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Low prevalence of methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to glycopeptides in Belgian hospitals

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ABSTRACT

Staphylococcus aureus strains with decreased susceptibility to glycopeptides (GISA) have been associated with increased risk of glycopeptide treatment failure. To assess the prevalence of these strains in hospitalised patients in Belgium, 455 methicillin-resistant *S. aureus* (MRSA) isolates collected in 2001 were screened by two assays: (i) growth on vancomycin agar screen (VAS; brain heart infusion agar (BHI) + vancomycin 6 mg/L); and (ii) a synergy/antagonism test with aztreonam/cefazolin on Mu3 agar (BHI + vancomycin 3 mg/mL). Isolates growing on VAS or Mu3 agar were characterised further by analysis of population susceptibility profiles. MICs of glycopeptides were determined by agar dilution, broth microdilution and Etest (low and high inocula) methods. The isolates were genotyped by pulsed-field gel electrophoresis (PFGE) and determination of staphylococcal cassette chromosome *mec* (SCC*mec*) type. No GISA isolates were found. Three (0.7%) hetero-vancomycin intermediate *S. aureus* (hVISA) and ten (2.2%) hetero-teicoplanin intermediate *S. aureus* (hTISA) isolates were identified by population analysis. All but one hetero-GISA isolate belonged to either epidemic PFGE group A/SCC*mec* type I (69%) or PFGE group D/SCC*mec* type I (23%), both of which were resistant to gentamicin. The sensitivity and specificity for the detection of hetero-GISA by the two assays were 15.4% and 99.8%, respectively, for VAS, and 84.6% and 95.9%, respectively, for Mu3. The data indicated that hetero-GISA strains were uncommon among Belgian MRSA isolates from hospitalised patients. Use of Mu3 agar was more sensitive, but less specific, than VAS as a screening method.

Keywords Belgium, GISA, glycopeptide resistance, MRSA, *Staphylococcus aureus*, vancomycin

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INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. For the past two decades, the prevalence of methicillin-resistant *S. aureus* (MRSA) has increased dramatically in many parts of the world. In Europe, considerable variations in the prevalence of MRSA are observed, ranging from <2% in Scandinavia and The Netherlands, to >30% in southern and western European countries [1]. In Belgium, the proportion of MRSA among *S. aureus* isolates from blood culture has risen from 23% in 1999 to 28% in 2002. Until now, glycopeptides have been considered as the treatment of choice for MRSA infections. In 1990, Kaatz *et al.* [2] described the first case of infection with a methicillin-susceptible *S. aureus* isolate with intermediate susceptibility to teicoplanin (TISA). Seven years later, the first infection caused by an MRSA isolate with intermediate susceptibility to vancomycin was reported in Japan [3]. Since then, at least 20 cases of infection caused by MRSA with intermediate susceptibility to both vancomycin and teicoplanin (GISA) have been reported worldwide [4,5]. In addition to GISA, strains that are borderline-susceptible to glycopeptides, but exhibit low-frequency resistance to glycopeptides ($\sim 10^{-6}$ subpopulation; hetero-GISA) have been described more frequently in Europe, Brazil and Asia [4–7].

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Although their clinical relevance is still questioned, such strains appear to be associated with a poor treatment outcome [8,9] and could represent the first step towards the emergence of glycopeptide-resistant mutants following further glycopeptide exposure.

The objectives of the present study were, first, to determine the prevalence and investigate the molecular epidemiology of MRSA with reduced susceptibility to glycopeptides in the national survey conducted in 2001 in Belgian hospitals, and second, to compare the diagnostic performance of two commercially available GISA/hetero-GISA screening agars.

MATERIALS AND METHODS

Resistance definitions

A GISA strain was defined as a *S. aureus* isolate with: (i) a vancomycin MIC of >4 mg/L and/or a teicoplanin MIC of >8 mg/L, and (ii) a population analysis profile similar to that of the VISA reference strain HIP5827 [10]. A hetero-GISA (h-GISA) strain was defined as a *S. aureus* isolate with: (i) a vancomycin MIC of ≤4 mg/L and/or a teicoplanin MIC of ≤8 mg/L, and (ii) a population analysis profile similar to that of the hetero-VISA reference strain Mu3 [7].

Bacteria

From January to December 2001, the Belgian MRSA Reference Laboratory invited all Belgian hospital laboratories ($n = 196$) to collect five non-duplicate, consecutive MRSA isolates from hospitalised patients [11]. Isolates from both routine clinical specimens and superficial screening cultures from mucocutaneous sites were included. The isolates were sent with a patient case-report form recording age, sex, type of specimen, hospital ward and type of acquisition (nosocomial or imported). A nosocomial acquisition was defined as acquisition of an MRSA strain at least 48 h after admission. In total, 455 isolates collected from 100 hospitals were confirmed as MRSA by both phenotypic (coagulase test and growth on agar containing oxacillin 6 mg/L) and genotypic (PCR for the 16S rRNA, *mecA* and *nuc* genes) methods [11].

Antimicrobial susceptibility

MIC determination

MICs were determined by the agar dilution method, according to NCCLS guidelines [12], for 15 antimicrobial agents: vancomycin, teicoplanin, oxacillin, erythromycin, clindamycin, quinupristin-dalfopristin, ciprofloxacin, gentamicin, tobramycin, minocycline, rifampicin, trimethoprim-sulphamethoxazole, fusidic acid, linezolid and mupirocin.

Glycopeptide susceptibility testing methods

All isolates were tested on vancomycin agar screen (VAS) and Mu3 agar (Becton Dickinson, Heidelberg, Germany). Briefly, for VAS, 10 μL of a 0.5× McFarland suspension was spotted on to brain heart infusion agar (BHI) supplemented with vancomycin 6 mg/L, and incubated at 35°C for a full 24 h [12]. For Mu3 agar, a 1× McFarland suspension was inoculated on to BHI agar supplemented with vancomycin 3 mg/L. Disks of cefazolin (30 μg) and aztreonam (60 μg) (Neo-Sensitabs; Rosco, Taastrup, Denmark) were placed on the plate. Following incubation for 48 h at 35°C, the plates were examined for an inhibition zone surrounded by a ring of satellite growth around the cefazolin disk, and a ring of heavy and confluent growth around the aztreonam disk [13].

Isolates with a vancomycin and/or teicoplanin MIC of ≥4 mg/L by agar dilution, or which grew on VAS or Mu3 agar, were characterised further by population analysis, broth microdilution tests and Etests (AB Biodisk, Solna, Sweden) with low and high inocula. MICs by the broth microdilution method were interpreted according to NCCLS recommendations [12]. Etest MICs were determined by two protocols: (i) 3 mL of a 0.5× McFarland suspension was flooded on to Mueller–Hinton agar (MH) and incubated for 24 h at 35°C [4]; and (ii) the 'Etest macromethod', in which 100 μL of a 2× McFarland suspension was inoculated on to BHI agar and incubated for 48 h at 35°C [14]. For the macromethod, the glycopeptide MICs were determined according to criteria provided by the manufacturer [14], in that isolates inhibited by both vancomycin and teicoplanin at ≥8 mg/L, or by teicoplanin alone at ≥12 mg/L, were considered to be putative hetero-GISA.

For population analysis, 100 μL of an overnight suspension (2× McFarland) was spread on to BHI agar supplemented with vancomycin 0, 2, 4, 6 or 8 mg/L, or teicoplanin 0, 4, 6, 8 and 16 mg/L [4]. Colony counts were determined after incubation for 48 h at 35°C. Control strains were included in each run: namely the vancomycin-susceptible *S. aureus* strain ATCC29213, hetero-VISA strain Mu3, and VISA strain HIP5827 [7,10].

Molecular typing

Chromosomal macrorestriction analysis using *Sma*I and pulsed-field gel electrophoresis (PFGE) was performed as described previously [11]. *Sma*I patterns were normalised and compared using the Dice coefficient and UPGMA clustering method with BioNumerics software v.2.5 (Applied Maths, Sint-Martens-Latem, Belgium). PFGE patterns were classified according to the following nomenclature [11]: (i) groups of patterns differing by ≥6 DNA fragments were designated by a capital letter (e.g., A); (ii) patterns within a group that differed by 3–6 DNA fragments were considered to form a 'type' and were designated by a numeral (e.g., A1); (iii) subtypes comprised any pattern profile within a type, and were designated by a lowercase letter suffix (e.g., A1a). Staphylococcal Cassette Chromosome *mec (SCCmec)* types were determined for GISA strains by PCR as described previously [15].

RESULTS

Glycopeptide susceptibility

All isolates were susceptible according to agar dilution to vancomycin (MICs of 0.25–4 mg/L) and teicoplanin (MICs of 0.06–8 mg/L). Six (1.3%)

isolates had vancomycin MICs of 4 mg/L, and 12 (2.6%) isolates had teicoplanin MICs of 4–8 mg/L. Only three (0.7%) isolates grew on VAS. Twenty-nine (6.4%) MRSA isolates grew on Mu3 agar, with an inhibition zone around the cefazolin disk and enhanced, usually confluent, growth around the aztreonam disk.

In total, 35 isolates showing either vancomycin or teicoplanin MICs of ≥4 mg/L, or which grew on VAS, or which were positive in screening tests on Mu3 agar, were characterised further by population analysis, broth microdilution and Etests. According to broth microdilution, all isolates were susceptible to glycopeptides, except one isolate which had intermediate susceptibility to teicoplanin (MIC of 16 mg/L). All isolates were susceptible to vancomycin and teicoplanin according to the Etest method with a low inoculum. However, by the Etest macromethod, 12 isolates showed an MIC of ≥8 mg/L for vancomycin and teicoplanin, or ≥12 mg/L for teicoplanin alone. Population analysis confirmed that three (0.7%) isolates had a heterogeneous-resistant subpopulation for vancomycin and teicoplanin, and ten (2.1%) isolates had a heterogeneous-resistant subpopulation for teicoplanin (Table 1; Figs 1 and 2).

The sensitivity and specificity for h-GISA detection were 15.4% and 99.8%, respectively, for VAS, vs. 84.6% and 95.9%, respectively, for Mu3 agar. The Etest macromethod detected 92% of h-GISA isolates with 100% specificity.

Table 1. Glycopeptide MICs and screen agar results of hGISA isolates (n = 13) from the Belgian National MRSA Survey 2001

Isolate	VAS screen	Mu3 agar screen	Agar dilution MIC (mg/L)		Broth microdilution MIC (mg/L)		Etest 0.5× McFa MIC (mg/L)		Etest macromethod[b] MIC (mg/L)		PAP
			TP	VA	TP	VA	TP	TP	VA	VA	
105	+	+	1	4	2	16	2	6	8	16	hGISA
124	–	–	4	4	2	4	2	4	4	8	hTISA
151	–	+	2	1	2	1	2	2	8	8	hGISA
202	–	+	2	4	2	8	2	4	4	16	hTISA
209	+	–	1	2	2	4	2	4	4	16	hTISA
242	–	+	2	2	2	8	2	4	4	16	hTISA
245	–	+	2	2	2	8	4	4	4	16	hTISA
254	–	+	2	2	2	8	2	4	4	16	hGISA
412	–	+	2	2	2	2	2	4	4	16	hTISA
421	–	+	2	4	1	4	2	4	4	16	hTISA
469	–	+	1	2	2	4	2	4	4	16	hTISA
632	–	+	2	8	4	8	2	6	8	16	hGISA
703	–	+	2	4	2	8	4	4	4	16	hTISA

VAS, vancomycin agar screen; VA, vancomycin; TP, teicoplanin; +, growth; – no growth; PAP, population analysis profile [a]0.5× McFarland suspension used as an inoculum [4]. [b]2.0× McFarland suspension used as an inoculum [14].

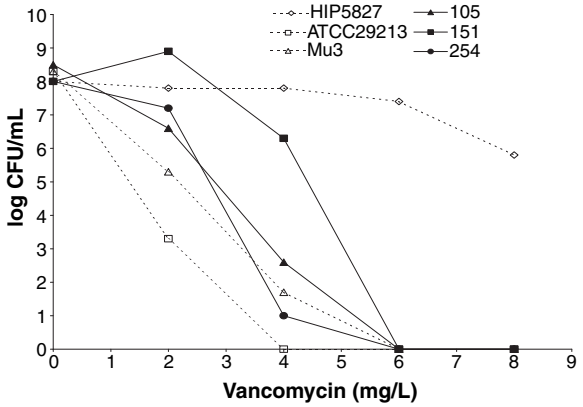

Fig. 1. Vancomycin population analysis of hetero-VISA strains.

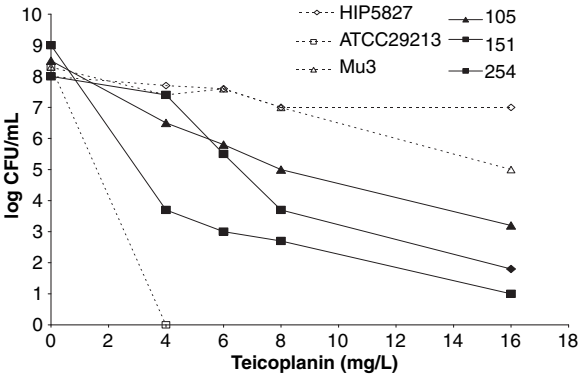

Fig. 2. Teicoplanin population analysis of hetero-VISA strains.

Demographic data

The 13 h-GISA isolates were recovered from 12 hospitals located in Brussels (n = 2), Wallonia (n = 6) and Flanders (n = 4). The median age of the patients from whom h-GISA were collected was 70 years (range, 48–90 years), and 85% of the patients were male. The patients were hospitalised in intensive care units (ICUs) (n = 4), surgical (n = 4), medical (n = 2), geriatric (n = 1) or other wards (n = 2) (Table 2). The h-GISA isolates were isolated from wounds and skin (n = 2), the respiratory tract (n = 3), blood (n = 2), nares (n = 2) or other sites (n = 4). Eleven (85%) of these isolates were acquired nosocomially.

Antimicrobial susceptibility and molecular typing

All except one of the 13 h-GISA isolates belonged to PFGE group A (n = 9) or D (n = 3), and carried

Table 2. Demographic data, molecular typing and resistance patterns for hGISA isolates (n = 13) from the Belgian National MRSA survey 2001

Isolate/ hospital	Type of unit	Origin of specimen	Nosocomial	PFGE type	SCCmec type	Resistance pattern
105/A	ICU	Nose	Yes	A4	I	OCEClGT
124/B	ICU	Respiratory	Yes	A19	I	OCEClGT
151/C	Surgical	Other	No	B2	IV	OC
202/D	Other	Wound	Yes	A19	I	OCEClGT
209/E	Medical	Other	Yes	D1	I	OCEClGT
242/F	Surgical	Respiratory	Yes	A1	I	OCEClGT
245/F	Other	Wound	No	A3	I	OCEClGT
254/G	ICU	Respiratory	Yes	A4	I	OCEClGT
412/H	Geriatric	Nose	Yes	D4	I	OCEClGT
421/I	ICU	Blood	Yes	A3	I	OCEClGT
469/J	Surgical	Other	Yes	D2	I	OCEClGT
632/K	Surgical	Other	Yes	A1	I	OCEClGT
703/L	Medical	Blood	Yes	A1	I	OCEClGT

O, oxacillin; C, ciprofloxacin; E, erythromycin, Cl, clindamycin; G, gentamicin; T, tobramycin; ICU, intensive care unit; PFGE, pulsed-field gel electrophoresis.

SCC*mec* type I (n = 12) (Table 2). MICs of oxacillin were ≥64 mg/L. All group PFGE A and D h-GISA isolates were resistant to ciprofloxacin, gentamicin, tobramycin, erythromycin and clindamycin (Table 2). All 13 h-GISA isolates were susceptible to quinupristin-dalfopristin, minocycline, linezolid, fusidic acid, mupirocin, rifampicin and trimethoprim-sulphamethoxazole.

DISCUSSION

Despite extensive glycopeptide susceptibility testing, no GISA strains were recovered in this survey of 455 MRSA isolates collected from 100 hospitals during the Belgian national survey. However, a low prevalence of isolates expressing hetero-resistance to teicoplanin (2.1%), or to both vancomycin and teicoplanin (0.7%), was found. The reported prevalence of h-GISA isolates ranges widely from one study to another, but was similarly low (<1%) in large surveys conducted in the UK, Italy, USA and Korea [16–19]. In contrast, a higher prevalence was observed among MRSA isolates analysed in Japan (5–22%), Germany (2–14%), The Netherlands (7.6%) and France (20%) [7,13,20,21], although other studies in France have reported lower incidences of h-GISA (0.6–5%) [22,23]. However, such differences may be more apparent than real because of marked differences in the study design and laboratory methods used for screening and confirming the hetero-resistance phenotype. Moreover, many of these studies were retrospective and analysed a limited number of selected isolates. Higher prevalence rates of h-GISA were reported in selected patient populations, e.g. oncology, surgery or ICU patients, that were more likely to be exposed to glycopeptide treatment [9,17,24]. Most studies have reported a higher prevalence of (hetero-) resistance of *S. aureus* to teicoplanin than to vancomycin, even in countries, such as the USA, where teicoplanin has not yet been licensed for clinical use [25–27].

In previous reports, GISA and h-GISA strains described in Europe belonged to a restricted range of epidemic MRSA strains, such as the UK EMRSA 15 and 16 strains, and the Brazilian and Iberian clones [20,25,26,28]. Molecular typing data from the present survey showed that all except one h-GISA isolate belonged to PFGE epidemic groups A or D, and carried SCC*mec* type I. Both of these clones had a similar resistance profile to multiple antimicrobial agents, including aminoglycosides and the macrolide-lincosamide-streptogramin B group. This association between h-GISA and a multiresistance phenotype has been reported previously [9,23,27,29]. By multilocus sequence typing (MLST), PFGE group A SCC*mec* type I isolates (n = 24) belong to the ST 247-MRSA-I clone (the 'Iberian clone') which has been disseminated widely in Europe and North America for an extended period of time [11,30]. In Belgium, this clone has been responsible for large hospital outbreaks since 1984, and was recovered from 80% of hospitals in the early 1990s. In 2001, this clone appeared to have been displaced by other epidemic clones, and particularly by new variants of PFGE group A (types A20 and A21) that are susceptible to gentamicin and carry a type IV SCC*mec* element. By MLST, such strains belong to the same clonal complex (CC 8), but differ by two alleles (ST8 vs. ST247). Interestingly, the present study did not recover any h-GISA among isolates belonging to gentamicin-susceptible PFGE group A – SCC*mec* type IV (n = 90). By MLST, PFGE group D – SCC*mec* type I isolates (n = 18) belong to the ST228-MRSA-I clone which has also been reported in Slovenia and Germany [30]. The PFGE group A and D SCC*mec* type I isolates, which were the main genotypes associated with the h-GISA phenotype in the present study, represented <10% of the isolates in the overall survey.

While GISA strains have been associated clearly with glycopeptide treatment failure, the clinical significance of h-GISA strains remains controversial. Some authors suggest that h-GISA strains could be precursors of GISA strains.

Moore *et al.* [31] found that hetero-resistance to vancomycin was associated with treatment failure in a rabbit model of endocarditis. In a similar model, Pavie *et al.* [32] did not observe the emergence of a resistant subpopulation following treatment with vancomycin, in contrast to teicoplanin, which seemed to be more prone to select for resistance [32]. Hetero-resistance has also been associated with glycopeptide treatment failure and a higher patient mortality rate [8,9]. However, a retrospective study of MRSA bacteraemia showed similar outcomes for patients infected by MRSA with reduced susceptibility to vancomycin or by vancomycin-susceptible MRSA [33]. Moreover, hetero-resistance does not seem be a common cause of persistent or recurrent bacteraemia [34]. Further prospective investigations are needed to better assess the clinical impact of hetero-resistance to glycopeptides.

Local outbreaks of infection caused by *S. aureus* with reduced susceptibility to glycopeptides have been described in several hospitals [23,35]. Nasal carriage of these strains has been detected among ICU staff [36]. In Belgium, an outbreak caused by a TISA strain has been reported in ICU patients (37th Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract J-122). Among the present collection of isolates, most (85%) h-GISA were acquired nosocomially. As the potential for dissemination of h-GISA has been demonstrated, guidelines for control of nosocomial transmission of MRSA should be followed strictly whenever such strains are detected.

The major problem encountered by clinical laboratories is the routine detection of isolates with reduced susceptibility to glycopeptides. Disk diffusion susceptibility testing is inadequate for detection of both GISA and h-GISA [37]. Most automated systems also fail to recognise staphylococci with reduced susceptibility to glycopeptides. The NCCLS recommends the use of BHI agar plates supplemented with vancomycin 6 mg/L, or MH agar supplemented with vancomycin 5 mg/L for the detection of GISA [38]. However, h-GISA strains can sometimes fail to grow on these media, as with VAS in the present study. Hiramatsu *et al.* [7] recommend the use of BHI agar supplemented with vancomycin 4 mg/L for the screening of h-GISA. This lower concentration of vancomycin allows the detection of h-VISA and VISA isolates, but this method lacks specificity [39]. Previous reports have described synergic activity between vancomycin and many β-lactams, except aztreonam, against GISA isolates [13]. The present study tested Mu3 agar – BHI agar supplemented with vancomycin 3 mg/L – with a modified disk diffusion method for detection of antagonism/synergism with β-lactams [13]. Of the 29 MRSA isolates showing enhanced zones of inhibition with a cefazolin disk, only 11 were confirmed as h-GISA by population analysis. The Etest macromethod developed by Walsh *et al.* [14], with breakpoints of vancomycin 8 mg/L and teicoplanin 8 mg/L or teicoplanin 12 mg/L, was found to be sensitive and specific for confirmation of h-GISA, but is too expensive for use in routine screening. Population analysis of susceptibility profiles is the reference method for confirming hetero-resistance, but is too laborious and time-consuming for routine use.

In conclusion, a low prevalence of h-GISA was found among nosocomial MRSA isolates collected from a large survey of Belgian hospitals in 2001. A higher proportion of isolates had a subpopulation resistant to teicoplanin (2.6%) than to vancomycin (0.7%). These h-GISA isolates were restricted largely to minor gentamicin-resistant clones. The Etest macromethod was nearly as accurate for confirmation of h-GISA isolates as analysis of population profiles. VAS showed a low sensitivity, and Mu3 a low specificity, as screening methods. Further studies should evaluate h-GISA screening methods and the clinical significance of these isolates in patients treated with glycopeptides.

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