นิพนธ์ต้นฉบับ

Enzyme-Linked immunosorbent assay for evaluation of measles immunity by using measles vaccine as a coating antigen.

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An enzyme - linked immunosorbent assay (ELISA), using measles vaccine as a coating antigen was standardized and evaluated for determining measles immune status. The optimal protein concentration of a coating antigen and the serum dilution used in the assay were equal to 3.75 μg/ml and 1:400, respectively. This condition maximized the different optical density values of the high-positive serum and the negative serum. The validity of the test was evaluated by using 51 immune sera and 32 susceptible sera. It was found that the test was able to distinguish immune individuals from susceptible individuals with 96.08% of the sensitivity and 87.50% of the specificity. The standardized ELISA was used to screen the measles antibody in 77 preimmunized sera and 77 postimmunized sera. The test was enough sensitive to detect measles antibody in low-titered preimmunized serum samples. The application of this assay for screening large numbers of samples to assess vaccine efficacy or for epidemiological studies in developing countries is discussed.

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พิพัฒน์ ลักษมีจรัลกุล, วิไล เกิดผล, วิชิต มธุรสภาษณ์, วิธีเอ็นซัยม์ลิงค์อิมมูโนซอบแบนท์ แอสเส เพื่อประเมินภูมิคุ้มกันต่อโรคหัดโดยใช้วัคซีนป้องกันโรคหัดเป็นแอนติเจน. จุฬาลงกรณ์เวชสาร 2532 กุมภาพันธ์; 33(2): 119-126

การศึกษานี้ได้ทำการประเมินประสิทธิภาพของวิธีเอ็นซัมย์ลิงค์อิมมูโนซอบเบนท์ แอสเส (อีไลซ่า) ในการตรวจหาสภาวะภูมิคุ้มกันต่อโรคหัดโดยใช้วัคซีนป้องกันโรคหัดเป็นแอนติเจน พบว่า ปริมาณความเข้มข้นโปรตีนของแอนติเจนที่เหมาะสมสำหรับตรวจเท่ากับ 3.75 ไมโครกรัม/มิลลิลิตร ขนาดความเจือจางของซีรั่มที่เหมาะสมในวิธีทดสอบเท่ากับ 1:400 ระดับความเข้มข้นแอนติเจนและความเจือจางของซีรั่มดังกล่าว ให้ความแตกต่างของค่าความเข้มของแสง ในซีรั่มบวกและซีรั่มลบ มากที่สุด เมื่อใช้วิธีดังกล่าวตรวจซีรั่มที่ได้จากบุคคลที่มีภูมิคุ้มกันต่อโรคหัด 51 ราย และซีรั่มที่ได้จากบุคคลที่ไม่มีภูมิคุ้มกันต่อโรคหัด 32 ราย ได้ค่าความไวของวิธีทดสอบ ร้อยละ 96.08 ความจำเพาะ ร้อยละ 87.50 และเมื่อนำไปทดสอบกับซีรั่มที่ได้จากบุคคลก่อนฉีดวัคซีนป้องกันโรคหัดและหลังฉีดวัคซีนป้องกันโรคหัด จำนวนอย่างละ 77 ราย พบว่า วิธีอีไลซ่านี้สามารถให้ผลบวกในซีรั่มที่มีแอนติบอดีระดับต่ำ ๆ ได้ ดังนั้นวิธีอีไลซ่าโดยใช้วัคซีนป้องกันโรคหัดเป็นแอนติเจนนี้ น่าจะเป็นวิธีที่ประหยัดและเหมาะสมสำหรับห้องปฏิบัติการทางน้ำเหลืองวิทยาทั่วไป เพื่อใช้ศึกษาสภาวะภูมิคุ้มกันต่อโรคหัดหรือระบาดวิทยาของโรคหัดในประเทศที่กำลังพัฒนาได้

Measles is an acute, highly contagious, viral disease characterized by a generalized maculopapular eruption.[1] The disease occures most commonly in children under fifteen years old. Although it is a mild disease for most children, it can result in severe complication including pneumonia, otitis media, diarrhea, convulsion and encephalitis.[2] Fifty-nine percent of cases occurred in children under 4 years of age and almost all children had measles by the age of 10 years.[3] Moreover, 10 to 13 percent of cases were reported in 3-to 11-month-infants.[2,4] In Thailand, the trend of disease has increased since 1975-1985.[5] The whole country annual reported cases in 1984 was about 47,205 cases and 70 death cases.[3] Parents had to spend about 612.40 bahts per case to treat their children with measles until recovery or death.[6] The economic loss, only on the part of the parents for measles treatment was about 29 million bahts per year. Therefore, measles immunization programme has been initiated to control the disease in Thailand since April, 1984.[7] The schedule of measles vaccination in a child varied from country to country.[8-11] It depends on the quantity of passive antibody from the child's mother and the severity of disease during the first year of life.[9,12] Seroconversion rates of 85% to 93% have been observed in 6- to 9-month infants in Kenya but only 65% in the USA at this age.[9] To resolve the controversies regarding measles vaccine failure, a sensitive method in headed for determining the low titer of maternal antibody which may interfere with the immune response from immunization. An enzyme-linked immunosorbent assay (ELISA) is a reproducible, sensitive, specific and easily interpretable test. It is much more feasible and more sensitive than the hemagglutination inhibition test.[13,14] However, the measles virus antigen used for coating ELISA microplate prepared from tissue culture of monolayer Vero cells may be too complicated for many laboratories in Thailand. Therefore, this study attempts to use the measles vaccine which is available in general hospitals, as a coating antigen in the measles ELISA. A standardization of the assay is made and the validity of the test is evaluated also.

MATERIALS AND METHODS

Human sera

1. Sera from 2 measles cases with the complement fixing antibody titer 1:128 or greater were pooled together and used for the high-positive control serum.

2. Sera from 32 normal children without a history of exposure to natural or vaccine measles virus and with the neutralizing antibody titer less than 1:4 were used as susceptible sera. Some of them were pooled together and used for the negative control serum.

3. Sera from 51 normal children with a previous history of measles illness, whose ages ranged from 14 to 15 years, were used for immune sera.

4. Seventy-seven serum samples of 9- to 10-month-old infants who had neither been illness with measles nor measles vaccination were used for preimmunized serum samples. Previous study showed that the 9-to 10-month-old infants had the lowest level of maternal antibody.[15] After 3 to 4 weeks of measles vaccination, these infants' blood were bled and used for postimmunized serum samples.

The high-positive serum and the negative serum, we used to standardize the method ; the immune sera, susceptible sera, preimmunized sera and postimmunized sera, we used to evaluate the utility of the test in screening the immune status. All serum samples were stored at −20°C until the ELISA was performed.

Antigen in ELISA

The attenuated Schwarz strain measles vaccine (ROUVAX, Institute Merieux, France), lot Y 1344, prepared in primary CEF culture medium was reconstituted in 1 ml of 0.85% normal saline solution. Serial dilutions beginning at 1:500 (protein concentration 15.00 μg/ml) were made and used as coating antigens in the assays.

Anti - human immunoglobulin conjugate and substrate :

The peroxidase - conjugated goat anti-human polyvalent immunoglobulins (Sigma Chemical Co USA, lot No 94 F 8940) and ortho-phenylenediamine (OPD)/ Hydrogen peroxide substrate were used in this assay.

Method of ELISA

The method of ELISA for determining measles antibody was an indirect method after Saunder and Clinard (1976).[16] A 50 μl of 2.5% fetal bovine serum in normal saline solution was added to each well of flat - bottom polystyrene microplates (Nunc) and incubated at 37°C for 18 hours. A 50 μl of 0.25% glutaraldehyde in phosphate buffer saline pH 7.2 was added and incubated at room temperature for 30 minutes. Microplates were washed 3 times with distilled water, a 50 μl of optimal measles antigen concentration in normal saline was added and incubated at 37°C for 18 hours. The microplates with coating antigen were kept in a desicator until use. The microplates were washed 3 times and 100 μl of 1:400 dilution of serum samples in phosphate buffer saline pH 7.4 containing 0.05% Tween-20 and 0.5% bovine serum albumin (from Chequerboard titration) was added. Then, the microplates were incubated at room temperature for 30 minutes and washed 3 times with normal saline-Tween-20 solution and shaken to dry. A 100 μl of

1:500 dilution of peroxidase-conjugated goat anti-human polyvalent immunoglobulins was added and incubated at room temperature for 30 minutes. The microplates were washed 3 times with normal saline-Tween-20 solution and 100 μl of the substrate solution was added. The reaction was allowed to proceed for 15 minutes at room temperature and was stopped with 50 μl of 4 N H_2SO_4. The optical density (OD) of the colored solution was measured by a spectrophotometer ELISA Minireader II (Dynatech Laboratory) at the wavelength of 490 nm.

The ELISA antibody level of a single specimen might be reflected by the OD value with the day to day variation. Therefore, the assay was standardized by comparing the OD values of test sera to the reference sera of high-positive and negative sera.[17] All test samples were assayed concomitantly with the high-positive and negative sera. The OD value for each tested serum was divided by the OD value of the high-positive serum for that day to determine an OD index (ODI) value as follows:

$$\text{ODI} = \frac{\text{OD of tested serum} \times \text{Mean OD of high-positive serum}}{\text{OD of high-positive serum on the same day}}$$

Mean OD of high-positive serum (N = 30)	= 1.26
Standard deviation of high-positive serum	= 0.15
Mean OD $\pm$ Standard deviation	= 1.11-1.41

In each assay if the OD value of the high-positive serum was greater or less than the Mean OD $\pm$ Standard deviation of the high-positive serum, the results of the assay were omitted and repeated.

RESULTS

Standardization of ELISA to determine measles antibody

The optimal concentrations of measles antigen, serum dilution and enzyme conjugate were determined by Chequerboard titration using the known high-positive control serum (Complement fixing antibody titer $\geq$ 1:128) and the negative control serum (Neutralizing antibody titer < 1:4). Dilutions Which maximized the different optical density values of the high-positive serum and the negative serum were chosen in the assay. It was found that the optimal dilutions of antigen and serum for determining measles antibody were 1:2000 (protein concentration = 3.75 μg/ml) and 1:400, respectively. The ratio of the optical density value of the high-positive serum to that of the negative serum was 6.33. (Table 1)

Table 1. Results chequerboard titration by using measles vaccine as a coating antigen in ELISA.

Dilutions of antigen (protein conc.)	Optical densities of reference sera								Antigen control	Buffer control
	High-positive serum				Negative serum					
	1:200	1:400	1:800	1:1600	1:200	1:400	1:800	1:1600		
1:500 (15.00 μg/ml)	1.79	0.96	0.58	0.31	0.32	0.21	0.11	0.09	0.03	0.03
1:1000 (7.50 μg/ml)	1.82	0.96	0.59	0.30	0.37	0.23	0.14	0.12	0.05	0.05
1:2000 (3.75 μg/ml)	1.83	1.14*	0.64	0.29	0.36	0.18*	0.13	0.10	0.04	0.01
1:4000 (1.88 μg/ml)	1.73	0.76	0.43	0.32	0.47	0.30	0.28	0.24	0.05	0.01

* The ratio of two reference sera was 6.33

The validity of ELISA for determining measles immune status

Fifty-one immune sera and 32 susceptible sera were used to evaluate the validity of the test. The mean ($\overline{X}$) $\pm$ standard deviation (SD) of optical density index (ODI) for 51 immune sera and 32 susceptible sera were 0.85 ± 0.29 and 0.26 ± 0.08 (Table 2).

The cut-off levels of positive results for measles immunity was at ODI above $\overline{X}$ + 2SD of susceptible sera (ODI > 0.42), the sensitivity and specificity of the test were 96.08% and 87.50%. (Table 3).

Table 2. Mean and standard deviation of the optical density index (ODI) of 51 immune sera and 32 susceptible sera.

Tested sera	No of tested	Statistics	
		Mean ($\overline{X}$)	Standard Deviation (SD)
Immune sera	51	0.85	0.29
Susceptible sera	32	0.26	0.08

$\overline{X}$ + 2SD of susceptible sera = 0.42

Table 3. The validity of an ELISA measuring measles antibody in 51 immune sera and 32 susceptible sera.

Results for ELISA	Immune status		Total
	Immune	Susceptible	
Positive*	49	4	53
Negative	2	28	30
Total	51	32	83

Sensitivity of test	= 96.08 %
Specificity of test	= 87.50 %
Predictive value of positive	= 92.45 %
Predictive value of negative	= 93.33 %
False positive	= 12.50 %
False negative	= 3.92 %
Efficiency of test	= 92.77 %

* Cut-off level of positive results for measles immunity was at ODI > 0.42

Screening for measles immune status

Sera from 51 immune sera, 32 susceptible sera, 77 pre-immunized serum samples and 77 post-immunized serum samples were determined for measles immune status with the standardized ELISA. Each assay included a high-positive serum and a negative serum for control in the test. When the ODI values of tested sera above 0.42 were considered positive for measles immunity, the percentages of positivity in immune sera, susceptible sera, post-immunized serum samples and pre-immunized serum samples were 96.08%, 12.50%, 88.31% and 35.06% (Table 4). The optical density index for individual serum was summarized and shown in Figure 1.

Table 4. The percentages of positivity for measles immunity in immune sera, susceptible sera, post-immunized serum samples and pre-immunized serum samples.

Tested sera	No of tested	Positive for measles immunity*	
		Number	Percentages
Immune sera	51	49	96.08
Susceptible sera	32	4	12.50
Post-immunized serum samples	77	68	88.31
Pre-immunized serum samples	77	27	35.06

* Cut-off level of positive results for measles immunity was at ODI > 0.42

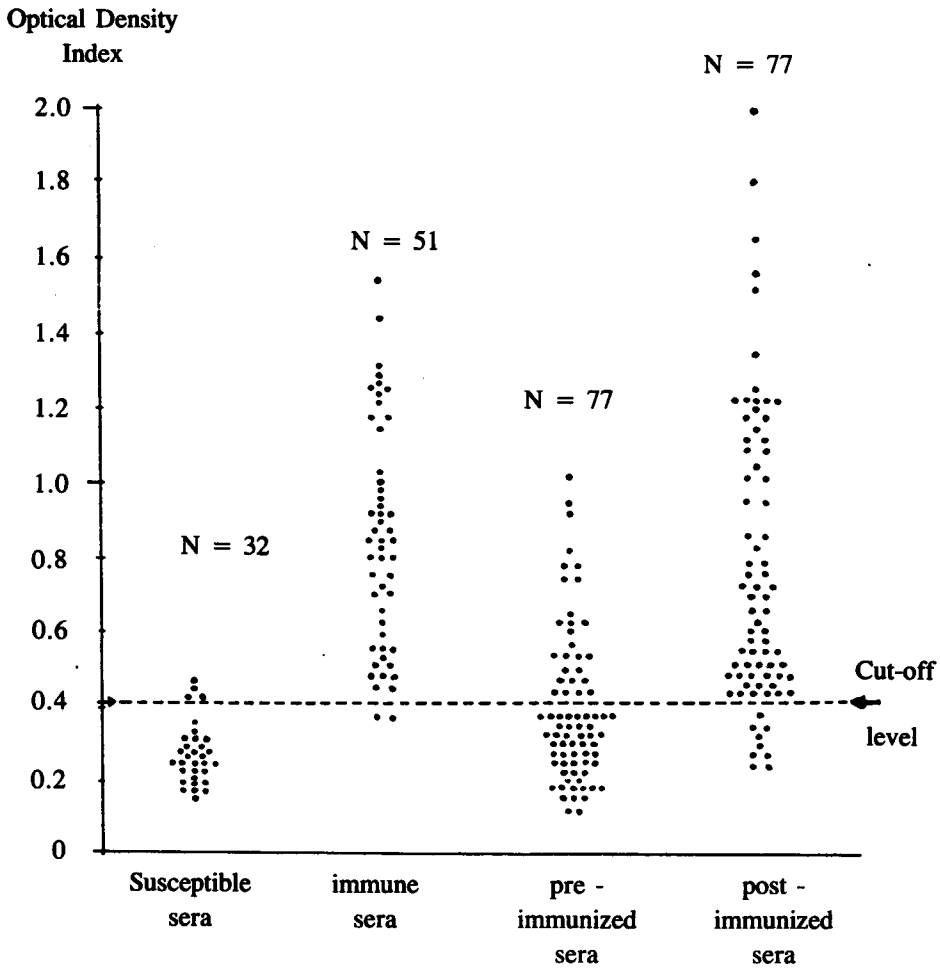

Figure 1. The optical density index for individual serum.

DISCUSSION

We have standardized and evaluated an ELISA for determining measles immune status by using measles vaccine as a coating antigen in the assay. The optimal protein concentration of a coating antigen was 3.75 μg/ml and the serum dilution used in ELISA was 1:400. This condition maximized the different optical density values of the high-positive control serum and the negative control serum. The ratio of the optical density value of the high-positive serum to that of the negative serum was 6.33 which was preferable for setting ELISA. The test was sensitive and specific enough to distinguish immune individuals from susceptible individuals. It was able to detect measles antibody in low-titre serum samples. However, the specificity of the test was rather less than in the previous study (99%)[14] which may have been due to the use of whole measles virion for the coating antigen. A previous study found that an ELISA was much more feasible and sensitive than the hemagglutination inhibition test in the testing of relatively low-titre serum.[14] The ELISA is appropriate for application in the community because it requires a very small volume of serum. As blood is collected from the fingertip, children will co-operate more than with the other methods.

The commercial kit of measles ELISA is rather expensive and the measles virus from tissue culture may be too complicated for many laboratories in Thailand and other developing countries. The measles ELISA by using measles vaccine as a coating antigen is cheaper and more available than the commercial kit or measles virus from tissue culture. Therefore, it may conveniently be used in small laboratories and may be an ideal test for screening large numbers of samples to assess vaccine efficacy or for epidemiological studies in developing countries.

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