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Study of Decreased Susceptibility to Vancomycin in Methicillin-Resistant *Staphylococcus aureus* Strains isolated from a Romanian Multidisciplinary Emergency Hospital

Studiul susceptibilității scăzute la vancomicină la tulpini de *Staphylococcus aureus* meticilino-rezistente izolate într-un spital de urgență multidisciplinar din România

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Abstract

The clinical relevance of Staphylococcus aureus strains with heterointermediate susceptibility to vancomycin (hVISA) is still controversial, however they could be responsible for treatment failures in patients treated with vancomycin. The lack of standardization and the complexity of testing methods are the main challenge in indentifying such strains. The aim of our study was to evaluate the frequency of hVISA strains in Targu-Mures Clinical Emergency Hospital. One hundred twenty-two, non-duplicate, methicillin-resistant S. aureus (MRSA) isolates susceptible to vancomycin using standard E-test (MIC≤2 mg/L) were screened for heteroresistance with Glycopeptide Resistance Detection test (E-test GRD). Population analysis profile-area under the curve (PAP/AUC) method was used for confirmation. Twenty-four strains (19.5%) were found positive with the screening method. Two of them (1.63%) were confirmed having hVISA phenotype and no strains with intermediate vancomycin susceptibility (VISA) were detected. In conclusion, the rate of MRSA strains with reduced vancomycin susceptibility was low. However, their monitoring may be useful, taking into consideration the wide usage of glycopeptides in the treatment of serious MRSA infections.

***Key words**: hVISA screening;glycopeptide resistance detection;population analysis*

Rezumat

Relevanța clinică a tulpinilor Staphylococcus aureus cu susceptibilitate heterointermediară la vancomicină (hVISA) nu este complet elucidată, totuși acestea pot fi responsabile pentru eșec terapeutic la pacienții tratați cu vancomicină. Lipsa standardizării și complexitatea metodelor de detectare sunt provocări importante in identificarea acestor tulpini. Scopul studiului a fost evaluarea frecvenței tulpinilor hVISA în Spitalul Clinic Județean de Urgență Târgu-Mureș. O sută douăzeci și două de tulpini de S. aureus meticilino-rezistente (MRSA),

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susceptibile la vancomicina cu E-test standard (CMI≤2 mg/L), au fost testate pentru heterorezistență cu metoda E-test GRD (Glycopeptide Resistance Detection). Pentru confirmare s-a folosit analiza populațională. Douăzeci și patru de tulpini (19.5%) au fost selectate cu metoda screening. Două dintre acestea (1.63%) au fost confirmate fiind hVISA, nici un izolat nu avea susceptibilitate intermediară la vancomicină (VISA). Rata tulpinilor hVISA a fost scazută. Monitorizarea lor poate fi totuși importanta, luând in considerare ca glicopeptidele sunt larg utilizate pentru tratamentul infecțiilor severe cauzate de MRSA.

Cuvinte cheie: *screening hVISA; detecția rezistenței la glicopeptide; analiza populațională*

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Introduction

Vancomycin is the first choice antibiotic for the treatment of severe methicillin-resistant *Staphylococcus aureus* (MRSA) infections such as nosocomial sepsis, endocarditis. Adaptation and development of resistance to widely used antibiotics is common among bacteria. Although full resistance to glycopeptides is still a rarity, infections caused by *Staphylococcus aureus* with reduced susceptibility are increasingly reported around the world (1). The majority of the strains were resistant to methicillin and were isolated from patients who previously underwent vancomycin therapy, but methicillin-susceptible *Staphylococcus aureus* (MSSA) strains with reduced susceptibility to glycopeptides were described as well (2; 3).

In contrast to the low frequency of fully vancomycin-resistant MRSA strains (VRSA), treatment failures with vancomycin were widely reported. Responsible for these could be the vancomycin-intermediate *S. aureus* (VISA) or the vancomycin heterointermediate *S. aureus* (hVISA) strains, which decrease the success rate of therapy without increasing mortality (4-6).

Since therapy failures were noted even in cases when the isolated *S. aureus* strain *in vitro* showed minimal inhibitory concentration (MIC) values for vancomycin within the susceptible range (MIC_{Va}<=4 mg/l), the Clinical and Laboratory Standards Institute (CLSI) lowered the vancomycin breakpoints in 2006, as follows: MIC_{Va}≤2 mg/L – susceptible, 4-8 mg/L – intermediate and ≥16 mg/L – resistant (7). Breakpoints set by EUCAST do not define a range for intermediate susceptibility, strains with MICs higher than 2 mg/L being classified already as resistant (8).

Heterointermediate VISA is defined as a *S. aureus* strain with an overall vancomycin MIC in the susceptible range (MIC≤2 mg/L) including small subpopulations (approximately 10^{-6}) able to grow in the presence of vancomycin at concentrations higher than 2 mg/L (9). Detection of heterointermediate resistance is difficult with current laboratory methods and there is no standardization.

In a previous study we characterized MRSA strains recovered from patients admitted to clinical wards with high risk for nosocomial infections, such as intensive care and surgical departments. All strains were susceptible to vancomycin (10). The aim of the present study was to evaluate the occurrence of hVISA among these strains, this being the first study investigating hVISA in Romania, in a large multidisciplinary university hospital.

Materials and methods

Clinical setting

The study was performed in Târgu-Mureș Clinical Emergency Hospital, a multidisciplinary hospital with 1084 beds.

Bacterial isolates

One hundred and twenty-two non-duplicate consecutive MRSA strains, isolated and identified by conventional microbiologic methods during routine diagnosis were collected during January-December 2010. Strains were stored at -70°C until further *in vitro* testing. MRSA ATCC 700698 (Mu3) as hVISA prototype and *Staphylococcus aureus* ATCC 29213 as vancomycin susceptible strain were used in the experiments as control strains.

Glycopeptide susceptibility testing

Standard vancomycin E-test (BioMérieux SA) was performed according to the manufacturer's recommendations. MICs were read after 24 h incubation at 35°C. If MIC endpoints were between two concentration values, results were reported rounding up to the next endpoint value.

For the broth microdilution method serial twofold dilutions of the vancomycin were prepared in 96-well cell culture plates (64 mg/L to 1 mg/L). A suspension of 0.5 McFarland in saline was prepared from an overnight culture of the tested strain, which was further adjusted to a cell count of 10^5 UFC/ml in double-concentrated cation-adjusted Mueller-Hinton broth; 100 µl of these were dispensed in each well containing 100 µl of the antibiotic solution. MICs were read after 24 h incubation at 35°C.

Glycopeptide Resistance Detection (GRD) E-test (BioMérieux SA) was used to screen for MRSA strains with decreased susceptibility to glycopeptides according to the manufacturer's recommendations. An inoculum of 0.5 McFarland was made from the overnight culture in cation-adjusted Mueller-Hinton broth and swabbed onto Mueller-Hinton agar supplemented with 5% sheep blood. Double-sided E-test strips with gradient for vancomycin and teicoplanin were placed on. Plates were read at 24 and 48 h, after incubation at 35°C. Results were interpreted according to the manufacturer's instructions: an isolate was considered positive for hVISA or VISA if the inhibition zone for vancomycin or teicoplanin was ≥8 µg/ml.

Population analysis profile – area under the curve (PAP/AUC), the gold standard method for confirmation of hVISA/VISA strains, was performed according to Wooton *et al.* (11) and Riederer *et al.* (12). A standard inoculum of 0.5 McFarland was made in saline from an overnight culture of the isolate to be tested. It was further diluted 10^3- and 10^5-fold in saline. Fifty microliters from each inoculum were plated with a glass rod onto brain heart infusion agar (BioMérioux SA) containing different concentrations of vancomycin (0, 0.5, 1, 2, 2.5, 4, 5, 6 mg/L). Colony count was performed at 48 h after incubation at 35 °C with Flash&Grow Petri Dish Colony Counter. The number of colony forming units per ml (CFU/ml) was calculated averaging the number of colonies grown at a given vancomycin concentration resulting from both sets of inocula and adjusting to the appropriate dilution. Graphic representation of the $\log_{10}$ CFU/ml plotted against the vancomycin concentrations and determination of the area under the curve (AUC) were done using GraphPad Prism software. The AUC of Mu3 was used for further interpretation, as follows: VISA if $AUC_{strain}/AUC_{Mu3} \geq 1.3$, hVISA if AUC_{strain}/AUC_{Mu3} was between 0.9 and 1.3 and VSSA if $AUC_{strain}/AUC_{Mu3} < 0.9$.

***agr* typing**

The PCR for detection of *agr* group was performed as described by Shopsin *et al*. (13). The *agr* type was determined according to the size of the resulting amplicons (*Table I*).

Results

All 122 non-duplicate MRSA isolates susceptible to vancomycin by standard E-test were screened by E-test GRD. Of these, twenty four strains (19.5%) were found positive. Their in-

Table I.: Primers used for *agr* typing and the sizes of the resulting amplicons

agr type	Primers	Amplicons (bp)
	F pan-agr 5_-ATGCACATGGTGCACATGC-3_	
I	R agr I, 5_-GTCACAAGTACTATAAGCTGCGAT-3_	440
II	R agr II, 5_-GTATTACTAATTGAAAAGTGCCATAGC-3_	572
III	R agr III, 5_-TGTTGAAAAAGTCAACTAAAAGCTC-3_	406
IV	R agr IV, 5_-CGATAATGCCGTAATAC CCG-3_	588

bp – base pairs

hibition zone values for vancomycin and teicoplanin ranged from 0.5 to 1.5 and from 8 to 16, respectively. None of them showed MIC for vancomycin >2 mg/L with standard E-test and broth microdilution method (*Table I* and *II*). Among these twenty four isolates only two (1.63%) were confirmed to be hVISA by PAP/AUC. The graphical representation of the PAP/AUC for one VSSA and one hVISA clinical isolate and the two control strains is shown in *figure 1*. All strains belonged to *agr* type I.

Discussion

In our study, 122 MRSA strains were evaluated for decreased susceptibility to vancomycin. Using GRD screening method 19.5% (n=24) of

Table II. Phenotypic characterization of the two heterointermediate *S. aureus* strains

Methods	Strain 1	Strain 2
E-test GRD inhibition zone value		
Vancomycin	1.5	1
Teicoplanin	12	16
Standard E-test MICs (mg/L)		
Vancomycin	1.5	1.5
Broth microdilution (mg/L)		
Vancomycin	1	1
PAP/AUC ratio	0.95	0.99

E-test GRD- Glycopeptide Resistance Detection, PAP/AUC – population analysis profile area under the curve

Table III. Phenotypic characterization of the vancomycin susceptible *S. aureus* strains

Nr.	BMD (MIC_{Va} mg/L)	Standard E-test (MIC_{Va} mg/L)	E-test GRD Va 48 h	E-test GRD Tp 48 h
1	1	0.75	0.75	8
2	1	1	1.5	12
3	1	1	0.75	12
4	1	0.75	0.75	8
5	1	1	0.75	8
6	1	1	0.75	8
7	1	1	0.75	8
8	1	0.5	1	8
9	1	1	0.75	8
10	2	1	0.5	8
11	1	1.5	0.75	8
12	1	0.75	0.75	8
13	1	0.5	0.50	8
14	1	0.75	0.75	8
15	1	0.75	0.75	10
16	1	0.75	0.75	8
17	1	0.75	0.75	8
18	1	1	1.50	8
19	1	1	0.75	12
20	1	1	0.75	8
21	2	1	0.75	8
22	1	0.75	0.5	8

BMD- broth microdilution, Va- vancomycin, Tp- teicoplanin, MIC- minimum inhibitory concentration, GRD- glycopeptid resistance detection

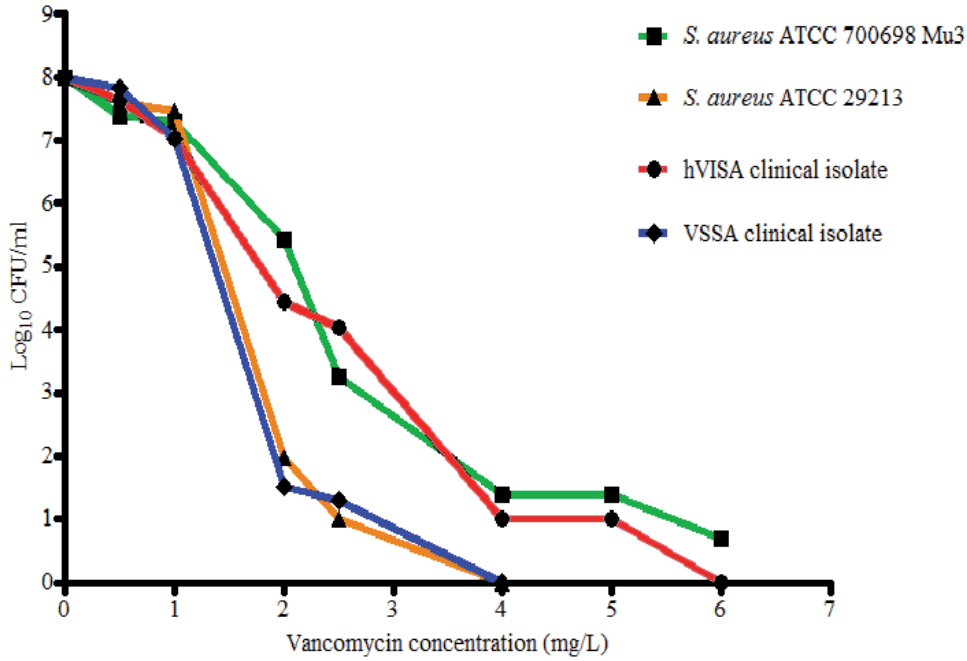

Figure 1. Population analysis profile of the control strains and representative clinical isolates

the isolates were suspected for hVISA/VISA. As confirmed by PAP/AUC, only two of them were hVISA (1.63%), and no VISA strains were detected. In accordance with our expectations, the frequency of MRSA strains with decreased susceptibility to glycopeptides was low, similarly to data reported by others.

In a meta-analysis performed by van Hal *et al*. the overall rate of hVISA among MRSA strains was approximated to 1.3%, with a variation between 0 and 73.7% (1). There are no recent data regarding the epidemiology of hVISA strains in Europe (9). According to a review by Howden, the prevalence of hVISA in European countries was low, below 2% among MRSA isolates (14). Kirby et al. evaluated 201 blood culture MRSA strains isolated between 2004 and 2006 in Liverpool. 2.5% of them showed reduced susceptibility to glycopeptides (15). Among 1284 MRSA isolates from different regions of Italy Campanile *et al*. detected 139 strains with vancomycin MICs between 1 and 2 mg/L. Of these, 36 strains (25.8%) were hVISA, accounting for 2.8% of all MRSA strains. In the same study, no VISA strains were found (16). In France and Belgium the prevalence of hVISA among MRSA strains was 0.6% and 0.7% or lower, respectively (17; 18; 19). When lowering macro E-test cut-off levels to 4 mg/L both for vancomycin and teicoplanin, Garnier *et al*. found 11% rate of hVISA among 2300 *S. aureus* strains. Seven of them were MSSA (3).

An evaluation through a 22 year period (1986-2007) in the USA showed an increase in the prevalence of hVISA isolates from 2.2 to 8.3%. The frequency of VISA strains in the same period ranged between 0.3 - 2.3% (20).

In China, Sun *et al*. identified an overall prevalence rate of 13.1% for hVISA and 0.5% for VISA from 200 bloodstream MRSA infections, with decreasing trend since 2002 to 2007. In Australia Howden *et al*. detected a prevalence

of S. aureus with reduced vancomycin susceptibility of 13% among blood culture isolates, but 50% when strains from all infection sites were considered (21).

There are no reports about hVISA from the neighboring countries, excepting Hungary. In 2008 Tóth *et al*. published a case report about a patient with fatal hVISA infection (22).

To our knowledge, there are no reports regarding glycopeptide susceptibility testing using other than standard tests, therefore hVISA occurrence could not have been documented so far in Romania. Using standard MIC determinations, no strains with reduced susceptibility to vancomycin were detected in studies performed in Iasi and Brasov (23; 24).

The different prevalence rates could partly be explained by the lack of standardization, although geographical particularities may also be present (14). Nosocomial spread of clones with reduced susceptibility could be responsible of increased prevalence in some hospitals (1).

Tests used to detect the VISA/hVISA phenotype show variable accuracy. Vancomycin broth MIC is appropriate to identify VISA, but not hVISA. However, the E-test methods can show 0.5 to 1 fold higher MIC values of those detected with microdilution (25).

To screen for hVISA, several methods were suggested, such as the E-test GRD and macro gradient test (MET) (9). In our study we used GRD for screening.

The specificity and sensitivity of GRD E-test – in the first reports – were 94 and 95%, respectively (26). Other studies described similar specificity but lower sensitivity (57-82%) (27). The negative predictive value of GRD was 97% (12). The MET, which uses a 2-McFarland inoculum and standard E-test strips, showed almost analogous precision as the GRD (12; 28; 29). To increase the accuracy of identification, combination of screening methods are recommended (14; 30; 31).

Beside one of the MIC methods, CDC suggests the use of vancomycin screening plates (BHI agar supplemented with 6 mg/L vancomycin, 0.5 McFarland standard inoculum) (31). This detects with higher accuracy VISA strains with vancomycin MIC $\geq$ 8 mg/L, than hVISA. The sensitivity and specificity of the test for hVISA detection was below 12% and between 68-100%, respectively (26; 32; 33). The Mueller-Hinton agar with 5 mg/L teicoplanin and 2 McFarland standard inoculum was applied by the ECDC as screening method for hVISA in the European Antimicrobial Resistance Surveillance Scheme (14; 34). This screening method detected the hVISA phenotype with 65-79% sensitivity and 35-95% specificity (26; 32; 33; 35).

Each bacterial strain found positive with any of the screening methods must be analyzed with a confirmatory test. Population analysis profile-area under the curve method, described by Wootton *et al*. in 2001 is the gold standard in confirmation of hVISA (11). This method is laborious and time consuming, therefore its use in the daily routine is not feasible.

Many phenotypic and genotypic features of hVISA were studied, including cell wall changes, autolytic activity, metabolic changes, and molecular mechanism of the resistance, respectively (14).

Sakoulas *et al*. found correlation between accessory gene regulator operon (agr) loss of function and reduced susceptibility to vacomycin (36). Initially it was taught, that only *agr* II is linked with hVISA phenotype (37), later it was demonstrated that S. aureus strains of each *agr* group (I-IV) can develop into hVISA after sub-therapeutic vancomycin exposure (38). Our strains belonged to *agr* type I.

As shown previously, most MRSA strains in our hospital belonged to the same PFGE group sharing spa type t030 and harbouring SCCmec gene cassette type III. The two MRSA strains found hVISA in this present study belonged to

the same major clonal group but their pulsotypes were not identical, showing 2 bands difference. Although both patients were admitted to the ICU, there was no epidemiological link between these two cases and no intrahospital spread of hVISA could be documented (10).

The clinical significance of hVISA strains is difficult to evaluate, because of the lack of controlled prospective studies. Although the prevalence of hVISA strains overall is low, there are several reports of glycopeptides treatment failures in patients with demonstrated hVISA infection (5″-6). Beside vancomycin treatment failure Casapao *et al*. noted persistent and/or recurrent bacteremia in patients with hVISA bloodstream infections (39). Infective endocarditis, osteomyelitis, prosthetic joint infections and deep abscesses occur more frequently with hVISA than with VSSA (4; 40; 41). Although VISA bacteremia can be correlated with the patient`s death, there are no statistically significant differences in overall mortality in deep-seated infection with hVISA and VSSA (1; 39; 42; 43). Others reported lower rates of infections, bacteremia and decreased capacity in inducing shock in case of S. aureus with reduced glycopeptide susceptibility, compared to VSSA (21; 44). Responsible for these may be the reduced virulence due to loss of function in the *agr* operon (45).

High rate of poor clinical outcomes can be associated with vancomycin MIC>2 mg/L, independently of hVISA or non-hVISA phenotype (46).

In conclusion, the rate of MRSA strains with reduced vancomycin susceptibility was low in our hospital. Although the clinical significance of hVISA strains is unclear, their monitoring may be useful, taking into consideration the wide usage of glycopeptides in the treatment of serious MRSA infections.

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