Paediatrica Indonesiana

VOLUME 48 January • 2008 NUMBER 1

Original Article

Difference of hemaglutinins between wild-type and vaccine measles virus in Indonesia

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

Abstract

Background Hemaglutinin (H) protein of measles virus is very important in the process of host cell infection. H protein is also able to induce specific antibodies which can neutralize measles virus and block the cell infection.

Objective This study aimed to explore the nucleotide and amino acid sequence differences between wild-type measles virus (G2, G3 and D9) with CAM-70, Schwarz and Edmonston-wt vaccine virus.

Methods The exctration and amplification of the gene were conducted in the laboratory using biomolecular technology. The gene and protein analysis were conducted using the bioinformatic technology.

Results The results showed that the differences in nucleotide sequences were highest between wild-type virus and CAM-70 vaccine virus (76-77 nucleotides), followed by Schwarz (61-64 nucleotides) and Edmonston (60-63 nucleotides). The differences in amino acid sequences were highest between wild-type virus and CAM-70 (24-29 residues), followed by Schwarz (13-20 residues) and Edmonston (12-19 residues).

Conclusion The Indonesian wild-type measles virus was genetically closer to Schwarz vaccine virus than CAM-70 vaccine virus, hence the neutralizing antibodies generated by Schwarz vaccine were more specific against Indonesian wild-type virus compared to CAM-70 vaccine. **[Paediatr Indones 2008;48:42-48]**.

Keywords: *measles, wilde-type, vaccine-type, hemagglutinin gene, hemagglutinin protein*

Acute infection caused by measles virus begins at upper respiratory tract followed by secondary viremia until the virus reaches other organs and tissues which leads to clinical symptoms. Measles virus can infect immune system cells such as lymphocytes and macrophages and therefore suppress temporarily the immune system.[1,2]

During the infection process, both transmembrane protein F and H need to cooperate together. F protein is responsible fusing the viral envelope with host cell membrane and viral penetration, while H protein is responsible for viral docking and interaction with receptors which reside in the surface of the host cell. Both F and H glycoproteins function is to fuse the virus with host cell membrane and facilitate the virus penetration into the cell.[4,5]

The immune system infected by the measles virus will respond to H proteins. Antibodies against H protein

From The Department of Child Health, Infectious Diseases Hospital Prof. Sulianti Saroso, Jakarta, Indonesia.[1] From The Department Microbiology, Medical School, University of Indonesia, Jakarta, Indonesia.[2] From The National Institute Health Research and Development, Indonesia.[3]

Reprint requests to: Made Setiawan, MD, Department of Child Health, Infectious Diseases Hospital Prof. Sulianti Saroso, Jakarta, Indonesia.

can be measured with infectivity neutralizing test in the tissue culture. The neutralizing antibodies plays an important role in preventing the disease.[6] The cellular immune response also responds to H proteins.[7] The variability of nucleotide sequence of H gene results in the various clades and genotypes of measles virus.[8] This variability leads to several structures of H protein from each clade or genotype which has different epitopes and will affect the specific immune response of the infected host cell.[9]

Currently in Indonesia, there are three known wild-type measles virus genotypes, i.e., G2, G3 and D9. The types of vaccine virus mostly being used are CAM-70 and Schwarz **(Table 1)**. The source of original CAM-70 vaccine virus was Tanabe strain from Japan, while the source of Schwarz vaccine was Edmonston strain isolated in 1954. It has been shown that nucleotide sequences of both vaccine virus are different.[10] The current wild-type measles virus has undergone significant genetic drift compared to the vaccine virus and isolates from 1950 and 1960.[11,12]

We aimed to explore the differences of nucleotide and amino acid sequence of H protein between wild-type measles virus and vaccine virus in Indonesia.

Methods

To analyze gene and protein of wild-type measles virus and measles vaccine virus, we used biology molecular and bioinformatics technology. Wild-type measles virus isolates were provided by Litbangkes RI. The genotypes of each isolates, which are G2, G3 and D9, have been determined by ICDC (Atlanta-USA). The genotypes of each isolates are also reexamined in Indonesia with same results.[13] The sequences of N gene for Schwarz and Edmonston-wt vaccine virus were downloaded from Genbank.

Viral RNA extraction was performed using QIAmp Viral RNA Mini Kit from QIAGEN (cat. no. 5204) according to the instruction from the manufacturer. The specimens used were culture products. Cells infected by the virus were taken from the bottle and were centrifuged by 1500X G at 4 degree C for 15 minutes. The supernatant was discarded, while the pellet was taken for further RNA extraction. RNA was extracted by means of pipetting 560 uL AVL buffer containing RNA Carrier into 1.5 ml Eppendorf tube. About 140 µL virus sample was added and was vortexed for 10-15 seconds, incubated for minimum of 10 minutes, and then centrifuged at 6000X g for 30 seconds. Five hundred and sixty µl absolute alcohol was added followed by further vortex and centrifugation. As much as 630 µL of the solution was transferred to the second spin column. Centrifugation was performed at 6000X for 1 minute. The collection tube containing the filtrate was disposed away and replaced by the new one. Five hundreds UI AW1 wash buffer was added, followed by centrifugation at the same speed for one minute. Collecting tube was then replaced with the new one with additional 500 Ul AW2 wash buffer added to the tube, and then another centrifugation at 20,000Xg was performed for 3 minutes. Collecting tube was replaced with the clean one, and then further centrifugation was performed at 20,000X g for one minute (to clean up the spin column for the buffer and alcohol). Collecting tube was replaced by 1.5 mL Eppendorf tube. AVE buffer was added as much as 60 µL (right in the middle of the filter). Incubation was performed at room temperature for one minute. Centrifugation was then performed at 6000X g for one minute. The RNA from elution was kept at temperature -20^{o} C or -70^{o} C.

Table 1. Wild-type isolates of measles virus

Code	Address	IgM	Age (yr)	Sex	Geno-type
MVi/INA/06.02/161Yo	Subang-Jabar	+	4.5	L	D9
MVi/INA/05.02/Ba	Gresik-Jatim	+	4	P	G3
Mvi/INA/03.04/362 Sep	Pekalongan	+	3.8	P	G2
Virus vaksin CAM-70	Bio Farma				
Virus Vaksin Schwarz*	NCBI Genbank				
Virus Edmonston-wt*	NCBI Genbank				

* Sequence data downloaded from NCBI Genbank

Table 2. Primers used for PCR and sequencing

No.	Primer Name		Nucleotide sequence	Position
1	Primer N	1	Cag ggA CAA gAg CAg gAT TA	75-95
		2	gTT CCT CAC CAC ATC CAA CC	751-770
2	Primer N	3	gAA ggT ggA TAA AgT ACA CCC AAC	688-711
		4	CTT TgA TCA CCg TgT AgA AAT gAT	1358-1382
3	Primer N	5	CAC ATT ggC ATC TgA ACT Cg	1241-1260
		6	CAA TgA Tgg Agg gTA ggC Ag	1714-1734

RNA was amplified by PCR machine using primers designed from the analysis of reference sequence from Edmonston-wt substrain AIK-C with accession number AB046218.[14] The program used to design the primer is Primer-3 **(Table 2 and Figure 1).**

Reverse transcription reaction was performed by SuperScript III Reverse Transcriptase (Invitrogen) used the protocol supplied by the manufacturer. The mixture of reverse transcription reaction was made by adding 1.5 µL random hexamer, 10 mM 1 µL dNTPs, 0.5 µL dH_20 and 10 µL RNA. It was heated at 65°C for five minutes, and then incubated in ice for one minute. Mixture was centrifuged for a moment, and then was mixed with 4 µL 5X first-Strand buffer, 1 uL 0.1 M DTT, 1 uL RNAsin, 1 uL Superscript III RT. The mixture was stirred carefully with a pipette, and then was incubated at 25°C for 5 minutes, at 50°C for 30-60 minutes, and the reaction was stopped by heating at 70°C for 15 minutes. The obtained cDNA was ready for used as template for PCR amplification.

The cDNA from reverse transcription reaction was amplified with PCR method using Platinum Taq DNA Polymerase (Invitrogen). The primers used are showed in **Table 2** and **Figure 1**. The reaction mixture was made by adding 10 uL cDNA, 1 uL 20 uM primer1, 1 uL 20 uM primer2, 1.5 uL MgCl2, 5 uL 10 mM dNTPs, 5 uL 10X PCR buffer and water/aquadest

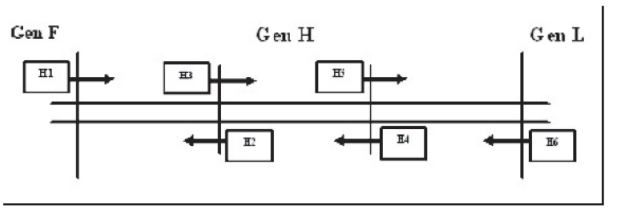

Figure 1. Strategy for overlaping sequencing of H gene

was added to fill up to 50 uL, 1 uL Platinum Tag, and was stirred carefully and was added with 1 drop of mineral oil. The reaction mixture was heated at 94°C for 2 minutes for denaturation process. The PCR reaction was performed for 40 cycles, consisted of denaturation for 30 second at 94°C, annealing for 60 seconds at 56°C, and extension for 120 seconds at 72°C, and ended with incubation at 72°C for 7 minute.

PCR product was analyzed using gel electrophoresis which detection was based on molecular weight or length of nucleotide chain. The H gene has DNA nucleotide with length of 1853 bp. To detect the PCR product, the conditions were 2% agarose gel mixed with 10 uL (1ug/mL) ethidium bromide, with 100 Volt voltages for 40 minutes (Coligan *et al*[15]).

DNA product was purified using QIAquick Gel Extraction Purification (QIAGEN catalog no. 28704), where the PCR product was run in the low melting agarose first before purification. The protocol used in the experiment was based on the instruction from the manufacturer.

The purified PCR product was cloned using TOPO TA Cloning kit from Invitrogen (catalog no. KA4500-01) according to the protocol supplied by the manufacturer. Plasmid extraction was performed using QIAprep Spin Miniprep (QIAGEN catalog no. 27104) according to the protocol from the manufac-turer.

The PCR product was sequenced by direct sequencing using Perkin Elmer ABI 337 Automatic DNA Sequencer according to Sanger method (dideoxy termination method). The primers used for sequencing were similar with those for PCR amplification with ABI PRISM Big Dye Terminator Cycle Ready Reaction Kit for the PCR sequencing reaction.

The raw data generated by the computer reading were reanalyzed manually by examining the electrophoresis trace and comparing with the available

sequence data (Edmonston-wt, CAM-70, and Schwarz). The examined and edited fragments were assembled to create the whole nucleotide sequence with the help of Genetyx and Bioedit software.

The completed gene sequences were compared with one another and with the sequence data obtained from NCBI Genbank. All comparison was performed using Genetyx, ClustalW and Bioedit, which can be downloaded from http://www.mbio.nesu.edu/Bioedit/bioedit.html.

The overall mean distance was performed with Mega 2.1 using Tamura-Nei method, while the amino acid overall distance was calculated using Poisson correction method.

Results

Nucleotide differences in H gene

Alignments of nucleotide sequences of H gene between G2, G3 and D9 against Edmonston-wt showed that G2 genotype and Edmonston-wt had 60 nucleotide differences (3.2%) leaded to 12 residues of amino acid substitutions. Alignment between G3 genotype and Edmonston-wt showed 62 nucleotide differences (3.3%) leaded to 15 residues of amino acid substitutions. Alignment between D9 genotype and Edmonston-wt showed 63 nucleotide differences (3.4%) leaded to 19 residues of amino acid substitutions **(Table 3)**.

Alignments of nucleotide sequences of H gene between G2, G3 and D9 against Schwarz showed that G2 genotype and Schwarz had 61nucleotide differences (3.3%) leaded to 13 residues of amino acid substitutions. Alignment between G3 genotype and Schwarz showed 63 nucleotide differences (3.4%) leaded to 16 residues of amino acid substitutions. Alignment between D9 genotype and Schwarz showed 64 nucleotide differences (3.5%) leaded to 20 residues of amino acid substitutions **(Table 3)**.

Alignments of nucleotide sequences of H gene between G2, G3 and D9 against CAM-70 showed that G2 genotype and CAM-70 had 77 nucleotide differences (4.2%)leaded to 24 residues of amino acid substitutions. Alignment between G3 genotype and CAM-70 showed 76 nucleotide differences (4.1%) leaded to 27 residues of amino acid substitutions. Alignment between D9 genotype and Schwarz showed 77 nucleotide differences (4.2%)leaded to 29 residues of amino acid substitutions. All three wild-type measles virus (G2, G3, and D9) had relatively same difference sequences with H-gene sequence of CAM-70 vaccine **(Table 3)**.

Those data showed that the greatest nucleotide differences was between G2, G3 and D9 genotypes and CAM-70 vaccine virus, followed by Schwarz and Edmonston-wt vaccine virus **(Table 3)**.

Amino acid differences in H protein

Differences in the amino acid sequences affect strongly the secondary structure of proteins because of the different nature of the amino acids. Generally, amino acids can be grouped into 2 categories, the hydrophobic and hydrophilic amino acids. The hydrophilic amino acids can be grouped further into non-charged and charged amino acids and the charged group can be categorized further into positively and negatively charged. If the differ amino acids belong to the same group, the effect may be minimal. However, if the differ amino acids belong to different groups, the secondary structure may be different. From 46 differ amino acid position of H protein, 29 belong to different groups (63%). These could lead to changes in the structure of H protein between wild-type and CAM-70 vaccine virus **(Table 4)**.

Differences of amino acid of H protein of wild-type and Schwarz virus occurred in 35 positions, with

Table 3. Nuclotide and amino acid different of wild-type and vaccine virus

Genotype of wild-type virus	Nucleotide differences			Amino acid differences		
	Edmons	Schwaz	CAM-70	Edmons	Schwarz	CAM-70
G2	60 (3.2%)	61 (3.3%)	77 (4.2%)	12 (1.9%)	13 (2.1%)	24 (3.9%)
G3	62 (3.3%)	63 (3.4%)	76 (4.1%)	15 (2.4%)	16 (3.6%)	27 (4.4%)
D9	63 (3.4%)	64 (6.5%)	77 (4.2%)	19 (3.1%)	20 (3.2%)	29 (4.7%)

Table 4. Amino acid differences of H proteins between wild-type virus and CAM-70 vaccine virus

No.	G2	G3	D9	Cam-70		No.	G2	G3	D9	Cam-70	
18	P	S	P	P	-	302	G	G	R	G	+
37	V	A	V	V	-	303	G	E	E	E	+
40	A	V	A	A	-	318	R	R	S	S	+
93	T	T	T	I	+	338	P	P	P	S	-
146	V	V	T	I	-	367	A	A	V	V	-
157	V	V	V	A	-	372	T	T	T	A	+
163	M	M	M	K	+	416	D	D	N	D	+
174	T	A	A	T	+	424	K	K	T	K	+
175	R	R	R	K	-	426	K	K	Q	K	+
176	T	T	A	T	+	428	A	A	C	A	+
183	S	A	A	A	+	451	V	V	M	V	-
195	S	S	R	R	+	455	T	T	T	N	-
211	S	S	S	G	+	476	L	L	F	F	-
203	L	L	L	P	+	481	N	N	N	Y	-
220	V	V	T	V	+	489	E	E	E	G	+
235	E	G	E	E	+	495	H	N	H	H	+
238	N	D	N	N	+	505	D	D	D	G	+
243	R	R	G	R	+	575	Q	Q	K	Q	+
252	Y	Y	H	Y	+	592	G	G	G	E	+
276	F	L	F	L	-	603	G	G	G	E	+
282	N	N	H	N	+	608	V	V	A	V	-
284	L	L	F	L	-	609	N	T	T	T	-
296	F	F	F	L	-	616	R	R	S	R	+

Note: (+) → Amino acid difference outside the group
(-) → Amino acid difference inside the group

Table 5. Amino acid differences of H proteins between wild-type virus and Schwarz vaccine virus

No.	G2	G3	D9	Cam-70		No.	G2	G3	D9	Schw.	
18	P	S	P	P	+	284	L	L	F	L	-
37	V	A	V	V	-	296	F	F	F	L	-
40	A	V	A	A	+	302	G	G	R	G	+
61	H	P	H	H	+	303	G	E	E	E	+
117	F	F	F	L	-	318	R	R	S	S	+
146	V	V	I	I	-	367	A	A	V	V	-
174	T	A	A	T	+	416	D	D	N	D	+
176	T	T	A	T	+	424	K	K	T	K	+
182	S	A	A	A	+	426	K	K	Q	K	+
195	S	S	R	R	+	428	A	A	C	A	-
216	S	S	S	G	+	451	V	V	M	V	-
220	V	V	T	V	+	476	L	L	F	F	-
235	E	G	E	E	+	481	N	N	N	Y	-
238	N	D	N	N	+	495	H	N	H	H	+
243	R	R	G	R	+	575	Q	Q	K	Q	+
252	Y	Y	H	Y	+	608	V	V	A	V	-
276	F	L	F	L	-	609	N	T	T	T	-
282	N	N	H	N	+						

Note: (+) → Amino acid difference outside the group
(-) → Amino acid difference inside the group

21 amino acids belong to different group (60%). These could lead to changes in the structure of H protein between wild-type and Schwarz vaccine virus. This analysis showed that Indonesian wild-type measles virus was closely related to Schwarz vaccine virus compared to CAM-70 vaccine virus **(Table 5)**.

Discussion

There were nucleotide sequence differences of the H gene between the wild-type measles virus and the vaccine virus examined in this experiment. The differences were about 60-77 nucleotides which accounted for amino acid

differences of 12-29 residues. The greatest differences were found between wild-type and CAM-70 vaccine virus (76-77 nucleotides which accounted for 24-29 residues). Around 63% of the amino acid differences had different properties, which led to the differences to the biological properties of the H protein.

The greater differences between the wild-type and CAM-70 compared to Schwarz indicated that the Indonesian wild-type virus were genetically closer to Schwarz compared to CAM-70 vaccine virus.

The measles infection can induce both hummoral and cellular immune responses. Although both glycoproteins of measles virus can induce neutralizing antibodies that prevent the disease in vivo, in order to recover, the T-cell response is needed. This can be seen as children suffering from a gamaglobulinemia could recover from measles, while children with T-cell abnormality would have complication when infected by measles virus. Therefore, T-cell has important roles in the recovery and prevention process against complication.[16] There are two known CTL epitopes of H protein.[17] Hu *et al*[18] found several B-cell epitopes on H protein. Obeid *et al*[19] also found the same things. To develop vaccines, both factors had to have special attention to ensure that the immune responses generated by the body are optimum and that the objectives of immunization program are met. Hence, both wild-type and vaccine virus should have the same nucleotide and amino acid sequences in B cell and CTL epitopes.

From the results of analysis of H-gene nucleotide sequences and H-protein amino acid sequences, it could be concluded that differences of the nucleotide sequences of H gene between wild-type and CAM-70 vaccine virus were greater compared to Schwarz and Edmonston-wt. Also the differences of amino acid sequences of H protein between wild-type and CAM-70 vaccine virus were greater compared to Schwarz and Edmonston-wt 60% of those differ amino acids had different properties; leading to changes in the conformation and antigenicity of the protein.

Hence, based on the nucleotide and amino acid sequences, wild-type measles virus (G2, G3 and D9) were genetically closer to Schwarz vaccine virus compared to CAM-70 vaccine virus.

Based on the results, we suggest to conduct a study on the configuration of H protein to observe the relationship between the variability of nucleotide and amino acid sequence with the protein structures. Both serologic and genetic analysis of vaccine virus should be done before deciding a vaccine that would be used nationally, in order to have vaccine that suitable with measles virus currently circulating in Indonesia.

Acknowledgments

The authors would like to thank Dr. I Nyoman Kandun MPH, the director of P2M & PLP who had funded this research. The authors would also like to thank Mr. Harun, Joko, Bambang at Litbangkes, Diah Iskandriati, Joko Pamungkas, Uus Saefullah, Silmi at PSSP IPB Bogor who had helped in finishing this study.

References

1. Nelson JD, Sandusky G, Peck FB. Measles skin test and serologic respons to intradermal measles antigen. JAMA 1966;198:185-6.
2. Fulginiti VA, ArthurJH. Altered reactivity to measles virus. J Pediatr 1969;75:609-16.
3. Parks CL, Lerch RA, Walpita P, Wang HP, Sidhu MS, Udem SA. Comparison of predicted amino acid sequences of measles virus strains in the Edmonston vaccine lineage. J Virol 2001;75:910-20.
4. Tyrell DIJ, Norrby F. Structural polypeptides of measles virus. J Gen Virol 1978;39:219-29.
5. Rima BK. The proteins of morbilli viruses. J Gen Virol 1983;64:1205-19.
6. Griffin DE, Ward BJ, Esolen LM. Pathogesis of measles virus infection: An hypothesis for altered immune responses. J Infec Dis 1994;170: S24-31.
7. Rose JW, Bellini WJ, McFarlin DE, McFarland HF. Human celluler immune response to measles virus polypeptides. J Virol 1984;49:988-91.
8. 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.
9. 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.
10. Bellini WJ, Rota JS, Rota PA. Virology of measles virus. J Infect Dis 1994;170:S15-23.

11. Wong TC, Ayata M, Ueda S, Hirano A. Role of biased hypermutation in evolution of subacute sclerosing panencephlitis virus from progenitor acute measles virus. J Virol 1991;65:2191-9.
12. Taylor MJ, Godfrey E, Baczko K, Ter meulen V, Wild TF, Rima BK. Identification of several different lineages of measles virus. J Gen Virol 1991;72:83-8.
13. Setiawan M. Analisis genetik dan antigenik virus campak liar dan virus vaksin di Indonesia [dissertation]. Jakarta: Fakultas Kedokteran Universitas Indonesia; 2005.
14. Komase K, Suzuki N, Nakayama T, Miki K, Kawanishi R, Fukuda K. Genom sequence of measles virus. NCBI no. accession: AB046218 (2001).
15. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. Current Protocols in Immunology Volume I. National Institute of Health; Published by Current Protocols Wiley, (1996).
16. Aicardi J, Goutieres F, Arseneo-Nunes ML, Lebon P. Acute measles encephalitis In children with immunosuppression. Pediatrics 1977 59:232-9.
17. Beauverger P, Chadwick J, Buckland R, Wild TF. Serotype-specific and canine distemper virus cross-reactive H-2KK-restricted cytotoxic T lymphocyte epitopes in the measles virus nucleoprotein. Virology 1994;203:172-7.
18. Hu A, Sheshberandaran H, Norbby E, Kovamees J. Moleculer characterization of epitope on the measles virus hemagglutinin protein. Virology 1993;192:351-4.
19. Obeid OE, Partidos CD, Steward MW. Analysis of the antigenic profile of measles virus haemagglutinin in mice and humans using overlapping synthetic peptides. Virus Res 1994;32:69-84.