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Characterization of antibiotic resistant and pathogenic *Escherichia coli* in irrigation water and vegetables in household farms

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ABSTRACT

This study aimed to characterize *Escherichia coli* present in irrigation water and vegetables from 16 household farms. Isolates were obtained from 50% of water (n = 210 isolates) and 38% of vegetable samples (n = 239). Phylogroups B1 (56% of isolates) and A (22%) were the most prevalent both in water and vegetables. Diarrheagenic strains were detected in vegetables. Irrespective of the source (i.e. water or vegetables), the most common antibiotic resistance was against streptomycin (89% resistant isolates) and tetracycline (24%). Common acquired genes (e.g. bla_{TEM}, *tetA*, *tetB*) were found in isolates from both sources. Class I integrons were detected in water (arrays *dfrA1-aadA1* and *dfr16-blaP1b-aadA2-ereA*) and vegetables (unknown arrays). *intI2* was detected in water (*dfrA1-sat2-aadA1*). Plasmids were detected in 14 isolates (IncFIC, IncFIB, IncFrep, IncI1 in both samples; IncY in vegetables). Plasmids from seven isolates were transferrable by conjugation, conferring resistance to antibiotics to the recipient strain. Multidrug-resistant (MDR) strains were isolated from water (12% of the unique isolates) and vegetables (21%). Predominant sequence types (STs) among MDR isolates were ST10, ST297 and ST2522. In some cases, the same STs and identical clones (as showed by rep-PCR typing) were detected in water and vegetables, suggesting cross-contamination. This study identified several risk factors in *E. coli* isolates from vegetables and irrigation water, raising health concerns. Also, results suggest that irrigation groundwater constitutes a source of *E. coli* that may enter the food chain through vegetables ingestion.

1. Introduction

In the past decades an extra effort in promoting balanced diets has been made by health authorities worldwide. Fresh produce, being a good source of essential components (i.e. vitamins, minerals and phytonutrients), protect against a range of illnesses such as cancers, cardiovascular diseases, diabetes and obesity (Pomerleau et al., 2006). In Europe, the Food and Agriculture Organization (FAO) indicates that fresh produce consumption has increased over the last four decades, and that in Southern Europe countries, including Portugal, the consumption of vegetables is higher than in Northern Europe (Elmadfa et al., 2009).

Fresh produce naturally carry a non-pathogenic microbial community but may become contaminated with human pathogens (Boehme et al., 2004; Edelstein et al., 2014). Contaminated fresh produce, and in particular leafy greens usually consumed raw or minimally processed, represent a risk of infection for consumers (FAO/WHO, 2008). Contamination can occur both at pre-harvest (i.e. through manure fertilization, irrigation water and wild animals) (Beuchat and Ryu, 1997) and post-harvest (i.e. by washing, handling and processing food) (Berger et al., 2010; De Roever, 1999). Despite the fact that tight food safety regulations were implemented in most countries, produce-associated outbreaks have been increasing in recent years (Jung et al., 2014). Factors contributing to the increase of produce-associated outbreaks include the production of vegetables in household farms, mainly for self-consumption, in which microbiological quality is rarely monitored.

Water used for irrigation has been identified as a main contributor to the contamination of fresh produce (EFSA BIOHAZ Panel, 2013; De Roever, 1999). Irrigation water may become contaminated either through direct contact with sewage and manure, or through nonpoint pollution sources, such as agricultural run-offs (Beuchat and Ryu, 1997; De Roever, 1999). Because water management and monitoring were inadequate in many member states, the European Union Water Framework Directive, adopted in 2000, established a framework of water policy (European Community, 2000). Groundwater use for irrigation in agriculture was stated as one important “hidden” resource. However, groundwater may also be impacted by anthropogenic activities and this impact may persist for a long period, even for several years after the

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eradication of the pollution source (European Commission, 2008).

Besides contributing to the spread of pathogens, irrigation water may play a key role in the dissemination of antibiotic resistance (De Roever, 1999; LeJeune et al., 2001). The selective pressure of contaminants in aquatic systems can potentiate and accelerate the transfer of genetic resistance determinants between antibiotic-resistant bacteria (ARB) and indigenous bacteria (Lupo et al., 2012; Tacão et al., 2012). The transfer of ARB and antibiotic resistance genes (ARGs) from the environment to humans represents a great concern and may occur for example through the consumption of contaminated fresh produce.

A wide spectrum of microorganisms including bacteria, viruses and protozoa have been associated with foodborne-outbreaks (De Roever, 1999). Enterobacteriaceae members are the most common bacterial agents causing food poisoning outbreaks, associated with the consumption of fresh and minimally processed vegetables (Beuchat, 2002; Friesema et al., 2008; Hamilton-Miller and Shah, 2001; Hilborn et al., 1999; Söderström et al., 2008). *Escherichia coli* is a key organism in foodborne illnesses (National Center for Emerging and Zoonotic Infectious Diseases, 2011) and some strains have been implicated in international scale outbreaks (Beuchat, 1996). It is also a common indicator organism of fecal contamination in aquatic systems, and is recognized as an important player in the spread of antibiotic resistance (Henriques et al., 2006; Szmolka and Nagy, 2013). The plasticity of this species is mainly due to a high aptitude to acquire genetic information through horizontal gene transfer. Pathogenic *E. coli*, particularly those that causes foodborne illness by disrupting the normal function of the intestines, as diarrheagenic *E. coli* (DEC) strains, possess virulence traits allowing their attachment to the human gut (Kaper et al., 2004; Nataro and Kaper, 1998). Multiple outbreaks of DEC infections linked to consumption of leafy green vegetables have been reported (Edelstein et al., 2014; Friesema et al., 2008; Hilborn et al., 1999; Söderström et al., 2008).

In Portugal, legislation for monitoring irrigation water quality includes physical (e.g. pH and salinity), chemical (e.g. Cl, SO_4, Mn) and biological (e.g. fecal coliforms) parameters (Ministério do Ambiente, 1998). Parameters related to antibiotic resistance or strain virulence are not included. In addition, domestic production of fresh produce is rarely (if ever) monitored. In Portugal, about 40% of the total population lives in rural areas, where domestic agriculture is of crucial significance (Direção-Geral De Agricultura, 2003). In this study, we characterized *E. coli* from irrigation water and vegetables from household producers, in terms of their antibiotic resistance phenotypes and genotypes, virulence determinants and the presence and diversity of mobile genetic elements. Results were analyzed in order to evaluate if irrigation groundwater represents a route of contamination of fresh produce.

2. Methods

2.1. Study area, sampling and water quality assessment

Sampling sites were located in Estarreja, a city in the North of Portugal (Fig. S1). Samples were picked from 16 household farms from June to September 2014. From each farm we sampled fresh vegetables and irrigation water used to irrigate those vegetables, from either shallow or deep wells with location never exceeding 50 m away from the vegetables cultivation site. Vegetables collected in each farm varied from collard, cucumber, lettuce, tomato and spinach, depending on their availability at the time of sampling. All samples were collected in sterile containers, stored under refrigeration, and processed within 24 h. Vegetable samples were processed following safe handling procedures recommended for human consumption purposes (e.g. handled after hand washing, trimmed of spoiled parts and washed thoroughly under running water).

Water quality was assessed through physical, chemical and microbiological parameters recommended by the Portuguese law (Ministério do Ambiente, 1998), as well as through the determination of additional parameters (e.g. NO_4, NO_2, K, Mg, Si, Ag, total coliforms and fecal enterococci).

2.2. Escherichia coli isolation

Five grams of each vegetable were aseptically weighed and washed with 40 mL of phosphate-buffered saline (PBS), at low speed for 10 min in a laboratory platform rocker. The volumes of 1 and 30 mL of the washing solution were filtered through 0.45 μm nitrocellulose membrane filters (Pall Corporation, USA). Water volumes of 100 and 500 mL were filtered through 0.45 μm membrane filters (Pall Corporation, USA). The filters were placed on HiCrome *E. coli* agar B (HEA) (Sigma-Aldrich, USA) plates and incubated at 44 °C during 18 to 24 h. After colony counting, characteristic colonies colored blue were selected and purified on HEA and Chromocult Coliform Agar (Merck, Germany) and stored in 15% glycerol at − 80 °C.

2.3. Genomic fingerprinting by rep-PCR

Whole-cell suspensions were prepared in 20 μL of sterile deionized water and 1 μL of each suspension was used as DNA template for BOX-PCR fingerprinting analysis. The PCR reaction and conditions were as previously described (Araújo et al., 2014). In each PCR assay, one positive control strain was added. Band patterns were analyzed using GelCompar II version 6.1 (Applied Maths, Belgium). The similarity between profiles was calculated with the Pearson coefficient and cluster analysis was performed using the unweighted pair group method using arithmetic averages (UPGMA). Isolates displaying different BOX-PCR profiles were considered non-clonal and used for further characterization. On the other hand, isolates displaying similar BOX-PCR profiles were subjected to further analysis with ERIC- and REP-PCR fingerprinting (Araújo et al., 2014) to confirm clonality.

2.4. Determination of E. coli phylogenetic groups

The quadruplex PCR assay developed and revised by Clermont et al. with primers for genes *arpA*, *chuA*, *yjaA*, and for the DNA fragment TspE4.C2 was performed, to assign each isolate to one of the eight *E. coli* phylogroups previously recognized (Clermont et al., 2013). For each PCR reaction, 3 μL of cell suspensions (prepared in 20 μL of sterile deionized water) were used as template. All PCR reactions were carried out in a 25 μL volume containing NZY*Taq* 2× Green Master Mix (2.5 mM $MgCl_2$; 200 μM dNTPs; 0.2 U/μL DNA polymerase) (NZYtech, Portugal). Primers concentration and PCR conditions were as previously described (Clermont et al., 2013). Positive and negative controls were included in each assay.

2.5. Antibiotic susceptibility testing

Escherichia coli isolates were tested for susceptibility against 16 antibiotics by the disk diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). The following antibiotics were used: amoxicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), piperacillin (30 μg), piperacillin/tazobactam (30/6 μg), cefepime (30 μg), ceftazidime (10 μg), cefotaxime (5 μg), imipenem (10 μg), aztreonam (30 μg), gentamicin (10 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) (Oxoid, UK). *Escherichia coli* ATCC 25922 was used as quality control. Isolates were classified as sensitive or resistant according to the EUCAST recommendations after 18-24 h incubation at 37 °C. Growth rank between sensitive and resistant values was considered as intermediate resistance and for calculation purposes as non-susceptible. Clinical and Laboratory Standards Institute (CLSI) recommendations were used when antibiotic breakpoints in EUCAST