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Genotyping of Rotavirus by Using RT-PCR Methods

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Abstract

There is a great diversity of rotavirus genotypes circulating worldwide, with dominant genotypes changing from year to year. Rotavirus genotyping was performed by using reverse transcription PCR with type-specific-primers. Since rotavirus is a RNA virus that has high mutation rate, there was a possibility of technical difficulty in genotyping due to mutation in the primer binding sites. During Indonesian rotavirus surveillance study 2006-2009, it was reported that 17% of samples subjected for G type and 21% of samples subjected for P type were untypeable. The objective of this study was to identify genotypes of the samples that were untypeable previously using RT-PCR based on the method described by Das *et al.* (1994) and Gentsch *et al.* (1992). There were 30 samples subjected to G type and 61 samples subjected to P type to be re-typed using method described by Gouvea *et al.* (1990) and Simmond *et al.* (2008) for G and P typing, respectively. By using another set of primer, the genotype of all samples was identified. This study highlights the importance of a constant reconsideration of primer sequences employed for the molecular typing of rotaviruses.

Key words: rotavirus, G typing, P typing

Introduction

Rotaviruses cause severe diarrhoea in children under 5 years old in developed and developing countries. Studies published between 1986 and 1999 showed that rotaviruses cause 22% of childhood diarrhoea hospitalization. This proportion increased to 39% in 2004. Approximately 611,000 children under 5 years old die every year from rotaviruses, mostly in developing countries (Parashar, *et al.*, 2006).

Rotaviruses are 70-nm icosahedral viruses that belong to the family *Reoviridae.* Rotaviruses consist of seven different serogroups of A – F. The most common rotaviruses that infect humans belong to group A. The particle of a rotavirus has a capsid that surrounds the double-stranded RNA genome consisting of 11 segments. Rotavirus genome segments encode 6 structural proteins (VP1-4, VP6, VP7) and 6 non-structural proteins (NSP1-6) (Dennehy, 2008).

A rotavirus has a capsid that is composed of three concentric layers of protein. The innermost layer is composed of VP2 protein, the middle layer is composed of VP6 protein and the outer layer is composed of VP7 glycoprotein and VP4 forms spikes that protrude from the surface of outer layer. VP4 and VP7 are essential proteins that elicit immune responses and used as the basis of a binary classification system. Typing based on VP7 protein (a glycoprotein = G type antigen) is known as the G-typing while based on VP4 protein (protease-sensitive protein = P-type antigen) is known as the P-typing (Greenberg and Estes, 2009).

Since rotavirus is a RNA virus that has high mutation rate, there is a great diversity

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of wild-type strains circulating in the world. The dominant strains always changes yearly. This changing is due to accumulation of point mutations that lead to antigenic change or new strain emergence (Kirkwood, 2010). Some hospital-based studies reported that 5 rotavirus genotypes, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[9] have been the most common causes of severe disease in children worldwide (Bishop, 2009).

Performing rotavirus surveillance is important since the strain circulated in the world is changing in every time and every place. Beside providing valuable data of circulating strains that are important for vaccine development, this surveillance can tract emergent type, and changes in strain diversity when vaccines are implemented (Widdowson *et al.*, 2009). To support rotavirus surveillance in the world, WHO provided a guideline for genotyping of rotavirus by using RT-PCR methods with different kind of primers applied in different geographical locations (WHO, 2009).

There were some studies to identify the genotype of rotavirus circulating in Indonesia, such as conducted by Soenarto *et al.* (2009) which based on RT-PCR method as described by Das *et al.* (1994) and Gentsch *et al.* (1992). It was reported that 17% of samples subjected for G typing and 21% of samples subjected for P typing were untypeable. The higher percentage of untypeable samples need a further study. So, the objective of this study is to identify the genotype of the samples that previously untypeable using RT-PCR based on the method described by Das *et al.* (1994) and Gentsch *et al.* (1992).

Materials and Methods

Stool samples

Stool samples were collected from children under five years old who hospitalized in Sardjito General Hospital, Yogyakarta; Kotamadya Yogyakarta General Hospital, Yogyakarta; Mataram General Hospital, Mataram; Sanglah General Hospital, Denpasar; Hasan Sadikin General Hospital, Bandung; Muhammad Husein General Hospital, Palembang, and Ciptomangunkusumo General Hospital, Jakarta during Rotavirus Surveillance in Indonesia 2006-2009. Stools samples were stored at -20^{0}C in Laboratory of Microbiology, Faculty of Medicine Universitas Gadjah Mada.

Rotavirus detection

All stool samples were tested for the presence of group A rotavirus using IDEIA™ Rotavirus (DakoCytomation) kit according to the manufacturer instructions. Rotavirus RNA was extracted from rotavirus positive stool samples and analyzed to determine both the VP7 (G-type) and VP4 (P-type) genotypes using the method described previously (Das *et al.*, 1994; Gentsch *et al.*, 1992). The samples that failed to yield detectable PCR product were classified as untypeable samples and be used in this research.

RNA extraction

Rotavirus RNA extracted from rotavirus positive stool samples using Minikit (Qiagen) according to the manufacture instructions.

RT-PCR genotyping

Untypeable samples were re-typed using method described by Gouvea *et al.* (1990) and Simmond *et al.* (2008) for G and P typing respectively. For G typing, consensus primers VP7F and VP7R were used in a first round RT-PCR (30 cycles) to generate a 881-bp VP7 gene segment. VP7F was then used in a second round PCR (30 cycles) with type specific primers aBT1 (G1), aCT-2 (G2), G3 (G3), aDT4(G4), aAT8(G8) and aFT9 (G9). For P-typing, consensus primer VP4F and VP4R were used in a first-round RT-PCR (30 cycles) to generate an 663-bp fragment of gene 4, VP4F was then used in a second round PCR (30 cycles) with type specific primers 1T-1D (P[8]), 2T-1 (P[4]), 3T-1(P[6]), 4T-1 (P[9]) and 5T-1 (P[10]). Primers correspond to VP7 and VP4 genes for rotavirus genotyping can be seen in Table 1. All PCR products were separated in a 2% agarose gel and visualized

Table 1. Primers correspond to VP7 and VP4 genes for rotavirus genotyping

Primer		Sequence (5′-3′)	Position	PCR product (bp)	References
G-typing first amplification described by Das *et al.* (1994)					
	9con1L	TAG CTC CTT TTA ATG TAT GGT AT	37 - 59	895	Das *et al.* (1994)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914 - 932		Iturriza-Gomara*et al.* (2001)
G-typing second amplification described by Das *et al.* (1994)					
G1	9T-1	TCT TGT CAA AGC AAA TAA TG	176 - 195	158	Das *et al.* (1994)
G2	9T-2	GTT AGA AAT GAT TCT CCA CT	262 - 281	244	Das *et al.* (1994)
G3	9T-3P	GTC CAG TTG CAG TGT AGC	484 - 501	464	Das *et al.* (1994)
G4	9T-4	GGG TCG ATG GAA AAT TCT	423 - 440	403	Das *et al.* (1994)
G9	9T-9B	TAT AAA GTC CAT TGC AC	131 - 147	110	Das *et al.* (1994)
G-typing first amplificationdescribed byGouvea*et al.* (1990)					
	VP7F	ATG TAT GGT ATT GAA TAT ACC AC	51 - 71	881	Iturriza-Gomara*et al.* (2001)
	VP7R	AAC TTG CCA CCA TTT TTT CC	914 - 932		Iturriza-Gomara et al. (2001)
G-typing second amplificationdescribed byGouvea*et al.* (1990)					
G1	aBT1	CAA GTA CTC AAA TCA ATG ATG G	314 - 335	618	Gouvea *et al.* (1990)
G2	aCT2	CAA TGA TAT TAA CAC ATT TTC TGT G	411 - 435	521	Gouvea *et al.* (1990)
G3	G3	ACG AAC TCA ACA CGA GAG G	250 - 259	682	Gouvea *et al.* (1990)
G4	aDT4	CGT TTC TGG TGA GGA GTT G	480 - 498	452	Gouvea *et al.* (1990)
G8	aAT8	GTC ACA CCA TTT GTA AAT TCG	178 - 198	754	Gouvea *et al.* (1990)
G9	aFT9	CTT GAT GTG ACT AYA AAT AC	757 - 776	179	Gouvea *et al.* (1990)
P-typing first amplificationdescribed byGentsch*et al.* (1992)					
	Con-3	TGG CTT CGC TCA TTT ATA GAC A	11 - 32	876	Gentsch *et al.* (1992)
	Con-2	ATT TCG GAC CAT TTA TAA CC	868 - 887		Gentsch *et al.* (1992)
P-typing second amplificationdescribed byGentsch*et al.* (1992)					
P[4]	2T-1	CTA TTG TTA GAG GTT AGA GTC	474 – 494	483	Gentsch *et al.* (1992)
P[6]	3T-1	TGT TGA TTA GTT GGA TTC AA	259 – 278	267	Gentsch *et al.* (1992)
P[8]	1T-1D	TCT ACT TGG ATA ACG TGC	339 – 356	345	Gentsch *et al.* (1992)
P[9]	4T-1	TGA GAC ATG CAA TTG GAC	385 – 402	391	Gentsch *et al.* (1992)
P[10]	5T-1	ATC ATA GTT AGT AGT CGG	575 – 594	583	Gentsch *et al.* (1992)
P-typing first amplificationdescribed bySimmond*et al.* (2008)					
	VP4F	TAT GCT CCA GTN AAT TGG	132 - 149	663	Simmond *et al.* (2008)
	VP4R	ATT GCA TTT CTT TCC ATA ATG	775 - 795		Simmond *et al.* (2008)
P-typing second amplification described bySimmond*et al.* (2008)					
P[4]	2T-1	CTA TTG TTA GAG GTT AGA GTC	474 – 494	362	Gentsch *et al.* (1992)
P[6]	3T-1	TGT TGA TTA GTT GGA TTC AA	259 – 278	146	Gentsch *et al.* (1992)
P[8]	1T-1D	TCT ACT TGG ATA ACG TGC	339 – 356	224	Gentsch *et al.* (1992)
P[9]	4T-1	TGA GAC ATG CAA TTG GAC	385 – 402	270	Gentsch *et al.* (1992)
P[10]	5T-1	ATC ATA GTT AGT AGT CGG	575 – 594	462	Gentsch *et al.* (1992)

with UV light after staining with ethidium bromide.

Results and Discussion

During Rotavirus Surveilance Study 2006-2009, there were 45 samples that has not been type detected for G typing using the method described by Das *et al.* (1994) and 86 samples for P typing using the method described by Gentsch *et al.* (1992). From these samples only 30 samples subjected to G typing and 61 samples subjected to P typing had sufficient volume to be re-typed using method described by Gouvea *et al.* (1990) and Simmond *et al.* (2008) for G and P typing, respectively. Figure 1A and 1B show the representative of RT PCR results of G typing and P typing respectively. Finally, the genotype of all samples could be identified and the result can be seen in Table 2.

Molecular methods for rotavirus genotyping provides increased sensitivity for

Table 2. Distribution of G and P genotype that previously untypeable using the method described by Das *et al.* (1994) and Gentsch *et al.* (1992).

Hospital	Untypeable G genotype (n)	G genotype after re-typing				Untypeable P genotype (n)	P genotype after re-typing			
		G1	G2	G9	G1+G9		P4	P6	P8	P6+P8
Sardjito	2	-	-	2	-	0	-	-	-	-
Muh. Hussein	3	1	-	2	-	2	-	-	2	-
Ciptomangun Kusumo	19	5	-	12	2	3	-	1	2	-
Hasan Sadikin	5	2	1	2	-	8	1	-	6	1
Sanglah	1	-	1	-	-	10	2	-	8	-
Mataram	0	-	-	-	-	9	-	1	8	-
Kodya Yogyakarta	0	-	-	-	-	29	-	-	29	-
Total (n)	30	8	1	19	2	61	3	2	55	1

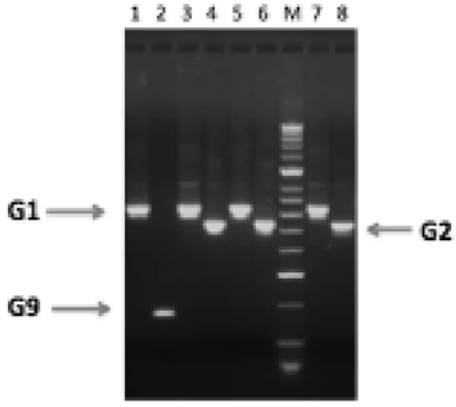

Figure 1A. G-genotyping of rotavirus using RT-PCR. Lane M, 100 bp DNA ladder; lane 1-8, samples from patients. Expected product sizes are 618 bp (G1), 521 bp (G2), 682 bp (G3), 452 bp (G4), 754 bp (G8), and 179 bp (G9).

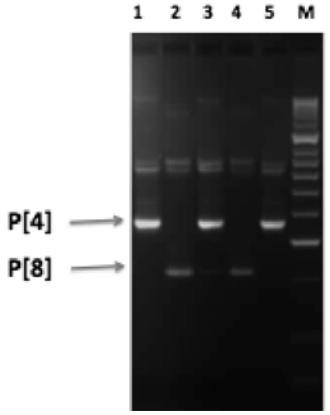

Figure 1B. P-genotyping of rotavirus using RT-PCR. Lane 1-5, samples from patients, lane M, 100 bp DNA ladder. Expected product sizes are 362 bp (P[4]), 146 bp (P[6]), 224 bp (P[8]), 270 bp (P[9]), and 462 bp (P[10]).

G and P genotyping. The methods allows the identification of putative reassortant strains. However, the reagents and methods used for genotyping needed to be closed monitored and up-dated since in nature, rotavirus is able to evolve rapidly by accumulation of point mutations through genetic drift and possibly zoonotic transmission and subsequent reassortment (Simmond *et al.*, 2008).

During rotavirus surveillance study conducted in 2006-2009, there were high proporsion of untypeable strain in every participating hospitals (Table 2). Since rotavirus is a RNA virus that has high mutation rate, there was possibility that this failure is cause by mutation in the primer binding sites. By changing the primers, this study succeeded to identify the genotype. As depicted in table 2, most of samples were G9 (63.3%), followed by G1 (26.7%), G2 (3.3%), and G1+9 (6.7%) respectively. For P typing, the most common genotypes were P[8] (90.1%), P[4] (4.9%), P[6] (3.3%) and P[6+8] (1.6%).

Nucleotide mismatches between the VP7 gene and their primers are associated with genotyping failure (Rahman *et al.*, 2005). When conducted a study in Bangladesh, Rahman *et al.* (2005) found some untypeable strains using the routine primer set described by Das *et al.* (1994). By sequence analysis, they found four nucleotide substitution at the G1 primer binding site which may have caused this genotyping failure. After using another primer set described by Gouvea *et al.* (1990), they could identify these untypeable strains.

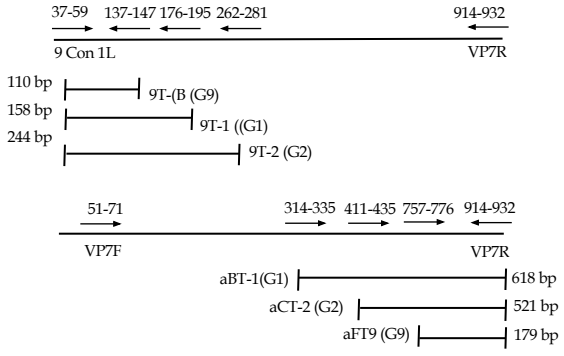

Figure 2. Schematic position of the VP7 primers on Das *et al.* (1994) and Gouvea *et al.* (1990).

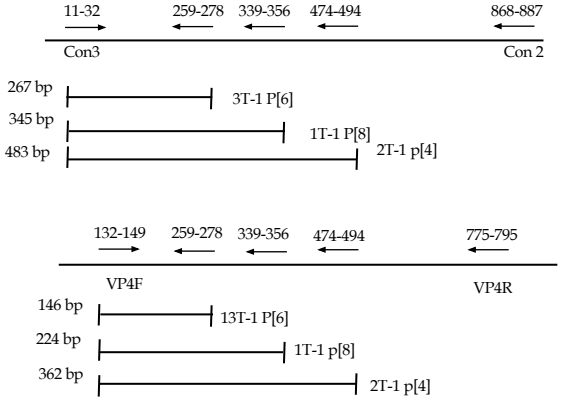

Figure 3. Schematic position of the VP4 primers on Gentsch *et al.* (1992) and Simmond *et al.* (2008)

This result supporting the study conducted by Simmond *et al.* (2008) who designed VP4F/VP4R primers. These primers provides increased sensitivity and allows typing the samples that previously untypeable using available methods. After re-tested using VP4F/VP4R primers, 63.7% of samples that previously untypeable using con3/con2 primers can be typed. Sequence analysis, shows that there are point mutations at the P-type-specific primer binding sites which may caused genotyping failure. The mutation in the primer binding site that cause nucleotide mismatches between the VP4 gene and primer sequence also reported by Adah *et al.* (1997), Iturriza-Gomara *et al.* (2000), and Iturriza-Gomara *et al.* (2004).

Figure 2 and 3 described the location of primer that were used in this study. For G typing, the location of Das's primers are totally different with Gouvea's primers. In contrast, Simmond's primers similar with Gentsch's primers but amplify shorter fragments of VP4 when compare with Gentsch's primer.

In this study, we found that by using VP4F/VP4 primers increased the sensitivity of P genotyping, comparing with con3/con2 primers. There are some reasons why VP4F/VP4R primers has higher sensitivity. Con3/con2 primers were designed by Gentsch from sequences derived from a limited number of cell-culture adapted rotavirus strains available in public databases at the time of the study (Gentsch *et al*., 1992). VP4F/VP4R primers were designed by Simmond based on the sequences derived from over 200 strains of different P types of human rotavirus strains isolated in the last decade. VP4F/VP4R increased efficiency of a PCR reaction since VP4F/VP4R amplifies 663 bp, a shorter fragment of the VP4 encoding gene, compare with con2/con3 that amplifies 876 bp (Simmond *et al*., 2008).

This study highlights the importance of a constant reconsideration of primer sequences employed for the molecular typing of rotaviruses. Since rotavirus is able to evolve rapidly by accumulation of point mutations, close monitoring of rotavirus genotyping methods is important.

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