



## EFFICACY OF SOME SECOND- AND THIRD-GENERATION FLUOROQUINOLONES AGAINST *BRUCELLA MELITENSIS* 16M IN BALB/C MICE

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### Summary

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Brucellosis is considered as a major endemic disease in Syria. There is a paucity of data regarding suitable antibiotic prophylaxis. BALB/c mice were challenged with either a low ( $10^4$  CFU) or a high ( $10^7$  CFU) concentration of *Brucella melitensis*. Antibiotics were administered prior to, post or at the same time as the bacterial challenge. Mice were killed either 48 hours or 30 days after the last injection of antibiotics. Efficacy of antibiotics to limit or control infection was determined by reduced bacterial burden in mice spleens. When a low concentration was injected, sparfloxacin, levofloxacin and doxycycline were effective 48 hours after the cessation of treatment. Sparfloxacin protection was observed 30 days after the cessation of treatment. After a high injected concentration, antibiotics were effective 48 h after the cessation of treatment just in prior to exposure groups. Only sparfloxacin was effective 30 days after the cessation of treatment in prior to exposure group. In conclusion, these results suggest that sparfloxacin and levofloxacin have almost the same protective efficacy as doxycycline against a low concentration of *B. melitensis* infections.

**Key words:** antibiotic prophylaxis, BALB/c mice, *Brucella melitensis*, quinolones

### INTRODUCTION

Brucellosis is a zoonotic disease with worldwide distribution (Corbel, 1997), but it is most frequent in the Mediterranean basin and South America (Young, 1995). *B. melitensis*, *B. abortus* and *B. suis* are the major causes of human brucellosis worldwide. Disease from marine species has also emerged (Sohn *et al.*, 2003). Its treatment remains complex and largely based on the principles applied

more than half a century ago. Doxycycline-rifampicin and doxycycline-streptomycin combinations still the recommended treatment of human brucellosis by the World Health Organization (Anonymous, 1986). Despite all regimens universally applied in clinical practice (Ariza *et al.*, 2007), relapses are still seen. The effective treatment of relapses is still unclear despite that its risk factors are well known

(Solera *et al.*, 1998; Ariza *et al.*, 1995). Thus, there is a need to evaluate other antibiotic treatments. The intracellular penetration and excellent *in vitro* activity of the fluoroquinolones make them attractive in treating intracellular infections such as brucellosis; and might have the potential to be useful in the prophylaxis against *Brucella* infection. The MIC<sub>90</sub> (minimum inhibitory concentration for 90% of the organisms) values of ofloxacin, sparfloxacin and levofloxacin, against *Brucella spp.* have been determined *in vitro* to be from 0.5 µg/mL (Kocagoz *et al.*, 2002) to 2 µg/mL (Trujillano-Martin *et al.*, 1999) for ofloxacin; 0.5 µg/mL (Trujillano-Martin *et al.*, 1999; Kocagoz *et al.*, 2002) for levofloxacin, and 0.12 µg/mL for sparfloxacin (Qadri *et al.* 1995; Kocagoz *et al.*, 2002).

Ideally, suitable prophylaxis treatment should use one antibiotic only applied orally for a short period (Atkins *et al.*, 2010). However, some efficacy against brucellosis in humans was observed using ciprofloxacin-doxycycline or ciprofloxacin-rifampicin combinations (Agalar *et al.*, 1999). On the other hand, neither ciprofloxacin nor ofloxacin showed a good protection against *Brucella* infections in murine model (Shasha *et al.*, 1992; Atkins *et al.*, 2009a). Newer fluoroquinolones have been developed in recent years. To our knowledge, the prophylaxis effects of the third generation of quinolones (such as sparfloxacin and levofloxacin), and some drugs of the second generation are not yet evaluated in murine models.

This study aimed to assess the role of sparfloxacin, ofloxacin and levofloxacin, compared with doxycycline, for the prophylaxis of *B. melitensis* infection using a murine model.

## MATERIALS AND METHODS

### *Bacteria*

*B. melitensis* strain 16M, obtained from the Laboratory of Microbiology and Immunology URBM (University of Namur, Belgium), was used as the inoculation strain in this study. *Brucella* were grown for 48 h in 2YT agar (peptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L; agar, 13 g/L [GibcoBRL]) at 37 °C. Bacteria were harvested into 20 mL of sterile phosphate-buffered saline (PBS) and the bacterial suspension was standardised to 10<sup>10</sup> colony-forming units (CFU)/mL prior to dilution to appropriate concentrations of inoculates. The concentrations were determined retrospectively by enumeration of ten-fold dilutions of the inoculates on 2YT plates. All experiments with live *Brucella* were performed in biosafety level 2 facilities.

### *Antibiotics*

Doxycycline, levofloxacin, ofloxacin, and sparfloxacin (all from Sigma, St. Louis, USA) were dissolved as per manufacturer's recommendations to a working concentration of 8 mg/mL. Antibiotics were prepared freshly each day and sterilised through a 0.2 µm filter.

### *Animals*

Two hundred sixty female BALB/c mice (7 to 8 weeks old, purchased from Charles River Laboratories, France) were randomly distributed into fifty-two experimental groups of five mice each. The mice were kept in conventional animal facilities and received water and food *ad libitum*. The experimental procedures on mice and the facilities used to hold the experimental animals were in accordance to the National law (Real Decreto 233/1988, in BOE number 67). The protocol of Atkins *et al.*

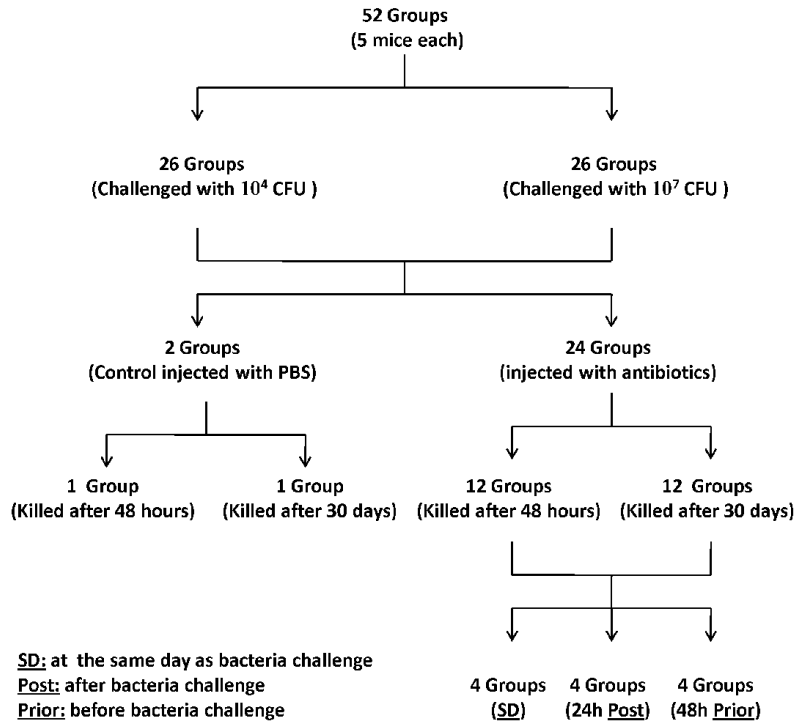


Fig. 1. Schematic design of experimental procedure.

(2009a) was used with some modifications. A scheme of experimental design is shown in Fig. 1. Briefly, mice were administered 100  $\mu$ L of PBS (positive control) or antibiotic solution (equivalent to 40 mg/kg in a 20 g mouse) twice daily by subcutaneous injection. The antibiotic treatment was started either 48 h prior to challenge (continued for 7 days), at the time of challenge (continued for 5 days) or 24 h after challenge (continued for 5 days). PBS was started at the time of challenge (continued for 5 days) for all four control groups. Mice were challenged with either a low dose ( $10^4$  CFU, 24 groups) or a high dose ( $10^7$  CFU, 24 groups) of *B. melitensis* 16M by intraperitoneal injection (100  $\mu$ L). Animals were culled either 48 h or 30 days after the final

antibiotic administration. Post mortem, spleens were removed and homogenised in 5 mL distilled water using a 80-Biomaster stomacher (Seward, England). Bacterial loads were determined following enumeration of ten-fold serial dilutions on 2YT plates (incubated for 3 days at 37 °C in air).

#### Statistical analyses

Data were transformed into  $\log_{10}$  CFU. Differences in CFU between the treated and untreated groups were evaluated by one way analysis of variance (ANOVA). All analyses were conducted with GraphPad Prism v.5.0. Bonferroni's post-test used to compare individual time points with the control. *P* values of 0.05 or less were considered statistically significant.

RESULTS

When a concentration of  $10^4$  CFU of *B. melitensis* 16M was injected, significant protection was observed in all mice groups treated with sparfloxacin, levofloxacin and doxycycline and killed 48 h after the cessation of antibiotic treatment (Table 1,  $P < 0.001$  in all cases). In addition, sparfloxacin protection was observed 30 days after the cessation of antibiotic treatment in all mice groups (Table 2,  $P < 0.001$  in all cases). Doxycycline was effective in only two groups, i.e. at the time of exposure and 24 h after exposure

(Table 2:  $P < 0.001$  in both); whereas levofloxacin was effective in the 24 h after exposure group only (Table 2,  $P < 0.001$ ). Finally, ofloxacin showed some protective effect 48 h after the cessation of antibiotic treatment in only two groups – at the time of exposure or 24 h after exposure (Table 1,  $P < 0.001$  for both).

Moreover, when a concentration of  $10^7$  CFU of *B. melitensis* 16M was injected, sparfloxacin protection was observed 48 h after the cessation of antibiotic treatment at the same day as exposure and prior to exposure groups (Table 3,  $P < 0.05$  and  $P < 0.001$ , respectively); and at

**Table 1.** Protective efficacy of doxycycline, sparfloxacin, ofloxacin and levofloxacin against *Brucella melitensis* in BALB/c mice challenged with  $10^4$  CFU of *B. melitensis* 16M and killed 48 hours after the last injection of the antibiotic. Data are presented as mean± standard deviation (n=5)

Antibiotics administration	Control	Doxycycline	Sparfloxacin	Ofloxacin	Levofloxacin
At the time of challenge	2.00±0.09	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
24 h after challenge	2.00±0.09	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
48 h before challenge	2.00±0.09	0 <sup>a</sup>	0 <sup>a</sup>	3.43±0.34	0 <sup>a</sup>

a:  $P < 0.001$  versus control.

**Table 2.** Protective efficacy of doxycycline, sparfloxacin, ofloxacin and levofloxacin against *Brucella melitensis* in BALB/c mice challenged with  $10^4$  CFU of *B. melitensis* 16M and killed 30 days after the last injection of the antibiotic. Data are presented as mean± standard deviation (n=5)

Antibiotics administration	Control	Doxycycline	Sparfloxacin	Ofloxacin	Levofloxacin
At the time of challenge	2.78±0.11	0 <sup>a</sup>	0 <sup>a</sup>	3.36±0.26	3.64±0.32
24 h after challenge	2.78±0.11	0 <sup>a</sup>	0 <sup>a</sup>	3.65±0.28	0 <sup>a</sup>
48 h before challenge	2.78±0.11	3.11±0.26	0 <sup>a</sup>	3.36±0.22	3.36±0.69

a:  $P < 0.001$  versus control.

**Table 3.** Protective efficacy of doxycycline, sparfloxacin, ofloxacin and levofloxacin against *Brucella melitensis* in BALB/c mice challenged with  $10^7$  CFU of *B. melitensis* 16M and killed 48 hours after the last injection of the antibiotic. Data are presented as mean± standard deviation (n=5)

Antibiotics administration	Control	Doxycycline	Sparfloxacin	Ofloxacin	Levofloxacin
At the time of challenge	3.89±0.036	4.06±0.36	3.18±0.21 <sup>b</sup>	3.21±0.49 <sup>b</sup>	4.1±0.44
24 h after challenge	3.89±0.036	0 <sup>a</sup>	3.8±0.50	3.33±0.71	0 <sup>a</sup>
48 h before challenge	3.89±0.036	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	2.29±0.50 <sup>c</sup>

a: P<0.001, b: P<0.05 and c: P<0.01 versus control.

**Table 4.** Protective efficacy of doxycycline, sparfloxacin, ofloxacin and levofloxacin against *Brucella melitensis* in BALB/c mice challenged with  $10^7$  CFU of *B. melitensis* 16M and killed 30 days after the last injection of the antibiotic. Data are presented as mean± standard deviation (n=5)

Antibiotics administration	Control	Doxycycline	Sparfloxacin	Ofloxacin	Levofloxacin
At the time of challenge	7.68±0.377	7.24±0.26	7.56±0.37	7.20±0.39	7.50±0.21
24 h after challenge	7.68±0.377	7.14±0.51	4.34±0.40 <sup>b</sup>	3.93±0.40 <sup>a</sup>	0 <sup>a</sup>
48 h before challenge	7.68±0.377	7.44±0.35	0 <sup>a</sup>	3.57±0.26 <sup>a</sup>	7.36±0.42

a: P<0.001, b: P<0.01 versus control

30 days after the cessation of antibiotic treatment in 24 h after exposure and prior to exposure groups (Table 4, P<0.01 and P<0.001, respectively). Levofloxacin and doxycycline were effective 48 h after the cessation of antibiotic treatment in two groups, i.e. 24 h after exposure group (Table 3, P<0.001 in both cases) and prior to exposure group (Table 3, P<0.01 and P<0.001, respectively). On the other hand, levofloxacin was effective 30 days after the cessation of antibiotic treatment in 24 h after exposure group (Table 4, P<0.001). Finally, ofloxacin showed some

protection effect 48 h after the cessation of antibiotic treatment in two groups, namely in the group treated at the same time as exposure or 48 h prior to exposure (Table 3, P<0.05 and P<0.001, respectively); as well as in 24 h after exposure and prior to exposure groups 30 days after the cessation of antibiotic treatment (Table 4; P<0.001 in both cases).

#### DISCUSSION

*Brucella* is considered to be susceptible to the antibiotics recommended by the WHO

for treatment of brucellosis. Relapses, at a rate of about 10 percent, usually occur in the first year after the infection, but they are caused in most cases by inadequate treatment (Pappas *et al.*, 2005). Strains resistant to the main antimicrobial agents may emerge and lead to treatment failure (Marianelli *et al.*, 2004). Many clinical studies and researches were performed to evaluate the efficacy of quinolones in such cases.

Data concerning the prophylactic efficacy of some quinolones were disappointing (Shasha *et al.* 1992; Atkins *et al.*, 2009b; Atkins *et al.*, 2010). Atkins *et al.* (2009a) were the only to highlight the potential ability of ciprofloxacin to provide a low level of protection against brucellosis, compared with doxycycline.

*In vitro* MICs data indicate that spar-floxacin, levofloxacin and ofloxacin are effective at killing *Brucella* spp. Qadri *et al.* (1995) found that spar-floxacin exhibited excellent *in vitro* activity against clinical isolates of *B. melitensis*. Alişkan *et al.* (2008) observed that in a total of 65 *B. melitensis* strains isolated from blood and bone marrow specimens, spar-floxacin was the most effective fluoroquinolone (MIC<sub>90</sub>=0.064 mg/L), followed by levofloxacin and ciprofloxacin (MIC<sub>90</sub>=0.125 mg/L), and ofloxacin (MIC<sub>90</sub>=0.50 mg/L). In a study performed on 60 *B. melitensis* isolates obtained from blood and fluids, Kilic *et al.* (2008) showed that levofloxacin was the most active fluoroquinolone agent (MIC<sub>90</sub>: 0.094 µg/mL), followed by moxifloxacin (MIC<sub>90</sub>: 0.125 µg/mL) and ciprofloxacin (MIC<sub>90</sub>: 0.19 µg/mL). In addition, Yamazhan *et al.* (2005) found that the conventional agent doxycycline (MIC<sub>90</sub>: 0.50 µg/mL) was more active than levofloxacin (MIC<sub>90</sub>: 2 µg/mL) against *B. melitensis*, *in vitro*. The results of Arda *et al.* (2004) indicated that

levofloxacin is ineffective in the treatment of experimental murine *Brucella abortus* infection either as monotherapy or in combination with rifampicin. On the other hand, in a prospective study performed on uncomplicated 118 patients, Ersoy *et al.* (2005) found that the use of combination therapy of ofloxacin plus rifampicin for 6 weeks was as effective as doxycycline plus rifampicin and doxycycline plus streptomycin, whereas Saltoglu *et al.* (2002) established that a 45-day course of doxycycline plus ofloxacin combination was as effective as the doxycycline plus rifampicin combination in patients with brucellosis.

Our data indicate that when the infection was performed with a high concentration of *B. melitensis* 16M (10<sup>7</sup> CFU), quinolones and doxycycline had a relatively poor efficacy, especially when antibiotics treatment started at the same time as infection. Only spar-floxacin was effective 30 days after the last injection of antibiotic when administrated prior to challenge. However, spar-floxacin and ofloxacin were more effective than doxycycline 30 days after the cessation of treatment in at the same day as infection group (P<0.001 in both cases) and 24 hours after exposure groups (P<0.001 in both cases); whereas, levofloxacin was more effective than doxycycline in 24 hours after exposure group (P<0.001). In contrast, doxycycline was more effective than spar-floxacin and ofloxacin 48 hours after the cessation of treatment in 24 hours after exposure group (P<0.001, in both cases); and it was more effective than levofloxacin in prior to exposure group (P<0.001).

On another hand, our results also revealed that spar-floxacin, and at a certain extent levofloxacin, had almost the same good efficacy as doxycycline when a low concentration of *B. melitensis* 16M

( $10^4$  CFU) was used. Sparfloxacin was effective in all mice groups 30 days after the last injection of antibiotic, whereas doxycycline was effective in two groups only. Nevertheless, doxycycline was more effective than levofloxacin 30 days after the cessation of treatment on the same day as infection group ( $P < 0.001$ ); and sparfloxacin was more effective than doxycycline 30 days after the end of treatment in prior to exposure group ( $P < 0.001$ ). Unfortunately, ofloxacin showed relatively inconsistent effects.

In another work of our group (Safi *et al.*, 2013) we found that doxycycline-ciprofloxacin and rifampicin-levofloxacin combinations have the potential to provide almost the same level of protection against a low concentration of *B. melitensis*, in comparison with the doxycycline-rifampicin combination.

In conclusion, our results highlight the potential of sparfloxacin and levofloxacin to provide almost the same level of protection against *B. melitensis* in comparison with doxycycline, especially when a low concentration of bacteria was used. Moreover, these quinolones seems to be more effective than doxycycline 30 days after the end of treatment when a high concentration of bacteria was used.

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