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Genetic diversity of *Streptococcus pneumoniae* in Tunisia

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ABSTRACT

Objectives: This study aimed to explore the genetic diversity of *Streptococcus pneumoniae* isolates in a Tunisian pneumology hospital.
Methods: A total of 141 *S. pneumoniae* strains isolated between 2009–2016 in the microbiology laboratory at A. Mami Hospital of Pneumology were investigated. Antimicrobial susceptibility testing was performed the disk diffusion method. MICs of penicillin G, amoxicillin and cefotaxime were determined by Etest. Serotyping was inferred from the results of multiplex PCR targeting 40 serotypes. Sequence types (STs) were determined by multilocus sequence typing (MLST).
Results: Among the 141 *S. pneumoniae* isolates, 98 (69.5%) were resistant to erythromycin. Evaluation of β-lactam susceptibility showed that 90 strains (63.8%) were non-susceptible to penicillin, whereas 48 (34.0%) had decreased susceptibility to amoxicillin and 21 (14.9%) to cefotaxime. Twenty-five serotypes were detected, and 10 isolates were classified as non-typeable. Vaccine coverage was 56.7%, 60.3% and 75.2% for pneumococcal conjugate vaccine 7 (PCV7), PCV10 and PCV13, respectively. Overall, 73 STs were identified, including 23 described for the first time. The most frequent STs were ST179 ($n=17$), ST3772 ($n=14$), ST2918 ($n=10$) and ST4003 ($n=5$), related to serotypes 19F, 19A, 14 and 23F, respectively. Moreover, 110 strains were classified within 45 STs. Three international antimicrobial-resistant clones were found, including Denmark^{14}-ST230 ($n=22$), Spain^{9V}-ST156 ($n=22$) and Portugal^{19F}-ST177 ($n=20$).
Conclusion: This study emphasises the clonal and international dissemination of antimicrobial-resistant *S. pneumoniae* clones. Significant differences in genetic variation were documented by MLST within the various serotypes identified.

1. Introduction

Streptococcus pneumoniae is a Gram-positive bacterium that colonises the nasopharynx of humans. Usually, episodes of colonisation are essentially asymptomatic and do not lead to illness. However, this balance between host and bacteria can be altered when innate and/or adaptive immune mechanisms are weakened, a situation that is more common in extreme ages and immunocompromised people. *Streptococcus pneumoniae* can cause various types of infections, with the most serious being meningitis and septicaemia [1,2].

The majority of pneumococcal strains have a polysaccharide capsule that acts as an important virulence factor. Based on antigenic characteristics of the polysaccharide capsule, more than 90 serotypes of *S. pneumoniae* have been identified [3]. Naturally susceptible to many antibiotic families, *S. pneumoniae* has gradually acquired resistance to sulfonamides (1943), tetracycline (1963), erythromycin (1967), penicillin (1967) and chloramphenicol (1970) [4].

Regarding pneumococcal vaccines, a vaccine composed of serotypes 1 and 2 was prepared in 1938. A 14-valent vaccine was marketed in the USA in 1977, and in 1983 a 23-valent vaccine was used in France. All of these vaccines were of polysaccharidic nature and were therefore not immunogenic in children <2 years of age. In 2000, a heptavalent conjugate vaccine was marketed containing the seven most common and antimicrobial-resistant serotypes of *S. pneumoniae*, which was immunogenic in children aged <2 years [5]. Currently, two pneumococcal conjugate vaccines (PCVs) are available, the 13-valent vaccine containing serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F, and the 10-valent vaccine containing serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F.

Typing methods for studying the epidemiology of *S. pneumoniae* were initially based on phenotypic markers such as optochin test, bile solubility, Gram staining, latex agglutination and serogrouping.

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However, these markers remain unsatisfactory and lack discriminatory power. Genotypic methods developed later used analysis of chromosomal DNA by gene amplification. This molecular typing method offers an optimal combination of technical feasibility, discriminatory power and ease of interpretation. However, the results obtained are not exploitable on a global scale and do not allow the phylogenetic placing of tested isolates. More recently, analysis of resistance gene polymorphism has emerged as an attractive approach to link epidemiology and resistance. Multilocus sequence typing (MLST) analysis is used to develop a macroepidemiological and phylogenetic analysis of *S. pneumoniae*. This technique is based on nucleotide sequence comparison of seven gene polymorphisms (*aroE, gdh, gki, recP, spi, xpt* and *ddl*) distributed over the bacterial chromosome and conserved during evolution. This technique makes it possible to determine the 'sequence type' (ST) as well as the clonal complex (CC) to which typed isolates belong. The sequences (and the alleles that they define) are exportable and can be gathered in a centralised database, accessible online (http://spneumoniae.mlst.net), which makes it possible to compare the strain that a laboratory has typed with international data available on the same species.

The aim of the present study was to characterise invasive and non-invasive *S. pneumoniae* isolates collected in a pneumology hospital in Tunisia from 2009–2016 based on analysis of serotypes, antimicrobial susceptibility patterns and STs obtained by MLST.

2. Materials and methods

2.1. Pneumococcal isolates

This study investigated 141 *S. pneumoniae* isolates obtained between 2009 and 2016 in the microbiology laboratory of A. Mami Hospital of Pneumology (Ariana, Tunisia).

Species identification was based on the following characteristics: α-haemolysis; Gram staining; optochin susceptibility; reactivity with the Slidex® Pneumo Kit (bioMérieux, Marcy-l'Étoile, France); and amplification of the *cpsA* gene as previously described [6]. All strains were stored at −80°C on porous beads until further processing.

2.2. DNA extraction

Genomic DNA was extracted from fresh bacterial cultures using a QIAamp® Tissue Kit (QIAGEN, Hilden, Germany).

2.3. Serotyping

Serotyping was inferred from the results of a multiplex PCR method able to discriminate 40 serotypes [1, 2, 3, 4, 5, 6A/B, 6C, 7F, 7C/7B/40, 8, 9N/L, 9V, 10A, 10F/10C/33C, 11A, 12F, 13, 14, 15A/F, 15B/C, 16F, 17F, 18, 19A, 19F, 20, 21, 22F/A, 23A, 23B, 23F, 24(A, B, F), 31, 33F, 34, 35A, 35B, 35F, 38/25F and 39] as described previously [7,8].

2.4. Multilocus sequence typing (MLST)

Streptococcus pneumoniae STs were identified by MLST. The pneumococcal typing system is based on the internal fragments of seven housekeeping genes, including: shikimate dehydrogenase (*aroE*); glucose-6-phosphate dehydrogenase (*gdh*); glucose kinase (*gki*); transketolase (*recP*); signal peptidase (*spi*); xanthine phosphoribosyltransferase (*xpt*); and D-alanine-D-alanine ligase (*ddl*). Primer pairs used to amplify these seven genes have been described previously by Adamiak et al. [9]. Allele numbers and STs were named according to the *S. pneumoniae* MLST database (https://pubmlst.org/spneumoniae). Phylogenetic relationships between STs were estimated using the freely available PHYLOViZ software [10].

CCs were assigned as groups of STs sharing six or more identical housekeeping alleles. STs belonged to the same CC if they shared five MLST allele numbers, with the CC being named after the putative founder of the cluster. ST profiles of PMEN (Pneumococcal Molecular Epidemiology Network) clones 1–43 were determined using the PMEN website (http://www.sph.emory.edu/PMEN). Isolates that could not be classified into any CC owing to lack of close relationships were designated as singletons.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the antibiogram method on 5% horse blood-enriched Mueller–Hinton agar as recommended by the Antibiogram Committee of the French Society for Microbiology (CA-SFM) 2013 guidelines. The antimicrobial agents tested included oxacillin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), lincomycin (15 µg), trimethoprim/sulfamethoxazole (SXT) (23.75/1.25 µg), vancomycin (30 µg), levofloxacin (5 µg) and rifampicin (5 µg). Isolates with an inhibition zone for the 5-µg oxacillin disk of <26 mm were screened for non-susceptibility to penicillin as follows. Minimum inhibitory concentrations (MICs) of penicillin G, amoxicillin and cefotaxime were determined by Etest (AB BIODISK, Stockholm, Sweden) for all putative penicillin-non-susceptible pneumococci. The MIC breakpoints used were those of the CA-SFM 2013, as follows: penicillin G, 0.06–1 mg/L; and amoxicillin and cefotaxime, 0.5–2 mg/L. An internal quality control was performed using *S. pneumoniae* ATCC 49619. Multidrug resistance was defined as resistance to three or more antibiotic families.

3. Results

3.1. Bacterial isolates

Strains from respiratory tract samples represented 83.7% (118/141) of cases, including 58.5% (69/118) obtained from protected respiratory samples in hospitalised patients with pneumonia (protected distal bronchial specimen and bronchoalveolar lavage) and 41.5% (49/118) of strains from unprotected pulmonary samples (sputum and lung aspirate). The remaining of 23 strains (16.3%) were isolated from deep pus, blood, pleural fluid and ear (Table 1). Among the 141 isolates, 106 (75.2%) and 35 (24.8%) were obtained from male and female patients, respectively (Table 2). Moreover, 123 strains (87.2%) were isolated from adults and 18 strains (12.8%) were from children aged <15 years (Table 2).

3.2. Serotype distribution

A total of 131 isolates could be classified within 25 distinct serotypes. The remaining 10 isolates (7.1%) were classified as non-typeable. The prevalent serotypes were 19F ($n = 28$; 19.9%), 14 ($n = 22$; 15.6%), 23F ($n = 16$; 11.3%), 19A ($n = 15$; 10.6%) and 6A/B ($n = 7$; 5.0%). The serotypes detected were covered by PVC7, PCV10 and PCV13 in 56.7%, 60.3% and 75.2% of isolates, respectively (Fig. 1).

3.3. Multilocus sequence typing

The 141 pneumococcal isolates tested in this study were distributed into 73 distinct STs (Fig. 2). Among the 73 STs identified, 23 were newly assigned (ST13520, ST13521, ST13522, ST13523, ST13524, ST13525, S13526, ST13527, ST13528, ST13529, ST13530, ST13531, ST13532, ST13533, ST13534, ST13535, ST13536, ST13537,

Table 1
Characteristics of 141 *Streptococcus pneumoniae* isolates with regard to penicillin G (PEN) and erythromycin (ERY) resistance, serotype and multilocus sequence typing (MLST) profile.

Predicted ST/CC (no. of isolates)	MLST	Associated PMEN clone	Source of isolation/year	Resistance		Serotype
				ERY	PEN	
230 ($n=22$)	ST230 (1)	Denmark^{14}-32	PDP/2009	22 R	22 PNSP	14
	ST276 (1)	Denmark^{14}-32 SLV	Ear			19A
	ST4253 (1)		Sputum/2015			24(A/B/F)
	ST2307 (3)	Denmark^{14}-32 DLV	PDP/2013 Sputum/2014 Sputum/2016			19F
	ST13527 (1)		Deep pus/2013			NT
	ST13536 (1)		PDP/2012			19F
	ST3772 (14)	Denmark^{14}-32 TLV	BAL/2012 BAL/2013 BAL/2015 Lung aspirate/2012 Lung aspirate/2016 ($n=2$) PDP/2015 Sputum/2014 ($n=4$) Sputum/2016 ($n=2$) Ear/2015			19A
156 ($n=22$)	ST156 (3)	Spain^{9V}-3	BAL/2010	R	PNSP	14
			Blood/2009	S	PNSP	
			Sputum/2009	R	S	9V
	ST334 (1)	Spain^{9V}-3 SLV	Sputum/2012	R	PNSP	
	ST838 (2)		Deep pus/2014	S	PNSP	
			BAL/2015	S	PNSP	
	ST2918 (10)		PDP/2011 Blood/2011 BAL/2012 BAL/2015 Deep pus/2015 Sputum/2013 Sputum/2014 ($n=2$) Sputum/2015 Lung aspirate/2016	10 R	2 S; 8 PNSP	14
	ST143 (3)	Spain^{9V}-3 DLV	PDP/2010	R	PNSP	
			Blood/2015	R	PNSP	
			PDP/2015	S	PNSP	
	ST2306 (1)		Sputum/2010	R	PNSP	
	ST13535 (1)		BAL/2016	R	PNSP	
	ST13542 (1)		Sputum/2010	R	PNSP	
177 ($n=20$)	ST179 (17)	Portugal^{19F}-21 SLV	Sputum/2016 Lung aspirate/2010 Lung aspirate/2013 Sputum/2010 Sputum/2011 Sputum/2015 ($n=4$) PDP/2010 BAL/2012 BAL/2015 ($n=4$) Pleural fluid/2012 Blood/2016	R	PNSP	19F
	ST3682 (1)		BAL/2013			
	ST13528 (1)	Portugal^{19F}-21 DLV	Lung aspirate/2014			
	ST13524 (1)	Portugal^{19F}-21 TLV	BAL/2015		S	
81 ($n=12$)	ST81 (3)	Spain^{23F}-1	PDP/2010	3 R	3 PNSP	23F
			PDP/2015	4 R		
			Lung aspirate/2013	5 R		
	ST326 (1)	Spain^{23F}-1, SLV	Lung aspirate/2015	R	PNSP	
	ST4003 (5)	Spain^{23F}-1, SLV	PDP/2010 PDP/2015 BAL/2016 Sputum/2009 Sputum/2015	5 R	5 PNSP	
	ST13522 (1)	Spain^{23F}-1, DLV	Sputum/2015	R	PNSP	
	ST13529 (1)	Spain^{23F}-1, SLV	Sputum/2014	R	PNSP	
	ST13532 (1)	Spain^{23F}-1, DLV	Sputum/2016	R	PNSP	
63 ($n=6$)	ST62 (3)	Sweden^{15A}-25, TLV	BAL/2015 ($n=2$) PDP/2015	3 S	3 S	11A
	ST63 (2)	Sweden^{15A}-25	Sputum/2014	2 R	S	15A/F
			Pleural fluid/2015		PNSP	23F
	ST408 (1)	Sweden^{15A}-25, TLV	BAL/2015	S	S	11A

(*continued on next page*)

Table 1 (*continued*)

Predicted ST/CC (no. of isolates)	MLST	Associated PMEN clone	Source of isolation/year	Resistance		Serotype
				ERY	PEN	
180 ($n=5$)	ST180 (4)	Netherlands^{3}-31	BAL/2013 ($n=2$) BAL/2015 Blood/2016	5 S	4 S	3
	ST505 (1)	Netherlands^{3}-31, DLV	BAL/2015		PNSP	
315 ($n=4$)	ST386 (3)	Poland^{6B}-20, DLV	PDP/2010 Sputum/2009 Sputum/2010	4 R	PNSP PNSP PNSP	6A/B
	ST13537(1)	Poland^{6B}-20, TLV	Blood/2016		S	
377 ($n=4$)	ST558 (4)	Utah^{35B}-24, SLV	BAL/2013 BAL/2014 Sputum/2015 Sputum/2015	4 R	PNSP S PNSP PNSP	35B
338 ($n=3$)	ST338 (2)	Colombia^{23F}-26	Sputum/2015 Blood/2016	R S	S S	23F
	ST360 (1)	Colombia^{23F}-26, DLV	Pleural fluid/2015	S	PNSP	23F
90 ($n=2$)	ST90 (2)	Spain^{6B}-2	PDP/2009 BAL/2009	2 R	2 PNSP	6A/B
218 ($n=2$)	ST3544 (2)	Denmark^{12F}-34, SLV	Blood/2014 PDP/2015	2 S	2 S	7F
113 ($n=1$)	ST113	Netherlands^{18C}-36	Lung aspirate/2016	R	PNSP	18
289 ($n=1$)	ST289	Colombia^{5}-19	Blood/2014	S	S	5
306 ($n=1$)	ST306	Sweden^{1}-28	Pleural fluid/2015	S	S	1
273 ($n=1$)	ST2325	Greece^{6B}-22, DLV	BAL/2016	R	PNSP	NT
9 ($n=1$)	ST2601	England^{14}-9, TLV	PDP/2015	S	PNSP	NT
37 ($n=1$)	ST8959	Tennessee^{23F}-4, TLV	BAL/2015	S	S	23B
91 ($n=1$)	ST9317	Netherlands^{7F}-39, SLV	BAL/2010	S	S	20
327 ($n=1$)	ST13525	Portugal^{6A}-41, TLV	PDP/2016	R	PNSP	19F
Singleton ($n=31$)	ST135	Singleton	Blood/2009	R	PNSP	20
	ST241	Singleton	Pleural fluid/2011	S	S	18
	ST346	Singleton	Sputum/2011	R	PNSP	15A/B
	ST404 (2)	Singleton	BAL/2009 BAL/2012	2 S	2 S	8
	ST1050	Singleton	BAL/2010	R	S	11A
	ST1220	Singleton	BAL/2010	S	S	3
	ST1766 (2)	Singleton	BAL/2009 BAL/2015	2 S	2S	31
	ST2358	Singleton	BAL/2013	S	S	4
	ST2567	Singleton	PDP/2014	S	S	NT
	ST3751	Singleton	PDP/2015	S	S	NT
	ST5969	Singleton	Deep pus/2009	S	S	NT
	ST8605	Singleton	Sputum/2015	R	S	11A
	ST8919	Singleton	PDP/2011	R	PNSP	
	ST9210	Singleton	BAL/2013	S	S	NT
	ST9338 (2)	Singleton	PDP/2009 BAL/2013	2 S	2 S	14
	ST13010	Singleton	BAL/2015	S	PNSP	17F
	ST13520	Singleton	PDP/2010	R	S	34
	ST13521	Singleton	Sputum/2013	R	PNSP	7C/7B/40
	ST13523	Singleton	PDP/2013	R	S	NT
	ST13526	Singleton	Sputum/2012	R	PNSP	19F
	ST13530	Singleton	Deep pus/2013	S	PNSP	17F
	ST13531	Singleton	BAL/2012	S	S	NT
	ST13533	Singleton	PDP/2010	S	S	14
	ST13534	Singleton	Ear/2009	R	PNSP	15A/F
	ST13538	Singleton	PDP/2010	S	S	5
	ST13539	Singleton	BAL/2010	S	PNSP	19F
	ST13540	Singleton	PDP/2010	S	S	19F
	ST13541	Singleton	BAL/2013	S	PNSP	6A/B

BAL, bronchoalveolar lavage; CC, clonal complex; DLV, double-locus variant; NT, non-typeable; PDP, protected distal bronchial specimen; PMEN, Pneumococcal Molecular Epidemiology Network; PNSP, penicillin-non-susceptible pneumococci; R, resistant; S, susceptible; SLV, single-locus variant; ST, sequence type; TLV, triple-locus variant.

Table 2
Patient characteristics and isolation of *Streptococcus pneumoniae*.

	Respiratory tract samples ($n=118$)	Non-respiratory samples ($n=23$)
Age		
Children aged <15 years ($n=18$; 12.8%)	18	0
Adults aged 15–85 years ($n=123$; 87.2%)	100	23
Sex		
Male ($n=106$; 75.2%)	88	18
Female ($n=35$; 24.8%)	30	5

ST13538, ST13539, ST13540, ST13541 and ST13542) (Table 1). The ST179 ($n=17$), ST3772 ($n=14$), ST2918 ($n=10$) and ST4003 ($n=5$) isolates were related to serotypes 19F, 19A, 14 and 23F, respectively. STs that exhibited more than one serotype included ST63 (serotypes 11A, 15A/F and 23F), ST156 (serotypes 9V and 14) and ST230 [serotypes 14, 19F, 19A and 24(A/B/F)]. A total of 110 strains were classified within 45 STs. These strains belonged to 19 clonal groups, whilst 28 STs (31 isolates) were singletons (Table 1). Among 43 PMEN clones, 19 included 110 isolates. Furthermore, CC230 ($n=22$ isolates), CC156 ($n=22$) and CC177 ($n=20$) were the most frequent CCs, constituting 45.4% of all tested isolates.

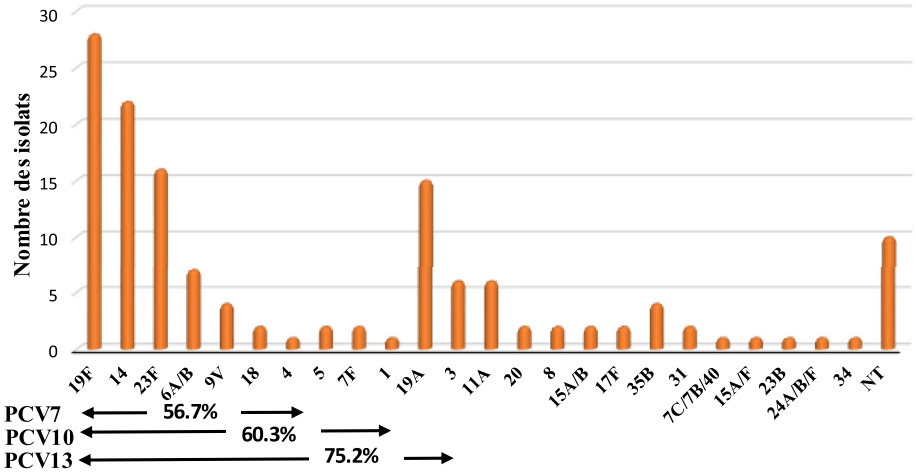

Fig. 1. Serotype distribution and vaccine coverage among studied *Streptococcus pneumoniae* isolates. PCV7, 7-valent pneumococcal conjugate vaccine; PCV10, 10-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; NT, non-typeable.

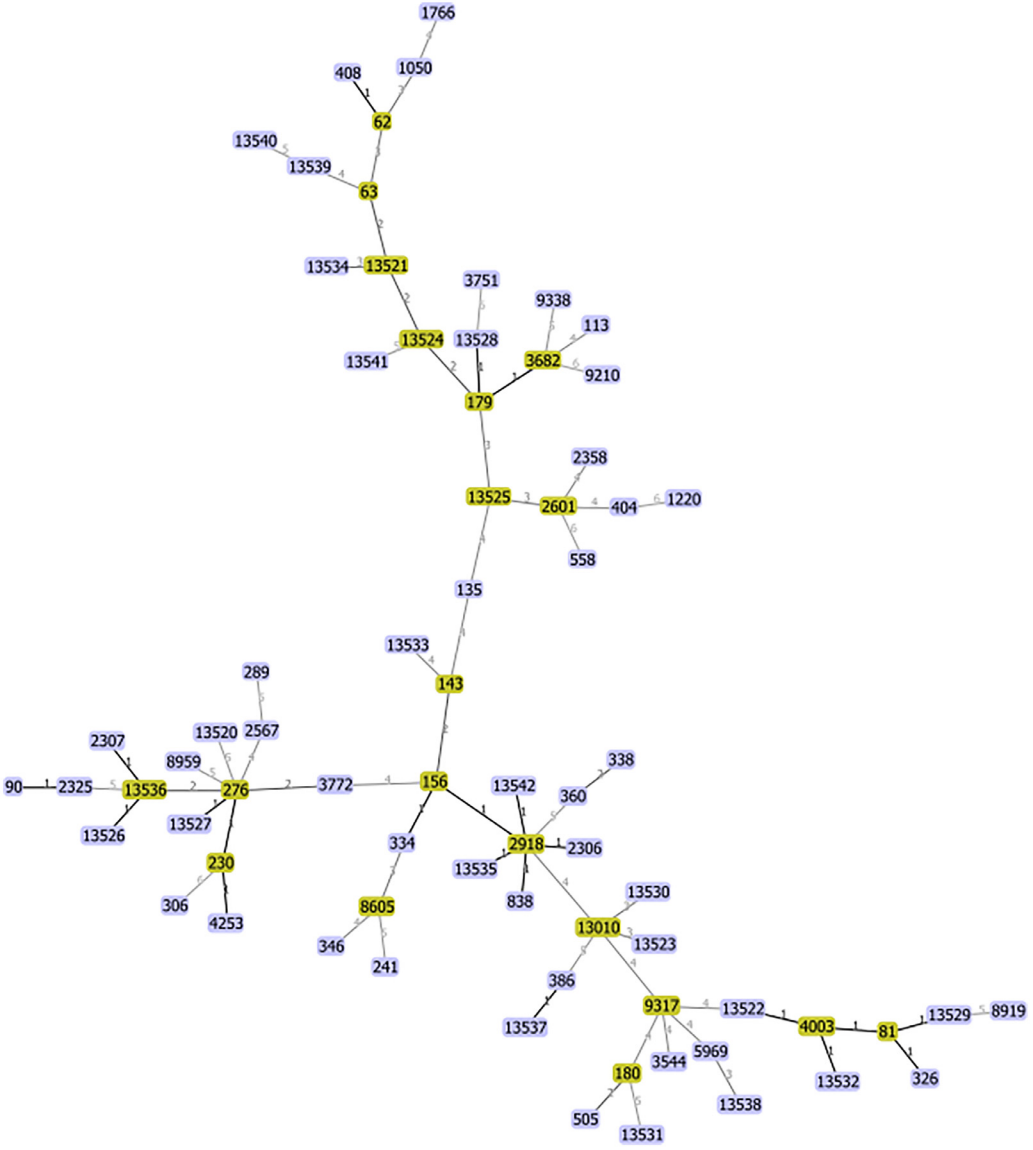

Fig. 2. Relationship between sequence types of pneumococcal isolates obtained in 2009–2016 constructed using goeBURST analysis.

3.4. Antimicrobial resistance

Among the 141 pneumococcal isolates, 90 (63.8%) were non-susceptible to penicillin G, whereas 48 (34.0%) exhibited decreased susceptibility to amoxicillin and 21 (14.9%) to cefotaxime. Regarding macrolides, 98 isolates (69.5%) were resistant to erythromycin, including inducible macrolide–lincosamide–streptogramin B ($iMLS_B$) phenotype (13.3%; 13/98), constitutive MLS_B ($cMLS_B$) phenotype (75.5%; 74/98) and M phenotype (11.2%; 11/98).

Of the 141 isolates, 93 (66.0%) of were multidrug-resistant (MDR). The most common MDR pattern was erythromycin/SXT/lincomycin/tetracycline/β-lactams.

Resistance to antimicrobials was more associated with ST than with serotype. Importantly, the second most frequent ST detected in this study (ST3772) represented 9.9% of tested isolates. ST3772 was associated with serotype 19A. In 2012–2016, serotype 19A isolates were resistant to both penicillin and erythromycin. CC230, CC156 and CC177 were the main resistant clones to penicillin and erythromycin.

4. Discussion

This study aimed to describe the genotypic diversity of pneumococcal isolates from invasive and non-invasive infections in Ariana, northern Tunisia. The predominant *S. pneumoniae* serotypes identified in this study were 19F ($n=28$; 19.9%), 14 ($n=22$; 15.6%), 23F ($n=16$; 11.3%), 19A ($n=15$; 10.6%) and 6A/B ($n=7$; 5.0%), accounting for 62.4% of the isolates. In a previous study carried out in Tunisia during 2000–2009, serotype 14 was the most frequently isolated (22.2%), followed by serotypes 19F (15.5%), 23F (10.3%) and 6B [11]. The predominant pneumococcal serotypes isolated from immunocompromised patients in Tunisia during 2005–2011 were 19F (17%), 23F (17%), 14 (13.5%) and 6B (10.1%), which is similar to the current findings [12]. However, in the current study the predominant serotypes included 19A from 2012. Following the introduction of PCV7, serotype replacement, especially serotype 19A, has been observed worldwide [13,14]. A high prevalence of serotype 19A has been noted in other Tunisian studies [11,15–17]. In Casablanca, Morocco, the most prevalent serotypes in descending order were 14 (16.1%), 6B (13.9%), 19A (11.4%), 19F (8.9%) and 23 F (8.9%) [18]. In a previous study, we also observed that among 187 pneumococcal isolates, the most common serotypes were 19F (31.6%), 19A (19.8%), 23F (11.2%), 6A (9.1%) and 14 (9.1%) [19]. However, in a systematic analysis of worldwide data, serotype 14 was the most common cause of invasive pneumococcal infections worldwide [20].

In the present study, the coverage rates of PVC7, PCV10 and PCV13 were 56.7%, 60.3% and 75.2% for total isolates, respectively. These findings are similar to recent results from Sfax, Tunisia [16]. PCV7 was introduced in Tunisia in 2008, then PVC10 and PVC13 became available in 2012–2013. The coverage rate of PCV13 was significantly higher than that of PCV10 because of the increased coverage of serotypes 3, 6A/B and 19A. The serotype coverage of PCV10 and PCV13 was statistically higher than the coverage of PCV7.

Using MLST, a wide genotypic diversity was identified among pneumococcal isolates from Tunisia. However, although 73 different STs were identified, isolates were assigned to only 19 CCs and there were large numbers of singletons (28 STs with 33 isolates). Also, 16.3% of isolates exhibited new STs. In this study, CC230 was the most frequent clonal complex, comprising seven STs including ST3772, ST2307, ST230, ST276 and others. The seven STs (22 isolates; 15.6%) were associated with international antimicrobial-resistant Denmark^{14}-32 clone ST230 (defined by the PMEN). The serotypes of strains included in CC230 were 14, 19A, 19F and 24(A/B/F), except for 1 non-typeable strain. CC156 included serotypes 14 and 9V isolates, which are related to Spain^{9V}-3 clone ST156. Likewise, the current results suggest that the pneumococcal clonal types identified in northern Tunisia are similar to those prevalent in many countries, including Portugal, Poland, Russia, Japan, Brazil, Italy and France, where high prevalences of Denmark^{14}-32 clone ST230 and Spain^{9V}-3 clone ST156 have been reported [21–27]. Furthermore, 20 isolates belonging to CC177 were classified into four STs, which are, in the database, mainly associated with serotype 19F strains and related to Portugal^{19F}-21 clone ST177. A study performed in 2015 in Tunisia had already detected this same clone [12]. In addition, the Portugal^{19F}-21 clone ST177 has been reported as the most frequent clone in Italy [26].

The emergence of STs including more than two serotypes owing to horizontal transfer of capsule genes has well been described [28] and there were several examples of capsular switching events in this study: ST63 (serotypes 11A, 15A/F and 23F); ST156 (serotypes 9V and 14); and ST230 [serotypes 14, 19F, 19A and 24(A/B/F)].

Among the 141 studied pneumococcal isolates, 90 (63.8%) were non-susceptible to penicillin. These results are similar to those in Tunisia where a study of susceptibility to β-lactams has shown that 75.3% of strains are non-susceptible to penicillin G [29]. This resistance level is much lower in *S. pneumoniae* strains isolated in France (25.7% for penicillin G) [30] and Iran (22% for penicillin G) [29]. In Brazil, 14% of pneumococci express non-susceptibility to penicillin G [31].

For erythromycin, the non-susceptible frequency in the current study was 69.5% (98/141). These results are similar to those obtained in Saudi Arabia where the non-susceptible rate was 72% [32] and in Korea (74.7%) [33]. In addition, erythromycin non-susceptibility is low in Brazil (6%) [31]. However, this rate is higher than the rate observed in Bulgaria (43.9%) [34].

In addition, in this study a predominance of $cMLS_B$ phenotype was observed (75.5%; 74/98), followed by the $iMLS_B$ phenotype (13.3%; 13/98). Finally, 11 strains (11.2%) exhibited the M phenotype. In parallel, these findings were also supported by an earlier study in northern Lebanon showing that $cMLS_B$ (68.9%; 31/45) was the highest prevalence resistance phenotype in macrolide-resistant *S. pneumoniae*, followed by M (28.9%; 13/45) and $iMLS_B$ (2.2%; 1/45) [35]. Whereas another study conducted in Tunisia reported $iMLS_B$ (80.5%; 33/41) as the predominant phenotype in macrolide-resistant *S. pneumoniae*, followed by M phenotype (9.8%; 4/41) [36].

Importantly, the second most frequent ST detected in the current study, representing 9.9% of tested isolates, was ST3772. ST3772 was associated with serotype 19A. In 2012–2016, Tunisian serotype 19A isolates were resistant to both penicillin and erythromycin, as was reported in France and Argentina [27,37]. There are many studies regarding the increase of serotype 19A isolates worldwide, mainly associated with vaccine-induced serotype replacement, antibiotic pressure, introduction of new clones and/or the increase of previously circulating clones [38].

Finally, dissemination of antimicrobial-resistant clones is recognised as an important factor in the emergence and prevalence of pneumococcal resistance. The evolution of antibiotic resistance has been greatly influenced by changes in serotype (e.g. 19A) distribution under vaccine pressure.

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Competing interests

None declared.

Ethical approval

Not required.

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