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Detection Methods of Glycopeptide-Resistant *Staphylococcus aureus* I

Susceptibility Testing

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1. Introduction

The breakpoint for resistance to vancomycin for *Staphylococcus aureus* is a minimum inhibitory concentration (MIC) of greater than 8 μg/mL ***(1)***. Isolation of the first strain of MRSA resistant to vancomycin (VRSA) Mu50 was made from a Japanese surgical patient with a wound infection who had failed with vancomycin therapy ***(2)***. A strain has been described that is heterogeneously resistant to vancomycin (Mu3 strain), and it was found to be susceptible to vancomycin (MIC 2 μg/mL by NCCLS criteria), but there were cells within the Mu3 population that resisted a vancomycin concentration of up to 9 μg/mL) ***(1)***. Most of the clinical *S. aureus* strains having reduced susceptibility to glycopeptide antibiotics are heterogeneous in their phenotypic resistance expression. The strains contain small subpopulations of cells that have different levels of glycopeptide resistance. They are designated hetero-resistant strains, and are defined by the population analysis (*see* below). MIC or paper disc susceptibility tests cannot detect hetero-resistant strains. It would appear that heterogeneously resistant VRSA is a preliminary stage that allows development into full resistance upon further exposure to vancomycin. Therefore, it seems reasonable to include VRSA and hetero-VRSA as possible risk factors for vancomycin therapeutic failure in MRSA infection.

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Further reports of VRSA associated with vancomycin therapeutic failure have been reported from USA, France, Turkey, and Korea. The resistance level of hetero-VRSA and VRSA is not yet comparable to that of vancomycin-resistant enterococci (VRE).

However, given the limited peak tissue concentration attained by intravenous vancomycin administration (e.g., only 2.46–2.49 μg/mL vancomycin in sputa after a 0.5 g single injection *(3)* or 5 μg/mL in an abscess after multiple 2.0 g injection *(4)*, it is not surprising that pneumonia or wound infections caused by VRSA do not respond to vancomycin therapy. Conventional susceptibility methods are not effective for detecting VRSA, so alternative techniques are required, and these are described in this chapter.

Gradient gel methods are convenient and simple methods to detect resistant strains *(5,6)*. The minimum inhibitory concentration (MIC) determination, generally used as an evaluation of antibiotic susceptibility of bacteria can only classify the bacteria into discontinuous stepwise levels of resistance. The method that we describe here, is able to detect more subtle differences in the resistance level by using the agar impregnated with continuously increasing concentration of antibiotic. Control strains with a known resistance level should always be used with test strains as a control to monitor batch-to-batch deviation of the gradient gel.

Population analysis is one of the most important methods employed in the study of glycopeptide resistance in *S. aureus (1,7)*. It allows heterogeneous resistance to be detected simply. It is found that vancomycin and β-lactam antibiotics antagonize each other in their action against hetero-VRSA strains in Japan *(8,9)*. Mu3 agar plate method utilizes this antagonism to detect the hetero-VRSA strains. Our studies have shown that any β-lactam antibiotic so far tested can be used in this method: forty-six commercially available β-lactam antibiotics were examined and all showed the same antagonistic effect *(8,9)*.

2. Materials

1. Tryptose soy broth (TSB).
2. Brain heart infusion broth (BHIB).
3. Brain heart infusion agar (BHIA).
4. Slope plate 10 × 14 × 1 cm, (Eiken, Tokyo, Japan).
5. Antibiotics: make fresh as required.
6. Vancomycin: stable at 4°C for up to 3 mo or at –20°C for up to 3 mo.
7. Resting medium: 1 m*M* glycine, 1 m*M* glutamic acid, 0.5 m*M*, D-L-alanyl-D-L-alanine, 0.2 m*M* L-lysine, 1 m*M* $MgCl_2$, 0.1 m*M* $MnCl_2$, 0.17 m*M*, uracil, 8.2 μ*M* nicotinamide, 80 m*M* K_2HPO_4, 3 μ*M* thiamine, 28.5 m*M* glucose. Filter with 0.22 μm pore size filter. Stable at 4°C for up to 1 mo.
8. Paper disc: 8 mm diameter thick disc.

9. Paper disc containing β-lactam antibiotics: make fresh as required or use a commercial source.
10. Mu3 agar plates (Becton-Dickinson, Tokyo, Japan).

3. Methods

3.1. Gradient Gel (Fig. 1)

1. Cultivate test and control strains overnight in TSB.
2. Adjust the OD 0.3 at 578 nm (about 10^8 CFU/mL) with fresh TSB (*see* **Note 1**).
3. Prepare a gradient agar plate as follows. Pour 40 mL of BHIA containing appropriate concentration of antibiotic into a slope plate at an angle of about 5 degrees.
4. Keep solidified for 30 min at room temperature. Then set the plate horizontally, and pour 45 mL of fresh BHIA on top of the solidified BHIA. Keep the solidified agar at room temperature for 120 min (*see* **Note 2**).
5. Streak the test and control strains onto the gradient agar plate with a cotton swab. The growth of the bacteria is observed after incubation at 37°C for 48 h.

3.2. Population Analysis (Fig. 2)

1. Culture test and control bacteria overnight in TSB.
2. Adjust the optical density to OD 0.3 at 578 nm (about 10^8 CFU/mL).
3. Make 10-fold serial dilutions from a portion (50 μL) of the cell suspension and spread with sterilized spreader onto the BHIA plates containing varied concentrations of vancomycin, for example, 0, 1, 2, . . .7, 8, 9, 10 μg/mL.
4. Leave the plates for 15 min on a clean bench before use to remove excess moisture on the agar surface (*see* **Note 3**)
5. Count the number of colonies growing on each plate after incubation at 37°C for 48 h.
6. Plot the colony counts on a semi-logarithmic graph with colony counts on the vertical axis and vancomycin concentration on the horizontal axis (*see* **Note 4**).

3.3. Mu3 Agar Method

1. Culture test and control bacteria overnight in TSB.
2. Adjust the optical density of the culture to OD_{578} of 0.3.
3. Inoculate onto the entire surface of a BHI agar plate containing 4 μg/mL of vancomycin and 100% of resting medium ***(10)*** with one stroke of a sterilized cotton wool swab (*see* **Notes 5** and **6**).
4. Place three paper discs (thick, 8 mm in diameter) containing 0.005, 0.05, and 0.5 μg of ceftizoxime, respectively, on the agar plate inoculated with the test organism.
5. Incubate the agar plate at 37°C for 24–48 h.
6. Suspect hetero-resistance if bacterial growth is observed around the discs (*see* **Fig. 3**). Ready-made Mu3 agar plates are commercially available from Becton-Dickinson, Tokyo, Japan.

4. Notes

1. Overnight culture is prepared in TSB to prevent the organism from forming clusters.
2. For the appropriate gradient of antibiotics to be formed in the agar plate, the plate must be kept for 120 min.

(1) Poure 40ml of agar medium containing X μ g/ml of antibiotic into the plate sloped with an angle of 5 degree

(2) Reset the plate horizontally after the agar is solidified completly, Pour 45 ml of drug-free agar medium on the solid agar .

(3) Streak the prepared bacterial cell suspension onto the gradient plate with a cotton swab, and incubate.

(4) Growth of cells after incubation at 37°C

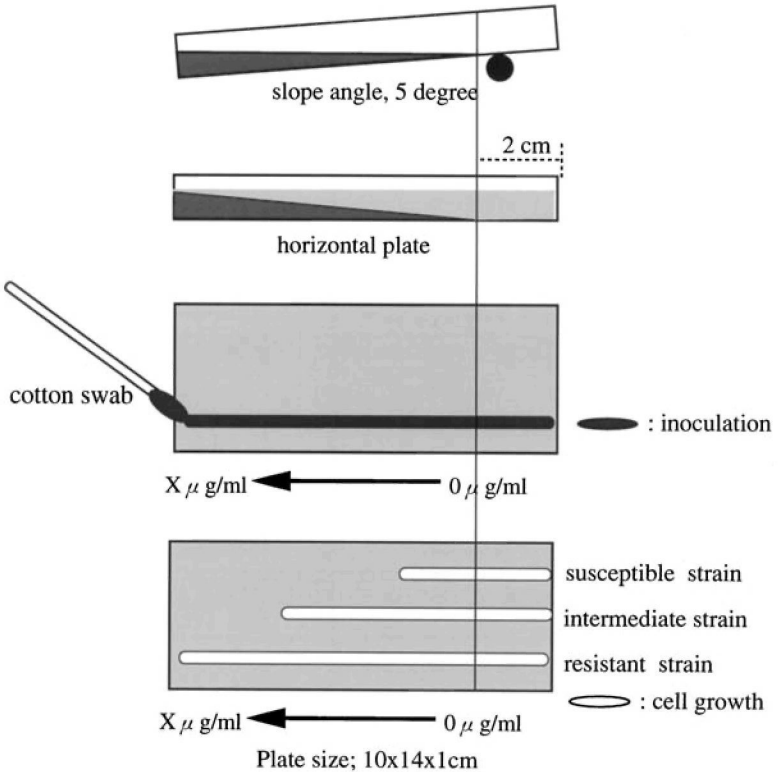

Fig. 1. Process of evaluating vanomycin resistance by gradient gel technology.

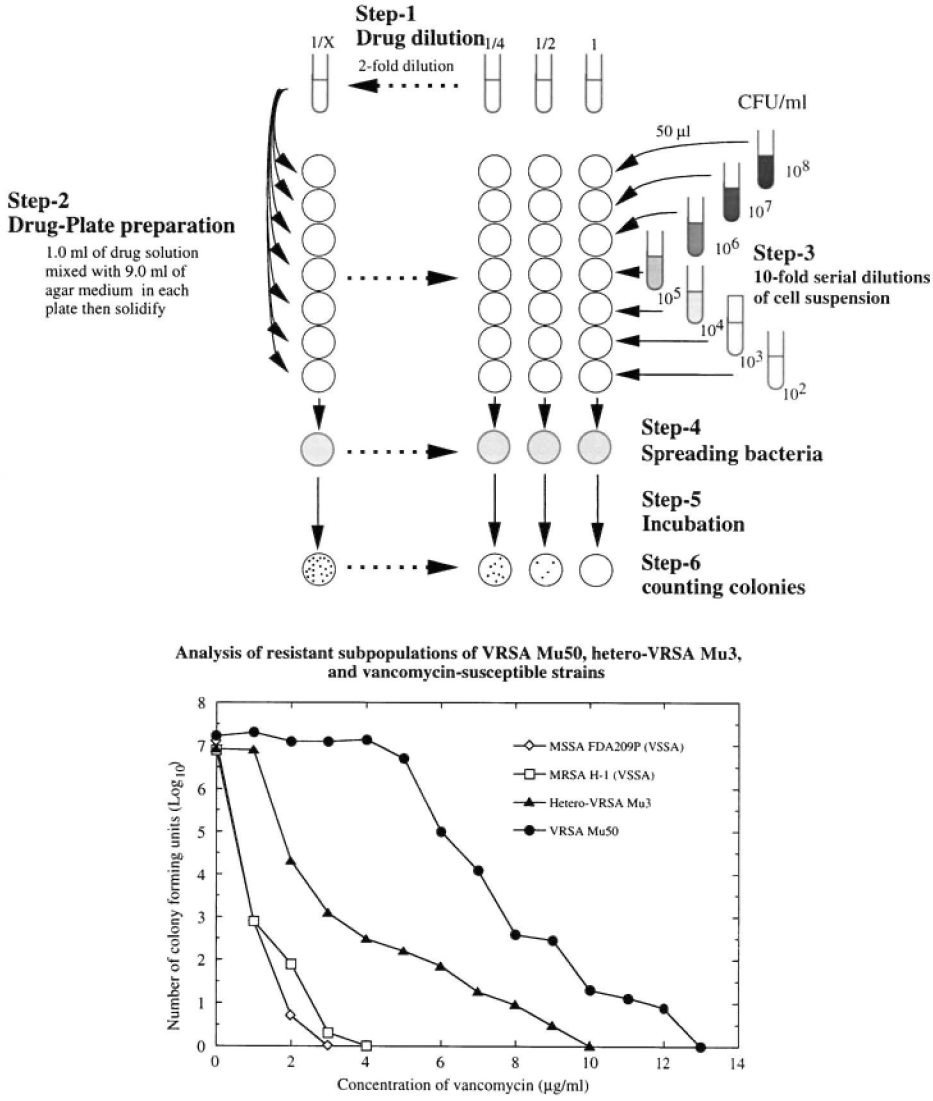

Fig. 2. Population analysis for vancomycin resistant *S. aureus*.

3. The surface of the agar inoculated with bacteria should be dried briefly before incubation, otherwise, patchy growth of "susceptible" cells will appear on the wet surface, which makes the cell count unreliable.
4. The maximum number of cells spread on the agar plate of 9 cm in diameter must be kept less than 10^7 cfu. Otherwise, reliable colony counts cannot be achieved because of some patchy growth of "susceptible" cells on the plate ***(1)***.

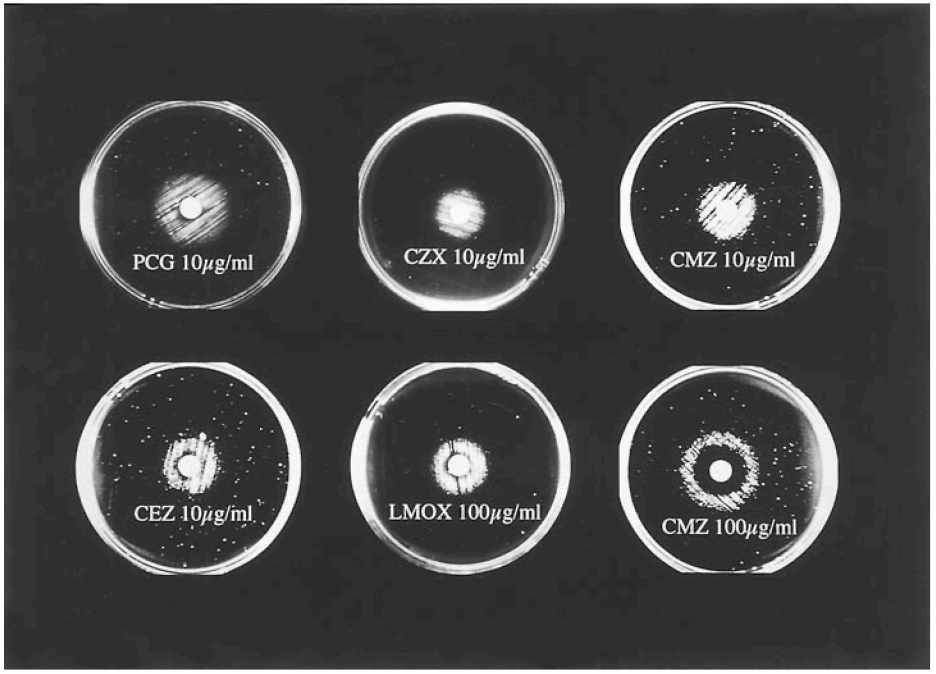

Fig. 3. Example of results using the Mu3 agar method.

5. Multiple inoculation of the cells on the Mu3 agar plate is not recommended. Heavy inoculum makes the result difficult to interpret because of nonspecific growth of the cells all over the plate
6. This method is valid to detect Mu3-type hetero-VRSA prevalent in Japan, but there are some hetero-VRSA (as defined by population analysis) strains in the United States and possibly in other countries which are not detectable by this method.

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