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Detection of Aichi virus genotype B in two lines of wastewater treatment processes

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ABSTRACT

Enteric viruses are released in important quantities into the environment where they can persist for a very long time. At very low doses, they can cause human gastroenteritis, and are responsible for a substantial number of waterborne diseases. The aims of this study were multiple: firstly, to study the circulation of Aichi viruses (AiV) in wastewater sampled at the scale of a pilot wastewater treatment plant; secondly, to evaluate the performance of two wastewater treatment procedures, as natural oxidizing lagoons and rotating Biodisks, concerning the AiV removal; and finally, to determine the different type of AiV genotype found during this study. Hence, the pilot wastewater treatment plant is principally irrigated by the wastewater of three neighbouring clinics. Wastewater samples were collected during 2011 from the two lines of biological treatment procedures. AiV detection in wastewater were achieved using the Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique, and the identification of AiV genotype was realized by the direct sequencing of PCR products. The result revealed that AiV strains were identified in 50% (n = 51) of the wastewater samples. A significant increase of the AiV detection frequency was registered from upstream to downstream of the five ponds constituting the natural oxidizing lagoon process, and at the exit of the rotating Biodisks procedure. All detected AiV strains showed the highest nucleotide sequence identity to genotype B that has been recently observed in patients in Asia. This finding represented the first Tunisian survey that revealed and mentioned the first detection of AiV genotype B in sewage and by the same argued for a noticeable resistance or survival of this type of virus in the two lines of treatment considered.

1. Introduction

Sewage pollution plays a major role in the transmission of different pathogenic enteric viruses, which are associated with gastrointestinal diseases in human populations. Aichi viruses (AiV) have emerged as viral agents that are associated with foodborne and waterborne non-bacterial acute gastroenteritis in humans [1]. AiV excreted with human faeces contribute to contaminating surface waters directly or after discharge into the natural environment of treated or non-treated sewage [2]. The resistance of these viruses to different treatment procedures facilitates their transmission directly by the faecal-oral route or indirectly by consumption of contaminated food or water [1]. Detection of AiV was reported in sewage and shellfish samples in Tunisia [3], in sewage and surface waters in Netherlands [2], in sewage in Italy [4], in surface waters in Venezuela [5], in sewage and river waters in Japan [6]. Viruses in sewage are assumed to reflect the viruses circulating in the human

Abbreviations: AiV, Aichi viruses; WTPP, Wastewater Treatment Pilot Plant; EC, Electrical Conductivity; COD, Chemical Oxygen Demand; BOD_5, Biological Oxygen Demand; SS, Suspended Solids; NH_4-N, Ammonium Nitrogen; NO_3-N, Nitrate Nitrogen; NO_2-N, Nitrous Nitrogen; P-PO_4, *Ortho*-Phosphate.

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population and originating from asymptomatic and symptomatic persons [7]. Hence, environmental surveillance studies are extremely useful to determine the circulation of viruses in the human population [7,8] and to obtain sequence information on the circulating AiV strains [4]. Laboratory diagnosis of AiV infection is difficult because the virus is not easily cultivable. The development of a broadly reactive reverse transcription polymerase chain reaction (RT-PCR) and a Real-Time RT-PCR (RT-qPCR) have facilitated its detection in both clinical and environmental samples [9–11].

AiV is a small non-enveloped virus with a single-stranded and positive-sense RNA genome, Aichi viruses belong to the *Picornaviridae* family, and recently it has been classified as a new enteric virus *Kobuvirus* genus [1,12,14]. AiV in humans was reported in 1989 in Japan from a sample collected during an oyster-associated gastroenteritis outbreak [12]. The complete nucleotide sequence of an AiV was described in 1998 [14]. Freshly, AiV was detected at a low incidence in children with gastrointestinal symptoms in Asia [15,16], in Europe [17–19], in South America [17] and in Tunisia [20,21]. AiV have mainly been detected by PCR targeting the 3 CD junction of the virus genome [22]. This junction region has been recognized as a conserved one. The viral protein VP1 is more genetically diversified [13,18,23]. On the other side, the VP1 sequence typing is considered as a standard method for the classification of Picornaviruses [24], but analysis of the 3 CD region has been used to divide AiV into 3 genotype categories A, B and C [18,22,25]. As well, several epidemiological studies indicated that various AiV genotype are circulated in different epidemiological settings, and that genotype A was the most predominant one in Japan, in Germany, in Tunisia and in France [15,17,18,21]. The genotype B was also found in Malaysia and in Brazil [17,22].

In the present study, wastewater samples were collected at the scale of two pilot secondary biological treatment procedures, a natural oxidizing pond and a rotating biodisks. These wastewater samples were analysed for the AiV detection, and for the genotype determination to evaluate the AiV removal by these last two sewage treatment procedures. Thereby, the monitoring of the AiV circulation in polluted wastewater should help improve guidelines and strategies for better prevention of the environmental contamination by this kind of pathogens.

2. Materials and methods

2.1. The Wastewater Treatment Pilot Plant

The different biological treatment processes of the Wastewater Treatment Pilot Plant (WTPP) and the sampling plan were previously described in the two studies of Ibrahim et al. [27,28]. Briefly, the two biological wastewater treatment procedures considered were the natural oxidizing ponds and the rotating biodisks. During the year 2011, 102 wastewater samples were collected. A volume of 1 L of wastewater was sampled at the exit of each pond (P_1, P_2, P_3, P_4 and P_5) of the natural oxidizing lagoons and of the rotating biodisks (D).

2.2. AiV detection by RT-PCR

Virus extraction: The virus extraction from the wastewater was achieved as recommended by the USA Environmental Protection Agency, using the method of beef extract and $AlCl_3$ [27–29]. The concentration of the viral particles was performed by polyethylene glycol 6000 (PEG 6000) [30], and the decontamination of the viral suspension was completed by purification using syringe filters.

Nucleic acid extraction: The viral RNA has been obtained from 800 μL of sewage extract by an automatic extractor NucliSENS® EasyMag™ platform (bioMérieux, Marcy L'Etoile, France), according to the manufacturer's instructions. The viral RNA was eluted in a final volume of 110 μL.

AiV detection: Wastewater samples were screened by RT-PCR using the primer sets Ai6261 and Ai6779, to amplify a 519 pb fragment at the 3 CD junction (viral protease and RNA-dependent RNA polymerase) [22]. RT-PCR was performed using a Qiagen One Step RT-PCR Kit according to the manufacturer's instructions and to the cycles of amplification given by the authors of each primer set. All the bacteriological and the physico-chemical parameters of wastewater samples were analysed according to the standard methods [31].

2.3. AiV typing

The AiV genotyping was completed by direct sequencing of the PCR products with the same primers mentioned above and by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The obtained nucleotide sequences were then compared to others of AiV strains from different genotype that are accessible in the GenBank database and in the National Reference Centre for Enteric viruses of Dijon (France) by using Fasta program. The alignment of sequences was carried out using Codon Aligner software. The BLAST program was used to compare and to determine the most related sequences from GenBank to the Tunisian ones. Phylogenetic analyses were assessed according to the neighbour-joining method with the MEGA software [32] and the PHYLIP package [33]. The pairwise distances among the sequences were calculated with the Kimura two-parameter distances. The tree was visualized with the Tree view program [34]. The nucleotide sequences obtained in the present study were submitted to GenBank under accession numbers KJ743959–KJ743985.

2.4. Statistical analysis

The numbers of AiV positive wastewater samples were separated by the least significant difference (LSD) according to the Waller-Duncan a, b test, and by using the SPSS program V19, for Windows.

3. Results

3.1. Physico-chemical and bacteriological parameters

The average value of physico-chemical and bacteriological parameters recorded during the year of wastewater sampling at the scale of the different oxidizing lagoons and of the rotating biodisks procedures were represented in Table 1.

3.2. The variation of AiV frequencies in the two treatment procedures

AiV were detected in 51 (50%) out of the 102 wastewater samples collected during the year of the survey. Forty-two of the positive wastewater samples (41%) were detected in the five natural oxidizing pond procedure (P_1 to P_5), and only nine of the positive sewage samples (9%) were registered at the exit of the rotating Biodisks process. The overall detection frequencies of AiV, in the various ponds processes and at the exit of the rotating biodisks, were distributed as follows: 4% (n = 4/102), 6% (n = 6/102), 12% (n = 12/102), 6% (n = 6/102), 14% (n = 14/102) and 9% (n = 9/102) in the P_1, P_2, P_3, P_4, P_5 ponds and in the rotating Biodisks (D), respectively. On the other hand, the detection frequencies of AiV in each pond (P_1 to P_5) and at the exit of the rotating Biodisks line (D) were 15% (n = 4/26), 28% (n = 6/21), 80% (n = 12/15), 50% (n = 6/12), 87% (n = 14/16) and 75% (n = 9/12), respectively (Fig. 1). The