Full Length Research Paper

Molecular characterization of norovirus from acute gastroenteritis patients in Malaysia

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Rotavirus and recently norovirus have been described as important and most common cause of acute gastroenteritis in children. The mode of transmission is fecal-oral. Diagnosis of both of these viruses can be made by rapid antigen detection of the viruses in stool specimens and strains can be further characterized by enzyme immunoassay or reverse transcriptase polymerase chain reaction. Stool specimens collected from various hospitals in Malaysia were examined for norovirus by both immunochromatography and reverse-transcription polymerase chain reaction techniques. Rotavirus antigen was screened by a commercially available latex agglutination test kit. Altogether, 168 stool samples were collected for both norovirus and rotavirus screening. Out of these, 77/168 (45.8%) were examined for rotavirus with 17/77 (22%) rotavirus antigen positive. Due to very small amount of fecal materials obtained in some cases, only 151/168 (89.8%) were sufficient for norovirus and 14/151 (9.3%) were positive for norovirus genogroup II (GII). This study highlights that rotavirus remains the main agent for acute gastroenteritis and identification for emerging norovirus among the children is becoming important for proper patient management.

Key words: Norovirus, rotavirus, immunochromatography, latex agglutination, RT-PCR, phylogenetic analysis.

INTRODUCTION

Noroviruses (NoV) are the worldwide major cause of viral gastroenteritis in human (Marshall et al., 2003). They belong to the Norovirus genus of the Caliciviridae family, which also includes the Sapovirus genus. NoV which is also called small round structured viruses (SRSVs) are non-enveloped, single-stranded positive sense RNA viruses (Frankhauser et al., 2002). The primary route of NoV transmission is through the fecal-oral route (Alice et al., 2004). Primary infection results from either the consumption of focal contaminated food or water (Rabanaeu et al., 2003). Currently, at least five NoV genogroups (GI, GII, GIII, GIV and GV) are being reported with GII, GIII and GIV commonly found in humans and GII the most predominant genogroup causing worldwide acute gastroenteritis infecting persons of all age groups (Frankhauser et al., 2002; Alice et al., 2004; Kirkwood and Bishop, 2001). Outbreaks of gastroenteritis in USA showed that GII strains were the predominant type accounting to 73% in all outbreaks (Frankhauser et al., 2002), 94% of positive specimens in children hospitalized with acute gastroenteritis in Australia (Kirkwood and Bishop, 2001) and 64% in Thailand (Guntapong et al., 2004).

Rotavirus (RV) forms the genus Rotavirus of the Reoviridae family which are often associated with children under the age of 5 years, (Parashar et al., 2006), whilst NoV is common in the adults and older children (Kirkwood and Bishop, 2001). However, in Malaysia, the aetiology and frequency of viral gastroenteritis due to NoV is still poorly investigated and understood unlike the vast studies already reported on rotavirus (Zuridah et al., 2010; Yap et al., 1998; Rasool et al., 1993). The aim of our study was to detect for both RV and NoV and further characterization of NoV for epidemiological purposes.

MATERIALS AND METHODS

Stool samples were obtained from 8 different hospitals in Malaysia. These samples were tested for RV antigen using the Rotalex kit.
For NoV, antigens were screened by immunochromatography (IC) test (The RIDA® QUICK Norovirus, R-Biopharm, Germany) and ELISA (The RIDASCREEN® Norovirus, R-Biopharm, Germany). The IC test is based on the principle that it uses specific monoclonal antibodies directed against NoV capsid protein. Results were interpreted visually after 15 min.

The ELISA is based on monoclonal antibodies bound on the surface of the microwell strips to capture the antigen in stool specimens and samples were assayed accordingly (R-Biopharm, 2006).

For RNA extraction, NoV ssRNA was treated with Ultraspec™ II RNA isolation system (BioTecx/USA). The eluted ssRNA were then transferred to new clean sterile tubes and frozen until further use (BioTecx/USA). Next, the extracted ssRNA was used as the template for reverse transcription (RT) (Qiagen, Germany protocol). Two microlitre RNA template was added to a 20 µl reaction mixture containing a final concentration of 10X reverse transcription buffer, 2.0 µl of 5 mM dNTPs mixture, 2.0 µl of 10 µM random primers, 1 µl Sensiscript RT, 2 µl of RNase-free water and 10 units/µl RNase inhibitor (Fermentas) and incubated at 37° C for 60 min using the random primers (Qiagen, 2004).

Further amplification was performed using primers NV O F2 and NV O R (Bull et al., 2005). Five microlitre of cDNA was added to 20 µl of a PCR mixture containing 2.0 µl of 10X PCR buffer (Fermentas), 1.2 µl of 25 mM MgCl₂, 0.4 µl of 10 mM dNTPs mix (Promega), 0.24 µl of 50 µM each primer, and 0.4 µl of 2.5 U Taq polymerase (Fermentas). Pre-denaturation was carried out at 95°C for 5 min followed by 40 cycles of PCR (95°C for 30 s, 55°C for another 30 s, and 72°C for another final 60 s). Electrophoresis of the amplicons was carried out in 1% agarose gel with 1X TBE buffer with a 100 bp ladder (Fermentas) included.

For phylogenetic studies, all the 14 positive amplicons coded as C7, C4, C11, C12, C14, D1, D3, D6, D7, E1, E2, E8, and E9 were sent to an independent laboratory. The nucleotide sequences obtained were then analyzed using the public database (BLAST programme). Phylogenetic analysis showed that the NoV analyzed was grouped into 2 distinct genotypes GII/4 and GII/3 (Figure 2). GII/4 was the most predominant genotype, accounting for 71.4% (10 of 14), and followed by 29% (4/14) GII/3. All the samples were more than 95% similar to other strains found in Asian countries, especially in Japan. NoV GII gene of patients C7, C4, C11, C12, C14, D1, D3, D6, D7, E1 and E2 were clustered in the same branch with those in Japan (GII/4Nagano). Meanwhile, samples from patients E8 and E9 were clustered in the same branch with strains in Argentina (GII/3Arg320/95/AR).

**RESULTS**

Of the 168 specimens, 77 samples (45.8%) that were screened for RV antigen were from patients less than 5 years old by latex agglutination test. For NoV, not all the 168 samples were screened for NoV. Only 151 samples were screened, of which 14/151 (9.3%) samples were positive.

The amplicons with 379 bp size were confirmed to be NoV genogroup II (GII) (Figure 1). The sequences were compared against the GenBank database using the BLAST programme. Phylogenetic analysis showed that the NoV analyzed was grouped into 2 distinct genotypes GII/4 and GII/3 (Figure 2). GII/4 was the most predominant genotype, accounting for 71.4% (10 of 14), and followed by 29% (4/14) GII/3. All the samples were more than 95% similar to other strains found in Asian countries, especially in Japan. NoV GII gene of patients C7, C4, C11, C12, C14, D1, D3, D6, D7, E1 and E2 were clustered in the same branch with those in Japan (GII/4Nagano). Meanwhile, samples from patients E8 and E9 were clustered in the same branch with strains in Argentina (GII/3Arg320/95/AR).

**DISCUSSION**

This study describes the incidence and genetic diversity of NoV in diarrheal patients in Malaysia. The incidence found in this study (9.3%) which confirms that infection rates in our study population are comparable to Thailand, Taiwan and Vietnam (14, 8.2, and 5.4%, respectively) (Hansman et al., 2004b; Chen et al., 2007; Hansman et al., 2004a).

The predominant rotavirus infection (22%) was in concordance with previous investigations on the viral etiology of pediatric gastroenteritis (Zuridah et al., 2010; Chen et al., 2007; Zuridah et al., 2009; Nguyen et al., 2007). However, due to sampling volumes, no mixed infections were investigated in this study. This should be pursued in future because many studies have reported...
Figure 2. Phylogenetic analysis of NoV local strains (labeled as C7, C4, C11, C12, C14, D1, D3, D6, D7, E1, E2, E8 and E9) and other global strains GII-4 Nagano/2007/JP from Japan, GII-3 Saitama from Japan, GII-3 Arg from Argentina, GII-2 X81879 Melksham from UK, GI/1 M87661 Norwalk from USA. OUTGROUP Manchester Sapovirus is used as an outgroup control.

that children are vulnerable to several viruses at the same time (Yap et al., 1998). Besides norovirus and rotavirus, adenovirus and astrovirus were also detected in small percentage (Tran et al., 2010).

To our knowledge, this is the first preliminary report on NoV infection in the hospitalized patients in Malaysia. Genetic diversity was observed, encompassing 2 genotypes, namely GII/3 and GII/4 with GII/4 the most prevalent in concordance to other reports worldwide (Nguyen et al., 2007; Bucardo et al., 2008).

However, in Japan, a change in distribution of predominant NoV genotypes, GII/4 to GII/3 in 2003 to 2004 and GII/3 to again GII/4 in 2004 to 2005 was observed in a surveillance conducted over a three year period (Phan et al., 2006a; Phan et al., 2006b). Therefore, continuous monitoring of NoV genetic property in Malaysian population is important for updating and comparing global trends of NoV infection. Great caution is needed in interpreting this result; moreover the prevalence strain cannot be concluded as final due to the small sample size analyzed thus this study warrants further investigation. In conclusion, this study concurs with other reports which indicate that NoV is another important enteric virus that can cause gastroenteritis.

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REFERENCES


