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Methicillin resistance and vancomycin heteroresistance in in cystic fibrosis patients

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► **To cite this version:**

V. Cafiso, T. Bertuccio, D. Spina, F. Campanile, D. Bongiorno, et al.. Methicillin resistance and vancomycin heteroresistance in in cystic fibrosis patients. *European Journal of Clinical Microbiology and Infectious Diseases*, 2010, 29 (10), pp.1277-1285. 10.1007/s10096-010-1000-5 . hal-00605684

HAL Id: hal-00605684

<https://hal.science/hal-00605684>

Submitted on 4 Jul 2011

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Editorial Manager(tm) for European Journal of Clinical Microbiology & Infectious
Diseases
Manuscript Draft

Manuscript Number: EJCMIID-D-09-00638R1

Title: Methicillin-resistance and vancomycin heteroresistance in Staphylococcus aureus in cystic fibrosis patients

Article Type: Article

Keywords: HA-MRSA; CA-MRSA; hVISA; cystic fibrosis

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Abstract: Methicillin-resistant Staphylococcus aureus (MRSA) infections are increasingly reported in cystic fibrosis (CF) populations worldwide. In this paper, we sought to look at the epidemiology, the molecular characterization and the antibiotic resistance of MRSA isolates in our cohort of CF patients. All MRSA were collected prospectively at the University Hospital of Catania (I) in a two-year study between mid 2005 - mid 2007 and underwent molecular, pathotype and susceptibility characterizations.

Our study demonstrates persisting infections with both HA- and CA-MRSA including PVL-positive strains in our CF population with an overall prevalence of 7.8%. We demonstrated that in these patients, persistence was sustained by either identical clones that underwent subtle changes in their toxin content or by different clones over time. The isolation of MRSA in our CF population aged 7-24, was associated with an increased severity of the disease even if, due to the small sample of patients included and the paucity of data on the clinical outcome, these results cannot be conclusive. Furthermore, 35.7% of strains were hVISA, questioning the use of glycopeptides in the treatment of MRSA infections in these patients.

Response to Reviewers: Dear Dr vanBelkum,
Thank you very much for the revision of our ms.

Please find below our answers to the referees' comments:

Referee 1

Specific comments

1) The referees' comments is absolutely pertinent. Our tests were all carried out under standard conditions, however, even if one of our aims was not the detection of SCVs by using specific conditions, we were fully aware that they could be present and we were very careful in the analysis of each colony for the eventual presence of these variants. We have added data to the Material and Methods section and commented on this in the discussion section.

2) The result have been modified accordingly

3) a. the reference has been added

b. done

4) a. done

b. as the patients only had samples collected during a recurrent attack, the number vary depending on the patient. This was added to the material and Methods section.

c. the mean number of the culture/patient was not included for the reason above described

d. done

e. yes, added

f. done

g. done

5) a. MRSA and MSSA. adde

b. this is true

6) done

Best regards

Stefania Stefani

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Methicillin-resistance and vancomycin heteroresistance in *Staphylococcus aureus* in cystic fibrosis patients

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Short title: MRSA in cystic fibrosis patients

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are increasingly reported in cystic fibrosis (CF) populations worldwide. In this paper, we sought to look at the epidemiology, the molecular characterization and the antibiotic resistance of MRSA isolates in our cohort of CF patients. All MRSA were collected prospectively at the University Hospital of Catania (I) in a two-year study between mid 2005 - mid 2007 and underwent molecular, pathotype and susceptibility characterizations.

Our study demonstrates persisting infections with both HA- and CA-MRSA including PVL-positive strains in our CF population with an overall prevalence of 7.8%. We demonstrated that in these patients, persistence was sustained by either identical clones that underwent subtle changes in their toxin content or by different clones over time. The isolation of MRSA in our CF population aged 7-24, was associated with an increased severity of the disease even if, due to the small sample of patients included and the paucity of data on the clinical outcome, these results cannot be conclusive. Furthermore, three strains were hVISA, questioning the use of glycopeptides in the treatment of MRSA infections in these patients.

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INTRODUCTION

There has recently been an increasing number of reports worldwide dealing with the isolation of *S.aureus*, particularly MRSA, in cystic fibrosis (FC) patients, with prevalences ranging from 0.1% in 1995 to 18.9% today [1-3].

Despite this increasing frequency of isolation and the serious potential morbidity from MRSA in these patients, very little is known about its clinical significance and even less is known about the molecular characteristics of virulence and resistance of these clones.

With regard to the clinical significance of MRSA in the lung of CF patients deterioration of the lung function and MRSA isolation was addressed in many papers but the importance of isolating this microorganism was poorly stressed; however, in a recent longitudinal study, the persistent presence of MRSA correlated with worsening X-ray images, leading to significantly more courses of intravenous antibiotics affecting patient growth, and eventually to a more rapid decline in lung function [4-9].

With regard to the biological characterization of the microorganism, MRSA is an extraordinarily flexible pathogen, able to cause infections in patients with known risk factors [10], but recently it was isolated in the community worldwide, causing infections in healthy people with rapid and severe onset and sometimes responsible for severe outcome. Community-associated methicillin-resistant *S.aureus* (CA-MRSA) are known to be genetically different from the nosocomial strains and to have enhanced virulence, due to a complex make up of toxins and enzymes. CA-MRSA generally harbor small mobile chromosomal *mec* cassettes (SCC*mec*) types IV, V, VI or VII and several subtypes especially type IV that mediate methicillin-resistance [11-13]. These cassettes, in these strains, are in some cases associated with the Panton Valentine Leucocidin (PVL) and other virulence factors, usually not found in hospital associated methicillin resistant *S.aureus* (HA-MRSA) [14,15].

1 In Europe, and in Italy, on the contrary to what has happened in the USA where the
2 diffusion of CA-MRSA is due to the almost exclusive spread of two major clones (ST1 USA400
3 and ST8 USA300), many different clones belonging to diverse STs have been isolated
4 [13,15,16-19]. This is true in many community infections but not in CF, in which many studies
5 have failed to demonstrate an association between PVL and MRSA or, when found, the strains
6 were not characterized [7,20,21]. A recent large cohort of Italian patients with CF and CA-
7 MRSA were all found to be negative for the PVL gene [22].

8 Furthermore, less is known about the source of the MRSA clones infecting these
9 patients who, due to their natural clinical history, are considered out-patients attending,
10 during their life, various specific centers or hospital wards. The lifestyle of these patients is
11 surely a risk factor for the acquisition of MRSA and the paucity of papers dealing with this
12 problem, stimulated us to carry out this research.

13 The purpose of this 2-year prospective study was to determine the frequency with
14 which MRSA was acquired in our CF population attending the University Hospital of Catania,
15 Italy, as well as evaluating persistence and carrying out the molecular characterization of the
16 strains including identifying their virulence content. MRSA were also evaluated for their
17 resistance to antibiotics and their presence and persistence was correlated with lung function
18 during the study period.

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MATERIALS AND METHODS

Study design and staphylococcal isolation

In the period between February 2005 – October 2007, 64 patients attending our CF center were screened during routine examinations. Respiratory cultures (217, collection was patient dependent) were plated onto horse blood, colistin nalidixic acid, MacConkey, mannitol salt agar and *Burkholderia cepacia* selective plates (Oxoid, Basingstoke, UK). Our purpose was to study only the *S.aureus* isolated, however all the colonies were carefully examined for the eventual presence of SCVs (none were found).

The FEV1 was registered for each patient.

Isolates were identified based on positive tube coagulase and API-Staph (Bio-Merieux, Marnes-la-Coquette, France), and a positive amplification of the 16S *S. aureus* rDNA (data not shown). The resistance to methicillin was confirmed by the presence of the *mecA* gene [23]. All patients positive for MRSA were longitudinally monitored and all MRSA serial isolates were included in the study.

Molecular characterization of MRSA isolates

To determine if the 14 MRSA isolates were CA and/or HA-MRSA associated clones the following were carried out: genomic macro-restriction profiling using PFGE [24], Multilocus Sequence Typing (MLST) as previously published [23], SCC*mec*-typing by multiplex polymerase chain reaction (PCR) followed by different multiplex-PCR protocols, focusing on the *mec* gene complex and the *ccr* gene complex [25-27].

Pathotype and biofilm determination

1 A complete pathotype including the capsular type, the membership to an *agr*-group
2 and the adhesin and toxin gene content was performed by using both conventional PCR and a
3 new protocol, established in our laboratory, of multiplex PCR as previously published [13,28].
4

5 Furthermore, due to the importance of biofilm production in CF patients, all strains
6 were tested for their ability to produce biofilm by a spectrophotometrical quantitative assay.
7 Each strain was grown in Tryptone Soy broth (Oxoid, Basingstoke, UK), with the addition of
8 0.25% glucose (TSBG). These assays were performed in microtitre plates as previously
9 published [29-32].

10 Antibiotic susceptibility testing and heteroresistance determination

11 Antibiotic susceptibility was determined for the following antibiotics: methicillin,
12 vancomycin, teicoplanin, tobramycin, levofloxacin (Sigma Chemical Com St Louis MO),
13 linezolid (Pfizer Inc New York NY), tigecycline (Wyeth Pharmaceutical, Collegeville, PE) and
14 daptomycin (Novartis, Basel, CH). MICs were performed in Cation Adjusted MH broth
15 following CLSI guidelines [33]. Heteroresistance was determined by macroEtest (AB-Biodisk
16 SE) and confirmed by population analysis (PAP) as previously described [34-36]. Briefly,
17 colonies from cultures grown overnight on tryptic soy agar were inoculated into tryptic soy
18 broth. After incubation for 24h, dilutions of 10^{-3} (10^5 CFU/ml) and 10^{-6} (10^2 CFU/ml) were
19 prepared in saline, and 50 μ l were inoculated onto BHI agar plates containing 4.0, 6.0, 8.0,
20 12.0, 16.0 mg of vancomycin/liter and 4.0, 8.0, 16.0, 32.0 mg of teicoplanin/liter, respectively.
21 Results were compared to the Mu3 (hVISA) and Mu50 (VISA) standard strains, kindly
22 provided by NARSA (Network on antimicrobial resistance in *Staphylococcus aureus*,
23 <http://www.narsa.net/>).

RESULTS

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During the study period, 5 out of the 64 patients were found to be infected only with MRSA (7.8%). In these five patients, co-colonization with MSSA was observed in patients 3, 4 and 5, whereas no co-infection with *Pseudomonas aeruginosa* and/or *Burkholderia cepacia* was observed. Table 1 shows the age, the FEV1 index and the antibiotic therapy of the 5 patients positive for MRSA, and the main genetic and virulence characteristics of the 14 isolates. All strains were *mecA* positive with oxacillin MICs varying from 2-4 (considered low-susceptible or resistant by CLSI) to > 256 mg/L and belonged to the *agr* type I. The isolates possessed the capsular antigen type 5 and showed different PFGE profiles when all patients were compared, but almost identical multiple isolates in the same patient. The comparison of the macrorestriction profiles with the STs showed that in two cases (patients 1 and 2), the MRSA isolated were nosocomial clones circulating in Italy and in Europe i.e. ST22 (EMRSA 15) and ST247, while the others (patients 4 and 5) corresponded to CA-MRSA isolates, belonging to different STs, i.e. ST8, 291 and 72; only in patient 3 (ST8) was it difficult to assign these isolates to HA- or CA-MRSA, only on the bases of their molecular features, considering this clone a bridge between hospital and community-associated strains. As previously observed in these patients, despite the use of the correct antibiotic courses, each clone tended to persist inside the lung, with diverse duration related to the reactivation of symptoms of each patient. The only exception was patient 5, in whom, after the initial isolation of a PVL-positive ST8-CA-MRSA IVa, identified as a USA300 clone variant (without the ACME genomic island), the strain was replaced by ST72 and these latter strains possessed an identical or closely related PFGE profile.

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All strains were medium or strong biofilm producers (table1), and this result seemed consistent with the ability of these microorganisms to adhere and persist in the lung of these patients.

1 The presence of *mecA*, *mecR/I* was determined and, while all strains possessed the
1
2 *mecA* gene, only seven contained Δ *mecR* (class B *mec*-complex) and the same strains had an
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4 SCC*mec* cassette organized similarly to the type IV (ST22), type IVc and IVa (ST8) all those
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7 4 possessing recombinases *ccrA₂B₂*, while type I (ST247) showed recombinases *ccrA₁B₁*. In the
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10 5 other seven strains it was impossible to define a structured SCC*mec*, at least by using the
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12 6 multiplex PCR protocols [25-27].
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15 7 Further studies involving sequencing will be necessary to determine the hypothetical
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17 8 structure of these *mec* regions.
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20 9 Concerning the analysis of virulence gene content, a core group of adhesins i.e. *icaA*,
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22 10 *clfA/B*, *fnbA*, *sdrE*, *spa*, and *atl* were present in all strains. With regard to the toxin core genes,
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25 11 hemolysins (hl-*s*) and leucocidin E (*lukE*) were always present, while the group of accessory
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27 12 toxin genes i.e. *sea*, *sec*, *spB*, *tst*, *lukS/F*-PVL, were present in various percentages inside the
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30 13 strains tested. Only patients 4 and 5 were infected by PVL positive strains at the beginning
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32 14 while, in both patients, the serial isolates, although MRSA, were subsequently found to be
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35 15 different. With regard to other toxins, enterotoxin A (*sea*) was present in isolates of patients
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37 16 1 and 3, while leucocidin E (*lukE*) was distributed in all isolates, as stated before. MRSA
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40 17 strains isolated in patient 4, despite belonging to the same ST and PFGE types, showed subtle
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42 18 changes in their virulence gene content, having lost the *lukS/F* gene present in the first isolate,
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45 19 but continuing to contribute to the worsening lung function of the patient even with the PVL
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47 20 negative strains. Furthermore, the MRSA ST291 isolated in this patient lacked the association
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50 21 between *spB* and *lukE*, commonly localized on the pathogenic genomic island vSa β [37]. A
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52 22 different situation was observed in patient 5, in whom after the first infection sustained by a
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55 23 variant of USA300 (ST8-IVa, ACME negative) PVL and TSST-1 positive, four strains belonging
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57 24 to ST72 were found. These strains were PVL negative, but one of them still maintained the
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1 toxic-shock syndrome toxin-1 (TSST-1). Generally speaking, among adhesion genes, *cna* was
2 never detected in our strains, while among toxin genes *eta*, *sed*, and *sej* were never detected.

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4 As shown in table 1, the age of the 5 patients ranged from 7 to 24 years and in patients
5 1, 2 and 5, a history of worsening pulmonary obstruction, as demonstrated by a decrease in
6
7 FEV1 index, was found.

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9 All MRSA strains were tested for their susceptibility to some anti Gram-positive
10 antibiotics. As shown in table 2, multiple isolates of the same patient maintained the same
11
12 level of susceptibility. All strains were resistant to tobramycin; 10/14 resistant to
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14 levofloxacin, all strains were susceptible to linezolid and tigecycline. Eight out of the 14
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16 strains showed MICs ≥ 1 mg/L to vancomycin and two strains showed MICs of 1.5 mg/L to
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18 daptomycin. MacroEtest and PAP analysis were performed to look for the possible presence of
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20 subpopulations in the presence of high concentrations of vancomycin and, due to the
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22 importance of one dilution difference in MIC for vancomycin, all isolates were included.
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24 Among the 14 strains, three isolates and their surviving replicates (see table) showed
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26 heteroresistant subpopulations with both methods and in two strains with MICs of 2-4 mg/L,
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28 a quasi-VISA behavior, i.e. similar to the MU50 strain, was found. It is interesting to note that
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30 in these patients, the hVISA strains were CA-MRSA belonging to ST8. The two strains with
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32 daptomycin MICs of 1 mg/L were not hVISA.

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DISCUSSION

This study on MRSA in the CF patients attending the Catania CF center, allowed us to make the following observations. First, 7.8% of our CF population harbored MRSA, among these we excluded the presence of SCVs due to the absence of typical phenotypic characteristics (small colony size, reduced growth rate and pigmentation, hemolysis negative growth on MSA, and weak coagulase production).

This prevalence is in agreement with data obtained in a recent survey in Italy [22], but less than that reported in studies from other countries [38].

Of the 14 longitudinally isolated strains in these patients, all were shown to be *agr*-type I and capsular type 5; 4 strains were HA-MRSA, 8 were CA-MRSA, with only 2 of them carrying the PVL gene, and 2 strains (3A and 3B) showed characteristics halfway between the ancient HA-MRSA and the contemporary CA-MRSA. Persistence was observed in all patients, two of them chronically infected as defined by three or more cultures containing the MRSA in the study period [7]. The homogeneous isolation of *agr*-I, *cap5* strains in the CF patients, demonstrated that they can colonize better the lung, surviving host defenses due to the anti-phagocyte properties of capsular type 5 strains and their biofilm production. With regard to the pathogenicity of MRSA, the strains showed strong adhesion properties demonstrated by the constant presence of *spa*, *clfA/B*, *atl* and *icaA*. This characteristic makes these strains able to adhere and invade host tissues where the sessile state is the best style in the lung of these patients.

Two patients were infected with two widespread HA-MRSA clones, i.e. the ST22-HA-MRSA-IV and ST247-HA-MRSA-I, while in the other three patients, different CA-MRSA were

1 detected. We are able to demonstrate in this paper, the isolation of ST8, ST291 and ST72
2 variably associated with *SCCmec* DNA type IVa and IVc but also with not-typeable
3 characteristics by using multiplex PCR protocols, as described. In these strains, recombination
4 events probably took place and further studies will be necessary to characterize these
5 *mecDNA* regions. A PVL positive USA300 variant (ACME negative) was found, for the first
6 time, in our CF population, being already present, even if rarely, in our country [15]. Patients 4
7 and 5 were infected with PVL positive strains at the beginning: interestingly in both cases, the
8 second cultures were negative, while all other cultures were positive again with PVL negative
9 CA-MRSA. In patients 1, 2 and 5, there was an association between persistent virulent MRSA
10 isolation and decreased lung functionality, as previously demonstrated [7, 39].

11 Some interesting comments can be made on these clones. ST247 is a well-known nosocomial
12 MRSA clone with a worldwide distribution, while ST22, although nosocomially associated, is
13 considered a bridge between the true nosocomial isolates and the community ones. With
14 regard to the community-associated strains, two different groups of organisms were found.
15 One is related to two different ST8 clones, in which only one was identified as a PVL positive
16 USA300 variant (ACME negative), while the other ST8-MRSA-IVc was a PVL negative strain.

17 The other group of CA-MRSA clones, i.e. ST291 and ST72, had different features in
18 common: first, their community origin, second the untypeability of the *SCCmec* DNA and a
19 low-level resistance to oxacillin, despite the presence of the *mecA* gene. ST291 is a double
20 locus variant of ST398, a PVL negative clone from swine origin, able to colonize or infect
21 humans [40]. Human infections sustained by this clone have been documented, in particular
22 endocarditis, ventilator-associated pneumonia and wound infections [41]. Some of the ST398
23 human isolates harbor the PVL gene, thereby illustrating the capacity of this clone to pick up
24 additional virulence factors. One of our ST291 strains was PVL positive while the subsequent
25 isolates were not. ST72 has been frequently isolated among methicillin-susceptible strains

1 worldwide, and ST72-MRSA-IV has been documented as a colonizer or a community-
2 associated strain, in infected patients from Asia and South America [42, 43]. Our ST72 MRSA
3 was untypeable, and, due to its characteristics, could be considered a precursor of the new
4 CA-MRSA clones that are becoming more diffuse all around the world.
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10 Regarding the antibiotic resistance characteristics of these 14 MRSA strains isolated in
11 CF patients, together with the characteristic of resistance to the usual drugs, 8 of these strains
12 showed a reduced susceptibility to vancomycin, furthermore, two strains showed a reduced
13 susceptibility to daptomycin.
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20 The presence of heteroresistance to vancomycin, described by other authors, was
21 demonstrated also in our CF patients in both ST8 clones, possessing, in two cases, a quasi-
22 VISA behavior if compared with the standard strain MU50 [44].
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27 As reported by many Authors, heteroresistance can be associated with clinical
28 vancomycin failure and this situation can be more severe in patients in whom persistence of
29 microorganisms in the lung is frequent [45, 12].
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35 In conclusion, the present study demonstrates persisting infections with both HA- and
36 CA-MRSA in our CF population, with an overall prevalence of 7.8%. The double colonization
37 by both hospital-associated and community-associated strains was already observed, but
38 these Authors did not check for persistence and did not find any association with declining
39 lung function [21]. We demonstrate that in these patients, persistence was sustained by either
40 identical clones that underwent subtle changes in their toxin content, or by different clones
41 over time. The isolation of MRSA in our CF population aged 7-24, was associated with an
42 increased severity of the disease even if, due to the small sample of patients included and the
43 paucity of data on the clinical outcome, these results cannot be conclusive.
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57 Furthermore, the presence of hVISA strains, questions the use of glycopeptides in the
58 treatment of MRSA infections in these patients.
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Acknowledgment

We gratefully acknowledge NARSA for providing Mu3 (NRS2) and Mu50 (NRS1) control strains, and Antony Bridgewood for the language revision of the manuscript. Partial support for this study was provided by grants from the Italian Minister of health and from MIUR Cofin 2007 -2007SCA9RK.

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Table 1. Methicillin-resistant *S.aureus* isolates: characteristics of patients and molecular properties of the strains

STRAINS*	DATE	PAT. AGE	FEV1 (%)	Patient Antibiotic therapy	OXA	agr	Capsular type	PFGE	ST	SCC TYPE	mecA	mecR/I	ccr	Biofilm	PATHOTYPE		
															Adhesins	Toxins	
1A	02/2005	21	56	TEC	>256	I	Cap5	G	22	IV	+	mecR	A ₂ B ₂	0.27	icaA fnbA sdrE spa atl clfA/B	hl-s sea sec spB lukE	
1B	05/2006			TEC	>256	I	Cap5	G	22	IV	+	mecR	A ₂ B ₂	0.27	icaA fnbA sdrE spa atl clfA/B	hl-s sea sec spB lukE	
2A	04/2005	24	67	TEC	>256	I	Cap5	A	247	I	+	mecR	A ₁ B ₁	0.49	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE	
2B	07/2006			52	TEC	>256	I	Cap5	A	247	I	+	mecR	A ₁ B ₁	0.49	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE
3A	02/2006	7	100	TEC	64	I	Cap5	σ ₂	8	IVc	+	mecR	A ₂ B ₂	0.30	icaA fnbA sdrE spa atl clfA/B	hl-s sea spB lukE	
3B	05/2006			TEC	64	I	Cap5	σ ₂	8	IVc	+	mecR	A ₂ B ₂	0.27	icaA fnbA sdrE spa atl clfA/B	hl-s sea spB lukE	
4A	04/2005	16	100	CIP	4	I	Cap5	γ	291	NT	+	-	-	0.25	icaA fnbA sdrE spa atl clfA/B	hl-s lukE lukS/F	
4B	01/2006			92	SXT	4	I	Cap5	γ	291	NT	+	-	-	0.24	icaA fnbA sdrE spa atl clfA/B	hl-s lukE
4C	05/2006			83	CIP	4	I	Cap5	γ	291	NT	+	-	-	0.28	icaA fnbA sdrE spa atl clfA/B	hl-s lukE
5A	02/2006	20	32	MEM + TOB	4	I	Cap5	USA300 ACME neg	8	IVa	+	mecR	A ₂ B ₂ -	0.18	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE tst lukS/F	
5B	10/2006			30	MEM + TOB	4	I	Cap5	ε ₂	72	NT	+	-	-	0.32	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE
5C	10/2006				MEM + TOB	2/4	I	Cap5	ε ₁	72	NT	+	-	-	0.50	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE
5D	02/2007				MEM + TOB	2/4	I	Cap5	ε ₂	72	NT	+	-	-	0.26	icaA fnbA sdrE spa atl clfA/B	hl-s tst spB lukE
5E	10/2007			LNZ	2/4	I	Cap5	ε ₂	72	NT	+	-	-	0.26	icaA fnbA sdrE spa atl clfA/B	hl-s spB lukE	

Legend: * The number indicates a patient and the letter indicates a different isolate

NT= Not Typeable

CIP= Ciprofloxacin

AMC= Amoxicillin/Clavulanate

SXT= Trimethoprim/Sulfamethoxazole

MEM= Meropenem

TOB= Tobramicin

LNZ= linezolid

TEC= teicoplanin

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Table 2. Minimal Inhibitory Concentrations (MICs mg/L), heteroresistance determinants (MacroEtest, PAP analyses) of the 14 MRSA isolates

STRAINS	Levofloxacin MIC (mg/L)	Tobramycin MIC (mg/L)	Linezolid MIC (mg/L)	Tigecycline MIC (mg/L)	Daptomycin MIC (mg/L)	Vancomycin MIC (mg/L)	Teicoplanin MIC (mg/L)	MacroEtest	PAP
1A	32	4	2	0.25	0.12	0.5	0.5	VSSA	VSSA
1B	32	4	2	0.25	0.12	0.5	0.5	VSSA	VSSA
2A	8	128	2	0.06	0.5	0.5	<0.06	VSSA	VSSA
2B	8	128	2	0.06	0.5	0.5	0.06	VSSA	VSSA
3A	4	128	2	0.25	0.5	1	1	hVISA	hVISA
3B	4	128	2	0.25	0.5	1	1	hVISA	hVISA
4A	0.5	8	4	0.25	0.5	0.5	0.12	VSSA	VSSA
4B	0.5	4	4	0.25	0.25	1	0.12	VSSA	VSSA
4C	0.5	8	4	0.25	0.5	0.5	0.25	hVISA	VSSA
5A	0.5	8	2	0.25	0.5	2-4	2	hVISA	hVISA
5B	8	16	4	0.06	0.25	2-4	4	hVISA	hVISA/VISA
5C	8	64	4	0.12	1.5	1-2	1	hVISA	VSSA
5D	8	32	4	0.12	1.5	1	1	VSSA	VSSA
5E	16	32	2	0.06	0.5	2	4	hVISA	hVISA/VISA

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