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# Molecular typing, virulence traits and antimicrobial resistance of diabetic foot staphylococci

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## Abstract

**Background:** Diabetes *mellitus* is a major chronic disease that continues to increase significantly. One of the most important and costly complications of diabetes are foot infections that may be colonized by pathogenic and antimicrobial resistant bacteria, harboring several virulence factors, that could impair its successful treatment. *Staphylococcus aureus* is one of the most prevalent isolate in diabetic foot infections, together with aerobes and anaerobes.

**Methods:** In this study, conducted in the Lisbon area, staphylococci isolated ( $n = 53$ ) from diabetic foot ulcers were identified, genotyped and screened for virulence and antimicrobial resistance traits. Genetic relationship amongst isolates was evaluated by pulsed-field-gel-electrophoresis with further multilocus sequence typing of the identified pulsotypes. PCR was applied for detection of 12 virulence genes and e-test technique was performed to determine minimal inhibitory concentration of ten antibiotics.

**Results:** Among the 53 isolates included in this study, 41 *Staphylococcus aureus* were identified. Staphylococcal isolates were positive for intercellular adhesins *icaA* and *icaD*, negative for biofilm associated protein *bap* and pantone-valentine leucocidin *pvl*. *S. aureus* quorum sensing genes *agrI* and *agrII* were identified and only one isolate was positive for toxic shock syndrome toxin *tst*.

36 % of staphylococci tested were multiresistant and higher rates of resistance were obtained for ciprofloxacin and erythromycin. Clonality analysis revealed high genomic diversity and numerous *S. aureus* sequence types, both community- and hospital-acquired, belonging mostly to clonal complexes CC5 and C22, widely diffused in Portugal nowadays.

**Conclusions:** This study shows that diabetic foot ulcer staphylococci are genomically diverse, present resistance to medically important antibiotics and harbour virulence determinants. These properties suggest staphylococci can contribute to persistence and severity of these infections, leading to treatment failure and to the possibility of transmitting these features to other microorganisms sharing the same niche. In this context, diabetic patients may become a transmission vehicle for microorganisms' clones between community and clinical environments.

**Keywords:** Diabetic foot staphylococci, PFGE, HA-MRSA, CA-MRSA, MLST, Virulence factors

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## Background

Foot ulcers are an increasing problem in patients with Diabetes *mellitus* and infection is a frequent complication that actually constitutes the most common cause of hospitalization in diabetic patients, often related to lower-extremity amputation [1]. Several studies have demonstrated that they represent an economic burden worldwide, comparable with the costs associated with cancer, depression, lung and musculoskeletal diseases [2, 3]. Diabetic foot infections (DFI) are often polymicrobial and can be caused by several pathogens, mainly Gram positive bacteria, being *Staphylococcus* the most predominant bacterial genus, as already described [4, 5].

*Staphylococcus* is a frequent commensal bacteria of human skin and mucosa, being one of the major cause of infections in humans, ranging from minor skin infections to severe infections such as septicemia, endocarditis and osteomyelitis [6]. These bacteria may produce several virulence factors, one of the most important being biofilm formation, which consists in adherent bacterial populations growing inside their polymeric structures that confer the ability of evasion to immune system and to multiple antibiotic treatments [7]. Several virulence genes are implicated in biofilm formation, like *icaA* and *icaD*, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase [8]. A biofilm associated protein, coded by the gene *bap*, was also described as essential in biofilm production of some *Staphylococcus* spp. isolated from nosocomial infections [9].

One of the bacterial properties that allow the development and growth of multicellular biofilm is cell communication and signalling, in which the bacterial signals reach a specific density or “quorum” activating regulatory genes that control some cellular processes [10]; the *S. aureus* accessory gene regulator (*agr*) was the first peptide signal discovered [11].

Many virulence determinants including toxins, tissue degrading enzymes and immune evasion factors, are secreted by staphylococci, particularly by *S. aureus* [12]. *Clfa* is a gene responsible for causing platelet activation through binding to fibrinogen and fibrin and for inhibiting phagocytosis in *S. aureus* [13]. One of the major threats in severe tissue necrosis is the presence of the cytotoxin panton-valentine leukocidin (*pvl*), whose locus is carried on a bacteriophage, manifesting commonly in strains isolated from community-acquired skin and soft tissue infections and especially from pneumonia [14]. Some *S. aureus* isolates also secrete the toxic shock syndrome toxin 1 (TSST-1), a superantigenic toxin responsible for staphylococcal scarlet fever and toxic shock syndrome, encoded by the *tst* gene [15]. *S. aureus* and coagulase-

negative staphylococci (CoNS) infections occur in the community or in healthcare settings and an extremely high percentage of these isolates are resistant to methicillin. In Europe, methicillin-resistant *S. aureus* (MRSA) are predominantly acquired in healthcare settings representing a major challenge to the control of antibiotic resistance in hospitals [16]. Portugal is one of the European countries presenting higher rates of MRSA in hospitals, reaching 53.8 % according to last report data [17], and hospital-associated MRSA (HA-MRSA) have been extensively characterized [18–20]. However, less is known about the epidemiology of MRSA in the community (CA-MRSA), which remains poorly understood [21]. Epidemic MRSA (EMRSA)-15 clone (ST22-IV), is currently the most predominant clone in Portuguese hospitals, accounting for 72 % of all MRSA isolates, followed by the NY/Japan clone (NY/JP) (ST5-II). More recently a variant of this clone (ST105) appeared as the second most predominant clone in Portuguese hospitals [20, 22].

In the last years the complications of DFI have raised due to the increased rate of multidrug-resistant (MDR) isolates, so a better knowledge of these bacteria is necessary in order to institute an effective antibiotic therapy [1, 5]. This study aimed to investigate the molecular types, virulence traits and antimicrobial susceptibility pattern of *Staphylococcus* spp. isolated from diabetic foot ulcers in Portugal.

## Methods

### Bacterial isolates

A total of 53 staphylococci clinical isolates from diabetic foot ulcers, belonging to 49 samples collected in a transversal observational study conducted at four clinical centers in Lisbon, from January 2010 to July 2010 [4], were used in this study. Only eight patients were hospitalized during the collection of samples. All isolates were processed, isolated and identified by standard methods [4]. Each isolate corresponds to a different patient, with the exception of following pairs, which belonged to the same patient: *S. aureus* A2-1a and A2-1b, *S. aureus* B3-2 and B3-3, *S. aureus* Z1-1 and Z1-2, *S. aureus* Z3-1 and Z3-2, *S. aureus* Z21-1 and Z-21-3, *S. aureus* Z27-2 and Z27-3 and *S. aureus* Z33-1 and Z33-2. Although being recovered from the same patient, such staphylococci were included in further analysis due to the distinct colony morphologies observed during isolation and purification procedures.

### Identification at species level

After inoculation in Columbia Agar + 5 % sheep blood (Biomerieux), plates were incubated at 37 °C for 24 h. Rapid DNA extraction was performed by suspending four to five bacterial colonies in 100 µL of TE (10 mM

Tris, 1 mM EDTA, pH 7.8) buffer and heating to 97 °C for seven min. After centrifugation at 15 000 g for five min, supernatant was collected and stored at -20 °C for subsequent PCR screening.

*Staphylococcus aureus* and *Staphylococcus epidermidis* identification was confirmed using a multiplex PCR protocol described elsewhere [23]. Amplified products were analysed by electrophoresis using 0.5X Tris-Borate-EDTA (TBE) buffer in a 2 % agarose gel (Bioline) stained with GreenSafe (NZYTech) and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA ladder VI (NZYTech) was used as a molecular weight marker. *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984 were used as PCR amplification controls.

For the remaining staphylococcal isolates, Biomerieux API Staph galleries were used for species identification.

#### Screening for virulence factors

The presence of virulence determinants was evaluated by PCR amplification using primers and protocols previously described. Genes tested included coagulase gene *coa* [24], protein A gene *spa* [24], adhesin genes *icaA* and *icaD* [25], biofilm associated protein gene *bap*, clumping factor *clfA* [24], accessory regulators genes *agrI*, *agrII*, *agrIII* and *agrIV* [26], toxic shock syndrome toxin 1 gene *tst* and panton-valentine leukocidin *pvl* [27].

*S. aureus* ATCC 25923 was used as an amplification control for *coa*, *spa* and *clfA* genes. *S. epidermidis* ATCC 35984 was used as *icaA* and *icaD* positive control. *S. aureus bap* positive control was kindly provided by Dr. Penadés (Cardenal Herrera University, Valencia, Spain), *agrI*, *agrII*, *agrIII* e *agrIV* control strains by Dr. Carmen Torres (Rioja University, Spain), and *tst* and *pvl* positive controls by Dr. Michèle Bes (Centre National de Reference des Staphylocoques, Lyon,France).

#### Evaluation of antibiotic susceptibility and detection of *mecA*

Minimal inhibitory concentrations (MIC) were determined for antibiotics: cefoxitin (Fox), ceftaroline (Cpt), ciprofloxacin (Cip), clindamycin (Cli), doxycycline (Dox), erythromycin (Ery), gentamicin (Gen), linezolid (Lzd), meropenem (Mem) and vancomycin (Van), by placing e-test strips (Biomérieux) on staphylococci inoculated on Mueller Hinton plates, incubated for 24 h at 37 °C. Test performance was monitored using *S. aureus* ATCC 29213.

Detection of *mecA* gene was performed as previously described [23]. Amplified products were analysed by electrophoresis with 0.5X Tris-Borate-EDTA (TBE) buffer in a 1.5 % agarose gel (Bioline) stained with GreenSafe (NZYTech) and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA ladder VI (NZYTech) was used as

molecular weight marker. MRSA control strain was kindly provided by Dr. Birgit Strommenger (Robert Koch Institute, Germany).

Staphylococci under analysis were defined as Methicillin Resistant Staphylococcus (MRS) if resistant by cefoxitin MIC or if *mecA* positive [28], and as Multi-drug Resistant (MDR) if resistant to three or more antimicrobials belonging to different antibiotic classes and bacterial targets [29].

#### Macrorestriction analysis by Pulsed-Field Gel

##### Electrophoresis (PFGE)

Molecular fingerprinting of staphylococci was performed by PFGE using a CHEF-DRIII apparatus (Bio-Rad Laboratories, San Diego, USA). Bacterial cultures were grown overnight on Columbia agar supplemented with 5 % sheep blood (BioMérieux) and a cellular suspension of  $5 \times 10^9$  CFU/mL incorporated into 1.5 % low melting point agarose (BioRad). Discs were immersed into a lysis solution with lysostaphin (Sigma-Aldrich) (50 µg/ml), lysozyme (Merck) (100 µg/ml) and RNase (Roche) (50 µg/ml) at 37 °C for 3 h. After lysis, discs were incubated with proteinase K (NZYTech, Portugal) (1 mg/ml) for 17 h at 50 °C, followed by overnight digestion with *SmaI* (Takara) at 25 °C. Digested DNA was submitted to electrophoresis in 1 % agarose gel (Seakem LE) for 23 h at 14 °C and 6 V/cm with pulse times of five to 35 s. Lambda Ladder PFG Marker (BioLabs) 50 µg/ml was used as molecular weight marker. Agarose gels were stained with ethidium bromide and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI- 500). BioNumerics 7.5 software (Applied Maths, Kortrijk, Belgium) was used to register macrorestriction patterns and clustering analysis was performed using DICE similarity coefficient and the unweighted-pair group method with arithmetic mean (UPGMA).

##### *S. aureus* multilocus sequence typing (MLST)

Amplification of seven housekeeping genes, including carbamate kinase *arcC*, shikimate dehydrogenase *aroE*, glycerol kinase *glpF*, guanylate kinase *gmk*, phosphate acetyltransferase *pta*, triosephosphate isomerase *tpi*, and acetyl coenzyme A acetyltransferase *yqiL*, was done according to the already published protocols [30]. DNA sequencing was performed by Stabvida (Portugal). MLST sequences were analysed using Bionumerics 7.5 software (Applied Maths, Kortrijk, Belgium) and sequence types (ST) assigned according to the *S. aureus* MLST database (<http://saureus.mlst.net>) The eBURST algorithm, available at (<http://eburst.mlst.net>), was used to classify different ST into clusters or clonal complexes (CC). A minimum spanning tree (MST) constructed with BioNumerics 7.5 software (Applied Maths, Kortrijk, Belgium)

using the concatenated seven gene fragments was also used to evaluate the phylogenetic relationships between isolates.

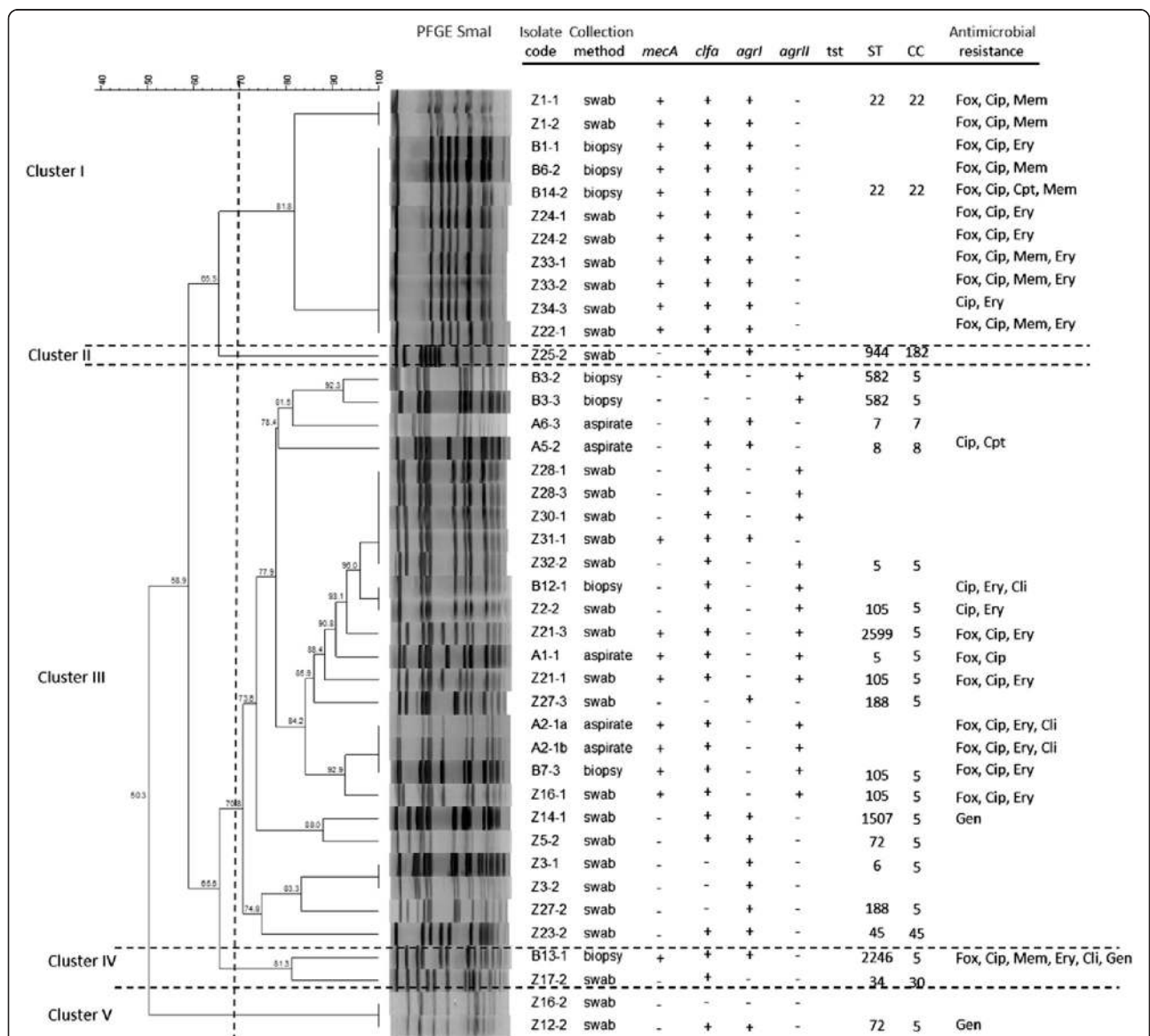
**Results**

**Identification at species level**

Among the 53 isolates included in the study, 41 were identified as *S.aureus* and six as *S. epidermidis* by multiplex PCR. The API galleries identified two isolates as *S. haemolyticus*, one as *S. schleiferi*, one as *S. caprae*, one as *S. simulans* and one as *Staphylococcus* sp.

**Screening for virulence factors**

All isolates were positive for *icaA* and *icaD* and negative for *bap* and *pvl*. The *clfa* gene was present in 70 % of the isolates (*S. aureus* n = 30, *S. epidermidis* n = 3 and *S. sp* n = 1). The *S. aureus* quorum sensing genes *agrI* and *agrII* were present in 60 % and 40 % of the *S. aureus* isolates respectively, and no *agrIII* or *agrIV* were found. Two *S. aureus* isolates did not harbour *agr*. With the exception of two isolates (one of which also *agr* negative), all *S. aureus* were positive for *spa*. As expected, all *S. aureus* isolates were *coa* positive, whilst only one *S. aureus* was positive for *tst* and it was MSSA (Fig. 1).



**Fig 1** Dendrogram based on *Smal*-PFGE patterns of the *S. aureus* diabetic foot isolates. The image also displays information regarding sample collection method, presence of virulence genes, ST/CC allocation and antimicrobial resistance profile. Fox - cefoxitin; Cip – ciprofloxacin; Mem – meropenem; Ery – erythromycin; Cpt – ceftaroline; Cli – clindamycin; Gen – gentamicin



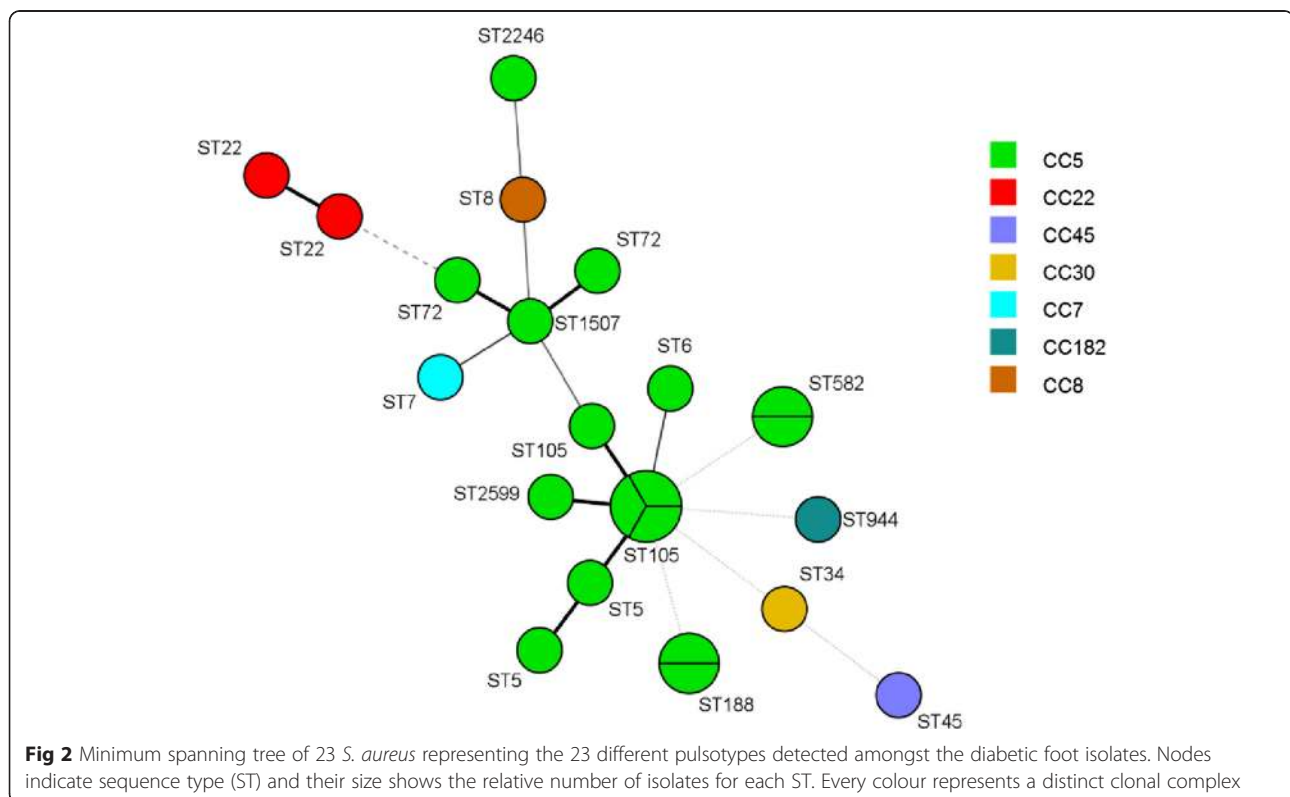
### Evaluation of antibiotic susceptibility and detection of *mecA*

All isolates were considered susceptible to vancomycin ( $MIC \leq 2 \mu\text{g/mL}$ ) and presented the same susceptibility to linezolid ( $MIC \leq 4 \mu\text{g/mL}$ ) and doxycycline ( $MIC \leq 4 \mu\text{g/mL}$ ) with the exception of one methicillin-resistant *S. epidermidis* (MRSE) isolate ( $MIC \geq 8 \mu\text{g/mL}$  and  $MIC \geq 16 \mu\text{g/mL}$  for linezolid and doxycycline respectively), which was resistant to six of the antibiotics tested. Ceftaroline MIC values were  $\leq 0.5 \mu\text{g/mL}$  and only two MRSA presented  $MIC \geq 4 \mu\text{g/mL}$  (ceftaroline-resistant). About 90 % of isolates obtained MIC values for clindamycin of  $\leq 0.25 \mu\text{g/ml}$  and for gentamicin  $4 \leq \mu\text{g/ml}$ . About 57 % of isolates were considered susceptible to ciprofloxacin ( $\leq 4 \mu\text{g/ml}$ ) and erythromycin ( $\leq 8 \mu\text{g/ml}$ ), presenting a resistance rate of 43 %. The percentage of MDR isolates was 36 % (Fig. 1).

Among the 41 *S. aureus* isolates tested, 20 were classified as MRSA (*mecA* positive) (Fig. 1), resulting in a prevalence of 48.7 % among *S. aureus* carriers; of these, 14 were cefoxitin resistant. Among the six *S. epidermidis* isolates, five were MRSE (*mecA* positive) and 3 were cefoxitin resistant. The other *Staphylococcus* isolates didn't carry the *mecA* gene and were cefoxitin susceptible. The total prevalence of methicillin-resistant isolates was 47 %.

### Macrorestriction analysis by Pulsed-Field Gel Electrophoresis -PFGE-

Analysis of the dendrogram displayed in Fig. 1 led to the selection of a 70 % similarity level for the assignment of PFGE genomic types (pulsotypes). Hence, *SmaI*-macrorestriction analysis revealed 18 distinct genomic patterns among the 41 *S. aureus* isolates examined. Cluster analysis allowed grouping the isolates into five main clusters at approximately 70 % similarity with one single member cluster (Fig. 1). All isolates included in cluster I were MRSA, *clfa* and *agrI* positives and belonged to ST22 (CC22). They were all resistant to ciprofloxacin and most of them also to erythromycin. Cluster II included only one isolate, sensitive to all antibiotics tested, *clfa* and *agrI* positive and belonging to ST944 (CC182). The *agrII* positive isolates were located only in cluster III that was the more diverse group because included different genotypes, most of them *clfa* positive belonging to CC5, both MRSA and MSSA. These MRSA isolates showed resistance to ciprofloxacin and erythromycin. The only one MSSA *agrII* isolate that was *tst* positive, belonged to this group. Cluster IV included two different genotypes, one of which stood out (B13-1), being resistant to six of the antibiotics tested. Cluster V included two MSSA isolates, one *clfa-agrI* positive and the other *clfa-agr* negative. Regarding the six *S. epidermidis* isolates, although the



number was inferior, four pulsotypes were observed and, noteworthy, two different pulsotypes corresponded to two isolates obtained from the same patient (data not shown).

#### ***S. aureus* multilocus sequence typing (MLST)**

High genetic diversity was revealed by MLST, as indicated by the detection of 15 ST among the 23 isolates tested (Fig. 1). Briefly, ST105 ( $n = 4$ ), ST5 ( $n = 2$ ), ST22 ( $n = 2$ ), ST188 ( $n = 2$  in the same patient with two different pulsotypes), ST582 ( $n = 2$  in the same patient with two different pulsotypes), ST6 ( $n = 1$ ), ST7 ( $n = 1$ ), ST8 ( $n = 1$ ), ST34 ( $n = 1$ ), ST45 ( $n = 1$ ), ST 72 ( $n = 1$ ), ST944 ( $n = 1$ ), ST1507 ( $n = 1$ ), ST2246 ( $n = 1$ ), ST2599 ( $n = 1$ , in a patient with a ST105 also) (Fig. 1 and Fig. 2). Based on sequence typing, isolates were assigned to seven MLST CC: CC5 ( $n = 17$ , including the two different pulsotypes found in two patients), CC22 ( $n = 2$ ), CC7 ( $n = 1$ ), CC8 ( $n = 1$ ), CC30 ( $n = 1$ ), CC45 ( $n = 1$ ), CC182 ( $n = 1$ ) (Fig. 1). MRSA lineages included ST105 (CC5), ST5 (CC5), ST22 (CC22) and ST2599 (CC5). The only MSSA *tst* positive isolate belonged to ST5. The minimum spanning tree (MST) shows the phylogenetic relationships among diabetic foot staphylococci (Fig. 2).

#### **Discussion**

Although previous studies reported *Pseudomonas aeruginosa* as the most common isolate in DFU [31, 32], many others authors from the late 1990s have shown that Gram positive cocci are the most predominant agents responsible for DFI, with *S. aureus* being the most commonly isolated pathogen with considerably high rates of MRSA [33, 34]. According to our results, most isolates were identified as *S. aureus* (77.3 %) and 48.7 % of them considered MRSA. A study conducted by Sotto et al. [35] reported a similar MRSA percentage but several studies showed lower rates [5, 32, 36]. The highest MRSA percentages in DFI, reaching 70 %, were found in India [37].

Regarding antimicrobial susceptibility patterns, 34 % of all staphylococcal isolates were cefoxitin resistant. However, *mecA* detection is considered the gold standard for methicillin-resistance by the CLSI [28] and 47 % of the studied isolates were *mecA* positive.

Only 10 % of all staphylococci showed resistance to meropenem but MRS isolates should be considered resistant to other  $\beta$ -lactam agents, therefore including also meropenem, because most cases of documented MRS infections have responded poorly to  $\beta$ -lactam therapy [28]. Cephalosporins with anti-MRSA activity include ceftaroline, the active metabolite of ceftaroline fosamil (Teflaro®, Forest Laboratories), a cephalosporin with an *in vitro* broad spectrum against MRSA and most enteric organisms [38]. Ceftaroline, approved by U.S. Food and Drug Administration (FDA) for treatment of

acute bacterial skin infections, displayed a very good efficacy in the studied isolates with MIC value  $\leq 0.5$   $\mu\text{g/mL}$ , with the exception of two resistant MRSA isolates. It is important to refer that one MRSA isolate that was resistant to six antibiotics studied showed sensitivity to ceftaroline. These results are in agreement with some recent studies that have already shown the excellent activity of ceftaroline, both *in vitro* [39] and *in vivo* [40].

Resistance to linezolid and doxycycline was detected only in one MRSE that showed resistance to six antibiotics. Linezolid-resistance in *S. epidermidis* has been already reported [41], possibly linked to a mutation in the V region of the 23S rRNA gene. MIC values for clindamycin and gentamicin showed susceptibility for 90 % of the isolates. These results suggest a good efficacy of linezolid, doxycycline, clindamycin and gentamicin for DFI treatment [42, 43]. As expected, all *Staphylococcus* tested were susceptible to vancomycin; until today only one case of vancomycin-resistant *S. aureus* was described in Europe, Portugal [44], and few cases worldwide, mostly in the USA [45].

About 43 % of the isolates were resistant to ciprofloxacin and erythromycin, two antibiotics largely used in clinical practice for these type of infections. Similar rates in ciprofloxacin and erythromycin resistance were found in a study conducted by Gadepalli et al. [36]. With the increasing use of quinolones in clinical practice, the development of resistance mutants has increased [46], pointing out for the importance to their careful administration in clinical settings. Several genes are implicated in macrolide resistance, especially in staphylococci and streptococci [47], explaining the low susceptibility rates of erythromycin in this bacterial genus.

It's important to remember that DFI are generally polymicrobial and the choice of antibiotic therapy often doesn't target specific pathogens. In fact, the present investigation revealed a high rate (36 %) of MDR isolates in DFI, which is in accordance with other reports [33, 36, 48] and should represent a serious warning for the control of this type of infections.

Virulence factors, like surface proteins and extracellular toxins, are widely distributed among staphylococci, potentially causing harmful pathogenic effects to the host [14]. In this study two *S. aureus* isolates were *spa* negative. Some studies have already reported the absence of *spa* protein with percentages of 3–5 % [49], that seems to be linked to point mutations. In a recent study we demonstrated that the staphylococcal isolates are able to form biofilm [50] which may explain why all isolates tested were positive for *icaA* and *icaD*. Otherwise, none of the isolates carried the *bap* gene, already described in some *Staphylococcus* spp. isolated from nosocomial infections [9].

The *clfA* gene was present in 70 % of our isolates including some *S. epidermidis*. The presence of *clfA* in *S. epidermidis* can be justified by the fact that in this

species the fibrinogen-binding proteins SdrG or Fbe, associated to adherence to fibrinogen, are highly similar to *S. aureus* clumping factors A and B [51].

The only *tst*-positive isolate was a MSSA, *agrII*-positive belonging to ST5. Jarraud et al. in [27] reported that most *tst*-positive *S. aureus* strains were associated with both community and hospital-acquired diseases and were all methicillin-sensitive *S. aureus* (MSSA).

None of our isolates was *pvl*-positive. The *pvl* locus is strongly associated to CA-MRSA and often to *agr* group III background [52]. In Portugal, it was related with one case of CA-MRSA in 2012, belonging to the USA300 epidemic clone (ST8-IVa, t008, *pvl* positive), the predominant CA-MRSA clone in USA at present [53]. The USA300 is a rare clone in Europe and its low prevalence in Portugal was expected [56].

The contribution of the *agr* system to *S. aureus* virulence by gene regulation has already been described, as well as the association of a particular *agr* type in clinical isolates harbouring important virulence factors [54]. The *agr* group I was identified in the majority of the analysed staphylococci, followed by *agrII*, as previously reported in numerous other studies [27, 55]. Neither *agrIII* nor *agrIV* were observed. Two *S. aureus* isolates were *agr*-negative, but it is known that these variants can occur both in vivo and in vitro [56].

The virulence profile of the studied DFI isolates was more similar to CA-MRS than HA-MRS strains. This is an unusual finding, considering that diabetic patients attend frequently healthcare facilities and may suggest an increasing lack of barrier between both settings: hospital and community.

Sotto et al. in [35] demonstrated that the virulence gene profiles of DFI *S. aureus* isolates enables to distinguish the grade of ulcers and to predict its outcome; more knowledge about the virulence features of DFI isolates would be very helpful in establishing a more accurate diagnosis and consequently an adequate therapy.

PFGE genomic typing demonstrated a high diversity of clones, detecting 18 *S. aureus* and four *S. epidermidis* pulsotypes, respectively. According to Tenover et al. 1995 [57], it is highly probable that *S. aureus* isolates grouping in the same pulsotype with 100 % similarity belong to the same ST, as determined by MLST. The correlation between PFGE and MLST showed that PFGE cluster I was the most homogeneous cluster, including only MRSA ST22 (CC22) isolates, the most common ST observed in this study. Portugal is the European country with the highest rate of MRSA (54.6 %) [3] and CC22 is a common and widespread clonal group from which different MRSA have emerged, like the pandemic ST22-MRSA-IV (UK-EMRSA 15), present in hospitals as well as in outpatients [58]. CC22 represents a major clone in Portugal hospitals since 2001, having replace the Brazilian

clone [59], and its prevalence has increased to more than 70 % of MRSA, likewise to what is observed in the United Kingdom, where this clone is believed to have originated [22]. All ST22 isolates were positive for *clfa*, another virulent factor that confers pathogenicity, and presented the quorum sensing *agr I* gene, already described as being common in ST22 staphylococci [59, 60].

The most common CC isolated in our study was CC5, present in PFGE clusters III, IV and V, and ST5 represented the second most frequent ST, after ST22. CC5 is another common and widespread clonal complex that includes a large number of different MRSA, some of which pandemic [58]. Shortly after the emergence of EMRSA-15, the New York-Japan (NY/JP) ST5-II and, more recently, a variant of this clone, ST105-II, appeared as the second most predominant clone in Portuguese hospitals [20]. Recently, a high percentage of MRSA (21.6 %) was also found in a community in Portugal, where EMRSA-15 or related clones were the predominant ones (77.2 %), followed by NY/JP or related clones (14.9 %) [61]. In this study, isolates belonging to CC5 presented mainly *agr* type II, particularly ST5, and included both MRSA and MSSA [18].

Besides ST5 and ST105, several ST belonging to CC5 were identified, namely MSSA *agrI* ST6, MSSA *agrI* ST72, MSSA *agrI* ST188, MSSA *agrI* ST582, MSSA *agrI* ST1507, MRSA *agrI* ST2246 and MRSA *agrII* ST2599. These less frequent ST have already been described in Portugal [20, 61, 62], with the exception of the ST1507 and ST2599, but little information is available regarding these ST. In fact, the only description found in the *S. aureus* MLST database (<http://saureus.mlst.net>), refers to a MRSA ST1507 isolated in 2006 in South Korea from a foodborne source and a MRSA ST2599 isolated from urine in 2013 in the USA. In our study the patient from which ST2599 (CC5) was recovered, also presented another *S. aureus* belonging to ST105 (CC5), being the only case where it was possible to identify two different ST in the same patient. In the other six cases in which the same patient showed two similar, but not identical pulsotypes, MLST revealed that they belonged to the same ST. Interestingly, some clones belonging to different CC presented a higher PFGE similarity than clones included in the same CC, as already observed [18].

Cluster II included only one isolate, MSSA *agrI* ST944 (CC182). MSSA ST944 was described in Switzerland being isolated from nasal swabs of healthy risk-free adult carriers [63] and in China, where it was present with high frequency in nasal carriage of healthy children in a kindergarten [64]. In the *S. aureus* MLST database, a MSSA ST944 has also been described in Norway, related with nasal swab carriage (<http://saureus.mlst.net>).

Cluster III was the most heterogeneous cluster, including mainly MSSA *agrI* isolates, belonging to the following ST: ST7 (CC7), ST8 (CC8), ST34 (CC30) and ST45 (CC45). In fact, a previous study concerning the population structure of MSSA in Portugal showed that these CC were, among others, the most predominant clonal types found between 1992 and 2011, both in the community and hospitals settings [20].

Patients with DFI constantly attend clinical centres for wounds healthcare, which may explain the high diversity of pulsotypes and ST found, including the main hospital-acquired clones present in Portugal (CC5 and CC22). It is important to refer that several less frequent clones, seldom described in literature and MLST database, were also found in this study. Therefore, diabetic patients can be important vehicles for clonal dissemination from the hospitals into the community and contrariwise, including less common clones.

## Conclusions

To our knowledge this is one of the few reports of staphylococci isolated from DFI that include information about the isolates origin, virulence factors and antimicrobial resistance profiles. Studies in DFI microbiology are scarce, as described recently by Zenelaj et al. [5], and further investigation of diabetic foot infections is urgent, allowing to adapt the therapeutic approach of these patients to the microbiological characteristics of the microorganisms involved.

## Abbreviations

*agr*: accessory gene regulator; CA-MRSA: community-acquired MRSA; CC: clonal complex; CIP: ciprofloxacin; CLI: clindamycin; CoNS: coagulase-negative staphylococci; CPT: ceftaroline; DFI: diabetic foot infections; DFU: diabetic foot ulcers; DOX: doxycycline; EMRSA: epidemic MRSA; ERY: erythromycin; FOX: ceftioxin; GEN: gentamicin; HA-MRSA: hospital-associated MRSA; ica: intercellular adhesion; LZD: linezolid; MDR: multidrug-resistant; MEM: meropenem; MIC: minimum inhibitory concentration; MLST: multilocus sequence typing; MRS: methicillin-resistant *Staphylococcus*; MRSA: methicillin-resistant *S. aureus*; MRSE: methicillin-resistant *S. epidermidis*; MST: minimum spanning tree; PFGE: pulsed-field gel electrophoresis; *pvl*: panton-valentine leukocidin; ST: sequence type; *S. aureus*: *Staphylococcus aureus*; *S. epidermidis*: *Staphylococcus epidermidis*; VAN: vancomycin.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

CM, MO and TS conceived and designed the experiments. CM performed the experiments and wrote the manuscript. CM and TS analysed the data. JJ contributed to the samples collection. MO contributed reagents, materials, and analysis tools and helped with the writing of the manuscript. TS, JJ, PCS, LT, and JMC contributed to data interpretation and helped with the writing of the manuscript. All authors read and approved of the final manuscript.

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