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BRIEF REPORT

Molecular detection of Aichi virus in raw sewage in Italy

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Abstract Aichi virus (AiV) is suspected to play a role in viral gastroenteritis in humans. In this study, we assessed the presence of AiV in untreated influent sewage samples collected at four wastewater treatment plants in central Italy. AiV was detected in 6 (12.5 %) of the 48 specimens and in all plants. All of the Italian strains showed the highest nucleotide and amino acid sequence identity to genotype B AiV detected recently in Asia, especially in China.

Aichi virus (AiV) was first recognized in 1989 as the cause of oyster-associated nonbacterial gastroenteritis in humans in Aichi Prefecture, Japan [25]. Based on its genomic organization and sequence similarities, the virus was later classified as a member of a new genus named *Kobuvirus* in the family *Picornaviridae* [9, 15].

AiV is a small, non-enveloped virus of approximately 27–30 nm in diameter with a single-stranded, positive-polarity RNA genome of 8,280 nucleotides (nt) in length. The single large open reading frame encodes a polyprotein of 2,432 amino acids that is cleaved into the structural proteins VP0, VP3, and VP1 and nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D [20, 27]. Based on the nucleotide sequence of the 3CD junction region, AiVs are currently classified into three genetically distinct genotypes (A, B and C) [2, 28].

AiV has been proposed to be a causative agent of gastroenteritis in humans. Several investigations worldwide have revealed that AiV is involved in 0.9-4.1 % of sporadic cases of pediatric gastroenteritis, with genotypes A and B being predominant [2, 7, 8, 17]. Furthermore, the high seroprevalence (80.0 % to 95.0 %) found in humans of different age groups [6, 13, 19, 22, 26] indicates widespread exposure and suggests that AiV infections are quite common [18].

Since their first identification, AiVs have been detected in Asia [14, 29], South America [1, 13], Africa [2, 23] and several European countries [2, 4, 7, 8, 13, 17].

AiV transmission is thought to occur through direct contact, by the oro-faecal route, or through consumption of contaminated food or water. In most cases, documented outbreaks associated with consumption of foods have involved oysters or other seafood [2, 11, 21, 25]. Recently, AiVs were found in raw and treated sewage samples in Venezuela and Tunisia [1, 21].

Analysis of untreated sewages appears to be a very useful methodology to reveal the presence of undetected faecal pathogens [3, 5, 12]. Raw sewage could contain enteric viruses shed from affected people that would reflect the actual state of the circulating viruses in different geographic areas.

To date, in Italy, there have been no published reports regarding the circulation of AiVs and their potential association with diarrheal cases or outbreaks.

In order to start filling this gap, we undertook a preliminary study to investigate the presence of AiVs in untreated sewage samples using molecular techniques. The sequences of the Italian strains detected were determined and analysed in detail to amass information on their genetic features.

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A total of 48 influent sewage samples were collected at four wastewater treatment plants located in the province of Teramo (Central Italy) in collaboration with the Ruzzo Reti SpA during sewage monitoring activity in 2010-2011. Daily flows in these plants range from 3,000 to 18,000 cubic meters, with a design capacity from 12,000 to 100,000 population equivalents. For each plant, sample collection was carried out by swabbing different surface points of the separation grids (influent bar screens and grit chambers) used for primary wastewater treatment. To minimize RNase contamination during sample collection, each swab was placed into a sterile container and shipped on ice. Disposable latex gloves were used and changed for each specimen collected. The sewage samples were transported to the laboratory under cold (+4 °C) conditions, diluted in phosphate-buffered saline (0.15 M, pH 7.2) to prepare a total of 1 ml of each specimen, and processed within 24 h after collection. RNA was extracted from 500 µl of the suspension using a QIAmp Viral RNA Kit (QIAGEN GmbH, Hilden, Germany) following the protocol of the manufacturer. The final RNA was eluted in 80 µl of RNase-free water and used directly in RT-PCR assays or stored at −80 °C.

The presence of AiV RNA was assessed using a broadly reactive primer pair, UNIV-kobu-F/UNIV-kobu-R [16], which can be used to amplify a 217-bp fragment of the 3D gene of members of all three kobuvirus species (*Aichivirus A, Aichivirus B* and *Aichivirus C*) and primer set Ai6261 and Ai6779 specific for AiVs, targeting a 519-bp fragment at the 3CD junction [28]. RT and PCR were performed in one-step procedure, using the SuperScript III a one-step system (Invitrogen, Ltd, Paisley, UK). The amplification products were analyzed by 1.5 % agarose gel electrophoresis and visualized by UV illumination after ethidium bromide staining.

All of the amplicons were purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and subjected to direct sequencing using BigDye terminator cycle sequencing chemistry and a 3730 DNA Analyzer (Applied Biosystems, Foster, CA). RT-PCR products for which no sequences were obtained by direct sequencing were cloned into the pCR2.1 vector (Invitrogen, Ltd, Paisley, UK). Plasmid DNA was purified using a miniprep kit (QIAGEN GmbH, Hiden, Germany), and three clones per product were sequenced.

Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov) and FASTA (http://www.ebi.ac.uk/fasta33) with default values were used to find homologous hits. Neighbor-joining phylogenetic analysis based on the 519-bp sequence of the 3CD junction was conducted using the MEGA software package, version 3.1 [10].

The AiV-like sequences detected in this study were made available in GenBank under the accession numbers KC488325-KC488328 and KC693051-KC693052 for the 3CD junction region and KC488329-KC488330 for the partial 3D gene.

Fig. 1 Neighbor-joining phylogenetic tree based on the 519-nt 3CD sequence of the AiVs. The tree was generated using the neighbor-joining method and the Kimura two-parameter distance correction, with statistical support provided bootstrapping with 1000 replicates. The scale bar indicates nucleotide substitutions per site. The markers denote AiV sequences detected in this study ▶

Out of 48 sewage samples, two (4.16 %) were found to be positive for AiV RNA using the kobuvirus universal primer set. A total of six specimens (12.5 %) were positive when re-screening the samples using the primer set Ai6261/Ai6779. The six AiV-like strains were distributed over the four wastewater treatment plants tested.

By FASTA and BLAST analysis of the partial 3D gene, the two Italian strains (AiV/8BF/2012/IT and AiV/4Ab/2012/IT) were found to be genetically related to the AiV like-sequences (89.0-99.0 % nucleotide [nt] sequence identity) identified in China [29], Japan [27, 28], Brazil [13], Germany [4, 13] and Hungary [17], showing the close relatedness with strains of genotype B.

Upon sequence analysis of the 3CD region with primers Ai6261/Ai6779, the AiV strains detected in this study (AiV/4A/2012/IT, AiV/3B/2012/IT, AiV/8BF/3CD/2012/IT, AiV/4AB/3CD/2012/IT, AiV/34AP/2012/IT and AiV/7C/2012/IT) shared 97.9-99.7 % identity with each other and displayed the closest relatedness (96.0-99.0 % nt sequence identity) with the AiVs previously found in patients with acute gastroenteritis in China (AiV/Chshc1-8) [29] and Germany (AiV/D/VI2244/2004 and AiV/D/VI2321/2004) [4]. Identity to the European AiV-like sequences detected in Sweden [7] and France [2] ranged from 88.0 % to 91.5 %. Based on inspection of the tree (Fig. 1), the six Italian sequences formed a tight cluster with the AiV strains from China [29], Germany [4], Brazil [13], Japan [24, 28] and Bangladesh [14], within genotype B [28].

To our knowledge, this is the first study in which evidence was collected for the occurrence of AiV in Italy. Using a combination of generic and specific primers, AiV-like strains were identified in a sample collection obtained from four wastewater treatment plants. Furthermore, the observation that the positive specimens were found in all of the plants tested supports the hypothesis of a wide distribution of this virus in the geographical area assessed. However, although the six strains detected in our study were collected at sites that were distant from each other, molecular analysis of the 3CD junction region revealed little genetic variation among them, suggesting limited strain heterogeneity.

By molecular analysis of the partial 3D and 3CD junction regions, the Italian sequences displayed the highest


EF466014/Aiv/J-4404/JPN
EF079150/AiV/J-4415/02/JPN
EF466015/Aiv/J-4318/JPN
EF466013/AiV/J-4383/JPN
EF079153/Aiv/J-4478/02/JPN
AB034650/AiV/A1471/96/JPN
AB092828/AiV/494/97/JPN
AB092829/AiV/177/96/JPN
GQ927711/AiV/VI2287/2004/D
AY747174/AiV/BAY1/03/D
AB092826/AiV/364/96/VIE
AB092824/Aiv/144/96/JPN
AB092825/AiV/359/96/JPN
DQ145762/AiV/R380/CD3/FR
EU159246/AiV/E1197/2006/FR
FJ872497/AiV/Monastir/27457/2003/TUN
EU159249/AiV/E1588/2006/FR
FJ872492/AiV/Monastir/2403/2005/TUN
DQ145761/AiV/R586/FR
EU159248/AiV/E1208/2006/FR
EF079154/AiV/J-4827/02/JPN
EF079162/AiV/VN-640/03/VIE
JX564249/Aiv/kvgh9901263 2/2010/Taiwan
gEU159250/AiV/E1818/2007/FR
FJ878790/AiV/sewage/10.06/2004/TUN
FJ878792/Aiv/sewage/05.01/2005/TUN
FJ878786/AiV/sewage/23.02/2004/TUN
AB034655/AiV/N128/91/JPN
AB079268/AiV/A1156/87/JPN
EF079161/AiV/VN-636/03/VIE
AB010145/AiV/JPN
AB034652/Aiv/A843/88/JPN
AB034651/AiV/A844/88/JPN
EF079158/AiV/B-370/05/Bangladesh
EF079160/Aiv/T-132/02/THA
AB034657/AiV/M166/92/JPN
AB034653/AiV/A942/89/JPN
AB034663/AiV/T132/90/JPN
AB034656/AiV/N528/92/JPN
AB092832/AiV/414/96/JPN
AB034654/AiV/N1277/91/JPN
AB034659/AiV/P803/90/JPN
AB034660/AiV/P832/90/JPN
EF079159/AiV/B-1055/05/Bangladesh
EF079156/AiV/B-101/05/Bangladesh
EF079155/AiV/B-5/05/Bangladesh
EF079157/AiV/B-171/05/Bangladesh
AB092834/AiV/700/98/JPN
AB092833/AiV/488/97/JPN
DQ028832/AiV/GO/03/01/Brazil
AB034658/AiV/P766/90/JPN
AB034662/AiV/P880/91/JPN
AB034661/AiV/P840/91/JPN
GQ927712/AiV/VI2244/2004/D
GQ927706/AiV/VI2321/2004/D
AB092830/AiV/139/96/JPN
FJ890519/AiV/Chshc1/CHN
GU459258/AiV/Fujian-1021/2008/CHN
EU715251/AiV/Qld/2008/204/Australia
GU339099/AiV/58174/CHN
GU339098/AiV/58118/CHN
KC438327/AiV/34AP/2012/IT
KC693051/AiV/4AB/3CD/2012/IT
KC693052/AiV/8BF/3CD/2012/IT
KC438328/AiV/7C/2012/IT
KC438325/AiV/4A/2012/IT
KC438326/AiV/3B/2012/IT
FJ890521/AiV/Chshc3/CHN
FJ890522/AiV/Chshc4/CHN
FJ890520/AiV/Chshc2/CHN
FJ890517/AiV/Chshc6/CHN
FJ890523/AiV/Chshc7/CHN
FJ890518/AiV/Chshc8/CHN
FJ890516/AiV/Chshc5/CHN
DQ145759/AiV/RN48/FR
AB084788/Bovine-kobuvirus/U-1
EU787450/Porcine-kobuvirus/S-1-HUN
Genotype A
Genotype B
Genotype C
0.05

identity to AiV strains that were previously detected in patients with gastroenteritis in China and Germany [4, 29].

Genetic analysis of the AiVs recently identified in outbreaks of diarrhoea in several European countries revealed that genotype A is the most common genotype circulating in Europe [2, 4, 7, 8, 11, 13, 17]. Interestingly, in our analysis, all of the AiV-like sequences detected grouped with strains of genotype B, which also includes sequences recently identified in Germany [4], confirming a co-circulation of both genotypes on the same continent. This may be accounted for by geographical changes in the circulation of AiV, which are likely due to increased migration, tourism, and international trade.

In conclusion, this preliminary study provides direct evidence on the presence of AiV in the environment in Italy. Further studies are warranted in order to examine the significance of these findings and to establish the clinical importance of AiVs in human enteric diseases.

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Conflict of interest All of the authors declare that there are no financial or other relationships that might lead to a conflict of interest. All of the authors have seen and approved the manuscript and have contributed significantly to the work.

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