ORIGINAL ARTICLE

Rotavirus RNA Electropherotype in Different States in Malaysia for the Year 2000 and 2001

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Summary

A total of 157 stool samples were examined for Group A rotaviruses in diarrheic children admitted to 8 different major hospitals in Malaysia. The overall incidence rate in this study was 19.7% (31 of 157) with a variation of 9.5% to 39.1% in different locations. Majority of the infections detected were in those under 2 years of age and there were fewer admissions in the older age group. The stool samples were initially screened for rotavirus Group A by latex agglutination method and followed by RNA electrophoresis. The size and the characteristics wheel-shaped morphology of the viral preparations when examined by electron-microscopy further confirmed the presence of rotaviruses in the positive stool samples. Analysis of the RNA pattern showed that majority of the isolates, 51.6% (16 of 31) were Type IIC ('long' with comigration of RNA segments 7 and 8), 35.5% (11 of 31) with Type IIG ('long' with comigration of segments 7,8,9), 9.7% (3 of 31) with Type IG ('short' with comigration of RNA segments 7,8,9) and 3.2% (1 of 31) of mixed or atypical pattern. It appeared that over a 12 year interval, only one new or unusual rotavirus electropherotype was found. This is the first comprehensive report on the electropherotypes of rotaviruses covering eight different geographical locations in Malaysia and the data obtained is useful for understanding the geographic distribution and types of rotaviruses transmitting in Malaysia.

Key Words: Group A rotavirus, Latex agglutination, Electron-microscopy, RNA-PAGE, Electropherotype

Introduction

Rotavirus is an important causative agent of acute gastroenterities among infants and young children , adults, immunocompromised individuals and also widespread in many species of domestic and wild animals[1]. Rotavirus belongs to the family Reoviridae, non-enveloped with a triple-layered icosahedral protein capsid and a genome of 11 double stranded RNA segments[2,3]. Rotaviruses have been classified into 7 groups, A to G,[4] based on the inner capsid protein, VP6. VP6 (encoded by the 6th RNA segment) also determines the subgroup specificity[5]. Rotavirus group A cause the majority of gastroenterities in humans and group B in human adults in China[6], while viruses in groups D, E, and F have so far only been found in animals[7]. Group C were first identified in pigs but recently it has been associated with outbreaks in humans[8]. The outer capsid proteins VP7 (major glycoprotein encoded by gene 7, 8 or 9) and VP4 (minor protease-sensitive spike protein encoded by gene

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4) induce neutralizing antibodies in infected individuals which have now been used to classify rotaviruses into G (VP7) and P (VP4) serotypes[9,10]. The group A rotaviruses have been classified into 14 G serotypes[11] and at least 11 P serotypes[12].

Besides identification of subgroup and serotype specificities, many studies on RNA migrations by PAGE for preliminary characterization of rotavirus isolates have been reported in Scotland[13], Indonesia[1], Hong Kong[14], Malaysia[15,16], Australia[17,18], India[19,20], Italy[21], Thailand[22], Kuwait[23] and Japan[24]. All group A rotavirus either exhibit a 'long' or 'short' pattern RNA pattern on electrophoresis depending on the fast or slow migration pattern of segment 11, respectively[25]. It has been observed that the majority of human rotavirus with subgroup I specificity have a 'short' or 'supershort' pattern whereas those with subgroup II specificity have a 'long' pattern[1,5,18]. Recently several strains of human rotavirus that exhibit subgroup I specificity but long pattern (properties that are typical of animal rotavirus), and other atypical associations between subgroup, serotype and electropherotype (e-type) have been described[5,19, 20,26,27,28,29].

We report here the first analysis of the rotavirus strains detected in eight different geographical locations throughout Malaysia. The distribution and diversity of RNA e-types were examined to see for any new or unusual strain that may have emerged.

Materials and Methods

Clinical specimens

Over a period of 10 months (September to November 2000 and January to July 2001), 157 stool specimens were collected from children aged up to 5 years. These children with acute gastroenterities were admitted to 8 government hospitals in various locations in East and West Malaysia namely, Hospital Umum Sarawak, Kuching, Hospital Miri, Sarawak, Hospital Kuala Lumpur, Hospital Seremban, Hospital Kota Bahru, Hospital Kangar, Hospital Malacca and Hospital Sultanah Aminah, Johor Bahru. The specimens were stored at –20°C until tested.

Detection of Rotavirus Group A antigen

Human Rotavirus group A were detected by latex agglutination (LA) method (Diarlex Rota kit, Orion Diagnostica, Finland) following the manufacturer's protocol. Diarlex Rota[R] is a rapid test based on the agglutination of dry spot latex.

Electron microscopy

Stools were examined by direct negative staining electron microscopy using a 10% stool suspension, placed onto Formvar-coated 400 mesh copper grid, stained with 2% methylamine tungstate (Bio-Rad) and viewed by H-7110 scanning system.

Extraction and detection of rotavirus RNA by PAGE

For RNA extraction, 0.25 g stool or 200ul of 10% stool homogenate in PBS was suspended in a microcentrifuge tube containing 0.5 ml of 0.1 M sodium acetate buffer, pH 5.0 and 1% w/v sodium dodecyl sulfate. An equal volume of a 3:2 phenol chloroform was then added. The mixture was shaken vigorously for 1 min on a vortex mixer and centrifuged at 6,000 rpm for 2 min. The clear upper aqueous layer containing double-stranded RNA was removed and kept frozen at –20°C[22,17]. For RNA electrophoresis, 40µl of RNA was mixed with 10µl sample buffer (0.5 M Tris pH 6.8, 25% v/v glycerol and 0.1% w/v bromophenol blue)[30] .

A 10% separating gel and 4% stacking gel without sodium dodecyl sulfate in all the buffers were casted in a Mini-PROTEAN II Dual slab cell to form a slab gel of 80 mm width, 85 mm long and 1.0 mm thick. Only 25µl of the RNA mixture was loaded into each well for electrophoresis at 100 V for 2 h. The gel was then stained by ethidium-bromide and photographed using the Bio-Rad Gel Doc 2000.

The variation in RNA migration pattern was compared with reference strains representing the 'short' (I pattern) or 'long' (II pattern). The RNAs that yielded identical patterns will be co-electrophoresed before confirming that the patterns are similar.

Reference RV Strains

For RNA-PAGE, the following reference strains were used in our study: DS-1 (subgroup I, serotype 2), Wa (subgroup II, serotype 1), P (subgroup II, serotype 3), ST3 (subgroup II, serotype 4), 69M (subgroup 1, proposed serotype 8), W161 (subgroup II, proposed serotype 9). These strains were provided by Dr Jon R Gentsch, Centre of Disease Control and Prevention, Atlanta, GA.

Results

Detection of Rotavirus by EM

Of the 7 samples that were antigen positive, 3 samples were further confirmed to contain rotavirus by electron microscopy. Figure 1 showed the distinct characteristics of rotavirus particles in the positive samples. It was noted that a shorter time was taken to locate these particles in samples that strongly agglutinated in the LA test and longer time to search for the rotavirus particles in those that were weakly positive. Thus, there seems to be some degree of correlation between the intensity of the reaction in the Rota[R] test and the number of rotavirus particles observed, however this was not proven statistically.

Incidence of Rotavirus infection in different age groups

The incidence of rotavirus infection in the different age groups is shown in Table I. The largest number of admission for diarrheal episode was seen in the less than 2 years age group with fewer admissions in the older children.

Distribution of Rotavirus in different hospitals in Malaysia by latex agglutination and RNA-PAGE

Figure 2 showed the e-types isolated at different locations in the study areas. The RNA segments 1 to 4, 5 and 6, 7 to 9 and 10 and 11 or the 4:2:3:2 migration pattern were seen in all the positive cases. Tam's classification method[14] was used based on the relative migrations of segments 10 and 11 and comigration of the other segments, whereby, pattern I with slower migration or pattern II with faster migration of segments 10 and 11. The I and II patterns were then subdivided into A when all the 11 segments were resolved, B, C, or G when the patterns showed comigration of segments 2 and 3, 7 and 8, or 7 to 9.

The distribution of the e-types and the rotavirus incidence rate are shown in Table II. The IIC e-type were detected in all the locations except Malacca and Johor Bahru. Type IIG was not detected in Kangar and Kota Bahru. Two and 1 IG e-type were detected in Kuala Lumpur and Johor Bahru, respectively. One mixed e-type was detected in Kuala Lumpur.

Table I: Incidence rate of Rotavirus group A by latex agglutination, RNA PAGE and electropherotypes in different locality

Place	No. of samples tested	Diagnostic method		Incidence (%)	Electropherotypes			
		LA[2] +	PAGE +		IIC	IIG	1G	Mixed
Kuching[1]	25	4	4	4 (16.0%)	3	1	-	-
Miri[1]	32	3	3	3 (9.4%)	2	1	-	-
Miri	19	ND	5	5 (26.3%)	2	3	-	-
Kuala Lumpur	23	9	9	9 (39.1%)	4	2	2	1
Kangar	7	2	2	2 (28.6%)	2	-	-	-
Kota Bahru	6	2	2	2 (33.3%)	2	-	-	-
Seremban	11	2	2	2 (18.2%)	1	1	-	-
Malacca	21	2	2	2 (9.5%)	-	2	-	-
Johor Bahru	13	ND	2	2 (15.4%)	-	1	1	-
Total	157	24 (32 ND)	31	31 (19.7%)	16 (51.6%)	11 (35.5%)	3 (9.7%)	1 (3.2%)

+, strong agglutination in the LA and typical rotavirus group A RNA profile in PAGE

ND, latex agglutination not done but identified by RNA PAGE

[1] Samples collected in Sep-Nov 2000 in Kuching and Miri (all the other samples were collected in the year 2001)

[2] A total of 34 samples that form non-specific agglutination in the LA test were negative by RNA-PAGE

Table II: Incidence of Rotavirus infection according to age group and corresponding electropherotypes

Age	Total no. tested	No. RV positive (%)	Electropherotypes			
			IIC	IIG	IG	Mixed
0- 6 months	49	6 (12.2 %)	5	1	-	-
7-12 months	48	11(22.9%)	4	5	1	1
13 mths - 2 year	33	6 (18.1%)	2	4	-	-
25 mths - 3 year	16	6 (37.5%)	3	1	2	-
37 mths - 4 year	8	2 (25.0%)	2	-	-	-
49 mths - 5 year	3	0	-	-	-	-
Total	157	31 (19.7%)	16 (51.6%)	11 (35.5%)	3 (9.7%)	1 (3.2%)

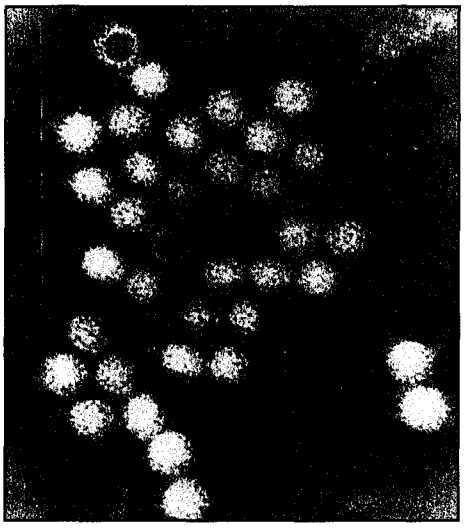

Fig. 1 : Rotavirus particles prepared from a positive sample as seen by electron microscopy 60,000 x magnification). This virus is approximately 70 nm in size and has a characteristic wheel-like appearance.

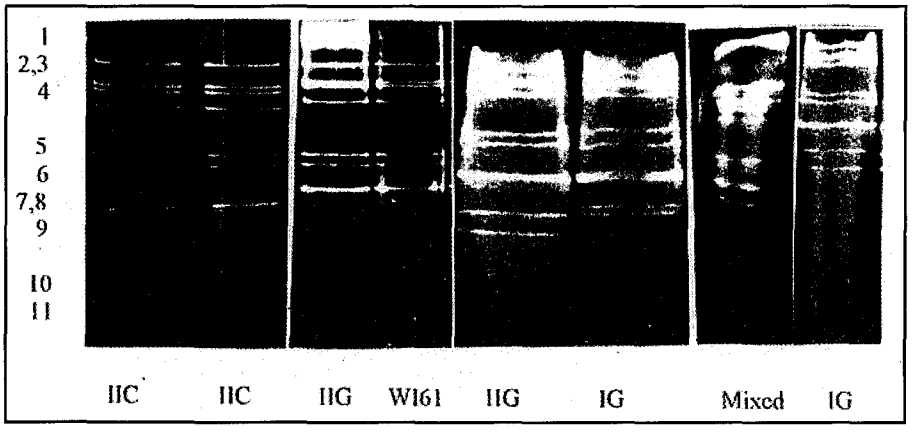

Fig. 2 : Comparative genome profile of Malaysian group A human rotavirus isolates and reference strain WI61. The position of RNA segments 1 through 11 are indicated on the left and the type of electropherotypes at the bottom of the gel.

Discussion

The occurrence of human rotavirus infections has been recorded continuously in Malaysia since 1983 and it has continued to cause outbreaks[15,16,31]. The isolation rates varies from 12% to 40% [31,16] and from 5.7% to 83.8% [15] and 19.7% in the present study. In comparison, a 28.5% in Hong Kong and 29.8 % in Bangkok were reported in 1985 to 1987[14,22].

Most infections occurs between 6 to 24 months of age, similar to other findings[14,32,33]. From Table I, it appears that there was no association of age and e-type, as reported by others[34]. Yap et al. (1992)[16] reported that children infected by 'short' e-type were significantly older than those infected by long pattern . Barker[35] reported an association of 'short' e-type with hypotonic dehydration and lethargy with 'long' e-type. A few recent report linking rotavirus virulence with gene segment 4 confirmed that subgroup specifity or e-type and severity of disease are unlikely to be linked[36,37,38].

In the RNA analysis, a replacement of one e-type by another e-type was noted. Between 1988 to 1989, it was shown that the predominant e-type was IIA (70.0%)[16]. This 'long' pattern strain was different from the IIC predominant strain detected from 1977 to 1988[15]. In our study we noted that the dominant type was IIC, thus indicating a cyclic change in the e-types.

The presence of a single predominant e-type in a given rotavirus epidemic and that the 'long' e-type is still dominating agrees with similar studies carried out elsewhere[22,18,23,26].

The co-circulation or replacement of one e-type by another e-type have been reported in other parts of the world[13,17]. This cycle varied from 2 to 3 years[17,18], 6 years[39] , 10 years[15] and 12 years in the present study. We could not explain why the cyclic changes were longer than that reported elsewhere. Since this is the first e-type study conducted in seven different locations in Malaysia, we therefore could not comment whether a similar shift occurred as in Kuala Lumpur. We noted that there was no Type IIC in Malacca and Johor Bahru. In Miri and Kuching no 'short' e-type was found. This finding was different from the previous study which showed a 72% of 'short' e-type in Sibu, Sarawak[35]. Generally, the short e-type were detected in every rotavirus outbreak but with much lower incidence[15,23,16].

The association of e-types and serotypes are rather conflicting. Some reported as no clear association of e-types and serotypes[25,34] whilst some authors have associated the 'short' e-type with serotype 2 and 'long' e-type with serotypes 1, 3 and 4[1,2,5,18,19]. Earlier work reported that the G4 predominated in Kuala Lumpur, Penang, Eastern Selangor and Kuching[40]. Here we did not report the serotype analysis of all the positive cases yet. As such, a correlation between e-type and serotypes cannot be presented. At present we are in the process of determining the G and P serotypes of all the 31 positive isolates in this study.

The electrophoresis of RNA is invaluable for the identification of group A rotavirus or atypical strains. The latex agglutination and RNA-PAGE were found to be equally sensitive.

In this study, the detection of Group A rotavirus by latex agglutination, electron-microscopy and RNA-PAGE showed no ambiguity. The combination of several techniques avoids the problems posed by some reports of rotaviruses without the group antigen[41] hence the basis of grouping and RNA electropherotyping should not be disregarded. Thus, our findings together with the work of previous authors and other global studies underscores the importance of continuos monitoring of circulating serotypes. Further molecular analysis of the strains should therefore be initiated.

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