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# Bacteriostatic or bactericidal effect of linezolid against multiresistant *Streptococcus pneumoniae*

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**SUMMARY**

The nasopharynx plays a critical role as the reservoir of *Streptococcus pneumoniae*, including drug-resistant strains particularly in children attending day care centers. A total 58 nasopharyngeal, multiresistant isolates of *S. pneumoniae* collected from healthy pre-school children were susceptible to linezolid (MIC = 0.25-1 mg/l), irrespective of serotype and drug resistance pattern. The majority of them (about 94%) were sensitive to the bactericidal effect of linezolid with MBCs = 0.5-4 mg/l. One isolate was killed at 8 mg/l of linezolid, while two at higher concentrations of this antibiotic with MBCs 16 or 32 mg/l, suggesting tolerance of linezolid. BOX-PCR fingerprinting data imply that two linezolid-tolerant strains belonged to distinct clones. Linezolid tolerance was confirmed by monitoring the viability of these isolates during exposure to 4 or 20 mg/l of this antibiotic. The linezolid-tolerant strains were sensitive to the bactericidal effect of vancomycin. Linezolid tolerance in clinical isolates of pneumococci may represent a potential therapeutic risk, especially in infections in which bactericidal activity of drug is critical for eradication of bacteria.

*KEY WORDS:* Linezolid, Bacteriostatic or bactericidal effect, *Streptococcus pneumoniae*

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## INTRODUCTION

*Streptococcus pneumoniae* is one of the most common bacterial pathogens causing not only upper and lower respiratory tract infections but also invasive infections, especially in children and elderly people (Kaplan and Mason, 1998). Recently, the emergence of drug resistance among *S. pneumoniae* isolates has become a problem worldwide (Varon *et al*., 2000; Tenover, 2001). This is generally attributed to inappropriate use of antibiotics and chemiotherapeutics (Varon *et al*. 2000; Zdziarski *et al*., 2003). Pediatric infections caused by drug-resistant pneumococci are an increasing concern with limited treatment options (Kaplan and Mason, 1998; Kaplan *et al*., 2003).

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Linezolid, belonging to oxazolidinones - a new class of antimicrobial agents, inhibits bacterial protein synthesis. Because of its unique mode of action, linezolid does not display cross-resistance with other classes of antimicrobial agents affecting protein synthesis in bacteria (Livermore, 2000; Wilson, 2001). Linezolid is active against several Gram-positive bacteria: staphylococci, streptococci and enterococci, including multiresistant strains (Johnson *et al*., 2000; Cercenado *et al*., 2001; Szczypa *et al*., 2001; Peric *et al*., 2002). This drug was shown to possess good antipneumococcal activity, causing mainly bactericidal effect (Zurenko *et al*., 1996; Kaplan and Mason, 1998). However, some bacteria, including *S. pneumoniae*, are able to survive in the presence of high concentrations of antibiotics without any changes in the MIC of the drugs. The ability of bacteria to escape lysis and killing by antibiotics of bactericidal activity is termed tolerance. This phenomenon was described for the first time over thirty years ago, when the tolerant clinical strain of *S. pneumoniae* with the defective, penicillin-induced autolysis was isolated

(Tomasz *et al.*, 1970). Antibiotic tolerance in clinical isolates may represent a potential health risk (Tuomanem *et al.*, 1986).
Several lines of epidemiological and microbiological studies point to the critical role of the nasopharynx of children, particularly those of preschool age and attending day care centers, not only as the reservoir of *S. pneumoniae* but also in the emergence and spread of drug-resistant strains (De Lencastre and Tomasz, 2002). Therefore, nasopharyngeal isolates of *S. pneumoniae* are useful in predicting drug resistant patterns of invading isolates in a given population (Ghaffar *et al.*, 1999). In this paper we assessed *in vitro* activity of linezolid against multiresistant strains of pneumococci isolated from nasopharynx of healthy pre-school children by determination of minimal inihibitory concentration (MIC) and minimal bactericidal concentration (MBC). Part of this study was presented during the 14th European Congress of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic (Malm *et al.*, 2004).

## MATERIALS AND METHODS

*Bacterial strains*
A total collection consisted of 58 nasopharyngeal, multiresistant isolates of *S. pneumoniae* from healthy pre-school children. A reference strain *S. pneumoniae* ATCC 49619 was included in these studies.

*Determination of susceptibility to antimicrobial agents*
Susceptibility to oxacillin (as a marker of penicillin sensitivity), erythromycin, clindamycin, tetracycline, chloramphenicol and cotrimoxazole (trimethoprim/sulphamethoxazole) was determined by the disc diffusion method according to the guidelines of Clinical Laboratory Standard Insitute (CLSI) (2004) (Hryniewicz *et al.*, 2004). Discs with antibiotics were obtained from Becton-Dickinson Company (New York, USA).

*Determination of the minimal inhibitory concentration (MIC) of penicillin*
Isolates exhibiting an inhibition zone of ≤19 mm around a 1 µg oxacillin disc were further tested by the E-test (AB Biodisk, Solna, Sweden), following the manufacturer's instructions, to determine the penicillin MIC. The following breakpoints were used: if MICs were ≤0.06 mg/l, organism was defined as susceptible; if they were 0.1-1.0 mg/l, the organism was defined as intermediate; and if they were ≥2 mg/l, the organism was defined as highly resistant to penicillin.

*Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of linezolid*
The MIC and the MBC of linezolid (Pfizer, Poland) were estimated by the broth microdilution method according to the guidelines of CLSI. The $MIC_{50}$ and the $MIC_{90}$ values were defined as the MIC inhibited 50% or 90% of the isolates, respectively. The $MBC_{50}$ and the $MBC_{90}$ values were defined as the MBC killed 50% or 90% of the isolates, respectively. Besides, the $(MBC/MIC)_{50}$ and the $(MBC/MIC)_{90}$ ratios were calculated, defined as the MBC/MIC ratio found in 50% or 90% of the isolates, respectively.

*Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of vancomycin or penicillin*
The MIC and the MBC of vancomycin (Sigma-Aldrich Corp., St. Louis, USA) or penicillin G (Sigma-Aldrich Corp., St. Louis, USA) were estimated by the broth microdilution method according to the guidelines of CLSI.

*Determination of the bactericidal effect of linezolid*
The bactericidal effect of linezolid was assessed by the time-kill assay using the method described by Zurenko *et al.* (1996). The time-kill reaction tubes contained supplemented proteose broth (Difco Laboratories, Detroit, MI, USA), linezolid at a final concentration of 4 or 20 mg/l and an inoculum of optical density corresponding approximately $10^7$ colony forming units (CFU) per ml. The tubes were incubated at 35°C in 5% $CO_2$ and the 100 µl samples were removed only at 0 and 6 h because of the autolytic nature of pneumococci during overnight incubation in broth. The appropriately diluted samples were applied on the surface of blood agar plates and incubated at 35°C in 5% $CO_2$. Colony counts were made after overnight incubation.

*Serotyping*
All pneumococci were serotyped on the basis of capsular swelling (Quellung reaction) observed in phase-contrast microscope after suspending in appropriate antisera (Statens Serum Institut, Copenhagen, Denmark).

*DNA isolation and BOX-PCR fingerprinting*
From 24-48 h *S. pneumoniae* cultures in Todd-Hewitt broth, the genomic DNA was isolated using the Genomic DNA Isolation and Purification Kit (Fermentas, Lithuania). Typing of the pneumococcal isolates by BOX-PCR fingerprinting was performed as described by Van Belkum *et al*. (1996). Briefly, 50 ng DNA were amplified by PCR (4 min at 94°C, predenaturation; 40 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 74°C, extension), using primer BOX-A (5'-AT-ACTCTTCGAAAATCTCTTCAAAC-3') designed from the primary structure of the pneumococcal BOX repeat motif. The amplified products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide, and the banding patterns were evaluated visually. Comparison of the banding patterns was performed by the unweighted-pair-group method using arithmetic averages and the Dice similarity coefficient applied to peaks. Computer-assisted analysis and methods and algorithms used in this study were carried out according to the BIO-PROFIL BioGene Windows Application V11.07, Vilber Lourmat, France. A tolerance of 2% in band position was applied during the comparison of the DNA patterns.

*Repeatability of the results*
All experiments were done in triplicate. The representative data are presented.

## RESULTS

A collection of nasopharyngeal, multiresistant isolates of *S. pneumoniae* chosen for these studies consisted of 22 isolates belonging to penicillin-sensitive *S. pneumoniae* (PSSP) and 36 isolates belonging to penicillin-insensitive *S. pneumoniae* (PNSP). All PNSP isolates were classified as in-

TABLE 1 - *Drug resistance patterns and serotypes of 22 multiresistant nasopharyngeal isolates of penicillin-susceptible Streptococcus pneumoniae (PSSP) in correlation with MICs, MBCs and MBC/MIC ratios for linezolid.*

| *Drug resistance pattern* | *Serotype* | *Number of strains* | *MIC (mg/l)* | *MBC (mg/l)* | *MBC/MIC* |
|---|---|---|---|---|---|
| ETeCC (n = 7) | 6 B | 1 | 0.25 | 1 | 4 |
| | 6 B | 3 | 0.5 | 1 | 2 |
| | 6 B | 1 | 0.5 | 2 | 4 |
| | 15 B | 1 | 0.5 | 1 | 2 |
| | 15 B | 1 | 0.5 | 2 | 4 |
| CSXTTe (n = 4) | 19 F | 1 | 0.25 | 2 | 8 |
| | 19 F | 2 | 0.5 | 2 | 4 |
| | 19 F | 1 | 1 | 2 | 2 |
| ESXTTeCC (n = 4) | 6 B | 2 | 0.5 | 1 | 2 |
| | 19 F | 1 | 0.5 | 2 | 4 |
| | 23 F | 1 | 0.5 | 16 | 32 |
| ECSXTTeCC (n = 7) | 19 F | 1 | 0.5 | 1 | 2 |
| | 19 F | 1 | 0.5 | 2 | 2 |
| | 19 F | 2 | 0.5 | 2 | 4 |
| | 19 F | 1 | 1 | 2 | 2 |
| | 9 V | 1 | 0.5 | 1 | 2 |
| | 23 F | 1 | 0.5 | 2 | 4 |

Legend: E = erythromycin, CC = clindamycin, Te = tetracycline, C = chloramphenicol, SXT = cotrimoxazole

TABLE 2 - *Drug resistance patterns and serotypes of 36 multiresistant nasopharyngeal isolates of penicillin-intermediate Streptococcus pneumoniae (PISP) in correlation with MICs, MBCs and MBC/MIC ratios for linezolid.*

| *Drug resistance pattern* | *Serotype* | *Number of strains* | *MIC (mg/l)* | *MBC (mg/l)* | *MBC/MIC* |
|---|---|---|---|---|---|
| PETeCC (n = 9) | 6 B | 1 | 0.25 | 0.5 | 2 |
| | 6 B | 1 | 0.5 | 0.5 | 1 |
| | 6 B | 3 | 0.5 | 1 | 2 |
| | 6 B | 3 | 0.5 | 2 | 4 |
| | 14 | 1 | 0.25 | 2 | 8 |
| PESXTTeCC (n = 1) | 19 F | 1 | 0.5 | 2 | 4 |
| PECSXTTeCC (n = 26) | 6 B | 1 | 0.25 | 1 | 4 |
| | 6 B | 1 | 0.25 | 2 | 8 |
| | 6 B | 5 | 0.5 | 1 | 2 |
| | 6 B | 6 | 0.5 | 2 | 4 |
| | 19 F | 1 | 0.5 | 0.5 | 1 |
| | 19 F | 1 | 0.5 | 1 | 2 |
| | 19 F | 5 | 0.5 | 2 | 4 |
| | 19 F | 1 | 0.5 | 4 | 8 |
| | 19 F | 1 | 1 | 2 | 2 |
| | 19 F | 1 | 1 | 4 | 4 |
| | 19 F | 2 | 1 | 8 | 8 |
| | 19 F | 1 | 0.5 | 32 | 64 |

Legend: P = penicillin, E = erythromycin, CC = clindamycin, Te = tetracycline, C = chloramphenicol, SXT = cotrimoxazole

termediate *S. pneumoniae* (PISP) with MICs of penicillin ranging from 0.1 to 1.0 mg/l.

All chosen isolates were multiresistant, *i.e.* insensitive to at least two non-beta-lactams drugs from different therapeutic classes - macrolides (erythromycin), lincosamides (clindamycin), tetracyclines (tetracycline), chloramphenicol or cotrimoxazole (trimethoprim/sulphametoxazole). The resistance patterns of the isolates are presented in Table 1 and Table 2.

All isolates were susceptible to linezolid with MICs ranging from 0.25 to 1 mg/l (Table 1, 2), irrespective of serotype and drug resistance pattern. The MIC value of linezolid for reference

TABLE 3 - *In vitro activity of linezolid against a total collection of multiresistant nasopharyngeal isolates of Streptococcus pneumoniae.*

| *Parameter* | *PSSP (n = 22)* | *PISP (n = 36)* | *Total (n = 58)* |
|---|---|---|---|
| $MIC_{50}$ (mg/l) | 0.5 | 0.5 | 0.5 |
| $MIC_{90}$ (mg/l) | 0.5 | 1 | 1 |
| $MBC_{50}$ (mg/l) | 2 | 2 | 2 |
| $MBC_{90}$ (mg/l) | 2 | 4 | 4 |
| $(MBC/MIC)_{50}$ | 2 | 4 | 4 |
| $(MBC/MIC)_{90}$ | 4 | 8 | 8 |

Legend: PSSP = penicillin-susceptible *S. pneumoniae*, PISP = penicillin-intermediate *S. pneumoniae*

strain *S. pneumoniae* ATCC 49619 was 0.5 mg/l. The $MIC_{50}$ and $MIC_{90}$ of linezolid for the PSSP isolates were 0.5 mg/l, while for PISP isolates 0.5 and 1 mg/l, respectively (Table 3). The majority of isolates (94.83%) were sensitive to the bactericidal effect of linezolid with MBCs ranging from 1 to 4 mg/l. The $MBC_{50}$ and $MBC_{90}$ of linezolid for the PSSP isolates were 2 mg/l, while for the PISP isolates 2 and 4 mg/l, respectively. The $(MBC/MIC)_{50}$ and $(MBC/MIC)_{90}$ ratio of linezolid for PSSP isolates were 2 and 4, while for PISP isolates 4 and 8, respectively (Table 3).

Two out of 58 isolates with MICs = 0.5 mg/l of linezolid were killed only at high concentrations of this drug with MBCs 16 or 32 mg/l and had a high MBC/MIC ratios 32 or 64. One tolerant isolate (T1) was PSSP with serotype 23F and drug resistance pattern - ESXTTeCC, while another tolerant isolate (T2) was PISP with serotype 19F and drug resistance pattern PECSXTTeCC (Tables 1, 2). The prevalence of linezolid tolerance among tested isolates was 3.45%.

Linezolid tolerance was confirmed by monitoring viability of these strains during exposure to 20 mg/l of this antibiotic (Table 4), similar to its maximal serum concentration after standard dosing; minimal serum concentration of linezolid is about 4 mg/l (Slater *et al*., 2001; Wilson, 2001). The time-kill assay was also performed for two of the non-tolerant isolates: one (NT1) from PSSP strains with serotype 19F and drug resistance pattern ESXTTeCC, showing MIC of linezolid 0.5 mg/l and MBC of linezolid 2 mg/l and another (NT2) from PISP strains with serotype 19F and drug resistance pattern PESXTTeCC, showing MIC of linezolid 1 mg/l and MBC of linezolid 2 mg/l. A bactericidal effect of linezolid for pneumococci was defined as a viable count reduction greater than 3 log colony forming units (CFU)/ml in the initial inoculum during 6 h incubation. Indeed, the degree of killing of non-tolerant strains by linezolid was high even at 4 mg/ml, without any or only slight effect against linezolid-tolerant strains even at 20 mg/ml (Table 4). Both strains that tolerated linezolid were sensitive to optochin and lysed with deoxycholate, which indicated undisturbed autolysin production (data not shown).

In the present study, BOX-PCR fingerprinting was used to estimate relatedness of linezolid-tolerant isolates; they had only 42% of genetic similarity (Figure 1). Also relatedness among 10 isolates

TABLE 4 - *Time-kill assay of selected, multiresistant nasopharyngeal isolates of Streptococcus pneumoniae in the presence of 4 or 20 mg/l of linezolid in the growth medium.*

| *Isolate* | *Linezolid (mg/l)* | *Bacterial population (log CFU/ml)* | | *The degree of bacterial population reduction after 6 h (log CFU/ml)* |
|---|---|---|---|---|
| | | *0 h* | *6 h* | |
| NT1 | 0 | 7.18 | 8.48 | - |
| | 4 | 7.18 | 2.67 | 4.51 |
| | 20 | 7.18 | 2.08 | 5.10 |
| NT2 | 0 | 7.15 | 8.40 | - |
| | 4 | 7.15 | 2.58 | 4.47 |
| | 20 | 7.15 | 2.10 | 5.05 |
| T1 | 0 | 7.12 | 8.30 | - |
| | 4 | 7.12 | 7.98 | - |
| | 20 | 7.12 | 6.30 | 0.82 |
| T2 | 0 | 7.16 | 8.45 | - |
| | 4 | 7.16 | 8.20 | - |
| | 20 | 7.16 | 7.78 | - |

Legend: CFU = colony forming units, NT1 and NT2 = linezolid non-tolerant *S. pneumoniae* strains, T1 and T2 = linezolid-tolerant *S. pneumoniae* strains

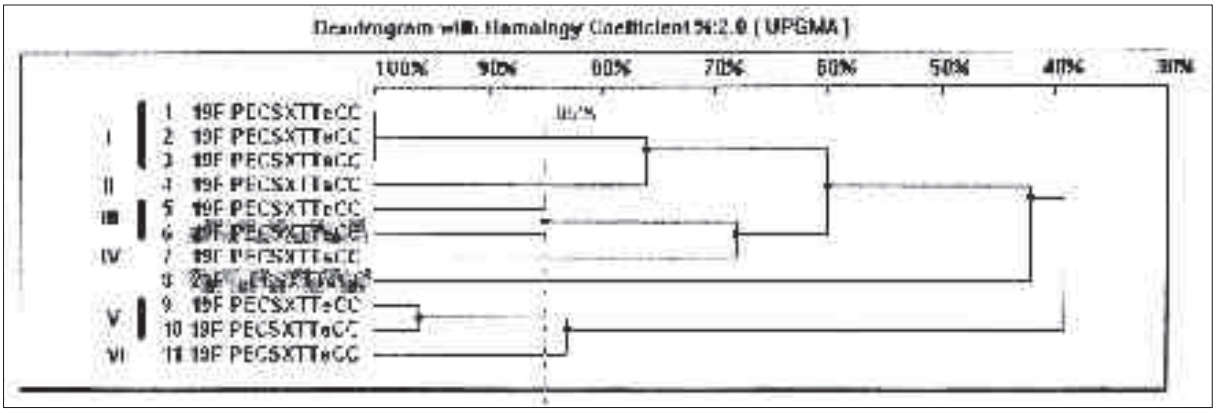


FIGURE 1 - *Dendrogram constructed from BOX-PCR banding patterns showing genetic relatedness of 11 pneumococcal isolates. Abbreviations as in Table 2. Linezolid-tolerant isolates are indicated by grey boxes.*

within the phenotype 19F PECSXTTeCC, to which one of the linezolid-tolerated strains belonged, was assessed. They were divided into six BOX-PCR types, when 85% level of similarity was used for grouping the isolates (Figure 1).
The two linezolid-tolerant strains were sensitive to the bactericidal activity of vancomycin with MIC = MBC = 0.5 mg/l, while PSSP isolate was also sensitive to the bactericidal activity of penicillin with MIC = MBC = 0.015 mg/l.

## DISCUSSION

Linezolid was well-tolerated and effective in treating serious pneumococcal infections not only in adults but also in children, being a promising therapeutic option in infections caused by pneumococci not susceptible to the available drugs (Kaplan *et al.*, 2003; Velissariou, 2006). Good antipneumococcal activity of linezolid against multiresistant nasopharyngeal isolates described in this paper is in agreement with studies by Szczypa *et al.* (2001) showing excellent activity of linezolid against *S. pneumoniae*, including both sensitive and resistant to penicillin ($MIC_{50}$ = 0.5 mg/l; $MIC_{90}$ = 0.5 or 1 mg/l), isolated from various clinical specimens in 2000 in 61 medical centers in Poland. Also data presented by other authors (Johnson *et al.*, 2000; Cercenado *et al.*, 2001; Peric *et al.*, 2002; Şener *et al.*, 2004; Ross *et al.*, 2007) indicate that linezolid remained highly active against pneumococci, irrespective of their serotype and resistance to other drugs, including protein synthesis inhibitors, *e.g.* erythromycin, clindamycin, tetracycline and chloramphenicol. It was found that linezolid shows bactericidal effect in pneumococci but not in other Gram-positive bacteria, *e.g.* staphylococci or enterococci (Zurenko *et al.*, 1996; Wilson, 2001). In our studies, the majority of nasopharyngeal pneumococci were sensitive to the bactericidal activity of linezolid. Only two strains could survive even at high concentrations of linezolid. Data presented by Lin *et al*. (2005) showed that although linezolid was mainly bacteriostatic against all studied *S. pneumoniae* strains at 2 x MIC after 24 h, significant killing at earlier periods was observed. However, these studies did not consider the autolytic nature of pneumococci during overnight incubation in broth.
Both linezolid-tolerant strains, described in this paper, remained sensitive to optochin and to deoxycholate, suggesting their normal ability to autolysin production. Therefore, it is probable that linezolid tolerance observed in nasopharyngeal isolates of *S. pneumoniae* may not be due to defective autolysin production *per se*, but this requires further studies. According to the literature (Novak *et al.*, 1999), vancomycin tolerance described in *S. pneumoniae* may be based on a defect in autolysin production, physiologically activated in the stationary phase of growth but unnaturally activated by antibiotics. Tolerance of *S. pneumoniae* also extended to other antimicrobial drugs such as beta-lactams, aminoglycosides and quinolones (Novak *et al.*, 1999). The prevalence of vancomycin tolerance in clinical isolates of *S.*

*pneumoniae* was assessed at the level of 3%, while penicillin tolerance was 8% (Normark *et al*., 2001).

Two linezolid-tolerant strains described in this paper had different serotypes and resistance patterns, therefore they greatly differed in the BOX-PCR fingerprinting pattern. Diversity both in serotype distribution and in the genotypic pattern was also found by other authors among penicillin tolerant isolates (Normark *et al*., 2001). In contrast, in 3 vancomycin tolerant clinical isolates of *S. pneumoniae* isolated from different geographic places, all were of serotype 9V and shared an almost identical BOX-PCR fingerprinting pattern as well as a similar pattern of susceptibility to six antibiotics (Normark *et al*., 2001).

In conclusion, so far only two linezolid-resistant *S. pneumoniae* isolates have been described, which were found to harbour novel ribosomal mutations (Farrell *et al*., 2004). However, our data indicate that some pneumococcal isolates may be insensitive to the bactericidal effect of this antibiotic. Linezolid tolerance, by analogy to tolerance to other antibiotics, may represent a potential risk of treatment failure, especially when bactericidal activity is critical for eradication of bacteria, *e.g.* in meningitis or sepsis (Normark *et al*., 2001). Linezolid tolerance may also be a favored background for acquisition of resistance to linezolid.

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