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## Comparison of pulsed-field gel electrophoresis and whole-genome-sequencing-based typing confirms the accuracy of pulsed-field gel electrophoresis for the investigation of local *Pseudomonas aeruginosa* outbreaks

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1 **Comparison of pulsed-field gel electrophoresis (PFGE) and WGS-based**  
2 **typing confirms the accuracy of PFGE for the investigation of local**  
3 ***Pseudomonas aeruginosa* outbreaks**

4  
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21 Declarations of interest: none

22

23 **Running title:** Comparison of PFGE and cgMLST

24

25 **Abstract**

26 In this study, we aimed to determine whether pulsed-field gel electrophoresis (PFGE)  
27 accurately recognizes isolates belonging to clusters defined by techniques based on whole  
28 genome sequencing (WGS) using *Pseudomonas aeruginosa* as a model.

29 We selected 65 isolates of ST395 *P. aeruginosa* isolated in seven European hospitals between  
30 1998 and 2012. Isolates were typed by PFGE and sequenced by WGS. A core genome MLST  
31 (cgMLST) analysis based on 3,831 genes was performed with a homemade pipeline. PFGE  
32 identified eight pulsotypes and cgMLST differentiated nine clusters and nine singletons. Five  
33 cgMLST clusters and pulsotypes (31/65 isolates) coincided perfectly. Isolates without evident  
34 epidemiological links grouped by PFGE were separated by cgMLST (16/65 isolates)  
35 differentiating cities, suggesting that PFGE should be kept for the investigation of local  
36 outbreaks. Importantly, hypermutator isolates still shared the pulsotype with their parents  
37 (16/65 isolates), whereas they were not recognized by cgMLST. This shows that PFGE was  
38 less affected than WGS-based typing by the accelerated genetic drift that occurs in epidemic  
39 *P. aeruginosa*.

40 In conclusion, although WGS-based typing has logically become the new gold standard, we  
41 show here that the PFGE can be used with confidence for the investigation of local outbreaks  
42 caused by *P. aeruginosa*.

43

44 **Keywords:** pulsed-field gel electrophoresis, whole genome sequencing, cgMLST, bacterial  
45 typing, *Pseudomonas aeruginosa*, outbreaks

## 46 **Introduction**

47 Pulsed-field gel electrophoresis (PFGE) has been used over the last three decades to decipher  
48 epidemiological links between bacterial isolates or between fungal isolates to identify  
49 reservoirs and routes of transmission. This technique has been steadily replaced by typing  
50 based on whole-genome sequencing (WGS), which provides highly supported phylogenetic  
51 trees and has become the new gold standard for bacterial typing. Previous studies have  
52 revealed its accuracy not only for epidemiological investigations in hospital settings [1,2] but  
53 also in wider settings, including isolates from different sources (*e.g.* environmental, food, or  
54 livestock) [3]. The recent implementation of WGS in clinical laboratories, easier access to the  
55 technique, and the growing number of publications using WGS-based typing have casted  
56 doubt on the utility of PFGE, now considered to be outdated.

57 The criteria for the clustering of epidemiologically-linked isolates based on their PFGE  
58 patterns was defined in 1995 and validated by numerous studies [4,5]. In contrast, the  
59 thresholds that cluster epidemiologically-linked isolates are harder to set with WGS-based  
60 typing and depend on the method used and on the strain collection. Molecular epidemiology  
61 often relies on single nucleotide polymorphism (SNP)-based analysis and on core genome  
62 multi-locus sequence typing (cgMLST) which consists of a gene-by-gene comparison of the  
63 core genome of the species [6]. The success of cgMLST spurred the definition of schemes for  
64 major pathogens in order to standardize the method [6].

65 A good accordance between WGS-based typing and PFGE was found in studies involving  
66 major pathogenic species such as *Escherichia coli*, *Klebsiella pneumoniae*, or *P. aeruginosa*  
67 [7–9]. Here, we further tested whether PFGE accurately differentiates *P. aeruginosa* isolates  
68 belonging to the same ST but from different outbreaks. ST395 is responsible for nosocomial  
69 outbreaks and was a good model. We analyzed on purpose a collection of isolates belonging  
70 to the same ST in order to challenge PFGE. A collection of *P. aeruginosa* was first typed

71 using PFGE which clustering was compared with that of cgMLST. This study aimed to show  
72 that PFGE effectively type isolates in local epidemiological surveys and that WGS is not  
73 always needed for bacterial typing.

74 **Materials and methods**

75 **Bacterial isolates.** We used a collection of 65 well-characterized clinical (n = 59) and  
76 environmental (n = 6) isolates of *P. aeruginosa* collected between 1998 and 2012 in the seven  
77 hospitals of Besançon (France), Birmingham (United Kingdom), Bordeaux (France), Lyon  
78 (France), Montpellier (France), and Lariboisière and Garches (Paris, France). We selected  
79 isolates belonging to ST395 and collected during various studies. A first study collected  
80 isolates from patients hospitalized in the intensive care units of six French hospitals in 2009.  
81 A second study was conducted in 2012 in Birmingham where *P. aeruginosa* were found from  
82 patients hospitalized in burns care units. A third study collected isolates of an epidemic strain  
83 retrieved in the University Hospital of Besancon between 1998 and 2012 [10–12,1].

84 Isolates were stored at the Centre de Ressources Biologiques – Filière Microbiologique at the  
85 University Hospital of Besançon (Supplemental Table I). All isolates were identified as  
86 *P. aeruginosa* by MALDI-TOF MS, with a log value  $\geq 2$  according to the manufacturer's  
87 recommendations. The ST of the 65 isolates was determined by multi-locus sequence typing  
88 (MLST) using a previously developed scheme [13] and all isolates were identified as ST395.

89 **PFGE.** We genotyped all the isolates by determining the macrorestriction profile of their total  
90 DNA (hereafter called pulsotype) by PFGE (CHEF DRIII, Bio-Rad) after restriction with  
91 *DraI* (Roche Diagnostics), according to a technique previously validated in the laboratory  
92 [14]. The software Bionumerics (Applied Math, Kortrijk, Belgium) built a DNA similitude  
93 matrix based on calculating the Dice coefficient for pairwise isolate comparisons. The  
94 dendrogram was built using the UPGMA (unweighted pair group using arithmetic averages)  
95 hierarchical algorithm. We used the *Staphylococcus aureus* NCTC 8325 strain as a reference  
96 to allow comparison between gels. The pulsotypes were defined using a similarity coefficient  
97 of 80%, as described in a previous study [15].

98 **Whole Genome Sequencing.** Bacterial DNA was isolated from an overnight culture on  
99 Mueller-Hinton agar (Bio-Rad, Marne la Coquette, France) and extracted with a QIAmp DNA  
100 Minikit (Qiagen). DNA was sequenced on an Illumina NextSeq, which generates 150-bp  
101 paired-end reads with a mean coverage of 80X. Reads were assembled using SPAdes  
102 v.3.13.0. We determined the cgMLST with the homemade pipeline pyMLST  
103 (<https://github.com/bvalot/pyMLST>) using a core genome of 3,831 previously defined genes  
104 [16]. A minimum spanning tree (MST) was built with GrapeTree [17] using distances  
105 calculated from genes present in all strains (n = 3,491). Isolates for which the genomes had  $\leq$   
106 20 alleles of difference were considered to epidemiologically linked.

107 **Identification of hypermutators.** Hypermutators are defined by a high rate of mutation, often  
108 due to a defective mismatch repair (MMR) system. We identified hypermutator candidates *in*  
109 *silico* by searching for non-synonymous mutations, insertions, or deletions in the MMR genes  
110 (*mutS*, *mutL*, or *uvrD*) relative to the PAO1 reference strain genome (NCBI accession number  
111 NC\_002516). We confirmed that isolates defective in the MMR system were hypermutators  
112 by comparing their mutation frequency with that of close isolates with an intact MMR system  
113 and that of the PAO1 reference strain using a previously described procedure [18]. We used  
114 Student's unpaired *t*-tests to determine the statistical significance of comparisons of the  
115 emergence frequencies of mutants resistant to rifampicin between ST395 isolates and the  
116 reference strain PAO1.

117 **Results and discussion**

118

119 The clusters defined by PFGE and cgMLST are presented in Figure 1. PFGE identified eight  
120 pulsotypes (A to H) and cgMLST distributed the isolates in nine clusters and nine singletons  
121 when using a threshold of 20 alleles.

122 Six pulsotypes (A, B, D, E, G, and F) were each specific to a single hospital, whereas  
123 pulsotypes C and H grouped isolates from four hospitals (Table I). Five pulsotypes (B, E, F,  
124 G, and H) perfectly coincided with the cgMLST clusters (31/65 isolates). The two pulsotype  
125 A isolates were separated in cgMLST as singletons with 34 alleles of difference. The 16  
126 isolates of pulsotype C were separated into four cgMLST clusters (of 6, 4, 3 and 2 isolates)  
127 and one singleton. Each cluster discriminated the hospital of isolation except for the isolates  
128 of Lyon which were separated in two clusters with at least 25 alleles of difference. The 16  
129 isolates of pulsotype D were distributed in one cgMLST cluster (11 isolates) and five  
130 genetically distant singletons with > 80 alleles of difference.

131 Previous studies have reported a good correlation between SNP-based typing and PFGE for  
132 the species *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterococcus faecium*, and  
133 *Escherichia coli* [8,9,19,20]. SNP-based and PFGE analysis used to discriminate close  
134 isolates of *P. aeruginosa* retrieved from intensive care units revealed perfect congruence  
135 between the two methods [7], showing the ability of PFGE to decipher the links between  
136 epidemiologically-related isolates. However, the stability of PFGE typing could not be tested  
137 by Parcell et al., as they compared isolates collected in a single hospital (Parcell *et al.* 2018).  
138 We deliberately included isolates of the same ST of *P. aeruginosa* to test the ability of PFGE  
139 to differentiate isolates of the same clonal group that are not epidemiologically linked and test  
140 the stability of the method. Here, the accuracy of PFGE is illustrated by the five cgMLST  
141 clusters matching those found with PFGE and by the fact that PFGE correctly differentiated



142 genetically distant isolates collected in the same ICU over the same time period (Table I) in  
143 the Lyon and Montpellier Hospitals (pulsotypes C, E, and H).

144 The genetic distance between the genomes of the isolates belonging to pulsotypes A and D led  
145 us to test whether they were hypermutators. Hypermutators are characterized by a defective  
146 MMR system, which involves the genes *mutL*, *mutS*, and *uvrD* [21]. We then compared the  
147 nucleotide sequences of these three genes of all the genomes of our dataset ( $n = 65$ ) with that  
148 of the reference strain *P. aeruginosa* PAO1 and found that the five genetically distant isolates  
149 of pulsotype D (but not the two pulsotype A isolates, data not shown) specifically harbored a  
150 defective MMR system. Pulsotype D isolates were collected throughout a 13-year outbreak at  
151 the University Hospital of Besancon [11]. The isolate DHS15 was retrieved in February 2002  
152 and had a non-synonymous mutation in *mutS*, leading to an amino-acid substitution in the  
153 protein MutS (Y433D). The four other isolates (210690101, 210690111, 210690101, B168)  
154 were retrieved in May 2009 and had the same mutation in *mutL*, creating a stop codon in  
155 MutL (R535X). We compared the mutation frequency of the five MMR-defective isolates  
156 with that of two isolates of pulsotype D with a wildtype MMR system and the reference strain  
157 PAO1 to confirm the phenotypic consequence of these mutations (Supplemental Figure 1).  
158 Our data confirmed that, while the pulsotype D isolates with an intact MMR system had a  
159 mutation frequency similar to that of PAO1, the mutation frequency of the five MMR-  
160 defective isolates was significantly higher ( $p < 0.05$ ) than that of PAO1 (Supplemental  
161 Figure 1). The hypermutability of the MMR-defective isolates most probably accounted for  
162 the genetic distances revealed by cgMLST. PFGE appears to be less affected than WGS-based  
163 typing by accelerated genetic drift that occurs in epidemic *P. aeruginosa*, depending or not on  
164 the MMR system.

165 Despite a good correlation between the two typing methods, PFGE can group isolates of  
166 different geographical origins. Hence, ST395 *P. aeruginosa* isolates from four distant

167 hospitals were separated by cgMLST but aggregated in pulsotype C (16/65 isolates). This  
168 confirms a previous study showing that PFGE may cluster temporally and geographically  
169 unrelated isolates, as well as isolates belonging to unrelated STs [20]. Pulsotype H was also  
170 consistent with the cgMLST clustering but grouped isolates of four different hospitals (Figure  
171 1). Although these results presumably reflect inter-hospital transmission events, we did not  
172 have access to the patient records that could have shown a transfer between hospitals. Data  
173 with pulsotypes C and H confirmed that PFGE should not be used for inter-hospital  
174 investigations without an obvious epidemiological link, as it could lead to uncertain  
175 clustering. It suggests that PFGE should only be used to compare isolates in a well-defined  
176 spatiotemporal context (*i.e.* for local outbreak investigations). More broadly, these  
177 observations stress the need for a systematic confrontation of bacterial typing results with  
178 epidemiological data. Overall, our results show that pulsotypes B, D, E, F, and G were  
179 consistent with cgMLST clustering, confirming that local outbreaks caused by *P. aeruginosa*  
180 can be investigated with confidence with PFGE.

181 PFGE results are easily interpreted since pulsotype clustering is based on the similitude  
182 coefficient [15]. The interpretation of cgMLST data is more complicated, for which the  
183 number of differences depends on the species, with no threshold defined for *P. aeruginosa*.  
184 Here, we set the threshold to 20 alleles of difference to delimit cgMLST clusters. A previous  
185 study chose a threshold of 14 alleles of difference for *P. aeruginosa* [16]. However, this  
186 threshold was not suitable for our study as it would have created too many clusters, especially  
187 for pulsotype D (data not shown). Indeed, it has been suggested that epidemiological data  
188 (*e.g.* the duration of the outbreak) needs to be considered for the choice of a suitable threshold  
189 [6].

190 The criteria for pulsotype clustering directly depends on the mutation rate of the pathogen  
191 tested. The good results obtained with PFGE for the investigation of local *P. aeruginosa*

192 outbreaks in this study suggest that it should be suitable for other clones of *P. aeruginosa*, as  
193 well as other pathogens with a comparable mutation rate, such as certain clones of  
194 *K. pneumoniae* or *Staphylococcus aureus* [22].

195 Over the last decade, WGS-based typing has challenged the PFGE. WGS-based typing  
196 sharply discriminates clonal populations [1,7] and the sequencing data allows the  
197 identification of resistance determinants, the deciphering of genome plasticity, the dating of  
198 the most recent common ancestor, the identification of plasmids, protein prediction, and the  
199 portability of the data [2,9,23]. Recombination events are less likely to modify the WGS-  
200 based clustering, whereas they can readily modify the PFGE pattern [24]. Despite its cost,  
201 WGS-based typing will overtake other typing methods. However, we confirmed here that  
202 PFGE is still appropriate for the investigation of local outbreaks. Finally, one may also ask  
203 whether there is not enough or too much WGS for outbreak investigations. Indeed, perhaps  
204 less data would be sufficient for this purpose.

205 Our study has some limitations. We used a single ST to challenge the PFGE typing, but  
206 consequently explored a small part of the *P. aeruginosa* diversity. Further experiments with a  
207 higher number of isolates, belonging to other clones which disseminate locally or globally  
208 could consolidate the evaluation of the PFGE performances.

209 In conclusion, although WGS-based typing has logically become the new gold standard, we  
210 show here that the PFGE can be used with confidence for local outbreak investigations.

211

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214 gift of ST395 isolates.

215

## 216 **Authors contribution**

217 DM, MS, BV, XB, and DH conceived and designed the experiments. DM, AM, PC and MT  
218 carried out the experiments. DM, AM, BV analyzed the data. DM, BV, XB, and DH wrote the  
219 manuscript.

220

221 **Conflict of interest**

222 None.

223

224 **Source of funding**

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226 commercial, or not-for-profit sectors.

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316 **Figure legends**

317

318 **Figure 1. Minimum spanning tree based on the cgMLST analysis of 65 *P. aeruginosa***  
319 **ST395 isolates.** The colors designate the hospitals from which the strains were isolated.  
320 Pulsotypes A to H are named inside the circles. Isolates with  $\leq 20$  different alleles are linked  
321 by black lines, whereas isolates with  $\geq 20$  different alleles are linked by dotted lines.

322 **Table I. Distribution and epidemiological data of pulsotypes and cgMLST clusters of 65 *P. aeruginosa* ST395 isolates.**

323

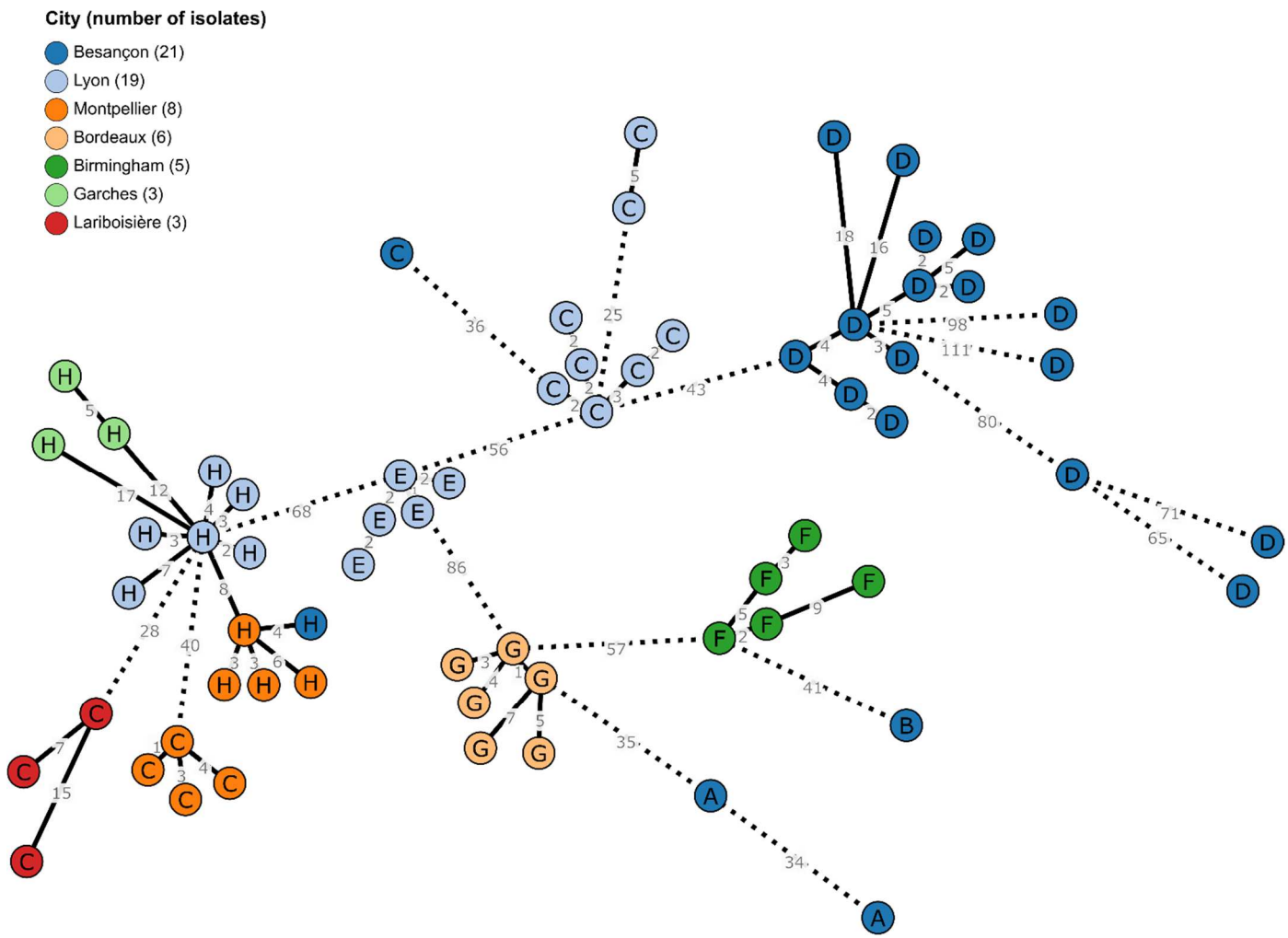
<b>Pulso- types</b>	<b>Number of isolates</b>	<b>Number of cgMLST clusters/singletons</b>	<b>Hospital(s)</b>	<b>Wards</b>	<b>Period of isolation</b>
A	2	0/2	Besançon	ICU	June 2009
B	1	0/1	Besançon	ICU	March 2009
C	16	4/1	Besançon, Lyon, Montpellier, Lariboisière	ICU	June 2009 to January 2010
D	16	1/5	Besançon	ICU, vascular surgery, neurology	January 1998 to April 2011
E	5	1/0	Lyon	ICU	April 2009 to May 2009
F	5	1/0	Birmingham	BCU	2012
G	6	1/0	Bordeaux	ICU	May 2009 to July 2009
H	14	1/0	Montpellier, Besançon, Lyon, Garches	ICU	April 2009 to October 2009

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325 ICU: intensive care unit, BCU: burn care unit



326 **Figure 1**



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