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ORIGINAL ARTICLE

Molecular Characterization of β-Lactam Resistant *Streptococcus pneumoniae*

Tarek E. S. El-Banna [1], Fatma I. Sonbol [1], Maysaa E. S. Zaki [2], Rana H. Abo-Hammam [3]

[1] *Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University, Egypt*
[2] *Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Egypt*
[3] *Forensic Medicine Authority, Ministry of Justice, Egypt*

SUMMARY

***Background: Streptococcus pneumoniae* (*S. pneumoniae)* is a commensal bacterium that normally colonizes the human nasopharyngeal cavity. Once disseminated, it can cause several diseases, ranging from non-invasive infections such as acute otitis media and sinusitis through to invasive infections with higher mortality. Antibiotic resistance among *S. pneumoniae* has increased dramatically and penicillin-resistant strains have spread worldwide with pneumococcus also being resistant to other types of antibiotics like erythromycin, tetracycline, and chloramphenicol. The aim of the present study was to study the susceptibility of the isolated strains to β-lactam and other antibiotics from different classes and to determine the prevalence of β-lactam resistance genes among *S. pneumoniae* clinical isolates.**

***Methods:* From a total of 178 sputum samples, isolates identified by standard microbiological method as *S. pneumoniae* were subjected to antibiotic susceptibility tests to β-lactam and non β-lactam antimicrobial agents by disk diffusion method. Biofilm formation was detected by microtitration plate and the resistance genotype was also determined using multiplex PCR technique with primers designed for PBP genes.**

***Results:* Out of 178 sputum samples, sixty isolates were recovered as *Streptococcus pneumoniae*. Most of isolates were multidrug-resistant (MDR) possessing a high (> 0.2) multiple antibiotic resistance index (MAR) value. Biofilm formation ability of isolates were strong, moderate, weak, and none, accounting for 21.67%, 45%, 25%, and 8.33% biofilm formers, respectively, and it was found that *pbp*1a, *pbp2b,* and *pbp*2x were present in 33 (55%), 25 (41.7%), and 45 (75%) of isolates, respectively.**

***Conclusions: Streptococcus pneumoniae* clinical isolates have an alteration in PBP resistance genes in response to β-lactam therapy which subsequently lead to increased MDR phenomena among these clinically important pathogens. These findings necessitate continuous monitoring of antimicrobial resistance to guide the empirical treatment of pneumococcal disease, as well as to encourage reflections to support public immunizations strategies.**

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Correspondence:
Rana Hashem Abo-Hammam
Forensic Medicine Authority
Ministry of Justice
Egypt
Phone: +20 1025453142
Fax: +20 502236678
Email: ranahashem111@hotmail.com

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INTRODUCTION

Streptococcus pneumoniae (*S. pneumoniae)* is a gram-positive diplococcus that was first isolated in 1881 by George Sternberg and Louis Pasteur. It is an infectious pathogen responsible for millions of deaths worldwide

[1]. It is more prevalent in young children, elderly, and immuno-compromised patients [2,3].
Since the identification of the first *Streptococcus pneumoniae* strain with decreased susceptibility to penicillin in the 1960s [4], antibiotic resistance among *S. pneumoniae* has increased dramatically and the mechanisms of resistance have begun to be revealed. Currently, penicillin-resistant strains have spread worldwide with pneumococcus also being resistant to other types of antibiotics like erythromycin, tetracycline, and chloramphenicol [5].
Biofilms are highly-structured communities of cells that produce an extracellular matrix and adhere to abiotic or biological surfaces [6,7]. The formation of bacterial biofilms confers increased resistance to antimicrobial agents [8,9]. In pneumococci, the decreased susceptibility to antimicrobial treatment during carriage is due to formation of biofilm communities in nasopharynx [9, 10].
The main mechanism of penicillin resistance in clinical isolates of *S. pneumoniae* involves the alteration of penicillin binding protein (PBPs) so as to reduce their affinity for the antibiotic molecule; mutations leading to resistance to penicillin are usually present in the transpeptidase-penicillin-binding domain [11].
Five PBPs of high molecular weight (PBPs 1a, 1b, 2x, 2a, and 2b) and one PBP of low molecular weight were identified in pneumococcus [12]. Three of these PBPs are mainly involved in the β-lactam resistance: PBP 1a, 2b, and 2x. PBP1a is the only bifunctional enzyme. PBP1a plays a key role in septum formation during the cell division cycle and its modification is essential for the development of high-level resistance to penicillins and cephalosporins [13]. Monofunctional transpeptidases are involved in peptidoglycan assembly, PBP2x participates in septal synthesis, while PBP2b functions in peripheral elongation [14].
The aim of the present study was to study the susceptibility of the isolated strains to β-lactam and other antibiotics from different classes and to determine the prevalence of β-lactam resistance genes among *S. pneumoniae* clinical isolates.

MATERIALS AND METHODS

Tested isolates

A total of 60 *Streptococcus pneumoniae* isolates were recovered from 178 sputum samples from patients with clinically and radiologically suspected patients with pneumonia at Mansoura Chest Hospital, Egypt during the period from January 2016 to February 2017.

Isolation and identification of *Streptococcus pneumoniae* isolates

Isolation and identification of pneumococci isolates were carried out according to Cheesbrough [15]. The collected samples of sputum were handled as follow: one loopful of each was mixed well with a drop of sterile saline and streaked onto blood agar plate and incubated overnight at 37°C with 5 - 10% CO_2. The recovered colonies were morphologically and microscopically examined. Suspected colonies were subjected to biochemical identification using catalase, optochin (5 µg disc, Oxoid, UK), and bile solubility test. Further confirmation of identity were carried out genotypically using a PCR technique to detect the autolysin gene (*lyt*A), PCR thermal cycling was done at initial denaturation temperature of 94°C for two minutes, followed by 25 amplification cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 15 seconds, and extensions at 72°C for one minute, then final elongation at 72°C for 5 minutes. The primers used were forward primer:
5'-CAACCGTACAGAATGAAGCGG-3',
reverse primer:
5'TTATT CGTGCAATACTCGTGCG-3'.
These were used to amplify a 319-bp fragment of the *lyt*A gene [16].

Antibiotic susceptibility

The antimicrobial susceptibility of *S. pneumoniae* to β-lactam as well as non β-lactam antibiotics was determined by the disk diffusion method on Mueller-Hinton agar in accordance with the standard of the Clinical and Laboratory Standards Institute (CLSI) [17], the tested antimicrobial agents were obtained from Oxoid, UK.

Multiple antibiotic resistance (MAR) index study and multidrug resistance (MDR) character calculation

Multiple antibiotic resistance (MAR) index is a tool that reveals the spread of resistant bacteria in a given population. The MAR index values for each isolate were calculated according to Mthembu [18].

$$\text{MAR index for isolates} = \frac{\text{Number of antibiotics to which the isolates was resistant}}{\text{Total number of antibiotics to which the isolate was exposed}}$$

The MDR character of the isolates was identified by observing the resistance patterns of the isolates to the tested antimicrobials. An isolate was considered multidrug resistant when it was non susceptible to at least one agent in ≥ 3 antimicrobial categories [19].

Analysis of β-lactam resistance among tested isolates

Phenotypical detection of biofilm production by the tested isolates

Biofilm formation was evaluated by culture plate method (microtitration plate) using crystal violet for semi-quantitative measurement of mature biofilms as described by Baldassarri et al. [20] with slight modifications. Suitable suspension (0.5 McFarland) of pneumococcal isolates were prepared using tryptic soy broth (TSB) with 1% glucose, diluted 1:100 in sterile TSB. Then 200 µL of the suspensions were inoculated into wells of 96-well polystyrene plate and incubated at 37°C in 5 - 10% CO_2 for 24 hours. Three wells were used for each isolate and the interpretation of biofilm production was done according to the criteria of Stepanovic et al., [21]

Table 1. Criteria of interpretation of biofilm production.

Biofilm production	Average optical density (OD)
No biofilm producer	≤ ODc
Weak biofilm producer	ODc < ~ ≤ 2 x ODc
Moderate biofilm producer	2 x ODc < ~ ≤ 4 x ODc
Strong biofilm producer	> 4 x ODc

Optical density cutoff value (ODc) = average OD of negative control + 3 x SD of negative control.

as shown in Table 1.

Molecular characterization of PBP genes among tested isolates

Extraction of total DNA of tested isolates

The tested *Streptococcus pneumoniae* were first treated with lysozyme (SIGMA-ALDRICH, USA) to weaken the rigid and multilayered cell wall. Total DNA extraction from tested isolates was performed using purification DNA kit (ABIO pure EXTRACTION) (Alliance Bio, USA) as recommended by manufacturer instructions.

Screening of tested isolates for antimicrobial resistance genes by multiplex PCR

Amplification of three penicillin binding protein genes (PBP) *pbp*1a, *pbp*2b, *pbp*2x in tested isolates were performed using uniplex or duplex PCR technique. The sequence of the primers used was summarized in Table 2. These techniques were performed as initialization step at 94°C for 3 minutes then 30 cycles at 94°C for 20 seconds, 57°C for 20 seconds, and 72°C for 15 seconds and a final elongation step at 72°C for 7 minutes [22]; the primer pairs designed were shown in Table 2. The amplified products were electrophoresed and the amplicons were stained by ethidium bromide and visualized on a trans-illuminator.

RESULTS

Isolate identification

Out of 178 sputum samples 60 (33.7%) *Streptococcus pneumoniae* isolates were recovered and determined to be gram positive diplococcus, catalase negative, optochin sensitive, bile soluble, and harbor the *lyt*A gene.

Antibiotic susceptibility

The susceptibility of isolates to 20 different antimicrobial agents (Table 3) revealed that 76.77%, 71.66%, and 70% of isolates were resistant to Ampicillin, Cefaclor, and Oxacillin, respectively. On the other hand, Linezolid, Clindamycin, Cefoperazone, Cefotaxime, Cephradine, and Ceftazidime were the most effective antimicrobials against *Streptococcus pneumoniae* isolates showing only resistance between 10 to 15%.

Multiple antibiotic resistance (MAR) index

The MAR index for all sixty isolates was found to range between 0.15 to 0.65. Most of tested isolates had a MAR index higher than 0.2.

S. pneumoniae isolates exhibited 11 major resistance patterns according to the number of resistance markers, and most of the isolates (93%) were resistant to 3 to 13 out of 20 tested antimicrobial agents (data not shown).

Biofilm formation

The pneumococcal isolates were screened for their ability to adhere to the wells of microtitration plates and hence biofilm production. Biofilm OD values were measured using an ELISA AutoReader at a wavelength of 570 nm.

For easier interpretation of the results, isolates were divided into four categories: non-biofilm formers, weak biofilm formers, moderate biofilm formers and strong biofilm formers. It was found that 21.67% of isolates were strong biofilm former, 45% of isolates were moderate biofilm formers, 25% of isolates were weak biofilm formers, and 8.33% of isolates were none-biofilm formers (Figure 1).

Genotypical detection of three penicillin binding protein genes by PCR

Amplification of three penicillin binding protein genes (PBP) *pbp*1a, *pbp*2b, *pbp*2x were performed using total DNA extract using PCR technique. Uniplex PCR technique was used for *pbp*1a gene. The amplified fragment length 430 bp of the *pbp*1a gene was present in only 33 (55%) out of the tested S. *pneumoniae* isolates.

Multiplex PCR technique for *pbp*2x and *pbp*2b genes revealed that the amplified fragment lengths of 292 bp corresponded to the *pbp*2x gene and the 77 bp corresponded to the *pbp*2b gene, and they were present in 45 (75%) and 25 (41.7%) of tested S. *pneumoniae* isolates, respectively. In general, all isolates contained at least one penicillin binding protein resistant gene except ten isolates (16.7%) which showed the resistance to three genes; *pbp*1a, pbp2b, and *pbp*2x, Figure 2.

DISCUSSION

Streptococcus pneumoniae was included as one of 12 priority pathogens in 2017. The continued high burden of disease and rising rates of resistance to penicillin and other antibiotics have renewed interest in prevention. In our study, the antimicrobial susceptibility patterns of *S. pneumoniae* clinical isolates were investigated using antibiotics belonging to various classes, and the results indicated an increase in MDR phenomena: about 93% of isolates were MDR while another study conducted in Japan showed that 46.4% of isolates were multidrug resistant (MDR) [23].

Table 2. Multiplex PCR primers and expected product sizes used for detection of β-lactam resistance among the resistant isolates.

Gene	Primer	Sequence	Amplicon size (bp)
*pbp*1a	Fw	5'AAACAAGGTCGGACTCAACC-3'	430
	Rv	5'AGGTGCTACAAATTGAGAGG-3'	
*pbp*2b	Fw	5'CCAGGTTCCACTATGAAAGTG-3'	292
	Rv	5'CATCCGTCAAACCGAAACGG-3'	
*pbp*2x	Fw	5'CAATCTAGAGTCTGCTATGGA-3'	77
	Rv	5'GGTCAATTCCTGTCGCAGTA-3'	

Table 3. Anti-microbial discs and prevalence of resistance of tested *S. pneumoniae* isolates to the studied antimicrobial drugs.

Antibiotic class	Antimicrobial agent (µg/disk)	No. (%) of resistant isolates
Penicillins	Oxacillin, OX (1 µg)	42 (70%)
	Ampicillin, AM (10 µg)	47 (78.33%)
	Ampicillin + sulbactam, SAM (20 µg)	41 (68.33%)
	Amoxicillin, AML (10 µg)	44 (73.3%)
	Amoxicillin + calvulonic acid, AMC (30 µg)	40 (66.66%)
Cephalosporins	Cephradine (1st), CRO (30 µg)	9 (15%)
	Cephalothine (1st), CL (30 µg)	16 (26.6%)
	Cefaclor (2nd), C (30 µg)	43 (71.66%)
	Cefoperazone (3rd), CFP (72 µg)	6 (10%)
	Ceftazidime (3rd), CAZ (30 µg)	10 (16.6%)
	Cefotaxime (3rd), CTX (30 µg)	8 (13.3%)
Macrolides	Erythromycin, E (15 µg)	32 (53.3%)
	Azithromycin, AZM (15 µg)	22 (36.6%)
Quinolons	Levofloxacin, LEV (5 µg)	21 (35%)
Lincomycin	Clindamycin, DA (2 µg)	10 (16.6%)
Glycopeptides	Vancomycin, VA (30 µg)	14 (23.3%)
Tetracyclines	Tetracycline, TE (30 µg)	25 (41.6%)
Others	Linezolid, LZD (30 µg)	10 (16.6%)
	Rifampicin, RD (5 µg)	25 (41.6%)
	Cotrimoxazole, SXT (25 µg)	24 (40%)

The emergence of multidrug-resistant *S. pneumoniae* (MDRSP) has been observed in various countries over the past decades with the increase in macrolide and penicillin resistance. Especially the parallel increasing frequency of MDRSP, concerns have been raised with respect to the treatment of pneumococcal diseases [24]. The MAR index is a tool to analyze health risk and is helpful to check the spread of bacterial resistance in a given population [25]. Interestingly, the MAR index in 55 (91.7%) of the isolates tested was higher than 0.2. As reported by Mthembu [18], this detected high MAR index revealed that these isolates originated from an environment where several antimicrobials were used.

Among the tested β-lactam, Ampicillin showed the highest (78.33%) prevalence of resistance. Although no beta lactamases previously were reported to be produced by pneumococci; however, adding sulbactam resulted in a decrease of resistance to 68.33%. This may be explained by the new finding of a metallo-β-lactamase involved in Ampicillin resistance of *Streptococcus pneumoniae* [26].

In the current study, resistance to chloramphenicol, cotrimoxazole, erythromycin, tetracycline, and vancomycin was 16.6%, 40%, 53.3%, 41.6%, and 23.3%, respec-

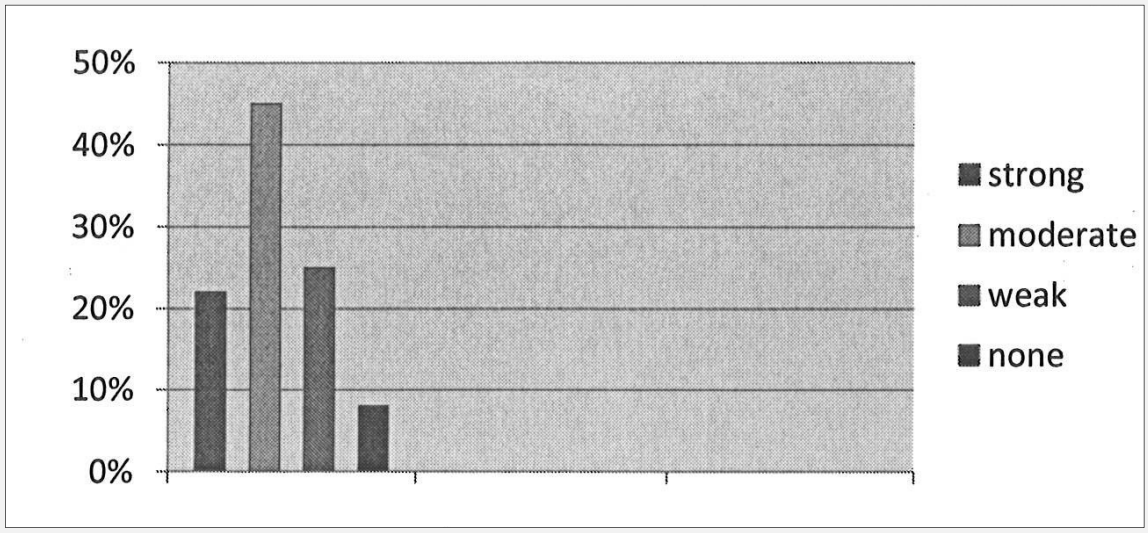

Figure 1. Biofilm forming ability of all isolates.

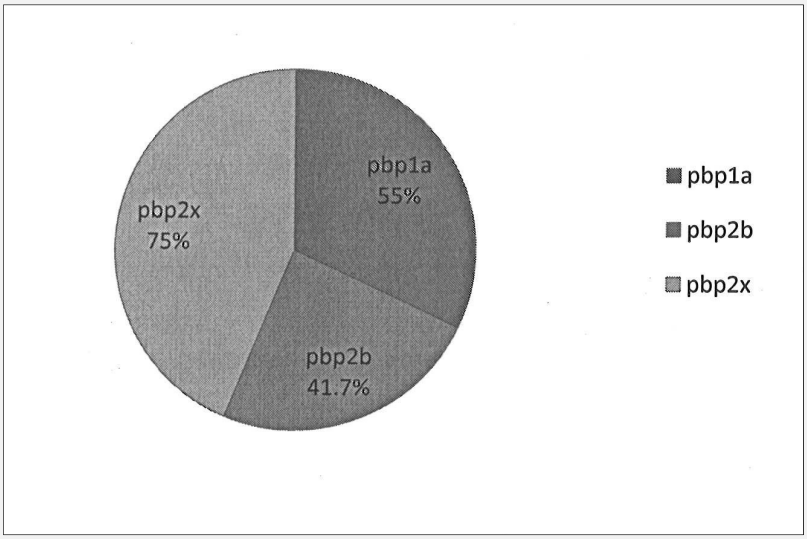

Figure 2. Prevalence of β-lactam genes among the tested isolates.

tively. Finding that is comparable to what was detected by El-Shafie and Taj-Aldeen [27] in Qatar. Resistance to chloramphenicol, co-trimoxazole, erythromycin, and tetracycline was 11.86%, 56.78%, 22.85%, and 38.14%, respectively. None of the isolates was resistant to vancomycin. Bahy et al. [28] found that resistance to tetracycline and co-trimoxazole was 55% and 43%, respecttively, slightly higher than the result reported in the present study, also reported that linezolid sensitivity was (84%) similar to the present study.

As colonization is a necessary step in pneumococcal pathogenesis [29,30] and there is evidence for the role of biofilms in disease [31], biofilm formation was detected in this study by microtitration plate and most isolates were able to form biofilm. Biofilm formation during colonization may provide one mechanism that the pneumococci utilize to persist during antibiotic exposure in the human host. It is worth mentioning that strong and moderate biofilm formers were found to harbor the *rrg*A gene (a gene encoding pilus protein in pneumococcus); however, this gene was not expressed as detected by scanning electron microscope (data not shown) this may be due to pilus-1 expression being regulated *in vivo* as high expression of pilus-1 is observed at early stages of colonization consistently with its role in adhesion and reduced expression during later stages of infection. Expression rates observed in clinical isolates *in vitro* may not reflect the actual rates during colonization/infection [32].

The PCR technique was used to detect the presence of the three PBP genes in our isolates. These genes were selected because they are mainly involved in β-lactam resistance. Our finding demonstrated that the *pbp*2x gene was the most prevalent gene being detected in 75% of isolates as it is the first target to be modified under antibiotic pressure [33] compared to 33 (55%) and 25 (41.7%) of *pbp*1a and *pbp*2b, respectively in the tested isolates. All isolates contained at least one penicillin binding protein resistant gene except ten isolates (16.7%) showed the three resistance genes. These isolates showed high MAR index and were strong and moderate biofilm formers. So, a high level of resistance is achieved through a combination of three altered PBPs.

Kawaguchiya et al. [23] reported that 35.8% of isolates had three altered genes, *pbp*1a, *pbp*2b, *pbp*2x (gPRSP), and 37.1% of isolates had *pbp*2x gene. Mosleh et al. [22] reported that 85% of the isolates had mutations in *pbp*2x, *pbp*2b, *pbp*1a and 100% of isolates had *pbp2x*. Tabatabaei et al. [36] reported that the *pbp*2x gene was identified in 80% of isolates while Habibian et al. [37] reported that the *pbp*2b gene was found in 4 (8%) of the total 50 samples in 2013.

CONCLUSION

Streptococcus pneumoniae clinical isolates are continuously altered in PBP resistance genes in response to β-lactam therapy which subsequently leads to increased MDR phenomena among this clinically important pathogen. These findings necessitate continuous monitoring of antimicrobial resistance to guide the empirical treatment of pneumococcal disease, as well as to encourage reflections to support public immunizations strategies.

Declaration of Interest:

There are no conflicts of interests for any of the authors.

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