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

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ABSTRACT

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

KEYWORDS

Aichi virus 1; groundwater; Kathmandu Valley; Picornaviridae

TEXT

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

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

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

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

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

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

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

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

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

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

Results of the detection of AiV-1 genomes in groundwater were quite different between the types of the wells: frequency of AiV-1 genome detection was significantly higher in shallow dug 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 taker 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

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

Nucleotide sequence accession numbers.

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

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REFERENCES

1. Haramoto, E., Katayama, H., Asami, M., & Akiba, M. (2012). Development of a novel method for simultaneous concentration of viruses and protozoa from a single water sample. *Journal of Virological Methods, 182,* 62–69.
2. Haramoto, E., Yamada, K., & Nishida, K. (2011). Prevalence of protozoa, viruses, coliphages and indicator bacteria in groundwater and river water in the Kathmandu Valley, Nepal. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 105,* 711–716.

3. Inoue, D., Hinoura, T., Suzuki, N., Pang, J., Malla, R., Shrestha, S., Chapagain, S. K., Matsuzawa, H., Nakamura, T., Tanaka, Y., Ike, M., Nishida, K., & Sei, K. (2014). High throughput DNA microarray detection of pathogenic bacteria in shallow well groundwater in the Kathmandu Valley, Nepal. *Current Microbiology*, *70*, 43–50.
4. Kitajima, M. & Gerba, C. P. (2015). Aichi virus 1: environmental occurrence and behavior. *Pathogens*, *4*, 256–268.
5. Kitajima, M., Hata, A., Yamashita, T., Haramoto, E., Minagawa, H., & Katayama, H. (2013). Development of a reverse transcription-quantitative PCR system for detection and genotyping of Aichi viruses in clinical and environmental samples. *Applied and Environmental Microbiology*, *79*, 3952–3958.
6. Kitajima, M., Iker, B. C., Pepper, I. L., & Gerba, C. P. (2014). Relative abundance and treatment reduction of viruses during wastewater treatment processes–identification of potential viral indicators. *Science of the Total Environment*, *488–489*, 290–296.
7. Ng, T. F. F., Marine, R., Wang, C., Simmonds, P., Kapusinszky, B., Bodhidatta, L., Oderinde, B. S., Wommack, K. E., & Delwart, E. (2012). High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. *Journal of Virology*, *86*, 12161–12175.
8. Ono, K., Rai, S. K., Chikahira, M., Fujimoto, T., Shibata, H., Wada, Y., Tsuji, H., Oda, Y., Rai, G., Shrestha, C. D., Masuda, K., Shrestha, H. G., Matsumura, T., Hotta, H., Kawamura, T., & Uga, S. (2001). Seasonal distribution of enteropathogens detected from diarrheal stool and water samples collected in Kathmandu, Nepal. *Southeast Asian Journal of Tropical Medicine and Public Health*, *32*, 520–526.

9. Reuter, G., Boros, A., & Pankovics, P. (2011). Kobuviruses – a comprehensive review. *Reviews in Medical Virology*, *21*, 32–41.
10. Shrestha, S., Haramoto, E., Malla, R., & Nishida, K. (2015). Risk of diarrhoea from shallow groundwater contaminated with enteropathogens in the Kathmandu Valley, Nepal. *Journal of Water and Health*, *13*, :259–269.
11. Shrestha, S., Haramoto, E., Sherchand, J. B., Hada, S., Rajbhandari, S., & Shindo, J. (2016). Prevalence of protozoa and indicator bacteria in wastewater irrigation sources in the Kathmandu Valley, Nepal: cases from Kirtipur, Bhaktapur and Madhyapur Thimi municipalities. *Journal of Water and Environment Technology*, *14*, 149–157.
12. Shrestha, S., Nakamura, T., Malla, R., & Nishida, K. (2014). Seasonal variation in the microbial quality of shallow groundwater in the Kathmandu Valley, Nepal. *Water Science & Technology: Water Supply*, 14, 390–397.
13. Tanaka, Y., Nishida, K., Nakamura, T., Chapagain, S. K., Inoue, D., Sei, K., Mori, K., Sakamoto, Y., & Kazama, F. (2012). Characterization of microbial communities distributed in the groundwater pumped from deep tube wells in the Kathmandu Valley of Nepal. *Journal of Water and Health*, *10*, 170–180.
14. Yamashita, T., Sugiyama, M., Tsuzuki, H., Sakae, K., Suzuki, Y., & Miyazaki, Y. (2000). Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. *Journal of Clinical Microbiology*, *38*, 2955–2961.

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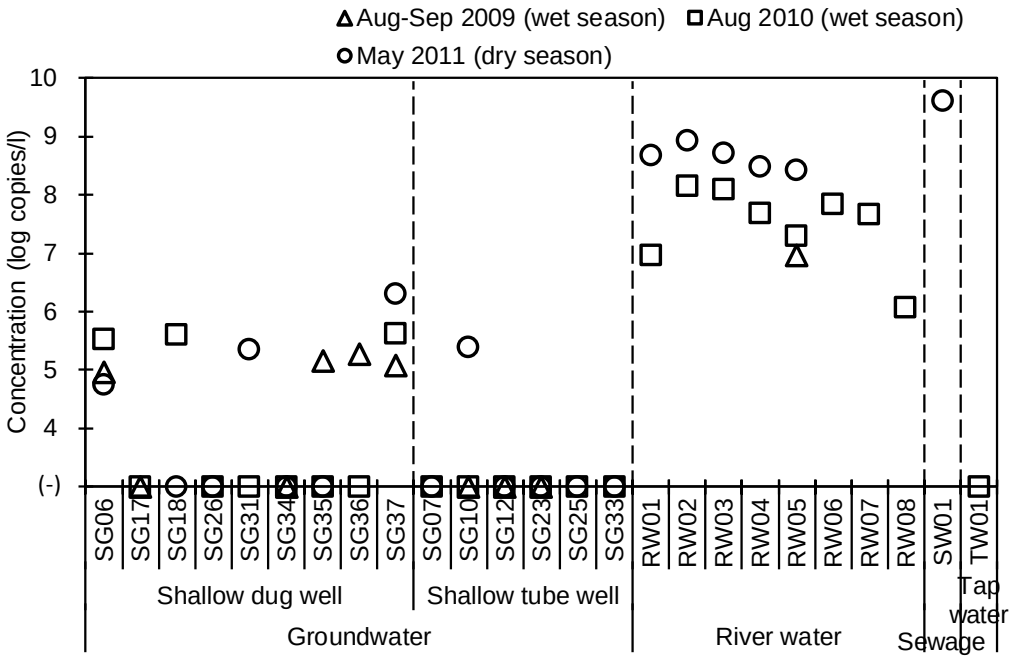

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179 Fig. 1 Concentrations of AiV-1 genomes in water samples

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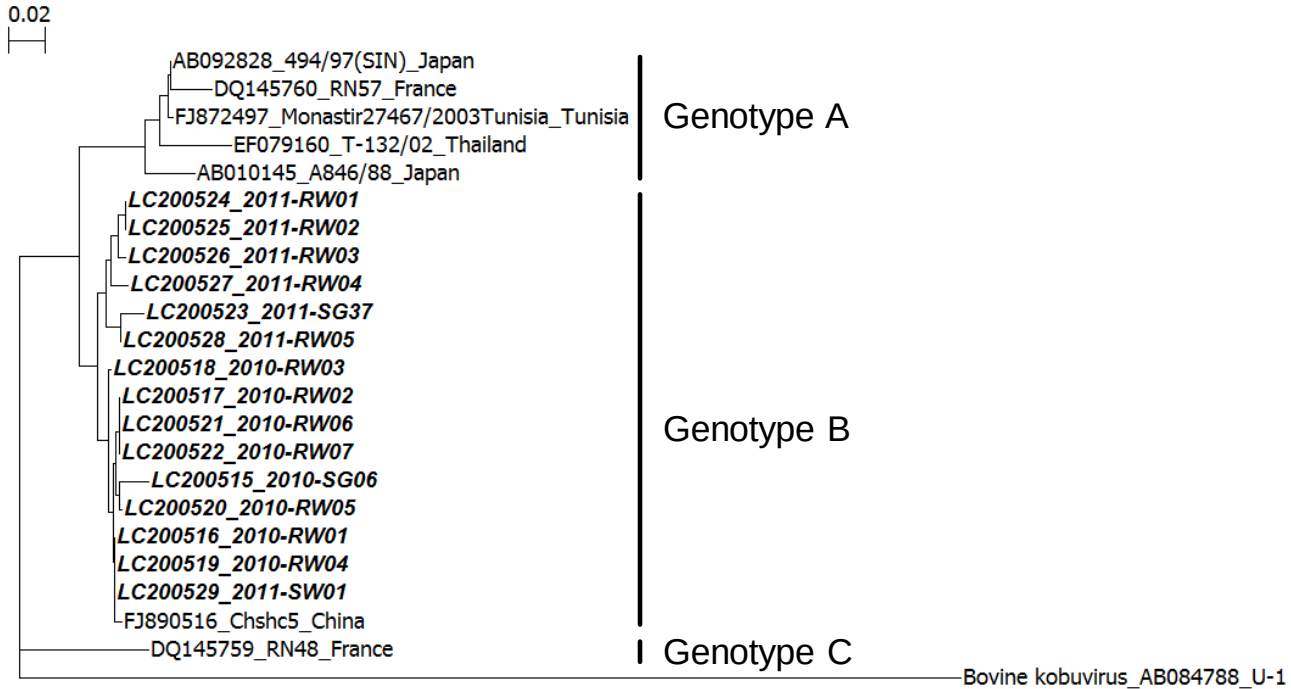

Fig. 2 Phylogenetic analysis of AiV-1 sequences identified in water samples. The tree was generated using the neighbor-joining method with 1,000 bootstrap replicates based on the 224-nt 3C-3D junction region sequences. The sequence of bovine kobuvirus (AB084788) was used as an outgroup. The scale bar 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.