

Quintuplex PCR to Detect Antibiotic Resistance Genes in *Streptococcus pneumoniae*

Navindra Kumari Palanisamy¹, Parasakthi Navaratnam², Shamala Devi Sekaran³

1 Faculty of Medicine, Universiti Teknologi MARA, Selangor, Malaysia

2 School of Medicine and Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Selangor, Malaysia

3 Faculty of Medicine, Universiti of Malaya, Kuala Lumpur, Malaysia

Received

3rd February 2016

Received in revised form

19th October 2016

Accepted

11th November 2016

Corresponding author:

Navindra Kumari Palanisamy, PhD

Core of Health and Wellbeing,

Faculty of Medicine,

Universiti Teknologi MARA (UiTM),

Sungai Buloh Campus,

Sungai Buloh, Selangor,

Malaysia.

Tel: +603-61267431 / +6012-6311224

Email: navindra87@yahoo.com

ABSTRACT

Introduction: *Streptococcus pneumoniae* is an important bacterial pathogen, causing respiratory infection. Penicillin resistance in *S. pneumoniae* is associated with alterations in the penicillin binding proteins, while resistance to macrolides is conferred either by the modification of the ribosomal target site or efflux mechanism. This study aimed to characterize *S. pneumoniae* and its antibiotic resistance genes using 2 sets of multiplex PCRs. **Methods:** A quintuplex and triplex PCR was used to characterize the *pbp1A*, *ermB*, *gyrA*, *ply*, and the *mefE* genes. Fifty-eight penicillin sensitive strains (PSSP), 36 penicillin intermediate strains (PISP) and 26 penicillin resistance strains (PRSP) were used. **Results:** Alteration in *pbp1A* was only observed in PISP and PRSP strains, while PCR amplification of the *ermB* or *mefE* was observed only in strains with reduced susceptibility to erythromycin. The assay was found to be sensitive as simulated blood cultures showed the lowest level of detection to be 10cfu. **Conclusions:** As predicted, the assay was able to differentiate penicillin susceptible from the non-susceptible strains based on the detection of the *pbp1A* gene, which correlated with the MIC value of the strains.

INTRODUCTION

Streptococcus pneumoniae is an important bacterial pathogen and is a major causative agent of respiratory infections. It also causes otitis media, pneumonia, sinusitis, meningitis, and septicemia and is frequently associated with significant morbidity and mortality [1].

During the early 1940's, isolates of *S. pneumoniae* were highly susceptible to penicillin, which was the drug of choice for suspected pneumococcal infections [2]. However, in the 1970's, penicillin-resistant and multidrug resistant pneumococcus was first reported in South Africa [3]. Since then, the rate of antibiotic resistance among pneumococci isolates to various antimicrobial agents has increased. *S. pneumoniae* resistance to penicillin is due to the alteration of the penicillin binding proteins (*pbp1A*, *pbp2B*, *pbp2X*), which reduces its binding affinity to β -lactams. Hence, cross resistance to other β -lactam drugs such as cephalosporins is not

uncommon. β -lactam resistance in *S. pneumoniae* has brought a need for alternative antibiotics such as macrolides and fluoroquinolones. These are frequently used as empirical therapy in respiratory tract infection, which has resulted in increased prevalence of macrolide resistant strains of *S. pneumoniae*.

Macrolide resistance in *S. pneumoniae* is a result of modification of the ribosomal target site adenine at position 2058 [4], as the A2058 nucleotide has a key function in the binding of this antibiotic. This modification results in a reduction of macrolide binding affinity to its target or modification of the conformation of binding site. This mechanism of resistance is assigned by the determinant gene, *ermB*. Macrolides resistance is also caused by another mechanism, which is the efflux mechanism of the drug and assigned as *mefE*.

Recently, strains of *S. pneumoniae* have developed resistance to fluoroquinolones, which have a

greater intrinsic activity against this organism. Previous studies have shown that the fluoroquinolones target the type II topoisomerase, DNA gyrase and topoisomerase IV [5, 6]. Amino acid substitution within the Quinolone Resistance Determining Region (QRDR) of these enzymes has resulted in resistance to fluoroquinolones in *S. pneumoniae* [6, 7].

The increase in incidence of multi-drug resistance among *S. pneumoniae* necessitates a need for a tool to identify the presence of these genes within the strains in order to institute appropriate antimicrobial therapy. In this study, we have used 2 sets of multiplex PCRs (quintuplex and triplex PCR) assay to identify and characterize the antibiotic resistance genes, *ermB*, *pbp1A*, *gyrA*, *mefE* simultaneously with the *S. pneumoniae* species specific pneumolysin gene and the common eubacteria gene.

METHODS

Bacterial Strains

One hundred and twenty pneumococcal isolates were obtained from clinical samples processed at the Microbiology Laboratory of the University Malaya Medical Centre, Malaysia from March 1999 to December 2003. The isolates were obtained from both pediatric and adult patients. The source of the isolates included blood, nasopharyngeal secretion, tracheal secretion, sputum and bronchoalveolar lavage. Samples were grown on 5 % horse blood agar and incubated at 37° C in 5 % CO₂ for 12 -15 hours prior to other biochemical and molecular assays.

Strain Identification

The strains were identified as *S. pneumoniae* using conventional microbiological methods including susceptibility to ethylhydrocupreine disc (optochin), catalase test and bile solubility.

Susceptibility Testing

The antibiotic susceptibility of the strains was tested on Mueller Hinton Agar (Oxoid) plates containing 5 % sheep blood (Oxoid), incubated at 37° C with 5 % CO₂ using the agar dilution method as described [8]. The antimicrobial agents used were penicillin, cefotaxime, ceftriaxone, and erythromycin. The antibiotics were

obtained from Sigma Aldrich (Sigma Chemical Co., St. Louis, Mo). *S. pneumoniae* ATCC 49619 was used as control. Strains were also tested against ciprofloxacin (Bayer, Germany), moxifloxacin (Bayer, Germany), gatifloxacin (Bristol-Myers Squibb, New Jersey, USA) and levofloxacin (Daiichi, Japan).

DNA Extraction

Genomic DNA was extracted from the bacterial culture using a previously described method [9]. Bacterial colonies suspended in 15 µl of dH₂O containing 50µg/mL were incubated at 37° C for 10 minutes. This was followed by addition of 10µg/mL Proteinase K and 0.1mM Tris HCL pH 7.5 and incubated at 37° C for another 10 minutes. Subsequently, the suspension was boiled for 5 minutes and finally centrifuged at 13000 rpm for 2 minutes. The supernatant obtained was used as the template in the PCR reaction. The extraction of DNA from blood samples was carried out using the standard phenol-chloroform extraction protocol [10].

PCR Amplification

The primers used in this study were extracted from previously published sequences [11]. The optimal PCR condition for a 50 µl reaction included 1X PCR buffer, 1.5mM MgCl₂, 0.2 mM dNTP mix, 2U Taq Polymerase (Fermentas) and 20 pmol of each primer. The PCR cycling parameters were as follows: An initial denaturation step at 94° C for 5 minutes, 15 cycles of amplification performed as follows: denaturation at 94° C for 30s, annealing temperature at 40° C for 30s and extension temperature at 72° C for 30s. Thereafter, another 20 cycles of amplification were completed as above but with an annealing temperature of 46° C and finally completed with an extension at 72° C for 2 minutes. The amplification reaction was performed in an Eppendorf Gradient Mastercycler. The PCR product was electrophoresed on a 2 % TAE agarose gel for 1 hour at 70V and the bands were analyzed using a UV trans illuminator.

Determination of Sensitivity and Specificity of the Assay

The sensitivity of the assay was determined using pure bacterial cultures and spiked blood samples. Colonies

from overnight grown pure bacterial cultures were suspended in 0.85 % NaCl and the turbidity adjusted to an OD equivalent to 0.5 McFarland standards, which is approximately 10^8 cfu / ml. Further 10 fold dilutions of the suspension were made up to 10 cfu / ml. DNA extraction was carried out on each dilution, which was later subjected to PCR amplification. The sensitivity of the assay in the clinical setting was carried out by simulating known concentration of bacterial cultures and human blood samples into blood culture bottles. This was carried out by inoculating 5 mL of healthy volunteer blood into an aerobic blood culture media (BD Biosciences, Becton Dickinson, USA), and the bottle was spiked with 100 μ L of diluted culture suspension, which ranged from 10^8 to 10^3 cfu. The final concentration of the bacteria in the blood culture bottles ranged from 10^6 to 10 cfu, considering 25 mL of the culture media (25 mL) in the blood culture bottles. The blood culture bottle was incubated overnight at 37° C, prior to DNA extraction and PCR amplification. Viable bacterial count in the blood culture media was determined before and after the incubation period. The DNA from blood cultures was extracted using the method previously described [10]. The DNA extracted from each blood culture bottle with known concentration of bacterial culture was subjected to PCR. The specificity of the assay was also evaluated using DNA extracted from other gram positive bacteria such as *Streptococcus sanguis* ATCC 10556, *Staphylococcus aureus* ATCC 25923 as well as gram negative bacteria including *Acinetobacter baumannii* ATCC 15308 and *Pseudomonas* sp.

RESULTS

The pneumococcal strains were assigned into 3 groups based on their susceptibility levels to penicillin i.e. penicillin sensitive *S. pneumoniae* (PSSP) for strains with penicillin MIC of < 0.125 μ g/mL, penicillin intermediate *S. pneumoniae* (PISP) for strains with penicillin MIC of $\geq 0.125 - < 1.0$ μ g/mL and penicillin resistant *S. pneumoniae* (PRSP) for strains with penicillin MIC of > 1.0 μ g / mL. There were 58 PSSP strains, 36 PISP strains and 26 PRSP strains. Out of the 120 pneumococcal isolates that were tested, 64 strains were sensitive to erythromycin, 12 intermediate to and 44 resistant to erythromycin.

MIC of representative strains used in this study is shown in Table 1. All 120 isolates were characterized using PCR. In Table 1, strains I18, R55, R88 and R100 are representative of PRSP strains, while strains R71, R81, R77, R72 and I99 represent the PISP strains. The control strains used in the study were *S. pneumoniae* ATCC 700676 and *S. pneumoniae* ATCC 49619 representing the PSSP and PISP strains respectively.

Table 1 MICs of the representative strains used in the study

STRAINS	MIC (μ g/ml)*			
	PEN	CRO	CTX	ERY
ATCC 700676	0.032 (S)	0.064 (S)	< 0.032 (S)	2.0 (R)
ATCC 49619	0.5 (I)	0.064 (S)	0.125 (S)	0.125 (S)
(I18)	2.0 (R)	1.0 (I)	2.0 (R)	128.0 (R)
(R71)	1.0 (I)	1.0 (I)	0.5 (S)	0.5 (I)
(R81)	1.0 (I)	1.0 (I)	1.0 (I)	2.0 (R)
(R77)	1.0 (I)	1.0 (I)	0.5 (S)	2.0 (R)
(R55)	2.0 (R)	2.0 (R)	2.0 (R)	1.0 (I)
(R88)	2.0 (R)	1.0 (I)	< 0.032 (S)	1.0 (I)
(R72)	1.0 (I)	1.0 (I)	0.5 (S)	> 1.0 (S)
(I99)	0.125 (I)	0.25 (S)	1.0 (I)	0.016 (S)
(R100)	2.0 (R)	1.0 (I)	1.0 (I)	2.0 (R)
(Direct Blood Culture)	S	S	Not tested	S

PEN (S: ≤ 0.06 , I: 0.12 – 1.0, R: ≥ 2.0), CRO (S: ≤ 0.5 , I: 1.0, R: ≥ 2.0), CTX (S: ≤ 0.5 , I: 1.0, R: ≥ 2.0), ERY (S: ≤ 0.25 , I: 0.5, R: ≥ 2.0)

Figure 1 illustrates the quintuplex PCR amplification assay on representative strains. The pneumolysin (*ply*) gene, which encodes the *S. pneumoniae* species specific gene, was amplified in all the *S. pneumoniae* strains and the common eubacterial gene, which acts as an internal control was also detected in all the strains. The *pbp1A* gene was not amplified in strains I18, R71, R81, R77, R55 and R100 but had positive amplification in strains R88, R72 and I99. The *ermB* gene was only detected in strains I18, R71 R77, R88, and I99. The eubacterial gene was amplified in all the strains. This confirms the presence of bacterial DNA. The pneumolysin gene (*ply*) was also amplified in all the *S. pneumoniae* strains which serve as *S. pneumoniae* species specific gene. Amplification of the *gyrA* gene in all the strains does not imply antibiotic resistance but acts as a housekeeping gene in this organism.

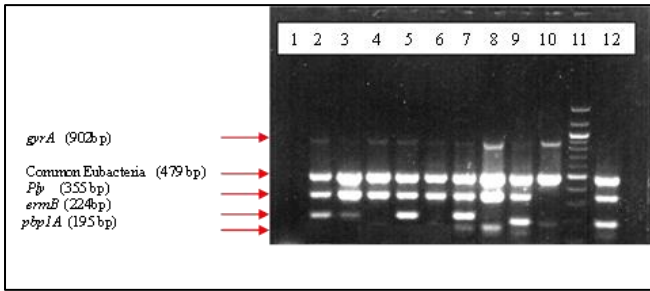


Figure 1 Screening of isolate using quintuplex PCR

- Lane 1: Negative control
- Lane 2: Strain I18
- Lane 3: Strain R71
- Lane 4: Strain R81
- Lane 5: Strain R77
- Lane 6: Strain R55
- Lane 7: Strain R88
- Lane 8: Strain R72
- Lane 9: Strain I99
- Lane 10: Strain R100
- Lane 11: 100 bp DNA ladder
- Lane 12: Direct blood culture isolate

The specificity of the quintuplex PCR was also tested against other gram negative and gram positive bacteria as shown in Figure 2. The *S. pneumoniae* species specific gene, pneumolysin (*ply*), was only detected in the *S. pneumoniae* strain R81 whereas this gene was not amplified in the other gram positive (*Streptococcus sanguis* ATCC 10556, *Staphylococcus aureus* ATCC 25923) and gram negative bacteria (*Acinetobacter baumannii* ATCC 15308, *Pseudomonas* sp.) that were tested. The gene encoding the common region of the eubacteria was detected in all the bacterial isolates tested. Similarly, the common eubacterial gene was also observed in the blood culture bottles that were spiked with known amount of bacterial suspension.

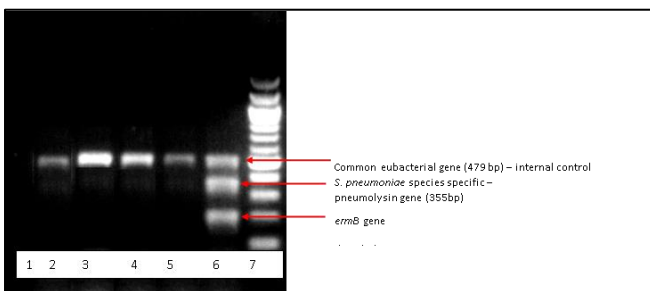


Figure 2 PCR amplification to test the specificity of the assay

- Lane 1: Negative control
- Lane 2: *Acinetobacter* sp.
- Lane 3: *Pseudomonas* sp.
- Lane 4: *Streptococcus sanguis*
- Lane 5: *Staphylococcus aureus* and) were tested.
- Lane 6: Lane *Streptococcus pneumoniae*
- Lane 7: 100bp DNA ladder

The lower limit of detection of the assay was determined to be as low as 10 CFU as illustrated in Figure 3. This was evident as the genes were not clearly observed in blood culture bottles spiked with lowest concentration of bacteria (Figure 3).

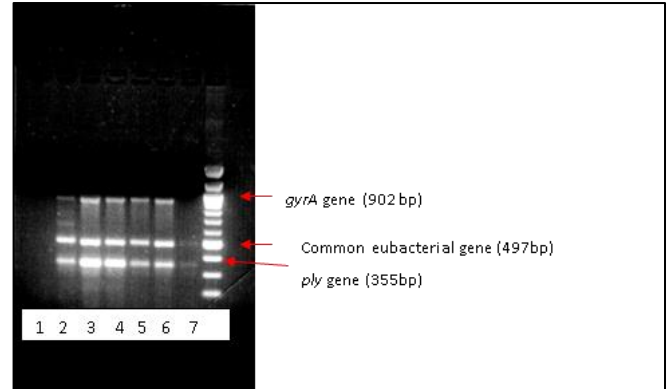


Figure 3 Amplification of the quintuplex PCR assay of simulated blood culture with known concentration of template to test the sensitivity of the assay

- Lane 1: Negative control
- Lane 2: Concentration of 10^6 cfu
- Lane 3: Concentration of 10^5 cfu
- Lane 4: Concentration of 10^4 cfu
- Lane 5: Concentration of 10^3 cfu
- Lane 6: Concentration of 10^2 cfu
- Lane 7: Concentration of 10 cfu
- Lane 8: 100 bp DNA ladder

The triplex PCR was used in addition to the quintuplex PCR to characterize the macrolide resistant strains, which did not harbour the *ermB* gene. Using the triplex PCR, only 33 of the 44 erythromycin resistant strains harboured the *ermB* gene, while 5 of 12 erythromycin intermediate strains harboured the *ermB* gene. Strains that were negative for the *ermB* gene were further tested for the presence of the *mefE* gene. Results obtained showed that the strains negative for *ermB* harboured the *mefE* gene instead. Figure 4 illustrates the PCR amplification of representative strains using the triplex PCR assay for the detection of *ermB* and *mefE* simultaneously.

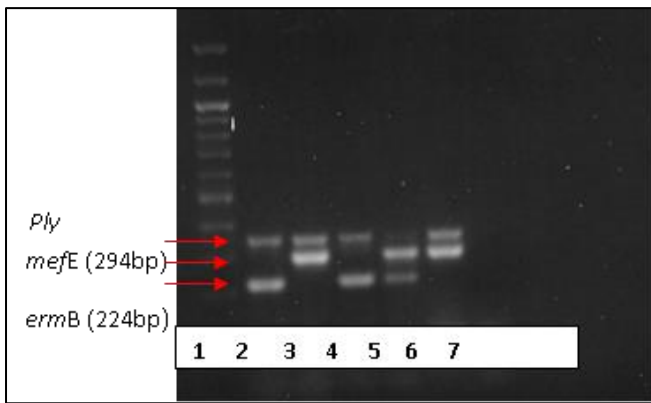


Figure 4 Triplex PCR of the macrolide resistance determinants and *S.pneumoniae* specific gene

Lane 1: 100 bp DNA ladder
 Lane 2: Strain I20
 Lane 3: Strain I28
 Lane 4: Strain R76
 Lane 5: Strain R17
 Lane 6: Strain R46
 Lane 7: Negative control

DISCUSSION

β -lactam antibiotics inhibit the growth of *S. pneumoniae* by inactivating the penicillin-binding proteins (PBPs), which are essential in cell wall synthesis. Resistance to β -lactams is due to alterations in the PBPs, resulting in reduced affinity of binding of the drug. In this study, the amplification of *pbp1A* gene is an indication of non-altered PBPs whereas no amplification of the *pbp1A* gene indicates alteration in the gene. Previous studies have shown that alteration in PBPs 2B, 2X and 1A confers penicillin resistance [12] but only alterations in *pbp2X* and 1A confers cefotaxime resistance [13]. However, alteration in *pbp1A* is essential to confer high-level resistance [14, 15, 16], hence the identification of *pbp1A* could be useful for detecting high-level penicillin resistance.

In this study, the *pbp1A* gene was detected by the quintuplex PCR in the 58 strains that were sensitive to penicillin but was not detected in all the 36 strains and 26 strains that were intermediate and resistant respectively to penicillin. The negative amplification of *pbp1A* gene indicates an alteration within the *pbp1A*, whereas presence of the gene indicates a susceptible strain with no alteration. An alteration in the penicillin binding proteins reduces its affinity of binding to β -lactams, hence reducing the susceptibility of the antibiotic to the organism. In our findings, amplification of the *pbp1A* gene in strains R88, R72 and

I99 indicates that there was no alteration in this gene. However, there could be alteration in the other PBPs that may have caused these strains to develop resistance to penicillin.

Using the quintuplex PCR, the *ermB* gene was detected in 38 strains, of which, 5 were intermediate and 33 were resistant to erythromycin. Some strains that harboured the *ermB* gene had also possessed the *mefE* gene (triplex PCR), which confers resistance to macrolides via the efflux mechanism and some of the strains that were negative for the presence the *ermB* gene, showed positive amplification of the *mefE* gene instead.

An attempt to incorporate the *mefE* gene into the quintuplex PCR was unsuccessful as it was inhibiting the detection of the other genes simultaneously. Macrolide resistance is conferred by the presence of the *ermB* or *mefE* or both the genes. The *ermB* was incorporated into the multiplex because of its high prevalence. The *ermB* determinant is borne by conjugative transposons related to Tn1545, Tn1545-like elements or a Tn 917-like element, that is part of a larger composite transposon, Tn 3872 [17,18]. This accounts for horizontal transfer of the element and results in a higher prevalence of the *ermB* determinant.

The detection of the *gyrA* gene by PCR in both fluoroquinolones susceptible and non-susceptible strains indicates no significance in the presence of the gene, with regards to the development of fluoroquinolones resistance. However, this gene acts as a housekeeping gene in *S. pneumoniae*. Fluoroquinolones resistance in *S. pneumoniae* is conferred by point mutations within the Quinolone Resistance Determining Region (QRDR). The targets of fluoroquinolones are the type II and type IV topoisomerases encoded by the *gyrA*, *gyrB*, *parC* and *parE* genes. Therefore, point mutations conferring fluoroquinolone resistance may occur in either one or more of these genes. In order to detect these mutations, PCR DNA sequencing needs to be carried out on all the strains.

Isolates from direct blood cultures that were confirmed gram positive by gram staining were also characterized using the two multiplex PCRs. In this study, Streptococcus group B and Viridans group Streptococci were used. The assay showed promising

results as only the internal control gene was amplified by the assay. This shows that the assay is 100 % genus and species specific. The lower limit of detection of the quintuplex PCR of up to 10 CFU/mL would allow characterization of *S. pneumoniae* strains from cultures containing low levels of bacterial load of infection.

The characterization of *S. pneumoniae* strains using multiplex PCR has an advantage with a shorter turnaround time. Detection of *S. pneumoniae* and its antibiotic resistance genes from a bacterial culture is possible within 3-4 hours using the multiplex PCR assays. The identification of the antibiotic resistance genes allows prediction of the appropriate drug therapy. Several investigators have evaluated PCR as a tool for diagnosis of pneumococcal infection [10, 19, 20]. However, previous researchers focused solely on identifying pneumococcal infections from infants and children. Most studies concentrated on detecting the pneumolysin gene in different sources of samples, such as sputum, urine and blood. In this study, we used the multiplex PCR assay to characterize the strains and identify genes encoding antibiotic resistance, which are commonly prescribed; penicillin and macrolides. Other researchers have also detected antibiotic resistance genes via PCR, whereby, PCR has been used to detect different classes of antibiotics separately in different tubes as compared to the multiplex PCR assay, which detects 3 different classes of antibiotics in one tube and also specifically detects *S. pneumoniae* via the detection of the *S. pneumoniae* species specific genes, pneumolysin (*ply*). Therefore, we conclude that characterization of *S. pneumoniae* strains could be carried out using multiplex PCR in order to shorten the turnaround time, hence avoiding unnecessary drug prescription.

Conflict of Interest

Authors declare none.

Acknowledgements

This work was supported by a grant provided by the Ministry of Science and Technology, Malaysia: 36-02-03-6027.

REFERENCES

1. Bartlett JG, Grundy LM. Community-acquired pneumonia. *New Engl J Med.* 1995; 333(24):1618-24.
2. Marton A, Gylyas RM, Munoz, Tomasz A. Extremely high incidence of antibiotic resistance in clinical isolates of *Streptococcus pneumoniae* in Hungary. *J Infect Dis.* 1991; 163(3):542-8.
3. Bacquero F. Pneumococcal resistance to β -lactam antibiotics: a global geographic overview. *Microb Drug Resist.* 1995; 1(2):115-20.
4. Weistblum B. Erythromycin resistance by ribosome modification. *Antimicrob Agents Chemother.* 1995; 39(3):577-85.
5. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev.* 1997; 61(3):377-92.
6. Pan X, Ambler J, Mehtar S, Fisher L. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother.* 1996; 42:2810-6.
7. Bast DJ, Low DE, Duncan CL, Kilburn L, Mandell LA, Davidson RJ, and JCS de Azavedo. Fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*: contributions of type II topoisomerase mutations and efflux to levels of resistance. *Antimicrob Agents Chemother.* 2002; 44(11):3049-54.
8. Wayne, PA. Performance standards for antimicrobial susceptibility testing: Fifteenth informational supplement. Document M100- S15 Clinical and Laboratory Standards Institute. 1995.
9. Unal S, Hoskins J, Flokowitsch JE, Wu CYE, Preston DA, Skatrud PL. Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. *J Clin Microbiol.* 1992; 30(7): 1685-91.
10. Dagan R, Shriker O, Hazan I, Leibovitz E, Greenberg D, Schlaefer F, Levy R. Prospective study to determine clinical relevance of detection of pneumococcal DNA in sera of children by PCR. *J Clin Microb.* 1998; 36(3): 669-73.
11. Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, Appelbaum PC.

- Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and β -lactam resistance, and to detect common macrolide resistance determinants. *J Antimicrob Chemother.* 2001; 48(6): 915-8.
12. Barcus VA, Ghanckar K, Yeo M, Coffey TJ, Dowson CG. Genetics of high level penicillin resistance in clinical isolates of *Streptococcus pneumoniae*. *FEMS Microbiol Lett.* 1995; 126(3):299-303.
 13. Munoz R, Dowson CG, Daniels M, Coffey TJ, Martin C, Hakenbeck R, Spratt BG. Genetics of resistance to third-generation cephalosporins in clinical isolates of *Streptococcus pneumoniae*. *Mol Microbiol.* 1992; 6(17):2461-5.
 14. Reichmann P, Konig A, Marton A, Hakenbeck R. Penicillin binding proteins as resistance determinants in clinical isolates of *Streptococcus pneumoniae*. *Microbial Drug Resist.* 1996; 2(2):177-81.
 15. Smith AM, Klugman KP. Non-penicillin-binding protein mediated high level penicillin and cephalosporin resistance in a Hungarian clone of *Streptococcus pneumoniae*. *Microbial Drug Resist.* 2000; 6(2):105-10.
 16. Smith AM, Klugman KP, Coffey TJ, Spratt BG. Genetic diversity of penicillin binding protein 2B and 2X genes from *Streptococcus pneumoniae* in South Africa. *Antimicrob Agents Chemother.* 1993; 37(9):1938-44.
 17. Courvalin P, Carlier C. Tn1545: a conjugative shuttle transposon. *Mol Gen Genet.* 1987; 206(2):259-64.
 18. McDougal LK, Tenover FC, Lee LN, Rasheed JK, Patterson JE, Jorgensen JH, LeBlanc DJ. Detection of Tn917-like sequences within a Tn916-like conjugative transposon (Tn3872) in erythromycin-resistant isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother.* 1998; 45:1595-8.
 19. Dominquez J, Gali N, Matas L, Pedroso P, Blanco S, Gimenez M, Prat C, Sopena N, Sabria M, Ausina V. PCR Detection of *Streptococcus pneumoniae* DNA in serum samples for pneumococcal pneumoniae diagnosis. *Clin Microbiol Infect.* 2001; 7(3):164-6.
 20. Isaacman DJ, Zhang Y, Reynolds EA, Ehrlich GD. Accuracy of a polymerase chain reaction-based assay for detection of pneumococcal bacteremia in children. *Pediatrics.* 1998; 101(5):813-6.