

Original Article

## Clinical optochin resistant *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* strains in Tunisia

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### Abstract

**Introduction:** *Streptococcus pneumoniae* can be responsible for severe human infections. Optochin resistance has been a potential cause of misidentification of pneumococcus and other members of the mitis group. Hence, rapid and easy optochin resistant (Opt<sup>r</sup>) *S. pneumoniae* identification is essential.

**Methodology:** Atypical pneumococci were characterized using optochin susceptibility, bile solubility based on spectrophotometric reading, serotyping, pulsed field gel electrophoresis (PFGE), 16S rRNA sequencing and PCR-based assays targeting pneumococcal genes *lytA*, *ply*, *pspA*, *cpsA*, *Spn9802* and *Spn9828*.

**Results:** Optical density values for the bile solubility test suggest the identification of four Opt<sup>r</sup> *S. pneumoniae* and one *Streptococcus pseudopneumoniae*. All Opt<sup>r</sup> pneumococci harbored *cpsA*, *lytA*, *ply*, *Spn9802*, *Spn9828* and *pspA* genes. Only *ply*, *spn9802* and *Spn9828* genes were detected in *S. pseudopneumoniae*. The 16S rRNA sequencing differentiates between these two species. Opt<sup>r</sup> *S. pneumoniae* strains belonged to different genotypes and serotypes (14, 19A, 3 and 9V). Three Opt<sup>r</sup> *S. pneumoniae* isolates were typed as *pspA* family 2, while one belonged to *pspA* family 1. Sequencing of the *atpA* and *atpC* gene of the Opt<sup>r</sup> variants revealed three mutations in the ATPase a-subunit (L99I, M23V and V52I) and one mutation in ATPase c-subunit (V48I).

**Conclusions:** Our data indicate that bile OD-values provides an accurate, fast and easy method to discriminate between Opt<sup>r</sup> *S. pneumoniae* and other *Streptococcus mitis* group. Moreover molecular techniques, confirming the bile test, can be used in order to prevent these atypical pneumococci and alert clinical microbiologists of the presence of these strains in the community.

**Key words:** *S. pneumoniae*; *S. pseudopneumoniae*; optochin resistance; bile solubility; molecular techniques.

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### Introduction

*Streptococcus pneumoniae* is one of the most important human pathogen worldwide and a leading cause of invasive and non-invasive infections in children and elderly. Differentiation between *S. pneumoniae* and the other alpha-hemolytic streptococci is conventionally based on biochemical and phenotypic characteristics, most commonly optochin susceptibility and bile solubility. However, since 1987, the emergence of optochin-resistance has been described from diverse geographic areas [1–8]. In Tunisia, Raddaoui *et al.* reported three Opt<sup>r</sup> *S. pneumoniae* strains isolated from immunocompromised patients between 2005 and 2011 [7].

Most of clinical microbiology laboratories used the identification procedure based on optochin susceptibility testing. Therefore, optochin resistance has been a potential cause of misidentification of

pneumococcus due to the complexity to distinguish Opt<sup>r</sup> *S. pneumoniae* from *S. pseudopneumoniae* or other closely related streptococci [9–18]. The low incidence and potential pathogenic role of *S. pseudopneumoniae* has been demonstrated in a murine model as well as in humans [11,13]. This bacterium may be associated with chronic obstructive pulmonary disease [12,18]. Thus, the differentiation of *S. pneumoniae* from *S. pseudopneumoniae* remains important. Despite having 99% 16S rRNA gene identity with *S. pneumoniae*, *S. pseudopneumoniae* is phenotypically distinct [9]. *S. pseudopneumoniae* is optochin resistant in the presence of 5% CO<sub>2</sub> and optochin susceptible in ambient atmosphere, bile insoluble and lacks the pneumococcal capsule [9].

As biochemical tests are often insufficient to distinguish between these species several molecular methods have been proposed. Among these methods

16S rRNA sequencing, PCR targeting the capsular polysaccharide biosynthesis gene A (*cpsA*) or specific pneumococcal virulence factors such as autolysin A (*lytA*), pneumolysin (*ply*), pneumococcal surface protein A (*pspA*), DNA fragment of unknown function (*Spn9828* and *Spn9802*), and competence-stimulating peptide ComC sequence (*comC*) were described [9,10,13,14,17]. In addition, Multilocus sequence typing (MLST) has already been established as important diagnostic approach, but this method is difficult to carry out for large-scale and routine clinical diagnosis.

The aim of this study is to use an accurate phenotypic and molecular testing for identification of optochin resistant *S. pneumoniae* and *S. pseudopneumoniae* isolates.

### Methodology

#### Clinical isolates

Five atypical Opt<sup>r</sup> pneumococci isolates were collected between 2014 and 2016 at Habib Bourguiba University Hospital Sfax-Tunisia. Of these strains, four isolates (Sp22, Sp125, Sp358, Sp345) were bile soluble and one strain (P1058) showed a difficulty to interpret the bile test based on visual evaluation. All strains were from non-invasive sites; sputum and ear exudates (Table 1). To ensure the phenotypic methods, two susceptible optochin *S. pneumoniae*, one *Streptococcus mitis* and one *Streptococcus oralis* strains were included in our study.

#### Phenotypical methods for bacterial identification

The identification of the isolates was carried out using conventional tests, including observation of Gram stain morphology, colony characteristics on blood agar plates, optochin susceptibility testing and bile solubility testing.

Optochin susceptibility was performed by disk diffusion, using commercially available optochin disks (5µg, BioRad, Hercules,USA) applied onto Mueller Hinton agar supplemented with 5% defibrinated horse blood and incubated simultaneously at 37 °C overnight

in 5% CO<sub>2</sub> atmosphere and in ambient atmosphere. Isolates were considered resistant to optochin if they displayed inhibition zones of less than 14 mm in 5% CO<sub>2</sub>.

Firstly bile-solubility test was performed in tube and interpreted by the naked eye. Colonies were transferred in 2 mL of saline solution and divided in two equal volumes. Deoxycholate was added to one tube and the two tubes were incubated at 37 °C for 30 minutes. The test was positive when clearing or loss of turbidity of the suspension has occurred in the deoxycholate tube.

Secondly, bile solubility testing was performed with the a densitometer as described previously [16]. Briefly, this technique is based on the measurement of the differences of absorbance in the test tube containing 10% sodium deoxycholate versus the control tube, after incubation for 10 minutes at 37 °C by spectrophotometer. The species with an optical density value (OD-value) of 2.1 or above were identified as *S. pneumoniae*, while finding an isolate with an OD-value in the range of 0.9 to 2.1 suggested the identification of *S. pseudopneumoniae* [16].

#### Molecular method for bacterial identification

Chromosomal DNA was extracted from the overnight cultures of the isolates using InstaGene kit (Biorad, Hercules, USA) according to the manufacturer’s instructions.

Detection of *cpsA*, *lytA*, *ply*, *pspA*, *Spn9828* and *Spn9802* genes, was achieved by PCR [14,19]. Amplification of approximately 1000bp of the 16S rRNA gene was carried out with the primer pair 536F (5’-CAGCAGCCGCGGTAATAC-3’) and rp2 (5’-ACGGCTACCTTGTTACGACTT-3’) [20]. Then, the PCR product was sequenced. The identification of species was determined by comparing the obtained sequence with that of existing sequences in the GenBank database using the BLAST program available at the National Center of Biotechnology Information Web site [21].

**Table 1.** Phenotypic and molecular characteristics of atypical pneumococci strains.

Strains	Serotype	Source of isolation <sup>a</sup>	species <sup>b</sup>	bile	OD value	<i>cpsA</i>	<i>lytA</i>	<i>ply</i>	<i>Spn 9828</i>	<i>Spn 9802</i>	<i>pspA</i> <sup>c</sup>	ATPA		ATPC	
												M23V	V52I	L99I	V48I
Sp22	14	2	Spn	+	2.8	+	+	+	+	+	2	+	+	-	-
Sp125	19A	2	Spn	+	2.8	+	+	+	+	+	1	-	-	+	-
Sp358	3	1	Spn	+	2.6	+	+	+	+	+	2	-	-	-	+
Sp345	9V/A	1	Spn	+	2.8	+	+	+	+	+	2	-	-	+	-
P1058	-	1	Spsp	+/-*	1.9	-	-	+	+	+	-	ND	ND	ND	ND

<sup>a</sup> 1: sputum; 2: ear exudates; <sup>b</sup> Spn: *S. pneumoniae*; Spsp: *S. pseudopneumoniae*; <sup>c</sup> *pspA* gene family; \* not clear; ND: not determined; *cpsA*, *lytA*, *ply*, *pspA*, *Spn9828* and *Spn9802* genes, were achieved by PCR.

**Serotyping**

Opt<sup>r</sup> and *cpsA* positive *S. pneumoniae* isolates were serotyped by a combination of multiplex PCRs targeting serotypes/serogroups 1, 3, 4, 5, 6A/B, 7F, 7C, 8, 9V, 10A, 11A,12F, 14, 15A, 15B/C, 16F, 17F, 18, 19A, 19F, 20, 22F, 23F, 31, 33, 34, 35B, 35F, and 38 using primers previously described by Pai et al. [22].

**Pulsed-field gel electrophoresis**

Pulsed-field gel electrophoresis (PFGE) of macrorestriction DNA fragments was performed as described by Lefevre et al. [23]. After macrorestriction using *SmaI* 30U (Promega), the fragments were separated in a GenePath system (Bio-Rad, Hercules, USA) according to the manufacturer’s recommendations.

***pspA* gene family typing**

The *pspA* genes were detected by PCR using primers previously described. R6 and TIGR4 isolates were used as control strains for *pspA* family 1 and family 2, respectively [19].

**ATPase**

PCR amplification was performed for *atpA* and *atpC* genes encoding H<sup>+</sup>-ATPase a- and c-subunit, respectively. Oligonucleotides *atpA*-F (5'-AATACATGGAACGAGAAGAAAAGG-3'), *atpA*-R(TGCATCAGTTACTCCTTTCTATTCC-3'), *atpC*-F (TAGCGGTTAAAAGTTGACAA-3') and *atpC*-R (5'-CCCTTTCTTCTCGTTCC-3') were used [24,25]. Sequencing results were analyzed by Clustal W and compared to *atpA* and *atpC* genes sequences of *S. pneumoniae* R6 susceptible to optochin.

**Ethical Approval**

This study was performed with approval from the Local Medical Committee of Habib Bourguiba Hospital, Sfax, Tunisia.

**Results**

**Bacterial identification**

Four atypical *S. pneumoniae* strains, resistant to optochin and bile soluble, were recovered between 2014 and 2016. Finding an OD-value in the range of 2.6 to 2.8 suggested the identification of *S. pneumoniae*. In addition to these strains, we recovered one α-hemolytic streptococcus resistant strain to optochin (P1058) with a bile test not clear on a visual interpretation. The OD-value of 1.9 implicate that could be *S. pseudopneumoniae*. For the control strains, the OD-values were 2.6 and 2.9 for optochin susceptible *S. pneumoniae* strains. None of the two other control strains were lysed by bile salts. The OD values were 1.6 and 0 for *S. mitis* and *S. oralis*, respectively.

The molecular methods showed that all Opt<sup>r</sup> pneumococci harbored *cpsA*, *lytA*, *ply*, *Spn9802*, *Spn9828* and *pspA* genes. *S. pseudopneumoniae* harbored only *ply*, *spn9802* and *Spn9828* genes. The 16S rRNA sequencing confirmed that the Opt<sup>r</sup> strain (P1058) was *S. pseudopneumoniae* (Figure 1).

**Molecular typing**

The pneumococcal isolates have different serotypes 14, 19A, 3 and 9V/A. The 19A strain was *pspA* family 1 and the three remaining strains were *pspA* family 2. As serotyping results, analysis of PFGE profiles indicated that the isolates were not genetically related to each other (Figure 2).

**Resistance to optochin**

Concerning the resistance to optochin, DNA sequencing demonstrated that two *S. pneumoniae* serotypes 19A and 9V/A had an ATPase-A mutation of Leu99Ile (L99I). It is noticeable that in the ATPase a-subunit we identified also two mutations; one of the helix1 (M23V) and a second mutation of Val52Ile in *S. pneumoniae* serotype 14. For ATPase c-subunit, only isolate with serotype 3 showed a mutation of Val48Ile (V48I).

**Discussion**

The emergence of optochin-resistant *S. pneumoniae* has been reported in recent years [1–8]. In the National Center of Bone Marrow Transplantation of Tunis-Tunisia, among 59 pneumococci recovered during 2005 to 2011 only three Opt<sup>r</sup> strains have been reported (5%)

**Figure 1.** Alignment of 16S rRNA sequence region of P1058 and SP22 with nucleotide sequence of *S. pneumoniae* (CP000918.1), *S. pseudopneumoniae* (EU156785) and *S. mitis* (AY518677.1) by Clustal V. Identical residues are indicated with an asterisk; Box represents region with mutation site.

<i>S. pneumoniae</i>	TAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTATG	641
<i>S. mitis</i>	TAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTATG	650
<i>S. pseudopneumoniae</i>	TAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTATG	552
P1058	TAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTATG	246
SP22	TAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAGTATG	199
*****		
<i>S. pneumoniae</i>	CTTGAGTGC AAGGGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGGTAGATATATG	701
<i>S. mitis</i>	CTTGAGTGC AAGGGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGGTAGATATATG	710
<i>S. pseudopneumoniae</i>	CTTGAGTGC AAGGGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGGTAGATATATG	612
P1058	CTTGAGTGC AAGGGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGGTAGATATATG	306
SP22	CTTGAGTGC AAGGGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGGTAGATATATG	259
*****		
<i>S. pneumoniae</i>	GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGC	761
<i>S. mitis</i>	GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGC	770
<i>S. pseudopneumoniae</i>	GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGC	672
P1058	GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGC	366
SP22	GAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGC	319
*****		
<i>S. pneumoniae</i>	GTGGGAGCAAAACAGGATTAGATACCCCTGGTAGTCCACGGT	821
<i>S. mitis</i>	GTGGGAGCAAAACAGGATTAGATACCCCTGGTAGTCCACGGT	830
<i>S. pseudopneumoniae</i>	GTGGGAGCAAAACAGGATTAGATACCCCTGGTAGTCCACGGT	732
P1058	GTGGGAGCAAAACAGGATTAGATACCCCTGGTAGTCCACGGT	426
SP22	GTGGGAGCAAAACAGGATTAGATACCCCTGGTAGTCCACGGT	379
*****		

[7]. In our study, we recovered four Opt<sup>r</sup> *S. pneumoniae* strains between 2014 and 2016. The isolation frequency of Opt<sup>r</sup> pneumococci varied in different countries; it is 2.1% in Portugal, 0.5% in United States and Argentina, 0.68% in Japan and 0.8% in Brazil [4,5,24,25]. The fifth Opt<sup>r</sup> strain included in our study showed a difficulty to interpret the bile test based on visual evaluation. The challenging identification of this bacterium is due to the subjective human evaluation of this technique. Recently, Slotved *et al.* presented cut-off OD-values that can be used for differentiating *S. pneumoniae* and *S. pseudopneumoniae* from other mitis group streptococci [16]. Also, Ercibengoa *et al.* recommended the use of bile-solubility test as a technique for *S. pneumoniae* identification, because of the emergence of optochin-resistant pneumococci and optochin susceptible *S. mitis* [26]. This method helped us to differentiate between *S. pneumoniae* and *S. pseudopneumoniae*. Molecular biology techniques have been developed and used to distinguish atypical *S. pneumoniae* from *S. pseudopneumoniae* or other closely related streptococci. In our study, 16S rRNA

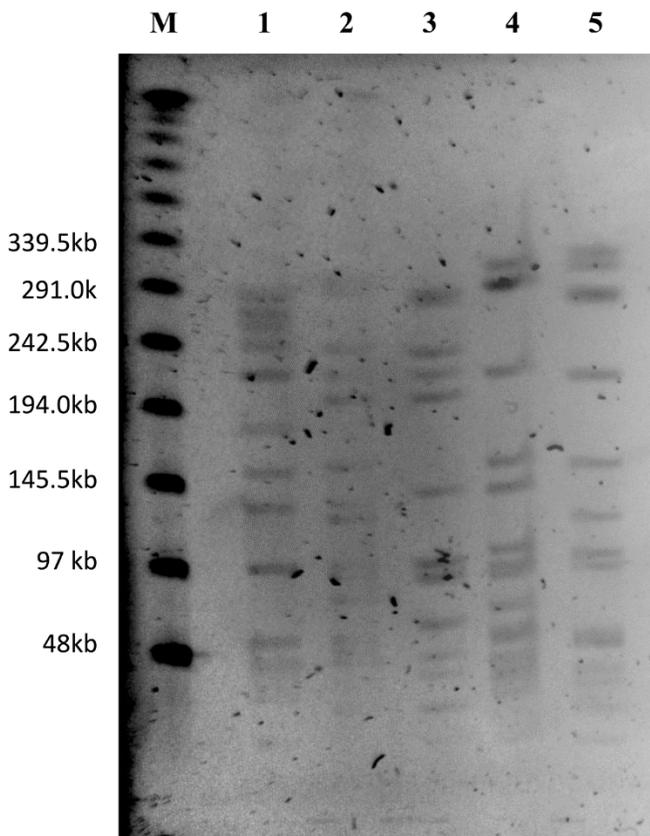
gene sequencing allowed us to distinguish *S. pneumoniae* from *S. pseudopneumoniae* by looking at the difference in 2 distinct positions that are described as sites for reliable *Streptococcus* species identification [10]. Indeed, El Aila *et al.* designed highly specific primers targeting a region of the 16S rRNA gene including these two sites that let to distinguish *S. pneumoniae* from other viridans group streptococci [10].

As reported previously, in our finding *ply* gene and Spn9802- and Spn9828-specific primers, known to be specific to *S. pneumoniae*, could not differentiate *S. pneumoniae* and *S. pseudopneumoniae* [14,27]. Only *cpsA*, *lytA* and *pspA* genes were useful for the discrimination of both species. Therefore, phenotypic and molecular methods based on PCR can easily be used in diagnostic procedures to distinguish *S. pneumoniae* from *S. pseudopneumoniae* and from two other *Streptococcus* species.

Our data indicate that Opt<sup>r</sup> *S. pneumoniae* strains possess different serotypes and genotypes (four PFGE profiles). Of note, Pinto *et al.* showed that optochin resistance is not due to clonal dissemination [6]. Pneumococcal strains were non invasive and belonged to a variety of capsular types (14, 19A, 3 and 9V/A). These serotypes were commonly found in our region, South of Tunisia [28]. It has been reported that serotype distribution among Opt<sup>r</sup> *S. pneumoniae* strains is mostly dependent on the epidemiological profile of each area [5–7]. In Tunis, North of Tunisia, three Opt<sup>r</sup> strains had different serotypes including 6C, 19F and 23F corresponding to the commonly types described in this region [7]. It was noticeable that distribution of *pspA* gene families among Opt<sup>r</sup> *S. pneumoniae* strains show *pspA* families 1 and 2 which are the common profiles identified in *S. pneumoniae* isolates [6,19].

The emergence of unusual phenotype Opt<sup>r</sup> *S. pneumoniae* is still limited. The mechanism leading to optochin resistance is essentially ascribed to ATPase c-subunit and rarely to ATPase a-subunit [7,29–31]. Majority of studies reported that the point mutations in amino acid residues 48, 49 or 50 of the H<sup>+</sup>-ATPase c-subunit confer optochin resistance. In our study among four Opt<sup>r</sup> *S. pneumoniae*, three isolates presented mutations located in the gene coding for the a-subunit of H<sup>+</sup>-ATPase. Two strains presented changes only in codon 99 (L99I) and one strain displayed two changes: one in codon 23 (M23V) and the second in codon 52 (V52I) in  $\alpha$  helices of the a-subunit. However the mutations in 23 (M23V), 52 (V52I) and 99 (L99I) of a-subunit of the H<sup>+</sup>-ATPase found in this study had never been observed before. Only one strain showed a

**Figure 2.** Pulsed field gel electrophoresis (PFGE) of SmaI-digested chromosomal DNA from optochin resistant isolates.



M: PFGE Lambda ladder; 1-4: Opt<sup>r</sup> *Streptococcus pneumoniae* (SP22, SP125, SP358, SP345); 5: *S. pseudopneumoniae* (P1058).

mutation previously described in codon 48 of the ATPase C. Recently Raddaoui *et al.* showed that for three Opt<sup>f</sup> strains recovered, only the sequencing of *atpA* and *atpC* genes showed modification in *atpC* gene [7].

## Conclusions

The occurrence of Opt<sup>f</sup> *S. pneumoniae* variants has hampered the phenotypic identification of pneumococci. Accurate identification of Opt<sup>f</sup> *S. pneumoniae* and differentiation of this emerging pathogen from *S. pseudopneumoniae* is important to ensure the correct diagnosis and treatment of patients. Despite the low number of isolates, this is the first time that we have isolated Opt<sup>f</sup> *S. pneumoniae* and *S. pseudopneumoniae* in the South of Tunisia. As for optochin susceptible *S. pneumoniae*, the bile solubility test based on spectrophotometric give a correct identification for Opt<sup>f</sup> *S. pneumoniae* and can differentiate it from other mitis group streptococci. In our study the Opt<sup>f</sup> *S. pneumoniae* strains belonged to serotypes 14, 19A, 3 and 9V/A, commonly found in our region. Constant vigilance must be applied to monitor the emergence of the Opt<sup>f</sup> strains of *S. pneumoniae* and *S. pseudopneumoniae*.

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