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Identification of the P Genotypes of rotavirus in children with acute diarrhea in Pekanbaru, Indonesia

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ABSTRACT

Aims: Acute diarrhea is still one of the major causes of death in children under five years old worldwide, including Indonesia. Data from the World Health Organization (WHO) Southeast Asia Region shows that acute diarrhea has caused approximately 10-11% of death in children under five years old and rotavirus (RV) is one of the major causes. This study is conducted to identify the profile and P genotypes of RV among children with acute diarrhea in Pekanbaru.
Methodology and results: Descriptive cross sectional study was conducted in children within the range of age 0-60 months with acute diarrhea who admitted to the government clinics and General Hospital of Riau Province from January to July 2015. Immunochrommatography and RT-PCR were used to identify the P genotype of RV from the stool specimens. The profile of patients was investigated including age, sex, hydration and nutritional status, also the exclusive breastfeeding. There were 71 children who suffered from acute diarrhea and 62.0% of stools specimens were positive by RT-PCR for RV infection. The highest rate was in the age group of 6-35 month (70.5%). Most of the patients were female (54.5%), the history of exclusive breastfeeding (54.5%), with moderate dehydration (54.5%) and good nutritional status (97.7%). The RV genotyping results showed the highest proportion was found in the P4 genotype (31.8%), followed by the P8 and P6 genotype, respectively 18.2% and 13.6%, P9 (11.4%), P10 (9.1%), P11 (2.3%) and indeterminate genotype (13.6%).
Conclusion, significance and impact of study: Rotavirus was found to be a considerable role as the etiology of diarrhea in children under five years in Pekanbaru and rotavirus P4 genotype was predominant. The result of this study is important for designing antigen-antibody diagnostics against RV.

Keywords: diarrhea, rotavirus, P genotypes, children

INTRODUCTION

Rotavirus (RV) is the leading cause of diarrhea in children with a high morbidity and mortality, particularly in developing countries. It is reported that most children aged 1-5 years have been infected by RV, with at least one type of the virus. Data shows that a human can be infected repeatedly by different strain of RV. However, children with repeated infections tend to show milder clinical symptoms. This situation shows the presence of a protective immune response against the virus (Widdowson *et al*., 2007).

Rotavirus infections generally occur in developing countries, while most death 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%. The viral infection is associated with 22% of hospital treatment due to diarrhea (Parashar *et al.*, 2006). It is estimated that diarrhea causes 2.1 million deaths in the worldwide and 440,000 cases are caused by RV. In industrialized countries, mortality caused by RV is rare, but morbidity is high enough and can spend more than US $ 1 billion per year (Kargar *et al.*, 2012; Parashar *et al.*, 2003; Parashar *et al.*, 2006).

The reports from a cohort study in Asia shows 13.5 million cases of diarrhea caused by RV with 1.9 million cases were actually treatable. The deaths reached 171,000 cases 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 (Fang *et al.*, 2005; Podewils *et al.*, 2005).

Rotavirus is transmitted through fecal-oral route and this virus has a short incubation period, only 1 to 3 days. The clinical manifestations characterized by watery diarrhea, fever, vomiting and sometimes end up with mild

to moderate dehydration. The process of infection takes place quickly and without an adequate treatment this type of diarrhea can lead to death. The greater risk of death are among children within the range of age 6 months to 2 years (Parashar *et al.*, 2003; Parashar *et al.*, 2006).

This virus belongs to the Reoviridae group. Its body structure consist of a capsid protein composed of three concentric structure that encloses the double stranded RNA (dsRNA). 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 consists of NS53, NS34, NS35, NS28 and NS26 (McDonald *et al.*, 2009).

VP1 is located in the core of the virus particle and is an RNA polymerase enzyme. VP2 forms the core layer of the virion and binds the RNA genome. VP3 is part of the inner core of the virion and is an enzyme called guanylyl transferase. The outer capsid of RV made up of a protease sensitive protein which protudes as a spike (VP4) and glycoprotein designated as VP7. VP6 forms the bulk of the capsid. Immunological analysis shows that VP4, VP6 and VP7 proteins are very immunologic, so that are commonly used for the development of vaccines and diagnostics (McDonald *et al.*, 2009; Bonkoungou *et al*., 2011). These outer capsid proteins of the virus, VP4 and VP7, elicits the production of neutralizing antibodies to RV and allows to define the G (for glycoprotein) and the P (for P sensitive) serotypes of the virus. (Bonkoungou *et al.*, 2011).

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 very dependent to sophisticated devices and also it requires highly trained personnels (Adlhoch *et al.*, 2011; Parashar *et al.*, 2003; Parashar *et al.*, 2006; Putnam *et al.*, 2007).

The accuracy of the diagnosis of RV by ELISA and RT-PCR are almost the same. Arguelles *et al.* (2000) found 62% and 68% of the samples were positive by ELISA and RT-PCR respectively. The use of ion-exchange chromatography has improved the accuracy of diagnosis of RV, but this method requires a difficult and expensive procedure (Olive *et al.*, 1989).

Rotavirus serotypes are based on the combination of P genotype and G genotype. Data from a meta-analysis study shows that there are 4 combinations P genotype and G genotype mostly found in human. P [8] G1 is the most common serotype found which reached 83% of the total RV cases, followed by P [4] G2 with 66.4% of cases, P [8] G3 with 8.3% of casesand P [8] G4 with 6.9% of cases. The rest are types with a small proportion, such as P [6] G9, P [8] G9, P [6] G1, P [4] G1 and P [8] G2 (Putnam *et al.,* 2007). A study of 435 samples in Algeria shows the type of G1 and P [8] has the largest proportion, respectively 37.5% and 80.8%. The main combinations are G1P [8] as much as 37.5% and G3P [8] with 25% of cases (Hassine-Zaafrane *et al.*, 2011).

Aside from regional related, the development of RV is also highly correlated with the seasons. A Multi-year analysis in the United States shows the changes in the pattern of RV G1P [8] genotype to the G3P [8] genotype within the period of 1976-1991 (McDonald *et al.*, 2009). Under these conditions, the most important one is the RV genotypes surveillance, because the genotype of virus is likely to change as a result of reassortment and the development of diagnostic methods based on the target protein of the dominant genotype.

This study is conducted to determine the genotype of RV as well as design-based diagnosis antigen-antibody against RV. In this study, we investigate the proportion of P genotypes of Rotavirus, the profile of patients with Rotavirus infections, and the predominant P Genotype of Rotavirus in Pekanbaru, Riau, Indonesia.

MATERIALS AND METHODS

We conducted a descriptive cross sectional study from January to July 2015 in children within the range of of age 0-60 months with acute diarrhea who admitted to some goverment clinics and Arifin Achmad General Hospital Riau Province. This study has already approved by Ethic Commite Medical Faculty Riau University.

Diagnostic methods

Immunoassay

The samples were taken from stool in a sufficient quantities (5-10 mL) then the sample is kept in a closed sterile plastic containers afterward. The stool samples then divided in two. The first group of samples were examined in the Laboratory of Microbiology Medical Faculty Riau University using the Enzyme Immunoassay for RV (Dakopatts, Dako Diagnostics Ltd., United Kingdom). While the second group of samples were stored in the refrigerator 4-8 °C to be examined in the Laboratory of Molecular Andalas University in Padang afterward.

Molecular analysis

Viral RNA was isolated using QIAamp RNA isolation kit (Qiagen) according to manufacturer's protocol. Semi nested RT-PCR method was used for the RV Genotyping using the One Step RT PCR kit (Qiagen). The early stages of RV were detected by using a universal primer (Con3 and Con2). RNA is converted into cDNA using reverse transcriptase that is already available in the kit. cDNA was amplified further at the first phase with universal primer and the positive result has obtained based on the size of 876 bp band. The analysis was based on the size of the band obtained, reffering to a study by Tamura *et al.* (2010) and also the Manual of

Rotavirus Detection and Characterization Methods (WHO, 2009).

Two-stage amplification performed with specific primers for P. Each type of amplification consisted of denaturation, annealing and extension and performed a total of 30 cycles for P genotypes, genotyping is intended to detect P[4], P[6], P[8], P[9], P[10], P [11]. The primers which used for the amplification is shown in Table 1.

The results were detected by using agarose gel electrophoresis and visualized by using 2% gel red (Biotium). P variation based on the size of the base pairs. The size of the base is not appropriate for all types of P categorized as indeterminate type (non typeable) and require further study.

Table 1: The primers which used for amplification Genotyping P Rotavirus

Primer	Sequences	Length (bp)	Type
Con3	tgg ctt cgc tca ttt ata gac a	876	Universal Primer Type P
Con2	att tcg gac cat tta taa cc		
1T-1	tct act tgg ata acg tgc	345	P[8]
2T-1	cta ttg tta gag gtt aga gtc	483	P[4]
3T-1	tgt tga tta gtt gga ttc aa	267	P[6]
4T-1	tga gac atg caa ttg gac	391	P[9]
5T-1	atc ata gtt agt agt cgg	583	P[10]
ND2	agc gaa ctc acc aat ctg	122	P[11]

RESULTS

Patients Characterization

There were 71 samples collected from some goverment clinics in Pekanbaru and Riau Province General Hospital. The samples were taken from stools of children with acute diarrhea and the hydration status, nutritional status, history of breastfeeding, age and sex were recorded (Table 2).

Laboratory Diagnostic

Of 71 stool samples, there were 42 patients (59.2%) with RV infection and 29 patients (40.8%) with non Rotavirus based on immunoassay method. By RT-PCR method, 44 (62.0%) samples are RV positive and 27 (38.0%) samples are negative for RV infection (Figure 1).

Rotavirus P Typing Result

The amplification is done by combining the primary Con3 with specific primers genotype. The template is the result of PCR products using a universal primer. RV genotyping results shows the highest proportion was P4 genotype (31.8%), followed by the P8 and P6 genotype, respectively 18.2% and 13.6%, P9 (11.4%), P10 (9.1%) and P11 (2.3%). In this study also found six samples (13.6%) were indeterminate genotypes (Figure 2).

Table 2: Patients characterization based on PCR method

Variable	Rotavirus (N = 44)		Non Rotavirus (N = 27)	
	N	%	N	%
Age (months)				
0-5	8	18.2	1	3.7
6-35	31	70.5	17	63.0
36-40	5	11.3	9	33.3
Sex				
Female	24	54.5	11	40.7
Male	20	45.5	16	59.3
History of Immunization				
Completed	24	54.5	20	74.0
Incompleted	20	45.5	7	26.0
Dehydration Status				
Mild	17	38.6	9	33.3
Moderate	24	54.5	18	66.7
Severe	3	6.9	0	0
History of Breastfeeding				
Exclusive Breastfeeding	24	54.5	19	70.4
Mix Exclusive Breastfeeding and Milk	14	31.8	7	25.9
Milk	6	13.7	1	3.7
Nutrition Status				
Under nutrition	1	2.3	0	0
Good nutrition	43	97.7	26	96.3
Over nutrition	0	0	1	3.7

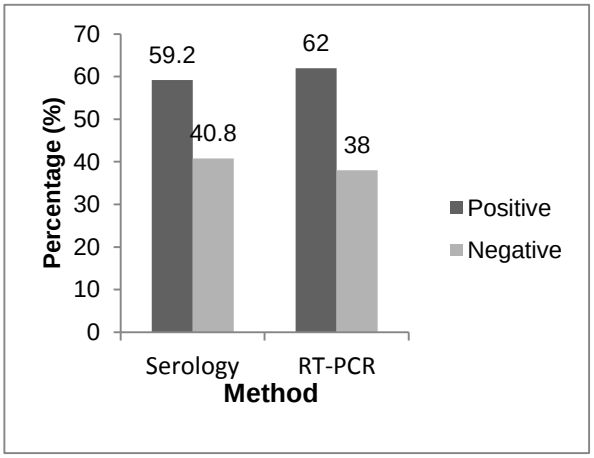

Figure 1: Distribution of Rotavirus diarrhea based on immunoassay and RT PCR Result.

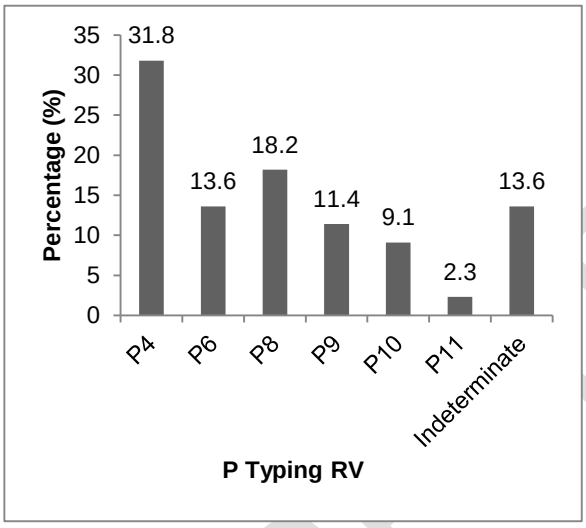

Figure 2: P Typing Rotavirus distribution from children with acute diarrhea.

DISCUSSION

Diarrhea related illness is a significant health problem in many different regions worldwide and RV is the leading cause of acute diarrhea in young children. Rotavirus infection is a common cause of acute diarrhea in pediatric especially in developing countries (Hassine-Zaafrane *et al*., 2011; John *et al*., 2014; Kargar *et al*., 2012). It is also the most common cause of non bacterial gastroenteritis in children both in developing and developed countries (Hassine-Zaafrane *et al*., 2011).

This study has revelaed 62.0% cases of acute diarrhea of children in Pekanbaru from January to July 2015 caused by RV. Previous study conducted in Sanglah Hospital, Denpasar Bali, Indonesia from April 2009 to December 2011 has also revealed that RV is the main etiologic agents of acute diarrhea, which is 49.8% cases of all acute gastroenteritis (Salim *et al*., 2014). The study in Surabaya Indonesia, conducted from April to December 2013 has revealed that 40% stools samples were positive for RV (Sudarmo *et al*., 2015). These results from various region in Indonesia are higher than the prevalence of RV attained in Tunisia (27.6%), and Saudi Arabia and Egypt (16% to 23%) (Hassine-Zaafrane *et al*., 2011). The different results are possibly related to the different method of sampling, analysis and seasons.

This study shows that the female group has a higher proportion of RV positive which is 54.5%. This finding was different from WHO scientific group that the number of affected males were up to 20% higher than females in some studies (Stebbins *et al*., 2007; Serchan *et al*., 2011). All of this finding does not reveal that female or male had greater susceptibility to RV infection or parents of affected sex group had more often seeking for medical care facilities.

In our study we found that children within the range of age 6-35 months are highly susceptible to both RV diarrhea (70.5%) and non RV diarrhea (63.0%). A similar result was obtained by a study in India involved children under 2 years conducted by John *et al.* (2014) and found that the most prevalent age for RV group is 6-15 months. Also a study by Sudarmo *et al.* (2015) indicated that most children who suffered from RV acute diarrhea were in the range of age 6-23 months (80.7%). Our study reveals the same result with the study conducted by Widowati *et al.* (2012) that in Jogjakarta Indonesia, the highest prevalence of RV infection found in children between 1-2 years (42.2%) and Sai *et al.* (2013) found in children between 13 to 36 months in Ji'nan China.

In our study, the children with exclusive breastfeeding (54.5%) and good nutritional status (97.7%) were the predominant in RV positive group. The same result was obtained by the study conducted by Sudarmo *et al.* (2015) in Surabaya which showed the majority of samples with RV positive were the children with exclusive breastfeeding and normal nutritional status. Our study also indicated that the majority of patients with RV infection had moderate dehydration (54.5%). The same result was obtained by the study conducted by Sudarmo *et al.* (2015) in Surabaya which also showed that most children with positive RV had moderate dehydration.

Our RV genotyping results showed the highest proportion was found in P4 genotype (31.8%), followed by P8 and P6 genotype, respectively 18.2% and 13.6%, P9 (11.4%), P10 (9.1%) and P11 (2.3%), also six samples (13.6%) were indeterminate genotype. These results were different with the study conducted by Doan which P8 genotype was the prominent (71.8%), followed by P4 (20%) and P6 (4.2%), and untypable strain for the P genotype was 1.5% (Doan *et al.,* 2003). The molecular characterization of Rotavirus group from various hospitals in Indonesia (Jakarta, Denpasar, Makassar and Mataram) in 2007 also showed that P8 was the predominant strain (39%) (Radji *et al.*, 2010). Another different result was obtained from a study conducted by Hassine-Zaafrane *et al.* (2011) in Tunisia that the P8 genotype was the predominant (80.8%) and Sai *et al.* (2013) found in Ji'nan China, P8 is also the common genotype (46.8%). The

similar result with our study was obtained by a study conducted by Sudarmo *et al.* (2015) in Surabaya in 2013 that the predominat type was the P4 genotype (31.8%). The two P genotypes (P4 and P8) have been reported as a significant etiology in the epidemiology of RV in human worldwide (Radji *et al.*, 2010, Santos *et al.*, 2005). These different results may indicate that the prevalence of different genotypes of RV may change periodically according to natural fluctuations (Sudarmo *et al.*, 2015).

In this study, we found that 13.6% samples are the indeterminate genotype. This kind of genotype is possibly a combination of P genotype with G genotype or mixture of human and animal P genotype. The same result was obtained from a study by Macedo *et al.* (2007) in Brazil, it showed there were 27.6% of mixture samples.

This study was limited to the P strain identification only. We did not involve the G strain identification yet, so that the combination of G and P strain were not identified. The identification of G strain will be conducted for the next study in order to find the predominant strain from the local area.

CONCLUSION

In conclusion, our study has done by involving children with acute diarrhea caused by rotavirus in Pekanbaru, Riau, Indonesia. The result shows that the RV infection was predominantly in female patient, children within the range of age 6-35 months are highly susceptible to both RV and non RV diarrhea. The majority of patients were children with exclusive breastfeeding and good nutritional status. The P4 genotype was the highest prevalence, followed by the P8, P6 and indeterminate genotypes. Further study is necessary to identify the G genotype and the combination of both genotypes in order to identify the prevalence genotypes of RV in Pekanbaru, Riau, Indonesia as well as design-based diagnosis antigen-antibody against RV.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

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