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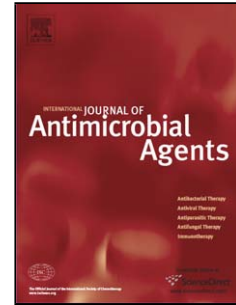
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1 **Heteroresistance to glycopeptides in Italian methicillin-resistant**

2 ***Staphylococcus aureus* isolates**

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25 **Abstract**

26 The prevalence and the molecular characterization of heteroresistant *S.aureus* strains
27 to vancomycin (hVISA) were determined in a large group of Italian strains, isolated from
28 2005 to mid 2007. Among the 1284 strains isolated from documented infections from
29 hospitalized patients (BSI, pneumonia and SSTIs), 139 *S.aureus* with MICs to vancomycin
30 between 1 and 2 mg/L were screened for the presence of hVISA using three different
31 methods, and confirmed by the population analysis profile (PAP). Thirty-six strains
32 (25.8%) were detected. Among the three screening methods used, the macroEtest
33 (MET) demonstrated 100% specificity and 75% sensitivity. hVISA strains were *agrI* and
34 II and belonged to the major nosocomial clones circulating in Italy (ST8, ST239, ST247,
35 and ST228); all strains were susceptible to quinupristin/dalfopristin, linezolid,
36 daptomycin, tigecycline and dalbavancin. In conclusion, we have demonstrated that
37 hVISA isolates are common in MRSA isolates with MICs between 1 and 2 mg/L, in Italy.
38 MET, with its high sensitivity and specificity should be used for an early detection of
39 hVISA, above all inpatients with serious or prolonged infections sustained by MRSA.
40

41 Finally, the most recent anti Gram-positive drugs maintained their full spectrum of *in*
42 *vitro* susceptibility against these strains.

43

44 First reports of *Staphylococcus aureus* with reduced susceptibility to vancomycin (RSV)
45 date back to 1997 [1] when Hiramatsu et al described the Mu50 (VISA) and the Mu3
46 (hVISA) strains in Japan, possessing MICs to vancomycin respectively of 8 and 4 mg/L,
47 isolated from patients in which vancomycin therapy failed. After these reports, it did not
48 take long for this resistance phenotype to be recognized all around the world,
49 predominantly in methicillin-resistant strains. Although the homogeneous resistance to

50 vancomycin (VISA) continues to be rare, there are increasing reports of strains showing
51 heteroresistance (hVISA), often with vancomycin MICs ≤ 2 mg/L [2,3,4]. Even if 1997 is
52 the official beginning of the isolation of strains with reduced vancomycin susceptibility
53 (RVS), a retrospective analysis of stored isolates detected previously unrecognized
54 hVISA/VISA strains at least back to 1987 both in the USA and in Europe [5].
55 Heteroresistance to glycopeptides, in which subpopulations with reduced susceptibility
56 (approximately 10^6 CFU/ml) coexist in a seemingly susceptible phenotype, has been
57 recently associated with clinical failure [6,7,8,9], and can be found predominantly in
58 MRSA strains, even if reports from methicillin-susceptible *S.aureus* have recently been
59 published [10].

60
61 One of the major difficulties in interpreting the literature on prevalence, epidemiology
62 and significance of hVISA itself, derives from the lack of standardized criteria for the
63 definition of hVISA and the use of different methodologies to detect them [11]. MIC
64 breakpoints are not sensitive enough to distinguish precursors of strains with reduced
65 susceptibility (hVISA) from the vancomycin-susceptible *S.aureus* isolates. For these
66 reasons, a large number of screening methods have been analyzed, and although a
67 number of them are useful for the detection of VISA, they are not adequate for hVISA
68 screening, in which resistant subpopulations can be found only at 10^{-5} to 10^{-6} CFU/ml, at
69 the limit of the inoculum size used in the MIC testing. Methods to detect hVISA therefore
70 tend to rely on the testing of higher inoculum (Etest 2Mc-Farland) or population
71 analysis profile (PAP) [2,11].

72
73 Significant controversy also exists also regarding the current and future roles of
74 vancomycin and teicoplanin in the treatment of serious MRSA infections, above all in

75 those strains in which vancomycin MICs creep towards the upper levels of susceptibility,
76 affecting the global susceptibility of the *S.aureus* population.

77

78 The resolution of all these problems requires a detailed understanding of the
79 epidemiological and clinical impact of changes in glycopeptide susceptibility of *S.aureus*
80 as well as criteria for detection and a full comprehension of the mechanisms at the basis
81 of reduced susceptibility (RS).

82

83 With this in mind, a retrospective analysis of MRSA isolates was performed in order to:

84 i) evaluate the prevalence of hVISA strains in a sample of 1284 sequential Italian MRSA
85 isolates, by using current methods for the screening of hVISA and VISA strains and
86 confirmed by PAP/AUC analysis; ii) perform the molecular characterization of these
87 strains with reduced susceptibility and their susceptibility to anti-Gram-positive drugs.

88

89 We screened 1284 sequential MRSA strains (isolated in a Italian surveillance study
90 involving 20 centres distributed throughout the country, between 2005 and mid 2007)
91 from documented bloodstream, pneumonia and skin-structure infections [12], and 139
92 strains with vancomycin MICs between 1 and 2 mg/L were selected for further studies.

93 Mu50 (VISA) and Mu3 (hVISA) were included in the study as control strains for
94 phenotypic and genotypic assays.

95

96 MIC determinations for glycopeptides, linezolid, dalbavancin, quinupristin/dalfopristin,
97 tigecycline and daptomycin, were performed according to CLSI [13].

98

99 Strains were screened using a rapid screening method as follows: BHIV₄ (containing 4
100 mg/L vancomycin, originally developed by Hiramatsu et al [1]) and BHIT₅ (containing 5
101 mg/L teicoplanin, described by Fitzgibbon et al as the best performing method used
102 [14]) compared with the macro Etest (MET), and confirmed by the reference PAP/AUC
103 method. Briefly, tests were performed as previously described [1,14], with the following
104 modifications: all isolates were screened on BHIT₅ and BHIV₄ using 10 µL-loop volumes
105 of bacterial suspensions with two densities, equivalent to a 0.5 and 2 McFarland
106 turbidity standard. Plates were incubated for 48 h at 37°C. *S. aureus* Mu3 (hVISA), Mu50
107 (VISA) and ATCC 29213 (vancomycin-susceptible *S. aureus*, VSSA) were used as control
108 strains.

109

110 To assay macro Etest (MET) procedures, all clinical isolates were grown overnight to a
111 2.0 McFarland standard in Mueller-Hinton broth; a 100µl sample was plated and
112 streaked onto BHI agar, and vancomycin and teicoplanin Etest strips were applied (AB
113 Biodisk, Sweden). Plates were incubated for 48h at 37°C and then evaluated for growth
114 following manufacturer's instructions (EAS003; AB Biodisk, Solna, Sweden) [15].

115

116 To perform population analysis (PAP/AUC), colonies from cultures grown overnight on
117 tryptic soy agar were inoculated into tryptic soy broth. After incubation for 24h,
118 dilutions of 10⁻³ (10⁵ CFU/ml) and 10⁻⁶ (10² CFU/ml) were prepared in saline, and 50µl
119 were inoculated onto BHI agar plates containing 4.0, 6.0, 8.0, 12.0, 16.0 mg of
120 vancomycin/liter and 4.0, 8.0, 16.0, 32.0 mg of teicoplanin/liter, respectively. After 48h
121 of incubation at 37°C, the colonies were counted and the log of the CFU/ml was plotted
122 against the vancomycin concentration by using Prism software (GraphPad Software Inc).

123 The ratio of AUC of the test isolates to the AUC of *S.aureus* Mu3 was calculated and
124 interpreted as previously published [11].

125

126 Molecular characterization of all the MRSA strains included in the study was conducted
127 by PFGE, MLST, SCCmec, and *agr*-typing; all techniques were performed as previously
128 described [12,16].

129

130 *Prevalence of hVISA in clinical specimens*

131 Among the 139 isolates with vancomycin MIC between 1 and 2 mg/L, PAP/AUC
132 identified 78 VSSA, and 36 hVISA strains, no VISA strains, as defined by CLSI
133 breakpoints, were detected. However, 9 out of the 36 hVISA, showed a population
134 profile similar to Mu50 (VISA), and these strains showed a higher number of cells with
135 MIC \geq 12 mg/L to vancomycin as determined by PAP.

136

137 Overall, the incidence of hVISA strains among the group of MRSA with MICs between 1
138 to 2 mg/L, was 25.8%, almost all isolated from blood and LRT infection specimens. As
139 reported in table 1, of the three screening methods used, the macro Etest detected all
140 strains with a specificity of 100% and a sensitivity of 75%. For the other two screening
141 methods BHIT₅ and BHIV₄, specificity was respectively 43.5 and 61.5%, while sensitivity
142 was respectively 75 and 66.6%.

143

144 *Molecular characterization of hVISA strains*

145 All our hVISA strains belonged to the *agr*I and *agr*II types and derived from the major
146 nosocomial pathogens circulating in Italy, i.e. MRSA ST8 (formerly the Archaic clone),
147 ST239 (formerly the Brazilian clone), ST247 (formerly the Rome clone), and ST228

148 (formerly the Iberian clone) (table 2). The strains were potently inhibited by all
149 antibiotics tested, i.e. linezolid, daptomycin, tigecycline, quinupristin/dalfopristin and
150 dalbavancin, with one or two-dilution variations considering the different clones.

151
152 Over the last few years, there has been significant interest regarding the changing
153 patterns of vancomycin MICs within the *S.aureus* population. This has been driven partly
154 by studies demonstrating poorer outcomes for vancomycin treatment of MRSA
155 infections with higher vancomycin MICs but still in the range of susceptibility
156 [7,8,17,18]. This change has directly impacted the proportion of hVISA, as has been
157 clearly demonstrated by many authors [19,20]. This “MIC creep” has been reported by
158 many centres [12,19,21,22,23,], while some others did not find any change over time
159 [24]. Therefore, changes in *S.aureus* vancomycin MICs can occur over time within
160 specific institutions.

161
162 The fact that the “MIC creep” obscures the presence of hVISA with subpopulations of
163 cells resistant to vancomycin, but also to teicoplanin [12], requires an accurate
164 assessment of the clinical significance of hVISA with reliable screening and confirmatory
165 tests.

166
167 In this study we surveyed nosocomial MRSA isolates sequentially collected from
168 different Italian hospitals during a period of 36 months. PAP/AUC analysis
169 demonstrated that, among strains with MICs between 1 and 2 mg/L, hVISA were found
170 in 25.8% of cases and these strains belonged to the major nosocomial clones circulating
171 in Italy. As demonstrated in many papers published in the last years, the prevalence of
172 hVISA has varied significantly. Some of these differences can be explained by differences

173 in laboratory definitions and testing strategies, however it appears that rates of hVISA
174 vary globally, which could be related to the amount of subpopulations present. Studies
175 reported rates ranging from less than 1% to as high as 50% [6,9,25].

176

177 In the present study, using MET at the manufacturer's criteria of ≥ 8 mg/L for
178 vancomycin and teicoplanin or ≥ 12 mg/L for teicoplanin alone, we found a good
179 correlation with the PAP/AUC, with a good sensitivity and a high specificity. BHIT5, as
180 demonstrated by other authors [14] is a useful method with a sensitivity comparable to
181 MET, but with a lower specificity. BHIV4, in this respect, is less useful.

182

183 Thirty-three out of the 36 hVISA strains emerged from the most diffused nosocomial
184 pandemic MDR MRSA clones. In Italy they belong to *agrI* and II type strains, as already
185 observed [26,27] and there is evidence of a potential association between a non-
186 functional *agr* locus and a reduced response to vancomycin therapy.

187

188 hVISA is susceptible to many antimicrobials currently available. Despite their MDR
189 phenotype, including resistance to fluoroquinolones, aminoglycosides, macrolides, these
190 strains were fully susceptible to linezolid, quinopristin/dalfopristin, tigecycline,
191 daptomycin and to the new investigational drug dalbavancin. There has been an
192 association between hVISA, VISA and reduced susceptibility to daptomycin [28,29,30],
193 however this appears to be strain specific and may be unstable [31]. Data from *in vitro*
194 studies suggest that daptomycin may have a lower rate on *in vitro* killing, still retaining
195 its potent bactericidal activity [32,33,34]. RVS strains maintain susceptibility to
196 linezolid, with no changes in the MIC distribution with respect to the VSSA. This drug is

197 essentially bacteriostatic, but it was used with success in the treatment of infections
198 sustained by MRSA, hVISA and VISA strains [35,36,37].
199 Tigecycline, the first glycycline to be available for clinical use, has a very good activity
200 against MSSA and MRSA: it has a very good *in vitro* activity against hVISA strains with
201 very low MIC values.

202
203 With regards to new drugs under development, the potent activity of dalbavancin was
204 demonstrated in our study. Data from the literature reported higher MICs of this drug in
205 hVISA or VISA strains, and an *in vitro* model [38] shows a need for the highest
206 concentrations of drug to achieve bactericidal activity, however, the clinical implications
207 of these findings are unclear.

208
209 In conclusion, we have demonstrated that hVISA isolates are common in MRSA isolates
210 with MICs between 1 and 2 mg/L, in Italy. Detection of hVISA requires specific and often
211 cumbersome laboratory tests, among them the optimal one has not yet been
212 determined. MET, with its high sensitivity and specificity should be used for an early
213 detection of hVISA, above all in patients with serious or prolonged infections sustained
214 by MRSA. Finally, the most recent anti Gram-positive drugs maintained their full
215 spectrum of *in vitro* susceptibility against these strains.

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224

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226

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230 was.

231

232

233

234 **Declarations**

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237 **Competing Interests:** SS has received grants, advisory invitations and speaking

238 invitations from many pharmaceutical companies. SS has received partial funding from

239 Pfizer Pharma to perform studies on linezolid (who also supplied the drug).

240 All other Authors have none to declare.

241 **Ethical Approval:** Not necessary

242

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390

391 Table 1. Sensitivity and specificity values for the three screening methods used, with respect
 392 to PAP/AUC (at 48h).

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METHODS	PAP hVISA N°36		% SENS	PAP VSSA N°78		% SPEC
	VSSA	hVISA		VSSA	hVISA	
BHI T5 2MF	9	27	75%	34	44	43.5%
BHI V4 2MF	12	24	66.6%	48	30	61.5%
Macro E-test 2MF	9	27	75%	78	0	100%

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398 Table 2. Genetic (ST, SCCmec, PFGE, and *agr* types) and phenotypic features of the 36
399 hVISA strains in study and MICs distribution (mg/L) of the major anti-Gram positive drugs
400 tested.
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	0.03	0.06	0.12	0.25	0.50	1	2	4
Archaic (3 hVISA)								
ST8-HA-MRSA-I agr-type I/IV								
Vancomycin	0	0	0	0	0	3	0	0
Teicoplanin	0	0	0	0	0	3	0	0
Daptomycin	0	0	0	1	2	0	0	0
Linezolid	0	0	0	0	0	2	1	0
Quinupristin/Dalfopristin	0	0	0	0	1	2	0	0
Tigecycline	0	0	0	1	2	0	0	0
Dalbavancin	1	2	0	0	0	0	0	0
Iberian (3 hVISA)								
ST247-HA-MRSA-IA agr-type I								
Vancomycin	0	0	0	0	0	3	0	0
Teicoplanin	0	0	0	0	0	0	1	2
Daptomycin	0	0	0	0	3	0	0	0
Linezolid	0	0	0	0	0	2	1	0
Quinupristin/Dalfopristin	0	0	0	2	1	0	0	0
Tigecycline	0	0	0	0	3	0	0	0
Dalbavancin	1	2	0	0	0	0	0	0
Rome (6 hVISA)								
ST247-HA-MRSA-I/IA agr-type I								
Vancomycin	0	0	0	0	0	1	5	0
Teicoplanin	0	0	0	0	0	1	1	4
Daptomycin	0	0	0	0	2	4	0	0
Linezolid	0	0	0	0	0	3	3	0
Quinupristin/Dalfopristin	0	0	0	2	4	0	0	0
Tigecycline	0	0	3	1	2	0	0	0
Dalbavancin	1	1	4	0	0	0	0	0
Rome (6 hVISA)								
ST247-HA-MRSA-I/IA agr-type I								
Vancomycin	0	0	0	0	0	0	6	0
Teicoplanin	0	0	0	0	0	0	4	2
Daptomycin	0	0	0	0	2	4	0	0
Linezolid	0	0	0	0	0	4	2	0
Quinupristin/Dalfopristin	0	0	0	2	4	0	0	0
Tigecycline	0	0	1	5	0	0	0	0
Dalbavancin	2	2	2	0	0	0	0	0
Brasilian (3 hVISA)								
ST239-HA-MRSA-IIIA agr-type I								
Vancomycin	0	0	0	0	0	3	0	0
Teicoplanin	0	0	0	0	0	3	0	0
Daptomycin	0	0	0	1	2	0	0	0
Linezolid	0	0	0	1	2	0	0	0
Quinupristin/Dalfopristin	0	0	0	1	2	0	0	0
Tigecycline	0	0	0	1	2	0	0	0
Dalbavancin	0	3	0	0	0	0	0	0
Italian (12 hVISA)								
ST228-HA-MRSA-I agr-type II								
Vancomycin	0	0	0	0	0	12	0	0
Teicoplanin	0	0	0	0	0	12	0	0
Daptomycin	0	0	0	0	9	3	0	0
Linezolid	0	0	0	0	0	6	3	3
Quinupristin/Dalfopristin	0	0	0	0	5	7	0	0
Tigecycline	0	0	2	0	10	0	0	0
Dalbavancin	0	12	0	0	0	0	0	0
Sporadic (3 hVISA)								
Vancomycin	0	0	0	0	0	3	0	0
Teicoplanin	0	0	0	0	0	1	0	2
Daptomycin	0	0	0	1	2	0	0	0
Linezolid	0	0	0	0	0	2	1	0
Quinupristin/Dalfopristin	0	0	0	1	1	1	0	0
Tigecycline	0	0	0	2	1	0	0	0
Dalbavancin	0	3	0	0	0	0	0	0

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