

# Effectiveness of bacteriophages in the sputum of cystic fibrosis patients

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## Abstract

Bacteriophages have been shown to be effective for treating acute infections of the respiratory tract caused by antibiotic-resistant bacteria in animal models, but no evidence has yet been presented of their activity against pathogens in complex biological samples from chronically infected patients. We assessed the efficacy of a cocktail of ten bacteriophages infecting *Pseudomonas aeruginosa* following its addition to 58 sputum samples from cystic fibrosis (CF) patients collected at three different hospitals. Ten samples that did not contain *P. aeruginosa* were not analysed further. In the remaining 48 samples, the addition of bacteriophages led to a significant decrease in the levels of *P. aeruginosa* strains, as shown by comparison with controls, taking two variables (time and bacteriophages) into account ( $p = 0.024$ ). In 45.8% of these samples, this decrease was accompanied by an increase in the number of bacteriophages. We also tested each of the ten bacteriophages individually against 20 colonies from each of these 48 samples and detected bacteriophage-susceptible bacteria in 64.6% of the samples. An analysis of the clinical data revealed no correlation between patient age, sex, duration of *P. aeruginosa* colonization, antibiotic treatment, FEV1 (forced expiratory volume in the first second) and the efficacy of bacteriophages. The demonstration that bacteriophages infect their bacterial hosts in the sputum environment, regardless of the clinical characteristics of the patients, represents a major step towards the development of bacteriophage therapy to treat chronic lung infections.

**Keywords:** Chronic infection, phage therapy, *Pseudomonas aeruginosa*, pulmonary infection

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## Introduction

Despite improvements in patient management, infection control policies, early detection and eradication therapies that have increased the mean life expectancy of cystic fibrosis (CF) patients to about 37 years, most of these patients eventually

succumb to chronic pulmonary bacterial infections [1–4]. The most prominent pathogen in CF patients, the gram-negative bacterium *Pseudomonas aeruginosa*, is becoming increasingly resistant to antibiotics [5], leading to a gradual decrease in the clinical benefits of antibiotic treatment over time.

In the environment, microbial communities are controlled by various mechanisms, including the antagonistic action of their specific viruses, through the combined activity of temperate and virulent bacteriophages [6–10]. Bacteriophages were used in medicine (phage therapy), back in the early 20th century, before the discovery of the first antibiotics [11,12]. With the current alarming increase in the frequency of infections caused by antibiotic-resistant pathogens and the lack

of new antibiotics, phage therapy is returning to the spotlight. There is recent support from experimental data and experience accumulated over 80 years in some European countries (Georgia, Russia and Poland) for the use of virulent bacteriophages for treating lung infections [13–20]. As a further step towards applications of bacteriophages in human medicine, we evaluated their potential to infect bacteria in the challenging environment of the lungs, by performing an *ex vivo* study on sputum samples from 58 chronically infected CF patients.

## Methods

### Study design

We carried out a multicentre cross-sectional study on sputum samples from 58 CF patients recruited from CF centres in Montpellier ( $n = 23$ ), Nancy ( $n = 20$ ) and the Necker Hospital in Paris ( $n = 15$ ). This study was approved by the regional ethics committee (Nîmes, registered under number 2011-A01197-34) and declared to ClinicalTrials.gov (no. NCT01818206).

### Bacteria and bacteriophage strains

We used the *P. aeruginosa* PAK strain to amplify bacteriophages PAK\_P1, PAK\_P2, PAK\_P3, PAK\_P4 and PAK\_P5; the CHA strain to amplify P3\_CHA[20] and CHA\_P1[19]; the

PAOI strain for PhiKZ and LUZ19; and the Aa245 strain for LBL3. Bacteriophages PhiKZ, LUZ19 and LBL3 were kindly provided by R. Lavigne, KU Leuven, Belgium. The four indicative strains (PAK, CHA, PAOI and Aa245) were cultured at 37°C in LB medium, with shaking, and bacteriophage lysates were prepared and purified as described elsewhere [20]. The cocktail of these 10 bacteriophages was freshly prepared from bacteriophage solutions, each of which had been titrated on the corresponding host the day before sample processing and on the day of processing. Bacteriophage titration was performed by serial dilutions spotted in triplicate on bacterial lawns. This cocktail was assembled from bacteriophages available in our laboratory without any prior knowledge of their efficacy on a large collection of CF *P. aeruginosa* strains.

New bacteriophages infecting colony #4 from sputum sample 04 were isolated as described elsewhere [18].

### Sputum sample processing

Four aliquots of sputum samples were used to evaluate the count of bacteria and bacteriophages before and after addition of the bacteriophage cocktail over 6 h (see Fig. 1 and Data S1). Bacteria were selected on cetrimide agar and bacteriophage counts were obtained using the four indicative strains.

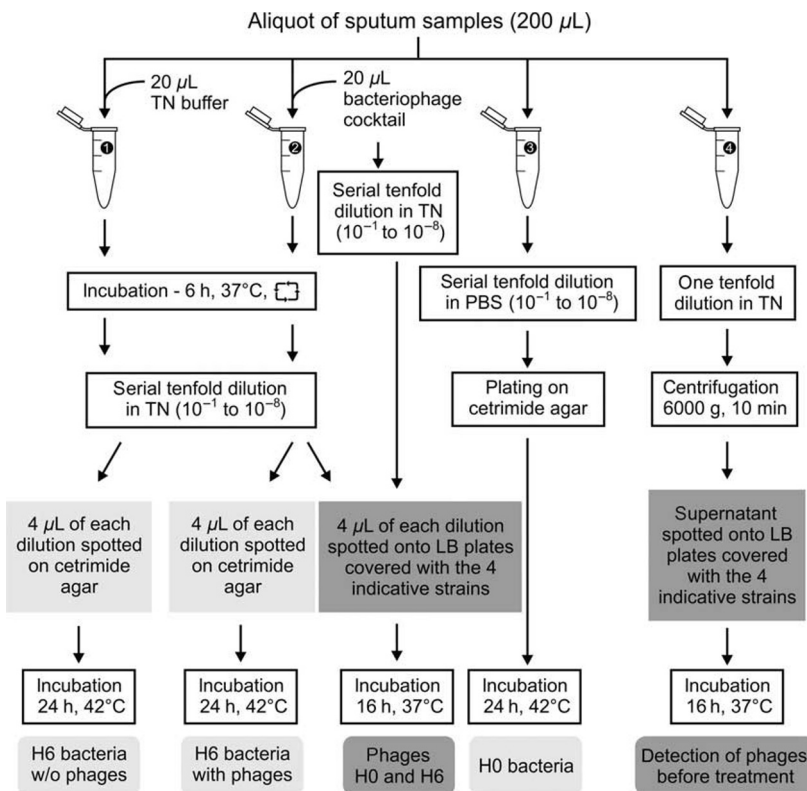


FIG. 1. Schematic diagram of the processing of sputum samples.

The reproducibility and accuracy of our counting procedure leading to the definition of threshold values are described in detail in Data S1.

#### Test of the sensitivity of individual colonies to each bacteriophage of the cocktail

We randomly selected 20 colonies (representative of the proportions of mucoid/non-mucoid and small/large colonies) from the cetrimide plates. The 15  $\mu\text{L}$  of each colony grown in broth were spotted on an LB plate, dried under a laminar flow hood and subsequently a 0.5- $\mu\text{L}$  drop of each bacteriophage suspension ( $1 \times 10^8$  pfu/mL) was spotted on top of the dried bacterial drops. Isolated plaques, confluent plaques or entirely clear areas were considered to indicate that the bacteria were susceptible to the phage tested, whereas the absence of plaques indicated full resistance (Data S1).

#### Molecular genotyping of *P. aeruginosa* strains

Five colonies from sputum samples 03, 04 and 33 were chosen for the molecular analysis (see Supplementary Excel file, genotyping tab). The 16S RNA gene was amplified as previously described [21], with a standard PCR kit (MP Biomedicals, Santa Ana, CA, USA), from a single colony. The clonality of these 15 isolates was assessed by repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) adapted to a semi-automated format (DiversiLab™ system, bioMérieux, Marcy l'Etoile, France) and fingerprinting profiles were analysed with web-based DiversiLab™ software v.3.4 (bioMérieux).

#### Data analysis

The primary criterion assessed was the change in counts for *P. aeruginosa* strains during the period of incubation in the presence of bacteriophages. We calculated that, to detect a 50% decrease in bacterial counts with a standardized effect size of 0.5, we would need to analyse  $n = 44$  paired observations, with an alpha risk of 0.05 and a beta risk of 0.10 (the standardized effect size to estimate the number of subjects is defined as the difference of the mean before and after divided by the standard deviation). Thus, taking missing data into account, we needed to enroll 55–60 consecutive patients in this study. The secondary criteria taken into account were the change in bacteriophage counts during the incubation period and the susceptibility of the 20 individual colonies tested per sample to each bacteriophage. Samples were then classified into five groups, defined as: negative, for samples in which no increase in bacteriophage counts was observed; group A, for samples in which bacterial counts decreased and bacteriophage-susceptible bacteria were present; group B, for samples displaying a decrease in bacterial counts and an absence of susceptible colonies; group C, for samples displaying no

decrease in bacterial counts and the presence of susceptible colonies; and group D for samples displaying no decrease in bacterial counts and an absence of susceptible colonies (Fig. 3).

#### Statistical analysis

Descriptive data are summarized as means  $\pm$  standard deviation (SD) or medians with interquartile ranges, according to the normality of the distribution, which was assessed with the Shapiro–Wilk test. We used Kruskal–Wallis tests or ANOVA tests for comparisons of quantitative variables. Categorical data are expressed as numbers and percentages, and were compared in chi-squared tests. Univariate and multivariate analyses were carried out with linear mixed-effect models for repeated measures. Variables were selected for inclusion in the multivariate model if they had p-values below 0.20 in univariate analysis, and a stepwise procedure was used to select the final variables included in the multivariate model. A p-value of  $\leq 0.05$  was considered statistically significant. Data were analysed with R.2.15.2 software.

## Results

#### Characteristics of the patients and treatment of the samples

The characteristics of the 58 CF patients enrolled in this study are reported in Table 1. Each of the 58 sputum samples collected was split into four aliquots. We added a cocktail of ten bacteriophages infecting *P. aeruginosa* to one of these aliquots and buffer alone to a second aliquot; the other two aliquots were used for subsequent analysis (Fig. 1; Methods). Bacterial counts were obtained on selective medium and bacteriophage counts were obtained on agar plates overlaid with four different indicative *P. aeruginosa* strains (Methods). Ten sputum samples (20.8%) contained no *P. aeruginosa* strains capable of growing on the selective medium and were not

**TABLE 1. Principal characteristics of the CF patients enrolled in this study**

Variable	Enrolled	<i>Pseudomonas aeruginosa</i> -positive <sup>a</sup>
Patients	58 <sup>b</sup>	48
Sex (female/male)	26/32	23/25
CFTR genotype	n (%)	n (%)
dF508/dF508	26 (45)	23 (48)
dF508/other	20 (34)	16 (33)
other/other	12 (21)	9 (19)
Age, yr (mean $\pm$ SD)	26 $\pm$ 10	27 $\pm$ 10
Baseline FEV1 (% predicted, mean $\pm$ SD)	52 $\pm$ 20	52 $\pm$ 19

<sup>a</sup>*Pseudomonas aeruginosa* colonization was first recorded between years 1983 and 2011 (at least 3 months before inclusion), with the majority, 31 patients, between years 1990 and 2000 and with only four patients in year 2011.

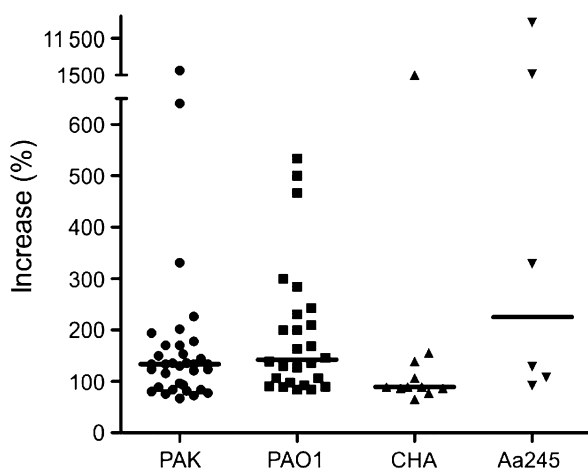
<sup>b</sup>Twenty-three patients from Montpellier, 15 from Paris (Necker Hospital) and 20 from Nancy.

analysed further (see Supplementary Excel file, bacteria numeration tab).

#### Analysis of the microbiological data (bacteria and bacteriophages)

In CF patients, *P. aeruginosa* populations displayed several phenotypes that could affect susceptibility to bacteriophages [22,23]. We then expected bacteria to grow when samples were incubated in the absence of bacteriophages, while significant growth attenuation was predicted in the presence of bacteriophages. A statistical analysis was performed on the 48 samples, in which bacterial counts (ranging from 33% to 6090% increase in the absence and from 18 to 98% reduction in the presence of bacteriophages) were compared in a mixed linear model with two parameters: time (0 h and 6 h) and bacteriophages (presence vs. absence). This analysis confirmed that the total number of bacteria increased during the incubation period in the absence of bacteriophages ( $p < 0.001$ ). However, despite this growth, the addition of bacteriophages significantly decreased the number of bacteria present ( $p 0.024$ ).

The secondary criterion considered was the difference between the number of bacteriophages added and the number recovered at the end of the incubation period. As bacteriophage counts can only increase if the bacteriophages infect bacterial hosts, any increase over the threshold level indicates that some of the bacteria in the sample were infected (Fig. 2, Methods; see Supplementary Excel file, threshold evaluation and phage numeration tabs). The results obtained for the four indicative strains were combined, and only seven samples



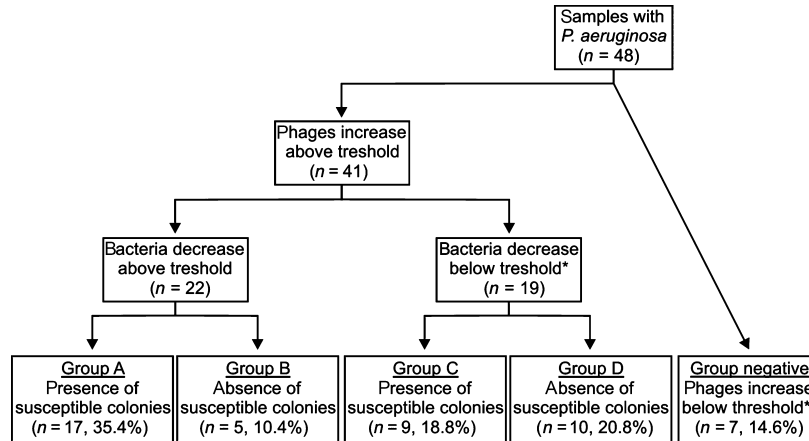
**FIG. 2.** Increase of bacteriophage counts following 6 h incubation. Individual data (% of increase between 0 and 6 h) are indicated for the 41 samples that have an increased value above the threshold from spot tests performed on each of the four indicative strains (PAK, PAO1, CHA and Aa245). The lines represent median values.

(14.6%) (Fig. 3; see Supplementary Excel file, analyses tab) displayed no increase in the number of bacteriophages over the threshold level (these seven samples were assigned to the negative group). The other 41 samples displayed an increase in bacteriophage numbers. We analysed these 41 samples by considering two additional criteria: (i) the decrease in bacterial counts and (ii) the susceptibility of 20 individual colonies to at least one of the ten bacteriophages (Methods; see Supplementary Excel file, susceptibility to phages tab). This led to the definition of four groups (Fig. 3, Methods): group A, which included 17 samples (35.4%), group B, which contained five samples (10.4%), group C, which contained nine samples (18.8%) and group D, which contained ten samples (20.8%) (see Methods and Supplementary Excel file, analyses tab including the results obtained for each indicative strain).

We used a dichotomous scoring system, no lysis vs. lysis (Methods), to analyse the susceptibility of 20 individual colonies from each sputum sample to each of ten bacteriophages (over 8900 tests). Bacteriophages PAK\_P5, LBL3, PAK\_P3 and PAK\_P4 were the most efficient, infecting 170, 156, 151 and 149 colonies, respectively, whereas bacteriophages CHA\_P1 and PhiKZ were the least efficient, infecting only 23 and 32 colonies, respectively, of a total of 892 (Table 2; see Supplementary Excel file, susceptibility to phage tab). The ten bacteriophages were able to infect up to 313 individual colonies, corresponding to a coverage of 35.1%, a low value, which could reflect the lack of initial selection for ability to infect *P. aeruginosa* strains from CF patients.

We also carried out a molecular analysis of five colonies isolated from each of three samples, one from group A, one from group B and one from group D. 16S RNA sequences confirmed that these 15 colonies belonged to *P. aeruginosa*. Genotyping confirmed that each set of five colonies belonged to the same group, suggesting that each of the corresponding patients was infected by one major genotype (Fig. 4). For the five colonies selected from the group D sample, all of which were resistant to all ten bacteriophages, we selected a single colony for the isolation of new bacteriophages from environmental sources. Three of these new bacteriophages, chosen at random, infected all five colonies from the group D sample, demonstrating that these colonies were not resistant to bacteriophage infection *per se*.

The presence of bacteriophages in the sputum samples before processing was assessed by checking for plaques following spotting of the sample on the four indicative strains (see Supplementary Excel file, prophages tab). Only a few plaques were observed from 11 samples, corresponding to no more than  $2 \times 10^3$  pfu/mL in each sample. Prophages were also detected during the testing of individual colonies for susceptibility to bacteriophages, in the form of tiny homoge-



**FIG. 3.** Diagram of the analysis of microbiological data. The 48 samples containing *P. aeruginosa* strains were classified into five groups according to counts of bacteriophages, counts of bacteria and susceptibility to individual colonies. \*, including bacteria increase; \*\*, including bacteriophages decrease. Amongst the seven samples of the negative group, one displayed an increase in bacteria counts, two a decrease and four a non-significant variation. Four samples out seven contained at least one colony susceptible to bacteriophages.

**TABLE 2.** Susceptibility of CF isolates to individual bacteriophages

Number of CF isolates	Resistant	Sensitive	Total
PAK-P1	813	79	892
PAK-P2	796	96	892
PAK-P3	741	151	892
PAK-P4	743	149	892
PAK-P5	722	170	892
P3-CHA	767	125	892
CHA-P1	869	23	892
PhiKZ	860	32	892
Luz19	797	95	892
LBL3	736	156	892
Total for the cocktail	7844	1076	8920

neous plaques covering the entire surface of the indicative strain spot. In total, 29 colonies from eight sputum samples presented such a phenotype (see Supplementary Excel file, prophages tab). The presence of these bacteriophages and prophages at such a low level did not prevent the determination of bacteriophage counts and colony sensitivity.

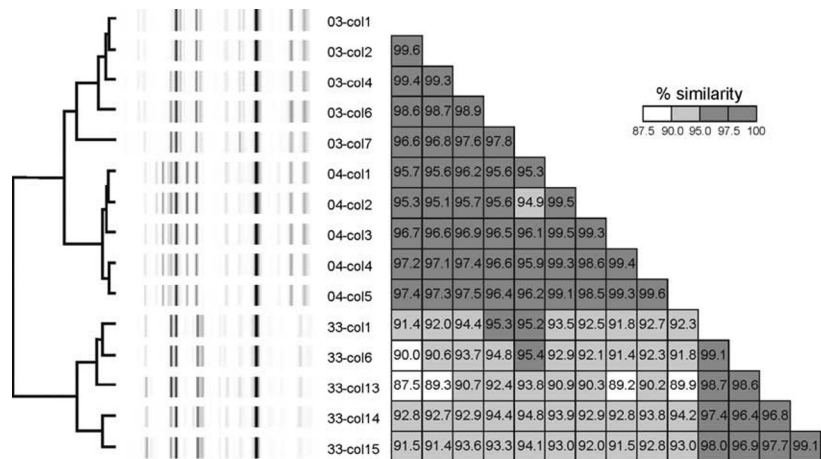
**Correlation between patient characteristics and microbiological data**

We investigated the correlation between group (negative, A, B, C or D) and clinical data (see Supplementary Excel file, clinical data tab), by performing a statistical analysis with several parameters (age, sex, FEV1, last antibiotic treatment and duration of colonization; Table 3). We found no link between these five groups, defined on the basis of microbiological data, and the clinical parameters tested (all p-values above 0.05).

**Discussion**

In the face of the growing threat posed by antibiotic-resistant strains and the particular microenvironment of the lungs of CF patients, the development of effective treatments is a major challenge. Mucus, pathogens (bacteria, fungi and viruses),

**FIG. 4.** Molecular typing of 15 *P. aeruginosa* colonies isolated from three sputum samples. Dendrogram, REP-PCR patterns and similarity matrix (colour-coded, based on percentage similarity) are represented for five randomly chosen colonies from samples 03, 04 and 33.





**TABLE 3.** Correlation between clinical and microbiological data for 48 patients

Variable	Negative group (n = 7)	Group A (n = 17)	Group B (n = 5)	Group C (n = 9)	Group D (n = 10)	p-value <sup>a</sup>	p-value <sup>b</sup>
Sex							
F	2 (28.57)	8 (47.06)	2 (40.00)	7 (77.78)	4 (40.00)	0.3571	0.3332
M	5 (71.43)	9 (52.9)	3 (60.00)	2 (22.22)	6 (60.00)		
Age	37.43 ± 18.24	24.53 ± 9.08	27.40 ± 6.35	28.78 ± 7.15	21.90 ± 3.81	0.0854	0.1891
FEV1 (%)	50.57 ± 13.88	54.00 ± 20.86	54.60 ± 25.02	44.78 ± 17.07	52.50 ± 20.39	0.9377	0.8334
Antibiotic							
A	0 (0.00)	2 (6.90)	1 (11.11)	0 (0.00)	2 (12.50)	0.5557	0.5220
Am	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (6.25)		
Az	1 (14.29)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)		
B	0 (0.00)	2 (6.90)	0 (0.00)	0 (0.00)	1 (6.25)		
C	1 (14.29)	3 (10.34)	2 (22.22)	5 (33.33)	1 (6.25)		
Cl	0 (0.00)	1 (3.45)	0 (0.00)	0 (0.00)	0 (0.00)		
Co	0 (0.00)	1 (3.45)	1 (11.11)	1 (6.67)	1 (6.25)		
F	1 (14.29)	4 (13.79)	1 (11.11)	1 (6.67)	1 (6.25)		
M	1 (14.29)	5 (17.24)	0 (0.00)	3 (20.00)	3 (18.75)		
N	4 (57.14)	9 (31.03)	2 (22.22)	3 (20.00)	2 (12.50)		
P	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (12.50)		
R	0 (0.00)	1 (3.45)	0 (0.00)	0 (0.00)	0 (0.00)		
T	1 (14.29)	0 (0.00)	1 (11.11)	2 (13.33)	0 (0.00)		
Tg	1 (14.29)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)		
To	2 (28.57)	1 (3.45)	1 (11.11)	0 (0.00)	2 (12.50)		
Length of colonization z(months)	160.71 ± 48.21	170.52 ± 86.44	157.00 ± 92.36	122.33 ± 95.29	127.20 ± 73.66	0.6413	0.5182

Last antibiotic administered to patients (A, amoxicillin + clavulanic acid; Am, amikacin; Az, aztreonam; B, cotrimoxazole; C, ciprofloxacin; Cl, ticarcillin + clavulanic acid; Co, colimycin; F, ceftazidime; M, meropenem; N, tobramycin/intravenous; P, pristinamycin; R, rifampicin; T, piperacillin + tazobactam; Tg, teicoplanin; To, tobramycin/aerosol).

<sup>a</sup>p-value for the five groups.

<sup>b</sup>p-value for the four groups (A,B,C,D). Statistical analyses were performed using the chi-square test for categorical variables and ANOVA or Kruskal–Wallis tests for quantitative variables.

extracellular materials (chemokines, DNA, proteases, etc.) and inflammatory cells form a barrier potentially preventing or interfering with antibacterial treatments. The renewed interest in phage therapy and its successful use against acute *P. aeruginosa* lung infections in mice led us to investigate whether bacteriophages could effectively infect bacteria in the sputum of CF patients. Using a set of 58 sputum samples collected from three hospitals in France, we demonstrated the efficacy of a cocktail of ten bacteriophages not specifically selected for their ability to infect *P. aeruginosa* strains in sputum. Thus, the microenvironment in the lungs of CF patients does not prevent bacteriophage activity as we found that the number of bacteriophages increased over the threshold value in 86.4% (41 out of 48 samples containing *P. aeruginosa* strains) of samples. Furthermore, none of the clinical parameters tested was associated, either positively or negatively, with bacteriophage efficacy. This patient-independent efficacy supports the further development of bacteriophage treatments.

However, this pioneering study had some limitations. First, only bacteria growing on the selective medium within 24 h were taken into account, but some *P. aeruginosa* strains require longer incubation times for colony formation. This may account for the increase in bacteriophage counts in seven of the ten samples classified as not containing *P. aeruginosa* strains. Second, we used a short incubation period, to optimize the detection of decreases in bacterial counts by bacteriophages, because we expected the sputum samples to contain a mixed population of bacteriophage-susceptible and bacteriophage-resistant colonies.

Group A samples gave the most straightforward results, whereas the results for group B can be explained by a shift in phenotype to bacteriophage susceptibility between the sputum and agar plate environments. Upon environmental signals it is known that bacterial gene expression can affect bacteriophage susceptibility [24,24]. In group C samples, the decrease in the bacteriophage-susceptible population was probably compensated for by an increase in the bacteriophage-resistant population. Group D samples probably contained a minor bacteriophage-susceptible population and a larger bacteriophage-resistant population. Finally, some negative group samples were found to contain isolated colonies susceptible to bacteriophages, suggesting that bacteria may be less susceptible to bacteriophage infection in the sample than on plates. Furthermore, patient factors (immune cells, proteases, etc.) and other microorganisms (fungi and viruses) may also hinder interactions between bacteriophages and bacteria.

In sputum samples, the amplification of individual bacteriophages could be evaluated only for LBL3 (infecting only one indicative strain), which gave the two highest levels of bacteriophage amplification (samples 50 and 58). LBL3 was also the second most active bacteriophage in analyses on isolated colonies (see Supplementary Excel file, phages numeration tab). The most active bacteriophage, PAK\_P5, is genetically closely related to PAK\_P3, P3\_CHA and CHA\_PI (the least active of the ten bacteriophages tested). These four bacteriophages had protein sequences that were about 90% identical. The genomic data of bacteriophages is therefore insufficient for predicting infectivity in clinical bacterial strains [19].

CF patients colonized by a single clonal population of *P. aeruginosa* displayed several phenotypes [22,23]. Consequently, it was not surprising to observe that the 20 colonies isolated from a single sputum sample were not equally infected by the ten individual bacteriophages, in any of the 48 samples (except those containing only resistant colonies). This confirms that various phenotypes can emerge from a unique genotype in the sputum of a patient, which can indeed affect bacterial susceptibility to bacteriophages. It is also an indication that genotyping may be to imprecise for the identification of clonal mutations relevant to bacteriophage susceptibility. Testing a set of bacteriophages against a large panel of isolated colonies for each patient would therefore be the best approach for formulating bacteriophage cocktails. However, the possibility of phenotypic variation between the sputum and agar plate environments represents an additional drawback. Nevertheless, our recent results clearly suggest that the most efficient bacteriophages *in vivo* are likely to be those isolated 'deliberately', using patients' strains, supporting the use of a personalized approach to achieve optimal treatment [19,26,27].

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## Author contributions

ES, IV, RC, FC, NM and LD contributed to the conception and design of the study. ES, IV, RC, BG and IS contributed to the acquisition of data. ES, IV, RC, IS, ND, JPP, DdV, NM and LD contributed to the analysis and interpretation of data.

## Transparency Declaration

The authors declare no conflicts of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Supplement to methods.

**Data S2.** Excel file with the entire set of data including tabs S1 to S8 designated as follows.

**Table S1.** Threshold evaluation.

**Table S2.** Phages numeration.

**Table S3.** Bacteria numeration.

**Table S4.** Prophages.

**Table S5.** Susceptibility to phages.

**Table S6.** Genotyping.

**Table S7.** Analyses.

**Table S8.** Clinical data.

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