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Protocols

A novel multiplex RT-PCR for identification of VP6 subgroups of human and porcine rotaviruses

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VP6 protein antigens allow classification of rotaviruses into at least four subgroups, depending on the presence or absence of SG-specific epitopes: SG I, SG II, SG (I + II), and SG non-(I + II). However, MAbs against epitopes on the VP6 protein of human and porcine rotaviruses, sometimes, do not recognize SG-specific epitopes or recognize irrelevant-SG epitopes and therefore result in the incorrect assignment of subgroups. In order to solve this problem, a novel multiplex RT-PCR was developed as an alternative tool to identify VP6 genogroups using newly designed primers which are specific for genogroup I or II. The sensitivity and specificity of the newly developed multiplex RT-PCR method was evaluated by testing with human and porcine rotaviruses of known SG I, SG II, SG (I + II), and SG non-(I + II) strains in comparison with monoplex RT-PCR and VP6 sequence analysis. The results show that the genogroups of both human and porcine rotaviruses as determined by the new multiplex RT-PCR method were in 100% agreement with those determined by monoplex RT-PCR and VP6 sequence analysis. The method was shown to be specific, sensitive, less-time consuming, and successful in genogrouping clinical isolates of rotaviruses circulating in children and piglets with acute diarrhea.

1. Introduction

Rotaviruses are the major cause of severe gastroenteritis in young children and young animals worldwide. Rotaviruses are a triple-layered particle of the Reoviridae family which are classified into groups (A–E) and subgroups (SG) according to the presence of epitopes on the middle-layered VP6 protein (Estes, 2001; Kapikian et al., 2001). Among them, group A rotavirus has been recognized as the most important group due to its highest prevalence and pathogenesis in human and various animal species (Gouvea et al., 1994; Estes, 2001). Two monoclonal antibodies (MAbs) that react specifically with subgroup I (SG I) (MAb 255/60) or subgroup II (SG II) (MAb 631/9) rotavirus strains were developed in early 1980s (Greenberg et al., 1983; Taniguchi et al., 1984) and have been used widely to characterize human and animal rotavirus strains (Kapikian and Chanock, 1996). The VP6 protein antigens allow the classification of these viruses into four subgroups: SG I, SG II, SG (I + II), and SG non-(I + II) depending on the presence or absence of SG-specific epitopes (Estes and Cohen, 1989; Hoshino et al., 1987; Gorziglia et al., 1988; Iturriza-Gómara et al., 2002). Subgrouping of rotaviruses by enzyme-linked immunosorbent assays (ELISAs) using SG-specific MAbs has been used extensively for epidemiological studies (Greenberg et al., 1983; Taniguchi et al., 1984). Most human rotaviruses belong to SG II, while animal rotaviruses belong to SG I (Gorziglia et al., 1988; Ito et al., 1997; Tang et al., 1997; Thongprachum et al., 2009). The unreliability of serological methods for subgrouping rotaviruses using SG-specific MAbs is a well-recognized problem. A study of human and porcine rotavirus strains circulating in children and in piglets with acute gastroenteritis in Chiang Mai has also revealed viruses with SG I, SG II, SG (I + II), and SG non-(I + II) specificities (Thongprachum et al., 2009). It is well established that accumulation of point mutations in the VP6 gene may lead to a change of amino acid residue in the VP6 protein, particularly on the epitope regions, thus making the virus unrecognizable by corresponding SG-specific MAbs. Recently, deduced amino acid sequences of human and porcine rotavirus strains with SG (I + II) and SG non-(I + II), were analyzed in comparison with those of SG I and SG II specificities. The data revealed that some human rotavirus strains were misidentified using MAbs; for example, strains which VP6 sequence analysis identified as genogroup II were misidentified as SG I and SG non-(I + II) by SG-specific MAbs. A similar phenomenon was observed in subgrouping of porcine

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rotaviruses; for instance, strains which VP6 sequence analysis identified as genogroup I were misidentified as SG II, SG (I + II), and SG non-(I + II) strains by SG-specific MAbs (Thongprachum et al., 2009). Based on these findings, it was interesting to develop a multiplex RT-PCR as an alternative method to identify genogroups of human and porcine rotaviruses.

2. Materials and methods

2.1. Clinical samples and rotavirus strains

This study includes 27 strains of human rotaviruses from the stool samples of children hospitalized with acute gastroenteritis in Chiang Mai, Thailand between May 2000 and December 2002. Of these, 3 were SG I, 21 were SG II, and 3 were SG non-(I + II) rotavirus strains. Additionally, 47 strains of human rotaviruses from pediatric patients in the year 2007 were also included in this study to evaluate the RT-PCR method developed in the present study.

Furthermore, 49 strains of porcine rotaviruses isolated from diarrheic piglets during the period of June 2000 to July 2003 from 6 different farms located in Chiang Mai, Thailand, were also included in this study. Among 49 strains of porcine rotaviruses, 4 were SG I, 3 were SG II, 24 were SG (I + II), and 18 were SG non-(I + II) rotavirus strains.

2.2. VP6 nucleotide sequencing

The VP6 full-length PCR products of human and porcine rotaviruses were gel-purified with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified products were sequenced in both directions using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 3100; Applied Biosystems). The VP6-F or VP6-R was used as a sequencing primer.

2.3. Designing oligonucleotide primers for VP6 genogrouping

The following reference sequences of SG I and SG II strains of human and porcine rotaviruses were used as the query sequences for blast alignment: S2 (DQ870488) and 1076 (D00325) for VP6 SG I human rotaviruses; TK159 (AY661888), RV3 (U04741), 116E (U85998), E210 (U36240), and Wa (K02086) for VP6 SG II human rotaviruses; OSU (AF317123), 4F (L29184), 4S (L29186), A131 (AF317124), A253 (AF317122), YM (X69487), and JL94 (AY538664) for VP6 SG I porcine rotaviruses; Gottfried (D00326) for VP6 SG II porcine rotavirus. In addition, the VP6 nucleotide sequences of human and porcine rotaviruses available in the GenBank database were also included in this analysis. Of these, 3 VP6 sequences of SG I human rotaviruses, 25 VP6 sequences of SG II human rotaviruses, 47 VP6 sequences of SG I porcine rotaviruses, and 2 VP6 sequences of SG II porcine rotaviruses were selected for comparative analyses.

The obvious strategy for designing primers specific for SG I or SG II rotaviruses was to look for the conserved regions within known sequences of VP6 gene with SG I or SG II specificity of those rotaviruses. After these conserved sequences were determined, they were used as the primer sequences and aligned against the known sequences of each VP6 subgroup and also used as queries in blast alignments. There are a large number of software packages available to perform structurally-based alignments, some of which are linked to the World Wide Web, for example http://primer3.sourceforge.net/ or http://www.basic.northwestern.edu. The program shifted along the sequence to evaluating chunks of the specified primer length based on the following criteria: melting temperature, GC content, and interactions with self and other primers. The specificity of the primer was checked by doing BLASTN search which revealed significant similarity to only each of the subgroup sequences.

2.4. VP6 genogrouping by monoplex and multiplex RT-PCR

Viral dsRNA was extracted from 20% fecal suspension with QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Before performing reverse transcription (RT), a mixture of 10 μl of viral RNA extract and 1 μl of 50% dimethyl sulfoxide (DMSO) was heated at 95 °C for 5 min to denature the dsRNA of the viral genome and then chilled immediately on ice.

For RT reaction, viral RNA was transcribed reversely according to the manufacturer's instruction (Fermentas, Vilnius, Lithuania). Briefly, the denatured RNA (10 μl) was added into 10 μl of RT mixture containing 3.9 μl of RNase free water, 3.0 μl of 5× reaction buffer (Fermentas, Vilnius, Lithuania), 0.4 μl of 20 μM each of forward and reverse primers (VP6-F and VP6-R; Table 2), 0.8 μl of 10 mM dNTPs mix (10 mM of each dNTPs), 0.5 μl of 1 U/μl RNase inhibitor (Invitrogen, Carlsbad, CA, USA), and 1 μl of 200 units/μl of RevertAid™ M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania). The full-length of the VP6 gene was reversely transcribed at 42 °C for 1 h and then heated at 70 °C for 10 min to denature the RT enzyme.

For monoplex RT-PCR of human rotaviruses, 2.5 μl of cDNA template from RT reaction was added to 22.5 μl of a mixture containing 17.75 μl of RNase free water, 2.5 μl of 10× PCR buffer, 0.75 μl of 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.5 μl of 10 mM dNTPs mix, 0.4 μl each of 20 μM of forward primer (VP6-F), reverse primer HG1-R (Table 2) for amplification of VP6 genogroup I or 0.4 μl of 20 μM of reverse primer HG2-R for amplification of VP6 genogroup II, and 0.2 μl of *Taq* polymerase (5 unit/μl, Invitrogen, Carlsbad, CA, USA). Then, PCR amplification was performed under the following thermocycling conditions: 30 cycles of 94 °C for 45 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min in a mastercycler (Eppendorf, Westbury, NY, USA).

For multiplex RT-PCR, the VP6 gene of human rotaviruses with genogroup I or II specificity was amplified with a combination of specific primers VP6-F, HG1-R, and HG2-R (Table 2). A suitable PCR condition was as follows: 2.5 μl of cDNA template from RT reaction was added to 22.5 μl of a mixture containing 17.55 μl of RNase free water, 2.5 μl of 10× PCR buffer, 0.75 μl of 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.5 μl of 10 mM dNTPs mix, 0.4 μl of 20 μM forward primer (VP6-F), 0.2 μl of 20 μM HG1-R reverse primer, 0.4 μl of 20 μM HG2-R reverse primer (Table 2), and 0.2 μl of *Taq* polymerase (5 unit/μl, Invitrogen, Carlsbad, CA, USA). Then, PCR amplification was performed under the following thermocycling conditions: 30 cycles of 94 °C for 45 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min in a mastercycler (Eppendorf, Westbury, NY, USA).

For the monoplex RT-PCR of porcine rotaviruses, the same protocol as the monoplex RT-PCR of human rotaviruses was used, except that cDNA template from RT reaction of porcine rotavirus, reverse primers PG1-R and PG2-R (Table 2) were used instead of HG1-R and HG2-R reverse primers. In addition, primer-annealing temperature of 45 °C for 30 s and primer extension at 72 °C for 40 s were used for VP6 genogrouping of porcine rotaviruses.

For the multiplex RT-PCR, the VP6 gene of porcine rotaviruses with genogroup I or II specificity was amplified using the combination of specific primers VP6-F, PG1-R, and PG2-R (Table 2). Again, the same protocol as the multiplex RT-PCR of human rotaviruses was used, except that cDNA template from RT reaction of porcine rotavirus, PG1-R and PG2-R reverse primers (Table 2) were used instead of HG1-R and HG2-R reverse primers. Additionally, the concentration of PG1-R used in VP6 genogrouping of porcine rotavirus was twice as high (0.4 μl of 20 μM) as those of HG1-R (0.2 μl of 20 μM). Furthermore, primer-annealing temperature of 45 °C for