In Vitro Activities of Ceftobiprole, Tigecycline, Daptomycin, and 19 Other Antimicrobials against Methicillin-Resistant \textit{Staphylococcus aureus} Strains from a National Survey of Belgian Hospitals

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The in vitro activities of 22 antimicrobial agents, including ceftobiprole, daptomycin, and tigecycline, against 511 methicillin-resistant \textit{Staphylococcus aureus} (MRSA) isolates from 112 Belgian hospitals were studied by using the CLSI agar dilution method. Isolates were characterized by pulsed-field gel electrophoresis (PFGE) analysis and by PCR detection of determinants of resistance to aminoglycosides, macrolides-lincosamides-streptogramins, and tetracyclines. A representative set of isolates with different PFGE genotypes was further characterized by multilocus sequence typing, determination of staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) type, and multiplex PCR for toxic shock syndrome type 1 (TSST-1) and Panton-Valentine leukocidin genes. MRSA isolates belonged to nine epidemic MRSA clones, of which sequence type 45 (ST45)-SCC\textit{mec} IV and ST8-SCC\textit{mec} IV were predominant, accounting for 49 and 20% of isolates, respectively. The distribution of antimicrobial resistance and TSST-1 genes was strongly linked to clonal types. Ceftobiprole, daptomycin, and tigecycline showed high activity against all isolates of these sporadic and epidemic MRSA clones, as indicated by MIC\textsubscript{90}s of 2 mg/liter, 0.5 mg/liter, and 0.25 mg/liter, respectively. The MIC distribution of daptomycin and tigecycline was not different in isolates with decreased susceptibility to glycopeptides or tetracyclines, respectively. Ceftobiprole MICs were not correlated with oxacillin and cefoxitin MICs. These data indicate excellent activity of the newly developed agents ceftobiprole, daptomycin, and tigecycline against MRSA isolates recently recovered from hospitalized patients in Belgium, supporting their therapeutic potential for nosocomial MRSA infections.

\textit{Staphylococcus aureus} is a leading cause of skin and soft tissue infections, surgical site and catheter infections, pneumonia, bacteremia, and osteoarticular infections (19). In the past two decades, methicillin-resistant \textit{S. aureus} (MRSA) has increased in incidence in many parts of the world as an agent of nosocomial infections. More recently, community-acquired infections caused by MRSA have been reported in the United States, Australia, and Europe (7, 37).

MRSA strains are frequently resistant to multiple classes of antimicrobial agents including aminoglycosides, macrolides-lincosamides-streptogramins (MLS), and tetracyclines (13). Until now, glycopeptides have been considered as the drugs of choice for the treatment of severe MRSA infections. Linezolid has recently been recommended as an alternative treatment for some of these infections (31). The requirement for effective new agents to treat MRSA infections is becoming increasingly apparent due to the emergence of strains with reduced susceptibility to glycopeptides and, more recently, of strains resistant to vancomycin by transfer of the \textit{vanA} gene from \textit{Enterococcus faecalis} (3, 15). New agents including tigecycline and daptomycin have recently been introduced into clinical practice for resistant gram-positive infections (27, 33). Ceftobiprole is a novel broad-spectrum cephalosporin that is in phase 3 of clinical development (2).

Since 1992, the Belgian Reference Laboratory for Staphylococci has organized epidemiological surveillances to monitor the evolution of genotypes and antimicrobial resistance profiles of MRSA strains isolated in acute-care hospitals (10). The last survey conducted in 2001 showed a genotypic diversification of MRSA strains into seven major epidemic clones disseminated in Belgian hospitals. The predominant clone was puls-field gel electrophoresis (PFGE) type B2-sequence type 45 (ST45)-\textit{mec} IV and was recovered from 81% of participating hospitals (8). The aims of the present study were to update the distribution of epidemic MRSA clones in 112 Belgian hospitals in 2003 and to determine their in vitro susceptibilities to 22 antimicrobial agents including the new antistaphylococcal drugs ceftobiprole, tigecycline, and daptomycin.


MATERIALS AND METHODS

Survey methods and collection of bacterial strains. From January to December 2003, laboratories serving all Belgian acute-care hospitals (n = 180 sites) were invited to collect up to five nonduplicate clinical MRSA isolates per hospital site. These strains were referred to the Reference Laboratory for Staphy-
Molecular typing. Bacterial isolates were genotyped by Smal macrorestriction analysis of genomic DNA resolved by PFGE and analyzed using BioNumerics software version 2.5 (Applied Maths, Belgium) (6, 10). PFGE profiles were compared to a database of all hospitals and community-acquired clones previously described during the last 11 years in Belgium (6–10).

Determination of SCCmec type was performed by multiplex PCR on a subsample of 92 MRSA strains stratified to represent all PFGE types in proportion to their frequency of occurrence (26). For the nine most frequent PFGE types in the collection, the SCCmec type was determined for 56 isolates from different hospitals with a range of 2 to 20 isolates per PFGE. The remaining 46 isolates from each represented less frequent PFGE types. A representative set of MRSA strains (7, 18) were included in each run as controls.

Antimicrobial susceptibility testing. All strains were tested on vancomycin screen agar (VSA) and teicoplanin screen agar (TSA) (4, 5). For VSA, 10 μl of a 0.5 McFarland standard inoculum was spotted onto brain heart infusion agar supplemented with 6 mg/liter vancomycin (Becton Dickinson, Heidelberg, Germany) and incubated for a full 24 h at 35°C. For TSA, 10 μl of a 2 McFarland standard inoculum was spotted onto MH agar supplemented with 5 mg/liter teicoplanin and incubated for a full 48 h at 35°C. Strains that had a MIC of ≥4 mg/liter for vancomycin and/or teicoplanin by agar dilution or strains that grew on VSA or TSA were further characterized by the E-test macromethod (AB Biodisk, Solna, Sweden) for vancomycin and teicoplanin (41). Results of glycopeptide inhibition concentration testing were interpreted according to the following criteria: strains inhibited by both vancomycin and teicoplanin at ≥8 mg/liter or by teicoplanin alone at ≥12 mg/liter were considered to be hetero-glycopeptide-resistant.

Resistance profile determination. Resistance genes encoding the tetracycline efflux pump system, tetK, or ribosomal protection protein, tetM; aminoglycoside-modifying enzymes encoded by aac(6′)-Ie-aph(3′)-Ia, ant(4′)-Ia, and aph(3′)-IIIa genes; ribosomal methylases encoded by ermA and ermC; and the macrolide efflux pumps encoded by msrA and msrB genes were tested by PCR (9, 23, 38).

Exotoxin gene detection. The presence of Panton-Valentine leukocidin (PVL) ( lukS-lukF PV) and toxic shock syndrome (TSS) type 1 (TST-1) genes was tested by PCR on a random subsample of isolates belonging to each of the nine most frequent PFGE types as well as to PFGE type J, which was previously described in Belgian community-acquired MRSA (CA-MRSA) PVL-positive strains (7, 18). At least 10 isolates from different hospitals per PFGE type were screened for toxin genes, except for the most frequent PFGE types (B2 and A20), for which a 20% sample of isolates was tested.

RESULTS

Hospital participation and bacterial isolates. Of 547 isolates collected from 112 hospitals, 511 (93%) were confirmed to be MRSA isolates. Another 36 isolates were excluded after being identified as coagulase-negative staphylococci (n = 10) and...
oxacillin-susceptible *S. aureus* (*n* = 16) or if they did not grow on subculture (*n* = 10).

**Genotype distribution.** PFGE patterns of SmaI macro-restriction fragments classified 511 isolates into 15 groups and 36 types (Table 1). Ninety percent of the isolates belonged to nine PFGE types, two of which were predominant: B2 (49%) and A20 (19%). These two epidemic types were found in 95 of 36 types (Table 1). Ninety percent of the isolates belonged to restriction fragments classified 511 isolates into 15 groups and the PFGE type D8 ST228-SCCmeC (Table 2). Cefotibiprole showed excellent activity in these isolates, as indicated by low MIC50 and MIC90 values of 0.5 mg/liter and 2 mg/liter, respectively. Likewise, tigecycline and daptomycin had excellent activity with MIC50 and MIC90 values of 0.25 and 0.5 mg/liter, respectively. Resistance to tetracycline was higher than the frequency of resistance to ciprofloxacin.

Forty-two (8.4%) MRSA isolates grew on TSA after 48 h, and only one isolate (0.2%) grew on VSA. By the E-test macro-method, no isolate had an MIC of ≥8 mg/liter for vancomycin and teicoplanin, whereas six isolates (1.1%) had MICs of ≥12 mg/liter for teicoplanin. These isolates were genotypically PFGE type D8 ST228-SCCmeC 1 (*n* = 3), PFGE type G10 ST5-SCCmeC IV (*n* = 2), and PFGE type A1 ST247-SCCmeC 1 (*n* = 1).

**Resistance gene distribution.** Among aminoglycoside-resistant isolates, 221 isolates (43%) carried the *ant(4’S)* gene, 27 (5%) carried the *aac(6’)-aph(2’)* gene, and 10 (2%) carried the *aph(3’)* gene (Table 1). The *aac(6’)-aph(2’)* gene was associated with the *ant(4’S)* gene in 14 isolates and with the *aph(3’)* gene in 9 isolates. Resistance to MLS was mediated mainly by the *ermA* gene (*n* = 172) (33%), the *ermC* gene (*n* = 126)
(24%), or both methylese genes (n = 4). One macrolide-resistant isolate harbored both msrA/B genes. Of tetracycline-resistant MRSA strains (n = 53), the tetM gene was detected in 44 (83%) isolates, and the tetK gene was detected in 9 (17%) isolates. Isolates carrying the tetM gene were resistant to tetracycline (MIC > 32 mg/liter) and had minocycline MICs that were two- to fourfold higher (≥4 mg/liter) than those of isolates either without detectable tet genes or harboring the tetK gene alone (MIC ≤ 0.5 mg/liter). The distribution of resistance genes was highly correlated to the MRSA clonal types.

Toxin gene distribution. The presence of TSST-1 and PVL genes was determined for representative strains (n = 156) of PFGE types B2 (n = 58), A20 (n = 23), A21 (n = 10), G10 (n = 11), C1 (n = 11), A1 (n = 11), C3 (n = 11), D8 (n = 10), L1 (n = 10), and J (n = 1). The majority of strains were negative for both toxin genes. A minority (n = 14) harbored the TSST-1 gene, and one isolate carried PVL genes. MRSA isolates carrying the TSST-1 gene belonged predominantly to PFGE type G10-ST5-SCCmec II (n = 8). The TSST-1 gene was also found in six strains of PFGE types A21, B2, C1, C3, and L1. The PVL-positive isolate showed PFGE type J-ST30-SCCmec IV.

DISCUSSION

Since 2001, we have reported the diversification of epidemic MRSA clones in Belgian hospitals (8). Nine major clones that belong to the four MRSA lineages (CC5, CC8, CC22, and CC45) associated with nosocomial infections worldwide were identified. The changes in the prevalence of epidemic MRSA genotypes led to a shift in resistance patterns with a decreased proportion of multidrug- and gentamicin-resistant MRSA strains compared to surveys conducted in the 1990s (6, 10). In this study, the genotypic distribution and the resistance rates of nosocomial MRSA isolates to MLS and aminoglycosides were similar to those from the last survey in 2001, except for a further decrease in gentamicin resistance from 11% to 5% (P < 0.001) and the expansion of genotype A20 (P = 0.02) (8). The low prevalence (1.1%) of the hetero-GISA phenotype was similar to that (2.6%) observed in nosocomial MRSA strains from Belgian hospitals in 2001 (24). In the present study, most of hetero-GISA isolates belonged to gentamicin-resistant ST247-SCCmec I and ST228-SCCmec I MRSA clones as previously reported (P < 0.001) (24).

Ceftobiprole (formerly BAL9141) is a novel parenteral cephalosporin that has antimicrobial activity against a broad spectrum of gram-negative and gram-positive bacteria, including methicillin-resistant staphylococci (2). The latter activity of ceftobiprole is due to its high affinity to penicillin binding protein 2a. In the present study, ceftobiprole showed excellent in vitro activity against a large collection of isolates representative of recent Belgian nosocomial MRSA isolates and belonging to four pandemic clones, consistent with previously reported values (2, 40). The MIC of ceftobiprole was not influenced by oxacillin and cefoxitin MICs, by the SCCmec type, or by reduced susceptibility to vancomycin. There was no significant difference in MICs between different epidemic clones. In vitro data have shown that prolonged serial passage in the presence of subinhibitory concentrations of ceftobiprole failed to select resistant mutants (2). Those data suggested that ceftobiprole is a promising broad-spectrum cephalosporin with excellent anti-MRSA activity, including those strains with decreased glycopeptide susceptibility.

Daptomycin belongs to a new class of antimicrobials, the lipopeptides, which disrupt bacterial cytoplasmic membrane potential in the presence of calcium ions (33). Daptomycin is highly effective against gram-positive bacteria including multiple-antibiotic-resistant strains. In the present survey, all MRSA strains were very susceptible at low drug concentrations, including strains with decreased susceptibility to glycopeptides, in agreement with previously reported data (12, 34). In vitro studies aimed at selecting spontaneous daptomycin-resistant mutants were unsuccessful (32). Recently, the first cases of infection with daptomycin-resistant strains of MRSA have been reported in patients with deep-seated septic thrombophlebitis and osteomyelitis (14, 21, 39).

The tetracyclines exert antibacterial activity by interacting with the bacterial 30S ribosomal subunit and thereby inhibiting protein synthesis. Tigecycline belongs to the glyyclcycline class of compounds by modification of the 9 position of minocycline (27). In vitro data have shown that tigecycline is active against both gram-positive and gram-negative bacteria, with the main exceptions of Pseudomonas aeruginosa and Proteus species. Two major mechanisms of tetracycline resistance in staphylococci have been described: (i) energy-dependent efflux systems encoded by plasmid-located genes, tetK and more rarely tetL, and (ii) ribosomal protection protein encoded by a transposon-located or chromosomal tetM determinant. These efflux systems confer resistance to tetracycline but not to minocycline, whereas the ribosomal protection protein confers cross-resistance to all tetracyclines. In the present study, the prevalence of tetracycline resistance among nosocomial MRSA strains was low compared to data from previous European surveys (30, 36). Most of the tetracycline-resistant isolates harbored the tetM gene only, and no isolate carrying both genes was found. In our series, tigecycline had equivalent in vitro activity against tetracycline-sensitive and tetracycline-resistant MRSA strains, with an MIC range of 0.06 to 0.5 mg/liter (median, 0.25 mg/liter), irrespective of the presence of tetM or tetK genes, confirming a previous report (28). In the present study, the distribution of tetM genes was strongly linked with genomic lineage: 100% of strains belonging to PFGE type G10-ST5-SCCmec II and 67% of strains belonging to PFGE type A1-ST247-SCCmec I harbored the tetM determinant.

In the present survey, we further analyzed toxin-associated genes and the genomic background of Belgian MRSA strains. The majority of strains carrying the TSST-1 gene belonged to MRSA PFGE type G10-ST5-SCCmec II. This clone is closely related to the “New York-Japan clone,” which has been associated with neonatal TSS-like exanthematous disease in Japanese hospitals and with TSS in Belgium (16, 17). TSST-1 genes have been found in different staphylococcal pathogenicity islands (25). The presence of large mobile genetic elements like staphylococcal pathogenicity islands could explain considerable differences in SmaI DNA fragment patterns between strains of PFGE types G10 and C1, which were otherwise indistinguishable by MLST and SCCmec type.

Since 2001, PFGE type A20-ST8-SCCmec IV was the second most common genotype among nosocomial Belgian MRSA isolates (8). ST8-SCCmec IV MRSA strains were reported in...
U.S. hospitals, among PVL-positive MRSA strains responsible for community outbreaks in the United States and The Netherlands, and for sporadic cases of CA-MRSA infections in Belgium (7, 35). Belgian hospital-acquired PFGE type A20-ST8-SCCmec IV strains differed from related PFGE type A23-ST8-SCCmec IV CA-MRSA strains by their lack of msrA/B and PVL genes and by the presence of the ant(4′) and ermC resistance genes (7). These observations suggest the parallel evolution of hospital- or community-acquired S. aureus strains belonging to the same successful lineage into distinct epidemic clones that have acquired different resistance genes and virulence factors in the health care and community settings. In the United States, a similar phenomenon was observed with the parallel emergence of two clones belonging to the same ST8-SCCmec IV lineage, USA300 isolates, associated predominately with community-onset infections, and USA500 isolates, associated with health care infections (22).

The PVL-positive isolate was susceptible to all antibiotics, including ciprofloxacin, and belonged to the ST30-SCCmec IV clone that was first isolated in native Australian populations and more recently described in Europe (7, 37). It is closely related to the EMRSA-16 (ST36-SCCmec IV) clone, which is endemic in hospitals in Great Britain and other European countries (29). This nosocomial clone was infrequently recovered in Belgian hospitals in 2001. In this survey, we did not find any MRSA strain belonging to the epidemic PVL-positive ST80-SCCmec IV clone widely disseminated in Europe, including Belgium (7, 37).

In conclusion, this study has confirmed that MRSA genotypes isolated from patients in Belgian hospitals belong to several international epidemic clones with the predominance of gentamicin-susceptible ST45-SCCmec IV and ST8-SCCmec IV types. Nosocomial isolates of ST8-SCCmec IV differ from Belgian CA-MRSA isolates of this lineage by a distinct constellation of horizontally acquired resistance or virulence genes, suggesting a divergent evolution in different populations and selective pressures. Moreover, we found one PVL-positive MRSA isolate belonging to the ST30-SCCmec IV clone, which was recently described as causing community infections in Belgium. The new antimicrobial drugs cefotiboprole, daptomycin, and tigecycline showed excellent activity against all MRSA strains that were recently recovered from patients in Belgian hospitals, including epidemic and sporadic clones and glycopeptide- as well as tetra-cycline-resistant strains.

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