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Francisco García Lázaro, R. Elisa Rodríguez-Tarazona, José Ángel García-Rodríguez, Juan Luis Muñoz-Bellido. Fluoroquinolone-resistant mutants obtained in vitro. *International Journal of Antimicrobial Agents*, 2009, 34 (3), pp.252. 10.1016/j.ijantimicag.2008.12.013 . hal-00556325

HAL Id: hal-00556325

<https://hal.science/hal-00556325>

Submitted on 16 Jan 2011

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PII: S0924-8579(09)00024-7
DOI: doi:10.1016/j.ijantimicag.2008.12.013
Reference: ANTAGE 2963

To appear in: *International Journal of Antimicrobial Agents*

Received date: 20-12-2008
Accepted date: 23-12-2008

Please cite this article as: Lázaro FG, Rodríguez-Tarazona RE, García-Rodríguez JÁ, Muñoz-Bellido JL, Fluoroquinolone-resistant *Brucella melitensis* mutants obtained in vitro, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2008.12.013

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Fluoroquinolone-resistant *Brucella melitensis* mutants obtained in vitro

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Received 20 December 2008; accepted 23 December 2008

Keywords: Fluoroquinolone; *Brucella*; *gyrA*

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Abstract

Three fluoroquinolone-resistant *Brucella melitensis* mutants were obtained after successive passages on norfloxacin-containing agar plates. The original strain was *B. melitensis* biovar 2 ATCC 23457. Mutants emerged at frequencies ranging between 1×10^{-6} and 3×10^{-8} . The three mutants showed a *gyrA* Ala71→Ser mutation as well as insertion of Ala340. No mutations were observed in the *parC* and *gyrB* genes. Minimum inhibitory concentrations (MICs) increased by four- to eight-fold from the wild-type to the first-step mutant for norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin. MICs of the second-step mutant increased significantly only for norfloxacin and those for the third-step mutant for norfloxacin and ciprofloxacin, suggesting the involvement of some efflux mechanism.

1. Introduction

Brucellosis remains an important problem in many 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. Although minimum inhibitory concentrations (MICs) of FQs against *Brucella* were higher than against other Gram-negative rods, their activity should be enough to achieve a good therapeutic response [5]. Nevertheless, further clinical studies showed high therapeutic failures and relapse rates of ca. 25% compared with <10% for conventional 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 pathogenic capacity is mainly associated with their capacity to survive in cells, especially in phagolysosomes where there is an acidic environment that might impair FQ activity [7,8]. Nevertheless, the involvement of topoisomerase mutations as a cause of FQ resistance had not been studied. Recently, Turkmani et al. [9] obtained FQ-resistant mutants in vitro from

Brucella abortus and *Brucella melitensis* by growing them in the presence of antibiotics on antibiotic gradient plates. Here we report the characteristics and mutation frequency for spontaneous FQ-resistant mutants obtained in vitro from a *B. melitensis* type strain and selected with norfloxacin.

2. Material and methods

The strain used was *B. melitensis* biovar 2 ATCC 23457. The FQs tested were norfloxacin (NOR) (Merck, Sharp & Dohme, Whitehouse Station, NJ), ciprofloxacin (CIP) (Bayer AG, Leverkusen, Germany), levofloxacin (LEV) (GlaxoSmithKline, Coraopolis, PA) and moxifloxacin (MOX) (Bayer AG), which were kindly provided by their respective manufacturers. The MICs of the antibiotics against *B. melitensis* ATCC 23457 were as follows: NOR, 1 µg/mL; CIP, 0.2 µg/mL; LEV, 0.1 µg/mL; and MOX, 0.2 µg/mL.

The *Brucella* strain was grown on trypticase soy agar (TSA) and then inoculated in tubes containing 8 mL of Wilkins-Chalgren broth. The tubes were then incubated at 37 °C for 48 h in an aerobic atmosphere with 10% CO₂ to achieve an inoculum size of ca. 1 McFarland turbidity standard [ca. 3 × 10⁸ colony-forming units (CFU)/mL]. Then, 1mL of this inoculum was plated onto chocolate PoliVitex plates (bioMérieux, Marcy L'Étoile, France) containing NOR at 4× MIC of *B. melitensis* ATCC 23457 and onto antibiotic-free agar chocolate PoliVitex for control and counting purposes. The colonies growing on NOR-containing plates were counted and tested against all four FQs by the Etest method on

Mueller–Hinton agar supplemented with 5% sheep blood (bioMérieux). The plates were incubated at 37 °C for 48 h in an aerobic atmosphere with 10% CO₂. Resistant mutants growing on NOR-containing plates were also frozen at –80 °C for further genetic analysis. In a second step, NOR-resistant mutants obtained in the first step were plated on TSA, inoculated again in Wilkins–Chalgren broth, incubated for 48 h and plated onto NOR-containing and antibiotic-free agar chocolate PoliVitex as previously, with the only difference that the NOR concentration was 4× MIC of the mutant inoculated instead of 4× MIC of the ATCC strain. The process was repeated until no resistant mutants were obtained when Mueller–Hinton broth cultures were plated onto antibiotic-containing plates.

DNA was obtained from resistant mutants by conventional methods and purified using ExoSAP-IT (GE Healthcare, Piscataway, NJ). Polymerase chain reaction (PCR) for the whole *gyrA* and *parC* genes was performed in an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) using PCR Master Mix (Promega Corp., Madison, WI). The primers used are shown in Table 1. The primers were designed on the basis of *gyrA* and *parC* sequences of *B. melitensis* biovar Abortus 2308 chromosome I (gi 82615033, Accession no. AM040264.1). 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 45 °C (*parC-5*), 48 °C (*gyrA-1*, *gyrA-7*, *parC-1*, *parC-2*, *parC-3* and *parC-4*), 50 °C (*gyrA-5*, *gyrA-6*, *parC-6* and *parC-7*), 54 °C (*gyrA-2*)

or 59 °C (*gyrA-3* and *gyrA-4*) 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* and *parC* sequences of *B. melitensis* biovar Abortus 2308 using BLAST software (National Center for Biotechnology Information, Bethesda, MD).

3. Results

The MICs of FQs against wild-type *B. melitensis* biovar Abortus 2308 and the resistant mutants obtained are shown in Table 2.

Results showed that FQ-resistant mutants can be easily obtained. The first selection step allowed the selection of a mutant whose MICs for NOR, CIP and LEV had increased by eight-fold, whilst the MIC of MOX had increased by four-fold. This mutant was selected with a selection rate of 1×10^{-6} . The second step selected a mutant whose MIC for NOR had again increased by eight-fold, but the MICs of CIP and MOX had increased by only two-fold and the MIC of LEV remained unchanged. The selection rate in this case was similar (8×10^{-7}). The third step selected a further mutant strain, although with a lower selection rate (3×10^{-8}). This mutant showed MICs of MOX and LEV only two-fold higher than the previous isolate, but its MIC for CIP increased by eight-fold and its MIC for NOR also increased by at least eight-fold.

The whole *gyrA*, *gyrB* and *parC* genes were amplified by PCR for reference strains and mutants. Despite their MIC differences, the mutants obtained in the three steps showed the same mutations: a transition from GCC to TCC in *gyrA* leading to the substitution Ala71→Ser (position corresponding to Ala67 in *Escherichia coli*) and an insertion of a GCG in the positions 1018–1020 leading to an insertion of an Ala340, corresponding to Leu336 in *E. coli* (Table 3). No mutations were observed in the *parC* and *gyrB* genes.

4. Discussion

Subunit A of DNA gyrase and DNA topoisomerase IV are the main targets for FQs in all bacteria in which FQ resistance has been described. Other mechanisms of resistance such as FQ efflux can play a role in some individual strains. Although FQs were initially considered a valuable alternative for the treatment of human brucellosis, high rates of clinical failures in the treatment of brucellosis with FQs were soon described [6]. Several causes have been suggested for these failures [7,8], but studies regarding the emergence of topoisomerase-resistant mutants had not been developed until recently. In 2008, Turkmani et al. [9] described the selection in vitro of four FQ-resistant mutants, three of which harboured mutations leading to a substitution of Asp91 to Tyr, Asp91 to Asn and Ala87 to Val. The mutation in Asp91 corresponds to Asp87 in *E. coli*, the second most frequent *gyrA* hot spot for mutations in FQ-

resistant *E. coli* after Ser83. The mutation in Asp87 would correspond to Ser83 in *E. coli*, the most frequently mutated position found in this species [10].

The results found in this study differ from results reported by these authors, since we found an Ala71 to Ser mutation in GyrA, corresponding to Ala67 in *E. coli*, and an Ala340 insertion also in GyrA, corresponding to Ala336 in *E. coli* (Table 3).

The Ala71→Ser mutation belongs to the so-called quinolone resistance-determining region (QRDR), where most mutations associated with FQ resistance appear, although it is not among the point mutations usually described.

Moreover, the Ala340 (336) insertion has not been previously reported as a FQ resistance-associated mutation and is away from the QRDR where most FQ resistance-associated mutations have been described.

These differences may derive from the different method used for obtaining mutants in vitro. Whilst Turkmani et al. [9] used gradient concentrations of CIP and ofloxacin, we used the traditional method of plating broth cultures on plates containing antibiotics at concentrations $\geq 4 \times$ MIC of the parent strain. Since for this method we use cultures containing $>10^8$ CFU/mL, instead of 10^4 CFU/mL, mutations emerging at a very low frequency might be more efficiently detected. This might explain why we already found resistant mutants at the first step and

then at the second and third steps further mutants with increasing FQ MICs were found.

Moreover, the FQ used for selection is probably important for the type of mutants selected. Other studies have shown that different mutations have different repercussions on the MICs of different molecules [11].

NOR is a highly hydrophilic FQ and is usually the FQ most affected by efflux pumps [12]. Some efflux pumps have been previously associated with FQ resistance in *Brucella*. The only member of the TolC family of transporters in *Brucella suis*, BepC, has been associated with resistance to ampicillin, tetracycline, macrolides and NOR [13].

FQ resistance in *Brucella* has been also associated with the expression of NorM1, an efflux pump that can affect FQs and probably aminoglycosides [14]. Although efflux pump involvement in resistance in our mutants is now being studied, some results suggest a role for this mechanism of resistance:

- the selection with NOR, a FQ whose activity can be seriously modified by efflux;
- the fact that the three resistant mutants obtained showed the same mutations despite the different MICs for some FQs; and
- whilst the MIC increases from the wild-type strain to the first-step mutant are similar for the four FQs tested (8× for NOR, CIP and LEV and 4× for

MOX), the second- and third-step mutants increase their MICs mainly for the FQs most affected by efflux in other studies [12].

Thus, FQ resistance in the first-step mutant might probably be associated with the *gyrA* mutations found, whilst the increase of NOR MICs in the second-step mutant and of NOR and CIP in the third-step mutants might be associated with efflux, since no additional mutations in *gyrA*, *gyrB* and *parC* were observed.

Our results suggest that FQ-resistant mutants can emerge in *B. melitensis* at least as easily as in other Gram-negative bacteria such as enterobacteria. Although previous studies suggest that mutations emerge mainly in *gyrA*, in positions equivalent to the most frequent mutations observed in *E. coli*, here we found mutations emerging in different positions, probably because of the FQ used as selector. The features of second- and third-step mutants suggest that the level of resistance shown by these mutants might be associated with efflux, since MICs of the most hydrophilic FQs increase significantly, whilst the increase in LEV and MOX MICs was much lower.

Otherwise, results obtained in vitro regarding FQ-resistant mutant emergence as a cause of therapeutic failures shall be confirmed by experimental findings in animal or macrophage culture models.

Funding: This study was financed by grant HUS4/04 from the Junta de Castilla y León, Spain.

Competing interests: None declared.

Ethical approval: Not required.

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Table 1

Primers and conditions used for *Brucella melitensis* *gyrA* and *parC* polymerase chain reaction

Primer name	Direct primer	Reverse primer	Fragment	Size (bp)
<i>gyrA</i> -1	5'GGCGGTTATAGGCGAGA	5'GCGCCATCTTTATGTGA	-179 to 187	374
<i>gyrA</i> -2	5'TCCATCAGTTCCGACAGTTTCG	5'TCGAGGAAATGCAGCGGTCT	44 to 632	589
<i>gyrA</i> -3	5'CGATCACGCGCCTTGTTGAGCAG	5'TGGTTCAGGCGGTATTGCGGTCG	510 to 1127	618
<i>gyrA</i> -4	5'GCGCACGATAGGTCGTGAGC	5'TCACGCTGCTCGACATGCTGC	1034 to 1654	621
<i>gyrA</i> -5	5'GCAGGATCGCCATCGA	5'ATATGGTCGTCACCGTCA	1589 to 2206	618
<i>gyrA</i> -6	5'CCATTGCCGTTTTCTCA	5' TCTTTGCTGGCCGCAA	2126 to 2711	586
<i>gyrA</i> -7	5'TGTGCGTCAGGTTGCA	5'GACCAAGGGTGTACGA	2613 to +221	369
<i>parC</i> -1	5'ACTGGAGTGAAACGAGGA	5'CAGCGCATCATAGATCGA	-147 to 287	435
<i>parC</i> -2	5'GACGGCGAAGAACACA	5'GCCGAACCGTTTGCA	28 to 536	509
<i>parC</i> -3	5'CTGGAAGGCATCAACGAA	5'TCTGCACCTGATAGGGAA	424 to 985	561
<i>parC</i> -4	5'TTCCCTATCAGGTGCAGA	5'GGTGCTGATCTTCTTCCA	879 to 1491	613
<i>parC</i> -5	5'AGAAGATCAGCACCGAA	5'CCGGAACAATGAAACCA	1478 to 1942	465
<i>parC</i> -6	5'CTTCGTGCACGATCCA	5'ACCCTTCGGCACCAGA	1875 to 2289	415
<i>parC</i> -7	5'GTTCGCAGCTTCGCAA	5'CTCGGCTATTGGGTCGA	2157 to +280	437

Table 2

Minimum inhibitory concentrations (MICs) of fluoroquinolones against wild-type *Brucella melitensis* biovar 2 ATCC 23457 and mutants obtained in vitro

Fluoroquinolone	MIC ($\mu\text{g/mL}$)			
	Wild-type	First-step mutant	Second-step mutant	Third-step mutant
Norfloxacin	2	16	128	>1024
Ciprofloxacin	0.2	2	4	32
Levofloxacin	0.1	1	1	2
Moxifloxacin	0.2	1	2	4

Table 3

Changes (shown in bold and underlined) observed in *gyrA* in resistant (R) mutants and correspondence with wild-type (WT)

Brucella melitensis biovar 2 ATCC 23457 and *Escherichia coli*

Strain	<i>gyrA</i> region
<i>E. coli</i> WT	46-RRVLYAMNVLGNDWNKAYKKS <u>A</u> RVVGDVIGKYHPHGDSAVY-86
<i>B. melitensis</i> WT	50-RRILHAMNEMNLAYNRPYRKS <u>A</u> GVVGEVMGKYHPHGDA SIY-90
<i>B. melitensis</i> R mutant	50-RRILHAMNEMNLAYNRPYRKS <u>S</u> GVVGEVMGKYHPHGDA SIY-90
<i>E. coli</i> WT	316-LNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRH-357
<i>B. melitensis</i> WT	320-LNQLYRYTPLQTSFGCNMVALNGGKPEQLTLLDMLRAFVAF-361
<i>B. melitensis</i> R mutant	320-LNQLYRYTPLQTSFGCNMVA <u>A</u> LNGGKPEQLTLLDMLRAFVAF-361