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Comparison of the In Vitro Efficacies of Moxifloxacin and Amoxicillin against *Listeria monocytogenes*▽†

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***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 to be 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 MIC of moxifloxacin for a large collection of *L. monocytogenes* strains of various origins (human, food, and environment) was 0.5 μg/ml (MIC range, 0.064 to 1 μg/ml). No differences were observed, irrespective of the origin of the strains. Moreover, no cross-resistance with fluoroquinolones was detected in strains that have been reported to be resistant to ciprofloxacin. The in vitro activities of moxifloxacin and amoxicillin were compared by time-kill curve and inhibition of intracellular growth experiments by 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 3 h and achieving complete broth sterilization within 24 h 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 the 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.**

Listeria monocytogenes is a gram-positive bacterium widely found in the environment (10). This facultative intracellular pathogen causes severe food-borne infections, septicemia 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 the use of effective treatment against *L. monocytogenes*, listeriosis is still associated with a high fatality rate (30%), especially when the CNS is infected (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 cases of first-line treatment failure or intolerance that counterindicate the use of beta-lactams (10, 13, 14, 23, 29, 32). Moreover, during the last few years increasing numbers of strains resistant to clinically relevant antibiotics have been reported (3, 4, 12, 28, 29). This underlines the need to anticipate the development of resistance when new antibiotics are validated as alternatives to current treatment.

The treatment of CNS listeriosis is complex, and the outcome depends on the early administration of antibiotics with rapid bactericidal activities 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 the formation of reservoirs within the cytoplasmic compartments of many eukaryotic cell types, including macrophages, by intracellular bacteria (2, 6, 20). Thus, there are few candidate molecules that meet these criteria (14, 32).

New fluoroquinolones with extended activity against gram-positive bacteria (34) seem to be promising (2, 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 to have been released on the market that is still commercially available and that combines rapid bactericidal activity against both extracellular and intracellular *L. monocytogenes* cells in vitro (2, 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 of moxifloxacin 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* cells 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),

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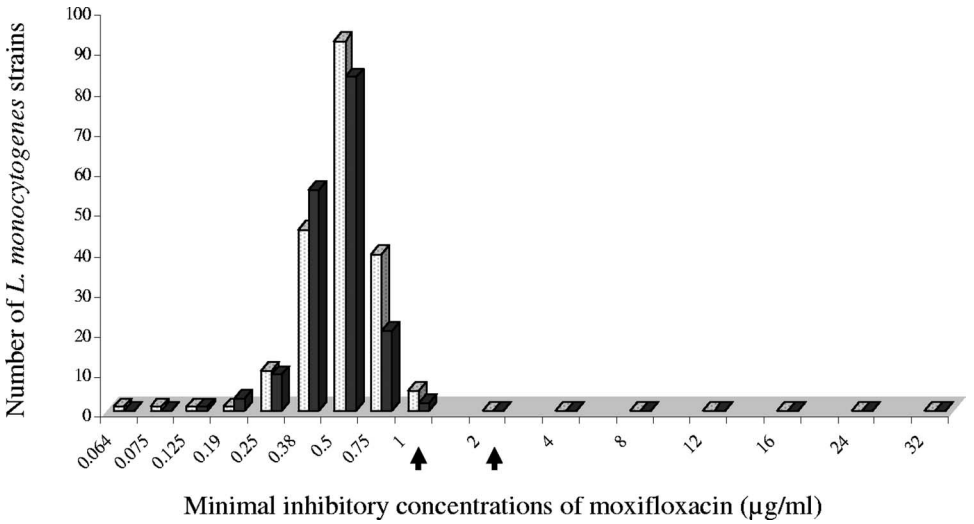

FIG. 1. Distribution of MICs of moxifloxacin for a collection of *L. monocytogenes* strains isolated in 2005 from humans (black) and food-processing environments (white). Arrows indicate the critical concentrations used for susceptibility interpretation classification (<1 and ≥2 μg/ml).

respectively. The 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 ($n = 16$) (see Table S1 in the supplemental material), *L. monocytogenes* strains isolated from humans in 2005 ($n = 205$), a set of randomly selected *L. monocytogenes* strains isolated from food and the environment in 2005 ($n = 183$), and *L. monocytogenes* strains resistant to ciprofloxacin isolated from humans since 2000 ($n = 8$).

Susceptibility testing. The MICs of moxifloxacin and ciprofloxacin were determined by the Etest procedure (AB Biodisk, Solna, Sweden), according to 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) (5). The isolates were categorized as susceptible, intermediate, or resistant according to the following breakpoints: 1 μg/ml ≤ MIC > 2 μg/ml.

Time-kill curves. The in vitro bactericidal activities of moxifloxacin and amoxicillin were determined against a virulent strain of *L. monocytogenes* (strain EGDe) (11). Five milliliters of Mueller-Hinton (MH) broth (Bio-Rad) was inoculated with 5×10^8 bacteria, and the mixture was incubated at 37°C. Moxifloxacin and amoxicillin were added to the MH broth suspension at various concentrations: 1× MIC, 4× MIC, 8× MIC, or 400× MIC. The last two concentrations correspond to the maximum serum concentration (C_{max}) after the administration of clinically relevant doses of moxifloxacin and amoxicillin to humans, respectively (31). Bacterial counts were determined in triplicate at the indicated times of incubation with antibiotics by subculturing 10 μl of serial 10-fold dilutions of the MH broth 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 and incubation for 48 h. The results were expressed as the number of CFU per milliliter and corresponded to the means ± standard errors from three experiments. Bactericidal activity was defined as the killing of more than 99.9% of the initial inoculum after 24 h of incubation (i.e., a ≥3-$\log_{10}$ CFU/ml decrease in viable counts). The killing rate was defined as the decrease in the initial inoculum within the first 3 h.

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 (age, 7 to 8 weeks; Elevage Janvier, Le Genest-St-Isle, France), as described previously (7). Bone marrow cells were maintained and cultured at 37°C under 10% CO_2 in complete medium: RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% decomplemented fetal calf serum and 10% L-cell conditioning medium (a source of macrophage colony-stimulating factor). After 7 days of differentiation, bone marrow-derived macrophages (3×10^5 cells/ml) were infected for 15 min with bacteria (*L. monocytogenes*-macrophage ratio of infection, 10:1), washed six times with RPMI 1640 medium, and preincubated in complete medium for 45 min. Thereafter, macrophages were incubated in complete medium with or without antibiotic for 24 h. At the indicated times of incubation, the 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 h. The results were expressed as the number of CFU per well and corresponded to the means ± standard errors 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. The equal distribution of MICs was analyzed by the Kolmogorov-Smirnov test with Stata software (version 8). *P* values of ≤0.05 were considered statistically significant.

RESULTS

Susceptibility to moxifloxacin. The results of MICs determined for all *Listeria* type strains and *L. monocytogenes* serovar reference strains showed that isolates of the *Listeria* genus are naturally susceptible to moxifloxacin, according to the chosen breakpoints (see Table S1 in the supplemental material).

The median MIC for the 205 *L. monocytogenes* strains isolated from patients (septicemia [52%], meningoencephalitis [28%], maternal-neonatal infections [17%], and focal infections [3%]) and collected by the NRC was 0.5 μg/ml (range, 0.064 to 1 μg/ml) (Fig. 1). These strains were distributed in the four PCR groups (8) as follows: IVb, 56%; IIa, 24%; IIb, 17%; and IIc, 3%. They were all susceptible to moxifloxacin. The MIC distribution was homogenous, with no association according to the PCR group or the clinical form.

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 μg/ml) (Fig. 1). These strains were distributed in the four PCR groups (8) as follows: IIa, 45%; IVb, 25%; IIb, 19%; and IIc, 11%. They were all susceptible to moxifloxacin. The MIC distribution was homogenous, with no association according to the PCR group.

Eight *L. monocytogenes* strains resistant to ciprofloxacin and

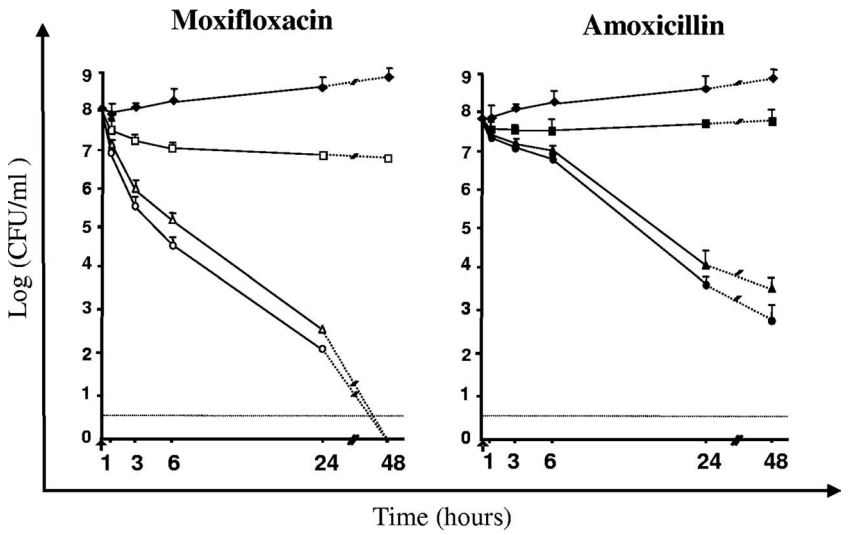

FIG. 2. In vitro efficacies of moxifloxacin and amoxicillin against extracellular forms of *L. monocytogenes*. Bactericidal activity was evaluated in time-kill curve experiments for the nontreated control (◆) and in MH broth supplemented with various concentrations of antibiotics: amoxicillin at 1× MIC (0.125 μg/ml; ■), amoxicillin at 4× MIC (0.5 μg/ml; ▲), amoxicillin at 400 × MIC (C_{max} or peak concentration, 50 μg/ml; ●), moxifloxacin at 1× MIC (0.5 μg/ml; □), moxifloxacin at 4× MIC (2 μg/ml; △), or moxifloxacin at 8× MIC (C_{max} or peak concentration, 4 μg/ml; ○). The results shown correspond to the means ± standard errors from three experiments. Arrows indicate the start of treatment.

isolated from patients since 2000 were also tested. The moxifloxacin MICs for these strains were not increased. Thus, no cross-resistance was detected between moxifloxacin and ciprofloxacin (see Table S1 in the supplemental material).

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

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

In MH broth, both antibiotics at concentrations above the MIC showed bactericidal activity against the extracellular forms of *L. monocytogenes* (Fig. 2). However, this bactericidal effect was obtained significantly more quickly with moxifloxacin than with amoxicillin: the initial inoculum was reduced by more than 3 $\log_{10}$ CFU/ml after 6 h 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 sterilization was observed after 24 h 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 the complete lysis of the macrophages 6 h after infection (Fig. 3). As early as after 3 h of incubation, the bacteria formed numerous filopodium-like projections within the cytosolic compartment of infected macrophages (Fig. 3).

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

By contrast, the formation of filopodium-like projections was not prevented by amoxicillin at the 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 3 h, 100% of the macrophages were still infected at this time, whatever the amoxicillin concentration used. Amoxicillin did not prevent the lysis of macrophages, which was also observed in control samples with no antibiotic (Fig. 3). This makes the interpretation of bacterial counts performed after 24 h of incubation difficult because of an alteration in the cellular monolayer. However, amoxicillin was bacteriostatic against the intracellular bacteria even after 24 h of incubation, if it was possible to obtain bacterial counts (Fig. 4).

Selection of strains resistant to moxifloxacin. No resistant strains were selected after 48 h 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 fluoroquinolones allows extended-spectrum activity against gram-positive bacteria (26, 34). Our epidemiological study with 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 resistance to moxifloxacin was detected, whereas resistance to

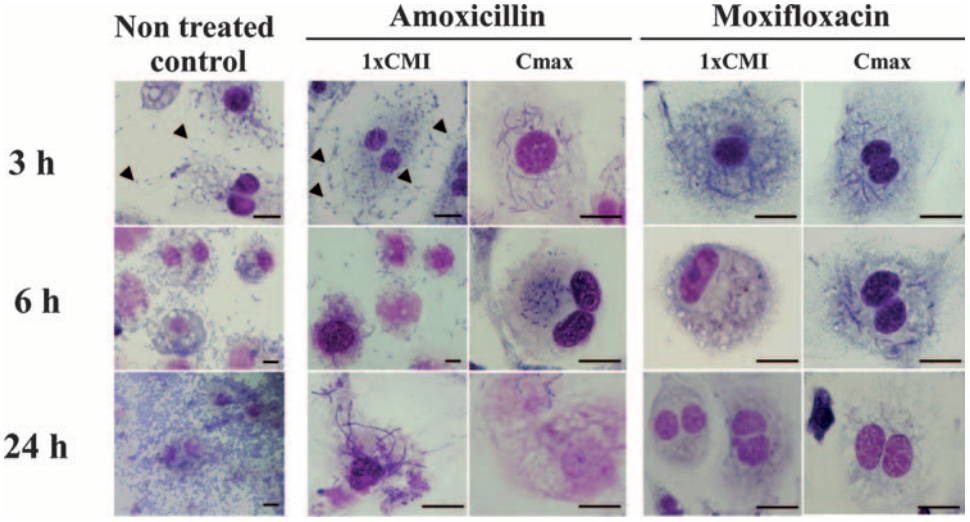

FIG. 3. Effects of amoxicillin and moxifloxacin on morphological aspects of macrophages infected with *L. monocytogenes*. Infected bone marrow-derived macrophages from BALB/c mice infected with *L. monocytogenes* were examined microscopically. Macrophages stained with May-Grunwald-Giemsa were observed at 3, 6, and 24 h of incubation with various concentrations of moxifloxacin or amoxicillin (1× MIC and C_{max}) or with no antibiotic for the nontreated control. C_{max} corresponds to the maximum serum concentration (or peak concentration) after the administration of clinically relevant doses of moxifloxacin and amoxicillin in humans (8× MIC and 400× MIC, respectively). Filopodium-like projections are shown by arrowheads. Bars, 15 μm.

ciprofloxacin is regularly detected among strains isolated from food, the environment, and humans (4, 12). The mechanism of resistance for these ciprofloxacin-resistant strains is due to the increased expression of *lde* (*Listeria* drug efflux) (12), which leads to the active and selective efflux of certain fluoroquinolones. Our result are consistent with the fact 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 alterations in the so-called quinolone resistance-determining regions. Moxifloxacin provides enhanced activity against DNA gyrase and topoisomerase IV due to a C-8 methoxyl group (26). The stepwise accumulation of mutations is therefore necessary for the expression of resistance to moxifloxacin, which thus prevents the selection of resistant bacteria, despite the use of large initial inocula in time-kill experiments (26, 35). Our results are consistent with the fact that this new fluoroquinolone exerts only weak selection pressure for resistance (26, 35).

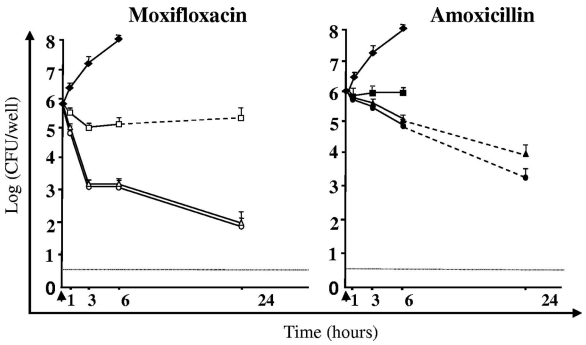

FIG. 4. In vitro efficacies of moxifloxacin and amoxicillin against intracellular reservoirs of *L. monocytogenes* cells. Bactericidal activity was evaluated for the nontreated control (◆) and in bone marrow-derived macrophages of BALB/c mice infected with *L. monocytogenes* and treated with various concentrations of antibiotics: amoxicillin at 1× MIC (0.125 μg/ml; ■), amoxicillin at 4× MIC (0.5 μg/ml; ▲), amoxicillin at 400× MIC (C_{max} or peak concentration, 50 μg/ml; ●), moxifloxacin 1× MIC (0.5 μg/ml; □), moxifloxacin at 4× MIC (2 μg/ml; △), or moxifloxacin at 8× MIC (C_{max} or peak concentration, 4 μg/ml; ○). The results shown correspond to the means ± standard errors from three experiments. Arrows represent the start of treatment. Dotted lines extrapolate the results of the bacterial counts after 24 h of incubation due to alterations in the macrophage monolayer.

Although both antibiotics kill extracellular forms of *L. monocytogenes*, moxifloxacin acts more quickly than amoxicillin (2, 30). Moreover, moxifloxacin achieved complete sterilization of cultures with large inocula after 24 h 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 favorable outcome.

However, the treatment of intracellular infections is complex. Antibiotic efficacy is dependent on the ability to eliminate the intracellular reservoirs of bacteria at the sites of infection (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 efficacies of new fluoroquinolones, including moxifloxacin, against the intracellular forms of *L. monocytogenes* in models of immortalized cell lines (J774 or THP-1 macrophages, HeLa and L929 cells) (2, 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 (1). Cellular pharmacokinetic parameters (intake, intracellular disposition in various subcellular compartments, accumulation, efflux) and pharmacodynamic parameters (bacterial responsiveness, coop-

eration with host defenses) govern the intracellular activities of antibiotics (1, 22, 25). 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 in cellular compartments, killing the intracytoplasmic forms of *L. monocytogenes* within the first 3 h (2, 22, 30). Despite the 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 polymerization, 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 filopodium-like projections observed even at the 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 spread of the bacteria to adjacent cells (6). Thus, moxifloxacin could be useful for preventing local spread at the site of infection 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, only a small proportion of which reaches the intracytoplasmic compartments of infected cells, presents weak and slow activity against intracytoplasmic bacterial growth (2, 17, 20). Enhanced effectiveness is observed with high doses of amoxicillin and a prolonged time of exposure of infected macrophages (2, 17, 20). The paradoxical activity of amoxicillin against intracellular bacteria may be explained by its action against extracellular bacteria released after cellular lysis, which thus prevents adjacent cells from becoming infected (2, 15, 17, 20). The paradoxical activity could also be explained by antibiotic phagocytosis, which has already been described for glycopeptide agents (17, 33). In this case, the restricted phagosomal localization of these antibiotics may explain why they do not prevent the formation of filopodium-like projections, as seen with amoxicillin in the early times of the present experiments. 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, which ensures the presence of sufficient concentrations at sites of infection, and because cellular immunity acts complementary to antibiotic treatment in the majority of cases (14, 25, 32).

Conclusions. Our results support the evidence for the 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 of this drug in humans.

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