Paediatrica Indonesiana

VOLUME 48 May • 2008 NUMBER 3

Original Article

Antigenic differences between wildtype measles viruses and vaccine viruses in Indonesia

Made Setiawan[1], Agus Sjahrurachman[2], Fera Ibrahim[2], Agus Suwandono[3]

Abstract

Background Measles virus has a single, negative strand RNA genome which codes 6 structural proteins: N, F, P, M, H and L. Currently there are several variances in the nucleotide sequences of N, F, M and H genes across wild type measles viruses, hence measles viruses can be categorized into clades and genotypes. The antigenicity of the previous genotype of measles is different from the current genotype.

Objective To determine the antigenic differences between wild type measles virus and measles vaccine virus.

Methods Analysis of the antigenic differences between wild type virus (G2, G3 and D9) and vaccine virus (CAM-70 and Schwarz) was performed by immunizing mice with the respective viruses. The serum was then tested with micro-cross-neutralization technique using the G2, G3, D9 and CAM-70 virus. Tests with cross ELISA examination technique were also performed using the same set of virus.

Results Analysis of the cross neutralization test and cross ELISA showed that the highest antigenicity reaction was found between wild type virus with antibody against wild type virus, while the lowest reaction was between wild type virus with antibody against CAM-70.

Conclusions We conclude that the antigenicity of antigenic protein from wild type virus is higher than antigenicity of vaccine virus protein. In addition, it was found that the antigenicity of proteins from Schwarz vaccine virus was higher than proteins CAM-70 vaccine virus. **[Paediatr Indones 2008;48:125-35]**.

Keywords: *measles virus, antigenic, measles vaccine, neutralization, ELISA*

Measles is a chilhood disease caused by infection of measles virus belongs to morbilli-virus genus and paramyxoviridae family. The virus has containment with negative, single strand RNA genome which codes 6 structural and non-structural proteins: nucleoprotein (N), P/V/C proteins, matrix (M), fusion (F), hemaglutinin (H), and large polymerase protein (L).[1]

Currently, in the world there are 8 clades and 22 genotypes wild type measles virus known,[2] while the those distributed in Indonesia are G2, G3 and D9 genotypes.[3,4] The type of measles vaccines circulating in the world are quite a lot, some are derived from Edmonston-vt virus such as Schwarz, Moraten, AIK-C, Zagreb, while some are from other strains such as Tanabe strains, including CAM-70 vaccine from Japan, Leningrad-16 from Russia and Shanghai-191 from China. Measles vaccines used frequently in Indonesia are CAM-70 and Schwarz.[5]

From the Department of Child Health, Infectious Disease Hospital Prof. Sulianti Saroso, Jakarta, Indonesia (MS).[1]The Department Microbiology, Medical School, University of Indonesia, Jakarta, Indonesia (AS, FI).[2] The National Institute Health Research and Development, Indonesia (AS).[3]

Reprint requests to: Made Setiawan,MD, Department of Child Health, Infectious Diseases Hospital Prof. Sulianti Saroso, Jl. Baru Sunter Permai Raya Jakarta-Utara 10340, Indonesia. Tel. 62-21-6506569

It has been known that there are genetic and antigenic differences between wild type virus and vaccine virus in United States.[6] The differences are found in the nucleotide sequences of N, H, F and M protein genes. These differences lead to the differences in the antigenicity between wild type measles virus and vaccine virus. Serum from current measles patients can neutralize current strain of measles four times better than it does to neutralizing current vaccine virus, while serum taken from people aged 50 years old or from people outside the measles endemic region can neutralize, the vaccine virus is the same as the wild type virus neutralization. This means that wild type measles virus has unique and more dominant epitopes compared to vaccine virus.[5]

During the year of 1988 to 1991, measles outbreak occurred in United States. Half of the babies and children infected with the disease had already received measles immunization.[7] In Indonesia, it was reported that about 15-30% of infected patients during outbreak period had received immunization.[8] Although the cause of the outbreak was not known, it was predicted that factors contributing to the causes were the failure of maintaining the coverage of immunization, vaccine still need a good cold chain, existence of maternal antibodies in the babies, and lower immunity raised by the vaccine compared to immunity raised by natural infection.[9] Other factor includes the antigenic differences between vaccine virus and wild type virus.[10]

The purpose of this study was to examine the antigenicity differences between vaccine viruses and wild type viruses in Indonesia.

Methods

This was an experimental laboratory study to determine the antigenic differences between several genotypes of wild type viruses and vaccine viruses in Indonesia by injecting each strain of measles virus to mice. Next, the serum containing built-up antibody was tested with neutralization test and cross ELISA to find out the reactivity of the antibody after immunization.

Tabel 1. The wild type virus isolates analyzed

No.	Code	Location/ Source	IgM	Age (yr)	Sex	Geno-type
1	MVi/INA/06.02/161Yo	Subang-Jabar	+	4.5	M	D9
2	MVi/INA/05.02/Ba	Gresik-Jatim	+	4	F	G3
3	Mvi/INA/03.04/362 Sep	Pekalongan	+	3.8	F	G2
4	CAM-70 vaccine virus	PT. BioFarma				
5	Schwarz vaccine virus	PT. Eurindo				

Samples

The current known strain of wild type viruses circulating in Indonesia are G2, G3 and D9 genotypes; the ones analyzed in this study were those 3 genotypes, which cultures had been sent to ICDC (Atlanta, USA). The genotype of the wild type virus was determined by ICDC and re-confirmed in Indonesia.[4] The vaccine viruses analyzed were CAM-70 and Schwarz (MMR) virus. The wild type viruses were obtained from Development and Research Center, Ministry of Health. The CAM-70 vaccine was obtained from Immunization Subdirectorate Ministry of Health, while the Schwarz (MMR) vaccine was obtained from PT Eurindo.

Measles virus stock preparation

The preparation of viruses stock was done according to instruction given by WHO.[11] First, the passage of B95a or Vero cell was performed as follows: the materials needed were 500 ml DMEM (Dulbecco's MEM) supplemented by penicillin 100 U/ml, streptomycin 100 μg/ml and Foetal Bovine Serum (FBS) 10% and culture bottles. The medium inside T-25 bottle was discarded. PBS (5 ml) was added for washing, and then discarded. Trypsin (1-2 ml) was added, and then left for a while without incubator, then discarded. Five ml DMEM was added and then the mixture was moved to other smaller tube to mix it evenly. Cell from one bottle are then divided into one-half, one-third, one-forth (no more than 1-4th). The cell was added to the prepared culture bottle filled with DMEM-PBS. The cell was then ready to be infected after 2 days.

When the monolayer cell in the bottle had grown successfully (around 75-80%), the liquid virus stock was inoculated into the culture bottles as much as 1.5-2 ml for T-25 bottle, incubated at 37^{o}C for 1 hour, and then observed under inverted microscope

to check for cell changes (such as floating cell or shape changes) as a result of intoxication. If this happened, the medium was replaced immediately. If all was good, DMEM (10 ml) containing 2% FBS and the antibiotics was added to the culture bottle. The culture bottles were then incubated at 37^{o}C. The cytopatic effect is examined every day. When the effect reached 75-80%, the virus was harvested, by scratching the wall of the bottle, centrifuged with 1500x g and filtered with Millipore 0.45 µl. The supernatant was stored as virus stock for further treatment.

Animal experiment

Animal used for the trials was BALB/c mouse, which gave good antibody response when injected with measles virus, and it was inbreed hence genetically identical. Thus the variances within and between groups can be reduced.[6] Each group consisted of 22 mice aged 8 months weighted around 25-28 grams mixed between male and female.

The trials consisted of 6 groups: group I was immunized with placebo as control, group II with G2 genotype, group III with G3 genotype, group IV with D9, group V with CAM-70 vaccine and group VI with Schwarz (MMR) vaccine (**Table 2**).

Blood (100 µl) was taken one week prior to immunization to make sure that the mice had not been infected with measles by using neutralization test, and another 1 ml of blood was taken two weeks after the third immunization for laboratory analysis. The laboratory analysis performed was antibody examination by ELISA method and cross neutralization test to find out the differences in antigenic property of each immunogenic protein.

Sample size

The animal trials consisted of six groups as mentioned above. Because a group would be tested with 4 different types of antigens, each group could be considered as two paired groups. Based on references, the mean and expected variance from the discrepancy of the results of neutralization tests reacted with wild type viruses were different with vaccine viruses. Hence, the calculation for the variance from the neutralization tests of wild type virus was splitted from the vaccine virus. This data was one of the important components in determining the sample size.

The sample size determination for each group was as follows:[12]

A. Animal group immunized with vaccine virus

The formula used to determine the animal sample size for neutralization test for each group receiving vaccine immunization is described below. The laboratory tests performed on the three groups is the same as on of the other groups. The data used to calculate the sample size in this group is counted with the equation:

$$N = \frac{(Z_\alpha + Z_\beta)^2 S_d^2}{d^2}$$

n = sample size
Z_α = normal standard deviation for α = 0.0025 (z_α two-side = 2.813)
Z_β = normal standard deviation for k β = 0.005 (Z_β=2.57)
S_d = predicted st andard deviation from average = 277.61
d = significant average difference between 2 group = 326

then n = 22.

B. Animal group immunized with wild type virus

The formula used is the same as above, while the data for calculation are as follow:

N = sample size
Z = 2.575 (normal standard derivative for α= 0.01)
Z_β = 1.960 (normal standard derivative for β= 0.025).
Sd = 277.61
d = 326
therefore N = 18.

To avoid the mismatched samples at the end of the experiments, samples were added to all groups of the 22th mice (20% addition).

Control Group

One of the groups was immunized with vaccine diluent or media solution as the control group. The ELISA test and cross neutralization test would then be performed on the serum of the group.

The categorization and selection of trial animals

The selection of trial animals was carried out by proportional random sampling with the same population size of male and female for each group.[13]

Table 2. Group of trial animals

Group	Size	Weeks								
		-I	I	II	III	IV	V	VI	VII	VIII IX
Placebo	22	♠	♥				♣		♣	♦
G2 genotype	22	♠	♥				♣		♣	♣
G3 genotype	22	♠	♥				♣		♣	♦
D9 genotype	22	♠	♥				♣		♣	♣
CAM-70 vaccine	22	♠	♥				♣		♣	♦
Schwarz vaccine	22	♠	♥				♣		♣	♣

Note :

♠ : Blood is taken for data base
♥ : Antigen injection
♣ : Booster
♦ : Blood is taken from the heart after anesthetized

The animal used is mice from BALB/c strain

Antigen for injection

As had been previously described, antigen injected for group A was media solution, group B was G2 genotype of wild type virus, group C was G3 genotype, group D was D9 genotype, group E was CAM-70 vaccine virus from Bio Farma with batch number 250463, and group F was Schwarz MMR TRIMOVAX vaccine from Aventis with batch number X6104-1.

Dosage of antigen

All groups were given the same titer of virus as follows: first injection was given 10^3 TCID50, while the first and second booster were $5x10^2$ TCID50. The virus was injected to the mouse intrapertoneally.[6]

Injection

Antigen was injected by blind method where the staff who was going to inject did not know the type of the antigen as all antigens were coded by number. In addition, the laboratory staff did not know the antigen of the obtained serum.

Blood Sampling

Blood was taken from the tails of the mice before immunization, while after immunization blood was taken from choroid plexus, continued from intracardiac by opening the mouse chest. Before the second blood sampling, the mice was anesthetized with ether.[14]

Micro-cross-neutralization test

The cross-neutralization test of the mice serum after immunization of G2, G3, D9, CAM-70 and Schwarz reacted with G2, G3, D9 and CAM-70 using method described by WHO,[15] Lennete and Schmid.[16] The principle of the test was that reaction between measles virus with specific antigen could be observed by searching for the formation of cytopatic effect on the cell (cultured cell).

The strains of measles virus used were G2, G3, D9 genotypes and CAM-70 vaccine viruses. The concentration was 100 TCID50. The titer of the virus was determined first before using the virus in the examination. The average of the titer was around $5x10^{3.5}$ to $10^{4.5}$/ml.

The cells used for cross neutralization test was continues cells line. The vero cell (ATCC CRL 1612) originated from green monkey kidney cell which had been adapted in the culture for more than a thousand time in vitro in the laboratory for CAM-70 test. The B95-8 (ATCC CCL81) was originated from limfoblast cell and used for neutralization test against wild type virus.

Antigen protein isolation

Virus was cutured with 1 liter solution, then centrifuge with speed of 1500 x g for 15 minutes. The supernatant were filtered with 0.45 nm paper filter. The filtered solution was centrifuged with 26000 x g for 3 hours at 4^{o}C to get the viral particle pellets. The pellets was suspended with Tris NaCl EDTA (TNE), and was purified further with column chromatography using Sepharose CL-4B beads, a cross-linked agarose with certain pore sizes. The eluent from the column chromatography was suspended in Tris NaCl EDTA (TNE) buffer, then recentrifuged with 6700X g for 90 minutes. The formed pellet was re-suspended in 100-300 µl TNE buffer and stored in 4^{o}C for overnight. At this step, the viral suspension or virion was ready to use for next experiment.[17,18] The antigen protein content was measured using BCA Protein Assay Kit with Bradford method. This method is quite sensitive, quick and stable. The standard solution used bovine serum albumin (BSA) with various concentration, and the standard curve was plotted on 560 nm wavelength. The antigen protein content could be calculated by measuring absorbance and comparing it to the standard curve.

Examination of IgG against measles virus antigen with ELISA

The examination of IgG against measles virus antigen was performed using non-direct ELISA method. We used antigen from G2, G3, D9 and CAM-70 genotypes. Antigen (100 µl or 1 µg/ml) was put into the microplate wells and incubated for 4 hours at 37^0 C. Blocking buffer was increased up to 300 µl, and then incubated for 2 hours in room temperature, and rewashed again. Serum containing antibody with a dilution of 1:1000 was added, and then incubated for 1.5 hours at room temperature, after that the remnant specimen was rinsed. Anti-immunoglobin anti-mouse labeled with peroxide enzyme 1:1000 was added to microplate wells, incubated for 1 hour in room temperatures and the left-over was flushed away. OPD (openyl diamine dihydrochloride) 100 µl/well was then added and then kept in dark room for 10 minutes so hydrolysis could occur on Ag-Ab-anti-IgE complex. The reaction was stopped with 50 µl 2N H_2SO_4. The amount of hydrolyzed substrate, which was proportional with to the amount of the enzyme on the complex, was measured on microplate reader. This result was used as the antibody titer parameter of the specimen.

Data analysis

Because each group of experimental subject was tested with 4 type of antigen (for ELISA) and 4 type of virus (cross-neutralization test), hence each group was considered as 4 matched pair groups. Analysis of the significant of numerical ELISA results was tested with ANOVA. When ANOVA tests showed differences, the analysis was then proceed to the double Least Significant Difference (LSD) tests.

When the data was not both normal nor homogen, the non-parametric Kruskal-Wallis test was used. The numerical data of the cross-neutralization test was transformed to Ln before being used to calculate geometric mean (GMT) and standard deviation (SD). The transformed data was analyzed by non-parametric Friedman test (connected group). When the significant differences was obtained from the test ($P<0.05$), the analysis was continued using Wilcoxon Signed Ranks Test to obtain the differences of each group.

Results

Neutralization test of mouse serum before immunization

To confirm that the mice were not infected by any measles virus before immunization, antibody titer tests against wild type virus were performed. The tests were carried out by using cross-neutralization test by reacting mouse serum with G2, G3, D9 and CAM-70 genotypes. The result of neutralization tests showed that all mice serum were unable to neutralize viral infection. All wells on microplate showed cytopatic effect. Hence, it can be concluded that none of the mice were exposed to measles virus.

ELISA tests for antibody titer against measles virus

The results of cross ELISA from mice immunized with G2 showed the highest average score obtained by the group reacted with G2 antigen with OD 0.9632, followed by G3 with OD 0.9526, D9 with OD 0.8742 and CAM-70 with 0.6832. The differences between G2 and CAM-70, G3 and CAM-70, D9 and CAM-70 were statistically significant ($P< 0.005$), while between G2 and G3, G2 and D9, G2 and G3 were statistically insignificant ($P>0.05$).

Results from mice immunized with G3 showed the highest OD obtained by the group reacted with G3 (0.9297) followed by G2 (0.8967), D9 (0.8544) and CAM-70 (0.8112). These results were statistically insignificant ($P>0.05$).

Results from mice immunized with D9 showed the highest OD obtained by the group reacted with D9 (1.3514) followed by G2 (1.0545), D9 (0.9015) and CAM-70 (0.8501). The differences between G3

Table 3. Results of cross neutralization test before immunization

Tested Serum		Genotypes (unit)			
		G2	G3	D9	CAM-70
G2	(GMT)	4.000	4.000	4.000	4.000
G3	(GMT)	4.000	4.000	4.000	4.000
D9	(GMT)	4.000	4.000	4.000	4.000
CAM-70	(GMT)	4.000	4.000	4.000	4.000
MMR	(GMT)	4.000	4.000	4.000	4.000
Plasebo	(GMT)	4.000	4.000	4.000	4.000

Tabel 4. Results of the ELISA examination of all serum from mice immunized with G2, G3, D9, CAM-70 and Schwarz measles virus

Tested Serum		Against genotype (OD=optical density)			
		G2	G3	D9	CAM-70
G2	Mean	0.9632	0.9526	0.8742	0.6832
	Sd	0.1493	0.2546	0.1821	0.1988
	P<0.05	A	A	A	b
G3	Mean	0.8967	0.9297	0.8544	0.8112
	Sd	0.2726	0.2594	0.2600	0.1417
	p>0.05	C	C	C	c
D9	Mean	1.0545	0.9015	1.3514	0.8501
	Sd	0.2912	0.2484	0.3912	0.2315
	P<0.05	E	Ef	G	f
CAM-70	Mean	0.0315	0.1254	0.1674	0.0484
	Sd	0.0239	0.1674	0.1026	0.0467
	P<0.05	H	J	J	k
MMR	Mean	0.0257	0.0424	0.1662	0.0198
	Sd	0.0625	0.0279	0.3085	0.0293
		M	M	L	m
P<0.05					
Plasebo	Mean	0.0300	0.0377	0.0308	0.0280
	Sd	0.0274	0.0228	0.0240	0.0173
	P>0.05	D	D	D	d

Note: Similar letters did not show statistically significant differences with P > 0.05.

and CAM-70, D9 and CAM-70, G3 and D9 were statistically significant (P<0.05) while between G2 and G3, G3 and CAM-70 were statistically insignificant (P>0.05).

However, results from mice immunized with CAM-70 showed the highest OD obtained by the group reacted with D9 (0.16736) followed by G3 (0.1254), CAM-70 (0.0484) and G2 (0.0315). The differences between G3 and CAM-70, G2 and CAM-70, G2 and D9 were statistically significant (P<0.05) while between G2 and D9 was statistically insignificant (P<0.05).

Similarly, results from mice immunized with Schwarz (MMR) showed the highest OD obtained by group reacted with D9 (0.1662) followed by G3 (0.0424), G2 (0.0257) and CAM-70 (0.0198). The differences between G3 and CAM-70, D9 and CAM-70, G2 and G3, G2 and D9 were statistically significant (P<0.05), while between G3 and D9, G3 and CAM-70, D9 and CAM-70, G2 and CAM-70 were statistically insignificant.

Figure 1 showed the results of ELISA examination on different groups with different antigens. The group immunized with D9 showed the highest results

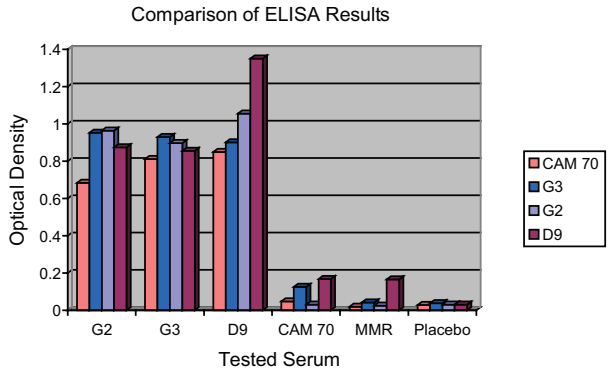

Figure 1. Comparison plot of ELISA results of all serum from immunized mice.

compared with those immunized with other genotypes. Additionally, the D9 immunized group showed highest result when reacted with antigen from D9 genotype.

Cross-neutralization tests

Geometric mean titer (GMT) of the results of neutralization tests of the serum obtained from mice which had been immunized with G2 genotype reacted with G2 virus was 256.00. This value was higher compared with other GMT value which reacted with other genotypes. The highest differences was found against CAM-70 vaccine with GMT of 68.59 followed by D9 with 90.51 and G3 with 132.51. The differences between G2 and CAM-70, G3 and CAM-70, D9 and CAM-70, G2 and G3, G2 and D9, G3 and D9 were statistically significant (P<0.05).

The cross-neutralization tests from serum obtained from mice immunized with G3 resulted with highest GMT (264.59) when reacted with G3 genotype, followed by G2 (210.01), D9 (141.32), and CAM-70 (119.82). The statistical tests between G3 and CAM-70, G2 and CAM-70, G2 and D9, G3 and D9 showed significant differences (P<0.05), while tests between G2 and G3, D9 and CAM-70 showed insignificant differences (P>0.05).

The serum obtained from mice immunized with D9 genotype gave highest GMT value (3183.42) when reacted with D9 genotype, followed by G3 (1922.93), G2 (1865.25) and CAM-70 (724). Tests of results between G3 and CAM-70, G2 and CAM-70, D9 and CAM-70, G3 and D9, G2 and D9 showed statistically significant differences (P<0.05) while

results between G3 and G2 were statistically not significant (P>0.05).

The serum obtained from mice immunized with CAM-70 vaccine virus resulted in lowest GMT value (29.96) when reacted with CAM-70 virus, while reaction with other genotypes gave comparable results (G3=41.67, G2=39.01, D9=40.317). Statistical test of results between G3 and CAM-70 showed significant differences (P<0.05), while results between G2 and G3, G2 and D9, G2 and CAM-70, D9 and CAM-70 showed insignificant differences (P>0.05).

Likewise, the serum from mice immunized with Schwarz vaccine resulted in highest GMT value (23.03) when reacted with G2 genotype, followed by D9 (20.66), G3 (19.92) and CAM-70 (9.96). Differences between G2 and CAM-70, G3 and CAM-70, D9 and CAM-70 were statistically significant (P<0.05), while between G2 and G3, G2 and D9, G3 and D9 were statistically insignificant (P>0.05).

The serum obtained from mice immunized with placebo did not show any indication of neutralization when reacted with all genotypes of measles virus. Cytopatic effect was observed in all wells of the microplate.

Antibody responses from each group of the mice after being immunized three times with the respective genotypes showed highest titter by group injected with D9 genotype, followed by G3, G2, CAM-70 and Schwarz (MMR).

Figure 2 showed the differences of the results of neutralization tests within each group. These differences may indicate the differences in the antigenicity of each antigen against the same antibody which reacted with those antigens. As example, the group immunized with D9 had the highest titter when tested with D9 genotype compared to other genotypes. The plot in figure 2 also showed the differences of the results of neutralization tests between groups immunized with G2, G3, D9 and CAM-70.

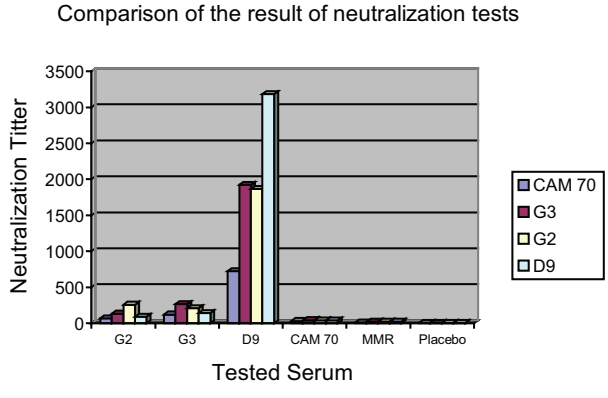

Figure 2. Comparison plot of the results of neutralization tests of all serum from immunized mice.

Discussion

ELISA results

After ELISA analysis was done on group of mouses that have been immunized with G2 genotype, it appeared that antibody to G2 genotype cross reacted with antigen of G3, D9 and CAM-70 vaccine virus. However, the ELISA cross-reaction result from each antigen showed different results. The highest score was from serum which was reacted with G2 virus antigen. While the serum reacted with CAM-70 vaccine virus showed the lowest score. The difference was statistically significant. The same with serum immunized with G3 genotype, it appeared that antibody against G3 genotype cross-reacted with antigen of G2, D9 and CAM-70. However, each reaction gave different result by ELISA. The highest score of ELISA result was found in serum reacted with G3 virus and the lowest was serum reacted with CAM-70. This proved that the antigenic property of antigen G3 genotype was higher than CAM-70 antigen, although the differences were not statistically significant.

The group of mice that was immunized with D9 appeared to cross-react with G2, G3 and CAM-70 antigen. However, there were differences in the ELISA result. The highest ELISA score when reacted with D9 antigen and the lowest when reacted with CAM-70 antigen. Statistically the differences were significant.

The antibody of mouse serum against CAM-70 antigen also cross reacted with antigen from G2, G3 and D9. However, ELISA result showed different scores. The highest score was when reacted with G2 antigen and the lowest was when reacted with CAM-70 and D9. The differences were statistically significant (P<0.05).

The antibody of mouse serum against antigen of Schwarz (MMR) vaccine also cross reacted with antigen from G2, G3, D9 and CAM-70. The highest score was obtained by the antigen of D9 genotype, while the lowest was obtained by cross-reaction with antigen of CAM-70 vaccine virus.

The ELISA result analysis found that reaction with antigen of CAM-70 always showed the lowest ELISA score. This might be because antigen protein of wildtype (G2, G3 and D9) had different antibody epitope from CAM-70 vaccine virus as a result of nucleotide sequence differences of the measles genes. Setiawan *et al*[20,21] found that there were many different amino acid sequences of the B-cell epitope. The epitope differences could affect the result of ELISA between mouse serum immunized by G2, G3, D9, CAM-70 and Schwarz with antigen of G2, G3, D9 and CAM-70. This was in accordance with the result of research done by Tamin *et al*[6] . Hu *et al*[19] also performed experiment against variant of LEC-WI strain of measles virus by using monoclonal antibody in-vitro. He found mutation after knew that the variant was resistant to reacted monoclonal antibody, while there was no mutation found on monoclonal antibody reaction which was not resistant. This means that monoclonal antibody only reacted with epitop peptide that was not undergo mutation and gave different reaction to the same epitope because of the differences in the amino acid sequences.

The result of the analysis of the amino acid sequences between G2, G3 and D9 genotype against CAM-70 in this research also showed different epitop view.[20,21] However, the CAM-70 antigen was still able to cross react with antibodies from wild type virus (G2, G3 and D9) with quite high score.

Dero *et al*[22] found that the result of ELISA test can differentiate two factors between epitope of mutated and non-mutated H 241-255 protein. So, the existence of the ELISA test differences between antigen of wild type measles virus and CAM-70 is likely because of the differences in the amino acid sequences of structural and non-structural proteins on each virus as a result of differences in the nucleotide sequences of the genes. This was proved by the existence of differences in the amino acid sequences of N, H and F proteins at B-cell and CTL epitopes between wild type virus (G2, G3 and D9) and CAM-70 vaccine virus in this research.[20]

The antibody titer in mice immunized with wild type virus was much higher compared to that in mice immunized with vaccine virus (CAM-70 and Schwarz). All mice immunized with wild type virus showed titer value (OD value) higher than 0.10. While mice immunized with CAM-70 only 8 mice (36%) showed OD value higher than 0.10 with highest OD of 0.830. Mice immunized with Schwarz was 7 (35%) that showed OD value higher than 0.10 with highest OD of 0.370. None of mice injected with placebo showed OD value higher than 0.10. The highest value was 0.087. It could be concluded that CAM-70 and Schwarz vaccine were less immunogenic compared with wild type virus.

Cross neutralization test

Neutralization test is the first technique to be used for detecting antibodies against virus. A lot of studies have been performed for years however the neutralization test is still the basic method to measure antivirus antibody and still the best test. The basic principal of this neutralization test is that animal serum infected by virus can neutralize the infecting virus if the serum and the virus were mixed to perform reaction for certain period. In this experiment, the neutralization were performed in the culture of Vero cell for CAM-70 vaccine virus and B95-8 cell for wild type virus (G2, G3 and D9). The neutralization was performed on different cell because CAM-70 was only able to grow and reproduce on Vero cell and was not able to grow on B95-8. Likewise, the wild type virus can only grow and reproduce on B95-8 and could not grow on Vero cell.[23] This was thought because of the different biological properties of wild type and vaccine virus, and thus might affect the result of the neutralization test.

To measure the immunologic response as the result of the differences in glycoprotein of G2, G3, D9 and CAM-70 measles virus, the neutralization test was performed on the serum of 6 groups of mice that had been immunized with placebo, G2, G3, D9, CAM-70 and Schwarz virus.

The result of the cross neutralization showed that the serum from the group immunized with G2 gave the highest result of neutralization test when reacted with antigen from G2 genotype virus, and gave the lowest result when reacted with antigen from CAM-70

virus. This means that G2 virus has different antigenic properties with CAM-70 vaccine virus.

Likewise, the serum from the mice group immunized with G3 genotype virus gave highest result of neutralization test when reacted with G3 antigen, and gave lowest result when reacted with CAM-70 antigen. This means that the antigenic properties of G3 are different compared to CAM-70 vaccine virus.

The serum from the mice group immunized with D9 genotype virus gave highest result of neutralization test when reacted with D9 antigen, and gave lowest result when reacted with CAM-70 antigen. This means that the antigenic properties of D9 are different compared to CAM-70 vaccine virus.

The serum from the mice group immunized with CAM-70 vaccine virus gave highest result of neutralization test when reacted with G3 genotype antigen, and gave lowest result when reacted with CAM-70 antigen. Tamin *et al*[6] also found the low result of the neutralization test from the serum of the children immunized with measles vaccine against vaccine virus compared to the wild type virus. This means that the antigenic properties of wild type measles virus (G2, G3 and D9) are more dominant to the extent of able to give higher result of neutralization test.

The serum from the mice group immunized with Schwarz vaccine virus (MMR) genotype virus gave highest result of neutralization test when reacted with G2 antigen, and gave lowest result when reacted with CAM-70 antigen. This means that the antigenic properties of wild type virus (G2, G3 and D9) are different compared to CAM-70 vaccine virus.

From the neutralization test, it can be concluded that the serum from mice immunized with wild type virus will always give higher result when reacted with wild type virus especially the homolog ones, compared to the serum from the mice immunized with CAM-70 vaccine virus. The differences in amino acid sequences at the epitope owing to the difference in the nucleotide sequences of H & F genes. The conclusion was consistent with that of by Tamin *et al*.[6]

This research found the differences in the amino acid sequences of F and H proteins between CAM-70 vaccine virus and wild type virus (G2, G3 and D9) distributing in Indonesia. The difference of the F protein between CAM-70 and the wild type was around 29-31 amino acids, while the difference of the H protein is around 24-29 amino acids. These differences are quite high that it was predicted, that the differences could lead to changes in the structure of both proteins. Beside that, the amino acid differences were also found at the B-cell epitopes that were important in neutralizing virus.[20,21]

Birrer *et al*[24] found differences in the epitope of H proteins between measles Edmonston-wt vaccine virus and wild type virus, by performing neutralization test using monoclonal antibody. Truong *et al*[25] also found the differences in the result of neutralization test between A, B3, D2 and D4 genotypes using monoclonal antibodies. However, Zhou *et al*[26] did not find any significant differences of the neutralization test between C1, D3, D5 and H1 against serum with high antibody titer. However, several D3 and H 1 virus could not be neutralized by antibodies with low titer. Kumada *et al*[27] did not find any significant differences either from the neutralization test of rabbit serum immunized with AIK-c vaccine virus against A, D3 and C1 genotype.

Xu *et al*[28] reported that changes of amino acids in H protein of wild type measles virus in China did not show any decreases of cross neutralization test between wild type strain and measles vaccine against antibodies after vaccination, and antibody of human serum after immunization could neutralize all wild type virus.

Sheshberadaran and Norrby[29] found that eventhough differences existed on several epitop of H protein, only small effect observed from the hemaglutination inhibition (HI) against anti-measles serum from human. It was concluded that changes in the epitope of H protein was not a major problem in the epidemiology especially the HI test.

Measles vaccine virus currently distributed was predicted to be effective across the globe eventhough genetic differences existed from the wild type virus.[30] However, we have to prepare when worst situation occurs where many mutations happen and causing the immune responses against vaccine unable to neutralize wild type measles virus. If wild type virus with hypermutation is found, then current vaccines may not be effective, hence vaccines have to be produced from the isolate which might take time and it is not possible to make new vaccine in short time. The main target of neutralization antibody and protective antibody is H protein.[31] Thus, if hypermutation happens in the important protective epitope of wild

type virus, it can reduce the effication of vaccine used for immunization. Beside that, existence of antigenic property changes and the decrease of body immune state can explain the increase of number of measles cases in several populated areas with high immunization coverage.[26]

Based on our findings, the antigenicity of measles virus currently distributing in Indonesia (G2, G3 and D9) is slightly different with that of CAM-70 vaccine virus. Besides, the CAM-70 vaccine virus is still able to give high result of neutralization test when reacted with antibody serum against wild type measles (G2, G3 and D9). Likewise, the antibody serum against vaccine virus can neutralize wild type virus. This means that despite the differences in the antigenic properties between wild type measles and CAM-70 vaccine virus, the antibody against CAM-70 can still neutralize the wild type measles virus infection.

The following conclusions could be made from our data:

1. CAM-70 and MMR antibodies were less reactive against antigen of G2, G3 and D9 genotypes. Antibodies from G2, G3 and D9 were also less reactive against antigen of CAM-70. This means that CAM-70 vaccine has lower antigenic properties than that of wild type virus (G2, G3 and D9 genotype).
2. CAM-70 and Schwarz vaccine virus were less immunogenic compared with wild type virus (G2, G3 and D9). This was proved by the result of the examination of the serum from mice immunized by CAM-70 and MMR vaccine virus, with ELISA and cross neutralization test using antigen from CAM-70, G2, G3 and D9, always showed much lower titer compared to the serum from mice immunized with wild type virus.
3. Although CAM-70 showed clear differences with G2, G3 and D9 genotypes, the antibody against CAM-70 vaccine virus is sill able to cross react with antigen from other genotype (G2, G3 and D9).

Because CAM-70 and Schwarz vaccine virus are less immunogenic, it is suggested to do clinical test in measle immunization twice in children on seperate time and be compared with the one measle immunized children based on clinical and serological epidemiology.

Acknowledgments

We would like to thank Dr. I Nyoman Kandum MPH, the Director of Communicable Disease Control and Environmental Helath Control who funded this research. We also would like to thank Mr. Harun, Joko, and Bambang of Litbangkes, Diah Iskandriati, Joko Pamungkas, Uus Saefullah, Silmi of PSSP IPB Bogor who helped us in finishing this study.

References

1. Griffin DE, Bellini WJ. Measles virus. In: Fields virology. 3rd ed. Philadelpia-New York: Lippincott-Raven; 1996. p. 1267-1312.
2. World Health Organization. Expanded program on Immunization. Standardization of the nomenclature for describing the genetic characterization of wild-type measles viruses. Weekly Epidemiological report 2001;76:241-8.
3. Litbangkes DepKes RI : Laporan hasil genotipe virus campak yang dikirim oleh WHO. 2002.
4. Setiawan M. Genotipe virus campak dan manfaatnya. 2007 (Submitted)
5. Bellini WJ, Rota JS, Rota PA. Virology of measles virus. J Infect Dis 1994;170:S15-23.
6. Tamin A, Rota PA, Wang Z, Heath JL, Anderson, LJ, Bellini WJ. Antigenic analysis of current wild type and vaccine strains of measles virus. J Infect Dis 1994;170:795-801.
7. Atkinson WL, Orenstein WA. The resurgence of measles in the United State, 1989-1990. Annu Rev Med 1992;43:451-61.
8. Litbangkes DepKes RI: Laporan hasil pengamatan KLB di beberapa wilayah di Indonesia. 1998
9. Gellin BG, Katz SL. Measles: state of the art and future directions. J Infect Dis 1994;170:S3-14.
10. Rota JS, Hummel KB, Rota PA, Bellini WJ. Genetic variability of the glycoprotein gene of current wild-type measles isolates. Virology 1992;188:135-42.
11. Rota J, Rota P. Bellini W. Isolation and identification of measles virus in cell culture: Manual revised July 24, 2002 for WHO/SEARO Inter-Country Training Course, Bangkok, August 2002.
12. Madiyono B, Moeslichan Mz, Sastroasmoro S, Budiman I, Purwanto H. Perkiraan besar sampel. In: Sastroasmoro S, Ismael S, editors. Dasar-dasar metodologi penelitian klinis. Jakarta: Binarupa Aksara; 1995. p. 187-212 .
13. Pratiknya, A.W. Dasar-dasar metodologi penelitian kedokteran dan kesehatan. 1st Ed. Jakarta: Penerbit CV

Rajawali; 1986.
14. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. Current Protocols in Immunology. Vol. I. Philadelphia: Current Protocols Wiley; 1996.
15. World Health Organization. Manual for the laboratory diagnosis of measles virus infection. GENEVA : Departemen of vaccines and biologicals;1999.
16. Lannete EH, Schmid NJ. Diagnostic procedures for viral and rickettsial infections; 4th ed. New York: AVO Broadway; 1969. p. 116-8.
17. Pamungkas J, Iskandriati D, Sajuthi D, Grant RF. Pembuatan kit serologis enzyme immunoassay dan Westernblot untuk pendeteksian anti SRV (Simian Retrovirus). Pusat Studi Satwa Primata. Bogor: Lembaga Penelitian-Institut Pertanian Bogor; 2003.
18. Iskandriati D. Identifikasi dan karakterisasi simian retrovirus tipe-D pada macaca fascicukaris, M.nemestrina dan presbitis SPP [Dissertation]. Bogor: Institut Pertanian Bogor; 2004.
19. Hu A, Sheshberandaran H, Norbby E, Kovamees J. Moleculer characterization of epitope on the measles virus hemagglutinin protein. Virology 1993;192:351-4.
20. Setiawan M, Syahrurochman A, Ibrahin F, Swandono A. Perbedaan sikuens gen protein H antara virus campak liar dan virus vaksin campak di Indonesia. 2005 (Submitted).
21. Setiawan M, Syahrurochman A, Ibrahin F, Swandono A. Perbedaan sikuens gen protein F antara virus campak liar dan virus vaksin campak di Indonesia. 2005 (Submitted).
22. S Deroo, KC El Kasmi, P Fournier, D Theisen, NH Brons, M Herrmann, *et al*. Enhanced antigenicity of a four-contact-residue epitope of the measles virus hemagglutinin protein by phage display libraries: evidence of helical structure in the putative active site. Molecular Imunnol 1998;35:435-43.
23. Rota PA, Liffick S, Rosenthal S, Heryanto B. Measles genotype in Indonesia and Malaysia. Lancet 2000;355:1557-8.
24. Birrer MJ, Udem S, Nathanson S, Bloom BR. Antegenic variants of measles virus. Nature 1981;293:67-9.
25. Truonga AH, Kreisa S, Ammerlaana W, Harttera HK, Adu F, Omilabuc SA, *et al.* Corresponding Author Contact Information. Genotype and antigenic characterization of hemaglutinin proteins of African measles virus isolates. Virus Res 1999;62:89-95.
26. Zhou J, Fujino M, Inou Y. Genotype of measles virus was detected in outbreacksin Japan after 2000. J Med Virol 2003;70:642-8.
27. Kumada A, Komase K, Nakayama T. Recobinant measles AIK-C strain expressing current wild-type hemagglutinin protein. Vaccine 2004;22:309-16.
28. Xu W, Tamin A, Rota JS, Zhang L, Bellini WJ, Rota PA. New genetic group of measles virus isolated in the People`s Republic of China. Virus Res 1998;54:147-56.
29. Sheshberadaran H, Norrby E. Characterization of epitopes on the measles virus hemaglutinin. Virology 1986;152:58-65.
30. Li Jina, Knowlesa WA, Rotab PA, Bellinib WJ, Browna DWG. Genetic and antigenic characterization of the hemagglutinin protein of measles virus strains recently circulating in the UK. Virus Res 1998;55:1071-83.
31. Fournier P, Brons NH, Berbers GA, Wiesmüller KH, Fleckenstein BT, Schneider F, *et al.* Antibody to a new linear site at the topographycal or fungtional interface between the hemagglutinin and fusion protein protect against encephalitis. J Gen Virol 1997;78: 1295-1302.