Antibiotic susceptibilities of *Legionella pneumophila* strain Paris in THP-1 cells as determined by real-time PCR assay

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**Objectives**: *Legionella* species are facultative intracellular bacteria. Evaluation of the activity of antibiotics against intracellular *L. pneumophila* is more predictive of their in vivo efficacy than MICs as determined in axenic medium. However, current methodologies are based on cfu count determination, and are tedious because of the slow growth of *Legionella* spp. We investigated antibiotic susceptibilities of *L. pneumophila* strain Paris in THP-1-derived macrophages, using a real-time PCR assay for evaluation of bacterial growth.

**Methods**: Intracellular activities of seven antibiotic compounds against two human isolates of *L. pneumophila* strain Paris were determined in THP-1-derived macrophages *in vitro*. Bacterial growth was evaluated using either cfu methodology or a real-time PCR protocol targeting the *mip* gene.

**Results**: Bacterial titres as determined using real-time PCR were well correlated with cfu counts. Antibiotic susceptibilities for the two *L. pneumophila* isolates tested were comparable when using either of the two techniques. MICs were also similar to those previously reported for other *L. pneumophila* serogroup 1 strains. In particular, rifampicin and the fluoroquinolones were the most active compounds, both in extracellular medium and in THP-1 cells. Real-time PCR, however, was much less laborious than the traditional cfu method.

**Conclusions**: Real-time PCR is better adapted than cfu-based methods to evaluating the antibiotic susceptibilities of large series of *Legionella* strains to newer antibiotic compounds.

Keywords: antibacterials, intracellular, macrophages, quantitative PCR

**Introduction**

*Legionella* species are the aetiological agents of legionellosis, a potentially life-threatening disease that manifests primarily as a pneumonia.1 Forty-eight species corresponding to 70 serogroups are currently recognized in the *Legionella* genus. However, the *Legionella pneumophila* serogroup 1 accounts for more than 90% of human infections,2–4 including the recently characterized *L. pneumophila* strain Paris, which is highly prevalent in France.5,6 *Legionella* spp. reside in aquatic environments, especially in biofilms and as symbionts of amoebae.1 Thus, legionellosis is usually acquired after exposure to environmentally generated aerosols. Infections are most frequently acquired in the community. Severe cases often occur in the elderly and in debilitated patients (e.g. patients with diabetes mellitus, chronic obstructive pulmonary disease, smokers, or immunosuppressed persons), but may also be observed in younger patients and in the healthy population. The global mortality rate of legionellosis is about 20–30%, but may be as high as 50% in immunocompromised patients despite appropriate antibiotic therapy.7 This contradicts the high in vitro susceptibility of *L. pneumophila* species to antibiotics. Thus, further evaluation of in vitro activity of newer antibiotic compounds against *Legionella* species is needed, both in axenic medium and in cell systems. However, current cfu-based methods remain tedious because of the slow growth of *Legionella* spp.

In this study, we investigated antibiotic susceptibilities of *L. pneumophila* strain Paris grown in THP-1-derived macrophage cells using real-time PCR technology to quantify the inhibition of bacterial growth.

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L. pneumophila antibiotic susceptibilities by real-time PCR

Materials and methods

Legionella strains

Two human isolates of L. pneumophila serogroup 1 strain Paris were used: CIP 107-629-T, a reference strain kindly provided by J. Etienne [French National Reference Center for Legionella (FNRLC), Lyon, France], and a clinical strain referred to as Paris-Gre1 isolate, cultured in our laboratory in 2001 from a broncho-alveolar washing fluid from a legionellosis case. This second isolate had been characterized as a Paris strain by pulsed field electrophoresis at FNRLC. Legionella strains were grown on buffered charcoal yeast extract medium, supplemented with α-ketoglutarate (BCYE-α) (Oxoid, Dardilly, France). Bacterial growth was harvested after 3–5 days incubation of cultures at 37°C, in a CO₂-enriched atmosphere.

Antibiotics

The following antibiotic compounds were tested: amoxicillin (GlaxoSmithKline), erythromycin (Abbott, Rungis, France), doxycycline (Sigma, Paris, France), ciprofloxacin and moxifloxacin (Bayer, Puteaux, France), levofloxacin and rifampicin (Aventis, Paris, France). Antibiotics were dissolved as recommended by manufacturers, diluted in sterile distilled water at concentrations ranging from 2 to 5 mg/mL, and stored at −80°C until used. Antibiotics were tested at 2-fold serial concentrations, ranging from 8 mg/L down to 0.001 mg/L depending on the antibiotic compound considered.

MIC and MBC determination in broth

MICs were determined in 96-well microtitre plates, using liquid BYE-α medium. BYE-α medium was prepared with 1% (v/v) yeast extract in sterile distilled water (Becton Dickinson, Pont de Claix, France) supplemented with 10% (w/v) Legionella growth supplement (SR110A; Oxoid) containing ACES [N-(2-acetamido)-2-aminoethanesulfonic acid] (100 g/L)/potassium hydroxide buffer, α-ketoglutarate (10 g/L), l-cysteine chloride hydrate (4 g/L), and ferric pyrophosphate (2.5 g/L).

A bacterial suspension of ~10⁵ cfu/mL was prepared in BYE-α medium, and 180 μL was dispensed in each well. Antibiotic concentrations (20 μL per well) were added at 10-fold the desired final concentration. MICs corresponded to the lowest antibiotic concentrations with no visible growth after 48 h incubation of the plates at 37°C. Antibiotic-free wells containing 200 μL of bacterial suspension were used as growth controls. MICCs determined in Mueller–Hinton broth for Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 served as antibiotic concentration controls.

Bacterial suspensions in wells with no visible growth were plated (after 1/10 dilution in sterile distilled water) onto BCYE-α media, which were incubated at 37°C in 5% CO₂ for 5 days. MBCs corresponded to the minimal antibiotic concentrations allowing 3 log reduction or more of bacterial titres compared with the primary inoculum. Experiments were carried out in duplicate, and repeated to confirm results.

Legionella pneumophila growth in macrophage-derived THP-1 cells

The human monocytic cell line THP-1 was kindly provided by J. L. Mege (Unité des Rickettsies, Marseille, France). Cells were grown in RPMI/10% SVF, i.e. RPMI 1640 medium (Invitrogen, Cergy Pontoise, France) supplemented with 2 mM L-glutamine (Invitrogen), 10 mM HEPES (Cambrex, Verviers, Belgium) and 10% fetal bovine serum (Cambrex). THP-1 cells were plated at a concentration of 5 × 10⁵ cells/mL in 24-well tissue culture plates (1 mL per well), and incubated for 24 h in RPMI/10% SVF containing 16 mM PMA (phorbol-12-myristate-13-acetate) at 37°C in 5% CO₂, for maturation into macrophage-like adherent cells.

THP-1-derived macrophages were then washed gently twice with RPMI/10% SVF in order to remove PMA. They were infected with an L. pneumophila suspension (~10⁵ cfu/mL, bacteria/cell ratio 1:1) in RPMI/10% SVF, and incubated for 2 h at room temperature to allow intracellular penetration of bacteria. Cell monolayers were then washed gently with RPMI/10% SVF to remove extracellular bacteria, and plates were incubated at 37°C, in a 5% CO₂-enriched atmosphere. Cell viability was more than 95% throughout experiments as determined by the Trypan Blue dye exclusion test (Gibco, Paisley, Scotland, UK). Bacterial counts were determined using both the cfu method and real-time PCR technology immediately following cell infection (H2), and after 1, 2 and 3 days incubation of cell cultures. Therefore, the cell monolayer and medium from each well were harvested after cell scraping using a sterile pipette. Cells were lysed by thermal shock (~80°C in dry ice, 37°C). Ten-fold serial dilutions of 0.5 mL of bacterial suspensions were then plated onto BCYE-α medium for cfu counts. The remaining 0.5 mL of bacterial suspensions served for DNA extraction for real-time PCR experiments.

3D confocal microscopy

THP-1-derived macrophages were grown on 15 mm diameter coverslips in 24-well tissue culture plates, as described above. They were infected with L. pneumophila CIP 107-629-T for 48 h, and then fixed for 10 min in 4% paraformaldehyde. Cell membranes were labelled using PKH26 Red Fluorescent Cell Linker Mini kit (Sigma), and bacteria were stained using a fluorescein-labelled anti-L. pneumophila LPS monoclonal antibody (Monofluo™ Legionella pneumophila IFA test kit, Bio-Rad). Cover slips were deposited on glass slides for fluorescence imaging. The confocal fluorescence imaging was performed using a LSM 510 laser scanning microscope (Carl Zeiss, Germany) equipped with a Plan-Neofluar 40x/1.3 Ph3 oil immersion objective at Institut Albert Boniot (IFR 73, Grenoble). The fluorophores PKH26 and fluorescein were sequentially excited with 543.5 nm (HeNe) and 488 nm (Ar 2+) laser lines, respectively. The intensity of each line was lowered using the acousto-optical modulator to avoid excessive bleaching of the fluorophore. The emission light was filtered with a NFT 545 beam splitter, long-pass filter LP 560 or band-pass BP 500–550. For the 3D z-stack acquisition, the pinhole settings were adjusted to allow optical sections thinner than 0.9 μm in both channels. The spatial shift between confocal excitation volumes of the two lasers was less than 0.2 μm in lateral and axial directions and was corrected after acquisition using LSM510 2.8 software.

MIEC determination in macrophage-derived THP-1 cells

THP-1-derived macrophages were prepared in 24-well tissue culture plates as described above. The cell monolayers were then infected with an L. pneumophila suspension (~10⁵ cfu/mL, bacteria/cell ratio 1:1) in RPMI/10% SVF, and incubated for 2 h at room temperature to allow intracellular penetration of bacteria. Cell monolayers were then washed gently with RPMI/10% SVF to remove extracellular bacteria, and antibiotic solutions were added to obtain the desired final concentrations. Bacterial counts were determined using both the cfu method and real-time PCR technology immediately following cell
infection (H2), and after 3 day incubation of cell cultures. MIECs corresponded to the minimal extracellular antibiotic concentration allowing complete inhibition of L. pneumophila intracellular multiplication, i.e. a bacterial count after 3 day incubation of cultures in the presence of the tested antibiotic less than or equal to that determined at H2.

Real-time PCR assays

L. pneumophila DNA was extracted from infected cell suspensions using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France), as recommended by the manufacturer. A real-time PCR assay targeting the mip gene (encoding a macrophage infectivity potentiator), previously described by Wellinghausen et al., was used. The specificity of this assay had been previously established. Forward and reverse primers were: Lp-mip-PT69 (5'-GCA TTG GTG CCG ATT TGG-3') and Lp-mip-PT70 (5'-GCTT TG 8 CCA TCA AAT CT- TCT GAA-3'). These primers allowed amplification of a 186 bp amplicon. Hybridization probes were: Lpneu LC Red (5'-LC Red 640-CCA TCT CAT TAA CAT CTA TGC C-3') and Lpneu FL (5'-CCA CTC ATA GGC TCT TGA ATG CTT TTA-fluorescein). Each reaction mixture contained 0.5 μM of each primer, 0.2 μM of each hybridization probe, 4 mM MgCl₂, 2 μL Fast Start Master Hybridization Probes reaction mix (Roche Diagnostics), 4.6 μL of sterile distilled water, and 5 μL of DNA extract, for a final volume of 20 μL. After an initial DNA denaturation step at 95°C for 8 min, the PCR profile corresponded to 45 cycles of denaturation at 95°C for 10 s, primer annealing at 57°C for 10 s, and primer extension at 72°C for 15 s, with a temperature transition step of 20°C/s in all segments. Each DNA extract was tested in duplicate to confirm reproducibility of the assays. A negative control was set by adding DNA-free sterile distilled water instead of DNA extract to the PCR reaction mixture. Genomic DNA extracted from an L. pneumophila ATCC 33152 suspension served both to construct standard calibration curves, and as a positive amplification control. The log of the concentration of each dilution series of this reference template DNA was plotted versus the cycle number at which the fluorescent signal increased above a threshold value (Ct value). The slope of the standard curve generated for each experiment allowed calculation of the reaction efficiency (e) according to the following equation: e = 10^-1/slope.

Results

Intracellular growth of L. pneumophila strain Paris

L. pneumophila Paris isolates did not grow in cell-free RPMI/10% SFV (Figure 1). They could be grown in THP-1-derived macrophage cells, with a ~3 log increase in bacterial titres after 72 h cell incubation (Figure 2). There was a good correlation between bacterial titres determined by cfu counts and those determined using real-time PCR assay (Figure 3). The intracellular location of L. pneumophila was demonstrated by the lack of multiplication of bacteria in cell-free RPMI/10% SFV, and by visualization of intracellular bacteria using confocal microscopy (Figure 4). L. pneumophila intracellular growth resulted in cell lysis after 3 day incubation of cultures.

MICs and MBCs as determined in BYE-α medium

MICs determined in Mueller–Hinton broth for S. aureus ATCC 25923 and E. coli ATCC 25922 strains were within the expected range. MICs were comparable for the two L. pneumophila Paris isolates (Table 1). Rifampicin was the most effective compound with an MIC ≤ 0.001 mg/L, whereas the fluoroquinolone compounds ciprofloxacin, levofloxacin and moxifloxacin gave comparable results (i.e. 0.03–0.06 mg/L). Erythromycin and doxycycline were less effective with MIECs as determined in THP-1-derived macrophages

MIECs were strictly the same either using the cfu method or real-time PCR assay. They were close to MIECs for fluoroquinolones, doxycycline and rifampicin (Table 1). MIEC was four times the MIC for erythromycin. The MIC and MIEC of amoxicillin for the two L. pneumophila strains tested were 1–2 mg/L and >4 mg/L, respectively.

Discussion

Legionellosis is a public health problem in industrialized countries because of the availability of hot water and aerosol-producing systems such as cooling towers.1 Macrolides, especially erythromycin, have historically been the first antibiotics recommended for treatment of legionellosis following the outbreak occurring during the American Legion conference in Philadelphia in 1976.9 More recently, newer macrolides, azalides (e.g. azithromycin), ketolides (e.g. telithromycin) and fluoroquinolones (e.g. levofloxacin, moxifloxacin) have proved to be superior in vitro to erythromycin10–12 and are currently recommended as alternatives to erythromycin. Although some authors recommend combining two active compounds in patients with severe and/or unresponsive disease (e.g. a macrolide with a fluoroquinolone or with rifampicin), the superiority of such antibiotic combinations has not been fully demonstrated in humans. The high mortality rates reported in debilitated patients despite appropriate antibiotic treatment justify the search for new drug therapies of higher efficacy. Because comparative clinical trials
are difficult to perform and may not be ethical, in vitro systems are still needed to test the susceptibilities of \textit{L. pneumophila} to newer antibiotic compounds. On the other hand, the antibiotic susceptibility determined in cell systems may be more predictive for \textit{in vivo} efficacy of antibiotics than MICs as determined in axenic medium. Eukaryotic cells may protect \textit{L. pneumophila} from the action of antibiotic compounds with poor intracellular penetration such as β-lactams. Also, it has been previously demonstrated that intraphagocytic growth of \textit{L. pneumophila} increases the antibiotic resistance of these bacteria.\textsuperscript{13}

Current methodologies are based on cfu count determination, and thus remain tedious because of the slow growth of \textit{Legionella} species. In this study, we investigated the antibiotic susceptibilities of \textit{L. pneumophila} strain Paris grown in THP-1-derived macrophage cells using real-time PCR technology to quantify the inhibition of bacterial growth. The THP-1 cell line can be differentiated into macrophages following stimulation with PMA. This cell line has previously been used for \textit{in vitro} culture of \textit{L. pneumophila}.\textsuperscript{14–16} MICs and MBCs determined for the two \textit{L. pneumophila} Paris isolates tested were within the range of results previously reported for this species when using a broth dilution susceptibility procedure with charcoal-free medium.\textsuperscript{17–20} Both isolates of \textit{L. pneumophila} strain Paris multiplied within THP-1-derived macrophage cells, leading to an increase in intracellular bacterial load of \( \sim 3 \) log after 3 day incubation of cultures. Prolonged incubation resulted in cell lysis. Because \textit{L. pneumophila} did not grow in cell-free RPMI/10% SVF medium, bacterial titres included both intracellular and extracellular forms of bacteria, the latter resulting from cell disruption following intracellular multiplication of \textit{L. pneumophila}. MIECs were defined as the minimum extracellular antibiotic concentration allowing complete inhibition of bacterial growth, i.e. no

Figure 2. Growth curves of the two \textit{L. pneumophila} Paris isolates CIP 107-629-T and Lp1 Paris–Gre1 in THP-1-derived macrophage cells, as determined by cfu counts or real-time PCR assays. A, B, C and D represent four independent experiments.

![Figure 2](image)

Figure 3. Correlations between cfu counts and genome copies (as determined by real-time PCR) for determination of bacterial titres in THP-1-derived macrophage cells.

![Figure 3](image)

Figure 4. \textit{L. pneumophila} CIP 107-629-T inside THP-1-derived macrophages, after 48h incubation of cultures. The intracytoplasmic location of \textit{Legionella pneumophila} was confirmed by confocal microscopy using general counterstaining of cellular membranes with the lipid marker PKH26. The bright spots represent bacteria (stained with fluorescein coupled anti-\textit{L. pneumophila} LPS antibody) against the faint grey background of cellular membranes (PKH 26). The 24 serial optical sections were measured from a 13 \( \mu \)m thickness fixed cell; each section was 38\( \times \)38\( \times \)0.5 \( \mu \)m.

![Figure 4](image)
difference in bacterial titres, as determined by real-time PCR assay, between the primary inoculum and bacterial titres following 3 day incubation of cultures. The real-time PCR protocol we used, targeting the \textit{L. pneumophila mip} gene, was previously described by Wellinghausen et al.\textsuperscript{8} Real-time PCR technology was used concomitantly with the traditional cfu method to determine the \textit{L. pneumophila} growth curve in THP-1-derived macrophage cells, with a good correlation between the two techniques, although titres were higher with the former technique. However, determination of bacterial titres using real-time PCR was much less laborious and time-consuming than with the cfu method.

To our knowledge, antibiotic susceptibilities of \textit{L. pneumophila} strain Paris have not been previously reported. MIECs and MIEC/MIC ratios were close to those previously described in THP-1-derived macrophages,\textsuperscript{15,16,21,22} and in other cell types.\textsuperscript{19} Rifampicin was the most active antibiotic compound \textit{in vitro}. However, single point mutations in the \textit{rpoB} gene have been associated with rifampicin resistance in \textit{L. pneumophila},\textsuperscript{23} and this antibiotic should not be used alone.\textsuperscript{11} Fluoroquinolone compounds were the second most active antibiotics. \textit{In vitro} selection of fluoroquinolone resistance in \textit{L. pneumophila} has been described and was related to single point mutation in the \textit{gyrA} gene, encoding the alpha subunit of DNA gyrase.\textsuperscript{24} Onody \textit{et al.} found no resistance against erythromycin, rifampicin, or ciprofloxacin among 98 \textit{L. pneumophila} clinical isolates.\textsuperscript{25} However, the possibility of \textit{in vivo} acquired resistance to fluoroquinolones in \textit{L. pneumophila} should be further evaluated. Doxycycline and erythromycin were less active, with MIECs close to maximum concentrations achievable in human serum. As for macrolide compounds, however, concentrations within phagocytic cells are much higher than those obtained in serum. Amoxicillin was not effective against intracellular \textit{L. pneumophila} despite good extracellular activity, and served as a negative control because of its poor intracellular penetration. The eukaryotic cell membrane is known to be poorly permeable to most beta-lactam compounds.

In conclusion, we were able to determine the intracellular activity of antibiotics against \textit{L. pneumophila} strain Paris in THP-1-derived macrophage cells using real-time PCR technology. Results were in accordance with those obtained with the traditional cfu method. Real-time PCR technology has been previously evaluated to test antibiotic susceptibilities of other intracellular pathogens, e.g. \textit{Rickettsia} species\textsuperscript{26} and \textit{Coxiella burnetii}.\textsuperscript{27} Because \textit{L. pneumophila} isolation from infected patients remains tedious, antibiotic therapy is usually empirical. Moreover, early administration of an effective antibiotic therapy is crucial to improve survival of patients. Our real-time PCR assay may not have a direct impact on management of legionellosis patients. However, it would provide easier and more efficient for determination of the intracellular activity of newer antibiotic compounds against \textit{L. pneumophila} than the cfu method in order to select compounds with improved activity.

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**References**