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Phylogenetic diversity, antibiotic resistance and virulence traits of *Aeromonas* spp. from untreated waters for human consumption

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ABSTRACT

It is well known that water constitutes an important contamination route for microorganisms. This is especially true for *Aeromonas* which are widespread in untreated and treated waters.
In this study, Portuguese untreated waters not regularly monitored were screened for the presence and diversity of aeromonads. A total of 206 isolates were discriminated by RAPD-PCR and 80 distinct strains were identified by *gyrB* based phylogenetic analysis. The most frequently detected species were *Aeromonas hydrophila*, *Aeromonas bestiarum* and *Aeromonas media*. The antibiotic susceptibility profile of these strains was determined and showed a typical profile of the genus. Nonetheless, the percentage of resistant strains to tetracycline, chloramphenicol and/or trimethoprim/sulfamethoxazole was lower than that reported for clinical isolates and isolates recovered from aquacultures and other environments historically subjected to antibiotic contamination. This suggests that the existence of such pressures in those environments selects for resistant *Aeromonas*. A similar trend for integron presence was found.
Genes coding for CphA and TEM, and *tet*(A), (E), (C) or (D) genes were found in 28%, 1%, and 10% of the strains, respectively. 10% of the strains contained an integron. Variable regions of seven class 1 integrons and one class 2 integron were characterised. Furthermore, strains displayed virulence related phenotypes such as extracellular lipolytic and proteolytic activities as well as aerolysin related genes (43% of strains). The *ascV* and *aexT* genes were found in 16% and 3% of strains respectively and, in some cases, concomitantly in the same specimen.
This study shows that diverse *Aeromonas* spp. presenting distinct antibiotic resistance features and putative virulence traits are frequently present in waters for human and animal consumption in Portugal. Genes associated to antibiotic resistance and microbial virulence previously identified in organisms with human health significance were detected in these aeromonads, suggesting that these waters may act as a pivotal route for infections.

1. Introduction

Clean and safe freshwater is vital to the survival of all living organisms, as it is to the smooth functioning of ecosystems, economies and communities (Un-Water, 2011). Concerns about water quality are huge and increasing attention has been given to this problem over the last years. In fact, it has been estimated that every year 1.8 million people die from diarrhoeal diseases attributed to unsafe water, from which 1.2 million are children under the age of 5 (WHO, 2004). Bacteria are among the organisms with higher contribution to these hazardous effects. For example, *Aeromonas* spp. have been frequently implicated in diarrhoeal episodes (Ahmed et al., 2012; Janda and Abbott, 2010).

Widespread in various habitats, *Aeromonas* are mainly present in aquatic environments (Di Bari et al., 2007; Figueira et al., 2011) but also in soil, food, and animals (Janda and Abbott, 2010). *Aeromonas* spp. are able to cause diseases in different animals, including humans, in which they are responsible for intestinal and extra intestinal infections such as gastroenteritis, bacteremia, skin and soft tissue infections and sepsis with lethal course (Khajanchi et al., 2010). Infection often occurs after contact or consumption of contaminated waters or food (Janda and Abbott, 2010), enhancing the potential health hazard that the presence of such microorganisms in water may represent (Figueira et al., 2011; Pablos et al., 2010). For these reasons, some countries have set standards for *Aeromonas* spp. in drinking waters (Pablos et al., 2010).

In fact, these organisms express an assortment of virulence factors which enable them to colonise, invade, establish in and infect different hosts (Galindo et al., 2006). Numerous extracellular proteins secreted by aeromonads were described, including hydrolytic enzymes

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such as lipases and proteases which help in invasiveness and in the establishment of the infection. Also, enterotoxic toxins and haemolysins such as aerolysin, Act and related toxins displaying cytotoxic, enterotoxic and haemolytic activities were mainly linked to diarrhoeal outcomes (Galindo et al., 2006). Type III secretion systems (T3SS), involved in the injection of effector toxins directly into the cytosol of host cells have been characterised in different *Aeromonas* spp. (Reith et al., 2008; Sha et al., 2007; Vilches et al., 2008). In some cases, genes encoding different effector toxins were concomitantly found in the same strain (Reith et al., 2008; Silver and Graf, 2009). Furthermore, other antibacterial compounds endowed with important ecological roles such as bacteriocins and bacteriocin-like substances have been reported. These compounds have been linked to the favouring of the microorganism in the competition for the colonisation of environmental, food and human microbial habitats and can, therefore, be considered a further component of virulence (Messi et al., 2003).

Resistance to diverse groups of antibiotics is another concerning characteristic of *Aeromonas* species (Figueira et al., 2011; Janda and Abbott, 2010). High resistance rates against penicillins and first generation cephalosporins have been described and are associated with the production of chromosomally encoded beta-lactamases (Janda and Abbott, 2010). Also, the *cphA*/*imiS* metallo-beta-lactamase and *tet* genes, have been primarily implicated in the resistance to carbapenems (Wu et al., 2012) and tetracyclines (Agersø et al., 2007). On the other hand, other clinically relevant *bla* genes usually encoded in mobile genetic elements although not commonly detected in *Aeromonas*, represent important resistance determinants (Wu et al., 2011). These were occasionally found among *Aeromonas* clinical strains (Pérez-Valdespino et al., 2009) and also in non-clinical isolates recovered from aquatic environments (Henriques et al., 2006a), animals (Barlow et al., 2008) and aquacultures (Jacobs and Chenia, 2007). Additionally, *Aeromonas* species have been shown to contain integrons, which can be responsible for the resistance to tetracyclines, aminoglycosides, chloramphenicol and trimethoprim (Chang et al., 2007; Kadlec et al., 2011; Moura et al., 2007).

Most studies regarding the screening of antibiotic resistance genes and putative virulence factors were restricted to *A. hydrophila*, *Aeromonas veronii*/*Aeromonas sobria* and *Aeromonas caviae* (Castilho et al., 2009; Khajanchi et al., 2010; Pablos et al., 2010; Wu et al., 2007), since these are the most commonly identified species in clinical samples (Ottaviani et al., 2011; Pablos et al., 2010). Hence, the real incidence of virulence determinants/antibiotic resistance in environmental isolates is still poorly described. Therefore the implications of human/animal contact with non-monitored waters are unknown. Taking into account the panoply of infections in which aeromonads are implicated, it is mandatory to conduct the surveillance of water of public use and to explore aeromonads' risk factors.

In Portugal, untreated and unmonitored waters are frequently used for direct human consumption as well as for agriculture and recreational purposes. In the present study, *Aeromonas* occurrence and diversity in untreated waters used for consumption and for agricultural and recreational activities in several regions of Portugal were investigated. Antibiotic resistance and virulence related traits of isolated strains were characterised in order to evaluate the potential of aeromonads in these waters as a public health risk.

2. Material and methods

2.1. Sampling and isolate recovering

Between June 2004 and January 2007, 96 untreated water samples were collected in Portugal, from either public or private sources, comprising a total of 86 sampling sites: fountains, wells, drilled wells and mines. These waters were used for drinking and other human activities as bathing, leisure or agriculture. Samples were collected in sterile 1 l glass bottles, transported at 4 °C to the laboratory and analysed within 5 h. Aeromonads were isolated by the membrane filter technique. Briefly, 100 ml of each sample was filtered through 0.45 μm pore size cellulose ester filters (Pall Life Sciences, USA). Membranes were incubated in glutamate starch phenol-red agar (GSP) (Merck, Germany) supplemented with 50 μg/ml of ampicillin (Merck, Germany) at 30 °C for 16 h–18 h. Yellow colonies were selected, purified and stored in 17% glycerol at −80 °C.

2.2. Genotyping, identification and phylogenetic analysis

DNA was extracted according to Fontes et al. (2011). For genotyping, random amplification of polymorphic DNA (RAPD) PCR was performed using primer OPA 16 (Lockhart et al., 1997). The reaction mixtures (25 μl) contained 1× PCR buffer (buffer with $MgCl_2$), 400 μM dNTPs, 4 μM of primer OPA16, 1 U of Taq ultratools DNA polymerase (Biotools B&M Labs, S.A., Spain) and 100–200 ng of genomic DNA. PCR amplifications were carried out in a PTC-100™ Peltier Thermal Cycler (MJ Research, INC, USA) as follows: initial denaturation (94 °C for 4 min), 40 cycles of denaturation (94 °C for 1 min), annealing (36 °C for 1 min) and extension (72 °C for 1 min) and a final extension (72 °C for 5 min). Amplicons were separated by electrophoresis in a 1.3% agarose gel-TAE buffer. Images were acquired with the Gel DocMega camera system 5.01 (Biosystematica, UK). Genetic profiles were visually analysed by intra-gel pattern comparison and isolates representative of each RAPD pattern were selected for subsequent analyses.

PCR amplification and sequencing of the *gyrB* gene were performed as described earlier (Fontes et al., 2011).

2.3. Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by the Kirby–Bauer disc diffusion method (CLSI, 2006, 2012). *Escherichia coli* ATCC25922 was used as a control strain. The following antimicrobials (Oxoid, UK) were tested: amoxicillin (AMX; 10 μg), amoxicillin/clavulanic acid (AMC; 30 μg), ticarcillin (TIC; 75 μg), ticarcillin/clavulanic acid (TIM; 85 μg), cefalotin (CEF; 30 μg), ceftazidime (CAZ; 30 μg), cefepime (FEP; 30 μg), aztreonam (ATM; 30 μg), imipenem (IPM; 10 μg), gentamicin (GEN; 10 μg), kanamycin (KAN; 30 μg), tobramycin (TOB; 10 μg), netilmicin (NET; 30 μg), tetracycline (TET; 30 μg), chloramphenicol (CHL; 30 μg), erythromycin (ERY; 15 μg), trimethoprim/sulfamethoxazole (SxT; 25 μg) and ciprofloxacin (CIP; 5 μg). Streptomycin (STR; 10 μg) was tested in strains containing aminoglycoside resistance genes encoded in class 1 integrons.

2.4. Screening of antibiotic resistance genes and integrons

The presence of β-lactamase genes bla_{TEM}, bla_{SHV}, bla_{OXA}, *cphA*/*imiS*, bla_{IMP} and bla_{VIM} and tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E) was analysed in all strains using primers and PCR conditions as described by Henriques et al. (2006a) and Nawaz et al. (2006), with slight modifications: on the contrary to the work of Nawaz et al. (2006), *tet* genes were independently amplified. Amplicons were confirmed by Southern blot hybridisation (Henriques et al., 2006b).

Strains were also screened for the presence of *intI1*, *intI2* and *intI3* by dot-blot hybridisation as described by Moura et al. (2007).

2.5. Integron characterisation

The genomic DNA of *intI1* positive isolates was submitted to PCR with primer sets targeting integrons' variable regions as described previously (Moura et al., 2007). The variable region of the class 2 integron was amplified using the Extensor HI-Fidelity PCR Master Mix (ABgene, United Kingdom) as follows: initial denaturation (94 °C for 5 min), 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (68 °C for 3 min) and a final extension (68 °C for 10 min). PCR products were analysed by electrophoresis. The band