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Chlorinated and ultraviolet radiation -treated reclaimed irrigation water is the source of *Aeromonas* found in vegetables used for human consumption

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ABSTRACT

Wastewater is increasingly being recognized as a key water resource, and reclaimed water (or treated wastewater) is used for irrigating vegetables destined for human consumption. The aim of the present study was to determine the diversity and prevalence of *Aeromonas* spp. both in reclaimed water used for irrigation and in the three types of vegetables irrigated with that water. Seven of the 11 (63.6%) samples of reclaimed water and all samples of vegetables were positive for the presence of *Aeromonas*. A total of 216 *Aeromonas* isolates were genotyped and corresponded to 132 different strains that after identification by sequencing the *rpoD* gene belonged to 10 different species. The prevalence of the species varied depending on the type of sample. In the secondary treated reclaimed water *A. caviae* and *A. media* dominated (91.4%) while *A. salmonicida*, *A. media*, *A. allosaccharophila* and *A. popoffii* represented 74.0% of the strains in the irrigation water. In vegetables, *A. caviae* (75.0%) was the most common species, among which a strain isolated from lettuce had the same genotype (ERIC pattern) as a strain recovered from the irrigation water. Furthermore, the same genotype of the species *A. sanarellii* was recovered from parsley and tomatoes demonstrating that irrigation water was the source of contamination and confirming the risk for public health.

1. Introduction

Water is an increasingly scarce resource and as a countermeasure wastewater is being treated to produce reclaimed water that can be reused mainly for agricultural irrigation, including fruits and vegetables that are destined for human consumption (Pianetti et al., 2004; Carvalho et al., 2012; Carey et al., 2016). The safety of reclaimed water and food products (shellfish, lettuces, meat etc.) is evaluated using bacteria indicators of fecal pollution (coliforms, *Escherichia coli,* etc.) during the stipulated controls fixed by legislation to determine their sanitary quality and potential health risk (Figueras and Borrego, 2010; Fernandez-Cassi et al., 2016). However, related illness outbreaks still occur worldwide mainly due to the failure of the fecal indicator organisms to predict the presence of pathogens (Figueras and Borrego, 2010). Among the emerging food and water borne pathogens the bacterial of the genus *Aeromonas* are of special interest because their significance in public health is still not clearly understood (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015; Teunis and Figueras, 2016).

The genus *Aeromonas* consists of Gram negative, oxidase positive bacilli that are considered autochthonous of aquatic environments and are commonly isolated from clinical and environmental samples (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013; Figueras and Beaz-Hidalgo, 2014, 2015). Several studies have shown that *Aeromonas* species are foodborne and waterborne pathogens of increasing importance (Abeyta et al., 1986; Granum et al., 1998; Altwegg et al., 1991; Demarta et al., 2000; Figueras and Borrego, 2010; Pablos et al., 2011; Khajanchi et al., 2010; Teunis and Figueras, 2016). *Aeromonas* spp. can be readily isolated from treated sewage, reclaimed water, sea water, fresh water and from drinking water distribution systems, where they appear to survive well, to proliferate at low temperatures and to be linked to pipe biofilms where populations may survive at high chlorine levels (Emekdas et al., 2006; Figueras and Borrego, 2010; Jjemba et al., 2010; Khajanchi et al., 2010; Martone-Rocha et al., 2010; Figueira et al., 2011; Igbinosa and Okoh, 2013; Robertson et al., 2014; Al-Jassim et al., 2015). Several species of *Aeromonas* are recognized to be opportunistic pathogens to humans and can affect both immunocompromised and immunocompetent individuals being the most frequent clinical presentation gastroenteritis followed by wound infections and bacteremia (Janda

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and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015; Humphries and Linscott, 2015). A clinical revision of studies from different countries performed by Figueras and Beaz-Hidalgo (2015) revealed that *Aeromonas* spp. recovered from feces represents up to 40.0% of the total recorded clinical isolates (n= 817). The gastroenteritis associated to *Aeromonas* ranges between 4.0 and 22.0% in developing countries, while this percentage does not exceed 10.0% in developed countries (Humphries and Linscott, 2015). Despite the role of *Aeromonas* spp. in diarrheal cases have been questioned in some studies, a recent publication has provided evidences that support the true enteropathogenicity of these bacteria (Teunis and Figueras, 2016). Recently, it was proved that *Aeromonas* spp. was the cause of two waterborne infections because strains showing the same genotypes were isolated from drinking water and from the feces of patients with diarrhea (Khajanchi et al., 2010; Pablos et al., 2011). Some studies have found *Aeromonas* spp. in reclaimed water used for agricultural irrigation, which is a risk for consumers when considering that this water can be the source of entry of the bacteria into the food chain (Pianetti et al., 2004; Al-Jassim et al., 2015; Latif-Eugenin, 2015; Fernandez-Cassi et al., 2016). However, so far, no studies have evaluated simultaneously the presence of *Aeromonas* spp. in the water used for irrigation and in the irrigated vegetables. Therefore, the aim of this study was to determine, using molecular methods (genotyping and sequencing the *rpoD* gene), the prevalence, diversity and epidemiological relationship of the *Aeromonas* isolates recovered from the ready to eat vegetables and the reclaimed water used for their irrigation.

2. Material and methods

2.1. Water and vegetables sampling

Eleven reclaimed water samples were collected from a wastewater treatment plant, located in Catalonia North-East of Spain. Three water samples were collected after the secondary treatment by activated sludge, three after tertiary treatment that involved chlorination (dose of 3–6 mg/L of sodium hypochlorite with a contact time of 30 to 90 min, to maintain a residual of 1–3 ppm) and ultraviolet radiation (dose of 25–30 mJ/cm^2) and five corresponded to irrigation water. The average range of pH and turbidity (NTU) of the secondary treated water were 7.23 (7.14–7.38) and 3.15 (0.54–4.71) respectively. For the tertiary treated water the values were 7.48 (7.26–7.62) and 3.12 (2.48–3.61) and for the irrigation water samples 7.18 (6.98–7.46) and 2.92 (2.34–3.90). The irrigation water came from a hose pipe that extracted water from a well, where the tertiary treated water was accumulated before being use for irrigation. In addition three irrigated vegetables samples i.e. lettuces, tomatoes and parsley were analyzed. Drip irrigation was applied to the tomatoes and spray irrigation to the lettuces and parsley. The frequency of the irrigation depended on weather conditions, but it was normally every two days.

2.2. Detection of faecal indicator bacteria

The determination of *E. coli* (EC) and intestinal enterococci (IE) was carried out using the 96-well microplate (BioRad, France) most probable number (MPN) methods ISO 9308-2 and ISO 7899-1 respectively. The detection of *E. coli* in the 96-well microplate is based on the expression β-D-glucoronidase enzyme, while the β-glucosidase is the target for intestinal enterococci.

2.3. Detection and isolation of Aeromonas

All samples were analysed by direct culture and after an enrichment step with Alkaline Peptone Water supplemented with Ampicillin (APW-A) at a concentration of 10 mg/l (Latif-Eugenín et al., 2016). For this, 10 ml of water samples were mixed with 90 ml of APW-A (1:10 vol/vol). For the vegetables, 10 g were homogenized in 90 ml of APW-A (1:10 wt/vol) using stomacher bags. The enrichment culture was incubated at 30 °C for 24 h, 10-fold serial dilutions were performed and then 100 µl were plated on 3 different culture media: Dextrin Ampicillin Agar (ADA, Havelaar et al., 1987), Starch Ampicillin Agar (SAA, Palumbo et al., 1985) and Bile Irgasan Brilliant Green-modified (BIBG-m, Neyts et al., 2000) and incubated at 30 °C for 24 h. Typical *Aeromonas* colonies were transferred to Trypticase Soy Agar (TSA, BD, France) to obtain a pure culture from which to perform the DNA extraction for the molecular genotyping and molecular identification. The density of *Aeromonas* spp. was determined by direct plate count and by the MPN method. For the direct plate count, 100 µl of water samples were directly inoculated on ADA medium. In the case of vegetables, 10 g were homogenized in Buffered Peptone Water (BPW, pH=7.0) and 100 µl of these homogenate were inoculated in ADA medium. In both cases, the plates were incubated at 30 °C for 24 h. For the MPN method, 500 µl of the water samples and 500 µl of the homogenized vegetable samples were inoculated in five replicate tubes containing 3.5 ml of APW-A and were incubated at 30 °C for 24 h. The verification of the positive tubes obtained with the MPN method was done with the ADA medium. The limit of detection for the direct plate count and the MPN methods were 35 cfu/100 ml and 20 MPN/100 ml, respectively.

2.4. Genotyping and molecular identification

DNA was extracted using InstaGene Matrix (BioRad, France) according to manufacturer's instructions. All isolates were identified to genus level using the PCR detection of Glicerophospholipid Cholesterol Acyltransferase (*gcat*) gene specific of the genus *Aeromonas* (Soler et al., 2002). To recognise isolates corresponding to a same genotype (=strain) and to avoid working with clones all isolates were genotyped with the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) technique using the primers and conditions described by Versalovic et al. (1991) and used for *Aeromonas* in several other studies (Soler et al., 2003a; Beaz-Hidalgo et al., 2012). Molecular identification to species level was performed by sequencing the *rpoD* gene using the primers and conditions described by Soler et al. (2004). Sequence corrections and analysis were performed with the DNAstar Seqman program (Lasergene, USA). The sequence alignments and the Neighbor Joining phylogenetic tree were performed using the MEGA program version 5.0 (Tamura et al., 2011).

2.5. Statistical analysis

The values obtained by the two analytical methods were transformed into $\log_{10}$ and the geometric mean was calculate with Excel (Microsoft Office 2007). A two-way ANOVA, followed by the Bonferroni post-hoc test were used for comparing the concentration of the bacteria indicators and aeromonads in the different types of samples. All the analyses were performed using SigmaPlot 11.0 (SSI, California, USA) and significance was fixed at $P < 0.05$.

3. Results

3.1. Detection of fecal indicator bacteria and Aeromonas

Reclaimed waters were positive for *E. coli* (EC) and intestinal enterococci (IE) in 45.5% of the samples (Table 1). The geometric mean of EC and IE in the secondary treated wastewater were reduced by the tertiary treatment in 3.35 and 3.43 logs (99.9% and 100% reduction) respectively (Table 1). Statistically significant differences were only observed for the densities of IE that were considerably reduced in the tertiary treated water and in the irrigation water ($P < 0.05$).

A total of 63.6% (7/11) water samples and all vegetables (3/3) were positive for the presence of *Aeromonas* spp. (Table 1). The four