

*pep*²⁷ and *lytA* in Vancomycin-Tolerant Pneumococci

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Received: May 23, 2011 / Revised: August 13, 2011 / Accepted: August 30, 2011

Vancomycin therapy failure due to the emergence of tolerance in pneumococci is increasing. The molecular mechanism of tolerance is not clear, but *lytA* and *pep*²⁷ are known to be involved. Our aim was to evaluate the expression of both genes in vancomycin-tolerant *Streptococcus pneumoniae* (VTSP) strains. Eleven VTSP strains from a total of 309 clinical isolates of *S. pneumoniae* from 1997 to 2006 were classified according to the criteria of Liu and Tomasz. All VTSP strains were evaluated for susceptibility according to CLSI criteria, serotype by the Quellung test, and clonality by PFGE. The expressions of *lytA* and *pep*²⁷ were analyzed in different growth phases by RT-PCR with and without vancomycin. Eighty-two percent of VTSP strains showed resistance to penicillin, and 100% were sensitive to vancomycin and cefotaxime. The most frequent serotypes of VTSP strains were 23F (4/11) and 6B (3/11). Clonal relationship was observed in only two strains. No significant changes were observed in *pep*²⁷ expression in the three phases of growth in VTSP strains with and without vancomycin. Interestingly, *pep*²⁷ expression in the stationary phase in the non-tolerant reference strain R6 was significantly higher. However, no significant differences in *lytA* expression were observed between VTSP and R6 strains during the phases of growth analyzed. The absence of changes in *pep*²⁷ expression in VTSP strains in the stationary phase may be related to their ability to tolerate high antibiotic concentrations, and thus, they survive and remain in the host under the antibiotic selective pressure reflected in therapeutic failure.

Keywords: Tolerance, vancomycin, *Streptococcus pneumoniae*, *pep*²⁷, *lytA*

Streptococcus pneumoniae is the most common cause of acute meningitis and respiratory tract infections in the young and elderly [8]. According to the World Health Organization, it is involved in approximately one million deaths annually in children worldwide [18].

For several years, penicillin has been successfully used to treat infections caused by *S. pneumoniae*, but therapy with this antibiotic has been compromised by the increasing prevalence of penicillin-resistant pneumococci. The appearance of strains with multiple antibiotic resistance has forced in some cases the inclusion of vancomycin as an initial treatment for meningitis, in spite of guidelines suggesting it as an antibiotic of last resort [3]. Currently, there are no reports of *S. pneumoniae* strains resistant to vancomycin, making the antibiotic an excellent alternative for the treatment of severe infections caused by this organism [4].

Before the antibiotic era, populations of antibiotic-sensitive bacteria contained a very small fraction (approximately 10⁻⁶) of antibiotic-tolerant cells [7]. At present, there is selective pressure on bacterial populations, due to the daily use of antibiotics such as vancomycin, resulting in the selection of tolerant bacteria. Bacterial tolerance to antibiotics is the ability to survive when the bacteria are under antibiotic selective pressure and do not show any apparent growth [14]. It was first described for β -lactam antibiotics such as penicillin and later for vancomycin. Bacterial tolerance has been proposed as a phenotype that could be a precursor to the phenotype of resistance [16]. In the last few years, tolerance to vancomycin has been described in clinical isolates of *S. pneumoniae* [11, 13].

R. Novak *et al.* [14] have explained that the molecular mechanism of tolerance to penicillin and vancomycin involves a defect in the activation of LytA, a murein hydrolase that mediates an endogenous process of death leading to cellular lysis. This defect involves a loss of enzymatic function due to a mutation in *vncS*, which

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encodes a two-component sensor system that may regulate a basic pathway triggering autolysis. Sung *et al.* [23] have also reported the lack of *LytA* and microbial tolerance in clinical strains.

On the other hand, R. Novak *et al.* [15] have observed a 27-amino-acid peptide, *Pep*²⁷, that is secreted by the ABC system and has an important role in controlling bacterial death. *Pep*²⁷ also offers an alternative way of explaining cell death by lysis, due to its responsibility for triggering the expression of *lytA*. In the current work, we investigated the effect of a high concentration of vancomycin on *pep*²⁷ and *lytA* expression in clinical isolates of *S. pneumoniae* with proven tolerance to vancomycin and determined their clonal relationship.

MATERIALS AND METHODS

Bacterial Strains

S. pneumoniae isolates were obtained from children aged from >3 to 144 months old (median=30 months) with different infectious diseases. *S. pneumoniae* strain R6 was employed as a vancomycin-intolerant reference strain [20]. All of the isolates were cultured on 5% sheep's blood agar plates (BBL, Franklin Lakes, NJ, USA) at 37°C in 5% CO₂.

Bacteriological Identification

Validation of isolates was accomplished through conventional bacteriological methods including colony and microscopic morphology, catalase test, optochin sensitivity (Taxo P), bile solubility, and positive coagglutination test (Phadebact; Pharmacia Diagnostics, Uppsala, Sweden). The strains were stored at -70°C in skim milk (Difco, Lawrence, KS, USA) until use.

Antimicrobial Susceptibility

All isolates were tested for susceptibility to vancomycin (Sigma-Aldrich, St. Louis, MO, USA) and penicillin G (Sigma-Aldrich). The minimum inhibitory concentration (MIC) was determined by the micro dilution method, using Mueller–Hinton broth (BBL) supplemented with 5% sheep's blood. Susceptibility was interpreted according to the criteria of the Clinical Laboratory and Standards Institute (CLSI) using *S. pneumoniae* ATCC49619 as a control [19].

Detection of Vancomycin-Tolerant *S. pneumoniae* (VTSP) Clinical Isolates

Selection of tolerance to vancomycin in all strains was determined according to the criteria established by Liu and Tomasz [10].

Bacterial Lysis

Four to five colonies of each clinical isolate were inoculated into 10 ml of Todd–Hewitt broth (BBL) supplemented with 1% yeast extract and incubated overnight at 37°C in a 5% CO₂ atmosphere. Vancomycin was added when the optical density (OD) of each culture reached 0.17 at 600 nm (approximately 10⁶–10⁷ CFU/ml) at a concentration of 5 µg/ml (10× MIC value). The OD was measured each hour for 4 h. Isolates that showed a decrease in OD of >50% after 2 h were defined as fast lysis, those with a decrease in OD of

>50% at 4 h were defined as moderate lysis, and those with a decrease in OD of <50% after 4 h were defined as negative lysis isolates.

Logarithmic Death Quantification

Serial dilutions of the *S. pneumoniae* strains showing negative and moderate lysis were made from the cultures exposed to vancomycin, and each dilution was grown on blood agar plates for 24 h at 37°C and in 5% CO₂. Logarithmic death was evaluated in triplicate as the log₁₀ decrease in counted viable cells. The tolerance breakpoint value was obtained by 15 independent determinations of the lysis pattern of the R6 strain. Logarithmic death of the R6 strain had a median value of 3.9 [standard deviation (SD), ± 0.5], whereas the limit defined for a moderately tolerant strain was >2 SD below that of R6 (logarithmic death <1.53) and the limit for a highly tolerant single strain was >3 SD below that of R6 (logarithmic death <1.13).

Characterization of Vancomycin-Tolerant *Streptococcus pneumoniae* Strains

Antimicrobial susceptibility. VTSP strains were tested for susceptibility to cefotaxime (Sigma-Aldrich), meropenem (AstraZeneca, Cheshire, UK), rifampicin (Sigma-Aldrich), erythromycin (MP Biomedical, Solon, OH, USA), trimethoprim (MP Biomedicals) with sulfamethoxazole (MP Biomedicals), clindamycin (Sigma-Aldrich), and linezolid (Pfizer Central Research, Groton, CT, USA), by MIC according to the CLSI guidelines [19].

Autolysin activity by deoxycholate technique (DOC_T). Autolysin activity in VTSP strains was determined according to a previously described technique [17]. The strains were considered to be positive for autolysin activity (Doc_T⁺) when the OD of the bacterial suspension decreased by more than 50% of the initial value.

Serotyping. VTSP strains were serotyped by the capsular Quellung method with commercial antisera (Statens Serum Institute, Copenhagen, Denmark).

Clonal relationship by pulsed-field gel electrophoresis (PFGE).

The clonal relationship of the strains was determined by PFGE of chromosomal DNA digested with *Sma*I as described by McEllistrem *et al.* [12], with modifications. Digestion was performed in a volume of 100 µl with 1× enzyme buffer and 30 U *Sma*I at 30°C for 4 h. The fragments were resolved by PFGE in two runs; the first in 1.3% agarose and the second in 1.6% agarose in 0.5× Tris-borate-EDTA buffer at 14°C and 6 V/cm in a CHEF Mapper system (Bio-Rad Laboratories). The parameters of the first run in block 1 were an initial pulse time of 1 s increased to 30 s for 19 h, and in block 2, 5 s increased to 9 s for 5 h. The parameters of the second run in block 1 were pulse times ramped from 2 to 20 s for 38 h. Both gels were stained with ethidium bromide at 0.5 µg/ml and then observed under UV light.

Growth curve. Growth curves for VTSP and R6 strains were determined to establish logarithmic, stationary, and death phase times using Todd–Hewitt broth supplemented with 1% yeast extract. The strains were incubated at 37°C in a 5% CO₂ atmosphere. The OD was measured each hour at a wavelength of 600 nm. VTSP strains were incubated to obtain a bacterial culture at the middle of each growth phase.

***lytA* and *pep*²⁷ expression analysis by RT-PCR endpoint.** All VTSP strains were subjected to RNA extraction by the TRIzol-method (Invitrogen, Carlsbad, CA, USA) during the three different growth phases described above. On the other hand, RNA from the

same VTSP strains was extracted after the addition of vancomycin at 5 µg/ml (10× MIC value) after 20 min of each growth phase. RT-PCR was performed using a GeneAmp AccuRT RNA PCR kit (Applied Biosystems, Foster City, CA, USA) with the following primers: pep27-1 (5'-ATGAGAAAGGAATTCACAACG-3') and pep27-2 (5'-TCACGGATCATCTCTT CATC-3'), designed for this work. The *lytA* gene was amplified using primers previously reported by Sung *et al.* [23]. As a constitutive control, 16S ribosomal RNA gene was used as previously described by Li-Korotky *et al.* [9]. RT-PCR products were separated on a 2% agarose gel, and the band density was measured using commercial software (Quantity One 4.1.1 Image Analyzer; Bio-Rad).

Statistical Analysis

The Statistic Package for the Social Sciences program (SPSS, version 10) was used for a non-parametric test (Wilcoxon and Friedman test). Categorical variables were described as percentages, and for continuous quantitative variables, median and minimum–maximum values were used.

PFGE analysis was done by considering the presence or absence of specific bands to obtain an estimate of similarity for each pair of isolates. The similarity was calculated using the Dice coefficient. The dendrogram was obtained by UPGMA and the relationship was supported by the cophenetic correlation coefficient using Mantel and a bootstrap test with 10,000 randomizations [22]. Multivariate statistical methods were carried out with the NTSYS-PC program (version 2.0; Exeter Software) [21].

RESULTS

A total of 309 strains of *S. pneumoniae* were collected as follows: 55 from cerebrospinal fluid (CSF), 71 from blood cultures, 47 from lower respiratory tract specimens, 64 from middle ear aspirates, and 110 from other specimens. All isolates were from patients no more than 5 years old. One hundred percent of the *S. pneumoniae* strains were

susceptible to vancomycin, whereas 63% were resistant to penicillin. The origin of the isolates is described in Table 1.

Vancomycin Tolerance in *S. pneumoniae*

All *S. pneumoniae* strains were classified according to the criteria of Liu and Tomasz [10] using a decrease in OD. Two hundred forty-one strains showed rapid lysis with a decrease in OD of $\geq 77.2\%$ (SD, $\pm 13.3\%$) 2 h after exposure to the antibiotic; 42 strains showed moderate lysis with a decrease of $\leq 19.76\%$ (SD, $\pm 15.8\%$) at 2 h and $\geq 67.83\%$ (SD, $\pm 15.1\%$) after 4 h of exposure; and 26 strains showed negative lysis without a significant decrease in OD ($\leq 10.61\%$ and SD $\pm 9.04\%$ at 2 h and $\leq 15.59\%$ and SD $\pm 11.04\%$) 4 h after exposure. Logarithmic death was assessed in the 68 strains that showed moderate and negative lysis. Eleven (3.6%) *S. pneumoniae* strains were tolerant to vancomycin. Five of these were moderately tolerant and six were highly tolerant, as shown in the scatter plot (Fig. 1).

Strain 261D, isolated from an eye discharge, showed a 29.67% increase in OD after 4 h of vancomycin exposure. This strain showed atypical features of phenotype (susceptible to taxo P and negative bile solubility). However, the strain was positive for *lytA* and 16S rRNA genes, so we conclude that this is an atypical *S. pneumoniae* strain [17].

Characterization of VTSP Strains

The average age of patients with VTSP isolates was 2.5 years, and 72% were male. Two of 11 strains were isolated from CSF with a diagnosis of meningitis, 2/11 with media otitis, and 3/11 were from blood with primary bacteremia (Table 1). Moreover, 54.5% cases had received at least one β -lactam antibiotic treatment in the last 3 months. None of the patients were attending kindergarden. VTSP strains showed resistance to penicillin, erythromycin, sulfamethoxazole-

Table 1. General characteristics of vancomycin-tolerant *S. pneumoniae* isolates from children, 1997 to 2006.

Strain	Year of isolation	Origin	Tolerance	Phenotype		
				Serotype	Doc _T	Resistance
35	1998	Blood	High	7F	+	Sensitive
36	1998	CSF	High	23F	+	SXT
43	1998	Otitis media	High	23F	+	PEN
55	1999	Otitis media	Moderate	28F	+	Sensitive
69	2000	CSF	Moderate	23F	+	PEN
103	2001	Blood	High	19F	+	PEN
134	2003	Conjunctive	High	15B	+	PEN
141	2003	Peritoneal fluid	High	23F	+	PEN, ERY, SXT, CLI ^a
167	2003	Blood	Moderate	6B	+	PEN
173	2004	Empyema	Moderate	6B	+	PEN, SXT
261	2004	eye discharge	Moderate	6B	-	ERY

STX: Trimethoprim–Sulfamethoxazole; PEN: Penicillin; ERY: Erythromycin; CLI: Clindamycin.

^aIsolate multiresistant.

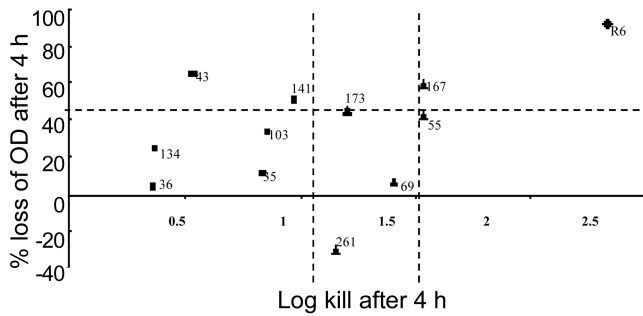


Fig. 1. Scatter plot classification of vancomycin-tolerant *S. pneumoniae* strains as a function of logarithmic death and decrease in OD.
 ■ Moderate tolerance; ▲ High tolerance; ◆ Non-tolerant R6 control strain.

trimethoprim, and clindamycin (82%, 18%, 27%, and 9%, respectively). One hundred percent of VTSP strains were susceptible to cefotaxime, meropenem, rifampicin, vancomycin, and linezolid. Ninety point nine percent of VTSP strains

showed the Doc_T⁺ phenotype (rapid lysis), the same as strain R6, while only strain 261D showed the Doc_T⁻ phenotype. The serotypes of the VTSP strains were 23F (4/11), 6B (3/11), and 7F, 28F, 19F, and 15F with one case each.

Among the 11 VTSP strains, 10 *Sma*I restriction patterns were found (Fig 2). The dendrogram revealed a close relationship between strains 167 and 173. These clones were isolated from different years, but they were the same serotype (6B).

Eight of the 11 VTSP strains reached the log phase 60 min after beginning the kinetic growth test, but strains 35, 69, 103, and R6 took 2 h to reach it. The log phase lasted 4 h for 9/11 VTSP and R6 strains and 2 h for 2/11 VTSP strains. The stationary phase ended at 20 h for all VTSP strains and 18 h for strain R6. The death phase continued for about 2 more hours. The RNA of each strain was obtained at the middle of the log and stationary phases, and from the start of the death phase.

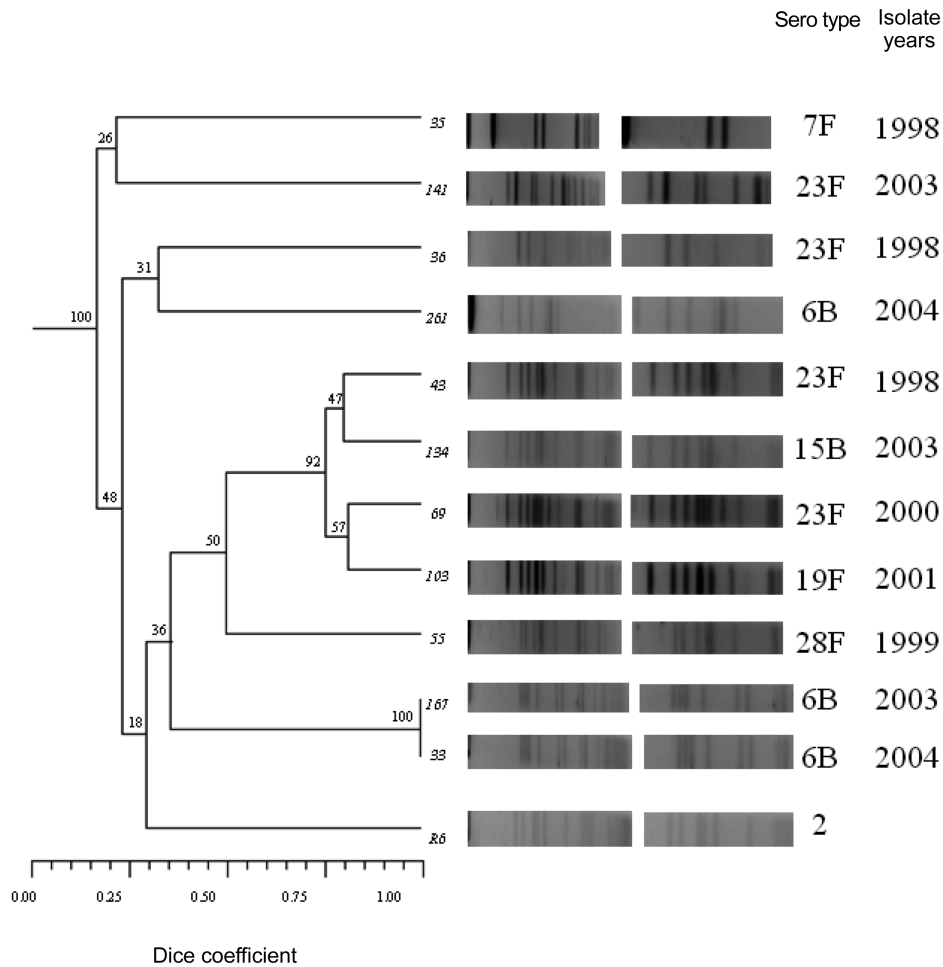


Fig. 2. Dendrogram of *Sma*I-PFGE of vancomycin-tolerant *S. pneumoniae* isolates from children attending a pediatric hospital from 1997 to 2006. CCCr = 0.937. P = 0.002. Bootstrap values are given at the nodes.

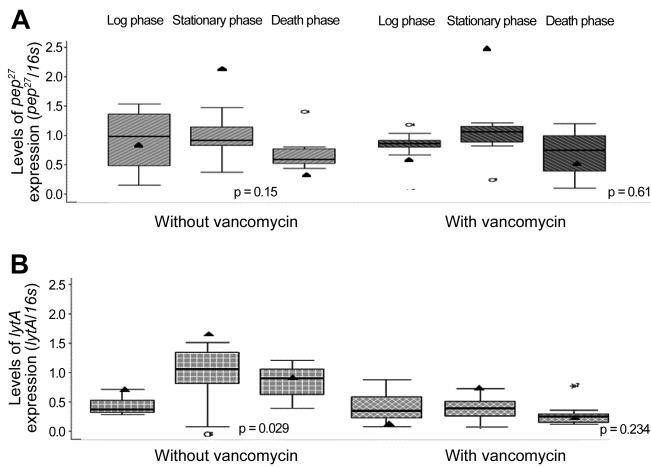


Fig. 3. Densitometry analysis by RT-PCR of *pep*²⁷ and *lytA* expressions in VTSP strains (**A** and **B**, respectively).

Left: gene expression at the middle of each growth phase. Right: gene expression at the middle of each growth phase in which vancomycin was added after 20 min. The ▲ represents the behavior of non-tolerant R6 control strain. The figure shows the maximum and minimum outcome analysis that was performed with the SPSS program using the Friedman test.

Fig. 3A shows *pep*²⁷ expression levels in VTSP strains. We did not observe statistically significant changes in any of the three phases of growth without vancomycin ($p=0.15$) and with vancomycin ($p=0.61$) according to the non-parametric Friedman test. Moreover, when comparing the gene expression levels with and without vancomycin in each phase (logarithmic, stationary, and death), we observed the same trend in expression using the Wilcoxon test ($p=0.379$, 0.859 , and 0.657 , respectively) (Fig. 4A). On the other hand, the non-tolerant reference strain R6 showed a significant increase in the expression of *pep*²⁷ in the stationary phase (from 0.761 to 2.0), which increased further when vancomycin was added (from 0.453 to 2.4).

The *lytA* expression levels in VTSP strains in the three phases of growth without vancomycin showed statistically significant differences ($p=0.029$, Friedman test) (Fig. 3B). However, when vancomycin was added, there was no change in the trend of expression ($p=0.234$). When we compared the *lytA* expression levels in each phase of growth with and without vancomycin, we observed a statistically significant decrease in the stationary phase ($p=0.004$) and death phase ($p=0.003$, Wilcoxon test) (Fig. 4B). Furthermore, in the non-tolerant reference strain R6, *lytA* expression was decreased in the presence of vancomycin.

DISCUSSION

The tolerant phenotype in Gram-positive bacteria is a mechanism of avoiding bacterial death when the cells are under selective pressure from a β -lactam antibiotic, and

has also been described for glycopeptides. In *S. aureus*, it has been suggested that tolerance is due to a faulty system of regulation of autolysins or changes in the composition of the cell wall [1]. However, the mechanism remains unclear. Nevertheless, the tolerant phenotype has acquired significance as the first step in therapeutic failure and development of antimicrobial resistance [1, 4].

The penicillin-tolerant phenotype was first recognized in 1985 in eight clinical isolates of pneumococci [10]. Five years later, the phenomenon of vancomycin tolerance was described [5, 6]. Subsequently, the tolerant phenotype was linked to relapse in a child with meningitis [6]. The present paper is the first report from Mexico regarding tolerance to vancomycin in clinical isolates of pneumococci that were all from children; two of these patients were associated with meningitis, and one of them died.

The LytA protein and Pep27 peptide both play an important role in the control of bacterial death. Novak *et al.* [15] have shown that the accumulation of Pep27 during late logarithmic and stationary phases reaches a critical point that is sensed by the two-component system VNCR/S, triggering different pathways leading to cell death [15]. Our findings showed no changes in the *pep*²⁷ expression level in VTSP strains in all growth phases with and without vancomycin. This phenomenon may be related to the ability of VTSP to tolerate a high antibiotic concentration.

Vancomycin tolerance in *S. pneumoniae* was previously reported and described as a decrease in lysis in strain Lyt 4-4, and one strain reported by Sung *et al.* [23], as the result of a dysfunction in the final production of the amidase. In our VTSP strain 261D, the Doc_T⁻ phenotype was observed with a 19% decrease in OD. From data not shown, we observed changes in the sequence of *vex2*. Perhaps the LytA of the VTSP strain 261D has an important change in its primary structure that can explain the tolerant phenotype observed [16].

The decrease in expression of *lytA* in the VTSP and R6 strains after the addition of vancomycin was 2.8 times greater in the stationary phase and 2 times greater in the death phase. However, LytA activity was intact for most tolerant strains; similar results were reported by Novak *et al.* [14]. These results suggest that the VTSP strains in the presence of the antibiotic have no cell division, and absence from cell death was due to the decreased expression principally of *pep*²⁷. For its part, the overexpression of *pep*²⁷ in strain R6 and the decrease in *lytA* expression in the presence of the antibiotic can explain how Pep²⁷ leads the regulation of cell death by other pathways not dependent on LytA [15].

To our knowledge, this is the first study of VTSP strains to be performed in a Mexican hospital with clinical isolates from a pediatric population. Similar studies have been conducted in other parts of the world, with VTSP

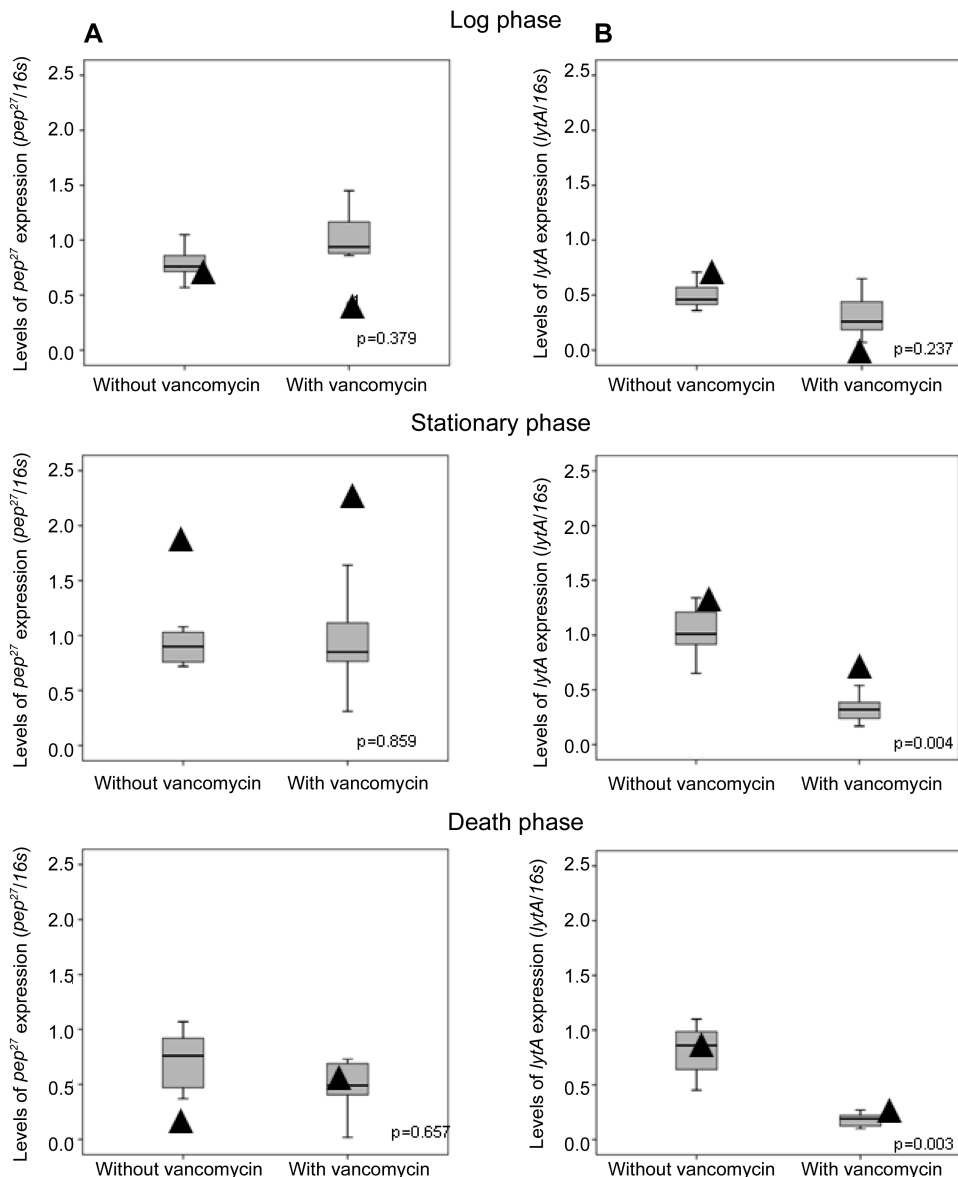


Fig. 4. Densitometric analysis by RT-PCR for the *pep²⁷* genes expression (A) and *lytA* (B) of VTSP strains without vancomycin, and 10 times the MIC value of vancomycin for 20 min.

The ▲ represents the behavior of the non-tolerant R6 strain. The figure shows the maximum and minimum outcome analysis that was performed with the SPSS program using the Wilcoxon test.

frequencies from 0 to 10.6% over diverse populations and etiologies, but we agree with other authors that it is vital to continue monitoring such isolates to prevent their possible emergence and dissemination [16, 20, 23].

Some studies have proposed that certain serotypes of pneumococci can be related to vancomycin-tolerant strains [2]. If these strains belong to the same serotype, they are phylogenetically more related, which might imply that tolerance is a result of natural polymorphism within the background of the species rather than a result of antibiotic treatment [5]. Among the VTSP strains in this study, we

observed a clonal relationship in only two strains, which were serotype 6B. However, other studies suggest that tolerance to vancomycin is independent of the serotype shown by the strains. A previous Mexican study showed that the frequent serotypes were 3, 6B, and 23F [24]. We found in our Mexican VTSP isolates that serotypes 6B and 23F were the most frequent.

The emergence of VTSP strains is a phenomenon that apparently is increasing, and the mechanism by which tolerance is induced in such strains has not been completely elucidated. Thus, it is necessary to complete further studies

on this type of bacteria and on the involvement of its genes to provide therapies that eliminate these strains or prevent their emergence. *pep*²⁷ is indeed a gene involved in several processes in these bacteria, and our results are related to the tolerance phenomenon.

Acknowledgments

The authors thank Gabriela Echaniz for the donation of the *Streptococcus pneumoniae* strain R6. This work was supported by federal resources (HIM/2006/021) from SSA, México and SALUD-2010-01-139945.

REFERENCES

- Bourgeois, I., M. Pestel-Caron, J. F. Lemeland, J. L. Pons, and F. Caron. 2007. Tolerance to the glycopeptides vancomycin and teicoplanin in coagulase-negative staphylococci. *Antimicrob. Agents Chemother.* **51**: 740–743.
- Fernebro, J., I. Andersson, J. Sublett, E. Morfeldt, R. Novak, E. Tuomanen, *et al.* 2004. Capsular expression in *Streptococcus pneumoniae* negatively affects spontaneous and antibiotic-induced lysis and contributes to antibiotic tolerance. *J. Infect. Dis.* **189**: 328–338.
- Gundián, G. P., P. J. Barreto, M. Á. Rodríguez, R. A. Machado, E. Mora, and M. Lescay. 1998. Glicopeptidos. *Acta Méd.* **8**: 54–57.
- Haas, W., J. Sublett, D. Kaushal, and E. I. Tuomanen. 2004. Revising the role of the pneumococcal *vex-vncRS* locus in vancomycin tolerance. *J. Bacteriol.* **186**: 8463–8471.
- Henriques Normark, B., R. Novak, A. Ortvqvist, G. Lallenius, E. Tuomanen, and S. Normark. 2001. Clinical isolates of *Streptococcus pneumoniae* that exhibit tolerance of vancomycin. *Clin. Infect. Dis.* **32**: 552–558.
- Henriques Normark, B. and S. Normark. 2002. Antibiotic tolerance in pneumococci. *Clin. Microbiol. Infect.* **8**: 613–622.
- Jayaraman, R. 2008. Bacterial persistence: Some new insights into an old phenomenon. *J. Biosci.* **33**: 795–805.
- Lanie, J. A., W. L. Ng, K. M. Kazmierczak, T. M. Andrzejewski, T. M. Davidsen, K. J. Wayne, *et al.* 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. *J. Bacteriol.* **189**: 38–51.
- Li-Korotky, H. S., L. A. Kelly, O. Piltcher, P. A. Hebda, and W. J. Doyle. 2007. Evaluation of microbial RNA extractions from *Streptococcus pneumoniae*. *J. Microbiol. Methods* **68**: 342–348.
- Liu, H. H. and A. Tomasz. 1985. Penicillin tolerance in multiply drug-resistant natural isolates of *Streptococcus pneumoniae*. *J. Infect. Dis.* **152**: 365–372.
- McCullers, J. A., B. K. English, and R. Novak. 2000. Isolation and characterization of vancomycin-tolerant *Streptococcus pneumoniae* from the cerebrospinal fluid of a patient who developed recrudescence meningitis. *J. Infect. Dis.* **181**: 369–373.
- McEllistrem, M. C., J. Stout, and L. H. Harrison. 2000. Simplified protocol for pulsed-field gel electrophoresis analysis of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* **38**: 351–353.
- Mitchell, L. and E. Tuomanen. 2001. Vancomycin-tolerant *Streptococcus pneumoniae* and its clinical significance. *Pediatr. Infect. Dis. J.* **20**: 531–533.
- Novak, R., B. Henriques, E. Charpentier, S. Normark, and E. Tuomanen. 1999. Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. *Nature* **399**: 590–593.
- Novak, R., E. Charpentier, J. S. Braun, and E. Tuomanen. 2000. Signal transduction by a death signal peptide: Uncovering the mechanism of bacterial killing by penicillin. *Mol. Cell* **5**: 49–57.
- Ortega, M., F. Marco, A. Soriano, E. García, J. A. Martínez, and J. Mensa. 2003. Lack of vancomycin tolerance in *Streptococcus pneumoniae* strains isolated in Barcelona, Spain, from 1999 to 2001. *Antimicrob. Agents Chemother.* **47**: 1976–1978.
- Obregón, V., P. García, E. García, A. Fenoll, R. López, and J. L. García. 2002. Molecular peculiarities of the *lytA* gene isolated from clinical pneumococcal strains that are bile insoluble. *J. Clin. Microbiol.* **40**: 2545–2554.
- World Health Organization. 2003. Pneumococcal vaccines. *Wkly. Epidemiol. Rec.* **78**: 110–119.
- National Committee for Clinical Laboratory Standards. 2005. *Performance Standards for Susceptibility Testing; Fifteenth Informational Supplement M100-S15*. NCCLS, Wayne, PA.
- Rodríguez, C. A., R. Atkinson, W. Bitar, C. G. Whitney, K. M. Edwards, L. Mitchell, *et al.* 2004. Tolerance to vancomycin in pneumococci: Detection with a molecular marker and assessment of clinical impact. *J. Infect. Dis.* **190**: 1481–1487.
- Rohlf, F. J. 1998. Numerical taxonomy and multivariate analysis system. Exeter Software Inc., New York, N.Y.
- Sneath, P. H. A. and R. R. Sokal. 1973. Taxonomic structure, pp. 188–305. *In: Numerical Taxonomy*. W. H. Freeman & Co., San Francisco, CA.
- Sung, H., H. B. Shin, M. N. Kim, K. Lee, E. C. Kim, W. Song, *et al.* 2006. Vancomycin-tolerant *Streptococcus pneumoniae* in Korea. *J. Clin. Microbiol.* **44**: 3524–3528.
- Villaseñor-Sierra, A., M. Lomas-Bautista, S. Aguilar-Benavides, and G. Martínez-Aguilar. 2008. Serotypes and susceptibility of *Streptococcus pneumoniae* strains isolated from children in Mexico. *Salud Pública Méx.* **50**: 330–333.