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Genotyping the G types of rotavirus and its clinical presentation in children under five years old with diarrhea in the government clinics in Pekanbaru, Indonesia

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ABSTRACT

Aims: Rotavirus (RV) is the most important etiological agent of diarrhea in children with high morbidity and mortality, mainly in developing countries. A big number of children from one to five year old have been infected by RV, with at least one type of virus. Data show that human body can be infected repeatedly by different strains of RV. It is predicted that there exist 16 types of G genotypes of RV. This study was aimed to identify the G genotypes of RV in children with diarrhea in Pekanbaru, Indonesia.
Methodology and results: This research is a cross-sectional study involving children aged zero to sixty months from January to July 2015. The stool samples were collected from diarrhea patients and the identification of RV was done by using the rapid test. The RNA isolation was performed on positive isolates. The identification of the G genotypes was performed by using *a semi nested reverse transcription PCR* method. The study involved 71 children with diarrhea. VP7-based RV detection showed 47 positive samples (66.1%). The predominant G type of the positive results are G1, G9 and G3 namely 36.2%, 25.5% and 12.8%. The average age of the subjects was 15.3±2.3 months. The majority of the subject were females (53.6%). Most of the diarrhea feces presented in this study were neither haemorrhagic nor liquefied. The subjects in this study are also presented with co-symptoms such as fever (100.0%), gastrointestinal disorder (94.6%) and dypsnea (16.1%).
Conclusion, significance and impact study: Based on this study, we conclude that the predominant G types is G1. This study was performed to identify the predominant G genotype of RV and designed the antigen-antibody based in diagnose RV.

Keywords: Diarrhea, rotavirus, children, G typing

INTRODUCTION

Rotaviruses are the most important etiological agents of severe diarrhea in infants and young children in the world, mainly in developing countries. Many children between 1-5 year-old had been infected by RV, at least with one type of virus. Data shows that a human can be infected repeatedly by different strain of RV. However, children with recurrent infections rarely show severe clinical symptoms because of the protective immune response to RV from the previous infection (WHO, 2011).

Rotavirus infections are common in developing countries most deaths cases are found in sub-Saharan region, South and Southeast Asia. Kargar *et al.* (2012) found that the proportion of RV diarrhea reached up to 34.8%. Each year the RV causes approximately 111 million cases of diarrhea in children resulting in 25 million cases of treatment at the clinic, 2 million cases of hospitalization and 352,000 to 592,000 deaths cases. In industrialized countries, death cases caused by RV are rare but the morbidity is high and it could cost over US $ 1 billion per year (Ramiq, 2004; Kargar *et al*., 2012; Widdowson *et al*., 2007).

The reports from a cohort study in Asia shows 13.5 million cases of diarrhea caused by RV 1.9 million cases were actually treatable. The death number reached 171,000 annually in children under five years old. As much as $ 191 billion is spent annually to treat diarrhea. India and China are the two countries with the highest RV cases (Podewlis *et al*., 2005; Fang *et al*., 2005).

Rotavirus transmission occurs through the fecal-oral route and the incubation period of this virus is about 2 days. The clinical manifestations are characterized by watery diarrhea, fever, vomiting, dehydration, electrolyte disorders, and the worst is shock to death. Oral and intravenous rehydration therapy to maintain osmotic and

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electrolyte balance is the most important thing in RV infections treatment. The risk of death increases among children aged 6 months to 2 years (WHO, 2011; Luchs and Timenetsky, 2016).

Rotavirus belongs to the Reoviridae group. Its body structure consists of a capsid protein composed of three concentric structure that encloses the double stranded RNA (dsRNA). The total genomic RV is 18,680 bp, which consists of 11 segments. These segments encode 6 structural proteins and 6 non-structural proteins. The structural proteins are VP1-VP7 while the non-structural consist of NS53, NS34, NS35, NS28 and NS26. Virion of the virus consists of VP2 proteins that encapsulate dsRNA as well as VP1 and VP3, the middle layer is composed by the VP6 protein and the outer portion contains the attached VP4 on VP7. Immunologic analysis showed that VP4, VP6 and VP7 proteins are highly immunologic, therefore they are widely used as a reference for vaccine and diagnostic development (Estes *et al*., 1989; McDonald *et al*., 2009).

Serotyping analysis shows that RV consists of 7 groups, A-G based on antigenic character of VP6. The surveillance data shows that only A, B and C groups can cause infection in humans. A group is the most infecting group whereas B group and C appear to be more sporadically and sometimes associated with RV outbreaks. A group is divided into two serotypes based on VP4 and VP7 DNA sequences. VP4 determines serotype G while VP7 determines serotype P (Parashar *et al*., 2006).

Rotavirus diagnosis can be confirmed by various methods, including viral culture, immunoserology (by ELISA) and molecular holdings. In between these methods, Reverse Transcriptation molecular examination using Polymerase Chain Reaction (RT-PCR) is considered to be the gold standard for the diagnosis of RV. The advantage of this method is its ability to identify the virus serotypes accurately. The disadvantages of this method are: it is very dependent to sophisticated devices and it also requires highly trained personnels (Parashar *et al*., 2006; Putnam *et al*., 2007; Adlhoch *et al*., 2011).

The accuracy of the diagnosis of RV by ELISA and RT-PCR are almost the same. Stockman *et al*. (2008) found that 18% of healthy groups were positively detectable with RT-PCR, whereas Arguelles *et al.* (2000) found thatfrom 62% positive samples with ELISA, 68% was positive with RT-PCR. The use of ion-exchange chromatography appears to increase the accuracy of RV diagnosis, but this method requires difficult and expensive procedures (Olive and Sethi, 1989). Under these conditions, there are two important things in RV placement, namely RV genotyping surveillances, these types of viruses tend to change due to reassortment and development of diagnostic methods based on target proteins of the dominant type (Olive and Sethi, 1989; Arguelles *et al*., 2000; Stockman *et al*., 2008).

Our previous study, Djojosugito *et al*. (2017), showed that out off 71 stool samples of children suffering from acute diarrhea in Pekanbaru, 44 samples (62.0%) were with positive RV, the predominant characteristic is female toddler patients (54.5%), and mostly found in children 6-35 months of age. In addition, it was found that the majority patients with acute diarrhea with positive RV were children with a history of exclusive breastfeeding (54.5%) and good nutritional status (97.7%). The study also showed the highest proportion of RV type P was P4 (31.8%) followed by P8, P6, P9, P10 and P11 respectively (Djojosugito *et al*., 2017). This research was aimed to identify the dominant G genotypes RV from stool isolates of diarrhea in children in Pekanbaru, Riau Province.

MATERIALS AND METHODS

Subject

The study involved 71 children under 5 years-old with acute diarrhea undertreatment by the Puskesmas (Goverment Clinic) and Arifin Achmad General Hospital in Riau Province from January to July 2015. This study was approved by "Universitas Riau Ethical Committee", and the informed consent was obtained from all parents. All patients were subject to thorough history taking and clinical examination at the time of specimen collection. An interview questionnaire was designed to obtain data regarding the age, sex, residence, duration of diarrhea and its frequency per day, the presence or absence of fever, vomiting and flu-like symptoms, the stool consistency, and the breast feeding history.

Sample collection

The stool specimens were obtained in a sterile container, resuspended with 1.5 mL Phosphate Buffer Saline (PBS) pH 7.2. The samples were centrifuged 8000 g for 10 min and supernatant were divided to 2 plastic tubes, for molecular and serological analysis). Place 500 µL feces supernatant in plastic tube and add RNA later to the tube with 1:1 ratio. The samples were transported the same day to the laboratory for molecular examination.

RNA extraction

RNA was extracted from stool using QIAamp Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's instruction. Viral RNA binds specifically to the QIAamp silica membrane while contaminants pass through. PCR inhibitors, such as divalent cations and proteins, are completely removed in two efficient wash steps, leaving pure viral RNA to be eluted in either water or a buffer provided with the kit.

Reverse transcription PCR for identification and genotyping

We have conducted 2 rounds/cycles of RNA amplification for detection using seminested RT PCR methods. For the first amplification, we used the concensus primers for RV (Con1 and Con2) and the second amplification, we used con1 as forward primer and VP7 genotype-specific primers (9T1, 9T2, 9T3, 9T4, 9T9) as reverse primers

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(WHO, 2011). Amplification using Superscript III one step RT PCR kit (Invitrogen, USA). The primers which are for amplification are shown in Table 1.

The first amplification has been prepared with 12.5 mL reaction mix, 1 µL template RNA, 0.5 µL each primers, both Con1 and Con2, 1 mL Platinum™ Taq Mix and molecular water to 25 µL. PCR system is 45 °C for 30 min for cDNA synthesis and was followed by immediatelly by 30 cycles PCR amplification (94 °C for 30 sec, 42 °C for 30 sec and 72 °C for 30 min) and the last extension was performed at 72 °C for 5 min.

RESULTS

Diagnose of rotavirus

The study was conducted involving 71 cases of children with acute diarrhea in Pekanbaru, Indonesia. The initial screening using rapid test for RV showed 42 cases (59.1%) positive result for RV and the remaining 29 cases (40.9%) were negative, allegedly related to other causes. A slightly different results were shown by a study using the nucleic acid amplification where the primary 9Con1/9Con 2 pair of VP7 RV and primary Con 2/Con 3 proteins on VP4 protein RV gave positive RV results in 48 cases (67.6%) and 42 cases (61.9%) respectively (Figure 1 and 2).

PCR typing was performed from dsDNA which was obtained from the first amplification using con 1 and con2 pair. Primers 3 µL of the dsDNA product served as the template for this second typing amplification. In this step, con1 acted as forward primer and VP7 genotype-specific primers as reverse. We have performed same reaction mix containing all genotype specific primers. The same PCR program was used with 30 cycles followed by a final extension at 72 °C for five min. The PCR products were resolved by 2% agarose gel electrophoresis and were visualized after ethidium bromide (0.5 µg/mL) staining, using an UV transilluminator

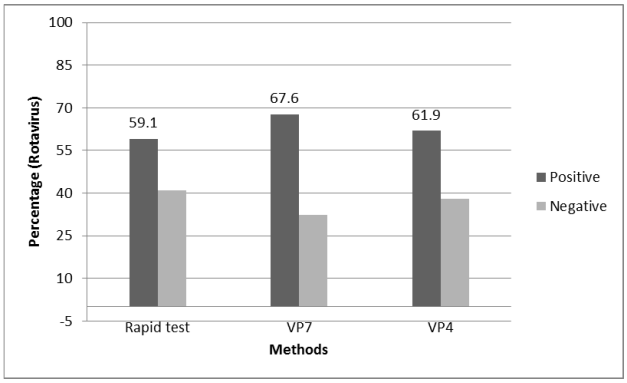

Figure 1: Distribution of Rotavirus diarrhea based on rapid test and amplification of VP7 and VP4.

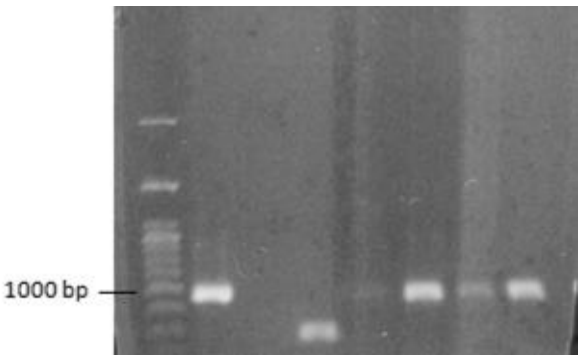

Figure 2: Amplification using primer con1 and con 2. Amplicon area was visible at 1000 bp (line 1, line 4, 5, 6 and 7).

Subtype G analyze

The subtype analysis was performed on 48 positive RV samples based on the amplification of VP7 RV area. The Rotavirus subtype is determined by the size of the band formed between the 9con1 primer and the specific primer subtype. The previous research data showed sub-type G1 size 159 bp, G2 242 bp, G3 464 bp, G4 404 bp and G9 111 bp. This study found different proportions among subtype RV, where the predominant subtype is G1 17 cases (35.4%) and G9 12 cases (25.0%). Other types respectively, the G4, G3 and G5, are found in smaller proportions, there are five cases for G4 and G3 followed by 3 cases for G2.

This study also found 4 cases (11.4%) with unidentified type (Non Typeable=NT). further research is needed to determine the unidentified subtype group, based on the number of subtypes G of the RV.

Clinical presentation

The obtained data in this study showed that the age average was 15.3±2.3 months, with the highest age was 3 years 2 months old and the lowest was 5 months. Based on gender, this study found that 52 children were males (46.4%) and 60 children were females (53.6%). There was no difference in the incidence of diarrhea based on sex group. The average of duration of diarrhea was 3.6±0.8 days with the frequency of diarrhea 3-5 times per day. The characteristics of subjects in this study were presented in Table 2.

Most of the presentation of the diarrhea in this study was non-bloody and watery diarrhea. Based on dehydration level, there were only 6 subjects (5.3%) having severe dehydration. This study also found most subjects were also presented with fever and gastrointestinal disorder (nausea, vomiting, bloated or abdominal pain) as many as 112 cases (100%) and 106 cases (94.6%), respectively. Dypsnea were found in 18 subjects (16.1%).

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Table 1: The primers which used for amplification Genotyping G Rotavirus.

Primer G typing	Sequences	Size (pb)	Type
9con1	tag ctc ctt tta atg tat gg	904	Primer universal tipe G
9con2	gta taa aat act tgc cac ca		
9T-1	tct tgt caa agc aaa taa tg	159	G1
9T-2	gtt aga aat gat tct cca ct	242	G2
9T-3	gtc cag ttg cag tgt agc	465	G3
9T-4	ggg tcg atg gaa aat tct	404	G4
9T-9	tat aaa gtc cat tgc ac	111	G9
G12	ccg atg gac gta acg ttg ta	384	G12

Table 2: Patients characterization based on clinical manifestation (n=112).

No	Description	Total	%
1	Age (months)	15.3±2.3	
2	Sex, males	52	46.4
3	Duration of diarrhea	3.6±0.8	
4	Frequency of diarrhea	4.2±1.1	
5	Diarrhea (severe)	6	5.3
6	Consistency of diarrhea	112	100.0
7	Bloody diarrhea	0	0.0
8	Immunization (completed)	110	98.2
9	Breastfeeding (Exclusive)	39	34.8
10	Fever	112	100.0
11	Dyspnea	18	16.1
12	Gastrointestinal disorder (nausea/vomiting)	106	94.6%

DISCUSSIONS

This study was aimed to analyze the rotavirus genotypes in children stool samples in Arifin Achmad Hospital Pekanbaru. The result showed that the RV proportion in children with diarrhea 59-67% depend on the diagnostic method used, where the largest proportion was found in the G1 group, 17 cases (35.4%), and G9 of 12 cases (25.0%). Other types were G4 and G3 found in 5 cases (10.4%) and G2 found in 2 cases (4.2%).

In general, the percentage of classical genotype, is 52.8% represented by 20 cases of G1 G4. These results are essentially lower than the global genotype pattern in Asia. Santos *et al.* (2005) reported that the proportion of G1-G4 in Asia and South America was about 68%, whereas in Africa 50% and Europe, North America and Australia reached up to 90-95% (Santos *et al.*, 2005).

Research from Linhares *et al* (2006) in South America reported that the proportion of G1-G4 was 69% where the largest was found in the G1 group that reached up to 59% and G9 which 29%. These results seemed higher than what we found, which were 32.4% for G1 and 23.5% for G9 respectively. Nevertheless, both studies showed an increasing trend of genotype G9, as a new subtype and widely developed in Asia and South America (Linhares *et al*., 2006; WHO, 2011).The prevalence of RV strains may differ from region to region due to geographical factors, even rare strains can be found in more developing countries.

The prevalence of RV infection varies among the age groups. In our study, the average age among the subjects was 15.3 months with the highest was on 3 years 2 months old and the lowest was on 5 months. Nguyen *et al.* (2004) found that children less than 2 years of age tend to get more infected than other age groups. Other studies have shown that the prevalence of RV infection in children less than 6 months of age was 15 to 20%. These findings showed that children in early childhood were more vulnerable to RV infection. This might be explained by the fact that children after 2 years-old got protective immunity against RV from the previous infection (Nguyen *et al*., 2004; Sai *et al*., 2013).

This study found that female children tend to have RV infection more than males. This finding differs from the previous studies. Ansari *et al.* (2013) in their study found that 64.2% children that had RV infection were males. Different result was also shown by a study in Nepal that 67.5% children with RV infection were males. The relationship between incidence of RV infection and sex group is still unknown until now (Shariff *et al*., 2003; Ansari *et al*., 2013; Ismaili-Jaha *et al*., 2014).

Most of the diarrhea in this study was presented with non-bloody and watery diarrhea. Review from Lundgren *et al.* (2001) considered that RV evokes intestinal secretion of fluid and electrolytes. Nguyen *et al* (2004) in their study

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have found 81.1% children with RV infection had watery diarrhea (Lundgren *et al*., 2001; Nguyen *et al*., 2004).

Our study also found most of subjects presented with fever and gastrointestinal disorder (nausea, vomiting, bloated or abdominal pain) as many as 112 cases (100%) and 106 cases (94.6%). The general manifestation of RV infection are: fever, vomiting and dehydration, they all tend to be more severe compared to other pathogens. Those symtoms can be found alone or in combination. In some cases ahospitalisation is needed. In this study, fever was the manifestation that was mostly found. The study has shown that fever is presented in RV infection as manas 45-84%. Alkali *et al.* (2015) reported fever was found among 51% cases. Vomiting is caused by disturbance of motoric activity of the stomach such as delayed emptying of fluid content that can cause mild to severe dehydration which is life-threatening children. (Nguyen *et al*., 2004; Alkali *et al*., 2015)

The analysis of clinical manifestations is based on several categories, including duration, frequency, diarrhea level and presence of blood and consistency. The results showed no differences between clinical manifestations in the RV and non-RV groups ($p > 0.05$) (Djojosugito *et al*., 2017). The similarity may occur because of the uneven distribution of the patient, In this case most patients are not hospitalized.

This study is limited to a small number of samples. In addition, further research on combination analysis for the development of antigen-antibody diagnostic designs for RVs is required and needed.

CONCLUSION

This study was conducted in 71 cases of children with acute diarrhea in Pekanbaru, Indonesia. Based on the results of this study, it can be concluded that RV proportion in diarrhea children was 59-67%, depense the type of diagnostic method being used. Genotype analysis showed that G1 had the greatest proportion and classical genotypic proportions was accounted for 35.1% cases. Further research is needed for the analysis of the combination and development of antigen-antibody-based diagnose design to detect RV.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

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