**Staphylococcus aureus** isolated from wastewater treatment plants in Tunisia: occurrence of human and animal associated lineages

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**ABSTRACT**

The objective was to characterize *Staphylococcus aureus* isolated from two wastewater treatment plants (WWTPs) located in Tunis City (Tunisia), during the period 2014–2015. Genetic lineages, antibiotic resistance mechanisms and virulence factors were determined for the recovered isolates. *S. aureus* isolates were recovered from 12 of the 62 wastewater samples tested (19.35%), and one isolate/sample was characterized, all of them being methicillin-susceptible (MSSA). Six spa types (t587, t674, t224, t127, t701 and t1534) were found among the 12 isolates, and the *spa*-t587, associated with the new sequence type ST3245, was the most predominant one (7 isolates). The remaining isolates were assigned to five clonal complexes (CC5, CC97, CC1, CC6 and CC522) according to the sequence-type determined and/or the *spa*-type detected. *S. aureus* isolates were ascribed to *agr* I (*n* = 3), *agr* II (*n* = 7) and *agr* III (*n* = 1); however, one isolate was non-typeable. *S. aureus* showed resistance to (number of isolates): penicillin (12), erythromycin (7), tetracycline (one) and clindamycin (one). Among the virulence factors investigated, only one isolate harboured the *tst* gene, encoding the TSST-1 (toxic shock syndrome toxin 1). Despite the low number of studied isolates, the present study reports the occurrence of both human- and animal-associated *S. aureus* clonal complexes in WWTPs in Tunisia.

**Key words** | animal and human lineages, *S. aureus*, wastewater

**INTRODUCTION**

The frequent use of antibiotics in hospitals, veterinary and agricultural settings has certainly contributed to the dissemination of resistant bacteria or their resistance genes in the environment. Furthermore, a large part of the antibiotics consumed are not absorbed and can end up in wastewater. It has been suggested that wastewater treatment plants (WWTPs) may play a role in the spread and the development of antibiotic-resistant bacteria (Börjesson et al. 2010; Rizzo et al. 2013). In addition, treated sewage water is reused in various ways, such as for agriculture, aquaculture and industrial purposes. During these activities, individuals may come into contact with reused wastewater and can be potentially exposed to resistant and virulent bacteria that may remain in the treated wastewater. *Staphylococcus aureus* is commonly found in the skin and the nails of humans and various animal species; however, it may also act as an opportunistic pathogen that causes minor to severe infections due to the production of different virulence factors. Nowadays, methicillin-resistant *S. aureus* (MRSA) is one of the most important threats to human and animal health worldwide (Cuny et al. 2015).
occurrence of *S. aureus* and MRSA in non-clinical environments such as wastewater has rarely been studied since the majority of wastewater studies are focused on microbial indicators of faecal contamination (George et al. 2002; Tran et al. 2015). Nonetheless, some studies have reported a low prevalence of *S. aureus* in hospital and municipal wastewater (Schwartz et al. 2003; Shannon et al. 2007). MRSA has been previously detected from wastewater (Börjesson et al. 2010; Kumar et al. 2015; Gómez et al. 2016).

The main aim of this study was to assess the occurrence of *S. aureus* in two WWTPs located in the suburbs of Tunis City (Tunisia), and to characterize the recovered isolates by determining their antibiotic susceptibility, the genes encoding antibiotic resistance and some virulence factors, and finally their clonal lineages.

**MATERIALS AND METHODS**

**Sampling and microbiological identification**

Sixty-two water samples were collected during the period of December 2014 to February 2015 from two WWTPs located in the north-east suburbs of Tunis City (Tunisia) (La Charguia: 59 samples and El Menzah: 3 samples). The two WWTPs received urban municipal wastewater from distinct areas in Tunis City. In the case of La Charguia WWTP, the sample collection was done at different points during the wastewater treatment process (at the entrance as influent, after the primary treatment, after the secondary treatment achieved by activated sludge, after the returned sludge, and lastly at the exit of the plant as effluent). In the case of El Menzah WWTP, only influent water was collected. Wastewater samples were recovered in a 500 mL sterile bottle, and transported under refrigeration condition of +4 °C to the laboratory for analysis.

Petri dishes containing Mannitol Salt Agar (MSA, Becton-Dickinson) were seeded by 1 mL of different wastewater samples, and incubated for 18–24 hours. One colony from each positive wastewater sample that showed typical *S. aureus* morphology was picked up for further study. Further identification of *S. aureus* was based mainly on colony morphology, Gram staining, the ability to coagulate rabbit plasma (BioMerieux, France) and the DNase activity. Identification of *S. aureus* was confirmed by a duplex polymerase chain reaction (PCR) that amplifies both the *nuc* gene and the methicillin-resistance genetic determinant *mecA* (CRL-AR 2009).

**Antimicrobial susceptibility testing**

Susceptibility to eight antimicrobial agents was performed using the disc-diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI 2015). Antimicrobial agents tested were (charge in µg/disc): penicillin (10 units), cefoxitin (30), tetracycline (30), gentamicin (10), tobramycin (10), ciprofloxacin (5), erythromycin (15) and clindamycin (2).

**Detection of antimicrobial resistance genes**

The presence of genes that confer resistance to penicillin (*blaZ*), tetracycline [*tet*(K), *tet*(L) and *tet*(M)], clindamycin [*lmu*(A), *lmu*(B), *vga*(A) and *vga*(C)] and erythromycin [*msr*(A)] was analysed by PCR (Gómez-Sanz et al. 2010; Lozano et al. 2012). Positive and negative controls from the collection of the University of La Rioja (Logroño, Spain) were used in all PCRs.

**Molecular typing methods of *S. aureus* isolates**

All *S. aureus* isolates obtained were characterized by *spa* typing (Harmsen et al. 2003), and *agr* typing as previously described (Shopsin et al. 2003). Multi-locus sequence typing was performed in selected *S. aureus* isolates (Enright et al. 2000). The clonal complex (CC) of the isolates was ascribed according to the sequence-type (ST) determined and/or *spa*-type detected.

**Virulence factors**

All isolates were tested by PCR for the presence of *tst* genes encoding the TSST-1 (toxic shock syndrome toxin), *eta* and *etb* genes encoding exfoliative toxins ETA and ETB, respectively (Jarraud et al. 2002) and *lukF/lukS-PV* genes encoding Panton–Valentine leucocidin (Lina et al. 1999).

**RESULTS AND DISCUSSION**

Very few data concerning the presence and the genetic lineages of *S. aureus* in wastewater are available in Tunisia.
Therefore, this study was undertaken to expand the knowledge about \textit{S. aureus} that might circulate among WWTPs. In this study, \textit{S. aureus} was detected in 12 of the 62 wastewater samples collected (19.35%), and one isolate/sample was characterized: 11 isolates from La Charguia WWTP and one from El Menzah WWTP (Table 1). The percentage of samples carrying \textit{S. aureus} detected in wastewater in this study is different from previous studies conducted in the United States and in Spain, where high rates (50–55%) of \textit{S. aureus} occurrence in wastewater were observed (Rosenberg Goldstein et al. 2012; Gómez et al. 2016). MRSA was not detected in this study, in dissimilarity with some European studies where MRSA has often been reported in wastewater (Börjesson et al. 2010; Porro et al. 2014). This finding might be related to the low rate or the absence of MRSA from healthy humans or animals in Tunisia (Ben Slama et al. 2011; Gharsa et al. 2015). Regarding antimicrobial resistance, all the isolates showed resistance to penicillin, which was encoded by the \textit{blaZ} gene in seven isolates. Indeed, penicillin resistance is widely disseminated among \textit{S. aureus} isolates worldwide (Lowy 2005). The \textit{msr}(A) gene was detected in the seven erythromycin-resistant isolates (Table 1). The \textit{msr}(A) gene codes for an ABC transporter protein that confers co-resistance to macrolides and streptogramin B antibiotics, and it has been reported from animal and human \textit{S. aureus} isolates (Schmitz et al. 2000; Wendlandt et al. 2013, 2015). The \textit{lnu}(A), \textit{lnu}(B), \textit{vga} (A) and \textit{vga}(C) genes were not detected in the clindamycin-resistant isolate. Only one isolate was resistant to tetracycline and harboured the \textit{tet}(K) gene. In fact, \textit{S. aureus} resistance to tetracycline was more common in MRSA than in the MSSA isolates (Sharma et al. 2013). In general, tetracycline resistance in \textit{S. aureus} is often encoded by the \textit{tet}(M), \textit{tet}(L) and \textit{tet}(K) genes (Wendlandt et al. 2013, 2015). Only one isolate harboured the \textit{tst} gene, and other virulence genes were not detected. Similarly, low rates of TSST-1 were earlier observed in \textit{S. aureus} of wastewater origin (Gómez et al. 2016), in contrast to animal and human \textit{S. aureus} isolates, where TSST-1 and other virulence factors are more frequent, especially in some specific genetic lineages (Monecke et al. 2009; Gharsa et al. 2012a).

Six \textit{spa} types (t587, t674, t224, t127, t1701 and t1534) were detected in this study (Table 1), the \textit{spa}-type t587 was the most predominant one (7 isolates, 58.3%). This \textit{spa}-type was associated with \textit{agr} type II in all but one of the isolates, and with a new ST named ST3245 (singleton) which differs from ST9 in \textit{glpF} and \textit{tpi} alleles. The ST9-CC9 is linked worldwide to livestock (Hasman et al. 2010). As far as we know, the \textit{spa}-type t587 has not been previously described in wastewater; nevertheless, the lineage t587-CC9

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Strains & WWTP samples$^a$ & \textit{spa}-type & ST/CC$^b$ & \textit{agr}-type & Phenotype of resistance$^c$ & Resistance genes & Virulence genes \\
\hline
C8300 & C-AS & t587 & ST3245 & NT & PEN, ERY & \textit{msr}(A) & – \\
C8302 & C-APT & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{blaZ}, \textit{msr}(A) & – \\
C8303 & C-AS & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{blaZ}, \textit{msr}(A) & – \\
C8304 & C-E & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{blaZ}, \textit{msr}(A) & – \\
C8305 & C-I & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{msr}(A) & – \\
C8306 & C-APT & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{blaZ}, \textit{msr}(A) & – \\
C8307 & C-RS & t587 & ST3245 & \textit{agr}II & PEN, ERY & \textit{blaZ}, \textit{msr}(A) & – \\
C8308 & C-I & t674 & ST15/CC5 & \textit{agr}II & PEN, TET & \textit{blaZ}, \textit{tet}(K) & – \\
C8309 & C-APT & t224 & (CC97) & \textit{agr}I & PEN & – & – \\
C8310 & M-I & t127 & (CC1) & \textit{agr}III & PEN, CLIN & \textit{blaZ} & – \\
C8311 & C-E & t701 & (CC6) & \textit{agr}I & PEN & – & – \\
C8313 & C-APT & t1534 & (CC522) & \textit{agr}I & PEN & – & \textit{tst} \\
\hline
\end{tabular}
\caption{Characteristics of the 12 \textit{S. aureus} isolates recovered in this study}
\end{table}

$^a$WWTP samples: the location of the WWTP (C: La Charguia; M: El Menzah) is indicated as well as the type of the samples (I, influent; APT, after primary treatment; AS, activated sludge; RS, returned sludge; E, effluent).

$^b$New ST is shown in bold. In some cases, the CC was assumed according to the \textit{spa}-type (in this case it is shown in parentheses).

$^c$PEN, penicillin; C, clindamycin; ERY, erythromycin; TET, tetracycline; NT, non-typeable.
S. aureus was detected in approximately 20% of wastewater samples of two WWTPs tested in Tunisia; we cannot discount that this relatively low frequency of detection could be influenced by the low volume of the sample (1 mL) used in this study. Different CCs associated with either animals or humans have been found. High rates of isolates showed resistance to penicillin and erythromycin, although resistance to tetracycline was also reported. The detection of one S. aureus isolate of lineage t1534 carrying the tst gene is of relevance and its impact on public health should be evaluated. Future studies, including a larger number of WWTPs of different geographic areas of the country, should be performed to expand the results obtained in this study.

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