RESEARCH NOTE

GENETIC CHARACTERIZATION OF GROUP A ROTAVIRUS VP6 GENE IN STRAINS FROM HOSPITALIZED CHILDREN WITH DIARRHEA, JAKARTA, INDONESIA

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Abstract. VP6 is a key protein for determination of rotavirus groups and for development of a vaccine because it can induce an effective immune protection. Thus, a 379-bp DNA fragment of VP6 gene of 15 rotavirus strains collected from stool samples of pediatric patients (<5 years old) with symptoms of acute diarrhea in three hospitals in Jakarta, Indonesia from February to July 2010 were characterized, of which 11 strains were assigned as VP6 genotype I2 with serogroup (SG) I characteristic, while 4 were VP6 genotype I1, SG II. When compared with two vaccine strains and two vaccine candidates, the 11 VP6 genotype I2, SG I strains were closely related to vaccine strain WC3, while the four VP6 genotype I1, SG II strains were closely related to vaccine strain AROLA490AB and vaccine candidates 116E and RV3. These findings of rotavirus VP6 genotypes and serogroups can provide information for appropriate application of rotavirus vaccines containing VP6 protein.

Keywords: rotavirus, genotype, serogroup, vaccine, VP6, Indonesia

INTRODUCTION

Globally, rotavirus accounts for 29-45% of all hospitalized children with diarrhea and causes approximately 611,000 deaths annually (Parashar *et al*, 2006). Rotavirus infection also is responsible for diarrhea in a variety of young mammals and birds (Wakuda *et al*, 2011). Rotavirus is member of the Reoviridae Family and has a genome comprising of 11 double-stranded (ds)RNA segments, which encode six structural proteins (VP1-4, VP6 and VP7) and six nonstructural proteins (NSP1-6) (Estes and Kapikian, 2007). VP6, a trimeric protein, is encoded by rotavirus gene 6 and is required for forming virion particles through its interactions with both the outer capsid proteins VP4 and VP7 and the core protein VP2 (Mathieu *et al*, 2001). When rotavirus enters a cell, VP4 and VP7 are removed to create a double-layer particle with a surface consisting of VP6 (Estes and Kapikian, 2007). The integrity of these double-layer particles plays an important role for viral RNA transcription (Ward and McNeal, 2010).

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Antigenic determinants of VP6 protein have been used to differentiate rotavirus into seven groups (A-G) (Estes and Kapikian, 2007). Identification of the virus groups is very important clinically as Group A rotavirus is the predominant causative agent of severe diseases in infants and young children (Nguyen *et al*, 2004). Reactivity of monoclonal antibodies (MAbs) with one and/or two specific epitopes of VP6 protein is used to differentiate Group A rotaviruses into four sub-groups (SG I, SG II, SG (I+II), and non-SG I and II (Estes and Cohen, 1989; Iturriza-Gomara *et al*, 2002). Based on nucleotide sequence of the whole rotavirus genome, 16 genotypes (I1-I16) have been identified (Matthijnssens *et al*, 2011), of which genotype I2 and I1 is associated with SG I and II, respectively (Kerin *et al*, 2007; Matthijnssens *et al*, 2011).

Two attenuated rotavirus vaccines, Rotarix® (a monovalent rotavirus vaccine) and RotaTeq® (a pentavalent rotavirus vaccine), are licensed worldwide; however, live vaccines have intrinsic limitations (Ward and McNeal, 2010). For example, an attenuated vaccine (Rotashield®) was withdrawn from the market in the United States in 1999, because the vaccine is associated with intussusceptions (CDC, 1999). Recent studies reported a significant increase in the rate of intussusceptions after vaccination with rotavirus vaccines (Weintraub *et al*, 2014; Yih *et al*, 2014). The causes of intussusceptions are unknown; it may have been induced by a non-structural protein (*eg*, NSP4) and/or other components of the virus (Kombo *et al*, 2001). Given the possible limitations of live vaccines, new alternative vaccines consisting of non-living rotavirus strains, such as viral-like particles (VLPs) and subunit VP6 protein, are needed to be developed (Ward and McNeal, 2010).

VP6 protein plays a key role in generating infection protection (Choi *et al*, 1999; Parez *et al*, 2004). O'Neal *et al* (1997) reported the induction of immune protection using VLPs composed of only VP6 and VP2 when administered intranasally with a toxin adjuvant. When compared with the neutralization proteins VP4 and VP7, VP6 protein could stimulate substantially more protection against rotavirus shedding when administered intranasally with a powerful adjuvant (Ward and McNeal, 2010). These data indicate that even though VP6 is not a neutralization protein, it has the potential of being used for developing a subunit vaccine.

Thus, it is important to analyze amino acid substitutions of VP6 protein of a variety of rotavirus strains from particular regions, including Indonesia, to predict appropriate vaccine applications. The present study analyzed nucleotide variations in a 379-bp DNA fragment of VP6 gene of 15 human rotavirus strains from Jakarta, Indonesia in comparison with other published rotavirus VP6 genes. This should allow us to evaluate the types of VP6 associated with outbreaks of rotavirus infection and the possibility of employing VP6 based-rotavirus vaccines.

MATERIALS AND METHODS

Clinical specimens

Fifty-four stool samples were collected from pediatric patients (<5 years old) with symptoms of acute diarrhea in three hospitals in Jakarta, Indonesia from February to July 2010. Samples were collected in sterile containers and transported at room temperature to the laboratory at Department of Microbiology, Faculty of Medicine, Universitas Indonesia within 4 hours. Upon arrival at the laboratory, samples were immediately centrifuged

at 1,780*g* (Universal 320 R, Andreas Hettich, Tufflingen, Germany) for 20 minutes and the supernatants were stored at -80°C until used.

The study was approved by the Ethics Committee of Hospital-Faculty of Medicine University of Indonesia-RSCM Hospital (no. 18/PT02.FK/ETIK/2010), and a parent of the children gave written consent before enrollment in the study.

RT-PCR and DNA sequencing

Viral RNA was extracted from stool supernatant (140 µl) using the QIAamp RNA Viral Mini Kit (Qiagen, Hilden, Germany) and 60 µl aliquot of RNA was stored at -80°C for not more than 1 week before used. PCR primers VP6-F (5′-GACGGVGCRACTACATGGT-3′) and VP6-R(5′-GTCCAATTCATNCCTGGTGG-3′) (where N = A/G/C/T, R = A/G, and V = A/G/C) (Kang *et al*, 2004) were used to amplify a 379-bp VP6 cDNA fragment from nt 747 to 1126 (GenBamk accession no DQ870488). RT-PCR was conducted using One Step RT-PCR Kit (Qiagen) (50 µl) consisting of 1X OneStep RT-PCR buffer, 2.5 mM $MgCl_2$, 1XQ solution, 400 µM dNTPs, 0.6 µM each primer, 2 µl of OneStep RT-PCR enzyme mix, 5 µl of RNA solution, and nuclease-free water. Thermocycling was performed in a BioRadiCycler (Bio-Rad Laboratories, Hercules, CA) as follows: 50°C for 30 minutes; 95°C for 10 minutes; 45 cycles of 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 1 minute; with a final heating at 72°C for 7 minutes. Amplicons were analyzed by 1.5% agarose gel-electrophoresis, purified from agarose gel using a QIAquick PCR Purification Kit (Qiagen) and directly sequenced using BigDye® Terminator Sequencing Standard Kit v3.1 (Applied Biosystems, Foster City, CA) with the same primers as used in PCR in an ABI 3130 Genetic Analyzer (Applied Biosystem). Sequences were deposited at GenBank, accession nos.KF732668-KF732682.

Phylogenetic tree construction

The phylogenetic tree was constructed using MEGA7 (Kumar *et al*, 2016). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in units of the number of base substitutions per site (Tamura *et al*, 2004).

RESULTS

Of the 54 samples, 16 (30%) samples were positive for Group A rotavirus while the remaining were negative. Overlapping editing of the sequences of RT-PCR amplicons of a fragment of VP6 using SeqScape v2.7 (Applied Biosystems) revealed 15 rotavirus strains suitable for subsequent analysis.

Fifteen rotavirus strains from Indonesia analyzed in this study clustered into two VP6 genotypes, 4 with genotype I1 and the remaining with I2, the former strains being closely related to the reference VP6 genotype I1 strain 2009727093, while the latter to the reference VP6 genotype I2 strain HC 12016 (Fig 1). The 11 I2 strains were closely related to vaccine strain WC3, while the 4 I1 strains to vaccine strain AROLA490AB and vaccine candidate strains 116E and RV3.

Analysis of VP6 deduced amino acid sequences (amino acids 249-361) showed those of genotype I2 differed from reference strain WC3 at amino acid 281 (Table 1). On the other hand, VP6 genotype I1 sequences differed from reference strain vaccine strain WC3 at 9 amino acid positions (252, 281, 291,305, 310, 315, 339, 342, 348), with 5 positions (291,305, 315, 342, and 348) being discordant amino acid

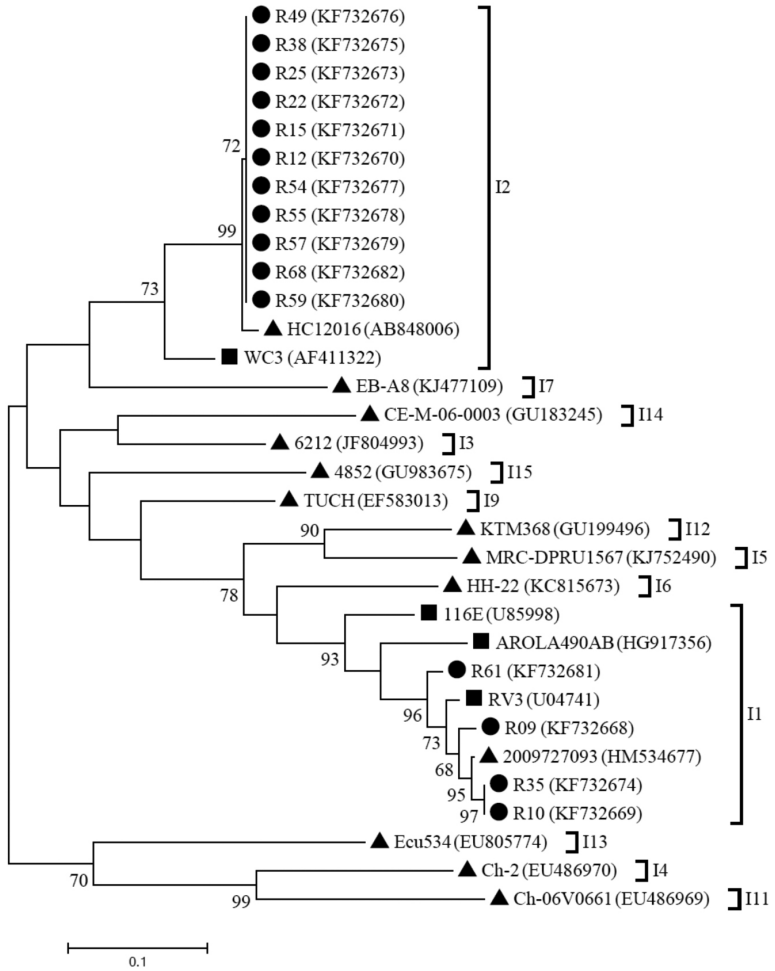

Fig 1–Phylogenetic tree of the 379-bp VP6 nucleotide sequences of the rotavirus strains. The strains analyzed in this study are indicated by filled circles. The reference strains genotypes I1-I15 retrieved from GenBank database are indicated by filled triangles. The vaccine and vaccine candidate strains are indicated by filled squares. GenBank accession numbers of the virus strains appear next to the strain names. Bootstrap values (>50%) of 500 replicates are indicated above the nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

changes. The 4VP6 genotype I1 strains showed identical amino acid sequences with the vaccine strain AROLA490AB and the vaccine candidates RV3 and 116E.

DISCUSSION

In developing countries, rotavirus associated diarrhea causes 20% of deaths in children <5 years of age (Parashar *et al*, 2003). For this reason, molecular studies have been performed, particularly the characterization of VP4 and VP7 proteins (Radji *et al*, 2010; Ansari *et al*, 2013; Moussa *et al*, 2016). In the present study, sequence analysis of a 379-bp DNA region (covering amino acid positions 249-361) of the VP6 gene of Group A rotaviruses obtained from hospitalized children with diarrhea in Jakarta revealed among genotype I2 VP6 sequences were highly conserved compared with those of genotype I1. This finding agrees with the report of Nyaga *et al* (2014), which characterized the full-length VP6 genes. It is worth noting that the phylogenetic tree constructed by Nyaga *et al* (2014) is similar to that generated in the current study, indicating that the 379-bp DNA segment of VP6 gene can be used to differentiate among VP6 genotypes.

Table 1
Alignment of the amino acid sequences of VP6 amino acid sequences of human rotavirus strains isolated from stool of pediatric patients (<5 years old)with symptoms of acute diarrhea in three hospitals in Jakarta, Indonesia from February to July 2010.

Strain	Genetype (I)*	Rotavirus VP6 amino acid position** from 249 to 361
		....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
		258 268 278 288 298 308 318 328 338 348 358
WC3 **(SGI)**	2	**FNPVILRPNN VEVEFLLNGQ IINTYQARFG TIIARNFDTI RLSFQLM<u>RPP N</u>MTPAV<u>AALF</u> PNAQPF<u>E</u>HHA TVGLTLRIES AVCESVLADA SETMLANVTS VRQEYAIPVG PVF**
R12	2	....V....... ....
R15	2	....V....... ....
R22	2	....V....... ....
R25	2	....V....... ....
R38	2	....V....... ....
R49	2	....V....... ....
R54	2	....V....... ....
R55	2	....V....... ....
R57	2	....V....... ....
R59	2	....V....... ....
R68	2	....V....... ....
AROLA490AB **(SGII)**	1	....L....... ......N... .Q....Q... .... N..L.....A ....
116E **(SGII)**	1	....N... .Q....Q... .... N..L.....A ....
RV3 **(SGII)**	1	...I...... ....N... .Q....Q... .... N..L.....A ....
R09	1	...I...... ....V....... ....N... .Q....Q... .... N..L.....A ....
R10	1	...I...... ....V....... ....N... .Q....Q... .... N..L.....A ....
R35	1	...I...... ....V....... ....N... .Q....Q... .... N..L.....A ....
R61	1	...I...... ....N... .Q....Q... .... N..L.....A ....

* VP6 genotype (I) determined by phylogenetic analysis of VP6 nucleotide sequences using the reference VP6 sequences of genotype I1-I15.
**Amino acid residues previously identified as defining serogroup (SG) I and SG II are in boldface (Lopez *et al*, 1994; Tang *et al*, 1997). R09-R68 are rotavirus strains analyzed in this study.Vaccine strains (WC3 and AROLA490AB) and vaccine candidates (116E and RV3) (Rippinger *et al*, 2010) are indicated in italic boldface. Dot indicates consensus amino acid.

It has been reported that amino acid substitutions of the VP6 protein at positions 296-299, 305, 306, 308, and 315 are capable of changing reactivity to SG I- and SG II-specific MAbs (Lopez *et al*, 1994; Tang *et al*, 1997). An A305 and the sequence between positions 296 and 299 contribute to reactivity to MAb 255/60 (SG I), while a mutation (E315Q) confers reactivity to MAb 631/9 (SG II) (Tang *et al*, 1997). In this study, the strains within VP6 genotype I2 showed the presence of A305, while all the strains within genotype I1 showed Q315, which was characteristic of SG II strains. These results are in good agreement with those of previous reports (Iturriza-Gomara *et al*, 2002; Thongprachum *et al*, 2009). Thus, it could be taken that the 11 isolated strains with VP6 genotype I2 and the 4 strains with VP6 genotype I1 belonged to SGI and II, respectively. These findings confirm previous reports from United States, South Africa, and Thailand (Kerin *et al*, 2007; Thongprachum *et al*, 2009; Nyaga *et al*, 2014).

In general, there were similarities in the VP6 amino acid sequences of the rotavirus strains isolated in Jakarta with those of the attenuated vaccine strains WC3 and AROLA490AB and vaccine candidates RV3 and E116 (Angel *et al*, 2007; Rippinger *et al*, 2010). However, the five discordant amino acid changes present in VP6 sequence of the isolated rotaviruses have the potential to influence the immunoreactivity of VP6 protein as have been reported by Lopez *et al* (1994) and Tang *et al* (1997) in which two changes (A305N and E315Q) are the main indications for changes in SG specificities as determined by reference mAbs. Choi *et al* (2000) reported that substitutions in VP6 amino acids 197-263 (CD1), amino acids 244-310 (CD2) and amino acids 332-397 (CD4) are able to decrease viral shedding by about 88%, 84%, and 92%, respectively. Substitutions found in the vaccine strain AROLA490AB, vaccine candidates RV3 and 116E, and the 4 isolated I1 strains were located in CD1, CD2, and/or CD4 regions. The effects of these substitutions on the protection against infection should be addressed in the future. Thus, the application of vaccine strain AROLA490AB and two vaccine candidates RV3 and E116 becomes questionable because of their significantly different amino acid sequences from most of the rotavirus strains analyzed in this study. However, the rotavirus strains, of course, are not representative of all strains in Indonesia.

In conclusion, this study highlights the need to characterize the VP6 protection-stimulating epitopes of rotavirus strains from many representative areas. This characterization will provide important information regarding more appropriate applications of rotavirus vaccines containing VP6 protein.

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