Pneumococcal empyema: Resistance patterns, fitness cost and serotype distribution

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ABSTRACT

Background: *Streptococcus pneumoniae* is a recognized etiology of invasive infections including parapneumonic empyema, and its resistance to antibiotics is evolving worldwide, raising concerns of encountering untreatable strains. This study measured the serotype distribution, antimicrobial susceptibility and biological cost incurred by resistance of pneumococci from pleural samples.

Methods: The serotype profiles, susceptibility results and growth rates were phenotypically determined for a panel of clinical strains of *S. pneumoniae* from cases of empyema between 2011 and 2019.

Results: Of 24 empyema cases, the isolated strains belonged to seven serotypes in the following descending order; 19A, 11A/D, 19F, 3, 7F, 1/6B while two strains remained non-typable. Penicillin susceptibility was shown in <80% of the isolates, while parenteral cephalosporins (cefuroxime and ceftriaxone) demonstrated activity in 83.3 and 95.8% respectively. High resistance frequency was noted for macrolides and sulfonamides, but the strains were uniformly sensitive to respiratory fluroquinolones, vancomycin and linezolid. The macrolide-resistant strain exhibited a high growth rate, suggesting a possible beneficial effect. Phenotypes with mono-resistance to sulfonamides and clindamycin were equally fit as the susceptible counterpart strains. Resistance to multiple antimicrobial agents resulted in a high degree of fitness deficit, while other resistant phenotypes were less fit.

Conclusions: The pneumococcal conjugate vaccine PCV13 serotypes still circulate in the community. The data indicate that resistance to certain antimicrobials incurs an apparent fitness cost in pneumococci which may limit the dissemination of such strains while low fitness cost, seen in case of resistance to macrolides, may contribute to the spread of resistant clones.

Keywords: Bacteria; Fitness; Pneumococci; Pneumonia; Resistance; Serotype; Empyema. **[Am J Med Sci 2022;364 (6):766–771.]**

INTRODUCTION

Parapneumonic effusions and empyema are estimated to complicate around 2 to 3% of pneumonia cases, with a recent incremental trend.[1,2] Although the incidence of empyema is not common, it can be associated with significant morbidity and mortality.[3,4] *Streptococcus pneumoniae* is among the pyogenic bacteria commonly implicated in causing empyema worldwide, and particularly in community-acquired cases.[5] Because of the difficulty to culture the fastidious organism, identification of pneumococcal serotypes that cause parapneumonic empyema is challenging.[6,7] It was proposed that serotype 1 possesses high invasive potential into the pleural space.[7-9] Further studies reported increases in the frequency of other serotypes (3, 7F, and 19A) amongst empyema cases in the post-vaccine era.[10,11] The PCV7 was nationally introduced in 2009, and then replaced by PCV13 in 2011. Currently, the Saudi Thoracic Society pneumococcal vaccination guidelines recommend the use of PPSV23 for all vaccine-naïve healthy individuals, while high-risk adults aged ≥50 years should receive one dose of PCV13 followed by PPSV23 after ≥ 1 year. Children aged between 2 and 6 years with high-risk conditions also receive one dose of PPSV23 at least 8 weeks after the last PCV13.[12] Introduction of vaccines has been linked not only to the reduction in the rate of the pneumococcal disease, but also the prevalence of drug-resistant pneumococcal strains.[13]

The high genomic plasticity of *S. pneumoniae* makes its resistance to various classes of antibiotics a great problem.[13] Acquisition of antimicrobial resistance by the pathogen can be associated with a physiological fitness cost that results in a decreased transmission rate.[14] The fitness of a pathogen is a measure of the organism's ability to survive, reproduce, and be transmitted. Some drug resistance-conferring mutations have less impact on bacterial fitness. To survive and restore the ability to be transmitted, a mutant may revert to either susceptible or compensatory mechanisms accumulate over time, with the second being more likely.[15] The fitness of a bacterium can be quantified in the laboratory by various *in vitro* models, including the average number of surviving

progeny of a particular genotype in direct competition with a susceptible strain.[16] Another approach to measure the fitness of resistant strains is to use the planktonic growth rate to quantify generation times against the wild type.[16] Such methods, when used on clinical strains, can provide an epidemiological insight into the fitness status of commonly encountered strains.

Data about fitness costs of resistant, invasive pneumococcal strains are very limited apart from few, inconclusive reports utilizing genetically transformed pneumococci rather than naturally occurring resistance in clinical isolates.[17-19] Whereas the *in vitro* experiments contribute to the understanding of the pneumococcal population changes in response to various mutations, estimating fitness parameters in relation to antimicrobial resistance in clinical strains will be informative, since the biological cost of resistance mutations by various antimicrobial agents contributes to the selective pressure of an agent.[16] In this study, pneumococcal serotypes that cause empyema in various age groups were evaluated along with their antimicrobial resistance and biological fitness patterns, to help improve the understanding of population dynamics of invasive pneumococcal isolates.

MATERIALS AND METHODS

Research settings and bacterial strains

This is a prospective, observational study conducted at an academic medical institution in Alkhobar, Eastern Region of Saudi Arabia, during the nine-year period between January 1, 2011 and December 31, 2019. The Institutional Review Board approval was obtained (IRB 2014-01-012)). All non-replicate, clinical strains of *S. pneumoniae* isolated from the pleural fluid or pleural biopsy of adults and children were included. Only cultures that were suggestive of true infections, rather than contamination, were considered based on clinical assessment, radiological findings and antimicrobial therapy. The samples were incubated on Columbia blood agar (CBA) (SPML, Saudi Arabia) in 5% CO_2 at 35 °C for 18 h. The strains were identified to the species level based on colonial morphology with α-hemolysis, negative catalase test and susceptibility to a 6 mm optochin disk (5 micrograms) placed at the junction of the primary inoculum and second streak. The identity of *S. pneumoniae* strains was then confirmed by either VITEK 2 automated system (bioMe´rieux Inc., Durham, NC, USA) based on biochemical signatures of the organisms or VITEK® MS (bioMe´rieux Inc., Durham, NC, USA), an automated mass spectrometry microbial identification system that uses the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) technology.

Minimal inhibitory concentration determination

E-test (AB-BIODISK, Sweden)-based minimal inhibitory concentration (MIC) was measured and interpreted following the manufacturer's instructions and the Clinical and Laboratory Standards Institute (CLSI 2019) guidance. Muller Hinton agar supplemented with 5% sheep blood (SPML, Saudi Arabia) was incubated overnight at 35 °C to determine the MIC using a 0.5 McFarland inoculum. The point of intersection of the inhibition ellipse on the E-test strip was recorded as the MIC. For the isolates where fitness studies were performed, the assays were performed in duplicate.

Serotype distribution

Serotyping of the isolates was carried out using the Pneumotest-Latex reaction based on a set of specific antisera in the Danish chessboard typing system (Statens Serum Institut, Copenhagen, Denmark) following manufacturer's recommendations. Briefly, an aliquot of 10 μL of a pneumococcal broth culture (BHI broth, SPML, Saudi Arabia) was mixed with 10 μL of latex reagent on a microscope slide, and manually rocked for 5−8 s. Positive reactions were read with the naked eye within 10 s, while agglutinations observed after more than 10 s were considered nonspecific and ignored.

Fitness estimation by generation time

The modified Youmans and Youmans method was used to measure the generation time in a planktonic growth rate model.[16] Briefly, pneumococcal isolates and a control strain were separately incubated on CBA at 35 °C for 18 h in 5% CO_2. Then, a single colony was inoculated in antibiotic-free BHI broth (5 mL), incubated for 4 h with frequent shaking to obtain exponential growth, and adjusted to turbidity comparable to a McFarland No. 0.5 standard. The culture was then diluted 1000-fold (approximately 10^5 CFU/ ml), loaded into the automated blood culture system BACTEC 9240 (BD Diagnostics, France) with a standard aerobic medium using 2 different dilutions, and incubated until they flagged positive. The time to positivity (TTP) was determined in minutes, followed by confirmation of purity by gram staining and spreading of one drop from the bottle contents onto CBA. The testing was done in triplicate, in two independent experiments. A modified Youmans and Youmans method was applied to calculate the growth rate constant (K) and generation time (G), using the formula $K = (\log A - \log B)/t$, where A is the higher dilution, B is the lower dilution and t is equal to the difference in the mean of TTP for three replicates. The generation time was then calculated using the equation: $G = \log 2/K$.[16]

Statistical analysis

Differences in fitness between the various phenotypes and control strains were analyzed by the one-way analysis of variance (ANOVA) test using the GraphPad Prism version 9.3.1 for Mac, which compares the three means. The null hypothesis was all population means were equal, and the alternative hypothesis was that at least one mean was different. A *p* value of less than 0.05

TABLE 1. The proportion of resistance among 24 respiratory strains of *Streptococcus pneumoniae* isolated from adults.

Drug	Susceptible number (%)
Penicillin: parenteral dose oral dose	19 (79.2%) 18 (75%)
Cefuroxime: parenteral dose oral dose	20 (83.3%) 19 (79.2%)
Cefotaxime	23 (95.8%)
Erythromycin	7 (29.6%)
Clindamycin	15 (62.5%)
Levofloxacin	24 (100%)
Moxifloxacin	24 (100%)
Doxycycline	17 (70.8%)
Trimethoprim-sulfamethoxazole	8 (33.3%)
Vancomycin	24 (100%)
Linezolid	24 (100%)

was considered significant in the calculated generation times of different strains.

RESULTS

In total, 24 isolates of *S. pneumoniae* were collected from invasive pleural samples exhibiting various susceptibility patterns (Table 1). All the pneumococcal isolates were susceptible to respiratory fluroquinolones, linezolid and the tested glycopeptide, vancomycin. They demonstrated variable resistance levels to other classes of antibiotics in the following ascending order: cefotaxime (a 3rd generation cephalosporin), parenteral cefuroxime, oral cefuroxime/parenteral penicillin, oral penicillin, doxycycline, clindamycin (lincosamide), and trimethoprim/sulfamethoxazole, with the highest resistance rate seen with macrolides (erythromycin) in 70.4% of the strains.

The serotypes detected were as follows: six 19A strains (25%), five 11A/D strains (20.8%), four 19F strains (16.7%), three serotype 3 (12.5%), two 7F strains (8.3%), one serotype 1 (4.2%), and one serotype 6B (4.2%) while two strains remained phenotypically non-typable (8.3%). The characteristics of the cases from whom the strains were obtained are illustrated in Table 2.

Isolates exhibiting monoresistance to each class of antimicrobials were further characterized by measuring their MICs as shown in Table 3, as well as an isolate exhibiting multidrug resistance (penicillin, erythromycin and trimethoprim-sulfamethoxazole).

Table 4 demonstrates the variable effects of antibiotic resistance patterns in pneumococci on their mean generation times measured in minutes. The strains were collected from unrelated patients and therefore represented nonclonal isolates as their epidemiological picture and serotypes suggested.

DISCUSSION

S. pneumoniae remains a leading cause of a wide range of invasive infections including empyema, even after the introduction of pneumococcal vaccines.[13]

TABLE 2. Clinical, epidemiological and laboratory characteristics of the 24 empyema cases.

Variable	Findings
Median age (years) ± SD	59.5 ± 14.3
< 18 years	10 (41.7%)
≥ 18 years	14 (58.3%)
Gender	
Male	15 (62.5%)
Female	9 (37.5%)
Blood culture	
Performed	22 (91.7%)
Positive	1 (4.2%)
Sputum culture	
Performed	24 (100%)
Positive	2 (8.3%)
Comorbidities	
Hypertension	4 (16.7%)
Diabetes mellitus	5 (20.8%)
Chronic cardiac condition	1 (4.2%)
Autoimmune diseases	1 (4.2%)
Malignancy	1 (4.2%)
Sickle cell disease	2 (8.3%)
Sepsis	3 (12.5%)
ICU admission	5 (20.8%)
LOS (days)	19.3 ± 13.6
In-hospital mortality	2 (8.3%)
90-day mortality	3 (12.5%)

Abbreviations: ICU, intensive care unit; LOS, length of stay.

Identifying invasive pneumococcal strains is a prerequisite for efficient implementation of regional and global immunization programs. Therefore, this study assessed the serotype distribution of pneumococci implicated in causing 24 empyema cases in a leading hospital in Eastern Saudi Arabia between 2011 and 2019. In an older multicenter study conducted in Saudi Arabia between 2000 and 2004 that included 350 pediatric pneumococcal isolates from various invasive sites, the most common serotype was 14, followed by serotypes 23F, 6B, and 19F accounting for > 80% of the invasive disease.[20] The common serotypes detected in order of frequency in the current study were 19A, 11A/D, 19F, 3, 7F, and 1/6B Notably, four of these six serotypes are included in the PCV13. It was reported earlier that serotype 3, a PCV13 vaccine strain, still circulates worldwide.[21,22] The non-PCV7 serotype 19A was the most prevalent in our cohort, and it has been also described as a cause of invasive pneumococcal disease following the use of PCV7.[23] Serotype 7F has been associated with increased morbidity and mortality seen with pneumococcal infections.[24]

Another focus of this work was to examine the susceptibility patterns of *S. pneumoniae* isolated from pleural samples. Early studies conducted in Saudi Arabia have shown high prevalence of drug-resistant *S. pneumoniae* isolates.[20,25,26] Table 1 shows that the resistance of pneumococci to B-lactams is less than the findings of previous reports. This can be explained by both the

TABLE 3. Quantitative antibiotic susceptibilities (μg/ml) of *Streptococcus pneumoniae* ATCC 49,619 and a panel of clinical strains (SN): * denotes resistance. MIC represents the minimal inhibitory concentration of antimicrobial agents tested against the organism.

Strain	ATCC 49,619	SN-24	SN-18	SN-109	SN-45	SN-30	SN-7	SN-149	SN-12
Serotype	19F	11A/D	19A	6B	1	19F	NT*	3	19A
Resistance Phenotype	Pansusceptible	Pansusceptible	Penicillin-resistant	Cefotaxime resistant	Macrolide-resistant	Macrolide-lincosamide resistant	Doxycycline-resistant	Sulfonamide monoresistant	MDR strain
Penicillin	0.03	0.06	3*	1	1.5	0.5	0.25	0.5	16*
Cefotaxime	0.25	0.125	0.25	6	0.25	0.25	0.25	0.125	0.5
Erythromycin	0.06	0.06	0.125	0.25	256*	4*	0.125	0.25	48*
Clindamycin	0.03	0.06	0.06	0.25	0.125	3*	0.125	0.25	16*
Levofloxacin	0.5	0.25	1	2	0.5	1.5	0.5	3	16*
Moxifloxacin	0.06	0.125	0.25	1	0.125	0.75	0.25	1	4
Doxycycline	0.06	0.06	0.125	0.25	0.125	0.25	16*	0.25	0.25
Trimethoprim-sulfamethoxazole	0.5/9.5	0.5/9.5	1/9	2/38	1/9	2/38	1/9	4/76*	4/76*
Vancomycin	0.12	0.003	0.003	0.5	0.5	0.003	0.006	0.25	0.5

TABLE 4. Association between antibiotic resistance patterns of pneumococci and their mean generation times measured in minutes.

Phenotype	Generation time in min +/- SEM	P value
ATCC 49619	28.7 (26.5 - 30.8)	–
Pansusceptible	31 (28.9 - 31.3)	0.33
Penicillin resistant strain	40.4 (32.8 - 47.3)	0.45
Cefotaxime-resistant strain	48.3 (42.9 - 55.4)	0.01
Macrolide-resistant strain	18.4 (17.5 – 19.4)	0.02
Macrolide-lincosamide resistant strain	28.2 (25.7 – 30.9)	0.39
Doxycycline-resistant strain	52.1 (49.9 – 53.7)	0.0002
Sulfonamide monoresistant strain	29.0 (26.3 - 32.9)	0.10
MDR strain	73.2 (65.2 - 77.6)	0.035

revision of the interpretative breakpoints of B-lactam resistance in non-meningeal pneumococci by CLSI in 2008, in addition to the possible impact of fitness cost on the survival of the resistant strains in the community (Table 3). The CLSI revisions resulted in the different categorizations based on the new guidelines examining the efficacy of double-dose penicillin on respiratory strains with intermediate MICs based on the older breakpoints.[27] Our resistance rates are higher than those described in the United States using current breakpoints, where at present 3.1% and 2.1% of non-meningeal pneumococcal strains are intermediate and resistant to penicillin respectively.[28] On the other hand, higher rates of penicillin resistance have been reported in Asian countries. A multi-center review of pneumococcal isolates (n = 685) from 14 centers in 11 Asian countries over eighteen months (January 2000 to June 2001) illustrated an overall 52% non-susceptibility to penicillin, with a rate of resistance as high as 74% in Vietnam.[29] In comparison, the resistance rates to both macrolide and sulfonamide groups of antibiotics (Table 1) are still alarming (70.3% and 66.6 respectively). The minimal fitness cost of drug resistance in these strains (GT = 18.4 and 29.0 min respectively) was also noted (Table 4). Pneumococcal resistance to sulfonamides is encountered in one third of the isolates in the United States, with emergence of drug-resistant replacement strains following the widespread use of the pneumococcal conjugate vaccine. The most prevalent replacement strain (serotype 19A) showed a high frequency of resistance to sulfonamides (83%) in comparison to other serotypes, 35B and 7F, that remained uniformly susceptible.[30] For macrolides, considerable geographical variation in pneumococcal resistance rates is noted.[28,31] Introduction of the conjugate vaccine didn't decrease the resistance patterns since the emergent replacement strains have a high rate of macrolide resistance.[32] Widespread use of fluoroquinolones increases the risk for emergence of resistance.[33] High rates of fluroquinolones resistance have been reported in Belgium, Croatia, and Hong Kong where 3

−13% of pneumococci were reported resistant.[34-36] In contrast, no resistance has been reported in Germany.[37] There was no resistance to fluroquinolones, linezolid or vancomycin detected in our cohort, although vancomycin tolerance has been described for some strains in a previous study leading to therapeutic failure.[38]

A limitation of the study is the small number of cases due to the low culture yield of *S. penumoniae* and thus culture-negative pneumococcal empyema could have been missed, driving an effect on the serotype epidemiology. Additionally, the vaccination status could not be retrieved for those cases. Despite the high vaccination rates in Saudi Arabia, the patients may have been older than the requisite age for routine vaccination when the PCV7 and PCV13 were introduced. Furthermore, pre-vaccination serotypes for empyema cases in the region are not known. Nevertheless, the data provided insights into the serotype distribution of empyema cases across a period of nine years.

CONCLUSIONS

In conclusion, the study has shed light on the overall resistance patterns and serotype distribution of pneumococcal empyema, and the fitness of resistant strains in comparison to the sensitive counterparts. The contribution of transmission to the spread of drug resistance is likely in cases of resistance to the classes of antimicrobials with minimal impact on organism's fitness. To the best of our knowledge, this is the first study where generation times of clinical pneumococcal strains are measured to provide evidence of biological burden of antimicrobial resistance. Surveillance of invasive pneumococcal strains is worth continuing to guide future vaccine development, and further work is suggested to assess the genotype-based fitness in circulating strains within the community.

DECLARATION OF COMPETING INTEREST

The author declares no conflicts of interest.

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AUTHOR CONTRIBUTIONS

A.A. conceived the idea, collected the data, performed the analysis, and wrote the paper.

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