Reduced susceptibility to vancomycin of nosocomial isolates of methicillin-resistant Staphylococcus aureus

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The MICs of vancomycin for 56 random nosocomial Staphylococcus aureus isolates homogeneously resistant to methicillin (homMRSA), 16 heterogeneously resistant isolates (hetMRSA) and 25 susceptible isolates (MSSA) were determined by a standard broth microdilution method. Representative isolates were also tested by an agar incorporation method, the Etest and population analysis. Although always in the susceptible range, MICs of vancomycin for homMRSA were significantly higher than those for hetMRSA or MSSA. Moreover, a homMRSA strain belonging to one of the major Greek MRSA clones contained a sub-population of cells that could grow in the presence of vancomycin 8 mg/L at a frequency of $6.7 \times 10^{-8}$.

Introduction

The isolation in Japan\textsuperscript{1,2} and the USA\textsuperscript{3,4} of clinical isolates of Staphylococcus aureus exhibiting vancomycin resistance, through new and as yet unidentified mechanisms, has rendered the threat of possible emergence of glycopeptide-resistant staphylococci an international public health emergency.\textsuperscript{5} The study of the prevalence of similar isolates in other parts of the world and the understanding of the underlying molecular mechanisms have thus become of great importance.

In order to investigate whether S. aureus with reduced susceptibility to vancomycin were present in Greece, a number of epidemiologically unrelated S. aureus (both methicillin-resistant and -susceptible) were examined.

Materials and methods

Bacterial isolates

Three groups of randomly selected non-replicate nosocomial S. aureus isolates, 56 homogeneously methicillin-resistant (homMRSA), 16 heterogeneously methicillin-resistant (hetMRSA) and 25 methicillin-susceptible (MSSA) were examined. All were isolated in 1994–1997 from active infections from nine Athens hospitals, and are presently held in the collection of the Department of Microbiology, University of Athens. Isolates were considered homogeneously resistant to methicillin when their inhibition zone diameter in a standard disc diffusion assay was $\leq 9$ mm, and heterogeneously resistant when the inhibition zone had a diameter of $>9$ mm, but one or more colonies were still growing within a zone of diameter of $\leq 9$ mm.

Susceptibility tests

The MIC of vancomycin for all isolates was determined by a broth microdilution method, according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS)\textsuperscript{6} with cation-adjusted Mueller–Hinton broth and two-fold dilutions of vancomycin concentrations. The initial inoculum contained $1.5 \times 10^6$ cfu (100 $\mu$L of a 1:100 dilution of a McFarland 0.5 cell suspension). For 37 representative homMRSA, all hetMRSA and all MSSA isolates, MICs were also determined by plating $3 \times 10^6$ cfu (10 $\mu$L of a suspension of bacterial cells corresponding to McFarland standard 1) on brain heart infusion (BHI) agar plates containing 0.5 mg/L increments of vancomycin in the range of 1–4 mg/L, as previously described.\textsuperscript{2} For 16 representative homMRSA isolates, the Etest (AB Biodisk, Solna, Sweden) was also used, following the recommendations of the manufacturers. In order to detect possible sub-populations with reduced susceptibility to vancomycin, a simplified population analysis was also performed.\textsuperscript{2} Fifty microlitres of a starting cell suspension (corresponding to McFarland standard 1) and ten ten-fold serial dilutions

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were spread on BHI agar plates containing concentrations (1–10 mg/L) of vancomycin increasing by 1 mg/L increments. The plates were incubated at 37°C for 48 h, then colonies were counted; the number of resistant cells contained in 50 µL of the starting cell suspension was calculated.

**Pulsed-field gel electrophoresis**

Preparation of chromosomal DNA, digestion with SmaI (New England Biolabs, Beverly, MA, USA) and pulsed-field gel electrophoresis (PFGE) (CHEF-DRIII; Bio-Rad, Hercules, CA, USA) were carried out as described previously, using lambda phage DNA concatamers (New England Biolabs) as size markers. Digital pictures (HeroLab, Wiesloch, Germany) of the gels were analysed with the GelCompar software (Applied Maths, Kortrijk, Belgium) for pattern similarity. The Dice coefficient, the UPGMA clustering algorithm and 1% tolerance of band difference were used.

**Statistical analysis**

Chi-square tests were used for comparison of the MIC values.

**Results**

All isolates tested were susceptible by broth microdilution to vancomycin by NCCLS standards, with most MICs between 0.5 and 1 mg/L. However, the MIC for the majority (70%) of homMRSA was 1 mg/L, while that for the majority of hetMRSA (56%) and MSSA (92%) was 0.5 mg/L (Figure 1). In addition, only in the homMRSA group was there an isolate with an MIC of 4 mg/L by the microdilution method. These differences were statistically significant ($P = 0.001$). Even more striking differences ($P < 0.001$) in the distribution of MICs among the three groups of isolates were observed with the agar incorporation method, which generally resulted in higher MICs (Figure 1), possibly resulting from the 20-fold higher inoculum required for this method. A distribution of MICs similar ($P = 0.07$) to that obtained by the agar incorporation method was obtained by the Etest (Figure 1).

Population analysis was performed on 11 isolates with the highest agar incorporation MICs: eight homMRSA (3.5 or 4 mg/L), two hetMRSA (3.5 mg/L) and one MSSA (2 mg/L). In this analysis, the MIC for all but one isolate differed at most by 1 mg/L from that obtained by agar incorporation. However, one homMRSA isolate, 3716, contained sub-populations that could grow in the presence of 5, 6, 7 and 8 mg/L vancomycin, with frequencies of $2 \times 10^{-7}$, $1.2 \times 10^{-7}$, $1 \times 10^{-7}$ and $6.7 \times 10^{-8}$, respectively. This isolate also gave the highest MIC by both the NCCLS method (4 mg/L) and the Etest (3.5 mg/L). When individual colonies of 3716 growing in 8 mg/L vancomycin were restested for susceptibility to vancomycin, by the NCCLS assay or the Etest, the MIC was identical to that of the parent strain, namely 4 or 3.5 mg/L, respectively, confirming the heterogeneity of this reduced susceptibility. However, selection of intermediately resistant populations could also be induced, since, by successive subculturing on agar plates incorporating increasing concentrations of vancomycin, colonies from the 3716 sub-population growing in 7 mg/L vancomycin could give rise to progeny growing in 9 mg/L vancomycin.

PFGE revealed that strain 3716 belonged to one of the major Greek MRSA clones (Kantzanou and Tassios, unpublished observation). Moreover, the PFGE pattern of this strain (Figure 2) was indistinguishable from that of two other homMRSA isolates, nos 3711 and 3715, with agar incorporation MICs of 4 and 3.5 mg/L, respectively. However, these two isolates clearly differed from 3716 in terms of their sub-population structure, since they did not contain sub-populations that could grow in concentrations of vancomycin significantly higher than their MIC.

![Figure 1. Distribution of vancomycin MICs among (a) homMRSA, (b) hetMRSA and (c) MSSA isolates. Hatched bars: NCCLS broth microdilution method, black bars: agar dilution test, white bars: Etest.](image-url)
Reduced vancomycin susceptibility in S. aureus

Discussion

Using either the NCCLS-recommended broth microdilution method or an agar incorporation method, we observed that the MICs of vancomycin were significantly higher among homMRSA than among hetMRSA and MSSA. This may be related to the observed increase of PBP2 production in strains intermediately resistant to vancomycin.1

One homMRSA isolate, 3716, for which the MIC of vancomycin was the highest observed (4 mg/L by the broth microdilution method), but still within the susceptible range, contained sub-populations of cells that were able to grow in vancomycin concentrations as high as 8 mg/L, i.e. in the range of intermediate resistance. This pattern of heterogeneous reduced susceptibility was similar to that of a number of Japanese and US isolates.2,4 Isolate 3716 belongs to one of the major Greek MRSA clones, as defined by PFGE. This was reminiscent of the situation both in Japan3 and the USA4, where strains intermediately resistant to vancomycin have been shown to have arisen within major MRSA clones. However, in the present study, other genotypically indistinguishable isolates belonging to the same clone, and therefore presumably sharing the same mechanisms of homogeneous methicillin resistance, did not display such heterogeneous reduced susceptibility to vancomycin. This could imply that methicillin resistance per se, though a predisposing factor, may not be entirely sufficient for development of reduced susceptibility to vancomycin.

Nevertheless, the existence of clinical isolates that have evolved reduced susceptibility to vancomycin in vivo is important, especially since it has been shown that continuous antibiotic pressure can select for high resistance among an initially heterogeneous population.2,3 Indeed, when colonies of the 3716 sub-population growing in the presence of 7 mg/L vancomycin were subcultured in increasing concentrations of vancomycin, progeny growing in the presence of 9 mg/L vancomycin could be selected.

Although we were unable to demonstrate intermediate resistance to vancomycin such as has been described in Japan1,2 or the USA3,4 (MIC of vancomycin 8 mg/L), strains which display heterogeneous expression of reduced vancomycin susceptibility may be regarded as a preliminary stage towards the development of resistance.

Acknowledgements

This work was supported in part by a grant from the Ministry of Health, Greece. We gratefully acknowledge Eli Lilly for providing vancomycin.

References


Received 3 August 1998; returned 6 November 1998; revised 30 November 1998; accepted 11 December 1998