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ANALYTICAL PROCEDURES

Digital PCR for Detection and Quantification of Fluoroquinolone Resistance in *Legionella pneumophila*

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ABSTRACT The emergence of fluoroquinolone (FQ)-resistant mutants of *Legionella pneumophila* in infected humans was previously reported using a next-generation DNA sequencing (NGS) approach. This finding could explain part of the therapeutic failures observed in legionellosis patients treated with these antibiotics. The aim of this study was to develop digital PCR (dPCR) assays allowing rapid and accurate detection and quantification of these resistant mutants in respiratory samples, especially when the proportion of mutants in a wild-type background is low. We designed three dPCRgyrA assays to detect and differentiate the wild-type and one of the three *gyrA* mutations previously described as associated with FQ resistance in *L. pneumophila*: at positions 248C→T (T83I), 259G→A (D87N), and 259G→C (D87H). To assess the performance of these assays, mixtures of FQ-resistant and -susceptible strains of *L. pneumophila* were analyzed, and the results were compared with those obtained with Sanger DNA sequencing and real-time quantitative PCR (qPCR) technologies. The dPCRgyrA assays were able to detect mutated *gyrA* sequences in the presence of wild-type sequences at up to 1:1,000 resistant/susceptible allele ratios. By comparison, Sanger DNA sequencing and qPCR were less sensitive, allowing the detection of *gyrA* mutants at up to 1:1 and 1:10 ratios, respectively. When testing 38 respiratory samples from 23 legionellosis patients (69.6% treated with an FQ), dPCRgyrA detected small amounts of *gyrA* mutants in four (10.5%) samples from three (13.0%) patients. These results demonstrate that dPCR is a highly sensitive alternative to quantify FQ resistance in *L. pneumophila*, and it could be used in clinical practice to detect patients that could be at higher risk of therapeutic failure.

KEYWORDS digital PCR, antibiotic resistance, fluoroquinolones, *gyrA*, *Legionella pneumophila*

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Legionella pneumophila, a Gram-negative, facultative, intracellular bacterium, is the causative agent of legionellosis, a severe pneumonia associated with mortality rates ranging from 5% to 25% (1). The diagnosis of this disease mainly relies on a urinary antigen test and culture and PCR testing of respiratory samples. The first-line drugs for the treatment of legionellosis are the macrolides and the fluoroquinolones (FQ) (2). *In vitro* antibiotic susceptibility testing of *Legionella* species strains is currently not recommended on a routine basis, especially because no standardized method is available from the CLSI (Clinical and Laboratory Standards Institute) or the EUCAST (European Committee on Antimicrobial Susceptibility Testing). However, therapeutic failures and

relapses are still reported (3–5), FQ-resistant mutants of *L. pneumophila* have been readily selected *in vitro* (6–9), and FQ-resistant mutants have also recently been found in legionellosis patients (10, 11). Indeed, Shadoud et al. recently characterized the *in vivo* selection of such FQ-resistant mutants of *L. pneumophila* in two legionellosis patients treated with these antibiotics (11).

Bacterial resistance to quinolones of clinical relevance is most often related to mutations in the genes encoding type II topoisomerases, also called DNA gyrase (encoded by *gyrA* and *gyrB* genes) and type IV topoisomerase (encoded by *parC* and *parE* genes) (12). Resistance-conferring mutations occur within specific regions of these genes, called the quinolone resistance-determining regions (QRDRs). The *in vitro* selection of *L. pneumophila* strains with high-level resistance to quinolones has been mainly related to mutations affecting codons 83 and 87 of the *gyrA* QRDR (7–9), although mutations in *gyrB* and *parC* were also described (9). Only mutations at codon position 83 of the *gyrA* gene [*gyrA*(*83*)] have been reported so far *in vivo* (10, 11). Using a next-generation DNA sequencing (NGS) approach, Shadoud et al. previously demonstrated that an *L. pneumophila* mutant population harboring the 248C→T (T83I) mutation was rapidly selected *in vivo* in two legionellosis patients treated with an FQ, representing from 1.05% of the total *L. pneumophila* lung population at the time of diagnosis up to 94% after a few days of FQ treatment (11). It can be hypothesized that a higher proportion of mutated *gyrA* sequences at the time of legionellosis diagnosis may represent a higher risk of FQ treatment failure. Also, the selection of the *gyrA*-mutated population by the administration of an FQ may occur faster when the proportion of mutants over wild-type strains is high at the time of treatment onset.

The prevalence of the *in vivo* selection of *L. pneumophila* FQ-resistant mutants in legionellosis patients treated with these antibiotics remains to be established. It cannot be determined by traditional phenotypic methods for several reasons. (i) *Legionella* cultures are negative in most legionellosis patients because of the fastidious nature of this bacterium. (ii) *L. pneumophila* isolation is even more difficult to obtain in patients under antibiotic treatment, even when patients experience treatment failure or relapses. (iii) A mixture of antibiotic-susceptible and a few resistant strains of *L. pneumophila* would certainly result in selecting the most susceptible population for antibiotic susceptibility testing. (iv) It is necessary to evaluate the respective proportion of such susceptible and resistant populations to evaluate the potential risk of treatment failure.

Digital PCR (dPCR) is an innovative PCR technology developed in the 2000s based on the partition of the sample to be analyzed into thousands to millions of individual PCRs. A major advantage of dPCR over real-time quantitative PCR (qPCR) is an increased sensitivity for the detection of a few mutants mixed with wild-type DNA sequences (13). dPCR has wide clinical applications in the oncology field (14) and ongoing applications in noninvasive prenatal diagnosis (15, 16) and organ transplant rejection monitoring (17). For infectious diseases, dPCR technology has been mainly used for the diagnosis of viral diseases (13), the quantification of bacteria in clinical samples (18–21), the detection and quantification of antibiotic resistance genes (22, 23), and the detection and quantification of bacterial toxins (24).

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

RESULTS

Sanger sequencing of the *gyrA* QRDR of the mutant-LPP mixtures. To assess the performance of Sanger sequencing, mixtures of FQ-resistant and -susceptible strains of *L. pneumophila* were analyzed. The mutated *gyrA* QRDR sequences were all detected at a 1:1 mutant/LPP (*L. pneumophila* serogroup 1 wild-type strain) ratio. By contrast, the mutated *gyrA* sequences were only occasionally detected at a 1:10 ratio and were not detected at 1:100 and 1:1,000 ratios. Figure 1A shows the sequencing chromatograms of the *gyrA* QRDR sequences for the D87H LPP mutant (LPPI5)-LPP mixtures. Similar results were obtained for the T83I LPP mutant (LPPI1)-LPP and the D87N LPP mutant

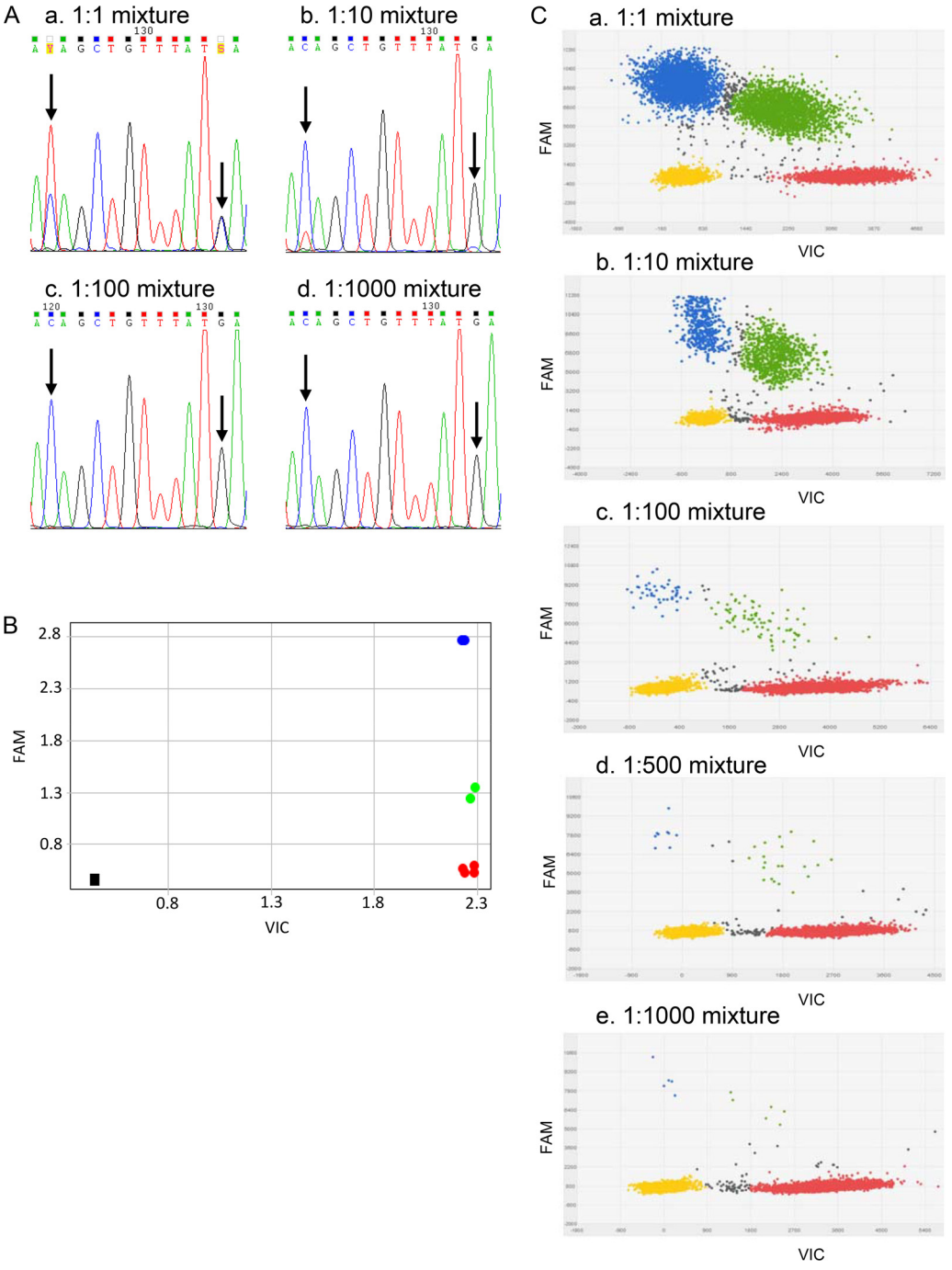

FIG 1 Comparison of Sanger sequencing, qPCRgyrA, and dPCRgyrA for mutant sequence detection in the LPPI5-LPP mixtures. (A) Sanger sequencing for the LPPI5-LPP mixtures: a, 1:1 mixture; b, 1:10 mixture; c, 1:100 mixture; d, 1:1,000 mixture. In the LPPI5-LPP 1:1 mixture, C/T and G/C heterozygous peaks were detected. In the LPPI5-LPP 1:10 mixture, a weak T peak was associated with the C peak, but the second mutation was not detected. For the 1:100 and 1:1,000 mixtures, sequencing chromatograms corresponded to the wild-type sequence. (B) qPCRgyrA allelic discrimination plot for the LPPI5-LPP mixtures. qPCRgyrA detected mutant sequences for 1:1 (in blue) and 1:10 mixtures (in green). The 1:100 (in red) and 1:1,000 (in red) mixtures were qualified as wild-type by qPCRgyrA. Black square is NTC. (C) dPCRgyrA scatter plots for the LPPI5:LPP mixtures: a, 1:1 mixture; b, 1:10 mixture; c, 1:100 mixture; d, 1:500 mixture; e, 1:1,000 mixture. Yellow dots represent wells with no amplification signal. Red dots represent wells with the VIC signal (corresponding to the wild-type *gyrA* QRDR sequences). Blue dots represent wells with the FAM signal (corresponding to mutated *gyrA* QRDR sequences). Green dots represent wells with the FAM plus VIC signal. Gray dots represent undetermined reaction wells. dPCRgyrA detected mutant sequences in up to 1:1,000 mixture.

(LPPI4)-LPP mixtures (data not shown). The Sanger sequencing sensitivity was evaluated at a 1:1 ratio (50% of mutated *gyrA* sequences).

Validation of the qPCRgyrA assays. The results of the experiments conducted for the validation of the three qPCRgyrA assays are shown in the supplemental material (Fig. S1, S2, and S3). The three assays were highly specific for their targets, although weak fluorescence signals were detected with the wild-type and mutant probes in the absence of the corresponding targets for the qPCRgyrA-D87N assay and with the wild-type probe for the qPCRgyrA-T83I assay. An adjustment of the thresholds allowed us to circumvent these nonspecific reactions. No signal was obtained with the no-template controls (NTCs).

Study of the mutant-LPP mixtures using the qPCRgyrA assays. To assess the performance of qPCRgyrA, mixtures of FQ-resistant and -susceptible strains of *L. pneumophila* were analyzed. The presence of mutated *gyrA* QRDR sequences was detected at 1:1 and 1:10 mutant/LPP ratios. By contrast, the mixtures at the 1:100 and 1:1,000 ratios were reported as wild-type DNA sequences by qPCRgyrA testing. The results for the LPPI5-LPP mixtures are shown in Fig. 1B. Similar results were obtained for the LPPI1-LPP and LPPI4-LPP mixtures (data not shown). The qPCRgyrA sensitivity was evaluated at a 1:10 ratio (10% of mutated *gyrA* sequences).

Validation of the dPCRgyrA assays. The results of the experiments conducted for the validation of the dPCRgyrA assays are shown in Table S3. As expected, dPCRgyrA quantified on average 1,000 target copies/μl. The three assays were highly specific for their targets, although the weak cross-hybridization reactions detected for qPCRgyrA assays were also visible in dPCRgyrA experiments as slight background noise. For the dPCRgyrA-T83I assay, the background noise was observed only for the mutant probe at 0.43 target copies/μl. For the dPCRgyrA-D87N assay, the background noise was observed with mutant and wild-type probes up to 0.69 target copies/μl. For the dPCRgyrA-D87H assay, the background noise was also observed with mutant and wild-type probes up to 1.27 target copies/μl. This small background noise was circumvented by the fixation of thresholds above these nonspecific signals. No signal was observed with the NTCs.

Study of the mutant-LPP mixtures using the dPCRgyrA assays. To assess the performance of dPCRgyrA, mixtures of FQ-resistant and -susceptible strains of *L. pneumophila* were analyzed. The percentages of mutated *gyrA* QRDR sequences in the mutant-LPP mixtures were calculated using the formula: mutant percentage = [mutant copies/(mutant copies + wild copies)] × 100. dPCRgyrA was able to detect the presence of mutated *gyrA* QRDR sequences at up to 1:1,000 mutant/LPP ratios. Taking into account the previously defined thresholds, the dPCRgyrA sensitivity was evaluated at a 1:1,000 ratio (0.1% of mutated *gyrA* sequences) for the dPCRgyrA-T83I and dPCRgyrA-D87N assays, and at a 1:500 ratio (0.2% of mutated *gyrA* sequences) for the dPCRgyrA-D87H assay. The results are shown in Table 1 for all the mixtures and in Fig. 1C for the LPPI5-LPP mixtures. Similar results were obtained for the LPPI1-LPP and LPPI4-LPP mixtures (data not shown).

Validation of the dPCRgyrA-T83I assay for application to human respiratory samples. dPCRgyrA-T83I assay specificity was evaluated by testing DNA extracts from other bacterial species and *Legionella*-negative respiratory samples. The dPCRgyrA-T83I assay showed no cross-reactivity against any of the non-*Legionella* strains or against the non-*pneumophila Legionella* species, except a weak signal with the *L. parisiensis* strain. No cross-reactivity was observed with the culture-positive but *Legionella*-free respiratory samples.

The linearity and interpretative criteria of a dPCRgyrA-T83I result were determined by testing a range of an *L. pneumophila* DNA concentrations (from 1.9 to 19,000 copies/μl as determined with the NanoDrop 2000c spectrophotometer) using the dPCRgyrA-T83I assay and *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip tests. The results from experiments conducted to determine the linearity and interpretative criteria of the dPCRgyrA-T83I assay are presented in Table S4. First, for the *L. pneumo-*

TABLE 1 dPCRgyrA results for the mutant-LPP mixtures[a]

Mutant strain (mutation)	Mutant/LPP ratio	No. of copies/μl						Mean % of mutants (SD)
		Wild type (VIC)			Mutant (FAM)			
		Chip 1	Chip 2	Mean (SD)	Chip 1	Chip 2	Mean (SD)	
LPPI1 (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	1,601.80	1,550.60	1,576.20 (36.20)	119.84	129.20	124.52 (6.62)	7.33 (0.52)
	1:100	1,393.90	1,385.60	1,389.75 (5.87)	9.88	6.87	8.38 (2.13)	0.60 (0.15)
	1:500	1,534.70	1,369.30	1,452.00 (116.96)	4.83	2.31	3.57 (1.78)	0.24 (0.10)
	1:1,000	1,249.90	1,325.00	1,287.45 (53.10)	0.83	1.27	1.05 (0.31)	0.08 (0.02)
LPPI4 (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	1,075.90	1,091.20	1,083.55 (10.82)	81.92	80.50	81.21 (1.00)	6.97 (0.14)
	1:100	1,353.00	1,303.70	1,328.35 (34.86)	9.88	8.43	9.16 (1.03)	0.68 (0.06)
	1:500	1,412.70	1,450.50	1,431.60 (26.73)	1.83	2.66	2.25 (0.59)	0.16 (0.04)
	1:1,000	1,462.10	1,418.80	1,440.45 (30.62)	0.94	0.87	0.91 (0.05)	0.06 (0.002)
LPPI5 (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	1,306.80	1,299.30	1,303.05 (5.30)	98.79	95.77	97.28 (2.14)	6.95 (0.12)
	1:100	1,099.90	1,284.50	1,192.20 (130.53)	7.89	8.45	8.17 (0.40)	0.68 (0.04)
	1:500	1,189.40	1,112.90	1,151.15 (54.09)	2.30	1.82	2.06 (0.34)	0.18 (0.02)
	1:1,000	1,311.20	1,282.70	1,296.95 (20.15)	1.33	0.87	1.10 (0.33)	0.08 (0.02)

[a]The results of each chip are presented, followed by the means and the standard deviations (SDs) from the duplicates.

phila DNA concentration at 1.9 DNA copies/μl as determined using the NanoDrop spectrophotometer, the dPCRgyrA-T83I assay quantified 0.49 copies of *Legionella* DNA/μl. This concentration corresponded to the background noise of the dPCRgyrA-T83I assay (which was evaluated as 0.43 target copies/μl). For this concentration, the *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip quantification cycle (C_q) values were nearly 36 cycles. Consequently, to obtain a dPCRgyrA-T83I signal above the background noise, we decided that only clinical samples with a *Legionella* sp. qPCR16S or *L. pneumophila* qPCRmip C_q of less than or equal to 35 cycles would be considered appropriate for dPCRgyrA-T83I testing. Moreover, we considered that, because the proportion of mutant sequences in a mixed mutant–wild-type population of *L. pneumophila* can be very low, a minimum total DNA concentration would be necessary to obtain a reliable evaluation of the percentage of mutant sequences. Therefore, we decided to set the minimum total DNA concentration required at 4 total DNA copies/μl (as determined by dPCRgyrA-T83I) to obtain an accurate analysis of the percentage of *L. pneumophila gyrA* mutants.

Correlation between dPCRgyrA and NGS results for respiratory samples. The dPCRgyrA-T83I assay was applied to DNA samples obtained from six legionellosis patients previously studied with NGS (11). In this previous work, NGS identified the *in vivo* selection of a T83I mutation in two patients (patients #2 and #4 [patient designations are from reference 11]), with proportions of mutated sequences ranging from 1.05% to 94%. dPCRgyrA confirmed the T83I mutation in all the samples collected from these two patients, in proportions ranging from 0.38% to 99.67%. Unfortunately, there was not a sufficient amount of material remaining from the sample collected from patient #2 at day 4 (the day after legionellosis diagnosis) to be tested by dPCR. For the four remaining patients, NGS found lower percentages of T83I mutations, ranging from 0.023% to 0.19%, which were considered nonsignificant (11). dPCRgyrA did not find any T83I mutations in these four patients. Table 2 compares the results obtained with the NGS and dPCRgyrA methods.

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

dPCRgyrA gave a significant amplification signal (DNA concentration higher than 4 copies/μl, as previously defined) for 34 (89.5%) samples from 20 (87.0%) patients. The

TABLE 2 Concordance between dPCRgyrA and NGS assays for clinical samples

Patient no.	Sampling day[a]	FQ treatment	% of T83I mutants from:	
			NGS[b]	dPCRgyrA-T83I
#2	0	No	2.9	1.68
	4	Yes	94	NP[c]
#4	0	Yes	1.05	0.38
	2	Yes	NP	81.65
	3	Yes	75	NP
	5	Yes	85	99.67
Negative for NGS (n = 4)	0	No	0.023 to 0.19	0

[a]Sampling day is given according to the day of legionellosis diagnosis.
[b]As determined by Shadoud et al. (11).
[c]NP, not performed.

results of the dPCR for these samples are presented in Table 3. For three samples, mutant sequences in proportions less than those of the dPCRgyrA-T83I assay sensitivity (0.1%) and/or background noise (0.43 FAM copies/μl) were found; these results were considered uninterpretable. For four samples collected in three patients, low percentages (0.13 to 1.13%) of T83I mutations were found. These three patients were treated with levofloxacin at the dose of 500 mg twice a day from the day of legionellosis diagnosis.

DISCUSSION

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

More recently, using an NGS approach, Shadoud et al. showed the *in vivo* selection of an FQ-resistant subpopulation of this pathogen in two legionellosis patients (11). Indeed, NGS allowed the detection over time of increasing percentages (from 1.05% to 94%) of *gyrA*(*83*) mutations in respiratory samples from these legionellosis patients (11). In these two patients, the most probable situation was the presence of a low level of resistant mutants that could have been missed by phenotypic tests but that dramati-

TABLE 3 dPCRgyrA-T83I assay results for clinical samples with a significant amplification signal

Patient no. (n = 20)	No. of respiratory samples (n = 34)		Sampling day[a]	% of T83I mutants
	FQ	No FQ		
4–20	19	7		0 or uninterpretable[b]
1	1		0	0
	1		10	0
	1		15	0
	1		22	0.13
2	1		1	0
	1		2	0.43
	1		5	1.13
3	1		5	0.83

[a]Sampling day is given according to the day of legionellosis diagnosis.
[b]Three samples with mutant sequences in proportions inferior to dPCRgyrA-T83I assay sensitivity (0.1%) and/or background noise (0.43 FAM copies/μl).

cally expanded after the administration of an FQ, leading to therapeutic failure (25). Defining ciprofloxacin resistance from molecular data is challenging. In this study, we considered FQ-resistant *L. pneumophila* strains harboring *gyrA*(*83*) mutations for three main reasons. First, these mutants were previously shown to display an 8-fold increase in ciprofloxacin MIC in comparison with their wild-type parental strain (9). Second, the strain isolated by Bruin et al. (10) also harbored a *gyrA*(*83*) mutation. Third, such a mutation is well known to be associated with high-level FQ resistance in many other Gram-negative bacterial species (12, 26, 27) and occurs at a high rate (12).

Consequently, it is of clinical interest to assess the presence of FQ-resistant mutants in an *L. pneumophila* population infecting a given legionellosis patient to adapt the antibiotic therapy. However, standard culture-based antibiotic susceptibility testing methods are not appropriate for *L. pneumophila* because of the fastidious nature of this pathogen. Therefore, the development of molecular techniques allowing the detection of FQ-resistant subpopulations of *L. pneumophila* in respiratory samples is warranted. Shadoud et al. previously demonstrated the usefulness of NGS for the detection, identification, and quantification of specific *gyrA* mutants within an *L. pneumophila* population (11). However, NGS technology remains expensive, and data interpretation is complex and time-consuming. This technique is not yet used on a routine basis in bacteriology laboratories. By contrast, dPCR is less expensive, easier to perform, and can also detect and quantify rare mutant DNA sequences in a mixed mutant–wild-type population.

In this work, we developed dPCRgyrA assays to detect and quantify FQ-resistant subpopulations of *L. pneumophila* in respiratory samples. As a proof of concept, three dPCRgyrA assays targeting the *gyrA* QRDR mutations previously characterized as responsible for FQ resistance in *L. pneumophila* were developed (8, 9, 11). The dPCRgyrA-T83I assay targeted the 248C→T mutation (T83I substitution), a mutation systematically observed in evolved FQ-resistant clones selected *in vitro* by Almahmoud et al. (9). It was also described *in vitro* by Jonas et al. (8) and *in vivo* in two legionellosis patients by Shadoud et al. (11). The 248C→T mutation was found alone or combined with other mutations in *gyrA* at codon position 87 (9). Thus, we designed two dPCRgyrA assays, the dPCRgyrA-D87N assay and the dPCRgyrA-D87H assay, targeting the mutations 259G→A and 259G→C, respectively.

A validation of the three dPCRgyrA assays showed high sensitivities. dPCRgyrA was able to detect *gyrA* mutant sequences mixed with wild-type sequences up to mutant/susceptible allele ratios of 1:1,000 (0.1%). By comparison, the Sanger sequencing and qPCR tests were less sensitive, because they detected mutant sequences only at 1:1 (50%) and 1:10 (10%) ratios, respectively. Consequently, the Sanger sequencing and qPCR tests may not be able to detect antibiotic-resistant subpopulations of *L. pneumophila* in the early stage of infection in legionellosis patients. The dPCRgyrA sensitivity levels observed in this study are similar to those previously reported by Pholwat et al. for the detection and quantification of isoniazid [*katG*(*315*) mutation], rifampin [*rpoB*(*531*) mutation], FQ [*gyrA*(*94,95*) mutations], and aminoglycoside [*rrs*(*1401*) mutation] resistance in *Mycobacterium tuberculosis* (sensitivity from 1% to 0.1%) (23). In the literature, the maximum sensitivity reported for dPCR is 0.001% (one mutant sequence for 100,000 wild-type sequences) (28–30). Such a high sensitivity may be obtained with a higher number of individual reactions (e.g., more than 1,000,000 individual reactions) and/or a different dPCR technique (droplet dPCR versus dPCR on a chip). dPCRgyrA could be considered a highly accurate method, because the percentages of mutant sequences quantified in the mixtures were very similar to the theoretical proportions. dPCRgyrA also produced highly reproducible results, since the quantitations of mutant sequences between the two chip replicates were very similar.

Currently, the resistance to FQ in *L. pneumophila* strains infecting humans has only been associated with *gyrA*(*83*) mutations (10, 11). Therefore, we applied the dPCRgyrA-T83I assay to respiratory samples collected from legionellosis patients. Additional validation steps for the dPCRgyrA-T83I assay were performed beforehand. Specificity was assessed by testing bacterial species belonging to genera other than *Legionella* and

Legionella species other than *L. pneumophila*. No cross-amplification was observed, except a slight cross-reactivity with *L. parisiensis.* However, only three cases of human infection with this species have been reported so far in the literature (31–33). We also determined that only respiratory samples with *Legionella* sp. qPCR16S or *L. pneumophila* qPCRmip *Cq* values of 35 cycles or less were considered eligible for a dPCRgyrA analysis because of a sufficient DNA amount. In addition, we considered 4 total DNA copies/μl as a threshold for reliable interpretation of the percentage of *gyrA* mutant sequences.

We then applied the dPCRgyrA-T83I assay to clinical samples from legionellosis patients previously studied with NGS (11). dPCRgyrA confirmed the *in vivo* selection of an FQ-resistant mutant in patient #4, with 0.38% T83I mutants at day 0 (D0) (versus 1.05% for NGS) and 99.67% at D5 (versus 85% for NGS).

We then retrospectively analyzed 38 clinical samples from 23 legionellosis patients using the dPCRgyrA-T83I assay. No sample harbored a majority of *gyrA* T83I mutants. This result was not unexpected, because FQ resistance selection in *L. pneumophila* seems a rare phenomenon (11) and most respiratory samples were collected early in the course of legionellosis for the majority of these patients, precluding visualization of *in vivo* selection of FQ-resistant mutants under FQ therapy. However, small amounts of *gyrA*(*83*) mutants (0.13 to 1.68%) were detected in four (10.5%) samples from three (13.0%) patients. Although higher than the cutoffs determined, these low percentages of mutant *gyrA* sequences must be interpreted with caution, because they may correspond to false-positive results of the dPCRgyrA assays, as well as for NGS (11). Alternatively, they may correspond to the presence of a low level of FQ-resistant mutants in the *Legionella* population infecting these patients. We only studied clinical samples collected on a routine basis for diagnostic purposes. The possibility of collecting several respiratory samples in legionellosis patients under FQ therapy would make it possible to better monitor the kinetics of the percentage of mutants. Indeed, an increase in the percentage of *gyrA* mutants would indicate an *in vivo* emergence of FQ-resistant mutants of *L. pneumophila*, and could be an alert to the possibility of the occurrence of a therapeutic failure. A demonstration was conducted with patient #4, for which dPCRgyrA detected 0.38% T83I mutants at D0 and 81.65% at D2, suggesting that the low level of mutant strain at D0 likely reflected true FQ-resistant mutants rather than false-positive results. This legionellosis patient might have been infected with a mixed *L. pneumophila* population containing a low level of T83I mutants, or these mutations might have occurred rapidly after infection.

The incidence of *in vivo* selection of FQ resistance in *L. pneumophila* is probably underestimated given that only a single FQ-resistant strain of *L. pneumophila* has been isolated so far in the clinical setting (10). dPCR exhibits a sensitivity level as good as that of NGS, but it is more adapted for routine diagnostic use. It is an inexpensive, easy-to-handle, rapid technology, and data interpretation is simple. Consequently, a good strategy for the characterization of antibiotic resistance in fastidious microorganisms would be to use NGS for the identification of the gene mutations involved, and then to develop dPCR tests to detect these resistances more easily. Although not found by Shadoud et al. (11) using NGS, it would be particularly interesting to look for the *gyrA* 247A→G mutation (T83A) previously described by Bruin et al. for the FQ-resistant strain isolated in a respiratory sample (10). Similarly, other mutations in DNA gyrase [e.g., *gyrA*(*83*), *gyrA*(*87*), and *gyrB*] and in topoisomerase IV-encoding genes should be searched for using dPCR, although they were only described *in vitro* in *L. pneumophila* (7–9). It would then be possible to develop multiplex dPCR assays to detect the most common mutations responsible for FQ resistance. Indeed, multiplex dPCR assays have already been developed, for example, in cancerology for *KRAS* mutations (34).

In conclusion, we developed dPCRgyrA assays to detect and quantify FQ-resistant subpopulations of *L. pneumophila* harboring mutations in the *gyrA* QRDR. We demonstrated that dPCR is a really powerful tool displaying high sensitivity, reproducibility, and precision and that this technique is suitable for daily practice in bacteriology laboratories. The major clinical interest of this technique was highlighted by confirming

TABLE 4 *L. pneumophila* strains used in this study

Strain	GyrA mutation description		FQ susceptibility (moxifloxacin MIC [mg/liter][b])	Reference or source
	Amino acid	Nucleotide		
LPP[a]	Wild type	Wild type	Susceptible (0.0625)	NRC for *Legionella* (Lyon, France)
LPPI1	GyrA83 (T83I)	248C→T	Resistant (0.5)	9
LPPI4	GyrA83 (T83I) plus GyrA87 (D87N)	248C→T plus 259G→A	Resistant (0.5)	9
LPPI5	GyrA83 (T83I) plus GyrA87 (D87H)	248C→T plus 259G→C	Resistant (0.5)	9

[a]LPP, *L. pneumophila* serogroup 1 strain Paris CIP107629T.
[b]According to Almahmoud et al. (9).

the *in vivo* emergence of FQ-resistant mutants of *L. pneumophila* in respiratory samples from a legionellosis patient, as previously characterized using NGS. The dPCR approach would be useful to set up a multicenter study of a larger cohort of patients to further define the prevalence of FQ-resistant mutants in legionellosis patients and evaluate their potential clinical impact. In case *gyrA* mutations in *L. pneumophila* are associated with a worsened prognosis, dPCR would be a useful diagnostic tool to predict the effectiveness of the FQ therapy in legionellosis patients.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *L. pneumophila* strains used in this study are listed in Table 4. The *L. pneumophila* sg 1 strain Paris CIP107629T (referred to as LPP) was used as an FQ-susceptible control. The FQ-resistant mutants LPPI1, LPPI4, and LPPI5 were derived from LPP by homologous recombination introducing the three main *gyrA* mutations previously selected and characterized *in vitro*, including the mutations 248C→T (T83I) for LPPI1, 248C→T and 259G→A (D87N) for LPPI4, and 248C→T and 259G→C (D87H) for LPPI5 (9).

For specificity purposes, 20 reference or clinical strains belonging to bacterial species other than *Legionella* sp. (see Table S1 in the supplemental material) and 19 strains of *Legionella* species other than *L. pneumophila*, provided by the French National Reference Center (NRC) for *Legionella* (see Table S2), were used.

Legionella strains were grown on BMPA medium (Oxoid, Cambridge, UK), corresponding to buffered charcoal yeast extract medium plus antimicrobial agents, at 37°C in a 5% CO_2-enriched atmosphere for 3 days. FQ-resistant *Legionella* strains were grown in a biosafety level 3 laboratory. The other bacterial species were grown on Columbia agar supplemented with 5% sheep blood or on chocolate agar supplemented with 5% PolyVitex (bioMérieux, Marcy l'Etoile, France) at 37°C in a 5% CO_2-enriched atmosphere for 24 h.

Patients and clinical samples. First, eight respiratory samples from six patients, previously studied with NGS (11), were analyzed by dPCR. These samples came from a previous single-center cohort of legionellosis patients admitted to an intensive care unit at Grenoble University Hospital (France) between 2006 and 2011. Samples from two patients that were previously detected as positive for T83I FQ-resistant mutants of *L. pneumophila* using NGS served as positive controls (11). NGS-negative samples from four patients served as negative controls (11). These controls were used for the validation of the dPCRgyrA assay developed targeting the T83I mutation.

Then, 38 respiratory samples from 23 patients with unknown NGS status were retrospectively evaluated for the presence of the T83I mutation. These patients were admitted to an intensive care unit at Grenoble University Hospital or Lyon University Hospital (France) between 2011 and 2016. They included 20 male patients and three female patients, with a mean age of 56 years (range, 23 to 83 years). During hospitalization, lower respiratory tract (LRT) (i.e., bronchoalveolar lavage fluid, bronchial aspirations, tracheal aspirations, or sputum) and urine samples were collected for diagnostic purposes. LRT samples were tested using routine bacterial cultures, *Legionella* cultures, and *Legionella* qPCR tests (qPCR16S for all *Legionella* species and qPCRmip for *L. pneumophila*, as previously described [35]) and then frozen at −80°C until used for this study. The 23 patients were selected because of positive *Legionella* qPCR tests (qPCR16S and qPCRmip) (see Results), while 21 patients also had positive urinary antigen tests for *L. pneumophila* sg 1, and 18 patients had positive cultures allowing isolation of *L. pneumophila* sg 1. All these patients were considered to be infected with an *L. pneumophila* strain because of the positive qPCRmip tests on LRT samples. Sixteen (69.6%) of the 23 patients were treated with an FQ.

In addition, to validate the clinical use of the dPCRgyrA targeting the T83I mutation, 10 respiratory samples from patients suffering from pneumonia other than legionellosis were used. These samples were negative by *Legionella* qPCR tests (qPCR16S and qPCRmip), while commensal and pathogenic bacterial species were detected by routine bacterial cultures.

The clinical sample collections of Grenoble and Lyon University Hospitals were declared to the French Ministry of Education and Research (numbers DC-2008-677 and DC-2008-176, respectively), and an ethics committee (CPP Sud-Est V) gave the authorization to use them for research purposes. Patient information

TABLE 5 Primers and probes for qPCRgyrA and dPCRgyrA experiments

Assay target	Primer or probe[a]	Sequence (5′→3′)[b]
T83I	T83I-F	ATGTCATCGGTAAATACCATCCTCAC
	T83I-R	GGTTGGGCCATACGAACAATG
	T83I-wt	VIC-AAACAGCTGTATCCCC
	T83I-mt	FAM-AAACAGCTATATCCCC
D87N	D87N-F	GTCATCGGTAAATACCATCCTCACG
	D87N-R	CGATTAAAAGGTAGCGCATGGAAAA
	D87N-wt	VIC-CAATGGTGTCATAAACA
	D87N-mt	FAM-ACAATGGTGTTATAAACA
D87H	D87H-F	GTCATCGGTAAATACCATCCTCACG
	D87H-R	CGATTAAAAGGTAGCGCATGGAAAA
	D87H-wt	VIC-CAATGGTGTCATAAACA
	D87H-mt	FAM-CAATGGTGTGATAAACA

[a]wt, wild-type; mt, mutant.
[b]Targeted nucleotides are underlined.

was provided through a hospital medical booklet, and only nonopposition on the part of the patients was needed.

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

DNA concentrations of LPP, LPPI1, LPPI4, and LPPI5 were assessed using Qubit fluorometric quantitation (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's recommendations and adjusted to 1 ng/μl. Then mixtures of DNA from LPPI1, LPPI4, or LPPI5 with DNA from the wild-type LPP strain were prepared at different mutant/LPP ratios, namely, 1:1, 1:10, 1:100, 1:500, and 1:1,000.

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

For the respiratory samples, DNA extraction was performed using either the EZ1 DNA tissues kit (Qiagen) or the MagNA Pure compact nucleic acid isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions.

Sanger sequencing of the *L. pneumophila gyrA* QRDR. The mutant-LPP mixtures were tested using a PCR-sequencing approach to assess the sensitivity of this technique for detecting mutated *gyrA* QRDR sequences in the presence of wild-type sequences.

A 383-bp fragment of the *gyrA* QRDR was amplified using the primers LgyrALubS7 (5′-GCGATGAG TGTCATTGTAG-3′) and LgyrALubASR2bis (5′-GTTTCATCATAGTTAGGGCTAAAATCAAC-3′) as previously described (11). Sequencing was performed using the same primers and the dye terminator method with the DTCS quick start kit (Beckman Coulter, Villepinte, France) on a CEQ 8000 sequencer (Beckman Coulter) as previously described (9). Amplicons were sequenced from both ends. The sequences obtained were visualized and analyzed with Chromas Lite (version 2.1.1; Technelysium) and aligned with Seaview4 (36).

Primers and probes for qPCRgyrA and dPCRgyrA. We followed the MIQE guidelines for nomenclature and qPCR protocol development (37). For qPCRgyrA and dPCRgyrA experiments, primers and probes targeting the wild-type and mutated sequences of the *gyrA* QRDR were designed using the custom TaqMan assay design tools (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA). Three assays were developed to detect the wild-type *gyrA* sequence and the *gyrA* QRDR mutations previously selected *in vitro* in *L. pneumophila* (9), including 248C→T (T83I), 259G→A (D87N), and 259G→C (D87H).

Primers and probes were provided by Life Technologies. Each assay mix contained PCR primers and two hydrolysis MGB probes, including a VIC-labeled probe complementary to the wild-type *gyrA* QRDR sequence and a FAM-labeled probe complementary to one of the three mutated *gyrA* QRDR sequences (Table 5).

qPCRgyrA validation and study of the mutant-LPP mixtures. Validation of the three qPCRgyrA assays for the specific detection of the wild-type and mutated (T83I, D87N, or D87H) *gyrA* QRDR sequences was performed by testing the LPP, LPPI1, LPPI4, and LPPI5 strains with the three assays. Each PCR run included NTCs to validate the absence of false-positive results caused by exogenous DNA contamination, autohydrolysis of the probes, or nonspecific hybridization.

Then, the mutant-LPP mixtures (at 1:1, 1:10, 1:100, and 1:1,000 ratios) were tested using the qPCRgyrA assays, in duplicate, to assess the sensitivity of this technique to detect mutated *gyrA* QRDR sequences in the presence of wild-type sequences.

Each of the PCR mixtures (25 μl) contained 12.5 μl of TaqMan genotyping master mix (Life Technologies, Thermo Fisher Scientific), 0.625 μl of 40× TaqMan assay mix (36 μM each primer, 8 μM wild-type probe, and 8 μM mutant probe), and 11.875 μl of diluted DNA (i.e., 5 to 10 ng of DNA per reaction). The qPCRgyrA assays were performed using a StepOnePlus system (Life Technologies, Thermo Fisher Scientific) with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of

denaturation at 92°C for 15 s and annealing and extension at 60°C for 1 min, and a final step of incubation at 60°C for 30 s. The results were analyzed at the endpoint with the StepOne software v2.3 (Life Technologies, Thermo Fisher Scientific).

dPCRgyrA validation and study of the mutant-LPP mixtures. As above, validation of the three dPCRgyrA assays for the specific detection of the wild-type *gyrA* QRDR sequence, T83I, D87N, and D87H mutations was performed by testing the LPP, LPPI1, LPPI4, and LPPI5 strains with the three assays. NTCs were also added to each run.

Then the mutant-LPP mixtures (at 1:1, 1:10, 1:100, 1:500, and 1:1,000 ratios) were analyzed in duplicates by the dPCRgyrA assays to assess the sensitivity of this technique to detect mutated *gyrA* QRDR sequences in the presence of wild-type sequences.

dPCRgyrA-T83I assay validation for application to human respiratory samples. Additional specificity testing, evaluation of linearity, and determination of interpretative criteria for a dPCRgyrA result were performed for the dPCRgyrA-T83I assay.

Given that respiratory samples may contain DNA from a number of commensal or pathogenic microorganisms other than *L. pneumophila*, as well as human DNA, additional specificity testing for the dPCRgyrA-T83I assay was performed. Therefore, 20 DNA extracts from non-*Legionella* bacterial species commonly isolated from respiratory samples (Table S1) and 19 DNA extracts from *Legionella* species other than *L. pneumophila* (Table S2) were tested. In addition, 10 DNA extracts from respiratory samples collected in nonlegionellosis patients containing commensal or pathogenic bacteria, but no *Legionella* sp. as determined by negative *Legionella* sp. qPCR16S and *L. pneumophila* qPCRmip tests, were tested.

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

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

The manufacturers' instructions specify that for any dPCR reaction, the maximum precision is obtained at 1.6 copies of target DNA per reaction. Therefore, DNA extracts obtained from bacterial strains tested (except *L. pneumophila* DNA of the concentration range) were diluted to reach the recommended concentrations, i.e., from 0.6 to 1.6 target DNA copies per reaction well, corresponding to 200 to 2,000 copies/μl in the final dPCR reaction mix. By contrast, the DNA extracts obtained from the clinical samples were tested by dPCR without diluting the samples.

Each of the dPCRgyrA mixtures (15 μl) contained 7.5 μl of 2× QuantStudio 3D digital PCR master mix (Life Technologies, Thermo Fisher Scientific), 0.375 μl of 40× TaqMan assay mix (containing 36 μM each primer, 8 μM wild-type probe, and 8 μM mutant probe), and 7.125 μl of diluted DNA. A QuantStudio 3D digital PCR 20K chip (Life Technologies, Thermo Fisher Scientific) was loaded with 14.5 μl of the PCR mixtures with a QuantStudio 3D digital PCR chip loader. dPCRgyrA was performed on a GeneAmp PCR system 9700 (Life Technologies, Thermo Fisher Scientific) with an initial denaturation step at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 10 s, and extension at 60°C for 30 s, and a final extension step at 60°C for 2 min.

Chips were read with the QuantStudio 3D digital PCR instrument (Life Technologies, Thermo Fisher Scientific), and the results were analyzed with the QuantStudio 3D AnalysisSuite software (Life Technologies, Thermo Fisher Scientific).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00628-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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Conception of the work was performed by S.B. and A.H. Experiments were performed by A.H. Analysis of the data was performed by S.B., A.H., and M.B. Clinical information about the patients was provided by S.J., L.B., and C.S. The manuscript was written by A.H., S.B., and M.M. All authors reviewed the manuscript before submission.

All the authors have no conflict of interest to declare.

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