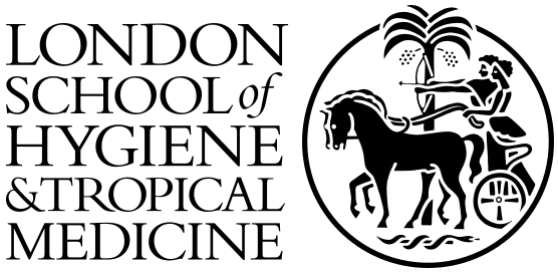

Commentary

Pathogen eradication" and *"Emerging pathogens"*: Difficult definitions in cystic fibrosis

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Abstract

Infection is a common complication of cystic fibrosis (CF) airways disease. Current treatment approaches include early intervention with the intent to eradicate pathogens in the hope of delaying development of chronic infection and chronic use of aerosolized antibiotics to suppress infection. The use of molecules that help restore CFTR function, modulate pulmonary inflammation, or improve pulmonary clearance, may also influence the microbial communities in the airways. As the pipeline of these new entities continues to expand, it is important to define when key pathogens are eradicated from the lungs of CF patients and equally important, when new pathogens might emerge as a result of these novel therapies.

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Predicted median life expectancy for people with cystic fibrosis (CF) is now reaching into the 5th decade (1). This dramatic rise is attributable to numerous research advances resulting in an improved understanding of the biology of CFTR (cystic fibrosis transmembrane conductance regulator) dysfunction and its consequences for innate immunity resulting in chronic infection, inflammation and lung damage. This knowledge has successfully translated into a variety of new treatments which have disease modifying potential.

Antimicrobial therapy to eradicate initial or repeated episodes of *Pseudomonas aeruginosa* positive sputum delays onset of chronic *P. aeruginosa* infection improving life expectancy (2,3). Other interventions that have contributed to improved life expectancy include: development of agents to help restore CFTR function; interventions to improve nutrition; using azithromycin as an immunomodulator; improving pulmonary clearance with ancillary mucoactive therapies such as hypertonic saline, DNAse and/or inhaled mannitol; infection control strategies; and as a last resort, lung transplantation (2,3,4). However, median predicted survival for CF patients is still substantially lower than that of the general population. The destruction of lung architecture, secondary to inflammation in response to chronic infection, is the major contributor to this shortened life span. Although advances in antimicrobial therapy have contributed significantly to increased life expectancy, they have also resulted in the emergence of multi-drug-resistant organisms that currently limits the long term effectiveness of this important treatment strategy (5).

As additional solutions for the care and treatment of CF patients are studied, an international working group of CF care providers, epidemiologists, and medical microbiologists gathered to address two questions which are important in considering the implementation of new antimicrobial agents: (i). when has a specific pathogen been eradicated from CF airways in an individual?; and (ii) when has an organism emerged as a pathogen in people with CF either *de novo* or as a result of these novel therapeutic approaches?

Defining “pathogen eradication” in individuals

Much of the improvement in life expectancy in people with CF is predicated on understanding how to prevent the establishment of chronic pulmonary infection. The major respiratory pathogen is a distinctive phenotype of *P. aeruginosa* referred to as "mucoid" (1). Mucoid strains of *P. aeruginosa* are highly adapted to grow in the CF airway. Key features of mucoid *P. aeruginosa* is a biofilm mode of growth that makes the organism refractory to innate immunity and antimicrobial therapy and a hypermutator phenotype which results in increased antimicrobial resistance (1, 6). The initial steps of the establishment of chronic infection with mucoid *P. aeruginosa* is colonization/infection with a non-mucoid strain (1,3). Genotyping studies suggest that initial colonization/infection is due to unique *P. aeruginosa* strains arising from the environment, although individuals may be infected with similar strains (6,7,8). However, what is less clear is whether individuals with similar strains obtained them from the environment or via cross-infection from another person with CF. Early studies showed that aggressive antimicrobial therapy of non-mucoid strains delayed the establishment of chronic infection (1,3). Subsequently two large studies, EPIC (9) and ELITE (10) determined the efficacy of eradication of *P. aeruginosa* using aerosolized tobramycin in different regimens with (EPIC) or without (ELITE) oral ciprofloxacin. Both were able to show that 28 days of aerosolized tobramycin alone led to negative cultures in approximately 90% of patients and the median time to next positive culture for *P. aeruginosa* was 2 years (9, 10). Adding ciprofloxacin, treating for longer duration (56 days), and routine treatment every 3 months did not improve outcomes. Further data from the EPIC cohort shows that individuals that had sustained eradication of *P. aeruginosa* were less likely to develop chronic infection compared to those with early recurrence of infection (9). The finding of a mucoid strain is a poor prognostic factor with a lower probability of eradication following therapy (11) and greater likelihood of having symptoms (2). Taken together, these data support the notion that regular culture of airways samples (surveillance cultures) beginning in infancy, with the express purpose of detecting *P. aeruginosa* during the early stages of infection, are an important standard of care for people with CF (2). When a positive airway culture occurs, the current practice is to use aerosolized antimicrobials for a fixed duration to eradicate *P. aeruginosa* (12). Registry data showing a striking decline in *P. aeruginosa* prevalence in a number of countries attest to the effectiveness of this eradication strategy (13, 14, 15). The optimal antibiotics and duration of treatment has not been fully established, but a

139 prolonged treatment duration of three months compared to one month and the
140 addition of oral antibiotics (16, 17) are not superior to one month of inhaled
141 tobramycin.

142 Other organisms that are pathogenic in the CF lung include members of the
143 *Burkholderia cepacia* complex particularly *Burkholderia cenocepacia,*
144 *Staphylococcus aureus* with methicillin resistant (MRSA) strains being observed with
145 increasing frequency, and *Mycobacterium* abscessus (1,3). MRSA is the only
146 organism for which a multi-center eradication study has been attempted (18). Here,
147 a complex eradication scheme of oral antimicrobials, nasal mupirocin, chlorohexidine
148 mouthwash and body wipes, and environmental cleaning including wiping
149 environmental surfaces and weekly washing of towels and linens was employed. It
150 was found that 54% of the CF subjects in the treatment arm remained free of MRSA
151 after 12 weeks compared to 10% in the control population. Owing to their low
152 prevalence and limited antimicrobial choices due to resistance, multi-center
153 eradication trials are not likely for either *B. cepacia* complex or *M. abscessus* (19,20)
154
155 Eradication presumes that a target organism has been eliminated from the airways.
156 To understand how eradication might be defined clinically, it is first important to
157 understand how chronic infection is defined. There have been a number of different
158 definitions of chronic CF lung infection (21). These definitions are based on the
159 persistent presence of a target organism e.g *P. aeruginosa*¸ and in some definitions,
160 an antibody response to the organism of interest (21). Since antibody tests are not
161 widely available or standardized (22), most definitions used in clinical trials of chronic
162 infections are based on sequential culture findings (9,10). The most widely used
163 definition of chronic CF lung infection is the Leed's criteria (5, 23). It has primarily
164 been used to define chronic *P. aeruginosa* infection in CF children. CF persons are
165 categorized as having no infection, being free of infection, intermittent infection, or
166 chronic infection. The initial definition was based on "monthly" cultures but has
167 evolved to one based upon the presence or absence of target organisms in four or
168 more respiratory specimens collected in a 12 month period (21,23). Patients defined
169 as having intermittent infection comprise those who have cultures positive for a
170 target organism in <50% of samples, with those defined as chronically infected target
171 organism culture positive in $\geq$50% of specimens.

Some investigators advocate for the use of qPCR as a surrogate for traditional culture for a specific target organism using the rationale that it is more sensitive than culture, especially in individuals who are unable to expectorate sputum (24, 25, 26). One study showed, however, that qPCR could not differentiate between subjects in whom eradication was successful and those who failed (25). As such, the utility of qPCR in eradication studies remains uncertain and requires further investigation.

It is important to recognize that the meaning of eradication in the research setting likely has different meanings for the research scientist compared to the research subject. The research subject should understand that eradication does not equal "cure" and that the primary goal of eradication treatment is to delay onset of chronic infection (9, 10, 11). Most subjects will "fail" eradication efforts at some point in the future. This failure may be the result of a recurrence of infection with the initial infecting agent or infection with a new strain of the same bacterial species (27).

In CF clinical trials, the definition of "eradication" varies widely from a negative culture at one week to three negative cultures over at least a six-month period after treatment cessation (10,11,12,18, 28). A definitive definition of "**eradication"**; would be valuable so that trials could be more easily compared. However, defining failure of eradication by a specific treatment is complicated by at least three factors. First, in non-sputum producers especially children under five years of age, oropharyngeal/deep throat swabs are often used for culture. Although these cultures tend to have good specificity, sensitivity is lacking meaning "false" eradication might be reported (29). Secondly, it may be difficult to differentiate "failure of eradication" with re-infection with a new genotype of the same target organism. Finally, the retention of indistinguishable genotypes in the oropharynx following successful lower airway antimicrobial therapy also raise important questions concerning upper airway reservoirs and how to best determine eradication in non-expectorating patients (7). Because oropharyngeal cultures are unreliable in reflecting what organisms are present in the lower airway, a second approach, a sinonasal washing may have value (29). Although a positive *P. aeruginosa* sinonasal culture has a strong correlation with the finding of the organism in the lower tract, it has a sensitivity of only 66% meaning one-third of individuals with *P. aeruginosa* in the lower tract will be culture negative. The accurate detection of *P. aeruginosa* in the lower respiratory tract using non-invasive techniques is challenging in the non-expectorating patient.

Eradication studies generally have not examined genotypes of the target organism prior to the initiation of therapy to enable comparison to the target organism isolate found post intervention. Moreover, they do not utilize multiple sampling strategies which assess both the upper and lower airway compartments. These approaches would be necessary for differentiating failure to eradicate (i.e. persistence of infection with the same strain) from re-infection either with the same or a different strain of the organism. This issue is further complicated by how many morphotypes should be genotyped pre- and post-intervention and what genotyping method should be used. Several PCR based methods have been used in molecular epidemiology studies of *P. aeruginosa* (7,8). The PCR-based method likely to offer the greatest discriminatory power is multi-locus sequence typing (MLST) (30). Another widely used method is pulsed field gel electrophoresis (PFGE) (30, 31) which has the advantage of being a widely accepted typing scheme that has been used for a variety of molecular epidemiology applications (32, 33). However, this technique is technically demanding and mutations, small insertions or deletions may cause organisms with the same genetic ancestry to appear to be distinct clones; whereas MLST is considered to be a more stable genotyping platform (30, 31). Overall, whole genome sequencing (WGS) is regarded as the most discriminatory of all typing methods (34). Ideally, as WGS becomes less expensive and more widely accessible, it will become the method of choice for bacterial strain typing. A major barrier to using WGS typing as epidemiologic tool is how to define if isolates belong to a specific clone. , The number of single nucleotide polymorphisms (SNPs) that defines a clone varies from organism to organism. For example, Marvig et al (6) reported that *P. aeruginosa* isolates recovered from CF children and young adults of the same clone type differed by 122 SNPs, while different clone types differed >10000 SNPs. By contrast, isolates of vancomycin-resistant enterococci (VRE) found to be indistinguishable by PFGE showed a diversity of <10 SNPs, indistinguishable MRSA isolates differed by <100 SNPs; whereas, unrelated VRE and MRSA PFGE types showed a divergence of approximately 4,000 and 20,000 SNPs, respectively (34). Before WGS method is adopted in clinical trials, clear definitions of what constitutes a clone and how many SNPs are necessary for isolates to be considered as unrelated clones needs to be determined.

As the development of novel CF therapies accelerate, a clear definition of what constitutes eradication.will allow for the design of rigorous studies that measure the effectiveness of these therapies and differentiate recurrence from re-infection with a different strain of the same organism. For now, the following recommendations should be considered:-

1. Eradication is best defined by obtaining multiple specimens (minimum of 3), over an extended time period (six months), all of which should all be negative for the target organism.
2. Genotyping of multiple colony types` of specific target organisms should be done at enrollment by a highly discriminatory technique (preferably WGS but PFGE or MLST may be reasonable substitutes) and compared to any target organisms found post-intervention. The number of isolates to be typed should be based on what is economically feasible.

Defining population-level pathogen emergence

The issue of when an organism "**emerges"** in a population has consequences for the use of novel therapeutics in CF patients. The term "**emerging pathogen"** is one that is greatly overused in the literature. This overuse most likely reflects the lack of a clear definition of what is an "emerging pathogen." Mathematically based definitions of "emerging pathogen", for example using segmented linear regression, are retrospective, but nevertheless offer a definition of greater precision (35). A widely used definition would be a "clinically distinct condition whose incidence in humans has increased" (36), but that suggests one should establish the time horizon over which this increase has happened, the population affected, and how much of an increase there has been when declaring "emergence (37)."

Pathogens that have emerged in CF patients according to this definition during the past four decades are members of the *B. cepacia* complex during the 1980s and MRSA during the first decade of this century. In the early 1980s, three CF centers in North America reported a new organism in CF patients called, at that time, *Pseudomonas cepacia* (19). This organism appeared to be truly novel causing a syndrome named the "cepacia syndrome" in which there was rapid pulmonary decline and in some cases bacteremia, a rare occurrence in people with CF,

resulting in death within months of infection but only rarely causing serious infection in other patient populations (20). This syndrome was found to be primarily due to a single species, *B. cenocepacia,* which is one of the 21 different species eventually characterized within the *B. cepacia* complex (19, 38). As molecular typing tools improved, centers where *B. cenocepacia* emerged were most often dominated by a single clone first recovered in a specific geographic locale (e.g. ET12 (Edinburgh/Toronto), PHDC, and Midwest strains) (18,20). The ET12 strain has subsequently been found throughout Canada, the United Kingdom and Ireland (18, 20,39). These *B. cenocepacia* strains, refractory to antimicrobial clearance, and able to be spread from person-to-person were eventually controlled by strict infection control practices.

The use of selective media to isolate members of the *B. cepacia* complex coupled with the use of genomic (DNA sequencing) and proteomic (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry or MALDI-TOF MS) identification techniques has resulted in the recognition of a number of "emerging" organisms (19,20). With the use of aerosolized tobramycin, the frequency with which *Stenotrophomonas maltophilia* and *Achromobacter spp*., two organisms resistant to tobramycin, are detected has increased (13,20). However, there is considerable debate as to whether either of these organisms are pathogens in chronic CF lung disease. Some studies suggest that *S. maltophilia* is a pathogen in settings where it can be proven that the patient is mounting a humoral response to the organism (40, 41,42); however, a large population-based study suggested otherwise (43). Similar data exists for *Achromobacter* spp suggesting that the organism is playing a pathogenic role in patients who mount a humoral response to it (20). Other much less frequently detected organisms (<1% prevalence) include *Burkholderia gladioli, Burkholderia pseudomallei*, *Ralstonia spp.*, *Chryseobacterium* sp., *Pandorea spp*., and *Inquilinus spp*.) (19,20). Insufficient longitudinal clinical data exists for people with CF infected with these organism in part due to the infrequency with which these organisms are recovered.

Another organism that has clearly emerged in CF persons has been MRSA in the United States during the first decade of this century. Chronic MRSA infection in CF is associated with declining lung function and premature death (44). Unlike *B.*

cenocepacia, the rise in MRSA in people with CF has paralleled the rise of MRSA in other US populations (13, 18, 45). Interestingly, MRSA has not increased in other countries with robust CF registry data such as the United Kingdom and Australia both of whom have been better able to control MRSA spread in the general population (15, 45)

Finally, the non-tuberculous mycobacteria (NTM) have also been described as "emerging" in people with CF. However the presence of NTM in people with CF was first described in the early 1990s based on data gathered in part in the 1980s (46). NTM found in people with CF predominantly comprise *Mycobacterium avium* complex and *M. abscessus* isolates. To say that an organism has "emerged," there needs to be evidence of an increase in its incidence. The problem in describing these organisms as 'emerging pathogens' is that there are historic data suggesting that it has been present in adult CF persons for at least 30 years. Increasing numbers of NTM infected CF persons in this century have been reported in the US, Israel, and Germany (47, 48, 49, 50). In the US, data has been gathered in a systematic manner only over the past five years. These data are further complicated by the fact that there is little standardization in how these organisms are detected and identified, thus making the data available of questionable value (47). Part of the increased detection of these organisms is likely due to both increased clinical awareness and improved genomic and proteomic based identification techniques (20). Another interesting possibility is that the combination of aggressive antimicrobial therapy over many years coupled with general improvements in overall health has created a CF adult population that is "primed" to be colonized/infected with environmental organisms that are highly resistant to antimicrobials. Examples of such organisms include *M. abscessus,* MRSA, *S. maltophilia* and *Achromobacter* spp.(19,20)

There are two intriguing observation concerning *M. abscessus* in CF lung disease that should be noted. First, there is a developing body of evidence that indicates that *M. abscessus* is associated with declining lung function in CF populations (49, 50) with the rate of pulmonary decline greater than seen with other CF pathogens (51). Secondly, a recent study has shown that a specific clone of *M. abscessus* may have spread globally (52). Could this clone be analogous to the *B. cenocepacia* clones that emerged in the 1980s and the MRSA US300 clone that emerged in the early part of this century in CF patients? Animal and *in vitro* studies suggest that this

strain demonstrates increased virulence when compared to other unique *M. abscessus* strains; however, further clinical and environmental studies are needed to determine its significance and origins (52).

An important recent finding is the recognition that patients with chronic CF lung disease have a unique microbiome which is resilient to antibiotic treatment (53). Within the context of this observation are two important findings. First, anaerobic bacteria and streptococci are frequently important components of this microbiome and changes in their relative abundance may be associated with pulmonary exacerbations (54,55,56,57). Secondly, as lung function declines, there is a decrease in airway microbial community diversity with certain organisms predominating (54,55,56,57,). Not surprisingly, these predominant organisms are those considered the major CF pathogens and include *P. aeruginosa, B. cepacia* complex and *S. aureus.* The role of NTM in the CF lung microbiome is presently unclear as challenges exist in their detection by current microbiome analysis methods.

Microbiome analysis by next generation sequencing will provide greater understanding of CF lung microbial communities and should eventually provide information on how organism interactions result in lung pathology. In the short term, the recognition of increased recovery of specific target organisms known to be associated with CF lung disease such as *P. aeruginosa,* MRSA*, S. aureus, B. cenocepacia,* and *M. abscessus* in patients receiving novel therapies will be important. Additionally investigators must be aware of the presence of organisms currently not associated with chronic CF lung infection which may be found with increasing prevalence in clinical trials of novel therapies.

The ability to accurately and reliably categorize CF patients, as having acquired an "emerging" pathogen or as having "eradicated" an existing infection, pivots on the intrinsic ability of clinical microbiology techniques to detect important shifts in patient microbiologic status. CF clinical microbiology laboratories are encouraged to follow best practice guidelines, as documented in the CUMITECH 43 guidelines (58), as well as the UK Cystic Fibrosis Trust Consensus Guidelines "Laboratory standards for processing microbiological samples from people with cystic fibrosis"(59). However,

application of these largely culture-based techniques may not be optimal to address issues of sensitivity and specificity to support microbiological status shifts, while other testing modalities (e.g. PCR or microbiome analysis) may allow greater precision.

As we move forward with clinical trials in CF lung disease it will be important for data safety monitoring boards to insist on the careful gathering of microbiology data.

1. Given the current technology and the understanding that specific organisms dominate the CF lung microbiome, predominant organisms even when they represent “normal flora” should be identified.
2. It will also be useful to establish reference laboratories similar to the national *Burkholderia cepacia* reference laboratories where organisms such as *P. aeruginosa,* MRSA, *M. abscessus* and perhaps others can be genotyped to determine if specific clones which may be more virulent are emerging as a result of specific therapies.
3. Microbiome analysis should be considered once a firm interpretative standard is available which can be used to determine if a particular therapy is associated with adverse alteration of the CF microbiome.

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