A multi-center study evaluating the current strategies for isolating

*Staphylococcus aureus* strains with reduced susceptibility to glycopeptides

Evaluation of GISA/hGISA detection methods.

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Abstract

Glycopeptide intermediate *Staphylococcus aureus* (GISA) and hetero (h) GISA strains are notoriously difficult to detect in the diagnostic laboratory. The clinical importance of GISA and in particular hGISA will only be obvious when a definitive detection method is available. A few novel GISA and hGISA detection methods have been proposed; however their validity has never been tested on a significant scale and in different laboratories. This study compares three screening methods for detecting GISA and hGISA strains in twelve laboratories using a blind panel of 48 strains of known glycopeptide susceptibility. The three screening methods used were Brain Heart Infusion agar with 6mg/L vancomycin (BHIA6V) (CDC/CLSI), Mueller-Hinton agar with 5mg/L teicoplanin (MHA5T) (EARSS) and the Macromethod Etest (MET) (EARSS) with Population Analysis Profile-Area Under Curve as a gold standard. Sensitivity and specificity were highest for MHA5T and MET identifying 82.5% and 85.9% of strains respectively. BHIA6V had poor sensitivity, particularly for hGISA (11.5% strains detected) and gave the largest inter-laboratory variability in performance. MET exhibited the least inter-laboratory variability. It is essential that laboratories use appropriate methods to detect GISA/hGISA so that prevalence and clinical importance of these strains can be properly assessed.
Introduction

The emergence of glycopeptide resistance in *Staphylococcus aureus* will have a significant impact on human health. In recent years, glycopeptide resistant *S. aureus* (GRSA) and both homogeneous intermediate resistance (GISA) and heterogeneous (hGISA) have increasingly been reported (18, 14, 3). The clinical significance of GRSA and GISA seem to be in little doubt, and there is mounting evidence that hetero-resistance is associated with failure of vancomycin therapy (7, 9, 13) However, in order to establish the prevalence and clinical relevance of GISA and in particular hGISA, a reliable method for their detection must be established.

GRSA strains exhibit vancomycin MICs of $\geq 32$mg/L, having acquired *vanA* from *Enterococcus faecalis/faecium* (3, 4). Their identification in-vitro is assumed to be straightforward using standard protocols, however; their identification with automated systems is reported to be questionable (16). GISA and hGISA isolates on the other hand exhibit vancomycin MICs of 4-8 and 2-4mg/L respectively and have a mechanism of resistance which has not been fully defined. Accordingly, the detection of GISA, and in particular hGISA, has been beset by problems due to unreliable methodologies (17, 7, 15). The current most reliable method for definitive identification of GISA/hGISA, and one that has been used in a number of surveillance studies, is the PAP-AUC method (19, 2, 8). This method is a modified population analysis method using an analysis protocol and criteria specifically designed to discriminate between GSSA, hGISA and GISA (19). Unfortunately, this method is labour-intensive and performance in a diagnostic laboratory on large numbers of strains is untenable. Thus a reliable screening agar would be preferable in terms of ease of use and cost. Several GISA/hGISA screening methods have been proposed including various screening agars (5, 6, 1) or interaction agars (H. Hanaki, S.
Ohkawa, Y. Inaba, T. Hashimoto, and K. Hiramatsu. Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother. Abstr. C132. 1998) and population studies (5). However these have not been assessed for comparability and inter-laboratory consistency. This study seeks to address this problem by examining three of the most commonly-used and recommended methodologies in a multi-centre, multinational comparison using the PAP-AUC method as a gold standard (19).
Methods

Study Design: The study was designed to evaluate the three most common identification methods for detecting *Staphylococcus aureus* isolates with reduced susceptibility to glycopeptides; a vancomycin screening agar as recommended by CDC and CLSI (www.cdc.gov/ncidod/dhqp/ar_visavrsa_labFAQ.html), a teicoplanin screening agar as recommended by EARSS (www.rivm.nl/earss/tools/-manual 2005), and the macromethod Etest (EAS 003, www.abbiodisk.com). Twelve laboratories participated, representing geographically diverse regions throughout the world; 2 UK, 3 USA, 2 Belgium, 1 Switzerland, 1 Sweden, 1 France, 1 Australia, 1 Russia. Each laboratory was furnished with 48 strains of known glycopeptide susceptibility (laboratories were blinded as to this data), protocols for three methods, and all media and antimicrobials necessary to complete the study. The strain set included three control strains: Mu50, Mu3, and ATCC 29213, and triplicates of clonally distinct clinical strains representing, 5 glycopeptide intermediate *Staphylococcus aureus* (GISA), 5 heterogeneous GISA (hGISA) and 5 glycopeptide susceptible (GSSA) (Table 1). All GISA strains exhibited a vancomycin MIC of 8mg/L or more using standard CLSI methods and a PAP-AUC value denoting GISA, i.e. ≥1.3 (19). hGISA strains exhibited a vancomycin MIC of 1.5-4mg/L using standard CLSI methods and a PAP-AUC value denoting hGISA, i.e. 0.9-1.29 (19). GSSA strains exhibited a vancomycin MIC of <2mg/L using standard CLSI methods and a PAP-AUC value denoting vancomycin susceptibility, i.e. <0.9 (19). The PAP-AUC method uses overnight cultures of test organism to inoculate, via a spiral plater, a range of plates containing 0 to 8mg/L vancomycin. After 48h incubation the resulting viable count is plotted against vancomycin concentration and the area under the curve compared to
that of the known hVISA Mu3 (18, 19). A results sheet was included to detail how
results should be noted.

Laboratory methods:

BHIA6V screening agar. In each participating laboratory Brain Heart Infusion Agar
(BHIA) (BBL, Becton Dickinson, MD, USA) plates were manufactured containing
6mg/L vancomycin (Eli Lilly, Basingstoke, UK). Strains were sub-cultured from
swabs onto blood agar and after overnight growth, several colonies suspended in 0.9% saline to obtain an inoculum with equivalent turbidity to McFarland 0.5. 10µl of inoculum was delivered onto the surface of the agar and the plate incubated at 35°C in air for 24 and 48hr. Growth of more than 1 colony was recorded at both 24 and 48hr. A strain was considered positive if growth of two or more colonies occurred after 24hr.

MHA5T screening agar. In each participating laboratory Mueller Hinton Agar (MHA) (Oxoid, Basingstoke, UK) plates were manufactured containing 5mg/L teicoplanin (Merrell Dow Pharmaceuticals, Staines, UK). Strains were sub-cultured from swabs onto blood agar and several colonies suspended in 0.9% saline to obtain an inoculum with equivalent turbidity to McFarland 2. 10µl of inoculum was delivered onto the surface of the agar and the plate incubated at 35°C in air for 24-48hr. Growth of more than 1 colony was recorded at both 24 and 48hr. A strain was considered positive if growth of one or more colonies occurred after 48hr.

MET. Performed according to manufacturers manual (EAS 003, www.abbiodisk.com), briefly several colonies were suspended in Mueller Hinton Broth (MHB) (Oxoid, Basingstoke, UK) to obtain an inoculum equivalent to 2 McFarland. 100µl of inoculum was evenly streaked onto a 90mm BHIA (BBL, Becton Dickinson, MD, USA) plate and allowed to dry. Both teicoplanin and
vancomycin Etest strips (ABBIODISK, Solna, Sweden) were applied to the surface of the agar and the plates incubated at 35°C in air for 24 and 48 hr. Zones were read at complete inhibition with care to visualize hazy growth and microcolonies. A strain was considered positive if readings were ≥8 mg/L for vancomycin and teicoplanin or ≥12 mg/L teicoplanin alone.

Analysis:
Each laboratory returned results in the form of a datasheet detailing screening method used, number of colonies grown at 24 h, number of colonies grown at 48 h, vancomycin Etest reading and teicoplanin Etest reading. For each laboratory and method, the 48 strains were designated phenotypes dependant upon screening agar criteria, which were then compared to the original phenotype as determined by PAP-AUC (19) (Table 1). Because the three methods used are not capable of distinguishing hGISA from GISA both phenotypes were classed as glycopeptide intermediate (GI).

Percentage correctly identified glycopeptide susceptible (GS) and glycopeptide intermediate (GI) strains were calculated along with method sensitivity, specificity and positive and negative predictive values. Sensitivity refers to how good a method is at identifying GI, specificity refers to how good a method is at identifying GS, positive predictive value refers to the probability that a positive result is correct and negative predictive value as the probability that a negative result is correct.
**Results**

The percentages of strains correctly identified are detailed in Table 2. Out of 16 GS strains in the set, BHIA6V, MHA5T and MET correctly identified a mean of 15.58, 12.17 and 14.08 strains respectively. However, out of a possible 32 GI strains (16 GISA and 16 hGISA) BHIA6V, MHA5T and MET were able to correctly identify a mean of 11.25, 27.58 and 26.83 strains respectively. The mean total percentage of strains correctly identified by BHIA6V, MHA5T and MET was 55.9%, 82.5% and 85.9% respectively.

The standard deviation (SD) was calculated to determine the variation between laboratories of results from each method. Mean SD was high for BHIA6V at 14.59 and lower for MHA5T (9.93) and MET (8.44) (Figure 1). The percentage of false positives identified in BHIA6V, MHA5T and MET were 0.42, 3.8 and 1.83, whilst the number of false negatives were 20.66, 4.42 and 5.25.

The means for correctly identifying GI strains can be separated further into GISA and hGISA. MHA5T and MET correctly identified 93.8% and 97.4% of GISA respectively and 70.3% and 78.7% of hGISA respectively, whilst BHIA6V only correctly identified 58.8% of GISA and only 11.5% hGISA strains.

When comparing how well each strain was identified in all methods with its PAP-AUC value (as a measure of the level of glycopeptide resistance) it was observed that BHIA6V only correctly identified strains with a high PAP-AUC (usually GISA >1.5). Whereas both MHA5T and MET appear to correctly identify GISA and hGISA strains with PAP-AUC >1.

In an attempt to optimise the two screening agars, we amended the criteria used to determine positives. Using BHIA6V the criteria for a positive test result was changed from growth of ≥2 colonies after 24h incubation to ≥1 colony after 48hr. For MHA5T
the criteria was changed from ≥1 colony after 48hr incubation to ≥2 colonies after 48hr. Changing the criteria improved the mean total percentage of correctly identified strains by 6.6% and 1.22% for BHIA6V and MHA5T respectively. No criteria amendments, including the lowering or raising of positive threshold readings, could be found to increase the numbers of strains correctly identified in MET.

Mean sensitivities for BHIA6V, MHA5T and MET are shown in Table 3. BHIA6V shows low mean sensitivity and negative predictive values but relatively high specificity and positive predictive values. MHA5T and MET show considerably higher sensitivity and negative predictive values but slightly lower specificity and positive predictive values. Overall, sensitivity, specificity and predictive values correlate with the analysis of percentage of strains correctly identified.
Discussion

This study evaluates the performance of three screening methods for detecting *S. aureus* with reduced susceptibility to glycopeptides. These methods were tested in twelve laboratories throughout the world using identical media, antimicrobials and isolates. Analysis of data from all laboratories shows that BHIA6V performed least effectively, with <60% of isolates correctly identified and sensitivity and specificity values of 35.16 and 97.4 respectively. BHIA6V also produced the greatest number of false negatives and showed the greatest variability between laboratories, with percentages of correctly identified strains ranging from 37.5% to 89.6%. A high degree of variability when using in-house produced BHIA6V has been previously reported but this study confirms this finding (16).

Overall, the total percentage of strains correctly identified, sensitivity and specificity were similar for MHA5T and MET. However, if the criteria used to identify positives for MHA5T was increased to 2 or more colonies at 48hr, instead of 1 or more colonies then the mean total percentage of strains correctly identified increased to 84.03% (from 82.9%), compared to 85.9% for MET. This increase in correct identification occurred in 6 of 12 laboratories, with the other 6 laboratories showed no change in correctly identified strains. MHA5T has slightly fewer false negatives but twice as many false positives as MET, although percentage numbers are small (1.8% and 3.8% respectively). Although sensitivity and specificity for MHA5T is slightly better at detecting GISA and especially hGISA (10% higher) than MET it is at the cost of falsely identifying GSSA as GI. Again, as with the numbers for false positives and negatives, the mean positive and negative predictive values for MHA5T and MET indicate that MHA5T is slightly better at predicting glycopeptide susceptibility but less accurate when predicting glycopeptide intermediate resistance.
Although these data show similar performance for MHA5T and MET, overall it seems that MET has the advantage of fewer false positives. The cost of introducing any screening test to a diagnostic laboratory is of importance, and both BHIA6V and MHA5T are relatively low in cost whilst MET has a greater impact on laboratory finances. However, laboratories should also be aware that any positive strain detected by a screening method would undergo confirmatory testing. This would incur additional cost and hence any reduction in the false positive rate would reduce unnecessary costs. Diagnostic laboratories must assess the relative benefits of the MET and MHA5T methods with respect to initial screening costs and the cost of confirmatory testing. MET also shows less variability of performance between laboratories, a significant factor when considering diagnostic methods.

Evaluation of screening methods for detecting these strains is of significant value if we are to fully understand the prevalence and hence clinical importance of GISA and in particular hGISA. Further, such investigations are crucial if data on the efficacy of new drugs, such as linezolid and daptomycin, directed against MRSA with reduced susceptibility to glycopeptides, is going to be at all meaningful.
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Dr Anne Bolmström (ABBiodisk, Sweden - Research).

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References


Table 1: Strain identities and origin

<table>
<thead>
<tr>
<th>GISA</th>
<th>ID</th>
<th>PAP-AUC</th>
<th>Ref</th>
<th>hGISA</th>
<th>ID</th>
<th>PAP-AUC</th>
<th>Ref</th>
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<tr>
<td></td>
<td>3759</td>
<td>1.57</td>
<td>Patron <em>et al.</em>, 2001</td>
<td>Duf France</td>
<td>1.16</td>
<td>Evelyne Lecaillon (Community Hospital, Perpignan, France. Personal communication)</td>
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<td></td>
<td>PC3</td>
<td>1.4</td>
<td>Sieradski <em>et al.</em>, 1999</td>
<td>AG Liverpool</td>
<td>1.29</td>
<td>John Corkill (Royal Liverpool and University Hospital, UK. Personal communication)</td>
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<td></td>
<td>LIM3</td>
<td>1.43</td>
<td>Ploy <em>et al.</em>, 1998</td>
<td>LIM1</td>
<td>1.14</td>
<td>Ploy <em>et al.</em>, 1998 (9)</td>
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<td></td>
<td>Michigan</td>
<td>1.9</td>
<td>Smith <em>et al.</em>, 1999</td>
<td>Southampton</td>
<td>1.04</td>
<td>Andy Tuck (PHL, Southampton General Hospital, UK. Personal communication)</td>
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<td></td>
<td>New Jersey</td>
<td>1.8</td>
<td>Smith <em>et al.</em>, 1999</td>
<td>Sweden</td>
<td>1.21</td>
<td>Ann Bolmström – Sweden (Personal communication)</td>
<td></td>
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<td></td>
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<td></td>
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<td>MDRSA79</td>
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Table 2: Percentage of correctly categorised strains for all three screening methods in all laboratories

<table>
<thead>
<tr>
<th>Screening Method</th>
<th>Percentage of GS(^a) strains correctly identified</th>
<th>Percentage of hGISA strains correctly identified</th>
<th>Percentage of GISA strains correctly identified</th>
<th>Percentage no. of hGISA and GISA strains correctly identified</th>
<th>Mean total percentage of strains correctly identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHIA6V(^b)</td>
<td>97.4 ± SD 7.3</td>
<td>11.5 ± SD 25.4</td>
<td>58.9 ± SD 29.7</td>
<td>35.2 ± SD 23.9</td>
<td>55.9 ± SD 14.59</td>
</tr>
<tr>
<td>MHA5T(^c)</td>
<td>76.0 ± SD 25.5</td>
<td>78.7 ± SD 20.4</td>
<td>93.8 ± SD 14.3</td>
<td>86.2 ± SD 15.9</td>
<td>82.8 ± SD 9.93</td>
</tr>
<tr>
<td>MET(^d)</td>
<td>88.0 ± SD 9.4</td>
<td>70.3 ± SD 23.3</td>
<td>97.4 ± SD 5.6</td>
<td>83.8 ± SD 13.2</td>
<td>85.9 ± SD 8.44</td>
</tr>
</tbody>
</table>

\(^a\)GS=Glycopeptide susceptible

\(^b\)BHIA6V=Screening agar using Brain Heart Infusion plus 6mg/L vancomycin

\(^c\)MHA5T=Screening agar using Mueller Hinton agar plus 5mg/L teicoplanin

\(^d\)MET= Macrodilution Etest
Table 3: Sensitivity, specificity, positive and negative predictive values for all three screening methods in all laboratories

<table>
<thead>
<tr>
<th>Screening Method</th>
<th>Mean sensitivity</th>
<th>Mean specificity</th>
<th>Mean + predictive value</th>
<th>Mean – predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHIA6V</td>
<td>35.16</td>
<td>11.47</td>
<td>97.4</td>
<td>21.37</td>
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<tr>
<td>MHA5T</td>
<td>85.95</td>
<td>79.71</td>
<td>75.55</td>
<td>79.89</td>
</tr>
<tr>
<td>MET</td>
<td>82.04</td>
<td>69.3</td>
<td>89.09</td>
<td>87.19</td>
</tr>
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</table>

aGI=Glycopeptide intermediate
Figure 1: Variation in performance of BHIA6V, MHA5T and MET in 12 laboratories
Figure 2: Comparison of methods in relation to glycopeptide susceptibility as determined by PAP-AUC

Full details of PAP-AUC protocol and criteria can be found in methods section.