Lanka. In addition, this is the first report of HPeV11 infection in patients with acute gastroenteritis. With the identification of six different genotypes of HPeV in the samples tested, a diversity of Sri Lankan HPeVs was found. Taken together with the findings from previous studies, it is suggested that HPeV should be included in the spectrum of viruses which are routinely screened for among infants and children with acute gastroenteritis.

Nucleotide sequence accession numbers. The nucleotide sequences of the Sri Lankan HPeV strains studied here have been deposited in GenBank under accession numbers HQ163869 to HQ163881 and HQ163883 to HQ163894.

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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. **Ahmed, K., R. Batuwanthudawe, T. G. Chandrasena, M. T. Mitui, S. Rajindrajith, G. Galagoda, S. B. Pun, R. Uchida, O. Kunii, K. Moji, N. Abeysinghe, A. Nishizono, and O. Nakagomi.** 2010. Rotavirus infections with multiple emerging genotypes in Sri Lanka. Arch. Virol. **155:**71–75.
3. **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.
4. **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.
5. **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.
6. **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. VandenBrouckeGrauls, M. G. Beld, and K. C. Wolthers.** 2006. Fourth human parechovirus serotype. Emerg. Infect. Dis. **12:**1572–1575.
7. **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.
8. **Boivin, G., Y. Abed, and F. D. Boucher.** 2005. Human parechovirus 3 and neonatal infections. Emerg. Infect. Dis. **11:**103–105.
9. **Boonyakiat, Y., P. J. Hughes, F. Ghazi, and G. Stanway.** 2001. Arginine-glycine-aspartic acid motif is critical for human parechovirus 1 entry. J. Virol. **75:**10000–10004.
10. **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.
11. **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.
12. **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.
13. **Kim Pham, N. T., Q. D. Trinh, S. Takanashi, C. Abeysekera, A. Abeygunawardene, H. Shimizu, P. Khamrin, S. Okitsu, M. Mizuguchi, and H. Ushijima.** 2010. Novel human parechovirus, Sri Lanka. Emerg. Infect. Dis. **16:**130–132.
14. **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.
15. **Nyambat, B., S. Gantuya, R. Batuwanthudawe, P. R. Wijesinghe, N. Abeysinghe, G. Galagoda, C. Kirkwood, N. Bogdanovic-Sakran, J. O. Kang, and P. E. Kilgore.** 2009. Epidemiology of rotavirus diarrhea in Mongolia and Sri Lanka, March 2005-February 2007. J. Infect. Dis. **200**(Suppl. 1):S160–S166.
16. **Oberste, M. S., S. M. Michele, K. Maher, D. Schnurr, D. Cisterna, N. Junttila, M. Uddin, J. J. Chomel, C. S. Lau, W. Ridha, S. al-Busaidy, H. Norder, L. O. Magnius, and M. A. Pallansch.** 2004. Molecular identification and characterization of two proposed new enterovirus serotypes, EV74 and EV75. J. Gen. Virol. **85:**3205–3212.
17. **Pham, N. T., W. Chan-It, P. Khamrin, S. Nishimura, H. Kikuta, K. Sugita, T. Baba, A. Yamamoto, H. Shimizu, S. Okitsu, M. Mizuguchi, and H. Ushijima.** Detection of human parechovirus in stool samples collected from children with acute gastroenteritis in Japan during 2007–2008. J. Med. Virol., in press.
18. **Pham, N. T., Q. D. Trinh, P. Khamrin, N. Maneekarn, H. Shimizu, S. Okitsu, M. Mizuguchi, and H. Ushijima.** 2010. Diversity of human parechoviruses isolated from stool samples collected from Thai children with acute gastroenteritis. J. Clin. Microbiol. **48:**115–119.
19. **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.
20. **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.
21. **World Health Organization.** 1995. The treatment of diarrhea: a manual for physicians and other senior health workers. World Health Organization, Geneva, Switzerland.
22. **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.
23. **Yan, H., F. Yagyu, S. Okitsu, O. Nishio, and H. Ushijima.** 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.

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Detection of Human Parechovirus in Stool Samples Collected From Children With Acute Gastroenteritis in Japan During 2007–2008

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Of 477 stool specimens, which had been screened for rotavirus, adenovirus, norovirus, sapovirus and astrovirus, 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, 247 negative samples (51.7%) were subjected to screening for human parechovirus. Human parechovirus (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 gene. HPeV was detected in 20 of 247 specimens tested, and the detection rate was found to be 8.1%. Seventeen of the 20 strains that tested positive for HPeV were sequenced successfully the VP1 gene. The majority of the HPeV strains (n = 15) could be identified as HPeV1, and the remaining 2 strains could be typed as HPeV3. By phylogenetic and identical matrix analyses of HPeV VP1 sequences, HPeV1 should be divided into two lineages, and all of the Japanese studied HPeV1 strains belong to the lineage 2 accordingly. This is the first report of the circulation of HPeV, especially HPeV1 in Japan. ***J. Med. Virol. 83:331–336, 2011.***

KEY WORDS: human parechovirus; acute gastroenteritis; Japan

INTRODUCTION

Human parechoviruses (HPeVs) belong to the large family of *Picornaviridae* which is a highly diverse family of important pathogens of humans and animals. 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) [Hyypia et al., 1992; Stanway et al., 1994].

The previous findings reveal the genetic variability of HPeVs and the number of newly identified HPeV genotypes has been on the increase. Based on VP1 sequence comparisons, HPeVs have been classified into 14 genotypes (HPeV1–14) (http://www.picornaviridae.com/parechovirus/hpev/hpev.htm), of these, nine HPeV genotypes (HPeV1–8 and 14) have been published up to date [Ito et al., 2004; Abed and Boivin, 2005; Boivin et al., 2005; Benschop et al., 2006a,b; Al-Sunaidi et al., 2007; Watanabe et al., 2007; Baumgarte et al., 2008; Benschop et al., 2008; de Vries et al., 2008; Drexler et al., 2009; Li et al., 2009].

It is established well that rotaviruses, adenoviruses, astroviruses, and caliciviruses are the most important etiologic agents of acute gastroenteritis, which is a common cause of morbidity and mortality worldwide [Glass et al., 2001]. However, there remains a "diagnostic gap," which has been attributed to less explored viral pathogens. The present study aimed to screen stool samples collected from Japanese children with acute gastroenteritis during 2007–2008 for HPeV infection, one of less explored viral pathogens which has been reported to be associated with diarrhea recently, and to characterize the detected HPeV strains.

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MATERIALS AND METHODS

Clinical Specimens

Of 477 stool specimens, which had been screened for rotavirus, adenovirus, norovirus, sapovirus and astrovirus [Chanit et al., 2009], collected from infants and children aged from 2 months to 15 years who sought treatment of diarrhea at pediatric clinics encompassing five localities (Sapporo, Maizuru, Tokyo, Saga, and Osaka) in Japan from July 2007 to June 2008, 247 negative samples (51.7%) were subjected to screening for HPeV.

RNA Extraction and Reverse Transcription (RT)

The RNA genome of HPeV 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. Then, for reverse transcription, 5 μl of the stored, extracted RNA was added to a reagent mixture consisting of 5× First Strand Buffer (Invitrogen, Carlsbad, CA), 10 mM dNTPs (Roche, Mannheim, Germany), 0.1 M DTT (Invitrogen), SuperScript Reverse Transcriptase III (200 U/μl) (Invitrogen), random primer (1 μg/μl) (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase Inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and 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 held at 4°C. The cDNA was stored at −30°C until using for PCR reactions [Yan et al., 2004; Phan et al., 2005].

Polymerase Chain Reaction (PCR) for Detection of HPeV

After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 5× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (10 mM), primers (20 μM), Taq DNA polymerase (5 U/μl) (Promega), and 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 I). 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.

Genotyping by VP1 Sequencing and Primer Designation

At first, to amplify the VP1 gene, the previously described primers developed by Benschop et al. [2006b] were used. Because of failure in obtaining PCR products of most HPeV-positive samples except for three, 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 et al. [2006b].

For primer designation, to obtain the full length of the VP1 gene, alignment of full genome sequences of reference strains of eight known HPeV genotypes available in GenBank databases was performed using Clustal X software to find the conserve 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 I.

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 I). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and DNA bands were visualized by SYBR Safe (Invitrogen, Tokyo, Japan) staining under ultraviolet light.

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 comparison analysis of the VP1 gene was conducted between the obtained HPeV strains and other reference HPeV strains of 9 known genotypes available in GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was conducted using MEGA version

TABLE I. Oligonucleotide Sequences of the Primers Used in This Study and Their Positions

Primer	Gene	Sequence 5′–3′	Sense	Position*	Amplicon	References
ev22(+)	5′TR	CYCACACAGCCATCCTC	+	312–328	270	Joki-Korpela and Hyypia [1998]
ev22(−)	5′TR	TRCGGGTACCTTCTGGG	−	581–565		
VP1-parEchoF1	VP1	CCAAAATTCRTGGGGTTC	+	2332–349	760	Benschop et al. [2006b]
VP1-parEchoR1	VP1	AAACCYCTRTCTAAATAWGC	−	3090–071		
Cap-parEcho-F	VP1	TCHACWTGGATGMGRAARAC	+	2162–181	1076	This study
Cap-parEcho-R	VP1	TCYARYTCACAYTCYTCYTC	−	3237–218		

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.

3.1 to determine the evolutionary relationship among studied sequences [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 (EU 024634), 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 (AM 235750), 653046 (FJ373170), K251176-02 (DQ315670); HPeV5: CT86-6760 (AF055846), T92-15 (AM235749), 676618 (FJ373175); HPeV6: 2005-823 (EU077518), NII 561-2000 (AB252582), BNI-67/03 (EU024629), 650045 (FJ373178); HPeV7: PAK5045 (EU556224); HPeV8: BR/217/2006 (EU716175); HPeV14: 451564 (FJ373179). For the studied Japanese strains, they are FJ648741-FJ648754, GQ149452, GQ203502, and GQ203503.

RESULTS

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 of the full-length VP1 capsid sequences. All 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 les than 18 months of age. Besides diarrhea, fever and vomiting were found in 30% and 15% of the patients, respectively. Coughing 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, in this study, HPeV was detected nearly the year round, except for April, July, and December, with the peak of incidence in February (data not shown).

Figure 1 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 1, the majority of the Japanese HPeV strains ($n = 15$) could be identified as HPeV1, and the remaining two strains could be typed as HPeV3. The figure shows clearly that HPeV1 strains clustered into two separate branches. The prototype strains of HPeV1, the Harris strain, clustered into 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 studied 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 (Fig. 1), 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 studied HPeV3 strains, and among reference strains of HPeV3, HPeV7, HPeV8, and HPeV14 as well (data not shown).

DISCUSSION

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

This is the first report on detection of HPeV in a large number of fecal samples collected from Japanese infants and children with acute gastroenteritis. In this study, a high detection rate of HPeV in the tested samples (8.1%) was found. However, it is not represent the percentage of HPeV-infected cases in all patients with acute gastroenteritis (477 cases) during 1-year period, because only

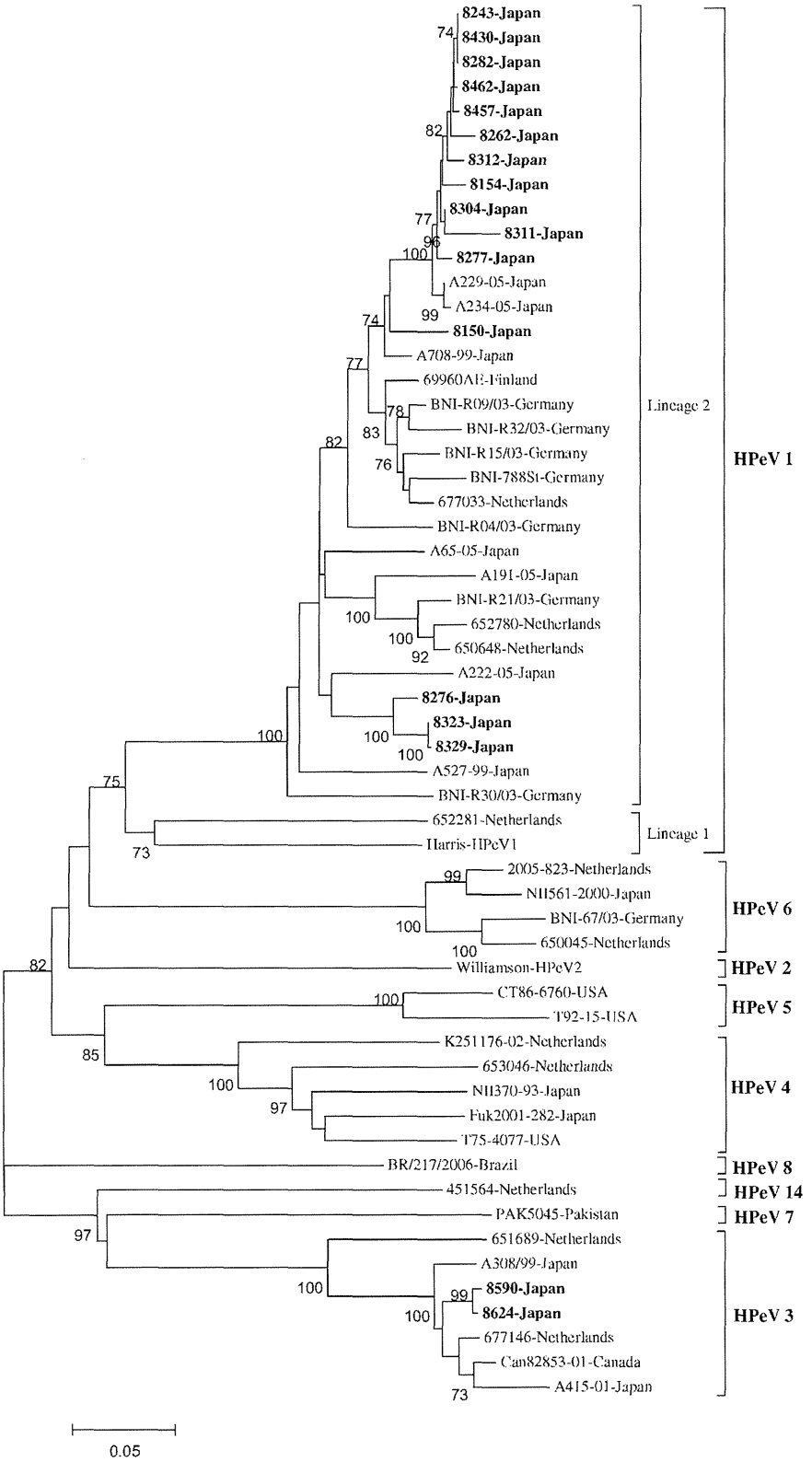

Fig. 1. Phylogenetic tree constructed from the 624 nucleotides of the VP1 gene of the strains studied and reference HPeV strains with 500 bootstrap repetitions. Percentage bootstrap values above 70% are shown at the branch nodes. The studied HPeV strains are in boldface type.

247 samples which had been known to be negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus were screened for HPeV. As a result, possible mixed infection of enteric viruses was excluded from analysis. The fact that this study did not include mixed infection of enteric viruses is a limitation of the study. Nevertheless, a proportion of 4.2% (20/477) of HPeV mono-infection was noted among the Japanese infants and children with acute gastroenteritis.

In this study, 702-base full length of the VP1 gene of the studied Japanese strains was obtained successfully. However, some of VP1 sequences of reference strains, especially the strain 451564 of the new genotype HPeV14 [Benschop et al., 2008], available in GenBank databases were partial VP1 sequences. Consequently, the phylogenetic tree was constructed based on 624 bases. The majority of the strains studied (15 strains) were identified as HPeV1, and two strains could be genotyped as HPeV3. In addition, the phylogenetic tree shows that HPeV1 strains clustered into two separate branches, one branch comprises of a limited number of HPeV1 strains including the prototype Harris strain, and the another branch consists of contemporary strains of HPeV1. Accordingly, incorporated with low amino acid identities between HPeV1 strains of the two branches, HPeV1 strains should be divided into two lineages as described in Results Section.

There have been four genotypes of HPeVs found in Japan up to date. They are HPeV1, HPeV3, HPeV4, and HPeV6 [Takao et al., 2001; Ito et al., 2004; Watanabe et al., 2007; Wakatsuki et al., 2008]. By this study, two different types of HPeVs, HPeV1 and HPeV3, were identified in Japanese infants and children with acute gastroenteritis, and the majority of the strains studied 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.

In a recent study, a high detection rate of HPeVs (14.6%) was also found in stool samples which were negative for rotavirus, adenovirus, norovirus, sapovirus, and astrovirus, collected from Thai children with acute gastroenteritis [Pham et al., in press]. In that study, four genotypes of HPeV, HPeV1–4, were identified with a predominance of HPeV1. Taken together with previous findings, these results suggest that HPeVs might be possible causative agents of acute gastroenteritis among the studied patients, and HPeV1 is the predominant genotype associated with acute gastroenteritis.

In conclusion, 15 HPeV1 and two HPeV3 strains were found in children with acute gastroenteritis in Japan during 2007–2008. This is the first report of the circulation of HPeV, especially HPeV1 in Japan. The study suggests that HPeV1 should be divided into two lineages.

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REFERENCES

Abed Y, Boivin G. 2005. Molecular characterization of a Canadian human parechovirus (HPeV)-3 isolate and its relationship to other HPeVs. J Med Virol 77:566–570.

Al-Sunaidi M, Williams CH, Hughes PJ, Schnurr DP, Stanway G. 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.

Baumgarte S, de Souza Luna LK, Grywna K, Panning M, Drexler JF, Karsten C, Huppertz HI, Drosten C. 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.

Benschop KS, Schinkel J, Luken ME, van den Broek PJ, Beersma MF, Menelik N, van Eijk HW, Zaaijer HL, VandenBroucke-Grauls CM, Beld MG, Wolthers KC. 2006a. Fourth human parechovirus serotype. Emerg Infect Dis 12:1572–1575.

Benschop KS, Schinkel J, Minnaar RP, Pajkrt D, Spanjerberg L, Kraakman HC, Berkhout B, Zaaijer HL, Beld MG, Wolthers KC. 2006b. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin Infect Dis 42:204–210.

Benschop K, Thomas X, Serpenti C, Molenkamp R, Wolthers K. 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.

Boivin G, Abed Y, Boucher FD. 2005. Human parechovirus 3 and neonatal infections. Emerg Infect Dis 11:103–105.

Chanit W, Thongprachum A, Khamrin P, Okitsu S, Mizuguchi M, Ushijima H. 2009. Intergenogroup recombinant sapovirus in Japan, 2007–2008. Emerg Infect Dis 15:1084–1087.

de Vries M, Pyrc K, Berkhout R, Vermeulen-Oost W, Dijkman R, Jebbink MF, Bruisten S, Berkhout B, van der Hoek L. 2008. Human parechovirus type 1, 3 4, 5 and 6 detection in picornavirus cultures. J Clin Microbiol 46:759–762.

Drexler JF, Grywna K, Stocker A, Almeida PS, Medrado-Ribeiro TC, Eschbach-Bludau M, Petersen N, da Costa-Ribeiro-Jr H, Drosten C. 2009. Novel human parechovirus from Brazil. Emerg Infect Dis 15:310–313.

Ehrnst A, Eriksson M. 1993. Epidemiological features of type 22 echovirus infection. Scand J Infect Dis 25:275–281.

Ehrnst A, Eriksson M. 1996. Echovirus type 23 observed as a nosocomial infection in infants. Scand J Infect Dis 28:205–206.

Figueroa JP, Ashley D, King D, Hull B. 1989. An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. J Med Virol 29:315–319.

Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, Noel J, Parashar U, Rosen B, Monroe SS. 2001. Gastroenteritis viruses: An overview. Novartis Found Symp 238:5–19; discussion 19–25.

Grist NR, Bell EJ, Assaad F. 1978. Enteroviruses in human disease. Prog Med Virol 24:114–157.

Hyypia T, Horsnell C, Maaronen M, Khan M, Kalkkinen N, Auvinen P, Kinnunen L, Stanway G. 1992. A distinct picornavirus group identified by sequence analysis. Proc Natl Acad Sci USA 89:8847–8851.

Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K. 2004. Isolation and identification of a novel human parechovirus. J Gen Virol 85:391–398.

Joki-Korpela P, Hyypia T. 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin Infect Dis 27:129–136.

Koskiniemi M, Paetau R, Linnavuori K. 1989. Severe encephalitis associated with disseminated echovirus 22 infection. Scand J Infect Dis 21:463–466.

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163.

Legay V, Chomel JJ, Fernandez E, Lina B, Aymard M, Khalfan S. 2002. Encephalomyelitis due to human parechovirus type 1. J Clin Virol 25:193–195.

Li L, Victoria J, Kapoor A, Naeem A, Shaukat S, Sharif S, Alam MM, Angez M, Zaidi SZ, Delwart E. 2009. Genomic characterization of novel human parechovirus type. Emerg Infect Dis 15:288–291.

Maller HM, Powars DF, Horowitz RE, Portnoy B. 1967. Fatal myocarditis associated with ECHO virus, type 22, infection in a

child with apparent immunological deficiency. J Pediatr 71:204–210.

O'Regan S, Robitaille P, Mongeau JG, McLaughlin B. 1980. The hemolytic uremic syndrome associated with ECHO 22 infection. Clin Pediatr (Phila) 19:125–127.

Pham NT, Trinh QD, Khamrin P, Maneekarn N, Shimizu H, Okitsu S, Mizuguchi M, Ushijima H. in press. Diversity of human parechoviruses isolated in stool samples collected from Thai children with acute gastroenteritis. J Clin Microbiol.

Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, Okitsu S, Muller WE, Ushijima H. 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.

Russell SJ, Bell EJ. 1970. Echoviruses and carditis. Lancet 1:784–785.

Stanway G, Hyypia T. 1999. Parechoviruses. J Virol 73:5249–5254.

Stanway G, Kalkkinen N, Roivainen M, Ghazi F, Khan M, Smyth M, Meurman O, Hyypia T. 1994. Molecular and biological characteristics of echovirus 22, a representative of a new picornavirus group. J Virol 68:8232–8238.

Stanway G, Joki-Korpela P, Hyypia T. 2000. Human parechoviruses—Biology and clinical significance. Rev Med Virol 10:57–69.

Takao S, Fukuda S, Shimazu Y, Noda M, Tokumoto S. 2001. The isolation of human parechovirus 1 from cases of acute respiratory illness in children. Jpn J Infect Dis 54:36–38.

Wakatsuki K, Kawamoto D, Hiwaki H, Watanabe K, Yoshida H. 2008. Identification and characterization of two strains of human parechovirus 4 isolated from two clinical cases in Fukuoka City, Japan. J Clin Microbiol 46:3144–3146.

Watanabe K, Oie M, Higuchi M, Nishikawa M, Fujii M. 2007. Isolation and characterization of novel human parechovirus from clinical samples. Emerg Infect Dis 13:889–895.

Yan H, Nguyen TA, Phan TG, Okitsu S, Li Y, Ushijima H. 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.

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Development of a Poliovirus Neutralization Test with Poliovirus Pseudovirus for Measurement of Neutralizing Antibody Titer in Human Serum▽

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In the Global Polio Eradication Initiative, laboratory diagnosis plays a critical role by isolating and identifying poliovirus (PV) from the stool samples from acute flaccid paralysis (AFP) cases. In recent years, reestablishment of PV circulation in countries where PV was previously eliminated has occurred because of decreased herd immunity, possibly due to poor vaccination coverage. To monitor the vulnerability of countries to PV circulation, surveillance of neutralizing-antibody titers against PV in susceptible populations is essential in the end game of the polio eradication program. In this study, we have developed a PV neutralization test with type 1, 2, and 3 PV pseudoviruses to determine the neutralizing-antibody titer against PV in human serum samples. With this test, the neutralizing-antibody titer against PV could be determined within 2 days by automated interpretation of luciferase signals without using infectious PV strains. We validated the pseudovirus PV neutralization test with 131 human serum samples collected from a wide range of age groups (ages 1 to >60 years) by comparison with a conventional neutralization test. We found good correlation in the neutralizing-antibody titers determined by these tests. These results suggest that a pseudovirus PV neutralization test would serve as a safe and simple procedure for the measurement of the neutralizing-antibody titer against PV.

In the Global Polio Eradication Initiative, laboratory diagnosis plays a critical role by isolating and identifying poliovirus (PV) from the stool samples from acute flaccid paralysis (AFP) cases for surveillance of PV circulation. In the World Health Organization (WHO) Global Polio Laboratory Network, PV isolation and identification have been performed at WHO national polio laboratories in a cell culture system (18, 19), followed by differentiation of the isolates into oral PV vaccine (OPV)-related PV, vaccine-derived PV (VDPV), and wild-type PV isolates by several methods at WHO regional reference laboratories (12, 19). Surveillance of PV is essential for monitoring the progress of PV eradication in countries where PV is endemic (4 countries as of 2011) and for the maintenance of the polio-free status of countries where PV is not endemic by preventing circulation of imported PVs or VDPVs from countries where PV is endemic through proper vaccination campaigns.

In the end game of the eradication program, surveillance of seroprevalence against PV in susceptible populations is essential for monitoring vulnerability to PV circulation in PV-free countries to sustain their PV-free status and the seroconversion rates in countries where PV is endemic to evaluate the effectiveness of vaccination strategies. In laboratories, the neutralizing-antibody titer has been determined by a conventional PV neutralization test (cPNT) using a susceptible cell culture system and infectious challenge virus (20). Characteristic requirements for a cPNT are as follows: (i) use of infectious virus (usually OPV strains are used), (ii) expertise of personnel (for observation of cytopathic effect [CPE] in inoculated cells), and (iii) extended time for results (5 to 7 days of culture). In Japan, surveillance of neutralizing antibody against PV has been performed every 2 or 3 years since 1974 for serum samples from healthy volunteers (about 1,100 to 1,800 individuals in 6 to 8 prefectures) in a wide range of ages (0 to >40 years) based on cPNTs in prefectural laboratories (http://idsc.nih.go.jp/yosoku/Polio/Year-P2009.html) (9). Considering the biosafety and expertise required for the test, a PV neutralization test that is safer and simpler than cPNT would be desirable in the end game of the eradication program.

In the present study, we have developed a novel PV neutralization test using non-self-proliferating PV pseudovirus, which encapsidated luciferase-encoding PV replicons with PV capsid proteins (2). In a pseudovirus PV neutralization test (pPNT), the neutralizing-antibody titer was determined based on the luciferase signals in inoculated cells within 2 days. The results suggested that pPNT would serve as a safe and simple procedure for the measurement of the neutralizing-antibody titer against PV.

MATERIALS AND METHODS

Cells, viruses, and human sera. RD cells (human rhabdomyosarcoma cells) and HEK293 cells (human embryonic kidney cells) were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Vero cells (African green monkey kidney cells) were cultured as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 0.11% bovine serum albumin (BSA) (fraction V; Sigma). RD cells were used for the titration of PV and for the pPNT. Vero cells were used for the

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pPNT. HEK293 cells were used for production of PV pseudoviruses. PV pseudoviruses, which encapsidated luciferase-encoding PV replicons with PV capsid proteins derived from $PV1_{Mahoney}$, $PV2_{MEF-1}$, and $PV3_{Saukett\ A}$, were prepared as reported previously (2) (see below for the construction of type 2 and 3 PV capsid protein expression vectors and preparation of PV pseudoviruses). Human sera were collected from healthy volunteers (aged 1 to 76 years) with informed consent. The experiments performed in the present study were approved by the Committee for Ethical Regulation of the National Institute of Infectious Diseases, Japan.

General methods of molecular cloning. *Escherichia coli* strain XL10gold (Stratagene) was used for the preparation of plasmids. Ligation of DNA fragments was performed using a quick ligation kit (New England BioLabs). PCR was performed using KOD Plus DNA polymerase (Toyobo). Reverse transcription-PCR (RT-PCR) was performed using a ReverTra Plus kit (Toyobo). DNA sequencing was performed using a BigDye Terminator v3.0 cycle-sequencing ready-reaction kit (Applied Biosystems) and then analyzed with a 3130 genetic analyzer (Applied Biosystems).

Construction of type 2 and 3 PV capsid expression vectors. For the construction of expression vectors of type 2 and 3 PV capsid proteins, we fused the enhanced green fluorescent protein (EGFP) gene to a PV capsid protein coding region of $PV2_{MEF-1}$ or of $PV3_{Saukett\ A}$ by PCR to measure the expression levels of PV capsid proteins. EGFP coding regions were amplified by PCR with primers 5′TGGTAAGCTTACCATGGGAGCTCTGAGCAA3′ and 5′ATGAGACTTG GGCGCCGTAGGTGGTCAGGC3′ (for fusion with the type 2 PV capsid protein coding region) or primers 5′TGGTAAGCTTACCATGGGAGCTCTGAG CAA3′ and 5′CGAAACTTGAGCTCCGTAGGTGGTCAGGCC3′ (for fusion with the type 3 PV capsid protein coding region) (HindIII sites in the primers are underlined) using pIRES2-EGFP (Clontech) as the template. The type 2 PV capsid protein coding region was amplified by RT-PCR with primers 5′GCCT GACCACCTACGGCGCCCAAGTCTCAT3′ and 5′TTAATCTAGATTAATA GGTTGTCAAGCCTT3′ (the XbaI site is underlined) using viral genomic RNA of $PV2_{MEF-1}$ as the template. The type 3 PV capsid protein coding region was amplified by RT-PCR with primers 5′GGCCTGACCACCTACGGAGCTCAA GTTTCG3′ and 5′TTAAGGATCCTTAATAGGTGGTCAAACCTT3′ (the BamHI site is underlined) using viral genomic RNA of $PV3_{Saukett\ A}$ as the template. Next, these DNA fragments of EGFP and PV capsid protein coding regions were fused by PCR using the primers 5′TGGTAAGCTTACCATGGG AGCTCTGAGCAA3′ and 5′TTAATCTAGATTAATAGGTTGTCAAGCC TT3′ (for type 2 PV) (the HindIII and XbaI sites are underlined) or primers 5′TGGTAAGCTTACCATGGGAGCTCTGAGCAA3′ and 5′TTAAGGATCC TTAATAGGTGGTCAAACCTT3′ (for type 3 PV) (the HindIII and BamHI sites are underlined). The fused DNA fragments were then cloned into the pKS435 expression vector (a kind gift from Koji Sakai, AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan) (2), following digestion by HindIII and XbaI (for type 2 PV) or by HindIII and BamHI (for type 3 PV). The resultant plasmids were named pKS435-EGFP-PV2(MEF-1) and pKS435-EGFP-PV3(Saukett A), respectively, and used as PV capsid protein expression vectors for production of PV pseudoviruses (1, 2).

Preparation of PV pseudovirus. PV pseudoviruses were prepared as reported previously with modifications (2). Briefly, a six-well plate (Falcon) with a 10% confluent monolayer of HEK293 cells was transfected with 2 μg of corresponding PV capsid expression vectors per well using a Lipofectamine 2000 reagent (Invitrogen), and the cells were incubated at 37°C in 2 ml DMEM supplemented with 10% FCS per well for 48 h. RNA transcripts of the PV replicon were obtained by using a RiboMax large-scale RNA production system-T7 kit (Promega) with DraI-linearized DNA of pPV-Fluc mc, which includes a PV replicon based on $PV1_{Mahoney}$ that has a firefly luciferase gene instead of the capsid-coding region, as the template. RNA transcripts were transfected into the monolayer of HEK293 cells transiently expressing PV capsid proteins at 48 h post-transfection using a Lipofectamine RNAiMax reagent (Invitrogen). Cells were harvested at 25 h posttransfection of the RNA transcripts, when most of the cells showed CPE, and then stored at −20°C.

cPNT. cPNTs were performed according to the standard procedure recommended by the WHO with modifications (20). First, 200 μl of human serum samples and standard anti-PV sera (positive control) were diluted 4-fold with EMEM (1/4 dilution), and then 2-fold dilution series were prepared with EMEM supplemented with 0.11% BSA, resulting in 1/4 to 1/1,024 dilution, and then 50 μl of diluted sera or EMEM supplemented with 0.11% BSA was added to three 96-well plates (2 wells for one dilution in 1 plate; a total of 6 wells for one dilution in 3 plates). Next, 50 μl of type 1, 2, or 3 PV Sabin strains (100 50% cell culture infective doses [$CCID_{50}$]) was added to each well of the plates (1 plate for each serotype of PV; a total of 3 plates) and then incubated at 37°C for 3 h. After incubation, 100 μl of Vero cell suspension in EMEM supplemented with 0.11% BSA (1.0×10^5 to 2.0×10^5 cells) was added to each well of the plates, and then the plates were incubated at 37°C for 7 days. The neutralizing-antibody titer of the serum against each type of PV was determined as the 50% endpoints of the serum that inhibited the PV infection observed by CPE of inoculated cells.

pPNT. For pPNT, first, 25 μl of human serum samples and standard anti-PV sera (positive control) was diluted with DMEM supplemented 4-fold with 1% FCS (1/4 dilution); 2-fold dilution series were made, resulting in 1/4 to 1/1,024 dilution; and then 5 μl of diluted sera or DMEM supplemented with 1% FCS (mock treatment for the negative control) was added to three 384-well plates (2 wells for one dilution in 1 plate; a total of 6 wells for one dilution in 3 plates) (catalog no. 781080; Greiner Bio-One). Next, 5 μl of type 1, 2, or 3 PV pseudovirus solution (400 infectious units [IU]) was added to each well of the plates (1 plate for each serotype of PV pseudovirus; a total of 3 plates), and then the plates were subjected to centrifugation (700 × *g*; 10 s; PlateSpin; Kubota). After centrifugation, the plates were sealed with a film (MicroAmp; Applied Biosystem) and then incubated at 4°C overnight. After incubation, 20 μl of RD cell suspension in DMEM supplemented with 5% FCS (5.0×10^3 cells) was added to each well of the plates, and then the plates were incubated at 37°C for 7 h. The luciferase activity of the infected cells was measured at 7 h postinfection (p.i.) with the Steady-Glo Luciferase Assay System (Promega) using a 2030 ARVO X luminometer (PerkinElmer) according to the manufacturer's instructions. PV pseudovirus infection was calculated as a percentage of the luciferase activity of the infected cells, where the luciferase activity in mock-treated cells was taken as 100% (mean signals, 1.4×10^5 to 1.8×10^5 cps; standard deviations, about 20% of the means). The neutralizing antibody titers of the serum against type 1, 2, and 3 PV were determined as the highest dilution of the serum that inhibited each type of PV pseudovirus infection at ≤20%, 2.5%, and 10%, respectively.

RESULTS

Development of a pseudovirus PV neutralization test for measurement of the neutralizing-antibody titer against PV in human sera. To establish a PV neutralization test using PV pseudovirus, we produced type 1, 2, and 3 PV pseudoviruses, which have a luciferase-encoding PV replicon in type 1, 2, and 3 PV capsid proteins, respectively. In our previous study, we established a production system for type 1 PV pseudovirus with $PV1_{Mahoney}$ capsid proteins (2). In the present study, we constructed PV capsid expression vectors that express capsid proteins of $PV2_{MEF-1}$ or $PV3_{Saukett\ A}$. With these expression vectors and a luciferase-encoding PV replicon, we obtained type 2 and 3 PV pseudoviruses with high titers (2.4×10^7 and 1.1×10^7 IU per ml, respectively) that were comparable to that of type 1 PV pseudovirus (4.4×10^7 IU per ml) (2). We performed serial passages of the type 2 and 3 PV pseudoviruses produced in an attempt to isolate infectious virus that might have emerged in the preparation (from 6.3×10^6 IU of PV pseudoviruses and 3 passages in HEp-2c cells, as previously performed for type 1 PV pseudovirus [2]), but no infectious virus was isolated from type 2 and 3 PV pseudoviruses (data not shown).

We analyzed the specificity of neutralization of PV pseudoviruses by using type-specific standard anti-PV sera and optimized the titer of PV pseudovirus for the neutralization test (Fig. 1). Different titers of PV pseudoviruses (200, 400, and 800 IU) were incubated with standard anti-PV sera (monkey antisera containing 13 units of neutralizing-antibody titer in 5 μl against type 1, 2, or 3 PV Sabin strains). Neutralization was performed at 4°C overnight according to the conditions used in cPNT. Standard anti-PV sera showed specific neutralization of corresponding types of PV pseudovirus in the range of titers of PV pseudoviruses. Similar neutralization curves were observed in a range of titers of PV pseudoviruses for all the serotypes. The neutralizing activity of the standard serum against type 1 PV pseudovirus was lower than those against type 2 and type 3

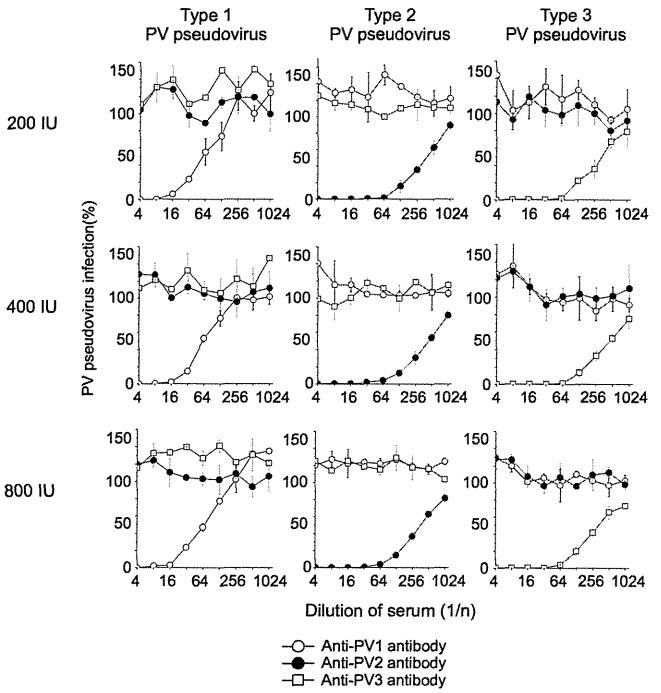

FIG. 1. Optimization of the PV pseudovirus titer for the pseudovirus PV neutralization test. Neutralization curves of PV pseudoviruses (200, 400, and 800 IU) with standard anti-PV sera are shown. The error bars indicate standard deviations.

PV pseudoviruses. Luciferase signals in the inoculated cells with 200, 400, and 800 IU of PV pseudoviruses were 1.2×10^7 to 1.5×10^7 cps, 2.7×10^7 to 3.3×10^7 cps, and 4.7×10^7 to 5.4×10^7 cps, with standard deviations of 13 to 15%, 7.5 to 14%, and 8.4 to 12% of the means, respectively. In this range of titers of PV pseudoviruses (200 to 800 IU), consistent results were obtained in the specificity and neutralization curves. Therefore, we determined that 400 IU of PV pseudoviruses was the optimal titer to give consistent results in pPNT. A summary of the procedure of pPNT with interpretation is shown in Fig. 2.

Measurement of the neutralizing-antibody titer against PV in human sera by cPNT and pPNT. We performed pPNT with 131 human serum samples and compared the results with those obtained by cPNT. In pPNT, the reciprocal of the highest dilution of serum that showed a value equal to or less than the threshold values was determined to be the neutralizing antibody titer (e.g., 64 for type 1 in Fig. 2B). Threshold values for each type of PV were determined by comparison with the results obtained by cPNT to give the anti-PV neutralizing-antibody-positive rates of samples (the percentage of samples that showed neutralizing-antibody titers of ≥8) closest to those determined by cPNT (Fig. 3A). The background signals in the tests corresponded to approximately 0.83% PV pseudovirus infection (standard deviation, 0.72%). Therefore, we could not set the threshold values below 2.5% PV pseudovirus infection for type 2 PV because of the high fluctuation near the background level. According to these criteria, threshold values for type 1, 2, and 3 PV were set at 20, 2.5, and 10% PV pseudovirus infection, respectively. With these threshold values, we observed good correlations in the neutralizing-antibody titers determined by cPNT and pPNT (R = 0.77, 0.90, and 0.88 for type 1, 2, and 3 PV, respectively) (Fig. 3B).

Finally, we compared the neutralizing-antibody titers determined by cPNT and pPNT among different age groups (Fig. 4). For type 2 and 3 PV, the distributions of neutralizing-antibody titers were consistent between cPNT and pPNT for all age groups. However, the distribution of titers for type 1 PV showed different profiles in cPNT and pPNT: lower titers in the young age group (1 to 19 years old) and higher titers in the elderly age group (over 60 years old) by pPNT. This suggested that anti-type 1 PV antibody has different properties among the age groups.

DISCUSSION

In this study, we developed a novel PV neutralization test using PV pseudoviruses instead of infectious PV strains. Pseudotyped viruses, especially enveloped viruses that require a high biosafety level for containment (e.g., severe acute respiratory syndrome coronavirus [SARS-CoV], lyssaviruses, Nipah virus, and highly pathogenic avian influenza A viruses), have been widely used in neutralization tests because of their safety (6, 7, 16, 17, 21). The advantages of pPNT compared to cPNT are as follows: (i) biosafety (pPNT uses non-self-proliferating PV pseudovirus versus infectious PV strains), (ii) simplicity of procedures (automated luciferase assay and interpretation versus observation requiring expertise), and (iii) shortened time to results (2 days versus 5 to 7 days). Moreover, pPNT requires less human serum than is required for cPNT (25 μl versus 200 μl) and thus could contribute to saving precious human serum resources to be used for biological tests. In the end game of the polio eradication program, biosafety and reduced burden in laboratory tests would have more importance than in areas where polio is endemic.

We observed good correlation in the overall results obtained by cPNT and pPNT for all types of PV. For pseudovirus neutralization tests, threshold values arbitrarily set in a range of 10 to 50% generally gave results that had good correlation with those obtained by conventional tests, especially for determining the neutralization-antibody-positive rates (6, 16, 17). Fine-tuning of threshold values might have benefit for linking the results obtained by different neutralization methods (e.g., suppression of CPE that could arise from 1 infectious unit of virus versus overall suppression of pseudovirus infection to threshold values). Therefore, we set the threshold values of pPNT in a range of the values examined for each type of PV to give antibody-positive rates closest to those obtained by cPNT (Fig. 3). PV pseudoviruses with capsid proteins derived from different strains in the same serotype (e.g., capsid proteins of the Sabin strain versus those of wild-type strains) might have different fine-tuned threshold values, in part depending on the variation in antigenicity and on the properties of the set of human sera examined without an absolute standard neutralizing antibody. The herd immunity required for PV elimination has been predicted to be about 80 to 97% from a circulation model, depending on the level of hygiene (5), or more than 70% from the emergence of circulating vaccine-derived PV in the Dominican Republic in 2000 and 2001, assuming there is no pocket of poor vaccination coverage (20 to 30%) in the

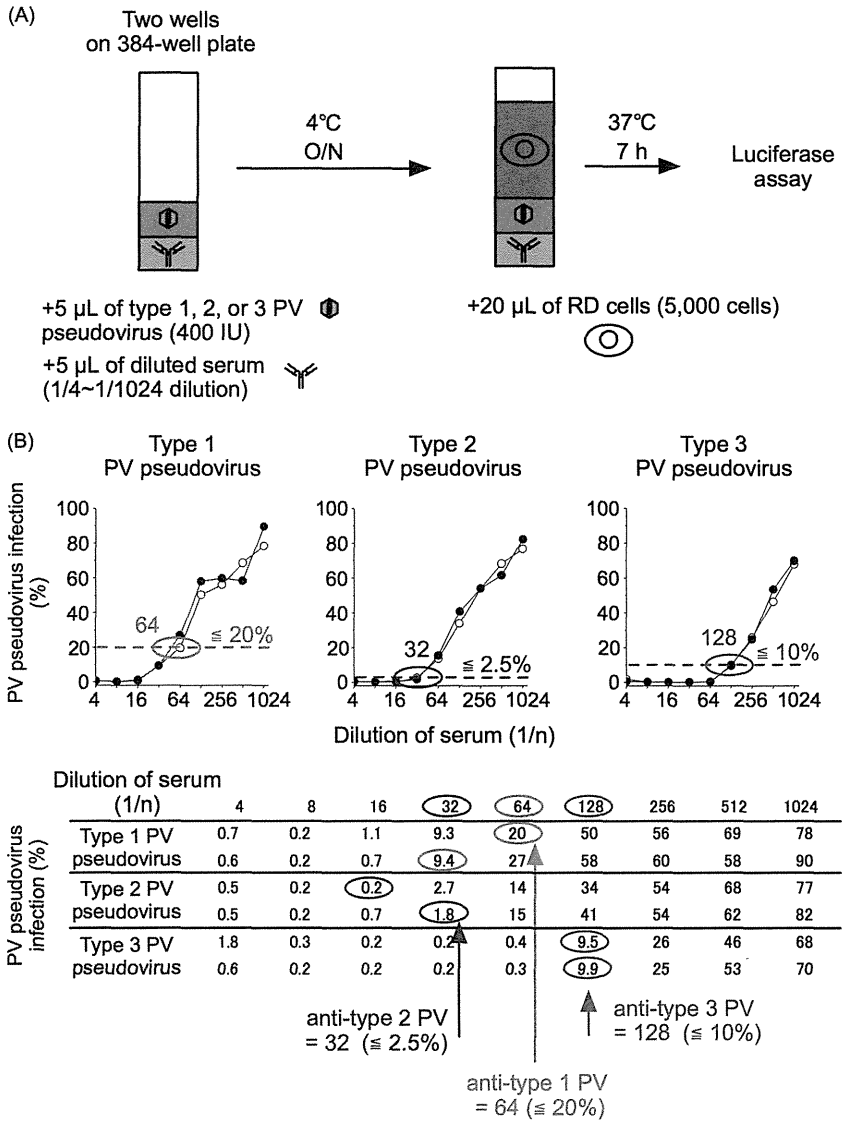

FIG. 2. Procedure for pPNT. (A) Schematic representation of the pPNT procedure. O/N, overnight. (B) Interpretation of pPNT. (Top) Neutralization curve of PV pseudoviruses with a human serum sample. The threshold levels for determination of the neutralizing-antibody titer are shown by the dotted lines (≤20%, 2.5%, and 10% for type 1, 2, and 3 PV, respectively), with neutralizing-antibody titers circled (64, 32, and 128 for type 1, 2, and 3 PV, respectively). (Bottom) Original data for the neutralization curve shown at the top. The wells that showed the highest dilution below the threshold values in two lines of measurement are circled. The wells that showed the highest dilutions below the threshold values in the measurement are indicated by arrows for each type of PV pseudovirus.

country (10). A neutralizing-antibody titer of ≥8 is considered to have a protective effect against paralytic poliomyelitis (reviewed in reference 13). Therefore, a wide range of threshold values (5 to 30% for type 1 PV, 2.5 to 10% for type 2, and 5.0 to 20% for type 3) in pPNT could be practically acceptable to estimate antibody-positive rates with an accuracy within 10% of those determined by cPNT.

We used type 1, 2, and 3 PV pseudoviruses that have capsid proteins of $PV1_{Mahoney}$, $PV2_{MEF-1}$, and $PV3_{Saukett\ A}$, respectively, for pPNT. These PV strains are virulent and are currently used as antigens of inactivated PV vaccine (IPV) (11). PV consists of 3 serotypes (14), although some antigenic variation could occur within each serotype after long circulation (for >10 years) (3). Therefore, to evaluate the neutralizing antibodies to wild-type PV strains, these PV pseudoviruses would have more benefit than using infectious PV vaccine strains. For neutralizing-antibody titers for type 1 PV, the overall results were consistent between cPNT and pPNT (Fig. 3). However, there was an age-dependent difference in the distribution of the titers: lower titers for the younger group and higher titers for the elderly group by pPNT (Fig. 4). In Japan, poliomyelitis outbreaks caused by wild-type strains mainly occurred before 1962 (>90% of the total reported cases from 1947 to date), before the introduction of OPV in 1961. Therefore, it is plausible that sera from the elderly group (≥60 years old; birth years before 1950) have higher neutralization activity against wild-type strains than vaccine strains. The low titers against type 1 PV observed by pPNT in the younger group

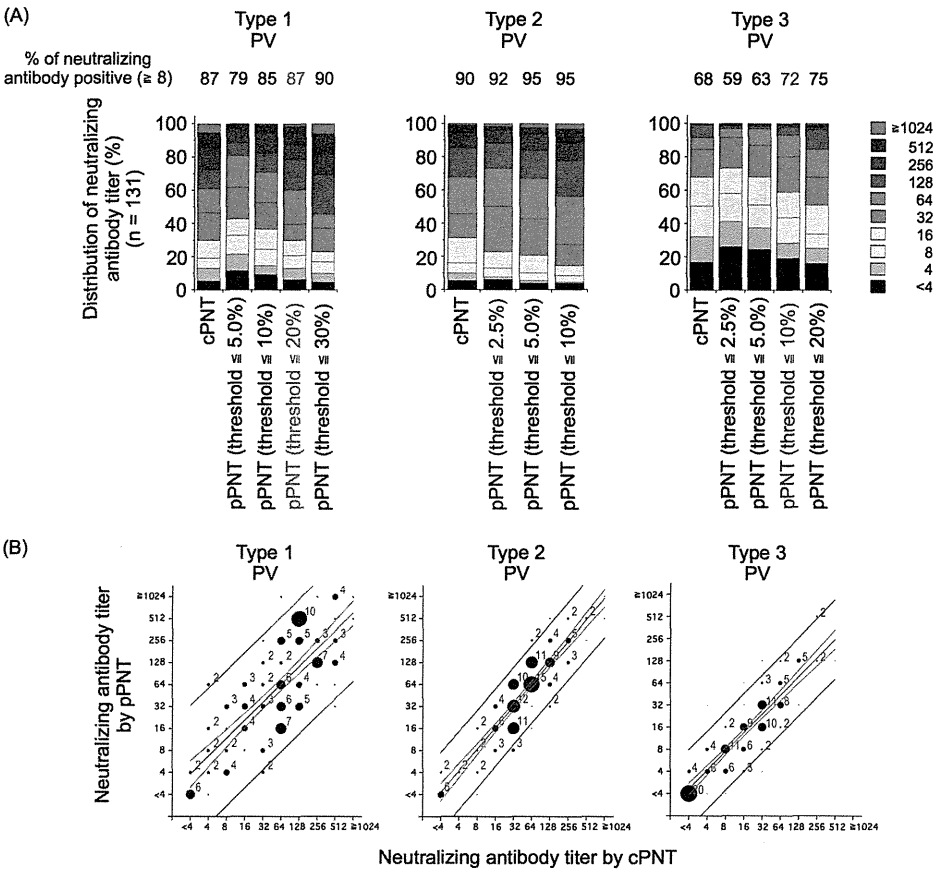

FIG. 3. Determination of threshold values for each type of PV in pPNT for human sera. (A) Distribution of neutralizing-antibody titers in human serum samples (n = 131) determined by pPNT in a range of threshold values. The results were compared with those obtained by cPNT. The neutralizing-antibody-positive rates of the samples were determined for each threshold value and are shown above the graphs. The threshold values that showed neutralizing-antibody-positive rates closest to those obtained by cPNT are highlighted with color for each type of PV (green, 20%; blue, 2.5%; and red,10% for type 1, 2, and 3 PV, respectively). (B) Scatter plots of neutralizing-antibody titers obtained by cPNT and pPNT. The threshold values used in pPNT were 20%, 2.5%, and 10% for type 1, 2, and 3 PV, respectively. The numbers of the serum samples in the corresponding spots are visualized by the sizes of the circles, along with the number of the sample for each spot when more than one sample was in the same spot. The regression line (red), 95% confidence interval (green), and 95% prediction interval (blue) of the analyses are shown.

suggested that antibodies induced by OPV apparently have higher titers for the vaccine strain than the parental $PV1_{Mahoney}$ strain or other type 1 attenuated strains (CHAT) (4, 8). Standard serum (monkey antisera raised against each Sabin strain) also showed relatively low neutralizing activity against type 1 PV pseudovirus compared to those against type 2 and 3 PV pseudoviruses (Fig. 1). Different levels of neutralizing-antibody titer against type 1 PV strains might have arisen

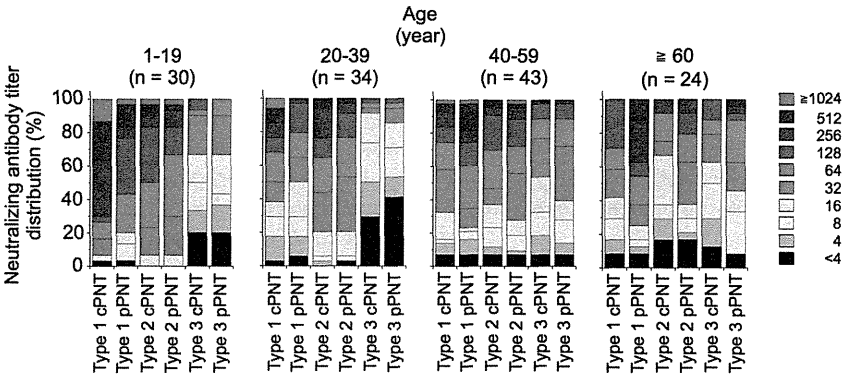

FIG. 4. Comparison of the neutralizing-antibody titers obtained by cPNT and pPNT in different age groups. The distributions of neutralizing-antibody titers obtained by cPNT and pPNT in the indicated age groups are shown. The numbers of serum samples examined for each age group are shown above the graphs in parentheses. The threshold values for pPNT were 20%, 2.5%, and 10% for type 1, 2, and 3 PV, respectively.

in part from differences in the epitopes of these strains recognized by the antibodies, as reported for those induced by OPV and IPV (antigenic sites 3 and 1, respectively) (15). Nevertheless, it is enigmatic that the distribution of neutralizing-antibody titers for a young age group (20 to 39 years old) showed only slight differences in the results obtained by cPNT and pPNT, in contrast to that of the 0-to-19-year age group. These age groups belong to the post-OPV introduction era, and thus, the properties of the antigen seem not to be the major determinant that caused this difference. This suggested that a transition in antibody properties occurred in long-term immune memory.

In summary, we developed a pseudovirus neutralization test for measurement of the neutralizing-antibody titer against PV in human serum. The pPNT would serve as a safe, simple, and rapid test for serosurveillance of PV.

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REFERENCES

1. **Arita, M., Y. Ami, T. Wakita, and H. Shimizu.** 2008. Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. J. Virol. **82:**1787–1797.
2. **Arita, M., N. Nagata, T. Sata, T. Miyamura, and H. Shimizu.** 2006. Quantitative analysis of poliomyelitis-like paralysis in mice induced by a poliovirus replicon. J. Gen. Virol. **87:**3317–3327.
3. **Blomqvist, S., et al.** 2004. Characterization of a highly evolved vaccine-derived poliovirus type 3 isolated from sewage in Estonia. J. Virol. **78:**4876–4883.
4. **Eggers, M., et al.** 2010. Immunity status of adults and children against poliomyelitis virus type 1 strains CHAT and Sabin (LSc-2ab) in Germany. BMC Infect. Dis. **10:**347.
5. **Fine, P. E.** 1993. Herd immunity: history, theory, practice. Epidemiol. Rev. **15:**265–302.
6. **Fukushi, S., et al.** 2006. Evaluation of a novel vesicular stomatitis virus pseudotype-based assay for detection of neutralizing antibody responses to SARS-CoV. J. Med. Virol. **78:**1509–1512.
7. **Han, D. P., H. G. Kim, Y. B. Kim, L. L. Poon, and M. W. Cho.** 2004. Development of a safe neutralization assay for SARS-CoV and characterization of S-glycoprotein. Virology **326:**140–149.
8. **Iwai, M., et al.** 2006. Assessment of efficacy of a live oral poliovirus vaccine for virulent Sabin-like poliovirus 1 strains in Japan. Acta Virol. **50:**139–143.
9. **Iwai, M., et al.** 2008. Evaluation of a two-dose administration of live oral poliovirus vaccine for wild and virulent vaccine-derived poliovirus type 1, 2, 3 strains in Japan. Scand. J. Infect. Dis. **40:**247–253.
10. **Kew, O., et al.** 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science **296:**356–359.
11. **Kew, O. M., R. W. Sutter, E. M. de Gourville, W. R. Dowdle, and M. A. Pallansch.** 2005. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu. Rev. Microbiol. **59:**587–635.
12. **Kilpatrick, D. R., et al.** 2009. Rapid group-, serotype-, and vaccine strain-specific identification of poliovirus isolates by real-time reverse transcription-PCR using degenerate primers and probes containing deoxyinosine residues. J. Clin. Microbiol. **47:**1939–1941.
13. **Nathanson, N.** 2005. David Bodian's contribution to the development of poliovirus vaccine. Am. J. Epidemiol. **161:**207–212.
14. **National Foundation for Infantile Paralysis Committee on Typing.** 1951. Immunologic classification of poliomyelitis viruses. I. A cooperative program for the typing of one hundred strains. Am. J. Hyg. **54:**191–204.
15. **Rezapkin, G., et al.** 2010. Repertoire of antibodies against type 1 poliovirus in human sera. J. Virol. Methods **169:**322–331.
16. **Tamin, A., et al.** 2009. Development of a neutralization assay for Nipah virus using pseudotype particles. J. Virol. Methods **160:**1–6.
17. **Wang, W., et al.** 2008. Establishment of retroviral pseudotypes with influenza hemagglutinins from H1, H3, and H5 subtypes for sensitive and specific detection of neutralizing antibodies. J. Virol. Methods **153:**111–119.
18. **Wood, D. J., and B. Hull.** 1999. L20B cells simplify culture of polioviruses from clinical samples. J. Med. Virol. **58:**188–192.
19. **World Health Organization.** 2004. Polio laboratory manual, 4th ed., WHO/IVB/04.10, and supplement to the WHO polio laboratory manual. World Health Organization, Geneva, Switzerland.
20. **World Health Organization.** 1995. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. WHO publication no. WHO/BLG/95.1. World Health Organization, Geneva, Switzerland.
21. **Wright, E., et al.** 2008. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. J. Gen. Virol. **89:**2204–2213.

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Phosphatidylinositol 4-Kinase III Beta Is a Target of Enviroxime-Like Compounds for Antipoliovirus Activity▽†

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Enviroxime is an antienterovirus compound that targets viral protein 3A and/or 3AB and suppresses a step in enterovirus replication by unknown mechanism. To date, four antienterovirus compounds, i.e., GW5074, Flt3 inhibitor II, TTP-8307, and AN-12-H5, are known to have similar mutations in the 3A protein-encoding region causing resistance to enviroxime (a G5318A [3A-Ala70Thr] mutation in poliovirus [PV]) and are considered enviroxime-like compounds. Recently, antienterovirus activity of a phosphatidylinositol 4-kinase III beta (PI4KB) inhibitor, PIK93, was reported, suggesting that PI4KB is an important host factor targetable by antienterovirus compounds (N. Y. Hsu et al., Cell 141:799–811, 2010). In this study, we analyzed the inhibitory effects of previously identified enviroxime-like compounds (GW5074 and AN-12-H5) and a newly identified antienterovirus compound, T-00127-HEV1, on phosphoinositide (PI) kinases. We found that T-00127-HEV1 inhibited PI4KB activity with a higher specificity for than other PI kinases, in contrast to GW5074, which had a broad specificity for PI kinases. In contrast, AN-12-H5 showed no inhibitory effect on PI4KB activity and only moderate inhibitory effects on PI 3-kinase activity. Small interfering RNA (siRNA) screening targeting PI kinases identified PI4KB is a target of GW5074 and T-00127-HEV1, but not of AN-12-H5, for anti-PV activity. Interestingly, T-00127-HEV1 and GW5074 did not inhibit hepatitis C virus (HCV) replication, in contrast to a strong inhibitory effect of AN-12-H5. These results suggested that PI4KB is an enterovirus-specific host factor required for the replication process and targeted by some enviroxime-like compounds (T-00127-HEV1 and GW5074) and that enviroxime-like compounds may have targets other than PI kinases for their antiviral effect.

Poliovirus (PV) is a small nonenveloped virus with a single-strand positive genomic RNA of about 7,500 nucleotides (nt) belonging to *Human enterovirus species C* in the genus *Enterovirus*, family *Picornaviridae*. PV is the causative agent of poliomyelitis, which is caused by the destruction of motor neurons by direct infection by PV in the cells (12, 19). With the established live attenuated oral PV vaccine (OPV) and inactivated PV vaccine (IPV) for PV (43, 44), the global eradication program for poliomyelitis has been continued by the Global Polio Eradication Initiative (GPEI) of the World Health Organization (WHO) since 1988. Currently, indigenous wild PVs are restricted to four countries of endemicity, with a substantial reduction of the cases in the major countries of endemicity, India and Nigeria (with only 40 and 11 cases, respectively, as of November, 2010). However, at least 10 countries are suffering from reestablishment of PV transmission or active outbreaks (e.g., there were more than 450 cases in Tajikistan) (http://www.polioeradication.org/casecount.asp). In the eradication program for poliomyelitis, antivirals for PV are anticipated to have roles in the PV posteradication era, i.e., in control of a circulating vaccine-derived PV (cVDPV) along with IPV and for treatment of patients chronically infected with PV and persons exposed to PV (17, 18). However, there is currently no antiviral available for PV infection.

Compounds with anti-PV activity can be classified as capsid-binding inhibitors, replication inhibitors, and encapsidation inhibitors in terms of the target stages in PV infection. Capsid-binding inhibitors target hydrophobic pockets on the virion and inhibit the PV uncoating process by stabilizing the virion or the human rhinovirus attachment process by inducing a conformational change of the virion (27, 40). Replication inhibitors target both the viral proteins and host proteins. Viral proteins 2A, 2C, 3A, 3C, and 3D have been identified as direct or indirect targets of anti-PV compounds, including elastase inhibitors (38), guanidine hydrochloride (GuHCl) (16), enviroxime (29, 50), rupintrivir (AG7088) (23, 39), and gliotoxin (42), respectively. An elastase inhibitor, methoxy-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MPCMK), also acted as a viral 2A protease inhibitor by forming a covalent bond with the active site of 2A protease (Cys109) and possibly the Val residue of MPCMK, and it reduced the production of infectious PV but not of infectious encephalomyocarditis virus (EMCV) (38, 49). GuHCl inhibits the initiation of negative-strand RNA synthesis by targeting viral proteins 2C and/or 2BC (8, 11, 16). GuHCl and some benzimidazole derivatives, including 2-(alpha-hydroxybenzyl)-benzimidazole (26), MRL-1237 (45), and TBZE-029 (22), belong to a group of 2C inhibitors in terms of the resistance mutations in the 2C-encoding region. Enviroxime inhibits positive-strand RNA synthesis by preventing normal formation of the replication complex, possibly by targeting viral proteins 3A and/or 3AB, although its direct interaction with the 3A protein was not detected (15, 29, 50).

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Recently, anti-PV compounds that have little structural similarity to enviroxime but induce common resistance mutations in the 3A-encoding region (enviroxime-like compounds) have been discovered, including TTP-8307 (24), some cellular protein kinase inhibitors (GW5074 and Flt3 inhibitor II) (4, 5), and the bifunctional antienterovirus compound AN-12-H5, which targets the replication processes of PV and enterovirus 71 (EV71) and also an early stage of EV71 infection (3). Rupintrivir was originally discovered as an irreversible inhibitor of human rhinovirus 3C protease by protein structure-based drug design methodologies (36). Inhibitory effects of the compounds on the function of viral proteins and/or evidence of direct interaction of viral proteins with the compounds suggested that these anti-PV compounds directly target viral proteins, except for enviroxime and enviroxime-like compounds (15, 36, 38, 41, 42).

Some host proteins have been identified as the targets of replication inhibitors as well as viral protein. To date, eIF4A, GBF1, and phosphatidylinositol 4-kinase III beta (PI4KB) have been identified as the targets of the replication inhibitors hippuristanol, brefeldin A, and PIK93, respectively (14, 30, 31, 37). Hippuristanol, a natural product of the coral *Isis hippuis*, suppresses initiation of translation by inhibiting RNA binding of eIF4A, and it delayed the expression of viral proteins in PV replication for 2 h (14). Brefeldin A blocks membrane traffic between the *cis*- and *trans*-Golgi compartments by targeting the cellular guanine nucleotide exchange factor GBF1 and inhibits PV replication but not EMCV replication (9, 21, 31, 37). Recently, a PI4KB inhibitor, PIK93, was identified as a potent anti-PV compound that targets PI4KB to suppress interaction of phosphatidylinositol 4-phosphate with viral 3D polymerase on the reorganized membrane vesicle for the formation of the viral replication complex (30). Encapsidation inhibitors target both viral and host proteins. Viral protein 2C and host protein Hsp90 have been identified as the targets of the encapsidation inhibitor hydantoin, which targets viral protein 2C and blocks polyprotein processing (46, 47), and of geldanamycin, which suppresses the encapsidation process by interfering with the folding of capsid proteins (28, 34), respectively. The emergence of mutants resistant to anti-PV compounds targeting host proteins is generally limited; no mutant resistant to geldanamycin was isolated in all attempts (28), and mutants resistant to brefeldin A were isolated after five passages in cultured cells (20). Mutants resistant to anti-PV compounds targeting host factors might have emerged by overcoming suppression of the specific target step by increasing corresponding viral protein activities as a result of the resistance mutation; brefeldin A resistance mutations conferred a resistant phenotype specific to brefeldin A treatment but not to other replication inhibitors (3).

In the present study, we have identified a novel anti-PV compound, T-00127-HEV1, by high-throughput screening with a large-scale chemical library (72,000 compounds). T-00127-HEV1 belonged to the enviroxime-like compounds based on the resistance mutation of PV. Surprisingly, we found that the PI4KB inhibitor PIK93 also belonged to the group of enviroxime-like compounds. This prompted us to analyze the inhibitory effects of enviroxime-like compounds on the activity of phosphoinositide (PI) kinases, including PI4KB. To analyze the specificity of enviroxime-like compounds to enterovirus, we examined the inhibitory effect of the compounds on the replication of another family of positive-strand RNA viruses, hepatitis C virus (HCV). The results suggested that PI4KB is a specific target of some enviroxime-like compounds, including GW5074 and T-00127-HEV1, but not AN-12-H5, for anti-PV activity. Strong anti-HCV activity of AN-12-H5, but not of GW5074 and T-00127-HEV1, suggested that enviroxime-like compounds may have targets other than PI kinases for their antiviral effect.

MATERIALS AND METHODS

Cells, viruses, and chemical library. RD cells (a human rhabdomyosarcoma cell line) and HEK293 cells (human embryonic kidney cells) were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). RD cells were used for titration of viruses and pseudoviruses and for screening. HEK293 cells were used for small interfering RNA (siRNA) screening to identify cellular targets of antienterovirus compounds. The Huh7.5.1 cell line was a kind gift from Frank Chisari (Scripps Research Institute). Huh7.5.1 cells were used for analysis of the inhibitory effects of anti-PV compounds on HCV replication. PV and EV71 pseudoviruses (TE-PV-Fluc mc and TE-EV71-Fluc mc), which encapsidated luciferase-encoding PV and EV71 replicons with capsid proteins derived from PV1(Mahoney) and EV71(Nagoya), respectively, were used for screening of antienterovirus compounds (1, 2, 5). PV1(Mahoney), EV71(Nagoya), and coxsackievirus B3 (CVB3) (Nancy) were used to analyze the inhibitory effects of identified compounds on enterovirus infection. PV pseudovirus mutants that have known drug resistance mutations, including G5318A (enviroxime and GW5074 resistance, 3A-Ala70Thr) (4, 29), U4614A (guanidine hydrochloride [GuHCl] resistance, 2C-Phe164Tyr) (7), and G4361A and C5190U (brefeldin A resistance, 2C-Val80Ile and 3A-Ala27Val) (20), were used for characterization of identified antienterovirus compounds (3). A diverse subset of 72,000 compounds from a chemical library at the University of Tokyo was used for screening. T-00127-HEV1 was supplied by Pharmeks Ltd. (Moscow, Russia), and the purity was checked by liquid chromatography-mass spectrometry (LC/MS). An siRNA library targeting human GBF1, ARF1, phosphatidylinositol 3-kinase-related kinase (ATM, ATR, FRAP1, PRKDC, SMG1, and TRRAP), and phosphoinositide (PI) kinase (PI4KA, PI4KB, PI4K2A, PI4K2B, PIK3C2A, PIK3C2B, PIK3C2G, PIK3C3, PIK3CA, PIK3CB, PIK3CD, PIK3CG, PIP4K2A, PIP4K2B, PIP4K2C, PIP5K1A, PIP5K1B, PIP5K1C, PIP5KL1, and PIP5K3) genes was purchased from Thermo Fisher Scientific as a form of siGENOME SMART pools, which contain four sets of different siRNAs for each mRNA and were validated as having over 75% knockdown efficiency for target mRNAs. As control siRNAs, siGLO cyclophilin B control siRNA, siGLO lamin A/C control siRNA, siGENOME nontargeting siRNAs 1 and 2, siGENOME RISC-FREE control siRNA, and siGENOME Tox transfection control were used in each experiment.

Screening of antienterovirus compounds. Five microliters of compound solution (60 μM; the final concentration of 10 μM) and 5 μl of PV pseudovirus solution (800 infectious units [IU]) were added to RD cells (5.0×10^3 cells per well in 20 μl medium) in 384-well plates (catalog no. 781080; Greiner Bio-One), and then the cells were incubated at 37°C for 7 h. Luciferase activity of the infected cells was measured at 7 h postinfection (p.i.) with the Steady-Glo luciferase assay system (Promega) using a 2030 ARVO X luminometer (Perkin-Elmer) according to the manufacturer's instructions. PV pseudovirus infection was calculated as a percentage of luciferase activity of the infected cells, where the luciferase activity in the infected cells in the absence of compounds was taken as 100%. The cutoff value of the inhibitory effect of candidate compounds was set to <10% of PV pseudovirus infection in the treated cells.

The cytotoxicity of the compounds was evaluated by viability of compound-treated cells in the screening. For candidate compounds, cytotoxicity was further evaluated by determination of the 50% cytotoxic concentration (CC_{50}) of the compounds. RD cells (1.0×10^4 cells per well in 50 μl medium) were cultured at 37°C in 96-well plates (Becton Dickinson), followed by addition of 50 μl compound solution (final concentrations of 10 μM for screening and 16 to 500 μM for determination of CC_{50}). The cells were incubated at 37°C for 2 days and then subjected to measurement of ATP as a marker of metabolically active cells by using a Cell Titer-Glo luminescent cell viability assay kit (Promega) according to the manufacturer's instructions. The cutoff value of screening for candidate compounds was set at >90% of viability of treated cells compared to that of mock-treated cells.

The inhibitory effect of anti-PV activity of the compounds was evaluated by

50% effective concentration (EC_{50}). RD cells (1.4×10^4 cells per well in 100 μl medium) in a 96-well plate were inoculated with 50 μl of PV pseudovirus (400 IU) and 50 μl of compound solution (final concentrations of 0.078 to 10 μM). The cells were incubated at 37°C for 7 h. Luciferase activity in the infected cells was measured at 7 h p.i. The EC_{50} values of a compound were obtained by nonlinear regression analysis of the dose-response curve.

Inhibitory effect of T-00127-HEV1 on enterovirus infection. The inhibitory effect of T-00127-HEV1 on PV, EV71, and CVB3 infection was evaluated by measurement of number of copies of the viral genome in the infected cells. RD cells (1.0×10^4 cells per well in 100 μl medium) in a 96-well plate were infected with PV1(Mahoney), EV71(Nagoya), or CVB3(Nancy) at multiplicities of infection (MOI) of 10, 1.0, and 0.1 at 37°C for 1 h in the absence of T-00127-HEV1. The cells were washed three times with 10% FCS–DMEM, followed by the addition of 100 μl of 10% FCS–DMEM containing 10 or 0 μM T-00127-HEV1. Cells were collected at 16 h p.i., and then viral RNA was extracted from the cells using a High Pure viral RNA purification kit (Roche). The number of copies of the viral genome was quantified using a real-time PCR system.

Quantification of viral RNA by real-time PCR. Real-time PCR was performed as previously described by Dierssen et al. with modification (25). Viral RNA was assayed in a 20-μl reaction mixture containing 5 μl of viral RNA by using the one-step SYBR PrimeScript Plus reverse transcription-PCR (RT-PCR) kit (TaKaRa) with primers EQ-1 and EQ-2 (25). Viral RNA of PV1(Sabin) was used to control the quantification of the number of copies. The mixtures were subjected to real-time PCR; the PCR conditions consisted of a reverse transcription step at 42°C for 30 min and 40 cycles of thermal cycling at 95°C for 3 s and 60°C for 30 s. The fluorescence emission of SYBR green I was monitored and analyzed by using an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems).

Inhibitory effect of antienterovirus compounds on *in vitro* activity of cellular protein kinases and PI kinases. The inhibitory effect of T-00127-HEV1 at a concentration of 10 μM on *in vitro* cellular protein kinase activities was assessed by kinase profiling with an ATP concentration near the K_m for each kinase (Carna Biosciences, Inc., Japan). Inhibitory effects of GW5074, AN-12-H5, and T-00127-HEV1 at a concentration of 10 μM on *in vitro* PI kinase activities were assessed by the SelectScreen kinase profiling service with an ATP concentration of 10 μM (Invitrogen). For T-00127-HEV1, the 50% inhibitory concentration (IC_{50}) for *in vitro* PI4KB activity was also measured with an ATP concentration of 10 μM.

Inhibitory effects of antienterovirus compounds on HCV replication. RNA transcripts of HCV replicons were obtained using a RiboMAX large-scale RNA production system T7 kit (Promega), using XbaI-linearized DNAs of the HCV replicon (the replicon clone pSGR-JFH-LucNeo-4 and a replication-defective clone with a disrupted polymerase motif [GDD to GND], JFHluci-GND) as the templates. The *in vitro*-synthesized RNA transcripts were transfected into Huh7.5.1 cells by electroporation as described previously with modifications (48). Ten micrograms of *in vitro*-synthesized RNA transcripts was mixed with 3.2×10^6 Huh7.5.1 cells in 400 μl Opti-MEM (Invitrogen). The mixture was transferred to an electroporation cuvette (Gene Pulser cuvette [0.4-cm electrode]; Bio-Rad) and then pulsed at 270 V and 950 μF using a Gene Pulser II apparatus (Bio-Rad). Transfected cells were immediately suspended in 10 ml of 10% FCS–DMEM and then transferred to 96-well plates (Microtest 96-well assay plate; Becton Dickinson) at 100 μl per well. The cells were incubated at 37°C for 1 h, and then 25 μl of antienterovirus compound solutions (final concentrations of 0.078 to 10 μM) or medium was added. The cells were incubated at 37°C for 23 h, and then the luciferase activity in the cells was measured at 24 h posttransfection (p.t.) with the Steady-Glo luciferase assay system (Promega) using a 2030 ARVO X luminometer (Perkin-Elmer) according to the manufacturer's instructions.

siRNA transfection. An RNA duplex of each siRNA (final concentration of 50 nM) was transfected into HEK293 cells (5.0×10^3 cells in 100 μl medium per well) in 96-well plates (Microtest 96-well assay plate; Becton Dickinson) by using DharmaFECT1 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were incubated at 37°C for 24 h, and then the supernatant was removed and replaced with 100 μl per well of 10% FCS–DMEM. The transfection efficiency of siRNA in the cells was evaluated by the efficiency of incorporation of fluorescence-labeled siRNA (siGLO control siRNAs) in the transfected cells at 24 h p.t. and by the efficiency of cell death in the cells transfected with siGENOME Tox transfection control at 96 h p.t. (cells transfected with this control reagent die by apoptosis). siRNA-transfected cells were used for experiments at 96 h p.t.

Western blot analysis. siRNA-treated cells in three wells of 96-well plates were collected at 96 h p.t. in 100 μl cell lysis buffer (21 mM HEPES buffer [pH 7.4], 1.8 mM disodium hydrogen phosphate, 137 mM NaCl, 4.8 mM KCl, 0.5% Nonidet P-40, and 0.5 mM EDTA) and then were subjected to 5 to 20% gradient polyacrylamide gel electrophoresis (e-PAGEL; Atto Corporation) in a Laemmli buffer system (33). The proteins in the gel were transferred to a polyvinylidene difluoride filter (Immobilon; Millipore) and blocked by using a blocking reagent (Qiagen) (for staining with anti-GBF1 and -ARF1 antibodies) or 5% nonfat dry milk (for staining with anti-PI4KB antibody). The filters were incubated with rabbit anti-GBF1, -ARF1, and -PI4KB antibodies (Abcam) (1:500, 1:250, and 1:50 dilutions, respectively) at room temperature for 1 h. The filters were washed with Tris-buffered saline (TBS) (20 mM Tris-HCl [pH 7.4], 137 mM NaCl) containing 0.1% Tween 20 (TBS-T) three times for 5 min each and then incubated with goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Pierce) (1:1,000 dilution) at room temperature for 1 h. The filters were washed with TBS-T three times for 5 min each, and then treated with Super-Signal West Femto Maximum Sensitivity substrate (Pierce) for the detection of the signal.

Target identification by siRNA sensitization (TISS) assay. siRNA-transfected cells were inoculated with 800 IU PV pseudovirus in the presence of suboptimal concentrations of anti-PV compounds or medium (as a mock-treated control) in a total of 200 μl per well at 96 h p.t. of siRNAs. The cells were incubated at 37°C for 7 h, and then the luciferase activity in the cells was measured with the Steady-Glo luciferase assay system (Promega) using a 2030 ARVO X luminometer (Perkin-Elmer) according to the manufacturer's instructions. The mean net relative light units (RLU) detected in mock-treated cells were 1.3×10^4 to 1.4×10^4 RLU, with standard deviations of 14% of the mean.

PV pseudovirus infection in drug-treated cells was calculated as a percentage of luciferase activity of the infected cells, where the luciferase activity in the infected cells in the absence of compounds was taken as 100% (for Fig. 1 and 5). PV pseudovirus infection in siRNA-transfected cells was calculated as a percentage of luciferase activity of the infected cells, where the luciferase activity in mock-transfected cells in the absence of compounds was taken as 100% (for Fig. 2). PV pseudovirus infection in drug-treated and siRNA-transfected cells was calculated as a percentage of luciferase activity of the infected cells, where the luciferase activity in mock-transfected cells in the presence of the compounds was taken as 100%.

To evaluate the direct effect of siRNA treatment, net PV pseudovirus infection, which is the ratio of PV pseudovirus infection in siRNA-transfected cells (percent) to cell viability (percent), was determined for each siRNA treatment (Fig. 2). The net PV pseudovirus infection in mock-transfected cells was 1. To evaluate sensitization of the cells to the compounds induced by siRNA treatment, normalized PV pseudovirus infection, which is the ratio of PV pseudovirus infection in drug-treated and siRNA-transfected cells (percent) to PV pseudovirus infection in siRNA-transfected cells in the absence of compounds (percent), was determined for each siRNA treatment. The normalized PV pseudovirus infection in mock-transfected cells was 1.

Statistical analysis. The results of experiments are shown as the averages with standard deviations. For comparison of the effect of siRNA treatment on sensitization of the cells to enviroxime-like compounds between two different siRNAs, a paired *t* test was performed with normalized PV pseudovirus infection obtained in four independent experiments. *P* values of less than 0.05 were considered to represent significant differences and are indicated by asterisks in the figures.

RESULTS

T-00127-HEV1 is a highly specific inhibitor for PI4KB. To identify potent anti-PV compounds that target conserved factors required for enterovirus replication, we performed a screening of 72,000 compounds by using a PV pseudovirus infection system (2, 5). We identified 488 compounds as initial hits in the screening. Candidate compounds were further selected from the initial hits according to the following criteria: (i) no apparent cytotoxicity was observed after 2 days of treatment with compounds at 10 μM (>90% viability with no morphological changes of the cells) (18 compounds were selected out of the 488 initial hits); (ii) compounds target the replication step (>90% inhibitory effect after the uncoating step) (8 compounds were selected out of the 18 noncytotoxic compounds); and (iii) compounds inhibit replication of enterovirus 71 (EV71), which belongs to *Human enterovirus species A*, a different species from PV (5 compounds were selected out of

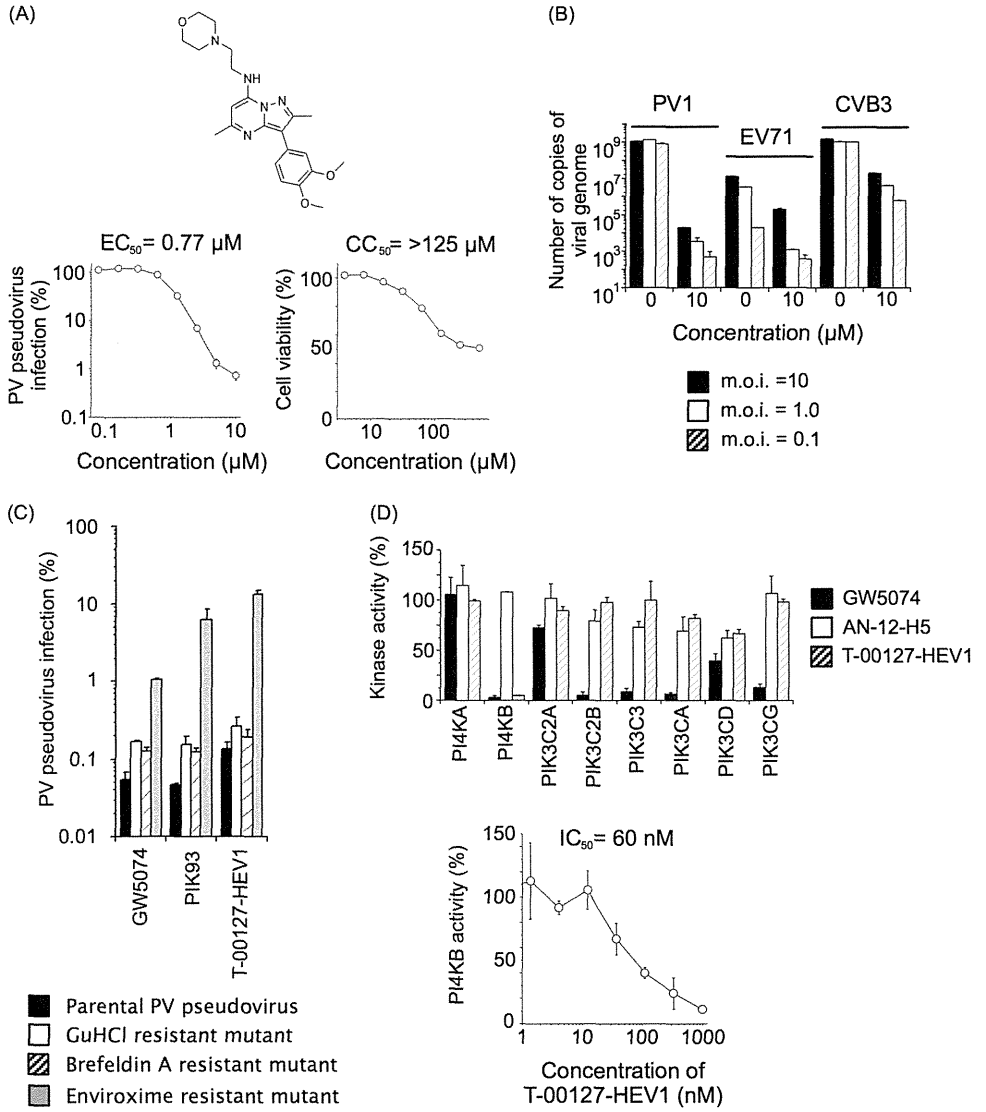

FIG. 1. Characterization of T-00127-HEV1 and inhibitory effects on PI kinases. (A) Characterization of T-00127-HEV1. Upper panel, structure of T-00127-HEV1. Lower panels, inhibitory effect of T-00127-HEV1 on PV pseudovirus (left panel) and viability of RD cells (right panel). PV pseudovirus infection in the absence of compounds was taken as 100%. (B) Inhibitory effect of T-00127-HEV1 on enterovirus infection. RD cells were infected with PV1(Mahoney), EV71(Nagoya), or CVB3(Nancy) at MOI of 10, 1.0, and 0.1 in the absence of T-00127-HEV1 and then were treated with 0 or 10 μM T-00127-HEV1 from 1 h p.i. The total number of copies of viral genomes in the cells at 16 h p.i. is shown. (C) Specificity of mutations causing resistance to antienterovirus compounds. RD cells were infected with PV pseudovirus mutants that have mutations causing resistance to GuHCl (U4614A), brefeldin A (G4361A plus C5190U), and enviroxime (G5318A) in the presence of antienterovirus compounds GW5074 (25 μM), PIK93 (1.3 μM), and T-00127-HEV1 (4.0 μM). PV pseudovirus infection in the absence of compounds was taken as 100%. (D) Inhibitory effects of enviroxime-like compounds on *in vitro* activities of PI kinases. Upper panel, *in vitro* activities of PI kinases were analyzed in the presence of enviroxime-like compounds (GW5074, AN-12-H5, and T-00127-HEV1 at a concentration of 10 μM) with an ATP concentration of 10 μM. Lower panel, inhibitory effect of T-00127-HEV1 on the *in vitro* PI4KB activity measured with an ATP concentration of 10 μM.

the 8 replication inhibitors). Thus, we finally identified 5 candidate compounds that met these criteria from the 72,000 compounds. Among these candidate compounds, T-00127-HEV1 showed more potent anti-PV activity (EC_{50} of 0.77 μM) than the other candidate compounds (EC_{50} of 1.7 to 4.7 μM), anti-EV71 activity (EC_{50} of 0.73 μM), a low cytotoxicity (CC_{50} of >125 μM), and a high selectivity index (SI) for PV (>162) (Fig. 1A and data not shown). The inhibitory effect of T-00127-HEV1 on enterovirus infection was analyzed with PV1(Mahoney), EV71(Nagoya), and CVB3(Nancy), which belongs to *Human enterovirus species B* (Fig. 1B). T-00127-HEV1 showed a broad inhibitory effect on infection with these viruses, with the highest activity against PV infection under the conditions examined.

To characterize the anti-PV activity of T-00127-HEV1 in terms of the resistance mutation of PV, we analyzed the inhibitory effect of T-00127-HEV1 on a panel of PV pseudovirus mutants with drug resistance mutations, including brefeldin A resistance, GuHCl resistance, and enviroxime resistance (Fig. 1C). In this characterization, we also examined the inhibitory

effect of the recently identified novel antienterovirus compound PIK93 (a PI4KB inhibitor) on this panel (30), because its virus resistance determinants have not been identified. Surprisingly, we found that both of the compounds belong to the group of enviroxime-like compounds targeting the 3A/3AB proteins of PV. This prompted us to analyze the inhibitory effects of previously identified enviroxime-like compounds, including GW5074 (5), AN-12-H5 (3), and T-00127-HEV1, on PI kinases (Fig. 1D). GW5074 and T-00127-HEV1 almost completely inhibited PI4KB kinase activity at 10 μM (3% and 5% of residual activity, respectively), in contrast to AN-12-H5 (108% of activity [no inhibition]). GW5074 inhibited most of the PI3 kinases, in contrast to T-00127-HEV1, which showed a high specificity to PI4KB with a moderate inhibitory effect on PIK3CD activity (67% of activity). AN-12-H5 showed only a moderate inhibitory effect on PI kinases, and no complete inhibition was observed.

To further characterize the specificity of T-00127-HEV1 for PI4KB, we analyzed the inhibitory effect of T-00127-HEV1 on *in vitro* activity of cellular protein kinases, including tyrosine kinases and serine/threonine kinases (a total of 150 kinases), because one of the enviroxime-like compounds, GW5074, targets several cellular protein kinases, including Raf-1 (6) (see Table S1 in the supplemental material). The inhibitory effect of T-00127-HEV1 on the examined kinases was weak, i.e., at most 29% inhibition for TTK kinase and <10% inhibition for other kinases at a concentration of 10 μM.

These results suggested that PI4KB is a target of some enviroxime-like compounds as well as PIK93 and that T-00127-HEV1 is a highly specific inhibitor of PI4KB.

PI4KB is a cellular target of GW5074 and T-00127-HEV1 for anti-PV activity. To analyze the cellular target of the anti-PV activities of enviroxime-like compounds, we first performed siRNA knockdown analysis targeting known PI kinases and related host factors for PV replication (GBF1 and ARF1), which are involved in membrane traffic, as well as PI4KB, on PV pseudovirus infection (Fig. 2). siRNA transfection efficiency was monitored by incorporation of fluorescence-labeled siRNAs and the Tox transfection control. Incorporation of fluorescence-labeled siRNAs was observed in virtually all the cells at 24 h p.t. (data not shown). The viability of cells treated with the siGENOME Tox transfection control was 11 to 15% of that of mock-treated cells at 96 h p.t. (Fig. 2A), suggesting a high transfection efficiency. To analyze the net inhibitory effect of siRNA treatment, net PV pseudovirus infection was determined by normalization of PV pseudovirus infection in siRNA-transfected cells to cell viability (Fig. 2B and C). siRNA targeting the GBF1 gene strongly inhibited the infection (0.089 to 0.1 of net PV pseudovirus infection), and a partial inhibition by siRNA targeting the PI4KA gene was observed (0.61 to 0.62 of net PV pseudovirus infection). For other siRNA treatments, including that targeting the PI4KB gene, significant inhibition of PV pseudovirus infection was not observed in normalized inhibition. To confirm specific reduction of the target proteins in the cells treated with the siRNAs, Western blot analysis was performed for siRNA-transfected cells targeted for the GBF1, ARF1, and PI4KB genes (Fig. 2D). The expression levels of the targeted proteins were substantially reduced, with specificity, by the corresponding siRNAs targeting the GBF1, ARF1, and PI4KB genes. These results suggested that reduction of PI

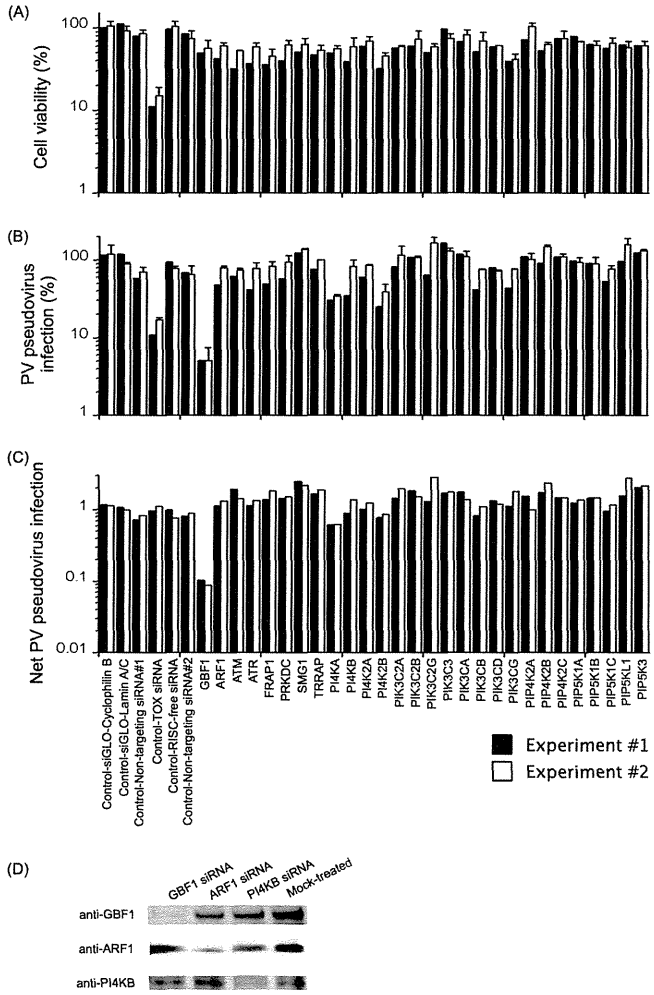

FIG. 2. Effect of siRNA treatment targeting PI kinases on PV pseudovirus infection. (A) Effect of siRNA treatment on cell viability. The viability of mock-treated cells was taken as 100%. (B) Effect of siRNA treatment on PV pseudovirus infection. PV pseudovirus infection in mock-transfected cells was taken as 100%. (C) Net PV pseudovirus infection in siRNA-treated cells. Net PV pseudovirus infection is the ratio of PV pseudovirus infection in siRNA-transfected cells (percent) to cell viability (percent). The net PV pseudovirus infection in mock-transfected cells was 1. (D) Western blot analysis of cell lysates of the cells transfected with siRNAs targeting the GBF1, ARF1, and PI4KB genes.

kinases by siRNA treatment was not sufficient to affect PV replication except in the case of reduction of GBF1.

To identify the cellular targets of GW5074, AN-12-H5, and T-00127-HEV1, we developed a target identification by siRNA sensitization (TISS) assay to identify genes that affect the sensitivity of the cells to these compounds. In this assay, the effect of siRNA treatment on the sensitivity of the cells to the compounds was analyzed to identify the target proteins of the compounds for their anti-PV activity, which could be increased by reduced expression levels of the target proteins. The sensitivity of the cells to the compounds was analyzed at suboptimal concentrations of the compounds (GW5074, AN-12-H5, and T-00127-HEV1), with which only moderate inhibition of PV pseudovirus infection was observed in mock-transfected cells

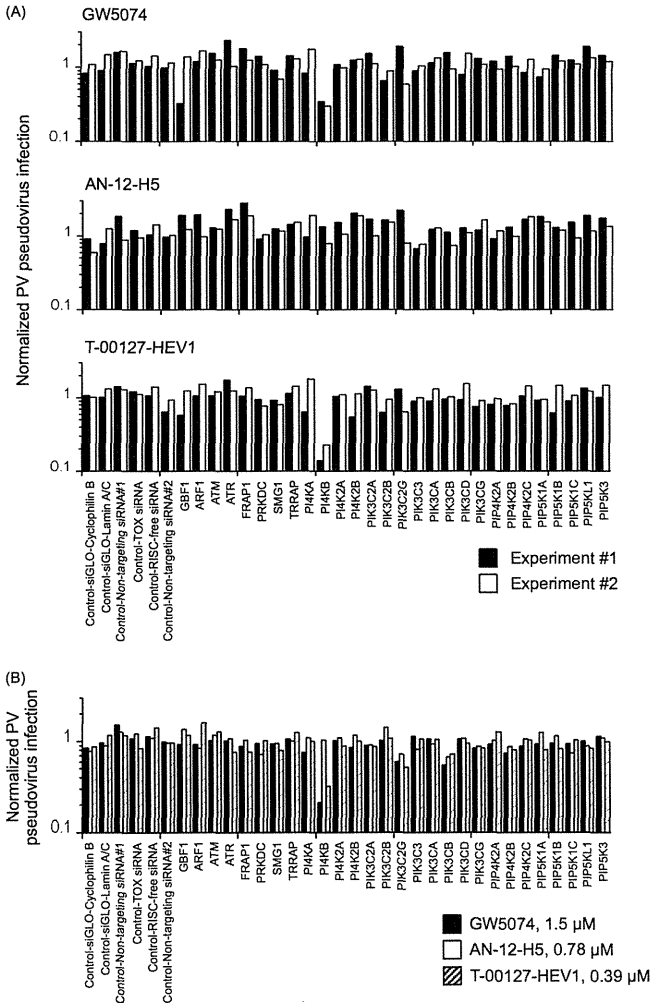

FIG. 3. Effects of enviroxime-like compounds on PV pseudovirus infection in siRNA-transfected cells. (A) Normalized PV pseudovirus infection in the presence of GW5074 (3.1 μM), AN-12-H5 (1.5 μM), and T-00127-HEV1 (0.78 μM). Normalized PV pseudovirus infection is the ratio of PV pseudovirus infection in drug-treated and siRNA-transfected cells (percent) to PV pseudovirus infection in siRNA-transfected cells in the absence of compounds (percent). The normalized PV pseudovirus infection in mock-transfected cells was 1. (B) Normalized PV infection in the presence of GW5074, AN-12-H5, and T-00127-HEV1 at lower concentrations (1.5, 0.78, and 0.39 μM).

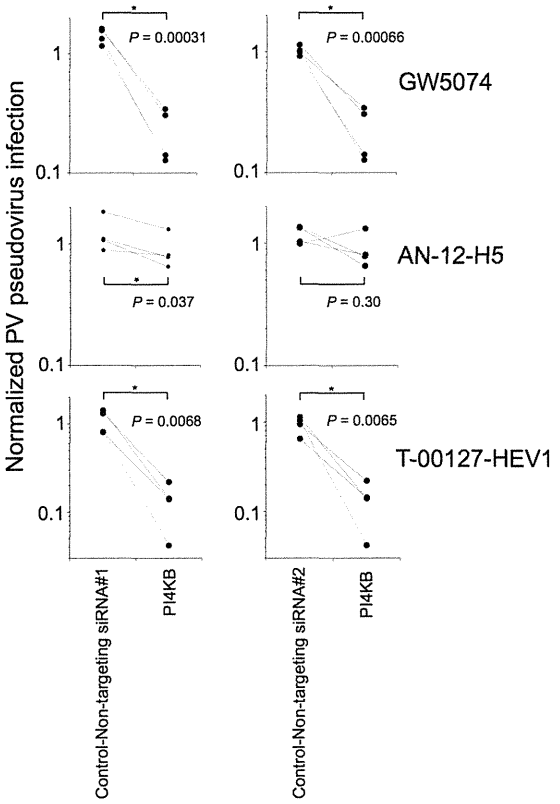

FIG. 4. Effect of enviroxime-like compounds on PV pseudovirus infection in PI4KB siRNA-transfected cells. HEK293 cells were transfected with siRNA targeting the PI4KB gene or with nontargeting siRNAs (nontargeting siRNAs 1 and 2), and then sensitization of the siRNA-transfected cells was analyzed in the presence of GW5074 (3.1 μM), AN-12-H5 (1.5 μM), and T-00127-HEV1 (0.78 μM). *, $P < 0.05$.

(22 to 47% and 57 to 83% of the infection level in mock-treated cells under the conditions used for Fig. 3A and B, respectively), but substantial inhibition could be observed in the cells transfected with siRNAs targeting the target proteins. In the two independent experiments, cells transfected with siRNA targeting the PI4KB gene showed increased sensitivity to GW5074 and T-00127-HEV1 (Fig. 3A). Increased sensitivity was also observed with lower concentrations of these compounds (Fig. 3B). However, in contrast to the case for GW5074 and T-00127-HEV1, cells transfected with the siRNAs examined did not show any increased sensitivity to AN-12-H5. The sensitization of the cells transfected with siRNA targeting the PI4KB gene to GW5074 and T-00127-HEV1 was specific and significant compared with that with nontargeting siRNA controls (Fig. 4). The increase of the sensitization of the cells to AN-12-H5 by treatment with siRNA targeting the PI4KB gene was modest (comparison with nontargeting siRNA 1) or not significant (comparison with nontargeting siRNA 2). These results suggested that GW5074 and T-00127-HEV1 target PI4KB for their anti-PV activities in the cells and that AN-12-H5 has another target(s) for its anti-PV activity.

Comparison of inhibitory effects of antienterovirus compounds on PV and HCV replication. PI4KB has been reported as a host factor for enterovirus replication (30), and its role in HCV replication has also been suggested from experiments using PIK93 and siRNA (13). On the other hand, Berger et al. showed that only PI4KA among the PI4Ks was required for HCV replication (10). To clarify the role of PI4KB in enterovirus and HCV replication, we analyzed the effects of identified anti-PV compounds on the replication of the HCV replicon (Fig. 5). PIK93 showed anti-PV activity and anti-HCV activity, consistent with previous reports (13, 30), although the observed anti-HCV activity was weaker that that previously reported (EC_{50} of 1.9 μM). The order of potency of the compounds on PV pseudovirus infection was PIK93 > T-00127-HEV1 > AN-12-H5 > GW5074. PIK93 showed the highest anti-PV activity among the compounds, with an EC_{50} of 0.14 μM for PV pseudovirus infection (Table 1). In contrast to the case for PIK93, no anti-HCV activity was observed for GW5074 and T-00127-HEV1. Surprisingly, AN-12-H5 showed

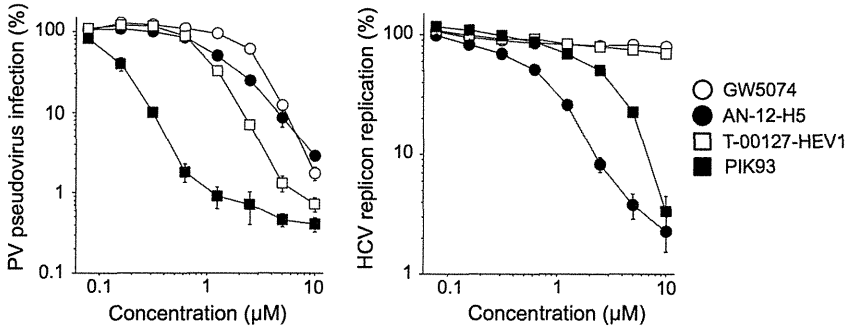

FIG. 5. Effects of enviroxime-like compounds on PV pseudovirus infection and HCV replicon replication. Inhibitory effects of enviroxime-like compounds on PV pseudovirus infection (left panel) and HCV replicon replication (right panel) are shown. PV pseudovirus infection or HCV replication in the absence of compounds was taken as 100%.

a strong anti-HCV activity even higher than that of PIK93. This suggested that AN-12-H5 has targets other than PI4KB for its anti-PV and anti-HCV activities.

DISCUSSION

In the present study, we performed high-throughput screening with a large-scale chemical library (72,000 compounds) and identified a novel enviroxime-like compound, T-00127-HEV1. T-00127-HEV1 was the most potent candidate compound, with broad specificity for enteroviruses (PV1, EV71, and CVB3) and low cytotoxicity. In the characterization of the anti-PV activity of T-00127-HEV1, we fortuitously found that T-00127-HEV1 and PI4KB inhibitor, PIK93, which also has a potent anti-PV activity (30), belong to the same group of anti-PV compounds, the enviroxime-like compounds, in terms of the resistance mutations (Fig. 1). PIK93 is a PI4KB inhibitor (IC_{50} of 19 nM) but also has an almost equal (for PIK3CA and PIK3CG) or lesser (for PIK3CB, PIK3CD, PIK3C2B, and PIK3C3) inhibitory effect on other PI kinases (32). Biological evidence (including physical complex with viral replication complex, colocalization with viral 3A protein, siRNA knockdown analysis targeting PI4KB, and binding activity of PI4P with viral polymerase) indicated the involvement of PI4KB in enterovirus replication (30). T-00127-HEV1 showed a less potent inhibitory effect on *in vitro* PI4KB activity (IC_{50} of 60 nM) than that of PIK93 but with a high specificity for PI4KB (at most 33% inhibition of PIK3CD activity and no inhibitory effect on other PI3 kinases with 10 μM T-00127-HEV1) (Fig. 1). T-00127-HEV1 did not show a significant inhibitory effect on cellular protein kinases examined, as reported for PIK93 (32) (see the supplemental material). The highly specific inhibitory effect of T-00127-HEV1 on PI4KB activity provides pharmacological evidence to support the importance of PI4KB activity in PV replication.

TABLE 1. Properties of antienterovirus compounds in PV and HCV replication[a]

Compound	CC_{50} (2 days), μM	Mean (SD) EC_{50}, μM		SI[b] (PV)
		PV	HCV	
GW5074	96	4.6 (0.85)	>10	21
AN-12-H5	78	1.1 (0.18)	0.69 (0.11)	74
T-00127-HEV1	>125[c]	0.77 (0.10)	>10	>162
PIK93	12	0.14 (0.0086)	1.9 (0.5)	85

[a] Data for GW5074 and AN-12-H5 were adapted in part from reference 3.
[b] SI, selectivity index (ratio of CC_{50} to EC_{50}).
[c] T-00127-HEV1 showed precipitation at above 125 μM.

To date, four enviroxime-like compounds have been reported, i.e., TTP-8307 (24), the cellular protein kinase inhibitors GW5074 and Flt3 inhibitor II, and the bifunctional enterovirus inhibitor AN-12-H5 (3–5, 24). To test the hypothesis that PI4KB is the target of enviroxime-like compounds, we analyzed the inhibitory effects of these enviroxime-like compounds on PI4KB activity *in vitro* and in an *in vivo* TISS assay (Fig. 1D, 3, and 4). In the *in vitro* assay, GW5074, but not AN-12-H5, showed a significant inhibitory effect on PI4KB activity. In the *in vivo* assay, we first analyzed the effect of siRNA treatment targeting the PI4KB gene on PV infection, but we found that the treatment was not sufficient to suppress PV infection, in contrast to that targeting the GBF gene, despite the efficient knockdown of PI4KB (Fig. 2C and D). This observation was not consistent with a previous report that showed about 70% inhibition of PV replication by siRNA knockdown of PI4KB (30). This apparent discrepancy might be caused by normalization of the observed PV infection by cell viability examined in this study, because siRNA treatment targeting the PI4KB gene affected cell viability under the conditions examined (39 to 60% of the viability of mock-treated cells), which had significant weight in the evaluation of net PV infection (Fig. 2C). This suggested that residual PI4KB activity was sufficient to support PV replication. The *in vivo* TISS assay showed that siRNA treatment targeting the PI4KB gene, but not those targeting other genes, conferred significant sensitivity to GW5074 and T-00127-HEV1 but not to AN-12-H5, suggesting that PI4KB is a target of some enviroxime-like compounds for their anti-PV activities (Fig. 3 and 4). Interestingly, we could not observe an effect of siRNA treatment targeting the GBF1 gene on the sensitivity to the examined compounds, which is inconsistent with a proposed role of GBF1 as a tether between viral protein 3A and PI4KB in a model of PV replication (30). However, strong suppression of PV infection by the siRNA treatment, to levels was even lower than that in Tox transfection-treated cells, suggested that the signal detected in the GBF1 knockdown cells might be derived from residual untransfected cells (Fig. 2B). These findings suggested that enviroxime-like compounds have PI4KB and some targets