Evaluation of a New Etest Vancomycin + Teicoplanin Strip for Detection of Glycopeptide Intermediate *Staphylococcus aureus* (GISA) and heterogeneous GISA

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Glycopeptide intermediate *Staphylococcus aureus* (GISA) and, in particular heterogeneous GISA (hGISA) are difficult to detect by standard MIC methods and thus an accurate detection method for clinical practice and surveillances is needed. Two prototype Etest strips designed for hGISA/GISA resistance detection (GRD) were evaluated using a worldwide collection of hGISA/GISA strains covering the five major clonal lineages. 150 strains comprising 15 GISA and 60 hGISA (defined by population analysis profiles – area under curve (PAP-AUC)), 70 glycopeptide susceptible *S. aureus* (GSSA), and 5 *S. aureus* ATCC® reference strains were tested. Standardised Etest VA MIC testing, the modified Etest macromethod (E-M) with VA and teicoplanin (TP) strips tested with a heavier inoculum using brain heart infusion agar (BHI), and two glycopeptide screening agar plates (6µg/ml VA/BHI and 5µg/ml MHA) were tested in parallel to the two new Etest GRD strips: VA 32- 0.5/ TP 32-0.5 µg/ml double-sided gradient (E-VA/TP), with one prototype overlaid with a nutrient (E-VA/TP+S) to enhance growth of hGISA. Etest GRD strips were tested with a standard 0.5 McFarland inoculum using Mueller Hinton agar ± 5% blood (MHA, MHB) and read at 18-24 and 48h. Interpretive cut-offs used for the new Etest GRD strips at 24 and 48h were: GISA: TP or VA ≥8 and standard VA MIC ≥6; hGISA: TP or VA ≥8 and standard VA MIC ≤4. Results on MHB at 48 h showed that E-VA/TP+S had high specificity (94%) and sensitivity (95%) in comparison to PAP-AUC, and was able to detect all GISA (n=15) and 98% of hGISA (n=60) strains. In contrast, the glycopeptide screening plates preformed poorly for hGISA. The new Etest GRD strip (E-VA/TP+S), utilising standard media and inoculums, comprises a simple and acceptable tool for detection of hGISA/GISA for clinical and epidemiologic purposes.
INTRODUCTION

The first reports of glycopeptide intermediate *Staphylococcus aureus* (GISA) raised some complex and challenging questions with respect to their clinical significance and methods for the detection of these phenotypes and definition of this “resistance” are issues that still remain today (10, 23). Additionally, a heterogeneous form of the resistance is frequently seen (hGISA) where only a small proportion of the population (approx. $10^6$) express the resistant phenotype creating even further detection dilemmas (10). Just as it took over 10 years for hMRSA to be simply considered the same phenotype (and genotype) as MRSA, so may the case prove to be for hGISA (4).

hGISA and GISA has been the subject of many recent reviews covering their mechanism of resistance, clinical relevance, treatment challenges, and detection methods (3, 7, 9, 15, 19, 20, 22, 23). It now appears that both hGISA and GISA have common structural and clinical features, inuring many researchers that they almost identical and should be reported as such (12). Generally speaking, both hGISA and GISA possess thickened cell walls, are associated with prolonged glycopeptide therapy and low glycopeptide serum concentrations, and often share a number of genetic expression markers e.g. atl, mrpB (8, 15, 18, 27, 28, 30). More importantly, there exist an increasing number of studies alluding to the clinical significance of hGISA and its association with vancomycin failures (5, 13, 14, 27).

The current differentiation of hGISA from GISA, appear to imply that GISA possess a homogenously vancomycin (VA) resistant population in contrast to hGISA. However, population studies of strains with reduced susceptibility to VA (MIC 2-16 µg/ml) show that this is indeed not the case and that GISA strains often present with a non-homogeneous or heterogeneous VA kill curve. The obvious difference seen between hGISA and GISA is that the GISA isolates more often produce enough of the sub-population expressing the
intermediate level of “resistance” such that these cells will now grow on VA at 4µg/ml when tested with standard inoculum equivalent to 0.5 McFarland standard. Accordingly, there is much debate over whether the current CLSI VA intermediate breakpoint of 4-8 µg/ml is appropriate for detecting all hGISA/GISA phenotypes. A recent study where VA MICs for 20 GISA, 157 h-GISA and 106 non-GISA were determined indicated that the current intermediate breakpoint of 4 should be reduced to 2 µg/ml to better identify the hGISA/GISA strains (31). This notion is further supported by the CDC which have revised their S. aureus /VA algorithm accommodating VISA stains not expressing high-levels of VA resistance (http://www.cdc.gov/ncidod/dhqp/ar-visavrsa-algo.html). This initiative was adopted by the CLSI in 2005 and amendments made to Table 2C, M2 and M7 (http://www.clsi.org).

Since their advent nearly ten years ago, there have been many methods advocated for detecting hGISA/GISA (6). Automated methods have been modified to try and detect high-level VA-resistant S. aureus (VRSA, MIC >32 µg/ml); however, these methods struggle to detect GISA and are inappropriate for detecting hGISA (1, 2). As disk diffusion testing was quickly recognised as unsuitable (16) other methods have been proposed such as glycopeptide screening plates (6) and various population studies (11, 29). Other techniques such as the modified Etest method (macromethod) have also been introduced but it requires non-standard media (Brain Heart Infusion) and 2.0 McFarland inoculum i.e. variations that beyond standard susceptibility testing praxis (26). Accordingly, we investigated a new Etest GRD strip (E-VA/TP), one with a growth nutrient and one without. The GRD strip uses a standard inoculum and agar and herein we test it against an international collection of hGISA/GISA/non-GISA comprising different MLST profiles and established genetic lineages (17).
MATERIALS AND METHODS

Strains. The following reference organisms were used: *S. aureus* ATCC: 29213 and 25923 (non-MRSA), 43300 (MRSA), 700698 (Mu3 - hGISA), and 700699 (Mu50 - GISA) were tested in quintuplicate. The clinically derived test strains comprised the following: 15 GISA, 60 hGISA (defined by a positive PAP-AUC) and 70 GSSA. These isolates comprised an international collection of unique strains (different clones) comprising all five major MRSA endemic clonal lineages (17).

Media and antibiotics. Brain-Heart Infusion (BHI) and Mueller-Hinton agar was obtained from BBL (Becton and Dickinson, Cockeysville, MD). Vancomycin (VA) and teicoplanin (TP) antibiotic powders were obtained from Fagron (Barsbuttel, Germany) and Molcan Corp (Richmond Hill, Canada), respectively.

Etest macromethod. The Etest macromethod (E-M) was performed as described by Walsh *et al.* (26). A suspension of colonies from an overnight culture on blood plate was prepared in MH broth and the turbidity adjusted to 2.0 McFarland after which 200 µl of the suspension was pipetted and evenly streaked out on the surface of a 90-mm BHI agar plate. The Etest standard procedure for MIC testing of VA was performed using an inoculum suspension in 0.9% saline (0.5 McFarland) that was streaked onto Mueller Hinton agar plates (BBL). After drying the plates for approx. 10 minutes, Etest strips (AB BIODISK, Solna, Sweden) for VA (0.016 to 256 µg/ml) and TP (0.016 to 256 µg/ml) were applied to the BHI plate and VA to the MH plate. Agar plates were incubated at 35 °C for 18-24 and 48h and read by two different laboratory technicians.
**Etest GRD strip.** The two new Etest prototype strips evaluated were: VA 32- 0.5/ TP 32-0.5 µg/ml double-sided gradient (E-VA/TP), with one prototype with a nutrient incorporated into the strip (E-VA/TP+S) to enhance the growth of hGISA. Both strips were tested with standard inoculum (0.5 McFarland) using Mueller-Hinton agar agar (BBL) ± 5% blood and read at 18-24 and 48h. The endpoints read from the Etest GRD strips should not be regarded as true MIC values but rather as modified results with interpretive cut-offs defined for the phenotypic detection of glycopeptide resistance phenotypes in *S. aureus*. Preliminary interpretive cut-offs used for the Etest GRD prototype strips read at 24 and 48h were: GISA: E-M values of TP or VA ≥8 and standard VA MIC ≥6; hGISA: E-M values of TP or VA ≥8 and standard VA MIC ≤4.

**Vancomycin screening plate.** The VA (6 µg/ml) BHI screening plate (21, 24, 26) recommended by the CDC (http://www.cdc.gov) was used. All plates were spot-inoculated with 10 µl of an inoculum suspension prepared with growth from an overnight blood agar plate, with a turbidity equivalent to 0.5 McFarland. Plates were incubated for 48h and growth was reported after both 24 and 48h.

**Teicoplanin screening plate.** The TP (5 µg/ml) Mueller-Hinton agar screening plate recommended by the CA-SFM (http://www.sfm.asso.fr) was used. All plates were spot-inoculated with 10 µl of an inoculum suspension prepared with growth from an overnight blood agar plate, with a turbidity equivalent to 2 McFarland. Plates were incubated for 48h and growth was reported after both 24 and 48h.

**Population analysis profile – area under the curve (PAP-AUC).** The method described by Wootton *et al.* was used (29). After 24 h incubation in tryptone soya broth (Oxoid, Basingstoke, Hampshire, UK), an undiluted culture and dilutions of 1/10^{-8} and 1/10^{-5} were inoculated using a spiral plater (Don Whitley, Shipley, UK) onto BHI agar (Oxoid) plates.
containing 0.5, 1, 2.5, 4, and 8 µg/ml of VA. Colonies were counted after 48h incubation. The number of CFU/ml was plotted against VA concentration by using GraphPad Prism software (San Diego, CA, USA). The area underneath the curve was plotted for each test strain and compared with the curves for Mu3, Mu50 and *S. aureus* ATCC control strains. A ratio was then calculated by dividing the AUC of the test strain by the AUC of Mu3. The isolates used herein had their PAP-AUC calculated prior to commencement of the study; however, a random sample (# 50 isolates) was chosen to ensure the PAP-AUC value i.e. the hGISA/GISA phenotype had been maintained.

**Statistical analysis.** The performance of each method in detecting hGISA/GISA was evaluated by comparison with the PAP-AUC ratio. Each method was assessed for its specificity and sensitivity in discriminating hGISA/GISA from GSSA as previously described (26). The specificity is based on the number of correct negative results i.e. the true number of GSSA strains that were correctly identified. The sensitivity is based on the number of hGISA/GISA that was correctly identified.
RESULTS

Figures 1-3 show examples of results with the new Etest VA/TP+S strips for S. aureus ATCC 29213, ATCC 700698 (Mu3) and ATCC 700699 (Mu50), respectively. Table 1 shows the MIC ranges for each of the methods. Typically, the GRD values for the negative control GSSA strain S. aureus ATCC 29213 for VA and TP varies between 0.5 -1 µg/ml on both Mueller-Hinton agar and Mueller-Hinton agar plus blood. For hGISA and GISA the MIC ranges were higher after 48hrs and the resistance enhanced by the presence of both the blood and growth supplement (Table 1). S. aureus ATCC 700698 (Mu3) gave low VA values and discernibly high TP values (≥ 32 µg/ml) and small colony variants (SCVs) were clearly visible within the TP inhibition ellipses on Mueller-Hinton agar and Mueller-Hinton agar plus blood. S. aureus ATCC 700699 (Mu50) gave high VA values (12 µg/ml) compared to Mu3, with SCVs clearly visible in the VA inhibition ellipse, particularly on Mueller-Hinton agar plus blood. Predictably, the Mu50 GISA phenotype had very high TP value (>32 µg/ml) and almost no inhibition ellipse.

Table 2 compares the sensitivity and specificity of the Etest GRD strips (E-VA/TP +/− S) with the E-M for the 150 PAP-AUC phenotypically characterised strains. For the detection of hGISA/GISA, the E-VA/TP + S strip tested on Mueller-Hinton agar plus blood and read at 48h had the highest sensitivity (94%), and was comparable to the E-M. The addition of 5% blood to Mueller-Hinton agar increased the 48h detection sensitivity for E-VA/TP and E-VA/TP+S, from 80 to 89%, and 84 to 94%, respectively. The sensitivity for 24h reading was appreciably lower than for 48h, confirming the need for extended incubation to optimise the detection of glycopeptide resistance. After 48h incubation, all methods gave a specificity of 95-96%.
The vanocmycin screening plate, VA/BHI, missed most hGISA/GISA strains (overall sensitivity of 27% - Table 2) with 12% sensitivity for hGISA and 87% sensitivity. The CA-SFM screening plate (TP/ Mueller-Hinton agar) was better than VA/BHI with an overall sensitivity of 65% and a specificity of 95%. However, whilst it was able to detect most GISA strains (93% sensitivity) it only had a sensitivity of 58% for hGISA strains.

All strains were independently tested in triplicate under blind conditions to examine the robustness of each method. Table 3 summarises the reproducibility of the Etest GRD strips (E-VA/TP +/- S) and E-M to the two agar screening plates. For the GISA strains (n=15), both the E-VA/TP, E-VA-TP+S strips and E-M had 100% reproducibility compared to the agar screens VA/BHI and TP/ Mueller-Hinton agar that had 87 and 93%, respectively. The screening plates were unreliable for the detection of hGISA (n=60) with VA/BHI and TP/Mueller-Hinton agar giving poor reproducibilities of 12% and 58%, respectively. The best performance for the repeated detection of hGISA was found with the E-VA/TP+S strip on Mueller-Hinton agar plus blood which had a reproducibility of 98% at 48h. The addition of blood to Mueller-Hinton agar proved very effective in increasing the reproducibility of hGISA detection from 81 to 95%, and 89 to 98%, for E-VA/TP and EVA/TP+S strips, respectively (Table 3).
DISCUSSION

The increasing debate over the clinical significance of GISA, and in particular h-GISA, has been compounded by the difficulty in detecting them. The clinical failure attributable to glycopeptide use may in part be due to their physical chemical properties, high protein binding, and potentially insufficient drug levels at the infection site due to poor pharmacokinetics properties of the drug and difficulties in optimising the dose and dosing regimen relative to concentration-related toxicity of the drugs (25). However, despite sub-therapeutic drug levels, it is now known that staphylococci can respond to the presence of the low levels of glycopeptide which can subsequently select for low-level resistance. This type of heterogeneous and variable resistance in staphylococci which is expressed at varying frequencies within the population is not detectable by either disk diffusion (16, 27) or automated susceptibility testing systems (8, 24) – a phenomenon recognised by numerous expert groups including the CDC (http://www.cdc.gov/ncidod/dhqp/ar-visavrsa.html), CLSI (http://www.CLSI.org) BSAC (http://www.bsac.org.uk) and CA-SFM (http://www.sfm.asso.fr). To address the limitations of disk diffusion and automated systems, agar screening plates have been developed as simple alternatives to screen for hGISA/GISA strains, since populations analysis profile methods are highly specialised and unsuitable for use in clinical laboratories. The PAP-AUC used as the reference method in this study, whilst labour intensive, is sensitive. The VA/BHI plate, initially developed for vancomycin resistance screen in Enterococci, is commercially available and widely used in the US.

However, this study and various others have clearly shown that this method is poor in detecting hGISA and occasional will fail to detect GISA strains. TP, a more sensitive marker for the detection of hGISA/GISA is not available as a commercial reagent in the US, thus, precluding the TP/ Mueller-Hinton agar plate as a viable option for screening of hGISA/GISA.
The E-M was first introduced as a potential screening method for detecting hGISA/GISA and thereafter further investigated in a controlled study (26). The new Etest GRD strip with double sided VA and TP concentration gradients across 7 dilutions (E-VA/TP) was designed to be used with normal media and at a standard inoculum, circumventing some of the perceived problems that arose with the macro-method. GISA and hGISA strain are notoriously slow growing and their standard feature is the thickened cell wall and their pleomorphic appearance – often involving SCVs. Accordingly, in order to detect all SCVs a growth supplement has been added which enhances their growth and thus their detection.

The two prototypes E-VA/TP and E-VA/TP+S were thus examined using the standard inoculum of 0.5 McFarland and on two different agar plates (Mueller-Hinton agar +/- blood) that are readily available in the clinical laboratory. The sensitivity at 48h is greater than that at 24h (Table 2) as the SCVs are larger enough to be seen with the naked eye and are visible as a light growth within the Etest ellipse (Fig. 1-3). Given that vancomycin is often used for an extended period (some cases report hGISA being treated for a period of 18 weeks), we feel that the 48h incubation period for improved sensitivity is appropriate will not overtly affect the clinical outcome (27). Results for E-VA/TP+S read at 18-24h incubation, if positive for hGISA/GISA can be reported as such, although negative results should be confirmed after 48h incubation since sensitivity was highest at 48h, detecting all GISA (n=15) and 98% of hGISA (n=60) strains on Mueller-Hinton agar plus blood.

Clinicians and laboratories alike are becoming increasingly aware that patients on long term vancomycin therapy and cases of recurrent MRSA bacteremia (5, 12) may signal the presence and potential spread of hGISA/GISA strains. The extent of the problem is still unknown as laboratories are not equipped with appropriate and sensitive test methods that can reliably detect these phenotypes in their clinical routines. Herein, we have presented a new method – the Etest GRD strip with vancomycin and teicoplanin - that performs as well.
as the E-M and the reference PAP-AUC assay, but with the simplicity of a standard routine diagnostic test which can be used daily for clinical and epidemiological purposes.
REFERENCES


28. **Wootton, M., P. M. Bennett, A. P. MacGowan, and T. R. Walsh.** 2005. Reduced expression of the *atl* autolysin gene and susceptibility to autolysis in clinical


Table 1. MIC ranges for GRD Etest and standard MICs compared with the Etest macro-method and PAP-AUC

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>GRD MHA$^1$</th>
<th>GRD MHB$^2$</th>
<th>GRD + S MHA$^3$</th>
<th>GRD + S MHB</th>
<th>Etest Macro-method</th>
<th>PAP-AUC</th>
<th>V-MIC$^6$</th>
<th>T-MIC$^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>T</td>
<td>V</td>
<td>T</td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSSA</td>
<td>0.5-0.75</td>
<td>1.0-1.5</td>
<td>0.5-1.0</td>
<td>0.75-2.0</td>
<td>0.5-0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRSA</td>
<td>0.5-0.75</td>
<td>1.0-1.5</td>
<td>1.5-2.0</td>
<td>0.75-1.0</td>
<td>0.5-0.75</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>hGISA</td>
<td>0.75-1.0</td>
<td>1.0-3.2</td>
<td>1.5-2.0</td>
<td>0.75-4.0</td>
<td>0.5-0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GISA</td>
<td>6-12</td>
<td>6-12</td>
<td>32-&gt;32</td>
<td>6-12</td>
<td>8-16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MIC ranges for GRD Etest and standard MICs compared with the Etest macro-method and PAP-AUC.
1 GRD with Muller Hinton Agar
2 GRD with Muller Hinton Agar plus 5% blood
3 GRD plus supplements with Muller Hinton Agar
4 GRD plus supplements with Muller Hinton Agar plus 5% blood
5 V: vancomycin; T: teicoplanin
6&7 MIC were read at 18hr
TABLE 2: Sensitivity and specificity of E-VA/TP+/-S on MHA and MHB, and E-M for detection of hGISA/GISA phenotypes characterised by PAP-AUC

<table>
<thead>
<tr>
<th>Detection (%)</th>
<th>Etest GRD strips</th>
<th>E-M</th>
<th>Agar screens</th>
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<tbody>
<tr>
<td></td>
<td>E-VA/TP</td>
<td>E-VA/TP +S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MHA</td>
<td>MHB</td>
<td>MHA</td>
</tr>
<tr>
<td>24h</td>
<td>24h</td>
<td>48h</td>
<td>48h</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>53</td>
<td>80</td>
<td>55</td>
</tr>
<tr>
<td>Specificity</td>
<td>100</td>
<td>95</td>
<td>100</td>
</tr>
</tbody>
</table>

¹Read at 48hrs
TABLE 3: Reproducibility of Etest GRD, E-M and agar screening for detection of hGISA/GISA/GSSA

<table>
<thead>
<tr>
<th>% Correct phenotype</th>
<th>Etest GRD (48h)</th>
<th>E-M</th>
<th>Agar screen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E-VA/TP</td>
<td>E-VA/TP+S</td>
<td></td>
</tr>
<tr>
<td>MH</td>
<td>MH</td>
<td>MH</td>
<td>BHI/2McF</td>
</tr>
<tr>
<td>GISA (15x3)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>hGISA (60x3)</td>
<td>81</td>
<td>95</td>
<td>89</td>
</tr>
<tr>
<td>5 ATCC (25x3)</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</table>
FIG. 1. E-VA/TP+/-S with MHA and MHB for *S. aureus* ATCC 29213
FIG 2. E-VA/TP+/-S with MHA and MHB for S. aureus ATCC 700698 (Mu3/hGISA)
FIG. 3. E-VA/TP+/-S with MHA and MHB for *S. aureus* ATCC 700699 (Mu50/GISA)