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# DETECTION OF HUMAN GROUP A AND C ROTAVIRUSES IN PEDIATRIC PATIENTS WITH ACUTE GASTROENTERITIS BY REAL TIME RT-PCR ASSAY: A PRELIMINARY STUDY

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## Abstract

Rotavirus causes 25–55% of all hospital admissions for diarrhea and approximately 611.000 deaths every year in developing countries. Clinically, it is not possible to recognize the diarrhea caused by rotavirus and other infections. To know a causative agent of rotavirus gastroenteritis, availability of an accurate diagnosis assay is necessary. Therefore, we developed real time RT-PCR assay (rRT-PCR) assay for confirmation of infections of Group A or C rotaviruses simultaneously. A total of 54 stool samples obtained from pediatric patients (< 5 years old) was used in this study. All samples were tested for Group A rotavirus by Serological rapid test. Result of serological rapid test was compared with rRT-PCR assay to obtain the test accuracies of both assays. Result of this study showed that rates of positive testing for Group A rotavirus by serological rapid test and the rRT-PCR assay were 22.22% and 18.50%, respectively. Forty-two serology-negative specimens for Group A rotavirus were also PCR negative (100% specificity). Two serology-positive specimens for Group A rotavirus was rRT-PCR negative (confirmed by electrophoresis gel); therefore, rRT-PCR assay represents the decrease of 3.70% in the number of specimens that are positive for Group A rotavirus. For Group C rotavirus, all tested samples were no rRT-PCR positive and the results need to be confirmed in the future.

*Keywords: real time RT-PCR, rotavirus, serological rapid test*

## Introduction

Human rotavirus is member of the *Reoviridae* Family and has 11 segments of double-stranded RNAs (dsRNA). There are seven species of rotavirus (Group A-G). Group A rotavirus is a predominantly causative agent of severe acute gastroenteritis disease in infants and young children. Infection of Group C rotavirus in humans also occurs in both children and adults, usually in sporadic cases or clustered outbreaks.[1,2]

In developing countries, diarrhea is the third most common cause of death with approximately 2 million deaths per year or 17% of all deaths in children younger than 5 years.[3] Of the incidences, rotavirus causes 18,7–55% of all hospital admissions for diarrhea and approximately 611.000 deaths every year.[4-7]

Clinically, gastroenteritis caused by rotavirus infection is characterized by profuse diarrhea, mild fever and vomiting, leading to mild to severe dehydration.[8] It is clinically not possible to recognize the diarrhea caused by rotavirus and other infections.[9] Therefore, to know a causative agent of rotavirus gastroenteritis, availability of an accurate diagnosis assay is necessary, because children with testing positive for rotavirus need not given any antibiotic except for other bacterial infections. Use of inappropriate antibiotics not only adds the cost of treatment and risks of adverse reactions, but also enhances the emergence of resistant bacteria.[8]

Several techniques have been developed to detect rotavirus in stool, including electron microscopy, polyacrylamide gel electrophoresis (PAGE) of viral nucleic acid, antibody-based assays, and PCR-based molecular assays.[1,10,11] An antibody-based assay, rapid test for detection of rotavirus antigen in stool sample, is commonly used because it is rapid, ingenuous, and inexpensive. Several rapid tests have been evaluated and showed the ranges of 54-82.9% sensitivity and 73.3-100% specificity.[12-16] The performance of those rapid tests indicated that the tests could only be used for screening but not for confirmation tests. Therefore, a high specific and sensitive test is mostly needed for confirmation of the rotavirus infection. For the reason, in this study we developed a molecular-based assay, real

time RT-PCR assay (rRT-PCR), for confirmation of infections of both Group A or C rotaviruses. This assay could also be used in epidemiological study to define particular outbreaks of both rotaviruses.

## Methods

**Clinical specimen and preparation.** A total of 54 stool samples were collected from pediatric patients (< 5 years old) with symptoms of diarrhea received at RSCM, THAMRIN, and PELNI hospitals, from February to July 2010. The samples were collected in sterile container and transported to Microbiology Laboratory, Faculty of Medicine University of Indonesia (FMUI) by using specimen transport box with condition of room temperature. The fresh samples were immediately tested by serologic assay. Thereafter, the samples were four-folded diluted in 0.9% NaCl solution, vortexed briefly and centrifuged at 4000 rpm for 20 min. The supernatant was stored at -80 °C until tested for rRT-PCR assay.

**Serologic assay.** Serologic test was performed by using SD BioLine Rotavirus Rapid Test (MT Promedt Consulting GmbH) following manufacturer's instruction. Principle of this test is an immunoassay for detection of antigen of Group A rotavirus in fecal specimens.

**Viral RNA extraction.** Viral RNA was extracted by QIAamp RNA Viral Mini Kit (Qiagen) following manufacturer's instruction. Viral RNA was suspended by a final elution volume of 60 µl. The final elute containing RNA was stored at -80 °C not more than one week. Five micro liter of elute was used as template for rRT-PCR assay.

**Primer and probe**. The primers and probes for Group C rotavirus were previously reported by Logan et al.,[11] whereas the primers for Group A rotavirus were reported by Kang et al.[10] The probe for Group A rotavirus was designed in this study. Ten nucleic acid sequences of Group A rotavirus were retrieved from GenBank (*www.ncbi.nlm. nih.gov*) and aligned by using the ClustalW (BioEdit version 7.0.9.0). The following was Accession No. of gene sequences in GeneBank used to generate the sequence alignments: AF531912.2, AF531913.1, AY601549.1, AY601550.1, AY601551.1, AY601552.1, AY601553.1, AY601554.1, AY787645.1, and DQ005110.1. The performance of designed probe was analyzed by Primer Designer Software. All primers were purchased from GeneCraft and probes were purchased from Applied Biosystems. The DNA sequences of primers and probes are showed in Tabel 1.

**rRT-PCR Assay.** The assay was performed in 25 µl of reaction mixture with the following compositions: 1x reaction mix, the appropriate primers and probes at concentrations as detailed in Table 1, 1 Unit of enzyme mix (Applied Biosystems)), and 5 µL of template RT-PCR reaction. The reaction was performed on LightCycler IQ5 (Bio-Rad) with the following thermal conditions: RT reaction at 50 °C for 30 min, initial denaturation at 95 °C for 10 min; 45 cycles of denaturation at 94 °C for 15 s, annealing/extension at 56 °C for 1 min.

**Minimal detection of rRT-PCR assay.** One sample that has been confirmed by DNA sequencing was used as to establish the minimal detection of rRT-PCR assay. Final elute of viral RNA extraction was serially ten-folded diluted in DEPC-treated water. Each dilution was tested for rRT-PCR assay.

**Cross-reactivity.** Cross-reactivity of primers and probes used in rRT-PCR assay was evaluated against pathogenic *Escheriachia coli* and non-pathogenic *Escheriachia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio eltor*, *Shigella dysenteriae*, *Klebsiella pneomoniae*, Herpes Simplex Virus, and Cytomegalo-virus. A total of 2-5 ng of viral or bacterial genomes were used as template for rRT-PCR assay.

**DNA Sequencing.** One sample positive for Group A rotavirus were confirmed by DNA sequencing. The DNA sequences were blasted in GeneBank to generate "taxonomy report" and "phylogenetic tree".

**Tabel 1. DNA sequences of primers and probes used for rRT-PCR assay**

| Reaction (target gene) | Primer or probe | Sequence (5'→3')[a] | Conc. (µM)[b] | Ref. |
|---|---|---|---|---|
| Rotavirus A (VP6) | RotaAF | GACGGVGCRACTACATGGT | 0.44 | 10 |
| | RotaAR | GTCCAATTCATNCCTGGTGG | 0.31 | 10 |
| | Probe-rotaA | *FAM*-ATTGARTCTGCAGTTTGTGA-*TAMRA* | 1.50 | Study |
| Rotavirus C (VP7) | RotaCF | TTAGATACTACAAGTAATGGAATCGGATGT | 0.31 | 11 |
| | RotaCR | TGGGTGTCATTTGATACAACTTCA | 0.31 | 11 |
| | Probe-rotaC | *HEX*-CAGCTAGTACAGAAACTT-*TAMRA* | 1.25 | 11 |

[a] FAM and HEX is the reporter dyes coupled on the 5' end of the probes, while TAMRA is coupled on the 3' end of the probes
[b] Concentration of primers or probes in rRT-PCR reaction

## Results and Discussion

**Cross-reactivity and minimal detection of rRT-PCR assay.** The primers and probes used in this study showed no cross-reactivity with genomes of pathogenic *Escheriachia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio eltor*, Herpes Simplex Virus, and Cytomegalovirus (Figure 1). The rRT-PCR assay could detect viral RNA in clinical sample at 1 : 100 dilution (data not shown). The ability of the assay to detect particular number of viral genomes in clinical sample was not determined in this study because we had no RNA standard to estimate the number of viral genomes in clinical samples.

Of 54 samples, serology tests showed rates of positive testing for Group A rotavirus were 22.22% (Tabel 2). The rRT-PCR assay was only able to detect 10 (18.50%). Forty-two serology-negative specimens for Group A rotavirus were also PCR negative; thus specificity of serological rapid test was 100% (Tabel 2). Two serology-positive specimens for Group A rotavirus was PCR negative. To ensure the false positive test results, we confirmed the rRT-PCR assay by gel electrophoresis gel and showed no DNA band of two samples (data not shown). Thus, rRT-PCR assay represent the decrease of 3.70% in the number of specimens that are positive for Group A rotavirus. For Group C rotavirus, we did not detect the virus for any clinical sample.

**Detection of target viruses in clinical specimens**. Applicability of rRT-PCR assay to clinical specimens was evaluated against 54 stool samples from pediatric patients with diarrhea (Figure 2). A comparison result between serologic and rRT-PCR assays is presented in Table 2.

**DNA Sequencing and phylogenetic analysis.** One sample positive for Group A rotavirus (RotA_R9) was confirmed by DNA sequencing. The result of DNA sequencing was blasted in GeneBank to generate a taxonomy report and the RotA_R9 was classified as Group A rotavirus (data not shown). The phylogenetic tree was also generated by using 25 strains of Group A rotavirus (Figure 3). The result showed that Group A rotavirus in this study (RotA_R9__study_) placed lineage 5. The virus was very closely related with viral strain from Korea (EU679386_1_Korea) and more closely related with viral strain from India (AY601549_1_India) (Figure 3). Based on countries as viral strain sources, there was no certain cluster because viral strains of one country scattered at different lineages (Figure 3).

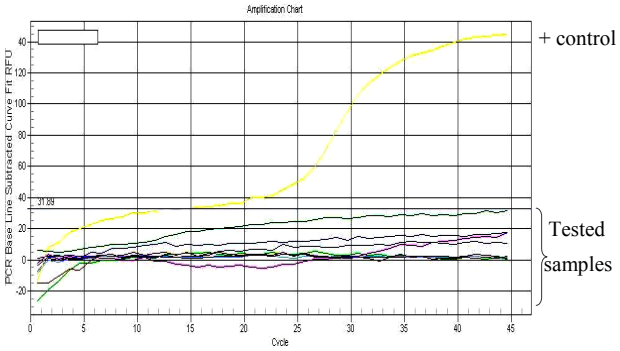


**Figure 1. The Specificity of the rRT-PCR Assay. The Assay Only Reacted with Group A Rotavirus (+ Control), and None of Assay Reactivity to Tested Samples (Other Lines)**

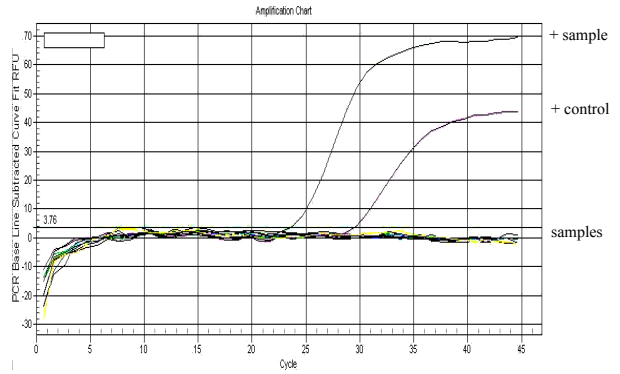


**Figure 2. An Result Example of rRT-dPCR Assay Tested Against Clinical Samples. + Control: Positive Control. + Sample: Sample with Positive Test. - Samples: Samples with Negative Tests**

**Tabel 2. Result of Comparing Serological Rapid Test to rRT-PCR Assay for 54 Samples from Pediatric Patients with A Gastroenteritis**

| Virus | No. (%) of specimens | | | | | |
|---|---|---|---|---|---|---|
| | RT-PCR +, Serology + | RT-PCR +, Serology - | RT-PCR -, Serology + | RT-PCR -, Serology - | Total Serology + | Total RT-PCR + |
| Rotavirus A | 10(18.50) | 0(0) | 2(3.70) | 42(77.78) | 12(22.22) | 10(18.50) |
| Rotavirus C | ND | 0(0) | 0(0) | 0(0) | 0(0) | 0(0) |

-: negative. +: positive. ND: not done

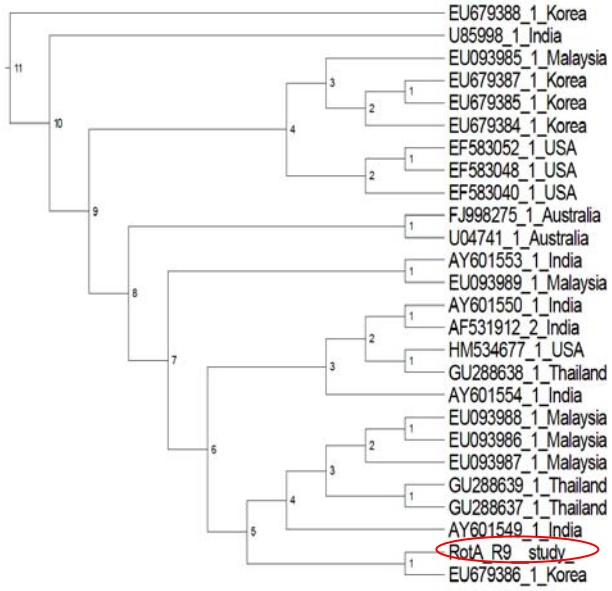


**Figure 3. Phylogenetic Tree Derived from Alignment of Nucleotide Sequences in this Study (RotA_R9__study__) with those Retrieved from GeneBank. Tree was Generated Using FigTree v1.3.1. DNA Sequences Obtained from GenBank are Indicated as the Accession Number and Virus Sources**

The main advantage of the rRT-PCR assay in this study is that it is able to detect any viral genomes of Gorup A and C rotavirus as an indication of the viral infection. Moreover, the duplex approach can saves time and reagents because of only performing a single reaction instead of two reactions for the detection of Gorup A and C rotavirus and is an ideal way for conserving samples in limited supply.[17] The most disadvantage of rRT-PCR assay is that the cost of the real-time PCR machine and reagents that lead the cost of test to be more expensive than other routine assays.

Rates of positive testing for Group A rotavirus by serological rapid test and rRT-PCR assay were 22.22% and 18.50%, respectively (Tabel 2). Both assays showed considerable test results, as having been reported that about 18.7–55% of all hospital admissions for diarrhea in developing countries were caused by rotavirus.[4-7,18,19] In addition, Group B rotavirus could also infect human even though in very rarely compared with Group A and C rotavirus,[20] in which Group B rotavirus was not detected in this study.

Forty-two rRT-PCR negative samples were also serological rapid test negative (100% specificity). However, results of Serological rapid tests showed 2 false positive test results while compared with rRT-PCR assay (Tabel 2). This comparison study indicated that rRT-PCR assays represent the decrease of 3.70% in the number of specimens that are positive for Group A rotavirus (Tabel 2). This finding was also reported by van Doorn et al.[21] To ensure the false positive test results, we confirmed the rRT-PCR assay by gel electrophoresis gel showing no DNA band of two samples (Figure 3). Therefore, the rRT-PCR is an appropriate assay applied to a confirmation assay, while serological rapid test should be used as a screening assay. This approach is also recommended by Gouvea et al.[22] and Jiang et al.[23]

During this study, we did not detect Group C rotavirus. Consequently, we did not perform the reaction optimization; however, we used the optimal condition such as primer and probe concentration as reported by Logan et al. that have designed the primer and probe.[11] None of detected Group C rotavirus may depict a true result, because the virus infects children and adults, usually in sporadic cases or clustered outbreaks.[1,2,23] Most of the outbreaks occurred in school aged people 6-18 years old.[24] Most of patients in this study were less than 2 years old and only 7 patients were more than 2 years old (data not shown). However, the thought should be confirmed and evaluated for larger samples.

According to phylogenetic analysis, DNA sequences of RotA_R9 viral strain (this study) showed very closely related with viral strain from Korea (EU679386_1_Korea) and followed by viral strain of India (AY601549_1_India) (Figure 3). Interestingly, RotA_R9 viral strain was not grouped into viral strains from Indonesia's neighboring countries such as Malaysia and Thailand; meaning that RotA_R9 strain was not lineaged from Malaysia and Thailand but even from Korea or India. However, in this study we just performed comparable gene homology for one sample (RotA_R9) that did not reflect representative strain of Indonesia. Further genetic analysis for strains from Indonesia is needed performed to obtain genetic information for development and evaluation of vaccines that is used in Indonesia.

## Conclusion

In this preliminary study, we successfully developed the rRT-PCR assay for detection of Group A and C rotavirus. The rRT-PCR assay represents the decrease of 3.70% in the number of specimens that are positive for Group A rotavirus by serological rapid test. For Group C rotavirus, all tested samples showed negative tests and the results need to be confirmed in the future. The findings in this study will be evaluated for larger samples in the future to know the actual accuracy of rRT-PCR assay.

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