นิพนธ์ต้นฉบับ

Comparative study on the results of rotavirus detection by Enzyme-Linked Immunosorbent Assay (ELISA) and Polyacrylamide Gel Electrophoresis (PAGE)

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Rotavirus is an important causative agent of acute gastroenteritis in infants. The diagnostic method used in the virus laboratory at Chulalongkorn Hospital is an enzyme-linked immunosorbent assay (ELISA) which detects only group A rotavirus. Other groups of rotavirus,such as group B and C, are also known to cause the disease in human but they can not be detected by ELISA. Therefore, polyacrylamide gel electrophoresis (PAGE) is the method of choice to diferentiate rotavirus into its group by determining the pattern of rotavirus RNA migration called electropherotype.

A total of 111 stool specimens were obtained from in-patients of the neonatal ward at Chulalongkorn Hospital and analyzed for the presence of human rotavirus by using both the ELISA and PAGE methods. There were 65 (58.6 %) specimens determined positive by ELISA and 57 (51.35 %) specimens determined positive by PAGE. Among the 57 PAGE-positive specimens,one specimen was from the 46(41.4 %) specimens with negative

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negative results by ELISA. In contrast, 9 specimens with positive results by ELISA were negative by PAGE. When the ELISA results were compared to those of PAGE, the correlation between the two methods was 91.0%. The electropherotype of rotavirus in 57 PAGE - positive samples was differentiated into 5 patterns: pattern 1,84.1% (48/57): pattern 2, 1.8% (1/57);pattern 3,1.8% (1/57);pattern 4,10.5% (6/57) and pattern 5,1.8 % (1/57). They were all group A will Long (L) pattern. Thus this study reveals that rotavirus group A is still the major cause of infantile diarrhoeal disease in Thailand.

Key words : Rotavirus, ELISA, Rotavirus electropherotypes, PAGE.

ปิยนท วิรัชศิลป์, ภาวพันธ์ ภัทรโกศล, วนิดา มั่งมี, วรรณา พรรณรักษา. การศึกษาเปรียบเทียบผลการตรวจหาไวรัสโรตาโดยวิธี Enzyme-linked Immunosorbent Assay และ Polyacrylamide Gel Electrophoresis. จุฬาลงกรณ์เวชสาร 2538 พฤศจิกายน ; 39(11): 823-831

ไวรัสโรตาเป็นสาเหตุสำคัญสาเหตุหนึ่งของโรคอุจจาระร่วงแบบเฉียบพลันในเด็กเล็ก วิธีการตรวจวินิจฉัยโรคที่ห้องปฏิบัติการไวรัสวิทยา โรงพยาบาลจุฬาลงกรณ์ คือ วิธี Enzyme-Linked Immunosorbent Assay (ELISA) ซึ่งตรวจได้เฉพาะไวรัสโรตาในกลุ่ม A เท่านั้น ไม่สามารถแยกไวรัสโรตาในกลุ่มอื่น เช่น กลุ่ม B และ C ซึ่งเป็นสาเหตุก่อโรคในคนได้ ดังนั้นวิธี Polyacrylamide Gel Electrophoresis (PAGE) จึงเป็นวิธีเลือกอีกวิธีหนึ่งในการตรวจหาเชื้อไวรัสโรตากลุ่มต่าง ๆ โดยดูรูปแบบการเรียงตัวของสารพันธุกรรมชนิด RNA ของไวรัสโรตา ที่เรียกว่า Electropherotype

ทำการศึกษาตัวอย่างอุจจาระผู้ป่วยภายในของโรงพยาบาลจุฬาลงกรณ์จากหน่วยทารกแรกคลอด จำนวน 111 รายที่ส่งตรวจหาไวรัสโรตาโดยวิธี ELISA และ PAGE ผลการตรวจพบไวรัสโรตาด้วยวิธี ELISA จำนวน 65 (58.6%) ราย และตรวจพบด้วยวิธี PAGE จำนวน 57 (51.35%) ราย ใน 57 ตัวอย่างที่ตรวจพบไวรัสโรตาด้วยวิธี PAGE มี 1 ตัวอย่างจาก 46 (41.4%) ตัวอย่างที่ให้ผลลบโดยวิธี ELISA แต่ในทางกลับกันพบว่า 9 ตัวอย่างที่ให้ผลบวกโดยวิธี ELISA ให้ผลลบ โดยวิธี PAGE เมื่อทำการเปรียบเทียบผลการตรวจทั้งสองวิธี คือ ELISA และ PAGE พบให้ผลสอดคล้องกัน 91% จากการศึกษา Electropherotype ของ RNA ที่ตรวจพบในตัวอย่างจำนวน 57 ราย โดยวิธี PAGE สามารถจัดแบ่งตามความแตกต่างได้ 5 แบบ โดยแบบที่ 1 พบ 48 (84.1%) ตัวอย่าง และแบบที่ 2 พบ 1(1.8%) ตัวอย่างแบบที่ 3 พบ 1 (1.8%) ตัวอย่าง แบบที่ 4 พบ 6 (10.5%) ตัวอย่าง และแบบที่ 5 พบ 1 (1.8%) ตัวอย่างทั้งหมดเป็นไวรัสโรตากลุ่ม A ที่รูปแบบเป็น Long (L)-pattern ในการศึกษานี้แสดงให้เห็นว่าไวรัสโรตากลุ่ม A ยังเป็นสาเหตุหลักที่ทำให้เกิดโรคอุจจาระร่วงแบบเฉียบพลันของเด็กเล็กในประเทศไทย

Rotaviruses have been found to be the most important cause of gastroenteritis among Thai infants and young children below 2 years of age.[1] Rotavirus is a member of the genus rotavirus in the family reoviridae. By electronmicroscopy (EM), the virus is seen to be about 70 nm in size and appears shaped as a wheel.[2] Rotaviruses are separated into several groups (group A-G) due to their antigenic differentiation. However, the most frequently isolated rotaviruses share the group A antigen, which can be classified into 2 subgroups (1 and 2) and at least 7 distinct serotypes by cross neutralization test.[3-6] There are many diagnostic methods used for detecting rotaviruses. The original gold standard test of EM to visualize rotavirus particles has been replaced by the enzyme-linked immunosorbent assay (ELISA) test for rotavirus-specific antigens. It was developed and is used in routine diagnosis at the Virology Laboratory Unit of Chulalongkorn Hospital.[7] Unfortunately, ELISA can not distinguish the other groups of rotavirus since it is specific to only the group A rotavirus. The application of polyacrylamide gel elec-trophoresis (PAGE) with silver stain to diagnose rotavirus infection offers attractive advantages.[8] By this technique, rotavirus-containing specimens analyzed on polyacrylamide gels reveal the characteristic rotavirus pattern of 11 segments of double-stranded (ds)-RNA. The identification of specific ds-RNA migration patterns, called electropherotype, is a special utility in epidemiological study. PAGE is

also a diagnostic test that distinguishes non-group A from group A rotaviruses.[9]

In this present study, comparison between the results of ELISA and those of PAGE for the detection of rotavirus in stool specimens was evaluated. Moreover, the electropherotypes of those PAGE-positive specimens were identified.

Materials and methods

Fecal Specimens

One hundred eleven fecal specimens from patients admitted to Chulalongkorn Hospital during the period of January to April, 1992 were examined for rotavirus antigens by ELISA and then kept frozen at $- 20°C$. Later, all 111 specimens were thawed and determined for RNA by PAGE.

ELISA Technique

Approximately 10% suspensions of stool were prepared in phosphate-buffered saline (PBS), pH 7.5. The suspension was mixed well and centrifuged at 1000 xg for 10 minutes. The supernatant fluid was then collected for ELISA antigen detection. The procedure for the ELISA test was as has previously been described.[7] In brief, rabbit anti-rotavirus antibody was coated on microtiter plates for 1.5 hours and washed five times, after which the supernatant fluid of each stool sample was applied. Normal rabbit serum was coated and run parallel to each sample as a control. After a two-hour incubation period at 37°C, the plates were washed and rabbit anti-rotavirus

conjugated horseradish peroxidase was added and then incubated for one hour.The plates were washed again and coloric development using o-phenylene diamine (OPD) and H_2O_2 as a substrate was carried out. The reaction time was 15 minutes; the reaction was stopped by 4 N H_2SO_4. The OD at 492 nm was determined. The sample was determined positive when the difference between the OD of sample in rabbit anti-rotavirus coated well and that of sample in normal rabbit serum coated well was equal to or greater than 0.2. This critical cut-off value was determined as described by Kurstak, E.[10]

PAGE Technique

The method has already been described in Werasakaumpai et al.[11] Fifty grams of stool or 0.3 ml of watery stool were mixed with 0.5 ml of 0.1 M sodium acetate containing 1% sodium dodecyl sulfate (SDS), pH 5.0. An equal volume of a 3:2 (vol/vol) phenol-chloroform mixture was added to each specimen and mixed for 1 minute. The mixture was then centrifuged at 7000 xg for two minutes. The upper layer, which contained the RNA extract, was removed and 40 ul of it was mixed with 10 ul of sample buffer (0.5 M Tris, pH 6.8, 20% glycerol, 0.1% bromphenol blue) before being applied to the gel for electrophoresis. Electrophoresis was done in vertical slab gel electrophoresis. A discontinuous system was used with Tris-glycine reservoir buffer, pH 8.3 and 3% acrylamide staking gel with 10%

separating gel. The plate was 19 cm long by 16.5 cm wide with 12 wells spaced 0.75 mm apart. Electrophoresis was carried out at 160 volts for 16 hours at room temperature.

Silver Staining

The separation of ds-RNA in slab gel was visualized by silver staining.[12] The gel was fixed in 10% ethanol with 0.5% acetic acid for 30 minutes, then immersed in 0.011 M silver nitrate for 30 minutes and rinsed twice with distilled water. After washing, developing solution (0.75 M NaOH containing 0.1 M formaldehyde) was applied to the gel for 5-10 minutes or until discrete bands could be seen. The reaction was stopped by pouring 5% acetic acid over the gel. For long-term storage, the gel should be transferred to 0.07 M Na_2CO_3.

Results

The 111 fecal specimens were tested for the presence of human rotavirus by the ELISA and PAGE methods. The results are shown in Table 1. Sixty five (58.6%) out of the 111 specimens were determined positive by ELISA and 46 (41.4%) samples were ELISA negative. Among the 65 ELISA positive samples, 56 (86.2%) samples were PAGE-positive and 9 (13.8%) samples were PAGE-negative by PAGE method. Within the 46 ELISA - negative samples, it was found that PAGE revealed one positive sample (2.2%).

Table 1. The results of 111 fecal specimens determined by ELISA and PAGE.

PAGE	ELISA		Total
	Positive	Negative	
Positive	56(86.2%)	1(2.2%)	57
Negative	9(13.8%)	45(97.8%)	54
Total	**65(58.6%)**	**46(41.4%)**	**111**

The PAGE technique's results are able to differentiate rotavirus into group and also electropherotypes due to the migration of 11 ds-RNA fragments.[8] Therefore, in this present study, 57 specimens determined positive by PAGE were shown to be rotavirus group A according to the pattern of ds-RNA electropherotypes which is 4-2-3-2 (Fig. 1).[13,14] They were all classified to be Long (L)-electropherotype with 5 distinct patterns (Fig 1). The most prominent was pattern 1, 84.1% (48/57) and followed by pattern 4, 12.5% (6/57) (Table 2). The rests were detected in only 1 (1.8%) sample each (Table 2).

Table 2. Electropherotypes of rotavirus group A.

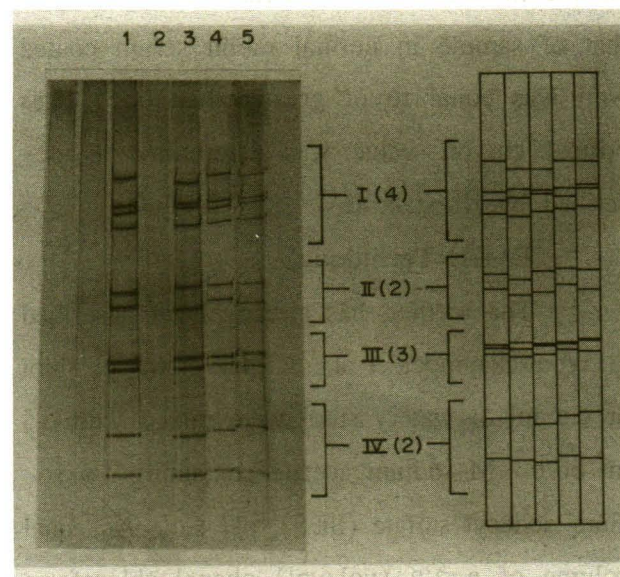| Electropherotypic pattern	No. of specimens.
1	48 (84.1%)
2	1 (1.8%)
3	1 (1.8%)
4	6 (12.5%)
5	1 (1.8%)
Total	57 (100%)

Figure 1. Schematic patterns of migration of 11 segments of ds-RNA of rotavirus. Lane 1-5 : 5 patterns of electropherotype of rotavirus group A with long (L)-pattern. Group I-IV with the numbers of migrated RNA (in parenthesis) are indicated.

Discussion

Human rotavirus infections are mostly caused by rotavirus group A which is known to cause acute gastroenteritis in infants 6-24 months of age.[1] Group B rotaviruses are much

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การศึกษาเปรียบเทียบผลการตรวจหาไวรัสโรตาโดยวิธี Enzyme-linked
Immunosorbent Assay และ Polyacrylamide Gel Electrophoresis.

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less common but have been responsible for some extensive waterborne outbreaks in China involving adults as well as children.[9] Group C rotaviruses occur mainly in pigs and affect human only occasionally.[15] The main diagnostic test in Thailand is based on commercial available reagents which can determine only rotavirus group A. At the Virology Laboratory Unit of Chulalongkorn Hospital. Punnarugsa V and Vutayakorn J[7] developed in-house ELISA procedures for detection of rotavirus antigens in stool specimens. The principle of the test is double-antibody sandwich method controlled by using normal rabbit immunoglobulin fraction instead of rabbit anti-rotavirus antibody as coating antigens. This ELISA procedure has been shown to detect only rotavirus group A. Its sensitivity is 97.7% and specificity is 96.1%.[7] The PAGE technique is a useful method in epidemiological studies but it is claimed to be a practical and informative diagnostic test.[8] The technique is quite laborious and requires specific apparatus. This PAGE technique proved to be more sensitive in detecting rotavirus in human stools than either the EM or ELISA procedures.[8]

In the present study, we compared the results from both ELISA and PAGE methods on 111 stool specimens. The results revealed that the ELISA technique could detect rotavirus in 65 specimens whereas PAGE detected it in 57 specimens (Table 1). One ELISA - negative result was proved to be false negative by PAGE. This might occur due to the presence

of a blocking factor in the stool specimen.[12] In addition, we could not conclude that 9 ELISA-positive specimens were false positive due to the negative result by PAGE (Table 1). The possible explanation for the 9 ELISA-positive specimens being false negative was that the specimens contained only a small amount of RNA, or during the preparation of RNA extraction, those viral RNA were somehow degraded. However, the correlation of results from both ELISA and PAGE was 91%. In 1992, we reported that the PAGE method could detect only 29 specimens from 35 ELISA-positive results which was a correlation rate of 82.86%.[11] Here, the same methods were used and revealed that 56 out of 65 (86.2%) ELISA-positive results could also be detected by PAGE. PAGE is more specific than ELISA while ELISA seems to be more sensitive than PAGE. However, the specificity and sensitivity of both ELISA and PAGE in this study cannot be calculated because the accepted standard method for detection of rotaviruses is detection of viral particles by EM which cannot be done.[2] Recently, Ginevskaya et al also reported that the PAGE technique detected 85.5% of ELISA-positive samples.[6] According to our data and Ginevskayas report, it seems that ELISA is more sensitive than PAGE.

The PAGE results revealed 5 electropherotypes from 57 specimens (Fig.1). All these specimens were collected between January and April of 1992. All of those isolates

were group A rotaviruses with Long (L) pattern. The electropherotypic patterns from the fecal specimens of patients admitted to Chulalongkorn Hospital during 1989-1991 were also shown to be rotavirus group A with 2 distinct L-patterns and 1 Short (S)-pattern.[11] Unfortunately, this study can not compare whether the 2 L-patterns were similar to any of the 5 L-patterns due to the lack of specimens. The different electropherotypes of rotavirus has been suggested to correlate with the severity of the disease.[8] Here, the relationship between electropherotype pattern and the severity of disease could not be done due to the lack of the patients clinical information.

Recently, the development of polymerase chain reaction (PCR) and other methods for the amplification of viral nucleic acid has remarkably enhanced the sensitivity of rotaviral detection and analysis. Most application of this technique is mainly for epidemiological study especially in serotyping[16] and molecular genetics.[17] Although PCR is proved to be most sensitive method when compared to ELISA and PAGE[17], the main drawback remains the cost of test and false positive results.[18] PAGE technique has advantage in ability to studying the epidemiology of the rotavirus and the detection of the other groups of rotavirus which can cause the diarrhoeal disease. But, PAGE requires more specific apparatus, takes more time to conduct, and must be performed by skilled technicians. Thus, both PCR and PAGE are not practical to use for routine diagnosis

of rotavirus in the hospital. Since this study shows that diarrhoeal disease in Thailand is usually caused by rotavirus group A, ELISA, being specifically intended for rotavirus Group A, is therefore still the most efficient method for laboratory diagnosis.

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