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Title:

Effectiveness of bacteriophages in the sputum of cystic fibrosis patients

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120 characters "take home message":

Chronically infected cystic fibrosis patients with P. aeruginosa can beneficiate from bacteriophages treatments.

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-sensitive bacteria in 64.6 % of the samples. An analysis of the clinical data revealed no correlation between patient age, sex, duration of *P. aeruginosa* colonisation, 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.

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 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. Recent support from experimental data and experience accumulated over 80 years in this field in some European countries (Georgia, Russia, Poland) are in favour of 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 (Nimes, registered under number 2011-A01197-34) and declared to ClinicalTrial.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 PAO1 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, PAO1, 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 on 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 bacteriophages cocktail during 6 h (see Figure 1 and supplemental methods). Bacteria were selected on cetrimide agar and bacteriophages counts were obtained using the 4 indicative strains.

The reproducibility and accuracy of our counting procedure leading to the definition of threshold values are described in details in the supplemental methods.

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. 15 µL of each colony were spotted on LB plate, dried under a laminar flow hood and subsequently a 0.5 µl drop of each bacteriophage suspension ($1x10^8$ 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 permissive to the phage tested, whereas the absence of plaques indicated full resistance.

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), 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) 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 standardised 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 standardised 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 enrol 55 to 60 consecutive patients in this study. The secondary criteria taken into account were the change in bacteriophage counts during the incubation period and the sensitivity 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-sensitive bacteria were present; group B, for samples displaying a decrease in bacterial counts and an absence of sensitive colonies; group C, for samples displaying no decrease in bacterial counts and the presence of sensitive colonies; group D for samples displaying no decrease in bacterial counts and an absence of sensitive colonies (Figure 3).

Statistical analysis

Descriptive data are summarised as means ± 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 Krukall-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 ≤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 (figure 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 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 which could affect permissivity 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 absence and from 18 to 98% reduction in presence of bacteriophages) were compared in a mixed linear model with two parameters: time (0 h and 6 h) and bacteriophages (presence versus 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 (Figure 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 (14.6 %) (Figure 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 sensitivity 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 (Figure 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 Supplementary Excel file, analyses tab including the results obtained for each indicative strain).

We used a dichotomous scoring system, no lysis versus lysis (methods), to analyse the sensitivity of 20 individual colonies from each sputum sample to each of ten bacteriophages (over 8,900 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 (figure 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 $2x10^3$ pfu/mL in each sample. Prophages were also detected during the testing of individual colonies for sensitivity to bacteriophages, in the form of tiny homogeneous 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 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 colonisation; 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), extracellular materials (chemokines, DNA, proteases etc.) and inflammatory cells form a barrier potentially preventing or interfering with antibacterial treatment. 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 48 samples containing *P. aeruginosa* strains) 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 optimise the detection of decreases in bacterial counts by

bacteriophages, because we expected the sputum samples to contain a mixed population of bacteriophage-sensitive and -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 sensitivity between the sputum and agar plate environments. Upon environmental signals it is know that bacterial gene expression can affect bacteriophage sensitivity [24, 25]. In group C samples, the decrease in the bacteriophage-sensitive population was probably compensated by an increase in the bacteriophage-resistant population. Group D samples probably contained a minor bacteriophage-sensitive population and a larger bacteriophage-resistant population. Finally, some negative group samples were found to contain isolated colonies sensitive to bacteriophages, suggesting that bacteria may be less permissive to bacteriophage infection in the sample than on plates. Furthermore, patient factors (immune cells, proteases etc.) and other microorganisms (fungi, 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_P1 (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 colonised 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 permissivity to bacteriophages. It is also an indication that genotyping may be to imprecise for the identification of clonal mutations relevant to bacteriophage permissivity. Testing a set of bacteriophages against a large panel of isolated colonies for each patient would therefore be the best approach to 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 patient's strains, supporting the use of a personalised approach to achieve optimal treatment [19, 26, 27].

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Authors contributions

Conception and design of the study: ES, IV, RC, FC, NM LD

Acquisition of data: ES, IV, RC, BG, IS

Analysis and interpretation of data: ES, IV, RC, IS, ND, JPP, DdV, NM, LD

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Table 1 Principal characteristics of the CF patients enrolled in this study

Variable	Enrolled	*P. aeruginosa*-positive[a]
Patients	**58**[b]	**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, yrs (mean ± SD)	**26 ± 10**	**27 ± 10**
Baseline FEV1 (% predicted, mean ± SD)	**52 ± 20**	**52 ± 19**

[a] : *P. aeruginosa* colonisation 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 4 patients in year 2011.

[b] : 23 patients from Montpellier, 15 from Paris (Necker hospital) and 20 from Nancy

387 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

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391 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[a]	*p*-value[b]
Sex						0.3571	0.3332
F	2 (28.57)	8 (47.06)	2 (40.00)	7 (77.78)	4 (40.00)		
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 (1)						0.5557	0.5220
A	0 (0.00)	2 (6.90)	1 (11.11)	0 (0.00)	2 (12.50)		
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 colonisation (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

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393 (a) *p*-value for the five groups

394 (b) *p*-value for the four groups (A,B,C,D)

395 Statistical analyses were performed using Chi square test for categorial variables and ANOVA or Kruskal Wallis tests for quantitative variables.

396

(1) 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).

Figure legends

Figure 1 Schematic diagram of the processing of sputum samples

Figure 2 Increase of bacteriophages counts following 6h incubation

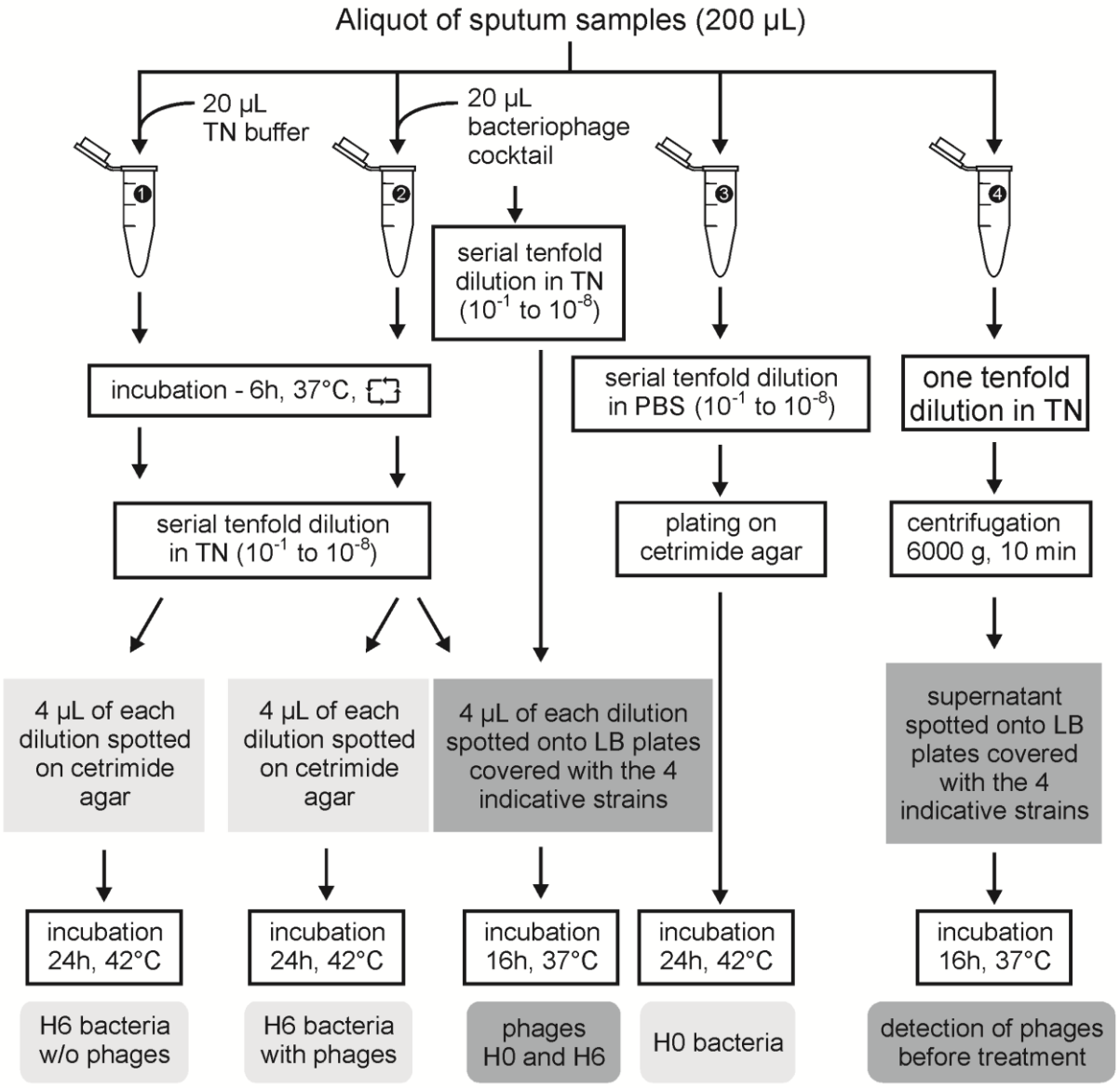
Individual data (% of increase between 0 and 6 hours) are indicated for the 41 samples which have an increased value above the threshold from spot tests performed on each of the four indicative strains (PAK, PAO1, CHA and Aa245). The lines represent median values.

Figure 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 sensitivity to individual colonies. *, including bacteria increase; **, including bacteriophages decrease. Amongst the 7 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 sensitive to bacteriophages.

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

425 Figure 1



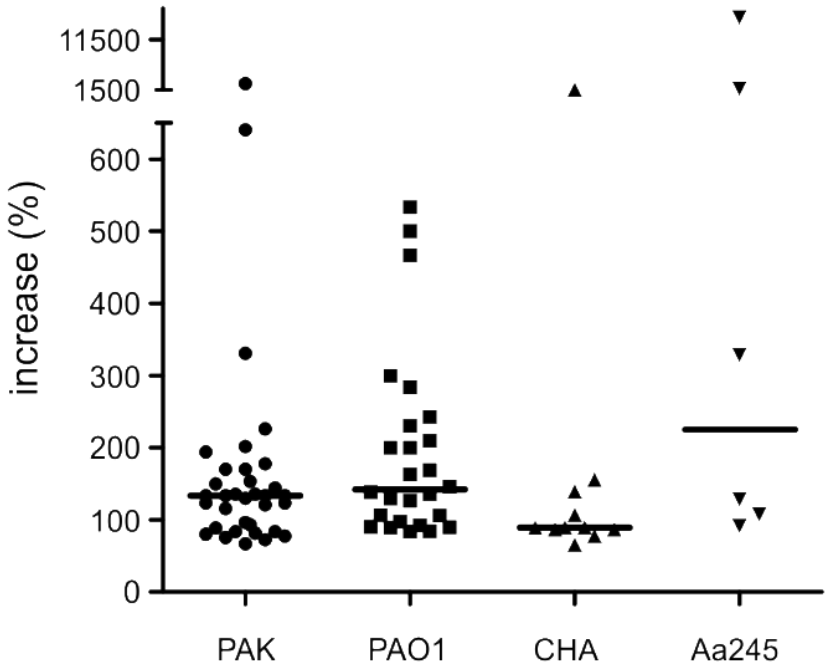
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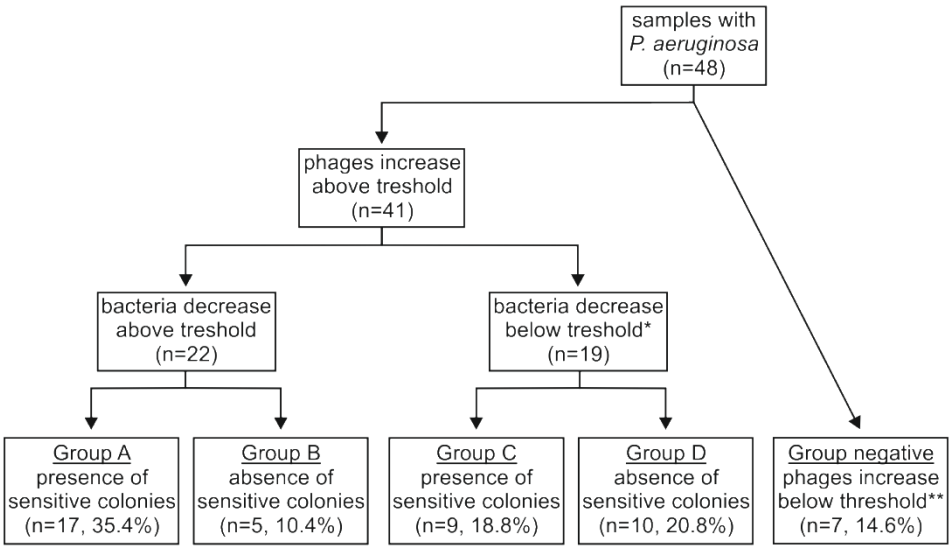
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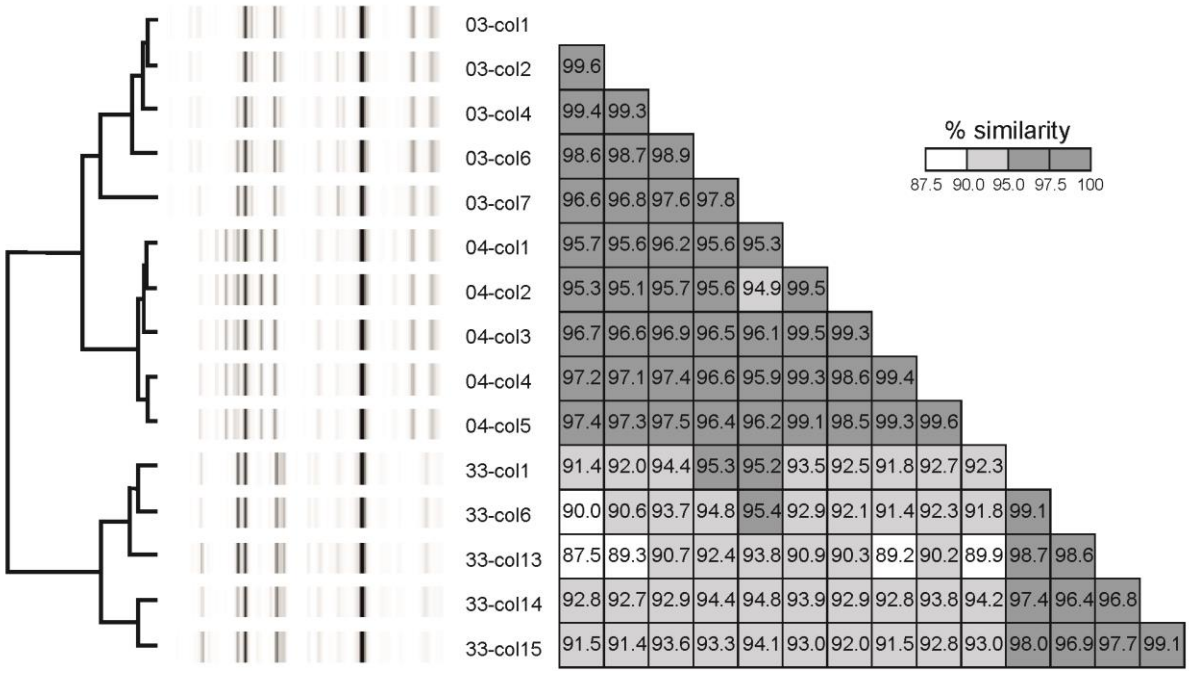


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Supplementary information

Supplement to 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 (Nimes, registered under number 2011-A01197-34) and declared to ClinicalTrial.gov (no. NCT01818206). CF was defined as positive results for the sweat chloride test and the presence of mutations of the CF gene. We asked patients with a stable clinical state that had previously tested positive for *P. aeruginosa* infection to participate in this study. These patients were provided with information about the study and they or their parents or representatives gave informed consent for participation before the collection of samples during the course of routine medical care. We reviewed the medical records of these patients and recorded their demographic and clinical characteristics.

Sputum sample processing

Between collection and processing, sputum samples were maintained at 4°C, for a maximum of three days. We first added an equal volume of Sputasol (Oxoid) to samples (0.1% dithiothreitol, final concentration) to ensure an even distribution between aliquots. The samples were incubated for 1 h at 4°C with shaking, and they were then each split into four aliquots of 200 µL each. We added 20 µL of TN buffer (10 mM Tris, 150 mM NaCl) to the first aliquot and 20 µL of the bacteriophage cocktail ($2x10^6$ pfu of each of the ten bacteriophages) to the second. Both these aliquots were incubated at 37°C for 6 h, with

shaking. Serial dilutions in PBS were prepared immediately from the third aliquot and plated on cetrimide agar (Sigma). The plates were then incubated at 42°C for 24 h, for the determination of bacterial counts and the subsequent selection of 20 individual colonies. The fourth aliquot was diluted 10-fold in TN, centrifuged (6000 x *g* for 10 minutes) and the supernatant was spotted onto LB agar plates already covered with the four indicative strains. The plates were incubated at 37°C overnight, for counting of the bacteriophages present in sputum samples before addition of the cocktail. After the aliquots had been incubated for 6 h, as described above, serial dilutions in TN were prepared from the first two aliquots and spotted (4 µL) onto cetrimide agar. The plates were then incubated at 42°C for 24 h. For the second aliquot only, we also spotted the same serial dilutions onto four LB plates, each covered by one of the four indicative strains, which we then incubated for 16 h at 37°C (figure 1).

Reliability of bacterial and bacteriophage counts

We assessed the reproducibility and accuracy of our counting procedure using 4 µL spots of serial dilutions of bacteria or bacteriophages, by performing the same counting procedure in triplicate, with known numbers of bacteria and bacteriophages. We observed 3.9 % to 8.0 % variability in the triplicate assays, with a mean of 6.7 % variability for bacteria and 9.6 % to 43.3 % variability, with a mean value of 30.1 %, for bacteriophages (see Supplementary Excel file, threshold tab). We considered there to be a significant increase in bacterial and bacteriophage counts if we observed an absolute change of more than twice the mean variability (13.4 % and 60.2 %, respectively). We increased these threshold values to 15% and 65%, respectively, for a more conservative analysis.

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. We streaked them onto LB plates, which we incubated at 37°C for 16 h. We then picked one clone from each colony with a toothpick and used it to inoculate 200 µl of LB broth in one of the wells of a 96-well plate. After 4 h of incubation at 37°C with shaking, we spotted ten 15 µL aliquots from each well to form a line on a square LB plate (10 x10 cm). Plates were then placed under a laminar flow hood (30 min) to dry, and we then dispensed a 0.5 µl drop of each bacteriophage suspension ($1x10^8$ pfu/mL) onto one of the dried bacterial drops (one suspension per drop). Plates were incubated at 37°C for 16 h. On each square plate, we were able to test the sensitivity of ten individual colonies to each of the ten individual bacteriophages. In the middle of the bacterial lawn, isolated plaques, confluent plaques or entirely clear areas were considered to indicate that the bacteria were permissive to the phage tested, whereas the absence of plaques indicated full resistance.