High prevalence in cystic fibrosis patients of multiresistant hospital-acquired methicillin-resistant *Staphylococcus aureus* ST228-SCCmecI capable of biofilm formation

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**Objectives:** Although methicillin-resistant *Staphylococcus aureus* (MRSA) is increasingly recognized in cystic fibrosis (CF) patients, it has not been sufficiently studied in large series. We analysed all MRSA isolates recovered from respiratory secretions of patients attending our CF unit (1994–2006).

**Methods:** Antibiotic susceptibilities were determined using both planktonic and sessile bacteria as inocula. Genetic relationships were determined by PFGE and multilocus sequence typing (MLST). SCCmec type and the presence of the *pvl* gene were also investigated.

**Results:** A total of 93 MRSA isolates (1–20 isolates per patient) were recovered from 18 of 77 CF patients with positive staphylococcal culture. Mean prevalence (4.4%) increased (*P* < 0.001) over time. All isolates were susceptible to linezolid, quinupristin/dalfopristin and co-trimoxazole but presented high resistance rates to amikacin (90%), gentamicin (85%), levofloxacin (81%) and erythromycin (69%). Except for macrolides and gentamicin, isolates were less susceptible growing in biofilms than in planktonic cultures. Fifteen different PFGE patterns were found, one of them consistently recovered for 6 years in the same patient. Identical clones were detected in several unrelated patients. MLST demonstrated that the international ST228 was the most frequent (67%) clone. The *pvl* gene was negative in all isolates and the SCCmec corresponded to types I (97%) and IV (3%). Strong mutators were not detected, but a considerable number were considered weak mutators.

**Conclusions:** Distinct microbiological and molecular features were detected among CF-MRSA isolates, probably due to adaptation to specific conditions in CF patients. Prevalence of MRSA increased among these patients, most of them colonized with a multiresistant biofilm-forming clone belonging to ST228-SCCmecI, suggesting cross-transmission or a common source.

Keywords: MLST, PFGE, MRSA, biofilms, mutation frequency

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**Introduction**

Recurrent and chronic pathogenic respiratory tract colonization is the most important cause of morbidity and mortality in cystic fibrosis (CF) patients, and *Staphylococcus aureus* is frequently one of the first organisms colonizing their respiratory tract.1 Recently, an increased prevalence of methicillin-resistant *S. aureus* (MRSA) isolates in these patients as well as its association with lower lung function and worse prognosis have been demonstrated.1–4 Moreover, the isolation of this pathogen can be the cause of a patient being rejected for pulmonary transplant and preventive strategies have been proposed for minimizing the risks of MRSA acquisition.7–9 Several studies suggest patient-to-patient transmission of the different CF pathogens, including MRSA.2,3,8,10

CF pathogens are well adapted to the CF pulmonary environment and are difficult to eradicate. As with *Pseudomonas aeruginosa*, *S. aureus* may persist for years in the same patient.11 It has been demonstrated that *S. aureus* isolates present a higher proportion of hypermutators in CF populations than those from...
non-CF populations, which might benefit antibiotic resistance and persistence. Moreover, the increased ability of CF pathogens to form biofilms makes the action of antimicrobial agents and pathogen eradication difficult. Neither of these features have been well studied in CF-MRSA isolates nor has the presence of virulence factors such as the pvl gene, recently detected as an emerging problem in CF patients.

The aim of this study was to characterize the MRSA population recovered from CF patients from our institution, determining their genetic relationships, their antibiotic susceptibility patterns in both planktonic and biofilm modes of growth and their mutational frequencies.

Materials and methods

Patients, bacterial isolates and microbiological study

A retrospective study of all patients (n = 152, median value 75.5 patients/year) who attended our CF unit between January 1994 and December 2006 was performed. From these, 77 patients presented positive staphylococcal sputum cultures, which corresponded to MRSA in 18 patients (equal number of males and females; median age of 23.6 years, range 5–33). Ninety-three MRSA isolates were recovered (1–20 isolates per patient) from different respiratory sources: sputum samples (88.2%), bronchial aspirates (9.6%) and bronchial biopsy (2.2%). Sputum samples were homogenized with N-acetyl-cysteine and processed by a modified quantitative technique. Columbia 5% blood, MacConkey, mannitol-salt and a selective Burkholderia cepacia agar medium were incubated in air for 24 h at 37°C and then for an additional period of 24 h at 25°C. In addition, bacitracin-chocolate agar was plated and incubated in 5% CO₂ for 48 h at 37°C. A culture was considered positive for S. aureus when growth of this organism was observed, irrespective of the bacterial count. All colonies with compatible morphology with S. aureus were subcultured and identified using the semiautomatic Wider System (Fco. Soria Melguizo, Madrid, Spain).

For antimicrobial susceptibility testing in biofilm, an additional control collection of 20 MRSA consecutive isolates recovered from blood cultures from non-CF patients attending our hospital in 2006 was included.

Population structure analysis

PFGE-SmaI was applied to analyse genetic relatedness of MRSA isolates. The CHEF DR-III apparatus (Bio-Rad, Birmingham, UK) was used for 24 h with the following settings applied at 6 V/cm²: 5–30 s and with the Lambda Ladder PFGE Marker (New England Biolabs, Inc., Beverly, MA, USA). Macrophragment fragments were compared and interpreted both visually and with the Phoretix 5.0 software, using the Dice coefficient for dendrogram construction and clone definition. The criteria used were: unrelated strains, ≤0.7 of Dice’s coefficient; probably related, 0.8–0.8; closely related, 0.8–0.9; and identical, ≥0.9. One isolate per clone were further typed by multilocus sequence typing (MLST) following a previously published scheme and also the web site www.mlst.net.

The staphylococcal chromosomal cassette (SCCmec) type was determined in all isolates using the scheme proposed by Yang et al. using the S. aureus ATCC 49775 strain as a positive control.

Antimicrobial susceptibility testing

Susceptibility to different antimicrobials was determined using the standard microdilution method using the Wider System. Methicillin resistance was confirmed with the cefoxitin disc diffusion test.

The antibiotic susceptibility of one isolate each of different PFGE patterns was also tested by standard procedures to determine the MIC; it was also tested in biofilm formation to assess the minimal biofilm inhibitory concentration (MBIC). Biofilm assay was performed as previously published, using brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) as the medium for biofilm growth. The ATCC MRSA 49476 strain was included as an internal control. All experiments were performed in duplicate and the median value of both experiments was calculated.

Determination of mutation frequencies

Mutation frequencies were determined in triplicate in rifampicin-susceptible MRSA isolates from an overnight culture in 10 mL of BHI. For total viable count determination, an aliquot of 100 µL was diluted in BHI broth and plated onto 5% sheep blood agar plates. The remaining culture was centrifuged for 5 min at 2500 g. The pellet was resuspended in 500 µL of BHI broth and seeded on 5% sheep blood agar plates containing 100 mg/L rifampicin. Mutation frequency values have been reported as the proportion of rifampicin-resistant colonies (detected after 72 h of incubation in a 5% CO₂ atmosphere) versus total viable counts. Results correspond to the mean value obtained in duplicate experiments, each with duplicate colony counts. ATCC 43300 and PAO1ΔmutS strains were used as nonmutator and hypermutator controls.

Statistical analysis

A linear regression using the SPSS software was applied to investigate the statistical significance of the prevalence increase observed.

Results

Prevalence of MRSA

Between 1994 and 2006, 152 patients attended the CF unit in our institution (mean 76 patients/year, range 57–94 patients/year). Eighteen of 77 CF patients (23%) with positive staphylococcal culture were infected/colonized with MRSA. This pathogen was not recovered during 1994 and 1995, but 93 MRSA isolates were detected in 18 patients from 1996 to 2006 (mean 9.7, range 1–20) (Table 1 and Figure 1). The overall prevalence of MRSA among patients followed by our CF unit was 4.4%, with a clear increasing trend during the last years, reaching the highest value in 2006 (8.4%) (Figure 1). Statistical significance (P < 0.001) of this prevalence increase was corroborated by a linear regression which showed an R² coefficient of 0.92.

Dynamics of MRSA population

Fifteen different PFGE patterns were found among the 93 isolates tested (Table 1 and Figure 2). Fourteen patients presented a single clone, whereas two different clones were observed in three patients, and one patient carried three different MRSA isolates. Homogeneous antibiotic resistance markers were detected in all isolates belonging to the same clone. Persistence of a single MRSA clone was detected in 77.8% of patients. It is of note that in one patient (no. 15) this persistence was documented from 1996 to 2006, with 24 MRSA-positive cultures during this period.
One isolate per clone was further characterized by MSLT. Five different sequence types (STs) were identified: ST228 (8 pulsotypes, 44 isolates and 12 patients), ST5 (2 pulsotypes, 5 isolates and 2 patients), ST247 (1 pulsotype, 3 isolates and 1 patient), ST72 (1 pulsotype, 1 isolate and 1 patient) and ST235 (1 pulsotype, 14 isolates and 2 patients). Two patients (nos 10 and 11) were brothers living together and shared the pulsotype B (ST228) (Table 1 and Figure 2). Unexpectedly, in isolates belonging to N and O pulsotypes (representing 26 isolates from three different patients), the MLST analysis was inapplicable, as we were unable to amplify any of the housekeeping genes used in MLST, except for the \textit{arc} gene corresponding to allele 1.

The bands obtained after the double \textit{Hin}fI and \textit{Bsm}I digestion of the \textit{ccrB}-PCR amplification corresponded to those proposed for SCC\textit{mec} types I (97%) and IV (3%). A positive \textit{pvl} gene amplification was not obtained in any CF-MRSA isolate.

### Table 1. MRSA population results in the 18 CF patients

<table>
<thead>
<tr>
<th>Sequence type</th>
<th>Pulsotype</th>
<th>No. of isolates</th>
<th>Patients (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST228</td>
<td>A</td>
<td>21</td>
<td>1 (4), 2 (3), 4 (1), 9 (6), 17 (7)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7</td>
<td>10 (3), 11 (1), 18 (3)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>5 (1)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>4</td>
<td>8 (4)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>4</td>
<td>3 (4)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2</td>
<td>3 (2)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>2</td>
<td>3 (2)</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>3</td>
<td>12 (3)</td>
</tr>
<tr>
<td>ST5</td>
<td>I</td>
<td>4</td>
<td>11 (4)</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>1</td>
<td>17 (1)</td>
</tr>
<tr>
<td>ST247</td>
<td>K</td>
<td>3</td>
<td>13 (3)</td>
</tr>
<tr>
<td>ST72</td>
<td>L</td>
<td>1</td>
<td>6 (1)</td>
</tr>
<tr>
<td>ST235</td>
<td>M</td>
<td>14</td>
<td>13 (12), 14 (2)</td>
</tr>
<tr>
<td>NT</td>
<td>N</td>
<td>1</td>
<td>7 (1)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>25</td>
<td>15 (20), 16 (5)</td>
</tr>
</tbody>
</table>

NT, not typeable.

### Antibiotic susceptibility

In the standard microdilution susceptibility testing, all 93 CF-MRSA isolates were susceptible to linezolid, quinupristin/dalfopristin, co-trimoxazole and glycopeptides, whereas variable resistance rates were detected to amikacin (90%), gentamicin (85%), levofoxacin (81%), erythromycin (69%), clindamycin (69%), rifampicin (29%) and fosfomycin (19%).

The ability of strains to form biofilms was corroborated in 14 of 15 MRSA CF pulsotypes, in all 20 blood culture isolates and also in the ATCC MRSA 49476 strain (Table 2). As expected, MBIC values were higher than the corresponding MIC values, especially when considering MBIC\textsubscript{90}, although some special features were detected. Interestingly, with regard to the MIC\textsubscript{50} and MBIC\textsubscript{50} values, CF isolates (but not isolates from blood cultures) were more susceptible to clindamycin, erythromycin, azithromycin and gentamicin in a biofilm mode of growth than in a planktonic mode of growth (Table 2).

### Mutation frequencies

Sixty-six of 93 CF-MRSA isolates were rifampicin-susceptible, thus allowing rifampicin mutation frequency determination. Strong hypermutable strains were not detected, although weak mutators were observed (Figure 3). Overall, the rifampicin mutation frequency ranged from $1.6 \times 10^{-11}$ to $1.9 \times 10^{-8}$ (mean: $1.3 \times 10^{-9}$). The analysis of mutation frequency distribution suggests a tri-modal profile, the first ($1 \times 10^{-11}$–$7.5 \times 10^{-10}$) corresponding to the normomutators, and including the majority of the isolates, whereas the second and the third modal peaks ($5 \times 10^{-9}$ and $5 \times 10^{-8}$) could be related to different types of weak hypermutable strains.

### Discussion

Special features, including a hypermutational phenotype, the ability to form biofilms and high antimicrobial resistance rates, have been demonstrated in CF pathogens. These peculiarities could be the result of an adaptive process in their special habitat conditions.\textsuperscript{12,15,16,18,19,25,26} Although different studies have
focused on CF patients or on MRSA isolates, until now these distinct features have not been examined in the CF-MRSA population. Our retrospective study included all patients with MRSA bronchial colonization in our CF unit (\( n = 18 \)) during a large follow-up period (1994–2006), which enabled the recovery of a considerable number of MRSA isolates (\( n = 93 \)).

MRSA is a major pathogen in the hospital setting causing serious infections that usually present multiresistance to many antibiotics. Moreover, the increased frequency of this organism in the community, especially with carriage of virulence factors, including \( pvl \), is a matter of concern. \(^{21,27} \) Although the real role of MRSA colonization in CF respiratory tract deterioration has not yet been clarified, its presence can be the cause of rejecting a patient for pulmonary transplant. Several risk factors have been identified and different epidemiological measures have been proposed for preventing MRSA acquisition in CF patients. \(^{7,8} \) Overall prevalence of MRSA isolation among our CF patients was 4.4%, but a rapid and significant increase was detected over recent years, reaching 8.5% in 2006. A similar tendency has been observed in other institutions but with higher prevalence figures, in keeping with the last available Annual Report from the Cystic Fibrosis Foundation Patient Registry (17.2%). \(^{3,6} \) This trend could be related to the current increased prevalence of MRSA in both hospitalized and non-hospitalized patients and/or circulation of more transmissible clones. \(^{17} \) In recent studies in Europe, the prevalence of MRSA isolates in CF patients ranged from 5% to 27%. \(^{3,6,18} \) Despite this increase, consensus protocols regarding MRSA eradication in CF patients have not yet been clearly defined. In our CF unit, MRSA colonization is individually evaluated and patients are only treated during exacerbation processes, but patient segregation protocols are not applied unless a patient is hospitalized.

The population analysis demonstrated that the STs found (Table 1) are those that are commonly found among MRSA. A recent study demonstrated that ST228, the predominant clone (67%) in our study, was also prevalent (18.3%) among MRSA circulating clones in Spain, presenting an antibiotic

Table 2. Antibiotic susceptibility in biofilm formation results of CF and blood isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Origin</th>
<th>MIC(_{50})</th>
<th>MBIC(_{50})</th>
<th>MIC(_{90})</th>
<th>MBIC(_{90})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linezolid</td>
<td>CF</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>8</td>
<td>16</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>CF (\geq 256)</td>
<td>8</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>(\leq 0.25)</td>
<td>(\leq 0.25)</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>CF (\geq 256)</td>
<td>32</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>128</td>
<td>128</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>CF (\geq 256)</td>
<td>16</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>CF 0.25</td>
<td>64</td>
<td>0.5</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>0.125 1</td>
<td>0.25</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>CF 16</td>
<td>4</td>
<td>64</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>16</td>
<td>64</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>CF (\geq 256)</td>
<td>16</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>4</td>
<td>16</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>CF 2</td>
<td>2</td>
<td>4</td>
<td>(\geq 256)</td>
<td>(\geq 256)</td>
</tr>
<tr>
<td></td>
<td>blood</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td>(\geq 256)</td>
</tr>
</tbody>
</table>

Bold formatting indicates MBIC values lower than MIC values.
multiresistance phenotype and SCC\textit{mec} type I similar to our isolates.\textsuperscript{28} In the same study, ST247 corresponded to 6\% of the Spanish population, whereas in our CF-MRSA isolates ST247 only represented 3\%. Moreover, both ST228 and ST247 have been identified as two of the most prevalent hospital-acquired MRSA (HA-MRSA) epidemic strains circulating in Europe.\textsuperscript{29–31}

In 15 of our 18 CF patients (83\%), colonization with a single MRSA clone was observed, and ST228 was the most frequently detected (61\%). Persistence of this clone during the period of study was demonstrated in 10 patients (56\%). In the other three patients, two different clones were detected (ST228/ST5 in two patients and ST235/ST247 in one patient) although without coexistence (Figure 2). Interestingly, several PFGE pattern differences were detected among the persistent clones within the same patient that might indicate, as with \textit{P. aeruginosa}, a particular evolution in the bronchial compartment. Moreover, this was also found with the same clone in different patients. Nearly 75\% of our patients shared MRSA isolates with similar PFGE belonging to ST228, suggesting cross-transmission or a common environmental source.

A total of 26 isolates corresponding to three patients and presenting two different PFGE patterns were unsuitable for MLST. PCR experiments were unsuccessfully repeated using different conditions, including newly designed primers (data not shown), but only one (\textit{arcc} gene) of the seven housekeeping genes was amplified. We suspected that these genes could suffer mutational or deleting events, and further studies are necessary to clarify this issue.

Recent typing studies have demonstrated that carriage of the \textit{mec} element is limited to a few MRSA clones,\textsuperscript{32,33} which include all of the clones detected in our study. The method used for SCC\textit{mec} typing was that published by Yang \textit{et al.}\textsuperscript{21} based on double digestion of a \textit{ccrB} amplified fragment. Using this method, two different patterns corresponding to types I (97\%) and IV (3\%) were observed in our isolates, although the band positions were not exactly the same. The special features of these CF strains were also reflected in the SCC\textit{mec}, which were quite different from those previously published.

HA-MRSA isolates are usually associated with SCC\textit{mec} types I, II and III, and tend to be resistant to all antimicrobials with the exception of glycopeptides. In contrast, community-acquired MRSA are linked to SCC\textit{mec} type IV, the presence of the \textit{pvl} gene and are usually susceptible to non-\textit{\beta}-lactam antibiotics. Our CF isolates fit with HA-MRSA, presenting resistance to multiple antibiotics (macrolides, aminoglycosides and quinolones). Moreover, we could not demonstrate the presence of \textit{pvl} in our population. This possibility has recently emerged in CF patients\textsuperscript{16} and will create a new threat as these isolates are generally associated with a poorer prognosis.\textsuperscript{27,34,35}

The biofilm experiments showed the great ability of MRSA to grow in this manner. In fact, all 20 blood culture isolates and 16 of the 17 CF isolates were biofilm formers ($P = 0.27$). This was in contrast to our experience with \textit{Streptococcus pneumoniae} isolates in which CF isolates were found to be significantly more able to form biofilms than those from blood.\textsuperscript{15}

The association among biofilm formation and HA-MRSA has been recently suggested and could give an advantage to this pathogen when chronically colonized.\textsuperscript{16} Although sessile growth usually implies an increase in the MIC values, in our experiments considerably lower values of MBIC\textsubscript{30} have been observed for macrolides and gentamicin. This suggests that these antibiotics are very active against biofilm formation, as previously reported for \textit{P. aeruginosa}.\textsuperscript{37}

Finally, a high proportion of weak hypermutator isolates among CF macrolide-resistant \textit{S. aureus} was detected when compared with non-CF isolates.\textsuperscript{12} The median mutation frequencies among our CF-MRSA isolates were $1.3 \times 10^{-9}$, whereas for the CF macrolide-resistant \textit{S. aureus} previously published the frequencies were $3.4 \times 10^{-6}$.\textsuperscript{12} These differences might be due to the different methodologies used or a particular characteristic of the MRSA population, which have a long cell-division period, and the growth curves are delayed in comparison with methicillin-susceptible \textit{S. aureus} (A. M., R. Del C. and R. C., unpublished results).

In conclusion, strains from the antibiotic multiresistant HA-MRSA ST228 clone colonized a significant proportion of our CF patients. All CF-MRSA isolates have special features including SCC\textit{mec} elements and mutation frequencies. Additional studies are needed to understand the dynamics of colonization/infection of MRSA in CF patients, and the role of this organism in their prognosis.

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Transparency declarations

None to declare.

References

MRSA from cystic fibrosis patients


