

# Wastewater Analysis Indicates that Genetically Diverse Astroviruses, Including Strains Belonging to Novel Clades MLB and VA, Are Circulating within Japanese Populations

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**Human astroviruses (HAsVs) are a common etiological agent of infantile gastroenteritis. Recent studies revealed that novel astrovirus (AstV) strains of the MLB clade (MLB-AstVs) and VA clade (VA-AstVs), which are genetically distinct from the classic HAsVs, are circulating in the human population. In the present study, we quantified classic HAsVs as well as carried out a genetic analysis of classic and novel HAsVs in wastewater in Japan. The concentration of classic HAsVs in the influent water samples ranged from 10<sup>4</sup> to 10<sup>5</sup> copies per liter, and the amount removed by wastewater treatment was determined to be 2.4 ± 0.3 log<sub>10</sub>. Four types of classic HAsV strains (HAsV types 1, 2, 5, and 4/8) as well as novel AstV strains belonging to the MLB-2, VA-1, and VA-2 clades were identified using reverse transcription-PCR (RT-PCR) assays, including assays newly developed for the detection of strains of the MLB and VA clades, followed by cloning and nucleotide sequencing. Our results suggest that genetically diverse AstV strains are circulating among the human population in Japan. The newly developed (semi)nested RT-PCR assays for these novel AstV clades are useful to identify and characterize the novel AstVs in environmental waters.**

Human astroviruses (HAsVs) are considered one of the most important causes of infantile gastroenteritis worldwide (1). The infections typically occur sporadically, and the cause of 2 to 10% of total viral gastroenteritis cases is attributed to HAsVs (1–6). Astroviruses (AstVs) possess nonenveloped, icosahedrally shaped virions containing approximately 6,800 nucleotides (nt) of single-stranded positive-sense RNA (7). It has been reported that the diameter of AstV particles shed in fecal specimens ranges from 28 to 31 nm, while the diameter of those grown in cell culture is 41 nm (8). Their viral genome consists of three open reading frames (ORFs), namely, ORF1a, ORF1b, and ORF2, encoding the serine protease, RNA-dependent RNA polymerase, and capsid proteins, respectively (7). HAsVs are antigenically or genetically classified into eight different types (HAsV type 1 [HAsV-1] to HAsV-8). Previous studies revealed that HAsV-1 is the most prevalent type among infected individuals and in the environment, followed by HAsV-2, -3, -4, and -5 (3, 5, 9–13).

Because HAsV can be excreted in the feces of infected individuals at a high concentration (up to 10<sup>15</sup> particles/g) (14), examination of municipal wastewater samples could be an effective approach to understand the actual prevalence and epidemiology of these viruses (15, 16). In spite of their importance as enteric pathogens, the occurrence and other characteristics of HAsVs in water compared to those of other enteric viruses, such as noroviruses, rotaviruses, and adenoviruses, have not been well documented (17, 18). Due to the scarcity of knowledge, the fate of HAsVs in water environments and wastewater treatment processes is not well understood.

Recent studies based on viral metagenomic analysis have identified some novel AstVs, namely, clade MLB AstVs (MLB-AstVs) and clade VA (HMO) AstVs (VA-AstVs), in human feces (19, 20). The novel AstV strains are genetically distinct from the classic HAsV strains (20–22); in fact, VA-AstVs are genetically more closely related to mink/ovine AstVs than to classic HAsVs (20, 22). To date, MLB-AstVs have been divided into 3 genotypes

(MLB-AstV genotype 1 [MLB-AstV-1] to MLB-AstV-3) and VA-AstVs have been divided into 4 genotypes (VA-AstV genotype 1 [VA-AstV-1] to VA-AstV-4) (7, 20, 23, 24). It has been suggested that VA-AstV-1 and MLB-AstV-1 are associated with gastroenteritis outbreaks (23, 25), but the prevalence and etiological role of the novel AstV strains in humans remain unclear (26, 27). Some recent studies reported on the frequent detection of the novel AstVs in fecal samples (24, 26). This prompted us to investigate the occurrence of HAsVs in wastewater, which contains viruses shed from all populations regardless of symptoms.

On the basis of the background presented above, we performed the present study to investigate the occurrence and genetic diversity of classic HAsVs as well as novel AstVs in wastewater in Japan. Classic HAsV genomes were quantified by real-time quantitative PCR (qPCR) assays to assess the abundance of classic HAsVs at a wastewater treatment plant (WWTP) and the level of reduction of classic HAsVs by the wastewater treatment process. In addition, we employed nested and seminested PCR assays, including assays newly designed for the detection of novel AstVs, to amplify the viral genomes. The strains were further characterized on the basis

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of the sequence of the 3' end of ORF1b or the sequence of the 5' end of ORF2.

## MATERIALS AND METHODS

**Collection and concentration of wastewater samples.** Wastewater samples were collected monthly from October 2007 to March 2008 at a WWTP in an urban area in Japan as described in our previous study (28). The characteristics of the WWTP are described in Table S1 in the supplemental material. This WWTP employs chlorination and sand filtration after the secondary treatment. During the 6-month study period, a total of 24 samples were collected from four locations in the treatment train: influent, after secondary treatment, after chlorination, and after sand filtration (effluent). The samples (100 ml for the influent samples and 1,000 ml for each of the other samples) were concentrated using an electronegative membrane (type HA; diameter, 90 mm; pore size, 0.45  $\mu\text{m}$ ; Millipore, Tokyo, Japan) and a centrifugal ultrafiltration device (Centriprep YM-50; Millipore) to obtain a final volume of approximately 0.7 ml, as previously described (29). As a process control,  $2.2 \times 10^7$  genome copies of murine norovirus (MNV) were inoculated into 140  $\mu\text{l}$  of the concentrate.

**Viral RNA extraction and RT.** Viral RNA was extracted from 140  $\mu\text{l}$  of the concentrated sample inoculated with MNV using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany) to obtain a final volume of 60  $\mu\text{l}$ , according to the manufacturer's protocol. The reverse transcription (RT) reaction was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Briefly, 10  $\mu\text{l}$  of extracted RNA was added to 10  $\mu\text{l}$  of an RT mixture containing 2  $\mu\text{l}$  of 10 $\times$  reverse transcription buffer, 0.8  $\mu\text{l}$  of deoxynucleoside triphosphates (dNTPs), 2  $\mu\text{l}$  of 10 $\times$  random hexamers, 50 U of MultiScribe reverse transcriptase, and 20 U of RNase inhibitor. The RT reaction mixture was incubated at 25°C for 10 min, followed by 37°C for 120 min and, finally, 85°C for 5 min to inactivate the enzyme.

**Quantification of classic HAstV genomes by qPCR.** TaqMan-based qPCR for classic HAstVs was performed with a LightCycler 480 real-time PCR instrument II (Roche Diagnostics, Mannheim, Germany) as described previously (30). Briefly, 5  $\mu\text{l}$  of cDNA was mixed with 20  $\mu\text{l}$  of a reaction buffer containing 12.5  $\mu\text{l}$  of a 2 $\times$  TaqMan gene expression master mix (Applied Biosystems), 400 nM (each) sense and antisense primers, 100 nM TaqMan minor groove binder (MGB) probe, and nuclease-free water. Real-time PCR was performed under the following thermal cycling conditions: initial denaturation at 95°C for 15 min to activate the DNA polymerase, followed by 50 cycles of amplification with denaturation at 94°C for 15 s and annealing and extension at 62°C for 60 s. Fluorescence readings were collected and analyzed with LightCycler 480 software (version 1.5; Roche Diagnostics). The genome copy numbers of classic HAstVs were determined on the basis of a standard curve prepared with 10-fold serial dilutions of plasmid DNA containing an insert of the 5' end of the ORF2 region of classic HAstV-1 (Oxford strain) at concentrations ranging from  $10^5$  to  $10^1$  genome copies per reaction mixture on the basis of the plasmid DNA concentration, determined by measuring the optical density at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Kanagawa, Japan). Two PCR tubes were used for each sample, and the average cDNA copy numbers for the two tubes were used for subsequent calculations. Negative controls were included to avoid false-positive results due to cross-contamination. No false-positive qPCR signal was observed. The qPCR assay was designed to detect only classic HAstV strains (30). In fact, numerous mismatches between the nucleotide sequences of the novel AstVs and the primer and TaqMan probe sequences were observed (see Table S2 in the supplemental material), indicating that the assay does not detect the novel AstVs.

**Determination of viral RNA extraction and RT-qPCR efficiency.** The inoculated MNV was recovered by RT-qPCR to determine the virus detection efficiency, as described previously (28). Briefly, the RNA extract was subjected to RT and qPCR as described above. A primer set and TaqMan probe designed by Kitajima et al. (31) were used for the reactions.

Plasmid DNA containing an insert of the target sequence was used for generating a standard curve as described above. The virus detection efficiency was determined by comparing the observed genome copy number in a sample to that in a positive control prepared by inoculating MNV into Milli-Q water. If the ratio between the amount of MNV quantified from a sample and that quantified from the positive-control sample was less than 10%, the quantification was regarded as unreliable due to low RNA extraction and/or RT-qPCR efficiencies. Since MNV was spiked to determine the viral RNA extraction and RT-qPCR efficiencies, the classic HAstV concentration obtained was not corrected even if the quantification was determined to be unreliable.

**Design of PCR primers for MLB-AstV and VA-AstV detection.** A set of seminested PCR primers for amplification of a 644-nt fragment in the 3' end of ORF1b of MLB-AstVs was designed on the basis of the nucleotide sequence alignment of 11 MLB-AstV strains in the GenBank database (see Fig. S1A in the supplemental material). Similarly, a set of nested PCR primers for amplification of a 618-nt fragment in the 5' end of ORF2 of VA-AstVs was designed on the basis of the nucleotide sequence alignment of 5 VA-AstV strains in the GenBank database (see Fig. S1B in the supplemental material). The accession numbers of the novel AstVs used for primer design were as follows: ET065575, ET065582, FJ222451, FJ227124, FJ227125, FJ227127, GQ502188, GQ502189, GQ502190, GQ502191, and GQ502192 for MLB-AstVs and FJ973620, GQ502193, GQ502194, GQ502195, and GQ502196 for VA-AstVs. The newly designed primers are shown in Table 1. A nucleotide BLAST search of each primer showed no significant homology to nontarget sequences (data not shown).

**(Semi)nested PCR.** For amplification of each AstV target, a nested PCR assay for classic HAstVs and the newly designed (semi)nested PCR assays for novel AstVs (Table 1) were performed separately using KOD plus (version 2; Toyobo, Osaka, Japan), a high-fidelity PCR polymerase. Briefly, the first round of PCR amplification was performed in 50  $\mu\text{l}$  of a reaction mixture containing 5  $\mu\text{l}$  of cDNA, 1.0 U of KOD plus polymerase, 5  $\mu\text{l}$  of 10 $\times$  buffer for KOD plus polymerase (version 2), 200 nM dNTPs, 1.5  $\mu\text{l}$  of 25 mM  $\text{MgSO}_4$ , 400 nM (each) sense and antisense primers, and nuclease-free water. Amplifications of all three target genes were performed on a Verti 96-well thermal cycler (Applied Biosystems) under the following thermal cycling conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of amplification with denaturation at 98°C for 10 s, primer annealing at 54°C for 30 s, and an extension reaction at 68°C for 1 min and then a final extension at 68°C for 7 min. The second round of PCR was performed in a 50- $\mu\text{l}$  reaction mixture containing 2  $\mu\text{l}$  of the product of the first round of PCR amplification, 1.0 U of KOD plus polymerase, 5  $\mu\text{l}$  of 10 $\times$  buffer for KOD plus polymerase (version 2), 200 nM dNTPs, 1.5  $\mu\text{l}$  of 25 mM  $\text{MgSO}_4$ , 400 nM (each) sense and antisense primers, and nuclease-free water. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification with denaturation at 98°C for 10 s, primer annealing at 54°C for 30 s, and extension reaction at 68°C for 1 min and then a final extension at 68°C for 7 min.

**Cloning, sequencing, and phylogenetic analysis.** The products from the second PCR amplification were separated by electrophoresis in a 1.5% agarose gel and visualized under a UV lamp after ethidium bromide staining. PCR products of the expected size (i.e., approximately 362 bp, 645 bp, and 618 bp for classic HAstVs, MLB-AstVs, and VA-AstVs, respectively) were excised from the gel and purified with a QIAquick gel extraction kit (Qiagen). The purified products were cloned into a Zero Blunt TOPO pCR 2.1 vector (Invitrogen, Carlsbad, CA), and the plasmid constructs were then transformed into *Escherichia coli* One Shot TOP10 chemically competent cells (Invitrogen). The transformants were incubated at 37°C on an LB agar plate containing 20  $\mu\text{g}/\text{ml}$  of kanamycin. Six to eight colonies were selected, and insertion sizes were checked by direct colony PCR amplification using KOD plus polymerase (version 2) and an M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAACAGCTATGAC-3') primer set. The PCR products were purified with a PCR purification kit (Qiagen), and both strands were sequenced with a BigDye

TABLE 1 Primers and TaqMan probe used in this study

Target	Assay	Primer or probe name	Function	Sequence (5' → 3') <sup>a</sup>	Location <sup>c</sup>	Reference or source
Classic HAstV	qPCR	HuAstVf2240	Sense primer	CAGGTAACCTGTTGAGGTCA	4349–4367	30
	qPCR	HuAstVf2140	Sense primer	GCAAGTYACTGTTGAGGTCA	4348–4367	30
	qPCR	HuAstVf2239T4	Sense primer	GAAGTCACTGTGGAGGTC	4349–4366	30
	qPCR	HuAstVr	Antisense primer	GTTTGGTCTCTGTGACACC	4544–4562	30
	qPCR	HuAstV/1-8/TP	TaqMan probe	FAM-TTACGGACACGYTGA-MGB-NFQ <sup>b</sup>	4501–4515	30
	Nested PCR (first round)	AHAstVF1	Sense primer	AATCACTCCATGGGAAGCTCCT	4139–4160	33
	Nested PCR (first round)	AHAstVR1	Antisense primer	CCTARCGCYTGCACDGG	4697–4713	33
	Nested PCR (second round)	AHAstVF2	Sense primer	CAGAAGAGCAACTCCATCGCAT	4280–4301	33
	Nested PCR (second round)	AHAstVR2	Antisense primer	GTRCTYCCWGTAGCRTCTTAAC	4664–4686	33
	MLB-AstV	Seminested PCR (first round)	SF0073	Sense primer	GAYTGGACWCGATTGATGGTAC	3110–3132
Nested PCR (first round)		AHMLBR1	Antisense primer	CAGGYTTAGGCCAGTTGTA	4016–4035	This study
Seminested PCR (second round)		AHMLBR2	Antisense primer	CGAGTGAAGCGCCTTGGAAG	3778–3798	This study
VA-AstV	Nested PCR (first round)	AHVAF1	Sense primer	TATGGGAARCTCCTTTGCTAYCGC	4025–4048	This study
	Nested PCR (first round)	AHVAR1	Antisense primer	ARTTTCTTGACAAACCACCAWCC	5211–5233	This study
	Nested PCR (second round)	AHVAF2	Sense primer	ATGCTGGATAGRCTTTGGAGGG	4172–4193	This study
	Nested PCR (second round)	AHVAR2	Antisense primer	SCTCCCTCTTCATTKGTRTCTGT	4812–4834	This study

<sup>a</sup> The mixed bases in degenerate primer and probe are as follows: Y, C or T; W, for A or T; R, A or G; D, A, G, or T; S, G or C; K, T or G.

<sup>b</sup> FAM, 6-carboxyfluorescein; MGB, minor groove binder; NFQ, nonfluorescent quencher.

<sup>c</sup> The corresponding nucleotide positions of classic HAstV-1 strain Oxford, MLB-AstV-1, and VA-AstV-1 (GenBank accession numbers L23513, FJ222451, and FJ973620, respectively).

cycle sequencing kit (version 3.1; Applied Biosystems) and a 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were assembled using the program Sequencher (version 4.2.2; Gene Codes Corporation, Ann Arbor, MI) and aligned by use of the Clustal W program (version 1.83; <http://clustalw.ddbj.nig.ac.jp/top-e.html>). The distances were calculated using Kimura's two-parameter method (32), and phylogenetic dendrograms from a bootstrap analysis with 1,000 replicates were generated by the neighbor-joining method.

**Nucleotide sequence accession numbers.** The nucleotide sequences determined in this study were deposited in GenBank under accession numbers LC009636 to LC009664 (classic HAstVs), LC009665 to LC009677 (MLB-AstVs), and LC009678 to LC009684 (VA-AstVs).

## RESULTS

### Quantification of classic HAstV genomes in wastewater by RT-qPCR.

The virus detection efficiencies obtained using inoculated

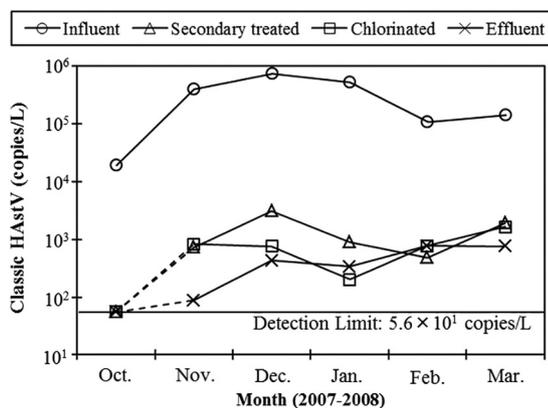


FIG 1 Concentrations of classic HAstVs in wastewater samples determined by RT-qPCR. The results for samples negative for classic HAstVs (secondary treated, chlorinated, and effluent samples collected in October 2007) were plotted on a line indicating the detection limit ( $5.6 \times 10^1$  copies per liter) and are connected by dashed lines.

MNV are summarized in Table S2 in the supplemental material. All samples showed at least 30% RNA extraction and RT-qPCR efficiencies, and these values are acceptably high, indicating that AstVs were detected at sufficient efficiencies.

All influent samples ( $n = 6$ ) were positive for classic HAstVs, with concentrations ranging from  $1.9 \times 10^4$  (October 2007) to  $7.5 \times 10^5$  (December 2007) copies per liter (Fig. 1). The partially or fully treated wastewater samples were also positive for classic HAstVs, except for the samples collected in October 2007, when the influent samples showed the lowest concentration. The concentrations of classic HAstV genomes in secondary treated and chlorinated wastewater samples ranged from  $10^2$  to  $10^3$  copies per liter, and those in effluent samples ranged from  $10^1$  to  $10^2$  copies per liter, except for the samples negative for classic HAstVs. Among the samples positive for classic HAstVs, the geometric mean reduction of classic HAstV genomes brought about by the whole wastewater treatment process was determined to be  $2.4 \pm 0.3 \log_{10}$ .

**Genetic diversity of classic HAstVs and MLB- and VA-AstVs in wastewater.** Among the influent and effluent samples tested (one sample of each type per month), classic HAstV-positive PCR products were obtained from all the influent samples and 4 effluent samples by nested RT-PCR; no PCR products were obtained from the effluent samples in October and November 2007, and of these samples, the October 2007 sample tested negative by qPCR as well, but the November 2007 sample was qPCR positive (Fig. 1). On the basis of the results of nucleotide sequencing analysis, a total of 29 classic HAstV strains were identified in the wastewater samples (Fig. 2). Type 2 and type 4/8 (types 4 and 8 could not be distinguished [33]) were found in both influent and effluent wastewater samples at relatively high frequencies. Type 1 and type 5 were found in 2 and 1 influent wastewater samples, respectively, but these types were not found in effluent wastewater samples (Fig. 2).

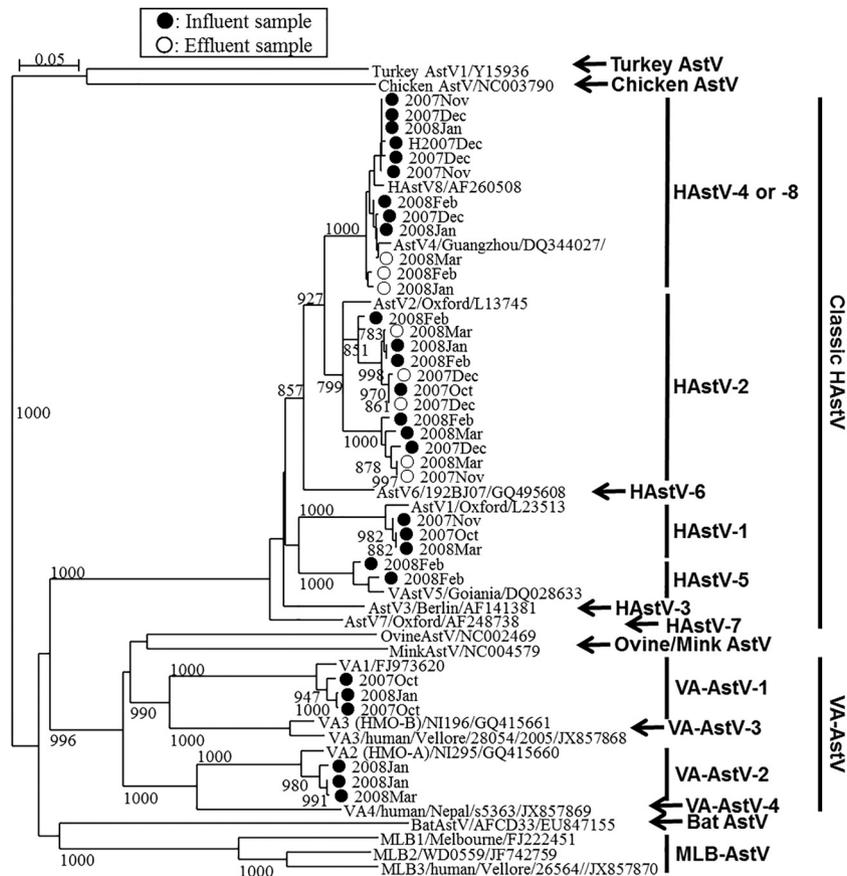


FIG 2 Phylogenetic tree of classic HAstV and VA-AstV strains obtained using an approximately 360-nt fragment at the 5' end of ORF2. Strains detected from influent samples (marked ●) and strains detected from effluent samples (marked ○), along with corresponding historical AstV strains, including MLB-AstVs and other animal AstVs strains, are included. Bootstrap values greater than 700 among 1,000 replicates are shown on each branch.

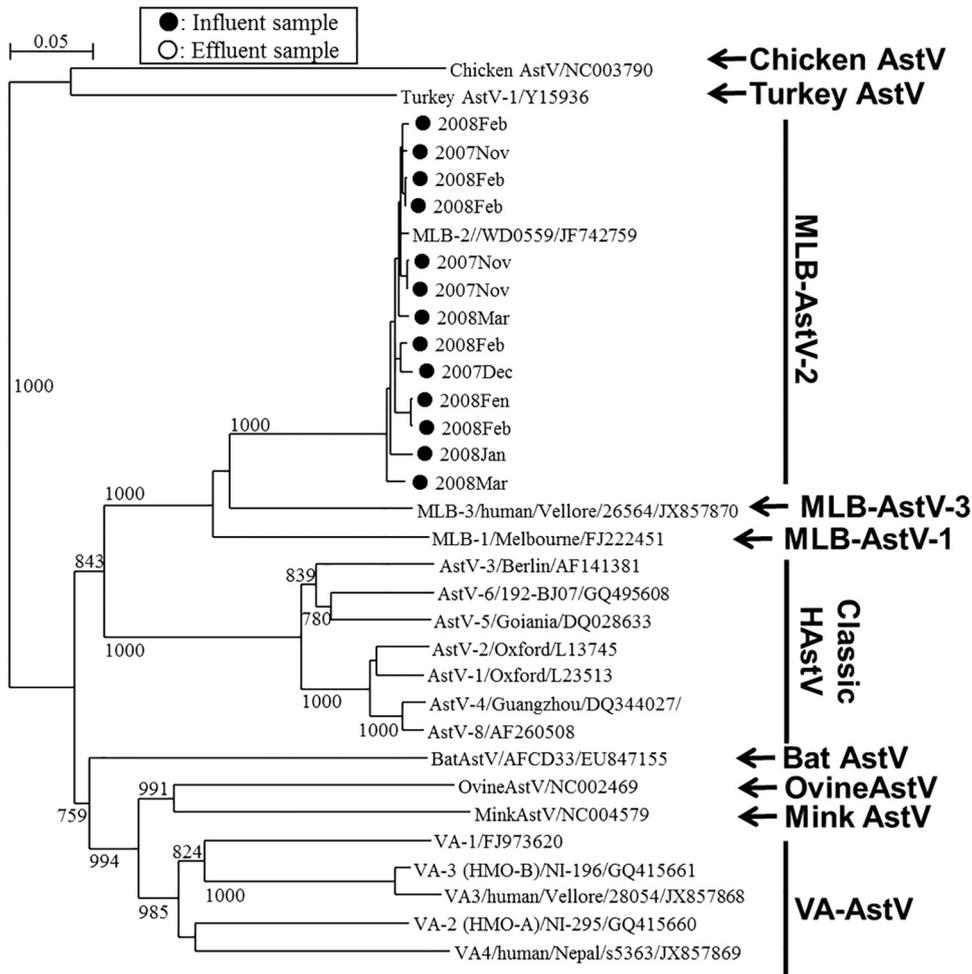
MLB- and VA-AstVs were identified in 5 and 3 influent samples, respectively, but they were not detected in any of the effluent samples (Fig. 2 and 3 and Table 2). Nucleotide sequence analysis of the 3' end of ORF1b (MLB-AstVs) and the 5' end of ORF2 (VA-AstVs) revealed that all the MLB-AstVs identified in the present study (13 strains) were closely related to type 2 and the VA-AstVs (6 strains) were classified as type 1 or type 2 (Fig. 2 and 3). An influent sample collected in January 2008 contained both type 1 and type 2 VA-AstVs.

## DISCUSSION

Classic HAstVs are responsible for 2 to 10% of cases of infantile viral gastroenteritis and are recognized to be important enteric pathogens (1). The goal of the present study was to reveal the prevalence and genetic diversity of human AstVs, including AstVs of emerging clades MLB and VA, in municipal wastewater. Since cell culture assays for classic HAstVs are time-consuming, less sensitive than RT-PCR, and not specific, RT-PCR-based assays are more effective for determination of the occurrences of classic HAstVs. In general, the amplification efficiency/sensitivity of PCR for environmental virus detection can be affected by several factors, such as the primer and probe sequences used and the sample matrix (34, 35). Because environmental samples contain multiple virus strains, selection of a broadly reactive RT-PCR assay is important to obtain representative results.

We first investigated the occurrence of classic HAstVs in waste-

water by using qPCR assays. We reported the concentration of classic HAstVs in wastewater samples determined by a TaqMan-based qPCR assay for classic HAstVs (developed by Le Cann et al. [36]) in our previous study (28). This assay has been widely used for the detection and quantification of classic HAstV genomes in clinical as well as environmental samples (37–39); however, we found that the primer and probe sequences of the assay (targeting the 3' end of ORF2) have considerable mismatches with the sequences of classic HAstV strains, based on the sequence alignment of a total of 281 classic HAstV nucleotide sequences (33). This prompted us to consider another TaqMan-based assay reported by Yokoi and Kitahashi (30), which targets the 5' end of ORF2 and shows substantially fewer mismatches, for the more accurate detection/quantification of genetically diverse classic HAstVs (see Fig. S2 in the supplemental material). Using this assay, we examined the same wastewater sample set used in the previous study and obtained a positive ratio (21 positive samples out of 24 samples tested) higher than that obtained in our previous study (11 positive samples out of 24 samples tested) using the less broadly reactive assay (36). Furthermore, the concentration observed in the present study was  $0.89 \pm 0.30 \log_{10}$  higher than that observed in the previous study for each sample (see Fig. S3 in the supplemental material). The assay targeting the 5' end of ORF2 (30) showed improved detection and quantification compared to those of the other assay targeting the 3' end of ORF2 (36). The genetic



**FIG 3** Phylogenetic tree of MLB-AstVs strains obtained using a 645-nt fragment at the 3' end of ORF1b. Strains detected from influent samples (marked ●) along with corresponding historical AstV strains, including VA-AstVs and other animal AstVs strains, are included. Bootstrap values greater than 700 among 1,000 replicates are shown on each branch.

sequences on the 5' end of the ORF2 region are more conserved than those on the 3' end of the ORF2 region among classic HAsTV strains (40). Thus, the concentration of classic HAsTVs determined in our previous study using the assay targeting the 3' end of ORF2 was underestimated. The concentrations of classic HAsTVs

in the influent samples ( $1.9 \times 10^4$  to  $7.5 \times 10^5$  copies per liter, as determined by the assay targeting the 5' end of ORF2) were similar to those of genotype II (GII) noroviruses ( $8.9 \times 10^4$  to  $9.5 \times 10^5$  copies per liter), the leading cause of viral gastroenteritis, according to the findings of our previous study which analyzed the same

**TABLE 2** Astrovirus genotypes identified in wastewater samples

Mo and yr of sample collection	Genotype(s) identified in:					
	Influent			Effluent		
	Classic HAsTV	MLB-AstV	VA-AstV	Classic HAsTV	MLB-AstV	VA-AstV
Oct. 2007	2, 4/8 <sup>a</sup>	ND <sup>b</sup>	1	ND	ND	ND
Nov. 2007	1, 4/8	2	ND	ND	ND	ND
Dec. 2007	2, 4/8	2	ND	2	ND	ND
Jan. 2008	4/8	2	1, 2	2, 4/8	ND	ND
Feb. 2008	2, 4/8, 5	2	ND	4/8	ND	ND
Mar. 2008	1, 2	2	2	2, 4/8	ND	ND
Positive ratio <sup>c</sup>	6/6	5/6	3/6	4/6	0/6	0/6

<sup>a</sup> Type 4 or 8.

<sup>b</sup> ND, not detected by RT-qPCR or RT-(semi)nested PCR.

<sup>c</sup> The data represent the number of samples in which the indicated genotype(s) was detected/total number of samples tested.

sample set analyzed in the present study (28). This suggests that classic HAstVs are widespread in humans in Japan, at least during the autumn and winter seasons. Classic HAstV infections are observed sporadically in general (1), while GII noroviruses frequently cause gastroenteritis outbreaks in winter (41). This indicates that there were considerable numbers of underreported cases that caused mild or asymptomatic infections in the sewage service area. The improved detection/quantification also implies that the risk of classic HAstV infection via water may be higher than was previously thought. It is possible that the risk of infection with HAstVs is comparable to that with other widespread viruses causing gastroenteritis, such as norovirus, rotavirus, and adenovirus.

The reduction of classic HAstVs by the conventional activated sludge process ( $2.4 \pm 0.3 \log_{10}$ ) was comparable to that of many of other enteric viruses tested in our previous study (28). A difference in the stability of different AstVs in the environment has been suggested. For example, Morsy El-Senousy et al. (42) reported that genogroup B classic HAstVs (types 6 and 7) are more resistant to wastewater treatments than other classic HAstV genogroups. In our current study, however, this genogroup was not detected in wastewater (Fig. 2), which agrees with the findings of previous clinical studies reporting that this genogroup is not prevalent among gastroenteritis patients in Japan (10, 43). Future studies should investigate using type/clade-specific qPCR assays to detect differences in the levels of reduction of different AstV genotypes/groups, including the novel clades, attained with treatment.

In our previous study, we designed nested PCR primers targeting the conserved 5' end of the ORF2 region (33). The assay showed sensitivity comparable to that of the broadly reactive RT-qPCR assay in this study (Fig. 2 and Table 2). Classic HAstV-1 has been the type that has been the most frequently found in clinical samples worldwide (10, 44, 45), as well as in urban wastewater and surface water samples (9, 46). In the present study, however, classic HAstV-1 was detected in only 2 influent samples, while classic HAstV-2 and classic HAstV-4/8 were more frequently detected in both influent and effluent samples. These results also suggest that classic HAstV infections, which may be caused by classic HAstV-2 and -4/8 in the study area, are underreported.

We developed seminested PCR primers targeting the 3' end of ORF1b and nested PCR primers targeting the 5' end of ORF2 of MLB- and VA-AstV, respectively, for the specific detection of each clade. We found that each region was conserved among genetically diverse MLB- and VA-AstV genotypes (see Fig. S1 in the supplemental material). We identified type 2 MLB-AstVs and type 1 and 2 VA-AstV strains, showing the considerable genetic diversity of AstVs in the wastewater samples (Fig. 2 and 3). Even though MLB-AstVs were detected in the influent samples at a relatively high positive ratio (5 positive samples of 6 samples tested), they were not detected in any of the effluent samples. On the contrary, classic HAstVs showed a high positive ratio in both influent samples (6 positive samples of 6 samples tested) and effluent samples (4 or 5 positive samples of 6 samples tested). This implies that MLB-AstVs are present at lower concentration in the influent samples and/or that MLB-AstVs are more readily reduced by the wastewater treatments. For further investigation of the occurrence of the novel AstVs and their levels of reduction with wastewater treatment, the application of quantitative assays which have not

yet been developed to a number of samples would be indispensable.

The presence of these novel AstVs has been documented in several countries, including Australia, China, India, Nepal, the Netherlands, Nigeria, Pakistan, and the United States, but not in Japan (19, 20, 22, 23, 47, 48). We identified MLB-AstVs in all influent samples collected between November 2007 and March 2008. This trend was similar to that for the classic HAstVs, which showed the lowest concentration in October 2007, indicating that the epidemiological trends for MLB-AstVs and classic HAstVs are similar. In contrast, VA-AstVs were less frequently detected in the influent samples, suggesting that VA-AstVs are not as prevalent as classic HAstVs or MLB-AstVs in humans. Similarly, clinical studies investigating infantile diarrheal samples in India, the United States, Egypt, and China reported the more frequent occurrence of MLB-AstVs than VA-AstVs (22, 49, 50). A previous clinical study reported the less frequent detection of MLB-AstV, but the study investigated samples from adults with diarrhea and children without diarrhea (20). These observations suggest that MLB-AstVs may be more prevalent than VA-AstVs among children with diarrheal disease. All MLB-AstV strains found in this study were classified as type 2 strains, which have been found in nasopharyngeal swab and plasma samples obtained from febrile children and have been hypothesized to infect extraenteric tissues (7, 50, 51). Our results suggest that the prevalence of nonenteric MLB-AstVs as well as enteric viruses can be assumed by investigating wastewater samples.

The occurrence of the novel AstVs in wastewater samples strongly implies their circulation in Japan and potential waterborne transmission. A previous study suggested an association of VA-AstVs with gastroenteritis outbreaks, although the pathogenicity of the novel AstVs has not been well documented to date (23). Finkbeiner et al. (22) pointed out that the novel AstVs could be the cause of gastroenteritis diseases whose etiologies are undetermined. Further studies are needed for characterization of the novel AstVs with respect to their pathogenicity, epidemiology, and fate in the water environment.

In summary, we identified genetically diverse AstV strains, including emerging MLB- and VA-AstV strains, in wastewater in Japan. To our knowledge, this is the first study describing the occurrence and genetic diversity of MLB- and VA-AstVs in wastewater. MLB-AstVs seemed to be prevalent during the autumn and winter seasons, while the seasonal prevalence of VA-AstVs remains unclear. Most AstV strains identified in the wastewater samples were clinically undetermined types, suggesting that these strains may be causing underreported (mild or asymptomatic) infections and that AstV strains which were found to be circulating in the study area are more genetically diverse than was previously appreciated.

Our results demonstrate the importance of investigation of water samples to explore the actual prevalence of AstVs among human populations. Future studies, such as a yearlong surveillance and quantitative detection of the novel AstVs, are required to obtain a better understanding of the epidemiology of AstVs.

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