

Prevalence and Genetic Diversity of Aichi Viruses in Wastewater and River Water in Japan[▽]

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Aichi virus (AiV) genomes were detected in 12 (100%) influent and 11 (92%) effluent wastewater and 36 (60%) river water samples. Among 260 strains identified, 255 were genotype A and 5 were genotype B. This is the first report describing the molecular characterization of AiVs in aquatic environments in Japan.

Aichi viruses (AiVs) are members of the genus *Kobuvirus* in the family *Picornaviridae* and possess single-stranded, positive-sense RNA genomes (24). Based on 3C and 3D (3CD) junction region nucleotide sequences, AiVs are currently classified into three genetically distinct genotypes (A to C) (2, 25). They have been identified in Asian (14, 15, 23, 26, 27), European (2, 7, 12, 13, 16, 17), South American (13), and African (19, 20) countries, suggesting their worldwide distribution. Seroprevalence studies performed in several countries demonstrated a high percentage (80 to 99%) of AiV antibodies in adults (3, 13, 18, 21, 24), indicating widespread human exposure. In contrast, AiV infection in patients with sporadic or epidemic gastroenteritis is low (2, 7, 12–17, 19, 20, 23, 27). These findings, however, do not reveal a role of AiVs in human enteric diseases, and thus their actual prevalence, pathogenesis, and molecular epidemiology remain unclear.

Fecal-oral AiV transmission through contaminated food or water is indicated by AiV detection in fecal samples of infected individuals (2, 7, 12–17, 19, 20, 23, 27) and raw and treated sewage (22), sewage-polluted river water (1), and shellfish samples (4, 12, 22). To further elucidate their prevalence and infection route, comprehensive documentation of their environmental occurrence and fate is essential. We investigated the prevalence and genetic diversity of AiVs in wastewater and river water in Japan for 1 year. The presence of AiV genomes in the water samples was determined by nested reverse transcription (RT)-PCR, and the strains were further characterized according to the 3CD junction region sequences.

Between March 2005 and February 2006, influent and effluent samples were collected monthly from a wastewater treatment plant, which utilized a conventional activated sludge process. These samples (100 ml, influent, and 1,000 ml, effluent) were concentrated by the adsorption-elution method using an electronegative filter (catalog no. HAWP-090-00; Millipore, Tokyo, Japan) with an acid rinse procedure (8). Between April 2003 and March 2004, 60 river water samples were collected monthly from five sites (sites 1 to 5) from upstream to down-

stream along the Tamagawa River in Japan. Further detailed information on the Tamagawa River basin and the locations of sampling sites has been published previously (10). These samples (500 ml) were concentrated using the cation-coated filter method (5, 6). The concentrated wastewater and river water samples were further concentrated using a Centriprep YM-50 device (Millipore) to obtain a 700- μ l final volume. Viral RNA was extracted from the concentrated samples using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany), followed by RT using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

Nested PCR was performed using the 6261/6779 and C94b/246k primer sets and Hot Start Ex *Taq* DNA polymerase (TaKaRa Bio Co., Shiga, Japan) to amplify the 266-bp 3CD junction region (25). Second-round PCR products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Subsequently, eight clones per sample were selected, and both strands were sequenced using the BigDye Terminator v3.1 cycle sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Nucleotide sequences were aligned using ClustalW version 1.83, and distances were calculated using Kimura's two-parameter method (9).

Among 12 influent and 12 effluent wastewater samples tested, AiVs were detected in all influent and 11 (92%) effluent samples by nested RT-PCR, demonstrating high AiV prevalence in the wastewater samples. Nested RT-PCR detected AiVs in 36 of 60 (60%) river water samples from one to four of the five sampling sites in each month, except for July 2003, when no AiVs were detected in any samples. AiVs were not detected in samples from the upstream area (site 1) but were detected in those from mid- to downstream areas (sites 2 to 5) in 8 of 12 (67%) months. Interestingly, the detection rate of AiVs in river water samples was higher than that of noroviruses or sapoviruses (10, 11).

By phylogenetic analysis, we identified 125 and 135 distinct AiV strains from wastewater and river water samples, respectively (Fig. 1 and 2). Genotype A strains were abundant in both wastewater and river water samples, whereas genotype B strains were identified in only one influent sample collected in February 2006 and one river water sample collected in April 2003 at site 3 (Fig. 1 and 2).

To date, only a few studies have detected AiVs in water

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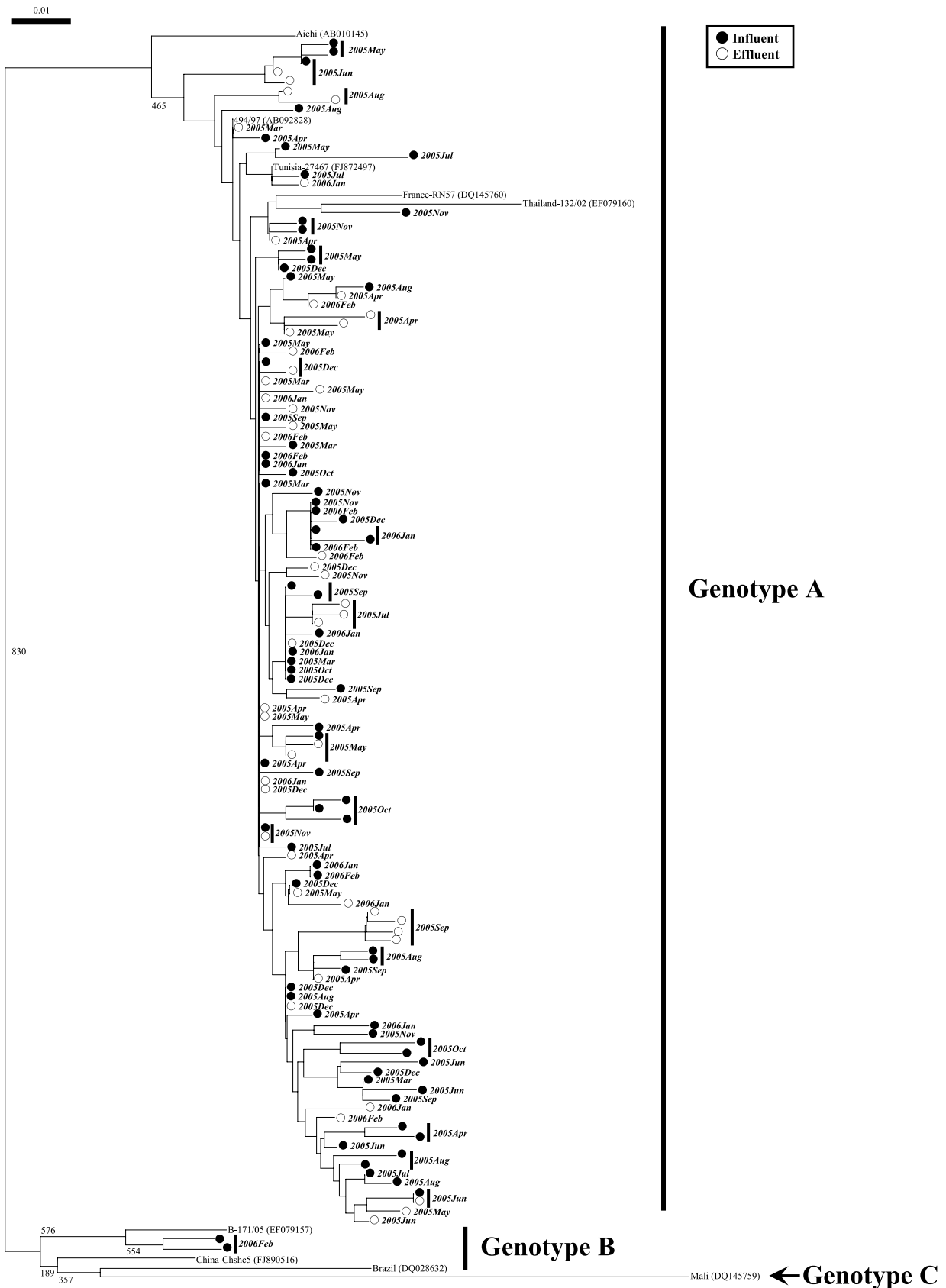


FIG. 1. Phylogenetic tree of AiV strains identified in wastewater samples. The tree was constructed on the basis of sequences of a 224-nucleotide stretch within the 3CD junction region. The numbers on each branch indicate bootstrap values for the genotype, and bootstrap values of 950 or greater were considered statistically significant for grouping. The scale represents nucleotide substitutions per site. Filled and open circles indicate AiV strains detected in influent and effluent wastewater samples, respectively. The year and month are shown in bold italics beside the symbols.

samples. Sdiri-Loulizi et al. (22) identified AiVs in 15 of 250 sewage samples (6%) in Tunisia, much lower than the detection rate reported in the present study. This may be because they performed single-round RT-PCR using the 6261/6779 “outer” primer set. We performed nested PCR utilizing an inner primer set, C94b/246k (25), to increase the sensitivity. Alcalá et al. (1) examined only 11 sewage-polluted river water samples for AiVs, far fewer than were studied here. Furthermore, these studies performed direct nucleotide sequencing without molecular cloning, and both studies identified only a single genotype: genotype A by Sdiri-Loulizi et al. (22) and genotype B by Alcalá et al. (1). In contrast, we cloned the second-round PCR products and sequenced eight clones per sample. Thus, we successfully identified both genotypes A and B even when they coexisted in a single sample (influent wastewater sample in February 2006).

This is the first report describing the molecular detection and characterization of AiVs in aquatic environments in Japan. A total of 260 distinct AiV strains were identified from wastewater and river water samples, with a considerable genetic diversity (Fig. 1 and 2), which was much larger than the number of AiV strains identified in any other previous environmental studies (1, 22). Among the strains identified, 255 were classified into genotype A and 5 were genotype B. Our novel findings based on environmental investigations demonstrate that more genetically diverse AiV strains are prevalent among human populations than previously appreciated. The present study highlights the importance of further studies on AiVs toward a better understanding of their distribution and etiological role, if any, in human enteric diseases.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers AB569644 to AB569903.

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