Clinical Isolates of *Streptococcus pneumoniae* That Exhibit Tolerance of Vancomycin

Birgitta Henriques Normark,1 Rodger Novak,2 Åke Örtqvist,2 Gunilla Källenius,1 Elaine Tuomanen,3 and Staffan Normark 1

1Swedish Institute for Infectious Disease Control, Microbiology and Tumor Biology Center, Karolinska Institutet; 2Karolinska Hospital, Karolinska Institutet, Sweden; and 3St Jude Children’s Research Hospital, Memphis

The ability of *Streptococcus pneumoniae* to escape lysis and killing by vancomycin, a property termed “tolerance,” has recently been noted in a laboratory strain of the species. Vancomycin tolerance in clinical isolates represents a potential new health risk. We determined the prevalence of vancomycin and penicillin tolerance among 116 clinical isolates of pneumococci by monitoring lysis and viability after exposure to the respective antibiotic for 4 hours. Eight percent of the strains were tolerant to penicillin and 3% were tolerant to vancomycin. The 3 vancomycin-tolerant isolates also had a high ratio of minimum bactericidal concentration to minimum inhibitory concentration, in contrast to nontolerant strains. They were of serotype 9V and had reduced susceptibility to penicillin. Only 1 was also tolerant to penicillin. Growth rate and ability to divide were not affected in the 3 vancomycin-tolerant strains, and they all lysed with deoxycholate, which indicates autolysin production. Vancomycin tolerance among clinical isolates of pneumococci will necessitate tracking to determine the magnitude of the evolving health risk, since tolerance may contribute to treatment failure (in particular, cases of meningitis, in which bactericidal activity is critical for eradication) and since it may also be a favored background for acquisition of resistance of vancomycin.

Resistance to β-lactams, such as penicillin, represents a major problem in the treatment of pneumococcal infections. Penicillin resistance has been found among up to 50% of clinical isolates, according to reports from many parts of the world [1]. In addition, an increasing amount of multidrug resistance makes the combat of these infections even more difficult. Even in a country like Sweden, where the frequency of pneumococci with reduced susceptibility to penicillin is low (3%–4%), multidrug resistance is found in a high proportion of penicillin-nonsusceptible pneumococcal isolates (authors’ unpublished observations). No resistance of the pneumococcus to the glycopeptide vancomycin has been reported yet, which is why this drug represents the last resort for the treatment of multidrug-resistant pneumococci [2, 3].

β-Lactam and glycopeptide antibiotics inhibit cell-wall synthesis, which results in arrest of bacterial growth. For example, penicillin binds covalently to penicillin-binding proteins (PBPs), which are membrane-bound enzymes that catalyze the terminal steps of the assembly of the cell wall. Acylation of the PBPs results in growth arrest. A second unknown step is required to achieve bacterial death. This step involves triggering activation of autolysins, enzymes that degrade the bacterial cell wall [4]. Thus, the bacteria themselves harbor the final executioner.

Resistance to antibiotics arises when the antibiotic does not reach or bind its bacterial target, thereby resulting in continued bacterial multiplication. Resistance
to penicillin is achieved by alterations in the PBPs that cause decreased affinity for penicillin. Another mechanism for escape from antibiotic-induced death is a process termed “antibiotic tolerance” [5], which targets the second (postantibiotic binding) step in killing. In contrast to the low binding affinity in resistant strains, antibiotics bind normally in tolerant strains, and the MIC is not altered. However, killing is dramatically reduced because autolysis is not triggered. Inactivation of the amidase gene \( \text{lytA} \) [5], which encodes the major autolysin at 37°C, or down-regulation of autolysin activity [6] results in tolerance. Recently, a mutant in \( \text{vncS} \), a putative histidine kinase gene of a 2-component regulatory system, was found to be vancomycin and penicillin tolerant, which suggests involvement of VncS in the signaling leading to the activation of the autolytic pathway [7].

Antibiotic tolerance results in defective lysis and greatly reduced killing of the bacteria. This translates to increased bacterial survival and regrowth when the antibiotic is removed [8, 9], which may lead to treatment failure in the clinical setting. Tolerance is also a favored precursor background for importation of determinants of resistance [7]. Although it has been described in the laboratory, tolerance is difficult to study in the clinic because of the lack of rapid diagnostic procedures. A large discrepancy between MIC and MBC has been used to suggest antibiotic tolerance, but this finding is weakened because of difficulties in reproducibility [10]. The only definitive method for detection of tolerance is to perform laborious time-kill curves [9].

In this study, clinical isolates of pneumococci were analyzed for tolerance of penicillin and vancomycin by means of time-kill curves. We report the first survey that showed the presence of pneumococci in the community with increased tolerance of vancomycin. Two of the 3 vancomycin-tolerant isolates were not tolerant to penicillin, a finding that shows that tolerance of these 2 lytic antibiotics may operate through separate mechanisms among clinical strains.

**METHODS**

**Strains of Pneumococci**

A total of 116 clinical isolates of pneumococci, which encompassed a variety of properties, were chosen from 4 different studies: (1) 19 blood isolates were from a study of invasive disease in Sweden, collected during 1987 and 1992 [11]; (k2) 33 blood isolates were from an international study of bacteremias (in which 5 different countries participated) and were collected during 1993–1995 [12]; (3) 27 isolates were from a study of consecutive isolates assessed by the Department of Bacteriology at Danderyd Hospital, Danderyd, Sweden during 1995 (unpublished data); and (4) 37 strains were from a Swedish study of pneumococci with reduced susceptibility to penicillin, and were collected from 1996 through 1997 (unpublished data). Sixty strains showed reduced susceptibility to penicillin (MIC, 0.125–2 mg/L). Fifty-nine strains were from the nasopharynx and 57 from blood (table 1). The isolates belonged to serotypes 23F, 14, and 9V. For controls, antibiotic-susceptible laboratory strain R6 and tolerant, autolysin-defective laboratory mutant Lyt 4-4 (defective in the \( \text{lytA} \) gene, encoding the major autolysin), were obtained from the Rockefeller University Collection (New York) [13].

Samples from frozen stock cultures were grown on blood agar plates for 16–20 h. Two loops of bacteria (~2 μL) were resuspended in 5 mL of glucose medium supplemented with 10% horse serum (Karolinska Hospital, Solna, Sweden). Aliquots of 0.5 mL were added to 10 mL of semisynthetic medium [14] supplemented with 0.1% yeast extract (Difco; this medium is referred to as c + y medium) and, without shaking, were kept in a water bath at 36°C.

**Lysis Rate and Viability Count**

When cultures reached an optical density (OD) of OD<sub>600</sub> 0.3 (corresponding to ~1 × 10<sup>8</sup> cfu/mL in both nontolerant and tolerant isolates), penicillin or vancomycin was added at 10 × MIC (penicillin, 0.1–20 mg/L; vancomycin, 5–10 mg/L). Turbidity (measured in a Sequoia-Turner spectrophotometer; absorbance, 620 nm; Barnstead/Thermolyne) and viability were monitored hourly for 4 h after addition of the antibiotic, a time that has been shown to clearly differentiate tolerant and nontolerant strains [15]. Viability counts were performed by diluting samples from each culture in PBS and plating on blood agar plates. Colony counts were made after overnight incubation in 5% CO<sub>2</sub> at 37°C. Strains that exhibited potential tolerance were tested at least 3 times. Killing-kinetics was performed for 1 vancomycin-tolerant and 1 nontolerant isolate, with use of a range of concentrations near the MIC.

All strains were tested for lysis by means of addition of 2% sodium deoxycholate to a bacterial suspension. The pH of the media was monitored in nontolerant and tolerant cultures. No significant differences in pH could be found between these cultures throughout the experiment.

**Definition of Tolerance**

The killing and lysis patterns of the laboratory strain R6 and its penicillin- and vancomycin-tolerant \( \text{lytA} \) mutant derivative Lyt 4-4, determined from 12 separate experiments, were used to establish the definition of tolerance. For R6, the mean loss of viability after 4 h of penicillin treatment was 3 log kill (SD ± 0.3), and the mean loss of OD was 86% (SD ± 3; figure 1, shaded region). In contrast, for Lyt 4-4 the mean log kill for penicillin was 2.1 (SD ± 0.3), and the mean log loss of OD was 6% (SD ± 5; figure 1, shaded region). The limits for penicillin tolerance were chosen as 2 SD from the mean of the penicillin-tolerant Lyt 4-4 (log kill ≤2.7; OD loss ≤16%).

The limits for vancomycin tolerance were defined in a similar...
manner (figure 2, shaded regions). For R6 the mean log kill was 3 (SD ± 0.3), and the mean loss of OD was 89% (SD ± 3); for Lyt 4-4 the log kill was 1.4 (SD ± 0.3) and the loss of OD was 19% (SD ± 12). The limits that defined vancomycin tolerance were a log kill of ≤2.0 and an OD loss of ≤43%.

Drug Susceptibility Testing

All isolates were tested for susceptibility to penicillin (benzylpenicillin; Astra) and vancomycin (Eli Lilly), as described elsewhere [16]. In brief, we used an agar dilution method in which serial 2-fold dilutions of each antibiotic were added to PDM II Sensitivity Test Agar (Biodisk) supplemented with 5% horse blood. The MIC was defined as the lowest concentration of an antibiotic that did not permit visible growth. For the isolates used in this study, the MIC range for penicillin was 0.008–2 mg/L, and that for vancomycin was 0.25–0.5 mg/L. MBC was determined according to the guidelines of the National Committee for Clinical Laboratory Standards [17].

DNA Techniques

**BOX fingerprinting.** BOX fingerprinting was done as described by Hermans et al. [18]. In brief, chromosomal DNA was cleaved by restriction enzyme *Pvu*II, subjected to agarose gel (0.8%) electrophoresis, denatured, and transferred onto a nylon membrane by means of vacuum blotting. Southern blot hybridization was performed with a 151-bp probe of a highly conserved DNA sequence within intergenic regions of the pneumococcal chromosome (BOX). The oligonucleotide primer pairs BR-A (5'-ATACTCTTGGAAATCTCTGAAC) and BR-C (5'-TATACTCAATGAAAATGAGCA) were used in PCR analysis to generate the probe.

**Single-strand conformation polymorphism (SSCP).** Six primer pairs labeled with alpha-32P[dATP] (Amersham) and designed to represent the entire histidine kinase gene vncS were used in 6 different PCR reactions on chromosomal DNA. SSCP was performed, according to the manufacturer’s protocol, with MDE gel solution (FMC Bioproducts).

**Sequencing.** Sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq DNA polymerase (PE Biosystems) on a 373A automated DNA sequencer (PE Biosystems).

RESULTS

Penicillin Tolerance among Clinical Isolates of *Streptococcus pneumoniae*

The pneumococcal isolates described in table 1 were analyzed for penicillin tolerance (table 2 and figure 1). Although tolerance occurred in virtually all categories, the typical tolerant strain was a type-23F isolate with reduced susceptibility to penicillin (〈•〉) and strains susceptible to penicillin (〈●〉) are shown. The mean value (×) for subtype R6, and the mean value (+) for Lyt 4-4 are also shown. The shaded areas show repeated values for R6 (×) and for Lyt 4-4 (+). The limit of tolerance is indicated by the dashed line.

**Table 1. Description of clinical isolates of pneumococci.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of isolates of serotype</th>
<th>Total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 (n = 41)</td>
<td>23F (n = 36)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Blood</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Reduced susceptibility to penicillin</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Susceptible to penicillin</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>
Vancomycin Tolerance by Pneumococci

Figure 2. Mean values (%) for loss of optical density (OD) and log kill 4 h after addition of vancomycin (10 × MIC) to 116 clinical strains of pneumococci. Strains with a reduced susceptibility to penicillin (♦) and strains susceptible to penicillin (●) are shown. The mean value for subtype R6 (×) and the mean value for Lyt 4-4 (+) are also shown. The shaded areas show repeated values for R6 (×) and for Lyt 4-4 (+). The limit of tolerance is indicated by the dashed line.

Table 2. Distribution of penicillin tolerance among 116 clinical isolates of pneumococci.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percentage of penicillin-tolerant isolates, by serotype</th>
<th>Total isolates, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates</td>
<td>14</td>
<td>23F</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>All isolates</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>Reduced susceptibility to penicillin</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>Susceptible to penicillin</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

Three of the isolates were vancomycin tolerant, showing degrees of killing and lysis similar to those of the autolysin-negative mutant Lyt 4-4 (figures 2 and 3). However, unlike the lytA mutant, 2 of the 3 vancomycin-tolerant clinical isolates were not tolerant to penicillin. Moreover, they all remained susceptible to deoxycholate. The MBC:MIC ratio for these 3 vancomycin-tolerant isolates was 32. For comparison, 15 nontolerant isolates were also examined and showed an MBC:MIC ratio of 1 to 2.

The 3 vancomycin-tolerant isolates, 1 from blood and 2 from the nasopharynx, all were of serotype 9V and had reduced susceptibility to penicillin. They were isolated from different geographic places (Barcelona, Spain; Örebro, Sweden; and Växjö, Sweden) and shared an almost identical BOX fingerprinting pattern as well as a similar pattern of susceptibility to 6 antibiotics. However, other 9V isolates with a similar BOX fingerprinting pattern were not necessarily tolerant (data not shown).

The only isolate that was both vancomycin and penicillin tolerant was recovered from a woman with bacteremic pneumonia who was treated with erythromycin and amoxicillin and who recovered. The other 2 vancomycin-tolerant isolates came from a child with recurrent otitis media and from a healthy child.

Vancomycin-killing kinetics were determined for 1 tolerant and 1 nontolerant isolate by use of vancomycin concentrations close to the MIC (0.5 mg/L). For the nontolerant isolate, killing was substantial even below the MIC, which was not the case for the tolerant isolate. For all concentrations and at most time points, there was a ≥2-log difference in viability between the tolerant and the nontolerant isolate (figure 4).

Loss-of-function mutation in the vncS gene that encodes a 2-component sensor-regulator system in pneumococci has recently been shown to produce tolerance of vancomycin and β-lactams [7]. SSCP was used to study potential polymorphism in the vncS gene of 9 clinical isolates, the 3 vancomycin-tolerant strains, and 6 nontolerant strains (figure 5). Different SSCP patterns among the 9 isolates could be detected only for the 3′ end of vncS. Nucleotide sequencing of this region showed a replacement of a valine for an alanine at position 440 of the vncS gene (figure 5).
isolate was tolerant to only 1 of these 2 lytic antibiotics. Altogether, this suggests that tolerance in the clinical setting might arise as the result of a defect in the antibiotic-induced triggering cascade that translates an antibiotic-induced halt in cell-wall synthesis into a signal to undergo the process of death.

Consistent with other studies [8, 9, 15, 24], penicillin tolerance was found in 8% of the 116 clinical isolates studied here, with a higher frequency of such tolerance found among isolates of serotype 23F than among those of serotypes 9V and 14. A wide diversity in the molecular fingerprinting pattern suggests the spread of this property in the pneumococcal population. Although very stringent criteria were used to define tolerance in this study, figure 1 and 2 show a wide spectrum of killing in clinical populations. However, the point at which penicillin tolerance becomes clinically significant is unknown.

During preparation of this report, a case was reported in which clinical failure followed vancomycin treatment of an infection caused by a vancomycin-tolerant isolate [25]. This raises concern because pneumococci are common, community-acquired pathogens and because vancomycin is the antibiotic of last resort against multiresistant strains. Here we present the first study in which a broad clinical collection of pneumococci was examined for vancomycin tolerance. Vancomycin tolerance was found in 3 serotype 9V isolates, all of which were also characterized by reduced susceptibility to penicillin. These 3 isolates underwent <2-log kill after 4 h in response to 10 × MIC of antibiotic. The 3 vancomycin-tolerant strains had similar molecular fingerprinting patterns, which suggests that they were highly related. Despite this close relationship, 2 were tolerant to vancomycin only and 1 to both vancomycin and penicillin, reinforcing the involvement of >1 pathway that leads to clinical tolerance against lytic antibiotics. Hence, the molecular basis for the mechanism of penicillin tolerance versus vancomycin tolerance might be different, implying that changes in different gene products could underlie the 2 traits.

In laboratory mutants, loss of function of the \( vncS \) histidine kinase, like an inactivation of the major autolysin gene \( \text{lytA} \), leads to tolerance of both penicillin and vancomycin, suggesting that this sensor might be involved in a signal transduction pathway required to activate preformed \( \text{lytA} \) [7]. A valine-to-alanine substitution was found in \( vncS \) in vancomycin-tolerant clinical isolates studied here. This replacement is conservative and outside the conserved regions for histidine kinases [26]. It is possible that polymorphism in \( vncS \) contributes to the tolerance phenotype in pneumococci and affects whether tolerance becomes restricted to vancomycin. To prove or disprove the involvement of the histidine kinase \( vncS \) in the tolerance phenomenon of clinical isolates, isogenic strains will have to be created bearing allelic variations in a single background.

Tolerance of penicillin was more common in certain serotypes. Therefore, isolates of serotype 23F were, in general, less

DISCUSSION

The morbidity and mortality rates associated with infections caused by pneumococci are high, and resistance, especially to penicillin, is causing increasing therapeutic problems [1, 2, 19–21]. However, a recent study of invasive disease in 5 countries did not show an increased mortality rate among patients with infections caused by pneumococcal strains with decreased susceptibility to penicillin versus patients with infections caused by penicillin-susceptible ones [12]. Multiresistance is found in a high proportion of isolates with reduced susceptibility to penicillin, even in countries where the percentage of penicillin-resistant isolates is low, as in Sweden (unpublished data). This emphasizes the need for understanding the mechanisms by which resistance to antibiotics develops.

Tolerance of lytic antibiotics has been suggested to operate as a platform for subsequent development to resistance. It is caused by a defective death response that is due either to a defective autolysin or to a defect in the signal transduction cascade that activates the autolysin. At 37°C the \( \text{lytA} \) amidase appears to be the major autolysin [5, 22, 23]. All tolerant clinical isolates studied here, in contrast to the \( \text{lytA} \) laboratory mutant strain, remained susceptible to deoxycholate, a finding that suggests the presence of autolysins that may be activated by this detergent [6]. In addition, whereas the \( \text{lytA} \) mutant was tolerant to both penicillin and vancomycin, all but 1 clinical isolate was tolerant to only 1 of these 2 lytic antibiotics. Altogether, this suggests that tolerance in the clinical setting might arise as the result of a defect in the antibiotic-induced triggering cascade that translates an antibiotic-induced halt in cell-wall synthesis into a signal to undergo the process of death.

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Vancomycin Tolerance by Pneumococci

Figure 4. Vancomycin-killing kinetics for 1 tolerant (I95, □) and 1 nontolerant (P16, ○) isolate of pneumococci, measured with the use of vancomycin concentrations near the MIC value (0.5 mg/L).

lytic to penicillin than were those of serotypes 9V and 14. Since strains belonging to the same serotype are phylogenetically more related, this might imply that tolerance is a result of natural polymorphism within the background of the species rather than a result of antibiotic selection. However, tolerant isolates would be expected to survive for a considerably longer period in the presence of lytic antibiotics. Here we have shown that at least for serotype 23F, penicillin tolerance was more common among strains with reduced susceptibility to penicillin. In addition, the three 9V strains tolerant to vancomycin had a reduced susceptibility to penicillin. It is therefore possible that tolerance acts as a favored background for acquisition of resistance to lytic antibiotics [7].

At concentrations near the MIC value, there is at least a 2-log difference in survival between the vancomycin-tolerant and nontolerant strains at different time points (figure 4). Therefore, a selection pressure close to the MIC might select for mutations leading to tolerance. Tolerance in the clinical situation may require a longer time at concentrations above the MIC to achieve eradication. The reported treatment failure occurred in a patient who had a vancomycin-tolerant isolate [25]. Furthermore, experimental meningitis caused by the vncS mutant failed to respond to vancomycin [7]. Therefore, a vancomycin-tolerant phenotype is likely to be clinically relevant in cases of meningitis. At this site where host defenses cannot compensate for lack of bactericidal activity, an altered dosage regimen in the treatment of meningitis with vancomycin-tolerant pneumococci should therefore be considered.

In addition to bacterial persistence, defective lysis may also affect the course of disease by influencing the inflammatory response. Peptidoglycan fragments released through bacterial lysis are known to be potent activators of the innate immune system [27, 28]. Autolysis has therefore been suggested to play a role in the course of pneumococcal meningitis in vivo, independent of the sensitivity of the bacterium to antibiotic-induced death. Therefore, in a rabbit model of meningitis, a lysis-defective strain grew to a higher titer before the onset of leukocytosis than did a normally lytic strain [15].

Vancomycin is an important drug in the management of multidrug-resistant pneumococci, often serving as the drug of...
last resort. No resistance of pneumococci to this drug has yet been reported. If vancomycin tolerance facilitates emergence of resistance or complicates the course of meningitis, it will be important to devise a test to monitor the frequency of vancomycin tolerance in various settings as an early alert indicator.

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**References**