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Emergence of quinolone-resistant, topoisomerase-mutant *Brucella* after treatment with fluoroquinolones in a macrophage experimental infection model



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ABSTRACT

Aim of the study: To determine the activity of fluoroquinolones (FQ) and the selection of FQ-resistant mutants in a macrophage experimental infection model (MEIM).

Material and methods: Canine macrophages were inoculated with *Brucella melitensis* ATCC 23457 (WT), achieving intracellular counts of around 105 CFU/mL. Cell cultures were incubated in the presence of ciprofloxacin (CIP), levofloxacin (LEV), moxifloxacin (MOX), and doxycycline (DOX). After cell lysis, surviving microorganisms were plated for count purposes, and plated onto antibiotics-containing media for mutant selection. Topoisomerases mutations were detected by PCR and sequencing.

Results: Bacterial counts after cell lysis were 14.3% (CIP), 65.3% (LEV), and 75% (MOX) lower compared to the control. Quinolone-resistant mutants emerged in cell cultures containing CIP and LEV with a frequency of around 0.5×10^{-3} . All mutants showed an Ala87Val change in *GyrA*. Mutants had FQs MICs around $10 \times$ WT. The ability of these mutants for infecting new macrophages and the intracellular lysis after antibiotic exposure did not change significantly. No 2nd step FQ-resistant mutants were selected from 1st step mutants.

Conclusions: Intracellular activity of FQs is low against WT and *gyrA*-mutant *Brucella*. FQs easily select *gyrA* mutants in MEIM. The ability of mutants for infecting new macrophages remains unchanged. In this MEIM, 2nd step mutants do not emerge.

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Selección de *Brucella* resistente a quinolonas por mutación en topoisomerasas, tras tratamiento con fluoroquinolonas, en un modelo experimental de infección en macrófagos

RESUMEN

Objetivo del estudio: Conocer la actividad de fluoroquinolonas (FQ) y la selección de mutantes resistentes (MR) a FQ en un modelo experimental de infección en macrófagos.

Material y métodos: Se inocularon macrófagos de origen canino con la cepa tipo *Brucella melitensis* ATCC 23457 (CT) hasta alcanzar recuentos intracelulares de aproximadamente 105 UFC/mL. Los cultivos celulares fueron incubados en presencia de ciprofloxacino, levofloxacino, moxifloxacino y doxiciclina. Una vez lisadas las células, los microorganismos supervivientes fueron sembrados en placas sin antibiótico para recuento, y en medios de cultivo conteniendo antimicrobianos para selección de MR. Los MR a topoisomerasas se caracterizaron mediante PCR y secuenciación.

Palabras clave:

Fluoroquinolonas

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Resultados: Los recuentos de microorganismos supervivientes tras el tratamiento con FQ y la lisis celular fueron: 14,3% ciprofloxacino, 65,3% levofloxacino y 75% moxifloxacino con respecto al control. Se seleccionaron MR a FQ en los cultivos celulares que contenían ciprofloxacino y levofloxacino, con una frecuencia en torno a $0,5 \times 10^{-3}$. Todos los MR seleccionados portaban un cambio Ala87Val en *gyrA*, y mostraban CIM de FQ en torno a 10x la de la CT. La capacidad de estos MR para infectar nuevos macrófagos y su lisis intracelular tras tratamiento antibiótico no se modificaron de manera significativa con respecto a la CT. Usando la misma metodología, no se seleccionaron MR de segundo nivel a partir de los MR de primer nivel obtenidos.

Conclusiones: La actividad intracelular de FQ frente a *Brucella* es baja, tanto frente a la CT como frente a mutantes con cambios en *gyrA*. Las FQ seleccionan con facilidad mutantes con cambios en *gyrA* en este modelo experimental de infección en macrófagos. La capacidad de estos mutantes para infectar nuevos macrófagos permanece intacta. En este modelo experimental de infección en macrófagos no se observó la selección de mutantes de segundo nivel.

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Introduction

Brucellosis remains an important problem in many geographic areas, including the Mediterranean basin. Usually recommended treatments, such as doxycycline plus rifampicin or doxycycline plus streptomycin, are efficacious but they are not free of side effects and need to be administered for several weeks.^{1–4}

When systemic fluoroquinolones (FQs) began to be available, they were considered a possible alternative for the treatment of brucellosis because of their activity against Gram-negative rods, good tissue levels and intracellular activity.⁵ Nevertheless, further clinical studies showed therapeutic failures and relapse rates of ca. 25% compared with <10% for conventional rifampicin or streptomycin plus doxycycline treatments.⁶

Some studies have suggested that these poor results might be associated with the reduced activity of FQs in an acidic environment. *Brucellae* are intracellular pathogens whose pathogenicity is mainly associated to their capacity to survive into cells, especially into phagolysosomes, where the acidic environment might impair FQ activity.^{7–9} Nevertheless, the involvement of topoisomerases mutations as a cause of FQ resistance has been seldom studied. The similarity between *Brucella* and *Escherichia coli gyrA* is only 70%, and the position equivalent to the serine 83 in *E. coli* is an alanine residue in *Brucella*. These differences between *Brucella* GyrA and the GyrA protein from other Gram negative bacteria more susceptible to FQs, such as enterobacteria, might explain the relatively high MIC (around 0.2–0.5 mg/L) of CIP against wild-type *Brucella*. Studies on human pathogens harboring a GyrA protein similar to *Brucella*, such as *Rickettsia* and *Bartonella*, show MICs of CIP around 0.5–1 mg/L, and MICs of 0.1–0.5 mg/l for other generally more active FQs such as sparfloxacino.^{10,11}

Turkmani et al.¹² obtained recently FQ-resistant mutants *in vitro* from *Brucella abortus* and *B. melitensis* by growing them in the presence of antibiotics on antibiotic gradient plates. We also published recently the emergence of Ala71 to Ser *gyrA* mutants from *B. melitensis* ATCC 23457.¹³

The role of intracellular environment and FQ-resistant mutants emergence in *Brucella* survival has not been studied in macrophage infection models. We have studied intraphagocytic survival of *Brucella* in presence of extracellular inhibitory concentrations of FQs, FQ-resistant mutants selection under these conditions, and capacity of FQ-resistant mutants selected for newly infecting phagocytes and for surviving inside them.

Material and methods

The strain used was *B. melitensis* biotype 2, strain 63/9 ATCC 23457. The microorganism was grown for 48 h, on chocolate

Polivtex[®] agar (bioMérieux, Le Balme Les Grottes, France), at 37 °C in a 5% CO₂ atmosphere.

The FQs tested were norfloxacin (NOR) (Merck, Sharp&Dohme, Whitehouse Station, NJ, USA), ciprofloxacin (CIP) (Bayer AG, Leverkusen, Germany), levofloxacin (LEV) (GlaxoSmithKline, Coraopolis, PA, USA) and moxifloxacin (MOX) (Bayer AG, Leverkusen, Germany), which were kindly provided by their respective manufacturers. MICs were determined by the agar-dilution method and by the E-test method, according CLSI (Clinical and Laboratory Standards Institute) general guidelines. The MICs of the antibiotics against *B. melitensis* ATCC 23457 were as follows: NOR, 1 µg/mL; CIP, 0.25 µg/mL; LEV, 0.12 µg/mL; and MOX, 0.25 µg/mL.

Doxycycline (DOX) (Pfizer, New York, USA) was kindly provided by the manufacturer. Gentamicin was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Antibiotic susceptibility was determined following previously described methods¹² and the general recommendations contained in the CLSI guidelines.¹⁴

Cell lines used were canine peritoneal macrophages (ATCC CRL-10389 DH82, Vircell, Granada, Spain). The cell culture medium used was Earle' balanced salt solution (Sigma–Aldrich, St. Louis, MO, USA) with 15% fetal calf serum (Sigma–Aldrich, St. Louis, MO, USA) and glutamine 2 mM (Sigma–Aldrich, St. Louis, MO, USA). The cell suspensions were placed in 25-cm² tissue-culture flasks (Costar, Cambridge, MA, USA), and macrophages were allowed to adhere for 24 h in a humidified CO₂ 5% incubator at 37 °C. Non-adhering cells were then washed off. Adhering cells were washed three times with 3 ml of complete medium. The adhering cells were then incubated in 3 ml of complete medium for 2 h. The number and viability of macrophages obtained by this procedure were determined by morphological examination and Trypan blue exclusion¹⁵.

B. melitensis biovar 2, ATCC 23457 from overnight cultures were opsonized at 37 °C for 60 min in ascitic fluid, previously sterilized by filtration (0.22 Sterivex-GP Millex-GV), containing a subagglutinating dilution (1 in 200 in complete RPMI 1640) of the mouse monoclonal antibody BmE 10-5 with specificity against the lipopolysaccharide (S-LPS) of *Brucella* spp.¹⁶ Bacterial suspensions were added to ascitic fluid and in the volume necessary for reaching the number of opsonized CFUs required. Opsonized bacteria were then centrifuged, washed and diluted in complete medium.

To infect macrophages, around 1×10^5 CFU/mL of opsonized *B. melitensis* biovar 2, ATCC 23457 in complete medium was added (bacteria/macrophage rate was around 100:1), and incubated for 90 min in a humidified CO₂ 5% incubator at 37 °C, to allow opsonized bacteria penetration into the macrophages. Non-phagocytosed bacteria were washed off. Then, 3 ml of complete medium containing gentamicin (100 µg/mL) were added to the macrophages to kill

any remaining extracellular bacteria.¹⁷ One flask was added only with gentamicin (control flask), while the remaining flasks were also added with CIP, LEV, MOX or DOX, at concentrations similar to serum concentrations reached at the usual dosage (6 µg/mL of CIP and LEV, 4 µg/mL of MOX and DOX).¹⁸

The macrophages were cultured for 2 h at 37 °C in a CO₂ 5% v/v incubator, and washed three times with complete medium. Complete medium from washing was then plated on chocolate Polivite^R agar to check the absence of extracellular microorganisms.

Cell cultures were then added with 5 ml of 0.5% Tween 20 (Sigma–Aldrich, St. Louis, MO, USA) sterilized by filtration, and incubated for 30 min at room temperature to lyse macrophages. The supernatant was collected, and 1 ml of supernatant, and of 1:10 and 1:100 dilutions of it in complete medium, were plated onto chocolate Polivite^R agar plates in order to count colonies forming units (CFUs) obtained, which would reflect the number of intracellular *B. melitensis* in the cell culture.

1 ml of supernatant, and of 1:10 and 1:100 dilutions of it in complete medium, were plated onto chocolate Polivite^R agar containing CIP, MOX and LEV at concentrations 4× their respective MICs for the ATCC 23457 strain, in order to detect hypothetical resistant mutants generated in the cell culture. MICs of the three FQs for the presumptive resistant mutants selected were checked by the agar dilution method. The mutant emergence rate was calculated and the quotient between the number of presumptive mutants detected on 4× MIC plates and the whole intracellular bacterial count in absence of FQs (bacterial count in control flask).

Then, the same mutants selection process was developed for obtaining 2nd step resistant mutants, using as infecting microorganisms the 1st step resistant mutants previously obtained. Chocolate Polivite^R agar plates inoculated for selecting 2nd step mutants contained CIP, LEV and MOX at 4× the 1st step mutants respective MICs.

DNA was obtained from resistant mutants by conventional methods and purified using ExoSAP-IT (GE Healthcare, Piscataway, NJ, USA). Polymerase chain reaction (PCR) for the whole *gyrA*, *gyrB* and *parC* genes was performed in an Eppendorf Mastercycler[®] (Eppendorf AG, Hamburg, Germany) using PCR Master Mix (Promega Corp., Madison, WI, USA). The primers used are shown in Table 1. The primers were designed on the basis of *gyrA*, *gyrB* and *parC* sequences of *B. melitensis* biovar 3 strain NI, chromosome I (Accession No. CP002931). The amplification protocol used was as follows: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 48 °C (*parC*-1, *parC*-3, *parC*-4 and *parC*-5), 50 °C (*gyrA*-1, *gyrA*-2, *parC*-2), 53 °C (*gyrB*-1, *gyrB*-2, *gyrB*-3, *gyrB*-5), 54 °C (*gyrB*-4) and 62 °C (*gyrA*-3,

Table 2

Intracellular counts of *B. melitensis* ATCC 23457 obtained in the control flask and in the flask added with FQs and DOX.

Flask	Extracellular concentration (mg/l)	Intracellular bacterial count (CFU/ml)
Control	–	4.9×10^4
CIP	6	4.2×10^4
LEV	6	1.7×10^4
MOX	4	1.2×10^4
DOX	4	0

Table 3

FQ-resistant mutants obtained from *B. melitensis* ATCC23457 on chocolate agar plates added with FQs at 4 × MIC concentrations.

Cell culture flask added with	Resistant mutants detection on agar containing 4 × MIC of		
	CIP	LEV	MOX
CIP	0.5×10^{-3}	0.6×10^{-3}	0.5×10^{-3}
LEV	0.6×10^{-3}	0.4×10^{-3}	0.5×10^{-3}
MOX	0	0	0

gyrA-4, *gyrA*-5), and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 3 min.

Amplified products were purified with ExoSAP-IT and sequenced. Sequences were aligned and compared with *gyrA*, *gyrB* and *parC* sequences of *B. melitensis* biovar 3 strain NI, chromosome I using BLAST software (National Center for Biotechnology Information, Bethesda, MD, USA).

Results

Intracellular counts of *Brucella* in absence of FQs and after FQs or DOX addition appear in Table 2. These results show that, after 2 h of incubation with CIP, intracellular bacterial count was similar to the control. LEV and MOX reduce the original bacterial count to 1/3–1/4, while DOX eradicated the whole intracellular bacterial population.

When FQ-resistant mutants emergence was determined among the intraphagocytic surviving populations, mutants were detected in the flasks treated with CIP and LEV, as appears in Table 3, while no mutants were obtained from the flask containing MOX, and from the flask that had been added only with gentamicin. FQ-resistant mutants selection rate with CIP and LEV was high, around 0.5×10^{-3} in all the cases when mutants were found.

Three to five colonies were picked from each plate for susceptibility and molecular study. Four susceptibility profiles were found, though MICs were similar for all mutants selected (Table 4), and further molecular analysis showed that all of them harbored the same topoisomerases mutations.

Table 1

Primers used for amplifying and sequencing topoisomerase *Brucella* genes.

Gene	Direct primer	Reverse primer	Fragment size (bp)
<i>gyrA</i> -1	5'CCATTGCGCTTTCTCTCA	5'TCTTTGCTGGCCGCAA	586
<i>gyrA</i> -2	5'GCGAGATCGCCATCGA	5'ATATGGTCGTACCGTCA	618
<i>gyrA</i> -3	5'GCGCAGATAGGTCTGTAGC	5'TCAGCTGTCTCGACATGCTGC	621
<i>gyrA</i> -4	5'CGATACGCGCTTGTGAGCAG	5'TGGTTTCAGGCGGTATTGCGGTGC	618
<i>gyrA</i> -5	5'TCCATCAGTTCGACAGTTTCG	5'TCGAGGAAATGCAGCGGTCT	589
<i>parC</i> -1	5'GACGCGAAGAACACA	5'GCCGAACCGTTTGA	509
<i>parC</i> -2	5'CTGGAAGGCATCAACGAA	5'TCTGCACCTGATAGGGAA	473
<i>parC</i> -3	5'TTCCCTATCAGGTGCAGA	5'GGTGTGATCTTCTTCCA	619
<i>parC</i> -4	5'AGAAGATCAGCACCGAA	5'CCGGAACAATGAAACCA	465
<i>parC</i> -5	5'CTTCGTGCACGATCCA	5'ACCTTCGGCACCAGA	415
<i>gyrB</i> -1	5'CCCATAGGCGCGAGA	5'ATGCAACGGCACAGCA	466
<i>gyrB</i> -2	5'TCGAGACGCTTGGCAA	5'GCACCTCCATCGGCAA	497
<i>gyrB</i> -3	5'AACGCACGCGTTCCA	5'CGACAGATCACGGGCTA	499
<i>gyrB</i> -4	5'CGTGATCTGTGGGTCA	5'GCTGTGATCTGGCTGA	514
<i>gyrB</i> -5	5'CCGGAACCTGTAGGAA	5'CCCGAGATGAATCCGAA	356

Table 4MIC (μg/mL) of FQs for *B. melitensis* ATCC 23457 and for mutants obtained.

Microorganisms	MIC (μg/ml)			
	Norfloxacin	Ciprofloxacin	Levofloxacin	Moxifloxacin
<i>B. melitensis</i> biovar 2 ATCC 23457	1	0.2	0.1	0.2
Resistant mutant 1	32	4	2	2
Resistant mutant 2	64	2	2	4
Resistant mutant 3	32	1	1	2
Resistant mutant 4	32	4	1	4

Table 5*gyrA* mutations observed in intracellular FQ-resistant mutants obtained from *B. melitensis* ATCC 23457.

Strains	<i>gyrA</i> region
<i>E. coli</i> WT	46-RRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDS ^a AVY-86
<i>B. melitensis</i> WT	50-RRILHAMNEMNLAYNRPYRKSAGVVGVMGKYHPHGDA ^b SIY-90
<i>B. melitensis</i> R mutant	50-RRILHAMNEMNLAYNRPYRKSAGVVGVMGKYHPHGDV ^c SIY-90

^a *E. coli gyrA* Ser-83.^b *B. melitensis gyrA* Ala-87.^c FQ-mutant *B. melitensis gyrA* Val-87.

All the resistant mutants obtained in macrophage cultures had a C to T mutation in the position 260 of *gyrA* gene, leading to a Ala (GCT) 87 to Val (GTT) change. Position 87 of GyrA from *B. melitensis* corresponds to position 83 of GyrA from *E. coli* (Accession No. X06373). This position corresponds to a Ser residue in wild-type *E. coli* gyrase subunit A, and is the main mutation point in topoisomerase-resistant *E. coli* (Table 5).

No significant changes were observed in *gyrB* and *parC* genes, as compared to wild-type strain.

Results obtained when the experiment of macrophage penetration was repeated using mutants obtained, instead of wild-type *B. melitensis* ATCC 2457, appear in Table 6.

No differences were observed in comparison to the behavior of the wild-type strain. Intracellular bacterial count, in absence of antibiotics, ranged from 2.8×10^4 to 5.3×10^4 CFU/mL; thus, the capacity of FQ-resistant mutants to penetrate into the phagocytes seems to remain unchanged. CIP led to very modest bacterial count reductions, while LEV and MOX reduced bacterial count to 1/3–1/4, and DOX cleared all the intraphagocytic microorganisms, as also happened when type strain was used.

When emergence of resistant mutants was determined among the intraphagocytic surviving populations, no 2nd step mutants were detected in any flask.

Discussion

Results obtained in the first experiment suggest that antibacterial activity of FQs against intraphagocytic *Brucellae* is moderate to low, much lower than DOX. Bacterial counts hardly decreased when cell cultures are treated with CIP, and decrease to 1/3–1/4 with LEV and MOX, while DOX eradicated all the intracellular *Brucellae*. That may explain the failures of FQs in the treatment of brucellosis, independently of the emergence of resistant mutants. This behavior may be due to the low pH, as has been suggested previously,^{7–9} or

might be associated to more complex factors. One important factor is probably the fact that *Brucella*, as happens to other microorganisms with GyrA proteins harboring significant differences with respect to conventional GyrA proteins found in enterobacteria,^{10,11} have a lower susceptibility to FQs. This way, *B. melitensis* ATCC 23457 has MICs of CIP, LEV and MOX of 0.1–0.2 mg/L, while expected MIC ranges of CIP and LEV against *E. coli* ATCC 25922 are 0.004–0.015 mg/L and 0.008–0.06 mg/L, respectively.¹⁴ Otherwise, this behavior enables one only 1st step *gyrA* mutation to be enough to get full resistance, as happens in this study if we consider the break points accepted for most Gram negative bacteria.

Moreover, the contact between *B. melitensis* and FQs inside the macrophage generates, at a high selection rate (around 0.5×10^{-3}), FQ-resistant mutants harboring *gyrA* mutations. The mutant selection rate was similar for CIP and LEV, and the *gyrA* mutation found was also the same. MOX did not select any mutant, but mutants selected by CIP and LEV were also MOX-resistant, MOX MIC increasing, as compared to wild-type strain, in a proportion similar to LEV and CIP (8–16 fold). We do not have a convincing explanation for the lack of mutants emergence in the presence of MOX. It does not seem to be associated to a higher intracellular activity, since bacterial count reduction in presence of MOX and LEV are similar. Otherwise, Ala-87-Val mutants selected in the presence of CIP or LEV increase their MIC of MOX in a similar way as they increase CIP and LEV MICs (8–16×). Thus, should these mutants emerge in the presence of MOX, it should have been detected. We can consider two possibilities: mutant emergence rate may be much lower in the presence of MOX than in the presence of CIP or LEV. This macrophage infection model may not be sensitive enough for low mutants emergence rates. Otherwise, MOX might select for other type of mutants with a more modest MIC increase, which might have been overlooked with the method used. Anyway, conventional *in vitro* selection studies with MOX might contribute to clarify this aspect.

Table 6Intracellular counts of FQ-resistant mutant *B. melitensis* obtained in the control flask and in the flask added with FQs and DOX.

Flask	Intracel. bacterial count (CFU/ml) Type strain	Intracel. bacterial count (CFU/ml) Resistant mutant No. 1	Intracel. bacterial count (CFU/ml) Resistant mutant No. 2	Intracel. bacterial count (CFU/ml) Resistant mutant No. 3	Intracel. bacterial count (CFU/ml) Resistant mutant No. 4
Control	4.9×10^4	3.8×10^4	2.9×10^4	3.2×10^4	5.3×10^4
Ciprofloxacin	4.2×10^4	3.5×10^4	2.5×10^4	3.0×10^4	4.0×10^4
Levofloxacin	1.7×10^4	1.4×10^4	1.8×10^4	1.7×10^4	1.7×10^4
Moxifloxacin	1.2×10^4	1.1×10^4	1.8×10^4	1.6×10^4	1.8×10^4
Doxycyclin	0	0	0	0	0

The mutant selection rate is high in comparison to other microorganisms, and to other studies on *Brucella*. Previous studies¹³ show that, in conventional mutant selection experiments, 1st step FQ-resistant mutant selection rate would be around 1×10^{-6} , while in this study the frequency is around 0.5×10^{-3} . The different selection rates might be associated to the methods of study. In conventional mutant selection experiments, mutants spontaneously emerging in a broth culture are selected by plating a broth aliquot on culture plates added with a selecting antibiotic at concentrations higher than the MIC of the original strain. In this experiment, the original strain underwent intracellular contact with different FQs for 2 hours, before being plated on antibiotic culture plates. This previous contact probably may promote resistant mutant generation, leading to a higher FQ-resistant mutant selection rate. Otherwise, in previous studies¹³ the selecting FQ used was norfloxacin, instead of CIP, LEV and MOX; thus, the results might not be exactly comparable.

There was no FQ-resistant mutants selection from the MOX-containing flasks. This result may probably be a bias associated to the number of *Brucella* CFUs inoculated in cell cultures. Since proportions *Brucella*/macrophages higher than 100:1 could not be used, because this damaged the cell culture, the inoculum had to be lower than that in conventional mutant selection experiments, and mutant selection rates lower than 5×10^{-4} may have remained undetected.

Though MICs of FQs for the mutants obtained were not identical, differences were small, and quinolone resistance-associated mutations found were the same for all the QR mutants selected. The Ala87Val mutation has been previously described by Turkmani et al.¹² These authors describe the selection *in vitro* of four FQ-resistant mutants, harboring mutations leading to Asp91Tyr, Asp91Asn and Ala87Val substitutions in GyrA. Asp91 corresponds to Asp87 in *E. coli*, the second most frequent *gyrA* hot point for mutations in FQ-resistant *E. coli*, after Ser83. Asp87 would correspond to Ser83 in *E. coli*, the most frequently mutated position found in this species.¹⁹

Other studies have found different FQ resistance-associated mutations, such as GyrA Ala71Ser, corresponding to Ala67 in *E. coli*, and GyrA Ala340 insertion.¹³

When these mutants were used as the initial inoculum for infecting macrophages, both the intracellular microorganisms count in the control flask and the microorganisms count after FQs addition were similar to the first experiment, suggesting that the capacity of FQ-resistant mutant for infecting macrophages, and for surviving inside them in the presence of FQs, are not significantly modified in comparison to wild-type strain.

In this study, no further FQ-resistant mutants were obtained when macrophages were infected with the 1st step mutant. Previous studies have shown that further 2nd and 3rd step FQ-resistant mutants can be selected in *Brucella*. These mutants showed higher MICs of norfloxacin and CIP the most hydrophilic FQs tested, but did not show any further mutation in *gyrA*, *gyrB* or *parC* genes, suggesting a role for efflux pumps in this resistance. These mutants were obtained at a low selection rate (8×10^{-7} and 3×10^{-8} , respectively); thus, the lower inoculum allowed for the study on macrophage cultures may have prevented the selection of these further mutants.

Conflict of interest

The authors declare no conflict of interest.

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