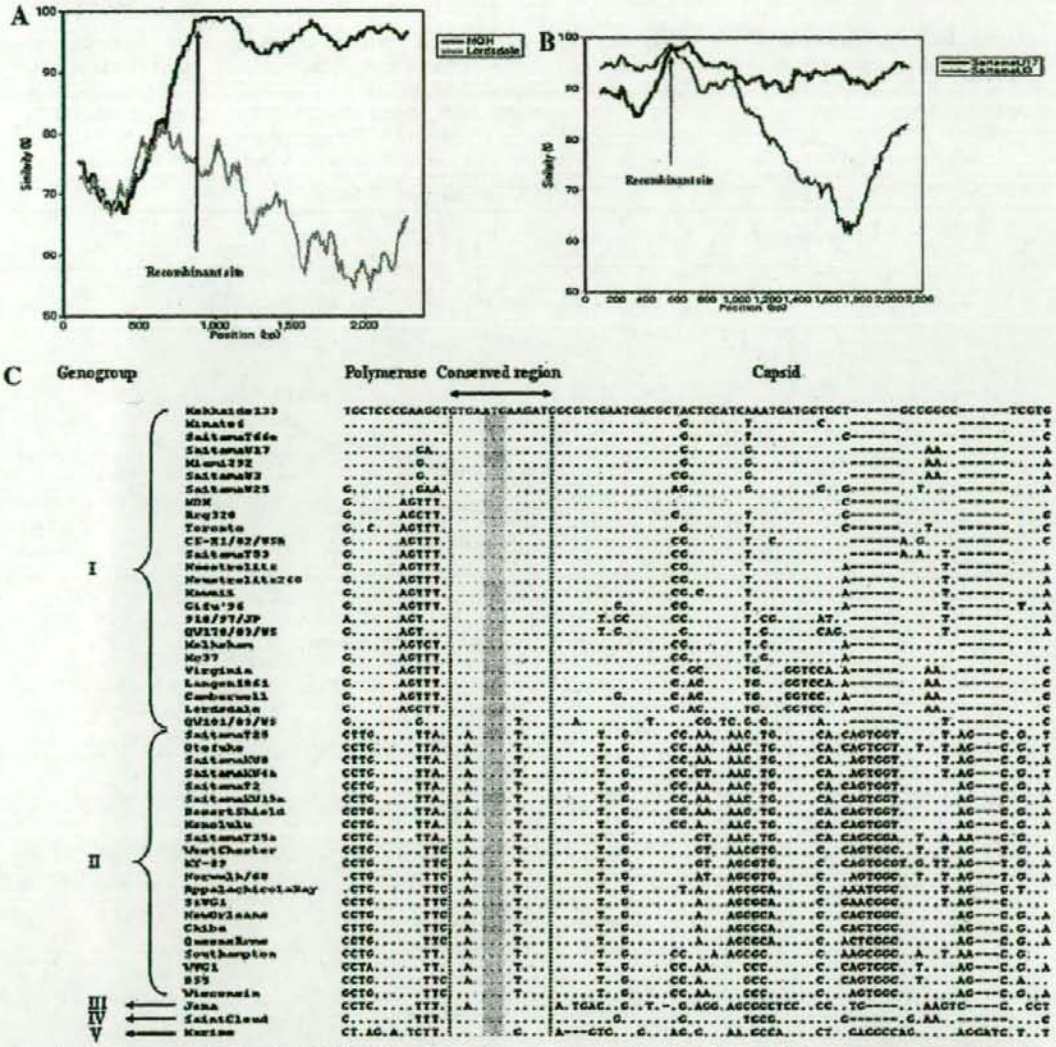

Fig. 4. Norovirus recombination in ORF1/ORF2 overlap. A: The Simplot analysis of the norovirus representative strain of the intergenotype recombination, the Hokkaido133 as the query strain, the reference strain MOH and the reference strain Lordsdale. The low and high homologies with the polymerase and capsid regions among them were found. B: The Simplot analysis of the norovirus strain of the intersubgenotype recombination, the Miami292 as the query strain, the reference strain SaitamaU17 and the reference strain SaitamaU3. The high and low homologies with the polymerase and capsid regions among them respectively were found. C: Direct inspection of the nucleotide sequence alignment of the norovirus polymerase and capsid regions. The 13 nucleotide-highly conserved region covering the partial ORF1/ORF2 overlap was found. The sequence data of the ORF1/ORF2 overlap of the genogroup VI and the genogroup VII were not available in GenBank.

Bull et al., 2005]. In the study, using Simplot the multiple novel intergenotype and intersubgenotype recombinations were also at this junction. Direct observation of the nucleotide sequence alignment of NoV polymerase and capsid genes revealed that the worldwide NoV strains, regardless different genogroups or different genotypes, shared the conserved region, covering this junction. This conserved region suggested that the recombination event occurred when two parental strains come into physical contact when both of them co-infected one cell. This conserved region might represent the break and rejoin site for recombination during viral replication.

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Isolation and Molecular Characterization of Aichi Viruses from Fecal Specimens Collected in Japan, Bangladesh, Thailand, and Vietnam▽

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Aichi virus is a new member of the family *Picornaviridae*, genus *Kobuvirus*, and is associated with human gastroenteritis. This study detected Aichi virus in 28 of 912 fecal specimens which were negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus and were collected in Japan, Bangladesh, Thailand, and Vietnam during 2002 to 2005.

Aichi virus, a small round virus about 30 nm in diameter, was first recognized in 1989 as the cause of oyster-associated nonbacterial gastroenteritis in humans (8–10). The virus was classified into a new genus named *Kobuvirus* of the family *Picornaviridae* (11), which contains nine genera, *Aphthovirus*, *Cardiovirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus* (which includes Aichi virus and bovine kobuvirus), *Parechovirus*, *Rhinovirus*, and *Teschovirus*. The complete Aichi virus genome was determined in 1998 and proved to be a single-stranded positive-sense RNA molecule with 8,251 bases, excluding a poly(A) tail; it contains a large open reading frame with 7,302 nucleotides that encodes a potential polyprotein precursor of 2,433 amino acids (11). In 2000, a reverse transcription (RT)-PCR method for the detection of Aichi virus was developed and a genetic analysis was performed with the 519-base RNA sequences at the putative junction between the C terminus of 3C and the N terminus of 3D. As a result, Aichi virus isolates have been divided into groups 1 (genotype A) and 2 (genotype B) (12).

Studies on Aichi virus were subsequently performed, and it was also detected in Brazil and Germany (2–7). However, there has been limited knowledge about the epidemiology of Aichi virus infection in Asian countries other than Japan and Pakistan. This study was performed to determine the prevalence of Aichi virus in Bangladesh, Thailand, Vietnam, and also in Japan and to provide a better understanding of the epidemiology and genetic relationships between the Aichi virus strains in the present study and the strains previously reported.

A total of 912 stored, extracted RNA samples from fecal specimens known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus by RT-PCR that were collected from patients with acute gastroenteritis in Japan (215 samples collected from July 2002 to June 2003), Bangladesh (405 samples collected from October 2004 to September 2005), Thailand (107 samples collected from March 2002 to December 2004), and Vietnam (185 samples collected from October 2002 to September 2003) were used in this study. First, RT was preformed by random priming. Then, to detect Aichi virus, a nested PCR was conducted with the primer sets for amplifying the 3CD junction region as described by Yamashita et al. (12). The first PCR was conducted with primers 6261 (5′-ACACTCCCACCTCCCGCCAGTA-3′) and 6779 (5′-GGAAGAGCTGGGTGTCAAGA) to amplify a 519-bp region between the C terminus of 3C and the N terminus of 3D. Next, a nested PCR was performed with the primer pair C94b-246k (C94b, 5′-GACTTCCCCGGAGTCGTCGTCT-3′; 246k, 5′-GACATCCGGTTGACGTTGAC-3′) to amplify a 223-bp segment within the 3C-3D junction region.

The products from the first PCR were then purified and sequenced in both directions. Phylogenetic analysis was performed by the neighbor-joining method within the MEGA 3.1 analytical package with a bootstrap of 1,000 replicates (1).

Of the 912 samples tested, 28 (3.1%) were positive for Aichi virus. Of these, 14 samples were collected from Japan, 10 were from Bangladesh, 3 were from Vietnam, and 1 was from Thailand. As a result, the prevalence rates of Aichi virus detected in each country were 6.5%, 2.5%, 1.6%, and 0.9%, respectively. Twenty Aichi virus strains were successfully sequenced, including 11 Japanese strains, 5 Bangladeshi strains, 3 Vietnamese strains, and 1 Thai strain.

The phylogenetic tree constructed from the 519-nucleotide sequences showed that 11 Japanese strains, 3 Vietnamese strains, 1 Thai strain, and 1 Bangladeshi strain were clustered into the same branch with Japanese reference strains A844/88, A848/88, 684/98, and N128/91 and belonged to genotype A. The four other Bangladeshi strains clustered into genotype B, together with Pakistani strains P832/90 and P840/91 (Fig. 1).

From previous studies, Aichi virus was known as one of the agents which cause acute gastroenteritis in human, especially associated with oysters (9, 10). In this study, Aichi virus was found in fecal specimens from diarrheal infants and children that were known to be negative for other common causative agents such as rotavirus, norovirus, adenovirus, astrovirus, and sapovirus. Thus, taken together with previous studies, these findings demonstrated clearly that Aichi virus was related to diarrhea in infants and young children in several Asian countries.

In this study, Aichi virus was detected in Asian countries

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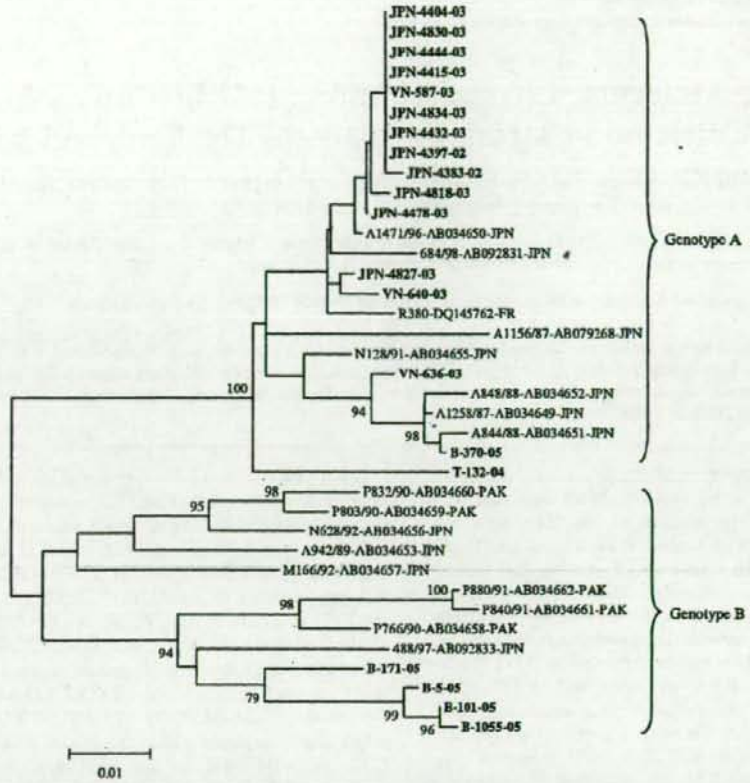

FIG. 1. Phylogenetic tree constructed from the 519-nucleotide sequences between the C terminus of 3C and the N terminus of 3D. Bootstrap values of greater than 70% are shown at the branch nodes. The Aichi virus strains in this study are in boldface. Abbreviations of locations: JPN, Japan; B, Bangladesh; VN, Vietnam; T, Thailand; FR, France; PAK, Pakistan. Aichi virus strains M166/92, N128/91, and N628/92 were isolated from Japanese travelers returning from Malaysia (M) and Indonesia (N).

other than Japan and Pakistan. This is the first finding of Aichi virus in fecal specimens from Bangladesh, Thailand, and Vietnam. These results also provide useful information for the epidemiological understanding of Aichi virus in Japan. Since Aichi virus is a new virus discovered recently, further genetic studies of it are definitely necessary.

Nucleotide sequence accession numbers. The nucleotide sequences of the strains studied here were assigned accession numbers EF079149 to EF079162 and EF466010 to EF466015.

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Amino Acid Substitutions in the VP7 Protein of Human Rotavirus G3 Isolated in China, Russia, Thailand, and Vietnam During 2001–2004

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The distribution of rotavirus G-types in the world appears to be changing, especially with the emergence of G3 and G9 in many countries. Sequence analysis of the VP7 gene was performed on the 27 human G3 rotavirus strains isolated in China, Russia, Thailand, and Vietnam during 2001–2004. All the strains studied were clustered into the same branch of the phylogenetic tree. The comparison of the G3 deduced amino acid sequences between the studied Chinese strains and the strains circulating in China during 1986–1992 showed a wide range of amino acid substitutions (up to 13 amino acids in the VP7 antigenic regions). The two considerable changes both from aspartic acid to asparagine were located at positions 96 in antigenic region A and 213 in antigenic region C. Those amino acid substitutions of the Chinese G3 strains might involve in the emergence of G3 rotavirus in China during 2001–2003. ***J. Med. Virol. 79:1611–1616, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: amino acid substitutions; rotavirus G3; China; Russia; Thailand; Vietnam

INTRODUCTION

Rotaviruses are the most important etiologic agents of severe diarrhea in infants and young children worldwide. It is estimated that rotavirus gastroenteritis causes the deaths of 611,000 infants and young children worldwide every year [Parashar et al., 2006]. Group A rotaviruses are most commonly detected and they have been divided into at least 15 genotypes called G-types (for glycoprotein) and 27 other genotypes called P-types (for protease-sensitive) based on the nucleotide sequences coding for the two capsid proteins VP7 and VP4, respectively [Rao et al., 2000; Kapikian et al., 2001; Varshney et al., 2002; Martella et al., 2006; Steyer et al., 2006; Khamrin et al., 2007]. Many epidemiological studies indicated that G1P[8], G2P[4], G3P[8], and G4P[8] are the most common G and P genotype associations [Desselberger et al., 2001; Santos and Hoshino, 2005].

Many of the earlier studies have focused on VP7, the protein that expresses the major neutralization antigen of rotavirus. By comparison of deduced amino acid sequences, nine variable regions (VR), VR1–VR9, have been defined in the entire VP7 sequence [Hoshino et al., 1994; Estes, 2001]. The three main antigenic regions: A (amino acid (aa) 87–101), B (aa 143–152), and C (aa 208–221), which correspond to VR5, VR7, and VR8 respectively, have been confirmed as the major rotavirus neutralization sites [Dyall-Smith et al., 1986; Taniguchi et al., 1988; Coulson and Kirkwood, 1991]. In addition, the three other antigenic regions have also been identified. They are region D (aa 291) [Coulson and Kirkwood, 1991; Dunn et al., 1993], region E (aa 190) [Dunn et al., 1993; Lazdins et al., 1995], and region F corresponding to VR9 (aa 235–242) [Kirkwood et al., 1993; Hoshino et al., 1994].

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Regarding the G-types, rotaviruses bearing G1, G2, G3, and G4 are the most common rotaviruses worldwide. Among these, rotavirus G1 has been the predominance in many countries for a long time [Santos and Hoshino, 2005]. Recently, different surveys showed that the distribution of rotavirus G-types seems to be changing. Rotavirus G1 has decreased, while rotavirus G3 has gradually increased and dominated over other G-types in some countries, such as Japan, China, Russia, and Ireland [Fang et al., 2002, 2005; Zhou et al., 2003; Lo et al., 2005; Reidy et al., 2005; Phan et al., 2006; Yoshinaga et al., 2006]. Rotavirus G9 has especially emerged as a common global strain [Ramachandran et al., 1998; Santos et al., 2001; Das et al., 2002; Armah et al., 2003; Kirkwood et al., 2003; Reidy et al., 2005; Khamrin et al., 2006]. Other rotavirus types such as G5, G8, G10, and G12 strains have increasingly been reported in different parts of the world [Beards and Graham, 1995; Santos et al., 1998; Das et al., 2003; Fischer et al., 2003; Laird et al., 2003; Castello et al., 2006; Samajdar et al., 2006]. However, no considerable change in the VP7 gene of rotavirus G1 isolated in Japan, China, Thailand, and Vietnam was noted in one previous study [Trinh et al., 2007]. Therefore, in the context of changing distribution of human rotavirus G-types in the world, especially the emergence of rotavirus G3, a sequence analysis was performed on several recent G3 rotavirus strains isolated in China, Russia, Thailand, and Vietnam during 2001–2004 to determine whether any change in the VP7 genes occurred.

MATERIALS AND METHODS

Clinical Specimens

A total of 27 G3 rotavirus strains from China (six strains, collected in Kunming, 2001–2003), Russia (seven strains, Khabarovsk, 2003–2004), Thailand (five strains, Chiang Mai, 2001), and Vietnam (nine strains, Children's Hospital 1–Ho Chi Minh City, 2002–2003) were subjected for the sequence analysis. The fecal specimens used in this study were known to be infected with rotavirus G3 by PCR using type-specific primer set [Gouvea et al., 1990].

RNA Extraction, RT-PCR, and Sequence Analysis

RNA extraction was first done using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany). The reverse transcription–polymerase chain reaction (RT-PCR) was then performed using the well-known primers Beg9 and End9 [Gouvea et al., 1990]. To obtain the amplicons necessary for sequencing and to amplify the first half of the VP7 gene, the newly designed reverse primer Mid1r (nucleotides 662–643), 5′-ATT CCT AAC GTT TGT GTA TT-3′ was used in combination with Beg9 (forward primer). A new forward primer Mid2 (nucleotides 517–537), 5′-GAT TTG ATA TTG AAT GAA TGG-3′ was designed to pair with End9 to obtain the remaining portion of the VP7 gene. Next, cycle sequence was conducted for both VP7 segments in both directions by using the four sequencing primers Beg9, Mid1r, End9, and Mid2 to ensure that the nucleotide sequences would be obtained correctly. Finally, the sequencing of the amplicons was performed using an automated sequencing system (ABI Prism 310, Perkin-Elmer Applied Biosystems, Inc., Foster City, CA). The comparison analysis was conducted on the obtained G3 strains and other G3 reference strains available in GenBank. The sequence data and the phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was also conducted using MEGA version 3.1 to determine the evolutionary relationship among studied sequences [Kumar et al., 2004]. The method was performed using heuristic search and close-neighbor interchange with a random option and with 500 bootstrap repetitions. Ninety-five percent confidence intervals were obtained using the mean and standard error values after 500 bootstrap resamplings using MEGA software.

Reference G3 rotavirus strains and accession numbers used in this study are the following: strain SA11, accession no. K02028; M, AB180974; 95-87, D86265; Ai-39, D86268; Ai-53, D86269; AU-1, D86271; AU-17, D86272; CH-32, D86273; CH-55, D86274; CH927, D86275; CHW17, D86276; CHW2, D86277; ITO, D86278; J-12, D86279; MO, D86280; TK08, D86281; YO, D86284; 107E1B, AB081594; 98/B31, AF260958.

RESULTS

The VP7 gene with 1,062 nucleotides in length of all the 27 G3 strains isolated in the four countries was successfully sequenced. Based on the global G3 strains available in the GenBank database for performing phylogenetic analysis, Figure 1 clearly showed that human rotavirus G3 strains including the strains studied clustered into two separate branches. One branch comprised of the Chinese strains isolated during the period of 1986–1992, except for the CH927 strain [Wen et al., 1997], and another branch included all the studied G3 strains from China, Russia, Thailand, and Vietnam and the global remaining strains.

The comparison analysis showed high homologies between the studied G3 strains (more than 98%) at nucleotide and amino acid levels. The homologies greater than 94.3% were noted between the strains studied and the human reference strains including the prototype strain M isolated in USA in 1976. Except for the Chinese strains isolated in 1986–1992, they were quite different from the remaining reference strains and the strains studied with the homologies ranging from 89.6% to 93.1% at amino acid level, and less than 90% (83% to 89.7%) at nucleotide level (data not shown).

Distance matrix analysis of the VP7 gene sequences of these strains combined with their phylogenetic distribution demonstrated two major lineages (designated as I and II) (Fig. 1 and Table I). Reference simian strain SA11 was comparably distantly related to the other strains at nucleotide level. The relationship among

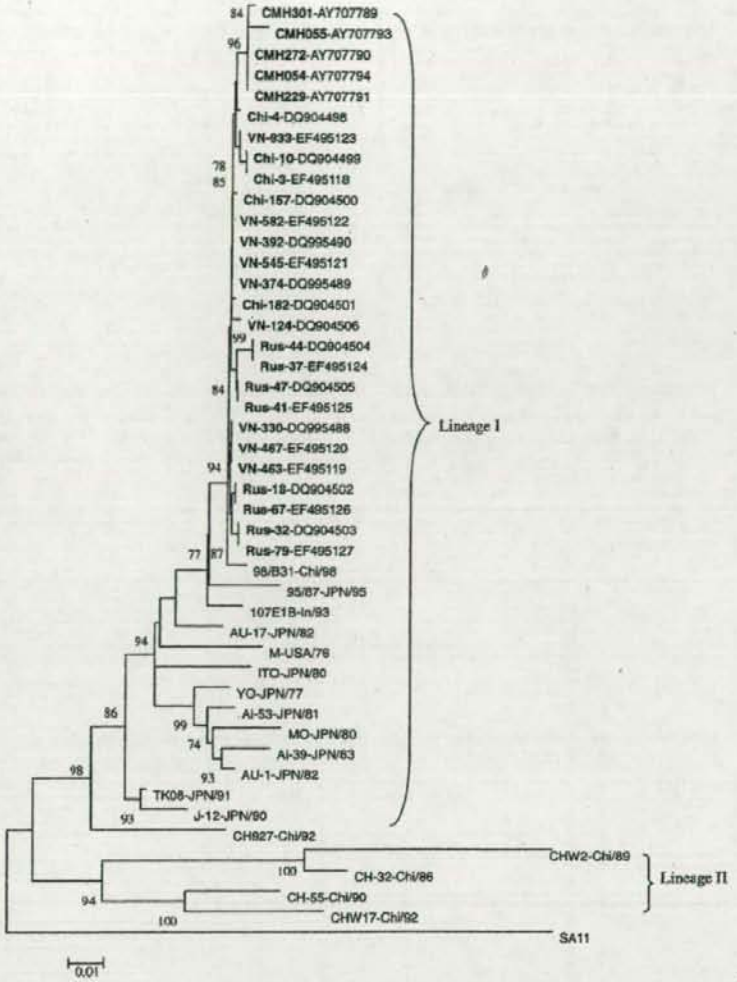

Fig. 1. Phylogenetic analysis of VP7 nucleotide sequences of the G3 strains. The phylogenetic tree was constructed based on 1,062 nucleotide sequences of the G3 VP7 genes. The strains in this study are in boldface type. Percentage bootstrap values above 70% are shown at the branch nodes. Abbreviations for locations: Chi for China, Rus for Russia, VN for Vietnam, CMH for Thailand, JPN for Japan, In for India. SA11: simian rotavirus strain, isolated in 1978. Isolation years of the strains studied: Chinese strains, 2001–2003; Russian strains, 2003–2004; Thai strains, 2001; Vietnamese strains, 2002–2003.

TABLE I. Percentages of Nucleotide Distances Among G3 Lineages

G3 lineage	Mean % nucleotide distance (95% confidence interval)		
	Lineage I	Lineage II	SA11
Lineage I	**3.5 (0.1–8.8)**		
Lineage II	15.5 (9.6–21.4)	**12.7 (6.6–18.7)**	
SA11	22.2 (21.2–23.2)	26.4 (24.2–28.5)	NA

Nucleotide distances within lineages are presented in boldface type. NA, not applicable.

lineages is clearly shown in Table I. The same lineages were defined by 3.5% and 12.7% nucleotide distances, while distances between lineages were higher than 15.5%.

Figure 2 showed the amino acid alignment of the strains studied and the reference G3 strains with respect to the prototype strain M. The strains studied from the four countries were almost identical in all of the six known antigenic regions. Two accumulated changes at positions 238 and 242 in antigenic region F were noted. In comparison to the simian prototype strain SA11, the G3 studied strains had three amino acid

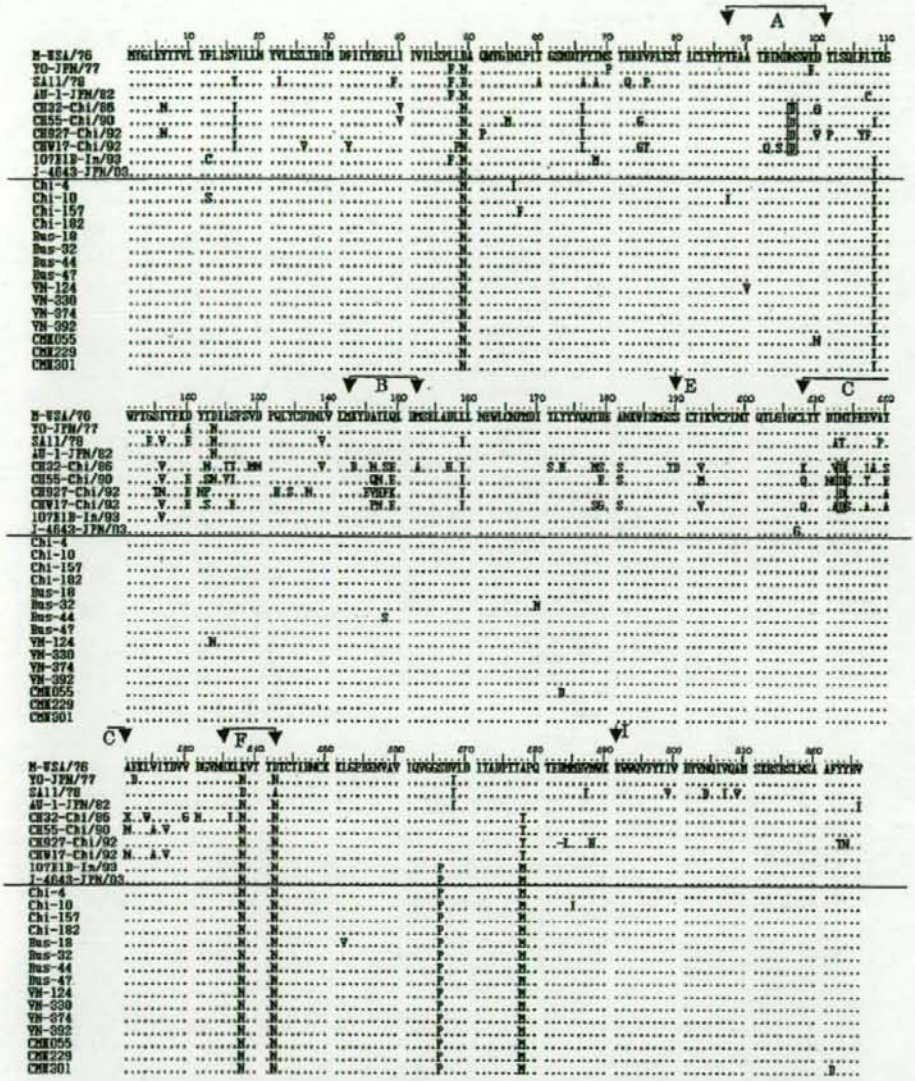

Fig. 2. Alignment of the deduced amino acid sequences of the VP7 gene between the studied G3 strains and the reference strains. SA11: simian rotavirus strain. The identical amino acids in each of the studied and reference strains with the strain M are identified by dots. Antigenic regions A–F are shown. Amino acids at positions 96 and 213 of the Chinese reference strains isolated in 1986–1992 are in boxes. Abbreviations for locations: Chi for China, Rus for Russia, VN for Vietnam, CMH for Thailand, JPN for Japan, In for India.

substitutions at positions 212, 213, and 219 in antigenic region C. However, there was a wide range of numbers of amino acid substitutions (up to 13) of the strains studied compared to the reference Chinese trains isolated in 1986–1992. Interestingly, there were two consistent amino acid changes at position 96 in antigenic region A and position 213 in antigenic region C, both from aspartic acid (Asp) to asparagine (Asn).

DISCUSSION

By the phylogenetic trèe and the G3 sequences presented nucleotide distance variations up to more than 20%, it is proposed that human G3 rotaviruses should be divided into two lineages: lineage I includes the global human G3 strains, lineage II includes the Chinese strains circulating during 1986–1992, except

for the CH927 strain [Wen et al., 1997] (Fig. 1 and Table I).

The G3 strains isolated in Russia, Thailand, and Vietnam could not be compared to the previous data, because this was the first time that the G3 VP7 genes from the three countries were sequenced. No dominance of rotavirus G3 in Thailand and Vietnam was noted. In Russia, however, rotavirus G3 was detected in a very high prevalence (86.6%) during the period of 2003–2004 [Phan et al., 2006]. Unfortunately, there were no reports on the distribution of rotavirus G-types in Russia prior to 2003. In China, before the year 2000, rotavirus G1 was the most prevalence. It was reported as high as 72.6% during 1998–2000. Meanwhile, G3 accounted for only 14.2%. In the following years, however, rotavirus G3 was identified with a very high detection rate (66.8%), it turned out to be the prevailing genotype in China [Qiao et al., 1999; Fang et al., 2002, 2005; Lo et al., 2005].

Several previous studies indicated that some particular amino acids in the six known antigenic regions, especially positions 94, 96, 147, 148, 190, 208, 211, 213, 238, and 291, are immunodominant epitopes by mapping of the neutralizing monoclonal antibody (N-MAb)-selected variants. By amino acid substitutions at those positions, with or without glycosylation change, human rotaviruses can change their antigenicity and escape host immunity [Mackow et al., 1988; Taniguchi et al., 1988; Gorziglia et al., 1990; Coulson and Kirkwood, 1991; Kobayashi et al., 1991; Green and Kapikian, 1992; Dunn et al., 1993; Kang et al., 1993; Kirkwood et al., 1993; Hoshino et al., 1994; Lazdins et al., 1995].

The comparison between the studied Chinese strains and the previous strains circulating in 1986–1992 revealed that although there was a diverse variation in the VP7 gene of the Chinese reference strains, two considerable changes were clearly noted at positions 96 in the region A and 213 in the region C. These two positions are commonly known as immunodominant sites of the protein VP7 and the most critical for reaction with neutralizing MAbs. Lazdins et al. [1995] reported that rotavirus with a mutation in the region C caused a 10-fold increase in resistance to neutralization by antiviral antiserum. The amino acid substitutions at these two positions were found to be some of the important changes that associated with failure to serotype, and responsible for the antigenic change [Nishikawa et al., 1989; Coulson et al., 1996; Gomara et al., 2001]. Recently, a report on those amino acid substitutions which associated with the emergence of rotavirus G3 isolated in Japan during 2003–2004 had also been proposed [Phan et al., 2007]. In summary, the findings of amino acid substitutions in the three main antigenic regions, especially at position 96 and position 213 might involve in the emergence of rotavirus G3 in China during 2001–2003, and also it was might be associated with the high detection rate of rotavirus G3 in Russia during 2003–2004. Surveillance of rotavirus infection should be done continuously to determine whether these strains emerge in Thailand and Vietnam, and continue to be dominant in China and Russia in future.

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Development and Validation of DNA Microarray for Genotyping Group A Rotavirus VP4 (P[4], P[6], P[8], P[9], and P[14]) and VP7 (G1 to G6, G8 to G10, and G12) Genes▽†

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Previously, we reported the development of a microarray-based method for the identification of five clinically relevant G genotypes (G1 to G4 and G9) (V. Chizhikov et al., J. Clin. Microbiol. 40:2398–2407, 2002). The expanded version of the rotavirus microarray assay presented herein is capable of identifying (i) five clinically relevant human rotavirus VP4 genotypes (P[4], P[6], P[8], P[9], and P[14]) and (ii) five additional human rotavirus VP7 genotypes (G5, G6, G8, G10, and G12) on one chip. Initially, a total of 80 cell culture-adapted human and animal reference rotavirus strains of known P (P[1] to P[12], P[14], P[16], and P[20]) and G (G1-6, G8 to G12, and G14) genotypes isolated in various parts of the world were employed to evaluate the new microarray assay. All rotavirus strains bearing P[4], P[6], P[8], P[9], or P[14] and/or G1 to G6, G8 to G10, or G12 specificity were identified correctly. In addition, cross-reactivity to viruses of genotype G11, G13, or G14 or P[1] to P[3], P[5], P[7], P[10] to P[12], P[16], or P[20] was not observed. Next, we analyzed a total of 128 rotavirus-positive human stool samples collected in three countries (Brazil, Ghana, and the United States) by this assay and validated its usefulness. The results of this study showed that the assay was sensitive and specific and capable of unambiguously discriminating mixed rotavirus infections from nonspecific cross-reactivity; the inability to discriminate mixed infections from nonspecific cross-reactivity is one of the inherent shortcomings of traditional multiplex reverse transcription-PCR genotyping. Moreover, because the hybridization patterns exhibited by rotavirus strains of different genotypes can vary, this method may be ideal for analyzing the genetic polymorphisms of the VP7 or VP4 genes of rotaviruses.

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Group A rotaviruses remain the single most important etiologic agents of severe diarrhea in infants and young children worldwide. Rotavirus diarrhea has been estimated to be responsible for a median of 611,000 deaths annually in children under 5 years of age, predominantly in developing countries (43). In the United States, approximately 2.7 million children are affected by rotavirus illness each year, resulting in about 20 deaths, 50,000 hospitalizations, 500,000 physician visits, and more than 1 billion U.S. dollars in societal costs (5, 19, 20, 30, 43). Because of the significant morbidity and mortality associated with rotavirus diarrhea, the development and implementation of a safe and effective rotavirus vaccine has been an important public health priority. Recently, two rotavirus vaccines have been licensed in many countries; the effectiveness of such vaccines in poor populations in certain sub-Saharan African as well as Southeast Asian countries remains to be determined (20).

Rotaviruses are nonenveloped, icosahedral viruses of the family *Reoviridae* with 11 genomic segments of double-stranded RNA, each encoding at least one structural or nonstructural protein. The rotavirus outer capsid proteins VP7 (which defines G types) and VP4 (which defines P types) evoke neutralizing antibodies independently (22, 25, 42). In addition, since neutralizing antibodies appear to play an important role in protection against rotavirus disease and infection in a serotype-specific manner (for reviews, see references 26 and 29), rotavirus strain surveillance (i.e., G and/or P type determination) has been conducted throughout the world. Various assays have been developed to determine G and P serotypes of rotaviruses, including type-specific-monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA) (2, 10, 55, 57) and a neutralization assay using type-specific polyclonal antibodies (18, 26, 60). However, because of the lack of ap-

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propriate and readily available reagents (e.g., G or P type-specific high-titer polyclonal antisera; MAb to G5, G6, G8, G9, or G10; and MAbs to various P types), serotyping is not an available option for processing a variety of rotavirus field samples. Genotyping by reverse transcription PCR (RT-PCR) developed in the early 1990s as a proxy method for serotyping, which employs a set of genotype-specific primers (multiplex RT-PCR), is rapid, simple, and sensitive (11, 14, 15, 21, 24, 58). Today, multiplex RT-PCR is the most widely used method for the identification of rotavirus G and P genotypes. However, there are several drawbacks in this method; for example, (i) because a majority of genotype-specific primers used in multiplex RT-PCR are designed from the sequences of strains isolated more than a decade ago, they may carry sequence mismatches in the primer binding regions of the target gene(s) of more-recent rotavirus strains, resulting in decreased sensitivity, and (ii) because a genotype is determined only by the size of PCR products in a gel, it is not uncommon to encounter a situation in which the possibility that certain spurious bands are present cannot be excluded or a mixed infection cannot be incriminated. Indeed, it has been reported that genotyping results could vary depending upon the primer pairs used in the multiplex RT-PCR due to the accumulation of point mutations in primer binding regions of the VP4 or VP7 gene (1, 4, 13, 27, 28, 38, 44, 48, 52). Not only for epidemiological rotavirus strain surveillance but also for effective rotavirus vaccine development, there is a need for sensitive and reliable diagnostic techniques which do not bear such disadvantages. Previously, we reported the development of a rapid and reliable method for the identification of clinically relevant human rotavirus G genotypes (i.e., G1 to G4 and G9) using oligonucleotide microarray hybridization (8). By using this method, which combines the sensitivity of PCR and the specificity of hybridization, we were successful in detecting and unambiguously identifying such G genotypes. Although 16 G genotypes and 28 P genotypes have been identified thus far (23, 36, 37, 46, 56), five G genotypes (G1 to G4 and G9) and two P genotypes (P[4] and P[8]) have been repeatedly shown worldwide to be of epidemiologic importance in humans (16, 17, 30, 32, 51). More recently, however, G genotypes other than G1 to G4 and G9 and P genotypes other than P[4] and P[8] have been detected in various parts of the world and include G5, G6, G8, G10, G12, P[6], P[9], and P[14] (for reviews, see references 17, 47, and 51). In this study, we modified the original microarray assay and expanded it to include the identification of (i) five clinically relevant human rotavirus VP4 genotypes (P[4], P[6], P[8], P[9], and P[14]) and (ii) five additional human rotavirus VP7 genotypes (G5, G6, G8, G10, and G12). The usefulness and validity of this assay were confirmed by analyzing a total of 128 stool rotaviruses collected between 1990 and 2004 from three countries. In addition, we evaluated whether this method could be applied to analyze the genetic polymorphism of the VP7 or VP4 genes of rotaviruses in a given G or P genotype isolated in different parts of the world.

MATERIALS AND METHODS

Rotavirus strains. A total of 80 cell culture-adapted human and animal reference rotavirus strains of known G and P genotypes were tested in this study (see Table S1 in the supplemental material). These strains, which were isolated from humans and eight different animal species in 19 countries on five continents between 1958 and 1999, represented collectively 13 G genotypes (G1 to G6 and G8 to G14) and 15 P genotypes (P[1] to P[12], P[14], P[16], P[18], and P[20]) (See Table S1 in the supplemental material). Each of the 80 rotavirus strains was plaque purified three times in MA104 monkey kidney cells prior to use.

Rotavirus-positive human stool samples. For further validation of this assay, we analyzed a total of 128 rotavirus-positive human stool field samples collected in three countries (Brazil, Ghana, and the United States) between 1990 and 2004, which were all previously analyzed by RT-PCR.

RNA extraction, RT-PCR, and nested PCR. Viral double-stranded RNA was extracted with TRIzol (Invitrogen) from infected cell culture lysates or stool suspensions (approximately 10%) as described previously (53). In order to increase the sensitivity of the microarray assay, gene amplifications were carried out in a first-round RT-PCR, followed by a nested PCR. RT was performed as follows: extracted double-stranded RNA (1 μl) was added to 10 μl of 1× first-strand buffer containing 10 mM Tris-HCl (pH 8.3); 40 mM KCl; 1.5 mM $MgCl_2$; a 200 μM concentration (each) of dATP, dCTP, dGTP, and dTTP; 5 mM dithiothreitol; 3.5% dimethyl sulfoxide; and a 100 pM concentration (each) of two primers (Beg9 and End9 for VP7 and F1 and C10 for VP4) listed in Table S2 in the supplemental material. The mixture was heated at 97°C for 5 min and placed on ice for 5 min, and then 2 U of SuperScript II (Invitrogen) was added. The tubes were set in a GeneAmp PCR system thermocycler (Applied Biosystems) and incubated at 42°C for 45 min. First-round PCR was performed in 25 μl of 1× storage buffer B containing 2.5 mM $MgCl_2$, a 200 nM concentration of each primer, a 100 μM concentration of each of the four deoxynucleoside triphosphates, 5 U of *Taq* DNA polymerase (Promega), and 1 μl of the RT product. Nested PCR was carried out in 25 μl of the same reaction mixture except that the different primer sets (LID3 and G922 for VP7 and F4 and C8 for VP4) and the first-round-PCR product (1 μl) were included. In both PCR steps, amplification was done under the following conditions: initial denaturation at 94°C for 2 min; 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min; and a final extension at 72°C 5 min. The PCR products were analyzed by electrophoresis in 1% agarose gel in 1× Tris-acetate-EDTA buffer containing 0.2 μg/ml of ethidium bromide.

Synthesis of Cy5-labeled DNA samples. The fluorescently labeled single-stranded DNA samples for hybridization were generated by the single primer extension (PE) reaction in the presence of a single forward primer used in the nested PCR. The reaction was performed in 25 μl of a reaction mixture containing 1× storage buffer B with 2.5 mM $MgCl_2$; a 600 nM concentration of each forward primer; a 200 nM concentration (each) of dGTP, dATP, and dTTP; 40 nM dCTP; 40 nM Cy5-dCTP; 5 U of *Taq* DNA polymerase (Promega); and 4 to 10 μl of a DNA template of the purified (QIAquick PCR purification kit) nested-PCR product. The following conditions were used in the PE reaction: 1 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 55°C, and 2 min at 72°C; and a final intubation for 10 min at 72°C. Cy5-labeled samples were separated from nonincorporated Cy5-dCTP by centrifugation on Centri-Sep columns (Princeton Separations).

Synthesis of a Cy3-labeled QC oligonucleotide. The Cy3-labeled quality control (QC) oligonucleotide was prepared as described previously (8).

Design of genotype-specific oligonucleotide probes. More than 720 VP7 gene sequences and 450 VP4/VP8* gene sequences obtained from GenBank were aligned and analyzed with MacVecor 6.0 (Accelrys). Sequences of highly conserved regions within a given G or P genotype were selected to design genotype-specific oligonucleotide probes which are listed in Table S2 in the supplemental material. The 5′ end of each probe bore an amino-link group to immobilize the probe effectively on silylated (aldehyde-coated) glass slides (CEL Associates, Inc.).

Microchip fabrication. Microchip fabrication was performed as previously described (8), with a slight modification. Microarray chips were printed using a contact microspotting robot (Cartesian Technologies, Inc.) and a ChipMaker microspotting device (TeleChem International, Inc.). The average spot diameter was 250 μm. The final spotting solution contained a 100 μM concentration of a genotype-specific oligonucleotide probe and a 20 μM concentration of a QC oligonucleotide in 0.25 M acetic acid. After the chips were printed, slides were dehydrated for 15 min at 80°C and incubated for 10 min in a freshly prepared 0.25% solution of $NaBH_4$. Then the slides were washed once for 5 min with 0.2% sodium dodecyl sulfate in water and five times for 1 min each with distilled water.

Hybridization. Hybridization was performed at 45°C for 30 min in an incubation chamber (ArrayIt). Immediately before hybridization, 1.8 μl of the Cy5-labeled sample was mixed with an equal volume of 2× hybridization buffer containing the QC oligonucleotide labeled with 0.15 μM Cy3. The mixture was heated at 99°C for 1 min to denature double-stranded DNA, followed by chilling on ice. The hybridization aliquot was applied to the array chip and covered with a plastic coverslip (4 by 7 mm) to prevent evaporation of the sample during

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incubation. After hybridization, the slide was washed once for 1 min with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) containing 0.2% Tween 20, once for 2 min with 6× SSC, and once for 2 min with 2× SSC and then dried by an air stream.

Microarray scanning and data analysis. Fluorescent images of the microchip were generated by scanning the slides by using a confocal GenePix 4100A personal fluorescence scanner (Axon Instruments). The fluorescent signals from each spot obtained at 570 nm (Cy3) and 694 nm (Cy5) were analyzed using GenePix Pro 5.0 software (Axon). Background signals obtained from the region surrounding each spot were subtracted, and the resulting absolute value of the Cy5 fluorescent signal from each probe was divided by the Cy3 signal from the QC probe of the same spot. Fluorescent signals with a statistically significant difference ($P < 0.01$) from the average background level were considered to be positive.

RESULTS

RT-PCR. A total of 80 rotavirus samples each of which bore one of 13 G genotypes (G1 to G6 and G8 to G14) and one of 15 P genotypes (P[1] to P[12], P[14], P[16], and P[20]) were analyzed by first-round and nested PCR. For first-round PCR, we used a conventional primer pair, Beg9 and End 9 (21), to synthesize full-length VP7 genes (1,062 bp). Seventy-five of the 80 (93.8%) samples were positive by first-round PCR. In order to increase the sensitivity, we carried out nested PCR on the first-round-PCR products using the primer pair LID3 and 922 (see Table S2 in the supplemental material) and successfully amplified all 80 samples. The size of amplicons generated by nested PCR was 894 bp. For the amplification of the VP4 genes, we designed four new PCR primers (F1, F4, C8, and C10) from conserved regions of the VP4 gene. Two conserved regions, from nucleotides 1 to 20 and 4 to 23, were used to design the forward primers F1 and F4, respectively, and two other conserved regions, from nucleotides 1446 to 1467 and 1595 to 1618, were selected to make the reverse primers C8 and C10, respectively (Table S2 in the supplemental material). The sensitivity of the first-round PCR using the F1 and C10 primer pair was 91.3% (73 of 80 samples). All 80 strains used in this study were amplified by nested PCR using the F4 and C8 primer pair. The sizes of amplicons generated by the first-round and nested PCRs were 1,618 bp and 1,464 bp, respectively.

Sensitivity of the oligonucleotide microarray assay. To determine the sensitivity of the oligonucleotide microarray assay, the nested-PCR products of the VP4 or VP7 gene of human rotavirus strain D (P[8]G1) were analyzed. Figure 1A shows a gel electrophoresis analysis of serially diluted (fivefold) cDNA amplicons of the VP7 (lanes 2 to 6) and VP4 (lanes 7 to 11) genes of strain D. The amount of cDNA in the initial reaction was 1 μg/μl (lanes 2 and 7); this was followed by a fivefold dilution series (lanes 2 to 6 and 8 to 11). Each diluted sample was purified and used as a template for the PE reaction under the conditions described in Materials and Methods. The scanned fluorescent images and fluorescence intensities of these samples are shown in Fig. 1B and C, respectively. The minimum detectable concentration of the nested-PCR product was 8 ng/μl, which corresponded to the minimum concentration that produced a visible band in a 1% agarose gel after electrophoresis.

Design and fabrication of G and P genotyping array chip. We previously reported the design and fabrication of an oligonucleotide microarray chip for the identification of five clinically relevant G genotypes (G1 to G4 and G9) of human rotaviruses (8). We recently designed genotype-specific oligonucleotide probes for five additional human rotavirus G genotypes (G5, G6, G8, G10, and G12). After eliminating cross-reactive probes, we selected seven probes for each G genotype.

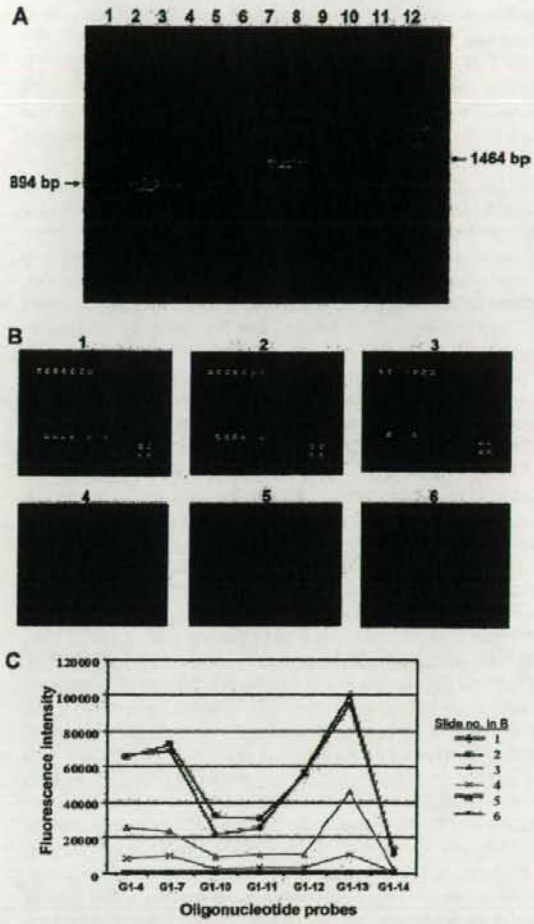

FIG. 1. Sensitivity of the oligonucleotide microarray hybridization assay. (A) Gel electrophoresis analysis of serially fivefold-diluted nested-PCR products of the VP7 gene (lanes 2 to 6) and VP4 gene (lanes 7 to 11) of human rotavirus D strain (G1,P[8]). A 10-μl sample of cDNA amplicons of each concentration was electrophoresed in a 0.8% agarose gel and stained with ethidium bromide. Lanes: 1 and 12, 1-kb DNA ladder mix; 2 and 7, 1 μM; 3 and 8, 200 nM; 4 and 9, 40 nM; 5 and 10, 8 nM; and 6 and 11, 1.6 nM. (B) Scanned images of fluorescently labeled cDNA samples of the VP7 or VP4 gene of the D strain hybridized to a microchip. Cy5-labeled samples of the VP7 and VP4 genes generated by PE were mixed immediately before hybridization. The concentration of cDNA amplicons used for PE were as follows: panel 1, 1 μM; panel 2, 200 nM; panel 3, 40 nM; panel 4, 8 nM; panel 5, 1.6 nM; and panel 6, 0 nM (distilled water). (C) Fluorescence intensities of the seven genotype G1-specific probes scanned from images in panel B. Numbers 1 to 6 in panel C correspond to images 1 to 6 in panel B.

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For P genotyping, we designed 9 to 12 genotype-specific oligonucleotide probes for each of five clinically relevant human rotavirus P genotypes (i.e., P[4], P[6], P[8], P[9], and P[14]) (see Table S1 in the supplemental material). Oligonucleotide probes complementary to the primers used for nested PCR served as positive controls. The sequences of anti-LID3 and anti-F4 were complementary to those of LID3 and F4, respectively (Table S2 in the supplemental material). Each genotype-specific probe and positive-control probe were mixed with QCprb (an oligonucleotide complementary to the Cy3-labeled QC probe) before the chips were printed onto glass slides. Thus, each spot on the glass slide contained QCprb not only for an evaluation of spotting reproducibility and hybridization efficiency but also for normalization of data. Figure 2A shows the newly designed rotavirus G and P genotyping microarray chip.

Analysis of fluorescent images. By analyzing scanned fluorescent images of microchips, we could detect and discriminate successfully all 78 samples belonging to one of 10 G genotypes (G1 to G6, G8 to G10, and G12) and all 52 samples bearing one of 5 P genotypes (P[4], P[6], P[8], P[9], and P[14]) (see Table S1 in the supplemental material). In addition, no significant cross-reactivity was detected from 3 strains belonging to the G11, G13, or G14 genotype and 28 strains belonging to the P[1] to P[3], P[5], P[7], P[10], P[11], P[12], P[16], or P[20] genotype, against which no specific probes were available on the chip. Figure 2B shows fluorescent images of microchips obtained from six rotavirus strains bearing one of five G (G2, G5, G6, G10, and G12) and one of five P (P[4], P[8], P[9], P[11], and P[14]) genotypes. In general, the specificities of all genotype-specific probes were high; however, there were a few cross-reactive probes that hybridized with samples of heterologous genotypes. For example, (i) probe G2-8 hybridized with two G10 strains (B223 [not shown] and KC-1 [Fig. 2B]) and (ii) probes G5-5, G6-4, and G9-5 hybridized with G3 strains Cat2, HCR3, and RRV, respectively. Among the P genotype-specific probes, only two probes, P4-1 and P14-6, cross-hybridized with a P[6] strain, US1205, and a P[8] strain, BD524, respectively. Of note was the finding that each cross-reactive probe hybridized with some but not all strains within a given genotype. Furthermore, there were no samples that hybridized with more than one heterologous probe, and in addition, the intensities of all cross-reactive signals were low.

Fluorescence intensity profiles. Figure 3 shows the quantitative fluorescence profiles of selected rotavirus strains bearing P[4], P[6], P[8], P[9], or P[14] specificity after analysis with GenePix Pro 5.0 software. The normalized maximum of quantitative fluorescence (Cy5) signals (percentage) of each oligonucleotide probe is shown on the *y* axis. In such profiles, the unique hybridization pattern of each rotavirus strain within a given G or P genotype can be recognized more clearly than in scanned fluorescent images (Fig. 2B). Because of spontaneous mutations in the probe binding regions of the VP4 or VP7 gene, different strains within the same genotype tended to display diverse patterns of hybridization.

Analysis of rotavirus-positive human field stool samples by microarray. In order to validate the microarray assay, we analyzed a total of 128 rotavirus-positive stool samples; 64 were from Brazil, 31 from Ghana, and 33 from the United States. Each of the samples was analyzed previously by RT-PCR in

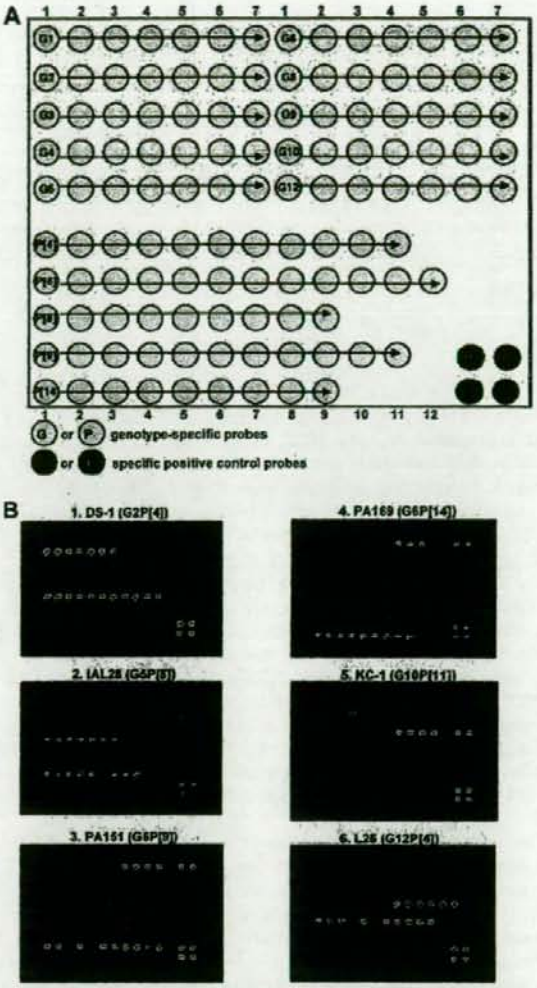

FIG. 2. (A) Layout of the rotavirus G (yellow) and P (blue) genotype-specific probes on the microarray chip. "G" and "P" in orange circles represent VP7 (anti-LID3) and VP4 (anti-F4) positive-control probes, respectively. (B) Scanned fluorescent images of hybridization patterns of six rotavirus strains each bearing various G-P combinations, as noted. Cy5 fluorescence-positive signals and Cy3 fluorescence QC signals were merged by using GenePix Pro 5.0 software.

each country. Tables 1 and 2 summarize the results. Several interesting features emerged from this study: (i) all the G and/or P genotypes that were previously nontypeable by RT-PCR were typed by microarray analysis, and (ii) a relatively large number of samples gave discordant genotyping results in the two assays. Because of this, we used the third assay, PCR-ELISA, and confirmed the microarray results. In the PCR-ELISA, the biotinylated VP7 or VP4 gene amplicons generated by RT-PCR were immobilized onto streptavidin-coated 96-well microplates and hybridized to digoxigenin-labeled G or

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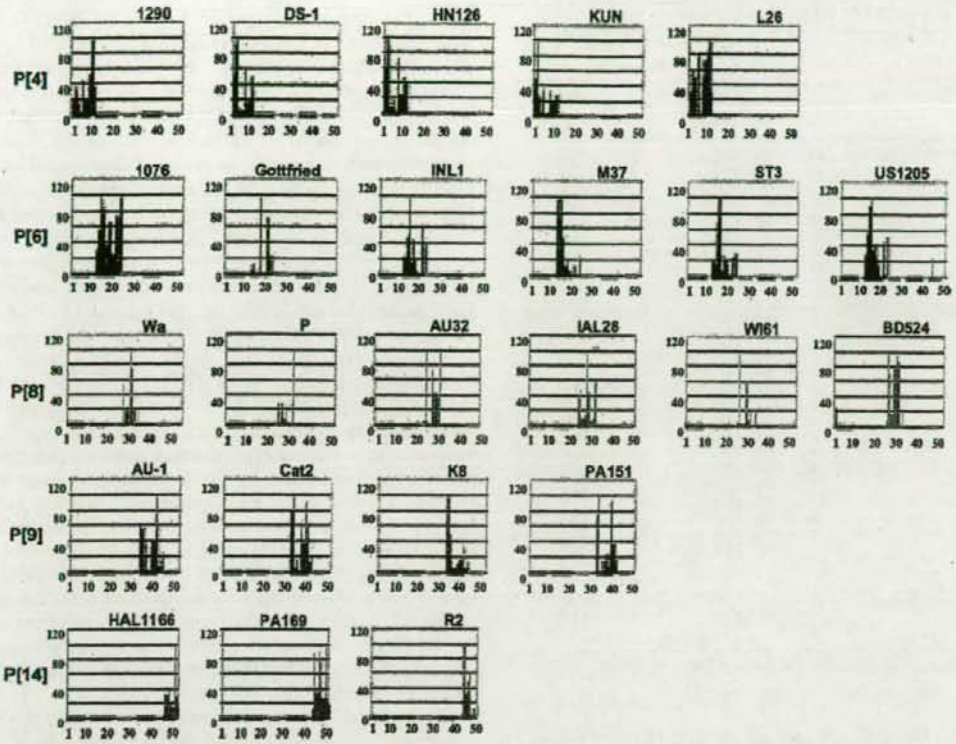

FIG. 3. Quantitative fluorescence (Cy5) profiles of rotavirus P genotype microarray of representative rotavirus strains belonging to P[4], P[6], P[8], P[9], or P[14]. Normalized percent fluorescent signals from each oligoprobe are shown on the *y* axis. Locations of probes are shown on the *x* axis and are marked with different colors: dark blue for P[4], orange for P[6], light blue for P[8], pink for P[9], and green for P[14].

P type-specific oligoprobes (three probes per type). The hybrids were then detected using antidigoxigenin Fab fragment labeled with peroxidase, and the reaction was measured spectrophotometrically (N. Santos et al., unpublished data).

DISCUSSION

Microarray-based techniques have been well established as powerful tools in various fields of molecular biology (6, 12, 45, 49). In virology, this method has been used for analyses of gene expression profiles of cells infected with various viruses. Recently, this assay has been applied for genotyping of selected viruses, including polioviruses, hepatitis B viruses, influenza viruses, hantaviruses, coxsackieviruses, papillomaviruses, measles virus, adenoviruses, flaviviruses, poxviruses, herpesviruses, and mumps viruses (3, 7–9, 31, 33, 34, 39–41, 50, 54, 59). We reported previously the successful development and evaluation of an oligonucleotide microarray hybridization methodology for the identification of five clinically relevant human rotavirus G genotypes (i.e., G1 to G4 and G9) (8). More recently, an increasing number of epidemiological studies have reported the detection in diarrheal patients of uncommon G types, including G5, G6, G8, G10, and G12, in both developing and developed countries. Thus, in this study, we modified the original microarray assay and expanded it to include the identification of such unusual G types as well as clinically relevant P genotypes (i.e., P[4], P[6], P[8], P[9], and P[14]). Hence, this microarray assay can now identify almost all G and P genotypes of human rotaviruses that have been detected thus far. Epidemiological surveillance of rotavirus G and P types before and after an introduction of a candidate rotavirus vaccine in various parts of the world is important in order to evaluate (i) temporal and geographic changes/fluctuations of rotavirus genotype distribution, (ii) relationships between vaccine efficacy and circulating rotavirus strains, and (iii) horizontal transmission of vaccine strains.

The analytical sensitivity of the microarray method reported in this study is very high. The minimum concentration of cDNA that could be detected and typed was 8 ng/μl. In general, samples that produced a visible band in an ethidium bromide-stained 1% agarose gel after the first-round or nested PCR were all successfully P or G genotyped. By applying nested PCR to the first-round-PCR products of strains that gave no visible bands (and therefore could not be typed), we could successfully amplify and genotype both the VP4 and VP7 genes of such rotavirus strains. Thus, because of the high sensitivity of primer pairs used in the nested PCR in this study, the combination of first-round and nested PCR was shown to

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TABLE 1. G and P genotypes of stool rotavirus samples determined by RT-PCR and microarray assays

Location (yr) where stool samples were collected	No. of samples analyzed	Genotype(s) determined by indicated assay[a]			
		RT-PCR		Microarray	
		G	P	G	P
Brazil (1990–2000)	14	G5	P[8]	G5	P[8]
	24	G1	P[8]	G1	P[8]
	1	G5	P[6]	G5	P[8]
	11	G1	NT	G1	P[8]
	1	G1	P[4]	G1	P[8]
	12	G1	P[8]	G9	P[8]
	1	G1	NT	G9	P[8]
Total	64				
Ghana (2003–2004)	1	G2	P[4]	G8	P[4]
	3	G2	P[6]	G2	P[6]
	1	G2	P[6]	G9	P[6]
	1	G2	P[6]	G8	P[4]
	1	G2	P[8]	G9	P[8]
	1	G2	P[6], P[10]	G1, G3	P[6], P[8]
	1	G2	P[6], P[10]	G1, G2	P[6]
	1	G2	P[6], P[10]	G2	P[6]
	2	G3	P[4]	G8	P[4]
	1	G3	P[6]	G3	P[6]
	1	G3	NT	G9	P[8]
	1	G9	P[6]	G2	P[6]
	11	G9	P[8]	G9	P[8]
	1	G9	P[8]	G2	P[8]
	1	G9	P[6], P[10]	G9	P[8]
	3	NT	P[8]	G9	P[8]
Total	31				
United States (1997–2000)	32	G1	P[8]	G1	P[8]
	1	G1	P[8]	G1	P[6]
Total	33				

[a] Discrepant G and/or P types are in boldface. NT, nontypeable.

have a potential of amplifying both the VP4 and VP7 genes of strains bearing not only common but also uncommon G or P specificity.

The specificity of this microarray method is also very high. We could correctly and unambiguously identify both P and G genotypes of all reference rotavirus strains belonging to genotypes against which the genotype-specific microarray probes were designed. In addition, no cross-reactivity with rotavirus G or P genotypes against which specific probes were unavailable on the chip was detected. Thus, strains which do not generate fluorescent signals can safely be considered to bear a G genotype other than G1 to G6, G8 to G10, and G12 and/or a P genotype other than P[4], P[6], P[8], P[9], and P[14].

Only four G and two P genotype-specific probes were found to be cross-reactive with samples of heterologous genotypes. Of note is the finding, however, that each of the samples that exhibited cross-reactivity reacted with only one heterologous probe. Moreover, the intensity of all cross-reactive signals was low. It has been well recognized that it is necessary to distinguish mixed rotavirus infections from cross-reactivity in multiplex RT-PCRs (14) by using a confirmatory assay for the individual genotypes. In contrast to PCR, the microarray method has the inherent ability to readily identify individual genotypes for the mixed infections. In such a case, unlike with cross-reactive signals, more than one probe of suspected G and/or P genotypes would produce strong positive fluorescence signals. Moreover, in this microarray assay, such cross-reactive signals can be utilized for accurate discrimination among various strains within the same genotype. For example, bovine G10 rotavirus KC-1 and B223 strains reacted with the heterologous G2-7 probe with low intensities, while human G10 rotavirus A64 strain exhibited no reactivity with the same probe.

While this study was in progress, Lovmar et al. reported (35) a genotyping method of five G (G1 to G4 and G9) and four P (P[4], P[6], P[8], and P[9]) types of rotavirus by using PE in a microarray format. Their method appears to differ from ours in various aspects. First, because discrimination of genotypes depends on the difference in the 3′ ends between genotype-specific probes and the target genome segments of samples, this method may not work well with strains that have a mutation in this region. For example, among the strains tested in the present study, (i) two G3 strains (HCR3 and Ro1843) which have 3′ ends different from that of each of three G3-specific probes (G3-1, G3-2, and G3-3) reported by Lovmar et al. could not be typed (false-negative result), and (ii) three or four sequence mismatches found between two G4 strains (Gottfried and SB1A) and the three G4-specific probes (G4-1, G4-2, and G4-3) described by Lovmar and coworkers may lead to a wrong conclusion (false-negative result). Second, in their assay, it may be difficult in some cases to distinguish mixed rotavirus infections from cross-reactions that may cause a false-positive result. For example, because there is only one base mismatch between the G10 strains (B223 and KC-1) tested in the present study and one of their G2-specific probes (G2-6) and because, in addition, 3′ ends are complementary to each other, such G10 samples may be misdiagnosed as a mixture of G10 and G2 viruses (false-positive result). Third, this method can provide only limited information about 3′-end differences among strains within the same genotype. Previously, we reported that our microarray method was capable of "subgenotyping" viruses in a given G genotype (G1 to G4 and G9) (8). In the present study, not only the VP7 gene but also the VP4 gene of each rotavirus strain was found to display a unique hybridization pattern that correlated closely with its nucleotide sequences. Thus, one of the major advantages of our new microarray

TABLE 2. Concordance and discordance in genotyping results determined by RT-PCR and microarray assays

Origin of samples (*n*)	No. (%) of G genotypes that were:			No. (%) of P genotypes that were:		
	Concordant[a]	Discordant[b]	Nontypeable[c]	Concordant	Discordant	Nontypeable
Brazil (64)	51 (79.7)	13 (20.3)	0 (0)	50 (78.1)	2 (3.1)	12 (18.8)
Ghana (31)	17 (54.8)	11 (35.5)	3 (9.7)	22 (71.0)	8 (25.8)	1 (3.2)
United States (33)	33 (100)	0 (0)	0 (0)	32 (97.0)	1 (3.0)	0 (0)

[a] Results were cocordant between RT-PCR and microarray analysis.
[b] Results were discordant between RT-PCR and microarray analysis.
[c] Strains were nontypeable by RT-PCR but typeable by microarray analysis.

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method is that it is ideal for analyzing such genetic polymorphisms of the VP7 and VP4 genes of various rotavirus strains within the same genotype. In addition, we found that the existence of even one nucleotide mismatch located near the 3′ or 5′ end of a probe changed its pattern of hybridization profile (data not shown). In general, rotavirus strains in a given genotype that are isolated (i) at different locations, (ii) in different years even at the same location, or (iii) from different animal species display different hybridization profiles. For example, two Japanese G2 strains, KUN and S2, displayed similar hybridization patterns which were distinct from that displayed by U.S. G2 strain DS-1 or Venezuelan G2 strain HN126 (data not shown). These results indicate that this assay can be applied to monitoring the evolutionary divergence and genetic drift of the selected rotavirus genes, including the VP7 and VP4 genes. Although sequencing of the target genome segment may be needed for detailed analysis, it is time-consuming, expensive, and not practical for analyses of a large number of field isolates. This microarray method is ideal for a rapid screening of a large number of rotavirus strains to detect and analyze genetic polymorphism before sequencing. Analyses by this assay of a possible genetic/antigenic drift of the VP7 and/or VP4 gene of G1P[8] viruses collected longitudinally in three countries (Brazil, Spain, and the United States) are under way in this laboratory.

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There is no conflict of interest to declare.

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ORIGINAL ARTICLE

Detection and Genetic Characterization of Norovirus in Oysters from China and Japan

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SUMMARY

A total of 225 oysters from China and Japan were collected during October 2005 to September 2006 and were then tested for the presence of norovirus by RT-nested PCR. The detection rate of norovirus was different between China and Japan, accounting for 14.6% (19 of 130) and 25.3% (24 of 95), respectively. In China, norovirus in oyster was detected continuously from July to February with the highest prevalence in August, October and November (each of 21%, 4 of 19). On the other hand, norovirus in Japan was found year-round with highest prevalence in March and October (each of 20.8%, 5 of 24). Norovirus strains detected were subjected to further characterization by sequence analysis. It was found that the norovirus strains belonged to only two distinct genotypes, the GII/3 (known as the Mexico virus cluster) and the GII/4 (known as the Lordsdale virus cluster). In China, the norovirus GII/4 was the most predominant, accounting for 78.9% (15 of 19). In contrast, it was interesting that both the norovirus GII/4 and the norovirus GII/3 were co-predominant with a prevalence of 50% (12 of 24) in Japan. Another interesting feature of the study was that the norovirus GII/4 strains in oysters from both countries were grouped into two distinct variant clusters known as the Farmington Hills variant and the Hunter variant. More than 10^2 copies of norovirus were detected in 41 of 43 oysters. This study provided additional evidence of the presence of norovirus in oysters and is also the first report to demonstrate the existence of norovirus variants in oysters. (Clin. Lab. 2007;53:405-412)

KEY WORD

norovirus, oysters, Japan, China

INTRODUCTION

Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality of children in both developed and developing countries. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries (1, 2). It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age. Despite much progress in the understanding of the pathogenesis and of management with the widespread use of oral rehydration therapies, acute gastroenteritis consistently ranks as one of the top causes of death worldwide (1-4). Norovirus is one of the leading agents of acute gastroenteritis worldwide and causes outbreaks in various epidemiological settings such as restaurants, schools, day care centers, hospitals, nursing homes, and cruise ships (5-10). Norovirus is the distinct genus within the family *Caliciviridae*. Norovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and 3). The ORF1 encodes non-structural proteins, the ORF 2 encodes the capsid protein, and the ORF3 encodes a small capsid protein (11).
The transmission modes of norovirus are classified into food-borne, water-borne, air-borne, person-to-person spread

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and might be by other unknown routes (5-8, 10, 12). Norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern. Oysters are filter feeders, and hence concentrate the viruses from contaminated water. As a result, consumption of virus-contaminated oysters represents a significant threat to oyster consumers as well as an economic threat to the seafood industry. Recently, oysters were reported to be responsible for viral outbreaks and sporadic cases of acute gastroenteritis around the world, and the number of cases of norovirus-related food poisoning has been increasing (13-16). For diarrheal disease control to be successful, continuous monitoring of the norovirus contamination of oysters is needed. Moreover, study of the molecular epidemiology of norovirus provides knowledge on the diversity of the specific types found in oysters, which is important in an effort to design suitable and effective norovirus control strategies.

Thus, in this study we determined the occurrence of norovirus contamination in oysters from China and in Japan during 2005-2006, characterized norovirus detected according to genogroup and genotype, and quantified the norovirus genome in these oysters.

MATERIALS AND METHODS

Oyster samples

A total of 225 oysters including 130 from Fujian Sheng, China and 95 from four different localities (Miyagi, Ibaraki, Mieken, and Nagoya) in Japan were collected during October 2005 to September 2006. The intestines of the oysters were removed by dissection and then weighed. Virus extraction and concentration were performed by using the modified method previously reported (17). The supernatants were collected and stored at –30 °C until use for the detection of norovirus.

Extraction of viral genome

The viral genomes were extracted from 140 µl of intestine suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Hilden, Germany).

Reverse transcription

For reverse transcription (RT), 4 µl of extracted viral genome was added to 4 µl of a reagent mixture consisting of 5X first strand buffer (Invitrogen, Carlsbad, CA, USA), dNTPs (10 mM/µl) (Roche, Mannheim, Germany), DTT (Invitrogen), superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total reaction mixture was 8 µl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C (18).

Table 1: Detection of norovirus in oysters from China and Japan during 2005-2006.

Country	Total of samples	Norovirus positives (%)
China	130	19 (14.6%)
Japan	95	24 (25.3%)

Polymerase chain reaction

Using nested polymerase chain reaction (PCR) with specific primers as previously reported resulted in the identification of norovirus (18). The RT-PCR was performed by using primers COG1F and G1SKR, and COG2F and G2SKR for amplification of the partial capsid genes of norovirus. The second amplification was performed using the first PCR product as the template with primers G1SKF and G1SKR, and G2SKF and G2SKR. These primers specifically generated five different sizes of amplicons of 330 bp and 344 bp for norovirus genogroup I and norovirus genogroup II, respectively. PCR was carried out with 1 µl of cDNA in 10 µl of the reagent mixture containing 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/µl), primers (33 pM), Taq DNA polymerase (5 U/µl) (Promega) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet light. The results were recorded by photography.

Quantitation of norovirus gemone

The norovirus capsid genes in oysters were quantified by using real-time PCR as previously described (14). The real-time PCR mixture contained 4 µl of cDNA, 17.5 µl of TaqMan universal PCR master mix (Applied Biosystem), a 100 pM concentration of each primer, and fluorogenic probes. The following PCR protocol was used: 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 95 °C for 15 s, and 56 °C for 60 s.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of PCR products (DNA) positive for norovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using the neighbor-joining method with