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Original article

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.

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

Palabras clave:
Fluoroquinolonas
Brucella
gyrA
Infección experimental 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.

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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.

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 sparfloxacin.[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 Polivitex^{R} agar (bioMérieux, Le Balme Les Grottes, France), at 37 °C in a 5% CO_2 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 methods[12] 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-cm^2 tissue-culture flasks (Costar, Cambridge, MA, USA), and macrophages were allowed to adhere for 24 h in a humidified CO_2 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 exclusion[15].

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 CO_2 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