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Characterization of staphylococci in urban wastewater treatment plants in Spain, with detection of methicillin resistant *Staphylococcus aureus* ST398☆

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ABSTRACT

The objective of this study was to determine the prevalence of *Staphylococcus* in urban wastewater treatment plants (UWTP) of La Rioja (Spain), and to characterize de obtained isolates. 16 wastewater samples (8 influent, 8 effluent) of six UWTPs were seeded on mannitol-salt-agar and oxacillin-resistance-screening-agar-base for staphylococci and methicillin-resistant *Staphylococcus aureus* recovery. Antimicrobial susceptibility profile was determined for 16 antibiotics and the presence of 35 antimicrobial resistance genes and 14 virulence genes by PCR. *S. aureus* was typed by *spa*, *agr*, and multilocus-sequence-typing, and the presence of immune-evasion-genes cluster was analyzed. *Staphylococcus* spp. were detected in 13 of 16 tested wastewater samples (81%), although the number of CFU/mL decreased after treatment. 40 staphylococci were recovered (1–5/sample), and 8 of them were identified as *S. aureus* being typed as (number of strains): *spa*-t011/*agr*-II/ST398 (1), *spa*-t002/*agr*-II/ST5 (2), *spa*-t3262/*agr*-II/ST5 (1), *spa*-t605/*agr*-II/ST126 (3), and *spa*-t878/*agr*-III/ST2849 (1). *S. aureus* ST398 strain was methicillin-resistant and showed a multidrug resistance phenotype. Virulence genes *tst*, *etd*, *sea*, *sec*, *seg*, *sei*, *sem*, *sen*, *seo*, and *seu,* were detected among *S. aureus* and only ST5 strains showed genes of immune evasion cluster. Thirty-two coagulase-negative *Staphylococcus* of 12 different species were recovered (number of strains): *Staphylococcus equorum* (7), *Staphylococcus vitulinus* (4), *Staphylococcus lentus* (4), *Staphylococcus sciuri* (4), *Staphylococcus fleurettii* (2), *Staphylococcus haemolyticus* (2), *Staphylococcus hominis* (2), *Staphylococcus saprophyticus* (2), *Staphylococcus succinus* (2), *Staphylococcus capitis* (1), *Staphylococcus cohnii* (1), and *Staphylococcus epidermidis* (1). Five presented a multidrug resistance phenotype. The following resistance and virulence genes were found: *mecA*, *lnu*(A), *vga*(A), *tet*(K), *erm*(C), *msr*(A)/(B), *mph*(C), *tst*, and *sem*. We found that *Staphylococcus* spp. are normal contaminants of urban wastewater, including different lineages of *S. aureus* and a high diversity of coagulase-negative species. The presence of multiple resistance and virulence genes, including *mecA*, in staphylococci of wastewater can be a concern for the public health.

1. Introduction

The extended use of antimicrobials for prophylaxis or treatment of human or animal infections, as well as the use in the past of these agents as animal growth promoters (now banned in EU), or the use in agriculture, have caused the spread of resistant bacteria or their resistance genes in different ecosystems, including the environment. The widespread use of these agents may act as selective pressure on natural bacteria population becoming an important determinant in resistance maintenance, development and dissemination. Moreover, some antimicrobials are very stable which might become in a long-term persistence of these active compounds (McArdell et al., 2003; Miao et al., 2004). On the other hand, some authors have proposed that the origin of virulence determinants could probably reside in the environmental microbiota (Martínez, 2013; Søborg et al., 2013).

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Conventional Urban Wastewater Treatment Plants (UWTP) could not completely remove antimicrobials or microorganisms. Previous studies have indicated that UWTP might be a vehicle for the dissemination of antimicrobials and antimicrobial resistant bacteria in the environment, mainly by effluent water or sludge (Kim and Aga, 2007; Rizzo et al., 2013). Additionally, water and sludge are reused in different ways, and the spread of resistant and virulent bacteria are of particular concern. The evaluation of an efficient treatment is important, and it is generally based on monitoring conventional water quality parameters that include heterotrophic bacterial counts and the abundance of coliforms.

Staphylococcus spp. are ubiquitous bacteria reported as normal microbiota of the mucous membranes and of the skin of mammals and birds; however, it may behave as opportunistic pathogens that can cause minor and severe infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important concerns in public health and it constitutes a serious threat. Although *S. aureus* is the most relevant species in the genus, coagulase-negative *Staphylococcus* (CoNS) is gaining interest due to its increased detection as responsible agents of infections (Becker et al., 2014).

Most studies on characterization of resistant bacteria in UWTPs are focused on fecal pollution indicator bacteria. Nevertheless, some studies have reported the presence of *S. aureus* (Porrero et al., 2014), MRSA (Börjesson et al., 2010; Kumar et al., 2015; Rosenberg Goldstein et al., 2012), or CoNS (Faria et al., 2009; Heβ and Gallert, 2014) isolates in UWTP or have reported the presence of antimicrobial resistance genes (Börjesson et al., 2009; Colomer-Lluch et al., 2014). In all of them, the presence of *S. aureus* after treatment seems to be low; however, MRSA is considered an emerging contaminant in water environments, and the fact that the isolates from wastewater can be more virulent and multidrug resistant has been speculated (Börjesson et al., 2010).

The aim of this study was to determine the prevalence of *Staphylococcus* in UWTPs of La Rioja (Spain), before and after treatment, as well as to perform the molecular characterization of the obtained isolates.

2. Material and methods

2.1. Sample collection

Sixteen water samples were collected during December-2012 to February-2013 from six different UWTPs (8 at influent points and 8 at effluent points) in La Rioja region (Logroño 2/2; Cornago 2/2, Aldeanueva de Ebro 1/1, Alfaro 1/1, Rincón de Soto 1/1, and Torrecilla en Cameros 1/1). Sample collection was performed in two different shots, but influent and effluent samples of each UWTP were recovered in the same day. In two of the UWTPs (Logroño and Cornago), samples were collected from both shots. Wastewater samples were obtained in sterile glass bottles (500 mL), and directly transported under refrigeration conditions to the microbiology laboratory for analysis.

2.2. Staphylococci colony count, isolation and identification

Different aliquots of wastewater samples were seeded in mannitol-salt-agar (MSA, BD, France) plates and oxacillin-resistance-screening-agar-base (ORSAB, Oxoid, England) plates supplemented with oxacillin (2 mg/L), for recovery of staphylococci and methicillin-resistant staphylococci (MRS), respectively. Plates were incubated at 37 °C for 48 h. *Staphylococcus* isolates that were grown in MSA plates were counted (CFU/mL).

In parallel, an aliquot of 100 μL of wastewater samples were pre-enriched by inoculation into brain heart infusion (BHI) broth (BD, France) with 6.5% NaCl and incubated at 37 °C for 24–48 h. After that, aliquots were inoculated in MSA and ORSAB media.

Up to six colonies per wastewater sample with staphylococci morphology were recovered, and initially identified by microbiological conventional methods (Gram staining, coagulase and DNase test). Identification was carried out by amplification of the species-specific *nuc* gene for *S. aureus* (Sasaki et al., 2010), and by amplification and sequencing of the *sodA* gene for CoNS (Poyart et al., 2001). Only strains showing different phenotypes of antimicrobial resistance of each species and of each sample were further characterized.

2.3. Antimicrobial susceptibility testing and detection of antimicrobial resistance genes

Susceptibility to penicillin, oxacillin, cefoxitin, kanamycin, gentamicin, tobramycin, streptomycin, tetracycline, chloramphenicol, erythromycin, clindamycin, ciprofloxacin, linezolid, trimethoprim/sulfamethoxazole, mupirocin, and fusidic acid, was performed by disk-diffusion method (CASFM, 2010; EUCAST, 2014). Cefoxitin resistance was used as phenotypic marker of methicillin resistance.

Detection of 35 antimicrobial resistance genes [*mecA*, *mecC*, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *tet*(O), *aac*(6′)-*aph*(2″), *fusB*, *fusC*, *erm*(A), *erm*(B), *erm*(C), *erm*(F), *msr*(A), *msr*(B), *mph*(C), *aph*(3′)-IIIa, *ant*(4′)-Ia, *lnu*(A), *lnu*(B), *lnu*(D), *lnu*(C), *vga*(A), *cfr*, *ant*(6)-Ia, *ant*(3″)(9), *str*, *dfr*(A), *dfr*(D), *dfr*(K), *dfr*(G), cat_{pc194}, cat_{pc221}, and cat_{pc223}] was performed by PCR (Benito et al., 2014; García-Álvarez et al., 2011; Gharsa et al., 2012; Lozano et al., 2012).

2.4. Virulence genotype and detection of immune evasion cluster genes

The presence of the genes encoding enterotoxins (*sea*, *seb*, *seg*, *sei*, *sem*, *sen*, *sec*, *seu*, and *seo*), exfoliative toxins (*eta, etb*, and *etd*), the Panton Valentine Leukocidin (PVL, *luk*F/S), and the toxic-shock syndrome toxin (*tst*) was studied by PCR (Benito et al., 2014; Gharsa et al., 2012).

Furthermore, the detection of genes of the immune evasion cluster system (IEC) [staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein (*chp*), staphylokinase (*sak*), enterotoxin A (*sea*) or enterotoxin P (*sep*)] which enables the classification into different IEC types (A–G), was performed in *S. aureus* isolates (van Wamel et al., 2006).

2.5. Molecular typing of S. aureus isolates

All *S. aureus* isolates obtained were characterized by *spa*-typing (www.ridom.com) and *agr* typing, as previously described (Shopsin et al., 2003). One isolate of each *spa*-type was selected for molecular characterization by multilocus sequence typing (MLST) (www.mlst.net).

3. Results

3.1. Staphylococcus recovered from wastewater

Staphylococcal isolates were detected in 13 of the 16 tested wastewater samples (81%), corresponding to 6 influent and 7 effluent samples. Colony counts performed of samples directly seeded in MSA plates revealed an average of staphylococci in influent samples of 6.8×10^2 CFU/mL versus 8×10^1 CFU/mL in effluent samples.

Forty *Staphylococcus* spp. isolates, 21 of them in influent samples [3 *S. aureus* y 18 CoNS] and 19 in effluent samples [5 *S. aureus* y 14