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Susceptibility trends including emergence of linezolid resistance among coagulase-negative staphylococci and meticillin-resistant *Staphylococcus aureus* from invasive infections☆

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ABSTRACT

Multiresistance in staphylococci constitutes a major challenge for the antimicrobial chemotherapy of invasive infections such as bacteraemia or bone and joint infections (BJIs). A nationwide prospective study was performed to detect antimicrobial resistance trends among staphylococci causing invasive infections. Between October 2011 and February 2012, 367 meticillin-resistant *Staphylococcus aureus* (MRSA) and 695 coagulase-negative staphylococci (CoNS) were collected from 37 French hospitals, mainly from bacteraemia (59.9%) and osteoarticular infections (29.0%). Minimum inhibitory concentrations (MICs) were determined by broth microdilution, and specific screening and confirmation tests were performed to detect heterogeneous vancomycin-intermediate *S. aureus* (hVISA). Staphylococcal isolates exhibiting a linezolid MIC > 4 mg/L were further characterised to determinate their clonal relationships and the mechanism of resistance. MRSA exhibited additional resistances, including levofloxacin (82% associated resistance), gentamicin (13.6%), fusidic acid (13.6%) and rifampicin (6.5%), compromising oral step-down therapy in BJIs. Only two hVISA strains (0.5%) were identified. Among the CoNS, mainly *Staphylococcus epidermidis* (506/695; 72.8%), resistance to first- and second-line agents was more common. Linezolid resistance was identified in 10 CoNS (1.4%). The most frequent linezolid resistance mechanism was the G2576T mutation in 23S rDNA (9/10). For the first time in France, the *cfr* gene was found in five related sequence type 2 (ST2) *S. epidermidis* from two different hospitals, in association with ribosomal RNA and L3 ribosomal protein mutations. These national data must be considered when selecting empirical treatment for invasive staphylococcal infections. Moreover, the emergence and spread of linezolid-resistant CoNS carrying the *cfr* gene is of concern.

1. Introduction

Staphylococci are responsible for a large portion of community and hospital-acquired bacteraemia, complicated skin and soft-tissue infections (SSTIs) and osteoarticular infections [1–4]. Thus, invasive staphylococcal infections constitute a public health problem and a therapeutic challenge especially for deep-seated infections, such as bone and joint infections (BJIs), due to multiresistant isolates. Next to *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS) cause an increasing number of infections, especially implant-associated infections that are particularly

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difficult to treat [5]. *Staphylococcus epidermidis* is the most frequently isolated CoNS species from clinical specimens; this opportunistic pathogen frequently carries antibiotic resistance genes leading to a multidrug-resistant (MDR) phenotype [5,6]. For 40 years vancomycin was the cornerstone of antistaphylococcal therapy; unfortunately, the end of the 20th century brought to light the emergence of isolates with decreasing susceptibility to glycopeptides [5,7]. Facing this worrying trend, new compounds targeting MDR Gram-positive bacteria were marketed, such as linezolid and daptomycin. Nevertheless, additional resistances to glycopeptides, lipopeptides and oxazolidinones appeared, demanding a thorough surveillance of clinical isolates [5,7]. The aim of this study was to determine the antibiotic susceptibilities of clinically relevant staphylococci responsible for invasive infections, focusing on well characterised CoNS and meticillin-resistant *S. aureus* (MRSA) isolates resistant to first-line agents.

2. Materials and methods

2.1. Study design, sites and clinical isolates

Between October 2011 and February 2012, the microbiological laboratories of 37 hospitals equally geographically distributed in France prospectively included a pre-defined number of consecutive MRSA and CoNS from invasive infections (Fig. 1) [8]. Susceptibility to meticillin (all isolates) and clindamycin (isolates from BJIs) was tested at the participating laboratory level according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/clinical_breakpoints/). Infections were considered as healthcare-associated if occurring in patients hospitalised for ≥3 days, carrying an intravenous or urinary catheter at the time of infection, or those on haemodialysis during the year before collection of the isolate. Catheter-related bloodstream infections were considered according to internationally recognised criteria (http://www.cdc.gov).

2.2. Antimicrobial susceptibility test method

The core laboratory identified all of the CoNS to species level by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) and determined the minimum inhibitory concentrations (MICs) of nine antibiotics by broth microdilution according to EUCAST recommendations (http://www.eucast.org/) using commercially prepared dry-form test panels (Sensititre® panels; TREK Diagnostic Systems, Cleveland, OH) including vancomycin, teicoplanin, linezolid, daptomycin, rifampicin, tigecycline, fusidic acid, levofloxacin and gentamicin. Calcium supplementation (50 mg/L final concentration) was used for testing daptomycin. *Staphylococcus aureus* ATCC 29213 was used as a quality control strain. Susceptibility testing results were interpreted in accordance with the EUCAST 2013 recommendations.

2.3. Detection of glycopeptide and linezolid resistance

A screening test for heterogeneous vancomycin-intermediate *S. aureus* (hVISA) was performed using the Etest macromethod (vancomycin and teicoplanin) as previously reported [9]. If positive, a population analysis profile–area under the curve (PAP-AUC) was performed at the French National Reference Centre for Staphylococci (Centre national de référence des Staphylocoques, Bron, France). All linezolid MICs above the EUCAST breakpoint (4 mg/L) were further confirmed by Etest and agar dilution methods.

2.4. Molecular analysis of linezolid-resistant isolates

The presence of mutations was investigated by sequencing domain V of 23S rDNA [10]. The number of mutated 23S rDNA copies was determined by pyrosequencing on a PyroMark Q24® Analyser using PyroMark Gold Q24® reagents (QIAGEN, Courtaboeuf, France). Briefly, to identify base changes in CoNS isolates, a 395-bp PCR product was generated using the primers rrn1-F (AGTTTGACTGGGGCGGTC) and rrn-1-R (TAGTACGAGAGGACCGG) and additional sequencing primers SP1 (TAAAAGCTACCCCGG) and SP2 (GCCCATTAAAGCGGTA). Mutations in the *rplC* and *rplD* genes that encode the L3 and L4 ribosomal proteins were investigated by sequencing as previously described [11]. For *Staphylococcus hominis* strains, the *rplD* gene was amplified by PCR using the primers L4*hominis*F-CGTGCTTCTTTACGCCAAGG and L4*homonis*R-ATCCGAGCACCTCCTCAACT. Nucleotide and deduced amino acid sequences were compared with the linezolid-susceptible *S. epidermidis* ATCC 12228 as well as linezolid-susceptible *Staphylococcus capitis* and linezolid-susceptible *S. hominis* isolated during this study. Presence of the *cfr* gene responsible for transferable resistance was tested by PCR as previously described [12].

The clonal relationship between isolates from the same species and their genetic background was characterised by pulsed-field gel electrophoresis (PFGE) and multilocus sequencing typing (MLST) methods as previously reported (http://sepidermidis.mlst.net) [8].

2.5. Statistical methods

Regarding the MRSA isolates, a comparison of antimicrobial susceptibility results was performed according to (i) their clinical origin, (ii) their vancomycin MIC (≤1 mg/L vs. >1 mg/L) and (iii) their clindamycin susceptibility. Regarding the CoNS isolates, a comparison of antimicrobial susceptibility results was performed according to (i) their clinical origin, (ii) their identification at the species level (*S. epidermidis* vs. other CoNS species) and (iii) their meticillin susceptibility. Statistical analysis was carried out using the χ^2 test, and a *P*-value of ≤0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the MRSA isolates

3.1.1. Clinical origins of the MRSA isolates

In total, 1062 staphylococcal isolates were collected, including 367 MRSA from either bacteraemia (197/367; 53.7%), BJI (136/367; 37.1%) and other invasive infections (34/367; 9.3%). MRSA from bacteraemia were mainly related to central (15.2%) and peripheral (11.2%) catheter infections, 73% being considered as hospital-acquired. Isolates from BJIs originated from primary (64.2%) and device-associated infections (35.8%); these infections were considered as hospital-acquired in 75% of cases.

3.1.2. Antimicrobial susceptibly of the MRSA isolates

MICs (MIC_{50}, MIC_{90} and range) and percent susceptibility of MRSA according to the clinical origins of the isolates are reported in Table 1. The only antibiotic compound for which the percentage of susceptible isolates varied in accordance with the origin of the isolate was levofloxacin: 15.2% of MRSA isolated from bacteraemia were susceptible to levofloxacin versus 29.4% of isolates from other invasive infections ($P = 0.0435$). All MRSA were susceptible to daptomycin, linezolid and tigecycline, with the MIC_{90} of the first two compounds approaching or reaching the breakpoint value: 0.5 mg/L for a daptomycin breakpoint of 1 mg/L; and 4 mg/L for a linezolid breakpoint of 4 mg/L. All but two MRSA were susceptible to glycopeptides; these two isolates were susceptible to vancomycin (MIC = 2 mg/L) but were resistant to teicoplanin