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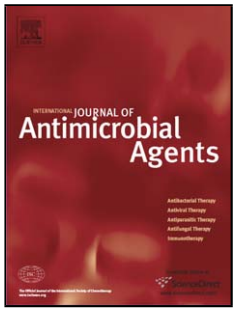

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Heteroresistance to glycopeptides in Italian methicillin-resistant *Staphylococcus aureus* isolates

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Abstract

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

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

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

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

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

Significant controversy also exists also regarding the current and future roles of 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

Strains were screened using a rapid screening method as follows: $BHIV_4$ (containing 4 mg/L vancomycin, originally developed by Hiramatsu et al [1]) and $BHIT_5$ (containing 5 mg/L teicoplanin, described by Fitzgibbon et al as the best performing method used [14]) compared with the macro Etest (MET), and confirmed by the reference PAP/AUC method. Briefly, tests were performed as previously described [1,14], with the following modifications: all isolates were screened on BHIT5 and BHIV4 using 10 µL-loop volumes of bacterial suspensions with two densities, equivalent to a 0.5 and 2 McFarland turbidity standard. Plates were incubated for 48 h at 37°C. *S. aureus* Mu3 (hVISA), Mu50 (VISA) and ATCC 29213 (vancomycin-susceptible *S. aureus*, VSSA) were used as control strains.

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

To perform population analysis (PAP/AUC), colonies from cultures grown overnight on tryptic soy agar were inoculated into tryptic soy broth. After incubation for 24h, dilutions of 10^{-3} (10^5 CFU/ml) and 10^{-6} (10^2 CFU/ml) were prepared in saline, and 50µl were inoculated onto BHI agar plates containing 4.0, 6.0, 8.0, 12.0, 16.0 mg of vancomycin/liter and 4.0, 8.0, 16.0, 32.0 mg of teicoplanin/liter, respectively. After 48h of incubation at 37°C, the colonies were counted and the log of the CFU/ml was plotted against the vancomycin concentration by using Prism software (GraphPad Software Inc).

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

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

Prevalence of hVISA in clinical specimens

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

Overall, the incidence of hVISA strains among the group of MRSA with MICs between 1 to 2 mg/L, was 25.8%, almost all isolated from blood and LRT infection specimens. As reported in table 1, of the three screening methods used, the macro Etest detected all strains with a specificity of 100% and a sensitivity of 75%. For the other two screening methods $BHIT_5$ and $BHIV_4$, specificity was respectively 43.5 and 61.5%, while sensitivity was respectively 75 and 66.6%.

Molecular characterization of hVISA strains

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

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

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

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

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

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

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

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

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

essentially bacteriostatic, but it was used with success in the treatment of infections sustained by MRSA, hVISA and VISA strains [35,36,37].

Tigecycline, the first glycylcycline to be available for clinical use, has a very good activity against MSSA and MRSA: it has a very good *in vitro* activity against hVISA strains with very low MIC values.

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

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

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Declarations

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Competing Interests: SS has received grants, advisory invitations and speaking invitations from many pharmaceutical companies. SS has received partial funding from Pfizer Pharma to perform studies on linezolid (who also supplied the drug).

All other Authors have none to declare.

Ethical Approval: Not necessary

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

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%**

Table 2. Genetic (ST, SCCmec, PFGE, and *agr* types) and phenotypic features of the 36 hVISA strains in study and MICs distribution (mg/L) of the major anti-Gram positive drugs tested.

	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