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PROPHYLACTIC EFFICACY OF SOME ANTIBIOTIC COMBINATIONS AGAINST *BRUCELLA MELITENSIS* 16M IN BALB/C MICE

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Summary

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Brucellosis is an endemic zoonosis in Syria, affecting both humans and animals. Data regarding suitable antibiotic combinations in post-exposure prophylaxis against *Brucella melitensis* infections are rare. Prophylactic effects of some antibiotic combinations were assessed in BALB/c mice, to limit or control infection by *B. melitensis* 16M. Antibiotics were administrated prior to (for 7 days), after or at the same time as (for 5 days) the bacterial administration. When a concentration of 10^4 CFU of bacteria was injected, doxycycline-rifampicin combination reduced the bacterial counts in the spleens of infected mice in all mice groups either 48 h our 30 days after the cessation of antibiotic treatment; whereas, all other combinations had almost good efficacy only 30 days after the cessation of antibiotic treatment. On another hand, only doxycycline-rifampicin and rifampicin-levofloxacin combinations had good efficacy 48 hours after the cessation of antibiotic treatment, when a concentration of 10^7 CFU of bacteria was injected. In conclusion, these results suggest that doxycycline-rifampicin combination, and may be doxycycline-ciprofloxacin and rifampicin-levofloxacin combinations, had good prophylactic efficacy against *B. melitensis* infections and may provide protection against these infections.

Key words: antibiotic combinations, *Brucella melitensis*, prophylaxis

INTRODUCTION

Brucellosis remains the commonest anthropozoonosis worldwide (Pappas *et al.*, 2006). *B. melitensis* is the major global cause of human disease, followed by *B. abortus* and *B. suis*. It is transmitted to humans through direct contact with infected animals, consumption of dairy products, or inhalation of aerosols.

Brucellosis is still hyperendemic in the Mediterranean basin, Middle East, Southwest Asia and parts of Latin America (Black, 2004). In 1986, the WHO (Anonymous, 1986) has released recommendations for use of doxycycline, combined with either rifampicin or streptomycin for treating human brucellosis. Different regimens have been universally applied in clinical practice (Ariza *et al*., 2007). Although *Brucella* isolates are generally considered susceptible to the antibiotics recommended by the WHO, sporadic cases of a kind of antibiotic resistance have been reported (Baykam *et al*., 2004; Lopez-Merino *et al*., 2004). Despite all

these regimens, a small percentage of relapses are still seen, ranging from 5% to 15% in uncomplicated cases. Risk factors for relapse have been assessed (Ariza *et al*., 1995; Solera *et al*., 1998), but it remains unclear what is the best regimen to be used in their presence.

The high incidence of relapses and therapeutic failures, in addition to the side effects of drug combination strategies, has led to the investigation of new treatment schemes of the disease. Fluoroquinolones, may serve as alternative drug choices (Kilic *et al*., 2008). Despite that clinical experience with fluoroquinolones, such as ciprofloxacin, for the treatment of brucellosis has been disappointing, this therapeutical group could be potentially useful for prophylaxis of *Brucella* infection. The efficacy of ciprofloxacin and levofloxacin against *Brucella spp*. has been determined *in vitro* in a number of studies that include reported MIC_{90} values (minimum inhibitory concentration for 90% of the organisms) of 0.19 μg/mL (Turkmani *et al*., 2006; Turan *et al*., 2007), 0.25 μg/mL for ciprofloxacin (Bodur *et al*., 2003); and 0.5 μg/mL (Trujillano-Martin *et al*., 1999) for levofloxacin.

Data are lacking regarding suitable post exposure antibiotic prophylaxis, which would ideally be a single-agent, short-course, and oral regimen (Atkins *et al*., 2010). However, ciprofloxacin administered with doxycycline or rifampicin appears to show some efficacy against brucellosis in humans (Agalar *et al*., 1999). Reports concerning the efficacy of ciprofloxacin (Shasha *et al*., 1992; Atkins *et al*. 2009a) and ofloxacin (Shasha *et al*., 1992) in the protection against brucellosis in murine model were disappointing. To our knowledge, in literature, no reports were found concerning the prophylactic role of antibiotic combination between one traditional drug with one quinolone against *B. melitensis* infection.

This study aimed to assess the prophylaxis with doxycycline-rifampicin, doxycycline-ciprofloxacin, doxycycline-levofloxacin, rifampicin-ciprofloxacin, and rifampicin-levofloxacin combinations against *B. melitensis* infection in BALB/C mice.

MATERIALS AND METHODS

Bacteria

B. melitensis strain 16M, obtained from the Laboratory of Microbiology and Immunology URBM (University of Namur, Belgium), was used as the challenge strain in this study. *Brucella* were grown for 48 h in 2YT agar (peptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L; agar, 13 g/L [GibcoBRL]) at 37 °C.

Bacteria were harvested into 20 mL of sterile phosphate-buffered saline (PBS) and the bacterial suspension was standardised to 10^{10} colony-forming units (CFU)/mL prior to dilution to appropriate concentrations of inoculates. The concentrations were determined retrospectively by enumeration of ten-fold dilutions of the inoculates on 2YT plates.

All experiments with live *Brucella* were performed in biosafety level 2 facilities.

Antibiotics

Doxycycline (Sigma, St. Louis, USA), rifampicin (Sigma), ciprofloxacin (Bayer, Istanbul, Turkey), and levofloxacin (Sigma) were dissolved as per manufacturer recommendations to a working concentration of 8 mg/mL. Antibiotics were prepared freshly each day and sterilised through a 0.2 μm filter.

Animals

Three hundred twenty females BALB/c mice (7 to 8 weeks old) were purchased from Charles River Laboratories, France. Animals were kept in cages, five mice per cage (sixty-four experimental groups in total), for 2 weeks before the start of the experiments (Table 1). The mice were kept in conventional animal facilities and received water and food *ad libitum*. The experimental procedures on mice and the facilities used to hold the experimental animals were in accordance to National law (Real Decreto 233/1988, in BOE number 67).

A total of 160 mice (32 groups) were inoculated intraperitoneally (i.p.) with $\approx 1\times10^4$ cfu/mouse of *B. melitensis* 16M strain in 100 µL of PBS, and another 160 mice (32 groups) were inoculated i.p. with $\approx 1\times10^7$ cfu/mouse of *B. melitensis* 16M strain in 100 µL of PBS. For each bacterial concentration, two groups were kept untreated as control and the remaining 30 groups were treated, twice a day, with 100 µL of different antibiotic combination solutions (equivalent to 40 mg/kg in a 20-g mouse). The antibiotic treatment was started either 48 h prior to challenge (continued for 7 days), at the time of challenge (continued for 5 days) or 24 h after challenge (continued for 5 days). PBS was started at the time of challenge (continued for 5 days) for control groups. Animals were culled at either 48 h or 30 days after the final antibiotic administration. Spleens were removed *post mortem* and homogenised in 5 mL of distilled water using a stomacher 80-Biomaster (Seward, England). Bacterial loads were determined following enumeration of ten-fold serial dilutions on 2YT plates (incubated for 3 days at 37 ^{o}C in air).

Statistical analyses

Data were transformed into $\log_{10}$ CFU. Differences in CFU between the treated and untreated groups were evaluated by one way analysis of variance (ANOVA). All analyses were conducted with version 5.0 GraphPad Prism. Bonferroni's *post hoc* test was used to compare individual time points with the control. *P* values of 0.05 or less were considered statistically significant.

Table 1. Number of mice groups depending on the injection protocol and the time of sacrifice

	Number of mice groups challenged with 10^4 cfu *B. melitensis*/mouse			
	48 h prior to challenge	at the time of challenge	24 h after challenge	control group
Animals culled 48 h after the final antibiotic administration	5	5	5	1
Animals culled 30 days after the final antibiotic administration	5	5	5	1
	Number of mice groups challenged with 10^7 cfu *B. melitensis*/mouse			
	48 h prior to challenge	at the time of challenge	24 h after challenge	control group
Animals culled 48 h after the final antibiotic administration	5	5	5	1
Animals culled 30 days after the final antibiotic administration	5	5	5	1

RESULTS

Figures 1 and 2 confirmed the utility of all antibiotic combinations for preventing *Brucella* infection 30 days after the cessation of treatment in all groups, when a concentration of 10^4 CFU of *B. melitensis* 16M was injected. However, the doxycycline-rifampicin combination was relatively more effective in prior to exposure and 24 h after exposure groups (Fig. 2B, C, P<0.0001) than other combinations. Significant protection was observed 48 hours after the cessation of antibiotic treatment in mice treated with doxycycline-rifampicin combination in all groups, i.e. either prior to exposure, at the same time as exposure or 24 h after exposure (Fig. 1, P<0.0001). However, doxycycline-ciprofloxacin combination protection was observed 48 hours after the cessation of antibiotic treatment in at the same time as exposure and prior to exposure groups (Fig. 1A, C, P<0.01 and P<0.0001, respectively), whereas doxycycline-levofloxacin combination was effective only in the 24 h after exposure group (Fig. 1B, P<0.0001) and rifampicin-levofloxacin combination was effective only in the prior to exposure group (Fig. 1C, P<0.0001). Finally, rifampicin-ciprofloxacin combination was ineffective.

Moreover, when a concentration of 10^7 CFU of *B. melitensis* 16M was injected, Fig. 3 revealed that doxycycline-rifampicin and rifampicin-levofloxacin combinations protection was observed 48 h after the cessation of antibiotic treatment in 24 h after exposure and prior to exposure groups (Fig. 3B, C, P<0.001 and P<0.0001, respectively). Thirty days after the cessation of antibiotic treatment, only doxycycline-ciprofloxacin and rifampicin-levofloxacin combinations in the 24 h after exposure group (Fig.4B, P<0.0001) were effective against *Brucella* infection.

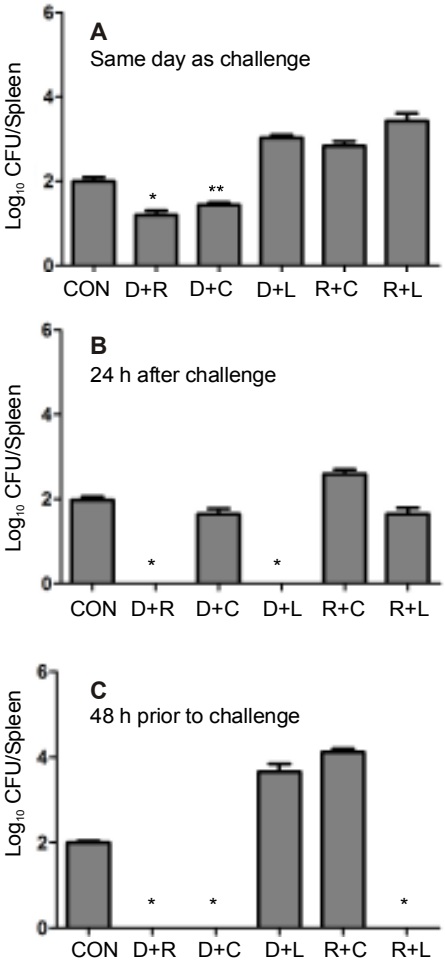

Fig. 1. Protective efficacy of doxycycline+rifampicin (D+R), doxycycline+ciprofloxacin (D+C), doxycycline+levofloxacin (D+L), rifampicin+ciprofloxacin (R+C) and rifampicin+levofloxacin (R+L) combinations against *B. melitensis* in groups of five BALB/c mice challenged with 10^4 CFU of *B. melitensis* 16M and killed 48 h after the last injection of the antibiotic. Treatment started at the same time as challenge (**A**); 24 hours after challenge (**B**) or 48 h prior to challenge (**C**). CON= control. *P<0.0001 and **P<0.01 vs control.

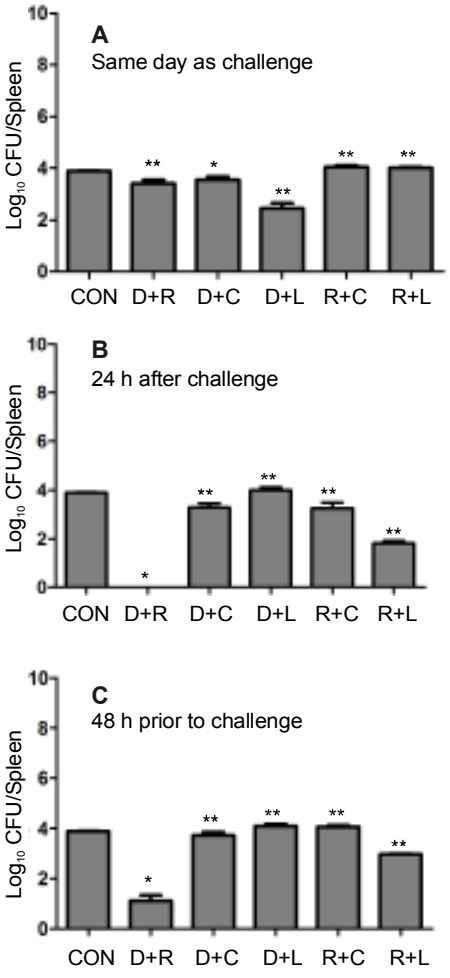

Fig. 2. Protective efficacy of doxycycline+rifampicin (D+R), doxycycline+ciprofloxacin (D+C), doxycycline+levofloxacin (D+L), rifampicin+ciprofloxacin (R+C) and rifampicin+levofloxacin (R+L) combinations against *B. melitensis* in groups of five BALB/c mice challenged with 10^4 CFU of *B. melitensis* 16M and killed 30 days after the last injection of the antibiotic. Treatment started at the same time as challenge (**A**); 24 hours after challenge (**B**) or 48 h prior to challenge (**C**). CON= control. *P<0.0001 and **P<0.001 vs controls.

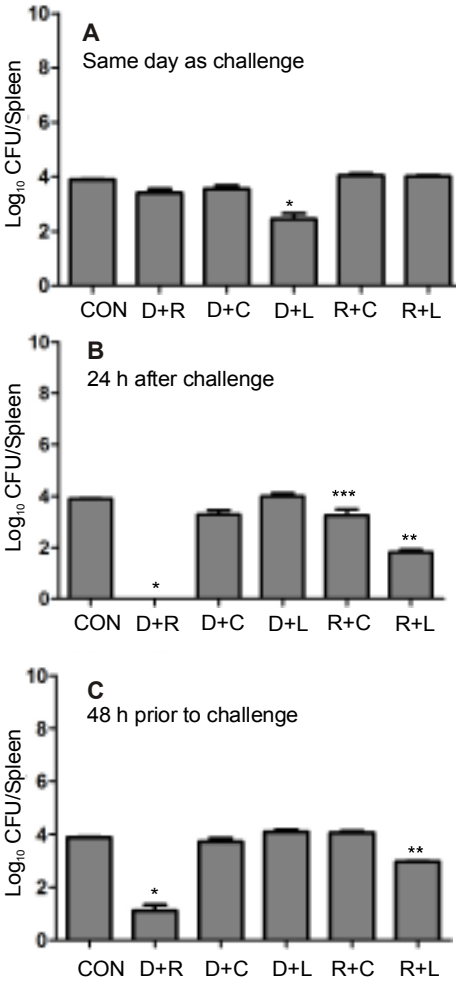

Fig. 3. Protective efficacy of doxycycline+rifampicin (D+R), doxycycline+ciprofloxacin (D+C), doxycycline+levofloxacin (D+L), rifampicin+ciprofloxacin (R+C) and rifampicin+levofloxacin (R+L) combinations against *B. melitensis* in groups of five BALB/c mice challenged with 10^7 CFU of *B. melitensis* 16M and killed 48 hours after the last injection of the antibiotic. Treatment started at the same time as challenge (**A**); 24 hours after challenge (**B**) or 48 h prior to challenge (**C**). CON= control. *P<0.0001, **P<0.001 and ***P<0.05 comparing with control.

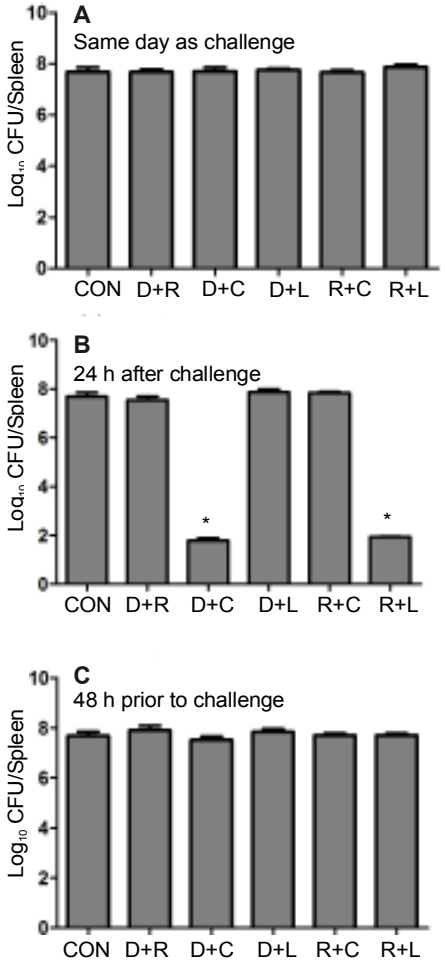

Fig. 4. Protective efficacy of doxycycline+rifampicin (D+R), doxycycline+ciprofloxacin (D+C), doxycycline+levofloxacin (D+L), rifampicin+ciprofloxacin (R+C) and rifampicin+levofloxacin (R+L) combinations against *B. melitensis* in groups of five BALB/c mice challenged with 10^7 CFU of *B. melitensis* 16M and killed 30 days after the last injection of the antibiotic. Treatment started at the same time as challenge (**A**); 24 hours after challenge (**B**) or 48 h prior to challenge (**C**). CON= control. *P<0.0001 vs control.

DISCUSSION

Antibiotic therapy for human brucellosis has been the objective of many studies. 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. The treatment of choice of human brucellosis caused by *B. melitensis* strains is a classical combination of long-acting tetracyclines and streptomycin (Solera *et al*., 1995). While streptomycin has been the aminoglycoside most frequently used, gentamicin offers a better efficacy–toxicity profile. Clinicians and laboratory researchers have performed several microbiological and clinical studies of the possible use of quinolones in the treatment of human brucellosis. The intracellular penetration and excellent *in vitro* activity of the fluoroquinolones make them attractive in treating intracellular infections such as brucellosis (Qadri *et al*., 1995). Bacteria can grow and multiply, infecting different parts of the body. Fluoroquinolones, such as levofloxacin (third generation) and ciprofloxacin (second generation), could stop multiplication of bacteria by preventing the reproduction and repair of their genetic material, DNA. On the other hand, doxycycline inhibits protein biosynthesis that causes cell death of the bacterial cell. It block bacterial translation by binding reversibly to the 30S subunit and distorting it in a way such that the anticodons of the charged tRNAs cannot align properly with the codons of the mRNA (Connel *et al.,* 2003). Rifampicin is thought to inhibit bacterial DNA-dependent RNA polymerase, which appears to occur as a result of drug binding in the polymerase subunit deep within the DNA/RNA channel, facilitating direct blocking of the elongating RNA (Campbell *et al.,* 2001). Moreover,

the need for a regimen that would eliminate disease relapse further necessitated the use of quinolones.

In literature, only some data regarding suitable antibiotic combinations post-exposure prophylaxis in murine models are available. The first experimental results showed that antibiotic combinations therapy with streptomycin plus aureomycin, terramycin, or sulfadiazine were definitely superior to any monotherapy by one of these drugs. Such combined therapy completely eradicated *Brucella* from the spleens of all but 1 of 100 mice treated with any of these combinations (Shaffer *et al*., 1953). The results of Lang *et al*. (1993) demonstrated that the combinations doxycycline-streptomycin and rifampicin-streptomycin are synergistic against *B. melitensis*, while the combination streptomycin-ciprofloxacin is indifferent and ineffective in the management of acute murine brucellosis. The results also appear to support the clinical superiority of combination drug therapy over monotherapy. On another hand, Grillo *et al*. (2006) found that the combinations doxycycline-gentamicin and doxycycline-rifampicin were effective in the clearance of Rev 1 infection, but only doxycycline-gentamicin combination improved significantly the therapeutic efficacy as compared with that of the antibiotics given alone. As a prophylactic agent against bioterrorism organisms, ciprofloxacin is recommended for post-exposure prophylaxis against *Yersinia pestis* (Russell *et al*., 1996), tularaemia (Russell *et al*., 1998), and systemic anthrax (Steward *et al*., 2004). Therefore, ciprofloxacin, and fluoroquinolones in general, might also be potentially useful for prophylaxis of *Brucella* infection (Atkins *et al*., 2009a).

Data reported by Shasha *et al*. (1992) indicate that mice treated with ciprofloxacin for 14 days or 21 days do not eliminate a *B. melitensis* infection. On the other hand, data reported by Atkins *et al.* (2009a) indicated the relatively poor efficacy of ciprofloxacin for treating brucellosis compared with doxycycline, but highlight the ability of ciprofloxacin potentially to provide a low level of protection. In another two studies, Atkins *et al.* suggested that, comparing with doxycycline, neither trovafloxacin nor grepafloxacin (Atkins *et al.,* 2010), neither moxifloxacin nor gatifloxacin (Atkins *et al.,* 2009b) would likely be valuable for post exposure prophylaxis of *Brucella* infection.

Our data indicate that when the infection was performed with a high concentration of *B. melitensis* 16M (10^7 CFU), all used combinations, with the exception of doxycycline-rifampicin and rifampicin-levofloxacin combinations in the groups that killed 48 h after the cessation of antibiotic treatment, had no prophylactic efficacy against *B. melitensis* infection. On the contrary, doxycycline-ciprofloxacin and rifampicin-levofloxacin combinations had almost the same good efficacy as doxycycline-rifampicin combination when a low concentration of *B. melitensis* 16M (10^4 CFU) was used. In addition, the doxycycline-levofloxacin combination showed a moderate prophylactic effect. Finally, the rifampicin-ciprofloxacin combination showed relatively good activity only in the groups that killed 30 days after the cessation of antibiotic treatment.

Nevertheless, Al Sibai *et al.* (1992), in a prospective study, reported high probabilities of brucellosis relapse after monotherapy with ciprofloxacin (26.7%). Also, in 480 patients with various forms of brucellosis, Aygen *et al.* (2002) revealed that the probabilities of relapse for the various treatment regimens were

4.6% for patients who received nonquinolone regimens and 17.9% for patients who received quinolone-based regimens (21.4% for ciprofloxacin monotherapy and 14.3% for the combinations of quinolones with other antibiotics). In addition, Tekkok *et al.* (1993) showed, in a retrospective study, that ofloxacin monotherapy led to a higher probability of brucellosis relapse than the combination of ofloxacin and rifampin in a small number of patients with spondylitis. Moreover, relapse rate was found to be 7.2% and 6.7% for ofloxacin plus rifampicin and doxycycline plus rifampicin, respectively (Saltoglu *et al.,* 2002). Finally, doxycycline plus ciprofloxacin found to be the most active combination *in vitro* (Al Dahouk *et al.,* 2005).

CONCLUSION

Our results highlight the potential of doxycycline-ciprofloxacin and rifampicin-levofloxacin combinations to provide almost the same level of protection against a low concentration of *B. melitensis*, in comparison with doxycycline-rifampicin combination. Almost no effect was seen when using these combinations as prophylactic agents against a high concentration of *B. melitensis* bacteria. If rifampicin could be replaced by ciprofloxacin, then rifampicin use could be restricted solely to the treatment of tuberculosis, which is considered as a big challenge in Syria. Finally, further and more specific studies on the favourable host, sheep, are recommended to determine the prophylactic efficacy of these combinations against *B. melitensis.*

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