Emergence of linezolid-resistant mutants in the susceptible cell population of methicillin-resistant

Staphylococcus aureus

Running title: linezolid-resistant MRSA in a susceptible population

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Footnote:

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Abstract

Methicillin-resistant *Staphylococcus aureus* with the MIC of linezolid 4 µg/ml, isolated from an unsuccessful linezolid therapy patient, yielded linezolid-resistant mutants in blood agar at 48 h of incubation. The resistant clones showed a MIC of linezolid ranging from 8 to 64 µg/ml and accumulated the T2500A mutation(s) of the ribosomal RNA genes. Emergence of these resistant clones appears to be facilitated by a cryptic mutation or mutations associated with chloramphenicol resistance.

Infection by methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious problem in hospitals and communities because this bacterium shows resistance to major chemotherapeutic agents. To combat MRSA infections, powerful antibiotics, such as vancomycin and linezolid, have been developed. However, vancomycin-resistant MRSA has emerged world-wide. Recently developed linezolid is probably one of a few choices for the treatment of vancomycin-resistant MRSA. Most *S. aureus* including MRSA is found to be linezolid-susceptible with a breakpoint of 4 µg/ml (3, 4). However, the emergence of linezolid-resistant MRSAs with an MIC of over 4 µg/ml has been reported recently (1, 5, 6, 8, 11, 12). The resistant cells were found to have either the G2576T (5, 11,
(12) or T2500A (8) mutation(s) in the gene(s) encoding the 23S ribosomal RNA (23S rRNA).

Moreover, some of the mutants accumulated the mutation in multiple copies of the rRNA genes rendering the cells increasingly resistant to linezolid (2, 6, 9). Other types of linezolid resistance reported are enzymatic methylation of the 23S rRNA by chloramphenicol methyltransferase (5) and a mutation in the ribosomal protein genes encoding, L3 and L4 (7, 13). We report here that linezolid-resistant cells, isolated from a patient in whom linezolid therapy was unsuccessful, were emerged from the susceptible cell population.

A patient with pyogenic spondylosis suffered from MRSA bacteremia, and was treated orally with 600 mg of linezolid twice a day for 68 consecutive days. However, S. aureus cells were frequently isolated from the blood resulting in the failure of linezolid chemotherapy. The MRSAs isolated from the pre-(HG503pre) and post-linezolid blood (HG503post) had the MIC of linezolid 2 and 4 µg/ml, respectively, determined by the Clinical Laboratory Standard Institute (CLSI) method (Table 1) (4). Therefore, both strains were classified as linezolid-susceptible MRSA. The relatedness of these strains was confirmed by comparing the pulsed-field-gel electrophoretogram of their chromosomal DNA treated with SmaI (not shown).

Since the MIC of linezolid in HG503post, a strain from the failure of linezolid treatment, was close to the border of the breakpoint and that of chloramphenicol was a 4-fold higher than that of HG503pre, we wondered whether or not this strain was genuinely linezolid-susceptible. Thus, the linezolid susceptibility of HG503post was examined again using the Etest™ (AB Biodisk, Solna, Sweden). The MIC of linezolid in the Mueller-Hinton (MH) agar was 0.19 µg/ml at 24 h of incubation, while that in MH agar supplemented with 5% sheep blood (MH-blood agar) appeared to be 1.5 and 4 µg/ml at 24 and 48 h, respectively. An important observation made was that many microcolonies appeared in the clear inhibitory zone in the MH-blood agar at 48 h (Fig. 1Ab). This result suggested that the linezolid-resistant mutants spontaneously emerged from the susceptible cell population, HG503post, while this phenomenon was not seen in HG503pre (Fig. 1Aa).
whether or not the resistant cells appeared only in the presence of linezolid, HG503post cells were streaked on antibiotic-free MH-blood agar and 20 randomly selected microcolonies were subjected to the Etest™. The result showed that all the subclones exhibited microcolonies in the clear inhibitory zone without exception, suggesting that they were segregated from a single susceptible cell (Fig. 1Ac and 1Ad).

To test the homogeneity of the HG503 cells, we analyzed the population distribution of HG503pre and HG503post in terms of linezolid susceptibility. The cells grown in antibiotic-free MH broth were plated on brain-heart-infusion (BHI) agar impregnated with various concentrations of linezolid. The MIC of the HG503post population ranged from 1 to 16 µg/ml of linezolid (Fig 1Bb), while that of HG503pre was restricted to within 1 to 2 µg/ml (Fig. 1Ba). These data suggest that the emergence of a resistant subpopulation in HG503post is due to a cryptic mutation or mutations. The colonies that appeared at the linezolid concentrations of 4 and 8 µg/ml in BHI agar were designated as HG503post-4R and HG503post-8R, respectively. Clinically, it is important to know how early such resistant mutants emerge during linezolid treatment. An in vitro experiment was designed to simulate such a situation by exposing HG503post to medium containing linezolid concentrations 1/4 MIC for 14 consecutive days (Fig. 2). The MIC of linezolid progressively increased to 8, 16, 64 and 128 µg/ml by the 4th, 5th, 6th and 7th day, respectively, as determined by the broth dilution method. The strain at the 7th day was designated as HG503post-7D. This experiment revealed that once the HG503post-like cell appeared, cells with high linezolid resistance emerged within a short time in the presence of a sub-MIC concentration of linezolid. A similar experiment with HG503pre revealed that the MIC of linezolid increased only a 2-fold.

The MIC of linezolid in HG503post, HG503post-4R, HG503post-8R and HG503post-7D appeared to be 4, 8, 16 and 64 µg/ml, respectively (Table 1) as determined by the CLSI method. MICs of other antibiotics were comparable among these strains, except for that of chloramphenicol (Supplemental Materials). Cross-resistance between chloramphenicol and linezolid was reported
earlier (10). Acquisition of resistance to chloramphenicol by the HG503post isolate is further
evidence for a mutation that could facilitate emergence of linezolid resistance.

Since the major factor associated with linezolid resistance was reported as a chromosomal
mutation in the gene(s) encoding the domain V region of the 23S rRNA, we analyzed this region by
PCR (refer to Supplemental Materials). The following results were obtained (Table 1). (i) Both
HG503pre and HG503post cells had a domain V sequence identical with that of the reference strain.
(ii) The HG503post-4R, HG503post-8R and HG503post-7D cells had the T2500A substitution in two,
three and four copies, respectively, of the rRNA genes. These results revealed that the MIC of
linezolid was closely associated with the number of mutations in the 23S rRNA genes.

In summary linezolid treatment failure was associated with a heterogeneously resistant
isolate. Although this isolate appeared to be susceptible by standard susceptibility test methods, a
linezolid-resistant subpopulation was detected by E-test after a 48-h incubation or with passage in
sub-MIC level of linezolid. Characterization of resistant clones showed that they had mutations in
multiple copies of the 23S rRNA gene. Although differences in growth rates were observed for more
resistance clones compared to the parent post-treatment isolate (Table 1 and Supplemental Materials),
these were relatively small. Moreover, testing of individual colonies of the post-treatment isolate
makes it unlikely that a mixed culture in the original post-treatment isolate accounted for presence of
the resistant subpopulation. A cryptic mutation (or mutations), outside of domain V of rRNA and
associated with a higher MIC to chloramphenicol, may account for this heterogeneous phenotype and
facilitate selection and emergence of higher-level resistant mutants with rRNA mutations.

References

   E. Murray, and J. P. Quinn. 2008. Clinical and microbiological aspects of linezolid


Table 1. Properties of the linezolid resistant clones isolated from HG503post

<table>
<thead>
<tr>
<th>Strain</th>
<th>Copy # of rRNA gene with T2500A mutation</th>
<th>MIC (µg/ml)</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LZD</td>
<td>CHL</td>
</tr>
<tr>
<td>HG503pre</td>
<td>none</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>HG503post</td>
<td>none</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>HG503post-4R</td>
<td>2</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>HG503post-8R</td>
<td>3</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>HG503post-7D</td>
<td>4</td>
<td>64</td>
<td>128</td>
</tr>
</tbody>
</table>

1, Both HG503pre and HG503post had five copies of the rRNA gene.

2, MICs of antibiotics were determined by the CLSI method.

Abbreviations: LZD, linezolid; CHL, chloramphenicol. MICs of other antibiotics were presented in the Supplemental Materials.

3, Doubling times were calculated from the growth curves in MH broth as shown in the Supplemental Materials.

Figure legends

Fig. 1. Emergence of linezolid-resistant subclones by Etest™ and their population analysis (A, Etest™) Cells were grown in MH broth overnight and a 100-µL aliquot was streaked on MH-blood agar with a linezolid-impregnated Etest™ strip. Plates were incubated at 35°C for 48 h. (a), HG503pre; (b), HG503post; (c and d), Randomly selected HG503post from the drug-free medium was subjected to Etest™. Only two subclones are shown.
(B, Population analysis) Cells were grown overnight in drug-free MH broth and a 100-µL aliquot were streaked on BHI agar impregnated with various concentrations of linezolid. Number of colonies on the plates were counted after incubation at 35°C for 48 h. (a) HG503pre; (b) HG503post

Fig. 2. In vitro simulation of the appearance of linezolid-resistant subclones in the presence of the sub-MIC level of linezolid.

The HG503post cells were grown in MH broth overnight, adjusted $A_{587\text{ nm}}$ to 0.3 ($10^8$ cfu/ml) and a 100-µL aliquot was inoculated into a fresh MH broth containing 1/4 MIC of linezolid and incubated overnight. MIC of linezolid in the culture was determined by the broth dilution method. Then, the cells grown in the culture were again exposed to a new 1/4 MIC of linezolid as above and this procedure was repeated for 14 consecutive days.

Symbols, △, HG503pre; ○, HG503post
Fig. 1

(A) E-test

(B) Population analysis