COMPARISON BETWEEN THE IN VITRO EFFICACY OF
MOXIFLOXACIN AND AMOXICILLIN AGAINST LISTERIA
MONOCYTOGENES

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ABSTRACT

Listeria monocytogenes is a facultative intracellular bacterium that causes severe infections associated with a high mortality rate. Moxifloxacin presents extended activity against gram-positive bacteria and has recently been suggested as a potential alternative in the treatment of listeriosis. We evaluated the in vitro efficacy of moxifloxacin against L. monocytogenes using a combination of epidemiological and experimental approaches. The median minimal inhibitory concentration of moxifloxacin for a large collection of L. monocytogenes strains of various origins (human, food, and environment) was 0.5 µg/ml [range: 0.064-1]. No differences were observed, irrespective of the origin of the strains. Moreover, no cross-resistances with fluoroquinolones were detected in strains that have been reported resistant to ciprofloxacin. The in vitro activity of moxifloxacin and amoxicillin were compared by time-kill curve and inhibition of intracellular growth experiments, using a model of bone marrow-derived mouse macrophages infected by L. monocytogenes EGDe. Both moxifloxacin and amoxicillin were bactericidal in broth against extracellular forms of L. monocytogenes. However, moxifloxacin acted much more rapidly, beginning to exert its effects in the first three hours and achieving complete broth sterilisation within 24 hours of incubation. Moxifloxacin has a rapid bactericidal effect against intracellular reservoirs of bacteria, whereas amoxicillin is only bacteriostatic, and appears to prevent cellular lysis and the subsequent bacterial spreading to adjacent cells. No resistant bacteria were selected during experimental in vitro experiments. Taken together, our results suggest that moxifloxacin is an interesting alternative to the reference treatment combining rapid and bactericidal activity, even against intracellular bacteria.
INTRODUCTION

Listeria monocytogenes is a gram-positive bacterium widely found in the environment (10). This facultative intracellular pathogen causes severe food-borne infections: septicaemia and central nervous system (CNS) infections primarily in elderly people and patients with impaired cellular immunity and abortion (10, 13, 18). The reference treatment currently associates high doses of aminopenicillin (ampicillin or amoxicillin) and gentamicin, administered intravenously (14, 32). Nevertheless, despite using effective treatment against L. monocytogenes, listeriosis is still associated with high fatality rate (30%), especially in CNS-infections (10, 13, 18, 23, 29). Prospective clinical studies on the best antibiotic regimen are not available as listeriosis is a rare disease in humans (15, 32). Thus, there is considerable diversity in the second-line treatment, in case of first-line treatment failure or intolerance that counter-indicates the use of beta-lactam (10, 13, 14, 23, 29, 32). Moreover, during the last few years increasing number of strains resistant to clinically relevant antibiotics have been reported (4, 5, 12, 28, 29). This underlines the need to anticipate the development of resistance validating new antibiotics as alternatives to current treatment.

Treatment of CNS-listeriosis is complex and outcome depends on the early administration of antibiotics with rapid bactericidal activity against L. monocytogenes and extensive diffusion in tissues, especially the cerebral parenchyma (13, 14, 18, 23, 29, 32). Furthermore, the efficacy of therapy is limited by intracellular bacteria forming reservoirs within the cytoplasmic compartments of many eukaryotic cell types, including macrophages (3, 6, 20). Thus, there are few candidate molecules that meet these criteria (14, 32).

New-generation fluoroquinolones with extended activity against gram-positive bacteria (34) seem to be promising (3, 19, 24, 30). These fluoroquinolones share several interesting pharmacokinetic properties in vivo and the ability to penetrate and concentrate intracellularly (30, 34). Moxifloxacin is the only one of these antibiotics released on the market and still commercially available that combines rapid bactericidal activity against both extracellular and
intracellular L. monocytogenes in vitro (3, 22, 30). However, no data are currently available on the susceptibility to moxifloxacin of a large collection of L. monocytogenes strains, whatever their origin and on the ability to select resistant strains during experiments.

We carried out an efficacy study combining epidemiological and experimental approaches to evaluate the activities of moxifloxacin and amoxicillin against extracellular and intracellular L. monocytogenes in a model of infected bone marrow-derived mouse macrophages.

**MATERIALS AND METHODS**

**Antibiotics.** Moxifloxacin and amoxicillin were provided by Bayer Pharma (Bayer AG, Wuppertal, Germany) and GlaxoSmithKline (Marly-le-Roi, France), respectively. Antibiotics were extemporaneously diluted to the appropriate concentration.

**Bacterial strains.** Antimicrobial susceptibility to moxifloxacin was determined for a representative selection of the collection strains from the French National Reference Centre for Listeria (NRC, Institut Pasteur, France). The strains studied included: Listeria Type strains and L. monocytogenes serovar Reference strains (n=16) (Table S1), L. monocytogenes strains isolated from humans in 2005 (n=205), a set of randomly selected L. monocytogenes strains isolated from food and environment in 2005 (n=183), and L. monocytogenes strains resistant to ciprofloxacin isolated from humans since 2000 (n=8).

**Susceptibility testing.** Minimal inhibitory concentrations (MICs) of moxifloxacin and ciprofloxacin were determined by the E-test procedure (AB Biodisk, Solna, Sweden) following the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM; http://www.sfm.asso.fr) To the best of our knowledge, there are no interpretative criteria for moxifloxacin and L. monocytogenes from any breakpoint committee (CA-SFM, EUCAST and CLSI) (1). Isolates were categorised as susceptible, intermediate or resistant according to the following breakpoints: 1 \leq MIC >2 \mu g/ml.
**Time-kill curves.** In vitro bactericidal activity of moxifloxacin and amoxicillin was determined against the virulent strain of *L. monocytogenes* (EGD-e) (11). Five millilitres of Mueller-Hinton broth (BioRad) were inoculated with 5x10^8 bacteria and incubated at 37°C. Moxifloxacin and amoxicillin were added to the MH-suspension at various concentrations: 1xMIC, 4xMIC, 8xMIC or 400xMIC. The last two concentrations correspond to the maximum serum concentration (Cmax) after administering clinically relevant doses of moxifloxacin and amoxicillin in humans, respectively (31). Bacterial counts were determined in triplicate at indicated times of incubation with antibiotics by subculturing 10 µl of serial 10-fold dilutions of the MH-suspension on brain-heart infusion (BHI, Becton Dickinson, Le Pont-de-Claix, France) agar plates and on BHI agar supplemented with 2 µg/ml of moxifloxacin incubated for 48 hours. Results were expressed as the number of colony forming units (CFU) per millilitre and corresponded to the mean ± standard error from three experiments. Bactericidal activity was defined by killing more than 99.9 % of the initial inoculum after 24 hours of incubation (i.e.: ≥ 3 Log_{10} CFU/ml decrease in viable counts). The killing rate was defined as the decrease in the initial inoculum within the first three hours.

**Bone marrow-derived mouse macrophages, infections and treatments.** Intracellular growth inhibition assays were performed with primary cultures of bone marrow-derived macrophages sampled from BALB/c mice, 7- to 8-weeks old (Elevage Janvier, Le Genest-St-Isle, France), as previously described (7). Bone marrow cells were maintained and cultured at 37°C under 10% CO₂ in complete medium: RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% decomplemented fetal calf serum and 10% growth factor (L-CSF). After seven days of differentiation, bone marrow-derived macrophages (3x10^5 cells/ml) were infected for 15 min with bacteria (*L. monocytogenes*-macrophage ratio of infection was 10:1), washed six times with RPMI and preincubated in complete medium for 45 min. Thereafter, macrophages were incubated in complete medium with or without antibiotic for 24 hours. At the indicated times of incubation, macrophages were washed twice with ice-cold
sterile phosphate-buffered saline and lysed with 1 ml of 0.1% Triton X-100. Serial 10-fold
dilutions of the lysates were plated in triplicate on BHI agar for bacterial counts and on BHI
agar supplemented with 2 µg/ml moxifloxacin for 48 hours. Results were expressed as the
number of CFU per well and corresponded to the mean ± standard error from three
experiments. Cellular integrity was checked each time and the rate of infection was
determined after microscopic examination of macrophages stained with May-Grunwald-
Giemsa.

Statistical analysis. Equal distribution of MICs was analysed with the Kolmogorov-Smirnov
test using Stata software (v.8). P values of ≤0.05 were considered to be statistically
significant.

RESULTS
Susceptibility to moxifloxacin. The results of MICs determined for all Listeria Type strains
and L. monocytogenes serovar Reference strains showed that the Listeria genus is naturally
susceptible to moxifloxacin according to the chosen breakpoints (Table S1).

The median MIC for the 205 L. monocytogenes strains isolated from patients (septicaemia
(52%), meningo-encephalitis (28%), materno-neonatal infections (17%), and focal infections
(3%)) and collected by the NRC, was 0.5 µg/ml [range: 0.064 to 1] (FIG. 1). These strains
were distributed in the four PCR-groups (8): IVb (56%), Ila (24%), IIb (17%), and IIc (3%).
They were all susceptible to moxifloxacin. MIC distribution was homogenous with no
association according to PCR-groups or clinical forms.

The median MIC for the 183 L. monocytogenes strains isolated from food and food-
processing environment collected by the NRC, was 0.5 µg/ml [range: 0.125 to 1] (FIG. 1).
These strains were distributed in the four PCR-groups (8): IIa (45%), IVb (25%), IIb (19%),
and IIc (11%). They were all susceptible to moxifloxacin. MIC distribution was homogenous
with no association according to PCR-groups.
Eight *L. monocytogenes* strains resistant to ciprofloxacin and isolated from patients since 2000 were also tested. MICs to moxifloxacin for these strains were not increased. Thus, no cross-resistances were detected between moxifloxacin and ciprofloxacin (Table S1).

We did not observe a significant difference in the distribution of MICs, irrespective of the origin of *L. monocytogenes* strains tested (*P*=0.316) (FIG. 1).

**Time-kill curves.** Moxifloxacin and amoxicillin activities against extracellular forms of *L. monocytogenes* were compared using the virulent *L. monocytogenes* strain (EGDe), which is susceptible to both antibiotics (MICs were 0.5 and 0.125 µg/ml for moxifloxacin and amoxicillin, respectively).

In MH-broth, both antibiotics at concentration above the MIC showed bactericidal activity against extracellular forms of *L. monocytogenes* (FIG. 2). However, this bactericidal effect was obtained significantly quicker with moxifloxacin than with amoxicillin: the initial inoculum was reduced by more than three Log₁₀ CFU/ml after six hours of incubation with moxifloxacin whereas the effect of amoxicillin on bacterial growth remained bacteriostatic during this period (FIG. 2). Moxifloxacin had a higher killing rate than amoxicillin. Moreover, complete broth sterilisation was observed after 24 hours of incubation with moxifloxacin, whereas this was never observed with amoxicillin.

**Inhibition of *L. monocytogenes* intracellular growth**

The infection of bone marrow-derived mouse macrophages by *L. monocytogenes* (EGDe) led to the rapid and total invasion of the well and completes lysis of macrophages six hours after infection (FIG. 3). As early as after three hours of incubation, bacteria formed numerous filopode-like projections within the cytosolic compartment of infected macrophages (FIG. 3).

Concentrations of moxifloxacin equal to and above the MIC prevented the formation of filopode-like projections and we also observed changes in the morphological aspects of intracellular bacteria. They were observed to be ghostly, chained, and elongated (FIG. 3). Moxifloxacin demonstrated a quick bactericidal activity, as the number of intracellular
bacteria was reduced by three $\log_{10}$ CFU/ml within three hours of incubation (FIG. 4). Moreover, moxifloxacin appears to have a protective effect against macrophage lysis, as many cells were still viable after 24 hours of incubation.

By contrast, the formation of filopode-like projections was not prevented by amoxicillin at MIC during the early stages of the experiment (FIG. 3), confirming a lack of activity against the intracytoplasmic reservoir of bacteria. Although, the number of bacteria observed within macrophages was reduced after the first three hours, 100% of macrophages were still infected at this time, whatever the amoxicillin concentration used. Amoxicillin did not prevent lysis of macrophages also observed in control samples with no antibiotic (FIG. 3). This makes the interpretation of bacterial counts performed after 24 hours of incubation difficult because of an alteration in the cellular monolayer. However, amoxicillin was bacteriostatic against intracellular bacteria even after 24 hours of incubation, if bacterial counts were possible (FIG. 4).

Selection of resistant strains to moxifloxacin. No resistant strains were selected after 48 hours of incubation with moxifloxacin. In addition, we detected no increase in the MICs of moxifloxacin for strains isolated during the early times of both assays.

**DISCUSSION**

The original structure of new generation fluoroquinolones allows extended spectrum against gram-positive bacteria (26, 34). Our epidemiological study on a large collection of *Listeria* strains showed that *Listeria* species are all naturally susceptible to moxifloxacin. Despite the selective pressure exerted by the intensive use of fluoroquinolones worldwide (34), no resistances to moxifloxacin were detected, whereas resistance to ciprofloxacin is regularly detected among strains isolated from food, environment and humans (5, 12). Mechanism for these ciprofloxacin-resistant strains is due to an increased expression of *lde* (*Listeria* drug efflux) (12) leading to an active and selective efflux of certain fluoroquinolones. Our result,
are consistent with that moxifloxacin is a poor substrate for active efflux in gram-positive bacteria including *L. monocytogenes* due to a 7-diazobicyclonyl group (26, 27).

In gram-positive bacteria, resistance can also result from mutational alteration in the so-called quinolone resistance-determining regions (QRDRs). Moxifloxacin provides enhanced activity against DNA gyrase and topoisomerase IV due to a C8-methoxyl group (26). Stepwise accumulation of mutations is therefore necessary for the expression of resistance to moxifloxacin, thus prevents the selection of resistant bacteria despite the use of high initial inoculums in time-kill experiments (26, 35). Our results are consistent with this new-generation fluoroquinolone exerting only weak selection pressure for resistance (26, 35).

Although both antibiotics kill extracellular forms of *L. monocytogenes*, moxifloxacin acts quicker than amoxicillin (3, 30). Moreover, moxifloxacin achieved complete sterilisation of cultures with high inoculums after 24 hours of incubation, whereas amoxicillin did not. As listeriosis primarily occurs in patients with severe underlying diseases including those with impaired cellular immunity (13, 14, 18, 32), the rapid bactericidal activity of moxifloxacin should be promising for a favourable outcome.

However, treatment against intracellular infections is complex. Antibiotic efficacy is dependent on the ability to cure the intracellular reservoirs of bacteria at infection sites (14, 20, 32) Thus antibiotics must rapidly reach the various intracellular compartments to attack intracytoplasmic bacteria (20). Several studies have recently shown the in vitro efficacy of new-generation fluoroquinolones against intracellular forms of *L. monocytogenes*, including moxifloxacin, in models of immortalised cell lines (J774 or THP-1 macrophages, Hela and L929 cells) (3, 19, 22, 24, 30). However, according to Carryn *et al.*, there are considerable quantitative differences in antibiotic activity depending of the type of host cell (2). The cellular pharmacokinetic (intake, intracellular disposition in various subcellular compartments, accumulation, efflux) and pharmacodynamic (bacterial responsiveness, cooperation with host defences) parameters govern the intracellular activity of antibiotics (2,
We thus developed and used a model of infected macrophages in primary culture derived from the bone-marrow of BALB/c mice (7), as opposed to transformed cell lines. In our model, moxifloxacin diffused and accumulated quickly into cellular compartments, killing intracytoplasmic forms of *L. monocytogenes* within the first three hours (3, 22, 30). Despite overexpression and/or increased activity of the MRP-like ciprofloxacin transporters, reported in ciprofloxacin-resistant J744 macrophages, the intracellular accumulation of moxifloxacin would not be significantly altered as moxifloxacin is only partially effluxed (21, 22).

Moxifloxacin had additional effects in preventing the intracellular expression of some virulence factors. Actin polymerisation, which depends on the expression of the protein ActA, allows the intracellular movement of bacteria, and can be used to detect their intracytoplasmic localization (6). The inhibition of the formation of filopode-like projections observed even at MIC could be due to the inhibition of ActA, thus preventing the cell-to-cell spreading mechanism (6, 9). Moreover, moxifloxacin appeared to prevent cellular lysis. This is of importance because cellular destruction leads to bacterial release and the subsequent spreading to adjacent cells (6). Thus, moxifloxacin could be useful for preventing local spreading at the infection site and probably distant bacterial dissemination. Indeed, previous studies highlighted the ability of *L. monocytogenes* to spread within infected phagocytes, especially into the cerebral parenchyma, leading to CNS-infection (9, 16).

By contrast, amoxicillin, of which only a small proportion of the drug reaches the intracytoplasmic compartments of infected cells, presents a weak and slow activity against intracytoplasmic bacterial growth (3, 17, 20). Enhanced effectiveness is observed with high doses of amoxicillin and a prolonged time of exposure of infected macrophages (3, 17, 20).

The paradoxical activity of amoxicillin against intracellular bacteria may be explained by acting against extracellular released of bacteria after cellular lysis, thus preventing adjacent cells from infection (3, 15, 17, 20). It could also be explained by antibiotic phagocytosis, already described for glycopeptide agents (17, 33). In this case, restricted phagosomal
localisation of these antibiotics may explain why they do not prevent the formation of filopode-like projections, as seen with amoxicillin in the early times of the experiment. Nevertheless, the synergistic association of amoxicillin with gentamicin sufficiently cures most *L. monocytogenes* infections (14, 32). The efficacy is explained by the use of high doses of amoxicillin ensuring sufficient concentrations at infection sites and because cellular immunity acts complementary to antibiotic treatment in the majority of cases (14, 25, 32).

Conclusions. Our results support the evidence of rapid bactericidal activity of moxifloxacin against extracellular and intracellular forms of *L. monocytogenes*. Thus, moxifloxacin constitutes a promising alternative for the treatment of listeriosis. However, as the in vitro activity does not always predict in vivo efficacy, these results will have to be confirmed by evaluating the activity of moxifloxacin in an animal model of listeriosis before any further use in humans.

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REFERENCE


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Figure legends

FIG. 1: Distribution of minimal inhibitory concentrations of moxifloxacin on a collection of *L. monocytogenes* strains isolated in 2005 from human (black) and food process environments (white). Arrows indicate the critical concentrations used for susceptibility interpretation classification (<1 and ≥ 2 µg/ml).

FIG. 2: In vitro efficacy of moxifloxacin and amoxicillin against extracellular forms of *L. monocytogenes*. Bactericidal activity was evaluated in time-kill curve experiments in Mueller-Hinton broth supplemented with various concentrations of antibiotics: amoxicillin at 1xMIC (0.125 µg/ml) ■, amoxicillin at 4xMIC (0.5 µg/ml) ▲, amoxicillin at 400xMIC (Cmax or peak: 50 µg/ml) ●, moxifloxacin 1xMIC (0.5 µg/ml) □, moxifloxacin at 4xMIC (2 µg/ml) Δ, moxifloxacin at 8xMIC (Cmax or peak: 4 µg/ml) ○; and non-treated control ♦. Results shown correspond to the mean ± standard error from 3 experiments. Arrows indicate the start of treatment.

FIG. 3: Effect of amoxicillin and moxifloxacin on morphological aspects of macrophages infected by *L. monocytogenes*. Microscopic examination of infected bone marrow-derived macrophages from BALB/c mice infected by *L. monocytogenes*. Macrophages stained with May Grunwald Giemsa, are observed at 3, 6 and 24 hours of incubation with various concentrations of moxifloxacin or amoxicillin (1xMIC and Cmax) or with no antibiotic for non-treated control. Cmax: corresponds to the maximum serum concentration (or peak) after the administration of clinically relevant doses of moxifloxacin and amoxicillin in humans, 8xCMI and 400xCMI, respectively. Filopode-like projections are showed by arrowheads. Bar, 15 µm.
FIG. 4: In vitro efficacy of moxifloxacin and amoxicillin against intracellular reservoir of *L. monocytogenes*.

Bactericidal activity was evaluated in bone marrow-derived macrophages of BALB/c mice infected by *L. monocytogenes* and treated with various concentrations of antibiotics: amoxicillin at 1xMIC (0.125 µg/ml) ■, amoxicillin at 4xMIC (0.5 µg/ml) ▲, amoxicillin at 400xMIC (Cmax or peak: 50 µg/ml) ●, moxifloxacin 1xMIC (0.5 µg/ml) □, moxifloxacin at 4xMIC (2 µg/ml) ∆, moxifloxacin at 8xMIC (Cmax or peak: 4 µg/ml) ○; non-treated control ♦. Results shown correspond to the mean ± standard error from three experiments. Arrows represent the start of treatment. Dotted lines extrapolate the results of bacterial counts after 24 hours of incubation due to alterations in the macrophage monolayer.
Figure 1

Minimal inhibitory concentrations of moxifloxacin (µg/ml)

Number of L. monocytogenes strains

0.064 0.075 0.125 0.19 0.25 0.38 0.5 0.75 1 2 4 8 12 16 24 32
Figure 2

[Graph showing the log (CFU/ml) levels over time (hours) for Moxifloxacin and Amoxicillin.]
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Figure 4

Moxifloxacin

Amoxicillin

Log (CFU/well)

Time (hours)