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***Streptococcus pseudopneumoniae*, an opportunistic pathogen in patients with cystic fibrosis**

Chloé Dupont<sup>a</sup>, Anne-Laure Michon<sup>b</sup>, Marion Normandin<sup>c</sup>, Guillaume Salom<sup>c</sup>, Marie Latypov<sup>d</sup>, Raphaël Chiron<sup>e</sup>, Hélène Marchandin<sup>f\*</sup>

<sup>a</sup> HydroSciences Montpellier, Université de Montpellier, CNRS, IRD, Laboratoire d'Ecologie Microbienne Hospitalière, Montpellier University Hospital, France

<sup>b</sup> Laboratory of Bacteriology, Montpellier University Hospital, France

<sup>c</sup> HydroSciences Montpellier, CNRS, IRD, University of Montpellier, France

<sup>d</sup> Centre de Ressources et de Compétences de la Mucoviscidose, Montpellier University Hospital, France

<sup>e</sup> HydroSciences Montpellier, Université de Montpellier, CNRS, IRD, Centre de Ressources et de Compétences de la Mucoviscidose, Montpellier University Hospital, France

<sup>f</sup> HydroSciences Montpellier, CNRS, IRD, University of Montpellier, Department of Microbiology, Nîmes University Hospital, France

\* Corresponding author: Prof. Hélène Marchandin, UMR 5569 HydroSciences Montpellier, Equipe Pathogènes Hydriques Santé Environnements, U.F.R. des Sciences Pharmaceutiques et Biologiques, Université de Montpellier, 15, avenue Charles Flahault, BP 14491, 34093 Montpellier Cedex 5, France

helene.marchandin@umontpellier.fr

## Abstract

The pathogenic power of *Streptococcus pseudopneumoniae* has been specified over years, particularly in case of chronic respiratory diseases; *S. pseudopneumoniae* isolation has however not been characterized before in CF patients. Identification of *S. pseudopneumoniae* remains challenging due to the high similarity level between species of the *Streptococcus mitis* group. Twenty CF patients with *S. pseudopneumoniae* were included. Isolates initially identified by phenotypic routine methods were subjected to both *recA* sequencing and amplification of *S. pseudopneumoniae* specific markers. Microbiological and clinical data were reviewed for patients with confirmed *S. pseudopneumoniae*. Thirteen isolates actually belong to *S. pseudopneumoniae*. *S. pseudopneumoniae* was associated with pulmonary exacerbation in 46% of the patients, either as the sole pathogen or as part of a polymicrobial infectious process. *S. pseudopneumoniae* has to be considered as an additional opportunistic pathogen in CF and additional studies are needed to increase knowledge of its epidemiology and clinical significance in CF.

**Key words:** Cystic fibrosis; *Streptococcus pseudopneumoniae*; opportunistic pathogen; exacerbation; identification; resistance.

## Introduction

*Streptococcus pseudopneumoniae*, first described in 2004, belongs to the “*mitis* group” within the *viridans* group of streptococci (VGS) [1]. An extremely high level of similarity, especially noted for *Streptococcus pneumoniae*, *Streptococcus mitis*, and *S. pseudopneumoniae*, characterizes the species of the *mitis* group. The identification of *S. pseudopneumoniae* remains therefore challenging [1-3].

The first reported isolates were from the lower respiratory tract but no clinical data were published. Further studies suggested that the species was an opportunistic pathogen involved in respiratory tract infections in patients with underlying chronic diseases [4]. However, in the chronic polymicrobial disease that Cystic Fibrosis (CF) represents, little attention has been paid to the non-pneumococcal VGS species. Therefore, both the implication and pathogenicity of *S. pseudopneumoniae* in this context remain unevaluated.

Here, we reviewed the clinical and microbiological data associated with *S. pseudopneumoniae* isolation in our CF center with the aim of specifying both the clinical impact and microbiological characteristics of *S. pseudopneumoniae* in CF patients.

## Materials and Methods

### *Study design and ethics statement*

We retrospectively analyzed the *S. pseudopneumoniae* isolates identified during the routinely performed analyses of respiratory tract samples of patients [5] attending the CF center at Montpellier University Hospital, France, over a 6-year period. Twenty isolates recovered in 20 patients were included. At the time of isolation, strains were identified on the basis of a combination of phenotypic tests (demonstration of optochin susceptibility in normal atmosphere compared to optochin resistance in a 5% CO<sub>2</sub>-enriched atmosphere, insolubility in bile, associated with a positive hybridization assay when performed). Antimicrobial

susceptibility testing was performed according to national guidelines [6]. Strains were stored frozen at -80°C.

The Institutional Review Board at Nîmes University Hospital had approved the study (IRB no. 19.02.01).

### *Clinical and microbiological data*

Clinical data and microbiological results associated with *S. pseudopneumoniae* isolation were recorded: demographic characteristics, clinical status with exacerbation of respiratory symptoms defined according to Fuchs criteria [7], bacteriological results (*S. pseudopneumoniae* load, antimicrobial susceptibility pattern, associated pathogens), antimicrobial therapy.

Isolates were analyzed by Matrix-Assisted Laser Desorption-Ionisation Time Of Flight (MALDI-TOF) mass spectrometry (MS) (VITEK MS®, bioMérieux). For molecular investigations, genomic DNAs were obtained using the MasterPure™ DNA purification kit (Epicentre). The *recA* gene was amplified, sequenced and analyzed as previously described [8]. The two markers SPS0002 and SPPN\_RS10375, recently described as specific for *S. pseudopneumoniae* [9,10], and the *S. pneumoniae* specific marker SPN0001 [9] were amplified as previously described [10]. The sequences were deposited on the GenBank database with accession numbers MK279301-MK279310 and MN219510-MN219519. Macrolides, lincosamides and streptogramin (MLS) resistance-encoding genes *ermB* and *mefA* were amplified as previously described [11].

## **Results**

Identification of *S. pseudopneumoniae* was confirmed by the combination of *recA*-based analysis and amplification of both SPS0002 and SPPN\_RS10375 species-specific markers for

13 out of the 20 isolates. The other 7 isolates had been misidentified as *S. pseudopneumoniae*, including 4 *S. pneumoniae* and 3 isolates that might have been members of either unrecognized or unknown taxa (Figure 1).

Nine of the *S. pseudopneumoniae* strains identified by molecular tools were available for evaluating the accuracy of identification using MALDI-TOF MS; 7 were identified as *S. pseudopneumoniae* and 2 were misidentified as *S. pneumoniae*. One of the strains not confirmed as *S. pseudopneumoniae* by molecular methods, *S. pneumoniae* strain P20-5287, was misidentified as *S. pseudopneumoniae* by MS.

*S. pseudopneumoniae* strains were susceptible to amoxicillin, cefotaxime, pristinamycin, rifampin, vancomycin, and teicoplanin. None displayed high-level resistance to aminoglycosides. Acquired resistance or decreased susceptibility was noted against penicillin G (n=2), tetracycline (n=4), erythromycin (n=7), clindamycin (n=2), and trimethoprim-sulfamethoxazole (n=1). The *mefA* gene was detected in the 5 isolates with isolated resistance to erythromycin suggestive of an efflux resistance mechanism while the *ermB* gene was found in the 2 isolates with co-resistance to erythromycin and clindamycin suggestive of methylation of the 23S rRNA.

Patients with confirmed *S. pseudopneumoniae* were mostly children (84.6% were under 10 years old) (Table 1). Eight isolates were recovered from sputum samples, four from nasopharyngeal aspirate specimens and one from bronchoalveolar lavage fluid. *S. pseudopneumoniae* was the dominant or a co-dominant microorganism in 77% of cases with loads up to  $10^8$  Colony Forming Units per ml. Six patients were chronically colonized by *Staphylococcus aureus* and one patient by *Pseudomonas aeruginosa*. Six patients (46%) experienced an episode of pulmonary exacerbation at the time of *S. pseudopneumoniae* isolation (Table 1). For three of them, filamentous fungi not previously identified were simultaneously cultivated while, for the three remaining patients, no other modification of

their microbial colonization profile (opportunistic pathogen, bacterial loads) was noted, *S. pseudopneumoniae* being co-isolated with the pathogen persistently colonizing the patient airways (*S. aureus* for 2 patients and *P. aeruginosa* for the third patient). Seven patients received an antimicrobial regimen that was effective on *S. pseudopneumoniae*, mainly co-amoxiclav towards *S. pseudopneumoniae* and/or associated *S. aureus*.

## Discussion

Identified during respiratory tract infections in patients with underlying diseases such as chronic obstructive pulmonary disease [4], *S. pseudopneumoniae* was subsequently reported from normally sterile sites including blood samples [12], transthoracic needle aspiration, cerebrospinal and ascitic fluids [2], and from late-onset bleb-related endophthalmitis [13]. Despite the fact that not all the above studies identified *S. pseudopneumoniae* using effective identification tools [4,13], recent studies based on genome sequence analysis have given accurate species identification for strains isolated during lower respiratory tract infections and from blood, thereby confirming the disease potential of the species [10]. Additionally, studies have reported antimicrobial resistance or decreased susceptibility of *S. pseudopneumoniae*, 20-60% to penicillin G, 29.5-42.9% to tetracycline, 42.6-100% to erythromycin, 20% to clindamycin, and 10-39.3% to trimethoprim-sulfamethoxazole [2,4,14,this study]. These observations have been reinforced by comparative genomics; *S. pseudopneumoniae* has been shown to be an intermediate species between *S. pneumoniae* and *S. mitis* with 48% of shared pangenome with *S. pneumoniae* [10] and the notable presence in its genome of several virulence factor- and antimicrobial resistance-encoding genes [10,15]. In this study, we showed that genes identical to those mediating MLS resistance in *S. pneumoniae* were present in *S. pseudopneumoniae* clinical isolates from CF patients.

The pathogen has thus raised real clinical concerns. However, VGS have received little attention in CF with the exception of the study by Maeda *et al.* who did not identify *S. pseudopneumoniae* [16]. Despite our study was monocentric, we showed that *S. pseudopneumoniae* that was associated with pulmonary exacerbation, either as the sole opportunistic pathogen or as part of a polymicrobial infectious process, should be considered as an additional pathogen in CF patients, mostly identified in children, as observed for the closely related species *S. pneumoniae* [17]. This may be related to the abundance of the species in the microbiota of tonsillar crypts in children and its significant association with children compared with adults [18]. Almost half of the patients presented with pulmonary exacerbation at the time of *S. pseudopneumoniae* isolation, a rate that can be compared with the 35% rate of exacerbation observed in cases of *S. pneumoniae* isolation [17].

Identification of *S. pseudopneumoniae* is still a matter of concern 15 years after its description as highlighted by successive studies, each proposing an identification scheme. We used *recA* gene sequencing, a method previously shown to be suitable for the identification of VGS [8,19] combined with the detection of *S. pseudopneumoniae*-specific molecular markers [9,10] that revealed several errors in species affiliation by identification tools routinely used in microbiology laboratories that could be problematic, as the patient might not benefit from suitable antimicrobial treatment. Lastly, our study revealed a still hidden diversity of taxa in the *mitis* group in CF, some of them being associated with pulmonary exacerbation.

*S. pseudopneumoniae* should be considered as an additional opportunistic pathogen in CF and our results warrant the need for additional studies to increase knowledge of the epidemiology and clinical significance of *S. pseudopneumoniae* in CF.

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### **Conflict of interest statement**

The authors have no conflict of interest to declare.

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## Legend to figure

**Fig.1.** Maximum-likelihood (ML) tree of 250-bp *recA* sequences showing the relationships between the 20 isolates of this study and species in the *Streptococcus mitis* group.

*recA* sequences of validated species *Streptococcus australis*, *Streptococcus sinensis*, *Streptococcus massiliensis*, *Streptococcus orisratti* and *Streptococcus oralis* subsp. *dentisani* in the *S. mitis* group were not available in the GenBank database. *recA* sequences of two putative novel species recently proposed in the *S. mitis* group on the basis of whole genome sequence (strain 596553 and strain LQJ-218) were included [20,21]. The phylogenetic tree was reconstructed using a GTR model plus gamma distribution and invariant sites as substitution model, in Seaview 4 package. Accession numbers are given in parentheses after the strain's title. ML bootstrap supports were calculated after 100 reiterations; only values exceeding 60% are shown. *Streptococcus pyogenes* was used as an extra group. Bar, 0.05 substitution per site.

Percentages of *recA* sequence identity are given for close relatives in the tree (the type strain of *S. pseudopneumoniae* shared 95.6% of *recA* gene sequence identity with *S. mitis* type strain and 93.2% with *S. pneumoniae* type strain in this analysis).

The detection of markers specific for *S. pseudopneumoniae*, SPS0002 and SPPN\_RS10375, is indicated by ① and ②, respectively; the detection of the *S. pneumoniae* specific marker SPN0001 is indicated by ③.

MALDI-TOF identifications are given where available (13 isolates): Sp, *S. pneumoniae*; Spp, *S. pseudopneumoniae* (framed).

**Table 1.** Clinical characteristics of CF patients at the time of *S. pseudopneumoniae* isolation, compared with patient data from our CF center.

	<i>S. pseudopneumoniae</i> identification	All patients*
Number of patients	13	205
Age	11.5 years (4 mo-59y)	20 (0-76y)
Sex (M/F)	7/6	111/94
CFTR genotype:		
- F508del / F508del	6	84
- F508del / other	3	75
- Other / other	4	46
Pancreatic insufficiency	8	161
Diabetes	1	31
Pulmonary exacerbation at <i>S. pseudopneumoniae</i> identification **	6	-
Associated pathogens (number of patients):		
<i>Staphylococcus aureus</i>	6	120
<i>Haemophilus influenzae</i>	2	40
<i>Pseudomonas aeruginosa</i>	2	100
<i>Achromobacter xylosoxidans</i>	1	20
Fungi	5	52
None	2	ND
Antibiotic treatment	7	-

\* Data were extracted from the French CF Registry.

\*\* Exacerbation of respiratory symptoms was defined according to the Fuchs criteria, which include clinical, spirometry and radiological criteria [7].

