AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

70 (2023) 4, 325–330

DOI:
10.1556/030.2023.02165


RESEARCH ARTICLE

# Accurate identification of *Streptococcus pseudopneumoniae* and other mitis group *Streptococci* identified as atypical *Streptococcus pneumoniae* in Tunisian pediatric population

SAMAR MHIMDI[1,2*], KHAOULA MEFTAH[1,2], AIDA BOUAFSOUN[1] and HANEN SMAOUI[1,2]

[1] Laboratory of Microbiology of Bechir Hamza Children's Hospital, Tunis, Tunisia

[2] Faculty of Medicine of Tunis, Tunis El-Manar University, LR18ES39, Tunis, Tunisia

Received: October 10, 2023 • Accepted: November 20, 2023
Published online: December 1, 2023

**ABSTRACT**

Accurate identification of Mitis group streptococci especially *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* seems difficult due to the lack of specific and sensitive tests. We performed an approach for the identification of atypical pneumococci in pediatric Tunisian population. In this study, 49 streptococcal isolates that were considered as atypical *S. pneumoniae* were analyzed by: optochin susceptibility in ambient and 5% $CO_2$ atmosphere, oxgall disk sensitivity, PCR targeting several genes and antimicrobial susceptibility.

The combined results of biochemical and molecular methods showed the presence of 23 *S. pneumoniae*, 7 *S. pseudopneumoniae*, and 19 other mitis group. Among *S. pseudopneumoniae*, all isolates were collected from respiratory tract samples and showed a high level of resistance to β-lactams with a MIC90 of 32 mg $L^{-1}$. Two isolates of *S. pseudopneumoniae* showed the typical phenotype of optochin resistance described in the literature. All isolates could be identified only by molecular tests. Among *Streptococcus pneumonaie*, all strains harbored the *lytA* gene and the Spn9802 fragment. But only 14 strains were encapsulated.

This study describes several assays for the identification of atypical pneumococci in order to gain insights on the nature of isolate and raise alert about the presence of these strains in the pediatric Tunisian community.

**KEYWORDS**

atypical *Streptococcus pneumoniae*, identification, *Streptococcus pseudopneumoniae*, Tunisia

## INTRODUCTION

The group mitis of streptococci includes many related species such as *Streptococcus pneumoniae* (pneumococci), *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, which differ in their pathogenic potential [1]. Among this group, *Streptococcus pneumoniae* remains the most virulent one and the leading cause of invasive and non-invasive bacterial infections among children worldwide [2]. Pneumococci is responsible of community-acquired pneumonia, meningitis, and bacteremia and is the most common cause of otitis media in infants and young children [3]. The daily identification of *S. pneumoniae* in the routine laboratory diagnostic relies on two features: solubility in bile (sodium deoxycholate), susceptibility to optochin upon 5% $CO_2$ additionally to phenotypic characteristics such as colony morphology and α-hemolysis on blood agar [4]. This pathogen is phenotypically closely related to *S. pseudopneumoniae* and they have some similar features [5]. Moreover, it has been reported in the literature atypical strains of *S. pneumoniae* which could

*Corresponding author. Laboratory of Microbiology of Bechir Hamza Children's Hospital, Faculty of Medicine of Tunis, Tunis El-Manar University, LR18ES39, Tunis, Tunisia.
Tel.: +216 52 819 397.
E-mail: samarmem@gmail.com

be optochin resistant for example [6, 7]. The first description of *S. pseudopneumoniae* was derived from isolate presumptively identified as *S. pneumoniae*. Its biochemical characteristics were slightly different from *S. pneumoniae*. Indeed, *S. pseudopneumoniae* lacks the pneumococcal capsule and is susceptible to optochin when incubated in ambient air but is resistant to it when incubated upon $CO_2$ atmosphere. Yet, the most specific test is the lack of solubility in bile for *S. pseudopneumonaie* [8]. All *S. pseudopneumoniae* strains described have been isolated from lower respiratory tract samples [9]. The clinical impact and pathogenic potential of this organism has not been clearly identified except in few studies that were performed on mouse models [10].

Phenotypic tests are often insufficient to distinguish between related species. So, molecular methods are recommended [11]. Several genes have been used to detect *S. pneumoniae* by a real-time PCR assay including genes encoding the pneumococcal surface protein A (*pspA*), the virulence factors pneumolysin (*ply*), autolysin (*lytA*), the DNA fragment Spn9802 of unknown function and the iron uptake ABC transporter lipoprotein *PiaA* gene [12–15]. Among these targets the Spn9802 detects both *Streptococcus pneuoniae* and *S. pseudopneumoniae* but not *S. mitis* and *S. oralis*, whereas the *lytA* and *piaA* allows detection of only *S. pneumoniae* [14]. Otherwise, PCR targeting the capsular polysaccharide biosynthesis gene A (*cps*A) specific of capsular *S. pneumoniae* was also used [16].

The aim of this Study is to differentiate *S. pneumoniae* from *S. pseudopneumoniae* and other mitis group species (MGS) in a collection of isolates identified as atypical pneumococci in pediatric population. We have combined a real time PCR targeting several genes with phenotypic assays in order to gain insights on the nature of such isolate.

## MATERIALS AND METHODS

### Bacterial isolates

We carried out an observational retrospective study of 49 non-duplicate streptococcal isolates that were considered as atypical *S. pneumoniae*. They were either non soluble in bile, optochin resistant and/or with a high level of resistance to beta lactams mainly third generation cephalosporins. These isolates were collected between June 2002 and December 2018, at the Children's Hospital of Tunis (Tunisia). Non-invasive isolates were recovered from clinical specimens such as sputum, tracheal aspirates, and ear exudates. Invasive isolates were obtained from clinical specimens from sterile sites (blood). Isolates were stored at −80 °C for further investigations.

### Bacterial identification

Pneumococcal identification was based on macroscopic appearance of colonies, microscopic examination (Gram staining), optochin susceptibility and bile solubility tests.

***Optochin susceptibility testing.*** The optochin susceptibility test was performed by overnight incubation of the isolate on blood agar plates with optochin tablets of 9 mm (bioMérieux, France) in 5% $CO_2$ and $O_2$ atmospheres. Optochin susceptibility was defined as a diameter zone of ≥15 mm.

***Bile solubility testing.*** This test was performed by the incubation of isolates overnight on blood agar plates with oxgall disks containing 1,000 µg oxgall (Rosco Diagnostica, Denmark) in a $CO_2$ atmosphere. Solubility was defined as a diameter zone of ≥19 mm, a diameter zone of ≤17 mm indicated the growth of a microorganism that is not soluble in bile and a diameter zone of 18 mm was considered an ambiguous result [14]. In the last case, the test was repeated twice.

### Antimicrobial susceptibility

The antimicrobial susceptibility of all isolates was determined by the disk diffusion method according to CASFM-EUCAST standards (www.sfm-microbiologie.org). The antibiotics tested were oxacillin, gentamicin, tetracycline, chloramphenicol, trimethoprim-sulfamethroxazole, erythromycin, vancomycin, teicoplanin and rifampicin. Minimum inhibitory concentrations (MICs) of penicillin G, amoxicillin, and cefotaxime were determined by E-test method (bioMérieux, France). *S. pneumoniae* ATCC 49619 was used as a quality control strain.

### Isolation of bacterial DNA

DNA was extracted and purified from bacterial suspension using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The extraction was performed according to the manufacturer's instructions.

### Real-Time PCR assay targeting *CpsA*, *lytA*, *Spn9802* and *piaA*

Detection of bacterial DNA was performed by qPCR using previously described primers and probes specific for the gene encoding the major *S. pneumoniae* autolysin *lytA* and fragment of unknown function Spn9802 [14, 17]. The primers and probe specific for the iron uptake ABC transporter lipoprotein *piaA* gene were also used [15]. Additional PCR reaction was elaborated to detect pneumococcal capsular polysaccharide *CpsA* gene as previously reported [18]. qPCRs were performed with simplex reactions in 25 µl volumes using TaqMan Universal Master Mix (Life Technologies, UK), 5 µl of DNA as a template plus primers and probes at 0.2 µM for *lytA* and *Spn9802*. For *piaA,* primers and probe were used at 0.15 and 0.175 µM respectively. Thermal cycling was performed in Light Cycler 480II/96 system (Roche Diagnostics, Switzerland) under the following conditions: an initial incubation step for 2 min at 55 °C, another step of activation Taq for 15 min at 95 °C followed by 45 cycles of 15s at 95 °C, and 1 min at 57 °C. Samples were considered positive when cycle threshold (Ct) values

were below 35. For each PCR reaction, a positive control (*S. pneumoniae* ATCC 49619) and a negative one, were systematically used.

## RESULTS

### Biochemical and molecular assays for *S. pneumoniae* and *S. pseudopneumoniae* identification

All isolates were grown on blood agar plates with alpha hemolysis. Macroscopic examination showed umbilicated colonies with central depression. They were observed with Smooth or rough appearance. Gram staining showed a Gram-positive, lanceolate cocci paired in candle flame or chains.

The results of real-time PCR assays showed that 23 of these isolates were *S. pneumoniae,* seven isolates were *S. pseudopneumoniae* and 19 isolates were from other MGS. For pneumococcal group, all strains harbored the Spn9802 fragment and *LytA* gene. While, the *PiaA* gene was detected in 65% of cases. Furthermore, 57% of isolates were encapsulated. The results of PCR showed the detection of seven *S. pseudopneumoniae* strains by the presence of *Spn9802* fragment only. About other *mitis* groups species, all strains didn't possess any targeted gene (Table 1).

For *S. pneumoniae* isolates, 10 strains were optochin susceptible in $CO_2$ and $O_2$ atmospheres. The remaining strains (13/23) were resistant upon $CO_2$ atmosphere. Four isolates of these were susceptible in $O_2$ atmosphere (Table 1).

Regarding *S. pseudopneumoniae* detected in this study, two strains were optochin resistant upon incubation in $CO_2$ but susceptible in $O_2$. While five isolates showed no concordant results in optochin sensitivity (Table 1).

The 19 strains of other MGS were either optochin susceptible or resistant giving the same result in $CO_2$ or $O_2$ (Table 1).

### Antibiotic susceptibility results

All *S. pseudopneumoniae* isolates were sensitive to teicoplanin, vancomycin and with a low level of resistance to gentamicin. Two strains were resistant to chloramphenicol and four strains showed resistance to cotrimoxazole and rifampicin. Resistance to erythromycin and tetracycline were found in five and three strains respectively. We found that all *S. pseudopneumoniae* had a high level of resistance to penicillin, amoxicillin and cefotaxime with a MIC90 of 32 mg $L^{-1}$.

All *S. pneumoniae* isolates were sensitive to teicoplanin, vancomycin and with a low level of resistance to gentamicin. One stain was resistant to chloramphenicol. Eighteen strains were resistant to erythromycin and 17 to cotrimoxazole. Resistance to tetracycline and rifampicin were detected in 11 and 12 isolates, respectively. Among *S. pneumoniae* strains, the high-level of resistance (MIC >2 mg $L^{-1}$) to amoxicillin and cefotaxime were 100% and 74% respectively.

### Epidemiological characteristics

All alpha hemolytic streptococcal isolates in this study were collected from hospitalized children all aged below 4 years (Table 2). All *S. pseudopneumoniae* strains were isolated from respiratory samples. While among *S. pneumoniae* strains, 2 were isolated from invasive samples (blood) and 21 collected from nonsterile sites (respiratory samples).

## DISCUSSION

Identification of *S. pneumoniae* in routine laboratory is usually based on phenotypic tests such as Gram staining,

*Table 1.* Results of biochemical assays compared to the analysis of *Spn9802, lytA, PiaA and CpsA* PCR results

| Number of isolates | Oxgall | OPT + $CO_2$ | OPT + $O_2$ | Results PCR | | | | Interpretation |
|---|---|---|---|---|---|---|---|---|
| | | | | *LYTA* | *Spn 9802* | *Pia A* | *CpsA* | |
| **5** | NS | S | S | + | + | + | + | *S. pneumoniae* |
| **1** | NS | S | S | + | + | + | – | *S. pneumoniae* |
| **2** | NS | S | S | + | + | – | + | *S. pneumoniae* |
| **2** | Ambiguous | S | S | + | + | + | + | *S. pneumoniae* |
| **1** | NS | R | S | + | + | + | – | *S. pneumoniae* |
| **2** | NS | R | S | + | + | – | – | *S. pneumoniae* |
| **1** | NS | R | S | + | + | – | + | *S. pneumoniae* |
| **3** | NS | R | R | + | + | + | + | *S. pneumoniae* |
| **3** | NS | R | R | + | + | + | – | *S. pneumoniae* |
| **2** | NS | R | R | + | + | – | – | *S. pneumoniae* |
| **1** | NS | R | R | + | + | – | + | *S. pneumoniae* |
| **2** | NS | R | S | – | + | – | – | *S. pseudopneumoniae* |
| **5** | NS | R | R | | | | | *S. pseudopneumoniae* |
| **7** | NS | S | S | – | – | – | – | *Non SP/SPP* |
| **12** | NS | R | R | | | | | *Non SP/SPP* |

NS: Not soluble; S: sensitive; R: resistant; *Non SP/SPP*: Non *S. pneumoniae*/*S. pseudopneumoniae*.

*Table 2.* Epidemiological characteristics of patients with PCR positive for *S. pneumoniae* and *S. pseudopneumoniae*

| Item | *S. pneumoniae* $N = 23$ N | *S. pseudopneumoniae* $N = 7$ N |
|---|---|---|
| **Age (Months)** | | |
| **Median** | 3.5 | 4 |
| **Gender** | | |
| Male | 16 | 3 |
| Female | 7 | 4 |
| **Site of samples** | | |
| Invasive | 2 | 0 |
| Non-invasive | 21 | 7 |

colonies morphology, hemolysis type and optochin resistance. *S. pseudopneumoniae* is a mischaracterized species of alpha hemolytic *Streptococci* that is usually overlooked during examination especially from sputum samples.

In this study we used biochemical and molecular assays to characterize 49 atypical *S. pneumoniae* isolates. We identified 23 *S. pneumoniae* isolates and 07 *S. pseudopneumoniae* isolates. The remaining 19 strains were of other MGS.

Only 10 *S. pneumoniae* isolates were sensitive to optochin. Indeed, we recovered 13 opt$^R$ *S. pneumoniae* strains in our collection. Mutations in the gene sequence of F0F1 ATP synthase subunit c have been associated with optochin resistance in pneumococci in some reports [19, 20].

As previously reported, *S. pseudopneumoniae* has been described to be resistant or to have indeterminate susceptibility to optochin when incubated in $CO_2$ but to be sensitive in $O_2$ atmosphere [1]. In our study, two *S. pseudopneumoniae* isolates displayed the typical phenotype whereas 5 isolates showed no concordant results in optochin sensitivity. In a Spanish study, only 50.8% of *S. pseudopneumoniae* isolates demonstrated the typical optochin phenotype [12].

In our study, all *S. pseudopneumoniae* isolates were collected from respiratory samples. The first description indicated the presence of *S. pseudopneumoniae* in sputum samples in patients with lower respiratory tract infection [21]. Although, its clinical relevance has not been clearly established. Several studies suggested that *S. pseudopneumoniae* was an opportunistic pathogen involved in respiratory tract infections in patients with underlying chronic diseases such as cystic fibrosis and chronic obstructive pulmonary disease but also in invasive diseases [12, 22, 23]. This makes this pathogen as a real clinical concern. It was described that the use of bile-solubility technique is an accurate method to discriminate between *S. pneumoniae* and other MGS species, because the increase of optochin resistant pneumococci and optochin susceptible *S. mitis* [24]. However, all *S. pneumoniae* isolates identified in our study were non soluble or considered ambiguous with oxgall disk method. The misidentification of these isolates is due to the low sensitivity of this technique. A study in Netherlands yields results that were not interpretable or ambiguous with this test [14].

The PCR assay targeting Spn9802, *lytA, PiaA and CpsA* was considered the most efficient method to differentiate *S. pseudopneumoniae*, *S. pneumoniae* and other MGS. Regarding to molecular identification, 14 *S. pneumoniae* isolates were encapsulated and 15 harbored the *PiaA* gene. All strains harbored the *lytA* gene and the Spn9802 fragment. Our finding was concordant with those from Netherlands which found the *lytA*-specific qPCR more sensitive than the molecular assay targeting *piaA* [15]. An Egyptian study described that all pneumococcal strains harbored the *lytA* gene, which was absent in *S. pseudopneumoniae* and MGS isolates [25]. In addition, Rolo et al. mentioned the presence of Spn9802 fragment in 72.7% of nontypeable *S. pneumoniae* [12]. Recently, a new real-time PCR assay targeting SP2020 (putative transcriptional regulator gene) for *S. pneumoniae* identification was described [26].

All *S. pseudopneumoniae* isolates lacked the *CpsA, LytA and PiaA* genes while Only Spn9802 fragment was detected by qPCR. These results have been recorded in previous studies [12, 14, 25]. The *lytA* gene has been considered the main markers to differentiate *S. pneumoniae* from *S. pseudopneumoniae*.

All *S. pseudopneumoniae* had a high level of resistance to penicillin, amoxicillin and cefotaxime with a MIC90 of 32 mg $L^{-1}$. While some studies reported high penicillin resistance rates in *S. pseudopneumoniae* (60.7%), no data was found on the MIC values in this species [12]. *S. pseudopneumoniae* showed resistance rates of 71%, 43% and 57% to erythromycin, tetracycline, and trimethoprim-sulfamethoxazole, respectively. Several studies have demonstrated that erythromycin resistance was the most common in *S. pseudopneumoniae* followed by resistance to tetracycline and trimethoprim-sulfamethoxazole [20, 21].

We recovered 100% and 74% of *S. pneumoniae* with high-level of resistance to amoxicillin and cefotaxime respectively. The high resistance rates described in our study has also documented in other ones [27, 28].

In summary, this study reported the biochemical and molecular methods for *S. pseudopneumoniae* discrimination from *S. pneumoniae* and other streptococci. The isolation of *S. pseudopneumoniae* from clinical samples together with the great percentage of antibiotic resistance observed in the present study should raise our attention to the clinical importance of this organism.

In our knowledge, this is the first study in a Tunisian pediatric population concerning the differentiation of *S. pneumoniae* and *S. pseudopneumoniae*. Despite the small number of strains included and the monocentric nature of our study, our results allowed to advance important conclusions for our laboratory practice. Overall, the real time PCR targeting several genes is the gold standard for the differentiation between *S. pneumoniae* and *S. pseudopneumoniae* in atypical clinical strains. For laboratory routine diagnosis, and to better identify atypical strains, it is important to combine several tests in order to overcome the

shortcomings of phenotypic methods and therefore to have a valid clinical interpretation of the results. Otherwise, differentiation among viridians group streptococcus including *S. pneumoniae* and *S. pseudopneumoniae* can be also conducted using genome sequencing methods or MALDI-TOFF method.

*Funding:* This work was supported by the Research and High Education Ministry of Tunisia (Research laboratory LR18ES39). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

*Conflict of interest:* None declared.

## ACKNOWLEDGEMENTS

The authors are grateful to the staff of the laboratory of Microbiology of Bechir Hamza Children's Hospital Of Tunis, Tunisia for their help in collecting samples.

## REFERENCES

1. Sistek V, Boissinot M, Boudreau DK, Huletsky A, Picard FJ, Bergeron MG. Development of a real-time PCR assay for the specific detection and identification of *Streptococcus pseudopneumoniae* using the recA gene. Clin Microbiol Infect 2012; 18(11): 1089–96. https://linkinghub.elsevier.com/retrieve/pii/S1198-743X(14)60744-8.
2. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009; 374(9693): 893–902. https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(09)61204-6/fulltext.
3. Ramdani-Bouguessa N, Ziane H, Bekhoucha S, Guechi Z, Azzam A, Touati D, et al. Evolution of antimicrobial resistance and serotype distribution of *Streptococcus pneumoniae* isolated from children with invasive and noninvasive pneumococcal diseases in Algeria from 2005 to 2012. New Microbes New Infect 2015; 6: 42–8. https://pubmed.ncbi.nlm.nih.gov/26106481/.
4. Ramirez M. Streptococcus pneumoniae. In: Tang YW, Sussman M, Liu D, Poxton I, Schwartzman J. editors. Molecular medical microbiology. 2nd ed. Boston: Academic Press; 2015, pp. 1529–46. https://www.sciencedirect.com/science/article/pii/B978012397169200086X.
5. Doern CD, Burnham CAD. It's not easy being green: the viridans group streptococci, with a focus on pediatric clinical manifestations. J Clin Microbiol 2010; 48(11): 3829–35. https://journals.asm.org/doi/10.1128/jcm.01563-10.
6. Cogné N, Claverys J, Denis F, Martin C. A novel mutation in the alpha-helix 1 of the C subunit of the F(1)/F(0) ATPase responsible for optochin resistance of a *Streptococcus pneumoniae* clinical isolate. Diagn Microbiol Infect Dis 2000; 38(2): 119–21. https://pubmed.ncbi.nlm.nih.gov/11035244/.
7. Raddaoui A, Ben Tanfous F, Achour W, Baaboura R, Ben Hassen A. Description of a novelmutation in the atpC gene in optochin-resistant *Streptococcus pneumoniae* strains isolates from Tunisia. Int J Antimicrob Agents 2018; 51(5): 803–5. https://pubmed.ncbi.nlm.nih.gov/29305958/.
8. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, Steigerwalt AG, et al. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J Clin Microbiol 2004; 42(10): 4686–96. https://pubmed.ncbi.nlm.nih.gov/15472328/.
9. Mohammadi JS, Dhanashree B. *Streptococcus pseudopneumoniae*: an emerging respiratory tract pathogen. Indian J Med Res 2012; 136(5): 877–80. https://pubmed.ncbi.nlm.nih.gov/23287138/.
10. Harf-Monteil C, Granello C, Le Brun C, Monteil H, Riegel P. Incidence and pathogenic effect of *Streptococcus pseudopneumoniae*. J Clin Microbiol 2006; 44(6): 2240–1. https://pubmed.ncbi.nlm.nih.gov/16757628/.
11. Ikryannikova LN, Lapin KN, Malakhova MV, Filimonova AV, Ilina EN, Dubovickaya VA, et al. Misidentification of alpha-hemolytic streptococci by routine tests in clinical practice. Infect Genet Evol 2011; 11(7): 1709–15. https://pubmed.ncbi.nlm.nih.gov/21798371/.
12. Rolo D, S Simões A, Domenech A, Fenoll A, Liñares J, de Lencastre H, et al. Disease isolates of *Streptococcus pseudopneumoniae* and non-typeable *S. pneumoniae* presumptively identified as atypical *S. pneumoniae* in Spain. PLoS One 2013; 8(2): e57047. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0057047.
13. Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, et al. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol 2007; 45(8): 2460–6. https://journals.asm.org/doi/10.1128/jcm.02498-06.
14. Wessels E, Schelfaut JJG, Bernards AT, Claas ECJ. Evaluation of several biochemical and molecular techniques for identification of *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* and their detection in respiratory samples. J Clin Microbiol 2012; 50(4): 1171–7. https://journals.asm.org/doi/10.1128/jcm.06609-11.
15. Trzciński K, Bogaert D, Wyllie A, Chu ML, van der Ende A, Bruin JP, et al. Superiority of trans-oral over trans-nasal sampling in detecting *Streptococcus pneumoniae* colonizationin adults. PLoS One 2013; 8(3): e60520. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0060520.
16. Park HK, Lee SJ, Yoon JW, Shin JW, Shin HS, Kook JK, et al. Identification of the cpsA gene as a specific marker for the discrimination of *Streptococcus pneumoniae* from viridans group streptococci. J Med Microbiol 2010; 59(Pt10): 1146–52. https://www.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.017798-0.
17. World Health Organization & Centers for Disease Control and Prevention (U.S.). Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis, Streptococcus pneumoniae*, and *Haemophilus influenzae*. WHO manual 2nd edition: World Health Organization; 2011; Report No.: WHO/IVB/11.09. https://apps.who.int/iris/handle/10665/70765.
18. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae*

isolates. J Clin Microbiol 2006; 44(1): 124–31. https://journals.asm.org/doi/10.1128/jcm.44.1.124-131.2006.

19. Souza ARV, de Pina SECM, Costa NS, Neves FPG, Merquior VLC, Peralta JM, et al. Description of optochin-resistant *Streptococcus pneumoniae* due to an uncommon mutation in the atpA gene and comparison with previously identified atpC mutants from Brazil. Sci Rep 2021; 11(1): 7936. https://www.nature.com/articles/s41598-021-87071-8.
20. Wen SCH, Anderson T, Murdoch D. Streptococcus pseudopneumoniae. Clin Microbiol Newsl 2014; 36(9): 65–71. https://www.sciencedirect.com/science/article/abs/pii/S0196439914000270.
21. Laurens C, Michon AL, Marchandin H, Bayette J, Didelot MN, Jean-Pierre H. Clinical and antimicrobial susceptibility data of 140 *Streptococcus pseudopneumoniae* isolates in France. Antimicrob Agents Chemother 2012; 56(8): 4504–7. https://journals.asm.org/doi/10.1128/aac.06374-11.
22. Dupont C, Michon AL, Normandin M, Salom G, Latypov M, Chiron R, et al. *Streptococcus pseudopneumoniae*, an opportunistic pathogen in patients with cystic fibrosis. J Cyst Fibros 2020; 19(4): e28–31. https://www.sciencedirect.com/science/article/pii/S156919931930966X.
23. Garriss G, Nannapaneni P, Simões AS, Browall S, Subramanian K, Sá-Leão R, et al. Genomic characterization of the emerging pathogen *Streptococcus pseudopneumoniae*. mBio 2019; 10(3): e01286–19 https://journals.asm.org/doi/10.1128/mBio.01286-19.
24. Ercibengoa M, Morales M, Alonso M, Ardanuy C, Marimón JM. Variants of the bile: solubility test to differentiate *Streptococcus pneumoniae* from other viridans group streptococci. Future Microbiol 2019; 14: 949–55. https://www.futuremedicine.com/doi/10.2217/fmb-2019-0073.
25. El-Kazzaz SS, El-Khier NTA, Arram EO. *Streptococcus pseudopneumoniae* as an emerging pathogen from patients with respiratory diseases.Afr J Microbiol Res 2017; 11(34): 1338–45. https://academicjournals.org/journal/AJMR/articleabstract/ECB197066073.
26. Tavares DA, Handem S, Carvalho RJ, Paulo AC, de Lencastre H, Hinds J, et al. Identification of *Streptococcus pneumoniae* by a real-time PCR assay targeting SP2020. Sci Rep 2019; 9(1): 1–11. https://www.nature.com/articles/s41598-019-39791-1.
27. Charfi F, Smaoui H, Kechrid A. Non-susceptibility trends and serotype coverage by conjugate pneumococcal vaccines in a Tunisian paediatric population: a 10-year study. Vaccine 2012; 30(Suppl 6): G18–24. https://linkinghub.elsevier.com/retrieve/pii/S0264410X12010195.
28. Zhou M, Yang Q, Kudinha T, Zhang L, Xiao M, Kong F, et al. Using matrix-assisted laser desorption ionization-time of flight (mALDI-tOF) complemented with selected 16S rRNA and gyrB genes sequencing to practically identify clinical important viridans group streptococci (VGS). Front Microbiol 2016; 7: 1328. https://www.frontiersin.org/articles/10.3389/fmicb.2016.01328/full.