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Molecular characterization of mutation in the parC and gyrB genes that confer fluoroquinolone resistance in Streptococcus pneumonia isolates

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Abstract

Resistance of Streptococcus pneumoniae to multiple antibacterial agents, including β-lactams, macrolides, tetracyclines, and co-trimoxazole, has emerged worldwide in the 1980s and 1990s and has emphasized the need for new therapeutic alternatives, such as newer fluoroquinolones. Older fluoroquinolones, such as ciprofloxacin and ofloxacin, have been widely used in the last 2 decades, but their activity against gram-positive pathogens is limited. Newer fluoroquinolones, such as levofloxacin, gatifloxacin, moxifloxacin, and gemifloxacin, have enhanced activity against most respiratory pathogens, and some are being more widely used to treat respiratory tract infections. Therefore, the emergence of fluoroquinolone-resistant S. pneumoniae strains, although worldwide prevalence is low, is a concern to clinicians who manage respiratory tract infections.

Aim of the work: The aim of this study was to determine the prevalence of fluoroquinolone resistance Streptococcus pneumoniae (FQRSP) and to examine the genetic relatedness of pneumococcal isolates with parC and gyrB genes mutations in different specimens.

Patients and Methods: In this study, Biometra Thermal Cyclar-T Gradient Software PCR system version 4 together with DNASIS 2.6 Sequence Analysis Programs were used to investigate the presence of mutations at quinolone resistance-determining regions of topoisomerase IV and DNA gyrase on 78 S. pneumoniae strains, Among 78 isolates 37 (47.4%) of S. pneumonia isolates were Fluroquinolones susceptible, 12 (15.4%) were with variable susceptibility and 29 (37.2%) were Fluroquinolones resistant.

Results: Our study illustrate the role of mutation in the parC & gyrB genes and the effect of mutations in the both genes in fluoroquinolone resistance among S. pneumoniae isolates.

Conclusion: Results indicated that there is a significant correlation between quinolone resistance development and mutations in the *parC* gene and in less significance in the *gyrB* genes .

Key words: GyrB, ParC, Streptococcus pneumonia, Fluoroquinolones.

Introduction

Antimicrobial resistance (AMR) is the ability of a microbe to resist the effects of medication previously used to treat them. This broader term also covers antibiotic resistance, which applies to bacteria and antibiotics. Resistance arises through one of three ways: natural resistance in certain types of bacteria, genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutations; or more commonly following gradual buildup over time, and because of misuse of antibiotics or antimicrobials. Resistant microbes are increasingly difficult to treat, requiring alternative medications or higher doses, both of which may be more expensive or more toxic. Microbes resistant to multiple antimicrobials are called multidrug resistant (MDR); or sometimes superbugs. Antimicrobial resistance is on the rise with millions of deaths every year. All classes of microbes

develop resistance: fungi develop antifungal resistance, viruses develop antiviral resistance, protozoa develop antiprotozoal resistance, and bacteria develop antibiotic resistance [(3)].

The rise in gram-positive pathogen resistance in recent years has prompted the pharmaceutical industry to develop fluoroquinolones with greater activity against these rapidly changing pathogens. Structural modifications to the basic fluoroquinolone nucleus have given rise to several new generations of compounds. With each new generation the potency against many gram-positive pathogens, including S. pneumoniae, has improved. Although the worldwide prevalence of fluoroquinolone-resistant S. pneumoniae remains low in relation to β-lactam resistance (<1%), the dissemination of successful resistant clones has nonetheless increased the prevalence in some countries [(4)].

Resistance to fluoroquinolones in S. pneumoniae arises in a stepwise fashion and results from alterations in the target binding site due to the acquisition of spontaneous mutations in the quinolone resistance-determining regions (QRDRs) of the topoisomerase IV and DNA gyrase genes. Although mutations usually occur in the QRDRs of parE and gyrA, a role for mutations in the parE and gyrA subunits in low-level resistance has been reported [(5)].

S. pneumoniae isolates with a mutation only in ParC usually remain susceptible or display only a modest increase in resistance. But these first-step mutants are associated with an increased risk for secondary mutations that may enhance resistance. Higher levels of fluoroquinolone resistance require mutations in parC. Resistance to fluoroquinolones can also occur through the overexpression of efflux systems, a phenotype frequently observed among resistant clinical pneumococci. During the past few years, Fluoroquinolone-Resistant Streptococcus pneumoniae (FQRSP) has been reported from several countries, although the prevalence remains low. Fluoroquinolone nonsusceptibility among pneumococci results mainly from point mutations in the quinolone resistance–determining regions QRDR topoisomerase genes [(6)].

Inappropriate use of any antibiotic can contribute to the emergence of resistance to that and related agents. So much work is needed to identify optimal strategies to prevent the emergence and spread of resistant pneumococcal strains in long-term care facilities, including potential use of pneumococcal conjugate vaccines, antimicrobial stewardship, and infection control interventions to interrupt transmission [(6)].

Aim of the work

The aim of this study was to determine the prevalence of fluoroquinolone resistance Streptococcus pneumoniae (FQRSP) and to examine the genetic relatedness of pneumococcal isolates with parC and gyrB genes mutations in different specimens.

Patients and Methods

This study was prospectively conducted over a period of 24 months between October 2015 and September 2017, at Sohag university hospital. During the study period, 78 patients hospitalized for a syndrome consistent with a diagnosis of CAP (community acquired pneumonia) included in this study with a mean age of 34.5 years (range, 2 to 67), 60% of whom were males.

Clinical specimens.

Fresh sputum samples were collected soon after collection of data from patients (75 specimens). Representative sputum originating from the lower respiratory tract was defined as that containing > 25 granulocytes and < 10 epithelial cells per low power field (lpf: total magnification: ×100).

Bronchoalveolar lavage (BAL) (3 specimens) as diagnostic techniques were used according to the clinical judgment of the physician in charge for some neonates.

Identification of Pneumococci and antimicrobial susceptibility testing.

Isolates were incubated in plates with increased CO2 (5-10%) in order to enhance the development of hemolytic zones of the pathogenic Streptococci and incubated for 18-24 hours. On a blood agar plate (BAP), colonies of S. pneumoniae appear as small, grey, moist (sometimes mucoidal), glistening round colonies, about 1 mm in diameter, and surrounded by a zone of alpha-hemolysis.

By gram stain isolates appear as lancet-shaped, Gram-positive diplococci or chains of cocci. The identification of bacteria in our samples was completed by the VITEK® 2 Compact System. As a commercial and standard system, its accuracy has been strictly evaluated.

Antimicrobial susceptibility testing and the MIC was determined by using VITEK® 2 Antimicrobial Susceptibility Tests (AST) according to the Clinical and Laboratory Standards Institute (CLSI).

Polymerase chain reaction (PCR):-

Simple PCR was performed for all strains to detect Fluoroquinolone Resistance Streptococcus pneumoniae (FQRSP) and to examine the genetic relatedness of pneumococcal isolates with par C, gyrB, parE and gyrA genes mutations.

i) Bacterial DNA extraction.

By the use of Quick-DNA™ Miniprep Kit (Catalog No D3024). Bacterial genomic DNA was extracted from several colonies of each isolate and extracted DNA was stored at 20 ○C prior to PCR amplification.

ii) DNA amplification.

The QRDRs of gyrA, gyrB, parC, and parE were amplified by Biometra Thermal Cyclar-T Gradient Software PCR system version 4. The primers for each loci were synthetized by (metabion international AG, Germany) as described by Maeda et al. 2011. The primers used For amplification gyrA gene, nucleotide sequence as follow:

F2-GACAAAGGAGATGAAGGCAAG, R2-GAAAATCTGGTCCAGGCAAG and for amplification gyrB gene: F-GGGAAATAGCGAAGTGGTCA, R-GTACGAATGTGGGCTCCAT and for amplification parC gene, F-CAAAACATGTCCCTGGAGGA, R-GCAGCATCTATGACCTCAGC and for amplification parE gene,:F-TCAAGTCTGCCATTACCAAG G,R-ACCCGCACCAATGGTATAAA.

PCR on lysates with primers as above using MyTaq ™ Red Mix, 2x (Bioline USA Inc., USA) was performed as follows: 1 cycle 95°C for 15 min for initial denaturation , 40 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by an extension step of 72°C for 10 min. The PCR products were separated by electrophoresis in a 2% agarose gel.

iii) DNA sequencing

Amplification products were purified with the QIAquick PCR purification kit (Qiagen). To identify mutations

DNA Sequencing reactions were prepared with ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit using conditions descriped by Zhanel et al. (8) with ABI 377 automated sequencer (PE Applied Biosystems, mississauga, ON).

Sequence analysis

DNA sequences were analyzed with DNASIS 2.6 Sequence Analysis Programs (Hitachi Software Engineering Co., Ltd., San Francisco, Calif.) against 1 of the 2 identical sequenced pneumococcal strains in the database (NC_008533 Streptococcus pneumoniae D39 and AE007317). D39 is a historically important serotype 2 strain that was used in experiments by Avery and coworkers to demonstrate that DNA is the genetic material.

Although isolated nearly a century ago, D39 remains extremely virulent in murine infection models and is perhaps the strain used most frequently in current studies of pneumococcal pathogenesis. To date, the complete genome sequences have been reported for only two S. pneumoniae strains: TIGR4 (The Institute for Genomic Research), a recent serotype 4 clinical isolate that derived from blood of a male Norwegian patient in 2001. Tettelin et al sequenced the complete genome of strain TIGR4 (9), and laboratory strain R6, an avirulent, unencapsulated derivative of strain D39 that was used in laboratory in 2001 by Hoskins et al (10).

Results

During the period from October 2015 and September 2017 our study was carried out in the Clinical Pathology Department, faculty of medicine, Sohag University Hospital, 78 participants included in our study, S. pneumoniae was isolated from 78 patients included in this study. The resistance percentages of all strains to tested antibiotics were as follows: 91% of isolates in our study were resistant to Ampicillin, 5.1% were intermediate and 3.8% were susceptible. Regarding Cefaclor 83.3% were resistant, 7.7% were intermediate and 9% were susceptible. Erythromycin was resistant in 82.1% of isolates, intermediate in 10.3%, and susceptible in 7.7%. Regarding Imipenem 10.3% of isolates were resistant. Tetracycline was resistant in 71.8% of isolates. Clarithromycin was resistant in only 6.4%, also 10.3% of our isolates were resistant to ceftriaxone. Trimethoprim/ sulfamethoxazole was resistant in 9% of our isolates.(table 1)

Table (1) Various antibiotics susceptibility of S. pneumoniae.

Variable	MIC (ug/dl)	No (%)
Ampicillin	Resistant ≥ 2	71(91%)
	Intermediate 0.12 – 1	4 (5.1%)
	Susceptible ≤ 0.06	3 (3.8%)
Cefaclor	Resistant > 16	65(83.3%)
	Intermediate 8 – 16	6(7.7%)
	Susceptible ≤ 4	7(9%)
Erythromycin	Resistant ≥ 1	64(82.1%)
	Intermediate 0.5	8(10.3%)
	Susceptible ≤ 0.25	6 (7.7%)
Imipenem	Resistant ≥ 1	8(10.3%)
	Intermediate 0.5	4(5.1%)
	Susceptible ≤ 0.25	66 (84.6%)
Tetracycline	Resistant ≥ 8	56 (71.8%)
	Intermediate 4	3 (3.8%)
	Susceptible ≤ 2	19 (24.4%)
Clarithromycin	Resistant ≥ 2	5 (6.4%)
	Intermediate 1	3(3.8%)
	Susceptible ≤ 0.5	70 (89.8%)
Ceftriaxone	Resistant ≥ 2	8 (10.3%)
	Intermediate 1	4 (5.1%)
	Susceptible ≤ 0.5	66 (84.6%)
Trimethoprim/ sulfamethoxazole	Resistant ≥ 4	7 (9%)
	Intermediate 1 – 2	3 (3.8%)
	Susceptible ≤ 0.5	68 (87.2%)

Break points of antibiotics "Ampicillin" ≥ 2 R & 0.12 – 1 I & ≤ 0.06 S , "Cefaclor" > 16 R & 8 – 16 I & ≤ 4 S, "Erythromycin" ≥ 1 R & 0.5 I & ≤ 0.25 S, "Imipenem" ≥ 1 R & 0.5 I & ≤ 0.25 S "Tetracyclin" ≥ 8 R & 4 I & ≤ 2 S, "Clarithromycin" ≥ 2 R & 1 I & ≤ 0.5 S, "Ceftriaxone" ≥ 2 R & 1 I & ≤ 0.5 S and "Trimethoprim-Sulfamethaxzole " ≥ 4 R & 1 – 2 I & ≤ 0.5 S.

Among 78 isolates 37 (47.4%) of S. pneumonia isolates were Fluroquinolones susceptible 12 (15.4%) were with variable susceptibility and 29 (37.2%) were Fluroquinolones resistant.

The MICs of Ciprofloxacin, Levofloxacin, Gatifloxacin and Moxifloxacin were measured and results were as follow, 44.9% of S. pneumonia isolates were resistant to ciprofloxacin, 11.5% were intermediate and 43.6% were sensitive. Regarding levofloxacin 42.3% of isolates were resistant, 9% were intermediate, and 48.7% were sensitive. Over forty six (46.1%) of our isolates were resistant to Gatfloxacin, 10.3% were intermediate, and 43.6% were sensitive. Regarding Moxifloxacin 46.2% of our isolates were resistant, 7.6% were intermediate, and 46.2% were sensitive (Table 2). Break points of fluroquinolones group "Ciprofloxacin" ≥ 4 R & 2 I & ≤ 1 S and "Levofloxacin" ≥ 8 R & 4 I & ≤ 2 S and "Gatfloxacin" ≥ 4 R & 2 I & ≤ 1 S "Moxifloxacin" ≥ 4 R & 2 I & ≤ 1 S.

Table (2) Fluroquinolones susceptibility of S. pneumoniae.

Variable	MIC (ug/dl)	no (%)
Ciprofloxacin (1st generation Fluoroquinolone)	Resistant ≥ 4	35(44.9%)
	Intermediate 2	9(11.5%)
	Susceptible ≤ 1	34 (43.6%)
Levofloxacin (2nd generation Fluoroquinolone)	Resistant ≥ 8	33(42.3%)
	Intermediate 4	7(9%)
	Susceptible ≤ 2	38(48.7%)
Gatfloxacin (3rd generation Fluoroquinolone)	Resistant ≥ 4	36(46.1%)
	Intermediate 2	8(10.3%)
	Susceptible ≤ 1	34 (43.6%)
Moxifloxacin (4thgeneration Fluoroquinolone)	Resistant ≥ 4	36(46.2%)
	Intermediate 2	6(7.6%)
	Susceptible ≤ 1	36(46.2%)

Sequencing of the Quinolone Resistance-Determining (QRDRs). .I

Of the 41 quinolone resistant and intermediate isolates, 9 (22.0%) had no substitutions in the QRDRs of either GyrB or ParC, 21 (51.2%) had a QRDR ParC substitution, while 16 (39.0%) had QRDRs substitutions in GyrB. The specific substitutions observed in **ParC** included: Ser79Ala, Ser79Phe, Ser79Tyr, and Asp83Asn. The combinations of substitutions observed in GyrA and ParC included: Ser81Phe and Ser79Phe, Ser81Tyr and Ser79phe, Glu85Lys and Ser79Phe, Ser81Phe and Ser79Tyr, Ser81Phe and Asp83Asn, the specific substitutions observed in **GyrB** were Ala583Ser and Arg545Asn The percent of isolates with each of the aforementioned substitutions is presented in table (3).

Table (3) Percent and types of the 41 Fluroquinolones-resistant S. pneumoniae isolates with resistance-associated QRDR substitutions.

Variable		no (%)
GyrB	Ala583Ser	5 (12.2%)
	Arg545Asn	3 (7.3%)
ParC	Asp83Asn	2 (4.9%)
	Ser79Ala	1 (2.4%)
	Ser79Phe	14 (34.1%)
	Ser79Tyr	4 (9.8%)

The most common ParC substitutions occurred at position Ser79, with Ser79Phe observed most frequently (34.1% of all resistant isolates).

As shown in table 4, at Ciprofloxacin MIC 2, 4, 8 and 16, 75%, 66.7%, 77.8% and 80% had no substitution in **Gyr B**, 22.2%, 11.1% and 20% at MIC 4, 8 and 16 had Ala583Ser substitution. 25%, 11.1% and 11.1% at MIC 2, 4 and 8 had Arg545Asn substitution.

Regarding Par C, at Ciprofloxacin MIC 2, 4, 8 and 16, 75%, 55.6%, 33.3% had no substitution in Par C, 11.1% and 10% at MIC 8 and 16 had Asp83Asn substitution. Only 10% at MIC 16 had Ser79Ala substitution. 25%, 33.3%, 33.3% and 70% at MIC 2, 4, 8 and 16 had Ser79Phe substitution. At MIC 4, 8 and 16, 11.1, 22.2% and 10% had Ser79Tyr substitution.

Table (4) MICs and substitutions observed in GyrB and ParC in Ciprofloxacin - resistant and intermediate S. pneumoniae isolates.

Variable (MIC)	2	4	8	16
GyrB				
No substitution	3 (75%)	6 (66.7%)	7 (77.8%)	8 (80%)
Ala583Ser	0 (0%)	2 (22.2%)	1 (11.1%)	2 (20%)
Arg545Asn	1 (25%)	1 (11.1%)	1 (11.1%)	0 (0%)
Par C				
No substitution	3 (75%)	5 (55.6%)	3 (33.3%)	0 (0%)
Asp83Asn	0 (0%)	0 (0%)	1 (11.1%)	1 (10%)
Ser79Ala	0 (0%)	0 (0%)	0 (0%)	1 (10%)
Ser79Phe	1 (25%)	3 (33.3%)	3 (33.3%)	7 (70%)
Ser79Tyr	0 (0%)	1 (11.1%)	2 (22.2%)	1 (10%)

As shown in table (5) at Levofloxacin MIC 4, 8 and 16, 75%, 61.5% and 85.7% had no substitution in **Gyr B**, 25%, 23.1% and 7.1% at MIC 4, 8 and 16 had Ala583Ser substitution. 15.4% and 7.1% at MIC 4 and 8 had Arg545Asn substitution.

Regarding Par C, at Levofloxacin MIC 4, 8 and 16, 75%, 30.7%, 14.3% had no substitution in Par C, 7.7% and 7.1% at MIC 8 and 16 had Asp83Asn substitution. Only 7.7% at MIC 8 had Ser79Ala substitution. 25%, 38.5% and 57.1% at MIC 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 15.4 and 14.4% had Ser79Tyr substitution. Only 7.1% at MIC 16 had Ser81Phe substitution.

Table (5) MICs and substitutions observed in GyrB and ParC in Levofloxacin-resistant and intermediate S. pneumoniae isolates.

Variable (MIC)	4	8	16
GyrB			
No substitution	3 (75%)	8 (61.5%)	12 (85.7%)
Ala583Ser	1 (25%)	3 (23.1%)	1 (7.1%)
Arg545Asn	0 (0%)	2 (15.4%)	1 (7.1%)
Par C			
No substitution	3 (75%)	4 (30.7%)	2 (14.3%)
Asp83Asn	0 (0%)	1 (7.7%)	1 (7.1%)
Ser79Ala	0 (0%)	1 (7.7%)	0 (0%)
Ser79Phe	1 (25%)	5 (38.5%)	8 (57.1%)
Ser79Tyr	0 (0%)	2 (15.4%)	2 (14.4%)
Ser81Phe	0 (0%)	0 (0%)	1 (7.1%)

As shown in table 6, at Gatfloxacin MIC 2, 4, 8 and 16, 50%, 66.7%, 66.7% and 100% had no substitution in **Gyr B**, 50% and 25% at MIC 2 and 8 had Ala583Ser substitution. 33.3% and 8.3% at MIC 4 and 8 had Arg545Asn substitution.

Regarding Par C, at Gatfloxacin MIC 2, 4, 8 and 16, 75%, 49.9%, 25% and 11.1% had no substitution in Par C, 16.7% at MIC 8 had Asp83Asn substitution. Also only 16.7% at MIC 4 had Ser79Ala substitution. 25%, 16.7%, 50% and 55.6% at MIC 2, 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 8.3% and 33.3% had Ser79Tyr substitution. Only 16.7% at MIC 4 had Ser81Phe substitution.

Table (6) MICs and substitutions observed in GyrB and ParC in Gatfloxacin-resistant and intermediate S. pneumoniae isolates

Variable (MIC)	2	4	8	16
GyrB				
No substitution	**2 (50%)**	**4 (66.7%)**	**8 (66.7%)**	**9 (100%)**
Ala583Ser	**2 (50%)**	**0 (0%)**	**3 (25%)**	**0 (0%)**
Arg545Asn	**0 (0%)**	**2 (33.3%)**	**1 (8.3%)**	**0 (0%)**
Par C				
No substitution	**3 (75%)**	**3 (49.9%)**	**3 (25%)**	**1 (11.1%)**
Asp83Asn	**0 (0%)**	**0 (0%)**	**2 (16.7%)**	**0 (0%)**
Ser79Ala	**0 (0%)**	**1 (16.7%)**	**0 (0%)**	**0 (0%)**
Ser79Phe	**1 (25%)**	**1 (16.7%)**	**6 (50%)**	**5 (55.6%)**
Ser79Tyr	**0 (0%)**	**0 (0%)**	**1 (8.3%)**	**3 (33.3%)**
Ser81Phe	**0 (0%)**	**1 (16.7%)**	**0 (0%)**	**0 (0%)**

As shown in table 7, at Moxifloxacin MIC 2, 4, 8 and 16, 100%, 44.4%, 10% and 12.5% had no substitution in **Gyr B**, 25%, 22.2% and 20% at MIC 2, 4 and 8 had Ala583Ser substitution. 25%, 11.1% and 12.5% at MIC 2, 4 and 16 had Arg545Asn substitution.

Regarding Par C, at Moxifloxacin MIC 2, 4, 8 and 16, 75%, 49.9%, 25% and 11.1% had no substitution in Par C, 11.2% and 10% at MIC 4 and 8 had Asp83Asn substitution. Also only 10% at MIC 8 had Ser79Ala substitution. 44.4%, 50% and 50% at MIC 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 20% and 25% had Ser79Tyr substitution.

Table (7) MICs and substitutions observed in GyrB and ParC in Moxifloxacin-resistant and intermediate S. pneumoniae isolates

Variable (MIC)	2	4	8	16
GyrB				
No substitution	2 (50%)	6 (66.7%)	8 (80%)	7 (87.5%)
Ala583Ser	1 (25%)	2 (22.2%)	2 (20%)	0 (0%)
Arg545Asn	1 (25%)	1 (11.1%)	0 (0%)	1 (12.5%)
Par C				
No substitution	4 (100%)	4 (44.4%)	1 (10%)	1 (12.5%)
Asp83Asn	0 (0%)	1 (11.2%)	1 (10%)	0 (0%)
Ser79Ala	0 (0%)	0 (0%)	1 (10%)	0 (0%)
Ser79Phe	0 (0%)	4 (44.4%)	5 (50%)	4 (50%)
Ser79Tyr	0 (0%)	0 (0%)	2 (20%)	2 (25%)

Discussion

Ninty one (91%) of isolates in our study were resistant to ampicillin, 5.1% were intermediate and 3.8% were susceptible. Regarding cefaclor 83.3% were resistant, 7.7% were intermediate and 9% were susceptible. Erythromycin was resistant in 82.1% of isolates, intermediate in 10.3%, and susceptible in 7.7%. Regarding imipenem 10.3% of isolates were resistant. Tetracycline was resistant in 71.8% of isolates. Clarithromycin was resistant in only 6.4%, also 10.3% of our isolates were resistant to ceftriaxone.Trimethoprim/sulfametho xazole was resistant in 9% of our isolates.

The MICs of Ciprofloxacin, Levofloxacin, Gatifloxacin and Moxifloxacin were measured in this study and we found that, 44.9% of S. pneumonia isolates were resistant to ciprofloxacin,11.5% were intermediate and 43.6% were sensitive. Regarding levofloxacin 42.3% of isolates were resistant, 9% were intermediate, and 48.7% were sensitive. Over forty six (46.1%) of our isolates were resistant to Gatfloxacin, 10.3% were intermediate, and 43.6% were sensitive. Regarding Moxifloxacin 46.2% of our isolates were resistant, 7.6% were intermediate, and 46.2% were sensitive. Also in study of Karger et al. [(11)] the resistance percentages of all strains to tested antibiotics were as follows: ciprofloxacin 73.33%, ofloxacin 53.33%, norfloxacin 48.89%, and levofloxacin 42.22%. The highest resistance was observed in patients in the age group of 31-40 years [(11)].

Regarding genetic substitution, we found that at ciprofloxacin MIC 2, 4, 8 and 16, 75%, 66.7%, 77.8% and 80% had no substitution in Gyr B, 22.2%, 11.1% and 20% at MIC 4, 8 and 16 had Ala583Ser substitution. 25%, 11.1% and 11.1% at MIC 2, 4 and 8 had Arg545Asn substitution.

In Par C, at ciprofloxacin MIC 2, 4, 8 and 16, 75%, 55.6%, 33.3% had no substitution in Par C, 11.1% and 10% at MIC 8 and 16 had Asp83Asn substitution. Only 10% at MIC 16 had Ser79Ala substitution. 25%, 33.3%, 33.3% and 70% at MIC 2, 4, 8 and 16 had Ser79Phe substitution. At MIC 4, 8 and 16, 11.1, 22.2% and 10% had Ser79Tyr substitution.

Similar to our results, in studies of Bast et al. [(12)], Broskey et al. [(13)], Brueggemann et al. [(14)] the most frequently observed substitutions in ParC were at positions Ser79 (Ala,

Phe or Tyr) (74% of ciprofloxacin-resistant isolates) and Asp83 (Ala, Asn, Gly or Tyr) (15% of ciprofloxacin-resistantisolates).Overall, the most common genotype observed was Ser79Phe (ParC) (35% of ciprofloxacinresistant isolates). The second most common genotype was isolates with a single ParC substitution (Ser79Phe) (13% of ciprofloxacin-resistant isolates). The high prevalence of these substitutions and their association with fluoroquinolone resistance are consistent with observations published by other investigators (Beekmann et al. [15], Jones et al. [16]). Also in study of Korzheva et al. [17] substitutions at Ser79 in ParC are believed to be the most commonly observed substitutions as these positions interact with the fluoroquinolone in the ternary complex.

Regarding levofloxacin, we found that at levofloxacin MIC 4, 8 and 16, 75%, 61.5% and 85.7% had no substitution in Gyr B, 25%, 23.1% and 7.1% at MIC 4, 8 and 16 had Ala583Ser substitution. 15.4% and 7.1% at MIC 4 and 8 had Arg545Asn substitution. In Par C, at levofloxacin MIC 4, 8 and 16, 75%, 30.7%, 14.3% had no substitution in Par C, 7.7% and 7.1% at MIC 8 and 16 had Asp83Asn substitution. Only 7.7% at MIC 8 had Ser79Ala substitution. 25%, 38.5% and 57.1% at MIC 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 15.4 and 14.4% had Ser79Tyr substitution. Only 7.1% at MIC 16 had Ser81Phe substitution. On the other hand, previous studies reported that between 59% and 71% of isolates with levofloxacin MICs of 2 pg/mL had QRDR substitutions in ParC [18, 19].

Regarding gatfloxacin, we found that in Gyr B, at gatfloxacin MIC 2, 4, 8 and 16, 50%, 66.7%, 66.7% and 100% had no substitution, 50% and 25% at MIC 2 and 8 had Ala583Ser substitution. 33.3% and 8.3% at MIC 4 and 8 had Arg545Asn substitution. In Par C, at gatfloxacin MIC 2, 4, 8 and 16, 75%, 49.9%, 25% and 11.1% had no substitution in Par C, 16.7% at MIC 8 had Asp83Asn substitution. Also only 16.7% at MIC 4 had Ser79Ala substitution. 25%, 16.7%, 50% and 55.6% at MIC 2, 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 8.3% and 33.3% had Ser79Tyr substitution. Only 16.7% at MIC 4 had Ser81Phe substitution.

Regarding moxifloxacin, we found that in Gyr B, at moxifloxacin MIC 2, 4, 8 and 16, 100%, 44.4%, 10% and 12.5% had no substitution, 25%, 22.2% and 20% at MIC 2, 4 and 8 had Ala583Ser substitution. 25%, 11.1% and 12.5% at MIC 2, 4 and 16 had Arg545Asn substitution. In Par C, at moxifloxacin MIC 2, 4, 8 and 16, 75%, 49.9%, 25% and 11.1% had no substitution in Par C, 11.2% and 10% at MIC 4 and 8 had Asp83Asn substitution. Also only 10% at MIC 8 had Ser79Ala substitution. 44.4%, 50% and 50% at MIC 4, 8 and 16 had Ser79Phe substitution. At MIC 8 and 16, 20% and 25% had Ser79Tyr substitution. Only 12.5% at MIC 16 had Ser81Phe substitution.

In study of Karger et al. [11] investigated the prevalence of mutations in the parC genes and their role in the development of quinolone resistance. Similar to the findings of other investigations, their results showed the highest prevalence of mutations regarded to the parC gene with 75.56%. The prevalence of this gene was reported to be 67.3% in 2001 in the United States [14], 21.9% in 2005 [20] and 70% in 2009 in Italy [21]. Sierra et al. [22] recently correlated mutagenic potency of the

fluoroquinolones to likelihood of mutant selection. They found levofloxacin and moxifloxacin to be less mutagenic than ciprofloxacin and gemifloxacin and resistant mutants to be selected most commonly by ciprofloxacin followed by gemifloxacin, moxifloxacin and levofloxacin [22].

In study of Brino et al. [23] most isolates had mutations at conventional sites in parC (codons for S79 or D83). However, 4 of 16 isolates with high-level resistance to one or more fluoroquinolones did not contain a mutation in the codon for S79 of ParC, the amino acid position most frequently reported to be associated with resistance of pneumococci to this class of agents. In addition, 3 of these 16 isolates had multiple mutations that included sites in gyrB. Fass et al. [24] found that only the MICs of levofloxacin and ofloxacin were increased with the introduction of this mutation into parE. However, when the same mutation occurred in gyrB, MICs were increased for all fluoroquinolones except moxifloxacin and gemifloxacin. Interestingly, GyrB mutants (changes of either E474K or D435E) were important for resistance to gatifloxacin but not to moxifloxacin. The chemical structures of these 8-methoxyquinolones differ only in their C-7 substituents [25, 26].

In addition to gyrB mutations, a novel amino acid change of A63T in ParC was detected in one isolate by Yoshida et al. [27]. Unlike most parC mutations in pneumococci, this alteration could not be selected on levofloxacin as a first-round transformant. However, when the parC gene fragment encoding the A63T change was introduced after the 4571 gyrB mutations, MICs of five fluoroquinolones increased two- to fourfold, indicating that mutations affecting this amino acid position make significant contributions to resistance. No alterations have been reported for this amino acid position in S. pneumonia [27]. Fukushima et al. [28] also reported the use of melting curve analysis for the detection of QRDR mutations in S. pneumoniae. They used two pairs of probes for the detection of the Ser79 and Asp83 mutations in ParC [28].

Conclusion

The present study provide an opportunity to view the predominant mutations conferring reduced susceptibility to FQs in clinical pneumococcal isolates.

Recommandations

We recommend:

1. Close attention to monitor fluoroquinolone susceptibility patterns and the association of multidrug resistance with fluoroquinolone resistance in isolates of S.pneumoniae.
2. The increased prescription of fluoroquinolones as first-line therapy for common infections such as respiratory tract infection will facilitate the emergence of resistance to this class of compounds and promote the emergence of multidrug-resistant strains and, therefore, should be discouraged as it will undermine the efficacy of fluoroquinolones to treat more-serious infections.
3. Continued surveillance of respiratory tract isolates and other pathogens is important, and appropriate clinical use of fluoroquinolones is imperative as they become more widely prescribed.
4. Further studies in larger numbers of patients are necessary to establish the role of gene substitution in (QRDRs) in S. pneumonia isolates and resistance to Fluoroquinlones.

5. Future prevalence studies will be able to track changes in the predominant mutations conferring resistance to FQs
6. In order to accurately analyze the increasing trend of fluoroquinolone resistance-associated substitutions in fluoroquinolone-susceptible S. pneumoniae isolates, the study of susceptible isolates will need to be repeated in a few years in different and variable hospitals and clinics in our country.
7. The maintenance of such surveillance is valuable in the preparation of future therapy guidelines and could lead to new therapeutic strategies for FQ-resistant S.Pneumoniae.

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