Study on Aichi virus, parechovirus, and bocavirus detected in children with acute gastroenteritis in Japan, Bangladesh, Thailand, Vietnam, and Sri Lanka

日本、バングラデシュ、タイ、ベトナム、スリランカの急性胃腸炎の小児で検出されたアイチウイルス、パルコウイルス、ボカウイルスに関する研究

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NTEROVIRUSES, AND HUMAN BOCAVIRUS

ABSTRACT

Acute gastroenteritis is one of the most common diseases in infants and children. It continues to be a significant cause of morbidity and mortality worldwide. Rotavirus, norovirus, sapovirus, adenovirus, and astrovirus have been established as the most important etiologic agents. However, the etiologic agents of more than half of acute gastroenteritis patients are undiagnosed. Recently, Aichi virus, human parechovirus (HPeV), and human bocavirus (HBoV) have also been considered to be agents of this illness. Thus, the aim of this study was to perform the molecular epidemiology of these viruses in children with acute gastroenteritis in Japan, Bangladesh, Thailand, Vietnam, and Sri Lanka.

The study was carried on 1,603 fecal specimens which had been shown to be negative for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus (except for 362 Sri Lankan samples which had not been screened for the 5 viruses yet) by reverse transcription - polymerase chain reaction (RT-PCR) and which were collected from infants and children with acute gastroenteritis. For study on Aichi virus, 912 samples were collected in Japan, Bangladesh, Thailand, and Vietnam during 2002-2005. For HPeV and HBoV, the study was done on 691 samples from Japan, Thailand, and Sri Lanka during 2005-2008. Screening of Aichi virus was done by a nested PCR using primer sets for amplifying the 3CD junction region. HPeV was detected by RT-PCR using a primer pair to amplify 5'UTR region of its genome and was genotyped by sequencing of the VP1 region. HBoV was screened by RT-PCR using a primer pair to amplify NP1 region of its genome and was genotyped by sequencing of the VP1/VP2 region.

Of the 912 samples tested, 28 (3.1%) were positive for Aichi virus. Of these, 14 (6.5%) samples were collected from Japan, 10 (2.5%) from Bangladesh, 3 (1.6%) from Vietnam, and 1 (0.9%) from Thailand. Aichi virus strains detected from Japan, Thailand, and Vietnam belonged to genotype A, while the majority of Bangladeshi strains belonged

to genotype B. Of the 28 positive samples, twelve were subjected to amplification and sequence analysis of the capsid gene of Aichi viruses. The phylogenetic tree constructed from nucleotide sequences of the capsid gene of the strains studied and reference strains demonstrated that Aichi virus strains clustered into two branches. A classification of Aichi viruses based on the capsid gene was proposed, in which lineage I consists of the Aichi virus strains detected from Japan, Thailand, Vietnam, and Germany, and lineage II includes Bangladeshi strains and a Brazilian strain. Alignment of nucleotide sequences of the capsid gene of the 17 Aichi virus strains studied and reference was done, then, 5 new primers were developed based on different nucleotides between two genotypes A and B. The newly developed primers were used in a nested PCR to examine 17 known-genotype Aichi virus strains. A nested polymerase chain reaction method using genotype-specific primers based on the capsid gene was developed to differentiate between genotypes A and B of Aichi viruses. The results showed that the PCR using newly designed genotype-specific primers could generate appropriate PCR products from all 17 samples tested, the newly developed primers could differentiate genotype A from genotype B, and all matched those obtained by nucleotide sequencing of the capsid regions. The nested PCR method using genotype-specific primers is useful and can be used for genotyping of Aichi viruses.

HPeV was detected in 8.1% (20/247), 14.6% (12/82), and 8.3% (30/362) of samples collected in Japan, Thailand, and Sri Lanka, respectively. Genotyping of the detected HPeV strains showed that in Japan eighteen of the 20 strains positive for HPeV were sequenced successfully for the VP1 gene and the majority of the HPeV strains (n=15) were identified as HPeV1, and the remaining 2 as HPeV3. For the detected Thai HPeV, the capsid VP1 gene of nine strains was successfully sequenced. The studied HPeV strains clustered into 4 different genotypes from HPeV1-4, and the majority of the strains studied (5 strains) belonged to HPeV1. In addition, a rare HPeV2 strain was also detected. In Sri Lanka, the capsid VP1 gene of twenty-seven strains was successfully sequenced. The studied HPeV

strains clustered into HPeV1 (11), HPeV3 (1), HPeV4 (5), HPeV5 (3). Five were determined as HPeV10, and the 2 remaining strains were identified as HPeV11. This is the first report of the circulation of HPeV in infants and children with acute gastroenteritis in Sri Lanka and the first report of HPeV10 and HPeV11. In addition, the diversity of Sri Lankan HPeV was also noted.

HBoV was found in 1.6% (4/247), and 1.2% (1/82) of samples collected in Japan and Thailand, respectively. Three Japanese strains were clustered into group 1, while the remaining Japanese strain and a unique Thai strain belonged to group 3.

At present, Aichi virus, HPeV, EVs and HBoV were detected by RT-monoplex PCR which use a single set of primers and can detect one target virus only. In contrast to RT-monoplex PCR, RT-multiplex PCR with different pairs of specific primers for amplifying different viral genomes in one reaction tube enables us to detect for two or more targets in a single test. In addition, RT-multiplex PCR assay is a simple and potential for rapid and cost-effective laboratory diagnosis. This study developed the RT-multiplex PCR method for detection of Aichi virus, HPeV, EVs, and HBoV in fecal specimens collected from infant and children with acute gastroenteritis.

In conclusion, this is the first finding of Aichi virus in fecal specimens from Bangladesh, Thailand, and Vietnam. This is also the first finding of HPeV from children with acute gastroenteritis in Thailand and Sri Lanka. In addition, from this study, a detection of novel HPeV10 and -11 strains were firstly reported.

This is the first report of detecting these kinds of viruses in fecal samples from infants and children with acute gastroenteritis by RT-multiplex PCR. The present study provides better understanding on molecular epidemiology of some less explored viral pathogens, Aichi virus, HPeV, and HBoV, detected from children with acute gastroenteritis in Asia.

ABBREVIATIONS

%	: Percent
ºC	: Degree Celsius
μg	: Microgram
μl	: Microliter
aa	: Amino acid
Ala	: Alanine
Asn	: Asparagine
Asp	: Aspartic acid
bp	: Base pair
cDNA	: Complementary deoxyribonucleic acid
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
ELISA	: Enzym-linked immunosorbent assay
EM	: Electron microscopy
EVs	: Enteroviruses
HBoV	: Human bocavirus
HPeV	: Human parechovirus
Leu	: Leucine
MEGA	: Molecular Evolutionary Genetics Analysis
min	: Minute
nm	: Nanometer
nt	: Nucleotide
NTR	: Non-translated region

ORF	: Open reading frame
P or Pro	: Proline
PCR	: Polymerase chain reaction
RNA	: Ribonucleic acid
RNase	: Ribonuclease
RT	: Reverse transcription
RT-PCR	: Reverse transcription-polymerase chain reaction
S or Ser	: Serine
Sec	: Second
STN	: Serine – Threonin - Asparagin
TSS	: Threonin – Serine - Serine
Thr	: Threonin
Val	: Valine

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LIST OF PUBLICATIONS

This thesis manuscript was written based on the following publication:

1. **Pham NT**, Takanashi S, Abeysekera C, Abeygunawardene A, Shimizu H, Khamrin P, Okitsu S, Mizuguchi M and Ushijima H. Human parechovirus infection in children hospitalized with acute gastroenteritis in Sri Lanka. J Clin Microbiol. 49: 364-366, Jan 2011.

2. **Pham NT**, Trinh QD, Chan-It W, Khamrin P, Shimizu H, Okitsu S, Mizuguchi M and Ushijima H. A novel RT-multiplex PCR for detection of Aichi virus, human parechovirus, enteroviruses, and human bocavirus among infants and children with acute gastroenteritis. J Virol Methods. 169: 193-197, August 2010.

3. **Pham NT**, Chan-It W, Khamrin P, Nishimura S, Sugita K, Maneekarn N, Okitsu S, Mizuguchi M and Ushijima H. Human bocavirus infection in children with acute gastroenteritis, Japan and Thailand. J Med Virol. 83: 286-290, Feb 2011.

4. **Pham NT**, Chan-It W, Khamrin P, Nishimura S, Kikuta H, Sugita K, Baba T, Yamamoto A, Okitsu S, Mizuguchi M and Ushijima H. Detection of human parechovirus in stool samples collected from children with acute gastroenteritis in Japan during 2007-2008. J Med Virol. 83: 331-336, Feb 2011.

5. **Pham NT**, Trinh QD, Takanashi S, Abeysekera C, Abeygunawardene A, Shimizu H, Khamrin P, Okitsu S, Mizuguchi M. and Ushijima H. Novel human parechovirus, Sri Lanka. Emerg Infect Dis. 16:130-132, January 2010.

6. **Pham NT**, Trinh QD, Khamrin P, Maneekarn N, Shimizu H, Okitsu S, Mizuguchi M. and Ushijima H, Diversity of human parechoviruses isolated from stool samples collected from Thai children with acute gastroenteritis. J Clin Microbiol. 48:115-119, January 2010.

7. **Pham NT**, Trinh QD, Nguyen TA, Dey SK, Phan TG, Hoang le P, Khamrin P, Maneekarn N, Okitsu S, Mizuguchi M, and Ushijima H. Development of genotype-

specific primers for differentiation of genotypes A and B of Aichi viruses. J Virol Methods. 156:107-110, March 2009.

8. **Pham NT**, Trinh QD, Khamrin P, Nguyen TA, Dey SK, Phan TG, Hoang le P, Maneekarn N, Okitsu S, Mizuguchi M, and Ushijima H. Sequence analysis of the capsid gene of Aichi viruses detected from Japan, Bangladesh, Thailand, and Vietnam. J Med Virol. 80:1222-1227, July 2008.

9. **Pham NT**, Khamrin P, Nguyen TA, Dey SK, Phan TG, Okitsu S, Ushijima H. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. J Clin Microbiol. 45:2287-2288, July 2007.

INTRODUCTION

Acute gastroenteritis is inflammation of the gastrointestinal tract, involving both the stomach and intestine and resulting in acute diarrhea with various accompanying symptoms, signs, such as nausea, vomiting, fever, or abdominal pain. It is one of the most common diseases in infants and children and continues to be a significant cause of morbidity and mortality worldwide [Musher and Musher, 2004]. It is reported that 3-5 billion cases of acute gastroenteritis and nearly 2 million deaths occur each year in children under 5 years [Elliott, 2007]. Etiologies include bacteria, viruses, parasites, toxins, and drugs. At least 75% of cases of acute gastroenteritis are caused by viruses [Goodgame, 2001]. Viral gastroenteritis ranges from a self-limited watery diarrheal illness (usually <1 week) associated with symptoms of nausea, vomiting, anorexia, malaise, or fever, to severe dehydration resulting in hospitalization or even death. Rotavirus, norovirus, sapovirus, adenovirus, and astrovirus have been identified as the most important etiologic agents [Glass et al., 2001; Marie-Cardine et al., 2002]. However, the etiologic agents of more than half of acute gastroenteritis patients are undiagnosed [Chanit et al., 2009; Olesen et al., 2005; Simpson et al., 2003]. Recently, Aichi virus, human parechovirus (HPeV), and human bocavirus (HBoV) have also been considered to be agents of this illness [Allander, 2008; Baumgarte et al., 2008; Calvo et al., 2008; Lau et al., 2007; Nakanishi et al., 2009; Reuter et al., 2009; Sdiri-Loulizi et al., 2008; Stanway et al., 2000].

Regarding Aichi virus, it was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in a Japanese patient. The virus belongs to a new genus, named Kobuvirus, of the family *Picornaviridae* [Yamashita et al., 1998]. Aichi virus was isolated in Vero cells from 6 (12.3%) of 47 patients in five gastroenteritis outbreaks in Japan. Five (0.7%) of 722 Japanese travelers returning from tours to Southeast Asian countries and complaining of gastrointestinal symptoms at the quarantine station of Nagoya International Airport in Japan, and 5 (2.3%) of 222 Pakistani children with gastroenteritis were found to be

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positive for Aichi virus [Yamashita et al., 1995]. In addition, by enzyme-linked immunosorbent assay (ELISA), 13 (23%) of 47 stool samples from adult patients in five oyster-associated gastroenteritis outbreaks were found to be positive for Aichi virus in Japan [Yamashita et al., 1993]. Recently, Aichi virus was also detected in Brazil and Germany [Oh et al., 2006]. In that study, Aichi virus was detected in three German stool samples and in five Brazilian samples. Those samples were obtained from patients involved in an outbreak of acute gastroenteritis.

For Parechoviruses, they belong to the large family of Picornaviridae, which is a highly diverse family of important pathogens of humans and animals [Stanway et al., 1994]. HPeV has been found to have at least 14 genotypes (HPeV1-14) [Harvala and Simmonds, 2009]. HPeV1 and -2, previously known as echovirus 22 and 23 respectively, were first isolated in 1956 from stool samples from children with diarrhea in USA [Wigand and Sabin, 1961]. HPeV4 was detected from stool samples of a neonate with febrile illness in the Netherlands in 2002 [Benschop et al., 2006a], and recently, HPeV8 was found from stool samples of Brazilian children with acute diarrhea [Drexler et al., 2009]. Although a variable spectrum of symptoms caused by HPeVs has been described up to date, the common symptoms are similar to those caused by some enteroviruses, including mostly enteritis with diarrhea, and respiratory disease [Baumgarte et al., 2008; Benschop et al., 2006b; Joki-Korpela and Hyypia, 1998; Stanway and Hyypia, 1999; Stanway et al., 2000]. Other symptoms and syndromes, such as meningoencephalitis, encephalomyelitis, flaccid transient paralysis, nosocomial infection, neonatal sepsis, myocarditis, myositis, lymphadenopathy, hand-foot-mouth disease, rash, fever of unknown origin, influenza-like illness, and Reye's syndrome, haemolytic uremic syndrome have also been reported [Boivin et al., 2005; Ehrnst and Eriksson, 1993; Ehrnst and Eriksson, 1996; Figueroa et al., 1989; Grist et al., 1978; Ito et al., 2004; Koskiniemi et al., 1989; Legay et al., 2002; Maller et al., 1967; O'Regan et al.,

1980; Russell and Bell, 1970; Watanabe et al., 2007]. In most of these, diarrhea has been the predominant symptom.

For human bocavirus (HBoV), it belongs to the genus Bocavirus of the subfamily Parvovirinae, family *Parvoviridae* [Allander et al., 2005]. The virus has been detected in children with respiratory tract infection in the world including Europe, Asia, Africa, America, and Australia. In these regions, the prevalence of HBoV infection varies considerably between 1.5-19% [Allander, 2008; Allander et al., 2007; Bastien et al., 2007; Calvo et al., 2008; Chieochansin et al., 2007; Jacques et al., 2008; Kesebir et al., 2006; Ma et al., 2006; Sloots et al., 2006; Smuts and Hardie, 2006; Smuts et al., 2008; Zeng et al., 2010; Zheng et al., 2010]. Recently, HBoV has been detected in 0.5-9.1% of fecal specimens obtained from children with acute gastroenteritis in Spain, Germany, Hong Kong, Korea, China, Japan, and Brazil [Albuquerque et al., 2007; Lau et al., 2007; Lee et al., 2007; Nakanishi et al., 2009; Vicente et al., 2007].

Having been considered as causative agents of diarrheal illness, the three mentioned viruses, Aichi virus, HPeV, and HBoV, were targeted in my studies based on stool specimens collected from infants and children under 5 years old with acute gastroenteritis in Japan, Bangladesh, Thailand, Vietnam, and Sri Lanka, due to limited or no information on their infection and molecular characteristics among the studied subjects in these Asian countries. The results showed that these viruses were present in samples negative for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus by RT-PCR. Especially, for HPeV, not only has the virus been found among the patients, its detection rates were found as high as from 8.1% to 14.6% in Japan and Thailand, respectively. In addition, by the present studies, clinical and molecular data of new HPeV genotypes 10 and 11 were firstly analyzed and published.

As new viruses discovered recently, development of new diagnostic methods or improvement of current laboratory approach tools for scientific research needs are definitely

necessary. In the present studies, I developed successfully a PCR method using newly developed primers for genotyping of Aichi viruses, and a multiplex PCR for detection of Aichi virus, HPeV, EVs, and HBoV from clinical specimens. These two PCR methods are useful diagnostic tools for researchers in this field.

AIMS OF THIS STUDY

The objectives of this study are:

1. To investigate the presence of Aichi virus in fecal specimens collected from children with acute gastroenteritis in Japan, Bangladesh, Thailand, and Vietnam

2. To do sequence analysis of the capsid gene of detected Aichi virus strains

3. To develop primers for genotyping of Aichi viruses by PCR

4. To investigate the presence of HPeV in fecal specimens collected from children with acute gastroenteritis in Japan, Thailand, and Sri Lanka; and to characterize the genetic diversity of the detected viruses by nucleotide sequencing and phylogenetic analysis

5. To investigate the presence of HBoV in fecal specimens collected from children with acute gastroenteritis in Japan, and Thailand; and to do sequence analysis of the detected viruses

6. To develop a RT-multiplex PCR method for detection of Aichi virus, HPeV, EVs, and HBoV from clinical specimens

Chapter I: STUDY ON AICHI VIRUS

A. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam

1. Introduction

Aichi virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in a Japanese patient. It is a small round virus with about 30 nm in diameter (Figure 1) [Yamashita et al., 1991]. The virus was classified into a new genus, named Kobuvirus, of the family *Picornaviridae* [Yamashita et al., 1998]. In 1993, a monoclonal antibody against Aichi strain was developed for detection of its antigen in stool samples. Furthermore, the prevalence rate for antibody against Aichi strain in healthy adults and children in Aichi Prefecture, Japan, was estimated by using a neutralization test. That prevalence rate was found to be 7.2% for infants and children aged 7 months to 4 years and increased with age to about 80% in persons 35 years old [Yamashita et al., 1993]. Aichi virus was then isolated in Vero cells from 6 (12.3%) of 47 patients in five gastroenteritis outbreaks in Japan. In addition, 5 (0.7%) of 722 Japanese travelers returning from tours to Southeast Asian countries and complaining of gastrointestinal symptoms at the quarantine station of Nagoya International Airport in Japan, and 5 (2.3%) of 222 Pakistani children with gastroenteritis were found to be positive for Aichi virus [Yamashita et al., 1995].

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 [Yamashita et al., 1998]. The structure of the genome includes 5'- NTR - leader protein - structural protein - nonstructural protein - 3' NTR - poly A (Figure 2).

By enzyme-linked immunosorbent assay (ELISA), 13 (23%) of 47 stool samples from adult patients in five oyster-associated gastroenteritis outbreaks were found to be positive for

Aichi virus in Japan. However, seroconversion against Aichi virus was observed in 20 (47%) of 43 patients involved in these five outbreaks by a neutralization test using paired sera [Yamashita et al., 1993]. These results suggested that ELISA was not sufficient for diagnosis of the Aichi virus infection. Accordingly, in the year 2000, a reverse transcription – polymerase chain reaction (RT-PCR) method for the detection of Aichi virus was developed and the genetic analysis was performed using 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 two groups: group 1 (genotype A) and group 2 (genotype B) [Yamashita et al., 2000].

Basic researches into Aichi virus were consecutively performed [Nagashima et al., 2003; Nagashima et al., 2005; Sasaki et al., 2001; Sasaki et al., 2003; Yamashita et al., 2001]. Recently, Aichi virus was also detected in Brazil and Germany [Oh et al., 2006]. In that study, Aichi virus was detected in three German stool samples and in five Brazilian samples. Those samples were obtained from patients involved in an outbreak of acute gastroenteritis. A panel of 485 German serum samples was also screened for antibody to Aichi virus, and a seroprevalence of 76% was found. Similar to data obtained from the Japanese seropanel, seroprevalence in Germany is age-dependent. However, age distributions between the two countries varied significantly, though. The majority of infections in Germany appeared to occur among children aged younger than 6 years, of whom, around 50% have the acquired antibody.

However, there has been a limited knowledge about 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 strains detected in the present study with the other strains previously reported.
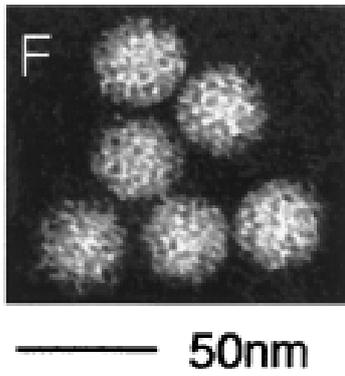
Figure 1. Appearance of Aichi virus visualized by electron microscopy [Yamashita et al., 1998].
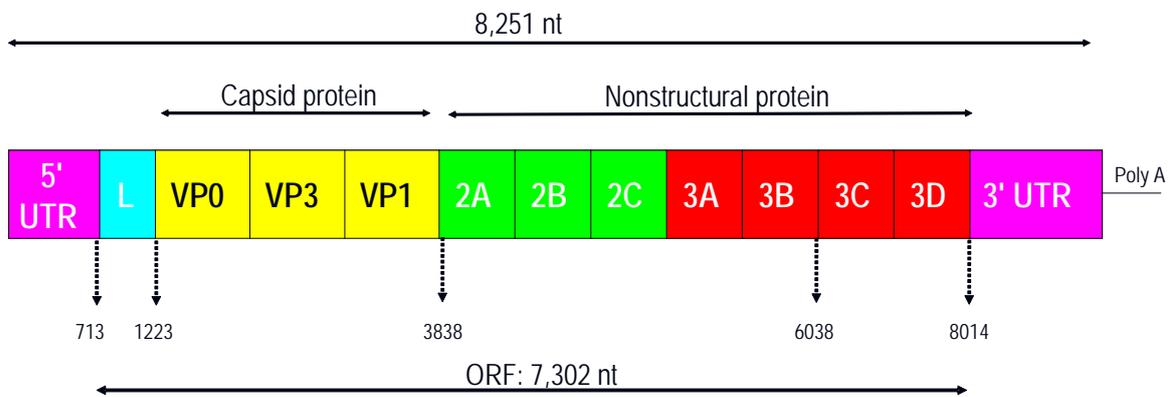
Figure 2. Genome organization of Aichi virus [Yamashita et al., 1998].

2. Materials and methods

Samples tested

A total of 912 stored, extracted RNA samples from fecal specimens known to be negative for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus by RT-PCR, which were collected from patients with acute gastroenteritis in Japan (215 samples, collected from July 2002 to June 2003), Bangladesh (405, collected from October 2004 to September 2005), Thailand (107, collected from March 2002 to December 2004), and Vietnam (185, collected from 2002 to 2003), were used in this study. These samples were collected from epidemiological studies / surveillances during the period of 2002 - 2005 from the four countries above [Dey et al., 2007; Khamrin et al., 2007; Nguyen et al., 2007; Phan et al., 2005a]. All original specimens were collected after the parents of the enrolled children had given the informed consents. The ethical approvals were obtained from the Scientific Committees of the involved hospitals or clinics in the four countries as well as from the Ethical Committee of the University of Tokyo, Japan.

RT-PCR

For reverse transcription, 5 μl of the stored, extracted RNA was added to a reagent mixture consisting of 3 μl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 0.8 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.8 μl of 0.1 M DTT (Invitrogen), 0.8 μl of SuperScript III Reverse Transcriptase (200 U/μl) (Invitrogen, Carlsbad, CA, USA), 0.8 μl of random primer (1 μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl of RNase Inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and 3.3 μl of distilled water. The total volume of reaction mixture was 15 μl. The reverse transcription reaction was carried out at $50^{o}C$ for 1 hr, followed by $95^{o}C$ for 5 min. The samples were then rapidly cooled on ice.

To detect Aichi virus, PCR was conducted using the primer sets for amplifying the 3CD junction region as described previously by Yamashita et al (Figure 3) [Yamashita et al., 2000]. After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 2.5 μl of 10x

Taq DNA polymerase buffer (Promega, Madison, WI, USA), 2 μl of dNTPs (2.5 mM/μl), 0.5 μl of each specific primer (33 μM), 0.1 μl of Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), and 17.4 μl of distilled water, the first PCR was conducted using the primer set C which included primer 6261 and primer 6779 to amplify a 519 bp region between the C terminus of 3C and the N terminus of 3D. The thermal cycler program was 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min [Yamashita et al., 2000]. Next, the nested PCR was performed using the primer pair C94b - 246k with the same thermal cycler program to amplify a 223 bp segment within the 3CD junction region of Aichi virus RNA. Analysis of the amplification products were performed by agarose gel electrophoresis, and the bands were visualized by ethidium bromide staining under ultraviolet light. The oligonucleotide sequences of these primers and their positions are described in Table 1.

Sequence analysis

The amplified product from the first PCR was then purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI Prism 310; Perkin Elmer-Applied Biosystems, Inc.). The two primers 6261 and 6779 were used as sequencing primers. Using BioEdit v7.0.5 software, comparison analyses at nucleotide and amino acid level were conducted between the detected Aichi virus strains in this study and reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database [Altschul et al., 1990]. The phylogenetic analyses were performed using the neighbor-joining method within the MEGA 3.1 analytical package with 500 bootstrap repetitions [Kumar et al., 2004].

Reference Aichi virus strains and their GenBank accession numbers used in this study are the following: strain A1258/87, accession no. AB034649; A1471/96, AB034650; A844/88, AB034651; A848/88, AB034652; A942/89, AB034653; N1277/91, AB034654;

N128/91, AB034655; N628/92, AB034656; M166/92, AB034657; P766/90, AB034658; P803/90, AB034659; P832/90, AB034660; P840/91, AB034661; P880/91, AB034662; T132/90, AB034663; AB079268, A1156/87; 144/96, AB092824; 359/96, AB092825; 364/96, AB092826; 132/96, AB092827; 494/97, AB092828; 177/96, AB092829; 139/96, AB092830; 684/98, AB092831; 414/96, AB092832; 488/97, AB092833; R380, DQ145762. The nucleotide sequences of the studied strains have been assigned as the following accession numbers: EF079149 to EF079162, and from EF466010 to EF466015.

Table 1. Oligonucleotide sequences of the 2 primer pairs used in the first and nested PCR for detection of Aichi virus. The primer pair of 6261 - 6779 was also used as sequencing primers.

Primer	Polarity	Sequence 5'to 3'	Gene	Position	Reference
6261	+	ACACTCCCACCTCCCGCCAGTA	3CD	6261-6282	Yamashita et al., 2000
6779	−	GGAAGAGCTGGGTGTCAAGA	3CD	6779-6760	Yamashita et al., 2000
C94b	+	GACTTCCCCGGAGTCGTCGTCT	3CD	6398-6419	Yamashita et al., 2000
246k	−	GACATCCGGTTGACGTTGAC	3CD	6663-6644	Yamashita et al., 2000
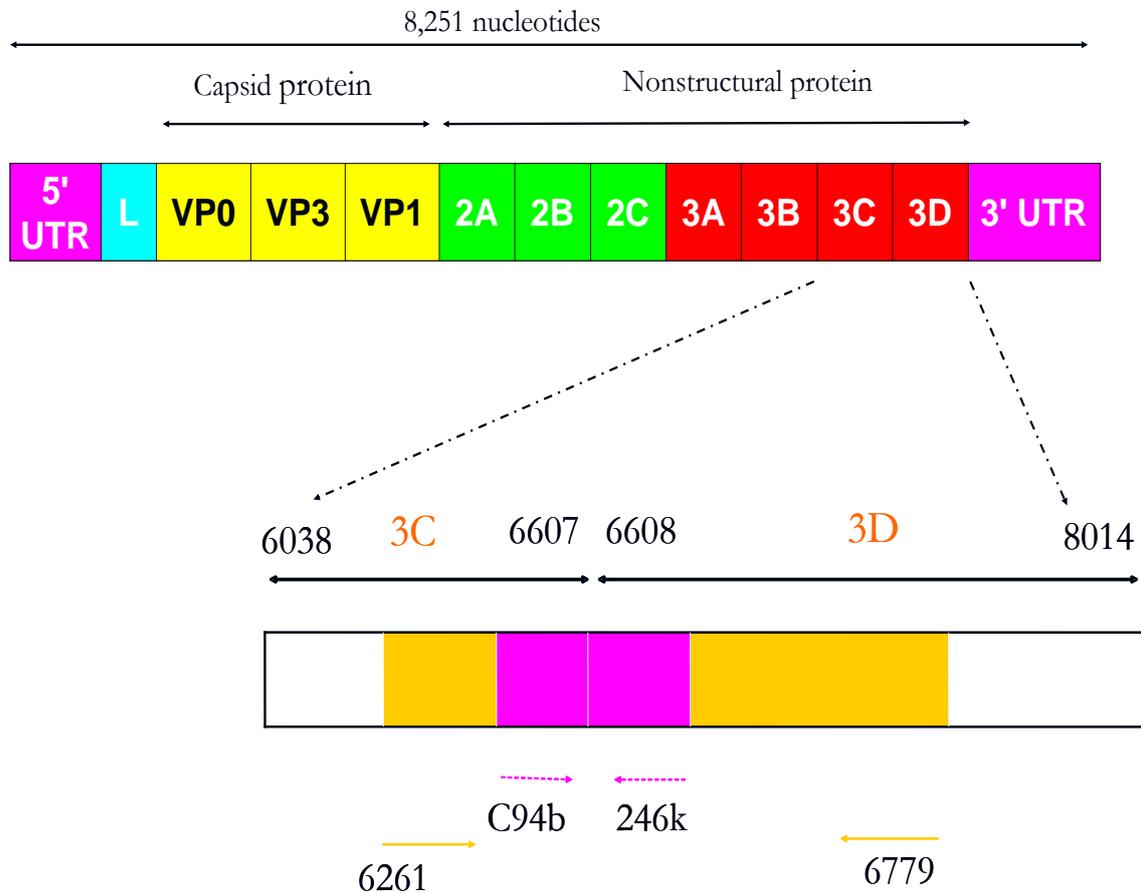
Figure 3. Genomic structure of Aichi virus and illustration on the binding sites of the two primer pairs used for detection of Aichi virus based on the 3CD junction region.

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3. Results

A total of 28 of the 912 samples tested (3.07%) were positive for Aichi virus. Of the 28, only 20 samples were positive by the first PCR, whereas 8 other samples showed the positive result by nested PCR. Of the 28 positive samples, 14 samples were collected from Japan, 10 were from Bangladesh, 3 were from Vietnam, and 1 was from Thailand. The percentages of Aichi virus positive samples among the specimens tested in those countries were 6.5%, 2.5%, 1.6%, and 0.9%, respectively. Twenty of the 28 Aichi strains were randomly selected for sequencing, of these, 11 strains were from Japan, 5 strains were from Bangladesh, 3 strains were from Vietnam, and 1 strain was from Thailand. The majority of their nucleotide sequences were 519bp in length.

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 the Japanese reference strains A844/88, A848/88, 684/98, together with strain N128/91 isolated from a traveler returning from Indonesia, and belonged to genotype A. The four other Bangladeshi strains were clustered into genotype B, together with the Pakistani strains P832/90 and P840/91 (Figure 4).

The similarities of more than 95.5% at nucleotide level and 97% at amino acid level were noted among the A genotype strains. The Japanese strains were quite identical to one another with the nucleotide and aa similarities greater than 98.6%. However, the Aichi virus strains were diverse in genotype B. The similarities among them were lower than 95%, even as low as 92.1% and 95.3% at nucleotide and aa levels, respectively. Among the Bangladeshi strains, the nucleotide similarities more than 96.9% and the aa similarities greater than 98.8 % were noted. The nucleotide similarities between genotypes A and B ranged from 90.5% to 93.8% (95.3% to 98.8% at aa level) (data not shown).

The alignment of the deduced amino acid sequences between the studied strains and the reference strains showed the followings: Although there were some positions possessing

different amino acids between genotypes A and B, they were inconsistent changes. Only one position with difference of amino acids was noted: The genotype A strains possessing Pro at positions 79, whereas the genotype B strains containing Leu. There was another position with a variable amino acid which was conserved within genotype A strains (Asp at position 59) but not in genotype B strains (either Ala or Val).
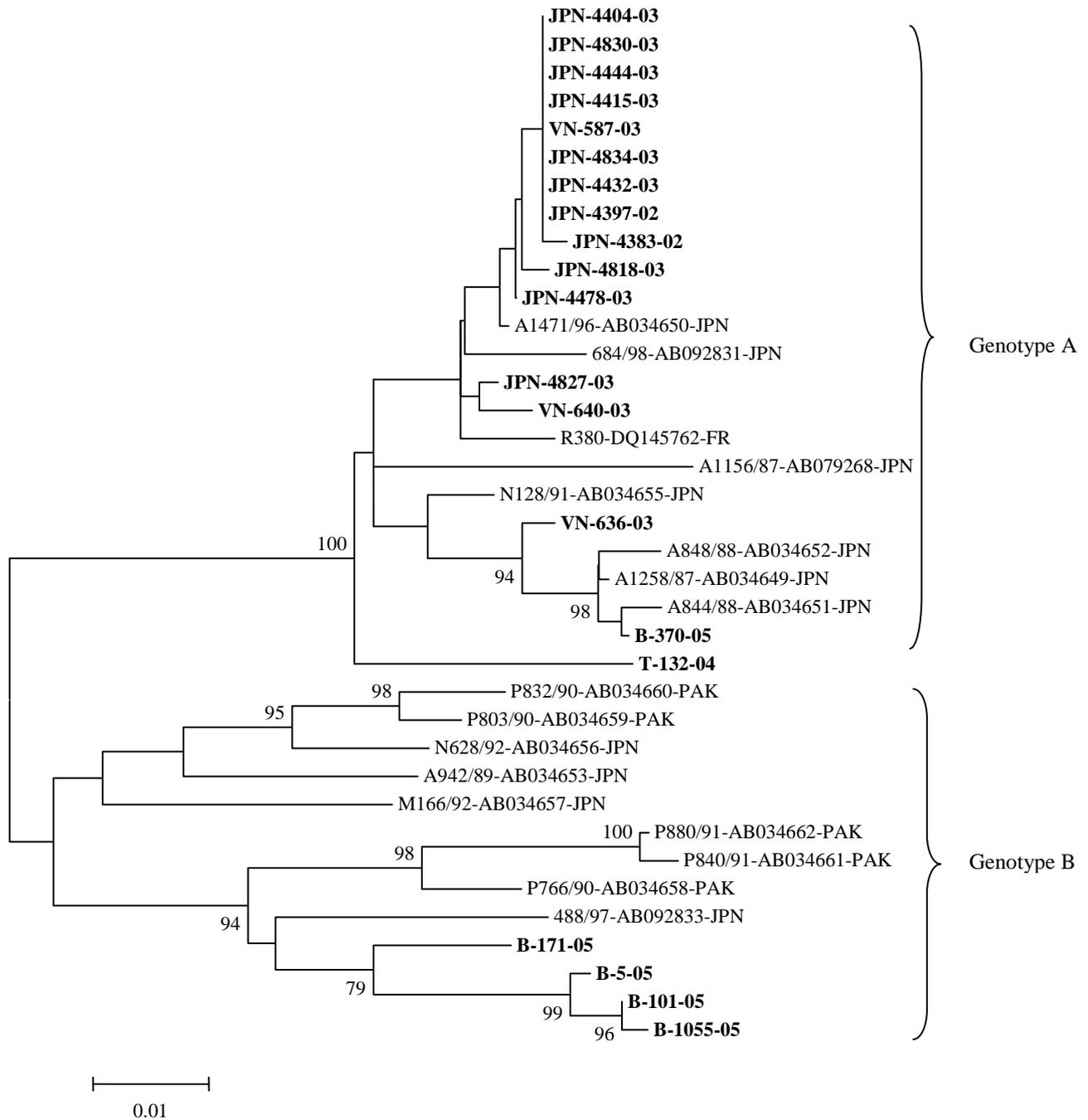
Figure 4. Phylogenetic tree constructed from the 519 nucleotide sequences between the C terminus of 3C and the N terminus of 3D with 500 bootstrap repetitions. Percentage bootstrap values above 70% are shown at the branch nodes. The Aichi virus strains in this study are in boldface type. Abbreviations for 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).

4. Discussion

From previous studies, Aichi virus has been known as one of the agents that causes acute gastroenteritis in adults aged from 15 to 34, especially associated with oyster [Yamashita et al., 1991; Yamashita et al., 1993; Yamashita et al., 1995]. In this study, Aichi virus was detected from diarrheal infants and children, whose fecal specimens had been known as negative for other common causative agents such as rotavirus, norovirus, adenovirus, astrovirus, and sapovirus by RT-PCR. Although Aichi virus related diarrhea was not common, the results from previous studies together with the present study clearly demonstrated that Aichi virus should be checked in fecal specimens collected from diarrheal patients. Once again, with the increasingly positive samples by nested PCR, the PCR method developed by Yamashita et al. clearly showed its usefulness in detection of Aichi virus from clinical specimens [Yamashita et al., 2003; Yamashita et al., 2000].

Up to date, Aichi virus strains isolated previously in Japan, Pakistan, German, and Brazil, have been divided into two genotypes (or groups), A and B [Oh et al., 2006; Yamashita et al., 2000]. In the study, the geographical characteristic of distribution of Aichi viruses seems to be recognized. The Japanese strains belonged to genotype A, as did most of the Japanese strains isolated previously. The majority of Bangladeshi strains clustered into genotype B together with Pakistani strains.

As results from this study have shown, Aichi virus could be detected in Asian countries 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 to the understanding about epidemiology of Aichi virus in Japan.

B. Sequence analysis of the capsid gene of Aichi viruses isolated from Japan, Thailand, Bangladesh, and Vietnam

1. Introduction

Aichi virus virions contain three (VP0, VP3, and VP1) capsid proteins of 42, 30, and 22 kDa [Yamashita et al., 1998]. In one previous study, the comparison of nucleotide sequences between the German and Brazilian Aichi strains with the Japanese strains revealed the VP1 coding region to be the most variable of the structural protein domains [Oh et al., 2006]. Nucleotide identities in this region were lower (86–87%) than in the 3CD section (minimum 88%). That finding is in agreement with the presumption of Yamashita et al., who suggested that sequence diversity in the structural protein coding regions may be higher than in the 3CD region [Yamashita et al., 1998]. In this region, as well as over the whole length of the ORF, the genotype A sequences were more closely related to each other than to that of the Brazilian type B isolate [Oh et al., 2006]. The findings indicate that genotype classification over the 3CD junction region is representative; however, the VP1 domain may offer a more distinct differentiation and therefore be even more appropriate. Molecular characterization of new Aichi strains should include this genomic region.

In previous studies, however, the detection and classification of Aichi virus were based on the 3CD region coding for the non-structural protein [Oh et al., 2006; Pham et al., 2007; Yamashita et al., 2000]. Therefore, in order to further research into Aichi virus genome, sequence analysis of the capsid gene of the detected Aichi virus in this study was performed as well.

2. Materials and methods

Clinical Specimens

Twelve fecal samples already known to be positive for Aichi virus from the previous step (Chapter IA) were used for doing PCR and sequence analysis of the capsid gene of Aichi virus. Of these 12 samples, 6 were from Japan, 3 from Bangladesh, 1 from Thailand, and 2 from Vietnam

RNA extraction, RT-PCR, and Primer Designation

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. To obtain the full length of 2,616 bp nucleotide sequence of the capsid gene, 5 segments were constituted and 10 primers were newly developed based on the 5 complete genomes of Aichi viruses available from GenBank and the sequence data obtained during the capsid gene characterization of Aichi virus strains isolated from the four countries (Figure 5). Five forward primers Cap F, Cap2, Cap3, Cap4, and CapE, used in combination with reverse primers Cap1R, Cap2R1, Cap3R, Cap4R and CapER, respectively (Table 2).

Sequence analysis

The primers designed for PCR were used as sequencing primers. The comparison analysis was conducted between the obtained Aichi virus strains and other Aichi virus reference strains from Japan, Germany, and Brazil available from GenBank.

The nucleotide sequences of the Aichi virus strains described in this study have been deposited in GenBank under accession numbers: from EU143271 to EU143279, EU143282, EU143286, and EU143287. Reference Aichi virus strains and their accession numbers are the followings: AB010145, AB040749, AY747174, NC001918, and DQ028632. The phylogenetic analyses were performed using the neighbor-joining method within the MEGA 3.1 analytical package with 500 bootstrap repetitions [Kumar et al., 2004].

Table 2. Oligonucleotide sequences of the 5 primer pairs used to amplify the whole segment of the capsid gene of Aichi virus

> R: A + G
>
> Y: C + T
>
> K: T + G

* Based on the Japanese reference strain with the accession number of AB010145

Primer	Polarity	Sequence 5'to 3'	Gene	Position	Reference
Cap F	+	CAGGTGCCTACCAAGCAAAGAC	L	1104-1125	This study
Cap1R	−	GGTGAACTCCTGGGACCAG	VP0	1786-1768	This study
Cap2	+	CCTCGCCTACCCCACCGCC	VP0	1666-1684	This study
Cap2R1	−	GAGACCGTGGAARGAGGAGTC	VP3	2317-2297	This study
Cap3	+	CATAGAGGTCCCYTAYATCTC	VP3	2149-2169	This study
Cap3R	−	CATACKGTGTATGTTCCGCGC	VP3	2760-2740	This study
Cap4	+	CAGTGGCGYGGTGRACTCG	VP1	2618-2636	This study
Cap4R	−	GCGATGTAYGTGAAGCACG	VP1	3303 3285	This study
Cap E	+	CTAGTCGGACCCCACACCGC	VP1	2897-2915	This study
CapER	−	GGATGGCCCAGTGGACGTAG	VP1	3854-3835	This study

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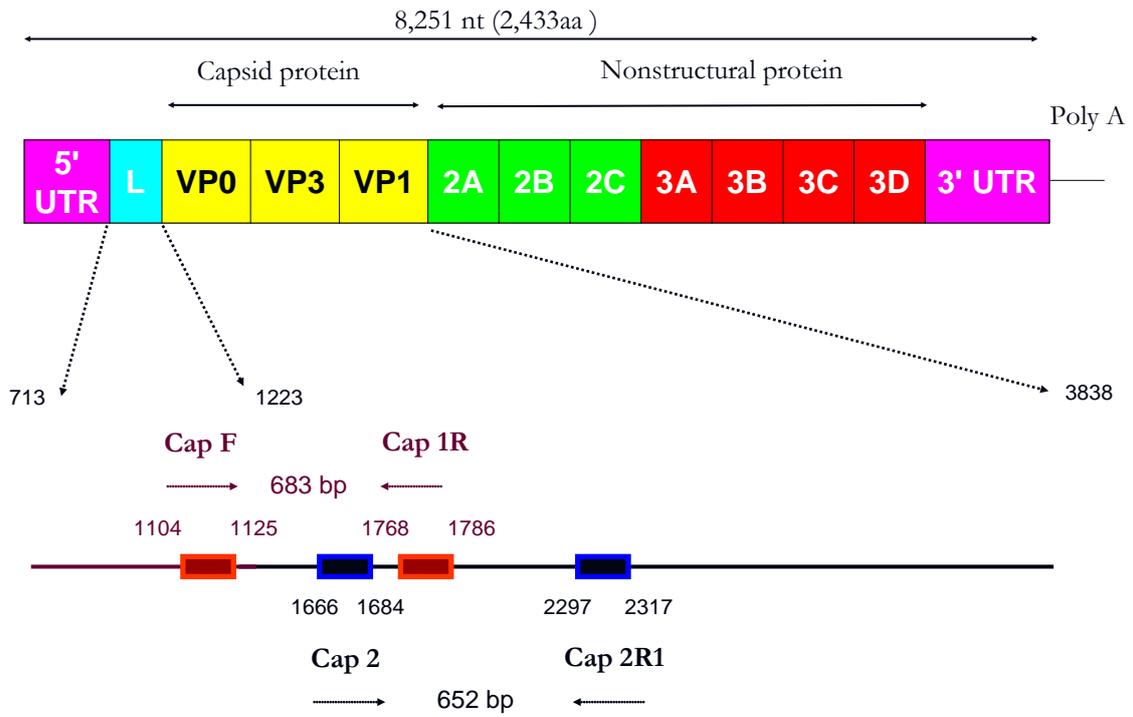
Figure 5. Illustration on the binding sites of the first two primer pairs used for amplifying the first two segments of the capsid gene.

3. Results

Twelve nucleotide sequences of the capsid gene of the Aichi virus strains from the four countries were obtained for comparison with global Aichi virus strains determined previously. The full length of the capsid gene of 9 strains from Japan, Thailand, and Vietnam was 2,616 bp, while three Bangladeshi sequences contained 2,619 bp.

The phylogenetic tree in Figure 6 clearly showed that all of Aichi virus strains were clustered into two major branches. One branch consisted of four sub-branches containing the strains from Japan, Thailand, Vietnam and reference strains from Japan, and Germany. The other branch consisted of the Bangladeshi strains and one reference strain from Brazil.

Distance matrix analysis of nucleotide sequences the capsid gene incorporated with their phylogenetic tree demonstrated two major lineages which could be designated as I and II (Figure 6 and Table 3). The relationship among lineages is clearly shown in Table 3. The same lineages showed 3.1% and 3.3% nucleotide distances, while distances between lineages were higher than 12.6%.

A comparison of the deduced amino acid sequences of the whole capsid gene of all Aichi virus strains showed that there were at least 14 amino acid substitutions which were lineage-specific. Especially, there were a different triple of amino acids at positions 220 - 222 (TSS of lineage I and STN in case of lineage II), and an insertion of P or S at position 224 of lineage II sequences which could not be found within lineage I sequences (Figure 7).

Table 3. Percentages of nucleotide distances among Aichi lineages and sub-lineages

_____

<div align="center">Mean % nucleotide distance</div>

_____

Aichi virus Lineage	Lineage I				Lineage II
	Ia	Ib	Ic	Id	
Lineage I			(3.3)		
Ia	0.6				
Ib	4.5	0.2			
Ic	4.3	5.8	3.4		
Id	4.8	6.6	5.1	NA	
Lineage II	**13.4**	**13.5**	**13.4**	**12.7**	(3.1)

_____

Nucleotide distances within lineage I and lineage II are presented in bracket, while distances between lineage II and every sub-lineage of lineage I are in boldface type.

NA, not applicable
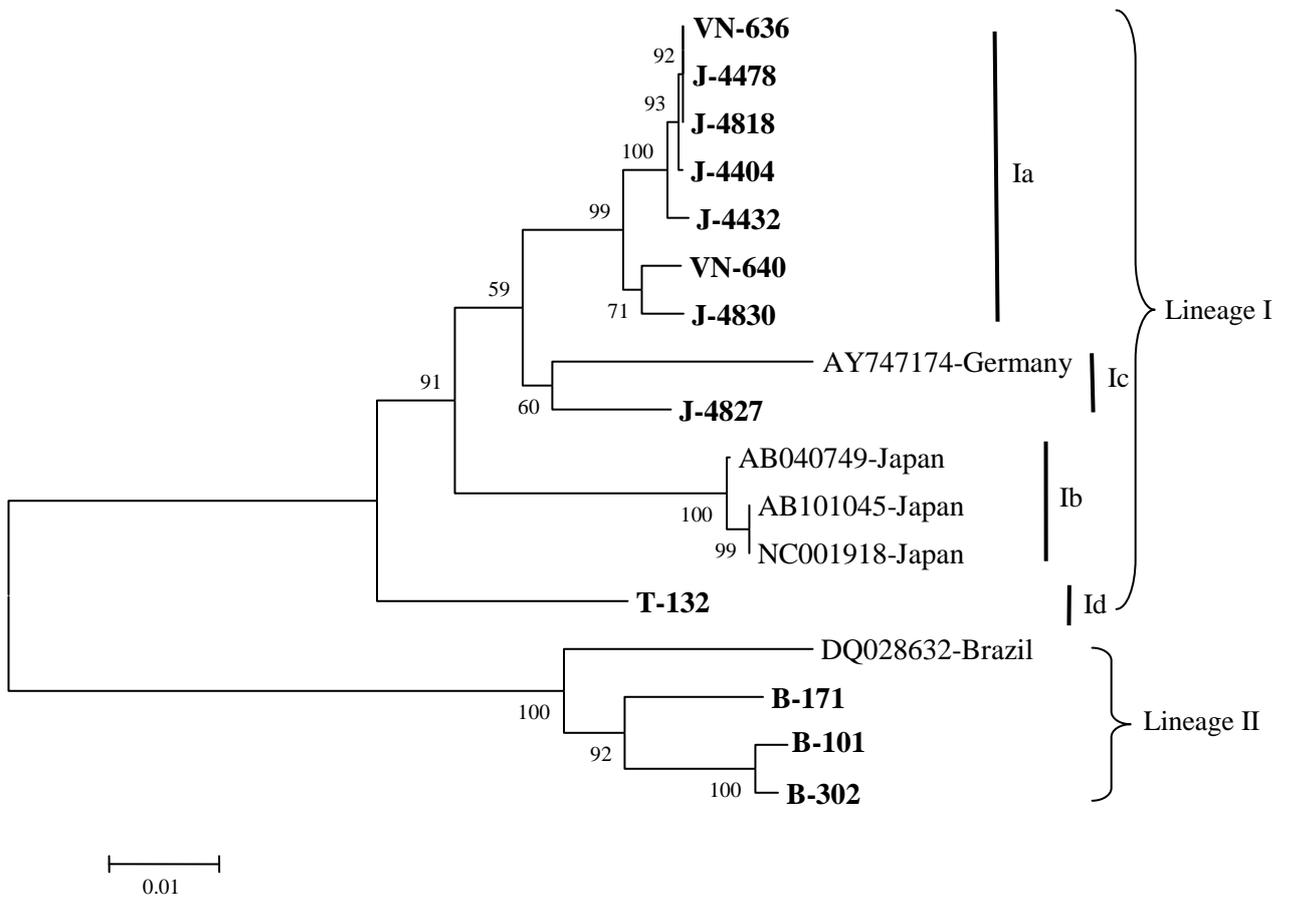
Figure 6. Phylogenetic tree constructed from the full length sequences of the capsid gene with 1000 bootstrap repetitions. The strains found in this study are in boldface type. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.

Figure 7.

```
                          10         20         30         40         50         60         70         80         90        100        110
AB010145-Japan   GNSVTNIYGN GNNVNTDVGA NGWAPTVSTG LGDGPVSCTP PTPSPGDTEV PPPRKPTTFP APPTKSGSRF SKWWEPAAAR ASESATDSAI EGIDAAGKAA SKAITRKLDR
AY747174-Germany ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
NC-001918-Japan
AB040749-Japan   ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
J-4432           ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
J-4478           ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
J-4818           ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
VN-636           ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
VN-640           ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
T-132            ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... ...S.....
DQ028632-Brazil  ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  G......... .........
B-101            ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D.,Y...... .........
B-171            ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
B-302            ..........  ...T..... ..........  ......ASA DS-L,.RSGG ASSE.TH.VS GSSN.V...  .......... .L.R.....  D......... .........
```


Figure 7. Amino acid sequence alignment of hepatitis E virus isolates (AB010145-Japan, AY747174-Germany, NC-001918-Japan, AB040749-Japan, J-4432, J-4478, J-4818, VN-636, VN-640, T-132, DQ028632-Brazil, B-101, B-171, B-302), residues 1–884.

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Figure 7. Alignment of the deduced amino acid sequences of the capsid gene between the studied strains and the reference strains. Different amino acids between the two major lineages are in boxes. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.

4. Discussion

The phylogenetic tree constructed based on the global Aichi strains including the strains studied and the reference strains showed that Aichi viruses could be divided into two major lineages: lineage I including the Aichi virus strains isolated in Japan, Germany, Thailand, and Vietnam, lineage II comprising strains from Bangladesh and Brazil. This proposal was also supported by the nucleotide distance variations up to more than 12.6% among Aichi strains between the two lineages (Table 3). Lineage I could be further divided into 4 sub-lineages from a to d. Sub-lineage Ia consists of Japanese and Vietnamese strains in this study, sub-lineage Ib contains Japanese reference strains, sub-lineages Ic comprises of the German reference strain and 1 studied Japanese strain, and sub-lineage Id includes the studied Thai strain (Figure 6 and Table 3).

The different amino acids between the two lineages could be found throughout the whole amino acid sequences. It could be presumed that the different antigenicity of the two lineages is attributed to the differences of the whole capsid protein. Furthermore, this classification of Aichi viruses seems to be similar to the classification based on the 3CD junction region, in which, the Japanese, Thai, and Vietnamese strains in this study belonged to genotype A and the three Bangladeshi strains to genotype B [Oh et al., 2006; Pham et al., 2007; Yamashita et al., 2000]. Therefore, the genetic differences between the two Aichi virus lineages seem to be not only due to the variation of the capsid gene, but also of the other regions of Aichi virus genome. Those hypotheses should be elucidated in further studies on Aichi viruses.

C. Development of genotype-specific primers for differentiation of genotypes A and B of Aichi viruses

1. Reason for the study:

Aichi virus virions contain three capsid proteins, VP0, VP3, and VP1. In comparison to genotype A strains, the capsid gene sequences of genotype B strains (including a sole strain from Brazil and three others from Bangladesh) have a triple nucleotide insertion within the VP0 region [Oh et al., 2006; Pham et al., 2008; Yamashita et al., 1998]. As described in Chapter IB, phylogenetic and sequence analyses of the capsid gene were undertaken and a high correlation of Aichi virus genotype to the 3CD junction region was demonstrated [Pham et al., 2008], and the genetic diversity of the capsid sequences of Aichi virus strains within each genotype was determined. The results showed that nucleotide sequence diversity within genotype A was higher than that of genotype B. In addition, the nucleotide distances between the two genotypes were equal to or greater than 12.7%, and the genomic differences between them could be found throughout the capsid sequences. At least 14 different deduced amino acids which were considered to be genotype-specific, especially the different triple of amino acids located within the VP0 region, were found and can be considered as the reference region for developing primers for genotyping (Pham et al., 2008).

For identification, Aichi virus genotype has been determined based only on sequence analysis of the 3CD junction region or the capsid gene, a critical method for genotyping of this virus. However, these procedures are complex and time consuming, requiring expensive reagents, equipment, and well-trained personnel. Therefore, the aim of this study was to develop a nested PCR method using genotype-specific primers based on the capsid gene for genotyping of Aichi virus.

2. Materials and methods

Primer designation

Reference nucleotide sequences of the capsid genes of 15 Aichi virus strains were obtained. Three of the 15 capsid sequences were derived from Aichi virus reference sequences available in the GenBank database under the accession numbers AB040749, AY747174, and DQ028632. The 12 remaining nucleotide sequences of the capsid gene were from the earlier study (Chapter IB) and their accession numbers are the following: EU143271 to EU143279, EU143282, EU143286, and EU143287 [Pham et al., 2008]. Alignment of the 15 capsid gene sequences using Clustal X software was performed to find the divergent and conserved regions and to create genotype-specific primers. Genotype-specific primers for A and B were designed based on the different sequences between A and B genotypes.

Specimens tested

Seventeen stored cDNAs known to be positive for Aichi virus were used in this study. The samples were collected from Japan (10 samples), Bangladesh (3 samples), Thailand (1 samples), and Vietnam (3 samples)[Dey et al., 2007; Khamrin et al., 2006; Nguyen et al., 2007; Phan et al., 2005a]. The genotypes of all 17 Aichi strains isolated from the above samples were known as genotype A or B from the earlier studies (described in Chapter IA and IB) by sequence analysis [Pham et al., 2007; Pham et al., 2008].

Differentiation of genotype A and B by nested PCR with genotype-specific primers

After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 5x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (10 mM), primers (20 μM), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), and distilled water, the first PCR was conducted using primers AiV-Cap-F and AiV-Cap-R (Table 4). The PCR protocol was 95°C for 1 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. Next, the nested PCR was carried

out using three primers: AiV-Cap-A, AiV-Cap-B, and AiV-Cap-ABR (Table 4) with the same thermal cycler program to generate a 336 bp product, which is a partial segment of VP3, specific for Aichi virus genotype A or an 879 bp product, which is a partial segment of VP0 and VP3, specific for Aichi virus genotype B (Figure 8). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and the bands were visualized by SYBR Safe (Invitrogen, Tokyo, Japan) staining under ultraviolet light. The results of genotyping by PCR were compared to the results determined by sequence analysis of the capsid gene.

Table 4. Oligonucleotide sequences of the newly developed primers for differentiation of genotype A and B of Aichi virus

Primer	Polarity	Sequence 5'to 3'	Gene	Position	Reference
AiV-Cap-F	+	GGCGAYCGCACCTGGTTG	VP0	1765-1782	This study
AiV-Cap-R	−	GAGATGAAGGGGATGGT	VP3	2837-2821	This study
AiV-Cap-A	+	TGCCTACTACCCCCCMAATG	VP0	2448-2467	This study
AiV-Cap-B	+	TCYACAAACTCTCCCACCGC	VP3	1905-1924	This study
AiV-Cap-ABR	−	GTGTATGTTCCGCGCAT	VP3	2783-2767	This study

Y means C or T, and M means C or A. Sequence position (*) is based on the full genome sequence of the Japanese reference strain with the accession number of AB040749.

3. Results

The oligonucleotide sequences of the new primers are shown in Table 4. The genotype A-specific primer (AiV-Cap-A) was designed based on six significant nucleotide mismatches between the capsid genes of the two genotypes at positions 2448-2467. The genotype B-specific primer (AiV-Cap-B) was developed at the positions 1905-1924 containing 8-10 nucleotide mismatches, especially including an insertion of CCC or TCC triple at positions 1917-1919 (located within the VP0 region) of the capsid sequences of genotype B strains when compared to those of genotype A strains. The primer binding sites of the two genotype-specific primers and the expected PCR products are shown in Figure 8.

Using 17 genotype-known reference strains, PCR genotyping was done using the newly developed primers. PCR products were successfully generated from all 17 samples; 14 were 336 bp segments identified as genotype A, and the 3 remaining were 879 bp segments identified as genotype B. In other words, 14 genotype A and 3 genotype B Aichi virus strains were recognized by using this genotyping PCR. The electrophoresis result is shown in Figure 9. These results were in agreement with those obtained by analyses of the capsid gene sequenced previously.

To test the specificity of the new genotype-specific primers, an additional PCR was carried out using genotype-determined samples with different genotype-specific primer for the second round of the genotyping PCR. For example, using genotype A-specific primer (AiV-Cap-A) for genotype B sample, or conversely, genotype B-specific primer (AiV-Cap-B) for genotype A sample. By such a PCR, no cross-reaction was observed. All samples were negative, except for two positive control reactions by using the right matches of the genotype-specific primers and genotype-known samples: sample A with primer AiV-Cap-A, and sample B with primer AiV-Cap-B (data not shown).
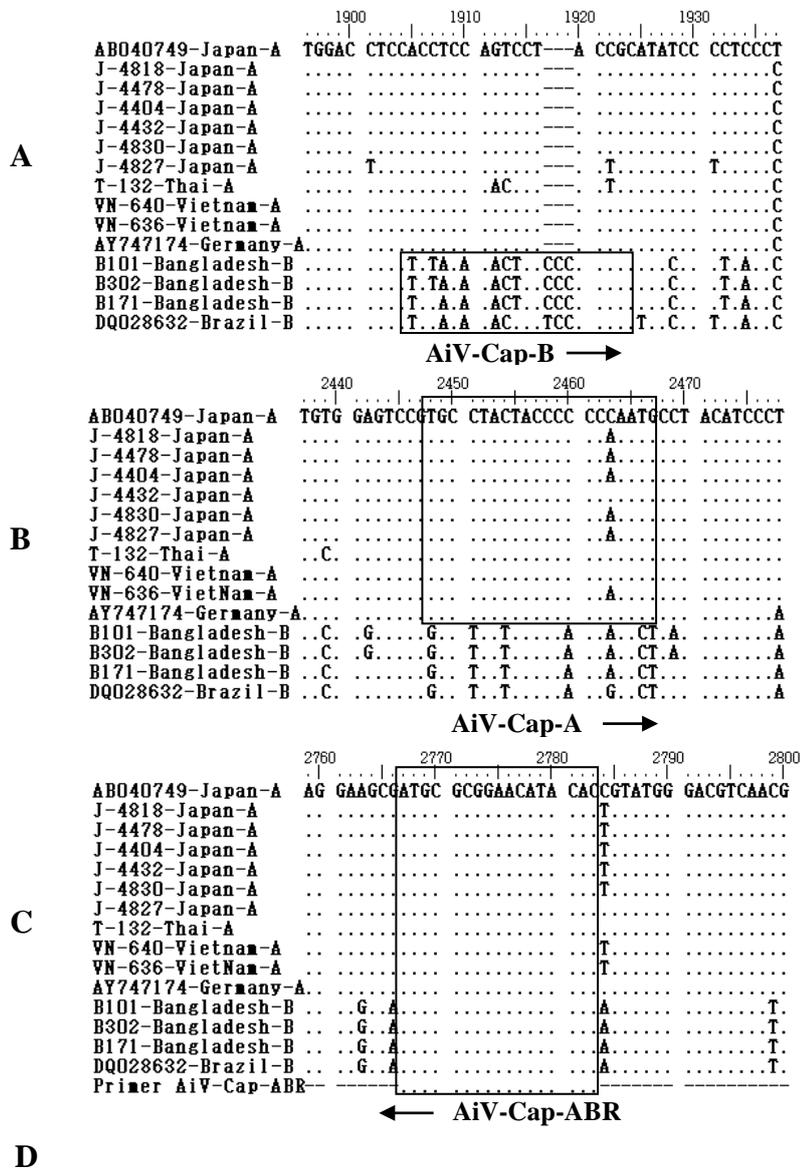
Figure 8.

A, B, and C. Extracts of alignment results of the 15 reference capsid gene sequences. The identical nucleotides in each Aichi reference strain with the Japanese reference strain under the accession number AB040749 are identified by dots. The primer binding areas are in boxes.

D. Schematic representation of the capsid gene of Aichi virus, and the expected PCR products.
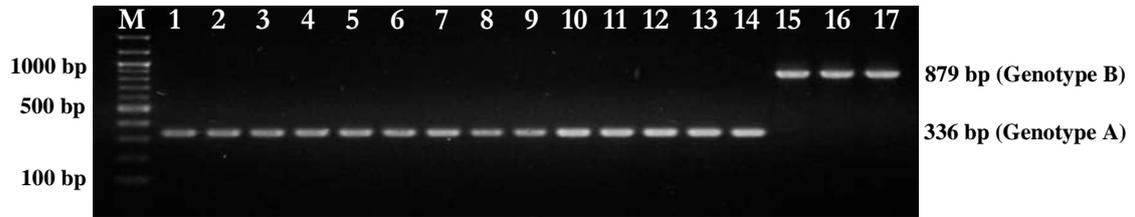
Figure 9. Results of genotyping of Aichi virus. The PCR fragments possess the expected molecular weights when compared to the molecular weight marker: (lanes 1-14) genotype A, (lanes 15-17) genotype B, (lane M) 100 bp ladder marker.

4. Discussion

These results demonstrate that nested PCR using the new primers generated appropriate PCR products from all 17 samples tested, and the new primers can separate genotype A from genotype B. As demonstrated in this study, the new primers are sensitive and specific enough for differentiation between genotypes A and B of Aichi viruses. The new primers functioned accurately and conclusively. Obviously, without the need for sequencing, the genotyping method by PCR is easier, faster, and more cost effective when compared to the critical method of sequence analysis of the 3CD junction region or the capsid region.

Briefly, nested PCR using genotype-specific primers is useful and can be used for genotyping of Aichi viruses isolated during epidemiological studies. However, although the new primers were developed based on all available full lengths of the capsid gene of Aichi viruses in GenBank up to the time when the study was conducted, it is noted that the number of reference capsid genes was limited to 15 sequences. Globally, there have been only three full lengths of the capsid gene which were derived from three full genomic sequences of reference strains (each of these strains isolated from Japan, German, and Brazil) and 12 others which were obtained in a previous study [Pham et al., 2008]. Therefore, the limitation of primer designation regarding reference strains of this study is also the limitation of global studies on the capsid gene of Aichi viruses. In addition, because of the low detection rate of Aichi virus from specimens, the number of samples positive for Aichi virus available for primer testing was small. Consequently, despite this study showing a strong capacity for differentiating between genotypes A and B of Aichi viruses, these new primers should be evaluated further with a larger number of Aichi virus strains.

Chapter II: STUDY ON PARECHOVIRUS

1. Introduction

Parechoviruses are small, non-enveloped, spherical, positive sense single-stranded RNA (ssRNA) viruses, about 30 nm in diameter (Figure 10), and belong to the large family of *Picornaviridae*, which is a highly diverse family of important pathogens of humans and animals [Stanway et al., 1994]. The Parechovirus genus has been defined in the early 1990s [Hyypia et al., 1992; Joki-Korpela and Hyypia, 2001]. The genus is composed of two species: Ljungan virus, isolated from bank voles [Niklasson et al., 1999] and human parechovirus (HPeV), a frequent human pathogen. The HPeV genome is approximately 7.3 kb nucleotides in length and contains a large open reading frame (ORF) coding for single polyprotein. The polyprotein is cleaved post-translationally into three structural proteins (VP0, VP3 and VP1) and seven non-structural proteins (2A-2C and 3A-3D) (Figure 11) [Hyypia et al., 1992; Stanway et al., 1994].

On the basis of studies using serological and genetic approach, HPeV has been found to have at least 14 genotypes (HPeV1-14) [Harvala and Simmonds, 2009]. HPeV1 and -2, previously known as echovirus 22 and 23 respectively, were first isolated in 1956 from stool samples from children with diarrhea in USA [Wigand and Sabin, 1961]. HPeV3 was identified in Japan from a one-year-old child with transient paralysis, fever, and diarrhea in 1999 [Ito et al., 2004], and almost simultaneously was also recovered from nasopharyngeal aspirates of neonates with suspected sepsis in Canada [Boivin et al., 2005]. HPeV4 was first detected from stool samples of a neonate with febrile illness in the Netherlands in 2002 [Benschop et al., 2006a], but has been shown to have circulated at least from 1970s [Al-Sunaidi et al., 2007; Schnurr et al., 1996]. HPeV5 was first identified in USA in 1986 from a 2-year-old child with high fever [Oberste et al., 1998], but this virus was originally assigned as HPeV2 based on neutralization data and has only recently been

reclassified as a fifth type based on genetic analysis [Al-Sunaidi et al., 2007]. HPeV6 was isolated from a cerebrospinal fluid specimen of a 1-year-old girl who died with Rey syndrome in Japan [Watanabe et al., 2007], and recently it was also identified in Europe [Baumgarte et al., 2008; de Vries et al., 2008]. Very recently, HPeV7 was identified from the stool samples of the healthy Pakistani child who had been in contact with a case of acute flaccid paralysis, and HPeV8 was detected from stool samples of Brazilian children with acute diarrhea. For the remaining HPeV types, HPeV14 infects human, and has been identified only very recently from Netherland [Benschop et al., 2008]. HPeV9-13 listed on http://www.picornaviridae.com/parechovirus/hpev/hpev.htm is uncertain.
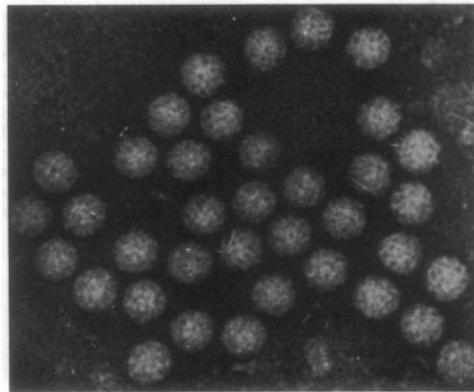
Figure 10. Electron micrograph of purified HPeV1 [Hyypia et al., 1992].
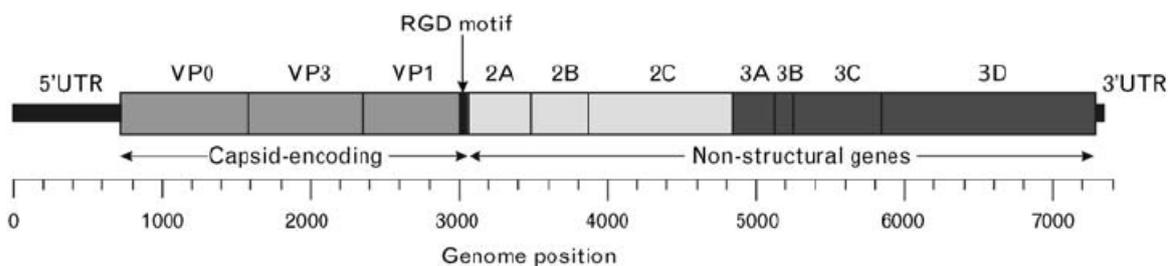
Figure 11. Genome organization of HPeV [Hyypia et al., 1992; Stanway G.,1994; King A.M.Q et al., 1999; Harvala et al., 2009].

Through detection of antibody to HPeV, it has been shown that 90% of children have been infected with at least one HPeV type by the age of 2 years [Joki-Korpela and Hyypia, 1998; Tauriainen et al., 2007].

Until the advent of molecular detection methods, HPeV was identified by neutralization assay following virus isolation in cell culture, using a panel of antisera. However, HPeV often grow poorly in culture, typing reagents are not widely available for the new types (HPeV3-14), and the technique is laborious and time consuming.

To date, a variable spectrum of symptoms caused by HPeVs has been described. The common symptoms are similar to those caused by some enteroviruses, including mostly enteritis with diarrhea, and respiratory disease [Baumgarte et al., 2008; Benschop et al., 2006b; Joki-Korpela and Hyypia, 1998; Stanway and Hyypia, 1999; Stanway et al., 2000]. Other symptoms and syndromes, such as meningoencephalitis, encephalomyelitis, flaccid transient paralysis, nosocomial infection, neonatal sepsis, myocarditis, myositis, lymphadenopathy, hand-foot-mouth disease, rash, fever of unknown origin, influenza-like illness, and Reye's syndrome, haemolytic uremic syndrome have also been reported [Boivin et al., 2005; Ehrnst and Eriksson, 1993; Ehrnst and Eriksson, 1996; Figueroa et al., 1989; Grist et al., 1978; Ito et al., 2004; Koskiniemi et al., 1989; Legay et al., 2002; Maller et al., 1967; O'Regan et al., 1980; Russell and Bell, 1970; Watanabe et al., 2007]. In most of these, diarrhea has been the predominant symptom. There has been no report on HPeV infection in Asian countries other than Japan such as Thailand and Sri Lanka up to date. In Japan, HPeV infection was noted; however, there has been no report of the circulation of HPeV among children with acute gastroenteritis. The present study aimed to screen stool samples collected from Japanese, Thai and Sri Lankan children with acute gastroenteritis during 2005-2008 for HPeV infection, one of less explored viral pathogens which has been reported to be associated with diarrhea recently, and to characterize the molecular properties of the detected HPeV strains.

2. Materials and methods

Clinical Specimens

A total of 691 fecal specimens were subjected to screening for HPeV. Of these, 362 were collected from infants and children hospitalized with acute gastroenteritis in Kandy, Sri Lanka from September 2005 to August 2006. The remaining 329 samples which had been known to be negative for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus, by RT-PCR, were collected from infants and children with acute gastroenteritis in Chiang Mai, Thailand from January to December 2005 (82 samples), and were from pediatric clinics encompassing five localities in Japan (Sapporo, Tokyo, Maizuru, Osaka, and Saga) from July 2007 to June 2008 (247 samples). All original specimens were collected after the parents of the enrolled children had given the informed consents. The fecal specimens from the outpatients were collected at the out-clinic wards or from the inpatients within 24h after admission and stored at -20°C until use. The ethical approvals were obtained from the Scientific Committees of the involved hospitals or clinics in Japan, Thailand, and Sri Lanka, as well as from the Ethical Committee of the University of Tokyo, Japan.

RNA extraction and Reverse Transcription (RT)

The RNA genome of HPeV was first extracted from 140 µl of 10% fecal suspension using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany) according to manufacture's instructions. Then, for reverse transcription, 5 µl of the stored, extracted RNA was added to a reagent mixture consisting of 3 µl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 0.8 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.8 µl of 0.1 M DTT (Invitrogen), 0.8 µl of SuperScript III Reverse Transcriptase (200 U/µl) (Invitrogen, Carlsbad, CA, USA), 0.8 µl of random primer (1 µg/µl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl of RNase Inhibitor (33 U/µl) (Toyobo, Osaka, Japan), and 3.3 µl of distilled water. The total volume

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of reaction mixture was 15 μl. RT reaction was carried out at 50°C for 1 hr, followed by 95°C for 5 min and then held at 4°C. The cDNA was stored at -30°C until using for PCR reactions [Phan et al., 2005c; Yan et al., 2004].

Polymerase Chain Reaction for detection of HPeV

After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 5 μl of 5x Taq DNA polymerase buffer (Promega, Madison, WI, USA), 2 μl of dNTPs (2.5 mM), 0.5 μl of each specific primer (20 μM), 0.1 μl of Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), and 14.9 μl of distilled water, screening PCR was conducted using primers ev22(+) and ev22(-) to amplify a 270-bp PCR product of 5'UTR region [Joki-Korpela and Hyypia, 1998] (Table 5). The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min.

For Sri Lankan samples, the samples positive for HPeV were tested further for common viral pathogens that cause diarrhea including rotavirus, adenovirus, norovirus, sapovirus, and astrovirus by RT-PCR as reported by Yan and colleagues [Yan et al., 2004; Yan et al., 2003], to see if there were co-infections between HPeV and these viruses.

Genotyping by VP1 sequencing and primer designation

At first, to amplify the VP1 segment, the previously described primers developed by Benschop and colleagues [Benschop et al., 2006b] were used. However, because of failure in obtaining PCR products of most HPeV-positive samples except for four, two new primers were designed for the first PCR. Then, for the nested PCR, it was performed using the inner primer pair described by Benschop and colleagues [Benschop et al., 2006b].

For primer designation, to obtain the full length of 702 bases of the VP1 capsid gene, alignment of full genome sequences of reference strains of 8 known HPeV genotypes available in GenBank databases was performed using Clustal X software to find the conserved regions, and the two new primers were designed outside the VP1 region.

Oligonucleotide sequences of the newly developed primers and their positions were described in Table 5.

The first PCR was done using the new designed primers and the thermal cycle program was as follows: 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The nested PCR was conducted using the known primer pair: VP1-parEchoF1 and VP1-parEchoR1 [Benschop et al., 2006b] at annealing temperature of 48°C to generate a 760 bp product (Table 5). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and the bands were visualized by SYBR Safe (Invitrogen, Tokyo, Japan) staining under ultraviolet light. The HPeV-positive samples were retested with another PCR by using a newly designed primer pair, 3DparEcho-F and 3DparEcho-R, derived from the 3D gene of the HPeV genome (Table 5).

Table 5. Oligonucleotide sequences of the primers used for detection and classification of HPeV. Y stands for C or T, R: G or A, W: A or T, H: A, C, or T, and M means C or A. Sequence position (*) is based on the full genome sequence of the prototype HPeV1 strain, Harris strain, with the accession number of L02971.

Primer	Polarity	Sequence 5'to 3'	Gene	Reference
ev22(+)	+	CYCACACAGCCATCCTC	5'UTR	Joki-Korpela et al., 1998
ev22(-)	−	TRCGGGTACCTTCTGGG	5'UTR	Joki-Korpela et al., 1998
VP1-parEchoF1	+	CCAAAATTCRTGGGGTTC	VP1	Benschop et al., 2006
VP1-parEchoR1	−	TCYACAAACTCTCCCACCGC	VP1	Benschop et al., 2006
Cap-parEcho-F	+	TCHACWTGGATGMGRAARAC	VP1	This study
Cap-parEcho-R	−	TCYARYTCACAYTCYTCYTC	VP1	This study
3DparEcho-F	+	GATTGGCACTTYATGATHAATG	3D	This study
3DparEcho-R	−	CAAATGAWTCTGCCATGAYAC	3D	This study

Sequence and Phylogenetic Analysis

The PCR amplicons of the VP1 gene were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers for amplification of VP1 gene were used as sequencing primers. The sequence data were collected by an ABI Prism 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The sequence of the VP1 segment from the HPeV strains obtained in the present study and other reference HPeV strains of nine known genotypes available in GenBank database were compared. The sequence data and the data from the phylogenetic analysis were analyzed using BioEdit (version 7.0.5) software. A parsimony analysis was also conducted by using MEGA (Molecular Evolutionary Genetics Analysis; version 3.1) program to determine the evolutionary relationship among sequences studied [Kumar et al., 2004]. The method was performed using close-neighbor interchange with a random option and with 500 bootstrap repetitions.

The nucleotide sequences of the reference HPeV strains described in this study have been deposited in GenBank under accession numbers: HPeV1: Harris (L02971), 652281 (FJ373120), BNI-R09/03 (EU024632), BNI-R32/03 (EU024636), BNI-R15/03 (EU024633), BNI-788St (EF051629), 677033 (FJ373136), 69960AE (AM933170), A229-05 (AB300968), A234-05 (AB300969), A708-99 (AB300935), BNI-R04/03 (EU024631), A65-05 (AB300963), A222-05 (AB300967), BNI-R21/03 (EU024634), 652780 (FJ373127), 650648 (FJ373108), A191-05 (AB300966), A527-99 (AB300928), BNI-90/03 (EU024630), BNI-R30/03 (EU024635); HPeV2: Williamson (AJ005695); HPeV3: Can82853-01 (AJ889918), 677146 (FJ373162), A415-01 (AB300945), A308/99 (AB084913), 651689 (FJ373153); HPeV4: Fuk2001-282 (AB433630), NII370-93 (AB434673), T75-4077 (AM235750), 653046 (FJ373170), K251176-02 (DQ315670); HPeV5: CT86-6760 (AF055846), T92-15 (AM235749), 676618 (FJ373175); HPeV6: 2005-823 (EU077518), NII561-2000 (AB252582), BNI-67/03 (EU024629), 650045

(FJ373178); HPeV7: PAK5045 (EU556224); HPeV8: BR/217/2006 (EU716175); HPeV14: 451564 (FJ373179).

For the studied trains, the accession numbers are: Japan, FJ648741-FJ648754, GQ149452, GQ203502, and GQ203503; Thailand, FJ648755-FJ648762 and GQ149453; and Sri Lanka, GQ402515, GQ402516, HQ163869-HQ163881 and HQ163883-HQ163894.

3. Results

a. Human parechovirus from Japanese samples

Of the 247 samples tested, 20 were positive for HPeV and the detection rate of HPeV was 8.1%. Of these, 17 strains were amplified and sequenced for the full length VP1 capsid sequences.

The 20 patients whose stool specimens showed positive for HPeV were infants and children aged from 5 to 51 months with the mean and median ages of 14.8 and 14 months, respectively. A half of the patients were less than 18 months of age. Besides diarrhea, fever and vomiting were found in 30% and 15% of the patients, respectively. Cough and coryza were present in 10% and 5% of the patients, respectively. No dehydration and neurological symptoms were noted. For seasonal pattern of HPeV infection, HPeV was detected nearly all the year round, except for April, July, and December, with the peak of incidence in February (data not shown).

Figure 12 shows the phylogenetic tree constructed from 624 bases of partial VP1 segment of reference HPeV strains and 17 Japanese strains found in this study. As shown in Figure 12, the majority of the Japanese HPeV strains (n=15) were identified as HPeV1, and the remaining 2 strains as HPeV3. The figure shows clearly that HPeV1 strains clustered into 2 separate branches. The prototype strains of HPeV1, the Harris strain, belonged to a branch (designed as lineage 1) together with a strain detected recently in the Netherlands, the 652281 strain. Fifteen studied Japanese HPeV1 strains were clustered into the remaining branch (designed as lineage 2) consisting of the majority of contemporary HPeV1 strains.

Identical matrix analysis of VP1 amino acid sequences of the Japanese strains and global reference strains was performed. For HPeV1 strains, amino acid identities among the 15 Japanese strains were greater than or equal to 91.5%. High amino acid similarities were noted between the strains studied and reference strains of the same lineage 2, and ranged from 91.0% to 99.5%, while those between the strains studied and the lineage 1 strains were

from 83.6% to 90.1%. Between the HPeV1 strains studied and those of other genotypes, amino acid similarities were less than 80% and varied from 55.7% (with genotype 3) to 79.6% (with genotype 6) (data not shown).

For the studied HPeV3 strains, they clustered closely to the Japanese HPeV3 prototype strain A308/99 and had 95.8% amino acid similarity to this strain. In comparison to the strain 651689 detected recently in the Netherlands and clustered separately from the HPeV3 prototype strain (Figure 12), the studied Japanese strains showed a mean amino acid similarity of 95.3%.

The alignment of deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeVs genotypes 1-8 and 14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for human parechovirus 1 entry, was present in all the studied HPeV1 strains. This RGD motif was not noted in the 2 HPeV3 strains studied, and among reference strains of HPeV3, HPeV7, HPeV8, and HPeV14 as well (data not shown).
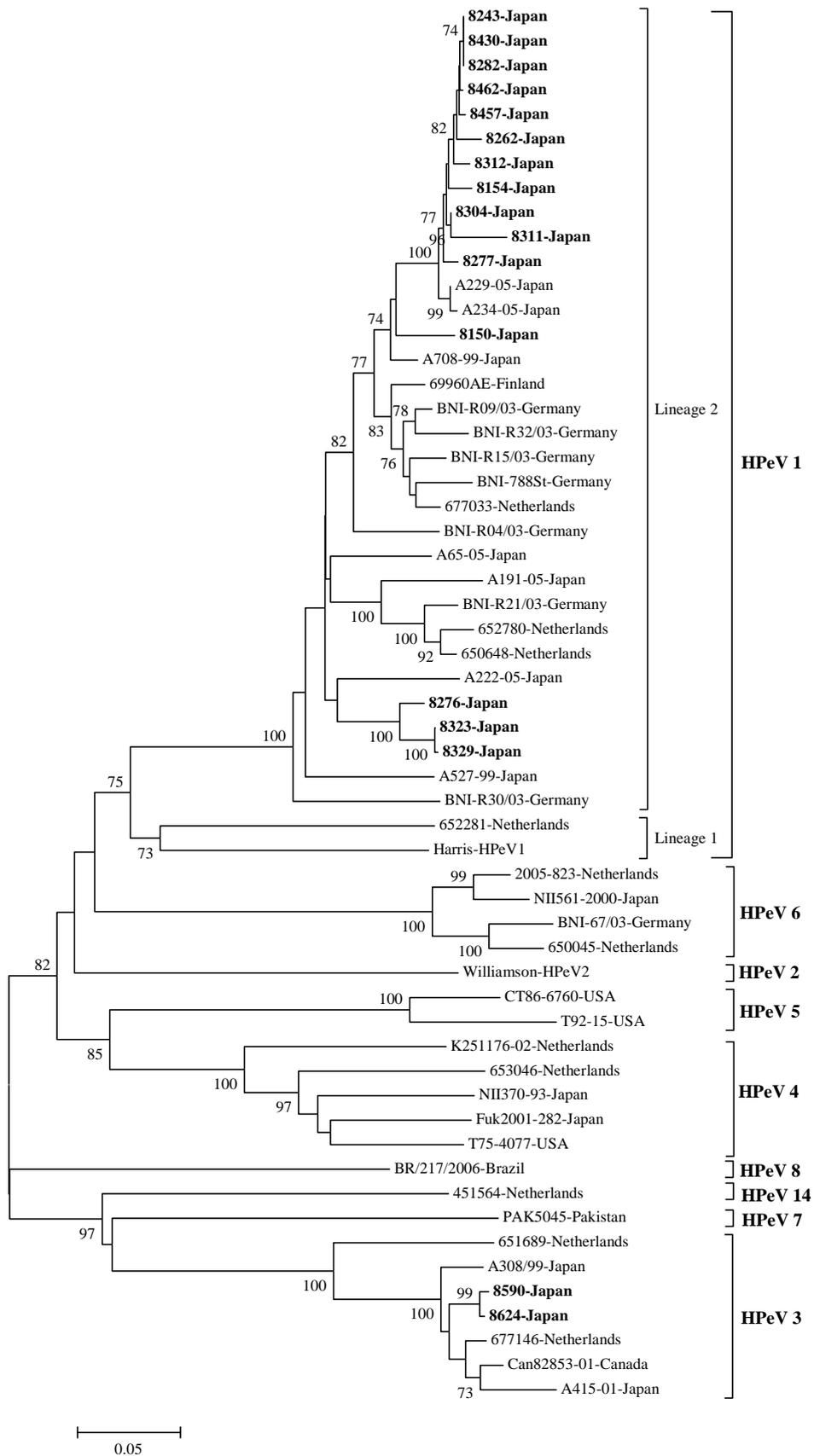
Figure 12. Phylogenetic tree constructed from the 624 nucleotides of the VP1 gene of the Japanese strains studied and reference HPeV strains with 500 bootstrap repetitions. Percentage bootstrap values above 70% are shown at the branch nodes. The HPeV strains studied are in boldface type.

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b. Human parechovirus from Thai samples

Of the 82 samples tested, 12 were positive for HPeV and the rate of detection of HPeV was 14.6%. All 12 patients whose stool samples were positive for HPeV were children aged from 6 to 24 months. Of these, 6 patients (50%) were from 6-18 months of age.

For genotyping, the full-length VP1 capsid sequences from only nine strains were successfully amplified and sequenced. The full length of the VP1 sequences of the nine studied strains was 702 bases.

Figure 13 shows the phylogenetic tree constructed from 624 nucleotide sequences of partial VP1 segment of reference HPeV strains and nine Thai strains found in this study. On the basis of the specific clustering of the isolates with known HPeV types obtained from GenBank database, the strains studied could be identified as HPeV genotypes 1 to 4. The majority of the Thai strains (five strains) belonged to HPeV1, the largest cluster of HPeVs. One Thai strain clustered together with the Williamson strain into HPeV2 cluster. The two other strains were HPeV3, the second largest cluster of HPeVs. The remaining strain was genotyped as HPeV4 and it clustered along with the K251176 strain, which was recently detected in Netherlands.

For the HPeV1 strains studied, three strains were found to cluster closely together with the prototype strain Harris and the amino acid similarities between the three strains and strain Harris ranged from 92.7% to 93.7%; while the two remaining HPeV1 Thai strains were in the larger cluster consisting of recently detected HPeV1 strains and the amino acid similarities between those two strains and the strain Harris were less than 90% (86.4% and 89.6%).

In the case of HPeV2 strains, the amino acid similarity between the Thai strain studied and the strain Williamson was 95.2%, while mean amino acid similarities between the Thai strain and strains of the other genotypes ranged from 63.8% (with HPeV3) and 74.2% (with HPeV1).
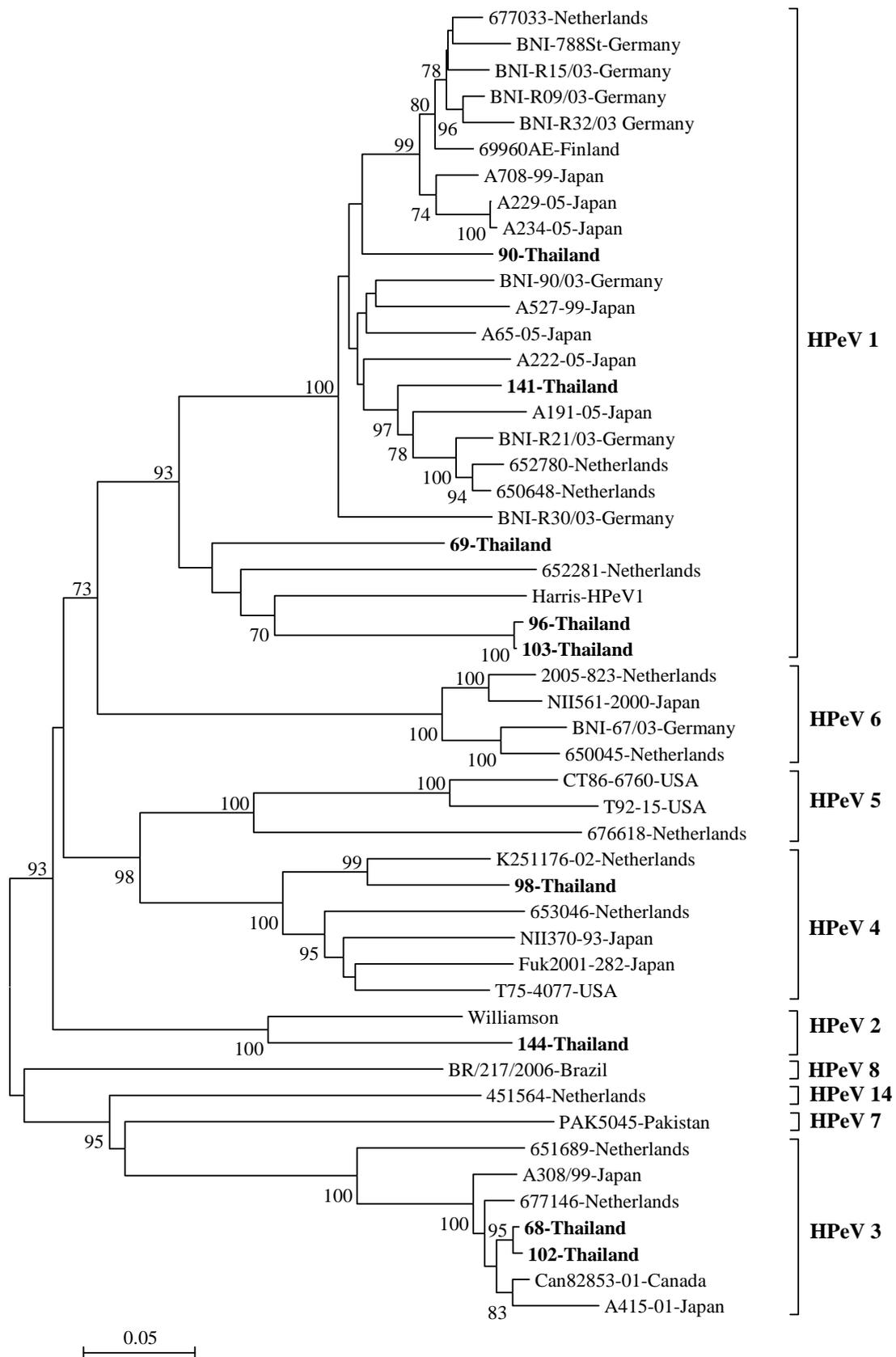
Figure 13. Phylogenetic tree constructed from the 624 nucleotide sequences of the VP1 gene of the Thai strains studied and reference HPeV strains with 500 bootstrap repetitions. Percentage bootstrap values above 70% are shown at the branch nodes. The HPeV strains studied are in boldface type.

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The two Thai HPeV3 strains studied clustered closely together with the Japanese strain A308/99, the prototype strain of HPeV3, and had 96.6% amino acid similarity to that strain.

Within HPeV4 cluster, the amino acid similarity between the Thai strain studied and other strains ranged from 97.8% to 99.1%.

Alignment of the deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeV genotypes 1-8 and 14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered critical for HPeV1 entry, was present in the HPeV1, HPeV2, HPeV4 strains studied but not in the HPeV3 strains studied (data not shown).

c. Human parechovirus from Sri Lankan samples

Of the 362 samples tested, 30 were positive for HPeV and the rate of detection of HPeV was 8.3%. All samples had been collected throughout the year and plotting of seasonal HPeV infection was shown in Figure14. Among the 30 positive samples, 20 showed co-infection with other viruses, such as rotavirus (14 samples, 46.7%), adenovirus (3 samples, 10%), norovirus GII (2 samples, 6.7%), norovirus GII and adenovirus (1 sample, 3.3%), and 10 samples (33.3%) showed single infection of HPeV.
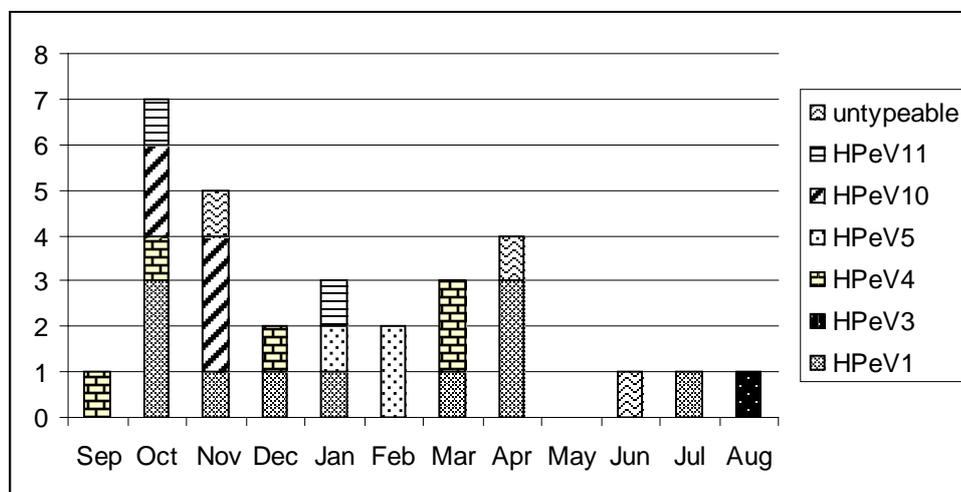
Figure 14. Distribution of HPeV-positive samples from September 2005 to August 2006 by genotype in Sri Lanka.

Clinical manifestations of children with HPeV in stool samples

The medical records of the 10 patients with samples positive only for HPeV were reviewed (Table 6). All the 10 patients were children aged from 2 to 26 months. Of these, five patients (50%) were less than 12 months of age. The male and female ratio was 1.5:1. Besides diarrhea, fever and vomiting were found in 30% and 40% of the patients, respectively. The respiratory tract symptoms, such as wheeze, cough, and coryza, were present in 30% of the patients. According to the WHO guidelines for assessing dehydration, the majority of the patients (9 out of 10 patients, 90%) suffered from dehydration. One patient experienced severe dehydration.

Prevalence of HPeV types

For genotyping, 27 of 30 HPeV positive samples were successfully amplified and sequenced of the VP1 region. Of these, 15 strains were successfully sequenced for the full VP1 segment, and the remaining 12 strains for 477 bases of partial VP1 gene.

Figure 15 shows the phylogenetic tree constructed from the full length VP1 segment of reference HPeV strains and 15 Sri Lankan strains found in this study. The strains studied were identified as HPeV1 (7 strains), and HPeV4 (4 strains). For the remaining 4 strains, VP1 sequences clustered together with none of the known 9 HPeV genotypes (HPeV1-8, and -14) in the phylogenetic analysis. They clustered into two groups (tentatively called groups A and B), group A consisting of strains LK-103 and LK-106, and group B including strains LK- 73 and LK-223 (Figure 15).

Identical matrix analysis of VP1 nucleotide sequences of these 4 strains and global reference strains of the 9 known genotypes available in GenBank databases was then performed. The results showed that the group A strains had highest mean nucleotide and amino acid similarities with HPeV3, 67.6% and 82.8%, respectively, and that the lowest mean nucleotide and amino acid similarities were found between group A strains and HPeV5, of 49.2% and 60.9%, respectively (Table 7). The group B strains had highest mean

nucleotide and amino acid similarities just only 64.7% and 73.6% with the group A strains, while lowest mean nucleotide and amino acid similarities of 54.8% and 68.1% were found between the group B strains and HPeV6 (Table 7). Therefore, these strains were expected to be classified into new or previously unpublished HPeV (HPeV 9, 11-13) genotypes based on previous proposed criteria to assign HPeV genotypes [Oberste et al., 2004].

The VP1 sequences of these strains studied were submitted to the International Committee on Taxonomy of Viruses Picornavirus Study Group in order to identify their genotype (http://www.picornastudygroup.com/types/index.html). These strains received the designation HPeV10 (strains LK-103 and LK-106) and HPeV11 (strains LK-73 and LK-223). The nucleotide and amino acid identities of 88.0% and 98.6% (strain LK-106) and 87.7% and 97.7% (strain LK-103) to the prototype HPeV10 (unpublished data) and the nucleotide and amino acid identities of 90.5% and 99.1% (strain LK-73) and 90% and 98.6% (strain LK-223) to the prototype HPeV11 (unpublished data).

The phylogenetic tree constructed based on partial VP1 segment of reference HPeV strains and all of the 27 Sri Lankan strains found in this study were then created and the result shows that studied strains were clustered into HPeV1 (11 strains), HPeV3 (1 strain), HPeV4 (5 strain), HPeV5 (3 strains), HPeV10 (5 strains), and HPeV11 (2 strains) (data not shown).

For the studied HPeV1, 4 strains were found to cluster closely together with the prototype Harris strain, and the amino acid similarities between the four strains and the Harris strain ranged from 95.4% (strain LK-69) to 96.9% (strain LK-82); while the seven others were in the larger cluster consisting of recently detected HPeV1 strains, and the amino acid similarities between the seven strains and the Harris strain were less than 90% (87.2% to 88.9%).

For the studied HPeV3 strain, one strain (LK-360) clustered into HPeV3 and amino acid similarity between prototype strain A308/99 and the LK-360 strain was 99.2%.

Within HPeV4 cluster, the amino acid similarity between the five Sri Lankan studied strains and other strains ranged from 99% (strains LK-46 and LK-148) to 99.7% (strains LK-49 and LK-276).

In the case of HPeV5 strains, the amino acid similarity between the three Sri Lankan studied strain and the reference strains was from 91.8% (strain LK-247) to 93.4% (strain LK-198).

Table 6. Clinical characteristics of HPeV positive patients [a]M, male; F, female.

Samples No	Month of detection	Gender [a]	Age (month)	Days of hospitalization	Diarrhea number of diarrheal stools/24h (Time)	Diarrhea duration of diarrhea (Day)	Dehydration No=0 1-5%=1 >6%=2	Fever	Vomiting	Wheeze Cough Coryza	Genotype of HPeV
69	Oct, 2005	F	26	3	6	4	0	-	-	-	1
73	Oct, 2005	F	6	8	10	3	1	+ (38^{0}C)	+	-	11
87	Oct, 2005	M	2	5	8	2	1	-	-	-	10
104	Nov, 2005	M	6	4	7	7	1	-	-	+	1
160	Jan, 2006	M	15	4	10	3	2	+ (38^{0}C)	+	-	1
223	Jan, 2006	M	14	1	10	5	1	-	+	-	11
247	Feb, 2006	M	7	4	4	3	1	+ (38.7^{0}C)	-	+	5
303	Apr, 2006	F	23	3	5	4	1	-	-	-	Unknown
345	Jul, 2006	M	12	1	8	14	1	-	-	+	1
360	Aug, 2006	F	18	2	4	3	1	-	+	+	10
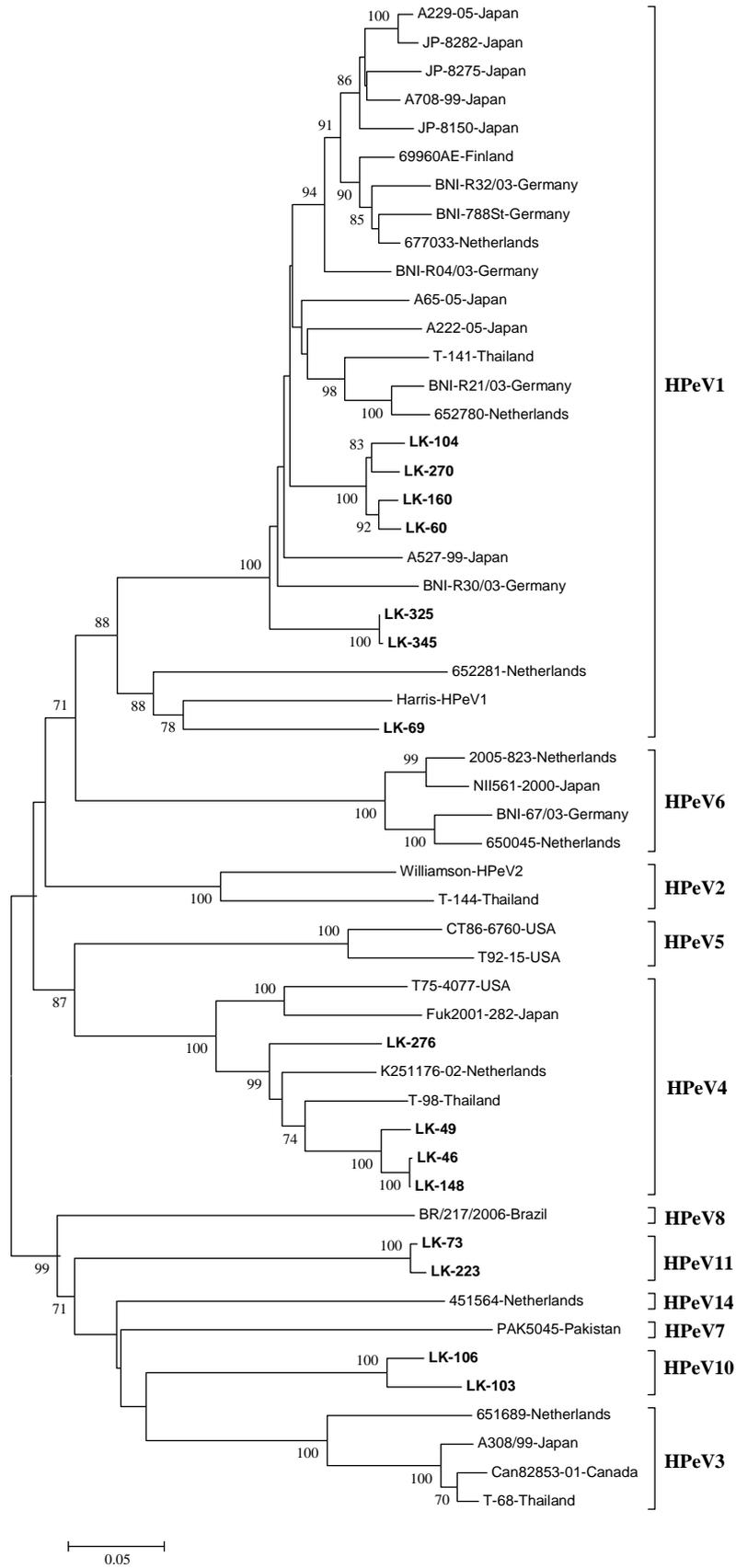
Figure 15. Phylogenetic tree constructed from nucleotide sequences of the full length VP1 segments of the Sri Lankan strains studied and reference HPeV strains with 500 bootstrap repetitions. Percentage bootstrap values above 70% are shown at the branch nodes. The studied HPeV strains are in boldface type.

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Table 7. Mean nucleotide similarities between HPeV genotypes

Mean nucleotide identities

Genotype	1	2	3	4	5	6	7	8	**10**	**11**	14
1											
2	61.9										
3	54.6	55.3									
4	61.1	61.9	56.7								
5	58.4	57.9	48.9	62.4							
6	61.8	57.3	57.8	58.4	56.7						
7	53.8	55.9	60.8	53.5	51.4	49.9					
8	58.0	57.6	58.2	58.1	53.2	55.1	58.9				
10	**56.9**	**51.5**	**67.6**	**56.8**	**49.2**	**51.3**	**63.7**	**60.5**			
11	**57.3**	**58.6**	**62.7**	**61.3**	**57.4**	**54.8**	**63.4**	**63.4**	**64.7**		
14	56.8	53.9	63.3	55.0	51.8	52.4	63.4	59.6	67.0	59.9	

4. Discussion

This is the first report of the circulation of HPeV in infants and children with acute gastroenteritis in Japan, Thailand, and Sri Lanka. In addition, high detection rates of HPeV in the tested samples of 8.1%, 14.6%, and 8.3% were found in the 3 countries, respectively. In the cases of Japan and Thailand, however, the detection rates do not represent the percentages of HPeV-infected cases in all patients with acute gastroenteritis during one-year period, because only samples which had been known to be negative for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus were screened for HPeV. In the 2 cases, mixed infection of enteric viruses was excluded. The study suggests that HPeV is a possible causative agent of acute gastroenteritis among the studied patients, and demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in the 3 countries.

To date, a spectrum of variable symptoms caused by HPeVs has been described. The common symptoms are similar to that caused by some enteroviruses, including enteritis with diarrhea, and respiratory disease [Baumgarte et al., 2008; Benschop et al., 2006b; Joki-Korpela and Hyypia, 1998; Stanway and Hyypia, 1999; Stanway et al., 2000]. Other symptoms such as rash, fever, flaccid transient paralysis, diseases such as meningoencephalitis, encephalomyelitis, myocarditis, myositis, lymphadenopathy, and syndromes such as Reye's syndrome, and haemolytic uremic syndrome have been reported also [Boivin et al., 2005; Ehrnst and Eriksson, 1993; Ehrnst and Eriksson, 1996; Figueroa et al., 1989; Grist et al., 1978; Ito et al., 2004; Koskiniemi et al., 1989; Legay et al., 2002; Maller et al., 1967; O'Regan et al., 1980; Russell and Bell, 1970; Watanabe et al., 2007]. In the present study, the clinical symptoms of the 20 Japanese patients whose fecal specimens were positive for HPeV and of the 10 Sri Lankan HPeV mono-infected cases do not seem to be different from those of other diarrheal diseases. The majority of HPeV isolates originated from infants and children aged under the age of 2 years.

Obviously, HPeV1 was found in high percentages compared to other HPeV genotypes. The finding was in good agreement with previous studies which reported that HPeV1 was predominant over other HPeVs found in patients with acute gastroenteritis [Baumgarte et al., 2008; Benschop et al., 2008; Benschop et al., 2006b; Drexler et al., 2009; Pham et al., 2010a; Pham et al., 2010d].

In this study, 5'UTR positive samples were retested and confirmed as positive. However, of a total of 62 samples positive for HPeVs in the 3 countries, only 54 samples could be genotyped. Therefore, it could not be excluded that unidentified HPeV genotypes, which the assay used was unable to pick up, were contained in the 8 ungenotypable samples (Japan: 2, Thailand: 3, and Sri Lanka: 3). In addition, a possibility of low viral load in the three ungenotypable samples could not be excluded as well.

JAPAN

The majority of the strains studied (15 strains) were identified as HPeV1, and 2 strains were genotyped as HPeV3. In addition, the phylogenetic tree shows that HPeV1 strains clustered into 2 separate branches, one branch consisting of a limited number of HPeV1 strains including the prototype Harris strain, and the other branch consisting of contemporary strains of HPeV1. Given the low amino acid identities between HPeV1 strains of the two branches, HPeV1 strains should be divided into 2 lineages as described in the results section.

There have been four genotypes of HPeVs found in Japan up to date. They are HPeV1, HPeV3, HPeV4, and HPeV6 [Ito et al., 2004; Takao et al., 2001; Wakatsuki et al., 2008; Watanabe et al., 2007]. This study identified two different types of HPeVs, HPeV1 and HPeV3, in Japanese infants and children with acute gastroenteritis, and the majority of the strains belonged to HPeV1. As a result, among the four HPeVs detected in Japan, HPeV1 has a largest number of strains reported and isolated mainly from children with acute gastroenteritis.

THAILAND

The results showed that 4 different HPeVs, HPeV1-4, were present in Thai infants and children with acute gastroenteritis, and more than a half of the detected strains belonged to HPeV1.

Among the well-known HPeVs, HPeV2 appears to be a rare genotype. Interestingly, one HPeV2 strain was found in this study. The finding is in support of the statement that HPeV2 infections rarely occur and are mostly associated with gastrointestinal symptoms [Abed and Boivin, 2006; Benschop et al., 2008; Ito et al., 2010].

For HPeV3, there were 2 strains isolated in the present study. According to previous reports, infection with HPeV3 is associated with younger age and more severe disease than infection with HPeV1 or HPeV2 [Benschop et al., 2006b; Benschop et al., 2008; Wolthers et al., 2008; Harvala and Simmonds, 2009]. Unfortunately, in this study, analysis and comparison on clinical symptoms related to HPeV3 infection could not be performed due to unavailable clinical data.

SRI LANKA

Various types of coinfections of HPeV with common diarrheal viruses such as rotavirus, norovirus, adenovirus were firstly noted. The co-infection rate of HPeV and these diarrheal viruses was as high as 66.7% (20/30).

In this study, the clinical symptoms of HPeV mono-infected cases were described. As listed in the Table 7, a half of the patients were infants. According to the WHO guidelines for assessing dehydration, the majority of the patients (9 [90%] out of 10) suffered from dehydration and 1 of these patients experienced severe dehydration. Ten episodes of diarrhea per day along with vomiting were noted in both cases of HPeV11 infection.

Phylogenetic analysis was performed based on partial VP1 sequences of the studied Sri Lankan strains studied and reference strains available in GenBank databases (data not

shown). The results showed that 6 different HPeVs, HPeV1, 3, 4, 5, 10 and 11, were present among Sri Lankan infants and children with acute gastroenteritis, and that 11 of the 27 detected strains belonged to HPeV1. Obviously, the circulation of various genotypes of HPeVs was noted among Sri Lankan infants and children in this study. In addition, HPeV10 and HPeV11-infected cases were firstly reported.

The alignment of deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeVs genotypes 1-8, 10 and 14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for human parechovirus 1 entry [Boonyakiat et al., 2001], was not present in the HPeV10 and -11 strains (data not shown). The lack of RGD motif in HPeV10, -11 may implicate that they have an RGD independent entry pathway.

Chapter III: STUDY ON BOCAVIRUS

1. Introduction

Human bocavirus (HBoV) is a newly discovered parvovirus that was first identified in pooled human respiratory tract specimens from Swedish children with a respiratory tract disease in 2005. HBoV is a small-sized, icosahedral non-enveloped particle (diameter 20-26 nm) (Figure 16), with a linear single-stranded DNA genome. The virus is classified into the genus Bocavirus of the subfamily Parvovirinae, family *Parvoviridae* [Allander et al., 2005]. HBoV genome is approximately 5.2 kb nucleotides in length and contains three open reading frames (ORF) encoding 2 non-structural proteins, NS1 and NP-1, and 2 capsid proteins, VP1 and VP2 (Figure 17).

Subsequently, the virus has been detected in children with respiratory tract infection in the world including Europe, Asia, Africa, America, and Australia. In these regions, the prevalence of HBoV infection varies considerably between 1.5-19% [Allander, 2008; Allander et al., 2007; Bastien et al., 2007; Calvo et al., 2008; Chieochansin et al., 2007; Jacques et al., 2008; Kesebir et al., 2006; Ma et al., 2006; Sloots et al., 2006; Smuts and Hardie, 2006; Smuts et al., 2008; Zeng et al., 2010; Zheng et al., 2010]. Recently, HBoV has been detected in 0.5-9.1% of fecal specimens obtained from children with acute gastroenteritis in Spain, Germany, Hong Kong, Korea, China, Japan, and Brazil [Albuquerque et al., 2007; Lau et al., 2007; Lee et al., 2007; Nakanishi et al., 2009; Vicente et al., 2007].

In a seroprevalence study in Japan, 204 serum samples were obtained from patients aged 0 months to 41 years, and an antibody to the VP1 protein was measured. The overall seroprevalence rate of antibodies against HBoV was 71.1%. The seropositive rate of HBoV was low (5.6%) in the 6 to 8 months age group and increased with age from 33.3% to 100% in persons from 9 months to 19 years old and 94.1% in the over 20 [Endo et al., 2007]. HBoV is detected most frequently in children between the ages of 6 and 24 months, and the

seroprevalence data from Japan [Endo et al., 2007] suggest that virtually all children have an infection during the first 5 years of life.

HBoV has been divided into three groups based on the VP1/VP2 region (capsid protein) or complete sequence [Chieochansin et al., 2007]. To date, the diagnosis of HBoV infection is based on the PCR amplification of viral genome fragments present in human respiratory, serum, stool and urine samples. A great number of different PCR techniques employing varying sets of primers specific for the viral genes NP1 ([Allander et al., 2005] [Simon et al., 2007], NS1 [Manning et al., 2006; Regamey et al., 2007], and VP1/VP2 [Bastien et al., 2007; Lee et al., 2007] have been described. In addition to the detection of viral genome by PCR, recent reports describe the detection of HBoV-specific antibodies in serum samples using Western blotting [Kantola et al., 2008] or immunofluorescence assay [Endo et al., 2007].

The present study aimed to screen stool samples collected from Japanese and Thai children with acute gastroenteritis during 2005-2008 for HBoV infection, one of less explored viral pathogens which has been reported to be associated with diarrhea recently, and to characterize the detected HBoV strains.
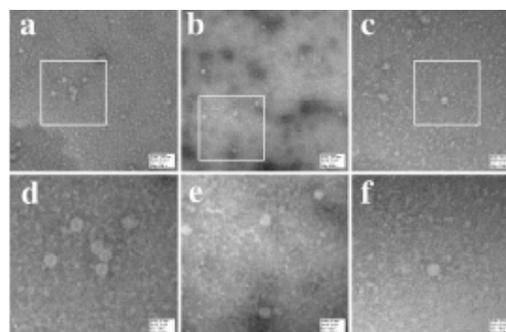
Figure 16. EM of HBoV in three different samples (a and d, b and e, and c and f) after negative staining with 4% uranyl acetate, observed at ×60,000 (a and b), ×100,000 (c), and ×150,000 magnification (d, e, and f). The boxes outlined in within miscrographs, a, b, and c are magnified in miscrographs d, e and f, respectively.
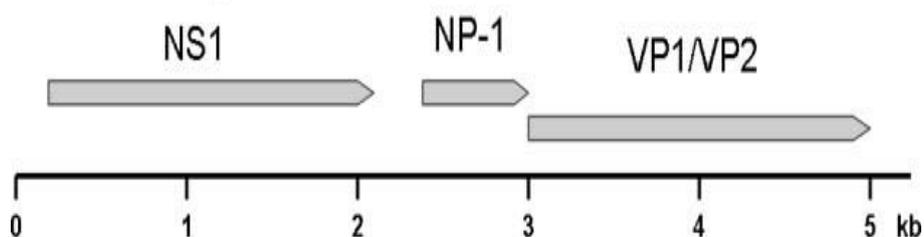
Figure 17. Genome organization of HBoV

2. Materials and methods

Clinical Specimens

Fecal specimens were collected from infants and children with acute gastroenteritis, aged from 2 months to 15 years, in pediatric clinics encompassing five localities (Sapporo, Tokyo, Maizuru, Osaka, and Saga) in Japan (247 samples collected from July 2007 to June 2008), Thailand (82 samples collected from January to December 2005). All the samples were shown to be negative for rotavirus, norovirus, sapovirus adenovirus, and astrovirus [Chanit et al., 2009; Khamrin et al., 2010], and were subjected to screening for HBoV. When HBoV was detected, the corresponding patients were identified and their clinical features, laboratory results, and outcome were analyzed retrospectively.

Detection and genotyping of the virus

Nucleic acid was extracted from 140 µl of 10% fecal suspension using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany) according to manufacturer's instructions. This product recovers both RNA and DNA from samples, allowing other less-explored enteric pathogens, such as Aichi virus and human parechovirus, being screened. Samples were then screened by PCR for the presence of HBoV by using the primers 188F and 542R to amplify a 354-bp PCR product of the NP-1 region (Table 8) [Allander et al., 2005]. In addition, these samples were screened for Aichi virus and human parechovirus also by RT-PCR with published primer sets [Pham et al., 2010b; Pham et al., 2010d; Yamashita et al., 2000].

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For genotyping of the detected virus, all HBoV positive samples were subjected to VP1/VP2 gene sequencing, in which the primers VP1/VP2F and VP1/VP2R were used to amplify an 819-bp segment of the VP1/VP2 gene (Table 8) [Kesebir et al., 2006].

Sequence Analysis

The PCR amplicons of the NP-1 and VP1/VP2 genes were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers for amplification of NP1 and VP1/VP2 genes were used as sequencing primers. The sequence data were collected by an ABI Prism 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The nucleotide sequences of the NP-1 and VP1/VP2 genes were compared to those of HBoV strains available in GenBank. The sequence data and phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was conducted using MEGA version 3.1 to determine the evolutionary relationship among studied sequences [Kumar et al., 2004].

The nucleotide sequences of the studied HBoV strains described in this study have been deposited in GenBank under accession numbers: GU563333-GU563338 (NP-1 gene) and GU563339-GU563344 (VP1/VP2 gene).

Table 8. Oligonucleotide sequences of the primers for detection and classification of HBoV

Primer	Polarity	Sequence 5'to 3'	Gene	Position	Reference
188F	+	GAGCTCTGTAAGTACTATTAC	NP1	2351-2371	Allander et al., 2005
542R	−	CTCTGTGTTGACTGAATACAG	NP1	2704-2684	Allander et al., 2005
VP1/VP2F	+	GGACCACAGTCATCAGAC	VP1/VP2	4370-87	Kesebir et al., 2006
VP1/VP2R	−	CCACTACCATCGGGCTG	VP1/VP2	5172-89	Kesebir et al., 2006

3. Results

Of the 329 samples tested, 6 (1.8%) were positive for HBoV. Of these, 5 samples were collected from Japan and 1 sample was from Thailand. As the result, the detection rates of HBoV in each country were 2% and 1.2%, respectively. For the 5 Japanese HBoV-positive samples, 3 were collected in Maizuru and the 2 remaining were from Osaka.

For clinical characterization, only data from 5 Japanese patients were available for analysis. All 5 Japanese children whose stool specimens showed positive for HBoV were infants aged from 8 to 19 months. All positive samples were detected in either April or May. Diarrhea in these patients lasted from 3-6 days, and the number of diarrheal episodes per day was less than 10. None of the patients was noted with fever, vomiting, dehydration, or respiratory symptoms. Co-detection of HBoV and HPeV or Aichi virus was not found (Table 9).

All positive PCR products of the NP-1 gene were confirmed by sequencing (GenBank). The virus detected in these patients was defined as HBoV by phylogenetic analysis (Figure 18A). For classification of the detected viruses, Figure 18B showed that 4 Japanese HBoV strains belonged to group 1, and the remaining 1 Japanese strain and one Thai strain belonged to group 2.

Table 9. Clinical characteristics of HBoV positive patients

M, male; F, female

Samples No	Month of detection	Gender	Age (month)	Diarrhea number of diarrheal stools/24h (Time)	duration of diarrhea (Day)	Fever	Vomiting	Wheeze Cough Coryza	Dehydration	Co-infection AiV HPeV	Group
8384	Apr	F	12	2	4	-	-	-	-	-	2
8387	Apr	M	12	10	3	-	-	-	-	-	1
8582	May	M	19	2	4	-	-	-	-	-	1
8583	May	M	8	4	4	-	-	-	-	-	1
8588	May	M	9	6	6	-	-	-	-	-	1
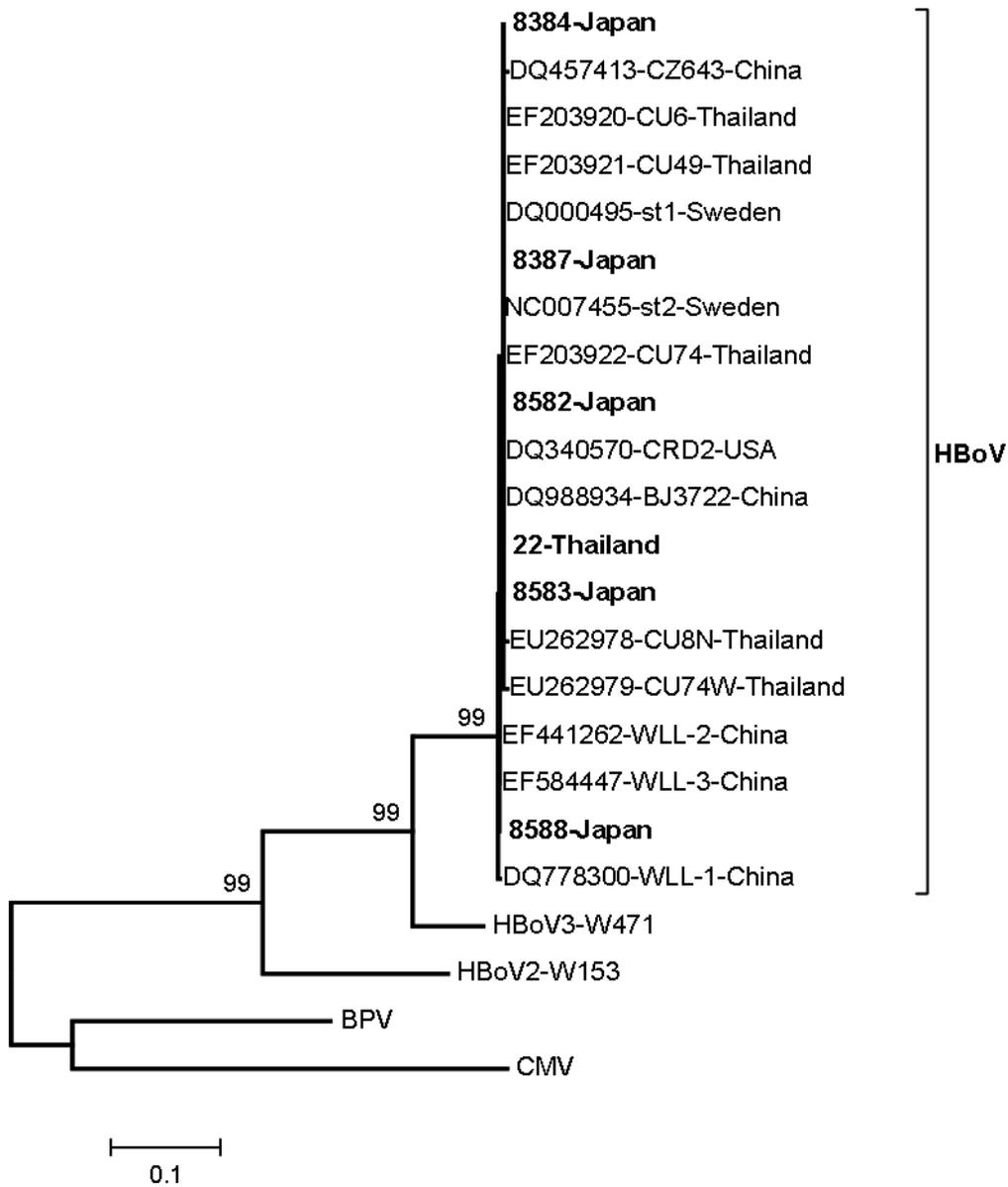
Figure 18A. Phylogenetic analysis of the NP-1 gene of HBoV in which the other members of *Bocavirus* genus, human bocavirus 2 (HBoV2), human bocavirus 3 (HBoV3), bovine parvovirus (BPV) and canine minute virus (CMV) were shown as the outgroup sequences.
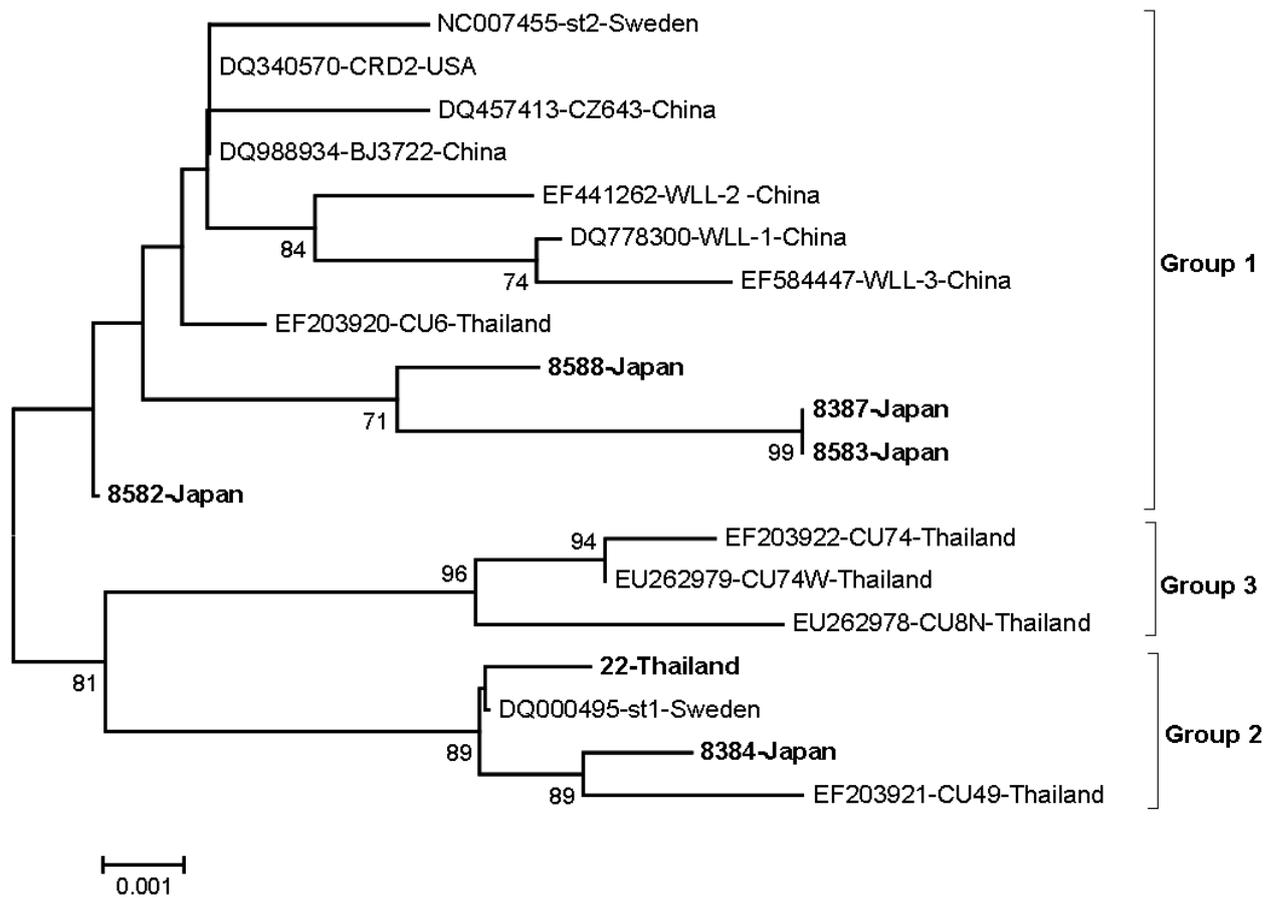
Figure 18B. Phylogenetic tree constructed from 819 nucleotide sequences of the VP1/VP2 gene of the studied and reference HBoV strains. The trees were created by Neighbor-Joining method and bootstrapped with 500 replicates. Percentage bootstrap values above 70% are shown at the branch nodes. The HBoV strains studied are in boldface type.

4. Discussion

In this study, HBoV was detected in fecal samples which had been collected from Japanese and Thai patients aged less than 24 months with acute gastroenteritis and known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus, and the detection rates of HBoV were 2% in Japanese samples and 1.2% in Thai samples. Nevertheless, the above detection rates do not represent the actual percentages of HBoV-infected cases in all patients with acute gastroenteritis during one-year period.

To date, HBoV has been detected in samples from respiratory tract, and has been associated with both upper and lower respiratory tract disease in infants and children [Allander, 2008; Allander et al., 2007; Bastien et al., 2007; Calvo et al., 2008; Chieochansin et al., 2007; Jacques et al., 2008; Kesebir et al., 2006; Ma et al., 2006; Sloots et al., 2006; Smuts and Hardie, 2006; Smuts et al., 2008; Zeng et al., 2010; Zheng et al., 2010]. HBoV was also present in gastrointestinal tract in children with gastroenteritis [Albuquerque et al., 2007; Lau et al., 2007; Lee et al., 2007; Nakanishi et al., 2009; Vicente et al., 2007]. However, its role in patients with acute gastroenteritis is unclear. In this study, HBoV was detected in stool samples collected from infants with acute gastroenteritis without symptoms of respiratory infection. In addition, the absence of any other intestinal pathogens, such as rotavirus, adenovirus, norovirus, sapovirus, astrovirus, human parechovirus, and Aichi virus, suggests that this virus is an enteric pathogen, a probable causative agent of diarrhea in the patients studied.

In Japan, the detection of HBoV in rectal swabs from children with acute gastroenteritis was reported once in Sapporo in northern Japan [Nakanishi et al., 2009]. In this study, 4 samples were positive for HBoV by RT-PCR with the detection rate of 0.5%, however, HBoV genotyping was not done in the 4 samples. In the present study, the virus was found in infants and children with acute gastroenteritis in Maizuru and Osaka located in southern Japan. In Thailand, HBoV detection in children with acute gastroenteritis was first reported in Bangkok (HBoV group 3, detection rate of 0.9%) [Chieochansin et al., 2007]. Taken together with the previous studies, the

findings suggest that this virus may be a possible and infrequent causative agent of Japanese and Thai infants and children with acute gastroenteritis.

To our knowledge, this is the first report of the circulating of HBoV groups 1 and 2, and of HBoV group 2 in infants and children with acute gastroenteritis in Japan and Thailand, respectively. The findings provide better understanding on epidemiological characteristics of HBoV infections in Japan and Thailand, and contribute useful data for future researches into HBoVs.

Chapter IV: DEVELOPMENT OF A NOVEL RT-MULTIPLEX PCR FOR DETECTION OF AICHI VIRUS, HUMAN PARECHOVIRUS, ENTEROVIRUSES, AND HUMAN BOCAVIRUS

1. Background

Acute gastroenteritis is one of the most common diseases in infants and children. It continues to be a significant cause of morbidity and mortality worldwide [Musher and Musher, 2004]. Rotavirus, norovirus, sapovirus, adenovirus, and astrovirus have been identified as the most important etiologic agents [Glass et al., 2001; Marie-Cardine et al., 2002]. However, the etiologic agents of more than half of acute gastroenteritis patients are undiagnosed [Chanit et al., 2009; Olesen et al., 2005; Simpson et al., 2003]. Recently, Aichi virus, human parechovirus (HPeV), enteroviruses (EVs), and human bocavirus (HBoV) have also been considered to be causative agents of this illness [Allander, 2008; Baumgarte et al., 2008; Calvo et al., 2008; Lau et al., 2007; Mark and Raymond, 2001; Nakanishi et al., 2009; Pham et al., 2007; Phan et al., 2005b; Reuter et al., 2009; Sdiri-Loulizi et al., 2008; Stanway et al., 2000].

For diagnostic purposes and for molecular epidemiological studies of the diversity of diarrheal viruses, the presence of eight types of common viral agents in fecal specimens have been determined by two sets of RT-multiplex PCR; set A was used to identify group A, B, and C rotaviruses and adenovirus, and set B was used to detect astrovirus, norovirus (GI, GII), and sapovirus [Yan et al., 2004; Yan et al., 2003]. The sensitivity and specificity of the two above sets of RT-multiplex PCR were demonstrated. Therefore, these two methods have been used widely to screen for these five common viruses.

At present, nevertheless, Aichi virus, HPeV, EVs, and HBoV are detected by RT-monoplex PCRs, which use a single set of primers and can detect one target virus only [Allander et al., 2005; Joki-Korpela and Hyypia, 1998; Yamashita et al., 2000; Zoll et al., 1992]. As a result,

using these PCR methods to screen clinical samples for these four viruses requires materials and time. Therefore, RT-monoplex PCR methods do not match well with clinical and research needs.

In contrast, RT-multiplex PCR with different pairs of specific primers for amplifying different viral genomes in one reaction tube can detect two or more targets in a single test. In addition, RT-multiplex PCR assay provides simple, rapid, and cost-effective laboratory diagnosis. This study develops a RT-multiplex PCR method to detect Aichi virus, HPeV, EVs, and HBoV in fecal specimens collected from infants and children with acute gastroenteritis.

2. Materials and methods

Fecal specimens

A total of 247 fecal specimens previously screened for rotavirus, norovirus, sapovirus, adenovirus, and astrovirus-negative [Chanit et al., 2009] were tested for Aichi virus, HPeV, EVs, and HBoV by RT-multiplex PCR. The samples were collected from infants and children aged 2 months to 15 years with acute gastroenteritis in pediatric clinics encompassing five localities (Sapporo, Tokyo, Maizuru, Osaka, and Saga) in Japan from July 2007 to June 2008.

Reverse Transcription (RT)

For reverse transcription, 5 μl of the stored, extracted RNA was added to a reagent mixture consisting of 3 μl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 0.8 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.8 μl of 0.1 M DTT (Invitrogen), 0.8 μl of SuperScript III Reverse Transcriptase (200 U/μl) (Invitrogen, Carlsbad, CA, USA), 0.8 μl of random primer (1 μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl of RNase Inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and 3.3 μl of distilled water. The total volume of reaction mixture was 15 μl. RT reaction was carried out at 50°C for 1 hr, followed by 95°C for 5 min and then rapid cooling on ice [Phan et al., 2005c; Yan et al., 2004].

PCR primers

Four pairs of specific primer for target viral genomes as published previously were used in the present study. To detect Aichi virus, a pair of primers 6261 and 6779 for amplifying the 3CD junction region of the virus genome, generating a 519 bp PCR product, was employed [Yamashita et al., 2000]. To detect HPeV, a 270 bp PCR product of 5'UTR region was generated using the two well-known primers ev22(+) and ev22(-) [Joki-Korpela and Hyypia, 1998]. Enteroviruses such as coxsackievirus, echovirus, enterovirus, and poliovirus were identified by the primer pair of F1 and R1 to generate a 440 bp PCR product of the 5'-NCR region of their genomes [Zoll et al., 1992]. Identification of HBoV was performed by PCR with the primers 188F and 542R to amplify a 354 bp PCR product of the viral NP-1 region [Allander et al., 2005]. The oligonucleotide

sequences of these primers and their positions are described in Table 10. The expected sizes of Aichi virus, HPeV, EVs, and HBoV were 519 bp, 270 bp, 440 bp, and 354 bp, respectively (Figure 19).

Monoplex PCR

In monoplex PCR, only one pair of primers was used to detect the target virus. After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 5 μl of 5x Taq DNA polymerase buffer (Promega, Madison, WI, USA), 2 μl of dNTPs (10 mM), 0.5 μl of each specific primer (20 μM), 0.1 μl of Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), and 14.9 μl of distilled water, screening PCR was conducted using specific primers to detect the target virus (Table 10). The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min.

Table 10. Specific primers for amplifying the genomes of Aichi virus, HPeV, EVs, and HBoV
Y means C or T, and R means A or G

Primer	Polarity	Sequence 5'to 3'	Gene	Position	Reference
Aichi virus					
6261	+	ACACTCCCACCTCCCGCCAGT	3D	6261-6282	Yamashita et al., 2000
6779	−	GGAAGAGCTGGGTGTCAAGA	3D	6779-6760	Yamashita et al., 2000
HPeV					
ev22(+)	+	CYCACACAGCCATCCTC	5'UTR	312-328	Joki-Korpela., 1998
ev22(-)	−	TRCGGGTACCTTCTGGG	5'UTR	581-565	Joki-Korpela., 1998
EVs					
F1	+	CAAGCACTTCTGTTTCCCCGG	5' NCR	160-180	Zoll et al., 1992
R1	−	ATTGTCACCATAAGCAGCCA	5' NCR	599-580	Zoll et al., 1992
HBoV					
188F	+	AGCTCTGTAAGTACTATTAC	NP1	2351-2371	Allander et al., 2005
542R	−	CTCTGTGTTGACTGAATACAG	NP1	2704-2684	Allander et al., 2005

Multiplex PCR

In the multiplex PCR, the four pairs of specific primers to detect Aichi virus, HPeV, EVs, and HBoV were mixed in a single test. The same volume and concentration of primers, reagents, and thermal cycler program described above were used.

| 6261 | Aichi virus | 6779 | **519 bp** |

| F1 | EVs | R1 | **440 bp** |

| 188F | HBoV | 542R | **354 bp** |

| ev22(+) | HPeV | ev22(-) | **270 bp** |
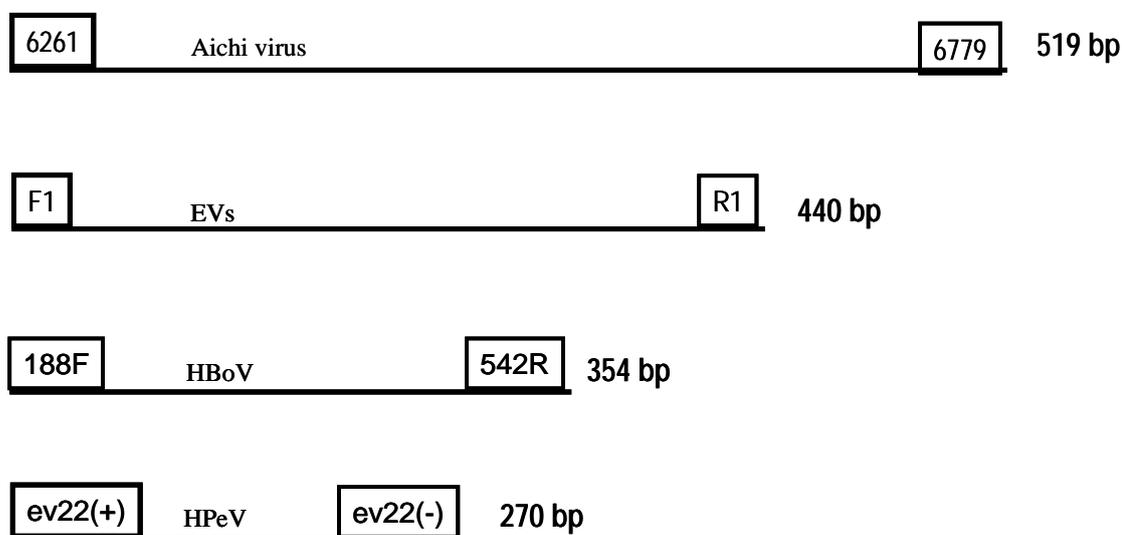
Figure 19. Diagram illustrating the PCR amplification of Aichivirus, EVs, HPeV, and HBoV.

Positive viral controls

The positive controls of Aichi virus and HPeV, enteroviruses, and HBoV used in this study were provided by Dr. Teruo Yamashita (Department of Microbiology, Aichi Prefecture Institute of Public Health, Japan), Dr. Hideaki Shimizu (Kawasaki City Institute of Public Health, Kawasaki, Japan), and Dr. Nobuhisa Ishiguro (Department of Pediatrics, Hokkaido University Graduate School of Medicine, Japan), respectively.

Negative viral controls

Distilled water and samples which showed mono-infection with one of the following viruses, group A rotavirus, adenovirus, norovirus (GI, GII), sapovirus, and astrovirus, were used as negative viral controls for the monoplex and multiplex PCRs.

Specificity testing of the four primer pairs

Specificity testing was performed for the positive controls of Aichi virus, HPeV, enteroviruses, and HBoV to determine the specificity of the mixture of four primer pairs. Two combinations were tested: i) the PCR mixture containing four primer pairs and each single template (cDNA), and ii) four primer pairs and four templates. Specificity testing was also carried out for negative controls of group A rotavirus, adenovirus, norovirus (GI, GII), sapovirus, and astrovirus.

Sensitivity testing of multiplex PCR and monoplex PCR

To compare the sensitivity level of multiplex PCR and monoplex PCR, 10-fold serial dilutions (10^{-1} to 10^{-4}) in distilled water of the four different viral cDNA of Aichi virus, HPeV, enteroviruses, and HBoV were tested by multiplex PCR and monoplex PCR. The multiplex PCR and monoplex PCR were carried out simultaneously for the same dilution series with the same PCR machine.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with SYBR Green for 20 min, then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Sequence analysis

The PCR products generated from the PCR reaction were purified and subjected to sequencing to confirm the amplicons of target sequences.

3. Results

Specificity testing of the four primer pairs

The specificity of the mixture of four primer pairs used in the present study was tested and shown in Figure 20. Each pair of primers amplified the viral genomes of positive controls and generated specifically and independently four different sizes of amplicons of 519, 270, 440, and 354 bp by 6261 and 6779 for Aichi virus, ev22(+) and ev22(-) for HPeV, F1 and R1 for EVs, and 188F and 542R for HBoV. No cross-reaction with non-targets was identified. For the negative controls of group A rotavirus, adenovirus, norovirus (GI, GII), sapovirus, and astrovirus, no amplicon was demonstrated (data not shown).

Sensitivity testing of mutiplex PCR and monoplex PCR

For the 10-fold dilution series, the highest dilutions at which mutiplex PCR showed positive results were 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-3} for Aichi virus, HPeV, EVs, and HBoV, respectively. On the other hand, the positive results of highest dilution in monoplex PCR were 10^{-3} for Aichi virus, 10^{-4} for HPeV, 10^{-4} for EVs, and 10^{-3} for HBoV (Figure 21). Taken together, the results indicated that the sensitivity of multiplex PCR decreased slightly for Aichi virus and HPeV compared to monoplex PCR.

Detection of target viruses in fecal specimens by RT-monoplex PCR and RT-multiplex PCR

A total of 247 fecal specimens previously screened for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus-negative were collected from infants and children with acute gastroenteritis in pediatric clinics encompassing five localities (Sapporo, Tokyo, Maizuru, Osaka, and Saga) in Japan from July 2007 to June 2008. All fecal specimens were tested for the presence of Aichi virus, HPeV, EVs, and HBoV by monoplex PCR and multiplex PCR with specific primers as previously published (Table 10). The results shown in Table 11 revealed that there was no difference of viral detection rate between monoplex PCR and multiplex PCR in clinical fecal specimens. HPeV was identified in 20 samples and the detection rate of HPeV was 8.1%

[Pham et al., 2010b]. Forty-one samples were positive for EVs and the detection rate of EVs was 16.6%. HBoV was detected in five samples with a detection rate of 2% [Pham et al., 2010c]. No Aichi virus was found among these subjects. In addition, mixed infection from more than one target virus was found in three samples, in which coinfection between EVs and either HPeV or HBoV were detected in one and two samples, respectively.

Table 11. Results of detection of target viruses in fecal specimens by monoplex PCR and multiplex PCR

Method	Samples	Monoinfection				Coinfection	
		Aichi virus	HPeV	EVs	HBoV	EVs-HPeV	EVs-HBoV
Monoplex	247	0	19	38	3	1	2
Multiplex	247	0	19	38	3	1	2
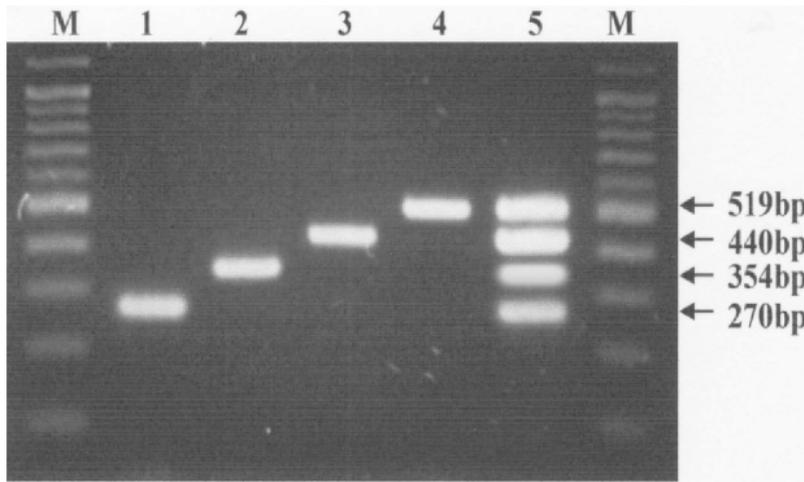
Figure 20. Specificity testing of the multiplex PCR assay with a mixture of four primer pairs for four positive controls.
Lane 1, HPeV; lane 2, HBoV; lane 3, EVs; lane 4, Aichi virus; lane 5, the mixture of Aichi virus, HPeV, EVs, and HBoV positive controls; M, marker.
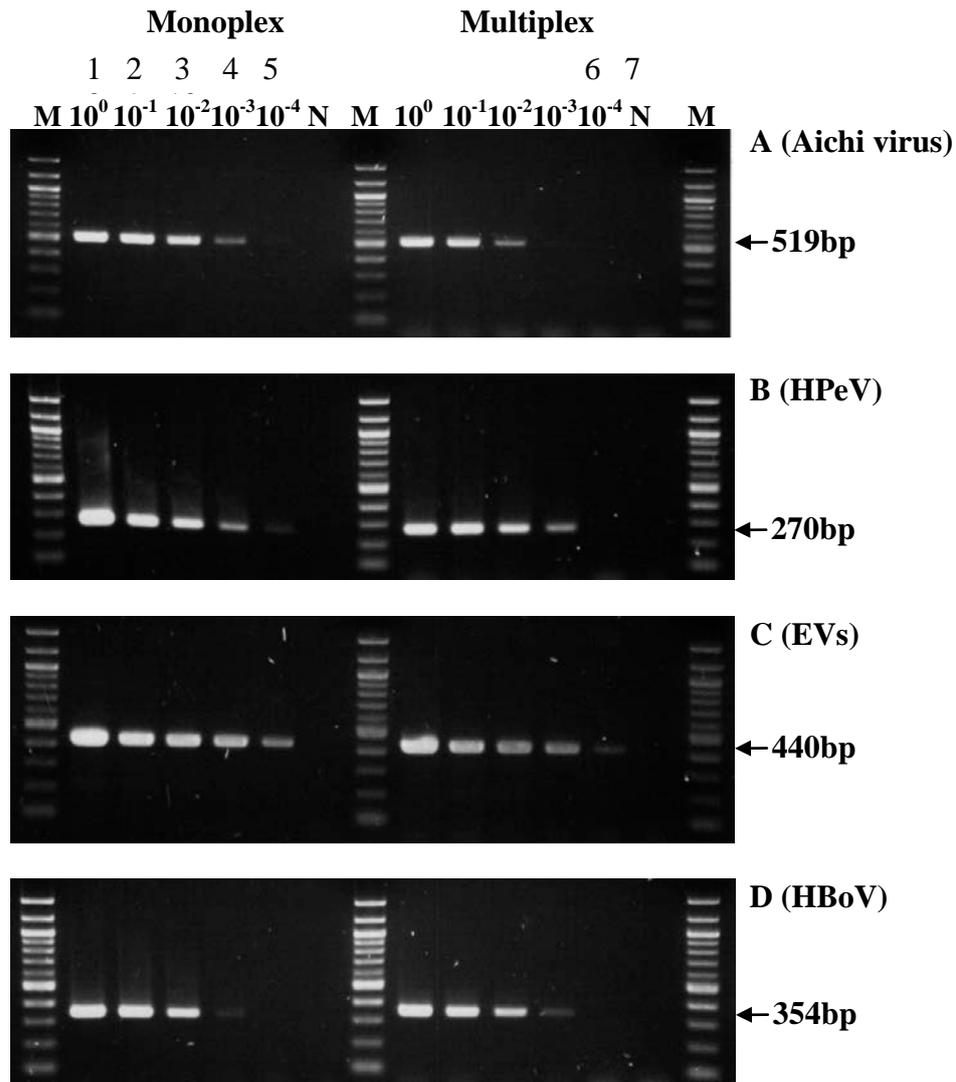
Figure 21. Comparison of the sensitivity between multiplex PCR and monoplex PCR for the 10-fold serial dilutions of positive controls.
A, Aichi virus; B, HPeV; C, EVs; D, HBoV; N, negative control; M, marker. Lanes 1-5, sensitivity testing of monoplex PCR; lanes 6-10, sensitivity testing of multiplex PCR.

4. Discussion

Currently, RT-PCR is considered to be a convenient, useful, and powerful method for molecular diagnosis, to detect pathogens from clinical specimens. It has been used widely. In addition, RT-multiplex PCR assay provides simple, rapid, and cost-effective laboratory diagnosis. In this study, a rapid assay to detect simultaneously Aichi virus, HPeV, EVs, and HBoV in fecal specimens was developed.

The results demonstrate that the newly developed multiplex PCR is highly specific in detecting the four viruses. As shown in Fig. 20, no cross-reaction with non-targets was identified. In addition, for the negative controls of group A rotavirus, norovirus (GI, GII), sapovirus, adenovirus, and astrovirus, no amplicon was observed (data not shown).

Despite the fact that the sensitivity of mutiplex PCR decreased slightly for Aichi virus and HPeV compared to monoplex PCR when using the 10-fold dilution series of templates (Fig. 2), the overall results revealed no difference in viral detection rates between monoplex PCR and multiplex PCR in clinical fecal specimens, as shown in Table 2.

However, in the case of monoplex PCR for detection of Aichi virus, it is a two-step PCR using 2 pairs of primers. With developed multiplex PCR, the Aichi virus detection was done by use of the first primer pair as described in the Chapter I. Therefore, there is a possibility in which Aichi virus positive samples were missed, or in other words, the detection rate of Aichi virus may be decreased by this multiplex PCR method.

As mentioned in the introduction, the presence of eight types of common viral agents in fecal specimens was determined by two sets of RT-multiplex PCR; set A was used to identify group A, B, and C rotaviruses and adenovirus, and set B was used to detect astrovirus, norovirus (GI, GII), and sapovirus [Yan et al., 2004; Yan et al., 2003]. However, for some less-explored or less frequent virus pathogens, such as Aichi virus, HPeV, EVs, and HBoV, which are increasing in reported frequency, detection is based basically on RT-

monoplex PCR, which uses a single set of primers and can detect one target virus only [Allander et al., 2005; Joki-Korpela and Hyypia, 1998; Pham et al., 2010b; Yamashita et al., 2000; Zoll et al., 1992]. This study developed a multiplex-PCR method using a set of four primer pairs (tentatively assigned as set C) which can detect simultaneously four other diarrheal viruses, Aichi virus, HPeV, EVs, and HBoV, with high specificity and sensitivity.

The sensitivity and specificity of the newly developed RT-multiplex PCR demonstrated a strong validation against RT-monoplex PCR. Obviously, the newly developed multiplex PCR is simple and less time consuming. It is truly a cost-effective laboratory method to detect these viruses.

The primer set suggested in this study is appropriate for screening for the four viruses in clinical specimens from infants and children with acute gastroenteritis in Asia, Europe, and America. However, it can be modified according to the situation in the target countries. In this study, Aichi virus was not found in clinical specimens. In our previous studies, as well as in other studies conducted in Asian, European, and American countries, low detection rates of Aichi virus were reported. However, Aichi virus was found in higher frequencies in African countries, such as Tunisia [Sdiri-Loulizi et al., 2008]. Its detection rate was found to be closer to, or even higher than, those of astrovirus, adenovirus, and sapovirus. In such a situation, the primer pair to detect Aichi virus can be excluded from the set C and added to the common virus primer list.

This is the first report of detecting Aichi virus, HPeV, EVs, and HBoV in fecal samples from infants and children with acute gastroenteritis by RT-multiplex PCR. With its high specificity and sensitivity, strong validation against RT-monoplex PCR, faster testing time, and reagent savings, this RT-multiplex PCR assay provides simple, rapid, and cost-effective laboratory diagnosis.

CONCLUSSION

1/. The percentages of Aichi virus positive samples among the specimens tested in Japan, Bangladesh, Thailand, and Vietnam, were 6.5%, 2.5%, 0.9%, and 1.6%, respectively.

This is the first finding of Aichi virus in fecal specimens from Bangladesh, Thailand, and Vietnam. These results also provide useful information to the understanding about epidemiology of Aichi virus in Japan.

2/. Based on sequence analysis of the capsid gene of the detected Aichi virus strains and reference strains, Aichi viruses could be divided into two major lineages: lineage I (genotype A) including the strains isolated in Japan, Germany, Thailand, and Vietnam, lineage II (genotype B) including strains from Bangladesh and Brazil. The nucleotide distances between the two genotypes were equal to or greater than 12.7 %, and the genomic differences between them could be found throughout the capsid sequences. At least 14 different deduced amino acids which were considered to be genotype-specific, especially the different triple of amino acids located within the VP0 region, were found. This is the first report of classification of Aichi viruses based on the capsid gene.

3/. Based on the genomic differences which were considered to be genotype-specific, a nested PCR method using the newly developed primers can separate genotype A from genotype B. As demonstrated in this study, the new primers are sensitive and specific enough for differentiation between genotypes A and B of Aichi viruses.

4/. High detection rates of HPeV of 8.1%, 14.6%, and 8.3% in clinical samples tested were found in Japan, Thailand, and Sri Lanka, respectively. This is the first report of the circulation of HPeV in infants and children with acute gastroenteritis in Japan, Thailand, and Sri Lanka. The study suggests that HPeV is a possible causative agent of acute gastroenteritis among the studied patients, and demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in the three countries. Taken together with the findings from previous studies, this study suggests that HPeV should be included

into the spectrum of viruses which are routinely screened for among infants and children with acute gastroenteritis.

Based on sequence analysis, with the identification of different genotypes of HPeVs in the samples tested, the diversity of Thai and Sri Lankan HPeVs was noted. In addition, clinical and molecular data of new HPeV genotypes 10 and 11 were firstly analyzed and published.

5/. The detection rates of HBoV in fecal samples which had been known to be negative for rotavirus, norovirus, adenovirus, sapovirus, and astrovirus collected from Japanese and Thai infants and children with acute gastroenteritis were found to be 2% and 1.2%, respectively. The findings suggest that this virus may be a possible and infrequent causative agent of Japanese and Thai infants and children with acute gastroenteritis.

This is the first report of the circulating of HBoV groups 1 and 2, and of HBoV group 2 in infants and children with acute gastroenteritis in Japan and Thailand, respectively. The findings provide better understanding on epidemiological characteristics of HBoV infections in Japan and Thailand.

6/. A novel RT-multiplex PCR for detection of Aichi virus, HPeV, EVs, and HBoV was successfully developed. This is the first report of detecting the four viruses in fecal samples from infants and children with acute gastroenteritis by RT-multiplex PCR. With its high specificity and sensitivity, strong validation against RT-monoplex PCR, faster testing time, and reagent savings, this RT-multiplex PCR assay provides simple, rapid, and cost-effective laboratory diagnosis.

With the first findings or reports of circulating of Aichi virus, HPeV, and HBoV in the studied countries, and especially, the first reports of HPeV10 and HPeV11 in the world, the studies contribute useful information and background on molecular epidemiology of the studied viruses. The studies suggest that these viruses should be included into the spectrum

of viruses which are routinely screened for among infants and children with acute gastroenteritis.

Besides providing the first classification of Aichi viruses based on the capsid gene, I developed successfully a nested PCR method using newly developed primers which can separate genotype A from genotype B. For classification of HPeV, in my studies, two new primers were designed for the first round PCR and could successfully amplified HPeV. In addition, I developed successfully a novel multiplex PCR for detection of Aichi virus, HPeV, EVs, and HBoV from clinical specimens. These newly developed PCRs are useful tools for scientists in researches and in clinical application as well.

PRESENT RESEARCHES RELATED TO FUTURE WORK

1. Study on recombination of HPeV isolated in Japan, Thailand and Sri Lanka

It has been said that recombination is a quite common phenomenon among HPeVs strains. In my studies, HPeVs have been identified among infants and children with acute gastroenteritis in Japan, Thailand and Sri Lanka, and the diversity of the detected HPeVs was noted. However, studies on recombination of detected HPeVs have not been done yet.

2. Sequence analysis of the complete genome of HPeV10 and HPeV11 isolated in Sri Lanka

HPeVs have been classified into 14 genotypes up to date. However, the genome sequences of HPeV 9-14 have not been determined yet. In my studies, 2 novel genotypes of HPeVs, HPeV 10 and HPeV11, have been identified. Obviously, there is a need of doing research on the full genome of these 2 genotypes.

3. Study on enterovirus infection in Asian countries such as Japan, Thailand, Vietnam, and Sri Lanka

Enterovirus belongs to Picornaviruses family and is believed to be the major etiologic agents causing outbreaks and sporadic cases of acute gastroenteritis. It is one of less-explored viral pathogens infecting billions of people worldwide. In my studies, a multiplex PCR for detection of 4 less-explored virus pathogens, Aichi virus, human parechovirus, enteroviruses and human bocavirus has been developed. Consequently, to pursue my plan of studying on such viruses, I will conduct a molecular epidemiological study targeting enterovirus from clinical specimens collected in Japan, Thailand, Vietnam, and Sri Lanka.

4. Development of a multiplex PCR for detection of viruses causing acute encephalitis

Viral encephalopathy/encephalitis remains a major medical concern with high morbidity and mortality, however, its incidence has been underestimated, especially in developing countries. The disease diagnosis is critical for clinical care and treatment. Due to limitations of present diagnostic methods, suitable approaches for determination of the causative agents have being developed.

I have been developing a multiplex PCR for detection of 10 viruses causing encephalopathy/encephalitis such as Japanese encephalitis virus, dengue virus, herpes simplex virus 1 and 2, human herpes virus 6, influenza A, enterovirus, human parechovirus, and adenovirus. Some first results showed that the method was able to detect the 10 viruses simultaneously. The method is considered a useful diagnostic approach of viral encephalitis, especially in developing countries. The method is simple, cost-effective, and possible to detect causative agents in early period of the disease without the need of culture.

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