Molecular Characterization and Epidemiology of Rotavirus Isolates Obtained from Children with Diarrhoea in Malaysia

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SUMMARY

This retrospective study examined the G/P type of rotavirus in RNA samples that have previously been e-typed by RNA-PAGE in 1996. The results were then compared to 2007 samples to ascertain the extent of changes that may have occurred in this 11-years time interval. The G and P genotypes were determined by hemi-nested PCR and further analysed by phylogenetic study. In 1996, the G/P combination G1P[8], G^{UT}P[8] and G1P^{UT} prevalence rate were 81%, 9% and 7%, respectively. As expected, the G9 genotype which has already emerged worldwide was identified in 42% of the 2007 samples with the remaining 33% G1P[8] and 25% G1P^{UT} Analysis of the RNA pattern showed that majority of the isolates were long e-type in both series, nevertheless minor differences within electropherotypes were observed. Genetic diversity in some strains of the human group A rotaviruses was analysed by phylogenetic methods. These findings will help in the decision to introduce rotavirus vaccines within the next decade.

KEY WORDS:
Group A rotavirus, G and P genotypes, Phylogenetic study

INTRODUCTION

Rotavirus (RV), from the family Reoviridae, is one of the leading causes of gastroenteritis among children less than 5 years of age with an estimated incidence of 27 per 10,000 population in Malaysia[1]. VP4 and VP7 (outer capsid proteins) are involved in virus neutralization, carry the serotype and genotype specificities and are implicated in the current dual classification of RV. The antigenic specificity carried by VP7 is termed the G serotype (for glycoprotein), and that by VP4 the P serotype (for protease-sensitive protein), respectively. Natural infection with RV as well as vaccine-induced immunity results in a homotypic protection. The success of rotavirus vaccines, therefore, depends on the antigenic similarities of both the G and P serotypes of vaccine strains with those of human rotaviruses circulating in nature[2].

Surveillance and continuous monitoring of RV serotypes will identify whether changes occur in serotype incidence and thus will increase awareness of emergence of rare or uncommon types[3]. In Malaysia, diarrhea was one of the top five leading causes of death in children aged 1-19 years from 1970 to 1980[1,4-7]. Throughout the world, four RV strains have been described: G1P[8], G2[P4], G3P[8] and G4[P]8, and are frequently recovered from children with diarrhea[8,9]. In Malaysia from 1977-1988, 71% G4 was reported[10], but recently 73% G9 were reported[1]. However, the distribution of G and P serotypes of human RV has not been documented routinely in Malaysia.

The objectives of the study was to characterize the strains of RV obtained from two different locations during different outbreaks in Malaysia and determine its epidemiology. The results obtained were compared with previous work done in this region.

MATERIALS AND METHODS

Viruses

This study was done at WHO Collaborating Centre for Child Health, Asian Rotavirus Surveillance Network Reference and Training Laboratory, Murdoch Childrens Research Institute (MCRI), Melbourne, Australia.

A total of 93 RNA samples from diarrhoeic children at Kuala Lumpur Hospital from April to December 1996, kept frozen after RNA-PAGE processes were used in this study. The 2007 samples (n=15) were collected from Hospital Batu Pahat, Johor and the stool specimens were tested for RV antigen by the latex agglutination test (LAT). All samples were brought to MCRI for analysis in proper cold-chain system and frozen until processed. Human rotavirus strains RV4 (long e-type,G1P[8]) and RV5 (short e-type, G2P[4]) were used as reference strains for RNA-PAGE.

Viral dsRNA and PAGE

Viral dsRNA from the 2007 series were extracted using 20% w/v fecal suspension in 0.01M Tris solution with NaCl and $CaCl_2$ and phenol-chloroform mixture and hydroxyapatite[11]. The eluted dsRNA were then transferred to fresh sterile tubes and frozen until further use. The RNA extraction for the 1996 samples were earlier performed following the method of Roger and Holmes[12].

All 15 extracted dsRNA from 2007 were electrophoresed using Laemmli's discontinous PAGE without SDS. Electrophoresis was done in the cold room using a 10% separating gel and a 4% stacking gel at 13mA for 16-18 hours. At the end of electrophoresis, the gels were stained with silver nitrate.

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G and P typing

The dsRNA extracted sample was used as the template for RT to synthesize cDNA copies from both strands by PCR. Full length gene 9 was amplified with primers VP7F and VP7R and G typed with primers VP7R and G1-G9[13]. These same samples were also P genotyped by using P specific primers VP4F and VP4R and P typed with primers VP4F and P mixture containing primers [P4], [P6], [P8], [P9], [P10] and [P11][14].

Ten microlitres of each PCR product were electrophoresed on a 1.2%-2% agarose gel (FMC Seakem) with suitable size marker in 0.5x TBE buffer, stained with ethidium bromide, then viewed and photographed on a UV transilluminator.

Rotavirus Sequencing and Phylogenetic Analysis

Three G1 samples were selected for sequence analysis: one from 2007 (coded as 21) and two from 1996 (coded as 137 and 105). The amplicons from full length VP7 DNA were electrophoresed in a low-melting point agarose gel, subsequently excised and extracted using the QIAquick gel extraction kit (QIAGEN). Cycle sequencing was carried out using the BigDye 1.1 kit with primers utilized in the PCR. The reactions were run on a 5100 ABI sequencer at University of Melbourne, Australia and the sequences analyzed using the sequencing program. Related sequences were obtained from public databases using the BLAST algorithm and an alignment was made with the ClustalW algorithm.

RESULTS

Detection of RV by LAT and RNA-PAGE

An electropherotype (e-type) profile (4-2-3-2) of rotavirus group A was demonstrated by PAGE in samples positive by LAT. Eleven of 15 (73%) tested samples from the 2007 series were positive by both assays. Most of the rotavirus strains detected by PAGE showed the long e-type (Fig. 1).

Hemi-nested PCR for Rotavirus G and P typing

Amplification for rotavirus G type (whole VP7 gene) with 1,062 bp and a fragment of the VP4 gene (865 bp) by RT-PCR were obtained (data not shown). It is interesting to note that all the 1996 RNA samples that have been kept for 11 years after examination for RV e-types could also be PCR-typed. None of these samples were G or P typed by PCR. In the earlier RNA-PAGE analysis, predominantly long e-type (94%) were reported[12]. Out of 93 samples, 20 were long e-types based on RNA-PAGE done at MCRI. It was observed that the variation in the long pattern was less compared to the 2007 RNAs (data not shown). One sample that was G^{UT}P[4] has a short e-type. All the G^{UT} and P^{UT} that was RNA-PAGE positive were re-amplified again for final confirmation. From the repeats, a few more positives were obtained and thus confirmed that only 9 (9.6%) cannot be G typed and 7(7.5%) cannot be P-typed.

For 1996 samples the following results were obtained: 81% G1P[8]; 9% G^{UT}P[8]; 7% G1P^{UT}; and 1% G2P[4], 1% $G^{UT}P^{UT}$ and 1% G3P[8]. For the 2007 samples, 42% G9P[8], 33% G1P[8] and 25% G1P^{UT} were obtained (Table I). Table I shows the results from this present study compared to studies done by other workers.

Phylogenetic analysis

The phylogenetic relationships of the VP genes among the rotaviruses were analyzed (Fig. 2). The dendogram of the VP7 genes of the three RV strains showed that they form the same lineage as G1 reference strain Wa. Strain 105 is clustered and very closely related to Bangladesh strain. Meanwhile, strain 137 is closely related to Thailand strain, and strain 21 is closely related to Taiwan strain. Other samples were not sequence due to insufficient extracted RNA samples. Rotavirus strains coded as Local (RP), Local (15) and Local (67) were sequences obtained from another study and were included to show its relationship with this present study. The results showed that strains 105, 137 and 21 were G1 and this result concurs with PCR typing. For samples RP, 15 and 67 no genotyping was carried out but from the phylogenetic analysis, it was confirmed as G4.

DISCUSSION

Gel electrophoresis, PCR and phylogenetic analysis have been widely used in analysis of diarrhoeal rotavirus samples all over the world. These techniques are important tools to rapidly reveal changes in migration of any of the eleven RV segments. RNA-PAGE does not, however, reveal the nature of the mutational event, which may be from a point mutation, recombination, genome rearrangement or segment reassortment[16]. Besides "long" and "short" e-types (differing in the rates of relative migration of RNA segments 10 and 11) various minor differences in the migration of corresponding segments have been recognized[17]. These differences have

Table I: Rotavirus strains identified in 1996 and 2007 compared to the strains obtained in studies from 1977-2003

RV strains	Present study		Other studies		
	1996	2007	1977-1988[10]	2000-2001[15]	2001-2003[1]
Common					
G1P[8]	81%	33%	14%	6.5%	3.8%
G3P[8]	1%	0%	3%	3%	0%
G4P[8]	0%	0%	71%	65%	0%
G2P[4]	1%	0%	0%	0%	4.5%
Uncommon					
G9P[8]	0%	42%	0%	0%	73.3%
G1P^{UT}	7%	25%	0%	0%	1%
G^{UT}P[8]	9%	0%	11%	19%	10%
Others	1%	0%	1%	6.5 %	7%
Total	**100%**	**100%**	**100%**	**100%**	**100%**

% in bold (dominant serotype)
UT = untypable

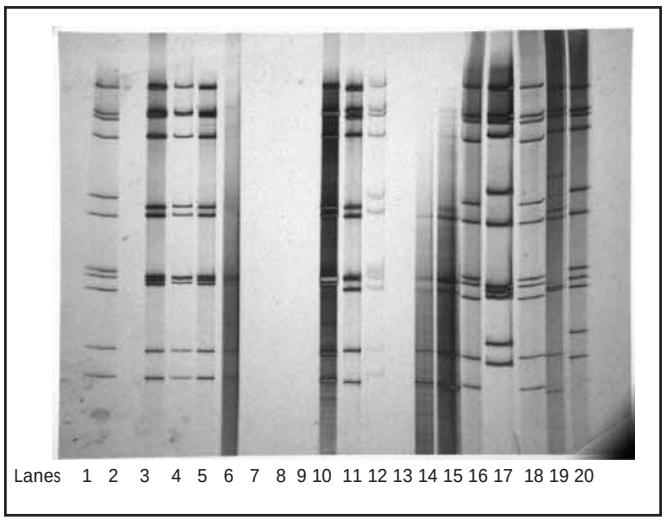

Fig. 1: Comparative genome profile of Malaysian Group A rotavirus isolates (Lanes 1 to 18), and reference strains RV4 (long e-type) (Lane 19) and RV5 (short e-type) (Lane 20)

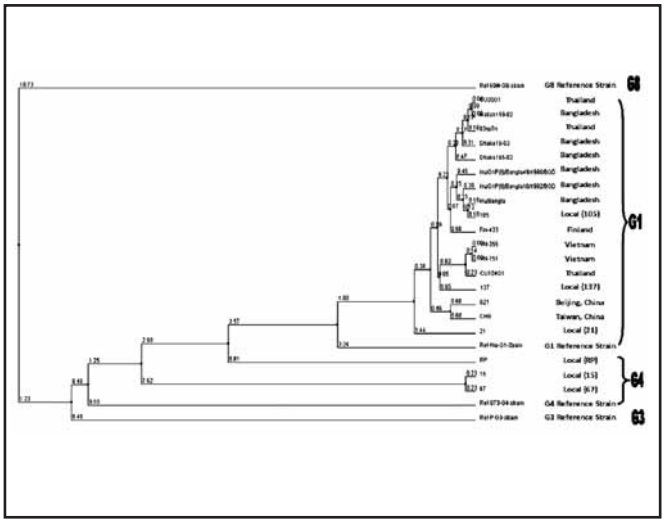

Fig. 2: Phylogenetic analysis of the nucleotide sequence of VP7 genes of rotavirus. The Malaysian strains are indicated by codings Local (105), Local (137) and Local (21). The G8 was included as an 'outgroup'.

been utilized extensively in epidemiological studies, but for vaccine development, serotyping remains essential. Monitoring prevalent serotypes using molecular methods is important to detect new strains before they become epidemic.

The tree (Fig. 2) shows that the Malaysian strains (105 and 137) compared to the reference strains and other strains from different regions of the world were both confirmed as G1 clustered together with reference strain, Wa. Both these G1 cluster were separated from the cluster formed by G4 strains. Within the G1 cluster, three groups were identified:105 very closely related to Bangladesh strain, 137 form the second group which was closely related to Thailand, and the third group (strain 21) which did not group with either of these two but exhibited a unique phylogenetic position within the G1 and closely related to Taiwan strain. This study has demonstrated that the rotaviruses responsible for the year 1996 (strain 105 and 137) are distinct from the strain in the year 2007 (strain 21). Genetic analysis of the VP7 genes revealed strain 21 from Batu Pahat was more closely related to the American strain Wa, which belonged to G1P[8] (GenBank accession number K02033).

Consistent with the findings of previous published studies, G1P[8] was the G/P type combinations frequently found in 1996. This pattern was similar to that seen in 2007: G1 58%. G1, which is most commonly found in other parts of the world is one of the types included in available vaccines. G9 (42%) was identified in 2007. These results reinforced the initial findings that in 2001-2003 serotype G9 persists in Malaysia[1], but has not become a predominant strain as yet. The common serotype G1 still accounts for 58% of strains in circulation. Previously, from 1977-1988[10], and subsequently from 2000-2001[15], serotype analysis on the distribution of rotavirus showed 71% and 65% G4, respectively. There was no G9 detected and no novel or rare strains were found. The G types (G1 and G9) and the common P type (P[8]) found in this study correlated with the common types identified in humans[17]. In comparison to other neonatal infections, P[6] have been reported in Australia,[18-19] but in the present study, P[6] was not identified in the Malaysian samples.

Over the years, from 1977 to 2007, serotype analysis showed that there has been a change in the predominant strain from serotype G4 to G1 and then G9. The appearance of G9 may have implications for vaccine development strategies and recommendations where protection against serotype G9 other than G1 may be required for a successful vaccination program in Malaysia. This would also mean that continuous surveillance is required for the detection of any changes that may take place over time. There is a need to identify and characterize rotavirus given the variety of G and P types observed.

The role of emerging G9 as the fifth globally important serotype in causing diarrhea among adults was highlighted in many studies[3,20-22]. This study signifies the role of emerging G9 serotype among adults since 1995 onwards. Rotavirus may spread to other members of family especially to siblings and to older members in whom rotavirus immunity has waned. The odd of an adult getting infection have been calculated as 2.9 times greater if the family contains a child less than two years than if it does not[23]. Adults can serve as reservoir to maintain rotavirus in the community and ensure its circulation to susceptible individuals.

Among Malaysian children, there is a significant burden associated with acute gastroenteritis and rotavirus-related hospitalizations and outpatient visits, and this burden potentially could be prevented by the use of rotavirus vaccines[24]. Together with this surveillance report and continuous monitoring of RV serotype in circulation, implementation of a rotavirus program will have a database to refer when there is a need to consider the choice of vaccines. Studies published during the past 20 years have documented that, in Malaysia, the range of proportion of

hospitalizations due to rotavirus was 28%-40%[25-26] and in the only community-based rotavirus study in Malaysia, rotavirus was detected in 12% of children[27].

In Malaysia due to the lack of immunological reagents (i.e. type-specific monoclonal antibodies), no in-house EIA technique is being carried out routinely. Most of the reports that have been done were based on RNA-PAGE and PCR technique[1,4,5,10,12,24-28]. This shortage can be overcome by collaborating with MCRI in the future. The EIA at MCRI employed a panel of MAbs specific for the major glycoprotein VP7 of the outer capsid of the five major group A human rotavirus serotypes G1, G2, G3, G4 and G9[29]. In the Asian Rotavirus Surveillance Network Report, the percentage rotavirus positive was 57% when it started the surveillance in the year 2001[30]. Earlier report in 1992 showed a lower positivity (28%)[27]. This present study that was undertaken at MCRI highlighted the need to collect data in Malaysia since an update is important. Furthermore, this study is relevant to promote the understanding and appreciation of the rotavirus disease as a significant cause of disease in children. The importance of vaccine program and its efficacy can also be learnt from the experience of the Australians. Finally, this collaborative research between Australian and Malaysian researchers should be continued to enable more efficient data analysis, facilitate training and serve as a platform for further understanding of rotavirus epidemiology in Malaysia.

In conclusion, this study demonstrated that (a) rotavirus G9 persists in Malaysia but has not become a predominant strain and that (b) G1 and G4 serotypes are still circulating in the country. Other uncommon strains may have been overlooked because of low prevalence, and lack of routine testing in the hospital settings.

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