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Highly diverse dynamics of *Pseudomonas aeruginosa* colonization from initial detection in cystic fibrosis patients: A 7-year longitudinal genetic diversity study[☆]

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ABSTRACT

In cystic fibrosis (CF), *Pseudomonas aeruginosa* (*Pa*) is a major pathogen that can persistently colonize patients. Genetic studies showed a high diversity of *Pa*, the success of widespread or 'international' clones and described epidemic clones in CF and Epidemic High-Risk (ERH) clones. Here, we characterized *Pa* genetic diversity over time after first colonization in CF patients, with the aim of accurately describing the dynamics of colonization in a context of scarce longitudinal studies including the first isolated *Pa* strain. Results represent the first genotyping data based on multilocus sequence typing (MLST) available for CF *Pa* in France. Forty-four CF patients with a first *Pa* colonization were included; 265 strains collected over 7 years in these patients were genotyped by multiplex rep-PCR, MLST, pulsed-field gel electrophoresis and/or whole genome sequencing. Forty-one sequence types were identified: 4 were unknown, 22 never previously reported for CF patients, and 6 corresponded to widespread clones colonizing 16 patients (36%). Unrelated strains were identified in 41 patients (93%). Twenty-six patients (59%) presented a recurrence during the study period. No specific clones were associated with transient, recurrent or persistent colonization. Our longitudinal study revealed that 9 of the 26 patients with recurrence (35%) harbored strains of different genotypes. Great genetic diversity was observed among initial *Pa* isolates excluding any cross-transmission. Persistent colonization may appear more complex than expected, imitating persistence, with successive colonization events by unrelated *Pa*.

1. Introduction

Pseudomonas aeruginosa (*Pa*) is a major pathogen in Cystic Fibrosis (CF) and 23.1% and 10.1% of children, as well as 46.5% and 23.8% of adult patients were respectively colonized by *Pa*, either intermittently or chronically, in 2021 according to European and French CF patient registries (European Cystic Fibrosis Society Patient Registry (ECFSPR), 2023; Registre français de la mucoviscidose – Bilan des données, 2021). The rate of colonized patients regularly increases with age, as described for the French CF population with at least one sputum analysis in 2021 which showed that 18% of patients aged under 4, 24% aged under 19

years and 38% aged under 39 were colonized by *Pa* (Registre français de la mucoviscidose – Bilan des données, 2021). This may be due to the cumulative risk of having a first colonization by *Pa* with age, and *Pa*'s ability to colonize CF airways persistently, despite the widespread implementation of antibiotic therapy to eradicate initial *Pa* acquisition. Indeed, acquiring *Pa* is shown to be associated with a significantly high rate of pulmonary exacerbation (Zemanick et al., 2015), supporting early antibiotic intervention to eradicate *Pa* as soon as it is first detected. Variable success rates for *Pa* eradication are reported in the literature depending on patient characteristics, the eradication regimen and the definition of successful eradication, i.e., *Pa*-free follow-up times. Studies

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based on large samples and long follow-up times have reported eradication success rates ranging from 39% to 72% (Ratjen et al., 2010; Taccetti et al., 2012; Cohen-Cyberknoh et al., 2016; Langton Hewer et al., 2020) and a median time to recurrence of *Pa* of about 26 months (Ratjen et al., 2010). Initial *Pa* treatment failures were associated with exacerbations in CF patients (Mayer-Hamblett et al., 2012). Over time, multiple recurrences reduce the chances of clearing *Pa* from CF airways and infection can become persistent and clinically significant with a progressive decline in lung function (Kalferstova et al., 2016). In Europe, chronic *Pa* infection is reported for 10.2% of children and 34.2% of adults with CF (European Cystic Fibrosis Society Patient Registry (ECFSPR), 2023) (20.1% of CF patients in France). Chronic colonization is usually defined according to the modified Leeds criteria, i.e., >50% of at least 4 samples collected over the past 12 months are positive for *Pa* (European Cystic Fibrosis Society Patient Registry (ECFSPR), 2023). This definition, based on the cultural detection of *Pa* during the standard care of CF patients without any information on the *Pa* strain isolated, may fail to distinguish patients in whom the same *Pa* strain reoccurred (relapse) from patients infected by a new strain unrelated to the one involved in the initial episode of infection (reinfection). *Pa* strain genotyping using molecular tools is essential for such purposes (Hall et al., 2014; Kalferstova et al., 2016). MultiLocus Sequence Typing (MLST), which assigns a Sequence Type (ST) to each strain, was shown to have the greatest predictive value in identifying *Pa* strains as unique and therefore was recognized as a first-pass discriminatory typing method (Waters et al., 2012). In addition, *Pa* genotyping addresses epidemiological issues by recognizing epidemic *Pa* lineages/clones, an indispensable prerequisite for specifying whether any of these clones displays specific characteristics in CF, e.g., successful spread, increased virulence. As an example, the first epidemic strain reported in CF, and one of the best-studied *Pa* clones in CF, is the Liverpool Epidemic Strain (LES) (ST146 or ST683), first discovered in 1996 in a children's center for CF where it colonized 60% of *Pa* positive patients (Cheng et al., 1996). In addition to the spread within centers, several studies across the world have also reported the spread of multi-resistant clones in various CF units (García-Castillo et al., 2011; Hansen et al., 2014; Johansen et al., 2008; Jones et al., 2001; Scott and Pitt, 2004) with the example of the Prairie Epidemic Strain (PES) (ST192) which colonized 29% of chronically infected patients referred from several Canadian centers for CF (Parkins et al., 2014). Finally, other genotypes of *Pa* displaying worldwide spread (widespread clones (WS) or international clones) including some causing frequent hospital-acquired infection outbreaks that have been defined as Epidemic High-Risk (EHR) clones, are spread among CF patients (Fothergill et al., 2012; Oliver et al., 2015; Parkins et al., 2018; Del Barrio-Tofiño et al., 2020). These successful clones are frequently multidrug-resistant and infection with these clones often portends a worse prognosis than infection with strains of other genotypes, including increased pulmonary exacerbation rate, exaggerated lung function decline, and progression towards end-stage lung disease (Parkins et al., 2018).

Although an abundant literature is available on the within-host adaptive microevolution of *Pa* during persistence, studies addressing the question of whether CF patients are recolonized by the same *Pa* strain or a different strain after the ever first colonization remain rare in the literature to date (Ratjen et al., 2010; Schelstraete et al., 2010; Taccetti et al., 2012; Tramper-Stranders et al., 2012; Kidd et al., 2015b; Cohen-Cyberknoh et al., 2016; Langton Hewer et al., 2020). In addition, there are still no genotyping studies based on MLST on *Pa* strains from CF patients available in France, as the very few French studies available to date are mostly based on multiple-locus variable-number tandem-repeat analysis (MLVA) (Vu-Thien et al., 2007; Sobral et al., 2012; Llanes et al., 2013). In the overall context described above, first *Pa* colonization events have been closely monitored for a long time at our center, with an immediate clinician alert as soon as *Pa* is first detected. This study was initiated based on the observation of first *Pa* colonization in 11 patients over a 10-month period, a highly atypical and worrying

observation at our center for CF, suggestive of cross-transmission events between patients or a common source of infection. The study was extended beyond that first observation and took place over a 7-year period. During that period, all additional patients with a first *Pa* colonization were also included, just as *Pa* strains isolated during the follow-up of all patients with a first *Pa* colonization. Consequently, this study aimed to i) describe the genetic diversity of *Pa* in 44 CF patients with a first *Pa* colonization, ii) compare *Pa* genotypes with the world MLST database and bring to light WS/EHR clones, and iii) study longitudinal genotype dynamics in the event of *Pa* recurrence after initial colonization.

2. Material and methods

2.1. Patients and *P. aeruginosa* strains

The CF center at Montpellier University Hospital is a regional center for CF in the South of France attending to approximately 250 CF patients at the time of the study. No patients are segregated at our center according to *Pa* colonization status but each patient occupies only one box and health professionals move from one box to another. In addition, there is an air purification system in all the boxes and surfaces are cleaned between patients as well as at the end of the day. Over a 7-year-period (2010–2016), all the CF patients who experienced a first *Pa* colonization were included in the study and followed during the study period. Sputum samples were collected at the CF center as part of the clinical care of these patients and routine sputum culture was performed according to national recommendations (Société Française de Microbiologie, 2022). Each *Pa* isolate (first *Pa* isolated in the patient and *Pa* subsequently isolated during patient follow-up if any) was stored frozen at -80°C . *Pa* was considered as eradicated if colonization or infection lasted less than a month. Chronic colonization was defined according to the aforementioned modified Leeds criteria (European Cystic Fibrosis Society Patient Registry (ECFSPR), 2023).

2.2. Genetic and genomic characterization of *P. aeruginosa* strain

The *P. aeruginosa* genotyping strategy is presented in Supplementary Fig. 1. All *Pa* strains recovered during the first episodes of *Pa* colonization/infection (including several colony morphotypes cultured from a sputum sample) were subjected to MLST according to Curran et al. (Curran et al., 2004). Sequence analyses were conducted using the PubMLST public database which included 4331 STs on April 19th, 2023 (<http://pubmlst.org/paeruginosa/>) of which 96 STs (444 strains) were deposited by our team for strains isolated from clinical or environmental samples in Montpellier. In the longitudinal part of our study, all sequential isolates from a same patient were first compared by multiplex-rep PCR (MR-PCR) according to Dupont et al. (Dupont et al., 2015); one strain per MR-PCR pattern was then selected for genotyping by MLST (Curran et al., 2004). Genetic diversity was determined based on the number of STs identified in the overall population and per patient. Relationships between STs identified in this study were evaluated by a global optimal eBURST analysis implemented by PHYLOViZ 2.0 software (Nascimento et al., 2017) with clustering of single- (SLV) or double-locus variants (DLV). Epidemic clones were inferred from previously reported STs: LES (ST146 or ST683), PES (ST192), Australian Epidemic Strains (AES1, 2, 3) (STs 649, 775 and 242), DK-1 and DK-2 lineages (ST386 and ST387), Dutch Epidemic Strain (ST406), PA14 clone (ST253), clone C (ST17), Manchester epidemic strain (ST217), Midlands 1 strain (ST148), ST439 (Strain B); other STs were also associated to EHR clones, particularly those of the worldwide top 10 *Pa* EHR clones (ST235, ST111, ST233, ST244, ST357, ST308, ST175, ST277, ST654, ST298) or WS clones like the international CF clone (ST274- clonal complex (CC) 2742) (Oliver et al., 2015; Parkins et al., 2018; Ambroa et al., 2020; Del Barrio-Tofiño et al., 2020). Data from this study were submitted to the PubMLST database (<http://pubmlst.org/>).

org/paeruginosa/).

When strains isolated from different patients shared a same ST, they were further subjected to genomic analyses to determine their epidemiological link. These strains were compared by Pulsed-Field Gel Electrophoresis (PFGE) after *SpeI*-DNA macrorestriction as previously described (Dupont et al., 2015). When there were identical or related PFGE profiles (<3 DNA fragments of difference), strains were subjected to whole genome sequencing (WGS) on NextSeq500 (Illumina, United States) as previously described and to core Single Nucleotide Polymorphism (SNP) analysis using Snippy v4.5.0 (<https://github.com/tseemann/snippy>) with default parameters after aligning the reads with the reference genome (RefSeq GCF_022976545.1). Raw data and assemblies were deposited in GenBank under the BioProject accession numbers PRJNA824006 and PRJNA902658.

3. Results

3.1. Baseline data on patients and *P. aeruginosa* strains included in the study

Over the 7-year study period (2010–2016), first *Pa* colonization was reported in 44 CF patients, representing an 18% overall rate of patients attending the CF center at our institution. Mean age at inclusion was 7.86 years and the sex ratio was 1.083. Five patients were adults (11.3%). Fourteen patients (32%) were homozygous genetic mutation

F508D and 18 (41%) were F508D heterozygous. A total of 265 *Pa* strains isolated from 134 samples in these 44 patients were available for further analysis as follows: 65 strains from first colonization events (1 to 4 colony morphotypes cultured per initial sputum sample) and 200 strains from recurrent colonization (1 to 35 strains and 1 to 14 samples per patient for the 26 patients with recurrence over the study period, 1 to 6 colony morphotypes per sample).

3.2. Expanded description of the *P. aeruginosa* genotype diversity in the CF population

MLST was performed on 125 *Pa* strains including the 65 strains from initial colonization events and 60 strains from recurrent episodes of colonization in 21 patients. These 60 strains were selected among the 200 subsequently isolated strains on the basis of distinct profiles in the MR-PCR analysis among strains from each patient. For the 125 *Pa* strains, 41 STs were identified (1 to 5 per patient), 36 of which had already been described and 5 (12%) unknown STs that were deposited in the PubMLST database as ST4375, ST4376, ST4377, ST4378 and ST4379. These five new STs corresponded to new combinations of known alleles and each shared 5 or 6 alleles with several previously described STs.

For 16 patients (36%), *Pa* strains were assigned to 6 WS clones: ST395 strains were identified in 8 patients, ST253 strains (PA14) were identified in 5 patients; ST17 (clone C) colonized 2 patients; and ST244,

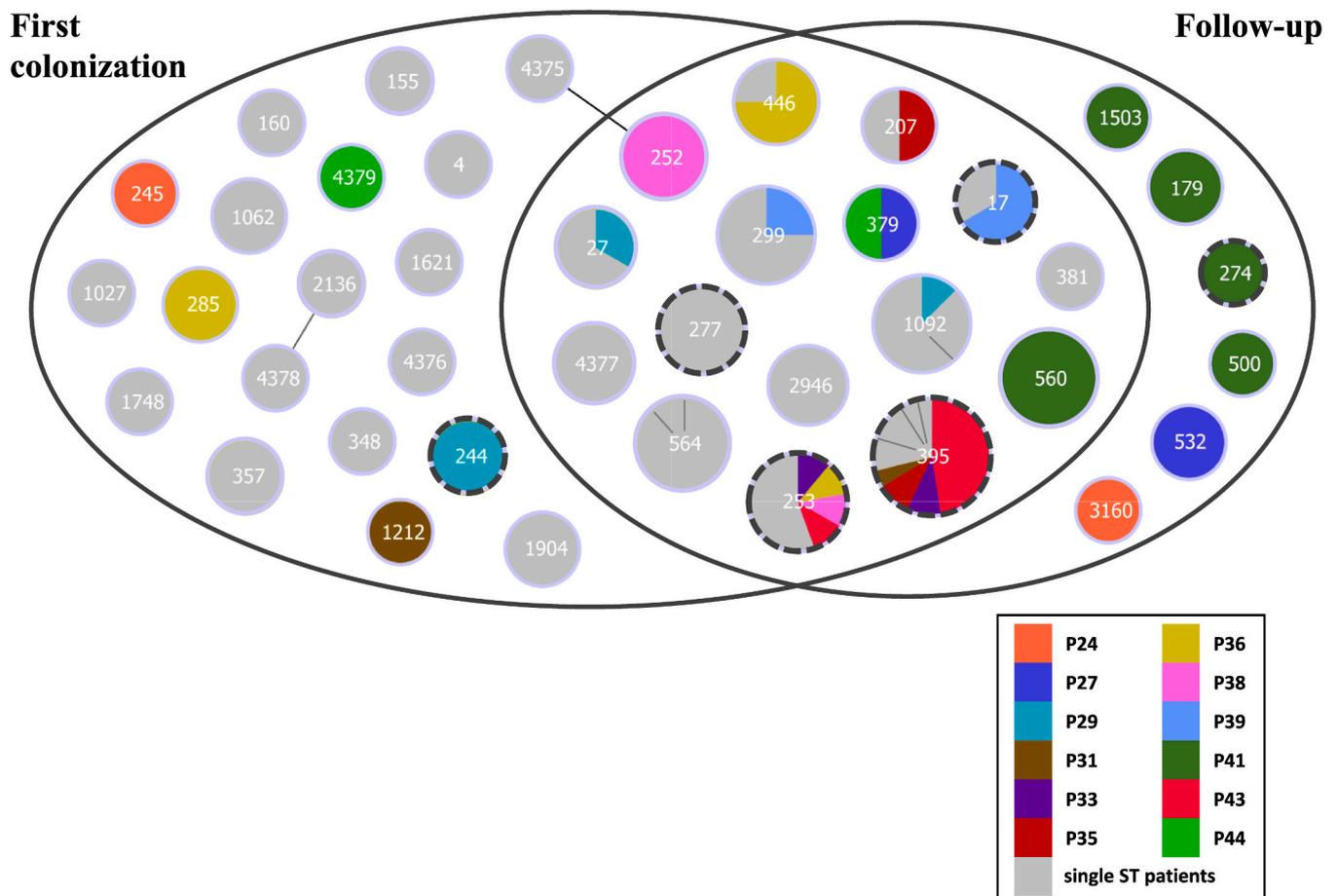


Fig. 1. GoeBURST diagram based on Multi Locus Sequence Typing data for *P. aeruginosa* strains included in the study.

Results are presented according to the time when the strain was isolated, first colonization or follow-up. Each circle corresponds to a sequence type (ST). The number given in the circle corresponds to the ST designation. Links between STs indicate single (SLV) or double locus variants (DLV). Widespread clones / Epidemic high-risk clones are surrounded by dotted lines. The size of each circle is proportional to the number of isolates for each ST. Each compartment in a circle represents one patient and compartment size within a circle is proportional to the number of strains of this genotype for each patient. The colors in the circles correspond to the patient's number as indicated in the box (P = patient).

ST274 (CC274) and ST277 strains each colonized one patient. Of these, ST244 and ST277 are part of the worldwide top 10 *Pa* EHR and ST274 is recognized as an international CF clone. Two patients (Patients 33 and 43) harbored strains belonging to two different WS clones (ST395 and ST253) during their follow-up. Other well-known epidemic clones in CF like LES, PES and AES, were not identified in our study.

Twenty of the 41 STs (49%) had never been reported before for strains of CF origin (Supplementary Table 1). Nineteen of the 41 STs (46%) were previously described in our institution for environmental and clinical strains from non-CF patients, including the EHR clones ST395, ST17 (clone C), ST244, and ST253 (unpublished data) (Supplementary Table 1). Ten STs (24.5%) were shared by strains isolated in several patients (2 to 8 patients), of which the EHR clone of ST395 was the most widespread in our population, identified in 8 patients, either at first colonization or during follow-up (Fig. 1). Nevertheless, cross-transmission or colonization from a common source could only be suspected for a pair of siblings (first colonization strains of Patients 17 and 37) and another patient (strain isolated during the follow-up of Patient 29) in whom the 3 ST1092 strains showed identical PFGE profiles and differed by 5–10 SNPs (genomes of *Pa* strains from siblings differed by 5 SNPs, the genome of *Pa* strains from Patient 29 differed by 5 and 10 SNPs from those of *Pa* strains from siblings). All other strains sharing an

identical ST displayed unrelated PFGE profiles (Supplementary Table 1). Finally, of the 41 STs, 19 were identified for first colonization strains only, 6 were identified for strains isolated during patient follow-up only, and 16 were shared by these two categories of strains.

3.3. A high diversity of first colonizing *P. aeruginosa* strains

A high diversity of initial colonizing *Pa* strains was highlighted with 35 STs identified for the 65 strains studied (Fig. 1). Considering that when two different colonial morphotypes from a same sputum sample were analyzed (37 strains from 16 patients' samples), strains systematically displayed the same ST, we can further consider that the 35 STs represented the overall genotype diversity observed for the 44 patients of the study (Supplementary Table 1). Twenty-four patients (54.5%) harbored strains with a unique genotype, not observed for any other strain in the study. Five corresponded to EHR clones that colonized 12 patients (27%), including unrelated strains of ST395 colonizing 6 patients (Supplementary Table 1).

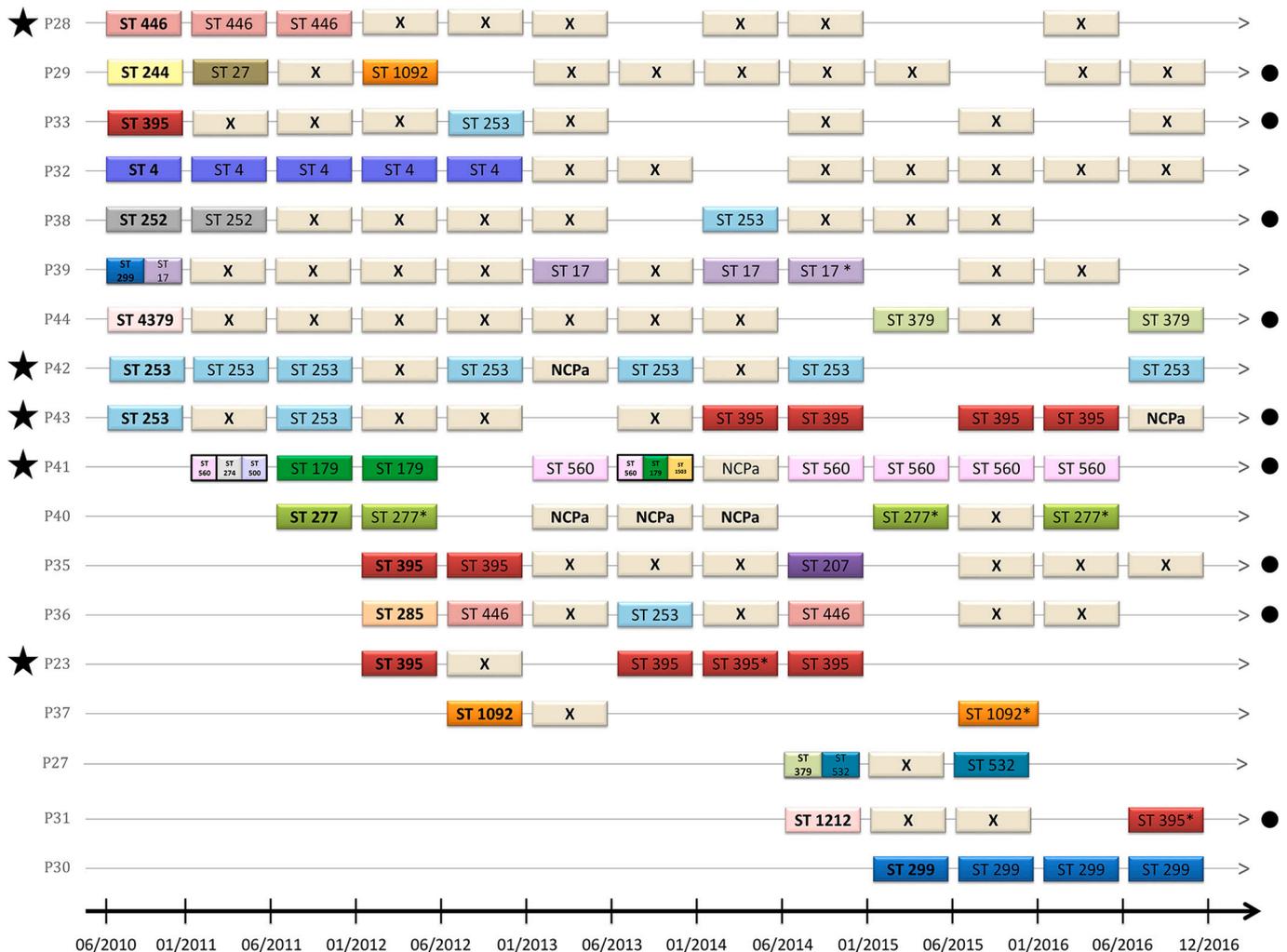


Fig. 2. *P. aeruginosa* MLST genotypes according to colonization timeline (MM/YYYY) for the 18 CF patients (P) with persistent colonization >1 year during the 7-year study period. Bold STs represent first colonization strains. Beige rectangles with X indicate samples without *Pa*. NCPa indicates a non-available *Pa* strain. *, nasopharyngeal specimen (swabbing). Black stars (on the left) indicate the 5 patients with chronic colonization according to the modified Leeds criteria. Black circles (on the right) indicate the 9 patients with first strain replacement.

3.4. Persistent *P. aeruginosa* colonization is not always sustained by a unique genotype

Pa was eradicated in 18 patients (41%) in the month following its first detection and up to the end of the study period whereas other *Pa* strains were cultured from subsequent samples in the 26 remaining patients (Supplementary Table 1). The mean time between the initial and the second *Pa* strain isolation in these patients was about 10.5 months (325 days, range: 57 days to 4.5 years). Eight of these patients with recurrence (30.8%) were then colonized for <1 year and 18 patients (69.2%) had a colonization time ranging from 1 to 6 years. Five of these patients strictly fulfilled the modified Leeds criteria for chronic colonization (11.4% of the cohort under study). Among the 18 persistently colonized patients, 9 patients (50%) were colonized by *Pa* of ST253 or ST395, two clones with worldwide environmental and clinical diffusion and high prevalence in water and clinical samples at our institution (Supplementary Table 1, Fig. 1). These ST colonized 60% of the 5 chronically colonized patients. In 17 of the 26 patients with a second *Pa* isolation (65%), the *Pa* genotype identified in the recurrent episode was identical to that of the strain initially colonizing the patient. In this case, the mean time to recurrence was 7.75 months.

Different STs were observed during the colonization time for 11 of the 18 persistently colonized patients (61%) (Fig. 2). Two of these patients (P27 and P39) were initially co-colonized by strains of two genotypes with persistence of one of these two strains only during follow-up (Fig. 2). In the 9 remaining cases (50%), a first strain replacement was observed with no specific advantage to WS/EHR clones identified (Fig. 2). In these latter cases, the mean time to recurrence was 15.7 months. For Patient 41, a highly complex colonization was highlighted with 5 different STs (ST560, ST274, ST500, ST179 and ST1503) identified during the 5-year colonization time and lung co-colonization involving up to 3 *Pa* of unrelated genotypes recovered from a sputum sample (Fig. 2). However, during the last one and a half years of *Pa* colonization in this patient, only ST560 strains were identified, corresponding to the first colonization genotype. This would suggest that this genotype had become dominant or was the only one colonizing the patient. Altogether, 50% of apparently persistent colonizations involved *Pa* of different genotypes and would have been misclassified as persistent in the absence of genotyping data as observed, for example, for Patient 29, whose apparent intermittent colonization from 2010 to 2012 corresponded to three episodes of sporadic colonization involving 3 different *Pa* strains. The 5 patients with colonization defined as chronic according to the modified Leeds criteria were truly colonized by a single *Pa* genotype over time; however, the true chronic colonization in patient P43 occurred after an initial period of colonization by a strain of distinct genotype and thus experienced a strain replacement (ST395 strain replaced ST253 strain) not suspected from this patient's apparent colonization profile (Fig. 2).

4. Discussion

4.1. A high diversity of *P. aeruginosa* strains reflecting the marked genetic diversity of the species and the diversity of sources of infection

Throughout this study, an important diversity of genotypes was observed with 41 STs identified for the 44 patients included, of which 35 STs were identified during the first episodes of *Pa* colonization. Although it was a single-center study, our study captured a wide genetic diversity of *Pa* strains and this result ties in with those of certain other studies using various typing methods like PFGE, MLST, MLVA, Enterobacterial Repetitive Intergenic Consensus-PCR, fluorescent amplified fragment length polymorphism, and single-nucleotide polymorphism typing using AT biochips (Clondiag Chip Technologies, Germany) (Jelsbak et al., 2007; Johansson et al., 2015; Kidd et al., 2015a; Llanes et al., 2013; López-Causapé et al., 2017; Scott and Pitt, 2004; Sobral et al., 2012; Vu-Thien et al., 2007). A total of 5 STs (12.2%) were

unknown at the time of the study, thus supporting the hitherto incompletely described diversity of *Pa* strains infecting CF patients, as previously observed by others (López-Causapé et al., 2017). However, new allele sequences were not observed and these 5 STs corresponded to new combinations of known alleles registered in the PubMLST database, reflecting the rapid genetic evolution of *Pa* through recombination events and allele exchange between strains. These observations are congruent with previous major findings published by Pirnay et al. highlighting: i) “the nonclonal epidemic *Pa* population structure, i.e., a superficially clonal structure with frequent recombination, in which occasionally highly successful epidemic clones arise”, and ii) that *Pa* genetic diversity in CF was similar to that of strains from other habitats (Piray et al., 2009). However, MLST cannot be used as the sole genotyping method. Indeed, combining MLST with PFGE, an accurate method for *Pa* genotyping compared with WGS, increases the discriminative power of the approach making it possible to accurately assign epidemiologically-related or unrelated strains (Kalferstova et al., 2016; Martak et al., 2020). In our study, 93% of patients were shown to be colonized by their own *Pa* strain after genotyping combining four methods applied in sequence. Indeed, strains from different patients with identical MLST genotypes mainly displayed different PFGE patterns. These results were congruent with another report, also including the major genotype identified in our study, the ST395 (Fernández-Olmos et al., 2013) but were different from results obtained from other centers showing potential cross-transmission or exposition to a common source for up to 34.5% of the patients studied (Vu-Thien et al., 2007; Abdul Wahab et al., 2014; Johansson et al., 2015; Milczewska et al., 2020). In our study, only 3 patients, two siblings and another patient, were colonized by a same *Pa* clone (MLST, PFGE and WGS). The siblings were living in the same house, a common source of contamination or a cross-transmission (directly or indirectly by contaminated objects) was therefore highly probable. In contrast, no link could be established with the third patient who was considered unrelated to the siblings, as the patients did not attend the center on the same days, discarding a potential cross-transmission during consultations. No other ST1092 strains had been previously identified at our institution and this genotype is not associated with environmental samples in the public PubMLST database. The origin of patients' contamination thus remains unknown and whether this clone displays specific capacities to colonize CF lungs has yet to be explored.

4.2. Success of worldwide-disseminated EHR clones in CF

We identified 6 WS/EHR (ST395, ST253 (PA14), ST17 (clone C), ST244, ST274 and ST277) that colonized about one third of the patients. We compared our results with those of a Spanish study that identified strains of 39 STs colonizing 36 CF patients (Fernández-Olmos et al., 2013). Interestingly, we found that only 9 STs were identified in both studies but 5 of these STs were WS/EHR clones (all WS/EHR clones identified in our study except for ST277) (Fernández-Olmos et al., 2013). The two STs most represented in our study were the ST395 (8 patients) and ST253 (5 patients). These two genotypes are registered in the PubMLST database for a panel of geographically unrelated strains originating from human clinical samples (CF and non-CF patients), animals and various environmental sources, including hospital water samples at our institution, confirming the broad distribution of the corresponding clones. A recent study also identified ST253/PA14 lineage as the second most frequent clone among 2882 *Pa* isolates from human and environmental origins and among strains from CF patients, all chronically colonized (Fischer et al., 2020). ST395 strains were previously shown to possess an arsenal of resistance determinants, probably contributing to their broad distribution through adaptation (Teixeira et al., 2016). Among them, copper resistance determinants possibly accounted for the clone's ability to spread during hospital outbreaks with water networks incriminated in the strain's dissemination (Petitjean et al., 2017). A Hungarian study about environmental *Pa*

strains (soil and water) observed that the majority of environmental isolates shared identical MLST genotypes with CF isolates, including ST253, and concluded that environmental *Pa* can be a potential risk to public health and cannot be excluded as an external (non-nosocomial) source of infection (Radó et al., 2017). It should be noted that the EHR clone of ST308 that was epidemic at our institution and identified for strains of clinical and environmental origin was not identified in our study (Abdouchakour et al., 2018).

4.3. Accurate identification of *P. aeruginosa* colonization type over time cannot be inferred from mere *Pa* recovery but requires molecular typing due to highly complex colonization dynamics

Among the 44 patients with a first *Pa* colonization in our study, 59% had a subsequent *Pa* isolate over two months later; this result falls within the range of nearly all other studies that included CF patients over variable study periods, and reporting 28% to 61% of patients with subsequent *Pa* colonization (Ratjen et al., 2010; Schelstraete et al., 2010; Taccetti et al., 2012; Tramper-Stranders et al., 2012; Kidd et al., 2015b; Cohen-Cyberknoh et al., 2016; Langton Hewer et al., 2020). Interestingly, the study by Munk et al. reported remarkable observations with the 19 patients included all having subsequent *Pa* colonization during their 3-year follow-up time (Munck et al., 2001). Mean time to recurrence was 10.5 months in our study, quite similar to the observation by Munck et al. showing an 8-month median time to recurrence of *Pa* (19 patients) but shorter than approximately 26 months as reported by Ratjen et al. (65 patients) (Munck et al., 2001; Ratjen et al., 2010). After recurrence, 11.4% of our cohort of patients developed chronic colonization as defined by the modified Leeds criteria, in line with a recent study reporting that 13% of 3580 patients with a first *Pa* colonization developed chronic colonization (Heltsh et al., 2018). Another recent study observed a higher rate of patients developing chronic colonization (one third of patients under study, 27/80) but the definition of chronic colonization was based on distinct, less stringent criteria, i.e., the European consensus criteria which consider a shorter period of colonization (6 months) (Jonckheere et al., 2018).

Genotyping made it possible to distinguish relapses from reinfections from a new source of contamination. In our study, 65% of patients with recurrence had a relapse and 35% had a reinfection, as observed in some of the few other studies that included *Pa* genotyping (Ratjen et al., 2010; Tramper-Stranders et al., 2012; Hall et al., 2014; Kidd et al., 2015b). However, other studies either reported higher rates of reinfection (73.7% of the 19 patients in the study by Munck et al. (2001) had acquired a new *Pa* strain with a distinct genotypic profile) or higher rates of relapse with 74% or 91% of the patients persistently colonized by a strain with a genotype identical to that involved in the first episode of colonization in the studies by Schelstraete et al. (2010) and Langton Hewer et al. (2020), respectively. Such a high variability between CF centers has been confirmed by a study comparing patients' colonization at two different CF centers, showing that over half of chronically colonized patients maintained their initially acquired clone for a prolonged period of time in one center (Hanover) whereas, at the other center (Copenhagen) two rare clones replaced the initially acquired individual clones in all but one patient (Cramer et al., 2012). The divergent epidemiology at these two sites was explained by their different hygiene and antipseudomonal chemotherapy practices, an explanation that can more largely support the differences observed in the overall literature. However, the discriminative power of the highly diverse genotyping methods used in studies including strain comparison, i.e., randomly amplified polymorphic DNA analysis (RAPD) (Schelstraete et al., 2010), variable number tandem repeat (VNTR)-based typing (Langton Hewer et al., 2020), etc., may also account for part of the variability observed. Here, we did not compare several specimen types but recognition of *Pa* eradication or strain replacement was shown to be achieved distinctly when bronchoalveolar or oropharyngeal samples were analyzed, raising questions about the best approach to define successful eradication of a

Pa strain (Kidd et al., 2015b). Finally, a mixed population of co-existing *Pa* strains can also be present in the patients' airways, as previously reported, and as in Patient 41 of our study (Cramer et al., 2010; Cramer et al., 2012; Williams et al., 2018). As previously observed, the co-colonization appeared in early stage, within the first three years of colonization (Cramer et al., 2010). However, because multiple colonies per timepoint were not systematically analyzed, the possibility that a mixed population is present but not recognized in the patient cannot be totally ruled out and also contributes to the challenge of asserting strain replacement. Despite these limitations, distinct times to recurrence were reported depending if recurrence corresponded to a relapse or a reinfection, being shorter when a relapse was identified while time during which the patient was free of *Pa* was longer in case of reinfection by a genetically unrelated strain. Schelstraete et al. and Jonckheere et al. also reported significantly shorter *Pa*-free time intervals between the very first and the second isolates for patients who became chronically colonized compared to non-chronically colonized patients (Schelstraete et al., 2010; Jonckheere et al., 2018).

The WS clones' success in strain replacement was not significant in our study. However, WS clones were identified in 60% of the patients with chronic colonization, suggesting that such strains may present selective advantages for long-term persistence in CF patients' airways. Accordingly, one study including five patients who experienced *Pa* strain replacement by a Prairie Epidemic Strain (PES) found that PES produced lower levels of proteases and elastases but was more resistant to antibiotics than the replaced non-EHR isolates (Duong et al., 2015). Similarly, it has been suggested that the Liverpool Epidemic Strain (LES) clone, shown as most likely to infect patients already infected by other *Pa* strains, has probably acquired mutations favoring transmission and, therefore, competitive ability towards other strains in patients' lung (Winstanley et al., 2016).

Persistent colonization is complex and *Pa* genotyping based on screening methods like MR-PCR must be recommended more widely in the management of CF patients to accurately classify the patient's colonization status and distinguish relapses from reinfection (Kalferstova et al., 2016; Scott and Pitt, 2004).

5. Conclusion

In this study, a huge diversity of *Pa* genotypes was observed, but no major cross-transmissions were observed between patients, apart from between siblings, and a potential other unrelated patient. According to the practices at our CF center, we showed that it is possible to avoid cross-transmission of *Pa* without segregating patients. We showed that worldwide-disseminated *Pa* clones are also prevalent in CF and major strains identified in chronically colonized patients. Finally, all observations underlined the striking complexity of *Pa* colonization in CF patients, which may account for persistent colonization being wrongly regarded as chronic colonization. This major finding supports the need to perform molecular typing of *Pa* strains more largely at first and subsequent isolations, because successive colonization events by unrelated *Pa* can mimic persistence. To this end, the typing strategy performed herein with a first stage of screening based on rep-PCR fingerprint typing may be useful and affordable for a number of laboratories, by limiting strains to be studied with more expensive, less generalized approaches. This appears even more crucial in the current context of wide use of cystic fibrosis transmembrane conductance regulator (CFTR) corrector and potentiator combinations. Indeed, the rate of CF patients colonized with *Pa* is decreasing worldwide since the implementation of these therapies. However, *Pa* remains a major pathogen and *Pa* colonization has still to be monitored to evaluate modifications in both *Pa* epidemiology and colonization dynamics in CF patients under treatment.

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Ethical statement

The Institutional Review Board (IRB) at Nîmes University Hospital had approved the study under IRB no. 19.02.01 (February 11th, 2019).

CRediT authorship contribution statement

Chloé Dupont: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft. **Fabien Aujoulat:** Data curation, Formal analysis, Investigation, Methodology, Supervision. **Massilia Benaoudia:** Formal analysis, Investigation. **Estelle Jumas-Bilak:** Resources, Writing – review & editing. **Raphaël Chiron:** Data curation, Writing – review & editing. **Hélène Marchandin:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Unknown Sequence Types identified in this study were deposited in the PubMLST database under ST4375, ST4376, ST4377, ST4378 and ST4379 designation (<http://pubmlst.org/paeruginosa/>).

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Appendix A. Supplementary data

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