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Original Article

The stability and immunogenicity of formalin-inactivated Enterovirus A71 whole virion vaccine after ten years of low temperature storage

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Dynamic light scattering;
Particle size distribution

Abstract *Background:* Vaccine stability is an important issue for vaccine development, which affects whether the vaccine product is effective within a certain period of time in each progress. Hand, foot, and mouth diseases (HFMD) is an epidemic disease in young children usually caused by Enterovirus A group viruses, and the Enterovirus A71 (EV-A71) had caused several pandemics and public health issues around the world. After two decades of research and development, formalin-inactivated EV-A71 (FI-EV-A71) vaccines are the first to complete the phase III clinical trials for protection against EV-A71 infection. Currently, the shelf life of FI-EV-A71 vaccine product is set to be within 18 months, but the stability and the effectiveness of the FI-EV-A71 whole virion when stored long-term at low temperature remains undetermined.
Methods: Assessing the long-term storage properties of viral particles facilitates flexibility in manufacturing of vaccine products. In this study, the stability profiles of FI-EV-A71 vaccine lots and bulks after long-term of low temperature storage were analyzed by protein tests, particle measurement and animal immunization study.
Results: After over ten years of storage, the reduction of protein concentration in the FI-EV-A71 bulk samples is less than 30 % and the antigenic content remained in a suspended,

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particulate state. Both the packed FI-EV-A71 final vaccine products and the FI-EV-A71 antigens adjuvant premix bulk could elicit strong neutralizing responses in mice.
Conclusion: After ten years of low temperature storage, the FI-EV-A71 vaccine still presents decent stability and good immunogenicity.

Introduction

Vaccine stability is an important issue for vaccine utilization and distribution, which could be affected by many factors including manufacturing process, cold-chain transportation, storage condition and the shelf life of the final vaccine products. Currently, the assessment of vaccine stability is proposed and listed in several guidelines.[1–3] It is suggested that liquid vaccines with an aluminum adjuvant may lose their potency when stored at freezing temperatures.[1–3] Inactivated viral bulks in a liquid state without aluminum adjuvants are generally kept at refrigerating temperature (2—8 °C), although whether or not they lose their potency when freeze is not known. Typically, most licensed vaccines have to be kept at 2—8 °C in refrigerator for storage.[1–5]

Hand, foot, and mouth disease (HFMD) is an epidemic viral infection, characterized by rashes on hands and feet, and herpangina in mouth, which commonly occurs in infants and young children.[5] Many Enterovirus A viruses can cause HFMD, and the infection may aggravate to neurological disease or death. A large portion of HFMD severe cases were reported in Enterovirus A71 (EV-A71) pandemics. Several pandemics of EV-A71-associated HFMD have been reported especially in the Asia countries during the past two decades.[6–9] To prevent EV-A71-associated HFMD, several types of EV-A71 vaccine candidates were developed, and the formalin-inactivated EV-A71 (FI-EV-A71) whole virion vaccine is the first vaccine that went through the human phase III clinical trials.[8–12] Currently, clinical trials of several FI-EV-A71 vaccines have been finished and four of them have been approved and locally distributed. The phase III clinical trials of the FI-EV-A71 vaccine (B4 subgenotype) have been conducted by two vaccine companies (ClinicalTrials.gov Identifier: NCT03865238 and NCT05099029), and two products have been approved in Taiwan (DHY05900014902 and DHY05900015204). Three FI-EV-A71 vaccine products (C4 subgenotype) have been approved (ClinicalTrials.gov Identifier: NCT01507857, NCT01508247 and NCT01569581) in mainland China.[8,9] All clinical trials of the FI-EV-A71 vaccines showed safety and good immunogenicity in young children population.

EV-A71 is a non-enveloped RNA virus that belongs to the Picornaviridae family and the *Enterovirus A* species. EV-A71 encodes four viral structural proteins (VP1, VP2, VP3, and VP4) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D).[7,13] Currently, the approved FI-EV-A71 vaccine products are made of whole virion compositions using mammalian cell manufacturing process, and the viral particles are recovered and purified using size-exclusion chromatography.[14] After EV-A71 particles were purified, the virus bulk was inactivated with a formaldehyde solution. Aluminum phosphate or aluminum hydroxide were mixed with FI-EV-A71 vaccine bulk for vaccine product formulation, and final products were filled in vials and stored at 2—8 °C.[10–12,15] In a previous study, the durability of inactivated poliovirus vaccine after long-time storage had been investigated.[16] Their results demonstrated that the poliovirus maintained its potency for at least four years without detectable loss when incorporated in the AL(OH)3-adsorbed Di-Te-Pol vaccine. Furthermore, two of the constituent poliovirus types remained potent at refrigeration temperatures for 20 years.[16] The stability test of the FI-EV-A71 vaccine has been reported for viral bulks and final products, which suggest the storage duration to be 12 months and 18 months respectively.[15] The profiles of the final FI-EV-A71 vaccine products were analyzed by regular protein tests, particle detection and animal studies. Currently, the shelf life of the FI-EV-A71 vaccine product is set to be 18 months. However, how long the FI-EV-A71 antigens can be stored at low temperature while keeping effective remains to be explored.

In this study, we reported the stability of FI-EV-A71 whole virions after long-term storage at 2—8 °C. Final vaccine products and bulks of FI-EV-A71 that had been manufactured and stored for over ten years were evaluated by protein and antigenic profiles, particle size evaluation and immunogenicity assays. These results provide valuable information to understand the stability of FI-EV-A71 vaccine after expiring the currently set shelf life and may prompt the possibility of long-term storage of enteroviral vaccines for managing the recurrence of enteroviral diseases.

Methods

Ethics statement

Animal protocols have been reviewed and approved by the Animal Care and Use Committee of the National Health Research Institutes (IACUC, NHRI) with the protocol code NHRI-IACUC-107095-A.

Cells, media and viruses

The human rhabdomyosarcoma cell line (RD) was obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). RD cells were culture in a DMEM medium (Gibco) supplemented with 10 % fetal bovine serum (FBS, Cytiva) and passaged twice weekly in T-flasks. The EV-A71

E59 strain (B4 genotype) was obtained from the Center of Disease Control, Taiwan.[17]

FI-EV-A71 vaccine lots, bulks and adjuvant

FI-EV-A71 vaccine lots and bulks were produced by the PIC/S cGMP facility of NHRI from 2009 to 2012 (Table 1). Four FI-EV-A71 vaccine lots (Lot. 1 to Lot. 4) were originally prepared for use in the phase I clinical trial (NCT01268787).[10,15] Four FI-EV-A71 bulks were manufactured following the phase I clinical trial for process practicing and as stocks for emergency utilization, which were labeled with sample-04 (S04), sample-05 (S05), sample-08 (S08) and sample-practice batch (SPB) and stored in the R&D laboratory, NHRI. A portion of these vaccine lots and bulks was kept in laboratory for research purpose, and were stored at 2—8 °C for over ten years. For comparison, we also included freshly prepared and purified EV-A71 solution by liquid chromatography (LC) as described in previous studies.[17—19] The empty particle (E-particle) and full particle (F-particle) of EV-A71 were also prepared using ultracentrifugation as described previously.[19,20] The total protein concentration of samples was determined by a BCA protein assay (Novagen). The adjuvant added in these vaccines is aluminum phosphate, which was prepared from the NHRI PIC/S cGMP facility.

SDS-PAGE and western blotting

The FI-EV-A71 samples were separated by 4—12 % Bis-Tris SDS-PAGE (Invitrogen) and were stained with silver staining. Western blotting analyses of EV-A71 antigens were performed as previously described.[18] After transferred onto a PVDF membrane (Invitrogen), the EV-A71 antigen was detected using EV-A71-specific monoclonal antibody mAb979 (Millipore).

Determination of viral particle size distribution using light scattering technique

The viral particle size distribution of FI-EV-A71 bulks was determined by the dynamic light scattering (DLS) technique using a Malvern Zetasizer Pro-blue instrument (Malvern Panalytical Ltd). A total 100 μL of sample was loaded into the disposable cuvette and measured at 25 °C by the DLS reader. Each sample was measured 3 times, and the Z-average diameter of viral particles was expressed relative to cumulants analysis.

Animal studies of FI-EV-A71 vaccine lots and bulks

Mouse studies were performed according to previous study.[15] Ten groups of 6—8 female BALB/c mice were intramuscularly immunized either with 0.2 mL of phosphate buffer solution (PBS)/aluminum (60 μg) as the control, the FI-EV71 vaccine lots (0.2 mL per dose),[15] or the viral bulk samples (0.2 mL of aluminum-absorbed 4 μg of EV-A71 antigen per dose).

Transmission electron microscopy inspection, virus neutralizing assay, and quantitative enzyme-linked immunosorbent assay (Q-ELISA)

The procedures for transmission electron microscopy (TEM) inspection,[21] virus neutralization assay,[21] and Q-ELISA[22] were performed as described previously. For details, please refer to the Supplementary information.

Results

Protein profiles of FI-EV-A71 vaccine lots and bulks after long-term storage

In previous study, the stability of several FI-EV-A71 vaccine lots and bulks had been measured.[15] These remaining vials of the FI-EV-A71 vaccine lots were kept in the original package and the manufactured viral bulks were not formulated and were both stored as research samples at 2—8 °C. For over ten years, we inspected these materials to determine the stability of FI-EV-A71 viral particles. Visual inspection of the packed vaccine vials did not find any color change, and the aluminum phosphate appeared white color and precipitated at the bottom of the vial in standing. These FI-EV-A71 bulks were also visually inspected and showed no sign of abnormality, precipitation, or aggregation situations. The residual protein concentration of these materials was determined by the BCA assay, and most of the samples show decreased total protein contents (Table 1). The differences in protein content between the initial detection and the recent detection range from 28.8 to 67.1 % in vaccine products, and 71.2—99.4 % in viral bulks. For the vaccine products, the initial protein contents were determined before the adjuvant formulation, while the latter assay (Mar, 2021) were done with the final products containing adjuvant, in which only the unabsorbed protein portion could be measured (Table 1). The VP2 epitope

Table 1 The protein content of FI-EV-A71 vaccine lots and bulks after long-term storage.

Sample mark	Vaccine products				Bulk samples (antigen only)			
	Lot.1	Lot.2	Lot.3	Lot.4	S04	S05	S08	SPB
Production time (Year-Month)	2009—Oct	2009—Oct	2010—Jun	2011—Apr	2010—Aug	2010—Nov	2012—May	2011—Feb
Initial value (μg/mL)	24.3[a]	27.7[a]	21.1[a]	26.7[a]	10.9	70.4	53.7	62.6
Recent value (μg/mL) (2021—Mar)	16.3[b]	11.2[b]	8.2[b]	7.7[b]	9.4	50.1	46.4	62.2

[a] Measured before adjuvant formulation.
[b] Measured with aluminum adjuvant formulated samples (unabsorbed protein).

contents were determined by the antigen-capture Q-ELISA. The antigenic VP2 was found to be ranged from 97 to 340 units in the vaccine products (Lot. 1 to 4, Table 2) and 877—2909 units in the bulk samples (S04, S05, S08, SPB) (Table 2). The protein profiles were analyzed by SDS-PAGE and all the samples showed the pattern of two major protein bands by silver stain (Fig. 1A). The VP2 and VP0 proteins could be detected by Western blot in these products and bulks (Fig. 1B). The content of VP0 was found to be decreased in the long-term stored bulks, but not in the fresh, LC-purified EV-A71 solution. These results show that the EV-A71 antigens can be detected in these samples without major degradation over ten years when stored at 2—8 °C storage.

The viral particle stability of FI-EV-A71 vaccine lots and bulks after long-term storage

The integrity of the viral particles and the immunogenicity of the FI-EV-A71 vaccine are critical factors for its effectiveness under long-term storage. To further investigate the viral particle stability of these FI-EV-A71 samples, TEM inspection and DLS detection were used to evaluate the samples of viral bulks. All samples of FI-EV-A71 bulks revealed 30—35 nm viral particles in solution under TEM observation (Fig. 2). Since TEM can only indicate a small portion of viral particles contained in the sample, the overall particle size distribution is an important indicator for the integrity of the sample. Samples of FI-EV-A17 bulks, along with the ultracentrifuge-isolated E-particle/F-particle, and the freshly prepared LC-purified EV-A71 solution were analyzed by the DLS assay. The results revealed a predominant particle size distribution in the range of 10—100 nm, with peak sizes from 37 to 44 nm (Fig. 3). PBS solution along did not cause a major interference to the measurement of particle size distribution in this study, and the peak observed above the 100 nm in the viral samples may come from the impurity in the PBS solution (Fig. 3). The Z-average size of these samples was summarized in Table 3. The average size of these viral particle samples

Table 2 The VP2 epitope contents of FI-EV-A71 vaccine lots and bulks estimated by Q-ELISA.

Sample mark	Vaccine products				Bulk samples (antigen only)			
	Lot.1	Lot.2	Lot.3	Lot.4	S04	S05	S08	SPB
VP2 epitope (Unit/mL)	97[a]	181[a]	340[a]	228[a]	877	1445	1048	2909

[a] Measured with aluminum adjuvant formulated samples (unabsorbed protein).

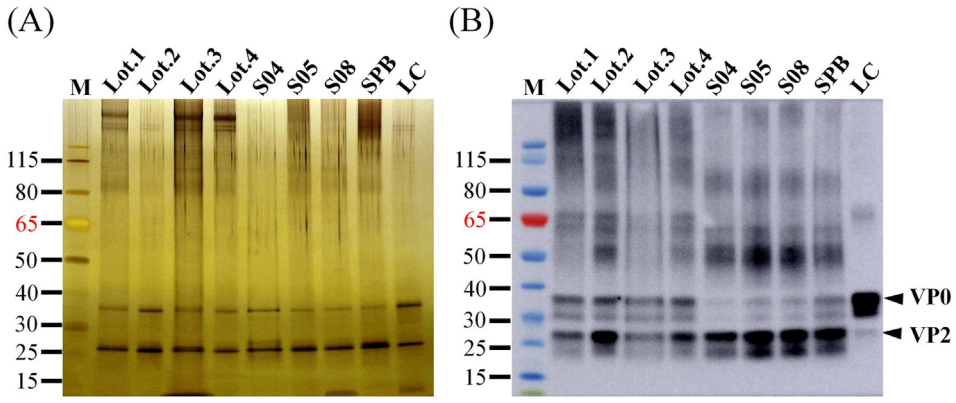

Figure 1. Protein component assay of FI-EV-A71 samples. (A) Silver-stained SDS-PAGE gel; (B) Western blotting assay with anti-VP2 mAb 979. Lane 1: molecular weight marker (M); lane 2: lot.1; lane 3: lot.2; lane 4: lot.3; lane 5: lot.4; lane 6: S04 bulk; lane 7: S05 bulk; lane 8: S08 bulk; lane 9: SPB bulk; lane 10: the LC-purified EV-A71 solution prepared in laboratory.

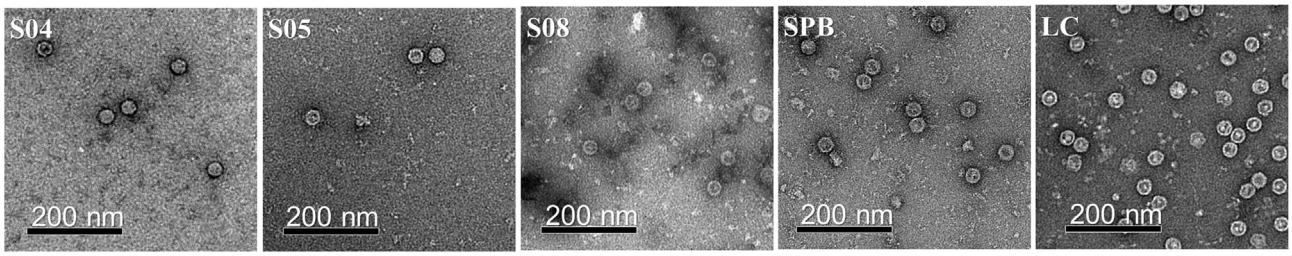

Figure 2. Transmission electron micrograph of FI-EV-A71 bulk samples after long-term storage. Four FI-EV-A71 bulks, including S04 bulk, S05 bulk, S08 bulk, and SPB bulk, and the LC-purified EV-A71 solution (LC) were inspected.

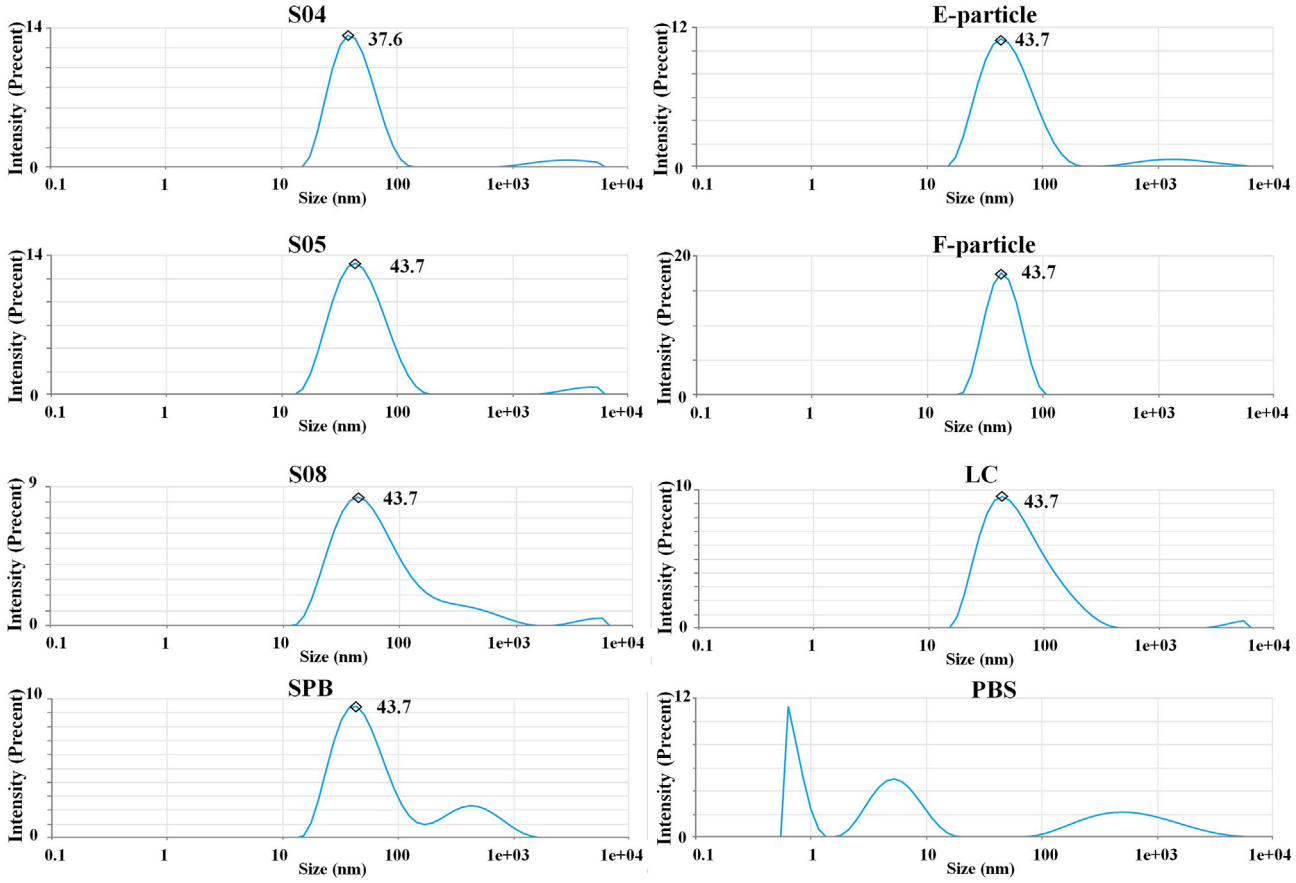

Figure 3. The particle size distribution of EV-A71 samples using dynamic light scattering. Four FI-EV-A71 bulks (S04, S05, S08, and SPB), E-particle, F-particle, the LC-purified EV-A71 solution (LC), and PBS were evaluated by DLS detection. The peak diameter value (◇) of each plot was indicated.

ranged from 40 to 52 nm. These results collectively showed that the long-term stored FI-EV-A71 bulks still retain the integrity of the virus particles, and indicate the stability of FI-EV-A71 viral particle in PBS for over 10 years at low temperature storage. In addition, DLS detection provides a rapid indication to confirm the presence and integrity of the viral particle.

Table 3 The Z-average diameter of EV-A71 samples using dynamic light scattering.

Sample name	Z-average (nm, n = 3)
S04	41.1 ± 0.6
S05	42.8 ± 0.5
S08	51.6 ± 2.1
SPB	51.6 ± 1.5
E-particle	47.3 ± 0.25
F-particle	42.6 ± 0.2
LC	50.9 ± 0.3
PBS	3.9 ± 0.5

Mouse immunogenicity studies of FI-EV-A71 vaccine lots and bulks after long-term storage

Ten groups of 6—8 mice were immunized with four FI-EV-A71 vaccine lots, four FI-EV-A71 bulks with adjuvant, laboratory prepared LC-purified EV-A71 solution with adjuvant, and PBS only, respectively. The results showed that the FI-EV-A71 vaccine lots (Lot.1 to Lot.4) and the FI-EV-A71 bulks (S04, S05, S08, SPB) revealed good neutralization titers (Nt titer, ranging from 1344 to 2399), while the LC-purified EV-A71 solution prepared in the laboratory showed a lower immunization response (Nt 507) (Fig. 4). These data showed that the immunogenicity of these long-term stored samples is well preserved, even after ten years.

Discussion

Given that the importance of conformational epitopes of EV-A71 in inducing immunogenicity has been elucidated by the study of EV-A71 neutralizing antibodies in infected children, the whole particle vaccine candidates have been

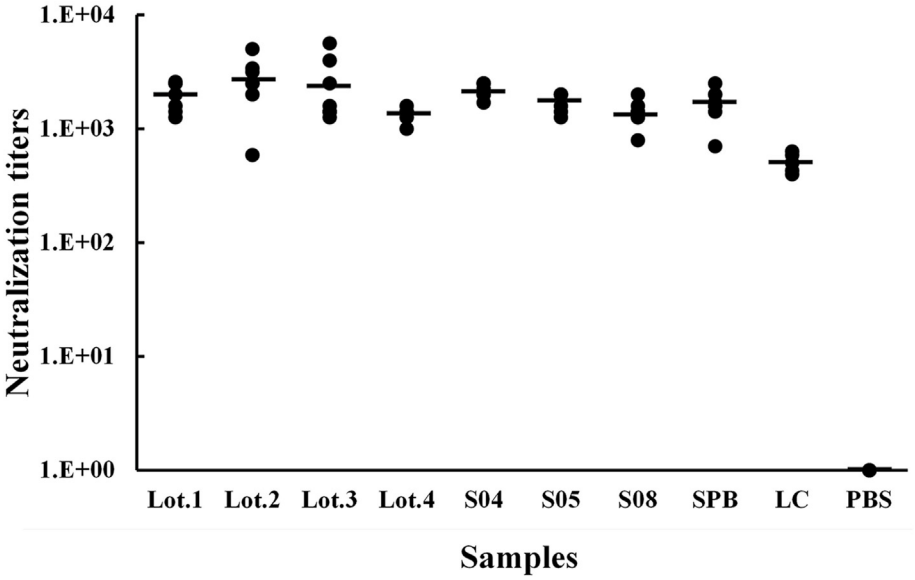

Figure 4. Neutralizing antibody responses of mice immunized with FI-EV-A71 samples against EV-A71 E59 strain. Four FI-EV-A71 vaccine lots (Lot.1, Lot.2, Lot.3, and Lot.4), four FI-EV-A71 bulks (S04, S05, S08, and SPB), the LC-purified EV-A71 solution (LC), and PBS were evaluated.

considered to be fundamental to induce effective immune response against EV-A71-associated diseases.[8,9,23] Phase I clinical trials of FI-EV-A71 vaccine candidates began in Q4 of 2010, and re-examination of these vaccine products will provide important information to understand the stability of these vaccines after long-term storage. In previous stability study of poliovirus vaccine, high temperatures affected the stability of inactivated poliovirus vaccine while the poliovirus D-antigen stored at −20 °C did not compromise its immunogenicity.[24,25] Moreover, the poliovirus was reported to remain potent when stored at refrigeration temperatures for 20 years.[16] In addition, the storage of H5N1 bulk for 4 years and AS03 adjuvant for 2.5 years were mixed to evaluate the immunogenicity and reactogenicity, and the result demonstrated that such duration did not appear to compromise the vaccine efficacy.[26] Currently, the FI-EV-A71 vaccine products are suggested to be used within 18 months after formulation. In this study, several FI-EV-A71 vaccine lots and bulks stored at low temperature for more than a decade have been shown to still retain its immunogenic efficacy in animal experiments. Such result may indicate the possibility of long-term storage of enteroviral vaccines for managing the recurrence of enteroviral diseases during a decade.

To evaluate the stability of FI-EV-A71 vaccine products and bulks after long-term storage, samples were analyzed by BCA protein assay, Q-ELISA, SDS-PAGE and Western blot. In previous studies, major protein compositions of FI-EV-A71 samples had been determined and the antigenicity had been confirmed by specific antibodies, such as the mAb979.[15,18,22] In samples of the formulated FI-EV-A71 vaccine lot, part of EV-A71 particles were absorbed by aluminum phosphate, while only the unabsorbed protein in solution could be detected by BCA assay and the amount was found to be lower than the initial measurement without adjuvant (Table 1). However, such result does not necessarily indicate that the viral particle degraded in the vaccine product and it had been shown that the unabsorbed protein was in a dynamic absorbed/unabsorbed condition in previous study.[15] In samples of the FI-EV-A71 bulks, decrease of the total protein concentration ranged from 0.6 to 29.9 % (Table 1). The VP2 antigenic content of all samples were confirmed by the Q-ELISA study (Table 2).[15,22] Despite the reduction of protein content, major antigenic proteins were detected in these samples by SDS-PAGE and western-blotting analysis (Fig. 1). These results collectively show that these long-term stored FI-EV-A71 vaccine products and bulks still retained most of the protein content and did not show significant signs of degradation.

Previous study has revealed the condition of protein components, but did not verify the integrity of viral particles of EV-A71 samples.[15] Aluminum phosphate forms heterogeneously sized aggregates (larger than several micrometers) that can adsorb antigens and use as vaccine adjuvant.[27] Since FI-EV-A71 particles were mixed with aluminum phosphate to formulate the final FI-EV-A71 vaccine product, these samples were unable to perform TEM inspection and DLS detection. In this study, the samples of FI-EV-A71 bulks were examined and the viral particles could be clearly identified in TEM inspection (Fig. 2), indicating the integrity of viral particles was well-preserved. Given that the TEM inspection presents only a partial evaluation of the samples, the DLS measurement provides the overall particle size distribution of the viral solution samples. In a previous study, DLS had been used to evaluate the particle integrity of EV-A71 VLP stored during 6 months at different storage temperatures (−80, −20, 4, 25, and 37 °C).[28] This technique was also used to measure the particle size of nano-emulsion adjuvant.[29,30] In this study, all samples of FI-EV-A71 bulks exhibited a predominant particle size distribution and major peak in the range of 37−44 nm using DLS detection. The particle size distribution of the purified E-

particles and F-particles of EV-A71 were located in the same range, which indicated the presence of homogeneous particles in these FI-EV-A71 bulk samples (Fig. 3). Solutions containing impurities other than enteroviral particles or degradations may present variable sizes distribution. The size variation of EV-A71 particles was about 20 % between TEM inspection and DLS detection, and this measurement inconsistency could be owing to the variation between these two methodologies and has been observed in previous studies.[28,30] The results of TEM inspection and DLS detection could provide an overall profile to investigate viral particle stability and integrity. The profile of particle distribution can be easily obtained by DLS measurement and provide a quantity value to assist TEM inspection for viral particles. These results show that the particle stability of FI-EV-A71 bulks did not decrease significantly after ten years of low temperature storage.

Mouse immunization studies summarized in Fig. 4 show that the long-term stored FI-EV-A71 vaccine lots and bulks can still elicit good immunogenic responses. No significant reduction in neutralization titers was observed for the same dosage of FI-EV-A71 vaccine lots (lot.1 and lot.3) compared to the 2012 study.[15] The long-term stored FI-EV-A71 bulks, when mixed with aluminum phosphate, also elicited good neutralizing responses similar to that of the packed vaccine lots. The freshly prepared FI-EV-A71 antigens in the laboratory induced lower neutralization titers than the long-term stored FI-EV-A71 antigen. The reason could be that the FI-EV-A71 antigens prepared in the laboratory were only roughly purified by the liquid chromatography with a small-size column, and the major immunogenic contents (the F-particles) were not as abundant as the samples prepared from the PIC/S cGMP facility (Fig. 1). The results showed that these FI-EV-A71 vaccine lots and bulks remain the ability to induce good immunogenicity after ten years of low temperature storage.

In conclusion, the results obtained from protein profiles, particle measurements, and mice study have demonstrated that the stability and immunogenicity performance of the FI-EV-A71 vaccine lots and bulks remain quite satisfactory after long-term of low temperature storage. Over ten years of storage, the protein concentration of FI-EV-A71 samples is not reduced by more than 30 % and the overall content remained in a viral particulate state. Both the packed FI-EV-A71 products and the FI-EV-A71 antigens bulks could induce significant neutralizing responses in mice. Inactivated FI-EV-A71 virions can be stored at 2—8 °C and remain stable for ten years, which provides flexibility and advantages in vaccine manufacturing, transportation and storage. Our results provide valuable information to understand the stability of FI-EV-A71 vaccine after expiring the currently set shelf life and may prompt the possibility of long-term storage of enteroviral vaccines for managing the recurrence of enteroviral diseases.

Declaration of competing interest

The authors have no financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.10.006.