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Regular paper

Daptomycin-resistant *Staphylococcus pettenkoferi* of human origin*

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The importance of nosocomial infections caused by coagulase-negative staphylococci is constantly growing. The threat primarily affects immunocompromised patients, the elderly and neonates, particularly after invasive surgery. The problem is fundamentally exacerbated by expanding antibacterial drug resistance. A case report is presented of an 86-year-old patient who underwent a ruptured abdominal aortic aneurysm surgery and developed septicaemia upon surgical wound infection. The causal agent was likely a carbapenem-resistant *Klebsiella pneumoniae*, however, daptomycin-resistant *Staphylococcus pettenkoferi* was identified in blood cultures in the absence of daptomycin treatment. To the authors' knowledge, the case study presented is the first published episode of daptomycin-resistant *S. pettenkoferi* strain.

Key words: daptomycin resistance, coagulase-negative staphylococci, *Staphylococcus pettenkoferi*, *Klebsiella pneumoniae*

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INTRODUCTION

Staphylococci are common commensals that frequently colonise skin and mucous membranes of healthy people. Nonetheless, these bacteria often behave as opportunistic pathogens which can lead to life-threatening local and systemic infections (Rasigade & Vandenesch, 2014). For historical reasons, clinical diagnosis is mainly focused on coagulase-positive staphylococci (CoPS), predominantly *Staphylococcus aureus*, which can lead to underestimation of coagulase-negative staphylococci (CoNS) pathogenicity (Morfin-Otero *et al.*, 2012). However, there is an increasing number of reports suggesting that CoNS are also an important cause of infections in hospitalised patients (Wójkowska-Mach *et al.*, 2014). This phenomenon may be exemplified by the case of *Staphylococcus pettenkoferi*. The latter is a coagulase-negative species forming smooth, slightly convex, glistening and opaque colonies (some isolates are yellow-orange pigmented), named in honor of Max von Pettenkofer (1818–1901), who was a pioneer in the field of hygiene and public health in the former kingdom of Bavaria. It was first described relatively recently (Trülzsch *et al.*, 2002; Trülzsch *et al.*, 2007) but has already been reported as an aetiological agent of wound (including those related to the diabetic foot) and bloodstream infections, osteomyelitis and bacteraemias in intensive care units (Krediet *et al.*, 2004; Loïez *et al.*, 2007; Song *et al.*, 2009; Mammina *et al.*, 2011; Mihaila *et al.*, 2012; Morfin-Otero *et al.*, 2012; Park *et al.*, 2015). The problem of CoNS infections in the clinical setting is made more severe as a result of antibiotic resistance spread (Davies & Davies, 2010; Rasigade *et al.*, 2012; Fluit *et al.*, 2013; Mkrtchyan *et al.*, 2013). Concerning staphylococci, the most alarming resistance phenotypes are those involving daptomycin, a drug which is one of only a few valid alternatives to treat diseases caused by methicillin- and vancomycin-resistant strains (Tótoli *et al.*, 2015). In the case study presented here, we report what appears to be a unique description of daptomycin-resistant *S. pettenkoferi* human isolate collected from blood.

CASE REPORT

Background. An 86-year-old male patient was admitted in June 2014 to the Civic Hospital of Pescara, Italy, due to an apparent wound infection (cultures were not performed), which had developed after ruptured abdominal aortic aneurysm surgery. A central venous catheter (CVC) was placed into the right internal jugular vein in order to administer systemic antibiotics. Despite several antimicrobial treatments, including ciprofloxacin, metronidazole meropenem, piperacillin/tazobactam, clindamycin, colistin, tigecycline and fluconazole, that the patient received in the two-month-period from hospitalization, his body temperature always kept between 36.8°C and 39.3°C, and no improvement in the wound aspect was observed. Nevertheless, the supervising clinicians did not request microbiological evaluation of the wound exudate, and instead the CVC was removed and sent for culture; it was found that its tip, particularly, grew a few carbapenem-resistant *Klebsiella pneumoniae* colonies (description of related methods is outside the scope of this paper). Concomitantly, blood samples were taken and sent to the Microbiology Laboratory, as described in detail below.

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*The results were presented at the 6th International Weigl Conference on Microbiology, Gdańsk, Poland (8–10 July, 2015).

Abbreviations: CoPS, coagulase-positive staphylococci; CoNS, coagulase-negative staphylococci; CVC, central venous catheter; EUCAST, European Committee for Antibiotic Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration; MALDI ToF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; HCCA, alpha-cyano-4-hydroxycinnamic acid; RFLP, restriction fragments length polymorphism; CA-MHB, cation adjusted Mueller Hinton broth; PCR, polymerase chain reaction

Despite treatment, the patient died due to septic shock 2 months after hospitalization.

Cultures. Two 4-mL blood samples were inoculated into two BacT/Alert bottles (bioMérieux, France, one for aerobes and one for anaerobes) and were scored as positive after 24 hour incubation. A drop from each bottle was inoculated onto agar-based media to cultivate both, aerobic and anaerobic organisms, if present, and incubated at 36 ± 1°C. Microscopic examination carried out at the same time revealed the presence of Gram-positive cocci in the aerobe-dedicated bottle. Catalase-positive, coagulase-negative, Gram-positive cocci were documented after 24-hour incubation and conclusively observed to represent a pure culture after 48 hours.

Identification and antibiotic susceptibility testing with Vitek2. The Vitek2 GP card (bioMeriéux, France) identified the isolate as *Staphylococcus capitis*, with 99% certainty (D'Azevedo *et al*., 2009). Also, the studied strain was found to be both, oxacillin- and daptomycin-resistant, by using the Vitek2 AST632 card (bioMeriéux, France).

Search for the *mec* genes. Real-Time Polymerase Chain Reaction performed with Xpert® MRSA Gen 3 (Cepheid, US) and targeting both, *mec*A and *mec*C genes, provided negative results, so the isolate was conclusively considered as non-methicillin/oxacillin-resistant, despite the above mentioned results.

Susceptibility testing by agar-based methods. An agar disc diffusion method based on EUCAST (European Committee for Antibiotic Susceptibility Testing) 2015 criteria (medium for testing, incubation temperature and incubation time were Mueller-Hinton agar, 35 ± 1°C and 18 ± 2 h, respectively) was carried out with a 30 µg cefoxitin disk and gave an inhibition zone diameter of 22 mm; the test was repeated in another laboratory, providing an inhibition zone diameter result of 23 mm; both were therefore in the resistant range according to CLSI 2015 (Clinical and Laboratory Standards Institute) and EUCAST 2015 guidelines. Also, determination of daptomycin and oxacillin MICs was performed using Liofilchem® MIC Test Strips and the values obtained turned out to be ≥1.5 mg/mL (Fig. 1) and 0.5 mg/mL, respectively. Hence, as reported by CLSI 2015 for oxacillin MIC interpretive criteria, cefoxitin screening might overestimate CoNS resistance to betalactams in some cases. Finally, daptomycin appeared to be ineffective, *in vitro*, on the studied strain.

Bruker Daltronic MALDI ToF identification. To allow further analysis, the staphylococcal isolate was re-identified using a MALDI ToF Biotyper**.** Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry. The MALDI Biotyper (Bruker, UK) utilises MALDI technology to measure ribosomal proteins and create a mass spectrum or 'fingerprint' unique to individual species of bacteria or fungi resulting in a high throughput and reliable method for the classification and identification of microorganisms (Seng *et al*., 2009; Dubois *et al*., 2010; Haigh *et al*., 2013).

A small amount of biological material was applied directly on a MALDI target plate in a thin smear. The thin microbial film was overlaid with 0.7 µl of a saturated alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution and air dried. The matrix protects the sample from being destroyed by the direct laser beam and facilitates vaporization and ionization. Inside the MALDI-TOF mass spectrometer, a 60 Hz nitrogen laser is fired 240 times at each sample and mass spectra are acquired between 2000 and 20000 Da automatically.

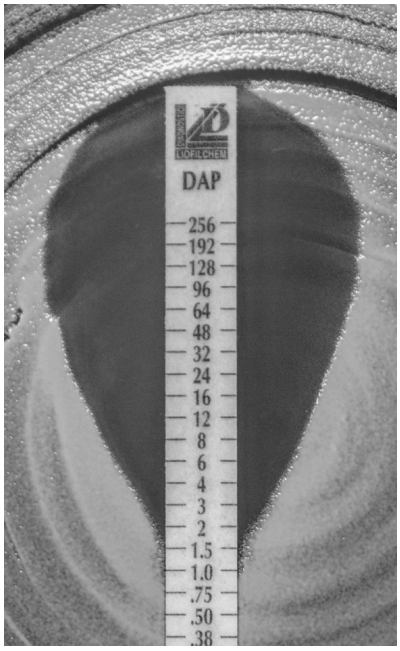

Figure 1. Determination of *S. pettenkoferi* s461 daptomycin resistance.
The minimal inhibitory concentration (MIC) is greater than 1 µg/ml, which allows for classification of the analysed strain as resistant, using the EUCAST and CLSI breakpoints.

The vaporized and ionised protein fragments are released into a tube (1–2 m in length), accelerated and allowed to drift towards a detector. Time-of-Flight (TOF) is proportional to the molecular weight of the fragment. After 240 laser shots, an accumulated spectrum of the sample will be produced.

The obtained mass spectrum will show a characteristic peak pattern known as the "fingerprint spectrum". "Pattern matching" the MS fingerprint spectrum with the reference library quickly identifies each microorganism. A matching score based on identified masses and the intensity correlation is generated and used for ranking of the results.

The tested strain gave an identification of *Staphylococcus pettenkoferi* and a matching score of 2.243 in the Bruker Daltronic realtime identification software with a strong match for strain *Staphylococcus pettenkoferi* KL906013_BK14723 DT UKH.

Vitek MS identification. In contrast to the identification obtained with the aforementioned Bruker-Daltronic MALDI-TOF technology, the one provided by bioMérieux (Vitek MS) was not able to achieve any characterization for the studied organism, as the latter was on more than one occasion labelled as 'non-identified'. Of course, this was due to the absence of *S. pettenkoferi* profile in the related software library.

Genetic identification. RFLP (restriction fragments length polymorphism) patterns were obtained for *gap* (Yugueros *et al*., 2000; Sa *et al*., 2001) and *sao*C genes (Bukowski *et al*., 2015) according to the methodology described by their authors in the referenced literature. For the absence of a reference strain in the widely accessible reference collections, the patterns were compared with *in silico* ones generated for aforementioned genes, whose sequences were obtained from the whole genome shotgun sequence of *S. pettenkoferi* VCU012 (GenBank accession: AGUA01000000), the only genomic sequence available for this species. The *in silico* patterns were obtained using CLC Main Workbench software (CLC bio, Qiagen)

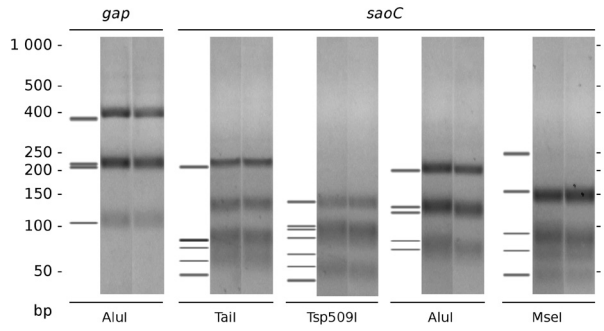

Figure 2. RFLP patterns for *gap* and *sao*C genes.
The patterns were obtained for the analysed *Staphylococcus* spp. strain in two replicas. First of each three lanes is an *in silico* pattern for the reference strain *S. pettenkoferi* VCU012. Below, restriction enzymes, corresponding to the patterns, are listed.

and compared to the *in vitro* ones using GelCompar II (Applied Biosystems). The *gap* patterns were identical between the reference and analysed strains (Fig. 2). For the *sao*C gene, 17 of 22 reference bands ideally corresponded to the obtained patterns, and for one of four different restriction enzymes, namely AluI, the pattern was identical to the reference one (Fig. 2). Importantly, the obtained patterns were unique when compared to those obtained for the other 26 staphylococci species (Bukowski *et al.*, 2015). Polymorphisms for the *sao*C gene suggest a distant phylogenetic relation with the reference strain. The results were validated by 16S rRNA and *rpo*B sequencing (Drancourt & Raoult, 2002; Becker *et al.*, 2004). For the former, the obtained sequence (GenBank accession: KT199298) was the most similar and 99% identical to the one for *S. pettenkofer*i B3117. The latter (GenBank accession: KT199299) was identical to the one for *S. pettenkofer*i CCM 7495. The results obtained using genetic methods clearly confirm the identity of the analysed strain as *S. pettenkoferi*, which was given a signature s461 (BioSample accession: SAMN03784716).

Broth dilution MIC Testing. A CLSI broth dilution method, following CLSI M100-S23 (2013) and CLSI M07-A9 (2012) guidelines was used to quantitatively determine the *in vitro* activity of daptomycin against the bacterial isolate. A microdilution broth method in which a sterile microtiter tray was inoculated with an antimicrobial dilution range was used, where the antimicrobial agent and a suspension of the tested organism were added together using an autoinoculator. After incubation, the MIC can be determined by examination of the plate for visible growth.

The antimicrobial potency for daptomycin was calculated using the purity value supplied, with corrections made for water content and the inactive fraction of salt preparations. All calculations were carried out according to the information supplied on the certificates of analysis. Stock solutions of antimicrobial agents and the tested substance were made using a specific mass (as stated on the manufacturer's certificate of analysis), calculated using the potency, required volume and stock concentration. A three step dilution scheme, as stated in CLSI M100-S23 (2013), was used to prepare the antimicrobial concentration range.

Once all required concentrations had been diluted at twice the required final concentration, they were loaded in 2.2 mL aliquots into polypropylene 96 well, deep well blocks. Deep well blocks were then used to dispense 50 μL aliquots into 96 well microtiter plates using an automated liquid handling system. These plates were then stored frozen at nominal –80°C, and thawed prior to testing.

As stated in CLSI and EUCAST guidelines, calcium ion concentration within the tested media is critical to ensure reproducibility and reliability of the results in case of daptomycin testing. The calcium ion content of the prepared broth microdilution plates (once inoculated) was calculated using an Olympus AU640 analyser. Calcium (Ca^{2+}) content in tested plates was 49.6 mg/L and falls within acceptable limits stated in the EUCAST guidelines.

Test plates were prepared using an inoculum solution containing $2–8 \times 10^5$ CFU/mL of the tested organism (colony forming units/mL). This solution was made firstly by selecting 3–5 colonies from an overnight subculture plate, and using that to prepare a 0.5 McFarland solution in 5 mL of cation adjusted Mueller Hinton broth (CA-MHB), which contained approximately 10^8 CFU/mL of the tested culture. 80 μL of this solution was added to 11mL CA-MHB to form an inoculum solution of approximately $2–8 \times 10^5$ CFU/mL. This was then added in 50 μL aliquots to the pre-prepared 96 well microtiter plates using an auto inoculating machine and incubated as per conditions stated in the CLSI guidelines.

Following incubation, MIC results were read by macroscopic evaluation, recorded on controlled paperwork and checked by a second scientist. The MIC result was 2 μg/ml, which allowed for classification of the analysed strain as resistant using EUCAST and CLSI breakpoints (CLSI and EUCAST: resistant breakpoint >1 μg/mL).

MIC results were confirmed using E-test strips following manufacturer's instructions and using Mueller Hinton agar (Fig. 1).

Putative mechanism of daptomycin resistance. Using the primers *mprF*-F: 5'-TAATTAGACATTAAGAATTGAGGATGAC-3'; and *mprF*-R: 5'-ACACTTAACGTCATAACCTCTTC-3'; a PCR product of *mprF* for the analysed *S. pettenkoferi* strain was obtained. Because of its length, the product was subjected to Sanger sequencing using two additional primers besides those aforementioned: *mprF*-in1-F: 5'-TTATTGTACCATCATTTCGAGTCTTG-3'; *mprF*-in2-F: 5'-TATGCATCTTATATTTCACTGCTATG-3'. The *mprF* gene codes for phosphatidylglycerol lysyltransferase, which is

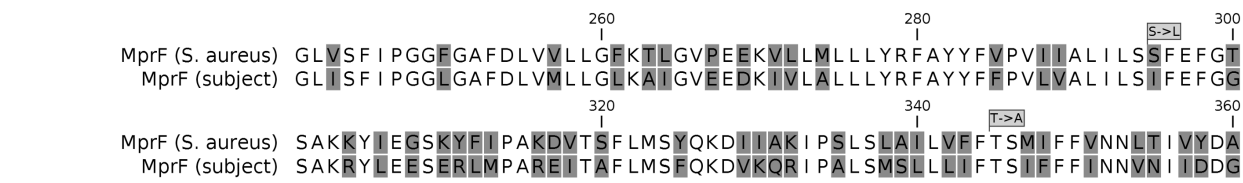

Figure 3. MprF sequence similarity between *S. aureus* and *S. pettenkoferi*.
The picture presents a fragment of an alignment between MprF sequence of *S. aureus* and the analysed *S. pettenkoferi* strain (subject). The varying amino acids are highlighted. The sites of the mutation mediating daptomycin resistance in *S. aureus* are marked above the sequence (S295L and T345A). Noticeably in the position 295 of the MprF sequence, the analysed *S. pettenkoferi* strain possesses isoleucine (I).

Figure 4. MprF sequence diversity within *S. pettenkoferi*.
The picture presents a fragment of an alignment between MprF sequence of *S. pettenkoferi* VCU012 (reference) and of the analysed *S. pettenkoferi* strain (subject). Two mutations leading to a charge change of two amino acid residues are highlighted (N734D, E760K).

responsible for phosphatidilloglicerol lysinilation. Missense mutations within *mpr*F may mediate membrane-charge-dependent resistance against daptomycin (Yang *et al.*, 2013; Bayer *et al.*, 2015; Yamaguchi *et al.*, 2015). As it was demonstrated for *S. aureus,* two missense mutation in the *mpr*F gene may lead to the resistant phenotype (Yang *et al.*, 2013). These mutations lead to serine (Ser, S) to leucine (Leu, L) change in the position 295 and threonine (Thr, T) to alanine (Ala, A) change in the position 345. However, the MprF sequence for *S. pettenkoferi* differs significantly from that for *S. aureus* (Fig. 3); the one obtained for the analysed strain (GenBank accession: KT199300) has isoleucine (Ile, I) in the position corresponding to the position 345 for *S. aureus*. The same difference is observed for *S. pettenkoferi* VCU012, the only strain of this species with known *mpr*F sequence (GenBank accession: AGUA01000000). Unfortunately, its susceptibility to daptomycin is unknown.

Nonetheless, when the *S. pettenkoferi* VCU012 MprF sequence is compared to the one for the analysed strain, two additional changes were identified (Fig. 4). These are asparagine (Asn, N) to aspartic acid (Asp, D) change in the position 734 and glutamic acid (Glu, E) to lysine (Lys, K) in the position 760. Such changes of the charged residues may alter MprF activity and may be involved in the daptomycin resistance. Such a phenomenon, related not only to daptomycin, may be an effect of cumulative missense mutations in *mpr*F, completely different from the two mentioned in the beginning of this section, and other genes, which leads to membrane structure changes (Young *et al.*, 2012). Undoubtedly, daptomycin resistance of the analysed *S. pettenkoferi* strain remains an attractive issue to be explored in future research.

DISCUSSION AND CONCLUSIONS

The exitus of the case presented was presumably due to an undetected carbapenem-resistant *K. pneumoniae* infection, while the bloodstream-related *S. pettenkoferi* isolate was a putative skin saprophyte accidentally introduced into the BacT/Alert bottle, possibly by virtue of inadequate preparation of the cutaneous venipuncture site.

Despite the fact that most likely the isolate represented a harmless contaminant, it was intriguing to observe that it displayed a daptomycin-resistant phenotype. As daptomycin resistance was observed in a methicillin/oxacillin-susceptible strain, it has been of further interest to note that these two resistance traits may not be necessarily related to each other or be concomitant. Moreover, such a finding was even more intriguing based on the fact that the patient had no noted prior daptomycin treatment. We could therefore speculate that the strain acquired the resistance trait elsewhere, before it reached the patient's skin and was transferred to the venipuncture site perhaps by means of caretakers' hands, and finally reached the bottle for blood collection due to the phlebotomy procedure. This is, however, no more than a hypothesis.

Another theory is that the *S. pettenkoferi* strain developed resistance whilst the patient was being treated for the *K. pneumoniae* infection, as antimicrobial levels reaching what is likely to have been a skin flora organism would most likely be sub-inhibitory, encouraging resistance development. Also, as the *K. pneumoniae* infection was notably resistant, a range of antibiotics may have been used to treat the patient (including prophylactic antibiotics for surgical preparation), increasing the chance of exposure of the flora to a wide range of compounds, and therefore increasing resistance development potential throughout the flora.

We believe a leading message may be taken home from the aforementioned observations; such a message focuses on CoNS behaviour as potential reservoirs for antibiotic resistance traits, independently from their commensal or pathogenic role. It is therefore important from both, a medical and epidemiological perspective, that clinical microbiologists who encounter CoNS isolates daily in the hospital practice, do not aprioristically dismiss them as innocent bystanders and that they investigate the resistant traits each strain may display. The importance of correct and complete identification of microbiological samples is also an issue of note. As with the case study presented here, a number of differing identification methods has been used and some either failed to produce a result or provided an incorrect result.

Finally, daptomycin resistance in CoNS is still an open field of research. It is then crucial that phenotypical observations should be supported by genome-based studies on molecular mechanisms behind a clinically evident lack of *in vitro* drug activity, especially when the latest compounds are involved, such as glycopeptides, linezolid, or daptomycin, that represent important weapons to face potentially life-threatening staphylococcal diseases throughout the hospital wards.

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