Efficacy of several antibiotic combinations against Brucella melitensis Rev 1 experimental infection in BALB/c mice

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Objectives: The objective of the present study was to compare the efficacy of gentamicin given alone or combined with doxycycline with that of standard combination therapies in BALB/c mice experimentally infected with the Brucella melitensis vaccine strain Rev 1.

Methods: A standard broth microdilution method was applied to determine the susceptibility of strain Rev 1 to the clinically most relevant aminoglycosides. Eight groups of BALB/c mice were inoculated intraperitoneally (i.p) with $1 \times 10^8$ cfu/mouse of strain Rev 1. While one group remained untreated, the other seven groups were treated 10 days later once a day for 14 days with (i) doxycycline given orally at 2 mg/day; (ii) streptomycin given i.p at 0.4 mg/day; (iii) gentamicin given i.p at 0.4 mg/day; (iv) rifampicin given orally at 0.5 mg/day; (v) doxycycline plus streptomycin; (vi) doxycycline plus gentamicin; and (vii) doxycycline plus rifampicin. The number of cfu per spleen and clearance of Rev 1 were assessed 34 days after inoculation.

Results: With the exception of streptomycin, strain Rev 1 was susceptible to all aminoglycosides tested. As expected, the combination doxycycline/streptomycin was ineffective against Rev 1 infection. In contrast, the combinations doxycycline/gentamicin and doxycycline/rifampicin were effective in the clearance of Rev 1 infection, but only the former improved significantly the therapeutic efficacy as compared with that of the antibiotics given alone.

Conclusions: Gentamicin may be used along with doxycycline when the classical combination is considered the first choice in the treatment of patients with brucellosis due to B. melitensis vaccine strain Rev 1.

Keywords: Human brucellosis, B. melitensis Rev 1 vaccine strain, antimicrobial therapy

Introduction

Brucellosis is an important public health problem, whose occurrence in humans is related directly with the prevalence of the infection in animals, and particularly in domestic ruminants. In endemic situations, vaccination is the only suitable way for controlling brucellosis in ruminants.1 Brucella melitensis is the main causative agent of brucellosis in both humans and small ruminants. The most effective and widely used vaccine against brucellosis in sheep and goats is the attenuated B. melitensis Rev 1 live vaccine strain.2 However, this vaccine strain can cause abortion and excretion in milk when sheep and goats are vaccinated during pregnancy in mass-vaccination programmes, increasing the risk of human infections due to Rev 1.1 Moreover, accidental Rev 1 inoculations are not rare in veterinarians and shepherds during the vaccination campaigns.3−5

The treatment of choice of human brucellosis caused by B. melitensis field strains is a classical combination of long-acting tetracyclines and streptomycin.6 However, other aminoglycosides such as gentamicin and netilmicin have shown better in vitro activity than streptomycin and clinical trials have demonstrated a similarly good efficacy.7 In the past years, some authors have recommended the use of gentamicin in these combination schedules based on its more favourable profile.8 Overall, doxycycline/aminoglycoside combinations have been considered the most effective treatment for human brucellosis, whereas the oral association of doxycycline and rifampicin may be a good alternative because of its more comfortable administration for non-complicated forms of the disease.6 However, the combination doxycycline–streptomycin is ineffective in human infections caused by Rev 1,3 probably due to the resistance of this vaccine strain to streptomycin as a logical
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consequence of its isolation procedure.\textsuperscript{2,9} Thus, in the case of being diagnosed, these infections due to Rev 1 are empirically treated with the combination doxycycline/rifampicin. Surprisingly, no clinical information exists to the possible use of other aminoglycosides in these patients, probably because of the assumption among clinicians of the existence of cross-resistances between streptomycin and other aminoglycosides. Based on the current knowledge of the mechanisms of resistance of Rev 1 strain to streptomycin, we speculated that other aminoglycosides may be active against this infection and therefore that the combination doxycycline–gentamicin may also be suitable for the treatment of patients infected with Rev 1.

In a first trial, and to exclude the possibility of cross-resistance, we have determined the susceptibility of *B. melitensis* Rev 1 vaccine strain against a selection of aminoglycosides in vitro. As the clinically most relevant aminoglycoside, the efficacy of gentamicin given alone or combined with doxycycline was determined in comparison with standard therapies in a second trial using BALB/c mice experimentally infected with the *B. melitensis* Rev 1 strain.

**Material and methods**

**Bacterial strain and suspensions**

The *B. melitensis* Rev 1 reference strain was kept freeze-dried in the *Brucella* Culture Collection of the CITA (Zaragoza, Spain). Before its use, *B. melitensis* Rev 1 cells were rehydrated in sterile Buffered Saline Solution (BSS; 0.85% NaCl, 0.1% KH$_2$PO$_4$, 0.2% K$_2$HPO$_4$; pH 6.85) and cultured onto Blood Agar Base (BAB No. 2; Difco, Becton Dickinson, USA) plates at 37°C for 5 days. The resulting culture was assessed for purity and absence of dissociation by the White and Wilson method\textsuperscript{10} and selected Rev 1 colonies subcultured in BAB for 24–48 h at 37°C. Cultures were harvested in BSS and adjusted by spectrophotometry to $10^5$ cfu/mL (OD$_{600}$ = 0.170) either in Mueller–Hinton broth (M–H; Difco, Becton Dickinson, USA)—for MIC determination—or in BSS—for inoculation in mice. For MIC assays, the bacterial suspension was properly diluted in the M–H broth to obtain a suspension containing $5 \times 10^5$ cfu/mL. For mice inoculations, two serial 10-fold dilutions of the adjusted suspension were performed in BSS to obtain a final bacterial suspension containing $1 \times 10^7$ cfu/mL.

**MIC assay**

A standard broth microdilution method was applied to determine the susceptibility of the *B. melitensis* Rev 1 vaccine strain to aminoglycosides other than streptomycin: kanamycin, amikacin, tobramycin, gentamicin, netilmicin and spectinomycin.\textsuperscript{11} Tetracyclines (tetracycline and doxycycline) and rifampicin (all obtained from Sigma–Aldrich, Spain) were used as controls. For this, 2-fold dilutions ranging from 256 to 0.25 mg/L of each antibiotic were prepared in M–H broth. A total of 0.1 mL of each antibiotic dilution plus 0.1 mL of the corresponding *B. melitensis* Rev 1 suspension containing $5 \times 10^6$ cfu/mL were dispensed in each of 96 wells on sterile polystyrene microplates (Maxisorp Nunc\textsuperscript{10}, Denmark). After homogenization, plates were incubated at 37°C for 5–7 days and the bacterial growth assessed by direct observation of absence of turbidity.

**Animal experiments**

Seven-week-old female BALB/c mice were obtained from Charles River Laboratories (Barcelona, Spain). Animals were kept in cages, in groups of 10 mice per cage, for 2 weeks before the start of the experiments, with water and food *ad libitum* and accommodated under biosafety conditions in the restricted-access facility at the CITA (Zaragoza, Spain; registration number ES 50 297 012 005). The experimental procedures on mice and the facilities used to hold the experimental animals are in accordance with the current European (Directive 86/609/EEC, in DOCE number 358), National (Real Decreto 233/1988, in BOE number 67) and Regional (Ley 11/2003, in BOA number 35) laws. A total of 56 mice were inoculated intraperitoneally (ip) with $1 \times 10^6$ cfu/mouse of *B. melitensis* Rev 1 strain in 0.1 mL of the adequate bacterial suspension (see above). Ten days later, mice were allotted randomly in eight groups each composed of seven mice. One group of mice was kept untreated as control and the remaining groups were treated once a day for 14 days, with the following antibiotics: (i) doxycycline (Vibravenosa\textsuperscript{18} 100 mg, Pfizer S.A., Madrid, Spain) given orally at 2 mg/day; (ii) streptomycin (Sulfato de estreptomicina Reig Jofré\textsuperscript{19} 1 g, Laboratorios Reig Jofré S.A., Barcelona, Spain) given ip at 0.4 mg/day; (iii) gentamicin (Gevramycin\textsuperscript{18} 20 mg, Schering Plough, Madrid, Spain) given ip at 0.4 mg/day; (iv) rifampicin (Rifaladin\textsuperscript{18} 20 mg/mL, Aventis Pharma, Madrid, Spain) given orally at 0.5 mg/day; (v) doxycycline/streptomycin; (vi) doxycycline/gentamicin; (vii) doxycycline/rifampicin. Combined treatments were applied with the same doses and routes as the corresponding antibiotics given alone. The selection of the antibiotic doses was made according to previous experimental brucellosis studies in mice.\textsuperscript{12–14} To avoid inhibitory effects due to tissue persistence of the antibiotics, the numbers of cfu/spleen of the *B. melitensis* Rev 1 strain were determined 10 days after the last treatment (i.e. 34 days after inoculation), as described elsewhere.\textsuperscript{15} Briefly, the spleens were aseptically removed, individually weighed, blended and diluted in 9 volumes (1:10; w:v) of BSS. Serial 10-fold dilutions of each spleen were performed in the same diluent, and 0.1 mL of the homogenate and of each dilution were smeared in triplicate on BAB culture plates. The number of Rev 1 cfu was determined after incubation of plates at 37°C in air for 5–7 days. The mean and SD (n = 7) of the log cfu/spleen for each group of mice as well as the ratio of mice cured (no colonies isolated in the first dilution of the spleen; limit of detection is <4 cfu/spleen) were calculated. A value of 1 cfu was considered when no cfu were detected in 0.3 mL of the spleen homogenate seeded. The statistical comparisons between mean values (n = 7) of log cfu/spleen were made using the one-way ANOVA and the Fisher’s Protected Least Significant Differences (PLSD) tests. The percentages of mice cured according to the different treatments were statistically compared using the $\chi^2$ test with the Fisher–Yates correction when required.

**Results**

As expected, the Rev 1 strain was not inhibited at concentrations of streptomycin as high as 2 mg/L, but it was at concentrations of 4 mg/L. Moreover, the Rev 1 strain was susceptible to the lowest concentration (0.25 mg/L) of the remaining aminoglycosides tested, thus showing no evidence for a common pattern of resistance of Rev 1 to the different aminoglycosides. The tetracyclines used as controls (tetracycline and doxycycline) inhibited the growth of Rev 1 at the lowest concentrations (0.25 mg/L) tested. The MIC for rifampicin, used also as a control, was 1 mg/L.

The results of the antibiotic treatments applied in BALB/c mice experimentally infected with *B. melitensis* Rev 1 are summarized in Table 1. When given alone, streptomycin was the only antibiotic unable to induce a significant reduction in the
controls, and no statistical differences were found in the log cfu of Rifampicin 0.96 – Streptomycin 3.45 alone but resulted in similar efficacy to rifampicin given alone. Either rifampicin or gentamicin resulted in a complete clearance in infection. In contrast, being equally effective in reducing the log cfu of Rev 1 infected mice were fully cleared of Rev 1 exclusively to doxycycline. Moreover, only four of the seven mice treated with this combination were fully cleared of Rev 1. Statistical differences between the percentage of cured mice by the Fisher’s PLSD test: a P < 0.002 versus doxycycline; b P < 0.02 versus gentamicin given alone; c P < 0.05 versus doxycycline-streptomycin treatment; d P < 0.05 versus untreated controls and streptomycin given alone.

Discussion

Antibiotic therapy for human brucellosis has been the objective of many studies but little attention has been directed to the infections induced by the attenuated B. melitensis Rev 1 vaccine strain. Doxycycline is one of the most widely used antibiotics for treating human brucellosis, but relapse rates are very high when it is used as monotherapy. For this reason, a combination with aminoglycosides is recommended in order to increase the efficacy of treatments and avoid relapses. While streptomycin has been the aminoglycoside most frequently used, gentamicin offers a better efficacy–toxicity profile.

Owing to the resistance of Rev 1 strain to streptomycin, the treatment of this infection can be problematic. Since a cross-resistance with other aminoglycosides has been assumed among clinicians, and in the case of being adequately diagnosed, Rev 1 infected patients are empirically treated with an oral combination of doxycycline and rifampicin, which is a good alternative, but less effective in complicated or focal forms of disease.

Clinical failures in brucellosis treatment are not related to the development of antimicrobial resistance, but are a consequence of the ability of Brucella to survive within the cells. This particular intracellular location protects brucellae against antimicrobial agents and probably explains why this bacterium has not required the evolutionary development of antimicrobial resistance as has occurred in most microorganisms. However, it has been reported that Normi multidrug efflux protein in B. melitensis confers resistance to gentamicin and other antimicrobial agents and the substrate specificity of Normi is highly similar to that of YdhE protein of Escherichia coli, which confers resistance to aminoglycosides such as kanamycin and streptomycin.

It has been reported that B. melitensis Rev 1 strain carries a mutation in the rpsL gene encoding the small subunit of the ribosomal protein S12, leading to an amino acid Pro-to-Leu change at codon 91. In fact, amino acid changes leading to chromosomally acquired streptomycin resistance in other bacteria have been reported at similar locations, conferring low-levels of resistance. In Salmonella Typhimurium, streptomycin-resistant mutants having this rpsL mutation acquire compensatory mutations, mainly in the rpsD, rpsE and rpsL genes encoding the ribosomal proteins S4, S5 and L19, respectively, but the existence of compensatory mutations in B. melitensis Rev 1 strain remains unknown. In ribosomal S12 mutants of E. coli there was no cross-resistance between streptomycin and the other aminoglycosides. In the present study we confirm similar findings for B. melitensis Rev 1 strain since all the aminoglycosides tested except streptomycin were effective in inhibiting the growth of this vaccine strain in vitro. The susceptibility against the other aminoglycosides and doxycycline (MIC ≤ 0.25 mg/L) and rifampicin (MIC 1 mg/L) used as controls was in agreement with the results obtained from B. melitensis field strains. However, the in vitro efficacy of some aminoglycosides does not necessarily correlate with their in vivo efficacy, because of the limited penetration of these antibiotics into eukaryotic cells, the niche of brucellae. As the comparatively high MIC of streptomycin for the vaccine strain suggests, treatment of mice with...
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streptomycin given alone even at relatively high doses (0.4 mg/ day) was fully ineffective against Rev 1 infection (Table 1). Accordingly, the moderate therapeutic effect obtained after treatment with the combination doxycycline–streptomycin should be exclusively attributable to the efficacy of doxycycline. This result was in agreement with that reported in Rev 1 infected patients.\textsuperscript{6}

To the best of our knowledge, no reports concerning the antibiotic therapy of Rev 1 infection in mice are available. The mouse model used was based on preliminary experiments conducted with virulent \emph{Brucella} strains,\textsuperscript{14,25} but adapted to the particular kinetics of the infection induced by the attenuated Rev 1 strain. Based on preliminary reports in mice,\textsuperscript{14,25} and the effective use of gentamicin in clinical practice,\textsuperscript{7} we selected gentamicin as the aminoglycoside of choice to be tested in mice. In this experiment we used a high dose of gentamicin of 0.4 mg/day, increased with respect to that used in previous experiments, showing that gentamicin doses of around 0.1–0.2 mg/day and pharmacokinetic parameters similar to those obtained with usual doses in humans are subtherapeutic for treating experimental brucellosis in mice.\textsuperscript{14,25} However, this gentamicin dose was equivalent to that used for streptomycin but in contrast to streptomycin when given alone reduced significantly the Rev 1 cfu with respect to untreated controls (Table 1). Treatments with doxycycline or rifampicin resulted also in a significant reduction of the levels of Rev 1 infection, but none of the antibiotics given alone was able to clear the infection in all animals treated (Table 1). The complete clearance of the bacteria between streptomycin and other aminoglycosides exclusively according the spleen weights. The extrapolation selecting the most adequate treatment.

The mean splenic counts were below the limit of detection of the method in both experimental groups and, thus, varying exclusively according the spleen weights. The extrapolation of these therapeutic results to human disease should be made with caution, and the goal of the present study was not to compare the efficacy of doxycycline–gentamicin combination with doxycycline–rifampicin combination in the infection due to Rev 1 vaccine strain, but to prove the absence of cross-resistance between streptomycin and other aminoglycosides \emph{in vitro} and in the mouse model. Our results strongly suggest that gentamicin may be used along with doxycycline when the classical combination of a tetracycline with an aminoglycoside is considered the first choice in the treatment of patients with brucellosis due to \emph{B. melitensis} Rev 1 vaccine strain.

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Transparency declarations

None to declare.

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