher than those without alum (Column B: Group 1 and 3).

After the 2nd immunization, by 1 µg with alum, this could raise the antibodies very much higher (Column C: Group 2) than those without alum at all week intervals (week 6, 8, 12, and 16), moreover, the levels were rather lower and remained the same as after the 1st injection alone (Column C: Group 1). Similarly, 2.5 µg with alum induced higher antibodies (Column C: Group 4) than those without alum (Column C: Group 3) at all week intervals studied.



**Figure 7:** Western-blot analyses with EV-A71 VP1-specific monoclonal antibody (A), positive bands in lanes 2 and 3 were VP1 which was estimated as 36 kDa. With VP2-specific monoclonal antibody (B), positive bands are of VP0 and VP2 in lanes 2 and 3, respectively. In both photographs, M is molecular weight markers, 2 and 3 are sample proteins from EV-A71 cultures in Vero cells, while in (A), 1 is standard VP1 protein.

**Table 2:** Mouse neutralization antibody titers in TCID<sub>50</sub> /ml against the homologous virus (EV-A71 THA-08-29961) after immunization of 1 or 2.5 µg total protein of purified inactivated EV-A71 virions, with or without alum, or PBS or PBS with alum. The antibody titers are shown before immunization (A), and at various week intervals after 1st immunization (B), and 2nd immunization (C).

| Animal group | Inactivated EV-A71 vaccine or placebo | Mouse neutralization titer (TCID <sub>50</sub> /ml) |  |  |         |         |          |
|--------------|---------------------------------------|---|--|--|---------|---------|----------|
|              |                                       | (A) Before immunization                             | (B) After 1 <sup>st</sup> immunization | (C) After 2 <sup>nd</sup> immunization |         |         |          |
|              |                                       |   |  | 2 weeks                                | 6 weeks | 8 weeks | 12 weeks |
| 1            | 1 µg total protein                    | 565   | 1,560                                  | 743                                    | 575     | 982     | 1,480    |
| 2            | 1 µg total protein + Alum             | 482   | 5,120                                  | 13,100                                 | 7,040   | 5,570   | 4,500    |
| 3            | 2.5 µg total protein                  | 482   | 2,160                                  | 2,040                                  | 1,320   | 1,100   | 2,270    |
| 4            | 2.5 µg total protein + Alum           | 482   | 4,090                                  | 7,990                                  | 5,750   | 7,020   | 5,450    |
| 5            | PBS                                   | 565   | 453                                    | 442                                    | 508     | 508     | 292      |
| 6            | PBS + Alum                            | 565   | 495                                    | 479                                    | 467     | 757     | 283      |

**Table 3:** Mouse neutralization antibody titers in TCID<sub>50</sub> /ml against the homologous virus (EV-A71 THA-08-29961) after 3rd immunization of purified 1 or 2.5 µg total protein of purified inactivated EV-A71, with or without alum, or PBS or PBS with alum, at 2, 4 and 6 weeks post 3rd immunization.

| Animal group | Inactivated EV-A71 vaccine or placebo | Mouse neutralization titer (TCID <sub>50</sub> /ml) after 3 <sup>rd</sup> immunization |         |         |
|--------------|---------------------------------------|--|---------|---------|
|              |                                       | 2 weeks  | 4 weeks | 6 weeks |
| 1            | 1 µg total protein                    | 16,300   | 17,723  | 15,090  |
| 2            | 1 µg total protein + Alum             | 17,300   | 24,525  | 11,450  |
| 3            | 2.5 µg total protein                  | 9,380  | 7,625   | 6,610   |
| 4            | 2.5 µg total protein + Alum           | 13,700   | 16,525  | 16,000  |
| 5            | PBS                                   | 321  | 565     | 374     |
| 6            | PBS + Alum                            | 405  | 565     | 343     |

For the groups of mice having the 3rd immunization, as shown in Table 3, whether with 1 or 2.5 µg, with or without alum, at week 2 the titers were drastically increased in all groups to 9,380 – 17,300 TCID<sub>50</sub>/ml comparable to those of 2nd immunization, and were highest at week 4 post 3rd injection as 24,525 TCID<sub>50</sub>/ml by 1 µg total protein with alum. Exclusively, 2.5 µg alone (Group 3) gave lower titer. However, after week 6, the titers started to decline a bit or quite stable except with 2.5 µg without alum which was very much lower (Group 3). On contrary, the groups injected with PBS and PBS with alum still showed very low background levels without any immune response.

## Discussion

Three structural capsid proteins VP1, VP2, and VP3 are considered to have immunologically reactive epitopes, but identified neutralizing antibodies are mainly induced by VP1. Since VP1 has been used for EV-A71 molecular genotyping to classify the virus into three genotypes A, B, and C and further divided into sub-genotypes B1-B5 and C1-C5, we analyzed the identities of VP1 nucleotide and amino acid sequences to other Thai thirteen strains of C4, C5 and B5 available in GenBank [5, 11, and 24]. Among these fourteen strains, the target C4 virus used showed both identities of nucleotides and amino acids quite

close to other C4, C5 and B5 studied, especially the amino acid sequence were as 97.2% – 100.0%, noteworthy, C4 and B5 were two major sub-genotypes circulating in Thailand during 2012-2014 reported, and deaths have been reported to associate with EV-A71 sub-genotype B5 in Thailand during 2012 [12, 25]. Since in this study, the C4 sub-genotype virus was used to develop EV-A71 vaccine candidate, these might be attributed to cross neutralization of the vaccine candidate to other sub-genotypes as some has been reported that other inactivated EV-A71 vaccine candidates could elicit cross-neutralizing antibody responses against EV71 sub-genotypes B1, B4, B5, and C4A [26].

Inactivated whole-virion EV71 vaccines have appeared to be the most potent vaccine candidates, and Vero cells have elicited a more effective immune response than recombinant VP1 protein or DNA vector vaccines [27, 28]. In this study, we developed an inactivated EV-A71 vaccine and since the virus was expected to be a candidate vaccine, we started development with EV-A71 isolation and the virus isolated was proofed to be a sole strain by plaque purification and immunostaining by IFA. To assay for EV-A71, typically, the virus are usually cultured in RD cells, but for preparing the virus to study in the aspect of vaccine candidate, we used Vero cells for virus production since Vero cell is one of GMP-certified cell lines for human vaccine productions and it is convenient for further scaling up in bioreactors. The EV-A71 strain used in this study could be propagated in Vero cells very fluently that highest virus amount as  $4.5 \times 10^9$  pfu/ml was achieved by day 3 - 4 post inoculation with the condition used (MOI  $10^{-5}$  and  $2 \times 10^7$  initial cells in 850 cm<sup>2</sup> roller bottles). Vero cells showed high susceptibility to the growth of this virus, similarly, Vero cells have been used in the four candidates among five inactivated EV-A71 vaccines that have been rapidly developed in the past few years [16].

For purification, all proteins from the cultures including serum and Vero cell proteins, nucleic acids and lipids of the size less than 100,000 Dalton were removed primarily by 100 kDa tangential flow filtration. Since the EV-A71 antigens were believed to intact as whole virions, after filtration, most of them were expected to be held in the retentate. After 10% – 50% sucrose gradient centrifugation, most of unwanted materials were removed and EV-A71 antigens were collected from the sediment of the very last fractions (details of purification of the inactivated EV-A71 virions will be discussed in further report).

With inactivated virus vaccines, generally, in order to induce antigenicity, it is necessary to use adjuvants and multiple inoculations. Aluminum compounds have been widely used as human vaccine adjuvants for more than 70 years. Its mechanism of action is believed to serve as a depot for slow antigen release which enhance uptake by immune cells [29, 30]. Since normal BALB/c mouse is not sensitive to EV-A71 infection that disease manifestations cannot occur, we then tested specific antibody raised in the normal mouse model. In our studies, purified inactivated EV-A71 virions clearly enhanced neutralizing antibody against the homologous pathogenic EV-A71, particularly when alum was added. Moreover, it indicated that the antibody titers were drastically much higher when second immunization

of the vaccines with alum were administered to the mice as compared to only a single immunization, while without alum the titers were similarly kept as those of single administrations even after 16 weeks (post first immunization). Similar finding has been reported by comparing with the vaccine strains without the adjuvant, the differences in immunogenicity among the vaccine strains absorbed with alum adjuvant produced by the three manufacturers were increased, especially at 14 and 28 days after immunization [31]. Anyway, even with the second immunization, we found all titers of all groups seemed to decline from time to time after that. However, more interestingly, our studies showed that after the third immunization, the titers of all vaccinated groups were reverted very much higher, particularly, 1 µg purified inactivated EV-A71 even without alum, could induce from lowest titer to around 12 times higher as compared to those after second immunization. These revealed similar or higher titers to those by 2.5 µg with or without alum, respectively, which seemed not to be dose dependent. Our findings were quite different from some previous report that two injections of various doses of purified inactivated EV71 has shown vaccine dose-dependent whether with or without alum to induce % seroconversion in BALB/c mice [32]. According to this difference, we may possibly assume that the amount of the vaccine used in our study whether 1 or 2.5 µg total purified inactivated EV-A71 protein might contain access amounts of the viral antigens that dose-dependent antibody titer could not be observed clearly. Nevertheless, our results also elucidated similarly to other report that the inactivated vaccine has several major disadvantages as immunogenicity is not long-lasting and requires multiple boosters. This is because inactivated vaccines only initiate the humoral immunity and lacks cellular immunity (CD8+ T cells) responses [33].

Although we did not test cross-neutralization of heterologous sub-genotypes strains of EV-A71 by mouse serum in vitro, however there have been a few studies that addressed whether the neutralizing antibody elicited by one EV-A71 sub-genotype could cross-neutralize other sub-genotypes or confer protection across genotypes or sub-genotypes. It has been reported that neutralizing antibodies elicited by strains of the C4 genotype in rabbits had variable cross-neutralizing effects against different strains of the same sub-genotype and the genotype A BrCr strain, while another study demonstrated that mice challenged with lethal doses of B3 genotype survived due to prior vaccination with a C4 genotype vaccine, Furthermore, there has been some report showed that C4 vaccine had good cross-neutralization and protection effect against various sub-genotypes of B4, B5, C2 and C5 [34, 35]. Our study revealed that by two booster doses of inactivated EV-A71 vaccine produced from Vero cells induced high neutralizing antibody titers in mice whether with or without alum. Since an inactivated EV-A71 vaccine is considered the safest viral vaccine, as there will be no reversion to the infectious wild type strain and from achievement of this inactivated EV-A71 vaccine candidate, toxicological study in an animal model, larger upstream manufacturing processes by the use of bioreactor system with micro-carriers, and efficient downstream purification steps will be expected to incorporate for further development.

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