Hidden Selection of Bacterial Resistance to Fluoroquinolones In Vivo: The Case of Legionella pneumophila and Humans

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1. Introduction

Acquired resistance to antibiotics in human bacterial pathogens has been extensively described and is associated with a high public health and economic burden (Martínez, 2008; Skippington and Ragan, 2011; Pendleton et al., 2013). In striking contrast, resistant mutants have been rarely, if ever, detected in humans for many bacterial species with strict or facultative intracellular lifestyles, including Rickettsia spp. (agent of rickettsioses), Coxiella burnetii (Q fever), Ehrlichia and Anaplasma species (ehrlichiosis and anaplasmosis), Chlamydia psittaci (psittacosis), Legionella pneumophila (legionellosis), Francisella tularensis (tularemia), and Brucella melitensis (brucellosis). However, mutants have been easily selected for when these pathogens were grown in vitro (Rolain and Raoult, 2005; Rouli et al., 2012; Maurin et al., 2001; Binet and Maurelli, 2005; Almahmoud et al., 2009; Sutera et al., 2014; Ravanel et al., 2009). These occasional human pathogens thrive in environmental or animal reservoirs, or both, and are not carried by, nor transmitted, among humans.

L. pneumophila is a Gram-negative facultative intracellular bacterium that multiplies in aquatic environments, especially in free-living protozoa and biofilms (Newton et al., 2010; Carratalá and García-Vidal, 2010). Legionellosis is an acute pneumonia caused by inhalation of aerosols containing Legionella spp., with L. pneumophila serogroup (sg) 1 accounting for more than 90% of human infections (Newton et al., 2010; Carratalá and García-Vidal, 2010). The macrolides (especially azithromycin) and the fluoroquinolones are first-line antibiotics for...
the treatment of legionellosis patients (Chidiac et al., 2012; Jespersen et al., 2010; Yu et al., 2009; Pedro-Botet and Yu, 2009; Burdet et al., 2014). However, treatment failures and relapses may occur despite administration of an appropriate antibiotic therapy (Chidiac et al., 2012; Jespersen et al., 2010; Yu et al., 2009; Pedro-Botet and Yu, 2009; Burdet et al., 2014). The mortality rate of legionellosis is 10% on average, but can be as high as 30% in immunocompromised patients (Chidiac et al., 2012; Jespersen et al., 2010; Yu et al., 2009). Acquired resistance to the macrolides and fluoroquinolones in L. pneumophila has been readily selected for in vitro (Almahmoud et al., 2009; Jonas et al., 2003; Nielsen et al., 2000). In striking contrast, no macrolide resistance (Jespersen et al., 2010; Yu et al., 2009; Pedro-Botet and Yu, 2009; Burdet et al., 2014), and only a single case of fluoroquinolone resistance has been reported in clinical strains (Bruin et al., 2014). In the latter case however, only one patient was studied with only microbiological approaches and, importantly the dynamics of fluoroquinolone-resistance during infection and hospitalization was not investigated. Here, we identified and further characterized, using molecular and genomic approaches, the in vivo selection of fluoroquinolone-resistance mutations in L. pneumophila during a large survey of 82 unrelated legionellosis cases.

2. Methods

2.1. Patients and Clinical Samples

We studied a cohort of 82 unrelated legionellosis patients including 64 males and 18 females (mean age: 55 years, age range: 27–97 years) that were admitted to the Grenoble University Hospital between 2006 and 2011. For diagnostic purposes, 139 lower respiratory tract samples were collected from these patients during hospitalization. After homogenization of the samples (appendix p 3), routine bacteriological tests (bacterial cultures. Legionella detection by culture and PCR) were performed. The homogenized samples were then frozen at −80 °C. Based on the criteria from the European Centre for Disease Prevention and Control (ECDC, 2012), legionellosis diagnosis was based on the combination of clinical and radiologic findings of pneumonia, and a positive urinary antigen test for L. pneumophila sg1 (Binax Now Legionella, Binax, Portland, ME, USA). In addition, we tried to further confirm diagnosis by isolation of a L. pneumophila strain from respiratory samples and detection of L. pneumophila DNA in the same samples using a previously described quantitative real-time PCR assay (qPCRmip assay) (Maurin et al., 2010).

2.2. Bacterial Strains and Media

We used four reference strains of L. pneumophila sg1 (Paris, Philadelphia, Lens, and Lorraine) (appendix p 5), and 27 strains belonging to 18 Legionella species other than L. pneumophila (appendix p 6), that are all fluoroquinolone-susceptible. We used three fluoroquinolone-resistant mutant strains (LPPP1, LPP4, and LPPI5) that we previously selected for in vitro (Almahmoud et al., 2009). All three have mutations in the gyrA Quinolone Resistance Determinant Region (QRDR). For specificity purposes, we used 33 non-Legionella bacterial strains that were previously isolated from human respiratory samples (appendix p 7). Legionella strains were grown on buffered charcoal yeast extract agar plates (BCYE, Oxoid, Basingstoke, UK) at 37 °C for 48–72 h. The other bacteria were grown on Columbia or chocolate blood agar supplemented with 5% Polyvitex (bioMérieux, Marcy l’Etoile, France).

2.3. Antibiotic Susceptibility Testing

Ciprofloxacin MICs were determined using the E-test method (bioMérieux). A bacterial inoculum of 1 McFarland turbidity standard was prepared in brain–heart infusion (BHI) broth (bioMérieux), and plated on BCYE plates carrying an E-test strip. Plates were incubated for 3–5 days at 35 °C in a 5%-CO2 atmosphere. The MIC is given on the strip at the inhibitory zone edge intersection (Rhomberg and Jones, 1994). We tested the 15 clinical strains of L. pneumophila sg1 sampled from the first respiratory samples of 15 patients (see Results section). The L. pneumophila Paris, Philadelphia, Lens, and Lorraine strains served as ciprofloxacin-susceptible controls, and the LPPP1, LPPI4, and LPPI5 strains as ciprofloxacin-resistant controls.

2.4. Quantitative Real-time PCR Assay Targeting the gyrA QRDR Mutations (qPCRGyrARLP)

In gram-negative bacteria, fluoroquinolone resistance is most often related to mutations occurring in the quinolone resistance determining region of gyrA, the gene encoding the sub-unit A of DNA gyrase, especially at the hot-spot codon positions gyrA83 and gyrA87 (Escherichia coli numbering system) (Davies and Davies, 2010; Komp Lindgren et al., 2003; Jacoby, 2005). We previously demonstrated that gyrA83 mutations could occur in vitro in L. pneumophila, and was associated with increased resistance to fluoroquinolones (Almahmoud et al., 2009). Thus, we developed a real-time PCR assay (qPCRgyrARLP) targeting mutations in the gyrA QRDR, especially at codon positions gyrA83 and gyrA87.

PCR and real-time PCR experiments were performed with genomic DNA from the relevant strains or from lower respiratory tract samples, after DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations (appendix p 3). We first PCR-amplified and sequenced a 383-bp region of the gyrA QRDR of 31 Legionella strains (appendix pp 3 and 6), as previously described (Almahmoud et al., 2009). We analyzed these sequences to design L. pneumophila-specific primers and probes to elaborate the qPCRGyrARLP assay. The sensitivity and specificity of the qPCRGyrARLP assay were first checked using a set of 67 strains, including 7 fluoroquinolone-susceptible or –resistant L. pneumophila control strains (appendix p 5), 27 strains belonging to other Legionella species (appendix p 6), and 33 strains belonging to other genera (appendix p 7).

2.5. Next-generation Deep-sequencing of the Legionella gyrA QRDR

We used high-throughput pyrosequencing (appendix p 4, table 1) to confirm and quantify the presence of gyrA QRDR mutated alleles in 11 human respiratory samples, including eight qPCRGyrARLP-positive samples from four patients (called patients #1 to #4), and three qPCRGyrARLP-negative samples from three patients selected randomly (called control patients).

2.6. Patient and Other Consents

We used respiratory samples and bacterial strains collected in the context of routine care of patients hospitalized at Grenoble University Hospital for severe legionellosis. We have declared this collection of human samples to the French Ministry of Education and Research (DC-2008-677), and we have the authorization to use it for research purposes from our ethics committee (CPP Sud-Est V). In accordance with the French law, patients’ information for this type of research is done through a hospital medical booklet and only a non-opposition of the patients is needed.

2.7. Role of the Funding Source

The study was sponsored by the French Centre National de la Recherche Scientifique (CNRS), Université Grenoble Alpes (UGA); and by the Direction de la Recherche Clinique et de l’Innovation (DRCI) Centre Hospitalier Universitaire de Grenoble.

The funding sources had no role in the study design; in data collection, analysis and interpretation; in writing of the manuscript or in the decision to submit it for publication. We did not receive any funds to write this article from a pharmaceutical company or other agencies.
3. Results

A positive urinary antigen test for *L. pneumophila* sg1 (Binax Now Legionella, Binax, Portland, ME, USA) was found for all 82 investigated pneumonic patients. Legionellosis diagnosis was further confirmed in 15 of the 82 patients by isolation of a *L. pneumophila* strain from the first collected respiratory sample. These 15 clinical strains of *L. pneumophila* displayed ciprofloxacin MICs ranging from 0.25 to 0.38 mg/L, whereas MICs were 0.38 mg/L for the susceptible *L. pneumophila* reference strains (Paris, Philadelphia, Lens, and Lorraine), 1.5 mg/L for the LPPH1 mutant with a single gyrA mutation, and 2 mg/L for the LPPH4 and LPPH5 mutants with two gyrA mutations (appendix p 5). We found no mutation in the gyrA QRDR of the 15 clinical strains. Legionellosis was also confirmed in 61 (74.4%) of the 82 legionellosis patients (including 13 patients with a positive *L. pneumophila* culture), by direct detection of *L. pneumophila* DNA in respiratory samples (112 of the 139 collected samples, 80.6%), using the qPCRmip assay (Maurin et al., 2010). The *L. pneumophila* DNA load in these samples varied from $5.8 \times 10^2$ to $6.8 \times 10^7$ genome units (GU)/mL of sample. For 19 patients, *L. pneumophila* culture and PCR assays were negative.

After standardization of the qPCRgyrAlp assay, a strong amplification signal of the gyrA QRDR was detected for all tested *L. pneumophila* strains. Furthermore, this assay could distinguish Legionella versus non-Legionella strains, *L. pneumophila* versus other Legionella species, fluoroquinolone-susceptible versus resistant mutants of *L. pneumophila*, and single versus double mutants (Fig. 1; appendix p 3; appendix p 5). When applied to the 139 respiratory samples collected from the 82 legionellosis patients, an amplification signal corresponding to the gyrA QRDR was detected in 114 samples (82%) from 62 patients (75.6%), including 63 and 11 samples collected from patients under and without fluoroquinolone therapy, respectively (Table 1). The qPCRgyrAlp assay was positive for 108 (96.4%) of the 112 samples showing a positive qPCRmip assay, but only for 7 of the other 27 qPCRmip-negative samples (25.9%, $p < 0.01$), suggesting that the DNA load in clinical samples was critical for detection of either the *mip* or gyrA gene. All qPCRgyrAlp-positive respiratory samples (104) collected from 58 patients gave identical results, their melting curve profiles suggesting a reference gyrA QRDR sequence (mean melting peak ± SD, 59.34 ± 0.41 °C) when compared to control strains (Fig. 1, Table 1). For 4 patients however (#1 to #4), we detected melting peaks of 56.35 ± 0.42 °C (three replicates per patient) for 6 of 11 respiratory samples, suggesting the presence of gyrA QRDR mutations.

To confirm the presence of gyrA83 or gyrA87 mutations in qPCRgyrALp-positive samples, we tried to PCR-amplify and sequence a 383-bp fragment of the *L. pneumophila* gyrA QRDR directly from eight respiratory samples of patients #1 to #4 (Table 1). Using next-generation deep sequencing (NGS) technology, we also determined the frequency of these gyrA QRDR mutations by testing the same respiratory samples from patients #1 to #4 and the qPCRgyrAlp-negative samples from three control patients (Table 1). We detected no PCR product for samples from patient #1 collected one and three days (D1 and D3) after the day of legionellosis diagnosis (D0), which may be related to low *L. pneumophila* DNA load ($< 10^3$ GU/mL sample, as determined by the qPCRmip assay). For patient #3, a PCR product was obtained from the D0 sample, in which we identified five mutations none of which affected the gyrA codons 83 or 87. Two silent mutations C219G and C264T (codons 73 and 88, respectively) occurred in the gyrA QRDR but outside the location of the sensor probe of the qPCRgyrAlp assay, and thus had no influence on the melting peak. The three others (T278C, A281G, and T285C at codons 93, 94, and 95, respectively) were localized in the region complementary to the anchor probe thereby explaining the observed melting peak decrease. Using the NGS method, we confirmed the lack of gyrA mutation at codons 83 and 87 for sample D0 of patient #3, and the five changes at codons 73 (C219 G), 88 (C264T), 93 (T278C), 94 (A281G), and 95 (T285C), at a frequency of 85.7% of 17,060 reads. More importantly, we detected low levels (0.023% to 0.19% of a total of 3267 to 14,716 reads) of C248T gyrA83 mutations (T83I GyrA change) in samples collected from control legionellosis patients before fluoroquinolone administration, as well as in the D3 sample (0.037% of 5258 reads) from patient #1. These later results suggested that, in some legionellosis patients, the gyrA83 mutants of *L. pneumophila* were present in the lower respiratory airways before administration of any antibiotic therapy.

For patients #2, a reference gyrA QRDR was detected in the D0 sample, but a C248T mutation resulting in the T83I GyrA change was found in the analyzed D4 sample, suggesting in vivo selection of fluoroquinolone-resistant mutants. Using NGS, we confirmed the presence of the C248T gyrA83 mutation in respiratory samples. It occurred at a frequency of 2.9% of 7205 reads at D0, increasing to 94% of 16,794 reads in the D4 sample after 4 days of treatment with levofloxacin and azithromycin (Table 1). The high proportion of gyrA83 mutants in sample D4 of patients #2 confirmed the melting curve profiles of the qPCRgyrAlp assay. Patient #2 recovered from legionellosis under levofloxacin and azithromycin therapy, after 31 days at ICU and a total of 79 days at hospital (Fig. 2).

For patients #4, a reference gyrA QRDR was detected in the D0 sample, but a C248T mutation resulting in the T83I GyrA change was found in both D3 and D5 samples, suggesting in vivo selection of fluoroquinolone-resistant mutants. Using NGS, the C248T gyrA83 mutation was detected in 1.05% of 8063 reads at D0, increasing to 7% of 15,824 reads and 85% of 16,050 reads at D3 and D5, respectively, while the patient was receiving ciprofloxacin and erythromycin in the
intensive care unit (ICU) (Table 1). The high proportion of gyrA83 mutants in samples D3 and D5 confirmed the melting curve profiles of the qPCRgyrAlp assay. For this patient, we investigated the dynamics of both the DNA load and gyrA83 allele by combining the qPCRmip and NGS approaches. While the global DNA load remained stable in respiratory samples from D0 to D5, the gyrA83 mutated alleles progressively replaced the wild-type alleles, suggesting an increase in the fluoroquinolone-resistant population of L. pneumophila, parallel to the reduction of the susceptible population (Fig. 3). Patient #4 was admitted to a hematologic ward for severe pneumonia and aplasia, and transferred after 11 days at ICU because of health worsening despite a 32-day hospital stay including 7 days at ICU. He was cured under ciprofloxacin and erythromycin therapy, with a fluoroquinolone-resistant strain of L. pneumophila isolated in a legionellosis patient treated with ciprofloxacin (Bruin et al., 2014). Very recently however, a fluoroquinolone-resistant strain of L. pneumophila was isolated in a legionellosis patient treated with ciprofloxacin (Bruin et al., 2014). This unique and preliminary observation raises the question of the dynamics and origin of fluoroquinolone resistance in L. pneumophila. It has been speculated that the environmental release of antibiotics may promote the emergence of antibiotic-resistant bacterial lineages (Davies and Davies, 2010), but this has never been demonstrated for Legionella species. Here, using a combination of molecular and genomic approaches, we investigated the infection dynamics of two legionellosis patients in a cohort of 82 during their hospitalization. We demonstrated

4. Discussion

It has commonly been assumed that L. pneumophila could not develop fluoroquinolone resistance (Jespersen et al., 2010; Yu et al., 2009; Pedro-Botet and Yu, 2009; Burdet et al., 2014). Very recently however, a fluoroquinolone-resistant strain of L. pneumophila was isolated in a legionellosis patient treated with ciprofloxacin (Bruin et al., 2014). This unique and preliminary observation raises the question of the dynamics and origin of fluoroquinolone resistance in L. pneumophila. It has been speculated that the environmental release of antibiotics may promote the emergence of antibiotic-resistant bacterial lineages (Davies and Davies, 2010), but this has never been demonstrated for Legionella species. Here, using a combination of molecular and genomic approaches, we investigated the infection dynamics of two legionellosis patients in a cohort of 82 during their hospitalization. We demonstrated

![Fig. 2. Clinical history and gyrA83 mutational dynamics for patients #2. The results of qPCRmip, qPCRgyrAlp and NGS assays are shown, together with the antibiotic therapy for patients #2 over his hospitalization stay. The number of days for relevant steps during hospitalization is given inside circles according to D0 which corresponds to the day of legionellosis diagnosis as determined by a positive urinary antigen test (UAT+). qPCR-mip and qPCRgyr ALp-wt indicate the proportion of the reads corresponding to the wild-type alleles and to the mutant-type alleles in respiratory samples, respectively. Results of the qPCRgyrAlp assay are indicated as “neg” (no amplification of the gyrA QRDR), “wt” and “mut” (a positive PCR assay with a reference and mutant-type melting peak, respectively). The proportion of the reads corresponding to the T83I gyrA83 mutation, determined by next-generation sequencing, is shown (NGS-mut.). Small arrows indicate the periods of antibiotic therapy. Lev: levofloxacin; Cp: ciprofloxacin; Azi: azithromycin; Ery: erythromycin; Amc: amoxicillin-clavulanate; Ca: cefazidime; Tc: teicoplanin.](image-url)
that infection occurred with a fluoroquinolone-susceptible population of \textit{L. pneumophila} that evolved in vivo toward a high load of fluoroquinolone-resistant \textit{gyrA}83 mutants after antibiotic therapy.

We designed a qPCRgyrALp assay that allowed us to detect a signal for the \textit{gyrA} QRDR in 114 samples from 62 of the 82 patients, including 63 samples collected in patients under fluoroquinolone treatment. We found \textit{gyrA}83 mutations in two patients (3.1%, patients #2 and #4). We did not seek mutations in either other \textit{gyrA} regions or other topoisomerase-encoding genes owing to the small amount of biological material, but such mutations may occur in vitro (Almahmoud et al., 2009). Thus, we have likely underestimated the incidence of in vivo selection of fluoroquinolone resistance in \textit{L. pneumophila}. We investigated the presence of a correlation between a positive qPCRgyrALp assay and higher legionellosis severity in the 82 patients of the cohort. There was a trend for the 62 patients with a positive qPCRgyrALp assay to be more frequently admitted to the ICU, and these patients more frequently received a combination of a macrolide and a fluoroquinolone than those with a negative qPCRgyrALp assay. These findings may reflect higher \textit{L. pneumophila} bacteria load in their respiratory samples, which we previously correlated with higher severity of the disease using the qPCRmip assay.

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Clinical history, and dynamics of \textit{L. pneumophila} DNA load and \textit{gyrA}83 mutations in respiratory samples from patient #4. (a) The results of qPCRmip, qPCRgyrALp and NGS assays are shown, together with the antibiotic therapy for patients #4 over his hospitalization stay (see legend of Fig. 2). (b) Dynamics of \textit{L. pneumophila} DNA load, expressed as log GU/mL clinical sample, in respiratory samples of patient #4 over time after admission in ICU. The total, reference, and mutant \textit{gyrA} QRDR DNA loads are shown by circles, triangles and squares, respectively. Data were extracted from the qPCRmip assay and NGS experiments. (c) Relative proportion of the reference (triangles) and mutant (squares) \textit{gyrA} QRDR allele in respiratory samples of patient #4 over time after admission in ICU. Data were extracted from the NGS experiments.}
\end{figure}

\begin{table}
\centering
\begin{tabular}{llllll}
\hline
 & qPCR gyrALp & qPCRmip & Legionellosis treatment & Hospital stay (mean days +/− SD) & ICU admission (n) & Death (n) \\
\hline
Positive & 62 & 58 & 4 & 22 & 20 & 18.1 +/− 16.1 & 21 & 5 \\
Negative & 20 & 3 & 17 & 11 & 0 & 14.3 +/− 11.7 & 2 & 0 \\
\hline
\end{tabular}
\caption{Analyses of potential correlations between parameters (qPCRgyrALp assay, qPCRmip assay, antibiotic treatment, duration of stay at hospital and ICU, death) for all 82 patients of the cohort.}
\end{table}

All numbers correspond to patients. 

| Parameter | Positive | Negative | FQ only (n) | FQ + MC (n) | p | \
|-----------|---------|----------|------------|-----------|---| 
| FQ: fluoroquinolone; MC: macrolide. 
| \(p < 0.001\) | 0.0004 | 0.23 | 0.07 | 0.32 | 

\(p\) Fisher exact tests were performed. 

\(\chi^2\) tests with Yates correction were performed. 

\(t\) tests were performed.
assay (Maurin et al., 2010). Indeed, there was a significant correlation between results of the qPCRgyrALP and qPCRmp1 assays.

A gyra83 mutation leading to the GyrA T83I substitution is likely to be clinically significant for two main reasons: first we previously demonstrated that it produced at least a four-fold increase of ciprofloxacin MIC in L. pneumophila Paris (Almahmoud et al., 2009), and second it is known to be associated with high-level fluorquinolone resistance in other Gram-negative bacteria (Komp Lindgren et al., 2003; Jacoby, 2005). We detected a rapid increase of gyra83 mutant alleles under fluoroquinolone treatment to 94% at D4 for patient #2 and 75% and 85% at D3 and D5, respectively, for patient #4. For this latter patient, a fluorquinolone-susceptible strain of L. pneumophila sg1 was isolated from respiratory samples at D0. During the stay of patient #4 at ICU, we observed a major in vivo shift in the structure of the L. pneumophila population with the replacement of fluorquinolone-susceptible by resistant cells, confirming that fluorquinolone therapy selected for in vivo fluorquinolone resistance during hospitalization. The two patients #2 and #4 suffered from a severe and prolonged course disease, but recovered under combined fluorquinolone and macrolide treatment. Additional cases will be needed to establish a statistical correlation between the disease severity and the in vivo selection of gyrA QRDR mutant strains of L. pneumophila. We found by NGS a very low percentage (<0.5%) of mutated alleles in respiratory samples from control patients and in sample D3 from patient #1, and a slightly higher percentage (1.05% and 2.9%, respectively) in sample D0 from patients #2 and #4. These sequences may correspond to false positives introduced during the PCR amplification step of the NGS process itself. Alternatively, they may reflect the spontaneous occurrence of fluorquinolone-resistance mutations in L. pneumophila, as previously described in other Gram-negative bacteria including E. coli (Komp Lindgren et al., 2003). Therefore, legionellosis patients might have been infected with a complex L. pneumophila population containing low abundance of gyra83 mutants, with subsequent selection of this population in patients #2 and #4 under fluorquinolone therapy. An alternative hypothesis could be the in vivo emergence of these mutants. The rapid increase in the proportion of mutated gyra83 alleles over a few days is more compatible with the former hypothesis. In both cases however, the selective sweeps resulting in the increased abundance of the gyra83 mutants occurred in vivo during fluoroquinolone treatment. We emphasize that patient #4 already received a three-day course of ciprofloxacin at D0.

We have shown that in vivo selection of gyrA mutations responsible for fluoroquinolone-resistance in L. pneumophila occurs in legionellosis patients during fluoroquinolone treatment. A likely hypothesis is that patients were infected with L. pneumophila populations containing low doses of gyra83 mutants. If so, it is puzzling that no fluorquinolone resistance has been so far characterized in environmental strains of L. pneumophila, and only a single fluorquinolone-resistant strain of this species has been isolated before in a clinical situation (Bruin et al., 2014). It should be emphasized that mutant strains of L. pneumophila have poor ability to propagate outside infected hosts because of the lack of human-to-human transmission and little opportunities to go back to their natural environmental reservoir. However, isolation of L. pneumophila from respiratory samples of legionellosis patients remains challenging, and almost impossible in patients under antibiotic therapy. We recommend the development and use of molecular tools to test the presence of resistance mutations in patients infected by L. pneumophila, especially in the case of poor response to antibiotic treatment. Our strategy should be optimized to allow more specific detection of gyra83 and gyrA87 mutations, and other previously characterized fluorquinolone-resistance mutations (Almahmoud et al., 2009; Jonas et al., 2003; Nielsen et al., 2000). This would allow to precisely define the prevalence of fluorquinolone-resistant mutants in legionellosis patients and their clinical consequences. The molecular tools could also serve to assess the emergence and spread of gyra83 mutants in environmental L. pneumophila strains. The possibility of in vivo selection for fluorquinolone-resistance mutations in L. pneumophila fully justifies the current practice of administration of combined antibiotic therapy in severe legionellosis cases (Roig and Rello, 2003). Our study showing in vivo L. pneumophila adaptation to antibiotic pressure paves the way for more intensive investigations of other intracellular bacterial species. This hidden resistance should indeed be intensively explored because it may contribute to the increasing public health threat of the gradual loss of effectiveness of antibiotic treatments.

In conclusion, although fluoroquinolones and macrolides are highly active against Legionella species, death rates of 10–15% are commonly reported in legionellosis patients. No correlation can be found in the literature between treatment failures or relapses and development of antibiotic resistance in Legionella spp. However, fluoroquinolone-resistant strains of L. pneumophila have been selected in vitro, and a fluoroquinolone-resistant isolate was recently obtained from a legionellosis patient. By using state-of-the-art molecular and genomic approaches, we identified, at high frequency, fluoroquinolone resistance mutations in lower respiratory samples from two additional legionellosis patients from a cohort of 82. The detected gyra83 mutation is associated with a significant increase in fluoroquinolones MICs in L. pneumophila. We demonstrated that selection for fluoroquinolone resistance occurred in vivo during fluoroquinolone therapy. These two patients required ICU admission and prolonged hospitalization, but recovered under combined fluoroquinolone and macrolide treatment. Although the exact clinical impact of our findings could not be statistically evaluated because only two out of 82 legionellosis patients were involved, the possibility of in vivo selection of fluoroquinolone resistance in L. pneumophila should now be considered both for prognostic evaluation and treatment optimization at least in patients with severe legionellosis.

**Contributors**

SJ, JE, JFT, DS and MM designed the experiments. LS and IA performed the experiments. LS and SL provided all clinical data. All authors had access to data and commented on the final version.

**Declaration of Interests**

We declare no competing interests.

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**Appendix A. Supplementary Data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.07.018.

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