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

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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). The

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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 [*lnu*(A), *lnu*(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 *S. aureus* that might circulate among WWTPs. In this study, *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 *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 *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; Porrero *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 *blaZ* gene in seven isolates. Indeed, penicillin resistance is widely disseminated among *S. aureus* isolates worldwide (Lowy 2003). The *msr*(A) gene was detected in the seven erythromycin-resistant isolates (Table 1). The *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 *S. aureus* isolates (Schmitz *et al.* 2000; Wendlandt *et al.* 2013, 2015). The *lnu*(A), *lnu*(B), *vga*(A) and *vga*(C) genes were not detected in the clindamycin-resistant isolate. Only one isolate was resistant to tetracycline and harboured the *tet*(K) gene. In fact, *S. aureus* resistance to tetracycline was more common in MRSA than in the MSSA isolates (Sharma *et al.* 2013). In general, tetracycline resistance in *S. aureus* is often encoded by the *tet*(M), *tet*(L) and *tet*(K) genes (Wendlandt *et al.* 2013, 2015). Only one isolate harboured the *tst* gene, and other virulence genes were not detected. Similarly, low rates of TSST-1 were earlier observed in *S. aureus* of wastewater origin (Gómez *et al.* 2016), in contrast to animal and human *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 *spa* types (t587, t674, t224, t127, t701 and t1534) were detected in this study (Table 1), the *spa*-type t587 was the most predominant one (7 isolates, 58.3%). This *spa*-type was associated with *agr* type II in all but one of the isolates, and with a new ST named ST3245 (singleton) which differs from ST9 in *glpF* and *tpi* alleles. The ST9-CC9 is linked worldwide to livestock (Hasman *et al.* 2010). As far as we know, the *spa*-type t587 has not been previously described in wastewater; nevertheless, the lineage t587-CC9

Table 1 | Characteristics of the 12 *S. aureus* isolates recovered in this study

Strains	WWTP samples[a]	*spa*-type	ST/CC[b]	*agr*-type	Phenotype of resistance[c]	Resistance genes	Virulence genes
C8300	C-AS	t587	**ST3245**	NT	PEN, ERY	*msr*(A)	–
C8302	C-APT	t587	ST3245	*agr*II	PEN, ERY	*blaZ*, *msr*(A)	–
C8303	C-AS	t587	ST3245	*agr*II	PEN, ERY	*blaZ*, *msr*(A)	–
C8304	C-E	t587	ST3245	*agr*II	PEN, ERY	*blaZ*, *msr*(A)	–
C8305	C-I	t587	ST3245	*agr*II	PEN, ERY	*msr*(A)	–
C8306	C-APT	t587	ST3245	*agr*II	PEN, ERY	*blaZ*, *msr*(A)	–
C8307	C-RS	t587	ST3245	*agr*II	PEN, ERY	*blaZ*, *msr*(A)	–
C8308	C-I	t674	ST15/CC5	*agr*II	PEN, TET	*blaZ*, *tet*(K)	–
C8309	C-APT	t224	(CC97)	*agr*I	PEN	–	–
C8310	M-I	t127	(CC1)	*agr*III	PEN, CLIN	*blaZ*	–
C8311	C-E	t701	(CC6)	*agr*I	PEN	–	–
C8313	C-APT	t1534	(CC522)	*agr*I	PEN	–	*tst*

[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 *spa*-type (in this case it is shown in parentheses).
[c]PEN, penicillin; CLI, clindamycin; ERY, erythromycin; TET, tetracycline; NT, non-typeable.

has been detected in bloodstream infections in French patients, and in intensive care unit patients from 14 hospitals in the Netherlands (Rijnders *et al.* 2009; Lamamy *et al.* 2013). Furthermore, two lineages, t224-CC97 and t1534-CC522, have been reported in bovine and small ruminant isolates, respectively (Hata *et al.* 2010; Porrero *et al.* 2012). The lineage CC1, associated with *spa*-type t127 and *agr*III, was detected in one isolate (Table 1); this CC is frequently detected in human isolates (Monecke *et al.* 2009; Lozano *et al.* 2011), although it has also been frequently reported in animals, including healthy donkeys in Tunisia (Gharsa *et al.* 2012b). The lineage t701-CC6, detected in one isolate, was previously reported in Tunisia among both human and animal isolates (Kechrid *et al.* 2010; Gharsa *et al.* 2012b). The lineage t674-ST15-CC5, found in one isolate, was previously detected in patients in Spain, but with different *spa* types (Lozano *et al.* 2015). It is worth noting that the CC5 is a human-associated lineage, frequently associated with MRSA strains (Nübel *et al.* 2008).

CONCLUSIONS

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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