Original Article

High immunogenicity of measles AIK-C vaccine produced in Vietnam

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Abstract. The goal of measles elimination has been set for 2012 in the WHO Western Pacific Region. To achieve this goal, an enhanced immunization strategy is required, and measles vaccines have been provided through the Expanded Programme on Immunization (EPI) in Vietnam. A large quantity of vaccines should be supplied for a two-dose immunization schedule with a supplementary catch-up campaign. The live measles vaccine AIK-C strain was produced in Vietnam through technical transfer from the Kitasato Institute, Japan. PolyVac I was produced from bulk materials imported from Japan and PolyVac II from the AIK-C seed. Two clinical trials were conducted using three vaccines: PolyVac I, and II, and the EPI vaccine (Rouvax) for the control. A total of 160 subjects were examined for the immunogenicity of PolyVac I and 57 for that of the EPI vaccine. All subjects became sero-converted in PolyVac I groups for three different Lots, but 54 (94.7%; 88.9-100%) of 57 recipients of the EPI vaccine became sero-positive. For PolyVac II, 118 initially sero-negatives became sero-converted. Whereas, 107 (95.5%; 91.7-99.3%) of 112 initially sero-negatives in the EPI group showed sero-conversion. The mean titers of post-immunization sera of sero-converted subjects receiving PolyVac I and II were lower than that for the EPI vaccine. No significant difference was observed in the incidence of adverse clinical events. Essentially the same results were obtained using PolyVac I and II, showing higher sero-conversion rates than the EPI vaccine, and PolyVac measles AIK-C will be favorable in Vietnam and Southeast Asian countries.

Key words: live attenuated measles vaccine, clinical trial, technical transfer

1. Introduction

Measles is still a major killer among infants in developing countries, and the World Health Organization announced that it aimed to reduce the number of measles deaths in 1999 by half by 2005 (1). The number of measles-related deaths decreased from 873,000 in 1999 to 345,000 in 2005, and to 242,000 in 2006, and so the tentative goal for 2005 was achieved on schedule (1,2).

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WHO/UNICEF estimates indicated that global routine measles vaccination coverage increased from 72% in 2000 to 82% for the first dose in 2007 (1-3). Indigenous outbreaks of measles were eliminated in the US by the implementation of a two-dose measles-mumps-rubella trivalent vaccine (MMR) program, and the sporadic cases reported in the US were caused by importation from Africa and Asian countries, including Japan (4,5). Even when measles virus was imported, the indigenous measles transmission chain was interrupted by high-level vaccine coverage and a reliable surveillance system (6-8). The WHO recommended the two-dose immunization strategy for countries with a high coverage of the first-dose measles vaccine, more than 95%. The proportion of countries offering children a second dose of the measles vaccine is increasing, and 168 (88%) countries now implement the two-dose

strategy (9). The new goal is to reduce measles mortality by 90% by 2010 compared with that before 2000 (9).

In Vietnam, the number of reported cases of measles was reduced to 2,245 cases in 2003 after the introduction of a measles vaccine campaign. Vaccine coverage at 9 months of age is now considered to be more than 95%, with approximately 90% sero-conversion rate. Now, a two-dose strategy is also implemented in Vietnam, but a large outbreak occurred in 2009. Measles vaccine has been supplied through the EPI, but very large doses would be required for the control of unexpected outbreaks. In preparing to meet the increase in demand for measles vaccine, domestic production in Vietnam would contribute greatly to the promotion of public health for infants and stimulate scientific activities in related fields, such as infectious diseases, biology, virology, and epidemiology. From this standpoint, mutual technical transfer between Vietnam and Japan was implemented for the production of measles vaccine. The AIK-C measles vaccine strain was independently developed from the wild-type Edmonston strain through plaque cloning in sheep kidney cells and chicken embryo cells at 32.5^{o}C (10-12). It shows optimal growth at 33^{o}C, but extremely poor or no growth at 39-40^{o}C, demonstrating temperature sensitivity (*ts*) (12). The AIK-C strain is one of the candidate vaccine strains for the Expanded Programme on Immunization (EPI) to overcome maternally conferred immunity, resulting in a high sero-conversion rate in young infants at 6 months of age (13-16). AIK-C has been administered at over 20 million doses, mainly in Japan, and the safety of the vaccine was reported after immunization during a ten-year period of Post Marketing Surveillance (PMS) (17). Measles vaccine for tropical and subtropical regions is required for a good thermostability. Human serum albumin and gelatin have been used for the stabilizer with different formulations because the failure in the cold-chain would occur during transporting the vaccines to the field. Objective of the study was to evaluate the stability and immunogenicity of the AIK-C measles vaccine produced by the Vietnam manufacturer in comparison with that of the EPI vaccine.

2. Materials and Methods

2.1. Vaccine production

The measles AIK-C vaccine strain was developed in the Kitasato Institute, Tokyo, Japan and has been used mainly in Japan (10-12). Two kinds of measles vaccine were produced in Vietnam: PolyVac I and II. PolyVac I was produced from bulk material imported from the Kitasato Institute, Tokyo, Japan, and PolyVac II from the seed strain supplied by the Kitasato Institute. PolyVac I and II have the same compositions, with some modification of the stabilizer of the vaccine used in Japan produced by the Kitasato Institute. The differences in compositions are shown in Table 1. PolyVac I and II measles vaccines comprised 10 doses in a single vial, containing 5,000 PFU/0.5 ml, and that produced in Japan is a single dose vial with the same infectivity. A major difference was the usage of hydrolyzed gelatin as a stabilizer. PolyVac I and II vaccines contained 0.36% hydrolyzed gelatin (Special LE200; GELITA, Eberbach, Germany), but there is no gelatin in the vaccine produced in Kitasato.

Table 1. Different compositions of measles AIK-C vaccine produced by PolyVac and Kitasato.

	PolyVac	Kitasato
Infectivity	5,000 PFU/ 0.5mL	5,000 PFU50/0.5 mL
Lactose	2.0 W/V%	5.0 W/V%
D-Sorbitol	0.72 W/V%	1.8 W/V%
L-Glutamate	0.4 W/V%	0.4 W/V%
Hydrolyzed gelatin	0.36 W/V%	--
Erthromycin	<10 ug	<10 ug
Kanamycin	<10 ug	<10 ug
Dose	10 dose	1 dose

2.2. Clinical trials

The study design of the clinical trials was discussed in the ethical committee of Military Academy of Medicine, Hanoi, Vietnam. A phase I study was performed, enrolling 27 and 30 adults for PolyVac I and II under organization by Military Academy of Medicine, Hanoi, Vietnam. They were examined during four weeks after immunization for the development of systemic illness and local reactions in addition with clinical blood examinations (blood cell counts and clinical biochemical tests) and there was no adverse event after vaccination. Phase III of clinical trial I was conducted in a randomized, double-blind manner, using three different lots of PolyVac I (M-0107, M-0207, and M-0307), and EPI vaccine (Rouvax, Sanofi-Pasteur-Merioux, France) for the control, in four different communes of Hai-Phong City. Including criteria was healthy young infants aged 9-11 months of

age who were not immunized with measles vaccine. Those having serious basic illness, taking immunosuppressive medication, and demonstrating acute illness were excluded. They were confirmed in a regional Health Care Center. A total of 241 young infants, were enrolled, randomized, and allocated under the orientation by Military Academy of Medicine, Hanoi, Vietnam. The key was opened after the fixation of clinical information and serological data. All written informed consent was obtained from either a parent or guardian.

Subjects were observed for 30 min after immunization for the appearance of anaphylaxis. To assess safety afterwards, health care workers visited their homes and checked the body temperature and for adverse clinical events based on the healthcare diary, every day for 28 days after immunization. Paired sera were obtained immediately before immunization and, in principle, 6-8 weeks after immunization.

Clinical trial II was conducted in 10 communes of Hai-Phong City using PolyVac II and EPI vaccine (Rouvax, Sanofi, France). A total of 263 young infants were recruited, and it was carried out in a similar manner as clinical trial I.

2.3. Serology

An enzyme-linked immunosorbent assay (EIA) kit, purchased from DENKA SEIKEN, Japan, was used for the evaluation of measles antibodies. Briefly, serum was diluted at 1:200 and added to the wells coated with purified measles virus particle antigens. Peroxidase-labelled anti-human IgG monoclonal antibodies were added, followed by enzymatic reaction with the substrate. The kit contained reference standard sera relevant to Neutralizing test (NT) titers, $2^{2.0,}$ $2^{3.0}$, $2^{4.0}$, $2^{5.0}$, $2^{6.0}$, and $2^{8.0}$. In each assay, these standard sera were assayed, and EIA units were calculated, employing a linear regression curve obtained from the reference standard sera. EIA units $>2^{2.0,}$ were considered positive (18). Seroconversion for the initially sero-positives was defined as four-hold or higher increase in EIA titers after vaccination in comparison with the initial EIA titers before vaccination.

Neutralization test (NT) antibody was examined based on 100% inhibition of the appearance of CPE in B95a cells. Briefly, serial two-fold dilutions of serum samples were mixed with an equal volume of challenge virus (approximately 100 TCID50). The mixture was placed on monolayers of B95a cells in 96-well plates and cultured for 7 days. The NT titer was expressed as the reciprocal of the highest serum dilutions which completely neutralized the appearance of CPE (18).

2.3. Statistical analysis

The sero-conversion rate and incidence of clinical adverse reactions were analyzed using the Fisher Extraction test, and the mean geometric mean titers of EIA were analyzed by the t student Welch method.

Table 2. Long-term stability at 2-8^{o}C and heat stability at 37^{o}C for 7 days.

Storage	2-8^{o}C *	37^{o} C 7days **
0	4.55 ± 0.07	-0.76
3 M	4.48 ± 0.06	-0.56
6 M	4.25 ± 0.09	-0.69
12 M	4.16 ± 0.13	-0.55
18 M	4.18 ± 0.06	-0.56
24 M	3.90 ± 0.12	-0.45

*: Vaccines were kept at 2-8^{o}C. Infectivity of three different lots is shown as log PFU/dose.
**: Freeze-dried vaccines stocked for each month were kept at 37^{o}C for 7days. Virus infectivity was assayed, and loss of infectivity is shown as a reduction of log 10. Thermostability regulation yielded less than a 1.0 reduction in infectivity at 37^{o}C for 7 days.

3. Results

3.1. Thermo-stability of PolyVac I and II

PolyVac I was produced in Vietnam from the bulk materials and PolyVac II from the seed, imported from the Kitasato Institute, Japan. The compositions were modified to meet with an EPI measles vaccine regulation, in which the infectivity decreases less than10^{-1} after one-week storage at 37^{o}C. The usage of gelatin is the major modification for tropical use to increase the stability, and the results of long-term and heat stability at 37 C are shown in Table 2. Lyophilized vaccines were stocked at 2-8^{o}C for 24 months for the three different lots, and infectivity was examined. After 24 months, infectivity was maintained within a $10^{0.65}$ drop. Vaccine materials stocked for 3, 6, 12, 18, and 24 months were examined for heat stability at 37^{o}C for 7 days. The drop in the rate of infectivity of each material was $10^{-0.45 \sim -0.76}$. Thus, the addition of hydrolyzed gelatin at 0.36% in a 10-dose vial increased the stability, and this measles vaccine formula was sufficient for EPI usage.

Table 3.Summary of serological responses of polyvac I and EPI vaccines

Vac	M-0107	M-0207	M-0307	EPI
No. Entry (Dropout)	60(16)	61(5)	60(6)	60(2)
No. Initially sero-positives(seroconvert)	2(0)	2(1)	0	1(0)
No. Initially sero-negatives	52	54	54	57
Sero-conversion rate (EIA>4)	52(100%)	54(100%)	54(100%)	54(94.74%)
95%CI				88.9%-100%
Sero positivity (EIA>8)	49(94.23%)	50(92.59%)	51(94.44%)	52(91.23%)
95%CI	87.9%-100%	85.6%-99.6%	88.2%-100%	83.9-98.6%
Mean titer of EIA (2^n)	4.84	4.77	4.68	5.16
95%CI	4.53-5.15	4.49-5.05	4.42-4.94	4.75-5.58
S.D.	1.126	1.042	0.969	1.553
Range	2.57-7.1	2.6-6.51	2.61-7.03	1.18-7.8

Seroconversion for the initially sero-positives was defined as four-hold or higher increase in EIA titers after vaccination in comparison with the initial EIA titers before vaccination.

Table 4. Summary of Serological responses of polyvac II and EPI vaccines

	PolyVacII	EPI
No. Entry (Drop out)	132(10)	131(15)
No. Initailly sero-positives (seroconvert)	4(2)	4(2)
No.Initailly sero-negatives	118	112
Sero-conversion rate (EIA>4)	118(100%)	107(95.5%)
95% C.I.		91.7-99.3%
Sero-positivity (EIA>8)	116(98.3%)	104 (92.9%)
95% C.I.	96.0-100%	88.1-97.7%
Mean titers of EIA (2^n)	5.301	5.749
95%CI	5.15-5.45	5.51-5.99
S.D.	0.839	1.281
range	2.84-7.41	1.13-7.62

Seroconversion for the initially sero-positives was defined as four-hold or higher increase in EIA titers after vaccination in comparison with the initial EIA titers before vaccination.

Table 5. Clinical adverse reactions

Vaccine	PolVac I M-0107,0207,0307	EPI (Trail I) (Rouvax)	PolyVac II	EPI (Trial II) (Rouvax)
No. Entry (drop out)	181(0)	60(0)	132(0)	131(0)
Age (months)	9-11	9-11	9-11	9-11
Anaphylaxic reaction	0	0	0	0
Fever	1.70%	3.30%	3.10%	3.80%
Rash	2.20%	0	0	2.30%
Runny nose	14.40%	13.30%	9.10%	8.40%
Diarrhea	13.30%	8.30%	0.80%	2.30%

3.2. Clinical trial I using PolyVac I produced from the bulk material imported from Japan

Clinical trial I was conducted and a summary of serological responses is shown in Table 3. Three consecutive lots of PolyVac I and EPI vaccine were used, and a total of 241 infants were enrolled in the study. Excluding the drop-out cases, initially sero-negatives, 52, 54, 54, and 57 subjects were examined for the immunogenicity of PolyVac I Lot M-0107, M-0207, and M-0307, and EPI vaccine, respectively. All subjects became sero-converted (EIA units $>2^{2.0}$) in PolyVac I groups for the three different Lots, but 54 out of 57 (94.7%; 88.9-100% of 95% CI) recipients of the EPI vaccine became sero-positive. When sero-conversion was defined as $>2^{3.0}$ EIA units, 49/52 (94.23%), 50/54 (92.59%), 51/54 (94.44%) and 52/57 (91.23%) became sero-converted for Lot M-0107, M-0207, and M-0307 and EPI vaccines, respectively. Mean EIA units are also shown also in Table 3. Mean EIA units (2^{n}) of sero-positives after immunization with Polyvac I Lot M-0107, M-0207, and M-0307 were 4.84, 4.77, and 4.68, respectively, without significant differences. However, these mean EIA units were lower than those observed after immunization with the EPI vaccine at 5.16. In recipients of PolyVac I, EIA antibodies were accumulated in 2 (4-6) units, and EPI vaccine in 2 (5-7) units.

In one commune, NT titers were examined together with the EIA assay for 45 sera after vaccination. Reference standard sera were assayed and EIA units were calculated by employing a linear regression line obtained from OD values and EIA titers of standard sera, and the results are shown in Fig. 1. EIA and NT

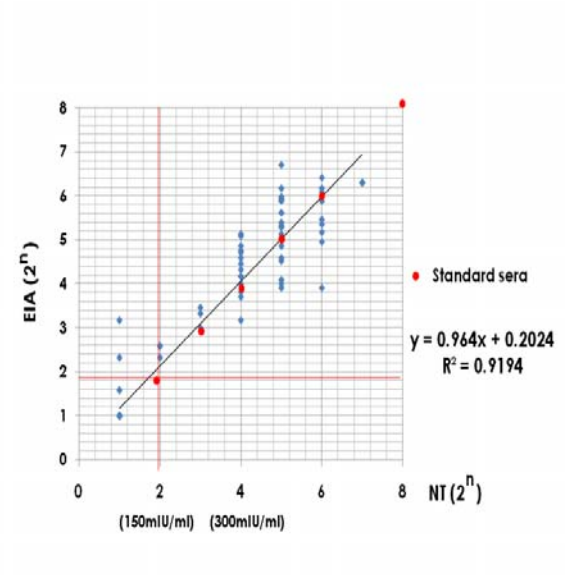

Fig. 1. Correlation between EIA and NT antibodies. 45 sera were obtained in one village after vaccination with PolyVac I. The Denka Seiken EIA kit has six reference sera. Red circles shows the standard reference sera and diamonds represent the 45 sera of the children vaccinated with PolyVac I. EIA units were calculated by employing a linear regression assay and compared with the NT titers.

showed a good correlation (r^2=0.9194), as described in a previous report (18).

Two sera showed <2.0 EIA units, and the NT titers of these sera were <2.0. Among three sera showing EIA weakly positive around 2 (2-3) units, one was NT negative. One serum sample $>2^{3}$ EIA units was negative for NT antibody and the others were positive for NT. International reference of measles NT serum was calibrated and 1:4 of NT assay in this study showed 150 mIU/ml.

EIA antibody responses after vaccination with PolyVac I and II measles vaccines

	PolyVac I	PolyVac II
Serological assay	160	118
Sero-conversion (EIA >2)	100%	100%
Sero-conversion (EIA >3)	93.75% (90-97.5%)	98.31% (96-100%)
Mean EIA units	4.765	5.306 ★ P<0.01
S.D.	1.049	0.839

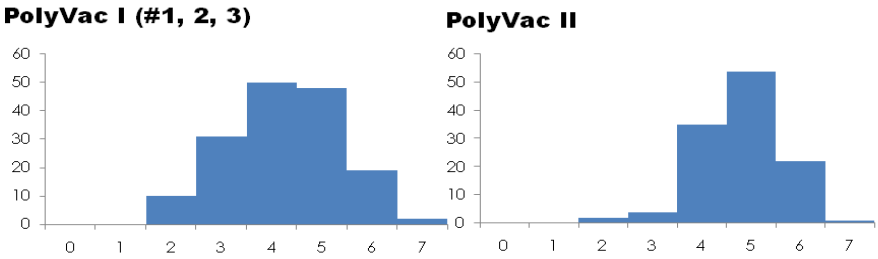

Fig.2. EIA antibody responses after vaccination with PolyVac I and II measles vaccines.
The upper panel shows the difference in immunogenicity, and the lower panel shows the distribution of EIA units after immunization with PolyVac I and II.

3.3. Clinical trial II using PolyVac II produced from seed material

As for the clinical trial of PolyVac II, measles vaccine was produced from the seed strain supplied from the Kitasato Institute and the final products were the same as for PolyVac I, with the same compositions. Clinical trial II was conducted in 10 communes in Hai-Phong City using PolyVac II and EPI (Rouvax) vaccine for the control, and the results are shown in Table 4. A total of 263 infants were enrolled, and 132 and 131 were immunized with PolyVac II or EPI vaccine, respectively. Among them, post-immunization sera were not obtained in 10 and 15 subjects for PolyVac II or EPI vaccine, respectively, because the parents refused to take a blood examination. And they completed the healthcare diary and they were included for the safety analysis. A total of 122 and 116 subjects were examined for serological responses, and four subjects were initially sero-positive in each group. Among 118 initially sero-negatives in the PolyVac II group, all subjects became sero-converted. Whereas, 107 (95.5%: 91.7-99.3%) out of 112 initially sero-negatives in the EPI group became sero-converted. When sero-conversion was defined as $>2^{3.0}$ units, 116/118 (98.3%; 96-100%) and 104/112 (92.9%; 88.1-97.7%) became sero-converted for PolyVac II and EPI vaccines, respectively. The sero-conversion rate for PolyVac II was higher than for the EPI Rouvac vaccine but not significant. The mean EIA unit after immunization with PolyVac II was $2^{5.301}$ (95%CI: 5.15-5.45, SD: 0.839) lower than that after immunization with EPI vaccine, $2^{5.749}$ EIA units (95% CI: 5.51-5.99, SD: 1.281). For each group, four subjects were initially sero-positive, and two in each group showed a significant serological response (more than 4-fold increase in EIA units).

3.4. Comparison of PolyVac I and II

In clinical trial I, 160 initially sero-negative subjects were examined for immunogenicity, and 118 in clinical trial II. The results are summarized in Fig. 2. All became sero-converted (EIA $>2^{2.0}$) and no significant difference was observed in sero-conversion rates (EIA $>2^{3.0}$). Mean EIA units were higher in the subjects immunized with PolyVac II and the distribution of EIA units shifted to high titers, as shown in Fig. 2.

3.5. Clinical adverse reactions

Regarding safety, clinical adverse reactions in clinical trials I and II are summarized in table 5. No serious adverse event occurred in any group. Approximately 3% of the recipients showed febrile reactions, with a high body temperature of

38-38.5°C, for 1-2 days duration. Several had running nose, and diarrhea. There was no significant difference in the incidence of clinical adverse reactions among the two clinical trials and EPI vaccine groups.

4. Discussion

Measles virus was first isolated in 1954 by Enders and Peebles from a patient, and the Edmonston strain was further passaged in human kidney or alantoic cells. Edmonston A and B vaccine strains were established after several passages in chicken embryo cells, developing attenuated strains (19). Currently available live attenuated vaccines are derived from Edmonston strain A or B. The AIK-C was developed from the wild-type Edmonston through small plaque cloning at low temperature at 33 C. The molecular backgrounds regarding the characteristics of the AIK-C strain were examined, showing that Leu at amino acid position 273 of the F protein was critical for small plaque formation and Pro at the position 439 of the P protein for the *ts* phenotype (20, 21). These two unique characteristics were probably closely related to the attenuation process of poor virus growth in the body, causing lower-level reactogenicity.

The immunogenicity of measles vaccine depends upon the strain and age at administration. Cell-mediated immunity was also induced in young infants at 6 months of age, as well as 1-5 years of age, who received the AIK-C strain (19, 20). Vaccination in infants < 6 months of age sometimes showed in sero-conversion failure because of the immaturity of the immune system and maternally conferred immunity. Based on the results of a large number of studies, the sero-conversion rate was 89.6% (82-95%) at the age of 8-9 months, and 99% (93-100%) at the age of 11-12 months following the first dose of measles vaccine (19). In this study, PolyVac I and II demonstrated 100% sero-conversion, whereas, approximately 95% of recipients receiving the EPI vaccine were sero-converted. Concerning the distribution of EIA units after immunization, PolyVac I and II induced relatively lower titers in comparison with Rouvax EPI vaccine (Schwarz strain). In several clinical trials, AIK-C has shown a high sero-conversion rate with slightly lower titers after immunization. This reflects the ts phenotype of the AIK-C that resulted in lower virus growth in the body.

Measles vaccine, besides the AIK-C strain, is not stable at a high temperature, and so 2.0 % hydrolyzed gelatin has been added as a stabilizer in the past to increase the stability for tropical use. This was effective to maintain the infectivity, but caused gelatin allergy (24). This thermo-stable AIK-C was, therefore, withdrawn from the market. After immunization with gelatin-containing measles, rubella, mumps, and chicken pox vaccines, anaphylactic reactions or hives were demonstrated from 1994 in Japan (25). At the same time as this was being reported in the U. S. (26), IgE antibodies against gelatin were detected in these cases. Gelatin as a stabilizer of live vaccines is not an essential factor, and IgE antibodies against gelatin should be present before the administration of gelatin-containing live vaccines. It was necessary to identify the cause(s) of sensitization before the immunization of live measles vaccine or other live vaccines. Nakayama et al. (24) reported that a trace amount of gelatin in diphtheria-tetanus-acellular pertussis (DTaP) was responsible for sensitization against gelatin. Gelatin, even though in trace amounts, enhanced the sensitization because DTaP vaccine, containing aluminium adjuvant, was administered three times before measles vaccination. Since 2000, all DTaP vaccines on the Japanese market have become free of gelatin, and, consequently, the incidence of gelatin allergy has decreased, along with the removal of gelatin from all live vaccines (27). In the U. S., trace amounts of hydrolyzed gelatin were used in DTaP produced by several big vaccine companies, using a 15-28 ug/dose of small molecule, hydrolyzed gelatin, but no marked increase in rate of anaphylaxis after MMR vaccination has been observed since the introduction of gelatin-containing DTaP (28). As for vaccine stability, gelatin or human serum albumin has been used as a stabilizer of measles vaccine. Measles AIK-C vaccine in Japan did not contain gelatin, but the formulation for Japanese use did not need to be stable at 37°C. Regarding the difference in the composition of PolyVac and Kitasato AIK-C, 0.36% hydrolyzed gelatin was used in PolyVac AIK-C. Employing this composition, it maintained stable infectivity even after 7 days incubation at 37°C, showing within a 1/10 reduction of the infectivity. The gelatin material is hydrolyzed to a MW of less than 4,000. In clinical trials, a total of 313 young infants were immunized with PolyVac I and II, and there was no serious adverse allergic event. All infants underwent previous immunization with a series of DPT. DPT is administered at 3, 4, and 5 months of age in Vietnam. DPT in Vietnam is the whole cell pertussis type, not containing gelatin. Gelatin used in PolyVac I and II was hydrolyzed gelatin with small molecules. Therefore, the risk of gelatin allergy was

theoretically reduced in comparison with gelatin allergy in Japan from 1994 to 2000. In these clinical trials, no serious adverse event, anaphylaxic reaction or shock, was observed.

PolyVac I and II measles vaccines produced in Vietnam demonstrated excellent stability properties with the addition of hydrolyzed gelatin as a stabilizer and the immunogenicity of the vaccines was at least equivalent to that of the standard EPI vaccine. Now, the annual number of newborns is 1.3 million in Vietnam, and the regular measles immunization schedule involves those at the ages of 9 months and 6 years. Recently, the incidence of measles in teenagers has been increasing because of a reduction in vaccine-acquired immunity. When outbreaks occur, additional vaccine administration for teenagers should be considered. Several million doses of PolyVac measles vaccine are ready to be produced under the regulation of GMP. Domestic production and vaccine supply would be beneficial for realizing an active immunization strategy in Southeast Asia, as well as in Vietnam, independent of EPI supply.

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