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Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia

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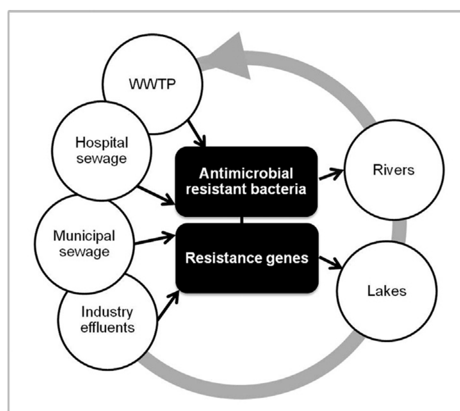
HIGHLIGHTS

- *E. faecium* was the most prevalent species, followed by *E. faecalis*.
- HLR-G enterococci were recovered in wastewater (19%) and surface-water (6%) samples.
- Diverse genetic lineages of HLR-G enterococci were detected in water in Tunisia.
- *esp* gene, putative marker of clinical enterococci, was detected in three isolates.
- Wastewater and surface-water could contribute to the spreading of HLR-G enterococci.

GRAPHICAL ABSTRACT



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ABSTRACT

One hundred-fourteen samples of wastewater (n = 64) and surface-water (n = 50) were inoculated in Slanetz–Bartley agar plates supplemented or not with gentamicin (SB-Gen and SB plates, respectively) for enterococci recovery. Enterococci were obtained from 75% of tested samples in SB media (72% in wastewater; 78% in surface-water), and 85 enterococcal isolates (one/positive-sample) were obtained. *Enterococcus faecium* was the most prevalent species (63.5%), followed by *Enterococcus faecalis* (20%), *Enterococcus hirae* (9.4%), *Enterococcus casseliflavus* (4.7%), and *Enterococcus gallinarum*/*Enterococcus durans* (2.4%). Antibiotic resistance detected among these enterococci was as follows [percentage/detected gene (number isolates)]: kanamycin [29%/*aph*(3′)-IIIa (n = 22)], streptomycin [8%/*ant*(6)-Ia (n = 4)], erythromycin [44%/*erm*(B) (n = 34)], tetracycline [18%/*tet*(M) (n = 6)/*tet*(M)-*tet*(L) (n = 9)], chloramphenicol [2%/*cat*(A) (n = 1)], ciprofloxacin [7%] and trimethoprim–sulfamethoxazole [94%]. High-level-gentamicin resistant (HLR-G) enterococci were recovered from 15 samples in SB-Gen or SB plates [12/64 samples of wastewater (19%) and 3/50 samples of surface-water (6%)]; HLR-G isolates were identified as *E. faecium* (n = 7), *E. faecalis* (n = 6), and *E. casseliflavus* (n = 2). These HLR-G enterococci carried the *aac*(6′)-Ie–*aph*(2″)-Ia and

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erm(B) genes, in addition to *aph*(3′)-IIIa (n = 10), *ant*(6)-Ia (n = 9), *tet*(M) (n = 13), *tet*(L) (n = 8) and *cat*(A) genes (n = 2). Three HLR-G enterococci carried the *esp* virulence gene. Sequence-types detected among HLR-G enterococci were as follows: *E. faecalis* (ST480, ST314, ST202, ST55, and the new ones ST531 and ST532) and *E. faecium* (ST327, ST12, ST296, and the new ones ST985 and ST986). Thirty-two different PFGE patterns were detected among 36 high-level-aminoglycoside-resistant enterococci recovered in water samples. Diverse genetic lineages of HLR-G enterococci were detected in wastewater and surface-water in Tunisia. Water can represent an important source for the dissemination of these antibiotic resistant microorganisms to other environments.
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1. Introduction

Enterococci are ubiquitous bacteria widely distributed in the environment, being also part of the normal intestinal microbiota of healthy humans and animals. For this reason, enterococci are good indicators of fecal pollution in water (Boehm and Sassoubre, 2014). Moreover, this genus is characterized by intrinsic resistance to many antibiotics and by its ability to acquire new resistance mechanisms. Intrinsic resistance affects to beta lactams (including a broad range of cephalosporin antibiotics), aminoglycosides (low level), clindamycin, trimethoprim/sulfamethoxazole or even vancomycin (in the case of the species *Enterococcus gallinarum* and *Enterococcus casseliflavus*, which contain the *vanC1*/*vanC2* genes) (Hollenbeck and Rice, 2012). Standard recommended therapy for systemic enterococcal infections consists in a combination of either penicillin or vancomycin with an aminoglycoside (gentamicin or streptomycin). The goal of this combination therapy is to achieve a synergistic bactericidal effect between the cell wall agent and the aminoglycoside (Gavaldà et al., 1999). However, resistance of these agents is being increasingly reported (Arias et al., 2010).

Vancomycin resistance can be encoded by acquired resistance genes such as *vanA* and *vanB2* (Hollenbeck and Rice, 2012). The acquisition of the genes (*aac*(6′)-Ie–*aph*(2″)-Ia, *aph*(3′)-IIIa, and *ant*(6)-Ia), which encode aminoglycoside modifying enzymes (AMEs), confers high level resistance to different aminoglycosides. In this sense, the bifunctional enzyme AAC(6′)–APH(2″) confers high level resistance to gentamicin (HLR-G) and to other aminoglycosides, except to streptomycin (del Campo et al., 2000). These genes are located on mobile genetic elements (plasmids or conjugative transposons) (Murray., 1992; Simjee and Gill., 1997), which are widespread among enterococci.

High-level aminoglycoside resistant (HLR-Ag) enterococci and vancomycin resistant enterococci (VRE) are important causes of human infections. In recent years, the HLR-Ag phenotype continues to increase in hospital settings eliminating the advantages of the combination therapy (Arias et al., 2010). Moreover, these resistant microorganisms have been found in non-clinical samples of different origins such as pets, wild animals, food or environmental samples (Cattoir and Leclerq, 2010; Torres et al., 2003). It has been previously suggested that water can contribute to the spread of resistant enterococci (Tejedor Junco et al., 2001). Thus, antibiotic resistant enterococci have been detected in the effluents of the wastewater treatment plants (WWTPs), in the effluents of hospitals and in raw municipal inflow (Varela et al., 2013), in surface water (Tejedor Junco et al., 2001), in coastal water (Zdragas et al., 2008), in treated wastewater used for irrigation (Ben Said et al., submitted; Beccera-Castro et al., 2015), or in wastewater treatment plant that provide effluent for reuse (Rosenberg Goldstein et al., 2014). Interestingly, one method for rapid and sensitive detection of *Enterococcus* sp. and mainly of *Enterococcus faecalis* and *Enterococcus faecium* isolates (the most predominant fecal enterococci) in potable water samples has been described (Maheux et al., 2011).

The aim of our study was to determine the rate and species of enterococci in wastewater and surface water in Tunisia, as well as to study the phenotypes and genotypes of antibiotic resistance in recovered isolates. In addition, to analyze the frequency of high-level-gentamicin resistant (HLR-G) enterococci and their molecular characteristics, in order to evaluate the role of wastewater and surface water as source of dissemination of these antibiotic resistant bacteria.

2. Material and methods

2.1. Sample collection

One-hundred-and-fourteen water samples, corresponding to wastewater (WW, n = 64), and surface-water (SW, n = 50), were collected and analyzed in this study. Wastewater samples were obtained from: a) wastewater treatment plants (WWTPs) (16 samples, from 8 WWTPs at inflow and outflow points); b) stagnant water (17 samples from 13 cities), c) municipal sewage (MS) (15 samples, from septic tanks of 5 cities of the north and east of the country); d) industry effluents (11 samples, obtained in 11 industries; and e) hospital sewage (HS) (5 samples, of effluents of 5 hospitals of Tunis). The 50 samples of surface-water were obtained from: a) rivers (35 samples, from 25 rivers); b) lakes (9 samples, from 9 lakes); and c) saline lakes (6 samples, from 6 saline lakes). All samples were obtained in the North and East of Tunisia during September 2011–July 2012. They were collected in sterile bottles and transported to the laboratory at 4 °C, and they were kept refrigerated and analyzed within 6 h.

2.2. Isolation and identification of bacterial strains

Water samples were processed according to the following protocol: 1 ml of water was added to 5 ml of Brain Heart Infusion (BHI) Broth (1:5), being incubated at 37 °C for 24 h. After that, several dilutions of the enrichment broth were seeded on Slanetz–Bartley agar (Scharlau, Spain) plates, either supplemented (SB-Gen) or not (SB) with gentamicin (120 $\mu g \cdot ml^{-1}$), that were incubated 48 h at 37 °C. One colony per plate, with typical enterococcal morphology, was picked from each plate and streaked onto BHI agar plates. The isolates were initially characterized as enterococci based on biochemical tests, including Gram staining, catalase reaction, hydrolysis of esculin in the presence of bile, and capacity to grow in hypersaline medium. The species identification was confirmed by polymerase chain reaction (PCR), using primers and conditions for different enterococcal species (Torres et al., 2003).

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility patterns to antibiotics, including vancomycin (30 μg), teicoplanin (30 μg), ampicillin (10 μg), trimethoprim–sulfamethoxazole (1.25 μg + 23.75 μg), chloramphenicol (30 μg), tetracycline (30 μg), erythromycin (15 μg), and ciprofloxacin (5 μg) were investigated using the disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2013). Detection of high-level aminoglycoside resistance was performed with high charge disks of gentamicin (500 μg), streptomycin (500 μg) and kanamycin (1000 μg) according to Antibiogram Committee of the French Society for Microbiology (CA-SFM, 2010). *E. faecalis* strain ATCC 29212 was used for quality control.

2.4. Detection of antibiotic resistance and virulence genes by PCR

The resistance genes for macrolides [*erm*(A), *erm*(B), *erm*(C)], tetracycline [*tet*(M), *tet*(L)], aminoglycosides [*aph*(3)-IIIa, *aac*(6′)-Ie–*aph*(2″)-Ia, *ant*(6)-Ia], and chloramphenicol [*cat*(A)] were tested by PCR in all the enterococcal isolates that showed resistance or

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