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Aurélie Hennebique, Marie Bidart, Sophie Jarraud, Laëtitia Beraud, Carole Schwebel, Max Maurin, Sandrine Boisset

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1 **Digital PCR for detection and quantification of fluoroquinolone resistance in *Legionella***
2 ***pneumophila*.**

3

4 Aurélie Hennebique^{a, b, #}, Marie Bidart^{a, c}, Sophie Jarraud^{d, e}, Laëtitia Beraud^d, Carole
5 Schwebel^f, Max Maurin^{a, b}, Sandrine Boisset^{a, b, #}.

6

7 ^a Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes,
8 Grenoble, France.

9 ^b Université Grenoble Alpes, TheREx, TIMC-IMAG, CNRS, UMR5525, Grenoble, France.

10 ^c Université Grenoble Alpes, Braintech Lab, CEA, INSERM UMR1205, Grenoble, France.

11 ^d Centre National de Référence des Légionelles, Institut des Agents Infectieux, Groupe
12 Hospitalier Nord, Hospices Civils de Lyon, Lyon, France.

13 ^e Centre International de Recherche en Infectiologie, INSERM U1111, Université Lyon 1,
14 CNRS, UMR5308, ENS, Lyon France

15 ^f Réanimation, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble, France ;
16 Radiopharmaceutiques Biocliniques, Inserm U1039, La Tronche, France.

17

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20

21 # Corresponding authors : Aurélie Hennebique: ahennebique@chu-grenoble.fr; Sandrine

22 Boisset: sboisset@chu-grenoble.fr.

23 **ABSTRACT**

24 The emergence of fluoroquinolone (FQ)-resistant mutants of *Legionella pneumophila* in
25 infected humans has been previously reported using a next-generation DNA sequencing
26 (NGS) approach. This finding could explain part of the therapeutic failures observed in
27 legionellosis patients treated with these antibiotics. The aim of this study was to develop
28 digital PCR (dPCR) assays allowing rapid and accurate detection and quantification of these
29 resistant mutants in respiratory samples, especially when the proportion of mutants in a
30 wild-type background is low. We designed three dPCRgyrA assays to detect and differentiate
31 the wild-type and one of the three *gyrA* mutations previously described as associated with
32 FQ resistance in *L. pneumophila*: 248C>T (T83I), 259G>A (D87N), and 259G>C (D87H). To
33 assess the performance of these assays, mixtures of FQ-resistant and -susceptible strains of
34 *L. pneumophila* were analyzed, and the results were compared with those obtained with
35 Sanger DNA sequencing and real-time quantitative PCR (qPCR) technologies. The dPCRgyrA
36 assays were able to detect mutated *gyrA* sequences in the presence of wild-type sequences
37 up to 1:1000 resistant:susceptible allele ratios. In comparison, Sanger DNA sequencing and
38 qPCR were less sensitive, allowing detection of *gyrA* mutants up to 1:1 and 1:10 ratios,
39 respectively. When testing 38 respiratory samples from 23 legionellosis patients (69.6%
40 treated with a FQ), dPCRgyrA detected small amounts of *gyrA* mutants in four (10.5%)
41 samples from three (13.0%) patients. These results demonstrate that dPCR is a highly
42 sensitive alternative to quantify FQ resistance in *L. pneumophila*, and it could be used in
43 clinical practice to detect patients that could be at higher risk of therapeutic failure.

44

45 **INTRODUCTION**

46 *Legionella pneumophila*, a Gram-negative, facultative intracellular bacterium, is the
47 causative agent of legionellosis, a severe pneumonia associated with mortality rates ranging
48 from 5% to 25% (1). Diagnosis of this disease mainly relies on urinary antigen test, and
49 culture and PCR testing of respiratory samples. The first-line drugs for treatment of
50 legionellosis are the macrolides and the fluoroquinolones (FQ) (2). *In vitro* antibiotic
51 susceptibility testing of *Legionella* sp. strains is currently not recommended on a routine
52 basis, especially because no standardized method is available from the CLSI (Clinical and
53 Laboratory Standards Institute) or the EUCAST (European Committee on Antimicrobial
54 Susceptibility Testing). However, therapeutic failures and relapses are still reported (3–5),
55 FQ-resistant mutants of *L. pneumophila* have been readily selected *in vitro* (6–9), and FQ-
56 resistant mutants have also recently been found in legionellosis patients (10, 11). Indeed,
57 Shadoud *et al.* recently characterized the *in vivo* selection of such FQ-resistant mutants of *L.*
58 *pneumophila* in two legionellosis patients treated with these antibiotics (11).

59 Bacterial resistance to quinolones of clinical relevance is most often related to
60 mutations in the genes encoding type II topoisomerases, also called DNA gyrase (encoded by
61 *gyrA* and *gyrB* genes) and type IV topoisomerase (encoded by *parC* and *parE* genes) (12).
62 Resistance-conferring mutations occur within specific regions of these genes, called the
63 quinolone resistance-determining regions (QRDRs). The *in vitro* selection of *L. pneumophila*
64 strains with high-level resistance to quinolones has been mainly related to mutations
65 affecting codons 83 and 87 of *gyrA* QRDR (7–9), although mutations in *gyrB* and *parC* were
66 also described (9). Only mutations at codon position 83 of the *gyrA* gene have been reported
67 so far *in vivo* (10, 11). Using a next-generation DNA sequencing (NGS) approach, Shadoud *et*
68 *al.* previously demonstrated that a *L. pneumophila* mutant population harboring the 248C>T

69 (T83I) mutation was rapidly selected *in vivo* in two legionellosis patients treated with a FQ,
70 representing from 1.05% of the total *L. pneumophila* lung population at the time of diagnosis
71 up to 94% after a few days of FQ treatment (11). It can be hypothesized that a higher
72 proportion of mutated *gyrA* sequences at the time of legionellosis diagnosis may represent a
73 higher risk of FQ treatment failure. Also, selection of the *gyrA* mutated population by the
74 administration of a FQ may occur faster when the proportion of mutants over wild-type
75 strains is high at the time of treatment onset.

76 The prevalence of the *in vivo* selection of *L. pneumophila* FQ-resistant mutants in
77 legionellosis patients treated with these antibiotics remains to be established. It cannot be
78 determined by traditional phenotypic methods for several reasons: 1/ *Legionella* cultures are
79 negative in most legionellosis patients because of the fastidious nature of this bacterium; 2/
80 *L. pneumophila* isolation is even more difficult to obtain in patients under antibiotic
81 treatment, even when patients experience treatment failure or relapses; 3/ a mixture of
82 antibiotic-susceptible and a few resistant strains of *L. pneumophila* would certainly result in
83 selecting the most susceptible population for antibiotic susceptibility testing; and 4/ it is
84 necessary to evaluate the respective proportion of such susceptible and resistant
85 populations to evaluate the potential risk of treatment failure.

86 Digital PCR (dPCR) is an innovative PCR technology developed in the 2000s, based in
87 the partition of the sample to be analyzed in thousands to millions of individual PCR
88 reaction. A major advantage of dPCR, compared to real-time quantitative PCR (qPCR) is an
89 increased sensitivity for detection of a few mutants mixed with wild-type DNA sequences
90 (13). dPCR has wide clinical applications in the oncology field (14), and ongoing applications
91 in noninvasive prenatal diagnosis (15, 16) and organ transplant rejection monitoring (17). For
92 infectious diseases, dPCR technology has been mainly used for diagnosis of viral diseases

93 (13), quantification of bacteria in clinical samples (18–21), detection and quantification of
94 antibiotic resistance genes (22, 23), and detection and quantification of bacterial toxins (24).

95 The aim of the present study was to develop dPCRgyrA assays for detection and
96 quantification of FQ-resistant mutants of *L. pneumophila* in respiratory samples collected
97 from legionellosis patients.

98

99

100 **MATERIALS AND METHODS:**

101 **Bacterial strains and culture conditions**

102 The *L. pneumophila* strains used in this study are listed in Table 1. The *L. pneumophila* sg 1
103 strain Paris CIP107629T (referred to as LPP) was used as a FQ-susceptible control. The FQ-
104 resistant mutants LPPI1, LPPI4, and LPPI5 were derived from LPP by homologous
105 recombination introducing the three main *gyrA* mutations previously selected and
106 characterized *in vitro*, including the mutations 248C>T (T83I) for LPPI1, 248C>T and 259G>A
107 (D87N) for LPPI4, and 248C>T and 259G>C (D87H) for LPPI5 (9).

108 For specificity purposes, 20 reference or clinical strains belonging to bacterial species other
109 than *Legionella* sp. (Table S1) and 19 strains of *Legionella* species other than *L. pneumophila*,
110 provided by the French National Reference Center (NRC) for *Legionella* (Table S2) were used.
111 *Legionella* strains were grown on BMPA medium (Oxoid, Cambridge, UK) at 37°C in 5% CO₂-
112 enriched atmosphere for 3 days. FQ-resistant *Legionella* strains were grown in a biosafety
113 level 3 laboratory. The other bacterial species were grown on Columbia agar supplemented
114 with 5% sheep blood, or on chocolate agar supplemented with 5% PolyVitex® (bioMérieux,
115 Marcy L’Etoile, France) at 37°C in 5% CO₂-enriched atmosphere for 24 h.

116

117 **Patients and clinical samples**

118 Firstly, eight respiratory samples from six patients, previously studied with NGS (11), were
119 analyzed by dPCR. These samples came from a previous single-center cohort of legionellosis
120 patients admitted to an intensive care unit at Grenoble University Hospital between 2006
121 and 2011. Two patients previously detected positive for T83I FQ-resistant mutants of *L.*
122 *pneumophila* using NGS served as positive controls (11). Four patients with NGS-negative
123 samples served as negative controls (11). These controls were used for validation of the
124 dPCRgyrA assay developed targeting the T83I mutation.

125

126 Then 38 respiratory samples from 23 patients with unknown NGS status were
127 retrospectively evaluated for the presence of the T83I mutation. These patients were
128 admitted to an intensive care unit at Grenoble University Hospital (France) or Lyon
129 University Hospital (France) between 2011 and 2016. They included 20 males and three
130 females, with a mean age of 56 years (range, 23–83 years). During hospitalization, lower
131 respiratory tract (LRT) (i.e., bronchoalveolar lavage or bronchial aspirations or tracheal
132 aspiration or sputum) and urine samples were collected for diagnostic purposes. LRT
133 samples were tested using routine bacterial cultures, *Legionella* cultures, and *Legionella*
134 qPCR tests (qPCR16S for all *Legionella* species and qPCRMip for *L. pneumophila*, as previously
135 described (25)), and then frozen at –80°C until use for this study. The 23 patients were
136 selected because of positive *Legionella* qPCR tests (qPCR16S and qPCRMip) (see Results
137 section), while 21 patients also had a positive urinary antigen test for *L. pneumophila* sg1,
138 and 18 patients had a positive culture allowing isolation of a *L. pneumophila* sg 1. All these
139 patients were considered infected with a *L. pneumophila* strain because of the positive
140 qPCRMip test on LRT samples. Sixteen (69.6%) out of the 23 patients were treated with a FQ.

141

142 In addition, to validate the clinical use of the dPCRgyrA targeting T83I mutation, ten
143 respiratory samples from patients suffering from pneumonia other than legionellosis were
144 used. These samples were negative for *Legionella* qPCR tests (qPCR16S and qPCRmip), while
145 commensal and pathogenic bacterial species were detected by routine bacterial cultures.

146

147 The clinical sample collections of Grenoble and Lyon University Hospitals were declared to
148 the French Ministry of Education and Research (number DC-2008-677 and DC-2008-176
149 respectively), and an ethics committee (CPP Sud-Est V) gave the authorization to use it for
150 research purposes. Patient information was provided through a hospital medical booklet and
151 only non-opposition on the part of the patients was needed.

152

153 **DNA extraction**

154 For bacterial strains, colonies were harvested and suspended in Tris-EDTA buffer solution
155 (Sigma-Aldrich, St. Louis, MO, USA). For FQ-resistant *Legionella* strains, bacteria were
156 inactivated by heating at 95°C for 15 min before DNA extraction. DNA was extracted using
157 the QIAamp DNA Mini kit (Qiagen, Courtaboeuf, France), according to the manufacturer's
158 recommendations.

159 DNA concentration of LPP, LPPI1, LPPI4, and LPPI5 was assessed using Qubit® Fluorometric
160 Quantitation (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's
161 recommendations and adjusted to 1 ng/μL. Then mixtures of DNA from LPPI1, LPPI4, or
162 LPPI5 with DNA from the wild-type LPP strain were prepared at different mutant:LPP ratios:
163 1:1, 1:10, 1:100, 1:500, and 1:1000.

164 DNA concentrations of suspensions of strains belonging to non-*Legionella* species and
165 *Legionella* species other than *L. pneumophila* were assessed using a NanoDrop® 2000c
166 spectrophotometer (Thermo Fisher Scientific), according to the manufacturer's
167 recommendations.

168 For the respiratory samples, DNA extraction was performed using either the EZ1® DNA
169 Tissues kit (Qiagen) or the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche
170 Diagnostics, Mannheim, Germany) according to the manufacturers' instructions.

171

172 **Sanger sequencing of the *L. pneumophila gyrA* QRDR**

173 The mutant:LPP mixtures were tested using a PCR-sequencing approach in order to assess
174 the sensitivity of this technique to detect mutated *gyrA* QRDR sequences in the presence of
175 wild-type sequences.

176 A 383-bp fragment of the *gyrA* QRDR was amplified using the primers LgyrALubS7 (5'-
177 GCGATGAGTGTCATTGTAG-3') and LgyrALubASR2bis
178 (5'GTTTCATCATAGTTAGGGCTAAAATCAAC-3') as previously described (11). Sequencing was
179 performed using the same primers and the dye terminator method, with the DTCS Quick
180 Start Kit (Beckman Coulter, Villepinte, France) on a CEQ 8000 sequencer (Beckman Coulter)
181 as previously described (9). Amplicons were sequenced from both ends. The sequences
182 obtained were visualized and analyzed with Chromas LITE (version 2.1.1, Technelysium) and
183 aligned with Seaview4 (26).

184

185 **Primers and probes for qPCR_{gyrA} and dPCR_{gyrA}**

186 We followed the MIQE Guidelines for nomenclature and qPCR protocol development (27).

187 For qPCR_{gyrA} and dPCR_{gyrA} experiments, primers and probes targeting the wild-type and

188 mutated sequences of the *gyrA* QRDR were designed using the Custom TaqMan® Assay
189 Design Tools (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA). Three assays
190 were developed to detect the wild-type *gyrA* sequence and the *gyrA* QRDR mutations
191 previously selected *in vitro* in *L. pneumophila* (9): 248C>T (T83I), 259G>A (D87N), and
192 259G>C (D87H).

193 Primers and probes were provided by Life Technologies. Each assay mix contained PCR
194 primers and two hydrolysis MGB probes, including a VIC-labeled probe complementary to
195 the wild-type *gyrA* QRDR sequence and a FAM-labeled probe complementary to one of the
196 three mutated *gyrA* QRDR sequences (Table 2).

197

198 **qPCR_{gyrA} validation and study of the mutant:LPP mixtures**

199 Validation of the three qPCR_{gyrA} assays for the specific detection of the wild-type and
200 mutated (T83I, D87N, or D87H) *gyrA* QRDR sequences was performed by testing the LPP,
201 LPPI1, LPPI4, and LPPI5 strains with the three assays. Each PCR run included no-template
202 controls (NTC) to validate the absence of false-positive results caused by exogenous DNA
203 contamination, autohydrolysis of the probes, or nonspecific hybridization.

204 Then the mutant:LPP mixtures (1:1, 1:10, 1:100, 1:1000) were tested using the qPCR_{gyrA}
205 assays, in duplicate, in order to assess the sensitivity of this technique to detect mutated
206 *gyrA* QRDR sequences in the presence of wild-type sequences.

207 The PCR mixture (25 µL) contained 12.5 µL of TaqMan® Genotyping Master Mix (Life
208 Technologies, Thermo Fisher Scientific), 0.625 µL of 40X TaqMan® assay mix (36 µM of each
209 primer, 8 µM of the wild-type probe, and 8 µM of the mutant probe), and 11.875 µL of
210 diluted DNA (i.e., 5–10 ng of DNA per reaction). The qPCR_{gyrA} assays were performed using
211 a StepOnePlus™ system (Life Technologies, Thermo Fisher Scientific) with an initial

212 denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s
213 and annealing and extension at 60°C for 1 min, and a final step of incubation at 60°C for 30 s.
214 The results were analyzed at end-point with the StepOne software v2.3 (Life Technologies,
215 Thermo Fisher Scientific).

216

217 **dPCRgyrA validation and study of the mutant:LPP mixtures**

218 As above, validation of the three dPCRgyrA assays for the specific detection of the wild-type
219 *gyrA* QRDR sequence, T83I, D87N, and D87H mutations, was performed by testing the LPP,
220 LPPI1, LPPI4, and LPPI5 strains with the three assays. NTCs were also added to each run.
221 Then the mutant:LPP mixtures (1:1, 1:10, 1:100, 1:500, and 1:1000) were analyzed in
222 duplicate by the dPCRgyrA assays in order to assess the sensitivity of this technique to detect
223 mutated *gyrA* QRDR sequences in the presence of wild-type sequences.

224

225 **dPCRgyrA-T83I assay validation for application to human respiratory samples**

226 Additional specificity testing, evaluation of linearity, and determination of interpretative
227 criteria for a dPCRgyrA result were performed for the dPCRgyrA-T83I assay.

228 Given that respiratory samples may contain DNA from a number of commensal or
229 pathogenic microorganisms other than *L. pneumophila*, as well as human DNA, additional
230 specificity testing for the dPCRgyrA-T83I assay was performed. Therefore, 20 DNA extracts
231 from non-*Legionella* bacterial species commonly isolated from respiratory samples (Table
232 S1) and 19 DNA extracts from *Legionella* species other than *L. pneumophila* (Table S2) were
233 tested. In addition, ten DNA extracts from respiratory samples collected in non-legionellosis
234 patients containing commensal or pathogenic bacteria, but no *Legionella* sp. as determined
235 by negative *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip tests, were tested.

236 The *Legionella* DNA concentration in clinical samples is difficult to determine accurately, and
237 thus it can hardly be standardized for dPCR testing. Therefore, it was necessary to evaluate
238 the linearity of the dPCRgyrA-T83I assay and the minimum DNA concentration required in a
239 respiratory sample to obtain interpretable results. For this purpose, a range of *L.*
240 *pneumophila* genome concentrations (from 1.9 to 19,000 genome copies/ μ L, as determined
241 using the NanoDrop[®] 2000c spectrophotometer) were tested using the dPCRgyrA-T83I assay
242 as well as the *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip assays.

243

244 **dPCRgyrA-T83I assay on respiratory samples**

245 The dPCRgyrA-T83I assay was applied to DNA extracts from respiratory samples collected in
246 legionellosis patients.

247

248 The manufacturers' instructions specify that for any dPCR reaction the maximum precision is
249 obtained at 1.6 copies of target DNA per reaction. Therefore, DNA extracts obtained from
250 bacterial strains tested (except *L. pneumophila* DNA of the concentration range) were
251 diluted in order to reach the recommended concentrations, i.e., from 0.6 to 1.6 target DNA
252 copies per reaction well, corresponding to 200–2000 copies/ μ L in the final dPCR reaction
253 mix. In contrast, the DNA extracts obtained from the clinical samples were tested by dPCR
254 without diluting the samples.

255

256 The dPCRgyrA mixture (15 μ L) contained 7.5 μ L of 2X QuantStudio[®]3D Digital PCR Master
257 Mix (Life Technologies, Thermo Fisher Scientific), 0.375 μ L of 40X TaqMan[®] assay mix
258 (containing 36 μ M of each primer, 8 μ M of wild-type probe, and 8 μ M of mutant probe), and
259 7.125 μ L of diluted DNA. A QuantStudio[®]3D Digital PCR 20K Chip (Life Technologies, Thermo

260 Fisher Scientific) was loaded with 14.5 μ L of the PCR mixture with a QuantStudio[®]3D Digital
261 PCR Chip Loader. dPCR_{gyrA} was performed on a GeneAmp[®] PCR System 9700 (Life
262 Technologies, Thermo Fisher Scientific) with an initial denaturation step at 98°C for 2 min,
263 followed by 40 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 10 s, extension
264 at 60°C for 30 s, and a final extension step at 60°C for 2 min.

265 Chips were read with the QuantStudio[®] 3D Digital PCR Instrument (Life Technologies,
266 Thermo Fisher Scientific) and the results were analyzed with the QuantStudio[®] 3D
267 AnalysisSuite™ software (Life Technologies, Thermo Fisher Scientific).

268

269

270 **RESULTS**

271 **Sanger sequencing of the *gyrA* QRDR of the mutant:LPP mixtures**

272 The mutated *gyrA* QRDR sequences were all detected at a 1:1 mutant:LPP ratio. In contrast,
273 the mutated *gyrA* sequences were only occasionally detected at a 1:10 ratio and were not
274 detected at 1:100 and 1:1000 ratios. Figure 1A shows the sequencing chromatograms of the
275 *gyrA* QRDR sequences for the LPPI5:LPP mixtures. Similar results were obtained for the
276 LPPI1:LPP and LPPI4:LPP mixtures (data not shown). The Sanger sequencing sensitivity was
277 evaluated at a 1:1 ratio (50% of mutated *gyrA* sequences).

278

279 **Validation of the qPCR_{gyrA} assays**

280 The results of the experiments conducted for validation of the three qPCR_{gyrA} assays are
281 shown in the additional data (Figures S1, S2, and S3). The three assays were highly specific
282 for their targets, although weak fluorescence signals were detected with the wild-type and
283 mutant probes in the absence of the corresponding targets for the qPCR_{gyrA}-D87N assay,

284 and with the wild-type probe for the qPCRgyrA-T83I assay. An adjustment of the thresholds
285 allowed us to circumvent these nonspecific reactions. No signal was obtained with the NTCs.

286

287 **Study of the mutant:LPP mixtures using the qPCRgyrA assays**

288 The presence of mutated *gyrA* QRDR sequences was detected at 1:1 and 1:10 mutant:LPP
289 ratios. In contrast, the mixtures at the 1:100 and 1:1000 ratios were reported as wild-type
290 DNA sequences by qPCRgyrA testing. The results for the LPP15:LPP mixtures are shown in
291 Figure 1B. Similar results were obtained for the LPP1:LPP and LPP4:LPP mixtures (data not
292 shown). The qPCRgyrA sensitivity was evaluated at a 1:10 ratio (10% of mutated *gyrA*
293 sequences).

294

295 **Validation of the dPCRgyrA assays**

296 The results of experiments conducted for validation of the dPCRgyrA assays are shown in
297 Table S3. As expected, dPCRgyrA quantified on average 1000 target copies/ μ L. The three
298 assays were highly specific for their targets, although the weak cross-hybridization reactions
299 detected for qPCRgyrA assays were also visible in dPCRgyrA experiments as a slight
300 background noise. For the dPCRgyrA-T83I assay, the background noise was observed only for
301 the mutant probe at 0.43 target copies/ μ L. For the dPCRgyrA-D87N assay, the background
302 noise was observed with mutant and wild-type probes up to 0.69 target copies/ μ L. For the
303 dPCRgyrA-D87H assay, the background noise was also observed with mutant and wild-type
304 probes up to 1.27 target copies/ μ L. This small background noise was circumvented by
305 fixation of thresholds above these nonspecific signals. No signal was observed with the NTCs.

306

307 **Study of the mutant:LPP mixtures using the dPCRgyrA assays**

308 The percentages of mutated *gyrA* QRDR sequences in the mutant:LPP mixtures were
309 calculated using the formula: mutant percentage = [mutant copies/(mutant copies + wild
310 copies)]*100. dPCR_{gyrA} was able to detect the presence of mutated *gyrA* QRDR sequences
311 up to 1:1000 mutant:LPP ratios. Taking into account the previously defined thresholds, the
312 dPCR_{gyrA} sensitivity was evaluated at a 1:1000 ratio (0.1% of mutated *gyrA* sequences) for
313 the dPCR_{gyrA}-T83I and dPCR_{gyrA}-D87N assays, and at a 1:500 ratio (0.2% of mutated *gyrA*
314 sequences) for the dPCR_{gyrA}-D87H assay. The results are shown in Table 3 for all the
315 mixtures and in Figure 1C for the LPP15:LPP mixtures. Similar results were obtained for the
316 LPP11:LPP and LPP14:LPP mixtures (data not shown).

317

318 **Validation of the dPCR_{gyrA}-T83I assay for application to human respiratory samples**

319 dPCR_{gyrA}-T83I assay specificity was evaluated by testing DNA extracts from other bacterial
320 species and *Legionella*-negative respiratory samples. The dPCR_{gyrA}-T83I assay showed no
321 cross-reactivity against any of the non-*Legionella* strains, or against the *Legionella* non-
322 *pneumophila* species, except a weak signal with the *L. parisiensis* strain. No cross-reactivity
323 was observed with the culture-positive but *Legionella*-free respiratory samples.

324 The linearity and interpretative criteria of a dPCR_{gyrA}-T83I result were determined by
325 testing a range of a *L. pneumophila* DNA concentrations (from 1.9 to 19,000 copies/μL as
326 determined with the NanoDrop® 2000c spectrophotometer) using the dPCR_{gyrA}-T83I assay
327 and *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip tests. Experiments conducted to
328 determine the linearity and interpretative criteria of the dPCR_{gyrA}-T83I assay are presented
329 in Table S4. Firstly, for the *L. pneumophila* DNA concentration at 1.9 DNA copies/μL as
330 determined using the NanoDrop spectrophotometer, the dPCR_{gyrA}-T83I assay quantified
331 0.49 copies of *Legionella* DNA/μL. This concentration corresponded to the background noise

332 of the dPCRgyrA-T83I assay (which was evaluated as 0.43 target copies/ μ L). For this
333 concentration, the *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip Cq (quantification
334 cycle) were close to 36 cycles. Consequently, to obtain a dPCRgyrA-T83I signal above the
335 background noise, we decided that only clinical samples with a *Legionella* sp. qPCR16S or *L.*
336 *pneumophila* qPCRmip Cq less than or equal to 35 cycles would be considered appropriate
337 for dPCRgyrA-T83I testing. Moreover, we considered that because the proportion of mutant
338 sequences in a mixed mutant:wild-type population of *L. pneumophila* can be very low, a
339 minimum total DNA concentration would be necessary to obtain a reliable evaluation of the
340 percentage of mutant sequences. Therefore, we decided to set at 4 total DNA copies/ μ L (as
341 determined by dPCRgyrA-T83I) the minimum total DNA concentration required to obtain
342 accurate analysis of the percentage of *L. pneumophila gyrA* mutants.

343

344 **Correlation between dPCRgyrA and NGS results for respiratory samples**

345 The dPCRgyrA-T83I assay was applied to DNA samples obtained from six legionellosis
346 patients previously studied with NGS (11). In this previous work, NGS identified the *in vivo*
347 selection of a T83I mutation in two patients (patients #2 and #4), with proportions of
348 mutated sequences ranging from 1.05% to 94%. dPCRgyrA confirmed the T83I mutation in all
349 the samples collected in these two patients, in proportions ranging from 0.38% to 99.67%.
350 Unfortunately, there was not enough remaining material from the sample collected from
351 patient #2 at day 4 (day after legionellosis diagnosis) to be tested by dPCR. For the four
352 remaining patients, NGS found lower percentages of T83I mutations, ranging from 0.023% to
353 0.19%, which were considered as nonsignificant (11). dPCRgyrA did not find any T83I
354 mutation in these four patients. Table 4 compares the results obtained with the NGS and
355 dPCRgyrA methods.

356

357 **Analysis of respiratory samples from other legionellosis patients**

358 Using the dPCRgyrA-T83I assay, 38 respiratory samples collected from 23 severe legionellosis
359 patients were retrospectively analyzed. These samples corresponded to the previously
360 defined selection criteria for a dPCRgyrA analysis: a positive *Legionella sp.*qPCR16S or *L.*
361 *pneumophila* qPCRmip test with a C_q less than or equal to 35 cycles.

362 dPCRgyrA gave a significant amplification signal (DNA concentration higher than 4 copies/μL,
363 as previously defined) for 34 (89.5%) samples from 20 (87.0%) patients. The results of dPCR
364 for these samples are presented in Table 5. For three samples, mutant sequences in
365 proportions less than the dPCRgyrA-T83I assay sensitivity (0.1%) and/or background noise
366 (0.43 FAM copies/μL) were found; these results were considered uninterpretable. For four
367 samples collected in three patients, low percentages (0.13–1.13%) of T83I mutation were
368 found. These three patients were treated with levofloxacin at the dose of 500mg twice a day
369 from the day of legionellosis diagnosis.

370

371

372 **DISCUSSION**

373 It has commonly been assumed that *L. pneumophila* could not develop FQ resistance.
374 However, Bruin *et al.* recently reported the isolation from a legionellosis patient of a *L.*
375 *pneumophila* strain displaying a ciprofloxacin MIC of 2mg/L, higher than the epidemiological
376 cutoff (1mg/L) he previously defined (10). Although, no FQ resistance breakpoint has been
377 currently established for *Legionella*, this strain was considered as FQ-resistant because
378 associated with a poor response to the antibiotic therapy and a prolonged hospitalization of
379 the patient.

380 More recently, using a NGS approach, Shadoud *et al.* showed the *in vivo* selection of
381 a FQ-resistant subpopulation of this pathogen in two legionellosis patients (11). Indeed, NGS
382 allowed the detection over time of increasing percentages (from 1.05% to 94%) of *gyrA83*
383 mutated sequences in respiratory samples from these legionellosis patients (11). In these
384 two patients, the most probable situation was the presence of a low level of resistant
385 mutants that could have been missed by phenotypic tests, but that dramatically expanded
386 after administration of a FQ, leading to therapeutic failure (28). Defining ciprofloxacin
387 resistance from molecular data is challenging. In this study, we considered as FQ-resistant *L.*
388 *pneumophila* strains harboring *gyrA83* mutations for three main reasons: first these mutants
389 were previously shown to display an eight-fold increase of ciprofloxacin MIC in comparison
390 with their wild-type parental strain (9); second the strain isolated by Bruin *et al.* (10) also
391 harbored a *gyrA83* mutation; and third such a mutation is well known to be associated with
392 high-level FQ resistance in many other Gram-negative bacterial species (12, 29, 30), and
393 occurs at a high rate (12).

394 Consequently, it is of clinical interest to assess the presence of FQ-resistant mutants
395 in a *L. pneumophila* population infecting a given legionellosis patient in order to adapt the
396 antibiotic therapy. However, standard culture-based antibiotic susceptibility testing methods
397 are not appropriate for *L. pneumophila* because of the fastidious nature of this pathogen.
398 Therefore, the development of molecular techniques allowing detection of FQ-resistant
399 subpopulations of *L. pneumophila* in respiratory samples is warranted. Shadoud *et al.*
400 previously demonstrated the usefulness of NGS for the detection, identification, and
401 quantification of specific *gyrA* mutants within a *L. pneumophila* population (11). However,
402 NGS technology remains expensive and data interpretation is complex and time-consuming.
403 This technique is not yet used on a routine basis in bacteriology laboratories. In contrast,

404 dPCR is less expensive, easier to perform, and can also detect and quantify rare mutant DNA
405 sequences in a mixed mutant–wild-type population.

406 In this work, we developed dPCR_{gyrA} assays to detect and quantify FQ-resistant
407 subpopulations of *L. pneumophila* in respiratory samples. As a proof of concept, three
408 dPCR_{gyrA} assays targeting the *gyrA* QRDR mutations previously characterized as responsible
409 for FQ resistance in *L. pneumophila* were developed (8, 9, 11). The dPCR_{gyrA}-T83I assay
410 targeted the 248C>T mutation (T83I substitution), a mutation systematically observed in
411 evolved FQ-resistant clones selected *in vitro* by Almahmoud *et al.* (9). It was also described *in*
412 *vitro* by Jonas *et al.* (8) and *in vivo* in two legionellosis patients by Shadoud *et al.* (11). The
413 248C>T mutation was found alone or combined with other mutations at codon position
414 GyrA87 (9). Thus, we designed two dPCR_{gyrA} assays, the dPCR_{gyrA}-D87N assay and the
415 dPCR_{gyrA}-D87H assay, targeting the mutations 259G>A and 259G>C, respectively.

416 Validation of the three dPCR_{gyrA} assays showed high sensitivity. dPCR_{gyrA} was able
417 to detect *gyrA* mutant sequences mixed with wild-type sequences up to mutant:susceptible
418 allele ratios of 1:1000 (0.1%). In comparison, the Sanger sequencing and qPCR tests were
419 less sensitive because they detected mutant sequences only at 1:1 (50%) and 1:10 (10%)
420 ratios, respectively. Consequently, the Sanger sequencing and qPCR tests may not be able to
421 detect antibiotic-resistant subpopulations of *L. pneumophila* in the early stage of infection in
422 legionellosis patients. The dPCR_{gyrA} sensitivity levels observed in this study are close to
423 those previously reported by Pholwat *et al.* for detection and quantification of isoniazid
424 (*katG315* mutation), rifampicin (*rpoB531* mutation), FQ (*gyrA94,95* mutations), and
425 aminoglycoside (*rrs1401* mutation) resistance in *Mycobacterium tuberculosis* (sensitivity
426 from 1% to 0.1%) (23). In the literature, the maximum sensitivity reported for dPCR is
427 0.001% (one mutant sequence for 100,000 wild-type sequences) (31–33). Such high

428 sensitivity may be obtained either with a higher number of individual reactions (e.g., more
429 than 1,000,000 individual reactions), a different dPCR technique (droplet dPCR *versus* dPCR
430 on a chip), or both. dPCR_{gyrA} could be considered a highly accurate method because the
431 percentages of mutant sequences quantified into the mixtures were very close to the
432 theoretical proportions. dPCR_{gyrA} also produced highly reproducible results since the
433 quantitation of mutant sequences between the two chip replicates were very close.

434 Currently, resistance to FQ in *L. pneumophila* strains infecting humans has only been
435 associated with mutations at codon *gyrA83* (10, 11). Therefore, we applied the dPCR_{gyrA}-
436 T83I assay to respiratory samples collected from legionellosis patients. Additional validation
437 steps for the dPCR_{gyrA}-T83I assay were performed beforehand. Specificity was assessed by
438 testing bacterial species belonging to genera other than *Legionella* and *Legionella* species
439 other than *L. pneumophila*. No cross-amplification was observed, except a slight cross-
440 reactivity with *L. parisiensis*. However, only three cases of human infection with this species
441 have been reported so far in the literature (34–36). We also determined that only
442 respiratory samples with a *Legionella* sp. qPCR16S or *L. pneumophila* qPCR_{mip} C_q of 35
443 cycles or less were considered eligible for a dPCR_{gyrA} analysis because of a sufficient DNA
444 amount. In addition, we considered 4 total DNA copies/μL as a threshold for reliable
445 interpretation of the percentage of *gyrA* mutant sequences.

446 We then applied the dPCR_{gyrA}-T83I assay to clinical samples from legionellosis patients
447 previously studied with NGS (11). dPCR_{gyrA} confirmed the *in vivo* selection of a FQ-resistant
448 mutant in patient #4, with 0.38% T83I mutants at D0 (*versus* 1.05% for NGS) and 99.67% at
449 D5 (*versus* 85% for NGS).

450 We then retrospectively analyzed 38 clinical samples from 23 legionellosis patients using the
451 dPCR_{gyrA}-T83I assay. No sample harbored a majority of *gyrA* T83I mutants. This result was

452 not unexpected because FQ resistance selection in *L. pneumophila* seems a rare
453 phenomenon (11), and most respiratory samples were collected early in the course of
454 legionellosis for the majority of these patients, precluding visualization of *in vivo* selection of
455 FQ-resistant mutants under FQ therapy. However, small amounts of *gyrA83* mutants (0.13–
456 1.68%) were detected in four (10.5%) samples from three (13.0%) patients. Although higher
457 than the cutoffs determined, these low percentages of mutant *gyrA* sequences must be
458 interpreted with caution because they may correspond to false-positive results of the
459 dPCR_{gyrA} assays, as well as for NGS (11). Alternatively, they may correspond to the presence
460 of a low level of FQ-resistant mutants in the *Legionella* population infecting these patients.
461 We only studied clinical samples collected on a routine basis for diagnostic purposes. The
462 possibility of collecting several respiratory samples in legionellosis patients under FQ therapy
463 would make possible to better monitor the kinetics of the percentage of mutants. Indeed, an
464 increase in the percentage of *gyrA* mutants would indicate an *in vivo* emergence of FQ-
465 resistant mutants of *L. pneumophila*, and could be an alert on the possibility of the
466 occurrence of a therapeutic failure. A demonstration was done with patient #4 for which
467 dPCR_{gyrA} detected 0.38% T83I mutant at D0 and 81.65% at D2, suggesting that the low level
468 of mutant strain at D0 likely reflected true FQ-resistant mutants rather than false-positive
469 results. This legionellosis patient might have been infected with a mixed *L. pneumophila*
470 population containing a low level of T83I mutant, or these mutants might have occurred
471 rapidly after infection.

472 The incidence of *in vivo* selection of FQ resistance in *L. pneumophila* is probably
473 underestimated given that only a single FQ-resistant strain of *L. pneumophila* has been
474 isolated so far in the clinical setting (10). dPCR exhibits a sensitivity level as good as that of
475 NGS, but it is more adapted for routine diagnostic use. It is an inexpensive, easy-to-handle,

476 and rapid technology, and data interpretation is simple. Consequently, a good strategy for
477 characterization of antibiotic resistance in fastidious microorganisms would be to use NGS
478 for identification of the gene mutations involved, and then to develop dPCR tests to detect
479 these resistances more easily. Although not found by Shadoud *et al.* (11) using NGS, it would
480 be particularly interesting to look for the *gyrA* 247A>G mutation (T83A) previously described
481 by Bruin *et al.* for the FQ-resistant strain isolated in a respiratory sample (10). Similarly,
482 other mutations in DNA gyrase (e.g., *gyrA83*, *gyrA87*, and *gyrB*) and in topoisomerase IV
483 encoding genes should be searched for using dPCR, although they were only described *in*
484 *vitro* in *L. pneumophila* (7–9). It would then be possible to develop multiplex dPCR assays to
485 detect the most common mutations responsible for FQ resistance. Indeed, multiplex dPCR
486 assays have already been developed, for example, in cancerology for *KRAS* mutation (37).

487

488 In conclusion, we developed dPCR_{gyrA} assays to detect and quantify FQ-resistant
489 subpopulations of *L. pneumophila* harboring mutations in the *gyrA* QRDR. We demonstrated
490 that dPCR is a really powerful tool displaying high sensitivity, reproducibility, and precision
491 and that this technique is suitable for daily practice in bacteriology laboratories. The major
492 clinical interest of this technique was highlighted by confirming the *in vivo* emergence of FQ-
493 resistant mutants of *L. pneumophila* in respiratory samples from a legionellosis patient, as
494 previously characterized using NGS. The dPCR approach would be useful to set up a
495 multicenter study of a larger cohort of patients to further define the prevalence of FQ-
496 resistant mutants in legionellosis patients and evaluate their potential clinical impact. In case
497 *gyrA* mutations in *L. pneumophila* are associated with a worsened prognosis, dPCR would be
498 a useful diagnostic tool to predict the effectiveness of the FQ therapy in legionellosis
499 patients.

500

501

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516

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629

630

631 **FIGURE LEGENDS**

632 **Figure 1. Comparison of Sanger sequencing, qPCRgyrA and dPCRgyrA for mutant sequences**
633 **detection in the LPPI5:LPP mixtures**

634 **(A) Sanger sequencing for the LPPI5:LPP mixtures.** a) 1:1 mixture. b) 1:10 mixture. c) 1:100
635 mixture. d) 1:1000 mixture. In the LPPI5:LPP 1:1 mixture, C/T and G/C heterozygous peaks
636 were detected. In the LPPI5:LPP 1:10 mixture, a weak peak T was associated with the C peak,
637 but the second mutation was not detected. For the 1:100 and 1:1000 mixtures, sequencing
638 chromatograms corresponded to the wild-type sequence.

639 **(B) qPCRgyrA allelic discrimination plot for the LPPI5:LPP mixtures.** qPCRgyrA detected
640 mutant sequences for 1:1 (in blue) and 1:10 mixtures (in green). The 1:100 (in red) and
641 1:1000 (in red) mixtures are qualified as wild-type by qPCRgyrA. Black square is NTC.

642 **(C) dPCRgyrA scatter plot for the LPPI5:LPP mixtures.** a) 1:1 mixture. b) 1:10 mixture. c)
643 1:100 mixture. d) 1:500 mixture. e) 1:1000 mixture. Yellow dots represent wells with no
644 amplification signal. Red dots represent wells with the VIC signal (corresponding to the wild-
645 type *gyrA* QRDR sequences). Blue dots represent wells with the FAM signal (corresponding
646 to mutated *gyrA* QRDR sequences). Green dots represent wells with the FAM+VIC signal.
647 Grey dots represent undetermined reaction well. dPCRgyrA detected mutant sequences up
648 to 1:1000 mixture.

649

650 **Table 1. *L. pneumophila* strains used in this study**

Strain	GyrA ^a	<i>gyrA</i> ^b	FQ susceptibility (Moxifloxacin MIC ^c)	Reference
<i>L. pneumophila</i> serogroup 1 strain Paris, CIP107629T (LPP)	Wild-type	Wild-type	Susceptible (MIC = 0.0625 mg/L)	NRC for <i>Legionella</i> (Lyon, France)
LPP11	LPP + GyrA83 (T83I)	248C>T	Resistant (MIC = 0.5 mg/L)	(9)
LPP14	LPP + GyrA83 (T83I) + GyrA87 (D87N)	248C>T+259G>A	Resistant (MIC = 0.5 mg/L)	(9)
LPP15	LPP + GyrA83 (T83I) + GyrA87*(D87H)	248C>T+259G>C	Resistant (MIC = 0.5 mg/L)	(9)

651

652 ^aChange in the amino acid sequence of the corresponding protein.

653 ^bChange in the nucleotide sequence of the gene.

654 ^cAccording to Almahmoud *et al.* (9).

655

656 **Table 2. Primers and probes for qPCRgyrA and dPCRgyrA experiments**

Assay	Primers and probes	Sequences
Assay T83I	T83I-F	5'-ATGCATCGGTAAATACCATCCTCAC-3'
	T83I-R	5'-GGTTGGGCCATACGAACAATG-3'
	T83I-wt	5'-VIC-AAACAGCT <u>G</u> TATCCCC-3'
	T83I-mt	5'-FAM-AAACAGCT <u>A</u> TATCCCC3'
Assay D87N	D87N-F	5'-GTCATCGGTAAATACCATCCTCACG-3'
	D87N-R	5'-CGATTAAAAGGTAGCGCATGGAAAA-3'
	D87N-wt	5'-VIC-CAATGGTGT <u>C</u> ATAAACA-3'
	D87N-mt	5'-FAM-ACAATGGTGT <u>T</u> ATAAACA-3'
Assay D87H	D87H-F	5'-GTCATCGGTAAATACCATCCTCACG-3'
	D87H-R	5'-CGATTAAAAGGTAGCGCATGGAAAA-3'
	D87H-wt	5'-VIC-CAATGGTGT <u>C</u> ATAAACA-3'
	D87H-mt	5'-FAM-CAATGGTGT <u>T</u> GATAAACA-3'

657

658 wt=wild-type

659 mt = mutant

660 **Table 3. dPCRgyrA results for the mutant:LPP mixtures**

661 The result of each chip is presented, followed by the mean and the standard deviation (SD) of the
 662 duplicate.

Mutant strain (mutation)	Mutant:LPP mixture	Wild copies/ μL (VIC)			Mutant copies/ μL (FAM)			% Mutant Mean (SD)
		Chip 1	Chip 2	Mean (SD)	Chip 1	Chip 2	Mean (SD)	
LPP11 (T83I)	1:1	660.31	482.67	571.49 (125.61)	655.22	651.94	653.58 (2.32)	53.63 (5.41)
	1:10	1601.80	1550.60	1576.20 (36.20)	119.84	129.20	124.52 (6.62)	7.33 (0.52)
	1:100	1393.90	1385.60	1389.75 (5.87)	9.88	6.87	8.38 (2.13)	0.60 (0.15)
	1:500	1534.70	1369.30	1452.00 (116.96)	4.83	2.31	3.57 (1.78)	0.24 (0.10)
	1:1000	1249.90	1325.00	1287.45 (53.10)	0.83	1.27	1.05 (0.31)	0.08 (0.02)
LPP14 (D87N)	1:1	434.52	432.43	433.48 (1.48)	365.80	353.98	359.89 (8.36)	45.36 (0.49)
	1:10	1075.90	1091.20	1083.55 (10.82)	81.92	80.50	81.21 (1.00)	6.97 (0.14)
	1:100	1353.00	1303.70	1328.35 (34.86)	9.88	8.43	9.16 (1.03)	0.68 (0.06)
	1:500	1412.70	1450.50	1431.60 (26.73)	1.83	2.66	2.25 (0.59)	0.16 (0.04)
	1:1000	1462.10	1418.80	1440.45 (30.62)	0.94	0.87	0.91 (0.05)	0.06 (0.002)
LPP15 (D87H)	1:1	633.68	650.10	641.89 (11.61)	510.10	533.82	521.96 (16.77)	44.84 (0.35)
	1:10	1306.80	1299.30	1303.05 (5.30)	98.79	95.77	97.28 (2.14)	6.95 (0.12)
	1:100	1099.90	1284.50	1192.20 (130.53)	7.89	8.45	8.17 (0.40)	0.68 (0.04)
	1:500	1189.40	1112.90	1151.15 (54.09)	2.30	1.82	2.06 (0.34)	0.18 (0.02)
	1:1000	1311.20	1282.70	1296.95 (20.15)	1.33	0.87	1.10 (0.33)	0.08 (0.02)

663

664 **Table 4. Concordance between dPCRgyrA and NGS on clinical samples**

Patient	Sampling day ^a	Fluoroquinolone treatment	Percentage of T83I mutant with NGS ^b	Percentage of T83I mutant with dPCRgyrA-T83I assay
#2	D0	no	2.9%	1.68%
	D4	yes	94%	NP
#4	D0	yes	1.05%	0.38%
	D2	yes	NP	81.65%
	D3	yes	75%	NP
	D5	yes	85%	99.67%
Negative for NGS (n = 4)	D0	no	0.023% to 0.19%	0%

665

666 ^aSampling day is given according to the day of legionellosis diagnosis.

667 ^bAs determined by Shadoud *et al.* (11)

668 NP = Not performed.

669

670 **Table 5. dPCRgyrA-T83I assay results for clinical samples with a significant amplification**
 671 **signal**

Patients (n=20)	Respiratory samples (n=34)		Sampling day ^a	Percentage of C248T mutant as determined by dPCRgyrA-T83I assay
	FQ	No FQ		
<i>n</i> = 17	19	7		0% or uninterpretable ^b
P1	1		D0	0%
	1		D10	0%
	1		D15	0%
	1		D22	0.13%
P2	1		D1	0%
	1		D2	0.43%
	1		D5	1.13%
P3	1		D5	0.83%

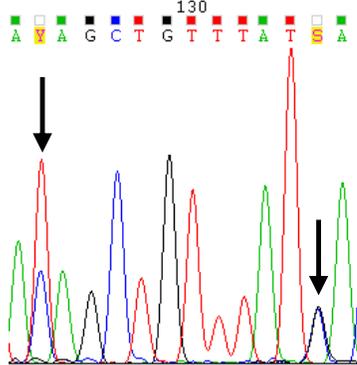
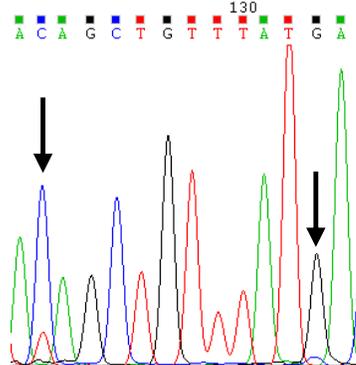
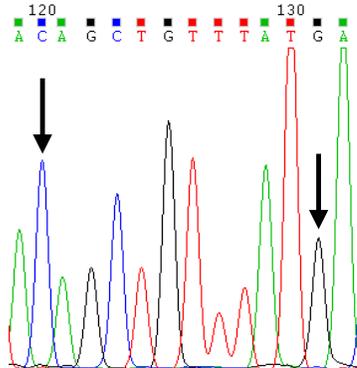
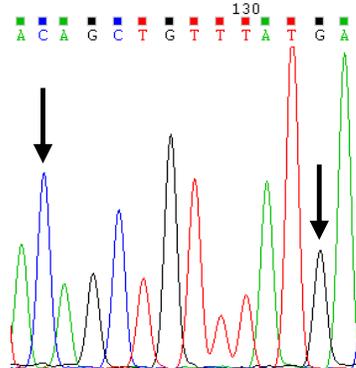
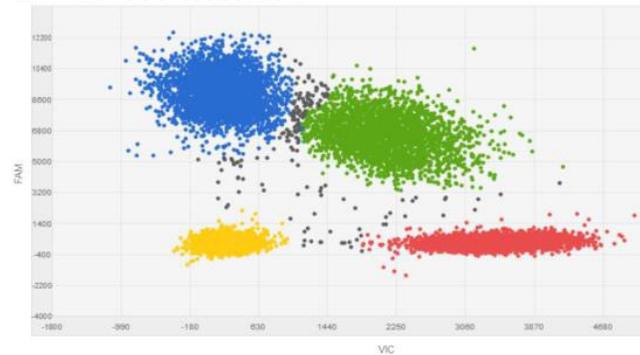
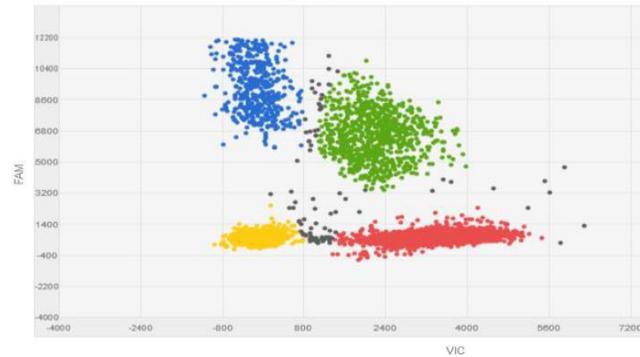
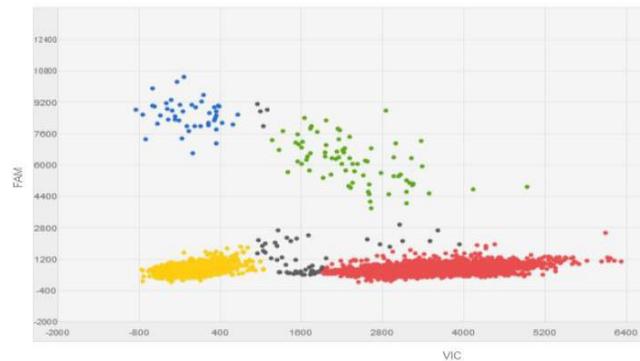
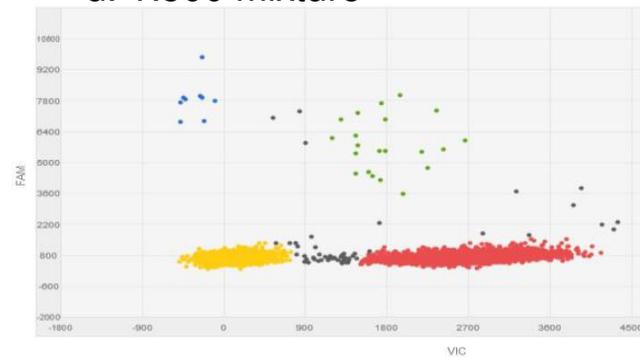
672

673 ^a Sampling day is given according to the day of legionellosis diagnosis (D0).

674 ^b Three samples with mutant sequences in proportions inferior to dPCRgyrA-T83I assay sensitivity

675 (0.1%) and/or background noise (0.43 FAM copies/ μ L).

676

A a. 1:1 mixture**b. 1:10 mixture****c. 1:100 mixture****d. 1:1000 mixture****B a. 1:1 mixture****b. 1:10 mixture****c. 1:100 mixture****d. 1:500 mixture****e. 1:1000 mixture**