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Unstable Vancomycin Heteroresistance Is Common among Clinical Isolates of Methiciliin-Resistant *Staphylococcus aureus*

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We tested 109 unique, vancomycin-susceptible, methicillin-resistant *Staphylococcus aureus* (MRSA) strains for vancomycin heteroresistance by a selection method, i.e., step-wise exposure of large inoculums to increasing concentrations of vancomycin. Although no strains demonstrated stable heteroresistance, 81 strains (74%) demonstrated unstable heteroresistance. Unstable heteroresistance is common among clinical isolates of MRSA and may represent a cause of therapeutic failure.

Heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hetero-VISA) strains are those for which MICs are conventional (≤4 μg/ml) except when high-density inocula are used; with such inocula, there are minority subpopulations for which MICs are in the intermediate range (8 to 16 μg/ml) (13). The detection of hetero-VISA requires inocula containing $>10^6$ total bacterial cells, since resistant clones can occur infrequently, being perhaps 1 in 1,000,000 (15). The MICs for the selected, stably heteroresistant clones are conventional, in the 4- to 8-μg/ml range (7, 17). In addition, unstable heteroresistance can be detected (12, 13). *S. aureus* clones demonstrating unstable heteroresistance grow in the presence of high concentrations of vancomycin (>4 μg/ml), as shown by step-wise exposure of large inoculums to increasing levels of drug, though conventional MICs for such clones are not elevated; i.e., nonsusceptible clones rapidly revert to normal phenotypes. Unstably hetero-VISA is not detected by usual laboratory antimicrobial tests. Our study was designed to determine the frequency of stable and unstable heteroresistance to vancomycin among unselected methicillin-resistant *Staphylococcus aureus* (MRSA) strains from adult and pediatric patients at two Chicago tertiary care hospitals.

Altogether, 109 MRSA strains from unique patients were randomly collected over a 1-year period, from 15 December 1998 to 14 December 1999. A total of 69 strains were from Evanston Northwestern Healthcare (two adult hospitals), and 40 strains were from the Children's Memorial Hospital.

All catalase-positive, gram-positive cocci in clusters were identified as MRSA by yielding positive results in an agglutination test for coagulase/protein A (Murex Biotech Ltd., Dartford, United Kingdom) and growing on 6-μg/ml oxacillin agar screening plates (Remel, Lenexa, Kans.). Vancomycin MIC testing was performed on the original unselected strains and the corresponding selected MRSA strains that grew on screening plates containing ≥16 μg/ml of vancomycin. MIC testing was performed with Microscan Pos MIC panels (Dade Behring, West Sacramento, Calif.) according to NCCLS methods (10). All panels were read after 24 h of incubation and, for all induced strains, again after 48 h.

MRSA strains were subcultured onto 5% sheep blood agar plates and incubated overnight at 35°C. A 0.1-ml sample from a McFarland 2.0 suspension in saline taken from overnight growth was spread evenly on brain heart infusion (BHI) agar containing 2-μg/ml vancomycin (Sigma Chemical Co., St. Louis, Mo.) and 4% NaCl, and an aztreonam paper disk (30 μg; Remel, Lenexa, Kans.) was placed in the center of the agar. All agar plates were prepared in-house. Colony counts confirmed that approximately 6×10^7 CFU were applied to each agar plate. The plates were incubated for 48 h at 35°C. Strains growing on vancomycin (2 μg/ml)-supplemented agar plates after 48 h were subcultured (multiple colonies) to a second vancomycin (2 μg/ml)-supplemented agar plate containing 4% NaCl (quadrant streaking for isolation), and the plates were incubated at 35°C overnight. Multiple colonies from the second vancomycin-supplemented agar plate were subcultured to blood agar plates (quadrant streaking for isolation), and the plates were incubated at 35°C overnight. The selection process was repeated with increasing vancomycin concentrations (4, 6, 10, 14, 16, 20, 24, and 26 μg/ml). The endpoint occurred when there was no growth on the vancomycin-supplemented agar plate containing the next highest concentration (5, 6).

Transmission electron microscopy (TEM) was performed on centrifuged pellets of staphylococcal cells to investigate whether cell wall thickening seen with stable heteroresistance was present in strains showing unstable heteroresistance. Cells were fixed in 2.5% glutaraldehyde–2% paraformaldehyde–0.75% sucrose at pH 7.4 and postfixed with 1% osmium tetraoxide. Dehydration steps were done with increasing concentrations of reagent ethanol. Cells were then embedded in 100% resin and incubated overnight at 70°C. The blocks were cut in 75- to 80-nm-wide strips, which were then stained with lead citrate and uranyl acetate (2, 11).

The MICs for all 109 original MRSA strains were ≤2 μg/ml.

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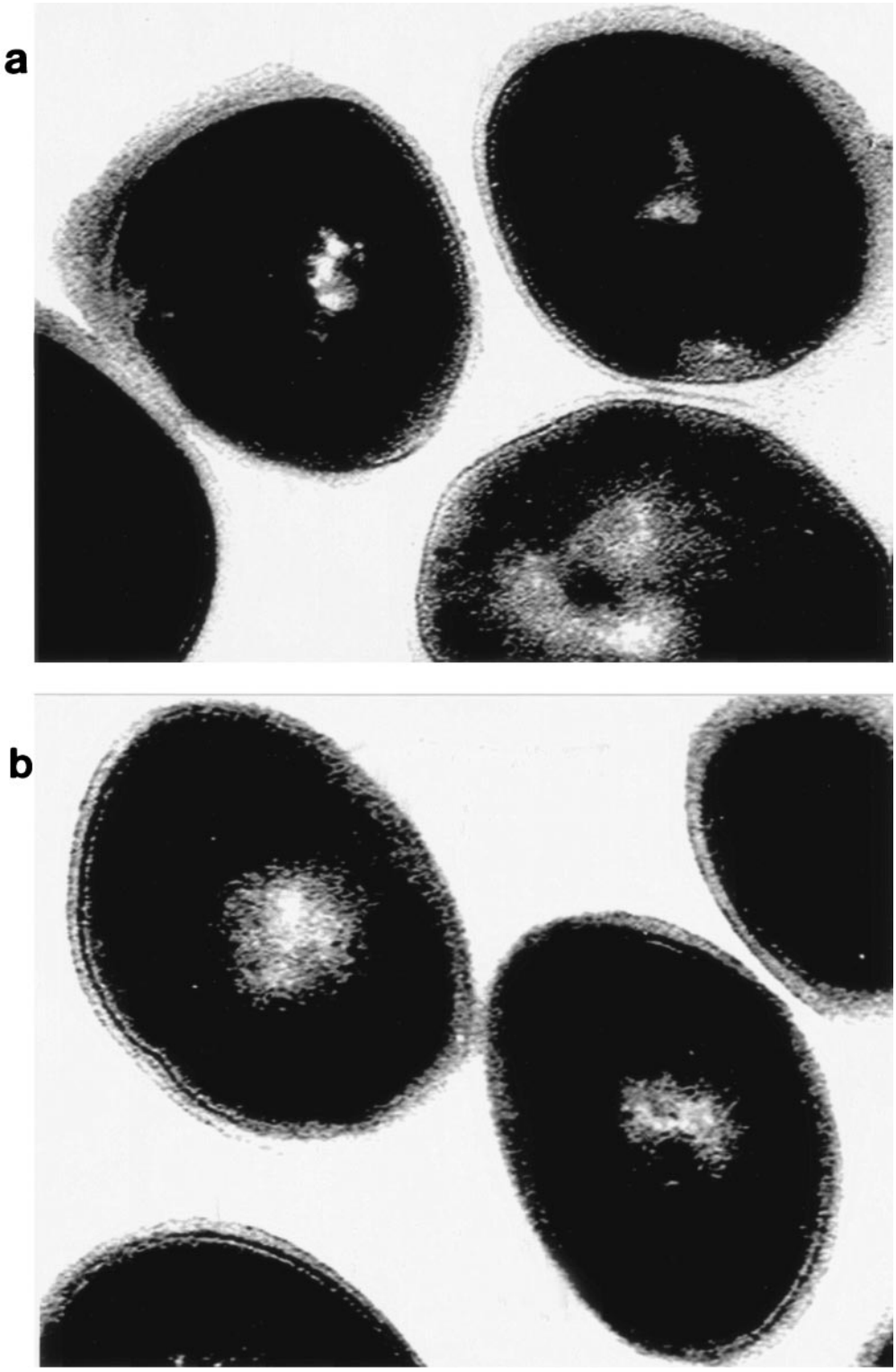

FIG. 1. Transmission electron microscopy showing *S. aureus* increased cell wall thickening and irregularity of postselection clone from a vancomycin (16 μg/ml) plate (a) and the same *S. aureus* strain preselection without cell wall thickening (b). Magnification, ×114,942.

TABLE 1. Screening for vancomycin heteroresistance

Specimen source	No. of strains	No. of *S. aureus* strains surviving at a vancomycin concentration of:								
		2 μg/ml	4 μg/ml	6 μg/ml	10 μg/ml	14 μg/ml	16 μg/ml	20 μg/ml	24 μg/ml	26 μg/ml
Pediatric patient	40	24	24	24	11	6	3	1	1	0
Adult patient	69	61	61	57	41	13	2	1	0	0
Total	109	85	85	81	52	19	5	2	1	0

A stable MIC of >4 μg/ml was not established for any vancomycin-exposed, selected clone. Of the 109 strains, 89 selected clones (74%) grew on plates containing vancomycin at concentrations of ≥6 μg/ml and were categorized as unstably hetero-VISA (Table 1). Unstably hetero-VISA strains were detected among both adult and pediatric isolates.

TEM was performed on three stains categorized as unstably hetero-VISA, all growing on selection plates containing vancomycin at concentrations that were ≥16 μg/ml. Conventional pre- and postselection vancomycin MICs were determined: for two of the strains, there was a change from ≤2 to 4 μg/ml, and for one strain, there was no change (the MIC remained at ≤2 μg/ml). The postselection cells of the two former strains showed increased thickening and irregularity of the cell wall compared to preselection cells (Fig. 1), suggesting that mechanisms for survival in increased concentrations of vancomycin by stably and unstably heteroresistant strains were similar. Measurements of representative cocci from these two strains showed cell wall thicknesses for preselected cells of 21.8 and 21.8 nm, respectively, compared to cell wall thicknesses of 34.8 and 26.1 nm, respectively, for cells selected from vancomycin-supplemented agar plates. The third strain, for which the MIC remained unchanged, showed no difference in cell wall thickness following selection.

Hetero-VISA strains display both stable and unstable phenotypes. We found that 74% of MRSA strains from adult and pediatric patients exhibited unstable heteroresistance. There were no strains with stable heteroresistance. Two of three strains characterized as unstably hetero-VISA and examined by TEM displayed the thickened cell walls that are characteristic of stably hetero-VISA. Controversy exists concerning the frequency of hetero-VISA. A recent report, using population analysis methods that differed from ours, found heteroresistance in 14% of MRSA isolates from patients with persistent or recurrent bacteremia (9).

In our study, BHI agar with an aztreonam disk and 4% NaCl was used to screen for hetero-VISA. BHI agar was selected to improve the growth of staphylococci (15). Selection of clones growing in elevated concentrations of vancomycin in the presence of NaCl and beta-lactams has been shown previously (7, 8, 16). Aztreonam was the beta-lactam used because it does not inhibit the growth of gram-positive cocci.

While some microbiology, infectious disease, and public health personnel have focused concern on VISA and vancomycin-resistant *S. aureus* characterized as such by conventional test methods, others consider the in vitro phenomenon of unstable vancomycin heteroresistance an explanation for the therapeutic failure of vancomycin in humans (1, 3, 4, 7, 11, 14, 17). Further evidence is needed to confirm this association, since not all patients harboring unstably hetero-VISA strains fail therapy. Such evidence could include TEM confirmation of cell wall thickening in clinical material, such as blood, and identification of cofactors contributing to therapeutic failure. If unstably heteroresistant strains are proven to contribute to therapeutic failure in specific clinical situations, novel in vitro testing methods may be needed for their detection.

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