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BRIEF REPORT

Genetically distinct genogroup IV norovirus strains identified in wastewater

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Abstract We investigated the prevalence and genetic diversity of genogroup IV norovirus (GIV NoV) strains in wastewater in Arizona, United States, over a 13-month period. Among 50 wastewater samples tested, GIV NoVs were identified in 13 (26 %) of the samples. A total of 47 different GIV NoV strains were identified, which were classified into two genetically distinct clusters: the GIV.1 human cluster and a unique genetic cluster closely related to strains previously identified in Japanese wastewater. The results provide additional evidence of the considerable genetic diversity among GIV NoV strains through the analysis of wastewater containing virus strains shed from all populations.

Keywords Norovirus · Wastewater treatment plant · Seminested PCR · Genetic diversity · Arizona

Noroviruses (NoVs) are the most significant pathogens associated with water- and food-borne outbreaks of non-bacterial acute gastroenteritis in humans worldwide [1]. They are members of the family *Caliciviridae* and possess a positive-sense, polyadenylated, single-stranded RNA genome with three open reading frames (ORFs) [1]. NoVs show considerable genetic diversity and are currently proposed to be divided into genogroups I–VI (GI–GVI), of which GI, GII, and GIV infect humans [2]. GII strains account for the majority of reported outbreaks of acute gastroenteritis due to NoVs worldwide, and GI strains cause a majority of the remaining cases [3]. GIV or "Alphatron-like" NoVs were first identified in stool samples from sporadic cases of gastroenteritis in the Netherlands [4], and they have been found occasionally in fecal specimens from gastroenteritis patients [5–10]. Interestingly, several studies have documented that GIV NoVs were also identified in carnivores, including domestic dogs and cats [11–14]; nevertheless, they are genetically distinct from the human GIV NoVs (genotype GIV.1) and classified as a separate genotype, GIV.2.

Recent studies also demonstrated the dissemination of GIV NoVs in municipal wastewater and river water in European and Asian countries [10, 15–22], suggesting that not only GI and GII but also GIV strains circulate worldwide and contaminate environmental waters. More importantly, Kitajima et al. identified several unique GIV NoV strains belonging to a novel genetic cluster in Japanese wastewater samples collected in 2006, which demonstrated considerable genetic diversity among GIV NoV strains [20].

In North America, no report is available on the occurrence of GIV NoVs in water, with limited clinical studies reporting the detection of GIV NoVs in feces of gastroenteritis patients [5]. On the basis of this background, we investigated the prevalence and genetic diversity of GIV NoVs in wastewater in Arizona, the United States, over a 13-month period. GIV NoV genomes in wastewater were detected using a seminested reverse transcription

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(RT)-PCR assay specific for GIV [19], and the strains were further characterized based on partial capsid gene sequences.

Between July 2011 and July 2012, a total of 50 wastewater grab samples were collected monthly from two wastewater treatment plants (WWTPs) (plants A and B, utilizing activated sludge and trickling filter, respectively) located in southern Arizona, which included 13 influent and 13 effluent samples from plant A and 12 influent and 12 effluent samples from plant B. Viruses in the wastewater samples (100 mL influent and 1000 mL effluent) were concentrated using an electronegative filter (cat. no. HAWP-090-00; Merck Millipore, Billerica, MA) and a Centriprep YM-50 device (Merck Millipore) to obtain a final volume of approximately 650 μL as described previously [23].

Viral RNA was extracted from the concentrated wastewater sample spiked with murine norovirus (MNV, S7-PP3 strain; kindly provided by Dr. Y. Tohya, Nihon University, Kanagawa, Japan), a molecular process control, using a ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The RT reaction was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The seminested PCR assay using COG4F, G4SKF, and G4SKR primers was then performed to amplify a 340-bp region of the GIV NoV partial capsid gene as described previously [19]. The second PCR products were separated by electrophoresis on a 2 % agarose gel and visualized under a UV lamp after ethidium bromide staining. All of the second PCR products with expected size were excised from the gel and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). At least eight colonies (clones) per sample were selected, and both strands of direct colony PCR products were sequenced using a BigDye Cycle Sequencing Kit version 3.1 and a 3730*xl* Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled using the program Sequencher™ version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned with Clustal W version 2.1 (http://clustalw.ddbj.nig.ac.jp/top-e.html). The distances were calculated by Kimura's two-parameter method [24], and a phylogenetic tree from a bootstrap analysis with 1000 replicates was generated by the neighbor-joining method. The nucleotide sequences determined in the present study have been deposited in GenBank under accession numbers LC150829–LC150875.

Among 50 wastewater samples tested, GIV NoVs were identified in a total of 13 (26 %) samples with the seminested RT-PCR (Table 1). The positive rate for plant A samples (54 % for influent and 15 % for effluent) was higher than for plant B samples (17 % for influent and 8 % for effluent). Use of MNV as a molecular process control, which was quantified with RT-qPCR [25], showed no substantial inhibition in the molecular detection process in any of the wastewater samples tested in this study (mean recovery efficiency of greater than 75 %; specific recovery efficiency data were reported in our previous study analyzing 48 of the 50 samples [23]).

Based on nucleotide sequencing analysis, a total of 47 GIV NoV sequences (1 to 6 different sequences per sample) were identified and grouped into two genetically distinct clusters: the GIV.1 human cluster and a unique genetic cluster (GIV.new) closely related to strains previously identified in Japanese wastewater reported by Kitajima et al.

Table 1 Detection of GIV NoVs in wastewater in Arizona, USA

Year/month	Plant A		Plant B	
	Influent	Effluent	Influent	Effluent
2011/July	GIV.new	–	NA	NA
Aug	–	–	–	–
Sept	GIV.new	–	–	–
Oct	–	–	–	–
Nov	–	–	–	–
Dec	–	GIV.new	–	–
2012/Jan	GIV.new	–	–	–
Feb	GIV.new	GIV.new	–	–
Mar	–	GIV.new	–	GIV.new
Apr	GIV.1 + GIV.new	–	GIV.1	–
May	–	–	–	–
June	GIV.new	–	GIV.new	–
July	GIV.new	–	–	–
% Positive	54 % (7/13)	23 % (3/13)	17 % (2/12)	8 % (1/12)

– Negative, *NA* not available

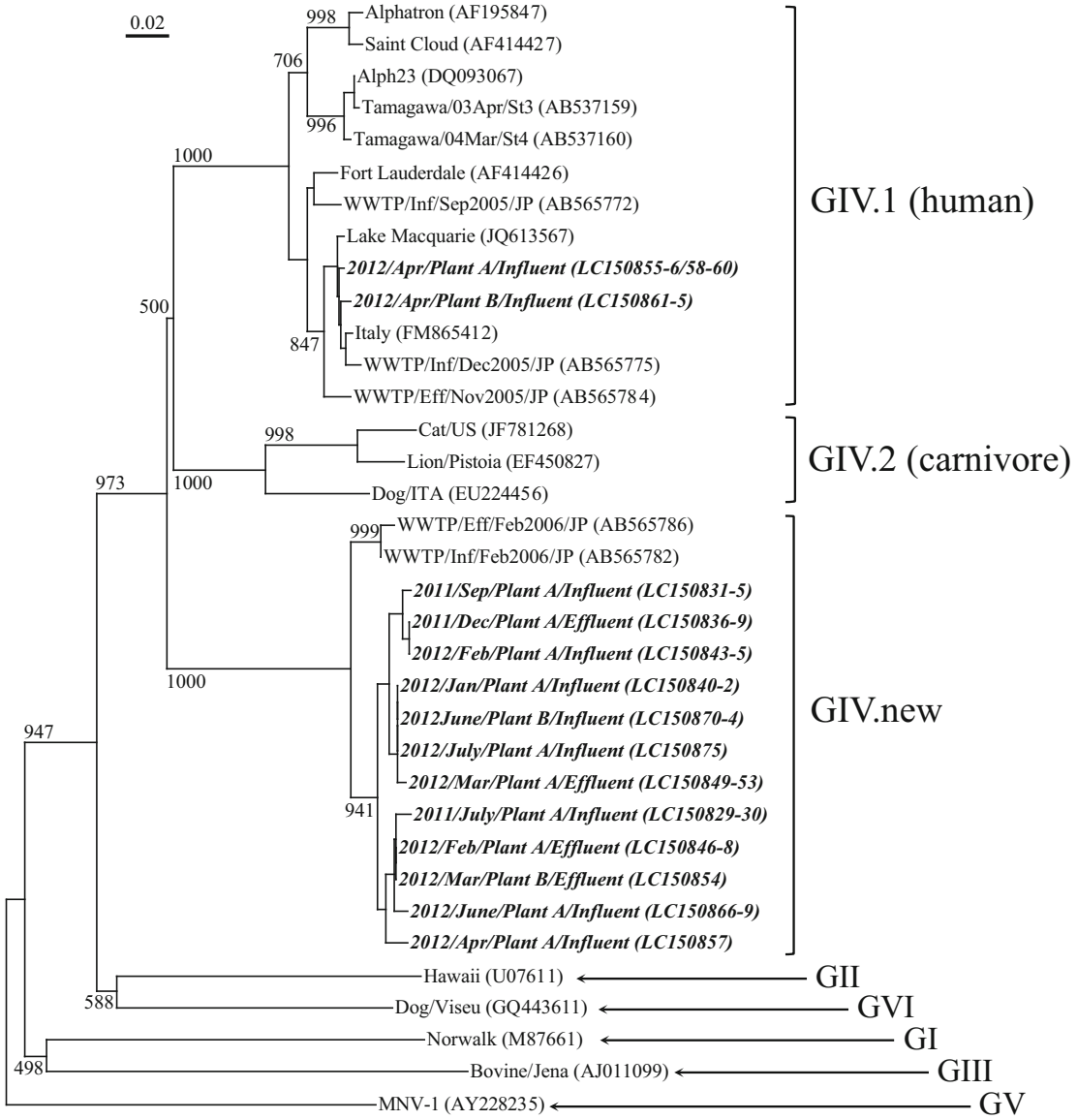

Fig. 1 Phylogenetic tree for GIV NoV strains using 282 nucleotides of the partial capsid gene sequences. The tree was generated by the neighbor-joining method with representative strains derived from wastewater and reference strains. The scale represents nucleotide substitutions per site. Strains shown in ***italic bold*** are representative GIV NoV strains identified in the present study, representing the year and month of sample collection, WWTP (plant A or B), sample type (influent or effluent), and GenBank accession number

[20] (Fig. 1). Interestingly, the strains belonging to the GIV.new cluster were identified in 11 of 13 GIV NoV-positive samples, whereas GIV.1 strains were identified in only two samples collected in April 2012. Influent samples collected from plant A in April 2012 contained both GIV.1 and GIV.new strains (Table 1 and Fig. 1), whereas other GIV NoV-positive samples contained either GIV.1 or GIV.new strains. The strains originating from the same sample in each genetic cluster shared high nucleotide sequence similarity (>98.2 % identity); therefore, only representative strains from each sample are shown in Figure 1. The representative GIV.1 and GIV.new strains identified in the present study (2012/Apr/Plant A/Influent [LC150855] and 2011/July/Plant A/Influent [LC150829], respectively) exhibited highest nucleotide identities of 99.6 % to Lake Macquarie (JQ613567) and 96.1 % to Wastewater/JPN (GIV strain identified in wastewater in Japan, AB565789), respectively, in the nucleotide sequence database (Table 2). The representative GIV.new strain (2011/July/Plant A/Influent [LC150829]) showed nucleotide sequence identities of only 81.9 % to Fort Lauderdale (GIV.1) and 77.9 % to Dog/ITA (GIV.2) (Table 2), demonstrating that this strain is genetically distinct from both of the previously described GIV genotypes.

Table 2 Nucleotide sequence similarity between the representative GIV NoV strains identified in the present study and previously described GIV NoV strains belonging to each genetic cluster, based on BLAST nucleotide search results

Cluster—representative strain	GIV.1			GIV.2		GIV.new
	Alphatron	Fort Lauderdale	Lake Macquarie	Dog/ITA	Cat/US	Wastewater/JPN
GIV.1—2012/Apr/plant A/influent	94.3 % (266/282)	97.5 % (274/281)	99.6 % (281/282)	84.2 % (235/279)	81.4 % (228/280)	81.7 % (227/278)
GIV.new—2011/July/plant A/influent	80.1 % (218/272)	81.9 % (221/270)	80.9 % (220/272)	77.9 % (218/280)	76.8 % (209/272)	96.1 % (271/282)

GenBank accession numbers for GIV NoV strains: 2012/Apr/Plant A/Influent, [LC150855]; 2011/July/Plant A/Influent [LC150829], Alphatron, AF195847; Fort Lauderdale, AF414426; Lake Macquarie, JQ613567; Dog/Italy, EU224456; Cat/US, JF781268; Wastewater/JPN, AB565789

The GIV NoV strains belonging to the unique genetic cluster (GIV.new strains) were closely related to the strains identified in Japanese wastewater samples collected in 2006 [20], but related virus strains have not been identified from human or animal stool specimens. Our results demonstrate that diverse GIV NoVs belonging to two genetically distinct clusters were circulating in Arizona, United States. Since wastewater contains viruses shed from all populations regardless of symptoms, virus strains and their genetic information obtained from wastewater should reflect more precisely the circulation of genetic variants. In the present study, we report the identification of genetically distinct GIV NoV strains in wastewater, which highlights the need for further clinical and environmental studies on GIV NoVs toward a better understanding of their prevalence, molecular epidemiology, and genetic evolution.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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