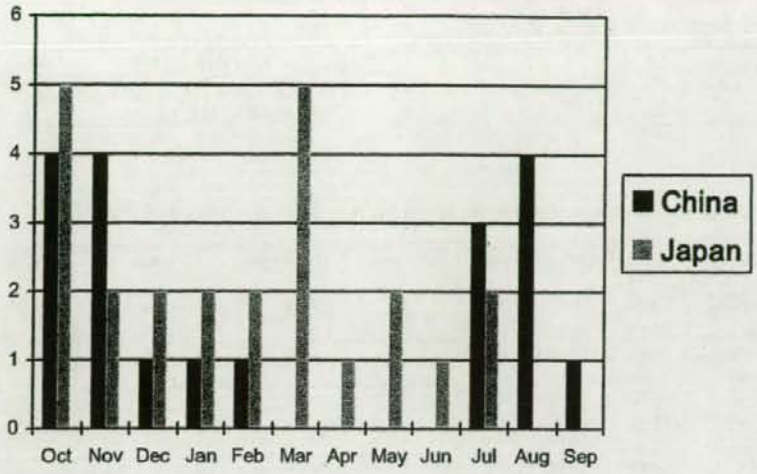

Figure 1: Monthly distribution of norovirus strains detected in oysters from China and Japan from October 2005 to September 2006.

CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Reference norovirus strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), Farmington Hills (AY502023), and Hunter284E/04O/AU (DQ078794).

RESULTS

Detection of norovirus contamination

A total of 225 oysters collected from China and Japan during October 2005 and September 2006 were examined for the presence of norovirus. Table 1 shows that norovirus was detected in 19 out of 130 oysters from China and in 24 out of 95 oysters from Japan, accounting for 14.6% and 25.3%, respectively. In China, norovirus in oysters 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, except for August and September, with highest prevalence in March and October (each of 20.8%, 5 of 24). Figure 1 demonstrates that no norovirus in oysters was detected from March to June and from August to September in China and Japan, respectively.

Distribution of norovirus genotypes

The partial nucleotide sequences of the capsid gene of norovirus detected in this study were compared to each other as well as to those of norovirus reference strains available in GenBank by BLAST. Table 2 shows that

Table 2: Genetic characterization of norovirus detected in oysters from China and Japan.

Countries	Norovirus genotypes		
	GII/3	GII/4	
China	4 (21.1%, 4 of 19)	15 (78.9%, 15 of 19)	
Japan	12 (50%, 12 of 24)	12 (50%, 12 of 24)	
		Farmington Hills variant (%)	Hunter variant (%)
China		8 (53.3%, 8 of 15)	7 (46.7%, 7 of 15)
Japan		3 (25%, 3 of 12)	9 (75%, 9 of 12)

Table 3: Characterization of norovirus strains detected in oysters from China and Japan during 2005-2006

Number	Strain	Place	Collection date	Genogroup	Genotype	Variant	Genome copy per oyster
1	1/oyster/CN	China	October 2005	II	4	Farmington Hills	$1.2x10^3$
2	4/oyster/CN	China	October 2005	II	4	Hunter	$4x10^2$
3	7/oyster/JP	Japan	October 2005	II	4	Hunter	$3.3x10^2$
4	9/oyster/JP	Japan	October 2005	II	3	-	$7.8x10^5$
5	16/oyster/CN	China	October 2005	II	3	-	$2.5x10^3$
6	18/oyster/CN	China	October 2005	II	3	-	$9x10^4$
7	22/oyster/JP	Japan	October 2005	II	3	-	$2.1x10^3$
8	24/oyster/JP	Japan	October 2005	II	3	-	$8x10^2$
9	25/oyster/JP	Japan	October 2005	II	3	-	$4.2x10^2$
10	26/oyster/CN	China	November 2005	II	3	-	$1.1x10^3$
11	27/oyster/CN	China	November 2005	II	3	-	$1.1x10^5$
12	31/oyster/JP	Japan	November 2005	II	4	Hunter	$2.6x10^3$
13	38/oyster/CN	China	November 2005	II	4	Hunter	$1.3x10^3$
14	40/oyster/CN	China	November 2005	II	4	Hunter	$1.2x10^2$
15	45/oyster/JP	Japan	November 2005	II	4	Hunter	$3.8x10^2$
16	51/oyster/JP	Japan	December 2005	II	4	Farmington Hills	$4.2x10^3$
17	55/oyster/JP	Japan	December 2005	II	4	Hunter	$6x10^1$
18	56/oyster/CN	China	December 2005	II	4	Farmington Hills	$4.1x10^3$
19	72/oyster/JP	Japan	January 2006	II	4	Hunter	$2.6x10^2$
20	76/oyster/CN	China	January 2006	II	4	Farmington Hills	$3x10^3$
21	84/oyster/JP	Japan	January 2006	II	4	Farmington Hills	$2x10^3$
22	89/oyster/CN	China	February 2006	II	4	Farmington Hills	$2.5x10^2$
23	91/oyster/JP	Japan	February 2006	II	4	Farmington Hills	$5.7x10^2$
24	104/oyster/JP	Japan	February 2006	II	4	Hunter	$3.5x10^2$
25	111/oyster/JP	Japan	March 2006	II	4	Hunter	$1.7x10^2$
26	121/oyster/JP	Japan	March 2006	II	3	-	$7.5x10^3$
27	122/oyster/JP	Japan	March 2006	II	3	-	$3.5x10^3$
28	123/oyster/JP	Japan	March 2006	II	3	-	$7.3x10^3$
29	125/oyster/JP	Japan	April 2006	II	3	-	$5x10^3$
30	139/oyster/JP	Japan	May 2006	II	3	-	$6.6x10^2$
31	146/oyster/JP	Japan	May 2006	II	3	-	$6x10^2$
32	168/oyster/JP	Japan	June 2006	II	3	-	$2.9x10^3$
33	177/oyster/JP	Japan	July 2006	II	3	-	$6.7x10^2$
34	186/oyster/CN	China	July 2006	II	4	Hunter	$4.4x10^2$
35	187/oyster/CN	China	July 2006	II	4	Hunter	$1.5x10^3$
36	189/oyster/CN	China	July 2006	II	4	Hunter	$5.5x10^2$
37	191/oyster/JP	Japan	July 2006	II	4	Hunter	$1.5x10^2$
38	192/oyster/JP	Japan	August 2006	II	4	Hunter	$2.2x10^2$
39	201/oyster/CN	China	August 2006	II	4	Farmington Hills	$3x10^2$
40	202/oyster/CN	China	August 2006	II	4	Hunter	$1x10^1$
41	203/oyster/CN	China	August 2006	II	4	Farmington Hills	$6.4x10^2$
42	205/oyster/CN	China	August 2006	II	4	Farmington Hills	$2.3x10^2$
43	216/oyster/CN	China	September 2006	II	4	Farmington Hills	$3.4x10^2$

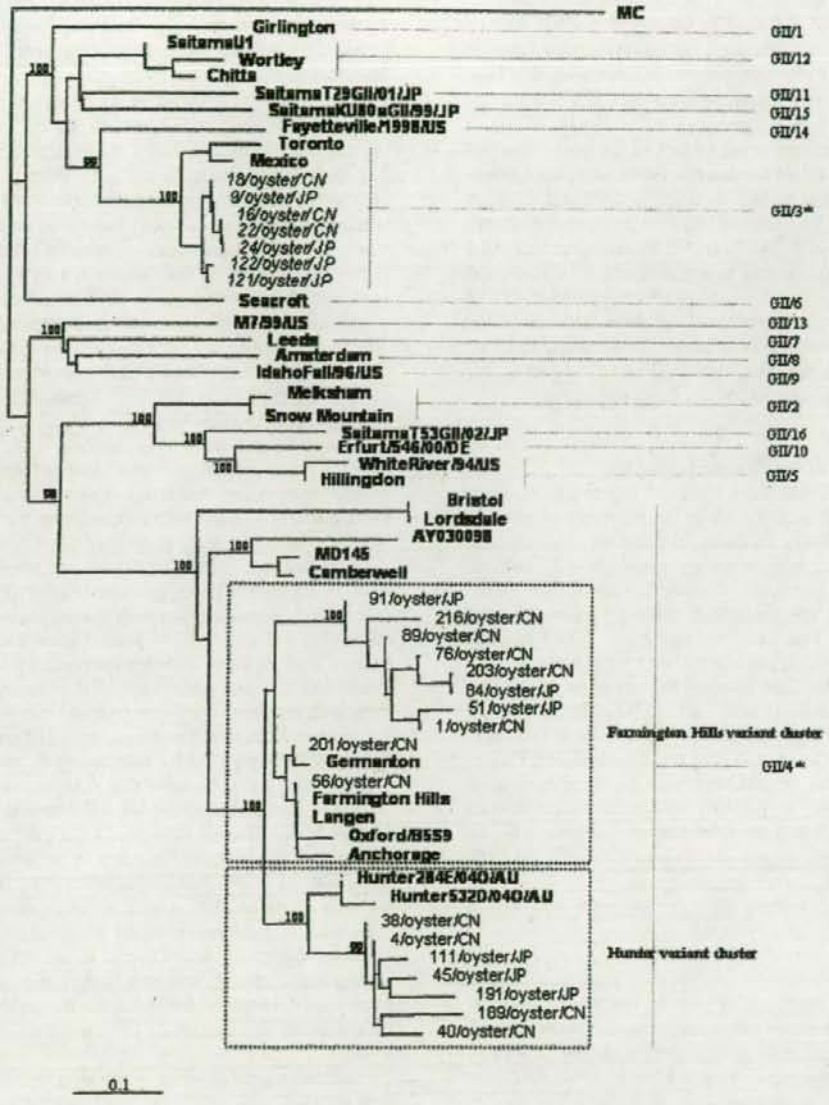

Figure 2

Figure 2: Phylogenetic tree obtained from nucleotide sequences of the norovirus capsid gene. Reference norovirus strains were selected from GenBank under the accession numbers indicated in the text. The norovirus strains detected in the study are highlighted in the italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. * indicates the genotype containing the norovirus detected in this study.

based on the sequence analysis of the capsid gene of these norovirus strains, norovirus was divided into two distinct genotypes, 3 and 4, within genogroup II (GII). In China, the norovirus GII/4 (known as the Lordsdale virus cluster) was the most predominant, accounting for 78.9% (15 of 19), followed by the norovirus GII/3 (known as the Mexico virus cluster) with a lower prevalence of 21.1% (4 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 (Figure 2). In China, the Farmington Hills-like norovirus predominated over the Hunter-like norovirus and these represented 42.1% (8 of 19) and 36.8% (7 of 19), respectively. In contrast, the detection rate of the Hunter-like norovirus was higher than that of the Farmington Hills-like norovirus in Japan, accounting for 37.5% (9 of 24) and 12.5% (3 of 24), respectively

Quantitation of the norovirus genome

Table 3 shows that more than 10^2 copies of the norovirus genome were found in the majority of oysters (95.3%, 41 of 43). In Japan, the lowest copy number was $6x10^1$ and the highest copy number was $7.8x10^5$. In China, the copy number of norovirus ranged from $1x10^1$ to $1.1x10^5$. It was found that the copy number of the norovirus GII/3 ranged from to $7.8x10^5$. The Farmington Hills-like norovirus strains had a copy number from $2.3x10^2$ to $2x10^5$. The Hunter-like norovirus strains had a copy number from $1x10^1$ to $2.6x10^3$. The number of oysters containing a large copy number (over 10^5) was identified in 7 cases (36.8%) and 10 cases (41.7%) in China and Japan, respectively. Of note, the highest copy number ($1.1x10^5$ to $7.8x10^5$) was found exclusively in norovirus GII/3 and the lowest copy number ($1x10^1$ to $6x10^1$) was only in the Hunter-like norovirus in both countries.

DISCUSSION

Norovirus is regarded as one of the most common causes of food-borne infections. Sporadic cases as well as outbreaks of acute gastroenteritis due to norovirus have been associated with the consumption of raw oysters grown in contaminated waters (19-23). In this study, the prevalence of norovirus contamination in oysters from China and in Japan was reported. Overall, the detection rate of norovirus was 19.1% in the total of 225 oyster samples included in this study. However, the detection rates of norovirus in oysters were quite different between China and Japan, representing 14.6% and 25.3%, respectively. Even though several studies conducted the detection on norovirus contamination in various different countries including Japan, reports documenting the occurrence of norovirus contamination in oysters from China are not available. To date, only one study on norovirus presence in oysters imported into Hong Kong from 11 countries over a 3-year period was published with the prevalence of 10.5% (24). Obviously, this is the first report, to the best of our knowledge, providing evidence for norovirus presence in oysters from China.

In some studies, the detection of norovirus contamination in oysters was predominant in the cold season, and several studies did not find a seasonal correlation (24-27). In this study it was found that norovirus was mainly detected continuously from October to May, months which are known as the cold season in Japan. In contrast, norovirus was mainly identified from July to November, known as the hot season in China. Taken together, these observations clearly indicate that the contamination of oysters with this virus occurred not only in the cold season but also in the hot season.

Genetic analysis revealed that the norovirus strains in our study belonged to only one distinct GII. This result was in line with other studies in which norovirus GII was the dominant genogroup in oysters (17, 28). It was found that the norovirus strains clustered into 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, norovirus GII/4 was the most predominant, accounting for 78.9%, followed by norovirus GII/3 with a lower prevalence of 21.1%. In contrast, it was interesting that both the norovirus GII/4 and the norovirus GII/3 were co-predominant with a prevalence of 50% 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. The Farmington Hills virus was associated with 64% of cruise ship outbreaks and 45% of land-based outbreaks in the United States in 2002 (15). Moreover, the Hunter virus was an important strain in causing 15.6% of sporadic cases as well as 42.9% of outbreaks of acute gastroenteritis during the year of 2004 in Australia (19). And the virus transmission by food was documented in these outbreaks (15). Quite possibly, these norovirus variants in oysters might play an important role in this diarrheal illness under these circumstances and our finding is the first to demonstrate evidence of the existence of norovirus variants in oysters.

To date, norovirus is still uncultivable by standard culture with different cell lines. The lack of a virus culture system has been a significant obstacle to the study of norovirus, but advances in the sequencing of norovirus have enabled its genomic characterization, and therefore genetic analysis becomes the principle method to classify norovirus. Recently, a quantitative real-time PCR method has been developed (14). In the previous reports, the sensitivity of nested PCR was higher than that of real-time PCR (27, 28). Therefore, the determination of the copy numbers of the norovirus genome was per-

formed only in norovirus-positive oysters by nested PCR. It was revealed that the majority of oysters (95.3%) had more than 10^2 copies of the norovirus genome. Interestingly, the number of oysters containing the large copy number (over 10^3) was found in 17 cases. Moreover, the highest copy number was 1.1×10^5 and 7.8×10^5 in Chinese and Japanese oysters. These findings were consistent with previous reports that oysters contained much smaller numbers of norovirus than the number of norovirus found in feces (30).

It has been reported that the distribution of norovirus genotypes in oysters was different to those in humans during the same period (24). However, it was interesting that the distribution of the norovirus genotype in Japanese oysters in this study was similar to the distribution of norovirus genotype in humans in Japan during 2005-2006, in which both norovirus GII/3 and GII/4 were co-predominant and only two, the Farmington Hills variant and the Hunter variant clusters, were identified within the GII/4 genotype (unpublished data). Obviously, there was an epidemiological link of the norovirus infection between humans and oysters in Japan.

In conclusion, these results are noteworthy because this is the first report, to the best of our knowledge, of the presence of norovirus variants in oysters. This observation improves our current knowledge of the genetic heterogeneity of norovirus in oysters as well as its epidemiology. Due to genetic diversity in different parts of the world, knowledge of molecular surveillance of norovirus in circulation in oysters is important in an effort to develop suitable and efficacious norovirus control strategies, and a continuous monitoring of the norovirus types in oysters is needed.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan.

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Clin. Lab. 2007;53:413-421
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ORIGINAL ARTICLE

Photocatalytic Inactivation of Diarrheal Viruses by Visible-Light-Catalytic Titanium Dioxide

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SUMMARY

Titanium dioxide (TiO_2) that had been irradiated with visible light (VL) was demonstrated to inactivate rotavirus, astrovirus, and feline calicivirus (FCV). The virus titers were dramatically reduced after exposure for 24 hrs to the VL-catalytic TiO_2. The addition of bovine serum albumin could protect the virus against inactivation by VL-catalytic TiO_2 in a dose-dependent manner. This finding implied that the VL-catalytic TiO_2 products might somehow interact initially with the viral proteins in the process of virus inactivation. Moreover, we showed partial degradation of the rotaviral dsRNA genome. This was more prominent when the virus was exposed to the VL-catalytic TiO_2 treatment for at least 2 days. An attempt was made to elucidate the mechanism underlying the inactivation of the viruses. It was found that upon activation of TiO_2 with VL by using a white fluorescent lamp, the reactive oxygen species such as superoxide anions (O_2^-) and hydroxyl radicals (·OH) were generated in a significant amount after stimulation for 8, 16, and 24 hrs. We therefore assume that virus inactivation by VL-catalytic TiO_2 might occur through the generation of O_2^- and OH followed by damage to the viral protein and genome. This is the first report, to the best of our knowledge, demonstrating the inactivation of rotavirus, astrovirus and FCV by the presence of TiO_2 film under VL as well as describing its mechanism. (Clin. Lab 2007;53:413-421)

KEY WORDS

diarrheal virus; photocatalysis; reactive oxygen; Ti O_2; visible light

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in infants and young children. Acute gastroenteritis is consistently ranked as one of the top causes of death in children [1-3]. The mortality among children due to acute gastroenteritis is greater in developing rather than in developed countries [3, 4]. Every year, approximately 3 to 6 million children die from diarrhea in Asia, Africa, and Latin America [5].

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Many different viruses can cause gastroenteritis, including rotavirus, adenovirus, sapovirus, astrovirus, and norovirus [1]. Among enteropathogenic viruses, rotavirus is recognized as the major etiologic agent of acute gastroenteritis in infants and children [6-8]. Rotavirus is a genus of the *Reoviridae* family with a particle size of 60 to 80 nm in diameter. Its genome consists of 11 double stranded RNA (dsRNA) segments, which encode six structural and six non-structural proteins. The non-structural proteins are involved in genome replication, the assortment of genome sets and the regulation of gene expression. The structural proteins form a triple-layered capsid around the dsRNA genome [6]. Despite much progress in understanding the pathogenesis and management of diarrheal illness with the widespread use of oral rehydration therapies, it remains one of the most important causes of global childhood mortality and morbidity [2-4].

Astrovirus is named so to describe the distinctive five- or six-pointed star visible on particles when viewed under an electron microscope. It is a small non-enveloped virus with a particle size of 28 nm. The virus particle contains a positive sense single stranded RNA genome with a single layered capsid protein. This virus has three open reading frames (ORFs), ORF1a, ORF1b, and ORF2. Moreover, ORF1a and ORF1b encode the viral protease and polymerase, respectively, whereas ORF2 encodes the capsid protein precursor [9]. Human astrovirus is classified into 8 serotypes (1 to 8), of which serotype 1 is the most common [10, 11].

Norovirus, a member of the human calicivirus genus, is another principal cause of nonbacterial acute gastroenteritis in all age groups and has been identified as an etiologic agent of waterborne outbreaks worldwide [12-14]. The virus particle ranges from 27 to 40 nm in diameter and has a single-stranded RNA genome with an icosahedral capsid structure [15]. There is no known animal or mammalian cell culture system that can be used for the cultivation of human norovirus. Because feline calicivirus (FCV) has a genome organization and capsid architecture similar to the human norovirus and can be easily grown in a cell culture, it has been used as a surrogate for the study of norovirus inactivation [16-18].

Since photoelectrochemical disinfection with platinum-doped titanium dioxide (TiO_2) was first introduced almost 20 years ago [19], many studies have utilized the strong oxidizing power of TiO_2 photocatalysts to purify water and air of environmentally toxic substances [20-22]. Furthermore, TiO_2 photocatalysts have also been applied to inactivate bacteria, phages, and cancer cells [23-25]. Hydroxyl radicals generated from the photocatalyst reactions were considered to play a significant role in microbial inactivation [26]. When water containing toxic substances or bacteria were treated by photocatalysis, a fine TiO_2 powder and strong light such as a mercury lamp were utilized. However, this system requires the recovery of TiO_2 power and a high cost of light. In most studies with the application of TiO_2 to inactivate microorganisms, the light source was an ultraviolet (UV) lamp or a black light lamp [27, 28]. In this study, we used a visible-light (VL) responsive TiO_2 instead of a conventional UV-catalytic TiO_2, whose mechanism of chemical photocatalytic degradation of viruses has been well documented. The VL-catalytic TiO_2 can be applied indoors as well as under sunlight. Therefore, the VL irradiation is more convenient, economical and safe for the inactivation of diarrheal viruses than UV catalysis. In addition, there is no available report on photocatalytic inactivation of enteropathogenic viruses.

Thus, the objective of this study was to investigate the role of complex photooxidants such as superoxide anions (O_2^-) and hydroxyl radicals ($\cdot OH$) in the inactivation of rotavirus, astrovirus, and FCV under VL irradiation. In addition, the mechanism of this inactivation process is described.

MATERIALS AND METHODS

Cell culture and viruses

The cell lines used in this study were: African green monkey kidney (MA104) cells, human colon carcinoma (Caco-2) cells and Crandell's feline kidney (CrFK) cells. MA104 cells and Caco-2 cells were cultured in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10% fetal calf serum (FCS), 0.03% glutamine, and 0.12% $NaHCO_3$. The CrFK cells were cultured in MEM supplemented with 8% FCS, 0.03% glutamine, and 0.12% $NaHCO_3$. All cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere incubator.

The following viruses were used: human rotavirus strain Odelia, a simian rotavirus strain SA11, human astrovirus serotype 1 (HAstV-1), and FCV strain F4. The Odelia and SA11 strains were propagated in MA104 cells in the presence of 2 μg/ml acetyltrypsin, while the HAstV-1 was propagated in Caco-2 cells with 5 μg/ml acetyltrypsin, and FCV was propagated in CrFK cells. All viruses were harvested from the cultured supernatants after two freeze-thaw cycles. The virus titers of Odelia, HAstV-1 and FCV were estimated by the neutralization test and expressed as 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) by the Reed-Muench method as described [29]. The SA11 virus titer was determined by a plaque assay and the titer was expressed as plaque forming units per ml (pfu/ml) [30].

TiO_2 film and chemicals

TiO_2 thin film was prepared from TiO_2 particles (EX-101, 5 g/liter; ECODEVICE Co., Saitama, Japan) by dip-coating on a nitoflon sheet (66 μg/cm^2). TiO_2 can absorb visible light of between 400 and 600 nm and show high activity [31]. Bovine serum albumin (BSA) globulin free grade and the other chemicals used in this study were all purchased from Wako, Ltd. (Osaka, Japan).

Light source and apparatus

Virus suspensions on TiO_2 film were irradiated with a 27-watt white fluorescent lamp (WL; model FPL27EX-N, Sanyo Electric Co. LTD., Osaka, Japan), which emitted light with wavelengths in the range of 400 to 720 nm. The cultured plate was placed 27 cm under the light source. The light intensity used in this study was 2 900 ± 100 lx, as measured by an illuminometer (Model 3423, Hioki E E Co. LTD., Nagano, Japan). The reaction temperature was controlled at 30 °C.

Table 1: Inactivation of human rotavirus (Odelia), simian rotavirus (SA11), human astrovirus (HAstV-1), and feline calicivirus (FCV) by VL-catalytic TiO_2 film

Treatment	Odelia		SA11		HAstV-1		FCV	
	Log_{10} $TCID_{50}$	Log_{10} reduction	Log_{10} PFU	Log_{10} reduction	Log_{10} $TCID_{50}$	Log_{10} reduction	Log_{10} $TCID_{50}$	Log_{10} reduction
No	3.40 ± 0.05	0	5.15 ± 0.09	0	4.54 ± 0.08	0	3.75 ± 0.05	0
WL	3.10 ± 0.03	0.30	4.27 ± 0.03	0.88	4.26 ± 0.09	0.28	3.25 ± 0.09	0.50
TiO_2	3.10 ± 0.03	0.30	4.27 ± 0.04	0.88	4.24 ± 0.09	0.30	3.57 ± 0.08	0.18
TiO_2 + WL	1.90 ± 0.03	1.50	2.37 ± 0.03	2.78	2.12 ± 0.08	2.42	1.80 ± 0.09	1.95

Odelia ($10^{3.4}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml), HAstV-1 ($10^{4.61}$ $TCID_{50}$/ml) and FCV ($10^{4.80}$$TCID_{50}$/ml) suspended in MEM were treated with either WL-irradiation or TiO_2 film alone, VL-catalytic TiO_2 film with WL-irradiation, or non-treated at 30°C for 24 hrs. After treatment, the virus suspensions were assayed for the surviving virus titers.

Table 2: Protection of human rotavirus (Odelia), simian rotavirus (SA11) and feline calicivirus (FCV) from inactivation by VL-catalytic TiO_2 film

BSA (mg/ml)	Log_{10} $TCID_{50}$ (Odelia)	Log_{10} PFU (SA11)	Log_{10} $TCID_{50}$ (FCV)
0	1.90 ± 0.02	2.37 ± 0.03	1.80 ± 0.08
0.1	2.20 ± 0.02	2.90 ± 0.02	2.40 ± 0.09
1	2.80 ± 0.04	3.95 ± 0.04	2.83 ± 0.09

Odelia ($10^{3.4}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml) and FCV ($10^{4.80}$$TCID_{50}$/ml) suspended in MEM were treated with VL-catalytic TiO_2 and WL-irradiation in the absence or presence of various amounts of BSA (0.1 to 1 mg/ml) at 30°C for 24 hr. After treatment, the virus suspensions were assayed for the surviving virus titers

Inactivation of the viruses

The virus suspensions (SA11 with $10^{5.19}$ pfu/ml, Odelia with $10^{3.40}$ $TCID_{50}$/ml, HAstV-1 with $10^{4.61}$ $TCID_{50}$/ml, and FCV with $10^{4.80}$ $TCID_{50}$/ml) of 200 µl each, were added to a dish (15 mm in diameter) in which a sheet of TiO_2-coated film (whole TiO_2 film, 176 mm^2) had been placed on the bottom. The dishes were then exposed to WL at 30 °C for 24 hrs and the virus suspensions were assayed for the surviving virus titer. All experiments were conducted in triplicate.

Effect of BSA on virus inactivation by VL-catalytic TiO_2

In order to investigate whether the inactivation of the viruses by VL-catalytic TiO_2 could be reversed by BSA, 200 µl of Odelia ($10^{3.40}$ $TCID_{50}$/ml), SA11 ($10^{5.19}$ pfu/ml), and FCV ($10^{4.80}$ $TCID_{50}$/ml) suspensions were treated with VL-catalytic TiO_2 in the absence or presence of two concentrations (0.1 and 1 mg/ml) of BSA and WL irradiation at 30 °C. After incubation for 24hrs, the titer of the surviving viruses was determined. All experiments were conducted in triplicate.

Analysis of rotavirus RNA by SDS-PAGE

The genomic dsRNA of rotavirus extracted by using phenol/chloroform extraction techniques was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described [32]. Briefly, 20 µl of dsRNA were mixed with 5 µl of bromophenol blue and run in 10% polyacrylamide gel with a 4% polyacrylamide stacking gel in Tris-glycine buffer (25 mM Tris and 192 mM glycine, pH 8.3) containing 3.5 mM SDS. After electrophoresis at 30 mA for 2 hrs, rotavirus RNA was visualized by staining with silver nitrate. The polyacrylamide gel was scanned directly and the bands were analyzed using the Scion Image for Windows (Scion Image version 4.0.2, Scion Co., MD, USA).

Determination of superoxide anions (O_2^-) and hydroxyl radicals (·OH)

The amount of O_2^- was determined according to the method described [33]. Briefly, TiO_2-film was submerged in 5 ml of MEM phenol red free PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 10 µM nitroblue-tetrazolium hydrochloride (NBT), and 0.1 mM EDTA. It was

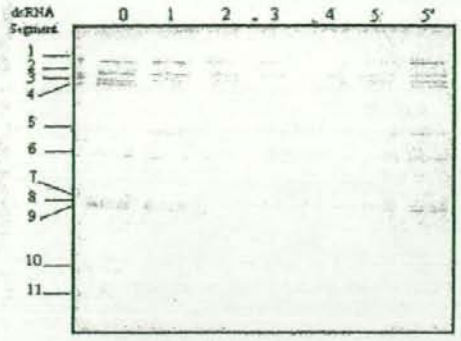

Figure 1: The SDS-PAGE of genomic dsRNA of simian rotavirus strain SA11 with or without VL-catalytic TiO_2 and WL-irradiated treatment. Lane 0, electrophoretic pattern of genomic dsRNA extracted from intact virus particles. Lanes 1-5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles after exposure to the treatment for 1, 2, 3, 4, and 5 days, respectively. Lane 5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles that were kept for 5 days in the same condition without treatment.

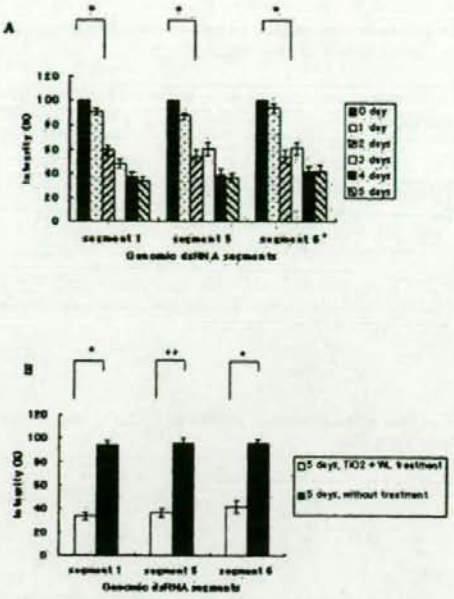

Figure 2: The integrity of segments 1, 5, and 6 of the genomic dsRNA of simian rotavirus strain SA11 after treatment with VL-catalytic TiO_2 and WL-irradiation. *$p< 0.01$ (A) Percentage integrity of segments 1, 5, and 6 of the genomic dsRNA with or without exposure to the treatment for 1, 2, 3, 4 and 5 days. (B) Percentage integrity of segments 1, 5, and 6 of the genomic dsRNA on day 5 with or without treatment. *: $p< 0.001$, **: $p=0.0001$

then irradiated with WL at room temperature for 0, 8, 16, and 24 hrs, respectively. The density of blue formazane on the film was determined by Scion Image analyzer software. The amount of hydroxyl radicals (·OH) was determined as previously described [34]. Briefly, TiO_2-film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 0.05 mM p-nitrosodimethylaniline, and then irradiated at room temperature for 0, 8, 16, and 24 hrs, respectively. The bleaching level of p-nitrosodimethylaniline was determined by measuring the OD (optical density) of the reaction mixture at 440 nm.

Statistics

Student's t-tests (independent t-test or paired t-test) were performed. If the p value was less than 0.05, the groups were considered to be significantly different.

RESULTS

Inactivation of Odelia and SA11 rotaviruses, HAstV-1, and FCV by VL-catalytic TiO_2

The viruses Odelia, SA11, HAstV-1, and FCV were treated with WL-irradiation alone, TiO_2 alone, VL-catalytic with WL-irradiation, or non-treated at 30 °C for 24 hrs. After treatment, the virus suspensions were assayed for the virus titers. The results shown in Table 1 indicate that the titers of the viruses in the suspensions remained almost the same (not significantly different) regardless of whether no treatment was given, or whether they were treated with WL-irradiation alone, or TiO_2 alone. The data showed that treatment of the viruses with WL-irradiation alone or TiO_2 alone had no effect on the virus. However, when the viruses were treated with TiO_2 and WL-irradiation, the titers of the viruses were dramatically reduced. The titer of Odelia was reduced by about 1.50 log from $10^{3.4}$ to $10^{1.9}$ $TCID_{50}$/ml, while the titer of SA11 was reduced by about 2.78 log from $10^{5.15}$ to $10^{2.37}$ pfu/ml. In addition, the titer of HastV-1 was reduced about by 2.42 log from $10^{4.61}$ to $10^{2.12}$ $TCID_{50}$/ml, whereas the titer of FCV was reduced by about 1.95 log from $10^{3.75}$ to $10^{1.80}$ $TCID_{50}$/ml. Taken together, these data clearly indicated that the VL-catalytic TiO_2 with WL-irradiation could dramatically inactivate the Odelia, SA11, HastV-1, and FCV.

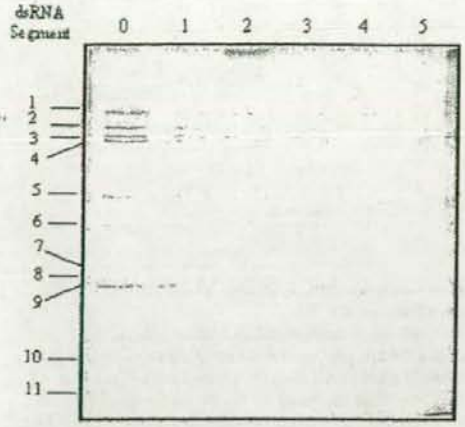

Figure 3: SDS-PAGE of the genomic dsRNA of simian rotavirus strain SA11 treated with VL-catalytic TiO_2 and WL- irradiation in the presence or absence of BSA. Lane 0, electrophoretic pattern of genomic dsRNA extracted from intact rotavirus particles. Lanes 1-5, electrophoretic pattern of genomic dsRNA extracted from rotavirus particles after exposure to the treatment for 1, 2, 3, 4 and 5 days, respectively.

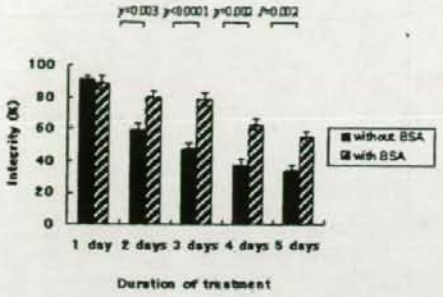

Figure 4: The integrity of the genomic dsRNA (segment 1) of simian rotavirus strain SA11 after treatment with VL-catalytic TiO_2 and WL-irradiation in the presence or absence of BSA (1 mg/ml) for 1, 2, 3, 4, and 5 days.

Protection of viruses by BSA against inactivation by VL-catalytic TiO_2 with WL-irradiation

In order to elucidate the underlying mechanisms of how the VL-catalytic TiO_2 inactivated the viruses, we hypothesized that the effect might take place on the capsid protein of the viruses. To test this hypothesis, the viruses Odelia, SA11, and FCV were treated with VL-catalytic TiO_2 and WL-irradiation in the absence or presence of different concentrations of BSA (0.1 and 1 mg/ml) at 30 °C for 24 hrs. After treatment, the titers of the surviving viruses were determined. The results shown in Table 2 revealed that in the absence of BSA the virus titer of Odelia was reduced from $10^{3.40}$ to $10^{1.90}$ $TCID_{50}$/ml, while in the presence of BSA at 0.1 and 1 mg/ml the titer of the surviving virus was increased from $10^{1.90}$ to $10^{2.20}$ and $10^{2.80}$ $TCID_{50}$/ml, respectively. This protective effect of BSA was also observed in the SA11 and FCV viruses. In the absence of BSA, the titer of the virus was reduced from $10^{5.15}$ to $10^{2.37}$ pfu/ml, while in the presence of BSA at 0.1 and 1 mg/ml the titer of surviving viruses was increased from $10^{2.37}$ to $10^{2.90}$ and $10^{3.95}$ pfu/ml, respectively. Furthermore, for FCV in the absence of BSA, the virus titer was reduced from $10^{4.80}$ to $10^{1.80}$ $TCID_{50}$/ml, whereas in the presence of 0.1 and 1 mg/ml of BSA the virus titer was increased from $10^{1.80}$ to $10^{2.40}$ and $10^{2.83}$ $TCID_{50}$/ml, respectively. Altogether, the data demonstrated that virus inactivation by VL-catalytic TiO_2 could be abolished by BSA in a dose dependent manner.

Analysis of rotavirus RNA by SDS-PAGE

In order to determine whether the RNA genome inside the rotavirus particles could be damaged by VL-catalytic TiO_2, the viral genome was extracted from rotavirus particles after inactivation with VL-catalytic TiO_2 and WL irradiation and then analyzed by SDS-PAGE. It was found that all 11 segments of the rotavirus genome were partially degraded, which was indicated by the intensity of the RNA bands, compared to those of the intact rotavirus (Figure 1). Then, the Scion Image Analyzer Software was used to analyze the intensity of dsRNA segment bands. Segments 1, 5, and 6 of SA11 genomic dsRNA were chosen for this analysis. The results shown in Figure 2A revealed that after treatment with VL-catalytic TiO_2 and WL-irradiation for 2 days, the intensity of segments 1, 5, and 6 of SA11 genomic dsRNA was decreased dramatically to 59.1%, 54.7% and 54.1%, respectively ($p< 0.01$). The decrease of the intensity between days 2 and 3 of treatment was not significantly different. However, on day 4 of treatment the percentage intensity of segments 1, 5, and 6 declined further to 37.5%, 39%, and 41.6%, respectively.

It might be possible that the decrease of the intensity of genomic dsRNA bands observed might have been due to auto-degradation of the genomic RNA molecule rather

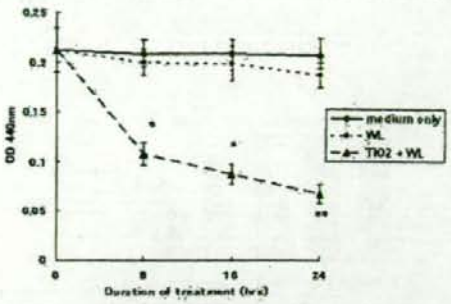

Figure 5: Generation of ·OH from VL-catalytic TiO_2 film after irradiation with WL in an aqueous medium. The TiO_2 film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, and 0.05 mM p-nitrosodimethylaniline and then irradiated with WL at 30 °C. The O.D. was measured at 440 nm after exposure for 0, 8, 16, and 24 hr. *: p=0.001, **: p<0.001 (against WL)

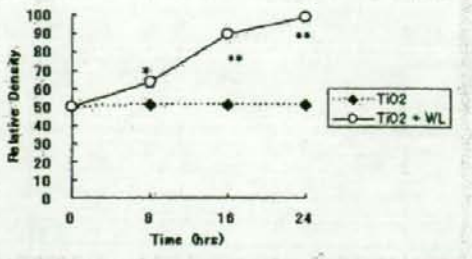

Figure 6: Generation of O_2^- on the VL-catalytic TiO_2 film after irradiation with WL.
The TiO_2-film was submerged in 5 ml of MEM PR(-) containing 0.03% glutamine, 0.12% $NaHCO_3$, 10 μM NBT, and 0.1 mM EDTA and then irradiated with WL at 30 °C. The film was then analyzed by the Scion Image Analyzer Software to calculate the relative density of O_2^-. *: p=0.001, **: p< 0.001

than the effect of VL-catalytic TiO_2 and WL-irradiated treatment. To solve this problem, the intensities of SA11 genomic dsRNA bands of segments 1, 5, and 6 treated with VL-catalytic TiO_2 and WL-irradiation were compared to those of the corresponding segments which were kept in the same condition without treatment. The results shown in Figure 2B demonstrated that the intensities of dsRNA bands of segments 1, 5, and 6 remained the same at more than 95%, while those treated with VL-catalytic TiO_2 were markedly decreased to 33.7%, 36.7%, and 42.4%, respectively. The data clearly indicated that the intensity decrease of genomic dsRNA bands of rotavirus was the result of VL-catalytic- TiO_2 and WL-irradiated treatment, and not due to auto-degradation of genomic RNA.

As shown in Table 2, BSA could reverse the inactivation of the viruses by VL-catalytic TiO_2 and WL irradiation: this raised the question of whether the BSA could also protect the viral RNA genome from degradation by VL-catalytic TiO_2 and WL-irradiated treatment. To answer this question, the genomic dsRNA (segment 1) of the SA11 strain treated with VL-catalytic TiO_2–WL irradiation for 1, 2, 3, 4, and 5 days in the presence of BSA (1 mg/ml) was analyzed by SDS-PAGE. The electrophoretic pattern of genomic dsRNA of simian rotavirus strain SA11 shown in Figure 3 revealed a relatively normal intensity of genomic dsRNA bands on days 1, 2, and 3. However, the intensity of the bands was low on days 4 and 5. In order to estimate the intensity of the bands more accurately, the Scion Image Software was used to analyze the intensity of the dsRNA band. Only the segment 1 band was used for this analysis.

The results shown in Figure 4 revealed that in the absence of BSA, the intensity of the genomic dsRNA electrophoretic band (segment 1), compared to that of the intact dsRNA was gradually reduced to 90.7% on day 1 and then strikingly reduced to 59.1%, 48.0%, 37.5% and 33.7% on days 2, 3, 4, and 5, respectively. In the presence of BSA (1mg/ml), the intensity of the genomic dsRNA band was recovered to 80.0% on day 2, 79.0% on day 3, 63.0% on day 4, and 55.0% on day 5. The data indicated that the intensity recovery of the dsRNA band ranged from approximately 21.0 to 31.0% (p< 0.01).

Generation of hydroxyl radical from VL-catalytic TiO_2 film with WL-irradiation in an aqueous medium

Attempts were made to elucidate the mechanism of VL-catalytic TiO_2 film with WL irradiation in the inactivation of the enteropathogenic viruses by looking for the generation of ·OH, which is toxic to viruses. The results shown in Figure 5 demonstrated that VL-catalytic TiO_2 irradiated with WL generates ·OH by bleaching the p-nitrosodimethylaniline to decrease the OD significantly after exposure to the treatment for 8, 16, and 24 hrs (at 8 hrs and 16 hrs: p=0.001, at 24 hrs: p< 0.001). In contrast, in medium alone or medium irradiated with WL, the OD remained at the same level after 8, 16, and 24 hrs of treatment.

Generation of superoxide anion on the VL-catalytic TiO_2 after irradiation with WL in an aqueous medium

Since O_2^- is another radical that is toxic to microorganisms, it was interesting to investigate whether O_2^- could also be generated from the VL-catalytic TiO_2 irradiated with WL. The generation of O_2^- was indicated by the formation of blue formazane on the VL-catalytic TiO_2 film. Since the blue formazane could not be eluted into the aqueous medium, the intensity of the blue formazane formation on the film was determined by using Scion Image Analyzer Software. The results shown in Figure 6 demonstrated that the density of blue formazane was markedly increased to 63.4% at 8 hrs, 89.7% at 16 hrs, and 99% after 24 hrs of treatment. In contrast, the formation of the blue formazane in the VL-catalytic TiO_2 film not irradiated with WL remained at the baseline regardless of whether the duration of the treatment was 8, 16, or 24 hrs. The results indicated that the VL-catalytic TiO_2 irradiated with WL indeed generates O_2^-.

DISCUSSION

Viral gastroenteritis is still a health burden in both developing and developed countries. Rotavirus and norovirus are the major causative agents of non-bacterial gastroenteritis and are associated with outbreaks as well as sporadic causes of this illness worldwide [1-4, 6, 35, 36]. The outbreak of diarrheal virus is sometimes associated with poor sanitation, especially in developing countries. Therefore, the discovery of a simple and effective method for inactivation of the viruses, particularly those contaminating environmental surfaces, would be a useful tool for the prevention of diarrheal virus transmission to humans.

The photocatalytic oxidation of TiO_2 occurred by electron transfer reactions and the generation of hydroxyl radicals [37]. The hydroxyl radical reacts with most biological molecules. The TiO_2 photocatalytic oxidation received wide attention and has been used for purification of water and clearing toxic substances from the environment [20-22]. Although the application of TiO_2 photocatalysts has been well established for inactivation of poliovirus and coliform bacteria [24, 38] as well as of cancer cells and other microorganisms [23, 25], there is no report on diarrheal viruses. The reactive oxygens ($\cdot OH$ [26] and O_2^-) generated from the photocatalytic reaction are considered to play a significant role in the microbial inactivation. The results of these studies were obtained from the use of UV-catalytic TiO_2.

This study is the first to apply photocatalytic TiO_2 under the activation by VL in the inactivation of viruses. The virus titers were dramatically reduced by up to 1.50-2.82 log10 after exposing the viruses to VL-catalytic TiO_2 for 24 hrs. It was also found that the inactivation of the viruses by VL-catalytic TiO_2 also continued after 24 hrs (data not shown). VL irradiation alone, or TiO_2 alone without VL irradiation, caused no harm to the virus, as indicated by the virus titers remaining at the same level as those without treatment. The data suggested that inactivation of the viruses by the VL-catalytic TiO_2 in this study is more likely caused by the radicals generated from the VL-catalytic TiO_2 reaction. In fact, we demonstrated that the reactive oxygen species, O_2^- and $\cdot OH$, were produced when TiO_2 was irradiated with VL. The hydroxyl radical ($\cdot OH$) has a high reactivity which makes it a very dangerous compound to the organism. It can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidetion) and amino acids (e.g. conversion of Phe to m-Tyrosine and o-Tyrosine). It reacts at diffusion rates with virtually any molecule found in its path including macromolecules such as DNA, membrane lipids, proteins, and carbohydrates. In terms of DNA, the hydroxyl radical can induce strand breaks as well as chemical changes in the deoxyribose and in the purine and pyrimidine bases. On the other hand, even superoxide anions (O_2^-) only exhibit mild oxidative properties. They have, however, a larger free diffusion path length and can undergo a series of interesting reactions. One of them might be dismutation to hydrogen peroxide, which then through Fenton chemistry, yields aggressive hydroxyl radicals [39]. Inactivation of diarrheal viruses required 24 hrs, which is much longer when compared to the 30 min required for the inactivation of poliovirus [38]. This might be due to the difference in the light source used in the inactivation of poliovirus 1, which was sunlight and black light with an emission spectrum of below 400nm. Therefore, the effect of disinfection may be enlarged by the inactivation ability of UV.

The electrophoretic analysis of genomic dsRNA rotavirus after treatment with VL-catalytic TiO_2 revealed a dramatic change in the intensity of the RNA bands, especially on days 2-5 of the treatment. The intensity reduction of electrophoretic RNA bands might be due to degradation of individual RNA segments rather than to random cleavage of the viral genome into smaller fragments, as observed in the phage PL-1 [28]. On the other hand, because of the triple-layered capsid protein structure of rotavirus, it could be possible to provide better protection of the viral genomic dsRNA from degradation by $\cdot OH$, generated from the VL-catalytic TiO_2. The degradation of genomic RNA segments of the rotavirus observed in this study is unlikely to have been due to the auto-degradation of the RNA molecule, since the genomic RNA kept in the same condition without treatment with VL-catalytic TiO_2 remained intact, with the intensity of the electrophoretic band being more than 95% of that extracted from the intact viral particles. This finding implies that the reactive oxygen species generated from VL-catalytic TiO_2 may somehow penetrate directly or indirectly into the capsid protein to cause damage to the genome of the virus.

The inactivation of rotavirus by VL-catalytic TiO_2 was partially recovered by the addition of BSA and the protection was in a dose-dependent manner. A similar observation was also found when other increasing doses of BSA such as 2 mg/ml, 3 mg/ml were tested (data not shown). The recovery rate was not completely restored, but rather stayed at about 21.0% - 31.0% no matter how long the genomic dsRNA of the rotavirus was treated with VL-catalytic TiO_2. It is possible that the inactivation effect exceeded the protecting effect of the BSA. On the other hand, it has been reported that the degradation of proteins occurred by photocatalytic TiO_2 with UV irradiation [38]. Our results suggested that the capsid protein might be the primary target of the reactive oxygen species in the initial phase of virus inactivation.

The light sources used in the inactivation of poliovirus 1, phage PL-1, and phage MS-2 were either a black light lamp or black-light blue lamp that emitted light with a spectrum of 300-400 nm, which was equivalent to that of the UV light [26, 28, 40]. The advantage of this study, regarding the light source, was the use of WL, which is simple and harmless to humans. The inactivation of enteropathogenic virus required a great deal of time, and was partially inhibited by the addition of BSA. As water may also be contaminated by other organic materials and micoorganisms in the real condition, the inactivation could be reduced. When considering safe long-time use of the reaction of TiO2 with VL, a potential application may be in places where there is no technical infrastructure, such as the walls of bathrooms and public facilities.

In conclusion, this is the first indication to demonstrate the inactivation of rotavirus, astrovirus and FCV by the presence of TiO_2 film under VL as well as describing its mechanism. This also underscores the potential as well as the importance of TiO_2 film in the future specific treatment of enteropathogenic virus infection.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan. Additionally, the study was also supported by the Heiwa Nakajima Foundation, the Mishima-Kaiun Foundation, and the Sumitomo Foundation in Japan.

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JOURNAL OF VIROLOGY, Sept. 2007, p. 10188–10194
0022-538X/07/$08.00+0 doi:10.1128/JVI.00337-07
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Vol. 81, No. 18

NOTES

Evidence of Intragenic Recombination in G1 Rotavirus VP7 Genes[▽]

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Received 15 February 2007/Accepted 25 June 2007

The G1 rotavirus is the most widespread genotype causing acute gastroenteritis in children. In an attempt to investigate the occurrence of intragenic recombination, 131 complete coding region sequences of VP7 genes of the G1 rotaviruses in GenBank were examined. Three hitherto-unreported intragenic recombinant rotaviruses were identified. It was noteworthy that two different types (interlineage and intersublineage) of intragenic recombination in rotaviruses were also found. This is the first report to demonstrate the existence of intragenic recombinations between interlineage and intersublineage in G1 rotaviruses.

Acute gastroenteritis is a significant cause of morbidity and mortality of children in both developed and developing countries. Despite much progress in the understanding of the pathogenesis of the disease and its management with the widespread use of oral rehydration therapies, acute gastroenteritis consistently ranks as one of the top causes of death worldwide (9, 10, 20). The rotaviruses, which comprise a genus in the family *Reoviridae*, are icosahedral in appearance. With VP4 spikes, the rotavirus is about 110 nm in diameter. The virion of this virus is a nonenveloped, triple-layered capsid containing 11 segments of double-stranded RNA genome. The rotavirus genome encodes six structural and six nonstructural proteins (3). This virus is estimated to be responsible for 111 million episodes of diarrhea requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 325,000 to 592,000 deaths every year in children under five years old (12). Over past decades, G1 rotaviruses have been the most widespread genotype causing acute gastroenteritis in children from many countries covering all continents of the world (18). Nucleotide substitution and genomic reassortment have been proposed to be the most important mechanisms of rotavirus evolution in nature (3, 4, 18). The rapidly increasing detection of G1 rotavirus, in association with the genetic heterogeneity, raises intriguing questions such as whether rotavirus evolution is driven by intragenic recombination. Thus, the objective of this study was to assess the occurrence of intragenic recombination in the VP7 genes of the G1 rotaviruses.

A total of 131 sequences of the G1 rotaviruses, including our 36 sequence data from China, Japan, and Vietnam (15, 22, 26), which did not include any gaps in the alignment for the entire coding region of VP7 genes, were collected from GenBank. Sequence alignment was performed using CLUSTAL X (21). Phylogenetic trees with 100 bootstrap replicates of the nucleotide alignment datasets were generated using the neighbor-joining method (17). Genetic distance was calculated using Kimura's two-parameter method (PHYLIP) (6). SimPlot was used to detect recombinant rotavirus sequences as well as the breakpoints (7).

All 131 sequences of the G1 rotaviruses in this study were classified into different lineages and sublineages (Fig. 1) according to the recent G1 rotavirus classification scheme in which the nucleotide homology of rotavirus strains within each sublineage ranged from 98% to 100%, indicating a genetic difference of only less than 2% among them; the nucleotide sequence divergence between sublineages within the same lineage was from 3% to 4%; and sequence variation among strains between lineages was considerably higher, ranging from 5% to 16% (15). Interestingly, by using SimPlot, we found the VP7 genes of three G1 rotavirus strains to have intragenic recombinations between interlineage and intersublineage.

Ban-59. The G1 rotavirus strain Ban-59 (U26366) was isolated from an infant with acute gastroenteritis in Bangladesh during 1988, and only this strain was assigned into lineage VII (15). Figure 2A shows evidence of the novel recombinant G1 rotavirus bearing different lineage sequence when the nucleotide sequence of strain Ban-59 was compared with that of strain JP421 belonging to lineage IV using SimPlot. The recombination breakpoints were estimated at positions 469 and 649. Before position 469 and after position 649, the identities of Ban-59 and JP421 were distinctly different, ranging from 90% to 93%. From positions 469 to 649, their identities were extremely high (100%). In contrast, the examination of the sequences for nucleotides 469 to 649 among G1 rotavirus lineages revealed the low identities, ranging from 82% to 96%. Of note, this region contained antigenic regions B (nucleotides 474 to 506) and E (nucleotides 615 to 620). Figure 2B also revealed that Ban-59 clustered into different lineages when the different part of VP7 gene-based grouping was performed. From nucleotides 49 to 468 and from nucleotides 650 to 1029, the lineage VII of Ban-59 remained. However, Ban-59 was

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▽ Published ahead of print on 3 July 2007.

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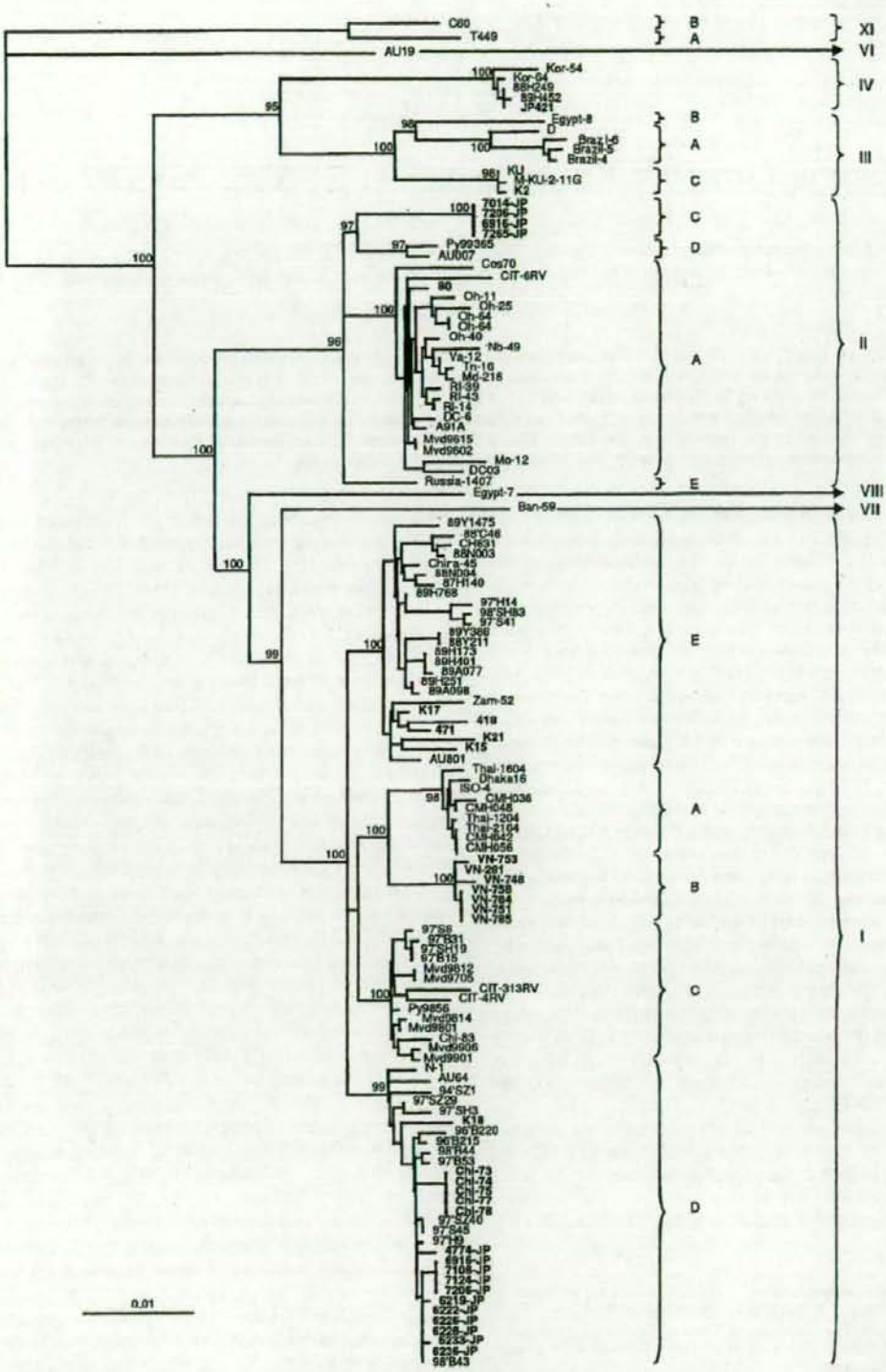

FIG. 1. Lineages and sublineages of 131 VP7 gene nucleotide sequences of G1 rotavirus used in the study. Our strains from China, Japan, and Vietnam are highlighted in boldface. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Bootstrap values of 70% or higher are considered significant for the grouping.

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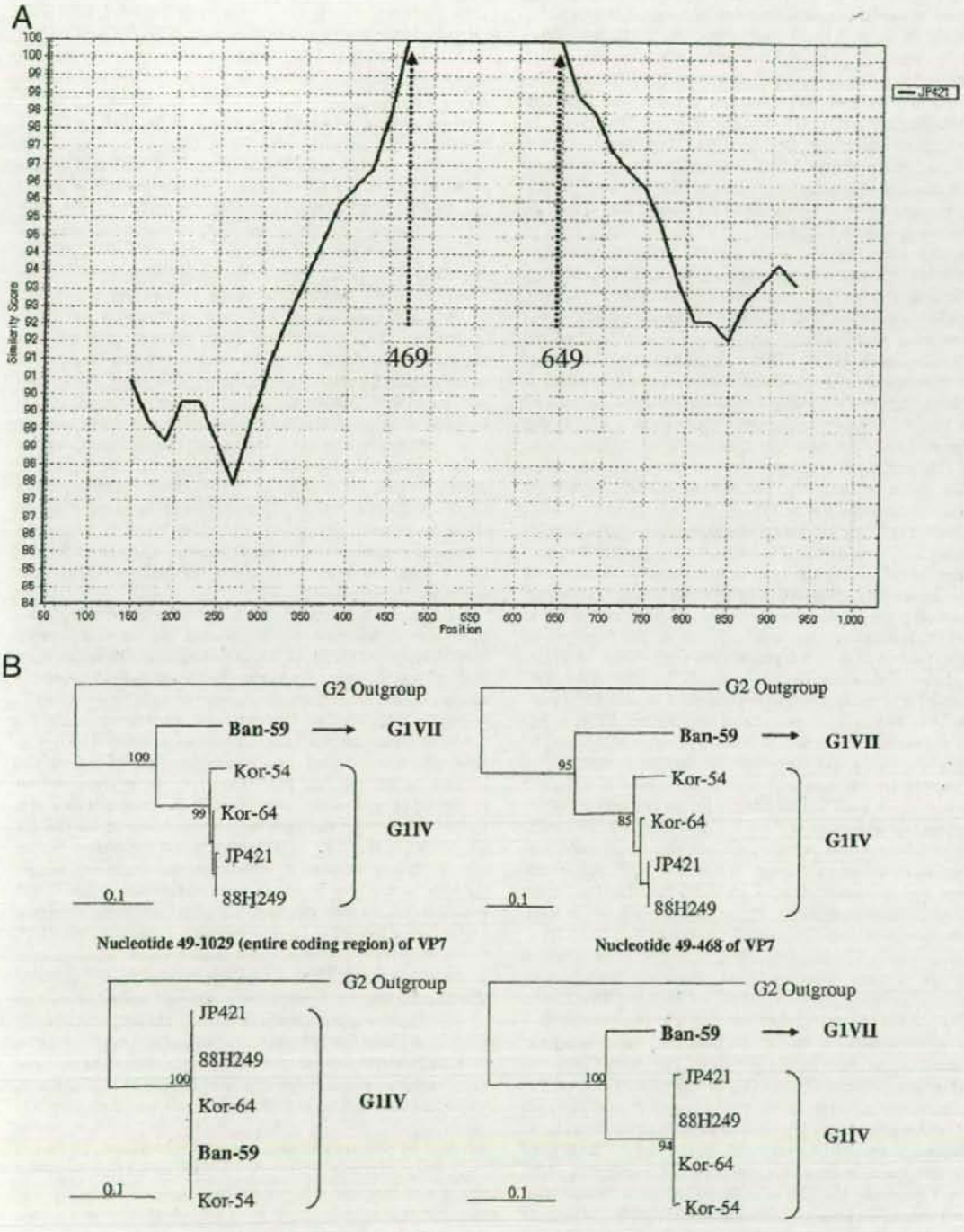

FIG. 2. Identification of novel recombinant G1 rotavirus strain Ban-59. (A) Nucleotide identity comparison of the complete coding regions of VP7 genes of Ban-59 (belonging to lineage VII as a query strain) with that of JP421 (belonging to lineage IV as a reference strain). (B) Observation of changes of genotypes of the Ban-59 on the basis of phylogenetic trees of nucleotide sequences. The scale indicates nucleotide substitutions per position. Numbers in the branches indicate bootstrap values. Bootstrap values of 70% or higher are considered significant for the grouping.

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classified into lineage IV. This kind of phenomenon was recognized as intragenic recombination between interlineage.

Strain D. More than 10 years before the isolation of strain Ban-59, strain D (AB118022) was identified in the United States in 1974. Strain D, together with three Brazilian strains isolated in 1991 and 1992 (Brazil-4, Brazil-5, and Brazil-6), formed sublineage IIIa (15). Using SimPlot, we determined the recombination event and the breakpoint at position 449 in the VP7 gene when strain D was compared with the reference strain Brazil-5, belonging to sublineage IIIa, and with the reference strain Egypt-8, belonging to sublineage IIIb (Fig. 3A). In comparison with Egypt-8, strain D shared a high level of nucleotide identity (98%) in the region from nucleotide 49 to nucleotide 448 and a lower level of the nucleotide identity (97%) in the region from nucleotides 449 to 1029. In contrast, strain D shared a low level of nucleotide identity (96%) in the region from nucleotides 49 to 448 and a high level of the nucleotide identity (98%) in the region from nucleotides 449 to 1029 with Brazil-5. Figure 3B demonstrated that strain D clustered into different sublineages when the different part of VP7 gene-based grouping was performed. Obviously, strain D was recognized as the intragenic recombinant between intersublineage IIIa and intersublineage IIb. It was found that the recombinant region (nucleotides 49 to 448) contained antigenic region A (nucleotides 309 to 353).

Russia-1407. The G1 rotavirus strain Russia-1407 (S83903) was originally detected in Russia, and it was assigned to sublineage IIe (15). When the nucleotide sequence of Russia-1407 was compared with that of Oh-64 by using SimPlot, an apparent site of genetic recombination was found at position 549 in the VP7 gene (Fig. 4A). Before this site, the identities of Russia-1407 and Oh-64 were rather low (only 96%). After this site, however, they were highly similar (99%). The results demonstrated that the nucleotide sequences of regions from positions 49 to 548 in these two strains were rather different, but their sequences from positions 549 to 1029 were identical. The phylogenetic trees also supported the SimPlot results. Figure 4B showed that Russia-1407 was grouped into two distinct sublineages, IIa and IIe, according to its low and high identities of nucleotide sequences to Oh-64. Taken together, the results clearly indicated that Russia-1407 was the novel intragenic recombinant of intersublineage. It was found that the recombinant region (nucleotides 549 to 1029) contained antigenic regions C (nucleotides 672 to 713), D (nucleotides 921 to 925), E (nucleotides 615 to 920), and F (nucleotides 747 to 776).

G1 rotaviruses have been reported as the most common genotype in many countries (8, 11, 18). Even though many studies have conducted surveillance on rotavirus infection in various countries, reports documenting intragenic recombination in rotaviruses are limited. To date, only three intragenic recombinant rotaviruses, CHW17 and CH55 from China (recombination between G1 and G3) and ArgRes1723 from Argentina (recombination between sublineages Ib and Ic within G4), were detected (14, 19). Remarkably, we found three novel intragenic recombinant rotaviruses, accounting for 2.3% (3 of 131). This seems to be a high frequency for rotaviruses, which are not generally thought to undergo intragenic recombination. Three novel intragenic recombinants were found in three different continents, including Asia (Ban-59), Europe (Russia-1407), and North America (D), where the prevalence of G1 rotavirus infection was very high, ranging from 60.3% to 73.7% (18). However, among three previously reported intragenic recombinants, two were found in Asia (CHW17 and CH55) and one was found in South America, where the prevalence of G3 and G4 rotavirus infection was low (only 6.5% and 8.8.3%, respectively) (18). Obviously, the prevalence of intragenic recombinants in G3 and G4 rotaviruses is higher than that in G1 rotavirus.

Phylogenetic analysis showed that these rotavirus strains clustered into different lineages and sublineages within the G1 genotype when gene-based grouping was performed for a different region of VP7. These observations suggested that there are two distinct kinds of intragenic recombination, interlineages and intersublineages. These results are noteworthy because this is the first report, to the best of our knowledge, showing intragenic recombination in interlineages and intersublineages of G1 rotaviruses. These phenomenons improve our current knowledge of the G1 rotavirus evolution as well as of the origin of genetic heterogeneity. Altogether, the recombination in rotaviruses could greatly affect phylogenetic groupings and confuse molecular epidemiological studies. Among three previously described intragenic recombinants, strains CHW17 and CH55 appeared to result from a double-crossover event (19) and strain ArgRes1723 appeared to result from a single-crossover event (14). Consistent with these findings, the single and double crossovers were also found in two novel intragenic recombinants (strain D and strain Russia-1407) and in one novel intragenic recombinant (strain Ban-59) detected in the study, respectively.

To date, two rotavirus vaccines, Rotarix and RotaTeq, have recently been released onto the market and licensed in more than 30 countries (1, 13, 16, 23, 24). Rotarix and RotaTeq have been proven to have significant clinical efficacies against G1 rotavirus gastroenteritis, with efficacy values of 96% and 95%, respectively (1, 23, 24). However, the occurrence of the G1 rotavirus gastroenteritis after immunization in children is possible. And these G1 rotavirus strains were different from the vaccine viruses (16, 23, 24). Therefore, the evidence of an intragenic recombination event in these cases should be investigated. Obviously, the intragenic recombination in the G1 rotaviruses in this study is important to aid the explanation of vaccine failure because it could produce immunity escape through exchanging antigenic regions between different G1 rotavirus strains with different antigenicities. Even antigenic regions were located in the recombinant regions of Ban-59, D, and Russia-1407; however, direct inspection of the alignment of the deduced sequences of antigenic regions of VP7 revealed that these antigenic regions are conserved among them and among the reference rotavirus strains used in the present study (15). It is likely that structural variation of the VP7 protein within a genotype develops by successive mutations in the gene (2). Therefore, the intragenic recombination in VP7 genes of three strains might induce the conformational changes of G1 rotavirus VP7 protein, which probably led to the changing antigenicity of these intragenic recombinant strains. To date, a study on localization of amino acids involved in conformational changes of rotavirus VP7 structure is not available (15). The identification of these amino acids is of significance and should be investigated by further studies.

Although the crucial contributions of genetic reassortment

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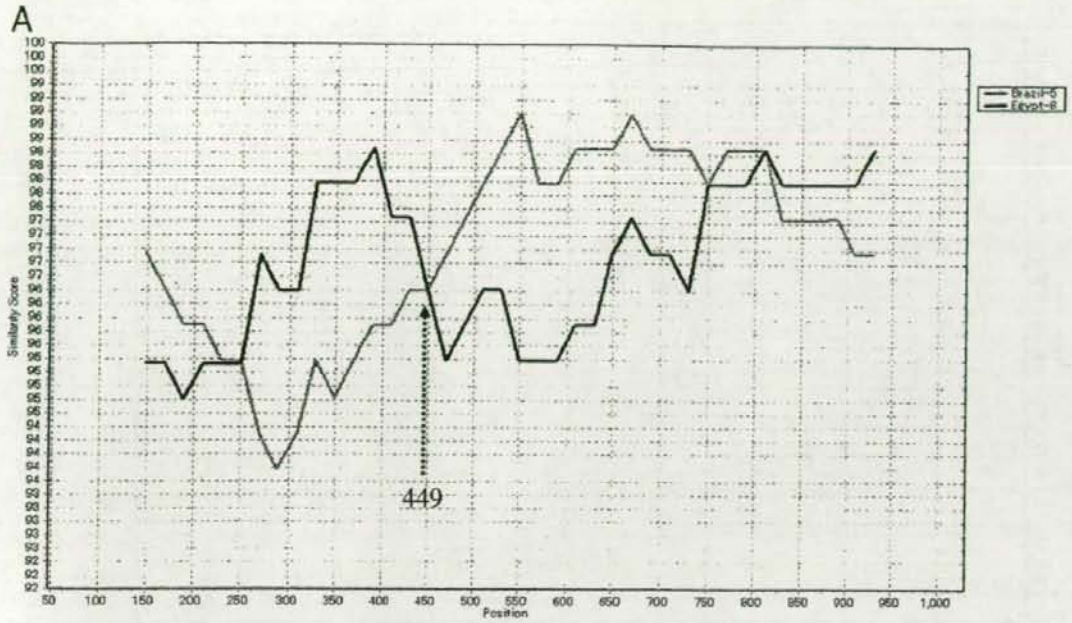

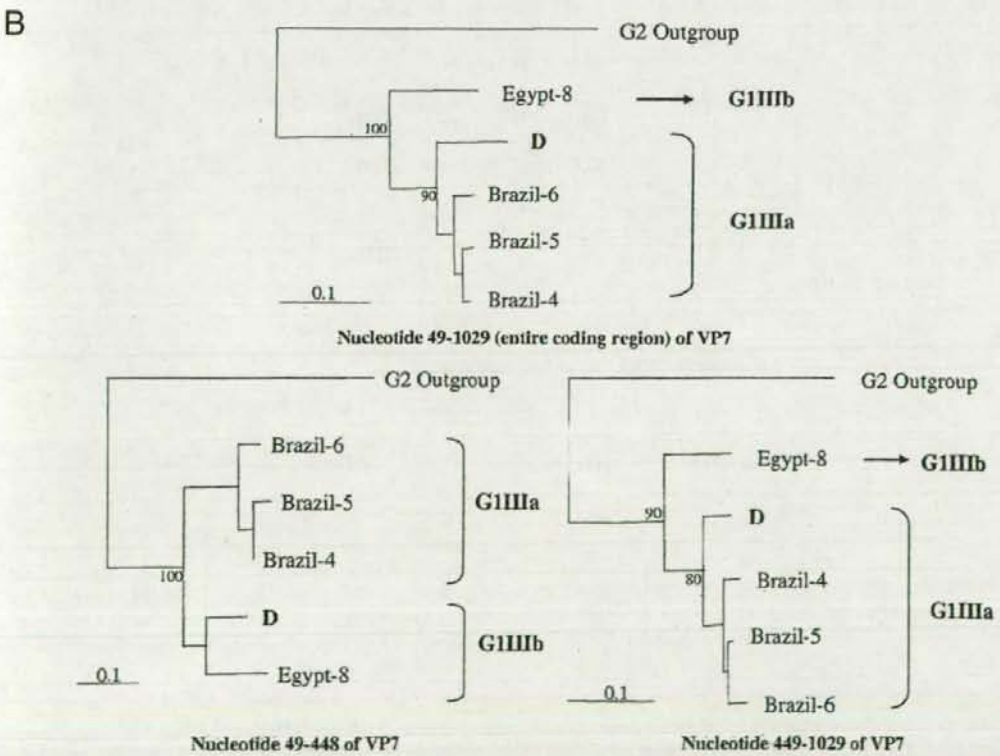

FIG. 3. Identification of novel recombinant G1 rotavirus strain D. (A) Nucleotide identity comparison of the complete coding regions of VP7 genes of strain D (belonging to lineage IIIb as a query strain) with those of the Egypt-8 (belonging to lineage IIIb) and Brazil-5 (belonging to lineage IIIa as reference strains). (B) Observation of changes of genotypes of D on the basis of phylogenetic trees of nucleotide sequences. The scale indicates nucleotide substitutions per position. Numbers in the branches indicate bootstrap values. Bootstrap values of 70% or higher are considered significant for the grouping.

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