Journal of Cystic Fibrosis xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

journal homepage: www.elsevier.com/locate/jcf

Original Article

Role of viable but non culturable cells in patients with cystic fibrosis in the era of highly effective modulator therapy

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ARTICLE INFO

Keywords:
VBNC
Lung infection
CF lung pathogens
Non-cultural methods

ABSTRACT

Background: Lung infections antibiotic treatment in Cystic Fibrosis patients (pwCF) is often complicated by bacterial persisters, including the so-called Viable but Non Culturable (VBNC) forms, live cells undetected by the routine cultural microbiological methods. This study investigated the occurrence of VBNC cells of five CF bacterial pathogens in 94 pwCF over one year and the possible associations with the patients' clinical features.
Methods: Sputum samples, recovered at routine visits and during exacerbation episodes, were analyzed for the presence of the five pathogens by both routine culture-based assays and species-specific qPCR. VBNC cells were estimated as the difference between molecular and cultural counts and their presence was matched with the clinical data in particular the therapeutic regimens.
Results: All but ten pwCF showed the presence of VBNC cells at least once during the study. *Pseudomonas aeruginosa* and methicillin-susceptible *Staphylococcus aureus* were the species most frequently found in the VBNC state. Only the former showed a significant association between chronic infection and VBNC cells presence; VBNC-MSSA positive patients significantly increased overtime. The presence of non culturable bacteria was generally concurrent with poor lung functionality and more frequent pulmonary exacerbations. No significant association with modulator treatment was evidenced.
Conclusions: The obtained data demonstrated the overwhelming occurrence of bacterial VBNC cells in CF lung infections, warranting a constant monitoring of pwCF and underlining the need of implementing the routine culture-based assays with culture-independent techniques. This is pivotal to understand the CF bacterial population dynamics and to efficiently contrast the lung infection progression and worsening.

1. Background

Cystic fibrosis (CF) is a genetic disease that results in the accumulation and the lack of clearance of dehydrated mucus, mostly in the respiratory system, and hence in bronchiectasis [1]. This thick mucus traps most inhaled bacteria and viruses in the airways, making patients more susceptible to infection. During the first years of life CF sputum cultures show the presence of *Haemophilus influenzae* and *Staphylococcus aureus*, presumed to be the source of the lung morbidity in children [2]. Meticulous daily management of lung infections combining airway

Abbreviations: CF, cystic fibrosis; pwCF, patients with cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; VBNC, viable but non culturable; PA, Pseudomonas aeruginosa; AXE, Achromobacter xylosoxidans; SM, Stenotrophomonas maltophilia; MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-susceptible Staphylococcus aureus; qPCR, quantitative polymerase chain reaction; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow over the middle one-half of the FVC; sd, standard deviation.

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https://doi.org/10.1016/j.jcf.2024.02.013
Received 21 November 2023; Received in revised form 15 February 2024; Accepted 20 February 2024
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clearance techniques together with prompt, aggressive antibiotic treatments are essential to prevent the progression of lung disease. Although highly effective modulator therapy (HEMT) has led to substantial reduction of disease progression with improvement of clinical outcomes, dramatically affecting the quality of life of these patients, the effects of these drugs on lung infections are still controversial [3]. In this regard, despite some evidence that modulators can act synergistically with antibiotics [4], there are no long-term data to advocate discontinuation of antibiotic therapy for individuals on CFTR modulators [5].

Patients with CF (pwCF) although undergoing antibiotic treatment, can repeatedly show positive cultures for the same bacterial species. Antibiotic ineffectiveness may lie in survival of resistant bacterial variants or specialized phenotypes unsusceptible to the drugs [6]. Patients are usually distinguished in chronic or intermittent lung infected, following defined criteria [7,8]. Chronic lung infections are characterized by periods of stability and often clear sputum cultures, interspersed by pulmonary exacerbations, characterized by at least 4 new clinical symptoms *e.g.,* increased cough, change in sputum, new or increased hemoptysis, radiographic changes indicative of pulmonary infection, temperature above 38 °C, anorexia), ultimately resulting in a progressive decline of respiratory function [9].

The routine culture-based microbiological diagnosis is often inefficient for chronic CF patients, since it could result in an unreliable identification of the pathogens responsible of the pulmonary exacerbation and of their drug susceptibility. The lack of correspondence between *in vitro* and *in vivo* data about antibiotic efficacy is, indeed, a commonplace. The recent development of culture-independent techniques has allowed a more reliable community profiling of bacteria involved in CF lung disease, a complex scenario often composed by different species, strains and strain subpopulations [10]. Among these, persister cells, quiescent forms able to endure stress conditions and resume proliferation after stress removal, deserve a special mention. A deeper stage of persisters is represented by the Viable but Non Culturable (VBNC) cells, unable to grow on cultural media, but still viable, potentially virulent and capable to regain culturability in presence of specific inducers [11]. There is experimental evidence that VBNC forms can occur *in vivo* and that they can play a role in recurrent, biofilm-related infections, such as the pulmonary CF disease [12]. Depletion of oxygen and nutrients, the immune inflammatory response and antibiotic treatment might induce VBNC cell development, thus impairing the infection eradication [13].

The rationale of this study arises from the evidence that a quote of patients with chronic lung infection due to typical CF pathogens such as *Pseudomonas aeruginosa* (PA), *Achromobacter* xylosoxidans (AXE), *Stenotrophomonas maltophilia* (SM), *methicillin-resistant (MRSA) and -susceptible (MSSA) Staphylococcus aureus* showed infection eradication for a period of time of their life, followed by symptoms recurrence and new positive cultures of the strain previously identified. Recent advances in CF microbiology confirmed the presence of VBNC bacterial forms in biofilms of CF sputum samples.

Our aims were: to confirm the occurrence of VBNC forms of these 5 typical lung pathogens in pwCF, and to assess their influence on the lung infection prognosis as well as the efficacy of the currently applied therapeutic protocols.

2. Methods

2.1. Patient enrolment

A total of 102 patients were enrolled from March 2021 to May 2021 and they were followed-up for 12 months. Inclusion criteria included CF diagnosis (based on clinical symptoms, genotypic detection of CF-causing mutations and sweat Cl^- concentrations higher than 60 mmol/L), the ability of producing sputum samples and a 2-years documented clinical/microbiological background. Exclusion criteria consisted in the inability of producing sputum samples. No gender- or age-based selections were applied. EC informed consent/assent was obtained by all patients and/or parents/guardians. The study was approved by the Ethic Committee of Marche Region on 14 Jan 2021 (protocol code 2020 386).

2.2. Sputum sample collection and cultural analysis

Sputum samples were collected for each subject during routine visits (*i.e.,* every 3–4 months), during pulmonary exacerbations episodes and after the related antibiotic treatment. Sample collection was performed in duplicate according to the Italian Cystic Fibrosis Society guidelines [14]. One sample was used for microbiological culture-based analyses, performed according to the recommendations of the Italian Cystic Fibrosis Society [14] and previously published guidelines [15,16]. Samples were diluted in ratio 1:1 with Sputasol (Oxoid) and stored at 2–8 °C till analysis, within 24 h.

Bacterial cultures were grown and quantified on a combination of McConkey, Mannitol Salt, Chocolate and Columbia blood agar plates. The species identification was carried out by MALDI TOF (VITEK-MS, BioMèrieux, Marcy-l'Étoile, France). The occurrence and abundance of PA, MSSA, MRSA, SM and AXE were recorded.

2.3. qPCR assays

The other sample was used in qPCR assays, performed adopting a combined DNA extraction-qPCR protocol demonstrated to avoid the interference of extracellular DNA, derived from dead cells [17]. Total DNA was extracted from the sample using the QIamp DNA minikit (Qiagen GmbH, Hilden, Germania). To reliably quantify staphylococcal cells, the sample lysis was performed in TE buffer additioned with lysozyme 2,5 mg/ml e lysostaphin 150 μg/ml. qPCR assays were performed using 10 μl of 2 × Rotor-Gene SYBR Green PCR master mix (Qiagen), 2 μl DNA and 0.2 μM of species-specific primers, listed in Table 1.

A calibration curve was built for each target species, using 10-fold dilutions of purified PCR amplicons of each target gene, and bacterial cells were quantified as previously described [17]. The obtained qPCR counts were compared to the cultural ones. Discrepancies ≥ 0.5log or a positive qPCR for culture-negative samples were considered as indicative of the presence of VBNC cells.

2.4. Clinical data

Patients were monitored even from the clinical point of view, recording the following parameters at each visit:

Table 1
Species-specific primer pairs used in qPCR assays to detect CF bacterial pathogens.

Target gene (target species)	Primer sequence (5′−3′)	Amplicon (bp)	Source
ecfX (PA)	ecfX-F AGCGTTCGTCCTGCACAAGT ecfX-R TCATCCTTCGCCTCCCTG	145	[17]
Sa442 (MSSA)	Sa442-F TCGGTACACGATATTCTTCAC Sa442-R ACTCTCGTATGACCAGCTTC	179	[18]
mecA (MRSA)	mecA-F TCCAGATTACAACTTCACCAGG mecA-R CCACTTCATATCTTGTAACG	162	[19]
smeT (SM)	Sme1-F GCATGATCTCCATSGTYTTG Sme1-R GGCACTTCAAGAACAAGAGC	192	[20]
16 s rDNA (AXE)	AXF1n AACTGACGGTACCTGCAGAATAA AXB1n CACGCTTTACGCCCAGTAAT	103	[21]

- Exacerbation episodes and recrudescence time;
- Antibiotic therapy (drug class, duration, route and number of therapeutic cycles);
- Total bacterial population evidenced in sputum samples (*i.e.,* all the identified bacterial species);
- Respiratory function by determining FEV1 (Forced Expiratory Volume in the first second), FVC (forced vital capacity) and FEF_{25-75} (forced expiratory flow over the middle one-half of the FVC);
- Nutritional status;
- Comorbidities.

The obtained data were correlated with the presence and/or the abundance variation of bacterial VBNC cells over time.

2.5. Statistical analysis

The number of patients recruited in the study ensured to estimate the prevalence of VBNC equal to 80% with a level of precision of 16.5, 95 Confidence Interval (95%CI) 70.8–87.3). Sample size was estimated using the method of confidence interval for a proportion, based on binomial distribution.

Quantitative variables were summarize using mean and standard deviation (sd) and comparisons among groups were performed by Student *t*-test for independent sample.

Qualitative variables were summarized using absolute and percentage frequencies and comparisons among groups were evaluated with Fisher Exact test. Cochran-Armitage test was used to evaluate a trend in the prevalence of VBNC across visits.

A significance level of 0.05 was used to assess the statistical significance. All the analyses were performed using the statistical software R.

3. Results

3.1. PwCF enrollment and features

Out of 102 enrolled patients, 94 completed the study (407 sputum samples in total). Eight patients were excluded because passed (1), lost to follow-up (5), transferred (1), unable to produce sputum samples after the start of CFTR modulator therapy (1).

Patients were equally distributed about gender (50% female/ male subjects), with a mean age of 26 years (sd 14.4 years) median age of 23.4 years (range: 5.4–70.9 years); 37.2% exhibited an FEV1 (% pred) higher than 90 at the beginning of the study, 57.4% an FEV1 between 40 and 89 and 5.3% an FEV1 lower than 39. Overall, 57 patients were treated with modulators (Ivacaftor, $n = 1$; Lumacaftor/Ivacaftor, $n = 2$; Tezacaftor/ Ivacaftor, $n = 5$; Elexacaftor/Tezacaftor/Ivacaftor, $n = 49$; duration of treatment, days: 14–2939), whereas 37 patients had never started therapy.

Forty-eight (51.0%) pwCF were chronically infected by PA, 79 (94.6%) by MSSA, 10 (10.6%) by MRSA and SM and 8 (8.5%) by AXE. 10 out of 94 patients (10.6%) were chronically co-infected with other CF lung pathogens (non-tuberculous *Mycobacteria,* $n = 3$; *Burkholderia* spp, $n = 2$; *Scedoporium* spp, $n = 3$; *Exophiala* spp, $n = 2$).

3.2. VBNC cell detection in pwCF

The occurrence of VBNC forms of the 5 investigated lung pathogens (*i.e.,* PA, MSSA, MRSA, SM and AXE), was sought in sputum samples collected at routine visits and/or during exacerbation episodes, by comparing the bacterial counts obtained by the conventional culture-based approach and species-specific qPCR assays. The specific Limit of Detection (LOD) was determined for each target gene (Supplementary Table 1). All but 10 pwCF exhibited the presence of VBNC cells at least once during the study (Table 2); specifically, unculturable PA forms were found in 119 (29.2%) sputum samples, MSSA in 149 (36.6%), MRSA in 50 (12.2%) SM in 19 (4. 6%) and AXE in 21 (5.1%). Cultural tests failed to detect the presence of the specific pathogen in 22, 26, 32, 10 and 5 samples, respectively.

Table 2
Prevalence of VBNC cells of the five pathogens at each visit.

		PA	AXE
Visit	n	% (95%CI)	% (95%CI)
Visit 1	94	30.9 (21.5–41.3)	7.4 (3.3–15.3)
Visit 2	94	33 (23.83–43.5)	5.3 (1.97–12.3)
Visit 3	94	27.7 (19.2–38.0)	5.3 (1.97–12.3)
Visit 4*	93	22.6 (14.8–32.7)	3.2 (0.8–9.8)
p		0.206	0.285
		SM	MSSA
Visit 1	94	3.2 (0.8–9.7)	27.7 (19.2–38)
Visit 2	94	4.3 (1.4–11.2)	29.8 (21–40.2)
Visit 3	94	6.4 (2.6–13.9)	47.9 (37.6–58.4)
Visit 4*	93	7.5 (3.3–15.4)	47.3 (37–57.9)
p		0.201	0.003
		MRSA	At least 1 VBNC
Visit 1	94	8.5 (4.0–16.6)	58.5 (47.9–68.4)
Visit 2	94	12.8 (7.1–21.6)	60.6 (50.0–70.4)
Visit 3	94	14.9 (8.7–24.1)	68.1 (57.6–77.1)
Visit 4*	93	11.8 (6.3–20.6)	65.6 (54.9–74.9)
p		0.478	0.258

PA: *Pseudomonas aeruginosa*, AXE: *Achromobacter xylosoxidans*, SM: *Stenotrophomonas maltophilia*, MSSA: methicillin-susceptible *Staphylococcus aureus*, MRSA: methicillin-resistant *Staphylococcus aureus*, CI: confidence interval; *: at visit 4 SS not available for one patient.

The species more prone to develop VBNC variants resulted PA and MSSA; only for the latter a significant increase ($p = 0.003$) over the year of the number of patients presenting VBNC cells was observed

Considering the infectious status of the patients (*i.e.,* absence/presence of chronic infection), only PA showed a significant association ($p = 0.0003$) between chronic infection and VBNC cells presence.

3.3. VBNC cell impact on CF infection progression

The influence of VBNC cells on the infection progression was first evaluated by comparing the FEV1 values obtained at each routine visit by the patients showing/not showing the occurrence of VBNC cells. Moreover, the analysis was performed with the focus on the CFTR-modulator therapy, comparing the values obtained at the first visit of treated and not treated subjects (Table 3).

PwCF in which VBNC cells were detected showed FEV1 values significantly lower than those not presenting unculturable cells at each visit (Table 3).

As regards the CFTR-modulator therapy, untreated patients showed a

Table 3
FEV1 (%pred) of pwCF carrying and not-carrying VBNC cells at each visit, and according to CFTR-modulator therapy.

	No VBNC		VBNC		
Visit	n.	FEV1 (%pred) mean (sd)	n.	FEV1 (%pred) mean (sd)	*p*
1	38	86.8 (21.3)	54	74.5 (23.4)	0.011
2	28	90.9 (18.4)	44	73.7 (25.2)	0.001
3	26	92.3 (18.7)	44	80.3 (26)	0.029
4	25	92.5 (23.9)	45	77.2 (25.4)	0.016

	No VBNC		VBNC		
CFTR modulator at visit 1	n.	FEV1 (%pred) mean (sd)	n.	FEV1 (%pred) mean (sd)	*p*
Not treated	18	92 (18.1)	37	79 (22.4)	0.037
Treated	20	79 (22.4)	17	65 (23.1)	0.070

n: number of patients; sd: standard deviation.

significant lower FEV1 value when presenting VBNC cells, while treated patients did not show significant differences due to the presence/absence of non culturable cells. However, the association between the modulator-based therapy and the FEV1 values seemed inconsistent.

PwCF were then characterized according to the occurrence of pulmonary exacerbations and the presence/absence of VBNC cells detected at the first routine visit (Fig. 1).

Patients who presented VBNC cells at the beginning of the study were significantly more affected by pulmonary exacerbations that those not showing unculturable cells, most likely due to their reactivation under favorable conditions.

The heterogeneity of the cohort prevented us from further associations, with the presence of comorbidities, antibiotic therapy or composition of the total bacterial community.

4. Discussion and conclusions

Bacterial persisters hamper the lung infection eradication and favor the exacerbation recurrence in pwCF. Among persisters phenotypes, the VBNC forms are particularly troublesome since undetected by routine cultural methods, leading to an underestimation of their clinical relevance. Most data on VBNC cells in the CF clinical setting are referred to the two main pathogens PA and MSSA/MRSA. Deschaght et al. were among the first to describe the insurgence and persistence of PA VBNC cells in hospitalized pwCF undergoing antibiotic treatment and showing an improvement of their clinical conditions [22]. Such results were mirrored in our previous study, with PA VBNC cells detected in both culture-positive and culture-negative samples of 88 pwCF [17].

Here we aimed to investigate the presence of VBNC cells of five main CF lung pathogens in CF sputum samples and to correlate their detection with the clinical status. All but 10/94 monitored patients harbored VBNC forms of at least one of the investigated bacterial pathogens at one or more sampling times over a year (Table 2). VBNC PA and MSSA were those mostly detected, in line with the frequent involvement of these two species in CF lung infections. Albeit less frequent, the finding of VBNC cells of SM and AXE is noteworthy, evidencing the ability of these two emerging pathogens to survive antibiotic treatments by dormancy and to develop chronic infections. The different limits of detection don't seem to represent an important bias in the detection rate of the investigated pathogens, at least as regards MSSA, which was the most frequently detected. As regards AXE, we can't exclude an underestimation of further positive samples, presenting a low pathogen abundance.

In most cases, the VBNC forms coexisted with culturable cells of the same species, however 95 samples (22, 26, 32, 10 and 5 per PA, MSSA, MRSA, SM and AXE, respectively) resulted negative by cultural tests were actually showed to carry VBNC cells of the investigated species. This dramatically questions the effectiveness of the currently adopted diagnostic approaches, since the infection could be erroneously considered as eradicated, despite the presence of alive bacterial cells, possibly able to resume full pathogenicity. This issue has been previously pointed out, evidencing the inability of the classical cultural assays to describe the real CF lung infection dynamics and the need to integrate them with reliable culture-independent techniques [23]. Accordingly, even the definitions of the infection stages (*i.e.,* intermittent *vs* chronic) and the related criteria might be revised on the bases of the results provided by culture-independent assays; this is a crucial issue for the correct interpretation of CF microbiological data, especially in the modulators era. In this perspective qPCR, already demonstrated to detect pathogens (*e.g.,* PA) before culture [24,25], can reliably discriminate between intermittent and chronic patients. Moreover, it is already adopted in clinical labs for virological diagnosis and genotyping and some companies already provide qPCR assays for defined respiratory bacterial pathogens.

The frequency of patients carrying VBNC cells did not show a regular pattern (increase or decrease) overtime, with the only exception of the increase of VBNC-MSSA carrying subjects. This can be explained by the cohort heterogeneity and the heavy involvement of MSSA in CF lung colonization. These results thus encourage further longitudinal studies focused on specific bacterial species to monitor their transforming ability into phenotypes difficult to detect/treat.

The clinical role of VBNC forms has been long debated, dividing the microbiologists between persistent *vs pre-mortem* cell variants [26]. Recent studies have demonstrated that *S. aureus* VBNC forms can still retain pathogenicity, overexpress virulence determinants (*e.g.,* toxins) and infect eukaryotic cell lines [27–29]. Therefore, a potential role of CF pathogens, even in the VBNC state, in the lung infection progression cannot be ruled out.

In our study, patients carrying VBNC cells were characterized by significantly lower FEV1 values than non VBNC-carrying patients at any

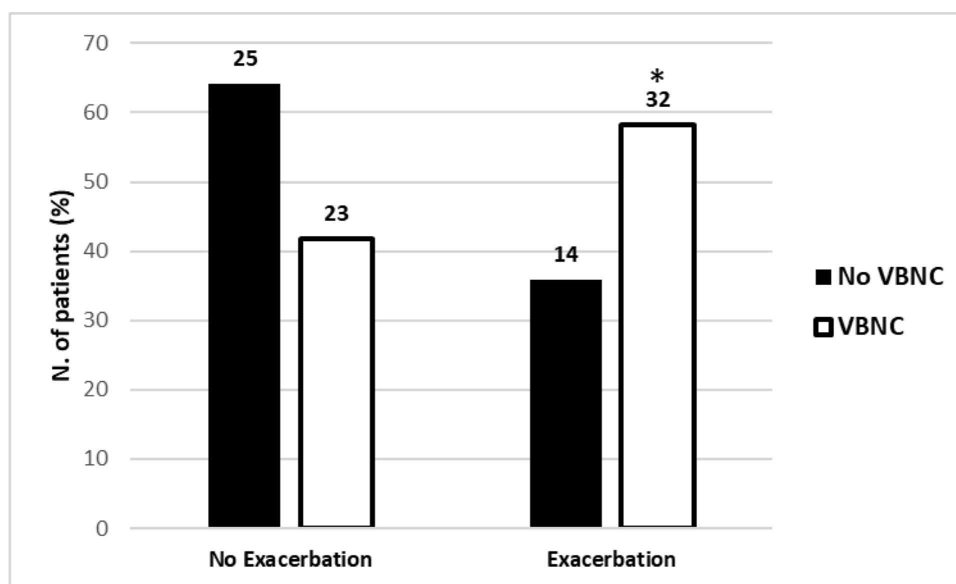

Fig. 1. Comparison of VBNC cell detection and pulmonary exacerbations in the 94 enrolled CF patients. The number (%) of patients without and with exacerbation not presenting (black column) or presenting (white columns) VBNC cells was compared. Data are presented as percentages of the total number of patients, while absolute numbers are indicated on the top of each column. *= $p < 0.05$.

routine visit, irrespective of the involved bacterial species (Table 3). Although requiring additional investigations, mainly to exclude the role of not sought pathogens, these data reveal the constant presence overtime of VBNC pathogenic forms in CF lung and suggest their possible contribution to the infection prognosis. Moreover, in the year of monitoring, pwCF carrying VBNC cells of the investigated pathogens resulted significantly ($p < 0.05$) more affected by pulmonary exacerbations than VBNC free subjects (Fig. 1), in line with the reported ability of VBNC forms to resume full metabolic and virulence activity [28].

The comparison of the number of pulmonary exacerbations between patients starting/not starting the modulator therapy evidenced a greater abundance of VBNC cells in the formers (data not shown). Although not statistically significant ($p = 0.610$), our results suggest that while the modulator therapy drastically reduces pulmonary exacerbations, lung infection is not cleared, likely persisting in a sub-clinical state, involving dormant cells. This agrees with real-world data, indicating contrasting results about the impact of the modulator therapy on the CF lung colonization and their inability to completely clear the infection [30]. The effect of the VBNC cells on clinical records resulted nevertheless milder in patients undergoingthe CFTR-modulator based therapy than in the untreated subjects (Table 3), who consistently exhibited lower FEV1 values. However, since such therapy is used for severe patients with low baseline lung function, the similarly low FEV1 values recorded in both VBNC-carrying and -free patients could be explained by concurrent factors like comorbidities or co-infections with other culturable pathogens. Such features, together with the high heterogeneity of the type of modulator and antibiotic treatment, often impaired the stratification of patients and hence the identification of significant correlations between the presence of VBNC cells and clinical data. However, for pwCF with a documented chronic lung infection by PA, a significant correlation between the presence of PA VBNC forms and the chronic infection condition ($p = 0.0003$) has been pointed out.

Our translational results provide evidence of the overwhelming occurrence of VBNC forms of different pathogens in the CF clinical settings and of their (in particular PA) impact on the lung infection progression. They also suggest that chronic/intermittent infections cannot be eradicated by the modulators therapy, underlining both the weaknesses of the routine cultural methods and the doubtful efficacy of the adopted therapeutic treatments to clear the infection.

While indicating an improvement of the CF lung conditions, the prevalence of VBNC forms in patients on modulators cannot *a priori* exclude a role of VBNC forms to induce pulmonary exacerbation under favorable conditions, an important issue which deserves more attention. qPCR provides reliable, quantitative and more valuable results for the routine diagnostics than the qualitative or semi-quantitative results provided by metagenomic analyses, which also require extensive time and costs. The possibility of easily adapting the reported molecular protocol to further species of interest and, thus, to routinely test the presence of VBNC cells in the lungs of pwCF, can help clinicians to improve the monitoring of the antibiotic treatments effectiveness for pulmonary exacerbations.

Overall, the evidences resulting from this pilot and monocentric study must be considered as a matter of further investigations for both clinicians and microbiologists, with the aim to reconsider the current definitions and guidelines for the diagnosis and management of the CF lung infection.

Author agreement statement

All the authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

Credit author statement

CN: conceptualization, methodology, validation, investigation, data curation, writing original draft and review and editing, supervision, project administration. SV, TV, DA, PA, CN, MM, FL: investigation. GR: conceptualization, methodology, formal analysis, writing original draft. TL: conceptualization, investigation, writing original draft. FB, VC: conceptualization, writing original draft. CF: conceptualization, methodology, formal analysis, writing original draft. BF, MG: conceptualization, methodology, validation, investigation, data curation, writing original draft and review and editing.

All authors made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Declaration of competing interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgments

We would like to thank patients and their families who participated in this trial, the Italian Cystic Fibrosis Research Foundation (FFC Ricerca) and CFF Delegations (Ascoli Piceno, Martinsicuro Teramo, Fabriano Ancona, Umbertide Città di Castello, Torino) who funded the project (FFC#22/2020).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2024.02.013.

References

[1] O'Sullivan BP, Freedman SD. Cystic fibrosis. Lancet 2009;373:1891–904. https://doi.org/10.1016/S0140-6736(09)60327-5.

[2] Smith A. Pathogenesis of bacterial bronchitis in cystic fibrosis. Pediatr Infect Dis J 1997;16:91–5. https://doi.org/10.1097/00006454-199701000-00030.

[3] Waters V. Beneath the surface: assessing cystic fibrosis pulmonary infections in the era of highly effective modulator therapy. J. Cyst. Fibros. 2021;20:723–4. https://doi.org/10.1016/j.jcf.2021.06.008.

[4] Rogers GB, Taylor SL, Hoffman LR, et al. The impact of CFTR modulator therapies on CF airway microbiology. J Cyst Fibros 2020;19:359–64. https://doi.org/10.1016/j.jcf.2019.07.008.

[5] Elborn JS, Blasi F, Burgel PR, et al. Role of inhaled antibiotics in the era of highly effective CFTR modulators. Eur Respir Rev 2023;32:220154. https://doi.org/10.1183/16000617.0154-2022.

[6] Flume PA, VanDevanter DR. Clinical applications of pulmonary delivery of antibiotics. Adv Drug Deliv Rev 2015;85:1–6. https://doi.org/10.1016/j.addr.2014.10.00.

[7] Döring G, Flume P, Heijerman H, et al. Consensus study group. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros 2012;11:461–79. https://doi.org/10.1016/j.jcf.2012.10.004.

[8] Proesmans M, Balinska-Miskiewicz W, Dupont L, et al. Evaluating the "Leeds criteria" for *Pseudomonas aeruginosa* infection in a cystic fibrosis centre. Eur Respir J 2006;27:937–43. https://doi.org/10.1183/09031936.06.00100805.

[9] Bilton D, Canny G, Conway S, et al. Pulmonary exacerbation: towards a definition for use in clinical trials. Report from the EuroCareCF working group on outcome parameters in clinical trials. J Cyst Fibros 2011;10(Suppl 2):S79–81. https://doi.org/10.1016/S1569-1993(11)60012-X.

[10] Sibley CD, Parkins MD, Rabin HR, et al. The relevance of the polymicrobial nature of airway infection in the acute and chronic management of patients with cystic fibrosis. Curr Opin Investig Drugs 2009;10:787–94.

[11] Ayrapetyan M, Williams T, Oliver JD. Relationship between the viable but nonculturable state and antibiotic persister cells. J. Bacteriol. 2018;200:e00249. https://doi.org/10.1128/JB.00249-18. -18.

[12] Li L, Mendis N, Trigui H, Oliver JD, et al. The importance of the viable but non-culturable state in human bacterial pathogens. Front. Microbiol. 2014;5:258. https://doi.org/10.3389/fmicb.2014.00258.

[13] Kvich L, Fritz B, Crone S, et al. Oxygen restriction generates difficult-to-culture *P. aeruginosa*. Front. Microbiol 2019;10:1992. https://doi.org/10.3389/fmicb.2019.01992.

[14] Raccomandazioni per le indagini microbiologiche dei campioni delle vie aeree di pazienti affetti da Fibrosi Cistica. 3° Edizione; 2022. www.sifc.it.

[15] Ed Gilligan P. Respiratory cultures from cystic fibrosis patients. In: Isenberg HD, Garcia LS, editors. Clinical microbiology procedures handbook. Wahington DC: ASM Press; 2007.

[16] UK cystic fibrosis trust microbiology laboratory standards working group laboratory standards for processing microbiological samples from people with cystic fibrosis. 1st edition 2010. September.

[17] Mangiaterra G., Amiri M., Di Cesare A., et al. Detection of viable but non-culturable *Pseudomonas aeruginosa* in cystic fibrosis by qPCR: a validation study. BMC Infect Dis 18(1):701 10.1186/s12879-018-3612-9.

[18] Sabet NS, Subramaniam G, Navaratnam P, et al. Simultaneous species identification and detection of methicillin resistance in staphylococci using triplex real-time PCR assay. Diagn Microbiol Infect Dis 2006;56:13–8. https://doi.org/10.1016/j.diagmicrobio.2006.02.013.

[19] Pasquaroli S, Citterio B, Cesare AD, et al. Role of daptomycin in the induction and persistence of the viable but non-culturable state of *Staphylococcus aureus* biofilms. Pathogens 2014;3:759–68. https://doi.org/10.3390/pathogens3030759.

[20] Okuno NT, Freire IR, Segundo RTRS, et al. Polymerase chain reaction assay for detection of *Stenotrophomonas maltophilia* in cheese samples based on the *smeT* gene. Curr. Microbiol. 2018;75:1555–9. https://doi.org/10.1007/s00284-018-1559-0.

[21] Adam P, Czapiewski P, Colak S, et al. Prevalence of *Achromobacter xylosoxidans* in pulmonary mucosa-associated lymphoid tissue lymphoma in different regions of Europe. Br J Haematol 2014;164:804–10. https://doi.org/10.1111/bjh.12703.

[22] Deschaght P, Schelstraete P, Van Simaey L, et al. Is the improvement of CF patients, hospitalized for pulmonary exacerbation, correlated to a decrease in bacterial load? PLoS ONE 2013;8:e79010. https://doi.org/10.1371/journal.pone.0079010.

[23] Gavillet H, Hatfield L, Rivett D, et al. Bacterial culture underestimates lung pathogen detection and infection status in cystic fibrosis. Microbiol Spectr 2022; 10:e0041922. https://doi.org/10.1128/spectrum.00419-22.

[24] Le Gall F, Le Berre R, Rosec S, et al. Proposal of a quantitative PCR-based protocol for an optimal *Pseudomonas aeruginosa* detection in patients with cystic fibrosis. BMC Microbiol 2013;13:143. https://doi.org/10.1186/1471-2180-13-143.

[25] Boutin S, Weitnauer M, Hassel S, et al. One time quantitative PCR detection of *Pseudomonas aeruginosa* to discriminate intermittent from chronic infection in cystic fibrosis. J Cyst Fibros 2018;17:348–55. https://doi.org/10.1016/j.jcf.2017.12.007.

[26] Signoretto C, Lleò MM, Tafi MC, et al. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl Environ Microbiol 2000;66:1953–9. https://doi.org/10.1128/AEM.66.5.1953-1959.2000.

[27] Magalhães AP, Grainha T, Sousa AM, et al. Viable but non-cultivable state: a strategy for *Staphylococcus aureus* survivable in dual-species biofilms with *Pseudomonas aeruginosa*? Environ Microbiol 2021;23:5639–49. https://doi.org/10.1111/1462-2920.15734.

[28] Liao X, Hu W, Liu D, et al. Stress resistance and pathogenicity of nonthermal-plasma-induced viable-but-nonculturable *Staphylococcus aureus* through energy suppression, oxidative stress defense, and immune-escape mechanisms. Appl Environ Microbiol 2021;87:e02380. https://doi.org/10.1128/AEM.02380-20. -20.

[29] Jiang H, Wang K, Yan M, et al. Pathogenic and virulence factor detection on viable but non-culturable methicillin-resistant *Staphylococcus aureus*. Front Microbiol 2021;12:630053. https://doi.org/10.3389/fmicb.2021.630053.

[30] Harvey C, Weldon S, Elborn S, et al. The effect of CFTR modulators on airway infection in cystic fibrosis. Int J Mol Sci 2022;23:3513. https://doi.org/10.3390/ijms23073513.