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Molecular Characterization of Sewage-Borne Pathogens and Detection of Sewage Markers in an Urban Stream in Caracas, Venezuela▽

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Molecular characterization of two sewage-borne pathogens identified hepatitis A virus (HAV) subgenotype IA and *Giardia duodenalis* assemblages A and B as predominant genotypes circulating in an urban area of Venezuela. This study reveals epidemiological features of human pathogens of worldwide distribution and the efficacy of molecular methods for accurate assessment of sewage pollution.

Multiple microbial pathogens may frequently be found in surface waters that receive uncontrolled municipal sewage discharges. The range and diversity of sewage-borne pathogens in surface waters are geographically specific and strongly dependent on the burden of infectious diseases in the population, the seasonal patterns of infectious diseases in the community, and the availability of sewage treatment processing (5, 7). The metropolitan city of Caracas, the capital and largest city of Venezuela, is located in northern South America near the Caribbean coast. Water pollution is a big issue in Caracas, like in many other cities in South America, where most of the human sewage (~97%) from overpopulated urbanized areas is discharged without any treatment into nearby rivers and coastal environments. Despite these facts, the seriousness of sewage-related health issues is not at the forefront of public concern in this country.

Giardia is the protozoan parasite most frequently detected in human fecal samples submitted to diagnostic laboratories from major cities in Venezuela. The frequency of giardiasis reported in the population varies between 21% and 45% but may increase up to 75% among school age children (8). Notwithstanding, the epidemiology of giardiasis in Venezuela remains unknown, and no previous studies have documented the distribution of species and genotype assemblages associated with human infections.

Hepatitis A virus (HAV), the etiological agent of hepatitis A in humans, has distinguishable epidemiological patterns of distribution and endemicity closely related to socioeconomic development (9, 12). Water- and food-borne outbreaks of HAV have been well documented worldwide (6, 19). Seroepidemiological studies conducted in selected populations in Venezuela have demonstrated high endemicity of hepatitis A infection among low socioeconomic population strata, with seroprevalences between 48% and 98% (13). Nevertheless, studies on HAV genotype circulation in major urban areas of the country are scarce, as is research on predominant exposure routes and potential transmission patterns through the environment.

The analysis of nucleic acid sequences of sewage-borne pathogens may provide relevant information on predominant species and genotypes of human-pathogenic viruses and parasites circulating in specific geographical areas (11, 12, 15). The molecular approach may be of relevance for countries lacking reliable disease surveillance programs and proper understanding of the potential transmission of specific human pathogens through the environment. In this research, *Giardia* cysts and HAV recovered from an urban stream were characterized by multiple molecular methods along with nucleotide sequence analysis to identify predominant genotypes circulating in a major urban area of Venezuela's capital. The strength and efficacy of multiple molecular methods for accurate assessment of human sewage pollution and risks of exposure to sewage-borne pathogens were also investigated.

Dry season sampling (October through March) was conducted in a heavily polluted urban stream ($>10^6$ fecal coliforms/100 ml) that flows in a southeast direction through the metropolitan city of Caracas (16). Water sample volumes of 100 ml were collected in three sterile centrifuge tubes two to four times per month. *Giardia* cysts were concentrated by centrifugation (100 ml at 1,500 × *g* for 15 min) followed by DNA extraction by the freeze-thaw method in the presence of Chelex-100 (3) for sucrose-purified cysts. Human-pathogenic assemblage occurrence was determined by nested PCR amplification and sequence analysis of the triosephosphate isomerase (*tpi*) gene (18). Multiple sequence alignments were performed with ClustalW (21), and phylogenetic analyses were conducted using MEGA4 software (20). The genetic diversity of *Giardia* isolates was inferred by the neighbor-joining method (17) using a bootstrap test of 1,000 replicates. *Giardia* cysts counts were obtained by fluorescence microscopy using BTF EasyStain monoclonal antibody stain (BTF Precise Microbiology, Inc.) and 4′6-diamidino-2-phenylindole (DAPI). The recovery efficiency of cysts was determined in five experiments using ColorSeed C&G spike suspensions as internal quality controls (14).

HAV particles were concentrated from 35 ml by ultracentrifugation and elution with 0.25 N glycine buffer following procedures previously described (16). Viral RNA was extracted from sample concentrates with Trizol (Invitrogen, Inc.,

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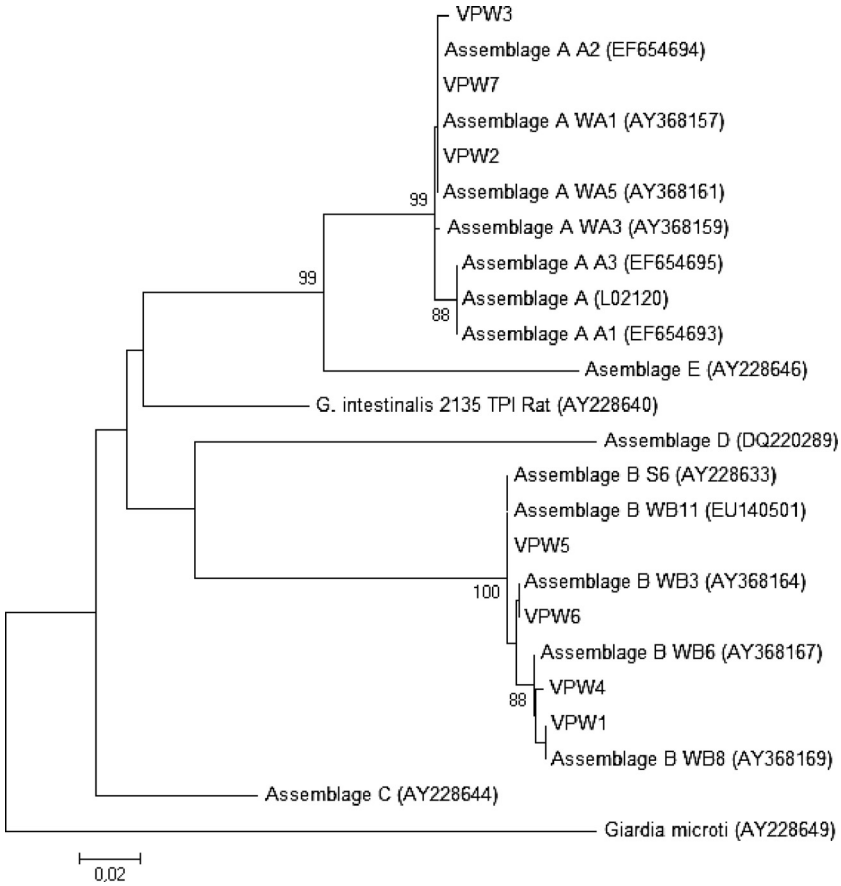

FIG. 1. Phylogenetic tree of *G. duodenalis* assemblages A (VPW2, VPW3, and VPW7) and B (VPW1, VPW4, VPW5, and VPW6) from urban stream samples forming two clusters in a neighbor-joining analysis of *tpi* nucleotide sequences. Only bootstrap values >80% are shown in the tree.

Carlsbad, CA) following the manufacturer's instructions. General detection of HAV was based on amplification of the 5′ nontranslated region (5′ NTR), while analysis of genetic diversity involved sequencing and phylogenetic analysis of the VP1 amino terminus and the full VP1 gene (2, 10). Sequence alignment was conducted with the DNAman software 5.2.2 (Lynnon BioSoft, Quebec, Canada) followed by phylogenetic analysis.

Molecular detection of sewage pollution was accomplished by PCR amplification of *Bacteroidales* human-specific 16S rRNA genes, *Bacteroides thetaiotamicron* 16S rRNA genes, and the *nifH* gene of *Methanobrevibacter smithii* using primers and PCR conditions originally described by Field et al. (4), Carson et al. (1), and Ufnar et al. (22), respectively.

Giardia duodenalis tpi nucleotide sequences amplified directly from urban stream waters were included after phylogenetic analysis into two well-defined clusters of assemblages A and B. These results were supported by high bootstrap values, as indicated in Fig. 1. The level of cysts recovered from these samples ranged from 10,400 to 62,000 cysts/liter; however, mean percent recoveries varied from 20% to 50%, which suggests that the urban stream may harbor and receive much higher loads of cysts.

Three genomic regions used for detection and characterization of HAV revealed the predominance of HAV strains belonging to subgenotype IA, the most frequent genotype associated with human disease worldwide (9). A neighbor-joining tree constructed from the alignment of nucleotide sequences from urban stream samples and sequences of HAV strains from case patients (unpublished data) was used to investigate the relationship between genotypes present in environmental and clinical samples. The comparative analysis indicated a high degree of identity (98 to 99%) between nucleotide sequences from the urban stream and the strains from sporadic HAV cases. The phylogenetic analysis grouped all of these sequences into two unique clades within subgenotype IA, strongly supported by significant bootstrap values (Fig. 2A, B, and C).

Three reliable published assays for detection of human-specific markers of fecal pollution identified and confirmed the predominant point source of water pollution. Sequence analysis of three randomly selected PCR products from each marker revealed ≥99% sequence identity with published sequences (GenBank) derived from different geographical areas, thus indicating the validity and specificity of the molecular markers as reliable indicators of human sewage pollution in Venezuela.

The results of this research demonstrate that the molecular assays applied for detection and characterization of sewage-borne pathogens in surface waters may have practical applications for epidemiological investigations on distribution of predominant human-specific genotypes circulating

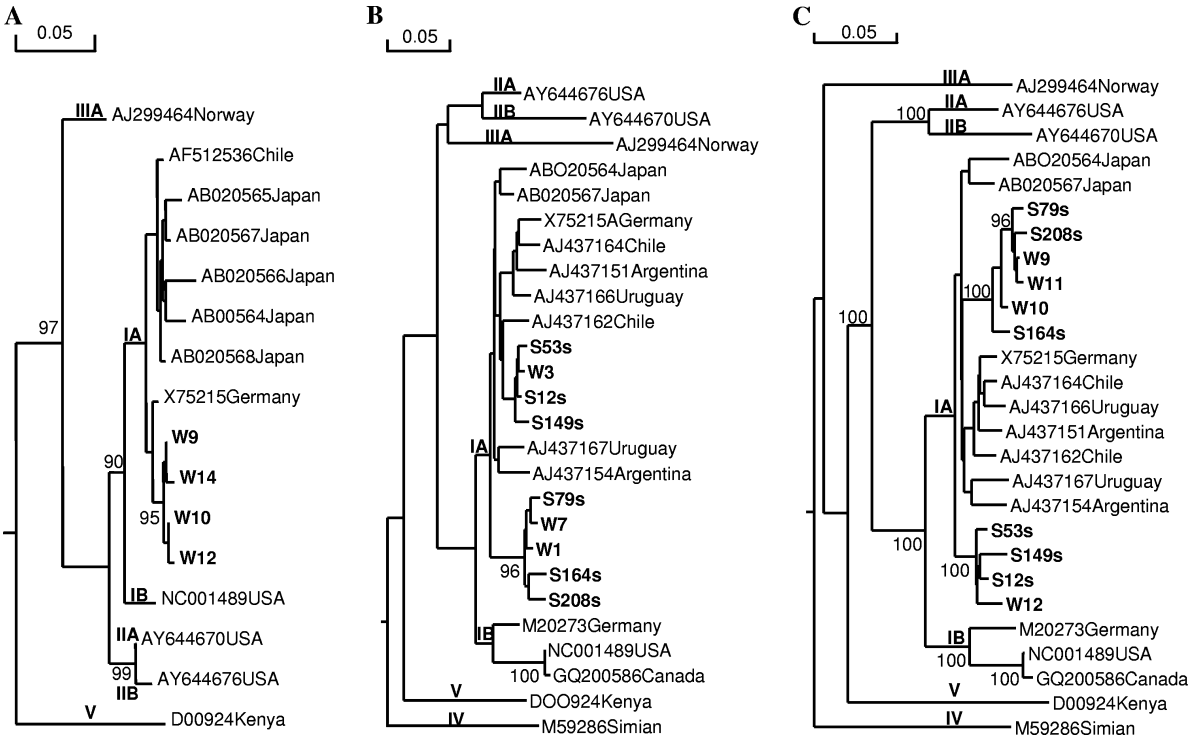

FIG. 2. Phylogenetic analysis of the HAV 5′ NTR (A; 284 nucleotides [nt]), VP1 amino terminus (B; 172 nt), and complete VP1 (C; 820 nt) regions. Nucleotide sequences of HAV reference strains are designated by their GenBank accession number, including the name of the country of origin, except for Venezuelan isolates, which are shown in bold. S, isolates derived from human sporadic cases; W, urban stream isolates. Phylogenetic analysis was performed by neighbor joining, and phylogenetic distances were calculated by the Kimura two-parameter test. Bootstrap values ≥90% are shown in the trees. Letters in bold indicate the subtype.

in urban populations. Previous studies identified the most predominant waterborne gastroenteritis viruses circulating in Metropolitan Caracas (16).

The molecular-based monitoring approach for rapid and precise identification of sewage-borne pathogens and sewage markers in surface waters has important implications for sewage-related health issues that require special attention in Venezuela and South America. Deficient sewerage coverage and lack of municipal wastewater treatment, commonly associated with informal settlements around densely populated urban areas, are responsible for many of the environmental degradation and public health problems that occur in these countries. The precise identification of human pathogens in the environment offers an appropriate and alternative approach for initial assessment of risks of exposure to waterborne pathogens. Current bacterial indicators of fecal pollution (fecal coliforms, *Escherichia coli*, and enterococci) do not allow identification of the relative sources of impacts (i.e., sewage, urban runoff, and agricultural waste) on surface waters. Thus, the molecular detection of sewage-borne pathogens and sewage markers in surface waters may be more effective than the bacterial indicator approach for forecasting pathogen distribution and for managing and reducing risks associated with inappropriate sewage disposal into natural waters in Venezuela and South America.

Nucleotide sequence accession numbers. The corresponding sequences of fragments of the *tpi* gene of *G. duodenalis* obtained from this study have been deposited in GenBank under accession no. GQ253432 to GQ253438. Nucleotide sequences of the partial and full VP1 gene of HAV have been deposited in GenBank under accession no. GU189565 to GU189575.

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