JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2007, p. 3263–3269 Vol. 45, No. 10
0095-1137/07/$08.00+0 doi:10.1128/JCM.00836-07


# Investigation of Reduced Susceptibility to Glycopeptides among Methicillin-Resistant *Staphylococcus aureus* Isolates from Patients in Ireland and Evaluation of Agar Screening Methods for Detection of Heterogeneously Glycopeptide-Intermediate *S. aureus*[▽]

Margaret M. Fitzgibbon, Angela S. Rossney,* and Brian O'Connell

*National MRSA Reference Laboratory, St James's Hospital, James's St., Dublin 8, Ireland, and Department of Clinical Microbiology, University of Dublin, Trinity College, Dublin 1, Ireland*

Received 19 April 2007/Returned for modification 5 June 2007/Accepted 1 August 2007

**Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates ($n$ = 3,189) from 2,990 patients were investigated by agar screening and by the Etest macromethod for reduced susceptibility to glycopeptide. No vancomycin-resistant *S. aureus* or glycopeptide-intermediate *S. aureus* (GISA) isolates were detected, but 178 isolates were confirmed as hetero-GISA (hGISA) by vancomycin population analysis profile (vPAP)-area under the curve (AUC) ratio determination and/or teicoplanin PAP (tPAP) methods. Of 139 isolates detected using the recommended Etest macromethod cutoff values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, 73 were confirmed as hGISA by vPAP-AUC, 95 were confirmed as hGISA by tPAP, and 108 were confirmed as hGISA by both methods. An Etest macromethod cutoff value of 8 mg/liter for teicoplanin alone detected a further 70 hGISA (17 were confirmed by vPAP-AUC and 70 were confirmed by tPAP). Agar screening utilizing brain heart infusion (BHI) agar containing 6 mg of vancomycin/liter (BHIV6) and Mueller-Hinton (MH) agar containing 8 mg of teicoplanin/liter (MHT8) failed to detect hGISA. MH agar containing 5 mg of teicoplanin/liter (MHT5) and BHI containing 5 mg of teicoplanin/liter (BHIT5) were evaluated using 10-μl volumes of three inoculum concentrations (with densities equivalent to 0.5 and 2.0 McFarland turbidity standards and stationary-phase BHI broth subcultures [$MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_S$, $BHIT5_{0.5}$, $BHIT5_{2.0}$, and $BHIT5_S$]). The sensitivity of all methods except $MHT5_{0.5}$ and $MHT5_{2.0}$ was 100%. The specificity ranged from 4 to 82%. $BHIT5_{0.5}$ yielded the best performance, with a specificity of 84% for detecting isolates with teicoplanin Etest macromethod values of ≥8 mg/liter. Screening on $BHIT5_{0.5}$ is useful where screen-positive isolates are investigated with the Etest macromethod and confirmed by vPAP-AUC and tPAP. The prevalence of hGISA among patients with blood culture isolates recovered in Irish hospitals between 1999 and 2003 was 2.6%, whereas the prevalence among patients with isolates from all specimen sites collected during a 2-week survey in 1999 was 12%. The prevalence in one hospital decreased from 5.3% in 2003 to 1.5% in 2004.**

Clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to glycopeptide were first described in Japan in 1997 (15). To date, three types of reduced susceptibility to glycopeptides have been described in *S. aureus*: vancomycin-resistant *S. aureus* (VRSA), glycopeptide-intermediate *S. aureus* (GISA), and hetero-GISA (hGISA). VRSA isolates exhibit vancomycin MICs of ≥16 mg/liter (5, 9, 13). According to the 2006 revision of the Clinical and Laboratory Standards Institute (CLSI) guidelines, isolates are considered GISA if they have vancomycin MICs of 4 to 8 mg/liter and/or teicoplanin MICs of 16 mg/liter, but current British and European breakpoint criteria define GISA as isolates with vancomycin MICs of 8 mg/liter (5, 9, 13). With isolates described as hGISA, the majority of bacteria exhibit susceptible MICs, but a minority population (perhaps as few as $10^{-6}$ cells) exhibit MICs in the intermediate category (15). The terms GISA or hGISA have been proposed to describe isolates with reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus* [VISA] or hetero-VISA [hVISA]) or to teicoplanin (teicoplanin-intermediate *S. aureus* [TISA] or hetero-TISA [hTISA]) or to both vancomycin and teicoplanin (6, 24).

Reports of VRSA, GISA, and hGISA among MRSA isolates are increasing (16). Between 2002 and 2005, six clinical VRSA isolates (MIC of ≥32 mg/liter) were reported from the United States, where resistance has been shown to be mediated by *vanA*, which encodes glycopeptide resistance in vancomycin-resistant enterococci (7). More recently, two VRSA isolates with vancomycin MICs of 32 to 64 mg/liter have been reported from India, but *vanA* genes were not detected (25). There has also been a report of VRSA from Jordan, where isolates were investigated by agar dilution and Etest MIC determination only (4). GISA isolates have been reported from many countries worldwide but are still relatively rare (6, 27). Isolates of hGISA occur more frequently with reported prevalence rates ranging from 0 to 74%; this variation reflects difficulties with definitions, screening methods, confirmatory techniques, and interpretative criteria used in different studies (6, 17, 27). Attempts to assess the clinical significance of GISA and especially hGISA are complicated by these problems, but infection with

* Corresponding author. Mailing address: National MRSA Reference Laboratory, St. James's Hospital, James's St., Dublin 8, Ireland. Phone: 353 1 410 3662. Fax: 353 1 410 3666. E-mail: arossney@stjames.ie.

▽ Published ahead of print on 8 August 2007.

TABLE 1. Numbers of isolates investigated by screening methods and by MIC determination

| MRSA collection no., name, yr (no. of isolates)[a] | No. of isolates tested (no. positive) by screening method[b]: | | | | | | No. of isolates tested for MIC as determined by: | |
|---|---|---|---|---|---|---|---|---|
| | BHIV4 | BHIV6 | MHT8 | $MHT5_S$ | $BHIT5_{0.5}$ | Etest macromethod | Etest | CLSI[c] |
| 1, H1 Q4, 1998 (188) | 188 (84) | 84 (0) | – | 84 (33) | – | 84 (7) | 7 | 84 |
| 2, N/S Study, 1999 (714) | 714 (489) | 489 (0) | – | 489 (178) | – | 489 (74) | 74 | 489 |
| 3, EARSS, 1999–2003 (1,580) | –[e] | 1580 (0) | – | 87[d] (30) | – | 1,580 (15) | 169 | 87[d] |
| 4, NMRSARL, 2000–2003 (97) | – | 97 (0) | – | – | – | 97 (19) | 19 | – |
| 5, H1 Q2, 2003 (330) | – | 330 (0) | 330 (0) | 330 (37) | 330 (73) | 73 (18) | 18 | – |
| 6, H1 Q4, 2004 (280) | – | 280 (0) | – | – | 280 (25) | 25 (6) | 6 | – |
| Total (3,189) | 902 (573) | 2,860 (0) | 330 (0) | 990 (278) | 610 (98) | 2,348 (139) | 293 | 660 |

[a] Abbreviations: N/S Study, North/South Study of MRSA in Ireland 1999.
[b] The numbers in parentheses indicate the numbers of strains determined to be positive by each screening method. For screening methods BHIV6, MHT8, $MHT5_S$, and $BHIT5_{0.5}$, growth of ≥1 colony indicated a positive result.
[c] CLSI broth microdilution.
[d] Isolates recovered between 1999 and 2002 exhibiting Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin.
[e] –, Not done.

GISA and hGISA has been associated with glycopeptide therapy failure (17).

Guidelines for the detection of VRSA and GISA have been published, but finding suitable guidance for the detection of hGISA is problematic (6, 17). Standard disk diffusion, broth microdilution MIC, and standard Etest MIC determinations all fail to detect hGISA, probably because the initial inoculum density used with these tests is too low to detect the resistant subpopulation (6, 26). The population heterogeneity of hGISA isolates is demonstrated when isolates are investigated by population analysis (15). A modified population analysis profile (PAP) method where the area under the curve (AUC) is calculated for each test isolate and compared to the AUC of the prototype hGISA isolate Mu3 (*S. aureus* ATCC 700698) has been described (29). This PAP-AUC ratio method has been reported to be a reliable and reproducible method of detecting hGISA but is time-consuming and unsuitable for use in clinical laboratories (27). Walsh et al. have described a modification of the Etest system—the Etest macromethod—wherein a heavy inoculum (200 μl of bacterial suspensions with densities equivalent to a 2.0 McFarland turbidity standard [$6 \times 10^8$ CFU/ml]; nutritious medium, i.e., brain heart infusion [BHI] agar) and prolonged incubation (48 h) have been shown to reliably detect isolates exhibiting reduced susceptibility to glycopeptides with sensitivity and specificity values of 96 and 97%, respectively, compared to the PAP-AUC method (26). However, widespread use of Etest strips for diagnostic laboratories is expensive, and a simple, reliable, cost-effective screening method for the detection of hGISA is needed for use in clinical laboratories.

Several agar screening methods that differ in medium composition, inoculum density, choice of glycopeptide (vancomycin or teicoplanin), and/or antibiotic concentration have been proposed (6, 10, 14, 15, 19). Hiramatsu et al. screened with BHI agar containing 4 mg of vancomycin/liter (BHIV4) and an inoculum density of $10^6$ CFU/ml and used population analysis for confirmatory testing (15). The sensitivity and specificity of screening on BHIV4 were shown to be 71 and 88%, respectively, compared to PAP-AUC ratio determination (26). In the same study, screening with Mueller-Hinton (MH) agar containing 5 mg of vancomycin/liter (MHV5) and BHI containing 6 mg of vancomycin/liter (BHIV6) failed to detect hGISA. Other studies suggested screening with BHI agar containing 6 mg of teicoplanin/liter (BHIT6) or MH agar containing 5 mg of teicoplanin/liter (MHT5) (10, 14). The European Antimicrobial Resistance Surveillance System (EARSS) recommends screening on MHT5 with a stationary-phase broth culture (using 10-μl volumes) and incubation for 48 h (12).

In Ireland, MRSA is a serious nosocomial problem, and the rates of methicillin resistance in *S. aureus* isolates recovered from blood are among the highest in Europe (20). A study in 1999 reported that there were no VRSA or GISA among 714 MRSA isolates investigated, and although 5% of the isolates yielded screening results suggestive of hGISA, the hGISA phenotype was not confirmed by PAP (22). In the present study, 3,189 MRSA isolates from 2,990 patients recovered between 1998 and 2004 were investigated to determine the prevalence of VRSA, GISA, and hGISA among MRSA isolates in Ireland and to evaluate agar screening methods for the detection of hGISA.

## MATERIALS AND METHODS

**Bacterial isolates.** MRSA isolates ($n$ = 3,189 isolates from 2,990 patients) from six collections of MRSA recovered between 1998 and 2004 were investigated. Isolates were collected from the following sources: (i) all MRSA isolates recovered in one 936-bed tertiary-referral adult university hospital (H1) during quarter 4 (Q4), 1998 ($n$ = 188) (21); (ii) all MRSA isolates recovered during a 2-week study period in 1999 from all hospitals that participated in the North/South Study of MRSA in Ireland ($n$ = 714) (22); and (iii) MRSA isolates from blood cultures (one isolate per patient per quarter) from Irish laboratories that participated in the EARSS project between 1999 and 2003 ($n$ = 1,580). In Ireland, participation in EARSS provides almost 100% population coverage, and all MRSA isolates reported are sent to the National MRSA Reference Laboratory (NMRSARL) for confirmation and further investigation (20). Collection 4 consisted of all MRSA isolates submitted to the NMRSARL between 2000 and 2003 for investigation of reduced susceptibility to glycopeptides ($n$ = 97). Collection 5 comprised all MRSA isolates recovered from H1 during Q2, 2003 ($n$ = 330) (20), while collection 6 consisted of all MRSA isolates recovered from H1 during a prospective study in Q4, 2004 ($n$ = 280). In general, one isolate per patient was included unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. Isolate details are summarized in Table 1.

Isolates were identified as MRSA as described previously and stored at −70°C

on cryoprotective beads (Protect Bacterial Preservers, Technical Service Consultants, Ltd., Hayward, United Kingdom) (11). All investigations were undertaken by using overnight subcultures on Columbia agar (LabM, Lab 1; International Diagnostics Group, plc, Bury, Lancashire, United Kingdom) containing 7% horse blood.

**Preliminary agar screening.** Isolates from collections 1 ($n$ = 188) and 2 ($n$ = 714) were screened on BHIV4 using a heavy inoculum (200-μl volumes of bacterial suspensions with densities equivalent to a 2.0 McFarland turbidity standard [$6 \times 10^8$ CFU/ml]) and incubation for 48 h at 35°C as described previously (11, 21). All isolates that grew on BHIV4 from collections 1 and 2 ($n$ = 84 and $n$ = 489, respectively) were included in the present study.

All isolates ($n$ = 2,860), the 573 isolates from collections 1 and 2 and the 2,287 isolates from collections 3 to 6 were screened with BHI agar (CM0375; Oxoid, Ltd., Basingstoke, England) containing 6 mg of vancomycin (vancomycin hydrochloride [V2002]; Sigma-Aldrich, Ltd., Tallaght, Ireland)/liter (BHIV6) as previously described (24). In a pilot study, all 330 isolates in collection 5 were screened on MH agar (catalog no. 225250; Becton-Dickinson and Company [BD], Columbia, MD) containing teicoplanin (teicoplanin sodium salt; Sanofi-Aventis, Paris, France) at a concentration of 8 mg/liter (MHT8) using an inoculum of $10^6$ CFU/ml (19).

All isolates that grew on BHIV4 from collections 1 and 2 ($n$ = 84 and $n$ = 489, respectively), all isolates from collection 3 recovered between 1999 and 2002 yielding Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin ($n$ = 87), and all isolates in collection 5 ($n$ = 330) were screened on MH agar containing 5 mg of teicoplanin/liter using 10-μl volumes of stationary-phase BHI broth (CM0225; Oxoid) subcultures ($MHT5_S$) delivered by using 10-μl loops (12). In all, 990 isolates were screened on $MHT5_S$. Plates were incubated at 35°C, and growth was observed after 24 and 48 h. Growth of ≥1 colony after 48 h of incubation indicated a positive result.

The following control strains were used: *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* Mu3 ATCC 700698, *S. aureus* Mu50 ATCC 700699, *Enterococcus faecalis* ATCC 29212, and *E. faecalis* ATCC 51299.

**Etest macromethod.** Etest macromethod values were determined according to the manufacturer's instructions using vancomycin and teicoplanin Etest strips (AB Biodisk, Ltd., Solna, Sweden) (2). Briefly, 200-μl volumes of bacterial suspensions prepared in saline to a density equivalent to 2.0 McFarland turbidity standard ($6 \times 10^8$ CFU/ml) were pipetted onto each BHI agar plate, and the inoculum was spread evenly with a swab. Plates were dried at room temperature, Etest strips were applied, and plates were incubated at 35°C for 48 h. The interpretative criteria for reduced susceptibility were values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone as recommended by the manufacturer (2, 26). All isolates from collections 1 and 2 that grew on BHIV4, all isolates in collections 3 and 4, and any isolate from collections 5 and 6 that grew on any screening media were investigated by the Etest macromethod. Details of the isolates investigated are summarized in Table 1.

**MIC determination.** Etest MICs for vancomycin and teicoplanin were performed according to the manufacturer's instructions on all isolates exhibiting Etest macromethod values ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone ($n$ = 139). Etest MICs were also determined for any isolate from collection 3 that yielded Etest macromethod values >4 mg/liter for vancomycin and/or teicoplanin ($n$ = 169). Broth microdilution MICs for vancomycin and teicoplanin were performed on all isolates requiring further investigation from collections 1 and 2 ($n$ = 84 and 489, respectively) and on all collection 3 isolates recovered between 1999 and 2002 yielding Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin ($n$ = 87) (8). The quality control strains used with MIC determinations were *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212. Details of the isolates investigated are summarized in Table 1.

**Population analysis profiling.** Colonies from cultures grown overnight on Columbia agar containing 7% horse blood were inoculated into tryptic soy broth (211825; BD). After incubation for 24 h, dilutions of $10^{-3}$ ($10^5$ CFU/ml) and $10^{-6}$ ($10^2$ CFU/ml) were prepared in saline. Volumes (50 μl) were inoculated onto BHI agar plates containing 0, 0.5, 1.0, 2.0, 2.5, 4.0, 8.0, 12.0, or 16.0 mg of vancomycin/liter and 0, 4.0, 6.0, 8.0, 12.0, or 16.0 mg of teicoplanin/liter, respectively, using a spiral plater (Don Whitley Scientific, Ltd., Shipley, United Kingdom) (18, 29). After 48 h of incubation at 35°C, the colonies were counted, and the log of the CFU/ml was plotted against the antibiotic concentration by using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) to obtain vancomycin PAPs (vPAP) and teicoplanin PAPs (tPAP). The vPAP graph was used to calculate the AUC of each isolate, and the ratio of the AUC of the test isolate to the AUC of *S. aureus* Mu3 (ATCC 700698) was calculated (29). The vPAP-AUC ratio criteria were as follows: glycopeptide-susceptible *S. aureus* (GSSA), <0.9; hGISA, 0.9 to 1.29; and GISA, ≥1.3. The criterion to define hTISA isolates using tPAP was any isolate with a resistant subpopulation that grew at concentrations above the susceptible breakpoint (>8 mg of teicoplanin/liter) (18). PAPs were performed on all isolates with Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone ($n$ = 139). In addition, PAPs were also established for any isolate yielding a teicoplanin macromethod value of 8 mg/liter ($n$ = 119). A pilot study of isolates yielding Etest macromethod values of 6 mg/liter ($n$ = 36) also had PAPs performed. Control isolates *S. aureus* Mu3 (ATCC 700698) (prototype hGISA strain), *S. aureus* Mu50 (ATCC 700699) (prototype GISA strain), and *S. aureus* ATCC 29213 (GSSA) were included with each batch of isolates tested.

**Evaluation of agar screening methods.** Six agar screen methods were investigated using 110 hGISA and 68 GSSA isolates. The hGISA isolates were detected by initial screening with the Etest macromethod using both vancomycin and teicoplanin and were confirmed by PAP methods using both vancomycin and teicoplanin. The 68 GSSA isolates comprised 24 isolates that yielded values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone by Etest macromethod screening and were negative by vPAP and tPAP methods and 44 isolates that yielded negative screening results with the Etest macromethod. The latter GSSA isolates included 10 with Etest macromethod values of ≥4 mg/liter and ≤8 mg/liter for both vancomycin and teicoplanin and 34 isolates with Etest macromethod values of ≤2 mg/liter for both vancomycin and teicoplanin. During preliminary agar screening, it had been noted that 51 isolates shown subsequently to be hGISA by vPAP and/or tPAP methods failed to grow on $MHT5_S$ when the inoculum was delivered by a 10-μl loop. Therefore, when evaluating the six agar screen methods, the inoculum was delivered by pipette.

The media evaluated were MHT5 and BHI agar containing 5 mg of teicoplanin/liter (BHIT5). Each medium was investigated by using 10-μl volumes from three inoculum preparations consisting of bacterial suspensions prepared in saline to densities equivalent to 0.5 and 2.0 McFarland turbidity standards, respectively (i.e., $MHT5_{0.5}$, $BHIT5_{0.5}$, $MHT5_{2.0}$, and $BHIT5_{2.0}$) and from stationary-phase subcultures grown in BHI broth ($10^8$ CFU/ml) ($MHT5_S$ and $BHIT5_S$). Twelve isolates were inoculated onto each plate, and screening tests were performed in duplicate. Plates were incubated at 35°C, and growth was observed after 24 and 48 h. Growth of ≥1 colony after 48 h of incubation indicated a positive result. After the evaluation, the medium with the best overall performance ($BHIT5_{0.5}$) was used to screen isolates from collections 5 ($n$ = 330) and 6 ($n$ = 280). Any isolate that yielded growth on $BHIT5_{0.5}$ was investigated further by the Etest macromethod as described above. The sensitivity, specificity, and positive and negative predictive values were calculated for all media evaluated.

## RESULTS

No VRSA or GISA isolates were detected among the 3,189 isolates investigated, but 5.6% of isolates (178 of 3,189) from 5.8% of patients (172 of 2,990) exhibited the hGISA phenotype (Table 2). The criterion used to define hGISA was any isolate recovered by any screening method with a vPAP-AUC ratio of 0.9 to 1.29 and/or a tPAP with a resistant subpopulation growing at concentrations >8 mg of teicoplanin/liter (18, 29).

**Preliminary agar screening.** The agar screening methods used are summarized in Table 1. No isolates grew on BHIV6 or MHT8 agar screen plates. Of 990 isolates screened on $MHT5_S$, 278 yielded growth. These isolates comprised 81 hGISA isolates, but an additional 51 hGISA isolates failed to grow on this medium.

**Etest macromethod.** The numbers of isolates yielding positive screening results by the Etest macromethod are shown in Tables 1 and 2. In total, 2,348 isolates were screened by the Etest macromethod and 139 yielded values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (Tables 1 and 2). When a cutoff value of 8 mg/liter for teicoplanin alone was accepted as the criterion for a positive macromethod result, an additional 119 isolates required further investigation by PAP methods (Table 2).

TABLE 2. Numbers of isolates yielding positive screening results as determined by the Etest macromethod and numbers of hGISA isolates detected by the vPAP and/or tPAP methods

| MRSA collection no., name, yr (no. of isolates/no. of patients)[a] | No. of isolates yielding positive screening results by the Etest macromethod | | | hGISA[e] | |
|---|---|---|---|---|---|
| | V and T ≥ 8 or T ≥ 12 (mg/liter)[b] | T = 8 (mg/liter)[c] | T ≥ 8 (mg/liter)[d] | No. of isolates (%) | No. of patients (%) |
| 1, H1 Q4, 1998 (188/169) | 7 | 8 | 15 | 10 (5.3) | 10 (5.9) |
| 2, N/S study, 1999 (714/682) | 74 | 62 | 136 | 81 (11) | 81 (12) |
| 3, EARSS, 1999–2003 (1,580/1,507) | 15 | 31 | 46 | 39 (2.5) | 39 (2.6) |
| 4, NMRSARL, 2000–2003 (97/59) | 19 | 11 | 30 | 28* (29) | 22* (37) |
| 5, H1 Q2, 2003 (330/303) | 18 | 7 | 25 | 16 (4.9) | 16 (5.3) |
| 6, H1 Q4, 2004 (280/270) | 6 | 0 | 6 | 4 (1.4) | 4 (1.5) |
| Total (3,189/2,990) | 139 | 119 | 258 | 178 (5.6) | 172 (5.8) |

[a] See Table 1, footnote *a*.
[b] Cutoff values of ≥8 mg/liter for both vancomycin (V) and teicoplanin (T) or ≥12 mg/liter for teicoplanin alone.
[c] Cutoff value of 8 mg/liter for teicoplanin (T) alone.
[d] Cutoff value of ≥8 mg/liter for teicoplanin (T).
[e] *, hGISA isolates comprised of 27 hGISA isolates from 22 patients and 1 isolate from an environmental source. Single isolates were recovered from 19 patients; two isolates were recovered from 1 patient, and three isolates were recovered from 2 patients.

**MIC determination.** Etest MIC results for both vancomycin and teicoplanin showed that all 293 isolates investigated had MICs ≤2 mg/liter for vancomycin and ≤8 mg/liter for teicoplanin. Similarly, all 660 isolates selected for testing by using the broth microdilution method yielded vancomycin and teicoplanin MICs of ≤2 mg/liter and ≤8 mg/liter, respectively. Hence, there were no VRSA or GISA strains among these MRSA isolates.

**Population analysis profiling.** Of 139 isolates yielding Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, 73 were confirmed by vPAP-AUC ratio to be hGISA (Table 3). When tPAP was used for confirmation of hGISA, an additional 35 isolates were recognized as hGISA (shown in Table 3). When the Etest macromethod cutoff value was decreased to 8 mg/liter for teicoplanin alone, a further 119 isolates required PAP and 70 additional hGISA were confirmed. Lowering the cutoff value to 6 mg/liter for teicoplanin did not yield any further hGISA. In total, 178 hGISA were detected comprising 13 hVISA, 88 hTISA, and 77 hVISA and hTISA isolates (shown in Tables 2 and 3). vPAP-AUC and/or tPAP analysis showed that 69% (178 of 258) of isolates with a macromethod value of ≥8 mg/liter for teicoplanin were hGISA.

TABLE 3. Numbers of hGISA isolates detected using vancomycin and/or teicoplanin population analysis profiling and breakdown into hVISA, hTISA, and hVISA and hTISA

| Group | No. of isolates investigated by PAP | No. of hGISA isolates detected | | | | | |
|---|---|---|---|---|---|---|---|
| | | vPAP-AUC | tPAP | Total hGISA | hVISA[c] | hTISA[d] | hVISA and hTISA |
| 1[a] | 139 | 73 | 95 | 108 | 13 | 35 | 60 |
| 2[b] | 119 | 17 | 70 | 70 | 0 | 53 | 17 |
| Total | 258 | 90 | 165 | 178 | 13 | 88 | 77 |

[a] Group 1 includes isolates exhibiting Etest macromethod values of ≥8 mg/liter for vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone.
[b] Group 2 includes additional isolates requiring PAP when the teicoplanin Etest macromethod cutoff value was decreased to 8 mg/liter.
[c] hVISA is defined as isolates with vPAP-AUC ratios ranging from 0.9 to 1.29.
[d] hTISA is defined as isolates with resistant subpopulations capable of growth at >8 mg of teicoplanin/liter.

**Evaluation of screening methods.** The sensitivity, specificity, and positive and negative predictive values of $MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_S$, $BHIT5_{0.5}$, $BHIT5_{2.0}$, and $BHIT5_S$ for the detection of hGISA are shown in Table 4. The sensitivity of all methods except $MHT5_{0.5}$ was ≥98% (the sensitivity of $MHT5_{0.5}$ was 66%), but the specificities ranged from 4 to 82%. During preliminary agar screening with $MHT5_S$, when the inoculum was delivered by using a 10-μl loop, the sensitivity and specificity of $MHT5_S$ were 61 and 77%, respectively. When the inoculum was delivered by pipette, sensitivity improved to 100%, but the specificity decreased to 6%.

When the specificity of each of the six agar screen methods to detect isolates with Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone was considered, the methods with the best performance were $BHIT5_{0.5}$ and $MHT5_{2.0}$, with sensitivities of 98 and 95% and specificities of 70 and 60%, respectively. In the course of the present study, the use of tPAP analysis and a criterion for a positive Etest macromethod result of 8 mg/liter for teicoplanin alone allowed detection of an additional 70 hGISA isolates. Using this criterion, the sensitivity and specificity of $BHIT5_{0.5}$ to detect isolates with Etest macromethod values of ≥8 mg/liter for teicoplanin were 99 and 84%, resepctively. The two isolates with positive Etest macromethod values that failed to grow on $BHIT5_{0.5}$ were shown by vPAP-AUC and tPAP analysis to be GSSA.

$BHIT5_{0.5}$ was used to screen all isolates in collections 5 and 6. Seventy-three isolates from collection 5 and 25 isolates from collection 6 were screen positive (Table 1). Of these, 25 isolates from collection 5 and 6 isolates from collection 6 yielded positive macromethod values, whereas 16 isolates from collection 5 and 4 from collection 6 were confirmed as hGISA (Table 2).

**Prevalence of VRSA, GISA, and hGISA.** No VRSA or GISA isolates were detected. Although the overall proportion of hGISA isolates was 5.6% (178 of 3,189), the proportions varied among different groups of isolates (Table 2). Among patients

TABLE 4. Evaluation of six agar screening methods to detect hGISA and isolates yielding positive Etest macromethod values

| Method | Determined value (%)[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | hGISA | | | | Etest macromethod (approach 1)[b] | | | | Etest macromethod (approach 2)[c] | | | |
| | Sens | Spec | PPV | NPV | Sens | Spec | PPV | NPV | Sens | Spec | PPV | NPV |
| $MHT5_{0.5}$ | 66 | 82 | 89 | 61 | 59 | 85 | 95 | 47 | 58 | 91 | 95 | 42 |
| $MHT5_{2.0}$ | 98 | 53 | 77 | 95 | 95 | 60 | 85 | 84 | 96 | 73 | 91 | 84 |
| $MHT5_S$ | 100 | 6 | 63 | 100 | 99 | 6 | 71 | 75 | 99 | 7 | 76 | 75 |
| $BHIT5_{0.5}$ | 100 | 57 | 79 | 100 | 98 | 70 | 88 | 95 | 99 | 84 | 95 | 95 |
| $BHIT5_{2.0}$ | 100 | 18 | 66 | 100 | 100 | 23 | 75 | 100 | 100 | 27 | 80 | 100 |
| $BHIT5_S$ | 100 | 4 | 63 | 100 | 100 | 6 | 71 | 100 | 100 | 7 | 77 | 100 |

[a] Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.
[b] Values for detecting isolates with Etest macromethod values of ≥8 mg/liter for vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone.
[c] Values for detecting isolates with Etest macromethod values of ≥8 mg/liter for teicoplanin alone.

with blood culture isolates investigated under the EARSS project, the prevalence was 2.6% (39 of 1,507), but among patients investigated during the North/South Study of MRSA in Ireland, 1999 prevalence was 12% (81 of 682). In H1, between Q2 2003 and Q4 2004, the proportion fell from 5.3% (16 of 303) to 1.5% (4 of 270).

## DISCUSSION

Since its emergence in 1997, the clinical significance of hGISA has been debated and, although it has been suggested that hGISA may be a laboratory-induced phenomenon, hGISA isolates have been associated with treatment failure and have been shown to be precursors of GISA (3, 15, 23, 28). Accurate assessment of the clinical significance of hGISA requires reliable screening and confirmatory methods (17). Studies have reported that the original method described by Hiramatsu et al. ($10^6$ CFU/ml inoculated onto BHIV4) did not reliably detect hGISA isolates (29). In preparation for the present study, the preliminary selection of isolates from collections 1 and 2 using screening with BHIV4 utilized an inoculum concentration of $10^8$ CFU/ml to ensure no hGISA isolates went undetected (11, 21).

In the clinical laboratory, it is likely that many hGISA isolates are unrecognized because the recommended screening methods present problems for diagnostic laboratories. The Etest macromethod is expensive if it is to be performed on all *S. aureus* isolates, and confirmatory testing with population analysis is labor-intensive, time-consuming, and unsuitable for routine use. In the present study, when the efficacy of various preliminary agar screen methods was assessed, the method of inoculum delivery was found to be a crucial factor. EARSS guidelines for detection of reduced susceptibility to glycopeptides suggest preliminary screening with MHT5 using a stationary-phase broth subculture inoculum and investigating any growth with the Etest macromethod (12). In the present study, the value of MHT5 as a preliminary screening method varied depending on whether the inoculum was delivered by a standard 10-μl loop when the sensitivity and specificity were 61 and 77%, respectively, or by pipette, where the sensitivity increased to 100% but the specificity fell to 6%. This poor specificity makes the method unsuitable for routine diagnostic use. Of the other agar screening media evaluated, $BHIT5_{0.5}$ using an inoculum of $10^6$ CFU/ml proved to be the most useful agar screen for detecting hGISA, with a sensitivity of 100% and a specificity of 57%. The specificity increased to 84% when the method was used as a screening technique to detect isolates with Etest teicoplanin macromethod values of ≥8 mg/liter. $BHIT5_{0.5}$ also has the practical advantage for the diagnostic laboratory that the inoculum density (equivalent to a 0.5 McFarland turbidity standard) is the same as that used for routine CLSI disk diffusion susceptibility testing.

Subsequent to the present study, further evaluation of $BHIT5_{0.5}$ was undertaken at the NMRSARL to investigate sensitivity and specificity when the criterion for a positive result was growth of >1 colony. Among 492 blood culture MRSA isolates screened with both the Etest macromethod and $BHIT5_{0.5}$ agar screening between June 2006 and May 2007, sensitivity and specificity values for the detection of (i) hGISA, (ii) isolates exhibiting Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, and (iii) isolates exhibiting teicoplanin Etest macromethod values of ≥8 mg/liter were 100 and 84% for all three categories when the criterion for a positive result was the growth of ≥1 colony. When the criterion was changed to >1 colony, the sensitivity and specificity values were 100 and 92% for categories i and ii and 95 and 94% for category iii, respectively. Population analysis profiling showed that the single isolate that exhibited a teicoplanin Etest macromethod value of 8 mg/liter and yielded one colony on $BHIT5_{0.5}$ was GSSA.

Recently, Wootton et al. compared three screening methods for detecting GISA and hGISA isolates and found that $MHT5_{2.0}$ and the Etest macromethod (using an 100-μl inoculum volume) yielded sensitivity and specificity values of 86 and 76% and 82 and 89%, respectively (30). In an earlier study in which the inoculum volume was 200 μl, the sensitivity and specificity of the Etest macromethod was reported to be 96 and 97% when the interpretative criteria for a positive screening result were ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (26). In the present study, 200-μl inoculum volumes were used as recommended by the manufacturer's technical guide for the detection of reduced susceptibility to glycopeptides, but there is a discrepancy between this recommendation and the recommendation to use a 100-μl volume inoculum in the manufacturer's Etest application sheet for staphylococci (1, 2). When the interpretative criterion of 8 mg/liter for teicoplanin alone for a positive Etest macromethod was used, the numbers of hGISA detected increased by 23% (17 of 73) when the vPAP-AUC ratio deter-

mination was used as the confirmatory method. The sensitivity and specificity of screening with $BHIT5_{0.5}$ to detect isolates with this positive Etest macromethod value were 99 and 84%, whereas screening on $MHT5_{2.0}$ yielded sensitivity and specificity values of 96 and 73%, respectively. Thus, a practical approach to detecting hGISA isolates in clinical laboratories is to screen on $BHIT5_{0.5}$ and to investigate positive results by the Etest macromethod, with final confirmation using PAP.

In the present study, when the Etest manufacturer's criteria of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone and vPAP-AUC only was used, the proportion of hGISA isolates detected was 2.3% (73 of 3,189). However, decreasing the teicoplanin Etest macromethod cutoff value to 8 mg/liter and including tPAP to confirm the hGISA phenotype increased the number of hGISA detected by 144% (105 of 73), bringing the overall proportion of hGISA to 5.6% (178 of 3,189). Rates varied from 1.5 to 37% depending on the patient population studied. A prevalence rate of 2.6% was found among blood culture isolates submitted to NMRSARL under the EARSS project where the protocol required data on the first isolate per patient per quarter. Since the hGISA phenotype tends to be associated with prolonged exposure to glycopeptide, a lower prevalence among this population might be expected (27). However, the lowest prevalence was observed in H1 during the prospective study in 2004 when isolates from all specimen sites were investigated. During that study, additional isolates were sought from patients on prolonged glycopeptide therapy, but only one further hGISA isolates was detected.

In summary, the present study has shown that $BHIT5_{0.5}$ is a useful method of screening for hGISA where screen-positive isolates are investigated by the Etest macromethod and confirmed by PAP. The currently recommended Etest macromethod cutoff criteria need to be reevaluated. tPAP is needed to ensure that the phenotype of all presumptive hGISA isolates is confirmed. A simpler hGISA confirmatory method suitable for use in clinical laboratories is required. The prevalence of hGISA among MRSA isolates recovered from blood culture in Ireland is 2.6%.

## ACKNOWLEDGMENTS

This study was supported by a grant from the Strategy for the Control of Antimicrobial Resistance in Ireland (SARI).

We thank the Bristol Centre for Antimicrobial Research and Evaluation for help and advice in setting up the PAP-AUC protocol. We also thank the Irish laboratories that participate in the EARSS project, the Irish EARSS Steering Committee, the staff of all laboratories that participated in the North/South Study of MRSA in Ireland, and the Steering Committee of the North/South Study. In addition, we thank Sanofi-Aventis for the gift of teicoplanin.

## REFERENCES

1. **AB Biodisk.** 2004. Etest application sheet 003 staphylococci. AB Biodisk, Ltd., Solna, Sweden. http://www.abbiodisk.com/pdf/eas/M0000143-MH0164.pdf.
2. **AB Biodisk.** 2004. Etest technical manual: 23 staphylococci. Etest for glycopeptides. AB Biodisk, Ltd., Solna, Sweden. http://www.abbiodisk.com/pdf/epl/M0000221_MH0271.pdf.
3. **Ariza, J., M. Pujol, J. Cabo, C. Pena, N. Fernandez, J. Linares, J. Ayats, and F. Gudiol.** 1999. Vancomycin in surgical infections due to methicillin-resistant *Staphylococcus aureus* with heterogeneous resistance to vancomycin. Lancet **353:**1587–1588.
4. **Bataineh, H. A.** 2006. Resistance of *Staphylococcus aureus* to vancomycin in Zaqra, Jordan. Pak. J. Med. Sci. **22:**144–148.
5. **British Society for Antimicrobial Chemotherapy.** 2006. BSAC methods for antimicrobial susceptibility testing, version 5, January 2006. BSAC, Birmingham, United Kingdom. www.bsac.org.uk/susceptibility_testing/bsac_standardized_disc_susceptibility_method.cfm.
6. **Brown, D. F., D. I. Edwards, P. M. Hawkey, D. Morrison, G. L. Ridgway, K. J. Towner, and M. W. D. Wren.** 2005. Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA). J. Antimicrob. Chemother. **56:**1000–1018.
7. **Centers for Disease Control and Prevention.** 2006. Investigation and control of vancomycin-intermediate and -resistant *Staphylococcus aureus* (VISA/VRSA). CDC, Atlanta, GA. http://www.cdc.gov/ncidod/dhqp/pdf/ar/visa_vrsa_guide.pdf. Accessed 14 February 2007.
8. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A7; 7th ed. CLSI, Wayne, PA.
9. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement M100-S16. CLSI, Wayne, PA.
10. **Comité de l'Antibiogramme de la Société Française de Microbiologie.** 2005. Recommendations du CASFM, Communiqué 2005. CASFM, Paris, France. http://www.sfm.asso.fr/nouv/general.php?pa=5. Accessed 14 February 2007.
11. **Department of Health and Children.** 2000. North/south study of MRSA in Ireland 1999. Irish Department of Health and Children, Dublin, Ireland. http://www.dohc.ie/publications/pdf/mrsa99.pdf. Accessed 14 February 2007.
12. **European Antimicrobial Resistance Surveillance System.** 2005. EARSS Manual 2005: EARSS protocol for testing *Staphylococcus aureus*: new and updated protocols for AST 2005. EARSS RIVM, Bilthoven, The Netherlands. http://www.rivm.nl/earss/tools/. Accessed 14 February 2007.
13. **European Committee on Antimicrobial Susceptibility Testing.** 2006. Glycopeptides: EUCAST clinical MIC breakpoints 2006-06-20 (v 1.3). EUCAST, Basel, Switzerland. www.srga.org/eucastwt/MICTAB/MICglycopeptides.html. Accessed 14 February 2007.
14. **Guerin, F., A. Buu-Hoi, J. L. Mainardi, G. Kac, N. Colardelle, S. Vaupre, L. Gutmann, and I. Podglajen.** 2000. Outbreak of methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to glycopeptides in a Parisian hospital. J. Clin. Microbiol. **38:**2985–2988.
15. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–1673.
16. **Howden, B. P., P. D. R. Johnson, P. B. Ward, T. P. Stinear, and J. K. Davies.** 2006. Isolates with low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. Antimicrob. Agents Chemother. **50:**3039–3047.
17. **Liu, C., and H. F. Chambers.** 2003. *Staphylococcus aureus* with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. Antimicrob. Agents Chemother. **47:**3040–3045.
18. **Nonhoff, C., O. Denis, and M. Struelens.** 2005. Low prevalence of methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to glycopeptides in Belgian hospitals. Clin. Microbiol. Infect. **11:**214–220.
19. **Park, Y.-J., M. Kim, E.-J. Oh, S.-O. Lee, B. K. Kim, and S. M. Kim.** 2000. Screening method for detecting staphylococci with reduced susceptibility to teicoplanin. J. Microbiol. Methods **40:**193–198.
20. **Rossney, A., M. J. Lawrence, P. M. Morgan, M. M. Fitzgibbon, A. Shore, D. Coleman, C. T. Keane, and B. O'Connell.** 2006. Epidemiological typing of MRSA isolates from blood cultures taken in Irish hospitals participating in the European Antimicrobial Resistance Surveillance System (1999–2003). Eur. J. Clin. Microbiol. Infect. Dis. **25:**79–89.
21. **Rossney, A. S., and C. T. Keane.** 2002. Strain variation in the MRSA population over a 10-year period in one Dublin hospital. Eur. J. Clin. Microbiol. Infect. Dis. **21:**123–126.
22. **Rossney, A. S., P. McDonald, H. Humphreys, G. M. Glynn, and C. T. Keane.** 2003. Antimicrobial resistance and epidemiological typing of methicillin-resistant *Staphylococcus aureus* in Ireland (North and South), 1999. Eur. J. Clin. Microbiol. Infect. Dis. **22:**379–381.
23. **Sieradzki, K., T. Leski, J. Dick, L. Borio, and A. Tomasz.** 2003. Evolution of a vancomycin-intermediate *Staphylococcus aureus* strain in vivo: multiple changes in the antibiotic resistance phenotypes of a single lineage of methicillin-resistant *S. aureus* under the impact of antibiotics administered for chemotherapy. J. Clin. Microbiol. **41:**1687–1693.
24. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, N. C. Clark, and K. Hiramatsu.** 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J. Clin. Microbiol. **36:**1020–1027.
25. **Tiwari, H. K., and M. R. Sen.** 2006. Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. Biomed Central, London, United Kingdom. Dis. BMC Infect. http://www.biomedcentral.com/1471–2334/6/156. Accessed 14 February 2007.
26. **Walsh, T. R., A. Bolmstrom, A. Qwarnstrom, P. Ho, M. Wootton, R. A. Howe, A. P. MacGowan, and D. Diekema.** 2001. Evaluation of current methods for

detection of staphylococci with reduced susceptibility to glycopeptides. J. Clin. Microbiol. **39:**2439–2444.

27. **Walsh, T. R., and R. A. Howe.** 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. Annu. Rev. Microbiol. **56:** 657–675.
28. **Ward, P. B., P. D. Johnson, E. A. Grabsch, B. C. Mayall, and M. L. Grayson.** 2001. Treatment failure due to methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to vancomycin. Med. J. Aust. **175:**480–483.
29. **Wootton, M., R. A. Howe, R. Hillman, T. R. Walsh, P. M. Bennett, and A. P. MacGowan.** 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. J. Antimicrob. Chemother. **47:**399–403.
30. **Wootton, M., A. P. MacGowan, T. R. Walsh, and R. A. Howe.** 2007. A multi-center study evaluating current strategies for isolating *Staphylococcus aureus* strains with reduced susceptibility to glycopeptides. J. Clin. Microbiol. **45:**329–332.