A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital

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One hundred methicillin-resistant *Staphylococcus aureus* (MRSA) strains, isolated between 1983 and 1999, were tested alongside the vancomycin hetero-resistant *S. aureus* (hVRSA) strain Mu 3, and vancomycin-resistant *S. aureus* (VRSA) strain Mu 50, for their resistance to vancomycin. This was achieved using the screening method described by Hiramatsu, gradient plates, agar incorporation, standard Etest, macrodilution Etest and a modified population analysis. Using Hiramatsu's screening method, 5% of the 100 MRSA were identified as VRSA and 5% identified as hVRSA, the gradient plates identified 7% hVRSA, and the standard and macrodilution Etests identified no hVRSA. Mu 3 appeared to be vancomycin-susceptible using both the agar incorporation and standard Etest methods, but was classified as hVRSA using the macrodilution Etest. The modified population analysis reliably detected vancomycin hetero-resistance in Mu 3 and identified no hVRSAs within the 100 MRSA sample.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infections, causing high morbidity and mortality in the UK and throughout the world. The proportion of MRSA has risen worldwide during the last two decades, with increasing epidemics in UK hospitals (EMRSA 15/16).[1] The recommended therapeutic agents for treating MRSA, which are often multi-resistant, are the glycopeptides; in particular vancomycin. Since the emergence of vancomycin resistance in enterococci in 1988,[2] in coagulase-negative staphylococci in 1987[3] and in MRSA in 1996[4], there has been speculation that the incidence of vancomycin-resistant *S. aureus* (VRSA) would increase. The first isolates to be reported were the VRSA, Mu 50 and Mu 3, a vancomycin hetero-resistant *S. aureus* (hVRSA) from Japan.[4] Recently, there have been further reports of both VRSA and hVRSA in the USA,[5,6] France,[7] Hong Kong,[8] Spain[9] and the UK.[10]

It is thought that two types of vancomycin resistance exist in *S. aureus.* VRSA strains such as Mu 50 have vancomycin MICs of ⩾8 mg/L, while in strains with heterogeneous resistance such as Mu 3, vancomycin MICs are 2–4 mg/L. The breakpoint of vancomycin for *S. aureus* is 4 mg/L in the UK,[11] while in the USA there is an upper breakpoint of 32 mg/L.[12] Using these breakpoints, Mu 50 (vancomycin MIC = 8 mg/L) would be classified as vancomycin resistant in the UK and as intermediate in the USA. However, the classification of hVRSA is less clear. As the reported VRSA and hVRSA have been associated with vancomycin treatment failure[9,12] and the precise proportion of VRSA and hVRSA in the MRSA population is unknown, current studies are focused on detecting vancomycin hetero-resistance in MRSA. Realistic results will only be attained through the use of an appropriate method, where detection of false positives and negatives are at a minimum.

The current methods of susceptibility testing of vancomycin in UK laboratories include the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc diffusion method[13] and Stokes' method.[14] Using a 5 μg disc, the zone diameter breakpoints with the former method are ⩽9 mm (resistant), 10–11 mm (intermediate) and ⩾12 mm (susceptible).[13] However, it has been reported that the Japanese strain Mu 50 exhibits susceptible results[15] in the US with NCCLS methods.[16] It has also been observed that the screening method described by Hiramatsu *et al.*[17] for detecting hVRSA yields false positives and negatives.[12,18]

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Other recommended laboratory methods for detection of vancomycin resistance include determination of MICs (by broth dilution, agar incorporation or Etest) and more specialized techniques such as population analysis profiles (PAPs),[19] gradient plates[20] and the addition of Mu 3 cell wall material to media.[21]

In this report, 100 historical MRSAs isolated between 1983 and 1999 have been screened using Hiramatsu's screening method, gradient plates, standard Etest, macrodilution Etest and a modified population analysis to assess the prevalence of hVRSA and VRSA in a UK hospital.

Materials and methods

Bacterial strains

One hundred MRSA strains from the collection at the Bristol Centre for Antimicrobial Research and Evaluation at Southmead Hospital, collected between 1983 and 1999, were used. Mu 50 and Mu 3[4] were used as positive control strains of homogeneous and heterogeneous vancomycin resistance, respectively.

Susceptibility testing

MIC determinations were performed using agar incorporation[11] and standard Etest procedures. Isosensitest agar plates (ISA; Oxoid, Basingstoke, UK) were inoculated with a 0.5 McFarland standard suspension of test organisms and a vancomycin E-strip was added. Plates were incubated at 37°C for 18 h.

Macrodilution Etests were performed as described by Bölstrum.[22] A 2 McFarland suspension (250 μL) of test isolate in saline solution was swabbed on to brain–heart infusion agar (BHIA; BBL, Cockeysville, MD, USA). The plates were allowed to dry and the vancomycin Etest strips applied. After incubation for 48 h at 37°C, the MICs were noted. The McFarland 2 suspension was also used to repeat the process for teicoplanin Etests. The criteria used to detect hVRSA when using the macrodilution Etest were MICs of ≥8 mg/L for both vancomycin and teicoplanin. Mu 3 had an MIC of 8 mg/L of vancomycin and 32 mg/L of teicoplanin.

The screening method was performed as described by Hiramatsu *et al.*[17] This involved inoculating 10 μL of a 0.5 McFarland standard broth on to BHIA plates containing 4 mg/L vancomycin (Lilly, Basingstoke, UK). Growth at 24 h denoted VRSA and growth at 48 h denoted hVRSA. The 100 MRSA were tested in batches of 10, with Mu 3 and Mu 50 used as positive controls and the Oxford *Staphylococcus* NCTC 6571 as a negative control with each batch.

Gradient plates were made by allowing 25 mL of BHIA containing 4 mg/L of vancomycin to set at a 12° angle in a 10 cm square Petri dish. This gradient slope was subsequently overlaid with 25 mL of BHIA and left to set horizontally. After 24 h incubation in tryptone soya broth

(TSB) (Oxoid, Basingstoke, UK), each culture was adjusted to a turbidity equal to McFarland 0.5, and a standard loopful (10 μL) of vancomycin from 0–4 mg/L was streaked across the gradient plate. Each plate held 10 test organisms plus Mu 3 as positive control, the repetition of Mu 3 providing reproducibility data for the method. The growth along the vancomycin gradient was measured after 48 h. The gradient plates were analysed using the ratio of the growth distance of the MRSA divided by the distance grown by Mu 3 on the same plate. This took into consideration any variability between plates. A ratio of ≥1 denotes an hVRSA.

Modified PAPs were performed as follows. After 24 h incubation in TSB, cultures were diluted in saline to 10^{-3} and 10^{-6}, and spiral plated (Don Whitley spiral platers, West Yorkshire, UK) on to BHIA plates containing 0.5, 1, 2, 2.5 and 4 mg/L vancomycin. Colonies were counted after 48 h incubation at 37°C and the viable count was plotted against vancomycin concentration using GraphPad Prism (GraphPad; San Diego, CA, USA). This was then used to calculate an area under the curve (AUC). To distinguish VRSA, hVRSA and vancomycin-susceptible MRSA, a ratio of the AUC of test MRSA divided by the corresponding AUC for Mu 3 was calculated. The criteria used for detection of hVRSAs were AUC ratios of ≥0.9. Again, MRSA were tested in batches of 10 with Mu 3 as a positive control. The ratio of test MRSA AUC divided by the corresponding Mu 3 AUC was calculated.

Results

Using Hiramatsu's screening method, five MRSA isolates exhibited one or more colonies at 24 h, suggesting homogeneous resistance (VRSA), and five exhibited one or more colonies at 48 h, suggesting that they were hVRSA. Mu 50 grew at 24 h in every test (100% accuracy), but Mu 3 only grew at 48 h in 80% of the tests, sometimes growing at 24 h and sometimes not growing at all (Table I).

Vancomycin MICs ranged from 0.38 to 2 mg/L for the 100 MRSA, 6 mg/L for Mu 50 and 3 mg/L for Mu 3, using the standard Etest protocol (Table II). These data suggest that Mu 3 is not vancomycin resistant. Using the macrodilution Etest method and the suggested criteria, Mu 3 had a vancomycin MIC of 8 mg/L and a teicoplanin MIC of 32 mg/L, and is classified as hVRSA. None of the 100 MRSA isolates were classified as hVRSA.

Seven out of 100 MRSA had ratios of ≥1 when using the gradient plates, indicating their similarity to Mu 3. Of these seven MRSA strains, only two corresponded to those positively identified by the other methods; one an hVRSA and the other a VRSA (Table III). The reproducibility of the measurements for Mu 3 were poor, ranging from 4.1 to 7.2 cm, giving a mean ± S.D. distance of 5.4 ± 0.8 cm. The range for MRSA was 1.7–6.6 cm and Mu 50 consistently gave a distance of 10 cm.

Table I. Accuracy of identification of the correct vancomycin resistance phentotype of Mu 50 (VRSA) and Mu 3 (hVRSA) using six methods

	MIC determination by agar incorporation and standard Etest[a]	Macrodilution Etest[b]	Gradient plates[c]	Screening method[d]	PAP[e]
Mu 3	no	yes	no	yes 80%/no 20%	yes 100%
Mu 50	yes	yes	yes	yes 100%	yes 100%

[a]Criteria used to detect hVRSA using agar incorporation and Etest was an MIC ≥ 4 mg/L.
[b]Criteria used to detect hVRSA were MICs of ≥ 8 mg/L for both vancomycin and teicoplanin.
[c]Gradient plate criterion used to detect hVRSA was a ratio of ≥ 1.
[d]Criteria used to detect VRSA and hVRSA using the screening method were growth at 24 and 48 h, respectively.
[e]Criterion used to detect VRSA using the modified PAP was a ratio of ≥ 0.9.

Table II. Distribution of vancomycin MICs of 100 MRSA isolates using the standard Etest method.

	MIC (mg/L)					
	0.38	0.5	0.75	1	1.5	2
No. of isolates	1	13	32	30	22	2

Table III. Number of isolates identified as hVRSA and VRSA from 100 MRSA isolates using all methods

Phenotype	MIC by agar incorporation and standard Etest	Macrodilution Etest	Screening method as described by Hiramatsu[17]	Gradient plates	Modified PAP
VRSA	0	0	5	0	0
HVRSA	0	0	5	7	0

The modified PAPs were inspected visually and a new analysis developed. Using the graph of $\log_{10}$ viable count versus vancomycin concentration, the AUC was calculated. The pooled AUC values calculated for Mu 50 on 10 separate occasions had a mean of 25.19 ± 0.68, which suggests good reproducibility. Mu 3 tested on 16 occasions also gave reproducible results, with a mean AUC of 21.06 ± 2.47 (Table I). The 100 MRSA tested individually had a mean AUC of 10.98 ± 2.94. These AUC data show good distinction between MRSA, hVRSA and VRSA. None of the test isolates had AUC ratios ≥ 0.9, and there was therefore no evidence of hetero-resistance to vancomycin.

Discussion

At present, the proportion of MRSA with reduced susceptibility to vancomycin (hVRSA) is unknown in many hospitals, but it is important for infection control reasons that this is established. To accomplish this, a sound method of detection is required instead of the current disc diffusion and Stokes' methods used in the UK and elsewhere, which fail to detect hVRSA. The other methods available, such as determination of MICs by broth dilution, agar incorporation or standard Etest,[23] as well as gradient plates and the screening method described by Hiramatsu, are not labour intensive, but would be an inappropriate means of screening for or detection of hVRSA, owing to the high number of false positives and negatives. The more specialized methods like population analysis and modified media plus β-lactam[21] interaction have been criticized for their labour intensity and the possibility that they may select rather than detect vancomycin resistance.[10] Given the number of MRSA to be tested in many laboratories, these methods would also be considered unsuitable.

Recent reports using Hiramatsu's screening method cite

$8\%^{24}$ and $4.8\%^{25}$ of the MRSA population as having reduced vancomycin susceptibility. However, these studies have been criticized for using poor methodology.[26] In this report, the gradient plates and screening method detected 7% hVRSA, and 5% hVRSA and 5% VRSA, respectively. These percentages of hVRSA would seem to correlate with those found in other countries; however, we consider these to be false positives because they do not correspond to the positives found using the other methods. It has also been found that the reproducibility of the screening method is very poor.[27]

The concern over the extent of hVRSA is understandable, especially with increasing reports of resistant isolates and their link with treatment failure.[9,12] However, so far these isolates have been susceptible to other antimicrobials, namely arbekacin and trimethoprim/sulphamethoxazole.[28] In light of the increased incidences of hVRSA, there have been calls for the monitoring and/or reduction of all vancomycin therapy plus improvement of laboratory methods to detect VRSA and hVRSA earlier and more accurately.

Recent research, using a macrodilution Etest[22] to screen for hVRSA in large numbers of MRSA, found 2% false positives.[29] Extending the results of this study to a working laboratory, it can be suggested that a macrodilution Etest should be performed initially, this providing a non-labour intensive screening method for many isolates but with few false positives. Alternatively, if the cost of screening all MRSA with Etest is too high, this could be limited to isolates from patients receiving glycopeptide treatment. Isolates suspected of showing resistance would then be subjected to analysis using the modified PAP to give a more accurate guide to vancomycin-resistance status.

References

1. Ayliffe, G. A. (1997). The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases* **24**, *Suppl. 1*, S74–9.

2. Nicas, T. I., Wu, C. Y., Hobbs, J. N. J., Preston, D. A. & Allen, N. E. (1989). Characterisation of vancomycin resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy* **33**, 1121–4.

3. Schwalbe, R. S., Stapleton, J. T. & Gilligan, P. H. (1987). Emergence of vancomycin resistance in coagulase-negative staphylococci. *New England Journal of Medicine* **316**, 927–31.

4. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. & Tenover, F. C. (1997). Methicillin resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* **40**, 135–6.

5. Centers for Disease Control and Prevention. (1997). *Staphylococcus aureus* with reduced susceptibility to vancomycin—United States. *Morbidity and Mortality Weekly Report* **46**, 765–6.

6. Smith, T. L., Pearson, M. L., Wilcox, K. R., Cosme Cruz, P. H., Lancaster, M. V., Robinson-Dunn, B. *et. al.* (1999). Emergence of vancomycin resistance in *Staphylococcus aureus*. *New England Journal of Medicine* **340**, 493–501.

7. Ploy, M. C., Grelaud, C., Martin, C., de Lumley, L. & Denis, F. (1998). First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. *Lancet* **351**, 1212.

8. Wong, S. S., Ho, P. L., Woo, P. C. & Yuen, K. Y. (1999). Bacteremia caused by staphylococci with inducible vancomycin heteroresistance. *Clinical Infectious Diseases* **29**, 760–7.

9. Ariza, J., Pujol, M., Cabo, J., Peña, C., Fernández, N., Liñares, J. et al. (1999). Vancomycin in surgical infections due to methicillin-resistant *Staphylococcus aureus* with heterogeneous resistance to vancomycin. *Lancet* **353**, 1587–8.

10. Howe, R. A., Bowker, K. E., Walsh, T. R., Feest, T. G. & MacGowan A. P. (1998). Vancomycin resistant *Staphylococcus aureus*. *Lancet* **351**, 602.

11. Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. (1991). A guide to sensitivity testing. *Journal of Antimicrobial Chemotherapy* **27**, *Suppl. D*, 1–50.

12. Howe, R. A., Wootton, M., Bennett P. M., MacGowan, A. P. & Walsh, T. R. (1999). Interactions between methicillin and vancomycin in methicillin resistant *Staphylococcus aureus* strains displaying different phenotypes of vancomycin susceptibility. *Journal of Clinical Microbiology* **37**, 3068–71.

13. British Society for Antimicrobial Chemotherapy Standardised Disc Sensitivity Testing Method. (1998). *Newsletter of the British Society for Antimicrobial Chemotherapy*, Summer.

14. Anonymous. (1997). Detecting vancomycin intermediate *Staphylococcus aureus*. *CDR Weekly* **7**, number 47.

15. Tenover, F. C., Lancaster, M. V., Hill, B. C. Steward, C. D., Stocker, S. A., Hancock, G. A. *et al.* (1998). Characterisation of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *Journal of Clinical Microbiology* **36**, 1020–7.

16. National Committee for Clinical Laboratory Standards. (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fourth Edition: Approved Standard M7-A4*. NCCLS, Villanova, PA.

17. Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hori, S., Hosoda, Y. *et al.* (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350**, 1670–3.

18. Wootton, M., Walsh, T. R., Howe, R. A., White, G., Andrews, J. M., Wise, R. *et al.* (1998). Methodological factors in the detection of hetero-geneously vancomycin resistant *Staphylococcus aureus*. (hVRSA). In *Program and Abstracts of the Thirty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, USA.* Abstract C-139. American Society for Microbiology, Washington, DC.

19. Berger-Bachi, B., Strassle, A. & Kayser, F. H. (1986). Characterisation of an isogenic set of methicillin-resistant and susceptible mutants of *Staphylococcus aureus*. *European Journal of Clinical Microbiology* **5**, 697–701.

20. Szybalski, W. (1952). Microbial selection I: Gradient plate technique for study of bacterial resistance. *Science* **116**, 46–8.

21. Hanaki, H., Ohkawa, S., Yoko, Y., Hashimoto, T. & Hiramatsu, K. (1998). Development of a medium (Mu 3) for detection of hetero-vancomycin resistant MRSA (hetero-VRSA). In *Program and Abstracts of the Thirty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, USA.* Abstract C-132. American Society for Microbiology, Washington, DC.

22. Bölstrum, A., Karlsson, A. & Wong, P. (1999). Macro-method conditions are optimal for detection of low level glycopeptide resistance in staphylococci. In *Program and Abstracts of the Ninth Congress of the European Society of Clinical Microbiology and Infectious Disease, Hamburg, Germany.* Abstract P0104.

23. Centers for Disease Control and Prevention. (1997). Interim guidelines for prevention and control of staphylococcal infection associated with reduced susceptibility to vancomycin. *Morbidity and Mortality Weekly Report* **46**, 626–35.

24. Geisel, R., Schmitz, F. J., Thomas, L., Berns, G., Zetsche, O., Ulrich, B. *et al.* (1999). Emergence of heterogeneous intermediate vancomycin resistance in *Staphylococcus aureus* isolates in the Dusseldorf area. *Journal of Antimicrobial Chemotherapy* **43**, 846–8.

25. Gulay, Z., Atay, T., Kucukguven, M. & Yulug, N. (1998). *Staphylococcus aureus* strains hetero-geneously resistant to vancomycin at a hospital in Turkey. In *Program and Abstracts of the Thirty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, USA.* Abstract C-136. American Society for Microbiology, Washington, DC.

26. Howe, R. A., Wootton, M., Walsh, T. R., Bennett, P. M. & Mac-Gowan, A. P. (2000). Heterogeneous resistance to vancomycin in *Staphylococcus* aureus. *Journal of Antimicrobial Chemotherapy* **45**, 130–1.

27. Howe, R. A., Wootton, M., Walsh, T. R., Bennett, P. M. & MacGowan, A. P. (1999). Expression and detection of hetero-vancomycin resistance in *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy* **44**, 675–8.

28. Centers for Disease Control and Prevention. (1997). Reduced susceptibility to *Staphylococcus aureus* to vancomycin—Japan 1996. *Morbidity and Mortality Weekly Report* **46**, 624–6.

29. Walsh, T. R., Bölstrum, A., Quarnstrom, A., Ho, P., Wootton, M., Howe, R. A. *et al.* (2001). Evaluation of current methods of detecting vancomycin resistance and hetero-resistance in *Staphylococcus aureus* and other staphylococci. *Journal of Clinical Microbiology*, in press.