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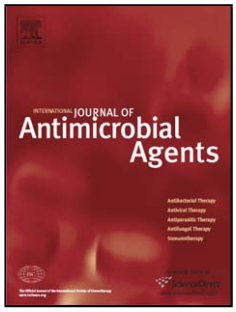

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In vitro selection of fluoroquinolone resistance in *Brucella melitensis*

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Abstract

Moxifloxacin-resistant mutants of *Brucella melitensis* 16M [moxifloxacin minimum inhibitory concentration (MIC) = 1 mg/L] were selected in order to characterise fluoroquinolone resistance mechanisms in this species. Eight independent mutants were obtained, with moxifloxacin MICs of 16–32 mg/L. The mutants displayed variable cross-resistance levels to other fluoroquinolone compounds, but no increased resistance to aminoglycosides, tetracycline, rifampicin, macrolides or co-trimoxazole. Sequencing of type II topoisomerase-encoding genes (*gyrA*, *gyrB*, *parC* and *parE*), which are natural targets for fluoroquinolones, revealed a *gyrA* mutation leading to the amino acid substitution Ala83Val (*Escherichia coli* numbering system) in five mutants with a moxifloxacin MIC of 32 mg/L, whereas no mutation was found in the remaining three mutants with an MIC of 16 mg/L. Phenylalanine-arginine-β-naphthylamide dihydrochloride, an efflux pump inhibitor, reduced moxifloxacin MICs by a factor of two to eight in all resistant mutants. In *B. melitensis*, fluoroquinolone resistance may arise from *gyrA* mutation and efflux pump overexpression mechanisms.

1. Introduction

Brucella spp. are aetiological agents of brucellosis, a worldwide zoonosis [1]. Terrestrial and marine mammals are natural reservoirs for these bacteria. Human contamination usually occurs via direct contact with infected domestic animals or via the digestive route after consumption of contaminated raw milk or milk products. Pasteurisation of milk and eradication of brucellosis in cattle has dramatically reduced the incidence of human brucellosis in developed countries. However, brucellosis remains endemic and a public health problem in many countries where these prophylactic measures have not been implemented, especially around the Mediterranean Sea, in the Middle East, in central Asia and in some parts of Africa and South America [2].

Two antibiotic combinations, i.e. doxycycline + rifampicin or doxycycline plus an aminoglycoside (e.g. streptomycin or gentamicin), remain the mainstay in brucellosis therapy [1]. Therapy alternatives may be needed in patients with severe adverse effects as well as in children <8 years old and in pregnant women in whom tetracyclines are contraindicated [1]. On the other hand, relapse rates of 10–15% are still observed with these antibiotic combinations despite prolonged therapy. Combinations of trimethoprim/sulphamethoxazole (co-trimoxazole) or a macrolide with either rifampicin or an aminoglycoside have been used for decades, but with higher relapse rates [1]. The fluoroquinolones have recently been considered as a promising alternative in the treatment of human brucellosis [3,4]. These antibiotics display low minimum inhibitory concentrations (MICs) against *Brucella* spp. in vitro [5–10] and have good oral bioavailability and a large volume of distribution including in eukaryotic cells where *Brucella* sp. multiply [11,12]. They are usually well tolerated

although, as for tetracyclines, they are contraindicated in young children and pregnant women. However, the use of fluoroquinolone monotherapy in brucellosis patients has led to frequent relapses and failures [13–15], and the combination of a fluoroquinolone with another antibiotic (especially rifampicin) is no more effective than currently available antibiotic regimens [3,4,16]. Thus, fluoroquinolones are currently not recommended as a first-line drug to treat brucellosis patients [3].

Several hypotheses have been raised to explain the discrepancies between the in vitro and in vivo activities of fluoroquinolones. First, fluoroquinolones have no bactericidal activity against *Brucella* spp. either in vitro [17,18] or in animal models [19,20]. Moreover, *Brucella* sp. multiply in acidic phagosomes both in professional and non-professional phagocytic cells [11,12]. Although fluoroquinolones are concentrated within eukaryotic cells, their activity is dramatically reduced at acidic pH [17,18]. Finally, in vivo selection of fluoroquinolone-resistant mutants in *Brucella* sp. has been evoked [21]. In vitro, only low variations in fluoroquinolone MICs between various *Brucella* strains has been reported [7–10,18], although there are rare exceptions [22].

Two major fluoroquinolones resistance mechanisms have been described so far [23,24]: (i) mutations in type II topoisomerase genes, encoding DNA gyrase and type IV topoisomerase, which are the natural targets of fluoroquinolone action; and (ii) overexpression of efflux pumps reducing drug accumulation in bacteria. Turkmani et al. [25] recently reported selection of *gyrA*-mediated fluoroquinolone resistance in *Brucella abortus*. In the present study, we further explored fluoroquinolone resistance mechanisms in *Brucella melitensis*.

2. Material and methods

2.1. Bacterial strains

Brucella melitensis 16M (ATCC 23456) was used in all experiments. *Brucella melitensis* 16M was grown on Columbia agar supplemented with 5% sheep blood (bioMérieux, Lyon, France) at 37 °C in a 5% CO_2-enriched atmosphere in a biosafety level 3 laboratory. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for MIC determination.

2.2. Antibiotics

The following antibiotics were tested: moxifloxacin (Bayer Pharma, Puteaux, France), ciprofloxacin (Bayer Pharma), levofloxacin (sanofi-aventis, Paris, France), doxycycline (Arrow, Lyon, France), rifampicin (sanofi-aventis), gentamicin (Dakota Pharma, Paris, France), co-trimoxazole (Roche, Neuilly-sur-Seine, France) and erythromycin (CSP, Cournon, France). Antibiotics were dissolved as recommended by the manufacturers, prepared in aqueous solutions at a concentration of 2 mg/mL and stored at –80 °C until use.

2.3. Selection of fluoroquinolone-resistant mutants

Brucella melitensis 16M was grown on Columbia agar with 5% sheep blood at 37 °C in 5% CO_2 atmosphere for 2 days. A bacterial inoculum was prepared in Mueller–Hinton broth (MHB) (AES Laboratory, Combourg, France) at 0.5 McFarland standard. Then, 20 μL of this suspension was added to each well of a 96-well microtitre plate.

MHB (180 μL/well) containing two-fold serial dilutions of moxifloxacin (0.06–128 mg/L of final concentrations) was added to each row of the microtitre plate. Thus, the experimental design allowed eight independent mutants to be selected. The microplate was incubated at 37 °C in a 5% CO_2 atmosphere. After 3–5 days incubation for the first 17 days of the experiment, or after 7–10 days incubation for the remaining 51 days of the experiment, the plate was visually examined for bacterial growth and moxifloxacin susceptibility values were noted. For each row, bacterial growth observed in the well containing the highest moxifloxacin concentration was harvested and dispensed (10 μL/well) in a new microtitre plate containing MHB (190 μL/well) with two-fold serial dilutions of moxifloxacin as above. Twelve subcultures were performed for a total period of 68 days, allowing progressive selection of eight moxifloxacin-resistant mutants. These mutants were then passaged eight times on antibiotic-free blood agar plates to test the stability of the resistant phenotypes and individual colonies were selected for further analysis. During the antibiotic resistance selection process, the intermediate-resistant populations were also harvested and kept frozen at –80 °C for further analysis.

2.4. Minimum inhibitory concentration determination

For *B. melitensis* 16M and isogenic resistant mutants, MICs were determined using a microdilution technique. For each strain, a 0.5 McFarland bacterial suspension was prepared in MHB and dispensed (180 μL/well) in 96-well microtitre plates. Antibiotics were added (20 μL/well) at ten times the desired final concentrations. Plates were incubated at 37 °C in a 5% CO_2 atmosphere and MICs were read after 3 days incubation. MICs were defined as the lowest antibiotic concentration that completely

inhibited visual growth. For *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, MICs were determined using the same procedure but the incubation time was 18 h.

2.5. DNA gyrase and topoisomerase IV gene sequencing

Bacterial suspensions were prepared by harvesting a single colony from each mutant in 1 mL of sterile distilled water. Bacteria were inactivated by heating at 90°C for 2 h. DNA was extracted using the QIAmp DNA Mini Kit (QIAGEN S.A., Courtaboeuf, France) according to manufacturer's instructions. Primers used for polymerase chain reaction (PCR) amplification and DNA sequencing are described in Table 1 and were purchased from MGW-Biotech (Roissy, France). PCR primers were first designed to amplify the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* using the *B. melitensis* 16M genome sequence available in GenBank (NC 003317 and NC 003318). For *B. melitensis* 16M and two fluoroquinolone-resistant mutants, whole DNA sequences of *gyrA* and *parC* genes were determined using the primer pairs described in Table 1. The specificity of the primers was checked using BLAST alignment (BLASTn; National Center for Biotechnology Information, Bethesda, MD). PCR was performed using the Pwo Master Kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Amplifications were carried out in 50 μL volumes containing 0.4 μM reverse and forward primers and 5 μL of DNA template. PCR was initiated by denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. The efficacy of PCR amplification was verified by agarose gel electrophoresis and ethidium bromide staining of PCR products. PCR products were then purified using Sephadex™-containing Millipore MAHV N45 plates (Sigma

Aldrich, Saint-Quentin-Fallavier, France). DNA sequencing was performed on a CEQ2000XL apparatus using GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, Roissy, France) and a CEQ2000 Dye Terminator Cycle Sequencing Protocol (Beckman Coulter). Sequence analysis was performed using the CEQ2000XL DNA analysis system (Beckman Coulter).

2.6. Evaluation of efflux pump overexpression

Moxifloxacin MICs of *B. melitensis* and the eight resistant mutants were determined in the presence or absence of efflux pump inhibitors (EPIs) (Sigma-Aldrich), including verapamil (25 μM), reserpine (32.8 μM), carbonyl cyanide-3-chlorophenylhydrazone (CCCP) (25–100 μM), sodium orthovanadate (50 μM), phenylalanine-arginine-β-naphthylamide (PAβN) (38.5 μM) and 1-naphthylmethyl-piperazine (88.4 μM). The activity of each EPI was also tested against *B. melitensis* 16M. Since PAβN was the only EPI that significantly reduced moxifloxacin MICs in resistant mutants, its activity was further tested at concentrations ranging from 38.5 μM to 616.5 μM on moxifloxacin, levofloxacin and ciprofloxacin MICs to determine a dose–effect relationship. All experiments with EPIs were performed three times to verify consistency.

2.7. NorMI and NorMII gene sequencing

NormI and NormII are currently the only efflux pumps that have been characterised as functionally active in *B. melitensis* 16M [26]. They belong to the MATE (multidrug and toxic extrusion) efflux pump family and have been shown to be specifically susceptible to PAβN [26]. Whole *NorMI* and *NorMII* genes plus a 250 bp upstream

DNA fragment were amplified and sequenced in *B. melitensis* 16M and two fluoroquinolone-resistant mutants using the primers indicated in Table 1 to check for the presence of mutations in the genes or in promoters.

3. Results

3.1. Selection of fluoroquinolone resistance in Brucella melitensis 16M

Following a total of 12 passages (i.e. 68 days) of *B. melitensis* 16M in the presence of moxifloxacin concentrations ranging from 0.25 mg/L to 256 mg/L, eight independent resistant mutants were selected, termed M1 to M8 (Table 2). First, five passages were performed every 3–5 days with no evident resistance selection. Then, seven additional passages were performed every 7–10 days and resistance selection rapidly occurred. Moxifloxacin resistance was stable in all mutants after eight further subcultures in antibiotic-free medium.

3.2. Minimum inhibitory concentration determination

In MHB, *B. melitensis* 16M displayed MICs of 0.125 mg/L for doxycycline, 0.5 mg/L for rifampicin, 0.5 mg/L for gentamicin, 1 mg/L for levofloxacin, ciprofloxacin and moxifloxacin, and 1.6 mg/L and 8 mg/L for trimethoprim and sulphamethoxazole, respectively. MICs of the same antibiotics for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were in the expected ranges.

Among the eight *B. melitensis* mutant strains, five displayed an MIC to moxifloxacin of 32 mg/L, whereas the remaining three displayed an MIC of 16 mg/L (Table 2). Variable levels of cross-resistance to the other fluoroquinolones tested were found

for the selected resistant mutants, with MICs ranging from 0.5 mg/L to 8 mg/L for ciprofloxacin and from 1 mg/L to 8 mg/L for levofloxacin (Table 2). Interestingly, ciprofloxacin and levofloxacin MICs remained unchanged for M7 despite a 16-fold increase in the moxifloxacin MIC. MICs to doxycycline, rifampicin, gentamicin and co-trimoxazole were unchanged in the eight moxifloxacin-resistant mutants compared with the *B. melitensis* 16M wild-type strain.

3.3. Determination of DNA gyrase and topoisomerase IV sequences

A single mutation in the *gyrA* QRDR was identified in the five mutants with the highest moxifloxacin MIC (32 mg/L) in codon 67 (GCT→GTT) leading to amino acid substitution Ala67Val (Table 2). Codon 67 in *B. melitensis* GyrA corresponds to codon 83 in the *E. coli* GyrA numbering system. No mutation was found in the *gyrA* QRDR of the remaining three mutants with moxifloxacin MICs of 16 mg/L. A 6 base insertion (i.e. CGCCAG) was found in the *parE* gene of mutant 8. This insertion led to two amino acid insertions, an alanine and a serine, at positions 515 and 516 in the corresponding protein. No mutations were found in *gyrB* or *parC* QRDR sequences. Determination of the whole *gyrA* sequence (2709 bp) in *B. melitensis* 16M and in M1 and M2 mutants (moxifloxacin MICs 16 mg/L and 32 mg/L, respectively) confirmed the presence of a single mutation at position 67 in M2 and the absence of any *gyrA* mutation in M1 compared with the *B. melitensis* 16M *gyrA* sequence. No mutation was found in the whole sequence of *parC* (2328 bp) in M1 and M2 mutants compared with that of *B. melitensis* 16M.

The intermediate-resistant populations were also checked for the presence of mutations in DNA gyrase- and topoisomerase-encoding genes. It was observed that

an Ala67Val substitution in GyrA occurred in the five mutants at different steps during the selection process (Table 2). The presence of Ala67Val substitution was correlated with a higher level of cross-resistance to levofloxacin and ciprofloxacin.

3.4. Evaluation of efflux pump overexpression

None of the tested EPIs displayed a bacteriostatic effect against *B. melitensis* 16M on its own, except PAβN at concentrations >154 μM, and CCCP that completely inhibited bacterial growth at concentrations ranging from 25 μM to 100 μM. CCCP activity could not be evaluated in resistant mutants. As shown in Table 3, only PAβN significantly reduced the moxifloxacin MIC (by a factor of two to eight) in all moxifloxacin-resistant mutants, but not in *B. melitensis* 16M. Furthermore, a dose–effect relationship was demonstrated (Table 3). PAβN only displayed a weak effect on ciprofloxacin and levofloxacin MICs in moxifloxacin-resistant mutants at a concentration of 154 βM (Table 3).

3.5. NorMI and NorMII gene sequencing

No mutations were found in *NorMI* and *NorMII* genes or in the 250 bp upstream of the genes for *B. melitensis* 16M and moxifloxacin-resistant mutants M2 and M6 (moxifloxacin MICs of 32 and 16 mg/L, respectively).

4. Discussion

The aim of the present study was to evaluate potential resistance mechanisms to fluoroquinolones in *Brucella* spp. A *B. melitensis* strain was studied because this

species is responsible for most human brucellosis cases in the world [1] and as potential fluoroquinolone resistance mechanisms have never been studied in this species. In vitro selection for fluoroquinolone resistance in *B. melitensis* 16M was easily achieved using moxifloxacin as the selecting drug, with a 16- to 32-fold increase in MICs in eight independent mutants. In particular, a small numbers of passages in antibiotic-containing medium was necessary to select resistant mutants when the incubation time between two passages was increased to 7–10 days. *Brucella melitensis* is a slow-growing bacterium. Moreover, moxifloxacin belongs to a new generation of fluoroquinolones with improved in vitro activity supposedly because of the presence of a methoxy group in C8. The combination of both characteristics may explain the need for prolonged incubation of *Brucella* cultures to select moxifloxacin-resistant mutants. Turkmani et al. [25] reported a similar observation when selecting resistance to ofloxacin and ciprofloxacin in *B. abortus*.

Mutations in topoisomerase-encoding genes are the leading cause of fluoroquinolone resistance in bacteria [23,24]. In Gram-negative bacteria, DNA gyrase is usually more susceptible to inhibition by fluoroquinolones than topoisomerase IV and thus resistance mutations occur first in the *gyrA* gene encoding subunit A of DNA gyrase (GyrA) [23,24]. These mutations usually lead to amino acid substitution in the protein sequence of the DNA-binding domain of GyrA, referred to as the QRDR, especially at hotspot positions 83 and 87 (*E. coli* numbering system). Frequent amino acid substitutions responsible for acquired resistance to fluoroquinolones in *E. coli* include Ser83Leu, Ser83Trp, Asp87Asn, Asp87Gly and Asp87Tyr [30]. These amino acid substitutions in the GyrA QRDR have been associated in *E. coli* with reduced affinity of fluoroquinolones with the enzyme–DNA complex [30]. Turkmani et al. [25] recently

reported three types of *gyrA* mutations in *B. abortus* leading to fluoroquinolone resistance. These mutations occurred in different mutants and led to the following amino acid substitutions: Asp91Asn or Asp91Tyr (corresponding to substitution in codon position 87 of *E. coli* GyrA); and Ala87Val (corresponding to substitution in codon position 83 of *E. coli* GyrA). In the five *B. melitensis* 16M mutants with an MIC of 32 mg/L to moxifloxacin, an amino acid substitution was found at codon position 67 (Ala67Val) corresponding to position 83 in the *E. coli* GyrA numbering system. Sequencing of the entire *gyrA* and *parC* genes in mutant M2 did not reveal any further mutation. Thus, it is highly probable that the observed *gyrA* QRDR mutation explains at least partially the acquisition of moxifloxacin resistance in these five mutants. Interestingly, a correlation between MICs to fluoroquinolones and sequence variation in the *gyrA* QRDR, especially at codon position 83, can be established. As shown in Table 2, a serine is found at GyrA codon position 83 in bacterial species with very low MICs to ciprofloxacin and ofloxacin, e.g. wild-type strains of *E. coli* [31] and *Mycobacterium fortuitum* [28]. Ser83Ala substitution has been reported in *E. coli* strains with a four-fold increase in MICs to these compounds [27]. An alanine residue at codon position 83 is found naturally in *Mycobacterium tuberculosis* [28], *B. abortus* [25] and *B. melitensis*, which display intermediate-level resistance to ciprofloxacin and ofloxacin. Finally, Ala83Val substitution has been reported in *M. tuberculosis* [29], *B. abortus* [25] and now in *B. melitensis*, leading to high-level resistance to fluoroquinolones. In both *M. tuberculosis* and *B. melitensis*, Ala83Val substitution induced four- to eight-fold increases in ciprofloxacin and ofloxacin MICs. The 6 bp insertion in *parE* of mutant M8 led to insertion of an alanine and a serine residue at codon positions 515 and 516 in the corresponding protein. This mutation is situated

outside the *parE* QRDR and it has not been previously associated with fluoroquinolone resistance in Gram-negative bacteria.

In the three resistant mutants M1, M6 and M7, with moxifloxacin MICs of 16 mg/L, no mutation was found in the QRDR of topoisomerases-encoding genes. Further sequencing of the entire *gyrA* and *parC* genes in mutant M1 did not reveal any mutation compared with the wild-type *B. melitensis* 16M DNA sequences. Moreover, in the five mutant lineages with Ala67Val substitution in GyrA, the substitution occurred in intermediate populations at moxifloxacin concentrations varying from 8 mg/L to 32 mg/L according to the mutant lineage considered (Table 2). These observations suggested that another fluoroquinolone resistance mechanism was involved and that it preceded Ala83Val substitution. Halling and Jensen [32] have previously shown that efflux systems play an important role in resistance to macrolides in *Brucella* spp. We investigated the possibility of efflux pump overexpression in moxifloxacin-resistant mutants by testing MICs to this antibiotic in the presence or absence of EPIs. Only PAβN significantly reduced moxifloxacin MICs in all resistant mutants with a dose–effect relationship, but not in *B. melitensis* 16M. PAβN is known to inhibit efflux pumps of the MATE family in different Gram-negative bacterial species [33]. It is a dipeptide amide that acts as an EPI by competing with the fluoroquinolone compounds, thus precluding extrusion of these antibiotics. MATE family efflux pumps have been described in *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Campylobacter* spp. and *Bacteroides thetaiotaomicron* [33]. Two putative MATE family efflux pumps have been recently described in *B. melitensis*, i.e. NorMI and NorMII [26]. Only NorMI has been fully characterised [26]. Thus, we can hypothesise that NorMI and/or NorMII are overexpressed in our *B. melitensis*

moxifloxacin-resistant mutants. Sequencing *NorMI* and *NorMII* genes in two mutants did not reveal any mutation. However, overexpression of efflux pumps is usually related to mutation in local or global regulator genes and less frequently in structural genes [33]. The former genes are currently uncharacterised in *B. melitensis*.

The moxifloxacin-resistant mutants bearing GyrA A67V substitution displayed only moderate levels of cross-resistance to other fluoroquinolone compounds such as levofloxacin and ciprofloxacin. Cross-resistance can be explained by a strong structural relationship between fluoroquinolone compounds. However, as previously reported [23,24], topoisomerase target preference may change according to the chemical structure of the quinolone compound considered. Also, PAβN had only a weak effect on ofloxacin and ciprofloxacin MICs in the eight mutants, suggesting the involved efflux pumps was more effective in extruding moxifloxacin. No cross-resistance was found for doxycycline, rifampicin, gentamicin and co-trimoxazole, which are first-line drugs in the antibiotic treatment of brucellosis. Although the major resistance mechanisms in bacteria to these antibiotics are different from those involved in fluoroquinolone resistance, their activity could have been altered by overexpression of efflux pump systems.

Altogether our results suggest that, as for most other Gram-negative bacteria [23,24], *B. melitensis* may become resistant to fluoroquinolone compounds by overexpression of efflux pumps and/or by mutations in the *gyrA* QRDR. The limitation of our study is that fluoroquinolone resistance mechanisms only in in vitro-selected mutant strains were characterised. Thus, the clinical relevance of our results awaits confirmation that fluoroquinolone resistance is truly occurring in clinical strains of *B.*

melitensis, in brucellosis patients receiving fluoroquinolone therapy, and that the involved resistance mechanisms are the same in clinical strains and in in vitro-selected mutant strains.

5. Conclusion

Our results show that moxifloxacin resistance in *B. melitensis* 16M first occurred by overexpression of efflux pumps. Higher resistance was then selected by mutation in the *gyrA* QRDR, leading to Ala67Val substitution in the GyrA QRDR. This substitution was responsible for cross-resistance to other fluoroquinolone compounds. Further work is needed to characterise better the *B. melitensis* efflux pumps involved in fluoroquinolone resistance, but the MATE family efflux pumps NorMI and NorMII are good candidates.

It should be emphasised that the alanine residue found at position 67 (83 in the *E. coli* numbering system) in wild-type *B. melitensis* GyrA is responsible for low-level resistance to fluoroquinolones, and that bacteria with low-level resistance to fluoroquinolones usually become fully resistant to these compounds through only a one-step mutation [25,29]. This strengthens current recommendations that fluoroquinolones should not be used as a first-line therapy in brucellosis patients [3].

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Table 1

Oligonucleotide primers used for polymerase chain reaction (PCR) amplification and DNA sequencing of genes encoding *Brucella melitensis* 16M topoisomerases and efflux pumps NorMI and NorMII

Gene	Forward primer (5′–3′ DNA sequence)	Reverse primer (5′–3′ DNA sequence)
gyrA QRDR	(Fw) ACGGCCTGAAGCCTGTGCAT	(Rv) CGACAGGAGGGATTCCGTGA
gyrA whole gene	(Fw1) CCATGAGCGTGATCGTGAG	(Rv1) CAGTTCGATGGCCGGATTG
	(Fw2) TTCAGGCGGTATTGCGGTC	(Rv2) CGCCTTGTTGAGCAGGAAC
	(Fw3) TGCAGACCTCGTTTGGCTG	(Rv3) CGCGAACGGCAATCATCTC
	(Fw4) CGATGAAGTGGCCGATGAG	(Rv4) CGTTGCGATTGACCTGCAC
	(Fw5) TTCGCCGCAACAAGCTGTC	(Rv5) GTAGCCGAATTCGCTGACC
	(Fw6) ATGCAGAAGCGGAGCTGTC	(Rv6) CCCCATTGCCGTTTTCCT
parC QRDR	(Fw3) TCGGCTCTTGAGGAACGTTA	(Rv3) CGATGGGTTCTTCGTCTTCC
parC whole gene	(Fw1) GGAAAAAGTCTGATTCCGCC	(Rv1) ATATTGGTGGCCATACCGAC
	(Fw2) ATGAGGAAGACGAAGAACCC	(Rv2) TCAGCTTGAACAGCGATTC
	(Fw3) GCTGCTTGACGATATTCGTG	(Rv3) TGAGGTCATCGAACTCCTTG

	(Fw4) CGTGCTTTTGAACTGACTG	(Rv4) AAGGCCGTCAGTATATCCTG
	(Fw5) CGACCGACAAGCTTTTGTTC	(Rv5) ACTCGACCAGTTCTTCCTTG
gyrB QRDR	(Fw) GTTGTCGAGAAGGTCATTCAGG	(Rv) GCGTTGAAGCCGTGCGTTTC
parE QRDR	(Fw4) AGGTGCGGAATTGTTCATCGTGG	(Rv4) CCAAGCCCCTTGAAACGTCC
NorMI whole gene	(Fw1) GACGGAACATTTGACGCGG	(Rv1) GCCCGCAAGTGCATTGAAG
	(Fw2) CGTCCTGCGGTCTTTCTTC	(Rv2) GACCCGTGGCATGAGAAC
	(Fw3) CCATTGCTTATGCGGGCTG	(Rv3) GCCAGCGTATCGTCATCAG
NorMII whole gene	(Fw1) CCGTTCGATGTCACGCATCG	(Rv1) GGATCGATATATAGGTCGCC
	(Fw2) CCACCTTCATGCATCCGACAC	(Rv2) CCGATGATCTGTTCAGCCG
	(Fw3) ATTTCTTCCTCGTGGCGGGC	(Rv3) TCGCCAGAAGCGTAATGCC

QRDR, quinolone resistance-determining region.

Table 2

Selection of resistant mutant strains M1 to M8 from *Brucella melitensis* 16M, with resulting minimum inhibitory concentrations (MICs) to moxifloxacin (MFX), ciprofloxacin (CIP) and levofloxacin (LVX), and characterised GyrA quinolone resistance-determining region (QRDR) substitutions

Strain	Highest moxifloxacin concentration (mg/L) allowing bacterial growth at Day:							MIC (mg/L)			GyrA QRDR amino acid substitution
	0–24	31	39	45	55	62	68	MFX	CIP	LVX	
M16								1	1	1	Wild-type
M1	1	2	4	4	8	8	16	16	4	4	None
M2	1	2	4	4	8	16	32 [a]	32	8	8	A67V
M3	1	2	4	8 [a]	32 [a]	64 [a]	64 [a]	32	4	2	A67V
M4	1	2	16 [a]	16 [a]	16 [a]	64 [a]	64 [a]	32	4	8	A67V
M5	1	2	8	8	8	32 [a]	64 [a]	32	8	8	A67V
M6	1	2	4	4	8	32	32	16	1	2	None
M7	1	4	8	8	16	32	32	16	0.5	1	None
M8	1	1	2	4	8 [a]	64 [a]	64 [a]	32	8	8	A67V

Species	GyrA 83 residue [b]	MIC (mg/L) [c]	GyrA 83 residue [b]	MIC (mg/L) [c]	MIC change	Reference

Escherichia coli	Serine	0.006	Alanine	0.032	×4	[27]
Mycobacterium fortuitum	Serine	0.06				[28]
Mycobacterium tuberculosis	Alanine	1	Valine	8	×8	[29]
Brucella abortus	Alanine	0.5	Valine	32/64	×64–128	[25]
B. melitensis	Alanine	1	Valine	4–8	×4–8	Present work

[a] Presence of GyrA A67V substitution.

[b] *Escherichia coli* numbering system (position GyrA 67 in *B. melitensis*).

[c] MIC to ofloxacin and/or ciprofloxacin.

Table 3

Moxifloxacin minimum inhibitory concentrations (MICs) (mg/L) of *Brucella melitensis* 16M and the eight moxifloxacin-resistant mutants in the presence or absence of efflux pump inhibitors (EPIs)

Strain	Moxifloxacin					Ciprofloxacin + PAβN (μM)			Levofloxacin + PAβN (μM)					
	No EPI	Ver 25 μM	Res 32.8 μM	SO 50 μM	NMP 88.4 μM	PAβN (μM)								
						38.5	77	154	38.5	77	154	38.5	77	154
16M	1	1	1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5
M1	16	16	8	8	8	8	4	2	2	2	2	2	2	2
M2	32	32	32	32	16	16	8	4	16	8	8	16	16	8
M3	32	32	32	32	16	16	8	4	16	8	8	16	16	8
M4	32	32	16	32	16	32	16	16	8	8	8	8	8	4
M5	32	32	16	32	16	16	8	4	16	8	8	8	8	4
M6	16	8	16	8	8	16	8	4	4	2	2	4	4	4
M7	16	16	16	16	8	8	4	2	< 0.5	< 0.5	< 0.5	1	1	<0.5
M8	32	32	32	32	16	8	8	4	8	8	4	8	8	4

Ver, verapamil; Res, reserpine; SO, sodium orthovanadate; NMP, 1-naphtylmethyl-piperazine; PAβN: phenylalanine-arginine-β-naphthylamide.