Chapter 3

# The Cystic Fibrosis Airway Microbiome and Pathogens

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**Abstract**

Cystic fibrosis (CF) is an autosomal recessive genetic disorder resulting from genetic defects in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR dysfunction in patients with CF leads to a number of pleiotropic manifestations with the prime pathology being mucus plugging in the airways and paranasal sinuses. Patients with CF are prone to polymicrobial infections and the airway microbiome in such patients changes continuously and evolves over time. The composition of the airway microbiome in CF patients is dependent on a number of factors including geographic variation, type of genetic mutation (e.g., ΔF508), antibiotic exposures, and chronic infection with certain pathogenic bacteria (e.g., *Pseudomonas aeruginosa*). Proteomic and genomic approaches to understanding the microbiome of patients with CF have provided new insights into the pathogenesis of this disease. High-throughput pyrosequencing, Sanger sequencing, and phylogenetic microarray analysis have enabled the recognition of multiple lineages and clonal populations of a single bacterial species within the same patient. This provides a unique opportunity to explore novel therapeutic approaches to this disease (for instance, use of probiotics and environmental manipulation) and potentially translate them into bedside clinical interventions.

**Keywords:** cystic fibrosis, microbiome, dysbiosis, *Pseudomonas aeruginosa*, burkholderia cenocepacia

## 1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene [1]. CF is most prevalent in the Caucasian population and is a common life-limiting disease [2]. CFTR is expressed on the apical surface of epithelial cells of the respiratory, gastrointestinal, pancreatic and reproductive tracts, and sweat glands [3]. The prime function of CFTR ion channel is to transport chloride ions across epithelial surfaces in order to maintain the osmotic gradient. Chloride ions are actively

pumped out into the luminal side of the gastrointestinal and respiratory tracts, which decrease water potential on the luminal side. Subsequently, water molecules move from a higher osmotic potential to a lower osmotic potential (down the osmotic gradient) and combine with mucin glycoproteins to keep them adequately hydrated. This in turn helps to maintain the thin consistency of the mucus layer, which is essential for optimal mucociliary function [4]. Thick and viscid mucus caused by a defect in chloride-conducting transmembrane channel results in stagnation of mucus. Moreover, CFTR channel also plays an important role in regulating the transepithelial transport of sodium and bicarbonate ions [5]. Defective CFTR functioning leads to an increase in pH of the mucus layer, which compromises the innate immune system and promotes inflammation. Defects in innate immunity and chronic inflammation predispose patients to recurrent pulmonary infections, which result in permanent lung damage—the prime cause of morbidity and mortality [6]. Pulmonary system is not the only organ-system affected in CF; endocrine, gastrointestinal, and reproductive systems are also involved in this multisystem disorder [3].

The human microbiome project aims to identify and characterize microbial flora of healthy and diseased individuals [7]. Understanding the role of infectious pathogens in the pathogenesis of CF in general and pulmonary exacerbations and lung damage in particular has enabled the scientific community to devise new treatment modalities for CF patients, which can potentially improve outcomes and survival in such patients. In patients with CF, different bacteria inhabit different parts of the lung at various stages of the disease and persistent inflammation in the lungs can change and modify the composition of the microbiome [8]. For instance, methicillin-sensitive *Staphylococcus aureus* (MSSA) and *Hemophilus influenzae* are common pathogens early in life of such patients [9]. As the disease progresses, more virulent pathogens—such as *Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MRSA)—invade the lung and cause pulmonary damage [10]. By understanding the evolution of the CF microbiome, we can gain further insights into the natural course of CF. This in turn can have important implications for developing interventions that can halt or reverse the course of progressive pulmonary damage and prolong survival and quality of life in CF patients [11]. In the following pages, we discuss the CF microbiome, its evolution and heterogeneity in CF patients, interaction between different bacteria within the CF lung and the factors that potentially affect the CF microbiome.

## 2. The microbiome

As mentioned previously, the human microbiome project aims to identify and characterize microbial flora of healthy and diseased individuals [7]. There is a diversity of microbes in every single human being i.e., diversity being defined as the number and distribution of a particular type of organism in a body habitat. Every human has particular and distinct microbes; dysbiosis (alteration in composition and balance) of these microbes is now thought to underlie the pathogenesis of many diseases, such as inflammatory bowel disease, *Clostridium difficile* (CD) colitis, bacterial vaginosis, obesity, and CF [12]. The human microbiome plays a very important role in human biology, defense mechanisms, metabolic processes (such as

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digestion, absorption, and assimilation) and even pathogenesis of acute and chronic diseases [13]. For instance, CD colitis is a disease that arises as a consequence of interaction of bacterial virulence factors, host immune mechanism and the intestinal microbiome [14]. Research studies have shown that variability in the innate host response may also impact upon the severity of CD colitis, and this variation may be accounted for by alterations in the gut microbiota [15]. Based on improved understanding of the pathogenesis of CD colitis, fecal microbiota transplantation (FMT) and other novel types of bacteriotherapy have become potentially effective treatment options for this deadly disease [16].

Another example of a disease where microbiota plays a major role in pathogenesis is Crohn's disease. The exact cause of Crohn's disease is unknown; however, evidence suggests that microbiota contribute to the underlying pathology and disease development [17]. No single bacterium has been convincingly shown to contribute to the overall pathogenesis of Crohn's disease. Instead, dysbiosis (bacterial imbalance) is more widely accepted as a leading factor in the disrupted host immune system cross-talk that results in subsequent intestinal inflammation [18]. Depletion of symbiont (beneficial) microbes (including Firmicutes, Bifidobacteriaceae, and Clostridia) in conjunction with an increase in pathobiont (harmful) microbes (such as Bacteroidetes and Enterobacteriaceae) is a striking feature observed in Crohn's disease. No single factor has been definitely identified as driving this dysbiosis; instead, a host of environmental factors—such as the diet, antibiotic exposures and possible early life infections—in the presence of underlying genetic susceptibilities may contribute to the overall pathogenesis of Crohn's disease [17].

In CF patients, composition of the microbiome of pulmonary and gastrointestinal tracts changes over time, presumably as a consequence of inflammation [19]. Most research studies have demonstrated the influence of inflammation in negatively selecting against potential pathogens. Moreover, some bacterial species may also have the ability to exploit inflammatory byproducts for their benefit, which may promote their natural selection in inflamed habitats [20]. Reactive nitrogen species produced during inflammatory responses can be exploited by pathogens for their growth. Moreover, inflammatory mediators can provide an environment for some bacteria to grow and use these inflammatory mediators for their survival [21]. Examples of such bacteria include *Escherichia coli* and *P. aeruginosa* in the gastrointestinal and respiratory tracts of CF patients, respectively. *P. aeruginosa* uses nitric oxide produced in the process of inflammation for its anaerobic respiration and promotes its growth in inflammatory environments. Likewise, *E. coli* uses increased nitrate in the environment for its anaerobic respiration and enhances its growth in the inflamed gut of CF patients [19].

## 3. Heterogeneity of the CF airway microbiome

Due to defects in innate immunity, CF patients are prone to polymicrobial infections and their airway microbiome changes continuously and evolves over time. The primary cause of death in CF patients is respiratory failure due to persistent and recurrent pulmonary infections with different pathogenic organisms [22]. Over the past decade, the median survival for such

patients stands at 37 years despite increases in life expectancy [23]. MSSA and *H. influenzae* are one of the most common pathogens cultured from sputum samples of affected children. *P. aeruginosa* has been associated with increased morbidity as most strains of this organism are multidrug resistant. Infections with bacteria of the Bukholderia cepacia complex (BCC) are associated with a worse prognosis [24]. Likewise, other multidrug resistant organisms, such as *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia*, can also be isolated from CF patients with end-stage pulmonary disease [25]. Nontuberculous mycobacterium (NTM) has also been identified as emerging causes of infections in patients with CF and their incidence may have been underestimated in the past [26]. More recently, research studies have shown that when sputum samples obtained from adults with CF are cultured, a significantly high density of anaerobic bacteria can be isolated—the most common of which are *Streptococcus milleri*, *Prevotella* spp., *Actinomyces*, and *Veillonella* [27].

Microbes of the lower airways in all humans exist in a dynamic state. Literature published on microbiome of CF patients has shown a complex and dynamic interaction between different organisms in the airways of such patients [28]. Organisms within a single patient are genetically and phenotypically diverse and heterogeneity is detectable even in different parts of the same lung. Over a period of time, community diversity of bacteria declines in CF patients as pulmonary function declines and lung disease progressively worsens. Studies have shown that diversity of microbial communities correlates positively with pulmonary function and outcome [29]. Such diversity was previously unrecognized as most studies relied solely on culture-based methods of culturing bacteria. However, novel state-of-the-art molecular techniques (such as Sanger sequencing of clone libraries, terminal restriction fragment length polymorphism [RFLP] analysis and microarray hybridization) have enabled the detection of subtle molecular diversity among seemingly similar bacterial species [30]. This diversity may be influenced by a number of factors including the patient's age, sex, type of CFTR mutation, antibiotic exposures, environmental factors, and extent and severity of lung disease. In a study by Zhao et al., sputum samples were collected from six CF patients over a period of 10 years. Of a total of 126 sputum samples, 662 operational taxonomic units (OTU) were identified and each patient had 5–114 different OTUs [29]. Similarly, in another observational study, sputum samples of patients with acute infective exacerbation of non-CF related bronchiectasis were collected. Sputum cultures from each patient contained large quantities of multiple bacterial species with a single predominant pathogenic species [31]. In one study, polymerase chain reaction (PCR)-temporal temperature gel electrophoresis (PCR-TTGE) was used to evaluate intraspecific and intragenomic 16S rDNA variability among commonly isolated respiratory pathogens from CF patients [32]. Significant discordance in intraspecific and intragenomic variability was noted among different bacterial species with *H. influenzae* displaying the highest level of intraspecific variability.

## 4. Composition of the CF microbiome and its determinants

The composition of the airway microbiome in CF patients is dependent on a number of factors including geographic variation (more common in white population), type of genetic

mutation (e.g., ΔF508), antibiotic exposures, and chronic infection with certain pathogenic bacteria (e.g., *P. aeruginosa*) [8]. Fetal lungs are sterile, just like fetal gastrointestinal tract, but they soon become colonized after birth. Fetal skin becomes colonized with microbes present in maternal reproductive and gastrointestinal tracts and lungs become colonized from gut flora of the child [33]. The common phyla found in healthy lungs include Bacteroides, Firmicutes, and Proteobacterium. Other genera include Prevotella, Veillonella, Streptococcus and Pseudomonas [34]. Many techniques have been used for the detection of microbes in CF patients. Some of these techniques include terminal RFLP profiling, microarray analysis, clone library sequencing, and pyrosequencing. The most frequently used samples from CF patients for analysis are expectorated sputum, tracheal aspirates, bronchial washings, and bronchoalveolar lavage (BAL).

The microbiome in patients with CF evolves as patients grow older, and this is a consequence of the wide adaptability of pathogenic bacteria. Clustering of phylogenetically similar bacterial communities and loss of the architectural diversity of the airway microbiome is a key feature of late-stage CF airway disease. Moreover, the type of bacterial species predominating at a particular age group is also of immense importance. In one study, phylogenetic diversity of CF airway microbiota in patients of different age groups was studied using microarray analysis [35]. *S. aureus* was detected in 65% of sputum samples and was more common in the pediatric population (72% of the pediatric sample). *Pseudomonas* spp. was found in 73% of samples and were most common in adults (91% of the adult sample). In the same study, older CF patients had reduced airway bacterial diversity and aggregation of relatively similar organisms; this process occurred in conjunction with a progressive decline in pulmonary function. *H. influenzae* was most prevalent in the pediatric population when the bacterial diversity was highest. Conversely, *P. aeruginosa* was most common in older individuals with a lower level of bacterial diversity. Likewise, members of the Mycobacteriaceae family and obligate intracellular pathogens (such as Chlamydia and Mycoplama spp.) were more prevalent in younger CF patients. Certain known or potential pathogens of CF patients, such as members of the Burkholderiaceae and Thermoactinomycetaceae families, were almost exclusively observed among adult patients.

In another study [29], CF patients with progressive lung disease were noted to have a decrease in bacterial diversity with increasing age, but the total bacterial density remained stable over time. Antibiotic exposures in conjunction with recurrent pulmonary exacerbations were proposed as a possible contributing factor toward this observation. In a study by Tunney et al., several anaerobic species (including a number of Veillonella and Prevotella species) constituted a significant portion of the CF airway microbiota [36]. In a unique study, next generation sequencing was used to study the microorganisms of gastric juice among patients with CF and non-CF controls [37]. CF gastric juice was noted to have an abundance of Pseudomonas spp. and a relative paucity of normal gut bacteria (such as Bacteroides and Faecalibacterium), which was in contrast with normal gastric juice samples. These results suggest that CF patients possess a unique aerodigestive microbiome that is inter-related. This explanation seems plausible as the factors that influence the airway microbiome (for instance, antibiotic exposures) are also likely to influence the microbiota of gut and other organ-systems of the body [38].

In patients with CF, different bacterial colony morphotypes can be isolated from a single sputum sample. There is some evidence to suggest that these different morphotypes arise from a single bacterial strain [39]. Microbes in the lungs of CF patients are capable of constantly adapting to selection pressures. Some of the mechanisms that enable the evolution of microbes include motility, type III secretion systems, lipopolysaccharide, plasmids (encoding for antibiotic resistance), biofilm formation, small colony variants, quorum sensing, and hypermutability. As a consequence of these mechanisms, different phenotypes arise from a single bacterial species and, over time, a single bacterial strain with dominating features may evolve [40]. Given that different bacterial strains have differing capacities to evolve, multiple lineages of bacterial colonies evolve and coexist [41]. Some studies have shown that complexity of bacterial communities inversely correlates with patient age, antibiotic exposures, and presence of *P. aeruginosa* [42]. In one study, heterozygosity for the ΔF508 mutation and presence of mutations other than the ΔF508 was associated with relative preservation of airway bacterial diversity over time [35]. This shows that apart from environmental exposures (such as antibiotic pressures), patients' genotype (type of mutation) also plays an important role in determining the composition of the CF airway microbiome. In terms of environmental exposures, antibiotic use has been shown to be the prime factor that adversely affects microbial diversity among CF patients [29]. Loss of bacterial diversity (under the selection pressure of antibiotics) has been associated with an increased risk of pneumonia in mechanically ventilated patients colonized with *P. aeruginosa* [43]. Smith et al. studied this further by performing whole genomic analysis of a single species of *P. aeruginosa* isolated from a patient with CF. Whole genomic sequencing was repeated multiple times during the course of the patient's illness, which enabled the detection of an overwhelming number of mutations. Based on these analyses, it was found that the strain of *P. aeruginosa* that inhabits patients with advanced CF differs significantly from wild-type *P. aeruginosa* [40].

The interaction among different bacterial colonies has also become a subject of intense research and genomic and proteomic approaches are currently being used to understand their interrelationships. In an experimental study, production of 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) by a strain of *P. aeruginosa* enhanced the aminoglycoside resistance of *S. aureus* [44]. This study provided some evidence of how bacterial interspecies interaction can alter the airway microbiome by selecting for resistant strains of a bacterial species. Previous studies have shown that HQNO is detectable in the sputum of infected CF patients. Therefore, an interaction between *P. aeruginosa* and *S. aureus* may account for the increased incidence of small colony variant (SCV) of *S. aureus* species in CF patients with advanced lung disease.

In the recent literature, an increasing number of unusual microbes have been reported as the cause of infective exacerbations of CF. Such bacteria include multidrug resistant pathogens like *S. maltophilia*, multidrug resistant *P. aeruginosa*, MRSA, *Burkholderia cenocepacia* and even NTM [45]. The emergence of such bacteria as members of the CF airway microbiome can have important implications for management and prognosis for patients. For instance, studies have shown that in CF patients with an acute exacerbation, there is discordance between the results of microbial sensitivity testing and response to antibacterial therapy [46]. Polymicrobial infections and presence of fastidious organisms may account for this observation. Moreover, such pathogenic bacteria can interact with other less virulent bacterial species and lead to

architectural distortion of the entire CF microbiome. In the following lines, we discuss common members of the CF airway microbiome, some of which are commonly implicated in infective exacerbations.

### 4.1. Methicillin-sensitive *Staphylococcus aureus*

*S. aureus* is a common colonizer of the anterior nares of adolescent and adult patients [47]. Among patients with CF, MSSA is one of the most common pathogens isolated from sputum samples obtained for culture and sensitivity testing. In the CF Foundation (CFF) patient registry (Bethesda, Maryland, USA), *S. aureus* was most commonly isolated from children and adolescents accounting for approximately 51% of the total samples. Moreover, the overall prevalence of *S. aureus* has been increasing over the past few decades. Infection with *S. aureus* has been associated with increased bronchial inflammation and decreasing pulmonary function [48]. Moreover, when coinfection with *P. aeruginosa* and MSSA occurs, mortality is increased manifold. Interestingly, studies have shown that MSSA is associated with more severe disease in children as compared to adults.

With the widespread use of antistaphylococcal antibiotics, incidence of Gram-negative infections among CF patients has increased and MSSA has become less common among adult patients. Overall, the most common cause of chronic lung infections in CF patients is *P. aeruginosa*, an oxidase-positive Gram-negative bacillus. Moreover, as CF patients grow older, MRSA becomes a more frequent cause of infective exacerbation than MSSA. Over the past few years, the incidence of MRSA infections has been steadily increasing, owing to increasing use of antistaphylococcal penicillins (such as oxacillin and nafcillin) [49]. More recently, a subtype of *S. aureus* species (viz. small colony variant) has been isolated more frequently from CF patients. The small colony variant of *S. aureus* species is fastidious and slow-growing, and it has also been associated with rapid decline in pulmonary function. As mentioned previously, selection of small colony variant species is promoted by HQNO—a product synthesized and secreted by *P. aeruginosa* species [44]**.** Increasing use of broad-spectrum antibiotics that select for multidrug resistant pathogens can explain this distortion in the composition of the airway microbiome in patients with CF.

### 4.2. Methicillin-resistant *Staphylococcus aureus*

*S. aureus* is typically the first bacterial pathogen to invade the pulmonary parenchyma in patients with CF. Chronic infection with this organism can persist in the airways of CF patients for several years. Acquisition of mecA gene mediates methicillin resistance in community-acquired MRSA by encoding for a mutated penicillin binding protein-2A (PBP-2A) [50]. The prevalence of MRSA has increased substantially over the past several years from an estimated 7.3% in 2001 to 22.6% in the year 2008 and 25.7% in 2012 [10]. This increase in prevalence of MRSA was noticed across CF patients of all age groups with the highest increase being in the adolescent age bracket. This increase in the prevalence of MRSA in CF patients has been directly linked to the increase in overall incidence of community-acquired MRSA in the general population [51]. In a study by Glikman et al., 22 of 34 (64.7%) MRSA isolates from patients with CF contained the gene SCCmec II—a typical feature of health-care associated

MRSA strains. On the other hand, 9 of 34 (26.5%) MRSA strains harbored the SCCmec IV gene, which characterizes them as community-acquired MRSA strains. Most patients with community-acquired MRSA were newly colonized with the strain. Additionally, children with CF were more likely to harbor MRSA isolates that were resistant to clindamycin and ciprofloxacin compared with strains from non-CF patients [52]. Other studies have reported persistent infections in CF patients with both hospital-acquired and community-acquired MRSA strains (including Panton-Valentine leukocidin-positive strains) with an overall prevalence of 7.8% [53]. In these studies, persistence was due to presence of different clones over time or identical clones that underwent minor modifications in their toxin content. Moreover, isolation of MRSA from CF patients aged 7–24 years has been associated with an increased severity of the disease. Alarmingly, some of these strains may be vancomycin-intermediate *S. aureus* (VISA), which implies that treatment with glycopeptides (such as vancomycin) may also be ineffective. Highly virulent strains, such as vancomycin-resistant *S. aureus* (VRSA), have also been reported to cause necrotizing pneumonia in a small number of CF patients [54]. Persistent infection with virulent strains of *S. aureus* has been associated with a rapid decline in pulmonary function [55]. In a case-control study, CF patients who were colonized with MRSA had a significantly higher rate of decline in $FEV_1$ (forced expiratory volume in first second) as compared to those who were not colonized with MRSA [56]. Moreover, MRSA-infected CF patients have been shown to have longer hospital stays than age- and sex-matched controls [57]. Serious manifestations of MRSA infections have also been described in various reports. Cavitary lesions have been described in two CF patients infected with Panton-Valentine leukocidin-positive MRSA strains [54]. This observation was consistent with other reports of serious pulmonary manifestations of community acquired MRSA infection [54, 58]. In a cohort study of longitudinal data, risk of death among CF patients who had at least one culture positive for MRSA was 1.27 times greater than for CF patients in whom MRSA was never detected [55]. In a meta-analysis of 76 studies, a clear and strong association was noted between exposure to antibiotics and isolation of MRSA [59]. The risk of acquiring MRSA was increased by 1.8-fold in patients who had taken antibiotics as compared to others. The risk ratios for quinolones, glycopeptides, cephalosporins, and other beta-lactam antibiotics were 3, 2.9, 2.2, and 1.9, respectively.

### 4.3. Hemophilus influenzae

*H. influenzae* is a facultative, anaerobic, Gram-negative bacillus. In many patients, this organism begins to colonize the upper respiratory tract since infancy. Approximately 20% of infants with CF are colonized by the end of first year of life and the rate is even higher for patients of older ages [60]. By the age of 5–6 years, more than 50% of children are colonized with this bacterium [61]. *H. influenzae* is a common pathogen of chronic lung infections and is frequently implicated in infective exacerbations of CF [62]. In children with CF, about 32% are colonized with this microorganism. However, as these patients grow older and are exposed to a wide range of broad-spectrum antibiotics, more virulent bacteria inhabit their respiratory tracts. Consequently, in adults with CF, the rate of colonization with *H. influenzae* is reported to be only 10–15%. Having said this, the prevalence of *H. influenzae* has increased from 10.3% in the year 1995 to 16.3% in the year 2008.

Similar to the general population, colonization of the upper respiratory tract of CF patients with *H. influenzae* is quite a dynamic process. Children will typically carry multiple strains of this bacterium simultaneously, whilst adults will be colonized with only one strain [63]; again, this is a natural consequence of the loss of microbial diversity induced by antibiotic selection pressures. Even in most healthy adults, the upper airway is colonized with *H. influenzae*; most strains in such healthy subjects are nontypeable. In particular, the nasopharynx is an area of the respiratory tract that serves as a potential reservoir of this bacterium. Eventually, the organism may spread from the nasopharynx to the lower respiratory tract and cause an infection of the pulmonary parenchyma [64]. Studies have shown that most CF patients are cocolonized with two or more distinct strains of *H. influenzae* [65].

*H. influenzae* is not considered a virulent pathogen in patients with CF. Interestingly, some studies have shown that colonization with *H. influenzae* is associated with a relatively preserved lung function. This is in sharp contrast to other microorganisms like *P. aeruginosa* and MRSA, whose colonization of the pulmonary parenchyma is strongly associated with a rapid decline in lung function [66]. In a prospective study, 27 patients with CF (under the age of 12 years) and 27 matched patients with asthma were followed up for 1 year [67]. The isolation rate of noncapsulated (nontypeable) strains of *H. influenzae* was significantly higher in the CF group as compared to that of the asthma group. During exacerbations, the isolation rate of *H. influenzae* in the CF group was significantly greater than at other times, whereas there was no significant difference in the control group. The distribution of biotypes of *H. influenzae* and *Hemophilus parainfluenzae* was similar in the two groups. In the CF group, biotype I was commonly detected and was associated with infective exacerbations of CF. In contrast, biotype V was more common in the asthma group, although it had no association with the development of infective exacerbations [67].

### 4.4. Pseudomonas aeruginosa

*P. aeruginosa* is an obligate aerobic, oxidase-positive, nonlactose fermenting Gram-negative rod. *P. aeruginosa* is the most common organism implicated in infective exacerbations in patients with CF. In the CFF patient registry (Bethesda, Maryland, USA), more than half of the patients (52.5%) were reported to be infected with *P. aeruginosa* in 1995. The risk of chronic infection with *P. aeruginosa* increased proportionately with increasing age. Moreover, the incidence of *P. aeruginosa* has been reported to be increasing in infants. Despite changes in the management of patients with CF, the frequency of persistent infection with *P. aeruginosa* has remained relatively stable over time [68]. In a study based on the CFF patient registry, prevalence of colonization with *P. aeruginosa* was 60% in 1995 and 56.1% in 2005 [69]. However, recent data suggest that the prevalence of P. aeruginosa is slowly decreasing over time and has been estimated to be 30.4% in the year 2015 [70].

The main reservoir of *P. aeruginosa* is the environment surrounding CF patients. It has been thought that among siblings with CF, prolonged exposure of young children to their older siblings with CF is a potential risk factor for acquisition of *P. aeruginosa*. A study published in 1991 reported that *P. aeruginosa* may be acquired by patients at CF recreation camps, clinics, and/or rehabilitation centers [71]. Studies on genotypes of *P. aeruginosa* performed using

conventional pyocin typing and DNA probe analysis reported that most CF patients harbored a persistent strain of *P. aeruginosa* in their lungs [72]. These studies suggested that cross-colonization possibly could occur among patients. Another study showed that 59% of CF patients harbored a clonal strain of *P. aeruginosa* and the dominant pulsotype was indistinguishable from nonclonal strains with respect to both colony morphology and resistance patterns [73]. Wolz et al. used DNA probe amplification assays and demonstrated that 46% of CF patients (who were initially uninfected) acquired P. aeruginosa infection at the end of a CF recreation camp [74]. Clear evidence of a cross-infection among patients attending a CF clinic was published in 2001 [75]. In this study, 22 of 154 patients attending an adult CF clinic were chronically infected with similar isolates (based on pyocin typing and pulsed-field gel electrophoresis [PFGE] analysis) of *P. aeruginosa* that shared unusual phenotypic features: lack of motility and pigmentation along with a remarkable resistance to many antibiotics. In another study from a large pediatric CF clinic from Australia, 65 patients (55%) were found to be infected with a similar strain of *P. aeruginosa*. These patients were more likely to have been hospitalized in the preceding 1 year for respiratory exacerbations [76]. On the other hand, a study conducted by Speert et al. in Vancouver (Canada) reported a low rate of transmission of *P. aeruginosa* from one CF patient to the other [77]. In this study, a total of 157 genetic types of *P. aeruginosa* were identified, of which 123 were unique to individual patients. These apparently conflicting findings may be accounted for by the highly adaptable nature of *P. aeruginosa* and its ability to evolve. In a study by Mahenthiralingam et al., different strains of *P. aeruginosa* were studied using genomic fingerprinting and random DNA amplification assays [78]. A total of 385 isolates from 20 patients were grouped into 35 random amplified polymorphic DNA (RAPD) strain types. Secretion of mucoid exopolysaccharide, loss of expression of RpoN-dependent surface factors and acquisition of serum-susceptible phenotypes in Pseudomonas were shown to be a specific adaptation to infection, rather than being acquired from a new bacterial strain. This explanation is also in congruence with observations from other studies that found different strains of *P. aeruginosa* in unrelated CF patients and identical or closely related strains among siblings [79]. The presence of distinct strains of *P. aeruginosa* in these studies reflects an absence of nosocomial transmission of organisms at respective CF centers [80]. This may be a consequence of strict hygiene measures and microbiologic surveillance instituted at most CF centers across the world following reports of nosocomial spread [75, 76].

The effects of *P. aeruginosa* infection on the CF lung are deleterious. In one observational study, outcomes of CF children colonized with *P. aeruginosa* were compared with those of noncolonized patients. Children colonized with *P. aeruginosa* had a worse outcome and experienced rapid decline in pulmonary function as measured by $FEV_1$ and $FEF_{25}$ (forced expiratory flow at 25% of vital capacity) [81]. In another longitudinal observational study, the temporal relationship between *P. aeruginosa* infection and pulmonary damage (as measured by $FEV_1$ and Wisconsin additive chest radiograph score) was explored. Acquisition of *P. aeruginosa* was independently associated with a worsening pulmonary status in children with CF [82]. Moreover, in these studies, decline in pulmonary function after colonization with *P. aeruginosa* was observed to be gradual. This decline in pulmonary function associated with *P. aeruginosa* infection is noted across all age groups. In another study, acquisition of mucoid

strains of *P. aeruginosa* was associated with an unfavorable prognosis [83]. From a pathologic perspective, *P. aeruginosa* causes repeated airway infections with eventual progression to chronic airway infection. This organism can also lead to necrotizing pneumonia, chronic bronchopneumonia, and chronic parenchymal lung disease. While the aggressive use of antipseudomonal antibiotics has been shown to delay the onset of chronic infection, prevalence rates of *P. aeruginosa* colonization have remained relatively stable over the past two decades [84, 85].

The CF airway provides a pathological milieu and a scaffold for chronic infection with resistant organisms, the most notable of them being *P. aeruginosa*. A number of virulent factors enable this resilient organism to establish it within the CF airways. One such virulence factor—overproduction of alginate slime capsule—characterizes the mucoid type of *P. aeruginosa*, which allows it to adhere firmly to the airway epithelium. Being encoded by the AlgT gene, alginate negatively regulates flagella, fimbriae, and quorum sensing. TTSS (injectosome) positively regulates alginate production indirectly through heat shock, osmotic, and oxidative stress responses [86]. In the inflamed CF airway, polymorphonuclear leukocytes (PMN) lead to the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) [87]. Moreover, mutated CF epithelial cells are unable to efflux glutathione (a potent free radical scavenger) and unable to absorb other dietary antioxidants. Production of ROS and RNI by PMN leads to DNA damage, lipid peroxidation and denaturation of proteins. At the same time, RNI and ROS lead to upregulation of alginate production by *P. aeruginosa*. The alginate slime capsule enables the bacterium to adhere firmly to the airway epithelial cells and results in persistence of this organism within the airways. At the same time, other virulence factors produced by *P. aeruginosa* (such as exotoxins) incur progressive pulmonary damage and help it to evade the (already impaired) host immune response. Over time, ROI and RNI lead to loss of microbial diversity and disruption of the airway microbiota. Simultaneously, such an environment favors the survival and selection of *P. aeruginosa* within the CF airway and leads to persistent infection with this organism [88, 89]. Moreover, antibiotic exposures select for multidrug resistant variants of the organism and allow them to predominate and colonize the airways [24, 90]. Alarmingly, recent reports from CF centers across the world have described certain strains of *P. aeruginosa* that exhibit resistance to all clinically relevant classes of antimicrobials ("pan-resistant" *P. aeruginosa*) [91]. This can explain the worse prognosis associated with this organism in most studies of CF patients.

### 4.5. Burkholderia cepacia complex

More than 60 species belonging to the genus Burkholderia are not pathogenic to humans, but some of the remaining species are implicated in serious infections in CF patients. Using 16S rDNA and recA gene analysis, 17 species of this genus have been grouped together as the Burkholderia cepacia complex (BCC). BCC is a group of virulent pathogens that are frequently implicated in infective exacerbations in CF patients with end-stage lung disease. Colonization with BCC in CF patients indicates a poor prognosis and has been shown to be associated with a requirement for lung transplantation. This worse prognosis is due to the inherent antibiotic resistance

possessed by these organisms and their ability to rapidly spread from patient to patient. In some cases, infection with BCC can lead to the development of cepacia syndrome—a rapid fulminating pneumonia that often leads to bacteremia and sepsis. Given their virulent nature, strict infection control measures are essential to prevent outbreaks of BCC in CF clinics and centers [92]. A report of rapid spread and outbreak of BCC infection was reported in a CF center in Toronto [93]. This center reported the development of cepacia syndrome in many patients, being characterized by rapidly deteriorating pulmonary function, fever, leukocytosis, elevated markers of inflammation, and BCC bacteremia. Furthermore, in another report, cepacia syndrome occurred in approximately 20% of infected patients and had a case fatality rate of 62% [93].

Outside of the BCC group, a few other species of the Burkholderia genus are also implicated in infective exacerbations. These species include *Burkholderia gladioli*, *Burkholderia fungorum*, *Burkholderia multivorans* and *Burkholderia pseudomallei* [94]. Of these, *B. gladioli* now accounts for a significant proportion of Burkholderia infections in CF patients [95]. In the United States, *B. multivorans* and *B. gladioli* together account for more than 50% of Burkholderia infections in CF patients.

Most infected CF patients harbor genotypically distinct strains of the BCC. Strains of Burkholderia spp. that are shared by multiple CF patients are very uncommon. This suggests that most Burkholderia infections in CF patients result from acquisition of strains from the natural environment [92, 96]. In this regard, *B. gladioli* and *B. cepacia* have been described as recognized plant pathogens. In one study, multilocus sequence typing of Burkholderia spp. revealed that more than 20% of CF isolates were identical to strains recovered from the environment [97].

In the CFF patient registry, prevalence of BCC was reported to have declined from 9% in 1985 to 4% in 2005. Incidence of BCC was also found to be reduced from 1.3% in 1995 to 0.8% in 2005 [69]. This has not changed significantly over the past decade as shown by data published in 2016 [70]. Ramette et al. analyzed 285 confirmed isolates of BCC using restriction analysis of recA and identified seven different BCC species in the environment [98]. Healthcare-associated outbreaks of BCC infections as a consequence of contaminated medical devices and products (such as mouthwashes, ultrasound gels, skin antiseptics, and medications) have been reported previously. While most of these outbreaks have generally involved non-CF patients, the potential for developing such outbreaks among CF patients remains a hazard [99]. Infection of the respiratory tract with BCC species in CF patients often results in a chronic persistent infection [100]. In most such cases, a single strain of Burkholderia spp. colonizes the respiratory tract.

Infection with BCC species has been associated with a worse prognosis. In one study, CF patients who were infected with *Burkholderia dolosa* had a rapid decline in $FEV_1$ over time [101]. In another study, patients colonized with *B. cenocepacia* had a worse outcome in terms of body mass index (BMI) and $FEV_1$ as compared to those colonized with *P. aeruginosa* or *B. multivorans* [102].

### 4.6. Anaerobic bacteria

Anaerobic bacteria have been described in the airways of people with healthy lungs and are generally not considered to be pathogenic. In patients with CF, anaerobic bacteria are persistent members of the lower airway community as the anaerobic conditions (and steep oxygen gradients) in the lower airways provide an ideal environment for their growth [88, 103].

However, in the CF lung, anaerobic bacteria can produce virulence factors and damage the lung parenchyma (perhaps as a consequence of impaired innate immunity), which may worsen pulmonary function and exacerbate the inflammatory response. Short-chain fatty acids produced by anaerobic bacteria can increase production of interleukin-8 (IL-8) by upregulating expression of the short-chain fatty acid receptor GPR41 [104]. Moreover, in the CF microbiome, anaerobic bacteria can interact with other established pathogens and lead to progressive pulmonary damage [105]. Previously, anaerobic bacteria were thought to be an infrequent cause of CF exacerbation; however, with the advent of novel (culture-independent) microbial detection methods [106–109], anaerobes have been isolated from more frequently. In one study, 23.8% of sputum specimens from CF patients grew more than $10^5$ colony forming units (CFU) per milliliter of anaerobic bacteria [110]. In another study, 15 genera of obligate anaerobes were identified in 91% of CF patients with counts (CFU/ml) being comparable to that of *P. aeruginosa* and *S. aureus* [111]. The most common anaerobes were *Staphylococcus saccharolyticus* and *Peptostreptococcus prevotii*. Some studies suggest that patients with lower aerobic and anaerobic bacterial load have worse pulmonary function and higher levels of inflammatory markers [112]. From a biological standpoint, lower quantity of aerobes and anaerobes may reflect disruption of the CF microbiota. Studies have shown that antibiotic therapy directed against *P. aeruginosa* during acute exacerbations does not affect anaerobes [111]. This observation could be explained by considering the resistance patterns of anaerobes. In 58% of patients, obligate anaerobes detected during acute infective exacerbations were resistant to antibiotics used for treatment. The chief obligate anaerobes in such cases were *Bacteroides* spp., *Porphyromonas* spp., *Prevotella* sp., *Veillonella*, *anaerobic Streptococcus* spp., *Proprionibacterium*, *Actinomyces*, *S. saccharolyticus* and *P. prevotii* [36, 111, 113]. Interestingly, infection with *P. aeruginosa* significantly increases the likelihood of isolating anaerobic bacteria from CF patients [36]. Some of these anaerobic bacteria (such as S. milleri) are now known to be associated with worse clinical outcomes. Furthermore, new anaerobic organisms have been detected for the first time from samples of CF patients. Such bacteria, for instance Gemella and Rothia mucilaginosa, have been found to be associated with dismal pulmonary outcomes. Most such patients are often coinfected with *P. aeruginosa* as well [114, 115].

### 4.7. Nontuberculous mycobacteria

Traditionally, the frequency of CF patients infected with NTM has been reportedly low. In the CFF patient registry, the prevalence of NTM infections among CF patients has been estimated to be 2.2%. Nevertheless, the prevalence of NTM has been increasing slowly over the past few decades. The prevalence of NTM infection in 1999 among CF patients was 0.85%, which increased to 2.18% in 2008 [116]. More recent data published in 2016 shows that the prevalence of NTM may be as high as 11.9% [70]. The most common NTM species have been reported to be Mycobacterium avium-intracellulare (MAI) complex and *Mycobacterium abscessus*. Factors associated with a culture positive for NTM are older age, greater $FEV_1$, higher frequency of MSSA colonization and lower frequency of *P. aeruginosa* infection [117]. In most patients, unique strains of NTM are detected by molecular typing, which suggests that neither person-to-person transmission nor nosocomial acquisition is implicated. In one study, the prevalence of NTM infection among 385 patients in three Parisian centers was 8.1%. *M. abscessus*

was isolated in all age groups. About 4.1% (16/385) of the study cohort met the American Thoracic Society (ATS) criteria for NTM-related lung disease [118]. In another multicenter study done in Israel [119], prevalence of NTM-related lung disease (as defined by the 2007 ATS criteria) was 10.8%. This study further suggested that the incidence of NTM infections is increasing over time. Other studies have demonstrated that the incidence of MAI complex infections in CF patients is decreasing with time, while that of *M. abscessus* complex is increasing [120]. Alarmingly, infection with *M. abscessus* complex has been associated with a worse impact on pulmonary function. Some researchers have proposed that eradication of *M. abscessus* complex may provide a significant improvement in terms of pulmonary outcome [121]. However, *M. abscessus* is difficult to manage, commonly affects younger children, and requires prolonged courses of intravenous antibiotics [122].

### 4.8. *Stenotrophomonas maltophilia*

*S. maltophilia* is a Gram-negative bacillus that is commonly implicated in nosocomial infections in non-CF patients. However, in patients with CF, *S. maltophilia* has been recognized as a cause of acute infective exacerbation. The medical importance of this pathogen is that it is inherently resistant to a wide range of broad-spectrum antibiotics (most notably carbapenems). The prevalence of infection with this organism has increased from 1 to 4% over a period of 20 years (1985–2005) [68]. In the CFF patient registry, the prevalence of *S. maltophilia* increased from 4.0% in 1996 to 12.4% in 2005 [69]. From 2005 till 2015, the prevalence of *S. maltophilia* seems to have plateaued [70]. *S. maltophilia* infections of the respiratory tract in CF patients tend to be acute and, in most cases, the organism does not persist in the lower airways (although recurrent infections can occur). Most isolates of this organism have been shown to be transmitted from patient-to-patient, especially among siblings, or those who are otherwise epidemiologically linked [123]. One-third of CF patients who experience recurrent infections with *S. maltophilia* harbor more than one strain of the organism [124]. The most important risk factors for acquiring *S. maltophilia* infections are therapy with carbapenems and central venous catheterization [125]. In one study, history of treatment with imipenem was 10 times more frequent among cases (who contracted *S. maltophilia*) than among controls [125]. Furthermore, all fatal infections with *S. maltophilia* occurred in patients who had received imipenem. Based on these results, it is advisable to cover *S. maltophilia* empirically in CF patients who develop super-infection while receiving imipenem therapy. In a report by Sanyal and Mokaddas [126], most strains of *S. maltophilia* were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole. Moreover, some evidence shows that CF patients infected with *S. maltophilia* were more likely to have been hospitalized for many days in the past one year [127]. Other factors associated with *S. maltophilia* acquisition were more than two courses of intravenous antibiotics, isolation of *Aspergillus fumigatus* or *P. aeruginosa* in sputum and oral steroid use [128]. *S. maltophilia* is also more common among CF patients who develop allergic bronchopulmonary aspergillosis (ABPA) [129]. While chronic infection with *S. maltophilia* is infrequent, it can occur in certain patients and requires repeated courses of antibiotics [130]. Chronic infection with *S. maltophilia* confers a threefold higher risk of mortality or the need for lung transplantation [131].

### 4.9. *Achromobacter xylosoxidans*

*A. xylosoxidans* has been recognized as a pathogen and cause of infective exacerbation in patients with CF [132]. In the CFF patient registry, the prevalence of *A. xylosoxidans* infection was 1.9% in 1995 [69]. In 2015, the prevalence had increased almost three-folds to 6.1% [70]. *A. xylosoxidans* is a ubiquitous organism that occurs widely in natural habitats. This organism is an opportunistic pathogen that affects only immunocompromised patients and those with CF. *A. xylosoxidans* is mostly implicated in nosocomial infections, such as hospital acquired pneumonia, catheter-associated urinary tract infection, and wound infections. Lung infections with this fastidious organism are difficult to eradicate. Most patients respond to antipseudomonal penicillins (such as piperacillin–tazobactam) and third- or fourth-generation cephalosporins [133]. In one report, two cases of *Achromobacter ruhlandii* developed after indirect contact between CF patients [134]. Another study from a French CF center reported that most isolates of Achromobacter spp. were resistant to fluoroquinolones and carbapenems [135]. In a retrospective study, CF patients who were chronically infected with *A. xylosoxidans* were more likely to have impaired pulmonary function. Additionally, the frequency of hospitalization was higher among such patients than others [136].

## 5. Implications for further research

Cystic fibrosis is a monogenetic multisystem disorder, but, pulmonary disease is the leading cause of morbidity and mortality. Recurrent pulmonary infections with pathogenic bacteria can lead to progressive pulmonary damage and eventually lead to death. Therefore, understanding the CF airway microbiome has immense importance for understanding the overall pathology of the disease. Disruption of the CF airway microbiome under the influence of environmental factors and antibiotic exposures is a crucial step in the development of end-stage pulmonary disease in such patients [40]. Colonization of the lower airways with pathogenic bacteria, such as *P. aeruginosa* [82] and *B. cenocepacia* [101], has been associated with end-stage pulmonary disease.

As the CF airway microbiome evolves under the influence of antibiotic exposures, microbes undergo a number of mutations and changes in their genome [137]. While these genetic mutations are an evolutionary mechanism for microorganisms (for instance, to acquire resistance to antibiotics), they create potential vulnerabilities that may be exploited in unique therapeutic approaches. Traditionally, the approach to management of CF pulmonary exacerbations has been through employment of antibiotics. While antibiotics are useful in the short run, multidrug resistant microbes eventually evolve and become a challenge to tackle. In view of this, novel approaches to the management of CF pulmonary disease have been proposed, which involve manipulating patients' microbial consortia [8]. From a theoretical perspective, such an approach aims to maintain the architecture of the CF airway microbiome and avoids the use of antimicrobials, thereby circumventing the problem of destroying the community structure of a patient's microbiome. Such a novel treatment approach is based on

the principles of personalized medicine and aims to tailor treatment according to each patient's individual microbiome [138]. By manipulating and restoring the structure of a patient's airway microbiome, the complex metabolomic profile of the patient's sputum (and other body fluids) can be altered, which may have long-lasting and pleiotropic consequences [139].

Novel treatment approaches for the treatment of CF patients hold theoretical promise, but their practical applicability and clinical efficacy remains to be established [140]. A recent pilot study compared the use of a probiotic (*Lactobacillus* spp.) versus placebo in pediatric CF patients. Patients receiving the probiotic demonstrated a significant reduction in hospitalization for pulmonary exacerbation and a beneficial effect on the gut in terms of reducing gastrointestinal inflammation [141]. Another clinical trial examined the efficacy of enteric probiotics in reducing the frequency and severity of pulmonary exacerbations in CF patients. Both studies reported that the use of enteric probiotics provided a significant reduction in the frequency of pulmonary exacerbations when compared to the placebo group [142]. Larger randomized controlled studies are needed to more fully evaluate the effect of probiotics on hard clinical endpoints [143]. Other treatment options based on these novel concepts need to be developed further, and they may help to improve the overall outcomes of patients with CF [144].

## Abbreviations

| | |
|---|---|
| ABPA | Allergic bronchopulmonary aspergillosis |
| ATS | American Thoracic Society |
| BAL | Bronchoalveolar lavage |
| BCC | Burkholderia cepacia complex |
| CD | Clostridium difficile |
| CF | Cystic fibrosis |
| CFF | Cystic Fibrosis Foundation |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CFU | Colony forming units |
| FAFLP | Fluorescent amplified fragment length polymorphism |
| $FEF_{25}$ | Forced expiratory flow at 25% of vital capacity |
| $FEV_1$ | Forced expiratory volume in first second |
| FMT | Fecal microbiota transplantation |
| HQNO | 4-Hydroxy-2-heptylquinoline-N-oxide |
| IL-8 | Interleukin-8 |
| MAI | Mycobacterium avium-intracellulare |
| MRSA | Methicillin-resistant Staphylococcus aureus |
| MSSA | Methicillin-sensitive Staphylococcus aureus |

| | |
|---|---|
| NTM | Non-tuberculous mycobacteria |
| OTU | Operational taxonomic units |
| PCR | Polymerase chain reaction |
| PBP-2A | Penicillin binding protein-2A |
| PFGE | Pulsed-field gel electrophoresis |
| PMN | Polymorphonuclear leukocyte |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction fragment length polymorphism |
| RNI | Reactive nitrogen intermediates |
| ROS | Reactive oxygen species |
| VISA | Vancomycin-intermediate Staphylococcus aureus |

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