# Clonal lineages detected amongst tetracycline-resistant meticillin-resistant *Staphylococcus aureus* isolates of a Tunisian hospital, with detection of lineage ST398

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Tetracycline resistance has been postulated as a potential phenotypic marker of livestock-associated lineage ST398 amongst meticillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates in some European hospitals. The objective of this study was to determine if this marker could also be applied to Maghrebian countries. In total, 99 MRSA isolates were collected in a Tunisian hospital during January 2011–October 2012, and 24 tetracycline-resistant MRSA isolates of this collection were characterized. All isolates were tested for antimicrobial resistance phenotypes and genotypes, molecular typing, and virulence genes. Multilocus sequence typing showed that the majority of the isolates (19/24) belonged to clonal complex CC8 (ST247, *n*=12 isolates; ST239, *n*=6 isolates; ST241, *n*=1 isolate). The remaining isolates belonged to CC398 (ST398, *n*=1 isolate), CC5 (ST5 and ST641, *n*=2 isolates), and CC80 (ST728, *n*=2 isolates). *Spa* typing discriminated MRSA in eight *spa* types: t052 (*n*=12 isolates), t037 (*n*=5 isolates), t044 (*n*=2 isolates), and t899, t129, t311, t1744 and the new t14712 (*n*=1 isolate each). Three *agr* groups were found amongst the studied isolates: *agr* group I (*n*=20 isolates), *agr* group II (*n*=2) and *agr* group III (*n*=2 isolates). We report the detection of one MRSA ST398–t899 isolate in the nasal sample of a farmer patient in Tunisia, representing the first report of ST398 in humans in Africa. Tetracycline resistance seems not to be a good phenotypic marker for MRSA ST398 strains in Tunisia, where CC8 was the most prevalent lineage. Continuous efforts to understand the changing epidemiology of this micro-organism are necessary not only for appropriate antimicrobial treatment and effective infection control, but also to monitor its evolution.

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## INTRODUCTION

Infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) are an important public health problem worldwide. MRSA was first identified in the early 1960s, a few years after the introduction of meticillin in therapeutics (Colley *et al.*, 1965). Since then, this resistant micro-organism has been spreading throughout the world. MRSA possesses a genetic

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Abbreviations: CA, community-acquired/associated; HA, hospital-acquired; LA, livestock-associated; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; PVL, Panton–Valentine leukocidin; SCC, staphylococcal cassette chromosome; $Tet^R$, tetracycline-resistant; TSST-1, toxic shock syndrome toxin 1

element called staphylococcal cassette chromosome *mec* (SCC*mec*), which contains the *mecA* gene that codes for the production of an altered penicillin-binding protein, PBP2a, that does not effectively bind β-lactam antimicrobials (Malachowa & DeLeo, 2010). As a result, MRSA is resistant to most of the currently available β-lactams (except some new cephalosporins). MRSA was considered primarily a nosocomial pathogen, affecting older adult patients in hospital or long-term care settings. However, in the past decade, infections have emerged in the community [community-acquired/associated (CA)-MRSA] and also from livestock [livestock-associated (LA)-MRSA]. Initially, CA-MRSA was genetically unrelated to hospital-acquired (HA)-MRSA strains and was more diverse than HA-MRSA (Hawkey & Jones, 2009). However, some reports have indicated that CA-MRSA may now be causing hospital cross-infection (Rehm, 2008). Moreover, it has been observed that companion and food animals act as reservoirs for MRSA, playing a key role in the epidemiology of this micro-organism (Catry *et al.*, 2010).

In recent years there has been increasing interest in certain genetic lineages associated with livestock animals, mainly in isolates belonging to clonal complex CC398 (Camoez *et al.*, 2013; Lozano *et al.*, 2012). This clonal lineage was first described in 2005 (Armand-Lefevre *et al.*, 2005; Voss *et al.*, 2005), and it has been found in different farm animals, especially in pigs, and in people in contact with these animals (Huijsdens *et al.*, 2006a, b; Khanna *et al.*, 2008; Witte *et al.*, 2007). Currently, there are no data about the presence of MRSA CC398 in Muslim countries, in which pork is not consumed. Thus, in Tunisia, some reports have described the detection of different clonal lineages of MRSA in samples from human and animal origins (Ben Nejma *et al.*, 2013; Ben Slama *et al.*, 2011; Gharsa *et al.*, 2012; Kechrid *et al.*, 2011; Mariem *et al.*, 2013). However, no MRSA ST398 isolates have been identified in hospitals in Tunisia. Previous studies carried out in Spain (Benito *et al.*, 2014; Camoez *et al.*, 2013; Lozano *et al.*, 2012) used tetracycline resistance as a phenotypic marker for the detection of LA-MRSA isolates, amongst them ST398, in hospital environments. The aims of this study were to perform molecular typing in a cohort of MRSA isolates obtained from patients hospitalized in the Military Hospital of Tunis (Tunisia), selected according to their phenotype of tetracycline resistance, as well as to determine if tetracycline resistance is a good marker for detection of CC398 in Tunisia.

## METHODS

**Sampling and microbiological isolation.** From January 2011 to October 2012, a total of 99 non-duplicated MRSA isolates (2011: $n=43$ isolates; 2012: $n=56$ isolates) were collected at the Microbiology Laboratory of the Military Hospital of Tunis (Tunisia). The incidence of MRSA was 25.6% (99/387) of the total *S. aureus* isolates recovered in this period. Thirty-nine strains amongst the 99 MRSA isolates showed tetracycline resistance (39.4%). Twenty-four of the 39 tetracycline-resistant MRSA (MRSA-Tet$^R$) isolates were included for analysis in the present study (the remaining isolates could not be recovered for the study).

**Antimicrobial susceptibility testing.** Susceptibility to 17 antimicrobial agents was performed using the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute recommendations (EUCAST, 2015; CLSI, 2012). Antimicrobial agents tested were [charge in μg (or U) per disc]: penicillin (10 U), oxacillin (1), cefoxitine (30), kanamycin (30), gentamicin (10), tobramycin (10), tetracycline (30), fosfomycin (50), trimethoprim–sulfamethoxazole (1.25/23.75), erythromycin (15), clindamycin (2), pristinamycin (15), ofloxacin (5), vancomycin (30), teicoplanin (30), mupirocin (200) and fusidic acid (10).

**Detection of antimicrobial resistance genes.** The presence of the *mec*A gene was studied by PCR in all isolates (Zhang *et al.*, 2004). The ribosomal methylases encoded by *erm*(A), *erm*(B), *erm*(C) and *erm*(T) genes, which confer resistance to erythromycin and clindamycin, and the efflux pump encoded by the *msr*(A) gene, which confers single resistance to erythromycin, were studied by PCR in the erythromycin-resistant isolates (Gharsa *et al.*, 2012). In addition, *tet*(K), *tet*(M), *tet*(L) and *tet*(O) genes conferring resistance to tetracycline, and *aph*(2)–*acc*(6) and *ant*(4′)–*Ia* genes responsible for aminoglycoside resistance were studied by PCR (Gómez-Sanz *et al.*, 2011). The double-disc diffusion test (D-test) with erythromycin and clindamycin discs was implemented in all isolates to detect inducible clindamycin resistance.

**Molecular typing methods of *S. aureus* isolates.** *Spa* typing was performed in all MRSA isolates, as described previously (Harmsen *et al.*, 2003).The polymorphic X region of the *spa* gene was amplified by PCR and sequences were analysed using Staph-Type software version 1.5.21 (Ridom), which automatically detected *spa* repeats and assigned a *spa* type according to the SpaServer database (http://spaserver.ridom.de/).

Identification of *agr* allele group (I–IV) was determined by multiplex PCR, as described previously (Shopsin *et al.*, 2003).

Multilocus sequence typing (MLST) was performed for all MRSA isolates studied. The allelic profile of each isolate was obtained by sequencing internal fragments of seven unlinked housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*). Alleles of the seven genes defined the allelic profile, which corresponded to a sequence type assigned by the MLST database (http://www.mlst.net).

**Detection of staphylococcal toxin genes.** All isolates were tested by PCR for the presence of the *tst* gene encoding the toxic shock syndrome toxin 1 (TSST-1) (Hwang *et al.*, 2007), *luk*S/*luk*F genes encoding Panton–Valentine leukocidin (PVL), as well as *eta* and *etb* genes encoding exfoliatins A and B (Gharsa *et al.*, 2012).

## RESULTS

### Characterization of antimicrobial resistance mechanisms and virulence genes

All 24 MRSA-Tet$^R$ isolates of this study harboured the *mecA* gene, and tetracycline resistance was mediated by *tet*(K), *tet*(L) and/or *tet*(M), with *tet*(M) being the predominant gene. Twelve different antibiotic resistance patterns were identified (Table 1), with 10 isolates showing the same profile (gentamicin, tobramycin, ofloxacin, erythromycin and clindamycin resistance). A high rate of aminoglycoside resistance (79%) was detected, and *aph*(2′)–*acc*(6′) and/or *ant*(4′)–*Ia* were identified. Macrolide/lincosamide resistance was identified in 63% of isolates [mediated in 14 of 15 isolates by *erm*(A); in one isolate the resistance gene

was unknown] and quinolone resistance in 71%. Five isolates showed fusidic acid resistance (21%) and one isolate showed fosfomycin resistance (4%). No teicoplanin or vancomycin resistance was detected amongst the studied isolates.

Regarding the presence of genes coding for virulence factors, only one isolate harboured the genes for PVL (*luk*S/*luk*F; Table 1). None of the MRSA isolates harboured the genes encoding exfoliative toxins (*eta* and *etb*) or TSST-1 (*tst*).

### Molecular typing

Using MLST, the 24 isolates of this study were grouped into six different sequence types: ST247 ($n$=12 isolates), ST239 ($n$=6 isolates), ST728 ($n$=2 isolates), ST241 ($n$=1 isolate), ST398 ($n$=1 isolate), ST5 ($n$=1 isolate) and ST641 ($n$=1 isolate) (Table 1).

*Spa* typing discriminated MRSA in eight *spa* types: t052 ($n$=12 isolates), t037 ($n$=5 isolates), t044 ($n$=2 isolates), t899 ($n$=1 isolate), t129 ($n$=1 isolate), t311 ($n$=1 isolate), t1744 ($n$=1 isolate) and a new *spa* type t14712 ($n$=1 isolate). Three *agr* groups were found amongst the studied isolates: *agr* group I ($n$=20 isolates), *agr* group II ($n$=2 isolates) and *agr* group III ($n$=2 isolates). The MRSA strain C6455 typed as *spa*–t899–ST398–*agr* I, was recovered from the nasal sample of a hospitalized patient and carried the *tet*(M) gene (Table 1).

## DISCUSSION

Amongst the 24 MRSA-Tet$^R$ isolates characterized, one isolate of lineage ST398 was identified. To the best of our knowledge, our study represents the first report of the detection of this clonal lineage in human MRSA isolates in Africa. This isolate was recovered from a nasal swab of a 74-year-old patient, suggesting nasal colonization; the clinical history of this patient revealed that he was a farmer, although no information about the specific type of farm was included. The lineage ST398 has been found primarily in Europe, where it was isolated from pigs and pig farmers (Armand-Lefevre *et al.*, 2005; Huijsdens *et al.*, 2006a; Voss *et al.*, 2005; Witte *et al.*, 2007). Subsequently, this clonal lineage was also identified in studies from the USA, South America, Asia and Canada (Cui *et al.*, 2009; Jímenez *et al.*, 2011; Khanna *et al.*, 2008; Smith *et al.*, 2009).

Human colonization or infection with the *S. aureus* ST398 clone has been associated with exposure mainly to pigs, but also to other animals (Armand-Lefevre *et al.*, 2005; Khanna *et al.*, 2008; Voss *et al.*, 2005; Witte *et al.*, 2007). Moreover, person-to-person transmission has been observed amongst household members and in the hospital settings (Huijsdens *et al.*, 2006a, b; Witte *et al.*, 2007). For example, a dramatic increase in people colonized as well as infected with the MRSA ST398 clone was reported in a Dutch hospital (van Rijen *et al.*, 2008). Due to the fact that MRSA ST398 isolates are usually resistant to tetracycline and with the aim to detect this clonal lineage in hospital environments, a previous study carried out in Spain already used tetracycline resistance as phenotypic marker (Lozano *et al.*, 2012). In that study, a high prevalence of ST398 was detected amongst MRSA-Tet$^R$ isolates (67%). In another study recently performed also in Spain (Camoez *et al.*, 2013), the rate of ST398 amongst MRSA-Tet$^R$ isolates increased according to the year studied, from no detection in 2000 to 44% in 2011. The low percentage detected in our study (4%, only one isolate amongst 24 studied isolates) could be explained by the food habits of the Tunisia population. Thus, the relationship of MRSA ST398 with pigs and the lack of consumption of pork in Tunisia could be the reason of this low prevalence. According to our results, tetracycline resistance seems not to be a good phenotypic marker for MRSA ST398 strains in Tunisia, which could be due to a low prevalence of this clonal lineage in this country; this study being the first to detect ST398 in human MRSA isolates. Although more studies would be needed to clarify this point, this situation could be similar in other Muslim countries (with no pork consumption by humans).

The MRSA ST398 isolate detected in our study presented the *spa* type t899, which has been previously associated with ST398 and ST9 (Lo *et al.*, 2012). Thus, it has been suggested that the detection of the same *spa* type in isolates belonging to two different sequence types is due to the exchange of genetic material amongst clones of animal origin (Camoez *et al.*, 2013). Moreover, t899 isolates have been recovered not only in pigs, but also in other animals, such as cattle, horses or poultry (Abdelbary *et al.*, 2014; Friese *et al.*, 2013; Graveland *et al.*, 2010; Smith & Pearson, 2011). Very recently, it has been also detected an MRSA strain ST398 of the spa type t4358 in a food sample of chichen origin in Tunisia (Chairat *et al.*, 2015).

Regarding the other clonal complexes detected in our study, genotypic characterization showed that the majority of the isolates (19/24) belonged to CC8 (ST247, $n$=12 isolates; ST239, $n$=6 isolates; ST241, $n$=1 isolate) and most of them presented a multiresistant phenotype. The percentage of MRSA-Tet$^R$ isolates detected in our study (39.4%) could be explained by the transmission and dissemination of some CA-MRSA isolates belonging to CC8. Interestingly, many of the ST247/CC8 (8/12) isolates showed identical phenotypic and genotypic characteristics, being resistant to gentamicin, tobramycin, ofloxacin, erythromycin and clindamycin. Moreover, some of these isolates were detected in the same wards (mainly the Intensive Care Unit and Surgery Unit). It seems possible that transmission of these MRSA isolates amongst the different patients may have occurred. The remaining isolates belonged to CC5 (ST5 and ST641, $n$=2 isolates) and CC80 (ST728, $n$=2 isolates).

Similar to our results, the occurrence of specific clones ST247, ST239 and ST5 in MRSA isolated at the Charles

**Table 1.** Characteristics of the 24 clinical MRSA-Tet$^R$ isolates included in this study

| Isolate | Ward* | Origin | MLST/clonal complex | *agr* group | *spa* type | PVL genes | Resistance phenotype for non-*β*-lactams† | Resistance genes |
|---|---|---|---|---|---|---|---|---|
| C6455 | SU | Nasal swab | ST398/CC398 | I | t899 | – | TET, OFX | *tet*(M) |
| C6460 | ICU | Pleural puncture | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC, FA, FOS | *tet*(M), *aph*(2″)–*acc*(6′), *erm*(A) |
| C6441 | ICU | Catheter | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C6457 | IMU | Wound | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C6444 | ICU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM, ERY, CC, FA | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A), |
| C6443 | SU | Wound | ST247/CC8 | I | t052 | – | TET, GM, TM, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C6447 | ICU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′) |
| C8028 | OTU | Wound | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C8032 | ICU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C8038 | ICU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C8041 | SU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C8045 | SU | Wound | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C8047 | SU | Blood | ST247/CC8 | I | t052 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′), *erm*(A) |
| C6462 | ICU | Blood | ST239/CC8 | I | t037 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *aph*(2″)–*acc*(6′), *erm*(A) |
| C6458 | ICU | Blood | ST239/CC8 | I | t037 | – | TET, GM, TM, OFX, FA | *tet*(K), *ant*(4′)–*Ia*, *aph*(2″)–*acc*(6′) |
| C6456 | DU | Wound | ST239/CC8 | I | t037 | – | TET, GM, TM, OFX, ERY, CC | *tet*(M), *aph*(2″)–*acc*(6′), *erm*(A) |
| C6442 | ICU | Blood | ST239/CC8 | I | t037 | – | TET, GM, TM, OFX | *tet*(M), *tet*(K), *aph*(2″)–*acc*(6′) |
| C6445 | HU | Catheter | ST239/CC8 | I | t037 | – | TET, GM, TM, FA | *tet*(M), *tet*(K), *aph*(2″)–*acc*(6′) |
| C8043 | OS | Blood | ST239/CC8 | I | t14712‡ | – | TET, GM, TM, OFX | *tet*(K), *tet*(M), *aph*(2″)–*acc*(6′) |
| C8049 | ICU | Blood | ST241/CC8 | I | t129 | – | TET, GM, TM, OFX, ERY, CC, SXT | *tet*(K), *tet*(M), *aph*(2″)–*acc*(6′), *erm*(A), *dfrA*, *dfrG* |
| C6446 | IMU | Blood | ST728/CC80 | III | t044 | + | TET, OFX | *tet*(K) |
| C8035 | OS | Abscess/wound | ST728/CC80 | III | t044 | – | TET, FA | *tet*(K) |
| C8031 | ICU | Blood | ST641/CC5‡ | II | t311 | – | TET, ERY, CC | *tet*(L) |
| C6461 | ICU | Blood | ST5/CC5 | II | t7144 | – | TET | *tet*(M) |

*ICU, Intensive Care Unit; IMU, Internal Medicine Unit; DU, Dermatology Unit; HU, Haemodialysis Unit; OTU, Organ Transplant Unit; OS, Orthopaedic Service; SU, Surgery Unit.
†Resistance phenotype in addition to meticillin and tetracycline resistance: GM, gentamicin; TM, tobramycin; OFX, ofloxacin; ERY, erythromycin; CC, clindamycin; FA, fusidic acid; FOS, fosfomycin. In all macrolide/lincosamide-resistant isolates, the double-disc diffusion test showed constitutive resistance to clindamycin.
‡This *spa* type is new and has been submitted to the appropriate database (see Methods).

Nicolle Hospital of Tunis has been reported (Mariem *et al.*, 2013). However, in that study, the majority of isolates belonged to ST80. This sequence type was not detected in our study, but two isolates belonging to CC80 with another sequence type (ST728) were found. Our results showed that all MRSA-Tet$^R$ isolates at the Intensive Care Unit, apart from two isolates, belonged to CC8 (ST247, $n$=6 isolates; ST239, $n$=3 isolates). Interestingly, CC8 and CC5 are the most prevalent clonal complexes worldwide. There are many different sequence types belonging to these clonal complexes and these sequence types have been identified in many countries and/or regions of the world (Stefani *et al.*, 2012). Single nucleotide polymorphism analyses of the CC5 (ST5) clone demonstrated that this clone appeared to have evolved many times, in many countries, through acquisition of the SCC*mec* cassette in a local meticillin-susceptible *S. aureus* CC5 (ST5) population (Nübel *et al.*, 2008). Phylogenetic evidence demonstrated the intercontinental spread and hospital transmission of CC8 isolates through North America, Europe, South America and Asia (Harris *et al.*, 2010). Therefore, in the 1990s, CC8 (ST239) was extended from South America to Europe and from Thailand to China (Gray *et al.*, 2011). Data from Africa are still limited, but also indicate a predominance of CC8 (ST239 and the infrequently described ST612), CC5 (ST5) and CC30 (ST36) lineages in Africa (Jansen van Rensburg et al., 2011; Breurec *et al.*, 2011; Moodley *et al.*, 2010). It is important to note that other CCs detected in our study (other than CC398), have also been detected in livestock animals; this is the case for CC5 and CC8 (Smith, 2015). An association between CC5 isolates and poultry samples has been strongly suggested (Smith, 2015).

However, our results showed one MRSA isolate typed as t044–ST728–CC80–PVL$^+$. Similar isolates have been mainly described in Central Europe (Aires de Sousa & de Lencastre, 2003; Deurenberg & Stobberingh, 2008). Kechrid *et al.* (2011) reported the spread of MRSA t044/t042–ST728–CC80 in isolates recovered from bacteraemic and osteomyelitis infections in children from Tunisia.

LA-MRSA rates have been increasing rapidly worldwide over recent years. Here, we reported the detection of one MRSA ST398 isolate in a patient for the first time in an African country. Continuous efforts to understand the changing epidemiology of this micro-organism are therefore necessary not only for appropriate antimicrobial treatment and effective infection control, but also to monitor its evolution.

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