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Genomic characterization of rotavirus isolates from Yangon Children's Hospital by Reverse Transcription Polymerase Chain Reaction

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The intentions of the study were to establish a reverse transcription polymerase chain reaction (RT-PCR) for G and P genotyping of rotavirus isolates and to determine the distribution of human rotavirus genotypes in Yangon. This study was done on stool samples collected from under five-year-old children admitted to the Yangon Children's Hospital for diarrhoea in 2004. After screening for rotavirus by enzyme immunoassay (EIA), 181 stool samples (32%) were randomly chosen from 564 rotavirus positive samples, for genotyping by using RT-PCR. The rotavirus positive samples were genotyped employing multiplex RT-PCR using G and P specific primers. Genotype G3 was the most common type identified (44% of samples) followed by G1 (33.3%). The genotype G1 was the predominant type in the early part of the year, but was replaced by genotype G3 from July to December 2004. The VP7 genotypes G2, G4 and G9 each represented as minor types (total < 13% of samples). Rotavirus P genotyping was attempted in 91 samples and P genotype can be ascertained in only 31 samples of which 74.2% were identified as P(8). There was no apparent pattern of P genotype distribution throughout the year. The common P and G type combinations were G1/P(8) and G3/P (8). Several unusual G and P type combinations were also identified, two of which were G1/P (4), and one sample was G3/P (4) and another belonging to G2/P (8). The distribution of G and P genotype provides important and valuable information for the development and introduction of rotavirus vaccines, the most effective strategy for the prevention of severe rotavirus diarrhoea.

INTRODUCTION

Rotavirus infection is the most common cause of acute gastroenteritis in young children, characterized by acute onset of watery diarrhoea, fever and vomiting [1]. Recent studies have estimated that 500,000 to 600,000 children die each year because of rotavirus gastroenteritis [2]. In response to this disease burden, several vaccines against rotavirus have been or are being developed. Before a rotavirus vaccine is introduced, common strains circulating in the community need to be explored. In many countries, however, strain characteristics of rotavirus are unknown because of a lack of adequate data or because no studies have been conducted recently. The lack of data is particularly notable in developing countries. The anticipated availability of an effective vaccine highlights the need for new data on the rotavirus disease in developing countries, where rotavirus-associated morbidity and mortality are high. Vaccination is the current strategy for control and prevention of severe rotavirus infections [2].

Rotavirus is a 100-nm virus with a characteristic wheel-shaped structure (rota)

and belongs to the family *Reoviridae*. The virus has three shells, an outer capsid, an inner capsid and a core. They surround 11 segments of double-stranded RNA, which encode for six structural proteins (VP1-VP4, VP6, VP7) and five non-structural proteins (NSP1 – NSP5). Two structural proteins, VP7 (the glycoprotein or G protein) and VP4 (the protease-cleaved protein or P protein), make up the outer shell and are considered important for vaccine development since they define the serotype of the virus and are the major antigens involved in virus neutralization [3]. Because the genes encoding these proteins segregate independently of each other during reassortment, a dual-serotyping system to account for the specificities of both VP7 and VP4 has been adopted [4]. Thus, the classification of rotaviruses is based on differences in the VP7 (G) and VP4 (P) capsid proteins. G serotypes 1-4, and P genotypes P(8) and P(4) predominate worldwide. The development of vaccines against severe rotavirus diarrhoea is based upon homotypic or heterotypic protection provided against either a single common G serotype (monovalent vaccines) or against multiple serotypes (multivalent vaccines) [5]. Candidate rotavirus vaccines have been prepared with reassortant strains specifically to protect against the 4 major rotavirus G serotypes (G1-4). The main reason for this study was to collect data that will facilitate and support the introduction of rotavirus vaccination, once a vaccine becomes available. The objectives of the study were to establish a Reverse Transcription Polymerase Chain Reaction (RT-PCR) for G and P genotyping rotavirus isolates and to determine the relative frequency of individual human rotavirus serotypes and genotypes prevailing in Yangon.

MATERIALS AND METHODS

This was a hospital-based, prospective study done from January through December 2004 in Yangon Children's Hospital, Yangon. Stool samples were collected from children under five years of age, admitted to the three medical wards of Yangon Children's Hospital for diarrhoea. A child admitted with a diagnosis of acute diarrhoea and who was less than 5 years of age were included in the study.

Approximately 10 ml of stool sample was collected from diarrhoeic children after admission using wide-mouth screw capped bottles and transported in cold boxes daily as soon as possible to the laboratory in the Virology Research Division of the Department of Medical Research (Lower Myanmar) and stored at -20º C until testing was done. The presence of rotavirus antigen was determined by Enzyme Immunoassay (EIA). Briefly, diluted stool samples, positive and negative controls were added to the wells of 96-well EIA plates precoated with anti-rotavirus antibody. The plates were incubated and then washed. Then anti-rotavirus antibody conjugated to horseradish-peroxidase was added and incubated. After washing, the enzyme substrate was added to develop colour in the wells. The absorbance (optical density) of the wells was read in an EIA reader at 450 nm wavelenghth. The cut-off value was calculated by adding 0.100 absorbance unit to the absorbance reading of the negative control. The positive control must have a value of greater than 0.500 absorbance units.

A subsample (~ one third) of the monthly rotavirus EIA positive stool samples was randomly chosen and G and P genotyped by Reverse Transcription Polymerase Chain Reaction (RT-PCR) [6,7]. Briefly, RNA was extracted from 10% stool suspensions in phosphate buffered saline (PBS) employing a phenol-chloroform-isoamyl alcohol mixture. After thorough vortexing and centrifugation, the aqueous suspension containing viral RNA was collected. Then, hydroxyappatite was added to allow RNA to bind to hydroxyappatite and then centrifuged. The pellet was washed and

double stranded (ds) RNA was eluted by 200mM potassium phosphate solution.

The extracted dsRNA was amplified by Reverse Transcription Polymerase Chain Reaction (RT-PCR) using specific oliginucleotide primers. The extracted RNA was used as a template to produce and amplify full-length complementary DNA (cDNA) of the VP7 and VP4 region by RT-PCR. The cDNA was used as a template for a second and subsequent rounds of PCR using genotype-specific primers to amplify cDNA. G types were identified by multiplex RT-PCR assay using consensus primers 9con1 and 9con2, and typing primers specific for the VP7 genes of G types 1- 4 and 9 (Table 1). P types were identified by multiplex RT-PCR assay using consensus primers con3 and con2, and primers specific for the VP4 genes of P types 4, 6, 8, 9, 10 and 11 (Table 2).

Table 1. Consensus and type-specific primers for G typing

Primer name	Sequence (5' to 3')	Strain/G type	PCR product (bp)
1st amp consensus primers			
9con1	TAG CTC CTT TTA ATG TAT GG	Wa/G1	
9con2	GTA TAA AAT ACT TGC CAC CA	Wa/G1	
2nd amp typing primers (9con1 was also included)			
9T-1	TCT TGT CAA AGC AAA TAA TG	Wa/G1	158
9T-2	GTT AGA AAT GAT TCT CCA CT	S2/G2	244
9T-3P	GTC CAG TTG CAG TGT AGC	107e1B/G3	464
9T-4	GGG TCG ATG GAA AAT TCT	ST3/G4	403
9T-9B	TAT AAA GTC CAT TGC AC	116E/G9	110

The amplified products together with a molecular weight marker were subjected to electrophoresis in a 2% agarose gel containing 0.5 µg of ethidium bromide per ml. The cDNA bands and the molecular weight marker were observed under the ultra violet light and photographed. Data were analyzed using Microsoft Excel program.

Table 2. Consensus and type-specific primers for P typing

Primer name	Sequence (5' to 3')	Strain/ [P] type	PCR product (bp)
1st amp consensus primers			
con3	TGG CTT CGC TCA TTT ATA GAC A	KU/P[8]	
con2	ATT TCG GAC CAT TTA TAA CC	KU/P[8]	
2nd amp primers (con3 was also included)			
1T-1	TCT ACT TGG ATA ACG TGC	KU/P[8]	345
2T-1	CTA TTG TTA GAG GTT AGA GTC	RV-5/P[4]	483
3T-1	TGT TGA TTA GTT GGA TTC AA	1076/P[6]	267
4T-1	TGA GAC ATG CAA TTG GAC	K8/P[9]	391
5T-1	ATC ATA GTT AGT AGT CGG	69M/P[10]	583

RESULT

During the year 2004, stool specimens were collected from 995 under five years old children who were admitted for diarrhoea. Among them, 564 (56.6%) samples were EIA positive for rotavirus, from which, 181 samples (32%) were randomly chosen for G and P genotyping by RT-PCR (Figures 1a and 1b).

Of the 181 rotavirus stool samples genotyped, genotype G3 was the most common genotype identified representing 44.8% of the samples and G1 strains were the second most common genotype accounting for 33.3% of isolates. The VP7 serotypes G2, G4 and G9 each represented minor types during the study period, being identified in less than 13% of samples. Seventeen stool samples (9.3%), although positive for rotavirus by EIA, either exhibited mixed G types or were non-typable.

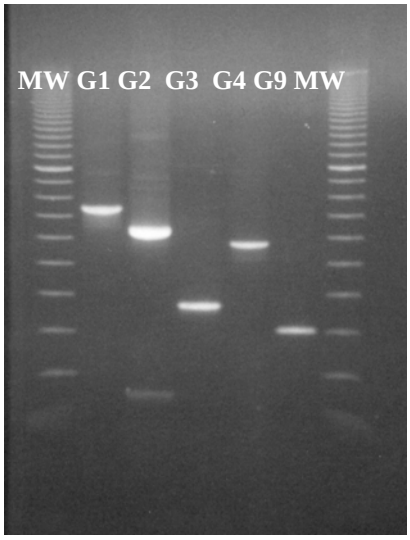

Fig.1a. G genotype cDNA bands in agarose gel

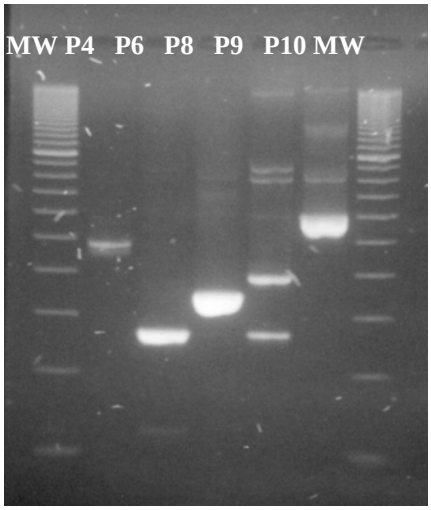

Fig.1b. P genotype cDNA bands in agarose gel

The rotavirus P genotype could be determined only in 31 samples of the 91 samples tested. Of the samples typed, 74.2% were identified as P(8).

Monthly distribution of VP7 types (G types) revealed that from January to June 2004, serotype G1 was the predominant type, but was replaced by serotype G3 from July to December 2004 (Fig. 2). The monthly distribution of P genotype showed no definite pattern. The G and P genotype combinations of individual rotavirus isolates could be determined in 25 samples, the most common combinations were G1/P(8) and G3/P(8). However, several unusual G and P type combinations were identified, two of which were G1/P(4), one sample was G3/P(4) and another belonging to G2/P(8) (Table 3).

Table 3. Distribution of G and P genotype combinations of 25 rotavirus isolates

G type	P type		
	P(4)	P(6)	P(8)
G1	2 (8%)	2 (8%)	7 (28%)
G2	2 (8%)	0	1 (4%)
G3	1 (4%)	0	7 (28%)
G4	0	0	3 (12%)
G9	0	0	0

DISCUSSION

Rotavirus strain surveillance has a high priority in disease control programmes worldwide. The continued identification of the most common G and P serotypes for inclusion in vaccines is an important priority. Subsequent to the introduction of a vaccine candidate, not only monitoring of circulating strains is recommended, but also surveillance of potential reassortment of animal rotavirus genes from the vaccine into human rotavirus strains is critical. Improved detection and characterization of strains will help to develop a comprehensive strain surveillance that may be required for tailoring effective rotavirus vaccines.

Epidemiologic surveillance of rotavirus VP7 (G) serotypes-genotypes conducted in various populations throughout the world has repeatedly shown that approximately 90% of the typable rotavirus isolates belong to G1-G4 [5]. Many studies using P (VP4) genotyping methods have indicated that, worldwide, rotavirus strains of the 4 common G serotypes are each associated with 1 P genotype: G1, G3, and G4 are associated with P(8), and G2 is associated with P(4). More recently, G serotypes-genotypes other than G1-G4, including G5, G8-G10, have been detected in various parts of the world [8, 9]. Also, G and P genotyping of rotavirus in specimens from India

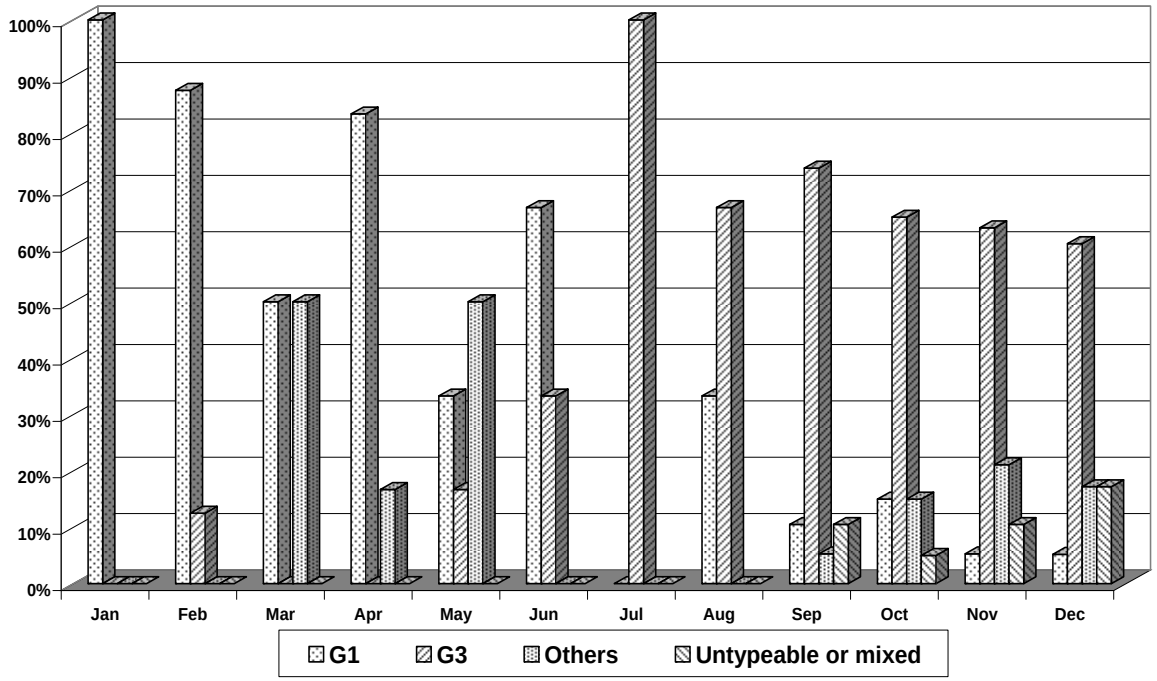

Fig. 2. Monthly distribution of G genotypes in Yangon, 2004

revealed that a high percentage of the childhood diarrhea strains belong to genotype P(6), and the most common strain had an unusual G serotype, G9 [10].

Similarly, in all regions surveyed in Brazil, apparent reassortants of genotype P(8) G5 were found in children with gastroenteritis [11]. These studies indicate that while rotavirus strains have limited diversity in many settings, reassortment between common and uncommon serotypes or animal strains can arise in some settings and, thus, leading to unusual diversity. In this study, the non-typable samples probably belonged to the G genotypes other than G 1-4 or G 9. Further characterization of these samples using primers other G 1-4 and G 9 are needed to assign them to specific G genotypes.

Of the G and P type combinations in this study, 27 samples (64%) were genotype P(8) with G1,G3, or G4 specificity and P (4) with G2 specificity. The finding is similar to those of previous studies which reported that 83.5% of the specimens were genotypes P(8) with G1,G3, or G4 specificity and P (4) with G2 specificity [5].

The common combinations of G and P genotypes detected in this study were P(8) G1 (28%); P(8) G3 (28%), followed by were P(8) G4 (12%). Several unusual G and P type combinations were also identified, two of which were G1/P(4), and one sample was G3/P (4) and another belonging to G2/P (8). These combinations might have resulted from natural reassortment between the common strains. Most of the samples (66.7%) could not be assigned a P genotype with the primers for the P types [P(4), P(6), P(8), P(9), P(10) and P(11)], probably because of mismatches in the primer binding region. This suggests that the VP4 gene of strains isolated in Myanmar may be different from those identified elsewhere. Therefore, more primers will be needed to be re-evaluated for the Myanmar rotavirus isolates.

This study demonstrates the diversity of rotavirus genotypes circulating in the community in a given year and the predominant genotype can change through the year, suggesting the vaccine developers should incorporate all the prevalent genotype-specific vaccines for efficacy against all the circulating genotypes.

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