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**Quantification and Genotyping of Aichi Virus 1 in Water Samples in the
Kathmandu Valley, Nepal**

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19 **ABSTRACT**

20

21 Aichi virus 1 genomes were detected by quantitative PCR in groundwater from shallow dug
22 (10/22) and tube wells (1/15), river water (14/14), and sewage (1/1), with the maximum
23 concentration of 4.0×10^9 copies/l. Nucleotide sequencing analysis demonstrated the prevalence of
24 genotype B in the virus positive samples.

25

26 **KEYWORDS**

27 Aichi virus 1; groundwater; Kathmandu Valley; Picornaviridae

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30 **TEXT**

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32 Aichi virus 1 (AiV-1) is a single-stranded, positive-sense RNA virus that belongs to the genus
33 *Kobuvirus* in the family *Picornaviridae* (Reuter et al. 2011). AiV-1 is considered as a potential
34 causative agent of viral gastroenteritis in humans, primarily transmitted through contaminated food
35 and water (Reuter et al. 2011).

36 Recent environmental studies have demonstrated a high prevalence of AiV-1 in various types
37 of water samples, such as raw and treated sewage, reclaimed water, and river water (Kitajima and
38 Gerba 2015). Quantitative PCR (qPCR) was employed in some of these studies, obtaining
39 quantitative data on the prevalence of AiV-1 in water, whereas nucleotide sequencing analysis
40 combined with conventional PCR was used to determine the distribution of AiV-1 genotypes
41 (Kitajima and Gerba 2015). Considering that a limited number of studies have been conducted so
42 far on the AiV-1 detection in water and that most of them were conducted in developed countries, it
43 is highly recommended to study the prevalence of AiV-1 in water samples in developing countries.

44 The Kathmandu Valley, the capital city area of Nepal, is well recognized as an area where
45 waterborne diseases are one of the most serious public health concerns, partially because of low
46 coverages of proper drinking water and wastewater treatment systems. People in the valley mainly
47 depend on groundwater for their domestic water use. Since contamination of groundwater by
48 waterborne pathogens could pose a health risk to humans, their prevalence in water needs to be
49 surveyed prior to the risk estimation. However, a limited number of studies in Nepal have been
50 reported on the prevalence of waterborne pathogens, such as pathogenic bacteria (Inoue et al. 2014;

51 Tanaka et al. 2012), protozoa (Haramoto et al. 2011; Ono et al. 2001, Shrestha et al. 2015; Shrestha
52 et al. 2016), and viruses (Haramoto et al. 2011), in water samples.

53 This study aimed to determine the prevalence of AiV-1 genomes in various types of water
54 samples, including groundwater, in the Kathmandu Valley. The first water sampling was conducted
55 in the wet season (August–September) of 2009 (Haramoto et al. 2011), followed by additional
56 sampling campaigns in both wet (August 2010) and dry seasons (May 2011). During the sampling
57 periods, a total of 53 water samples were collected from 9 shallow dug wells ($n = 22$), 6 shallow
58 tube wells ($n = 15$), 8 sites along the Bagmati River and its tributaries ($n = 14$), a tap in a house
59 supplied with tanker water ($n = 1$), and a sewage pipe ($n = 1$).

60 The water samples, except for the sewage sample, were concentrated to 12 ml by the
61 electronegative membrane-vortex method using a mixed cellulose ester membrane filter (pore size
62 of 0.45 μm and diameter of 47 mm; Merck Millipore, Billerica, MA, USA) (Haramoto et al. 2012),
63 as described previously (Haramoto et al. 2011). The volumes filtered were 50 or 100 ml for river
64 water, 3,000 ml for tap water, and 1,000 ml for groundwater, except for one sample that allowed
65 filtration of only 50 ml due to membrane clogging. No concentration step was done for the sewage
66 sample, but a supernatant after centrifugation was used for the subsequent analysis.

67 Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany),
68 followed by reverse transcription (RT) using a High Capacity cDNA Reverse Transcription Kit
69 (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (Haramoto et al. 2011).
70 Subsequently, 5- μl aliquot of the resulting cDNA was subjected to TaqMan MGB-based quantitative
71 PCR (qPCR) targeting approximately 101-bp VP1 region sequence of AiV-1 genomes (Kitajima et

72 al. 2013). All water and standard samples (plasmid DNA containing the qPCR target sequence with
73 known gene copy numbers) and negative controls were analyzed in duplicate using a Thermal
74 Cycler Dice Real Time System TP800 (Takara Bio, Otsu, Japan). Prior to RNA extraction, murine
75 norovirus (S7-PP3 strain), kindly provided by Dr. Y. Tohya (Nihon University, Fujisawa, Japan),
76 was added to the sample as a molecular process control, resulting in high extraction-RT-qPCR
77 efficiency values of $78.3 \pm 26.9\%$ ($n = 53$).

78 Furthermore, for genotype identification, samples positive for the AiV-1 qPCR were subjected
79 to nested PCR targeting the 3C-3D junction region (Yamashita et al. 2000), followed by agarose gel
80 electrophoresis, direct sequencing using a 3730xl DNA Analyzer (Thermo Fisher Scientific), and
81 phylogenetic analysis of the 224-bp amplified sequences, excluding primer sequences, using a
82 Genetyx software version 9.1.0 (Genetyx, Tokyo, Japan).

83 AiV-1 genomes were detected by qPCR in 26 (49%) of the 53 samples tested. As shown in
84 Figure 1, the highest concentration of AiV-1 genomes (4.0×10^9 copies/l) was obtained from the
85 sewage sample. AiV-1 genomes were also detected in all the river water samples, with high
86 concentrations ranging from 1.2×10^6 to 1.4×10^8 copies/l, which were greater than those in
87 influent of wastewater treatment plants in Japan and United States (Kitajima et al. 2013, 2014). This
88 can be explained by the situation where most untreated sewage is discharged directly to the river in
89 the Kathmandu Valley.

90 Results of the detection of AiV-1 genomes in groundwater were quite different between the
91 types of the wells: frequency of AiV-1 genome detection was significantly higher in shallow dug
92 wells (10/22, 45%) than in shallow tube wells (1/15, 7%) (χ^2 -test, $P < 0.05$). More vulnerable

93 structure of dug wells, which are usually made of brick or stone, than tube wells may have
94 attributed to the difference in detection frequency. Concentrations of AiV-1 genomes in the positive
95 samples ranged from 5.6×10^4 to 2.0×10^6 copies/l, with a geometric mean concentration of $2.3 \times$
96 10^5 copies/l (Fig. 1). AiV-1 genomes were detected in all the three samples collected at each of two
97 shallow dug wells (SG6 and SG37). The concentration of *Escherichia coli* at SG6 (1.1×10^2 – $2.6 \times$
98 10^2 MPN/100 ml) was within the normal range found in shallow groundwater in the Kathmandu
99 Valley, whereas that at SG37 (1.4×10^3 – 1.1×10^4 MPN/100 ml) was relatively higher than average
100 (Shrestha et al. 2014).

101 Unlike groundwater samples, AiV-1 genomes were not detected in the tap water sample
102 although it was contaminated with *E. coli* (1.1×10^2 MPN/100 ml). Since tanker water is one of the
103 important drinking water sources in the Kathmandu Valley, further studies need to be conducted to
104 evaluate the prevalence of AiV-1 genomes in tanker water samples, as well as their water sources.

105 Fifteen (58%) of 26 AiV-1 qPCR-positive samples were also positive for AiV-1-specific nested
106 PCR, and subsequent nucleotide sequencing analysis successfully classified all of these samples as
107 genotype B (Fig. 2). According to previous studies conducted so far, genotype A has been identified
108 more frequently in water and environmental samples than genotype B, but the trend was different
109 among the regions studied (Kitajima and Gerba 2015).

110 Although AiV-1 sequences were found in an untreated sewage sample collected in Kathmandu
111 by viral metagenomics (Ng et al. 2012), there has been no information available on the incidence of
112 AiV-1 in human fecal specimens in the Kathmandu Valley. The results of this study indicate that
113 AiV-1 of genotype B is circulating in human populations in the valley. Further studies are needed to

114 understand more deeply the prevalence and genotype distribution of AiV-1 in the environments.

115 **Nucleotide sequence accession numbers.**

116 The nucleotide sequences determined in this study have been deposited in the GenBank
117 database under accession numbers LC200515–LC200529.

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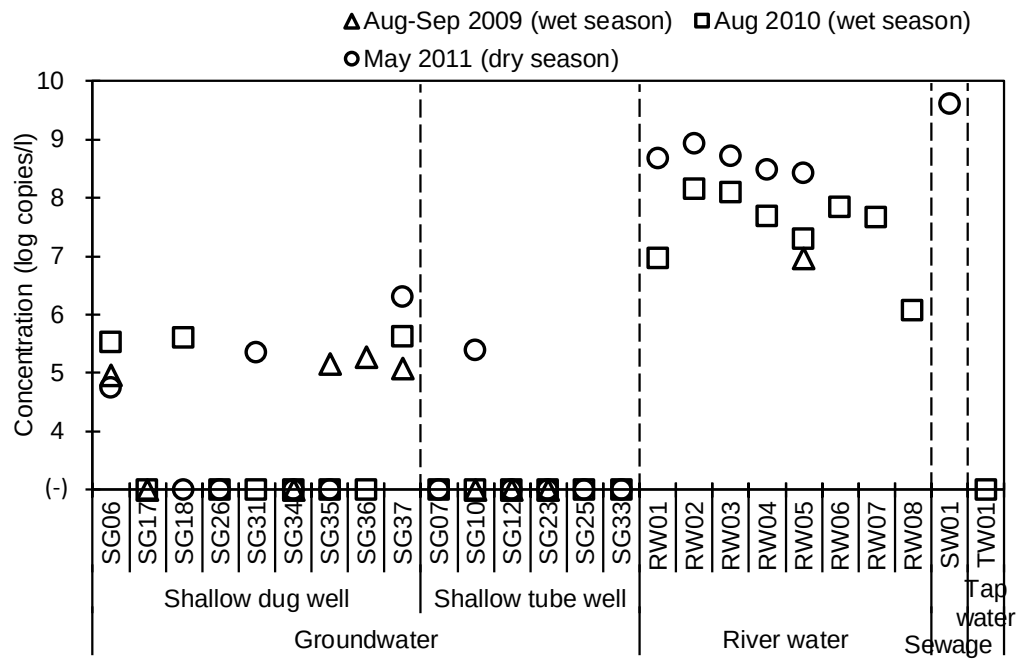


Fig. 1 Concentrations of AiV-1 genomes in water samples

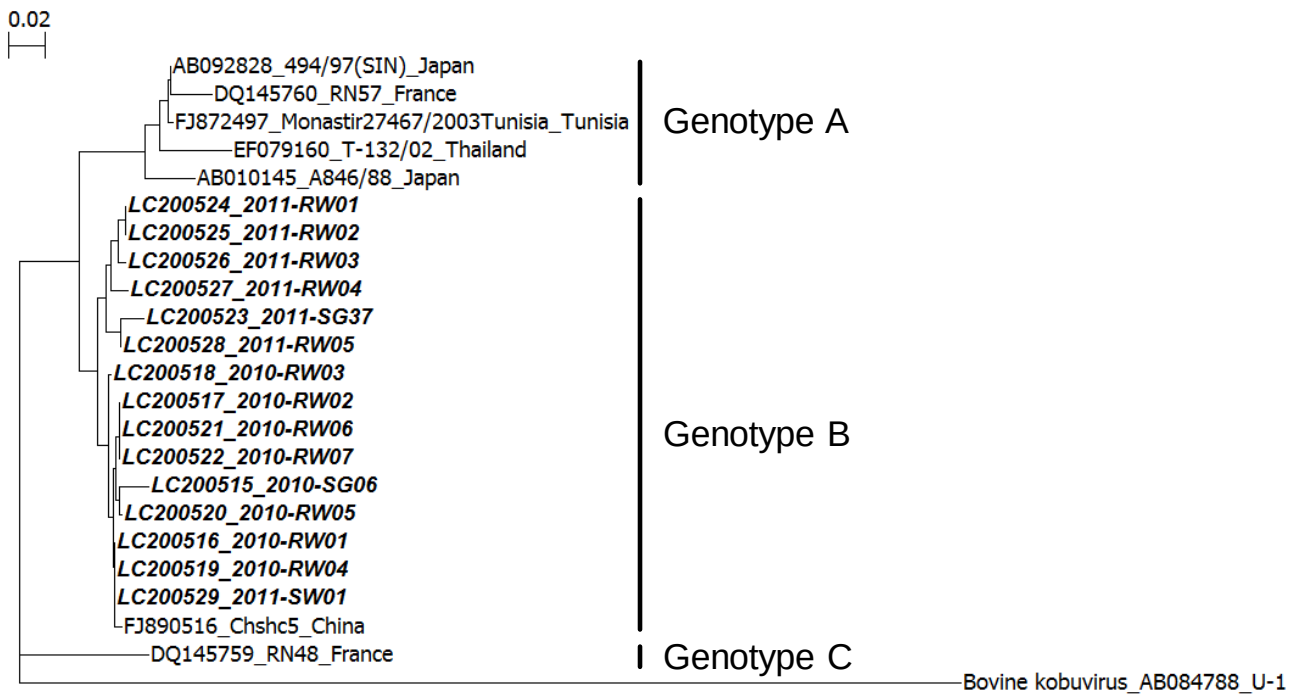


Fig. 2 Phylogenetic analysis of AiV-1 sequences identified in water samples. The tree was generated using the neighbor-joining method with 1,000 bootstrap replicates based on the 224-nt 3C-3D junction region sequences. The sequence of bovine kobuvirus (AB084788) was used as an outgroup. The scale bar represents the number of nucleotide substitutions per position. AiV-1 sequences obtained in this study are labeled with bold italics and indicate the GenBank accession number and sample identification.