Original Article

Polymerase chain reaction used to detect *Streptococcus pneumoniae* resistance to penicillin*

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Background: *Streptococcus pneumoniae* is the most common etiologic agent of community-acquired respiratory infections. In recent years, *S. pneumoniae* resistance to antimicrobial agents has increased. Minimum inhibitory concentration (MIC) is routinely used to determine resistance. Polymerase chain reaction (PCR) detects the genes responsible for *Streptococcus pneumoniae* resistance to penicillin within approximately 8 hours.

Objective: To compare the PCR and MIC methods in determining *Streptococcus pneumoniae* resistance to penicillin.

Method: A total of 153 Streptococcus pneumoniae samples, isolated from various anatomical sites, were evaluated in order to detect mutations in the genes encoding pbp1a, pbp2a and pbp2x, which are responsible for *Streptococcus pneumoniae* penicillin resistance. A correlation was found between mutations and penicillin MIP, as determined by the agar diffusion method.

Results: Overal *Streptococcus pneumoniae* resistance to penicillin was 22.8% (16.3% intermediate resistance and 6.5% high resistance). In a statistically significant finding, we observed no mutations in the penicillin-sensitive samples and only one mutation, typically in the gene encoding pbp2x, among the samples with intermediate resistance, whereas mutations in all three genes studied were observed in the high-resistance samples.

Conclusion: For determining *Streptococcus pneumoniae* resistance to penicillin, PCR is a rapid method of detection that could well be used in clinical practice.

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Key words: Streptococcus pneumoniae. Penicillin resistance. Polymerase chain reaction/methods.

*Study carried out in in the Molecular Biology Laboratory of the *Instituto de Pesquisas Biomédicas da Pontifícia Universidade Católica do Rio Grande do Sul*, PUCRS

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INTRODUCTION

Worldwide, *Streptococcus pneumoniae* is the most common etiologic agent of community-acquired respiratory infections and accounts for 3 to 5 million deaths annually[1]. In the United States alone, *S. pneumoniae* is annually responsible for 500,000 cases of pneumonia, 50,000 cases of bacteremia, 3000 cases of meningitis and approximately 7 million cases of acute otitis media[2,3].

Pneumococcal resistance to penicillin has been increasing significantly in recent years, especially in European countries such as Spain, France and Hungary, where it has reached up to 71%[4,5]. In some states of the USA, resistance to penicillin has reached 44%[6,7], whereas in Asia we can find alarming rates ranging from 70% to 78% in Hong Kong, South Korea and Taiwan[8-10].

In Brazil, some studies have shown levels of resistance near 20%[11-13]. However, a recent study detected 42.1% resistance to penicillin (26.8% intermediate resistance and 15.3% high resistance) among strains isolated in three Latin-American countries (Argentina, Brazil and Mexico)[14].

S. pneumoniae resistance to beta-lactam antibiotics is due exclusively to mutations in their natural target, the penicillin-binding proteins (PBPs), which prevent binding and make them indifferent to beta-lactam, that is, decrease their binding affinity with these drugs. In highly resistant strains there is a reduction in the capacity to bind to the molecules of the antibiotics in at least three of the five existing PBPs: pbp1a, pbp2x and pbp2b[15-17].

It is common practice to assess resistance by determining the minimum inhibitory concentration (MIC) of the antibiotic being tested. These tests are carried out in laboratories using methods such as the agar dilution, which is recommended by the *National Committee for Clinical Laboratory Standards*. The main drawback in the use of these methods, however, is the long time it takes to obtain results, usually over 48 hours[18], whereas polymerase chain reaction (PCR) detects mutations responsible for pneumococcus resistance to penicillin in a much shorter time, within approximately 8 hours[19].

With the current increase in pneumococcal resistance, it is imperative to develop a fast and reliable diagnosis technique capable of providing early and appropriate therapy in cases of infection caused by resistant strains. Therefore, the main objective of this study was to compare the PCR method to agar dilution MIC testing in the determination of *S. pneumoniae* resistance to penicillin.

METHODS

This transversal study of prevalence was carried out in the Molecular Biology Laboratory of the *Instituto de Pesquisas Biomédicas* (Biomedical Research Institute) of the *Pontifícia Universidade Católica of Rio Grande do Sul* (Pontifical Catholic University of Rio Grande do Sul) from January 1997 to September 2000. We studied 197 clinical samples of *S. pneumoniae,* isolated from any anatomical site and identified by conventional laboratory techniques. Evaluations were performed in the microbiology laboratories of seven collaborating hospitals, representative of the local communities and chosen out of convenience, in the city of Porto Alegre (in the state of Rio Grande do Sul): *Hospital de Clínicas de Porto Alegre*, *Irmandade Santa Casa de Misericórdia de Porto Alegre*, *Hospital da Criança Santo Antônio*, *Hospital Mãe Deus*, *Hospital Moinhos de Vento*, *Hospital Nossa Senhora da Conceição* and *Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul*[18]. Samples collected simultaneously or on different occasions from one single patient were analyzed. Samples not identified as pneumococcus through the standard methods of the National Committee for Clinical Laboratory Standards[18], as well as samples in which the PCR, conducted according to the technique described herein, did not detect the *lytA* gene, were excluded from the study.

The penicillin MIC was measured through the agar dilution method on Mueller-Hinton agar (Oxoid Unipath Ltd.; Hampshire, England) containing 5% defibrinated sheep blood. The cut-off points were those published by the National Committee for Clinical Laboratory Standards[18]: sensitive (MIC £ 0.06 mg/mL); intermediate resistance (MIC 0.12 to 1.0 mg/mL); and high resistance (MIC³ 2.0 mg/mL). The *S. pneumoniae* strain ATCC 49619 was used for quality control of the susceptibility test[18].

S. pneumoniae strains isolated in culture media were simultaneously submitted to PCR for detection of the *lytA* gene as described by Ubukata et al.[19]

in order to confirm bacterial identification and mutations in pbp1a, pbp2b and pbp2x, in accordance with the protocol introduced by Jalal et al.[20] and subsequently described herein.

Isolated *S. pneumoniae* colonies were removed from the culture medium through scraping with a platinum spatula and placed into a tube with 50 ml of distilled water for extraction of bacterial DNA. Subsequently, 50 ml of proteinase K were added and the material was incubated at 50°C for 16 hours, followed by inactivation of proteinase K at 95°C for 10 minutes.

Oligonucleotide initiators (primers) were used in the selection of initiators derived from the *lytA* gene of *S. pneumoniae* and from the genes encoding pbp2b and pbp2x of penicillin-sensitive *S. pneumoniae* and pbp1a of penicillin-resistant *S. pneumoniae*. These presented the following sequences:

Code	Gene	Sequence (5'-3')
B1	pbp2b	ACT CAG GCT TAC GGT CAT T
B2	pbp2b	ACG AGG AGC CAC ACG AAC AC
X1	pbp2x	GTC ATG CTG GAG CCT AAA TT
X2	pbp2x	AAC CCG ACT AGA TAA CCA CC
A1	pbp1a	AGG TCG GTC CTA GAT AGA GCT
A2	pbp1a	GAG CTA CAT AGC CAG TGT CTC
SPN1	*lytA*	TGA AGC GGA TTA TCA CTG GC
SPN2	*lytA*	GCT AAA CTC CCT GTA TCA AGC G

Two reaction mixtures were prepared, one containing the primers for amplification of the pbp2b and pbp2x genes and another containing the primers for the pbp1a and *lytA* genes, described as follows: 5 ml of the buffer specific for Taq DNA polymerase, 2 mM of $MgCl_2$, 1 U of Taq DNA polymerase 5 U/ml (Gibco, Gaithersburg, MD, USA), 200 mM of an equimolar mixture of nucleotide triphosphates and 50 pmol of each of the two pairs of primers.

For the preparation of the sample to be amplified and of the negative and positive controls, 2 ml of the DNA harvested from *S. pneumoniae* were mixed with 48 ml of the master mixture. As a negative control of the reaction, 2 ml of bidistilled water were added to 48 ml of the reaction mixture. As a positive control, in mixture 1, we used 2 ml of DNA extracted from the *S. pneumoniae* sample previously identified as penicillin sensitive, and, in mixture 2, 2 ml of DNA extracted from a sample identified as penicillin resistant. Each was added to 48 ml of the respective reaction mixtures.

Regarding the amplification cycles, the samples prepared with the respective positive and negative controls were placed in the thermocycler and submitted to 35 cycles, after an initial denaturation at 94°C for 3 minutes. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 2 minutes. After the 35 amplification cycles, the tubes were submitted to a final extension at 72°C for 7 minutes.

Electrophoresis was performed by separating 20 ml of the resulting solution, which were applied to a 2% agarose gel (Sigma, St. Louis, MO, USA) containing 2 mg/ml of ethidium bromide (Sigma). Electrophoresis was performed at 15 volts/cm for approximately 30 minutes. The gel was viewed under ultraviolet light and the image was captured with a Gel Doc1000, using the Molecular Analyst software (Bio-Rad, Hercules, CA, USA). The positive samples produced a visible band in the following sizes: pbp2b, 359 primer pairs; pbp2x, 277 primer pairs; pbp1a, 423 primer pairs; *lytA* gene, 273 base pairs (Figure 1).

The statistical analysis was carried out at first by relating the number of mutations, as determined by PCR, in each sample to their degree of resistance, as determined by their MIC. In each group of samples with differing numbers of mutations (no mutation, one mutation, two mutations or three mutations), a paired comparison was made between degrees of resistance (sensitive vs. intermediate, sensitive vs. resistant and intermediate vs. resistant), using a single-degree-of-freedom chi-square test to compare proportions (heterogeneity), with Yates' correction, considering a level of 95% ($p < 0.05$) as statistically significant. Subsequently, we identified relationships between the mutations in each of the three PBPs studied and the MIC-determined degree of resistance of the strains. In each group of samples with mutations in a determined PBP (pbp1a, pbp2b or pbp2x), a paired comparison was carried out between their degree of resistance (sensitive vs. intermediate, sensitive vs. resistant and intermediate vs. resistant), also using a single-degree-of-freedom chi-square test to compare proportions (heterogeneity), with Yates' correction, also considering a level of 95% ($p < 0.05$) as statistically significant.

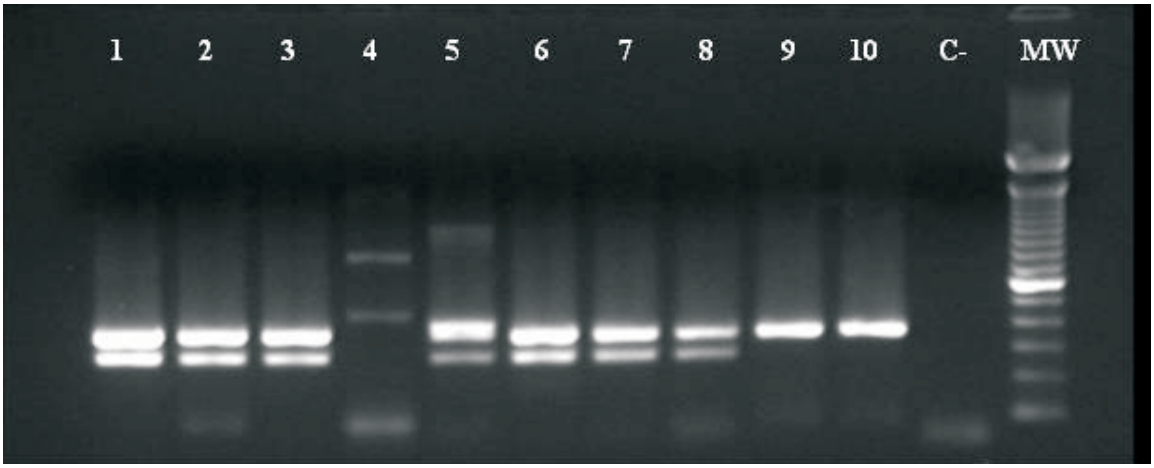

Figura 1 – Eletroforese em gel de agarose dos produtos da PCR amplificados usando *primers* derivados dos genes das PLP 2b e PLP 2x de *S. pneumoniae* sensíveis à penicilina. Os tamanhos destes produtos da PCR são de 359 e 277 pares de bases respectivamente. Colunas 1, 2, 3, 5, 6, 7 e 8: amostras sensíveis; colunas 9 e 10: amostras com resistência intermediária; coluna 4: amostra com alta resistência; coluna C-: controle negativo; coluna MW: marcador de peso molecular.

The study was approved by the Ethics in Research Committee of the Pontifical Catholic University of Rio Grande do Sul.

RESULTS

Of the 197 samples initially identified as *S. pneumoniae* in the microbiology laboratories of their hospitals of origin, 34 (17.2%) were excluded from the study for not presenting any growth in culture medium after defrosting (bacterial death) or because the identity of the bacterial species was not confirmed in the microbiological testing.

The remaining 163 samples were simultaneously submitted to MIC determination tests and PCR to detect mutations in the pbp1a, pbp2b and pbp2x and amplification of the *lytA* gene for confirmation of the bacterial identification. The *lytA* gene was detected in 153 samples, and the 10 samples that did not present this gene were eliminated from the final analysis.

Distribution of *S. pneumoniae* resistance in the 153 remaining samples in the study, as determined by the agar dilution test in accordance with their MIC, is shown in Table 1.

Initially, the number of mutations in each sample was related to the degree of resistance, as determined by the MIC, in order to obtain the proportion of sensitive, intermediately resistant and highly resistant strains with a determined number of mutations, and we found the differences to be statistically significant (Table 2).

There were no resistant strains in the group of samples without mutations. The difference between sensitive and intermediately resistant strains was significant ($\chi^2 = 33.89$; $p < 0.0005$).

We found no highly resistant strains in the group of samples in which there were mutations. The difference between sensitive and intermediately resistant strains was significant, with a predominance of sensitive strains ($\chi^2 = 16.277$; $p < 0.0005$).

In the samples with two and three mutations, we found no sensitive strains and a significant difference between samples presenting intermediate resistance and those presenting high resistance. There was a predominance of intermediately resistant strains in the group presenting two mutations ($\chi^2 = 0.006$; $p = 0.942$) and a predominance of highly resistant strains in the group presenting three mutations ($\chi^2 = 21.843$; $p < 0.0005$).

Subsequently, the presence of mutations in each of the three isolated PBPs was correlated to the indices of resistance determined by the MIC, and the statistical test was applied to analyze the significance of the differences found (Table 3).

The pbp2b mutation resulted in a lack of sensitive strains. There was a predominance of high resistance over intermediate resistance ($\chi^2 = 18.308$; $p < 0.0005$).

In samples with pbp2x mutations, the difference between sensitive and intermediately resistant strains was significant ($\chi^2 = 25.304$; $p < 0.0005$); the difference between sensitive and resistant strains was also significant ($\chi^2 = 18.388$ ($p < 0.0005$); and the difference between intermediately resistant and resistant strains was less than significant ($\chi^2 = 0.572$; $p = 0.4539$).

There were no sensitive strains found in relation to mutations in pbp1a, although there was a predominance of high resistance (intermediate vs. resistant: $\chi^2 = 21.843$; $p < 0.0005$) (Table 3).

DISCUSSION

In recent years, *S. pneumoniae* resistance to penicillin has increased considerably in Porto Alegre. In a study conducted by Chatkin et al.[21] in 1989, the rate of resistance in the city was reported to be only 3.2%, although it reached 22.8% during our study period. This index is similar to those found by Sessegolo et al.[11] and Levin et al.[12] in the state of São Paulo in the 1990s (24%). More recently, Mendes et al.[14] studied pneumococcal strains isolated in three countries in Latin America (Argentina, Brazil and Mexico) and found indices of resistance that were even more elevated (42.1%).

It is important to reassert that, among the resistant strains found, the majority (16.3% of the total number of strains tested) showed intermediate resistance to penicillin, compared with 6.5% presenting high resistance. As previously described by several authors, the samples with intermediate resistance presented good clinical response to penicillin in high doses[22,23], whereas highly resistant strains may cause greater morbidity and mortality[24,25].

In comparison to studies conducted in other countries, pneumoccocal resistance in Porto Alegre is still relatively low. For example, in the most recent epidemiological study carried out in the USA, Karlowsky et al.[6] evaluated 27,828 strains of *S. pneumoniae* isolated in various states and demonstrated an 18.4% rate of high resistance to penicillin.

In the present study, we found a significant association between the presence of mutations in the genes encoding *S. pneumoniae* PBPs and the indices of *in vitro* resistance to penicillin determined by MIC. In a statistically significant proportion of cases ($p < 0.05$), the sensitive strains were characterized by the absence of mutations in the PBPs. In each of the intermediately resistant strains, we detected only one mutation, usually in pbp2x. The highly resistant strains presented mutations in all three PBPs. An isolated mutation in pbp1a or pbp2b occurred in a significantly higher number of strains presenting high resistance than in those presenting intermediate resistance, whereas it was not possible to identify the pbp2x mutation in strains presenting intermediate or high resistance. We found a significant correlation between the combination of mutations in two or three PBPs and the expression of high resistance to penicillin. These results are in accordance with

TABELA 1
Níveis de resistência das amostras de *S. pneumoniae*

Nível de resistência	Número de amostras (%)
Sensível (CIM ≤ 0,06 mg/mL)	118 (77,2%)
Intermediária (CIM 0,12 a 1,0 mg/mL)	25 (16,3%)
Alta (CIM ≥ 2,0 mg/mL)	10 (6,5%)
Total:	153

CIM: concentração inibitória mínima.

TABELA 2
Relação entre o número de mutações e o nível de resistência

	Sensível	Intermediária	Alta
Sem mutações	86 (73%)*	2 (8%)	0 (0%)
1 mutação	32 (27%)	18 (72%)*	0 (0%)
2 mutações	0 (0%)	4 (16%)**	1 (10%)
3 mutações	0 (0%)	1 (4%)	9 (90%)***
Total	**118**	**25**	**10**

* comparação sensíveis x intermediárias: p < 0,05. ** comparação intermediárias x alta resistência: p = não significativo. *** comparação intermediárias x alta resistência: p < 0,05

TABELA 3
Relação entre as mutações em cada PLP e o nível de resistência

	Sensível	Intermediária	Alta
PLP 2b	0 (0%)	7 (28%)	10 (100%)*
PLP 2x	32 (27%)	21 (84%)**	10 (100%)***
PLP 1a	0 (0%)	1 (4%)	9 (90%)****
Total	118	25	10

* comparação intermediárias x alta resistência: p < 0,05
** comparação sensíveis x intermediárias: p < 0,05
*** comparação sensíveis x alta resistência: p < 0,05; comparação intermediárias x alta resistência: p = não significativo
**** comparação intermediárias x alta resistência: p < 0,05
PLP: proteína ligadora de penicilina.

those of other studies, which have demonstrated that isolated mutations in the pbp2x genome result in a low level of resistance to penicillin, whereas high resistance to penicillin requires genetic alterations in pbp1a and pbp2b as well[26-30].

Ubukata et al.[19] used PCR to identify mutations in the pbp2b genes of 1062 clinical samples of *S. pneumoniae* and found a correlation between such mutations and penicillin MICs in 98.9% of the sensitive strains and in 70.3% of the resistant strains.

Nagai et al.[30] evaluated the presence of mutations in the pbp2b and pbp2x genes of 218 samples of *S. pneumoniae* isolated from children in Japan. Mutations in pbp2x were observed in several strains presenting intermediate resistance to penicillin. Mutations in the pbp2x gene were found in 41.3% of strains sensitive to cefotaxime, which suggests that, even in strains susceptible to antimicrobials, the mechanism of resistance may be activated and may precede the resistance detected *in vitro*. This finding was reproduced in our study, and the pbp2x mutation was found in 84% of samples presenting intermediate resistance to penicillin, indicating that this may be a marker of lower resistance. Therefore, using isolated alterations in pbp2x, it was not possible to discriminate between strains presenting intermediate resistance to penicillin and those presenting high resistance to penicillin. However, it was possible to find significant differences between the sensitive strains and those presenting some degree of resistance (intermediate or high).

Du Pleiss et al.[29] used PCR to detect pbp1a in 183 clinically-isolated strains of *S. pneumoniae* and reported a 98.3% concordance between the PCR results and the MICs of penicillin data. The positive and negative predictive values of this molecular technique were 100% in the detection of samples with MIC[3] 1 mg/mL. Similarly, in our samples presenting high resistance to penicillin, we found the pbp1a mutation in 90% (9/10). We were unable to detect the pbp1a alteration in only one sample, which was classified as highly resistant to penicillin according to the guidelines of the National Committee for Clinical Laboratory Standards. This sample presented a MIC of 2.0 mg/mL, a value found precisely at the cut-off point that defines the strains as intermediately resistant or highly resistant to penicillin. However, this difference between the phenotypic and genotypic methods, in this isolated case, did not alter the effectiveness of the method.

The only previous study in which mutations in the three PBPs (pbp1a, pbp2b and pbp2x) were analyzed simultaneously, relating them to bacterial resistance, was conducted by Jalal et al.[20] The authors studied 230 clinically-isolated strains of *S. pneumoniae* and, using PCR, identified mutations in 93% of sensitive strains, in 85% of intermediately resistant strains, and in 100% of highly resistant strains. However, the mutations in pbp1a did not correlate well with *in vitro* resistance and were excluded from the data analysis, which was limited to the evaluation of pbp2b and pbp2x. Nevertheless, despite the lack of a deeper statistical analysis, the results achieved were considered potentially useful in clinical practice.

A very important factor in the treatment of patients with pneumococcal infection is the early introduction of the antimicrobial therapeutics, which may be decisive in the evolution and prognosis of the disease[31,32]. Through conventional microbiologic methods, the growth and identification of the microbe in culture requires a minimum of 24 hours and there is another 24-hour wait for the results of susceptibility tests[18]. All steps of the PCR technique described in the present study can be carried out within 8 hours. In addition, using PCR, the etiologic agent can be identified and its level of resistance determined simultaneously. These advantages make it possible to design a more appropriate treatment regimen and to initiate that treatment more rapidly.

Our results demonstrate that the PCR technique is rapid and is easily performed in adequately equipped laboratories. Therefore, PCR has potential clinical applications in the early detection of *S. pneumoniae* bacterial resistance.

REFERENCES

1. Tomasz A. Antibiotic resistance in *Streptococcus pneumoniae*. Clin Infect Dis. 1997;24(Suppl 1):S85-8.
2. Marston BJ, Plouffe JF, File TM, Hackman, BA, Salstrom SJ, Lipman HB, et al. Incidence of community-acquired pneumonia requiring hospitalization: results of a population-based active surveillance study in Ohio. JAMA. 1997;157:1709-18.
3. Ruiz-Gonzales A, Falguera M, Nogues A, Rubio-Caballero M. Is *Streptococcus pneumoniae* the leading cause of pneumonia of unknown etiology? A microbiologic study of lung aspirates in consecutive patients with community-acquired pneumonia. Am J Med. 1999;106:385-90.

4. Jones ME, Blosser-Middleton RS, Critchley IA, Karlowsky JA, Thornsberry C, Sahm DF. In vitro susceptibility of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*: a European multicenter study during 2000-2001. Clin Microb Infect. 2003;9:590-9.
5. Dobay O, Rozgonyi F, Hajdu E, Nagy E, Knausz M, Amyes SG. Antibiotic susceptibility and serotypes of *Streptococcus pneumoniae* isolates from Hungary. J Antimicrob Chemother. 2003;51:887-93.
6. Karlowsky, JA, Thornsberry C, Jones ME, Evangelista AT, Critchley IA, Sahm DF. Factors associated with relative rates of antimicrobial resistance among *Streptococcus pneumoniae* in the United States: results from the TRUST Surveillance Program (1998-2002). Clin Infect Dis. 2003;36:963-70.
7. Hoban D, Waites K, Felmingham D. Antimicrobial susceptibility of community-acquired respiratory tract pathogens in North America in 1999-2000: findings of the PROTEKT surveillance study. Diagn Microbiol Infect Dis. 2003;45:251-9.
8. Lee HJ, Park JY, Jang SH. High incidence of resistance to multiple antimicrobials in clinical isolates of *Streptococcus pneumoniae* from a university hospital in Korea. Clin Infect Dis. 1995;20:826-35.
9. Kim SN, Kim SW, Choi IH. High incidence of multidrug-resistant *Streptococcus pneumoniae* in South Korea. Microb Drug Resist. 1996;2:401-6.
10. Lo WT, Wang CC, Yu CM, Chu ML. Rate of nasopharyngeal carriage, antimicrobial resistance and serotype of *Streptococcus pneumoniae* among children in northern Taiwan. J Microbiol Immunol Infect. 2003;36,175-81.
11. Sessegolo JF, Levin AS, Levy CE, Asensi M, Facklam RR, Teixeira LM. Distribution of serotypes and antimicrobial resistance of *Streptococcus pneumoniae* strains isolated in Brazil from 1988 to 1992. J Clin Microbiol. 1994;12:906-11.
12. Levin AS, Teixeira LM, Sessegolo JF, Barone AA. Resistance of *Streptococcus pneumoniae* to antimicrobials in São Paulo, Brazil: clinical features and serotypes. Rev Inst Med Trop São Paulo. 1996;38:187-92.
13. Sader HS, Jones RN, Gales AC, Winokur P, Kugler KC, Pfaller MA, et al. Antimicrobial susceptibility patterns for pathogens isolated from patients in Latin American medical centers with a diagnosis of pneumonia: results from the SENTRY Antimicrobial Surveillance Program (1997). Diagn Microbiol Infect Dis. 1998;32:289-301.
14. Mendes C, Marin ME, Quinones F, Sifuentes-Osornio J, Siller CC, Castanheira M, et al. Antibacterial resistance of community-acquired respiratory tract pathogens recovered from patients in Latin America: results from the PROTEKT surveillance study (1999-2000). Braz J Infect Dis. 2003;7:44-61.
15. Appelbaum PC. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clin Infect Dis. 1992;15:77-83.
16. Dowson CG, Coffey TJ, Spratt BG. Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to beta-lactam antibiotics. Trends Microbiol. 1994;2:361-5.
17. Ikeda F, Yokota Y, Ikemoto A, Teratani N, Shimomura K, Kanno H. Interaction of beta-lactam antibiotics with the penicillin-binding proteins of penicillin-resistant *Streptococcus pneumoniae*. Chemotherapy. 1995;41:159-64.
18. Ruoff KL, Whiley RA, Beighton D. Streptococcus. In: Murray PR, Baron EJ, Pfaller MA, editors. Manual of clinical microbiology. Washington (DC): ASM Press; 1999. p.283-96.
19. Ubukata K, Asahi Y, Yamane A, Konno M. Combinational detection of autolysin and penicillin-binding protein 2B genes of *Streptococcus pneumoniae* by PCR. J Clin Microbiol. 1996;34:592-6.
20. Jalal H, Organji S, Reynolds J, Bennett D, O'Mason E Jr, Millar MR. Determination of penicillin susceptibility of *Streptococcus pneumoniae* using the polymerase chain reaction. Mol Pathol. 1997;50:45-50.
21. Chatkin JM, Fritscher CC, Rodrigues LF. Sensibilidade do *Streptococcus pneumoniae* aos antimicrobianos: resultados preliminares. Rev Med PUCRS. 1989;1:81-6.
22. Ewig S, Ruiz M, Torres A, Marco F, Martinez JA, Sanchez, M, et al. Pneumonia acquired in the community through drug-resistant *Streptococcus pneumoniae*. Am J Respir Crit Care Med. 1999;159,1835-42.
23. File TM Jr. Treating community-acquired pneumonia caused by penicillin-resistant *Streptococcus pneumoniae*. J Respir Dis. 1999;20:833-42.
24. Dowell, SF, Butler JC, Giebink GS, Jacobs MR, Jernigan D, Musher DM, et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance – a report from the Drug-Resistant *S. pneumoniae* Therapeutic Working Group. Pediatr Infect Dis J. 1999;8:1-9.
25. Pachecho TR, Cooper CK, Hardy DJ, Betts RF, Bonnez W. Failure of cefotaxime treatment in an adult with *Streptococcus pneumoniae* meningitis. Am J Med. 1997;102:303-5.
26. Smith AM, Klugman KP, Coffey TJ. Genetic diversity of penicillin-binding proteins 2B e 2X genes from *Streptococcus pneumoniae* in South Africa. Antimicrob Agents Chemother. 1993;37:1938-44.
27. Barcus VA, Ghanekar K, Yeo M, Coffey TJ, Dowson CG. Genetics of high-level penicillin resistance in clinical isolates of *Streptococcus pneumoniae*. FEMS Microbiol Lett. 1995;126:299-304.
28. Smith AM, Klugman KP. Alterations in PBP 1a essential for high-level penicillin resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 1998;42:1329-33.
29. du Pleiss M, Smith AM, Klugman KP. Application of pbp1A PCR in identification of penicillin-resistant *Streptococcus pneumoniae*. J Clin Microbiol. 1999;37:628-32.
30. Nagai K, Matsuo Y, Tsumura N, Sakata Y, Kato H. Antimicrobial susceptibilities and serotypes of *Streptococcus pneumoniae* in southwestern Japan and correlation of penicillin-binding proteins 2b and 2x mutations in susceptibilities of penicillin G and cefotaxime. Diagn Microbiol Infect Dis. 2000;37:107-13.
31. Bartlett JG, Mundy L. Community-acquired pneumonia. N Engl J Med. 1995;333:1618-24.
32. Bohte R, Hermans J, Broek PJ. Early recognition of *Streptococcus pneumoniae* in patients with community-acquired pneumonia. Eur J Clin Microbiol Infect Dis. 1996;15:201-5.