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1 **Title:**

2 Effectiveness of bacteriophages in the sputum of cystic fibrosis patients

3

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31

32

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35

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

38 bacteriophages treatments.

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40

41

42 **Abstract**

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

60

61 **Introduction**

62 Despite improvements in patient management, infection control policies, early detection and
63 eradication therapies that have increased the mean life expectancy of cystic fibrosis (CF) patients
64 to about 37 years, most of these patients eventually succumb to chronic pulmonary bacterial
65 infections [1-4]. The most prominent pathogen in CF patients, the gram-negative bacterium
66 *Pseudomonas aeruginosa*, is becoming increasingly resistant to antibiotics [5], leading to a
67 gradual decrease in the clinical benefits of antibiotic treatment over time.

68
69 In the environment, microbial communities are controlled by various mechanisms, including the
70 antagonistic action of their specific viruses, through the combined activity of temperate and
71 virulent bacteriophages [6-10]. Bacteriophages were used in medicine (phage therapy), back in
72 early 20th century, before the discovery of the first antibiotics [11, 12]. With the current alarming
73 increase in the frequency of infections caused by antibiotic-resistant pathogens and the lack of
74 new antibiotics, phage therapy is returning to the spotlight. Recent support from experimental
75 data and experience accumulated over 80 years in this field in some European countries (Georgia,
76 Russia, Poland) are in favour of the use of virulent bacteriophages for treating lung infections
77 [13-20]. As a further step towards applications of bacteriophages in human medicine, we
78 evaluated their potential to infect bacteria in the challenging environment of the lungs, by
79 performing an *ex vivo* study on sputum samples from 58 chronically infected CF patients.

80

81

82 **Methods**

83 *Study design*

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

88

89 *Bacteria and bacteriophage strains*

90 We used the *P. aeruginosa* PAK strain to amplify bacteriophages PAK_P1, PAK_P2, PAK_P3,
91 PAK_P4 and PAK_P5; the CHA strain to amplify P3_CHA [20] and CHA_P1[19]; the PAO1
92 strain for PhiKZ and LUZ19; and the Aa245 strain for LBL3. Bacteriophages PhiKZ, LUZ19 and
93 LBL3 were kindly provided by R. Lavigne, KU Leuven, Belgium. The four indicative strains
94 (PAK, CHA, PAO1, Aa245) were cultured at 37°C in LB medium, with shaking, and
95 bacteriophage lysates were prepared and purified as described elsewhere [20]. The cocktail of
96 these 10 bacteriophages was freshly prepared from bacteriophage solutions, each of which had
97 been titrated on the corresponding host the day before sample processing and on the day of
98 processing. Bacteriophage titration was performed by serial dilutions spotted in triplicate on
99 bacterial lawns. This cocktail was assembled from bacteriophages available in our laboratory
100 without any prior knowledge on their efficacy on a large collection of CF *P. aeruginosa* strains.
101 New bacteriophages infecting colony #4 from sputum sample 04 were isolated as described
102 elsewhere [18].

103

104 *Sputum sample processing*

105 Four aliquots of sputum samples were used to evaluate the count of bacteria and bacteriophages
106 before and after addition of the bacteriophages cocktail during 6 h (see Figure 1 and supplemental
107 methods). Bacteria were selected on cetrimide agar and bacteriophages counts were obtained
108 using the 4 indicative strains.

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

111

112 *Test of the sensitivity of individual colonies to each bacteriophage of the cocktail*

113 We randomly selected 20 colonies (representative of the proportions of mucoid/non mucoid and
114 small/large colonies) from the cetrimide plates. 15 µL of each colony were spotted on LB plate,
115 dried under a laminar flow hood and subsequently a 0.5 µl drop of each bacteriophage suspension
116 (1×10^8 pfu/mL) was spotted on top of the dried bacterial drops. Isolated plaques, confluent
117 plaques or entirely clear areas were considered to indicate that the bacteria were permissive to the
118 phage tested, whereas the absence of plaques indicated full resistance.

119

120 *Molecular genotyping of P. aeruginosa strains*

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

127

128 *Data analysis*

129 The primary criterion assessed was the change in counts for *P. aeruginosa* strains during the
130 period of incubation in the presence of bacteriophages. We calculated that, to detect a 50 %
131 decrease in bacterial counts with a standardised effect size of 0.5, we would need to analyse $n=44$
132 paired observations, with an alpha risk of 0.05 and a beta risk of 0.10 (the standardised effect size
133 to estimate the number of subjects is defined as the difference of the mean before and after
134 divided by the standard deviation). Thus, taking missing data into account, we needed to enrol 55
135 to 60 consecutive patients in this study. The secondary criteria taken into account were the
136 change in bacteriophage counts during the incubation period and the sensitivity of the 20
137 individual colonies tested per sample to each bacteriophage. Samples were then classified into
138 five groups, defined as: negative, for samples in which no increase in bacteriophage counts was
139 observed; group A, for samples in which bacterial counts decreased and bacteriophage-sensitive
140 bacteria were present; group B, for samples displaying a decrease in bacterial counts and an
141 absence of sensitive colonies; group C, for samples displaying no decrease in bacterial counts and
142 the presence of sensitive colonies; group D for samples displaying no decrease in bacterial counts
143 and an absence of sensitive colonies (Figure 3).

144

145 *Statistical analysis*

146 Descriptive data are summarised as means \pm standard deviation (SD) or medians with
147 interquartile ranges, according to the normality of the distribution, which was assessed with the
148 Shapiro-Wilk test. We used Kruskal-Wallis tests or ANOVA tests for comparisons of quantitative
149 variables. Categorical data are expressed as numbers and percentages, and were compared in chi-

150 squared tests. Univariate and multivariate analyses were carried out with linear mixed-effect
151 models for repeated measures. Variables were selected for inclusion in the multivariate model if
152 they had p -values below 0.20 in univariate analysis, and a stepwise procedure was used to select
153 the final variables included in the multivariate model. A p -value of ≤ 0.05 was considered
154 statistically significant. Data were analysed with R.2.15.2 software.

155

156

157 **Results**

158 **Characteristics of the patients and treatment of the samples**

159 The characteristics of the 58 CF patients enrolled in this study are reported in Table 1. Each of
160 the 58 sputum samples collected was split into four aliquots. We added a cocktail of ten
161 bacteriophages infecting *P. aeruginosa* to one of these aliquots and buffer alone to a second
162 aliquot; the other two aliquots were used for subsequent analysis (figure 1; methods). Bacterial
163 counts were obtained on selective medium and bacteriophage counts were obtained on agar plates
164 overlaid with four different indicative *P. aeruginosa* strains (methods). Ten sputum samples (20.8
165 %) contained no *P. aeruginosa* strains capable of growing on the selective medium and were not
166 analysed further (see Supplementary Excel file, bacteria numeration tab).

167

168 **Analysis of the microbiological data (bacteria and bacteriophages)**

169 In CF patients, *P. aeruginosa* populations displayed several phenotypes which could affect
170 permissivity to bacteriophages [22, 23]. We then expected bacteria to grow when samples were
171 incubated in the absence of bacteriophages, while significant growth attenuation was predicted in
172 the presence of bacteriophages. A statistical analysis was performed on the 48 samples, in which
173 bacterial counts (ranging from 33% to 6090% increase in absence and from 18 to 98% reduction
174 in presence of bacteriophages) were compared in a mixed linear model with two parameters: time
175 (0 h and 6 h) and bacteriophages (presence versus absence). This analysis confirmed that the total
176 number of bacteria increased during the incubation period in the absence of bacteriophages
177 ($p < 0.001$). However, despite this growth, the addition of bacteriophages significantly decreased
178 the number of bacteria present ($p = 0.024$).

179
180 The secondary criterion considered was the difference between the number of bacteriophages
181 added and the number recovered at the end of the incubation period. As bacteriophage counts can
182 only increase if the bacteriophages infect bacterial hosts, any increase over the threshold level
183 indicates that some of the bacteria in the sample were infected (Figure 2, methods; see
184 Supplementary Excel file, threshold evaluation and phage numeration tabs). The results obtained
185 for the four indicative strains were combined, and only seven samples (14.6 %) (Figure 3; see
186 Supplementary Excel file, analyses tab) displayed no increase in the number of bacteriophages
187 over the threshold level (these seven samples were assigned to the negative group). The other 41
188 samples displayed an increase in bacteriophage numbers. We analysed these 41 samples by
189 considering two additional criteria: i) the decrease in bacterial counts and ii) the sensitivity of 20
190 individual colonies to at least one of the ten bacteriophages (methods; see Supplementary Excel
191 file, susceptibility to phages tab). This led to the definition of four groups (Figure 3, methods):
192 group A, which included 17 samples (35.4 %), group B, which contained five samples (10.4 %),
193 group C, which contained nine samples (18.8 %) and group D, which contained ten samples (20.8
194 %) (see Supplementary Excel file, analyses tab including the results obtained for each indicative
195 strain).

196
197 We used a dichotomous scoring system, no lysis versus lysis (methods), to analyse the sensitivity
198 of 20 individual colonies from each sputum sample to each of ten bacteriophages (over 8,900
199 tests). Bacteriophages PAK_P5, LBL3, PAK_P3 and PAK_P4 were the most efficient, infecting
200 170, 156, 151 and 149 colonies, respectively, whereas bacteriophages CHA_P1 and PhiKZ were
201 the least efficient, infecting only 23 and 32 colonies, respectively, of a total of 892 (Table 2; see
202 Supplementary Excel file, susceptibility to phage tab). The ten bacteriophages were able to infect

203 up to 313 individual colonies, corresponding to a coverage of 35.1 %, a low value, which could
204 reflect the lack of initial selection for ability to infect *P. aeruginosa* strains from CF patients.

205
206 We also carried out a molecular analysis of five colonies isolated from each of three samples, one
207 from group A, one from group B and one from group D. 16S RNA sequences confirmed that
208 these 15 colonies belonged to *P. aeruginosa*. Genotyping confirmed that each set of five colonies
209 belonged to the same group, suggesting that each of the corresponding patients was infected by
210 one major genotype (figure 4). For the five colonies selected from the group D sample, all of
211 which were resistant to all ten bacteriophages, we selected a single colony for the isolation of
212 new bacteriophages from environmental sources. Three of these new bacteriophages, chosen at
213 random, infected all five colonies from the group D sample, demonstrating that these colonies
214 were not resistant to bacteriophage infection *per se*.

215
216 The presence of bacteriophages in the sputum samples before processing was assessed by
217 checking for plaques following spotting of the sample on the four indicative strains (see
218 Supplementary Excel file, prophages tab). Only a few plaques were observed from 11 samples,
219 corresponding to no more than 2×10^3 pfu/mL in each sample. Prophages were also detected
220 during the testing of individual colonies for sensitivity to bacteriophages, in the form of tiny
221 homogeneous plaques covering the entire surface of the indicative strain spot. In total, 29
222 colonies from eight sputum samples presented such a phenotype (see Supplementary Excel file,
223 prophages tab). The presence of these bacteriophages and prophages at such low level did not
224 prevent the determination of bacteriophage counts and colony sensitivity.

225

226 **Correlation between patient characteristics and microbiological data**

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

232

233

234 **Discussion**

235 In the face of the growing threat posed by antibiotic-resistant strains and the particular
236 microenvironment of the lungs of CF patients, the development of effective treatments is a major
237 challenge. Mucus, pathogens (bacteria, fungi and viruses), extracellular materials (chemokines,
238 DNA, proteases etc.) and inflammatory cells form a barrier potentially preventing or interfering
239 with antibacterial treatment. The renewed interest in phage therapy and its successful use against
240 acute *P. aeruginosa* lung infections in mice led us to investigate whether bacteriophages could
241 effectively infect bacteria in the sputum of CF patients. Using a set of 58 sputum samples
242 collected from three hospitals in France, we demonstrated the efficacy of a cocktail of ten
243 bacteriophages not specifically selected for their ability to infect *P. aeruginosa* strains in sputum.
244 Thus, the microenvironment in the lungs of CF patients does not prevent bacteriophage activity
245 as we found that the number of bacteriophages increased over the threshold value in 86.4% (41
246 out 48 samples containing *P. aeruginosa* strains) samples. Furthermore, none of the clinical
247 parameters tested was associated, either positively or negatively, with bacteriophage efficacy.
248 This patient-independent efficacy supports the further development of bacteriophage treatments.

249
250 However, this pioneering study had some limitations. First, only bacteria growing on the
251 selective medium within 24 h were taken into account, but some *P. aeruginosa* strains require
252 longer incubation times for colony formation. This may account for the increase in bacteriophage
253 counts in seven of the ten samples classified as not containing *P. aeruginosa* strains. Second, we
254 used a short incubation period, to optimise the detection of decreases in bacterial counts by

255 bacteriophages, because we expected the sputum samples to contain a mixed population of
256 bacteriophage-sensitive and -resistant colonies.

257
258 Group A samples gave the most straightforward results, whereas the results for group B can be
259 explained by a shift in phenotype to bacteriophage sensitivity between the sputum and agar plate
260 environments. Upon environmental signals it is known that bacterial gene expression can affect
261 bacteriophage sensitivity [24, 25]. In group C samples, the decrease in the bacteriophage-
262 sensitive population was probably compensated by an increase in the bacteriophage-resistant
263 population. Group D samples probably contained a minor bacteriophage-sensitive population and
264 a larger bacteriophage-resistant population. Finally, some negative group samples were found to
265 contain isolated colonies sensitive to bacteriophages, suggesting that bacteria may be less
266 permissive to bacteriophage infection in the sample than on plates. Furthermore, patient factors
267 (immune cells, proteases etc.) and other microorganisms (fungi, viruses) may also hinder
268 interactions between bacteriophages and bacteria.

269
270 In sputum samples, the amplification of individual bacteriophages could be evaluated only for
271 LBL3 (infecting only one indicative strain), which gave the two highest levels of bacteriophage
272 amplification (samples 50 and 58). LBL3 was also the second most active bacteriophage in
273 analyses on isolated colonies (see Supplementary Excel file, phages numeration tab). The most
274 active bacteriophage, PAK_P5, is genetically closely related to PAK_P3, P3_CHA and CHA_P1
275 (the least active of the ten bacteriophages tested). These four bacteriophages had protein
276 sequences that were about 90% identical. The genomic data of bacteriophages is therefore
277 insufficient for predicting infectivity in clinical bacterial strains [19].

278

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

292

293

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296 collecting the sputum samples.

297

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303

304 **Authors contributions**

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

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

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

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376

377

378

379

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

Variable	Enrolled	<i>P. aeruginosa</i> -positive ^a
Patients	58^b	48
Sex (female / male)	26 / 32	23 / 25
CFTR genotype	<i>n</i> (%)	<i>n</i> (%)
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

381 ^a: *P. aeruginosa* colonisation was first recorded between years 1983 and 2011 (at least 3 months
382 before inclusion), with the majority, 31 patients, between years 1990 and 2000 and with only 4
383 patients in year 2011.

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

385

386

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

388

389

390

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

392

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 categorical variables and ANOVA or Kruskal Wallis tests for quantitative variables.

396

397

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

401

402

403 **Figure legends**

404

405 Figure 1 Schematic diagram of the processing of sputum samples

406

407 Figure 2 Increase of bacteriophages counts following 6h incubation

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

411

412 Figure 3 Diagram of the analysis of microbiological data

413 The 48 samples containing *P. aeruginosa* strains were classified into five groups according to
414 counts of bacteriophages, counts of bacteria and sensitivity to individual colonies. *, including
415 bacteria increase; **, including bacteriophages decrease. Amongst the 7 samples of the
416 negative group, one displayed an increase in bacteria counts, two a decrease and four a non-
417 significant variation. Four samples out seven contained at least one colony sensitive to
418 bacteriophages.

419

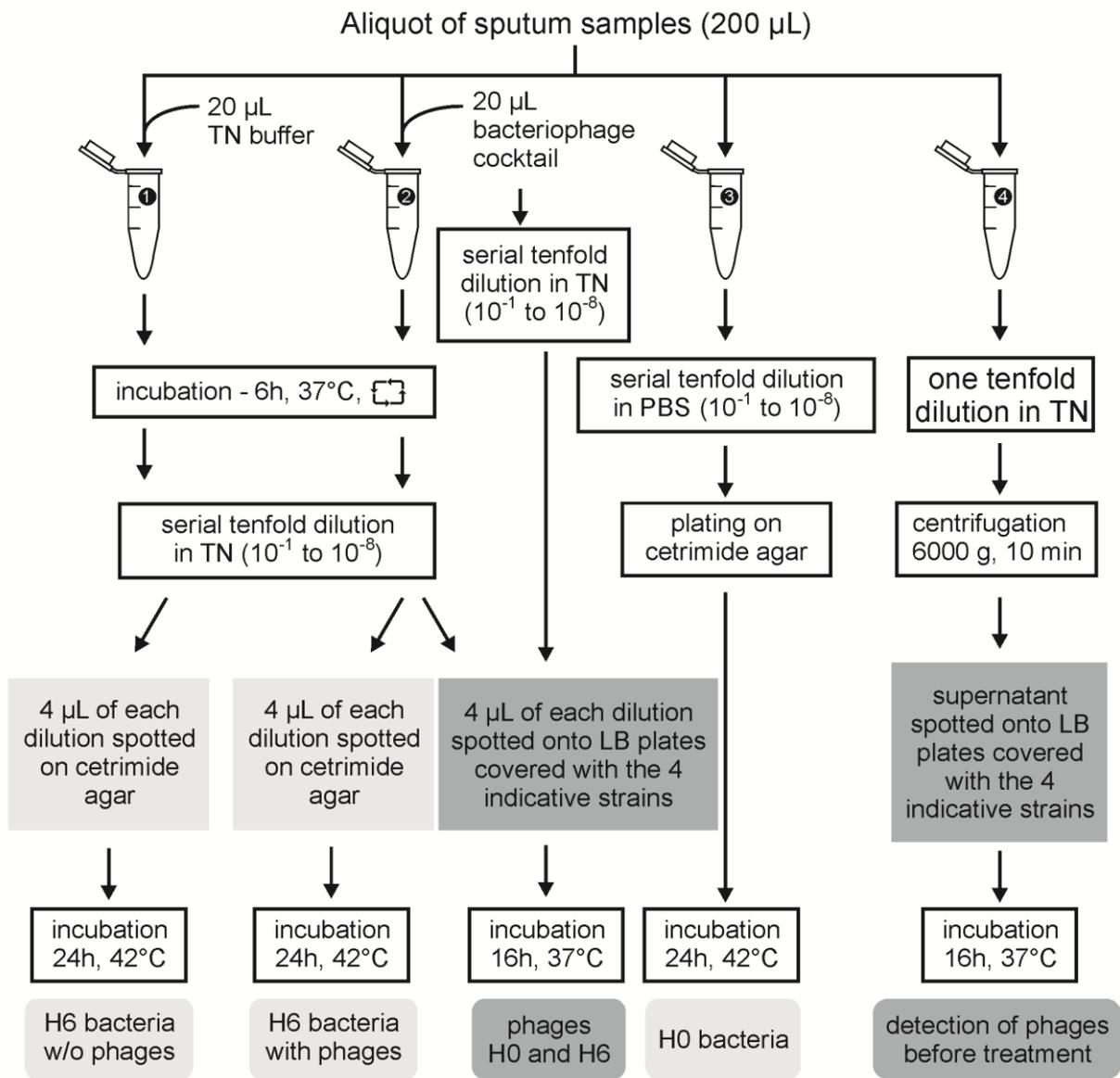
420 Figure 4: Molecular typing of 15 *P. aeruginosa* colonies isolated from three sputum samples.

421 Dendrogram, REP-PCR patterns and similarity matrix (color-coded, based on percentage
422 similarity) are represented for five randomly chosen colonies from samples 03, 04 and 33.

423

424

425 Figure 1



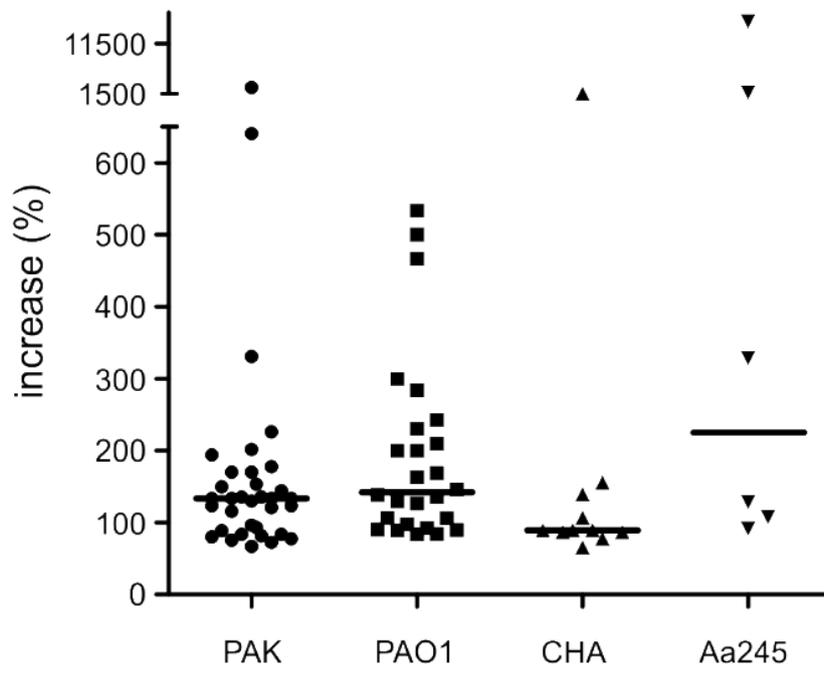
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427

428

429 Figure 2

430



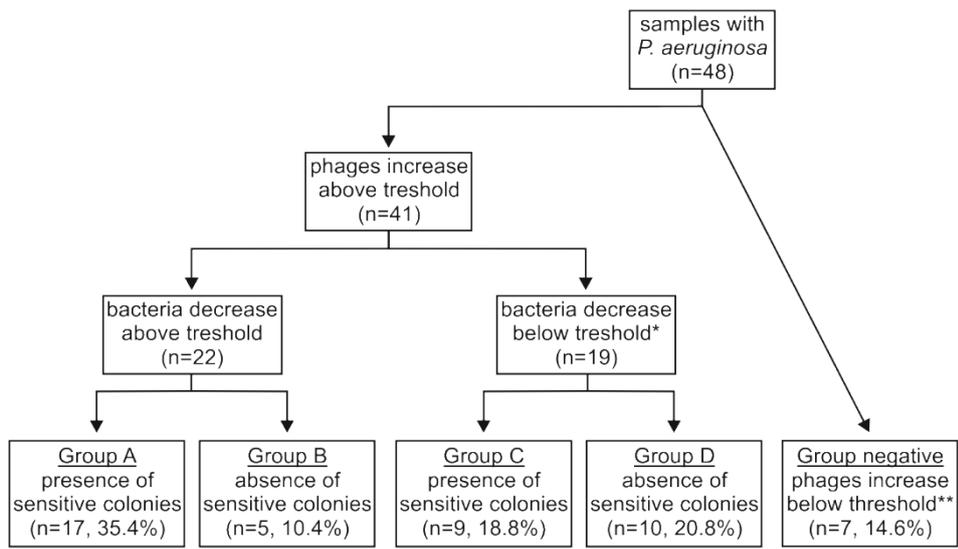
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434 Figure 3

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444 Supplementary information

445

446 Supplement to methods

447

448 *Study design*

449 We carried out a multicentre cross-sectional study on sputum samples from 58 CF patients
450 recruited from CF centres in Montpellier ($n=23$), Nancy ($n=20$) and the Necker Hospital in
451 Paris ($n=15$). This study was approved by the regional ethics committee (Nimes, registered
452 under number 2011-A01197-34) and declared to ClinicalTrial.gov (no. NCT01818206). CF
453 was defined as positive results for the sweat chloride test and the presence of mutations of the
454 CF gene. We asked patients with a stable clinical state that had previously tested positive for
455 *P. aeruginosa* infection to participate in this study. These patients were provided with
456 information about the study and they or their parents or representatives gave informed consent
457 for participation before the collection of samples during the course of routine medical care.
458 We reviewed the medical records of these patients and recorded their demographic and
459 clinical characteristics.

460

461 *Sputum sample processing*

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

469 shaking. Serial dilutions in PBS were prepared immediately from the third aliquot and plated
470 on cetrimide agar (Sigma). The plates were then incubated at 42°C for 24 h, for the
471 determination of bacterial counts and the subsequent selection of 20 individual colonies. The
472 fourth aliquot was diluted 10-fold in TN, centrifuged (6000 x g for 10 minutes) and the
473 supernatant was spotted onto LB agar plates already covered with the four indicative strains.
474 The plates were incubated at 37°C overnight, for counting of the bacteriophages present in
475 sputum samples before addition of the cocktail. After the aliquots had been incubated for 6 h,
476 as described above, serial dilutions in TN were prepared from the first two aliquots and
477 spotted (4 µL) onto cetrimide agar. The plates were then incubated at 42°C for 24 h. For the
478 second aliquot only, we also spotted the same serial dilutions onto four LB plates, each
479 covered by one of the four indicative strains, which we then incubated for 16 h at 37°C (figure
480 1).

481

482 *Reliability of bacterial and bacteriophage counts*

483 We assessed the reproducibility and accuracy of our counting procedure using 4 µL spots of
484 serial dilutions of bacteria or bacteriophages, by performing the same counting procedure in
485 triplicate, with known numbers of bacteria and bacteriophages. We observed 3.9 % to 8.0 %
486 variability in the triplicate assays, with a mean of 6.7 % variability for bacteria and 9.6 % to
487 43.3 % variability, with a mean value of 30.1 %, for bacteriophages (see Supplementary Excel
488 file, threshold tab). We considered there to be a significant increase in bacterial and
489 bacteriophage counts if we observed an absolute change of more than twice the mean
490 variability (13.4 % and 60.2 %, respectively). We increased these threshold values to 15% and
491 65%, respectively, for a more conservative analysis.

492

493 *Test of the sensitivity of individual colonies to each bacteriophage of the cocktail*

494 We randomly selected 20 colonies (representative of the proportions of mucoid/non mucoid
495 and small/large colonies) from the cetrimide plates. We streaked them onto LB plates, which
496 we incubated at 37°C for 16 h. We then picked one clone from each colony with a toothpick
497 and used it to inoculate 200 µl of LB broth in one of the wells of a 96-well plate. After 4 h of
498 incubation at 37°C with shaking, we spotted ten 15 µL aliquots from each well to form a line
499 on a square LB plate (10 x10 cm). Plates were then placed under a laminar flow hood (30
500 min) to dry, and we then dispensed a 0.5 µl drop of each bacteriophage suspension (1×10^8
501 pfu/mL) onto one of the dried bacterial drops (one suspension per drop). Plates were
502 incubated at 37°C for 16 h. On each square plate, we were able to test the sensitivity of ten
503 individual colonies to each of the ten individual bacteriophages. In the middle of the bacterial
504 lawn, isolated plaques, confluent plaques or entirely clear areas were considered to indicate
505 that the bacteria were permissive to the phage tested, whereas the absence of plaques
506 indicated full resistance.

507

508