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Molecular Detection and Characterization of Aichi Viruses in Sewage-Polluted Waters of Venezuela▽

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The circulation of Aichi virus in a major urban area was demonstrated using molecular detection with samples recovered from a major river polluted with sewage discharges in Caracas, Venezuela. Five out of 11 water samples studied were positive, being classified by phylogenetic analysis as genotype B. Analysis of sewage waters appears to be a useful methodology to uncover the presence of a hitherto undetected fecal pathogen in a given geographical area.

Sewage pollution plays a major role in the transmission of multiple viral pathogens associated with gastrointestinal diseases in human populations (10a). Understanding the distribution and persistence of sewage-borne viral pathogens in highly contaminated waters from different geographical areas may provide relevant information on the epidemiology of enteric viral disease worldwide (20). Aichi viruses have emerged as viral agents associated with food-borne nonbacterial acute gastroenteritis in humans. Seroprevalence studies conducted in Japan and Europe suggest that infections with this agent are quite frequent (16, 26). In the Western hemisphere, Aichi virus has been described only in Brazil, in clinical samples (7, 14, 16, 17, 19, 22, 24, 25, 27). In Venezuela, it is unknown whether Aichi viruses circulate in the population, and their eventual associations with diarrheal cases or sporadic outbreaks have not been documented.

Aichi viruses are small round viruses that are about 30 nm in diameter and have an RNA genome of positive polarity, with a length of approximately 8.3 kb. These viruses, together with the bovine and porcine kobuviruses, are classified into the genus *Kobuvirus* of the *Picornaviridae* family (11, 13, 18). Three genotypes of the Aichi virus (A to C) have been proposed based on sequence analysis of 519 nucleotides at the 3CD junction region (1, 24, 27).

The molecular detection and characterization of Aichi virus have been conducted so far in isolates from clinical samples (16, 17, 19, 24) and sea food (8), but to our knowledge, there are no reports of detection in water samples.

The present study was conducted in order to determine the occurrence and circulation of Aichi virus in a major urban area of Venezuela, using molecular detection and sequence analysis of viral particles recovered from surface waters heavily polluted with sewage discharges. A total of 11 samples were collected in an urban stream known as Guaire River, with samplings made twice a month from October 2007 to February 2008, with three samplings in February. The description of the sampling location and predominant point pollution source has been documented in previous publications (2, 21). The recovery of viral particles and RNA extraction followed procedures previously described (21). The molecular detection and characterization of Aichi viruses involved reverse transcription and nested PCR, using primers and cycling conditions that amplify a 519-bp fragment located between the 3CD region and the N terminus, 3D, of the Aichi virus genome (24). A new set of primers identified as AiF2 ($_{6283}$5′-CAA GGA CTT GCG GCG CTT CAT CG-3′$_{6305}$) and AiR2b ($_{6672}$5′-GCA CCC YTC YCC CGC CTA CGG TG-3′$_{6694}$) was designed for a second round of amplification, with an expected product of 411 bp. These primers anneal to an inner region of the first amplicon, and their sequence was based on the consensus sequence generated after multiple alignments of four complete genome sequences of Aichi virus available in the GenBank database (accession numbers DQ028632, FJ890523, NC001918, and AB010145). The nucleotide position reported for each new primer was based on the genomic sequence of the reference strain AB010145. For reverse transcription, 10 μl of the extracted RNA was mixed with 15 μl of molecular grade water, incubated at 95°C for 5 min, and placed on ice for RNA denaturation. The denatured RNA was reverse transcribed with random primers (0,02 μg) in the presence of avian myeloblastosis virus (AMV) reverse transcriptase (10 U), deoxynucleoside triphosphate mix (0.2 mM), and RNasin (40 U) in reverse transcription buffer to a final volume of 50 μl. The mixture was incubated at 42°C for 1 h followed by incubation at 95°C for 15 min. The first round of PCR amplification included 10 μl of cDNA, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate [dNTP]), 20 pmol of each primer (6261/6779), and 2.5 U of Platinum *Taq* polymerase in PCR buffer to a final volume of 50 μl. Cycling conditions included 40 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min. The second round of amplification was conducted under the same cycling conditions with the new primer set and 1 μl of the first round amplification product. All reagents were purchased from Invitrogen (Carlsbad, CA), except AMV, which was supplied by Promega (Madison, WI).

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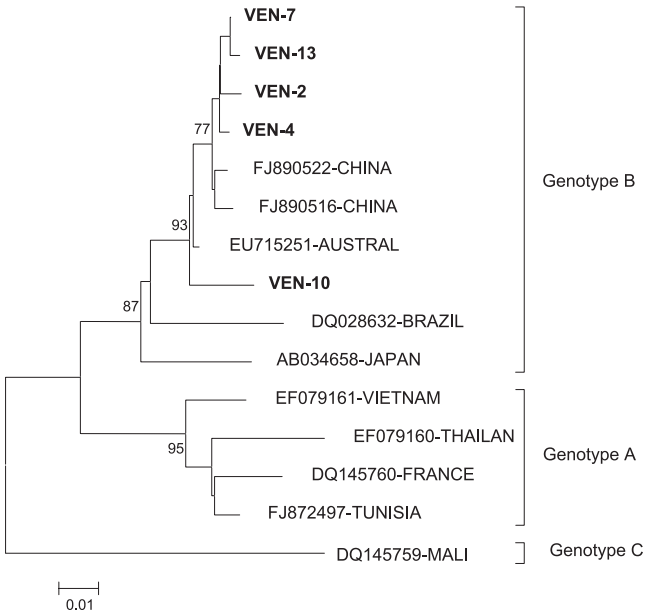

FIG. 1. Phylogenetic analysis of the Aichi viruses recovered from urban sewage, based on a 391-bp fragment of the genomic 3CD region junction, corresponding to positions 6322 to 6713 of the genomic sequence of the reference strain AB010145. The tree was constructed using the minimum evolution method as implemented in the package MEGA 4. The bar is in units of base substitutions per site. Bootstrap values (>75) are indicated for the corresponding nodes, based on a resampling analysis of 1,000 replicates. Venezuelan isolates are indicated in boldface type.

PCR products were purified with the PCR purification kit (Qiagen GmbH, Germany), by following the manufacturer's instructions, and sequenced commercially by Macrogen Sequencing Service (Macrogen, South Korea). Both strands were sequenced, and the resultant nucleotide sequences were compared with reference sequences of strains available in the GenBank database. Sequence assembly and alignment were conducted with the DNAMan software 5.2.2 (Lynnon Biosoft, Quebec, Canada) and the phylogenetic analysis with the MEGA 4.1 software (23).

Aichi virus was detected by the reverse transcription-PCR (RT-PCR) screening method in 5 of the 11 water samples tested. Nucleotide sequences of the partial 3CD region of five Aichi virus-positive samples (Ven7, Ven13, Ven2, Ven4, Ven10) were highly conserved (99.2% to 97%), with amino acid identities of 99 to 100%. Phylogenetic analysis of partial 3CD nucleotide sequences of Aichi virus strains from Venezuela and reference sequences from GenBank is shown in Fig. 1. The phylogenetic tree indicated that all five virus strains belonged to group B of Aichi virus, according to the classification proposed by Yamashita et al. (24). All five Venezuelan isolates are located in the same cluster, with one sample (Ven10), with a 2.6 to 2.8% nucleotide divergence from the other samples, located on a different minor branch, indicating some degree of variability among Venezuelan isolates. In addition, the nucleotide sequences of Aichi viruses from the sewage-polluted river in Venezuela shared a close similarity with Aichi virus strains from different geographical areas, thereby evidencing the circulation of closely related strains worldwide (12).

Although more sampling is required in order to make inferences about the prevalence of Aichi virus infection in the study area, the high proportion of positive samples may indicate a widespread circulation of Aichi virus among the Venezuelan population. Viral RNA of Aichi virus has been detected in variable proportions (0.3 to 20.5%) in human fecal samples (1, 12, 22, 26), and with a high seroprevalence in different countries (7, 16, 26). Further studies are warranted in order to elucidate the significance of these findings and to establish the clinical importance of Aichi virus and other enterically transmitted viruses, such as hepatitis E virus, in Venezuela.

Recently, various agents putatively associated with human or animal diarrhea have been described: klassevirus, bocavirus, salivirus, parechovirus, and torque teno virus (3–5, 10, 15). Klassevirus and bocavirus have been detected in sewage (3, 7) and torque teno virus in river waters (9). Sewage represents a natural pool of thousands of individual specimens, so the analysis of sewage waters appears to be a very useful methodology to uncover the presence of a hitherto undetected fecal pathogen in a given geographical area.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in the GenBank database under the following accession numbers: GU807430 (Ven2), GU807429 (Ven4), GU807431 (Ven7), GU807432 (Ven10), and GU807433 (Ven13).

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