genotypes 1 to 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, and HPeV4 strains studied but not the HPeV3 strains studied (data not shown).

DISCUSSION

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 (3, 6, 17, 31, 32). Other symptoms and syndromes as a result of HPeV infection, such as meningoencephalitis, encephalomyelitis, flaccid transient paralysis, nosocomial infection, neonatal sepsis, myocarditis, myositis, lymphadenopathy, hand-and-mouth disease, rash, fever of unknown origin, influenza-like illness, Reye's syndrome, and hemolytic-uremic syndrome, have also been reported (7, 10–13, 16, 21, 23, 26, 28, 30, 34).

By screening fecal samples known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus for human parechovirus, this study provides one more piece of evidence that HPeV infection is associated with acute gastroenteritis. In addition, with a detection rate of 14.6% among the samples tested, the study demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in Thailand.

In this study, the full length of the 702-base VP1 gene of the Thai strains studied was successfully obtained. However, some of VP1 sequences of the reference strains, especially strain 451564 of new genotype HPeV14 (4), available in the GenBank database were partial VP1 sequences. Consequently, the phylogenetic tree was constructed on the basis of 624 bases. The results showed that four different HPeV genotypes, HPeV1 to HPeV4, were present among Thai infants and children with acute gastroenteritis, and more than a half of the strains detected belonged to HPeV1. That finding was in good agreement with the findings of previous studies, which reported that HPeV1 was predominant over other the HPeV genotypes found in patients with acute gastroenteritis (3, 4, 6, 9).

Among the well-known HPeV genotypes, HPeV2 appears to be a rare genotype. Interestingly, one HPeV2 strain was found in this study. This finding is in support of the statement that HPeV2 infections rarely occur and are mostly associated with gastrointestinal symptoms.

Two HPeV3 strains were 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. Unfortunately, in this study, analysis and comparison of the clinical symptoms related to HPeV3 infection could not be performed due to the lack of availability of clinical data.

In this study, samples positive for HPeV were retested and were confirmed to be HPeV positive. However, of the 12 samples positive for HPeVs, the virus from only 9 samples could be genotyped. Therefore, the possibility that the three samples containing unidentifiable HPeV genotypes contained virus whose genotypes could not be detected by the assay used cannot be excluded. In addition, the possibility that these three samples contained low viral loads also cannot be excluded. Therefore, a real-time PCR method is more appropriate for the screening of fecal specimens for HPeVs. The fact that this study did not employ real-time PCR is also a limitation of this study.

In conclusion, this is the first report of HPeV infections among infants and children with acute gastroenteritis in Thailand. The diversity of the Thai HPeVs was found by the identification of the four different genotypes of HPeV (HPeV1 to HPeV4) in the samples tested. Taken together with the findings from previous studies, it is suggested that HPeV should be included in the spectrum of viruses for which children with acute gastroenteritis are routinely screened.

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REFERENCES

1. **Abed, Y., and G. Boivin.** 2005. Molecular characterization of a Canadian human parechovirus (HPeV)-3 isolate and its relationship to other HPeVs. J. Med. Virol. **77**:566–570.
2. **Al-Sunaidi, M., C. H. Williams, P. J. Hughes, D. P. Schnurr, and G. Stanway.** 2007. Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. J. Virol. **81**:1013–1021.
3. **Baumgarte, S., L. K. de Souza Luna, K. Grywna, M. Panning, J. F. Drexler, C. Karsten, H. I. Huppertz, and C. Drosten.** 2008. Prevalence, types, and RNA concentrations of human parechoviruses, including a sixth parechovirus type, in stool samples from patients with acute enteritis. J. Clin. Microbiol. **46**:242–248.
4. **Benschop, K., X. Thomas, C. Serpenti, R. Molenkamp, and K. Wolthers.** 2008. High prevalence of human parechovirus (HPeV) genotypes in the Amsterdam region and identification of specific HPeV variants by direct genotyping of stool samples. J. Clin. Microbiol. **46**:3965–3970.
5. **Benschop, K. S., J. Schinkel, M. E. Luken, P. J. van den Broek, M. F. Beersma, N. Menelik, H. W. van Eijk, H. L. Zaaijer, C. M. VandenBroucke-Grauls, M. G. Beld, and K. C. Wolthers.** 2006. Fourth human parechovirus serotype. Emerg. Infect. Dis. **12**:1572–1575.
6. **Benschop, K. S., J. Schinkel, R. P. Minnaar, D. Pajkrt, L. Spanjerberg, H. C. Kraakman, B. Berkhout, H. L. Zaaijer, M. G. Beld, and K. C. Wolthers.** 2006. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin. Infect. Dis. **42**:204–210.
7. **Boivin, G., Y. Abed, and F. D. Boucher.** 2005. Human parechovirus 3 and neonatal infections. Emerg. Infect. Dis. **11**:103–105.
8. **de Vries, M., K. Pyrc, R. Berkhout, W. Vermeulen-Oost, R. Dijkman, M. F. Jebbink, S. Bruisten, B. Berkhout, and L. van der Hoek.** 2008. Human parechovirus type 1, 3, 4, 5, and 6 detection in picornavirus cultures. J. Clin. Microbiol. **46**:759–762.
9. **Drexler, J. F., K. Grywna, A. Stocker, P. S. Almeida, T. C. Medrado-Ribeiro, M. Eschbach-Bludau, N. Petersen, H. da Costa-Ribeiro, Jr., and C. Drosten.** 2009. Novel human parechovirus from Brazil. Emerg. Infect. Dis. **15**:310–313.
10. **Ehrnst, A., and M. Eriksson.** 1996. Echovirus type 23 observed as a nosocomial infection in infants. Scand. J. Infect. Dis. **28**:205–206.
11. **Ehrnst, A., and M. Eriksson.** 1993. Epidemiological features of type 22 echovirus infection. Scand. J. Infect. Dis. **25**:275–281.
12. **Figueroa, J. P., D. Ashley, D. King, and B. Hull.** 1989. An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. J. Med. Virol. **29**:315–319.
13. **Grist, N. R., E. J. Bell, and F. Assaad.** 1978. Enteroviruses in human disease. Prog. Med. Virol. **24**:114–157.
14. **Hansman, G. S., K. Katayama, N. Maneekarn, S. Peerakome, P. Khamrin, S. Tonusin, S. Okitsu, O. Nishio, N. Takeda, and H. Ushijima.** 2004. Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand. J. Clin. Microbiol. **42**:1305–1307.
15. **Hyypia, T., C. Horsnell, M. Maaronen, M. Khan, N. Kalkkinen, P. Auvinen, L. Kinnunen, and G. Stanway.** 1992. A distinct picornavirus group identified by sequence analysis. Proc. Natl. Acad. Sci. U. S. A. **89**:8847–8851.
16. **Ito, M., T. Yamashita, H. Tsuzuki, N. Takeda, and K. Sakae.** 2004. Isolation and identification of a novel human parechovirus. J. Gen. Virol. **85**:391–398.

17. **Joki-Korpela, P., and T. Hyypia.** 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin. Infect. Dis. **27:**129–136.
18. **Joki-Korpela, P., and T. Hyypia.** 2001. Parechoviruses, a novel group of human picornaviruses. Ann. Med. **33:**466–471.
19. **Khamrin, P., N. Maneekarn, S. Peerakome, S. Tonusin, R. Malasao, M. Mizuguchi, S. Okitsu, and H. Ushijima.** 2007. Genetic diversity of noroviruses and sapoviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. J. Med. Virol. **79:**1921–1926.
20. **Kittigul, L., K. Pombubpa, Y. Taweekate, T. Yeephoo, P. Khamrin, and H. Ushijima.** 2009. Molecular characterization of rotaviruses, noroviruses, sapovirus, and adenoviruses in patients with acute gastroenteritis in Thailand. J. Med. Virol. **81:**345–353.
21. **Koskiniemi, M., R. Paetau, and K. Linnavuori.** 1989. Severe encephalitis associated with disseminated echovirus 22 infection. Scand. J. Infect. Dis. **21:**463–466.
22. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
23. **Legay, V., J. J. Chomel, E. Fernandez, B. Lina, M. Aymard, and S. Khalfan.** 2002. Encephalomyelitis due to human parechovirus type 1. J. Clin. Virol. **25:**193–195.
24. **Li, L., J. Victoria, A. Kapoor, A. Naeem, S. Shaukat, S. Sharif, M. M. Alam, M. Angez, S. Z. Zaidi, and E. Delwart.** 2009. Genomic characterization of novel human parechovirus type. Emerg. Infect. Dis. **15:**288–291.
25. **Malasao, R., N. Maneekarn, P. Khamrin, C. Pantip, S. Tonusin, H. Ushijima, and S. Peerakome.** 2008. Genetic diversity of norovirus, sapovirus, and astrovirus isolated from children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. J. Med. Virol. **80:**1749–1755.
26. **Maller, H. M., D. F. Powars, R. E. Horowitz, and B. Portnoy.** 1967. Fatal myocarditis associated with ECHO virus, type 22, infection in a child with apparent immunological deficiency. J. Pediatr. **71:**204–210.
27. **Niklasson, B., L. Kinnunen, B. Hornfeldt, J. Horling, C. Benemar, K. O. Hedlund, L. Matskova, T. Hyypia, and G. Winberg.** 1999. A new picornavirus isolated from bank voles (Clethrionomys glareolus). Virology **255:**86–93.
28. **O'Regan, S., P. Robitaille, J. G. Mongeau, and B. McLaughlin.** 1980. The hemolytic uremic syndrome associated with ECHO 22 infection. Clin. Pediatr. (Phila.) **19:**125–127.
29. **Phan, T. G., T. A. Nguyen, H. Yan, F. Yagyu, V. Kozlov, A. Kozlov, S. Okitsu, W. E. Muller, and H. Ushuijma.** 2005. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in eastern Russia. Clin. Lab. **51:**429–435.
30. **Russell, S. J., and E. J. Bell.** 1970. Echoviruses and carditis. Lancet **i:**784–785.
31. **Stanway, G., and T. Hyypia.** 1999. Parechoviruses. J. Virol. **73:**5249–5254.
32. **Stanway, G., P. Joki-Korpela, and T. Hyypia.** 2000. Human parechoviruses—biology and clinical significance. Rev. Med. Virol. **10:**57–69.
33. **Stanway, G., N. Kalkkinen, M. Roivainen, F. Ghazi, M. Khan, M. Smyth, O. Meurman, and T. Hyypia.** 1994. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J. Virol. **68:**8232–8238.
34. **Watanabe, K., M. Oie, M. Higuchi, M. Nishikawa, and M. Fujii.** 2007. Isolation and characterization of novel human parechovirus from clinical samples. Emerg. Infect. Dis. **13:**889–895.
35. **Yan, H., T. A. Nguyen, T. G. Phan, S. Okitsu, Y. Li, and H. Ushijima.** 2004. Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens from children in China. Kansenshogaku Zasshi **78:**699–709.

Brief Report

Subtyping and env C2/V3 Sequence Analysis of HIV-1 Isolated from HIV-Infected Children Hospitalized in Children Hospital 1, Vietnam during 2004–2005

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Summary

A molecular epidemiological study was conducted on 104 HIV-1 strains isolated from HIV-infected children hospitalized in Children Hospital 1 in Ho Chi Minh City, Vietnam during 2004–2005. Genetic subtyping based on env C2/V3 sequences revealed that CRF01-AE was the sole circulating recombinant form found in this study. Sequence analysis of the V3 loop showed that GPGQ tetramer was the most common V3 loop core motif identified in the HIV-1 strains studied (89.5%). The findings raise great concern about HIV-infected children in Vietnam and provide up-to-date molecular epidemiological information of HIV-1 circulating in Vietnam during the study period.

Key words: HIV-1, HIV-infected children, Vietnam.

Introduction

Despite the recent success of anti-HIV-1 therapy in controlling disease progression, HIV type-1 infection remains one of the most serious infectious diseases across the world. According to the Joint United Nation Program on HIV/AIDS (UNAIDS), an estimated 33.2 millions of people had been living with HIV worldwide as of December 2007, of these, 2.5 millions were children under 15 years of age [1].

In Vietnam, the first case of HIV was diagnosed in 1990 and since then, prevalence has climbed steadily. The estimated number of people living with HIV in Vietnam had more than doubled between 2000 and 2005 from 120 000 to 260 000 [1]. Although newly acquired pediatric HIV infections have virtually been eliminated in the developed world, with mother-to-child transmission (MTCT) rates reduced to ~1–2% since the advent of highly active antiretroviral therapy (HAART), pediatric HIV infection still remains a significant public health problem in developing countries, such as Vietnam. The number of HIV-infected children seeking health facilities, especially Children's Hospital 1, a central pediatric hospital responsible for treatment of children in Southern Vietnam, has been on the increase recently.

This study aimed to describe briefly the population of HIV-1 infected children who were hospitalized in Children Hospital 1, Ho Chi Minh City, Vietnam during October 2004 to September 2005, to determine subtypes/circulating recombinant forms (CRFs) of detected HIV-1 strains, and also to characterize their env C2/V3 gene.

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Materials and Methods

With ethical approval obtained from the Scientific Research Board of Children Hospital 1 in Vietnam and from the Ethical Committee of the University of Tokyo, Japan, the study was conducted on HIV-infected patients hospitalized in Children Hospital 1, Ho Chi Minh City, Vietnam during October 2004 to September 2005. After obtaining informed consent from the parents who signed permission for their children to participate in the study, whole blood samples were collected from 104 HIV-infected children during the study period. First, proviral HIV DNA was extracted from stored whole blood samples using DNAeasy Tissue Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. HIV-1 env C2/V3 region was then amplified by nested PCR using two sets of primers, the outer primer pair: 5′v3I, 5′-ATGCACACATGGAA TTAAGCCAGT-3′ and 3′v3I, 5′-TTGCAATAGAA AAATTCCCCTCTACAA-3′ (bases 6962–6985, and 7355–7381), and the inner primer pair: 5′v3II, 5′-TCAATTGCTGTTAAATGGCAGTCTAGCA-3′ and 3′v3II, 5′-TGCATTGTAATTTCTAGATCTCC TCCTGA-3′ (bases 6995–7022, and 7317–7345) [2]. The env C2/V3 PCR products were purified on QIAmp columns (Qiagen, Tokyo, Japan) and sequenced using the inner primers. Sequences were aligned with HIV-1 strains described previously of various subtypes available in GenBank databases. Tree was drawn using MEGA 3.1 program [3]. For HIV-1 subtyping, either sequence analysis of the env C2/V3 gene or PCR subtyping using subtype-specific primers as described previously [2] was used.

Results and Discussion

Of 104 infants and children enrolled in the study, 46 (44.2%) were male and 58 (55.8%) were female. Age distribution ranged from 1.5 months to 5 years, however, more than a half of patients were infants less than 6 months of age (57/104, 54.8%). For geographical distribution, the patients were from 21 provinces located in a half part of Southern Vietnam (data not shown). Of these, the three most industrialized provinces with largest numbers of patients were Ho Chi Minh City (44/104, 42.3%), Vung Tau (16/104, 15.3%) and Dong Nai (11/104, 10.6%). None of the patients was treated with anti-HIV drugs during their hospitalization. It is noted that a history of blood transfusion was recorded in two cases due to hemophilia or traffic accident. Although no direct evidence of HIV infection related to the history of blood transfusion was established in the two cases, the finding raises more concern about the safety of blood supply systems from HIV in Vietnam.

For subtyping, CRF01-AE was the sole circulating recombinant form found in 104 Vietnamese HIV-1 strains. The CRF01-AE was determined in 67 strains by using sequence analysis of env C2/V3 region, and that of the 37 remaining strains was identified by PCR subtyping due to the high cost of sequencing. The HIV-1 CRF01-AE viruses were also largely predominant found in cross-sectional molecular epidemiological studies previously conducted in Vietnam since 1996 [4–12]. The finding demonstrates that CRF01-AE is stably circulating in Vietnam.

Sequence analysis of the V3 loop showed that GPGQ tetramer was the most common V3 loop core motif identified in the HIV-1 strains studied (89.5%). Other motifs at the top of the V3 loop were GPGK (6%) and GPGR (4.5%). The motifs were preceded by either an isoleucine (I) (82.1%) or a methionine (M) (13.4%), valine (V) (3%), phenylalanine (F) (1.5%) residue (data not shown). The I residue is observed in reference CRF01-AE strains in Asia countries; while the M residue and V residue, which were only found in the north of Vietnam in previous studies [4, 6, 10], were two amino-acid substitutions also identified in Southern Vietnam.

In the recent past, a study estimating the global and regional distribution of HIV-1 subtypes and recombinants in the period 2000–2004 was conducted [13]. The study combined molecular epidemiology data on HIV-1 subtype distribution in individual countries with WHO/UNAIDS estimates of HIV prevalence. This work generated important insights into the global spread of HIV variants and was found very useful by many people in the HIV field. The present study contributes useful data for new analyses in detecting trends in regional and global HIV subtype distribution.

In addition, this is the first study describing a special and sensitive population directly affected by HIV/AIDS epidemic in Vietnam, HIV-infected infants and children. It is also noted that all of the HIV-infected patients in this study did not receive antiretroviral drugs during the study period due to non-availability of anti-HIV drugs. This points to a real HIV-infected population in need of anti-HIV therapy and raises great concern about HIV-infected children in Vietnam.

References

1. UNAIDS. UNAIDS/WHO 2007 AIDS epidemic update. Geneva: United Nations, www.UNAIDS.org (28 December 2007, date last accessed).
2. Yagyu F, Okitsu S, Tanamoto K, Ushijima H. Determination of HIV-1 subtypes (A-D, F, G, CRF01_AE) by PCR in the transmembrane region (gp41) with novel primers. J Med Virol 2005;76:16–23.
3. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5: 150–163.
4. Caumont A, Lan NT, Uyen NT, *et al.* Sequence analysis of env C2/V3, gag p17/p24, and pol protease regions of 25 HIV type 1 isolates from Ho Chi Minh

City, Vietnam. AIDS Res Hum Retroviruses 2001;17: 1285–1291.
5. Kato K, Kusagawa S, Motomura K, *et al.* Closely related HIV-1 CRF01_AE variant among injecting drug users in northern Vietnam: evidence of HIV spread across the Vietnam-China border. AIDS Res Hum Retroviruses 2001;17:113–123.
6. Kato K, Shiino T, Kusagawa S, *et al.* Genetic similarity of HIV type 1 subtype E in a recent outbreak among injecting drug users in northern Vietnam to strains in Guangxi Province of southern China. AIDS Res Hum Retroviruses 1999;15:1157–1168.
7. Lan NT, Recordon-Pinson P, Hung PV, *et al.* HIV type 1 isolates from 200 untreated individuals in Ho Chi Minh City (Vietnam): ANRS 1257 Study. Large predominance of CRF01_AE and presence of major resistance mutations to antiretroviral drugs. AIDS Res Hum Retroviruses 2003;19:925–928.
8. Menu E, Truong TX, Lafon ME, *et al.* HIV type 1 Thai subtype E is predominant in South Vietnam. AIDS Res Hum Retroviruses 1996;12:629–633.
9. Nerurkar VR, Nguyen HT, Dashwood WM, *et al.* HIV type 1 subtype E in commercial sex workers and injection drug users in southern Vietnam. AIDS Res Hum Retroviruses 1996;12:841–843.
10. Nerurkar VR, Nguyen HT, Woodward CL, *et al.* Sequence and phylogenetic analyses of HIV-1 infection in Vietnam: subtype E in commercial sex workers (CSW) and injection drug users (IDU). Cell Mol Biol (Noisy-le-grand) 1997;43:959–968.
11. Nguyen TH, Masquelier B, Pham VH, *et al.* Further characterization of HIV-1 isolates from Ho Chi Minh City, Vietnam. J Acquir Immune Defic Syndr Hum Retrovirol 1999;20:93–95.
12. Tran TT, Maljkovic I, Swartling S, *et al.* HIV-1 CRF01_AE in intravenous drug users in Hanoi, Vietnam. AIDS Res Hum Retroviruses 2004;20: 341–345.
13. Hemelaar J, Gouws E, Ghys PD, *et al.* Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 2006;20:W13–W23.

Novel Human Parechovirus, Sri Lanka

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Of 362 fecal samples collected from children with acute gastroenteritis in Sri Lanka during 2005–2006, 30 (8.3%) were positive for human parechovirus (HPeV) by reverse transcription–PCR. A novel HPeV, designated as HPeV10, was identified in 2 samples by sequence analysis of the viral protein 1 gene of the detected HPeVs.

Parechoviruses are small, nonenveloped, positive-sense, single-stranded RNA viruses belonging to the large family of *Picornaviridae*, a highly diverse family of important pathogens of humans and animals. The genus *Parechovirus* is composed of 2 species: *Ljungan virus*, isolated from bank voles (*1*), and *human parechovirus* (HPeV), a frequent human pathogen. The HPeV genome is ≈7.3 kb long and contains a large open reading frame coding for a single polyprotein. The polyprotein is cleaved posttranslationally into 3 structural proteins (viral protein [VP] 0, VP3, and VP1) and 7 nonstructural proteins (2A–2C and 3A–3D) (*2*,*3*).

Previous findings have shown the genetic variability of HPeVs, and the number of newly identified HPeV genotypes has been on the increase (*4*–*6*). To date there have been 9 published HPeV types assigned as types 1–8 and 14 (www.picornaviridae.com/parechovirus/hpev/hpev.htm). We identified a novel HPeV designated as HPeV10 that was detected in the stool samples of children in Sri Lanka who had acute gastroenteritis.

The Study

We used reverse transcription–PCR to screen 362 fecal samples collected from child inpatients with acute gastroenteritis at a hospital in Kandy, Sri Lanka, during September 2005 through August 2006 for HPeV. Informed consent was obtained from the mothers of all enrolled patients. The study was approved by the University of Peradeniya's Committee on Research and Ethical Review. Reverse transcription was performed by using random primer, and PCR was conducted by using primers ev22(+) and ev22(–) to amplify a 270-bp PCR product of the 5′ untranslated region (*7*).

For genotyping, samples positive for HPeV by the screening PCR were subjected to a 2-step PCR to amplify the VP1 sequence. The first PCR was done by using 2 newly developed primers, Cap-parEcho-F (5′-TCHACWTGGATGMGRAARAC-3′) and Cap-parEcho-R (5′-TCYARYTCACAYTCYTCYTC-3′), which were designed outside the VP1 region, whereas the nested PCR was performed by using the inner primer pair, VP1-parEchoF1 and VP1-parEchoR1, described by Benschop et al. (*8*). The PCR amplicons of the VP1 gene were purified and sequenced in both directions by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA, USA). The inner 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.).

Comparison analysis of the VP1 sequence was conducted between the obtained HPeV strains and reference HPeV strains of the 9 defined genotypes (HPeV1-8 and HPeV14) available in the GenBank database. The sequence data and the phylogenesis were analyzed by using BioEdit version 7.0.5 (www.mbio.ncsu.edu/BioEdit/bioedit.html). A parsimony analysis was also conducted by using MEGA version 3.1 to determine the evolutionary relationship among studied sequences (*9*). The method was performed using close-neighbor interchange with a random option and with 500 bootstrap repetitions.

Of the 362 samples tested, 30 were positive for HPeV; detection rate was 8.3%. Of these, 12 isolates were selected for amplification and sequencing of the VP1 gene. Ten of the 12 sequenced strains were of known and well-characterized genotypes (genotype 1, 7 samples; genotype 4, 3 samples). These strains were not further analyzed. Two remaining strains (LK-103 and LK-106, accession nos. GQ402515 and GQ402516, respectively) showed VP1 sequences that clustered together with none of the known 9 HPeV genotypes (HPeV1-8, 14) in the phylogenetic analysis (Figure). Nucleotide and amino acid similarities between these 2 strains were 94.4% and 99.5%, respectively (data not shown).

Identical matrix analysis of VP1 nucleotide sequences of the 2 strains from Sri Lanka and global reference strains of the 9 known genotypes available in GenBank databases was then performed. The results showed that the 2 studied strains had highest mean nucleotide and amino acid simi-

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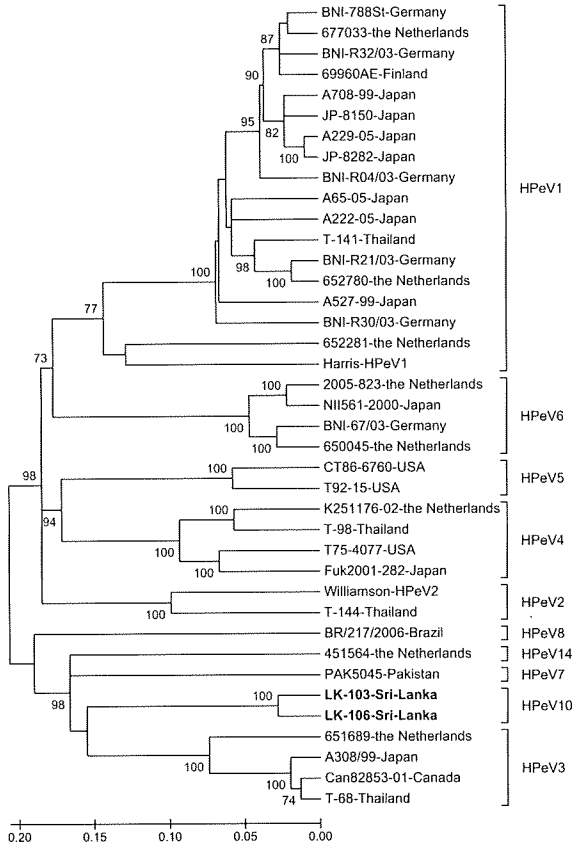

Figure. Phylogenetic tree constructed from nucleotide sequences of the structural viral protein gene of the strains studied and reference human parechovirus (HPeV) strains with 500 bootstrap repetitions. Percentage bootstrap values >70% are shown at the branch nodes. The studied HPeV strains are in **boldface**; their nucleotide sequences have been deposited in GenBank under accession nos. GQ402515 and GQ402516. Scale bar indicates nucleotide substitutions per site.

larities with HPeV3, 69.1% and 82.8%, respectively, and that the lowest mean nucleotide and amino acid similarities were found between the 2 studied strains and HPeV5, of 54.5% and 60.9%, respectively (Table). Therefore, these strains were expected to be classified into a new or previously unpublished HPeV (HPeV 9-13) genotype according to proposed criteria for assigning HPeV genotypes (*10*).

The VP1 sequences of the strains studied were submitted to the International Committee on Taxonomy of Viruses Picornavirus Study Group (www.picornastudygroup.com/types/index.html) to identify their genotype. These 2 strains were designated HPeV10 with their 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 BAN2004-10903 (M.S. Oberste et al., unpub. data).

The alignment of deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeVs genotypes 1–8 and 14 showed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for HPeV1 entry (*11*), was neither present in the strains studied nor among reference strains of HPeV3, HPeV7, HPeV8, and HPeV14 (*4–6,12*). Therefore, like HPeV3, HPeV7, HPeV8, and HPeV14, the lack of RGD motif in HPeV10 may imply that HPeV10 has an RGD-independent entry pathway.

Conclusions

We found HPeV in stool samples collected from hospitalized children in Sri Lanka who had acute gastroenteritis. The identified HPeV10 in this study was more closely genetically related to HPeV3 than to the remaining published HPeVs. Together with the unpublished findings of Oberste et al., this study provides basic data for future research into HPeV10. In addition, when taken together with other previous findings, our findings suggest that HPeV should be included in the spectrum of viruses for which routine screening is conducted among children with acute gastroenteritis.

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Table. Mean percentage nucleotide similarities between HPeV genotypes*

HPeV genotype	1	2	3	4	5	6	7	8	10	14
1										
2	64.1									
3	58.0	59.0								
4	63. 9	63.2	59.5							
5	61.6	60.7	53.5	65.8						
6	64.7	60.1	61.0	61.8	60.8					
7	57.3	59.9	63.8	58.7	56.0	54.1				
8	61.6	61.7	61.2	62.3	57.7	59.1	62.1			
10	60.5	56.8	69.1	60.9	54.5	55.9	66.0	63.6		
14	59.8	58.4	65.9	58.4	56.2	56.6	66.2	62.5	68.8	

*HPeV, human parechovirus.

Ms Pham is a physician and a PhD candidate at Tokyo University, Japan. Her current research interest focuses on less-explored viral pathogens of acute gastroenteritis in humans.

References

1. Niklasson B, Kinnunen L, Hornfeldt B, Horling J, Benemar C, Hedlund KO, et al. A new picornavirus isolated from bank voles (*Clethrionomys glareolus*). Virology. 1999;255:86–93. DOI: 10.1006/viro.1998.9557
2. Hyypia T, Horsnell C, Maaronen M, Khan M, Kalkkinen N, Auvinen P, et al. A distinct picornavirus group identified by sequence analysis. Proc Natl Acad Sci U S A. 1992;89:8847–51. DOI: 10.1073/pnas.89.18.8847
3. Stanway G, Kalkkinen N, Roivainen M, Ghazi F, Khan M, Smyth M, et al. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J Virol. 1994;68:8232–8.
4. Benschop K, Thomas X, Serpenti C, Molenkamp R, Wolthers K. High prevalence of human parechovirus (HPeV) genotypes in the Amsterdam region and identification of specific HPeV variants by direct genotyping of stool samples. J Clin Microbiol. 2008;46:3965–70. DOI: 10.1128/JCM.01379-08
5. Drexler JF, Grywna K, Stocker A, Almeida PS, Medrado-Ribeiro TC, Eschbach-Bludau M, et al. Novel human parechovirus from Brazil. Emerg Infect Dis. 2009;15:310–3. DOI: 10.3201/eid1502.081028
6. Li L, Victoria J, Kapoor A, Naeem A, Shaukat S, Sharif S, et al. Genomic characterization of novel human parechovirus type. Emerg Infect Dis. 2009;15:288–91. DOI: 10.3201/eid1502.080341
7. Joki-Korpela P, Hyypia T. Diagnosis and epidemiology of echovirus 22 infections. Clin Infect Dis. 1998;27:129–36. DOI: 10.1086/514615
8. Benschop KS, Schinkel J, Minnaar RP, Pajkrt D, Spanjerberg L, Kraakman HC, et al. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin Infect Dis. 2006;42:204–10. DOI: 10.1086/498905
9. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 2004;5:150–63. DOI: 10.1093/bib/5.2.150
10. Oberste MS, Michele SM, Maher K, Schnurr D, Cisterna D, Junttila N, et al. Molecular identification and characterization of two proposed new enterovirus serotypes, EV74 and EV75. J Gen Virol. 2004;85:3205–12. DOI: 10.1099/vir.0.80148-0
11. Boonyakiat Y, Hughes PJ, Ghazi F, Stanway G. Arginine-glycine-aspartic acid motif is critical for human parechovirus 1 entry. J Virol. 2001;75:10000–4. DOI: 10.1128/JVI.75.20.10000-10004.2001
12. Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K. Isolation and identification of a novel human parechovirus. J Gen Virol. 2004;85:391–8. DOI: 10.1099/vir.0.19456-0

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Emergence of New Norovirus Variants and Genetic Heterogeneity of Noroviruses and Sapoviruses in Children Admitted to Hospital With Diarrhea in Thailand

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Enteric caliciviruses, including noroviruses (NoVs) and sapoviruses (SaVs), are recognized as important etiologic agents of acute gastroenteritis with considerable genetic diversity. In this study, fecal specimens collected from 147 infants and young children admitted to hospital with acute gastroenteritis in 2005 in Chiang Mai, Thailand, were screened for NoVs and SaVs by reverse transcription-multiplex polymerase chain reaction (RT-multiplex PCR). Ten isolates (6.8%) belonged to NoV GII genogroup and five (3.4%) were positive for SaVs. GII/4 was the most predominant genotype of NoVs, followed by GII/15, GII/6, and GII/12. Surprisingly, NoVs GII/1, GII/2, GII/3, GII/7, and GII/16 genotypes, which were detected previously during the 2002–2004 survey, were not detected in 2005. Conversely, NoVs GII/6, GII/12, and GII/15 appeared in 2005 but were not detected during the 2002–2004 survey. The only genotype found to be common, as the most predominant genotype, in both surveys was NoV GII/4. Similar findings were also observed for SaVs, that is, the GI/2 and GIV detected during 2002–2004 were not detected in 2005, while GI/5 and GII/3 detected in 2005 were not detected previously during the 2002–2004 surveillance. In addition, comprehensive genetic evolutionary analysis of NoV GII/4 genotype demonstrated that the majority of GII/4 detected in this study (CMH002/05, CMH005/05, CMH042/05, and CMH083/05) were classified as new NoV variants and fell into subtype GII/4-d (Hunter'04-like cluster). Only one NoV GII/4 strain (CMH142/05) belonged to subtype GII/4-e. The data indicated heterogeneity and highly dynamic genotypic distribution of NoVs and SaVs circulating in children admitted to hospital with acute gastroenteritis in Chiang Mai, Thailand. ***J. Med. Virol. 82:289–296, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: norovirus; sapovirus; genetic variant; acute gastroenteritis; Thailand

INTRODUCTION

Viral gastroenteritis affects millions of people worldwide. The major etiologic agents of viral diarrhea include rotaviruses, astroviruses, adenoviruses, noroviruses (NoVs), and sapoviruses (SaVs) [Wilhelmi et al., 2003; Clark and McKendrick, 2004]. These viruses are transmitted mainly by the fecal–oral route by person-to-person contact or consumption of contaminated food and water. Among viruses causing diarrhea, rotaviruses are the most common and are the major cause of severe diarrhea in infants and young children worldwide [Parashar et al., 2006]. Recently, however, NoVs and SaVs have been recognized as the major causes of non-bacterial acute gastroenteritis in all age groups and also as important in childhood diarrhea. The NoVs and SaVs, members of the family *Caliciviridae*, are non-enveloped

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viruses with positive-sense single-stranded RNA genome [Green, 2007].

Because of a lack of a tissue culture system for NoVs and SaVs, characterization and classification of NoVs and SaVs are based mainly on reverse transcription-polymerase chain reaction (RT-PCR), genome sequencing, and phylogenetic analysis [Kageyama et al., 2004; Phan et al., 2007a]. The genetic analysis of the polymerase and capsid genes of NoVs and SaVs provided evidence for huge genetic diversity [Okada et al., 2007; Phan et al., 2007b]. The latest scheme for NoV nomenclature classifies NoVs into five separate genogroups (GI–GV) and at least 29 genotypes have been identified [Zheng et al., 2006]. Several epidemiological studies clearly indicate that NoV GII, particularly GII/4, is the most prevalent genotype in humans worldwide during the past decade [Hansman et al., 2004; Phan et al., 2006; Tseng et al., 2007]. Similarly, based on genetic analysis, SaVs have been classified into five genogroups (GI–GV) and at least 13 genetic clusters or genotypes have been reported. Of these, SaVs GI, GII, GIV, and GV are known to infect humans, whereas SaV GIII infects swine [Farkas et al., 2004; Wang et al., 2006; Hansman et al., 2007; Phan et al., 2007a]. NoVs that infect humans are highly diverse genetically. The diversity of NoVs is increased not only by recombination but also by point mutation, which leads to the emergence of new NoV variants. Since NoVs were isolated in the mid-1990s, new variants of the GII/4 genotype have been detected in numerous populations [Lopman et al., 2004; Ho et al., 2007; Johansen et al., 2008; Verhoef et al., 2008]. Recently, new NoV GII/4 variants, named Hunter'04-like and Farmington Hills-like clusters, have been reported as etiological agents of large epidemics of gastroenteritis in Australia, Japan, and Taiwan in 2004 and 2005 [Okada et al., 2005; Bull et al., 2006; Phan et al., 2006; Wu et al., 2006]. Phylogenetic evolutionary analysis by Okada et al. [2005] proposed temporal clustering of GII/4 subtypes into five major lineages (GII/4-a, GII/4-b, GII/4-c, GII/4-d, and GII/4-e). Recently, however, Nguyen et al. [2007] and Okada et al. [2007] reported one additional GII/4 subtype (GII/4-f) by comparing NoV GII/4 strains that had circulated in Vietnam and Japan, respectively.

In Thailand, even though few molecular epidemiological studies of NoVs and SaVs have been conducted, various genotypes have been reported in different epidemiological settings. The frequency of NoVs and SaVs detection rates ranged from 6.5% to 17.5% and 0.8% to 15.0%, respectively, in the hospitalized cases [Guntapong et al., 2004; Hansman et al., 2004; Veeravigrom et al., 2004; Malasao et al., 2008; Kittigul et al., 2009]. A study conducted in Chiang Mai, Thailand, from 2002 to 2004 demonstrated that NoVs and SaVs cocirculated in this area in children admitted to hospital with diarrhea at 14.1% and 1.2%, respectively [Khamrin et al., 2007]. In that study, only NoV GII was detected and GII/4 (62.8%) was the most predominant genotype, followed by GII/3 (20%), GII/1 (5.7%), GII/7 (5.7%), GII/2 (2.9%), and GII/16 (2.9%). In addition, only 3 of 248 specimens (1.2%) were positive for SaVs; one belonged to the GIV genogroup while the other two were genotypes GI/1 and GI/2.

The present study describes a follow-up survey of the incidence of NoVs and SaVs infections in pediatric patients admitted to hospital with diarrhea in Chiang Mai, Thailand, in 2005. The comprehensive genetic backgrounds of the NoVs and SaVs strains detected were determined by sequence and phylogenetic analyses.

MATERIALS AND METHODS

Specimen Collection

A total of 147 fecal specimens were collected from children hospitalized with acute gastroenteritis at the McCormick Hospital, Chiang Mai, Thailand. Only the patients who had a clinical diagnosis of acute gastroenteritis with watery diarrhea were included. The stool samples with mucus or blood were excluded from this study. The ages of the patients ranged from neonate up to 5 years old. The study period was 1 year, from January through December 2005.

RNA Extraction and RT-PCR

The RNA genomes of NoVs and SaVs were extracted from a supernatant of 10% fecal suspension using a QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of NoVs and SaVs in fecal specimens was detected by RT-PCR using the protocol described previously [Yan et al., 2003]. All of the detected viruses were genotyped by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR products of partial capsid genes of NoVs and SaVs were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer–Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer–Applied Biosystems, Inc.). The primers used for amplification of the partial capsid genes were also used as sequencing primers. The sequences obtained were compared to those of NoV and SaV reference strains deposited in the GenBank using BLAST searches. The genotypes of NoVs and SaVs were then classified using the clustering methods described previously by Kageyama et al. [2004] and Phan et al. [2007a].

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under accession numbers EU872281–EU872290 for NoV strains and EU872291–EU872295 for SaV strains.

TABLE I. Genotype Distributions of Noroviruses and Sapoviruses Detected in Pediatric Patients Admitted to Hospital With Acute Gastroenteritis in Chiang Mai, Thailand From 2002 to 2005

Year	No. of samples tested	No. of samples positive for viruses													
		Norovirus									Sapovirus				
		GII/1	GII/2	GII/3	GII/4	GII/6	GII/7	GII/12	GII/15	GII/16	GI/1	GI/2	GI/5	GII/3	GIV
2002[a]	43	—	—	—	4	—	1	—	—	—	—	—	—	—	—
2003[a]	45	1	1	1	1	—	—	—	—	1	1	—	—	—	1
2004[a]	160	1	—	5	16	—	2	—	—	—	—	1	—	—	—
2005	147	—	—	—	5	1	—	1	3	—	1	2	1	1	—

[a]The data were published previously by Khamrin et al. [2007].

RESULTS

Detection and Genotypic Distribution of Noroviruses

Of a total of 147 fecal specimens tested, 10 (6.8%) were positive for NoV and all belonged to the NoV GII genogroup. No mixed infections of NoV and SaV were found. Based on the analysis of partial sequence of capsid region, these NoV GII strains were classified further into four distinct genotypes, including GII/4, GII/6, GII/12, and GII/15 (Table I). NoV GII/4 was the most predominant genotype and accounted for the majority (5 out of 10) of NoV genotypes detected in the 2005 survey. NoV GII/15 was detected as the second most common genotype (3 out of 10). The prevalence rates of GII/6 and GII/12 genotypes were equal at 1 each out of 10.

Phylogenetic analysis of these NoVs revealed that GII/4 strains (CMH002/05, CMH005/05, CMH042/05, CMH083/05, and CMH142/05) detected in Chiang Mai, Thailand, in 2005 were closely related to the GII/4 strains detected in Japan during 2004–2005 (Chiba/04-1008/2004, Inba/050590/2005, Funabashi/050601/2005, and Sanbu/050878/2005) and formed a cluster separated from the GII/4 strain isolated from Bristol, England (Fig. 1). In addition, NoVs GII/6 (CMH010/05) and GII/12 (CMH145/05) were closely related to the NoV strains isolated from Ho Chi Minh City, Vietnam (HCMC204/2000 and HCMC91/2006), while the NoV GII/15 (CMH076/05, CMH104/05, and CMH113/05) formed a cluster as a sublineage of GII/15 separated from a cluster of Fayette/1999, Vannes L23/1999, and Saitama KU80a strains.

Over the last decade, the NoV GII/4 genotype has been recognized as one of the most common genotypes responsible for acute gastroenteritis caused by NoV worldwide [Hansman et al., 2004; Phan et al., 2006; Green, 2007; Tseng et al., 2007]. In Thailand, NoV GII/4 was also found to be the most predominant genotype since 2000 [Hansman et al., 2004; Khamrin et al., 2007; Malasao et al., 2008; Kittigul et al., 2009]. However, comprehensive genetic evolution of this NoV GII/4 genotype had never been analyzed for the Thai NoV samples. In this study, five strains of NoV GII/4 have been detected. As shown in Figure 2, one strain of NoV GII/4 (CMH142/05) belonged to subtype GII/4-e, while the majority of GII/4 detected in this study (CMH002/05, CMH005/05, CMH042/05, and CMH083/05) were classified into subtype GII/4-d. In addition, within subtype GII/4-d, the recent strains isolated in this study were identified as a new variant of GII/4 and fell into the same cluster as the new variant Hunter NoV reference strain. It is interesting that even though four other representative strains isolated previously in the same area in 2002 and 2003 (CMH038/02, CMH041/02, CMH043/02, and CMH043/03) also belonged to the GII/4-d subtype, they all fell into a different lineage from the NoVs GII/4 isolated in 2005 and clustered in a monophyletic branch with a Farmington Hills reference strain. It was of note that all of the GII/4 representative stains isolated previously in 2004 belonged to the GII/4-e subtype and appeared to be not related so closely to the variants causing epidemics in 2002–2003 and 2005. In this study, phylogenetic analysis for the NoV GII/4 subtype demonstrated that genetic drift, which led to the emergence of NoV variants in Thailand, occurred year on year.

Detection and Genotypic Distribution of Sapoviruses

In the same set of 147 fecal specimens tested for NoVs, 5 (3.4%) were positive for SaVs. The detection rate of SaVs was about half of that for NoVs (6.8%) (Table I). All of the SaV-positive samples were identified as a single SaV infection, with none being a mixed infection between NoV and SaV. Based on the analysis of partial sequence of capsid gene, one SaV isolate belonged to SaV GII/3, while the other four SaV strains belonged to the SaV GI genogroup. Of these, two were identified as GI/2, one was GI/1, and one was GI/5.

Phylogenetic analysis of these SaV strains (Fig. 3) revealed that SaV GI/2 (CMH024/05 and CMH082/05) detected in Chiang Mai, Thailand, in 2005 were closely related to the strain isolated from the same geographical area in 2004 (CMH013/04) as well as to the strain isolated from Chiba, Japan, in 2004 (Chiba/041413/2004). The SaV GI/5 (CMH087/05) was closely related to the GI/5 detected in Karachi, Pakistan, in 1990 (Karachi/1017/1990). The SaV GI/1 (CMH106/05) was most closely related to GI/1 Mc114 and CMH037/03, which were detected previously

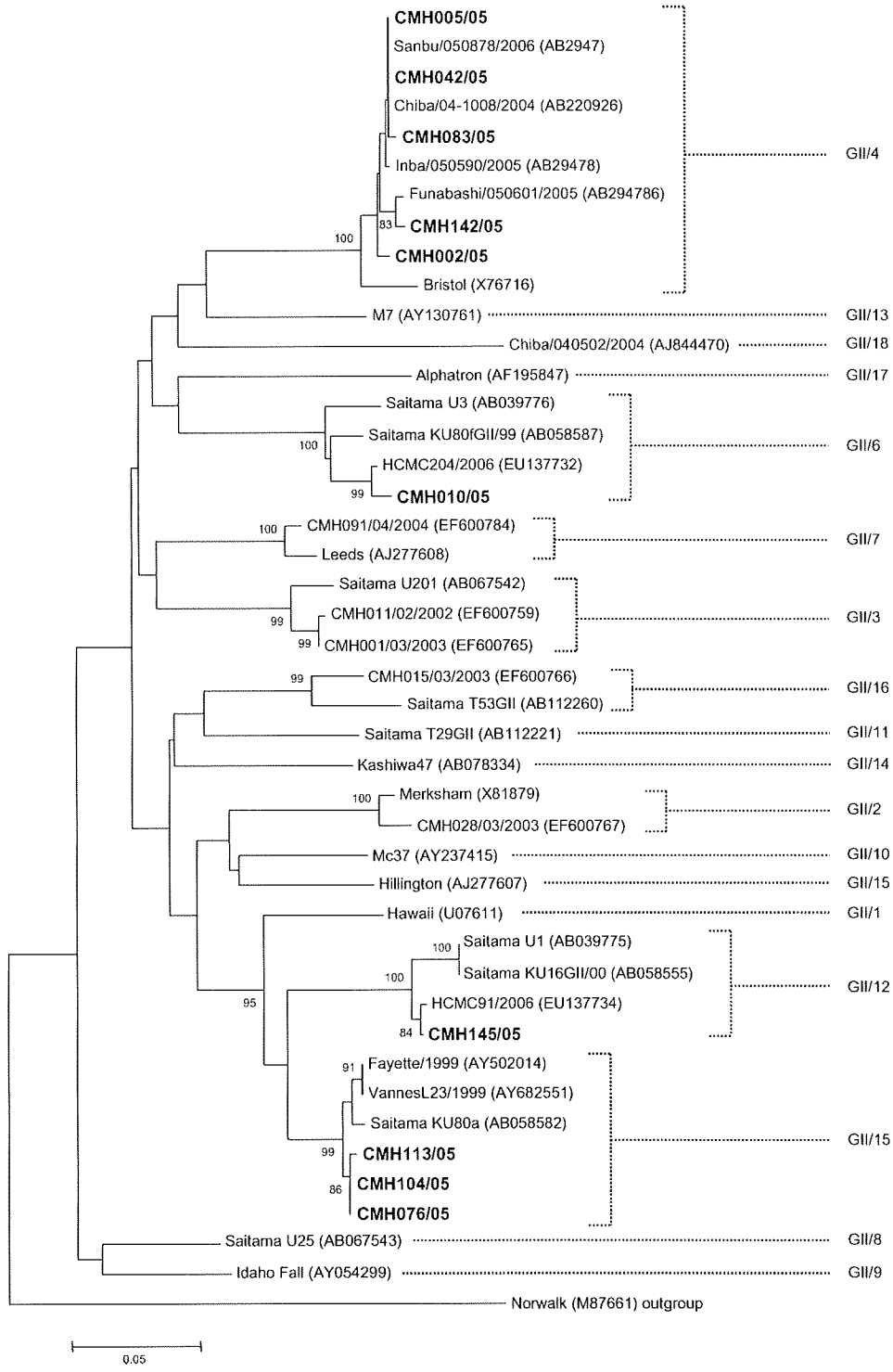

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoVs detected in pediatric patients admitted to hospital with acute gastroenteritis in Chiang Mai, Thailand, in 2005. The tree was constructed by the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The NoV strains detected in the present study are presented in boldface. The GenBank accession numbers of reference stains are given in parentheses.

in the same epidemic area (Chiang Mai) in 2001 and 2003, respectively, and to the Manchester strain, rather than to the GI/1 strain isolated previously in Sapporo, Japan. Moreover, the SaV GII/3 detected in the present study in 2005 (CMH047/05) clustered in a monophyletic branch with the SaV GII/3 strain (cruise ship/2000) isolated in the USA in 2000.

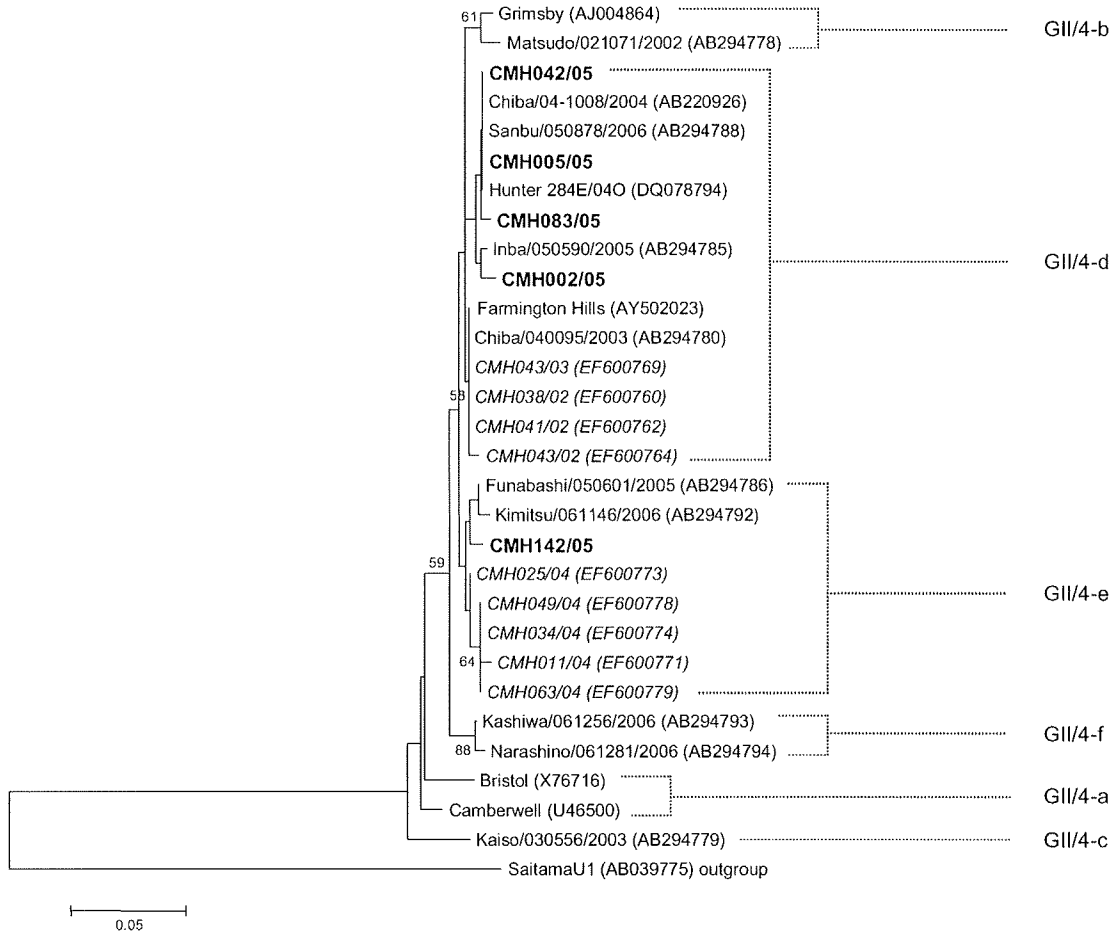

Fig. 2. Phylogenetic analysis of partial capsid sequences (238 bp) of NoVs for GII/4 subtype identification. The tree was constructed by the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The NoV strains detected in the present study are presented in boldface. The GII/4 strains reported previously in the year 2002–2004 are presented in italic. The GenBank accession numbers of reference stains are given in parentheses.

DISCUSSION

In nature, NoVs and SaVs, which infect humans, are very diverse genetically. Two major forces that drive the evolutions of NoVs and SaVs, point mutation and recombination, have been suggested [Bull et al., 2007; Reuter et al., 2008]. Epidemiological studies of NoV and SaV infections revealed that several different genotypes are circulating currently within the same or different geographical areas.

In Japan, consecutive survey studies of NoV and SaV infections in 2004–2005, in five different cities including Maizuru, Osaka, Sapporo, Saga, and Tokyo, demonstrated that NoV GII/4 was the most common genotype, followed by GII/3, GII/6, GI/1, and GII/1 genotypes. Interestingly, in the 2005–2006 survey, the distribution pattern of NoV genotypes was changed dramatically. The NoV GII/3 was detected as the most predominant genotype while GII/4 was detected as the second most common genotype, followed by GII/6, GII/1, GII/2, and GII/7. In addition, the emergence of rare SaV genotype and intragenotype recombination was also reported [Phan et al., 2007a,b]. The data from these studies indicate that the distribution of NoV and SaV genotypes circulating in Japan varies over time.

In Thailand, the prevalence of NoV and SaV infections was first studied in Chiang Mai during the period of 2000–2001 [Hansman et al., 2004]. The prevalence of NoV and SaV infections was 8.6% and 4.8%, respectively. Thereafter, Khamrin et al. [2007] demonstrated in the survey of 2002–2004 that the prevalence of NoV infection had increased from 8.6% to 14.1%, whereas SaV had decreased from 4.8% to 1.2%. In the same study, NoV GII/4 was shown to be the most predominant genotype and accounted for 62.8%, followed by GII/3 (20%), GII/1 and GII/7 (each at 5.7%), and GII/2 and GII/16 (each at 2.9%). It is important to emphasize that the distribution of NoV genotypes circulated in this geographical area has changed dramatically during the past 5 years. It appears that only NoVs GII (GII/1, GII/2, GII/3, GII/7, and GII/16) were detected during the 2002–2004 surveillance [Khamrin et al., 2007], whereas NoV GI was not detected even though both NoVs GI (GI/3, GI/7, and GI/8) and GII (GII/7, GII/8, and GII/10)

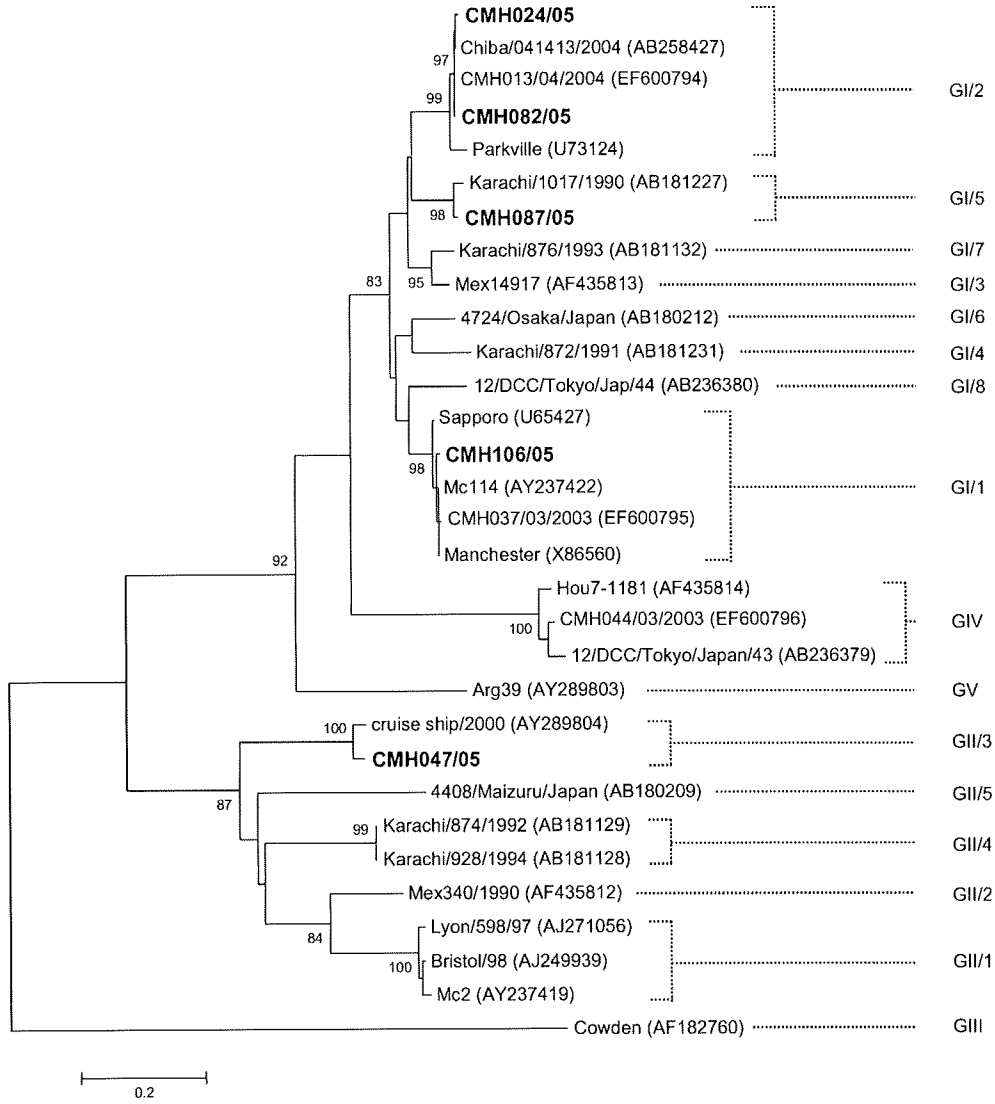

Fig. 3. Phylogenetic analysis of partial capsid sequences of SaVs detected in pediatric patients admitted to hospital with acute gastroenteritis in Chiang Mai, Thailand, in 2005. The tree was constructed by the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The SaV strains detected in the present study are presented in boldface. The GenBank accession numbers of reference strains are given in parentheses.

were detected previously during the 2000–2001 survey [Hansman et al., 2004]. The disappearance of NoV GI is confirmed in the present study, in which NoV GI remained undetectable in the 2005 epidemic season. Furthermore, several other genotypes of NoV GII (GII/6, GII/12, and GII/15) emerged, and many NoV GII strains (GII/1, GII/2, GII/3, GII/7, and GII/16) which were detected previously during the 2002–2004 survey disappeared in 2005. Additionally, it is interesting to point out that no GII/3 strain was detected in the 2005 survey even though it was the second most predominant genotype during five consecutive years of surveillance during 2000–2004 [Hansman et al., 2004; Khamrin et al., 2007; Malasao et al., 2008].

The NoV GII/4 strains have been reported as the most predominant genotype not only in Japan and Thailand but also in many other countries [Bull et al., 2006; Tu et al., 2007; Buesa et al., 2008; Reuter et al., 2008]. This observation may be attributed to a unique characteristic of relatively high genomic mutation of NoV GII/4 compared to other GII genotypes [Lopman et al., 2004; Reuter et al., 2008]. Collective accumulation of point mutations of the genome sequence may accelerate the emergence of new variants of NoV GII/4 to which humans lack immunity, thereby allowing those variants to infect and to circulate in humans.

NoV GII/4 Hunter variant strains were first identified as a cause of NoV outbreaks in several institutional settings in New South Wales, Australia, in 2004 [Bull et al., 2006]. Genetic evolutionary analysis demonstrated that the 2004 New South Wales NoV strains formed their own cluster, termed the Hunter cluster,

within the GII/4 subtype and distinct from other NoV GII/4 sequences. Therefore, the Hunter NoV strains were defined as a new GII/4 variant. Later, the Hunter strains were included in the GII/4-d subtype, named Hunter'04-like cluster [Okada et al., 2007]. Thereafter, the NoV GII/4 Hunter'04-like subtype (GII/4-d) was reported to be associated with several outbreaks in many countries on different continents worldwide [Phan et al., 2006; Wu et al., 2006; Okada et al., 2007; Siebenga et al., 2007]. In the present study, we also report the detection of NoV GII/4 variants (Hunter'04-like) (CMH002/05, CMH005/05, CMH042/05, and CMH083/05) (Fig. 2) in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, in 2005, 1 year after the worldwide epidemics of 2004. The detection of NoV GII/4-d variants in several countries at almost the same time indicates that these new variants are spread widely and play a significant role as human pathogens associated with acute diarrhea.

Molecular techniques have revolutionized the diagnosis of enteric caliciviruses and led to the recognition of enteric caliciviruses that are responsible for the majority of non-bacterial gastroenteritis pathogens in humans of all age groups [Wilhelmi et al., 2003; Clark and McKendrick, 2004; Green, 2007]. However, little is known about SaV infection in Thailand. Only five studies of SaV infections in Thailand have been reported [Guntapong et al., 2004; Hansman et al., 2004; Khamrin et al., 2007; Malasao et al., 2008; Kittigul et al., 2009]. Data from those reports indicate that SaVs circulating in Thailand are also diverse genetically and are less common, compared to NoV infection, with detection rate ranging from 0.8% to 15.0%. In the present study, the SaV detection rate in Chiang Mai in 2005 was higher (3.4%) than in the 2002–2004 survey (1.2%). It should be noted, however, that only pediatric patients with a clinical diagnosis of acute gastroenteritis with watery stool samples were included in the present study, while stool samples with mucus or blood were excluded.

In conclusion, the present study describes the emergence of new variants of NoVs and describes the genetic heterogeneity and changing pattern of genotypic distribution of NoV and SaV infections in children admitted to hospital with acute diarrhea in Thailand.

REFERENCES

Buesa J, Montava R, Abu-Mallouh R, Fos M, Ribes JM, Bartolomé R, Vanaclocha H, Torner N, Domínguez A. 2008. Sequential evolution of genotype GII.4 norovirus variants causing gastroenteritis outbreaks from 2001 to 2006 in Eastern Spain. J Med Virol 80: 1288–1295.

Bull RA, Tu ET, McIver CJ, Rawlinson WD, White PA. 2006. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. J Clin Microbiol 44:327–333.

Bull RA, Tanaka MM, White PA. 2007. Norovirus recombination. J Gen Virol 88:3347–3359.

Clark B, McKendrick MA. 2004. Review of viral gastroenteritis. Curr Opin Infect Dis 17:461–469.

Farkas T, Zhong WM, Jing Y, Huang PW, Espinosa SM, Martinez N, Morrow AL, Ruiz-Palacios GM, Pickering LK, Jiang X. 2004. Genetic diversity among sapoviruses. Arch Virol 149:1309–1323.

Green KY. 2007. The noroviruses. In: Knipe DM, Howley PM, editors. Fields virology. 5th edition. Philadelphia: Lippincott Williams & Wilkins. pp 949–979.

Guntapong R, Hansman GS, Oka T, Ogawa S, Kageyama T, Pongsuwanna Y, Katayama K. 2004. Norovirus and sapovirus infections in Thailand. Jpn J Infect Dis 57:276–278.

Hansman GS, Katayama K, Maneekarn N, Peerakome S, Khamrin P, Tonusin S, Okitsu S, Nishio O, Takeda N, Ushijima H. 2004. Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand. J Clin Microbiol 42:1305–1307.

Hansman GS, Oka T, Katayama K, Takeda N. 2007. Human sapoviruses: Genetic diversity, recombination, and classification. Rev Med Virol 17:133–141.

Ho EC, Cheng PK, Lau AW, Wong AH, Lim WW. 2007. Atypical norovirus epidemic in Hong Kong during summer of 2006 caused by a new genogroup II/4 variant. J Clin Microbiol 45:2205–2211.

Johansen K, Mannerqvist K, Allard A, Andersson Y, Burman LG, Dillner L, Hedlund KO, Jönsson K, Kumlin U, Leitner T, Lysén M, Thorhagen M, Tiveljung-Lindell A, Wahlström C, Zweygberg-Wirgart B, Widell A. 2008. Norovirus strains belonging to the GII.4 genotype dominate as a cause of nosocomial outbreaks of viral gastroenteritis in Sweden 1997–2005 arrival of new variants is associated with large nation-wide epidemics. J Clin Virol 42:129–134.

Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K. 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol 42:2988–2995.

Khamrin P, Maneekarn N, Peerakome S, Tonusin S, Malasao R, Mizuguchi M, Okitsu S, Ushijima H. 2007. Genetic diversity of noroviruses and sapoviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. J Med Virol 79:1921–1926.

Kittigul L, Pombubpa K, Taweekate Y, Yeephoo T, Khamrin P, Ushijima H. 2009. Molecular characterization of rotaviruses, noroviruses, sapovirus, and adenoviruses in patients with acute gastroenteritis in Thailand. J Med Virol 81:345–353.

Lopman B, Vennema H, Kohli E, Pothier P, Sanchez A, Negredo A, Buesa J, Schreier E, Reacher M, Brown D, Gray J, Iturriza M, Gallimore C, Bottiger B, Hedlund KO, Torvén M, von Bonsdorff CH, Maunula L, Poljsak-Prijatelj M, Zimsek J, Reuter G, Szücs G, Melegh B, Svennson L, van Duijnhoven Y, Koopmans M. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet 363:682–688.

Malasao R, Maneekarn N, Khamrin P, Pantip C, Tonusin S, Ushijima H, Peerakome S. 2008. Genetic diversity of norovirus, sapovirus, and astrovirus isolated from children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. J Med Virol 80:1749–1755.

Nguyen TA, Khamrin P, Takanashi S, Le Hoang P, Pham Le K, Hoang KT, Satou K, Masuoka Y, Okitsu S, Ushijima H. 2007. Evaluation of immunochromatography tests for detection of rotavirus and norovirus among Vietnamese children with acute gastroenteritis and the emergence of a novel norovirus GII.4 variant. J Trop Pediatr 53:264–269.

Okada M, Ogawa T, Kaiho I, Shinozaki K. 2005. Genetic analysis of noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. J Clin Microbiol 43:4391–4401.

Okada M, Ogawa T, Yoshizumi H, Kubonoya H, Shinozaki K. 2007. Genetic variation of the norovirus GII-4 genotype associated with a large number of outbreaks in Chiba prefecture, Japan. Arch Virol 152:2249–2252.

Parashar UD, Gibson CJ, Bresse JS, Glass RI. 2006. Rotavirus and severe childhood diarrhea. Emerg Infect Dis 12:304–306.

Phan TG, Takanashi S, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Sugita K, Nishimura T, Yamamoto A, Yagyu F, Okitsu S, Maneekarn N, Ushijima H. 2006. Detection and genetic characterization of norovirus strains circulating among infants and children with acute gastroenteritis in Japan during 2004–2005. Clin Lab 52:519–525.

Phan TG, Trinh QD, Yagyu F, Okitsu S, Ushijima H. 2007a. Emergence of rare sapovirus genotype among infants and children with acute gastroenteritis in Japan. Eur J Clin Microbiol Infect Dis 26:21–27.

Phan TG, Khamrin P, Quang TD, Dey SK, Takanashi S, Okitsu S, Maneekarn N, Ushijima H. 2007b. Emergence of intragenotype recombinant sapovirus in Japan. Infect Genet Evol 7:542–546.

Reuter G, Pankovics P, Szucs G. 2008. Genetic drift of norovirus genotype GII-4 in seven consecutive epidemic seasons in Hungary. J Clin Virol 42:135–140.

Siebenga JJ, Vennema H, Duizer E, Koopmans MP. 2007. Gastroenteritis caused by norovirus GGII.4, The Netherlands, 1994–2005. Emerg Infect Dis 13:144–146.

Tseng FC, Leon JS, MacCormack JN, Maillard JM, Moe CL. 2007. Molecular epidemiology of norovirus gastroenteritis outbreaks in North Carolina, United States: 1995–2000. J Med Virol 79:84–91.

Tu ET, Nguyen T, Lee P, Bull RA, Musto J, Hansman G, White PA, Rawlinson WD, McIver CJ. 2007. Norovirus GII.4 strains and outbreaks, Australia. Emerg Infect Dis 13:1128–1130.

Veeravigrom M, Theamboonlers A, Poovorawan Y. 2004. Epidemiology and clinical manifestation of rotavirus and norwalk-like viruses in Thai children. J Med Assoc Thai 87:50–54.

Verhoef L, Depoortere E, Boxman I, Duizer E, van Duynhoven Y, Harris J, Johnsen C, Kroneman A, Le Guyader S, Lim W, Maunula L, Meldal H, Ratcliff R, Reuter G, Schreier E, Siebenga J, Vainio K, Varela C, Vennema H, Koopmans M, Food Borne Viruses in Europe Network. 2008. Emergence of new norovirus variants on spring cruise ships and prediction of winter epidemics. Emerg Infect Dis 14:238–243.

Wang QH, Souza M, Funk JA, Zhang W, Saif LJ. 2006. Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assays. J Clin Microbiol 44:2057–2062.

Wilhelmi I, Roman E, Sánchez-Fauquier A. 2003. Viruses causing gastroenteritis. Clin Microbiol Infect 9:247–262.

Wu FT, Oka T, Katayama K, Wu HS, Donald Jiang DS, Miyamura T, Takeda N, Hansman GS. 2006. Genetic diversity of noroviruses in Taiwan between November 2004 and March 2005. Arch Virol 151:1319–1327.

Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. 2003. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 114:37–44.

Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. 2006. Norovirus classification and proposed strain nomenclature. Virology 346:312–323.

SEASONAL TREND AND SEROTYPE DISTRIBUTION OF ROTAVIRUS INFECTION IN JAPAN, 1981–2008

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Abstract: A total of 10,917 fecal specimens from infants and children with gastroenteritis in seven different regions of Japan in the last 3 decades were examined for rotavirus. We observed that the rotavirus peak shifted gradually from January to April (winter to early spring) during 17 seasons and the G1P[8] combination was the most predominant genotype in the last 28 years in Japan.

Key Words: rotavirus, seasonality, G-type, P-type, Japan

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Rotavirus exhibits distinct seasonality, and has been known as "winter diarrhea" in some parts of the world. "Winter gastroenteritis" and "winter diarrhea" were recognized illnesses of early childhood before rotavirus was identified and found to be their cause.[1] The most recent review of the global seasonality of rotavirus infections concluded that the winter seasonality of rotavirus infections is too simple a generalization. In developed countries with temperate climates, peak incidence is in winter; however, in developing countries with tropical or subtropical climates, the virus circulates year-round.[2–4] In Japan, there have been many studies of acute gastroenteritis in children since 1910, even before the discovery of rotavirus. Kasei-shoni-kolera (pseudocholera infantum) or Toki-nyuyoji-gerisho (infantile diarrhea in winter) are forms of gastroenteritis with symptoms of vomiting, slight fever, dehydration, and whitish, watery stools,[5] generally occurring in infants and young children.

Rotaviruses are members of the family *Reoviridae* classified into 7 groups (A–G) on the basis of distinct antigenic and genetic properties. Of these, group A rotaviruses are the most important, being a major cause of severe gastroenteritis in infants and young children worldwide.[4] On the basis of neutralization assay and/or sequence analysis, the VP7 and VP4 proteins and/or corresponding encoding genes of group A rotaviruses have been classified into G- and/or P-serotypes and/or genotypes, and a total of 15 G and 26 P-genotypes have been documented. Although numerous G/P combinations have been reported, a recent review revealed G1P[8], G2P[4], G3P[8], and G4P[8] as 4 globally important rotavirus G/P combinations.[4]

The objectives of this study were: to determine the seasonal trends and serotype distribution of rotavirus infection in Japan in the last 28 years.

METHODS

A total of 10,917 fecal specimens were collected from infants and children with acute gastroenteritis in 7 regions from the north to the south of Japan (Sapporo, Tokyo, Maizuru, Osaka, Saga, Kagawa and Kurume) between 1981 and 2008. We used the combined rotavirus serotyping data from 1981 to 2008[6–8] for analysis of long-term trend of rotavirus infection in Japan. But for seasonal trends of rotavirus infection, we used data from 1981 to 1986 and 1996 to 2008. Screening for rotavirus was conducted by RNA-PAGE, latex agglutination method, enzyme linked immunosorbant assay (1981–1994), by reverse transcriptase polymerase chain reaction (RT-PCR), latex agglutination (1995–2006), and by RT-PCR immunochromatography (2006–2008). Rotavirus was further characterized for serotypes according to the instructions provided in the enzyme linked immunosorbant assay kit insert with serotype-specific monoclonal antibodies (Mabs) (Serotec Company, Japan) and by RT-PCR with G and P serotype-specific primers. Monthly distribution of rotavirus was defined after adjusting the epidemic curves according to a 3-month unweighted moving average (mean).[2] The "peak" month during the rotavirus season was then defined as that, during which the greatest number of rotavirus-positive specimens were collected. We analyzed the laboratory-confirmed rotavirus cases between 1981 and 2008. Seasonal distribution data of rotavirus during 1987–1995 was not available. We used December as the start month of the rotavirus season for convenience, given that the annual rotavirus season began between November and December during the study period.

To ascertain any shift in the peak, Mann-Whitney *U* Test was used for analysis of relationship between duration from December to the peak month (beginning peak duration) during the 17 seasons. Furthermore we divided our study into 3 periods and compared mean beginning peak duration among 3 periods (1981/1982–1985/1986, 1996/1997–2001/2002, and 2002/2003–2007/2008). Interperiod differences in the mean were tested by 1-way analysis of variance followed by Mann-Whitney *U* Test. All calculations were performed with SPSS for Windows version 13.0, and significance was set at <0.05.

RESULTS

Between 1981 and 2008, 10,917 stool samples from 17,348 patients under 15 years of age with acute gastroenteritis were received for microbiologic study. Rotavirus antigen was detected in 2054 patients (18.8%). The annual number of detected patients with rotavirus in 1981–1986 was 27, 112, 78, 91 and 52, respectively, and in 1996–2008 was 98, 126, 195, 210,176, 137, 150, 83, 82, 117, 123, and 100, respectively (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/A213). The percentage of rotavirus infections detected in patients with rotavirus gastroenteritis was similar in the 3 study periods: 18.4% in the 1981–986 period, 18.9% in the 1996–2002 period, and 19.1% in the 2003–2008 period. The highest detection rates of rotavirus were in April (538) followed by March (507) and February (348) and the lowest detection rates were in September (0) followed by October[2] and August.[4] Seasonal increase in rotavirus diarrhea occurred annually in Japan (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/A213). Peak rotavirus activity occurred between December and January during 1981–1996, shifting to January to February during 1996–2002 ($P = 0.0001$) but peak was extended to March to April in 2003–2008 ($P = 0.0001$) (Table 1) (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/A213). The comparisons of

TABLE 1. The Comparisons of Rotavirus Positive Cases (Mean) in Each Month During 3 Periods

Month	1981–1986		1996–2002		2003–2008	
	Z	P	Z	P	Z	P
July vs. August	−1.000	0.317	−0.350	0.727	−1.477	0.140
August vs. September	0.000	1.000	−1.483	0.138	0.000	1.000
September vs. October	0.000	1.000	−1.000	0.317	−1.000	0.317
October vs. November	−1.964	0.050	−1.968	0.049	−0.738	0.461
November vs. December	−2.652	0.008	−0.890	0.373	−1.592	0.111
December vs. January	−0.730	0.465	−1.529	0.126	−1.451	0.147
January vs. February	−0.419	0.675	−1.446	0.148	−1.928	0.054
February vs. March	−1.567	0.117	−1.524	0.128	−2.727	0.006
March vs. April	−2.417	0.016	−0.320	0.749	−2.173	0.030
April vs. May	−1.063	0.288	−2.406	0.016	−2.887	0.004
May vs. June	−0.149	0.881	−2.173	0.030	−2.486	0.013
June vs. July	−0.149	0.881	−1.844	0.065	−1.800	0.072

Mann-Whitney *U* Test; *P* value <0.05 means significant.

rotavirus positive cases (mean) in each month during 3 periods showed that there is no significant difference among 3 periods from July to November. During the first period (1982–1986), the beginning of peak cases started from November. In the second period (1996–2002), the beginning of peak cases started from December to January while in the third period (2003–2008), the beginning of peak cases started from January to February (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/A213).

DISCUSSION

Many research groups in Japan have confirmed most of gastroenteritis cases to be caused by rotaviruses and noted that infection often appears related to the ambient temperature. Our analysis based on laboratory studies during the past 28 years shows that a temporal shift in peak rotavirus activity in Japan, from winter to early spring (from January to April), has occurred with statistical significance ($P = 0.0001$), in line with laboratory data from 7 regions of Japan for 1981 to 2008. As has been observed in other temperate regions of the world,[1–3] rotavirus infection was present throughout the year, but was more frequent in cold months. However, over the 28-year study period, important changes were observed in the seasonal pattern. We observed that rotavirus peak shifted gradually from January to April (winter to early spring) during 17 seasons. In most studies, seasonality did not vary much over long time periods, although variations in the timing of peak activity were reported.[1,3] The typical pattern of regular epidemics that appear in cold months in industrialized countries is similar to that observed in our region in the 1996–2008 period of this study. However, the seasonal pattern observed in the 1980s recalls the pattern reported in less-developed countries with a tropical climate.[1,4] Seasonality in disease incidence often reflects associations with weather factors.[2]

The coolest months (January and February) in winter have not changed, but annual average temperatures have increased gradually during the past 100 years with global warming in Japan,[2] apparently coinciding with a temporal shift in the peak rotavirus activity, from January to April. Our observations suggest a determining role for a single climatic factor, temperature, to explain the temporal trend.

Data of 1984 through 2006 previously published showed that rotavirus was a universal cause of acute gastroenteritis among Japanese infants and children.[6–8] During the period of 25 years, G1 increased gradually from 42.1% (1984–1985) to 86.0% (1998–1999), and became the predominant strain in Japan. However, a changed prevalence pattern of rotavirus G serotype was found in the year of 1999–2000 with an increase of G9 and G2 and a decrease of G1 (63.0%), although G1 remained the prevailing serotype. An unexpected decrease of G1 to 43.8% in 2000–2001 and 20.0% in 2001–2002 occurred. In contrast, G2 strains increased rapidly up to 33.3% and 37.8% in the same period. An exceptionally high frequency of rotavirus G3 was found at 29% during 2002/2003 and at 97.6% during 2003/2004. During the period of 2004/2005–2007/2008, G1 (around 58%) remained the predominant serotype among the Japanese pediatric population, with G2 second, and followed by G3, G9, and G4. On the other hand, P[8] (more than 80%) was the most predominant genotype in the most of the year, followed by P[4], P[9], P[6] and some other unusual P types. Regarding G and P combinations, G1P[8] combination was the most predominant genotype followed by G2P[4], G3P[8], G9P[8] were found in 92% of cases. Some unusual G and P combinations such as G1P[4], G2P[8], G3P[4], G3P[9], G1G3P[8], and G2G3P[8] were found during this periods. It was noteworthy to point out the shift of the dominant strain from G1 to G2 during 2001–2002. The overall pattern of serotypes was similar to those described in other countries of Asia.[4,8,9] It's clear that rotaviruses show great genomic diversity.

We found that rotavirus has been mostly associated with gastroeneteritis in infants and children less than 2 years of age in Japan. Over a 28-year period, the present study collected information on cases of rotavirus infection diagnosed and their epidemiological features in a specific geographic area and well-definded population, children under 15 years of age. The cases studied do not represent every case of rotavirus gastroeneteritis occurring in the study area.

REFERENCES

1. Cook SM, Glass RI, LeBaron CW, et al. Global seasonality of rotavirus infections. *Bull World Health Organ.* 1990;68:171–177.
2. Suzuki H, Sakai T, Tanabe N, et al. Peak rotavirus activity shifted from winter to early spring in Japan. *Pediatr Infect Dis J.* 2005;24:257–260.
3. Cilla G, Pérez-Trallero E, López-Lopategui MC, et al. Incidence, seasonality and serotypes of rotavirus in Gipuzkoa (Basque Country), Spain. A 14-year study. *Epidemiol Infect.* 2000;125:677–683.
4. Dey SK, Hayakawa Y, Rahman M, et al. G2 strain of rotavirus among infants and children, Bangladesh. *Emerg Infect Dis.* 2009;15:91–94.
5. Ito S. Pseudocholera infantum. *Jikazasshi.* 1910;125:751–757.
6. Zhou Y, Li L, Kim B, et al. Rotavirus infection in children in Japan. *Pediatr Int.* 2000;42:428–439.
7. Yoshinaga M, Phan TG, Nguyen TA, et al. Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan. *Arch Virol.* 2006;151:183–192.
8. Phan TG, Khamrin P, Quang TD, et al. Detection and genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan. *J Virol.* 2007;81:4645–4653.
9. Centers for Disease Control and Prevention. Rotavirus surveillance–worldwide, 2001–2008. *MMWR Morb Mortal Wkly Rep.* 2008;57:1255–1257.

Broad usage spectrum of G protein-coupled receptors as coreceptors by primary isolates of HIV

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Objective: HIV-1 can use various G protein-coupled receptors (GPCRs) in addition to CCR5 and CXCR4 as coreceptors; however, this type of HIV-1 infection has hardly been detected *in vivo*. The objective of this study was to elucidate the spectrum of GPCR usage by HIV-1 populations *in vivo*.

Design: CD4-expressing glioma cell line, NP-2/CD4, becomes highly susceptible to HIV-1 when the cells express GPCRs with coreceptor activities. This cell system was advantageous for detecting the inefficient use of GPCRs by HIV-1.

Methods: We developed NP-2/CD4/GPCR cells that express each of 23 GPCRs: 21 chemokine receptors (CCR1, CCR2b, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9B, CCR10, CCR11, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1, XCR1, D6, and DARC) and two other GPCRs (a formylpeptide receptor, FPRL1, and an orphan GPCR, GPR1). NP-2/CD4/GPCR cells were directly cocultured with HIV-1-positive peripheral blood lymphocytes and HIV-1 infection was detected.

Results: Primary HIV-1 isolates were obtained from NP-2/CD4/GPCR cells expressing CCR5, CXCR4, FPRL1, or GPR1 cocultured with 11 of 17 peripheral blood lymphocytes. Surprisingly, these isolates showed extremely expanded GPCR usage, such as CCR1, CCR3, CCR5, CCR8, CXCR4, D6, FPRL1, and GPR1 as coreceptors. We found that CCR9B, CCR10, and XCR1 also work as novel HIV-1 coreceptors.

Conclusion: FPRL1 and GPR1 have the potential to work as significant HIV-1 coreceptors *in vivo* next to CCR5 and CXCR4. HIV-1 populations that can use various GPCRs as coreceptors are already circulating *in vivo*, even in the early stage of HIV-1 infection.

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Keywords: AIDS, G protein-coupled receptor, HIV, infection, lentivirus, receptors

Introduction

One of the marked characteristics of HIV-1 is its genetic variability [1,2]. The high rate mutation of the HIV-1 genome is closely linked to its frequent phenotypic changes [3–5]. HIV-1 infection is mediated by CD4 cell and a coreceptor belonging to G protein-coupled receptors (GPCRs), including chemokine receptors (CKRs) [6]. Two CKRs, CCR5 and CXCR4, are believed to play major roles in HIV-1 infection *in vivo* [7,8]. HIV-1 strains that use CCR5 (i.e. R5 strain) infect lymphocytes in the early stage of HIV-1 infection [9]. During the progression to AIDS, HIV-1 strains that can use both CCR5 and CXCR4 as coreceptors (i.e. R5-X4

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strain) emerge. HIV-1 strains that efficiently use CXCR4 (i.e. X4 strain) then also emerge and expand the populations *in vivo* [10].

Along with the change of coreceptor use from CCR5 to CXCR4, HIV-1 probably expands GPCR usage [11]. Some primary HIV-1 isolates have been reported to use CCR2b [12], CCR3 [13], CCR8 [14], CXCR1 [15], CXCR2 [15], CXCR5 [16], CXCR6 [17], CXCR7/RDC1 [18], CX3CR1 [19], D6 [20], APJ [21], and GPR1 [22]; however, the roles of these coreceptors in HIV-1 infection *in vivo* have hardly been elucidated.

Some cell lines, such as CEM [23], GHOST3 [24], and MAGI [25], have been used to determine the coreceptor usages of HIV-1; however, these cells have already expressed CCR5, CXCR4, or GPR1, even though the levels are low [15,22,23]. To more sensitively detect GPCR usage as a coreceptor by HIV-1, we established an NP-2/CD4 cell line by transducing the CD4 gene into an HIV-1-resistant human glioma-derived cell line, NP-2 [15]. NP-2/CD4 cells transduced with a GPCR with coreceptor activity became susceptible to HIV-1 strains that use GPCR.

To elucidate the spectrum of GPCR usage by HIV-1 populations *in vivo*, we tried to isolate HIV-1 from peripheral blood lymphocytes (PBLs) of HIV-1-positive patients by coculturing them with NP-2/CD4 cells expressing each of 23 GPCRs (NP-2/CD4/GPCR cells). Primary HIV-1 isolates were obtained from NP-2/CD4/GPCR cells expressing CCR5, CXCR4, formyl-peptide receptor 1 (FPRL1), or GPR1. These isolates could use various GPCRs as coreceptors, including novel ones. Our results raised the possibility that HIV-1 populations with the potential to use various GPCRs as coreceptors are circulating *in vivo*.

Materials and methods

Cells

The human glioma cell line transduced with human CD4 gene, NP-2/CD4, was established as previously described [15]. Twenty-three sublines of NP-2/CD4 cells expressing each one of 23 GPCRs (see below) were cultured in Eagle's minimum essential medium (NISSUI Co. Ltd., Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS). Seventeen PBLs, 05JAGU-#01, #02, #03, #04, #05, #06, #07, #08, #09, #10, #11, #12, #13, #14, #15, #16, and #17 were isolated from the blood of 17 HIV-1-positive patients by Ficoll-Paque density gradient centrifugation and cultured in RPMI1640 medium (NISSUI Co. Ltd.) supplemented with 10% FCS. The human T cell line, C8166, was maintained in RPMI1640 medium containing 10% FCS.

Cloning of G protein-coupled receptor genes

DNA fragments containing the entire open reading frame (ORF) of CCR4, CCR6, CCR7, CCR9B, CCR10, CCR11, CXCR3, CXCR6, D6, or FPRL1 were amplified by reverse transcriptase (RT)-PCR using the cDNA made by total RNAs of PBLs or human T cell line C8166 as templates, and primer pairs (Sigma-Aldrich K. K., Tokyo, Japan) with 30 nucleotides of 5′-terminal and 3′-terminal sequences of ORFs. The DNA sequence of each GPCR was obtained from the DNA Data Bank of Japan/European Molecular Biology Laboratory/Genbank database as follows: CCR4 (accession number, X85740), CCR6 (U60000), CCR7 (L08176), CCR9B (AJ132337), CCR10 (AF215981), CCR11 (AF110640), CX3CR1 (U20350), CXCR3 (X95876), CXCR6 (AF007545), D6 (U94888), FPRL1 (M76672), and XCR1 (BC069075). The ORF DNA of each GPCR was cloned into the TA-cloning plasmid pDrive (Qiagen Co. Ltd., Tokyo, Japan) and recloned into the expression plasmid pCX-bsr [26]. The cloned GPCR genes were sequenced to determine their identities and orientation in the plasmids.

Establishment of NP-2/CD4 G protein-coupled receptor cells

NP-2/CD4 cells expressing CCR1, CCR2b, CCR3, CCR5, CCR8, CXCR1, CXCR2, CXCR4, CXCR5, CX3CR1, DARC, or GPR1, namely NP-2/CD4/CCR1, NP-2/CD4/CCR2b [15], NP-2/CD4/CCR3 [15], NP-2/CD4/CCR5 [15], NP-2/CD4/CCR8 [14], NP-2/CD4/CXCR1 [15], NP-2/CD4/CXCR2 [15], NP-2/CD4/CXCR4 [15], NP-2/CD4/CXCR5 [16], NP-2/CD4/CX3CR1 [15], NP-2/CD4/DARC [15], or NP-2/CD4/GPR1 [22] cells have been established. NP-2/CD4 cells expressing CCR4, CCR6, CCR7, CCR9B, CCR10, CCR11, CXCR3, CXCR6, D6, and FPRL1 were made by the method described elsewhere [15] and designated NP-2/CD4/CCR4, NP-2/CD4/CCR6, NP-2/CD4/CCR7, NP-2/CD4/CCR9B, NP-2/CD4/CCR10, NP-2/CD4/CCR11, NP-2/CD4/CXCR3, NP-2/CD4/CXCR6, NP-2/CD4/D6, and NP-2/CD4/FPRL1 cells, respectively. The expression of each GPCR in these cells was detected by RT-PCR using total RNA and the PCR primers pairs that were used to clone each GPCR as described above. As a control, expression levels of glyceraldehyde 3 phosphate dehydrogenase (GAPDH; (BT006893) mRNA in each cDNA preparation were determined by reverse transcriptase-PCR using the following PCR primers: GAPDHF, 5′-TGAAGGGTCGGAGTCAACGGATTTGGT-3′ (sense, 11th–36th) and GAPDHR, 5′-TAGACGGCAGGTCAGGTCCACCAC-3′ (antisense, 724th–747th).

Isolation of HIV-1

NP-2/CD4/GPCR cells (1×10^5) were seeded into a 24-well culture plate using RPMI1640 medium supplemented with 10% FCS 24 h before coculture with PBLs.