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Rotavirus Detection and the Characteristics of NSP4 as Enterotoxin to Induce Invagination in Children

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Abstract: Invagination is a disease occurs in children that requires emergency action. Factors associated with the occurrence of invagination can't be exactly determined because 95% of the cause of invagination is unknown (idiopathic), 5% for causal and rotavirus is estimated as one of the risk factors for the occurrence of invagination. **Methods:** After obtaining approval from the Ethics Committee of the Faculty of Medicine, USU, the parents of the patients were given a detailed explanation of the purpose / benefits of the research, and asked for the consent. Stool examination is divided into two groups which are invagination and diarrhea group. Stool samples are then examined in the laboratory by PCR to determine the presence rotavirus. **Results:** 83.3% overnourished children have invagination. Chi square analysis showed that there is no correlation between sex and ethnic group and invagination (p>0,05). 72.7% of Batak ethnic group has invagination and the rest 57.6% of the patient is not. There are 82.1% patients found positive with rotavirus type A whereas patients with diarrhea found as many as 17.9 %. There is a significant correlation between the incidence of rotavirus infection and invagination (p= 0.004). There was no significant relationship between NSP4 and invagination (p = 1.000). **Conclusion:** There is a significant relationship between nutrition status with incidence of invagination (p=0.034). The OR value is 1.542 (95% CI 1.074 – 2.214). This means that the children with overnutrition were at risk to have invagination 1.542 times more than the children with malnutrition. As many as 83.3% children with overnutrition suffer from invagination. No significant relationship between NSP4 and the incidence of invagination (p = 1.000). A total of 62.5% of children are found to have NSP4 invagination, whereas the group of children suffering from invagination with no founded NSP4 is about 63.8%.
Keyword : Invagination, Rotavirus, nutrient, Ethnic, NSP4.

Introduction

Invagination is a disease occurs in children that requires emergency action. Definite diagnosis for invagination in children is hard to be determined because the specific signs of invagination, Triad of Invagination, are not always found during history taking from the parents or physical examination.[1] Seventy five percents (75%) of cases was found at the age of under 3 years, 40% of them were found at the age between

4 and 12 months. Incidence of invagination was predicted to reach 1 in 2,000 children. Male patients dominate female with the ratio of 3:2 until 2:1.[2]

The result of a WHO research done in three main cities in Indonesia, published in 2000, showed that there were about 29 cases of invagination in Medan, found at the age of 2 months to 2 years and most of them were under 1 year (95%) with the sex ratio of male to female about 2:1. In the other cities, such as Jakarta and Yogyakarta, there were about 103 cases (86% were children under 1 year) and 35 cases (61% were children under 1 year) with the sex ratio of male to female about, respectively, 2:1 and 1:1.[3]

Research performed in Haji Adam Malik Medan National Hospital and Pirngadi General Hospital Medan in 2010 showed that the number of children diagnosed with invagination is about 49 children.[4] In 2007, a research was performed in Yogyakarta to determine causes of diarrhea where 90% of them were caused by rotavirus.[5] The observation performed in University Childrens Hospital, Basel, Switzerland in 2006 found that 25% cases invagination were preceded by diarrhea.[6]

Rotavirus is the most significant cause of diarrhea in infants and some animals. Rotavirus clearly infected mature enterocyte vili of the intestine.[7] The factors associated with invagination can't be exactly determined because 95% of the cause of invagination is unknown (idiopathic), 5% of known cause and rotavirus is predicted as one of the risk factors for invagination.[8]

Rotavirus had been detected in 3 (41%) patients with invagination. Even though only a few controlled research is performed, rotavirus infection causes lymphadenopathy and thickening of distal ileal wall in which those can be the cause of invagination. Because of that, rotavirus was thought to have associated with invagination in any cases.[9]

Rotavirus infected small bowel and cause severe diarrhea in children. Rotavirus infection often found in children under 1 year old as a risk factor for invagination.[10] Until this time, researches performed hasn't been showing fixed association between rotavirus infection with the incidence of invagination, except a case reported by dr. Nakagomi in Japan at the end of year 1970.[8] Rotavirus is composed of 11 segments. Each segment contains double-chained DNA, where each code has six structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and five non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5). Among those, NSP4 protein is a protein produced by rotaviral enterotoxin.[11,12] Natural toxic material of NSP4 and protein antigens likely involve in the development of the invagination with unknown additional interaction in host.

Diarrhea caused by rotavirus was first considered as malabsorption. Since 1996, various ideas had sprung up among statements that NSP4 had an important role in the secretion of fluids and electrolytes, therefore this presented a new viral enterotoxin secretion.[13] Incidence of invagination in children was one of the causes of morbidity and mortality in children.

Until now, the etiology and pathogenesis is not clear. However, some studies have reported that rotavirus enterotoxin (NSP4) potentially induces invagination in children. One of the alleged cause of invagination in children is rotavirus infection through the mechanism of viral enterotoxin (non-structural protein 4 (NSP4). The purpose of this research was to find a relation between rotavirus infection and the occurrence of invagination in children.

Material and Methods

The research design is observational analytic that assess the relationship between Rotavirus and invagination in children. This research is a Cross Sectional Study. The research is a Cross Sectional Study, performed by comparing the feces in children with invagination and rotavirus in children. The independent variable in this research is feces positive for rotavirus and dependent variable is invagination. The parameter used is gender and age.

The research was performed on October 2013-March 2014. The collecting research sample performed in any hospital such as Haji Adam Malik General Hospital, Malahayati Hospital, Mitra Sejati Hospital, Methodist Hospital, Santa Elisabeth Hospital, Sari Mutiara Hospital and Herna Hospital. Population of this research is children age from 1 month – 3 years old. Population is divided into 2 groups: Population I: Children age 1 month-3 years with suspected invagination. Population II: (Control) Children age 1 month-3 years with diarrhea.

Research sample is collected with inclusion and exclusion criteria in nominal data form. Sample for data analytic category uses the formula of *Sample Size Determination in Multinomial Logistic Regression* which is : *Rule of thumbs* : Sample is 10 times more than the amount of independent variable that is used in the research.[14] Because at this research there were 3 independent variable, so the large of sample: 30 minimum subjects.

Patients age 1 month – 3 years with suspected invagination and diarrhea are prepared to participate in this research. If invagination is found during surgery, then it fits on the inclusion criteria for the research. Patients with more than one diagnosis, present with congenital anomaly, refuse to participate in the research are excluded from the research.

Obtain a permit to perform the research from Health Research Committee of North Sumatera and hospital where the research take place. Obtain license and permission from the parents or next of kin of the children with suspected invagination and diarrhea who agreed to be the subject of the research. Through history taking with the patient's parents or next of kin, researcher filled the medical record that is used in the hospital where the research take place (name, age, sex, etc). Begin identification of children with suspected invagination (invagination triad) and diarrhea from age 1 month –3 years that visited the hospital. Perform physical examination in children with suspected invagination and diarrhea who agreed to be subject of the research. Feces collected from the patients with suspected invagination and diarrhea will then be examined using PCR method. Children with suspected invagination will undergo laparotomy exploration (invagination operation procedure)

Examination of faeces sample of patients with invagination and diarrhea with PCR

1. **Material and Tools that required:** Faeces was collected from children with suspected with invagination and children suffering from diarrhea. Then the specimens were taken up to be saved (vial) in clean and dry place. Faeces is taken about 1-2 ml or 1-2 mg. The sample should be brought to laboratory or should be saved in temperature of 2 to 8°C and must be examined within 48 hours (2 days), otherwise it should be frozen in temperature of -70 to -80°C.

2. **Working Methods:** Sample Preparation: Mix faeces with water smoothly. Take about 400 ul of sample (liquid faeces sample). Vortex for 15 second. centrifuge about 12,000 rpm for 10 minute. Take 140 ul supernatant into 1,5 mL mikro tube.

Extraction of RNA Viruses

Furthermore, viral RNA was extracted using the QIAmp Viral RNA Mini Kit (Qiagen, Cat Number: 52 904) with the following stages: 560 ul buffer AVL is added to the stool supernatant, then 6 ul of RNA carrier (Qiagen) is added then mix by Vortex for 15 seconds, incubate in the room temperature (15-25 ° C) for 10 minutes, centrifuge for 15 seconds to remove the pulp from inside of the lid , add 560 ul of ethanol (96-100%) , Mix by pulse-vortex for 15 seconds, centrifuge tube for 15 seconds to remove the pulp from the inside of the lid, apply 500 ul the solution to the QIAamp Mini spin column without wetting the rim, centrifuge tube with a speed of 8000 rpm, for 1 minute. Put QIAamp into a 2 ml clean collection tube (Discard the tube with filtrate), Repeat this step for better results and add 500 ul Buffer AW1, centrifuge the neck of the tube with the speed of 8000 rpm for 1 minute, Place the QIAamp neck 15th player to a 2 ml collection tube clean (Discard the tube containing the filtrate) , Add 500 ul Buffer AW2 , centrifuge tube's neck with a speed of 12000 rpm, for 3 minutes. Take the filtrate and place it in back to the neck of QIAamp Mini at the same tube (Discard the filtrate), centrifuge empty tube neck with a speed of 12000 rpm, for 1 minute, add 50 ul Buffer AVE, Incubate in room temperature for 2 minutes, centrifuge tube's neck with a speed of 12000 rpm, for 2 minutes.

Note: If not directly in RT-PCR, the results of elution can be stored at -800 C for no more than 5 days. (It is recommended to perform RT-PCR reaction on fresh samples).

RT-PCR Rotavirus A, B, and C

1. Duplex RT-PCR Rotavirus A and C (OneStep RT-PCR Kit, Qiagen; Cat 210210 (100 reaction). All of the conditions are prepared on ice. Before RNA was added into the RT-PCR mix, RNA is heated at 92^0C for 2 minutes, then immediately put into the ice [so double-stranded RNA remained separated], and then put RNA into the RT-PCR mixture). Primers:[15] Beg9 : 5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3'. VP7-1 : 5'-ACTGATCCTGTT GGCCATCCTTT-3'. G8NS1: 5'-ATTATGCTCAGACTATCGCCAC-3'. G8NA2 :

5'-GTT TCTGTACTAGCTGGTGAA C-3'. PCR Cycle: 50^0C (30 min), 95^0C (15 min), 94^0C (30 sec), 55^0C (30 sec) 40X, 72^0C (60 sec), 72^0C (7 min). The results of RT-PCR were analyzed on a 1.5% agarose gel: Positive Rotavirus A: 395 bp. Positif Rotavirus C: 352 bp.

2. RT-PCR Rotavirus B (OneStep RT-PCR Kit, Qiagen; Cat number: 210210 (100 reaksi). All conditions are prepared on ice. Before RNA is added to the RT-PCR mixture, RNA is heated at 92^0C for 2 minutes, then immediately put on ice [so single stranded DNA does not return to double stranded DNA], then put RNA into RT-PCR mixture). Primer [Gouvea *et al*., 1991; Sen *et al*., 2000]: Bl:-CTATTCAGTGTGTCGTG AGAGG 5'-3' B4: 5'-CGTGGCTTTGGAAAATTCTTG-3'. The results of RT-PCR were analyzed on a 1.5% agarose gel: Positive Rotavirus B: 489 bp

Statistical Analysis:

a. Descriptive Analysis

Data collection was done with a careful history taking and filling medical records of the study subjects who meet the inclusion and exclusion criteria until a minimum sample size is met. Subject characteristic data obtained from data collection includes: age, sex, results of stool examination (rotavirus +/-) and findings during operation (invagination +/-) then descriptive analysis is performed by determining the average value, standard deviation and prevalence ratio (PR).

b. Inferential Statistics

b.1. Univariate Analysis

Univariate analysis is the first step of the statistical analysis which is performed on each variables of the study results. In this analysis, all independent variables such as rotavirus, age, gender and the dependent variables findings during exploration laparotomy surgery (invagination +/-) are described.

b.2. Bivariate analysis

In this case we used the Chi Square test with 95% significance level. In this analysis there were two variables to be tested for the correlations, such as:

1. Children infected/not infected with rotavirus with findings of invagination/no invagination during operation.
2. Age o with the findings of invagination / no invagination during operations.
3. Gender with findings of invagination / no invagination during operation.

b.3. Multivariate analysis

Multivariate analysis is conducted on more than two variables, multivariate analysis selected is logistic regression. This analysis describes the correlation between:

1. The dependent variable/result → invagination (invagination type) with free variables /causes (independent variables) → rotavirus, age, gender.
2. The results of these observations were then tabulated.

Results and Discussion

This study was followed by 55 pediatric patients who have met the inclusion criteria. Of the 55 patients obtained 35 pediatric patients were found to suffer from invagination and 20 pediatric patients were diagnosed with diarrhea. The majority of respondents in both groups were boys, there were 25 children (65.8%) with the invagination and 13 children (34.2) with diarrhea. The mean age of patients with invagination is 6.74 months (SD = 5.04) and those with diarrhea is 6.35 years (SD = 2.98). Both groups of respondents are mostly patients with acute diarrhea, 52.7%. The most common invagination found was ileo-colica invagination. Found on 21 patients (38.2%) followed by ileo-caecal invagination on 6 patients (10.9%).

Table 1 Characteristics of Respondents

Characteristics	Group		p	OR	95% CI
	Invagination	Diarrhea			
Sex					
Male	25 (65.8)	13 (34.2)	0.620	1.118	0.415 – 4.362
Female	10 (58.8)	7 (41.2)			
Nutrition					
Over	15 (83.3)	3 (16.7)	0.034	1.542	1.074 – 2.214
Malnutrition	20 (54.1)	17 (4.9)			
Ethnic group					
Batak	16 (72.7)	6 (27.3)	0.252	1.263	0.856 – 1.864
Except Batak	19 (57.6)	14 (42.4)			

By using chi square test we found a significant relationship between nutrition status with invagination (p=0.034). The OR value was 1.542 (95% CI 1.074 – 2.214). This meant that the children with overnutrition were at risk to have invagination 1.542 times more than the children with malnutrition. As many as 83.3% children with overnutrition had invagination. Chi square analysis showed that there was no relation between sex and ethnic group with the incidence of invagination (p>0,05).

Table 2 Relationship of rotavirus infection with invagination

Rotavirus A	Group		p	OR	95% CI
	Invagination	Diarrhea			
+	23 (82.1)	5 (17.9)	0,004	1,848	1.172– 2.915
-	12 (4.4)	15 (55.6)			

Chi square analysis showed a relation between rotavirus with the incidence of invagination (p=0.004) , OR 1.848 (95% CI 1.172 – 2.915). That meant the children with rotavirus in faeces were found to be at risk of invagination about 1.848 times more than the children with no rotavirus found on their faeces.

Table 3 Relationship NSP 4 with invagination

NSP4	Group		p	OR	95% CI
	Invagination	Diarhea			
+	5 (62.5)	3 (37.5)	1.000	0.979	0.549 – 1.746
-	30 (63.8)	17 (36.2)			

By using the Fisher's exact test we obtained no significant relationship between NSP4 and the incidence of invagination (p = 1.000). A total of 62.5% of children were found to have NSP4 invagination, whereas the group of children suffering from invagination with no founded NSP4 was about 63.8%.

Conclusion

1. There is a significant relationship between nutrition status with incidence of invagination (p=0.034). The OR value is 1.542 (95% CI 1.074 – 2.214). This means that the children with overnutrition were at risk to have invagination 1.542 times more than the children with malnutrition. As many as 83.3% children with overnutrition suffer from invagination.

2. Chi square analysis shows that there is no relation between sex and ethnic group with the incidence of invagination (p>0,05).

3. In patients with invagination, there are as many as 23 children (82.1) with type A rotavirus positive whereas in patients with diarrhea, there are as many as 5 children (17.9). There is a significant relationship between rotavirus infection with the incidence of invagination (p = 0.004).

4. No significant relationship between NSP4 and the incidence of invagination (p = 1.000). A total of 62.5% of children are found to have NSP4 invagination, whereas the group of children suffering from

invagination with no founded NSP4 is about 63.8%.

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