UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF HUMAN GROUP A ROTAVIRUS ISOLATES FROM MALAYSIA AND DEVELOPMENT OF A COLORIMETRIC PCR-BASED TEST FOR THE DETECTION OF P[8] GENOTYPE

ZURIDAH HASSAN

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By

ZURIDAH HASSAN

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Rotavirus has been recognized as a leading cause of the diarrhoeal illness in children under 5 years of age in the developing world. Latex agglutination test was used to detect group A rotavirus from 157 in-patients from different hospitals in Malaysia during 2000 to 2001. Diarrhoea was detected in 31 (19.7%) children and majority were under two years of age.

When viewed under electron microscope by negative staining, rotavirus was seen as both double-shelled and single-shelled particles. Thirty one rotavirus antigen

positive samples with typical group A electropherotype were further characterized into their G or P types by polymerase chain reaction (PCR) assay. The two common electropherotypes were IIC (51.6%) and IIG (35.5%). The most prevalent VP4 genotype was 25 (80.6%) P[8] and 1 (3.2 %) P[6]. Genotype P[4] and P[9] were not isolated and 5 (16.1 %) were P untypable (P^{UT}). Regarding the VP7 genotype, G4 was the most prevalent (64.5 %), followed by G1 (6.45%), G2 (6.45%) and G3 (3.2 %). Neither G8 nor G9 was found and 6 (19.4 %) were G untypable (G^{UT}). Studies in many countries found that G1P[8], G4P[8], G2P[4] and G3P[8] are the group A rotavirus strains more commonly seen in children. However from this present study, the common strains in Malaysia were G4P[8], G1P[8] and G3P[8]. One G^{UT}P[6] strain (designated as 7W) was identified for the first time in Kuala Lumpur. Restriction endonuclease *Hae*III and *Sau*96I were also used to characterize the VP7 gene of the local 7W strain. However a restriction profile could not be assigned. The P[8] and P[6] local strains (represented by 67F and 7W, respectively) were also characterized by nucleotide sequence analysis. Phylogenetic analysis revealed that the VP4 genes of the 67F and 7W formed a distinct lineage.

The P[8] and P[6] are encoded by distinct VP4 gene alleles. The main diagnostic problem is the genetic diversity of these alleles among different rotavirus

strains. To overcome this problem, a method that employs non-radioactive dot hybridization was successfully developed for P[8] and P[6]. VP4 cDNA rotavirus-specific probes were prepared and labelled with digoxigenin (DIG). Anti-DIG-alkaline phosphatase and the substrate NBT/BCIP were used to detect the binding of the probe to target sequence.

A simple, practical, sensitive and specific assay based on polymerase chain reaction (PCR) and a colorimetric detection method (ELISA) for the typing of rotavirus in infected faeces has been developed succesfully. A set of oligonucleotides was employed for a single-tube reverse-transcription nested PCR (RT-nPCR). Upon synthesis of the first strand cDNA, a first stage of 10 cycles of PCR amplification was run to generate an 876-bp dsDNA from the 5' terminal third of gene 4. The process was completed in the same tube by performing another 35 cycles of second stage amplification incorporating a biotinylated and digoxigenin 5'-end labelled primers. The RT-nPCR produced a 180-bp amplicon representing the VP4 P[8] type. The sensitivity of the RT-nPCR method was compared to non-nested PCR method and nested PCR was found to be 100 times more sensitive. To further increase the sensitivity, the enzyme-linked immunoassay (ELISA) was incorporated into the system. Streptavidin-coated microtitre plate was used to capture the biotinylated PCR-amplified products. This RT-nPCR ELISA was able

to detect RNA as low as 4 pg nucleic acid. It was designed to type the single most epidemiologically important human rotavirus VP4 P[8] type which is often associated with rotavirus G1, G3 and G4 types. Monoclonal antibodies (Mabs) were used for G serotyping, but no Mabs were availabe for P serotyping. Therefore, the RT-nPCR ELISA method is a very useful technique to detect rotavirus.

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PENCIRIAN MOLEKULAR ROTAVIRUS KUMPULAN A DARI MANUSIA DI MALAYSIA DAN PEMBENTUKAN UJIAN KOLORIMETRIK BERASASKAN PCR UNTUK MENGESAN P[8] GENOTIP

Oleh

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Rotavirus telah dikenalpasti sebagai penyebab utama penyakit cirit-birit di kalangan kanak-kanak berumur kurang dari 5 tahun di negara membangun. Agglutinasi latex telah digunakan untuk mengenalpasti rotavirus kumpulan A dari 157 pesakit dari berlainan hospital di Malaysia sepanjang 2000 ke 2001. Cirit-birit telah dipencil daripada 31 (19.7%) kanak-kanak dan kebanyakannya adalah berumur kurang dari dua tahun.

Apabila dilihat menggunakan mikroskop elektron kaedah pencelupan negatif, rotavirus yang mengandungi dua selaput dan satu selaput dapat dilihat. Tiga puluh satu sampel rotavirus antigen positif yang menunjukkan elektroferotaip

khusus untuk group A telah dicirikan kepada jenis G atau P melalui reaksi rantaian polimerasi (PCR). Dua elektroferotaip yang sering ditemui adalah IIC (51.6%) dan IIG (35.5%). VP4 genotip yang terbanyak adalah 25 P[8] (80.6%) dan 1 (3.2%) P[6]. Genotip P[4] dan P[9] tidak ditemui dan 5 (16.1%) adalah P yang tidak boleh digenotip (P^{UT}). Berkaitan VP7 genotip, G4 adalah terbanyak (64.5%), diikuti oleh G1 (6.45%), G2 (6.45%) dan G3 (3.2%). G8 dan G9 tidak ditemui dan 6 (19.4%) adalah G yang tidak boleh digenotip (G^{UT}). Kajian di beberapa negara menunjukkan bahawa G1P[8], G4P[8], G2P[4] dan G3P[8] rotavirus kumpulan A sering ditemui dikalangan kanak-kanak. Walau bagaimanapun, dari kajian ini jenis yang selalu ditemui di Malaysia adalah G4P[8], G1P[8] dan G3P[8]. Satu strain G^{UT}P[6] (dikenalpasti sebagai 7W) dari kajian ini telah dilaporkan buat pertama kalinya dari Kuala Lumpur. Enzim pembatas *Hae*III dan *Sau*96I juga telah digunakan untuk mengkaji gen VP7 strain tempatan 7W tetapi profil pembatas tidak dapat ditentukan. Strain P[8] dan P[6] tempatan (diwakili sebagai 67F dan 7W) juga dikaji menggunakan jujukan nukleotid. Analisis filigenesis menunjukkan gen VP4 67F dan 7W membentuk kumpulan berlainan.

P[8] dan P[6] dienkod oleh gen VP4 allel yang berlainan. Masalah utama untuk mengenalpasti ialah kepelbagaian allel genetic di dalam strain. Untuk mengatasinya, dot hybridization tanpa-radioaktif telah dicipta untuk P[8] dan P[6]. Prob spesifik VP4 cDNA telah disedia dan dilabelkan dengan digoxigenin (DIG).

Anti-DIG-alkaline phosphatase dan substrat NBT/BCIP telah digunakan untuk mengesan prob yang terlekat pada jujukan yang disasarkan.

Satu lagi kaedah yang menggunakan teknik reaksi rantaian polimerasi (PCR) dan pengesanan 'colorimetrik' (ELISA) yang mudah, praktikal, sensitif dan spesifik untuk mengesan rotavirus telah berjaya dibentuk. Satu set oligonukleotida telah diguna dalam kaedah 'single-tube reverse-transcription nested polymerase chain reaction' (RT-nPCR). Di dalam pembentukan stran pertama cDNA di dalam tindakbalas RT, amplifikasi PCR pertama sebanyak 10 pusingan menghasilkan 876 bp dsDNA dari pangkal 5' gen 4. Proses ini disempurnakan dengan 35 pusingan amplifikasi kedua di mana primers yang dilabel dengan biotin dan DIG pada pangkal 5' digunakan. RT-nPCR menghasilkan amplikon 180 bp mewakili VP4 P[8]. Sensitiviti kaedah RT-nPCR didapati 100 kali lebih sensitif berbanding dengan kaedah tanpa-nPCR. Untuk menambahkan sensitiviti, kaedah ELISA telah dimasukan. Plat mikrotiter yang disalut dengan streptavidin telah digunakan untuk memerangkap produk PCR yang berbiotin. RT-nPCR ELISA boleh mengesan RNA sehingga 4 pg. Ia telah dicipta untuk mengesan VP4 gen P[8] yang mempunyai kepentingan epidemiologi dan lazimnya dikaitkan dengan rotavirus G1, G3 dan G4. Sebelum ini antibodi monoklonal (Mabs) digunakan untuk G serotyping, tetapi Mabs untuk P serotyping belum diwujudkan lagi. Maka, dengan itu, RT-nPCR ELISA adalah satu kaedah penting untuk mengesan rotavirus.

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I certify that an Examination Committee met on 22nd September 2004 to conduct the final examination of Zuridah Hassan on her Doctor of Philosophy thesis entitled "Molecular Characterization of Human Group A Rotavirus Isolates From Malaysia and Development of a Colorimetric PCR-based Test for the Detection of P[8] Genotype" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

ZURIDAH HASSAN

Date: 2004

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LISTS OF ABBREVIATIONS

aa	-	amino acid
ABTS	-	2,2'-Azido-di(3-ethyl)benzthiazoline sulphonic acid
Arg	-	arginine
bp	-	base pairs
$CaCl_2$	-	calcium chloride
cDNA	-	complementary deoxyribonucleic acid
CF11	-	cellulose fiber
COOH	-	carboxy end
$CsCl_2$	-	caesium chloride
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleic acid
ds	-	double stranded
dsp	-	double shelled particle
DEPC	-	diethyl pyrocarbonate
DIG	-	digoxigenin
EDTA	-	ethylene diamine tetra acetic acid
ELISA	-	enzyme-linked immunoassay
EM	-	electron microscope
e-type	-	electropherotype
*Hae*III	-	*Haemophilus influenzae* III
HCl	-	hydrochloric acid
H_2O_2	-	hydrogen peroxide
IBDV	-	Infectious Bursal Disease Virus
KCl	-	potassium chloride
KDa	-	kilodalton
Mg	-	magnesium
$MgCl_2$	-	magnesium chloride
MTP	-	microtiter plate
NaCl	-	sodium chloride
Na_2CO_3	-	sodium carbonate
$NaHCO_3$	-	sodium bicarbonate
NaOH	-	sodium hydroxide
NBT-BCIP	-	nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl-phosphate
NH_2 terminal	-	amino terminal
NDV	-	Newcastle disease virus
OD	-	optical density
ORF	-	open reading frame
PBS	-	phosphate buffered saline

PCR	-	polymerase chain reaction
PD	-	primer dimer
pH	-	negative logarithm of hydrogen ion
P^{32}	-	phosphorous^{32}
rpm	-	rotation per minute
RNA	-	ribonucleic acid
RNA-PAGE	-	RNA polyacrylamide gel electrophoresis
RT-nPCR	-	Reverse-transcription nested Polymerase Chain Reaction
RT-PCR	-	reverse trancriptase polymerase chain reaction
*Sau*961	-	*Staphylococcus aureus* 961
SD	-	standard deviation
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssp	-	single shelled particle
STE	-	saline tris EDTA
TAE	-	Tris acetic acid
TBE	-	Tris borate EDTA
TBS	-	tris buffered saline
TE	-	Tris EDTA
TEMED	-	tetramethylethylenediamine
UT	-	untypable
UPM	-	Universiti Putra Malaysia
VP	-	viral protein