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Comparative Efficacy of Some Quinolones and Doxycycline Against Chronic Infection of *Brucella melitensis* 16M in BALB/c Mice

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Abstract. This study was under taken to observe various treatment methods for brucellosis caused by *Brucella melitensis*. The effect of therapeutic regimens with ciprofloxacin, ofloxacin and levofloxacin alone or in combination with doxycycline was assessed against *B. melitensis* chronic infection using 200 mice. Doxycycline alone or in combination with ciprofloxacin was significantly found to reduce the infection till 135 days post-infection ($p<0.0001$). Moreover, doxycycline was more effective than ciprofloxacin and ofloxacin 135 days post-infection ($p = 0.04$ and $p = 0.02$, respectively). However, treatment with quinolone-doxycycline combinations revealed synergistic effects as they were able to reduce the splenic cell forming unit (CFU) from day 45 post-infection. Similarly, doxycycline treatment reduced the splenic colony forming unit (CFU) from day 90 post-infection. In conclusion, doxycycline seems to be the most effective agent against *Brucella* chronic infection.

Keywords: doxycycline, quinolones, *Brucella melitensis*, BALB/c mice

Introduction

Brucellosis remains the most common and serious problem in some parts of the world (Pappas *et al*., 2006). Ingestion of unpasteurized dairy products, as well as occupational exposure to infected animals are the major causes of brucellosis. Many *Brucella* species infect animals through direct contact. However, human could be infected when exposed to *B. abortus*, *B. melitensis*, or *B. suis* and the exhausting disease could become, over time, a chronic disease that affects several organs. In addition, some species could be used in a bioterrorist attack (Ariza *et al*., 2001).

In ruminants, no symptoms were seen after the first abortion; but these infected animals might develop a chronic disease, become chronic carrier, and *Brucella* could be found in milk and uterine discharges of these carriers during subsequent pregnancies (Díaz *et al*., 2006).

In humans, undulant fever recovers completely within three to twelve months with only few patients suffering from a chronic disease. However, relapses could be seen months after the initial symptoms. The most common complications occasionally seen in the undulant and chronic forms are arthritis, spondylitis, meningitis and chronic fatigue (Hendaus *et al*., 2015; Bosilkovski *et al*., 2007; Morata *et al*., 2003).

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In mice, the most infected organs are spleen and liver which are rapidly colonised with *Brucella* in splenectomised mice. During the chronic phases, the higher *Brucella* colonisation of the spleen protects the liver from infection (Grilló *et al*., 2012). Therefore, spleen is the best target organ to study *Brucella* infections. Chronic steady phase, where bacterial counts were stable, commonly started at 21 days and lasting till 56-77 days post-infection. At this phase, the maximum number of CFU within spleen is recorded. On the contrary, a slow elimination of the bacteria from target organs is observed during the chronic declining phase which may last beyond 252 days (High *et al*., 2007). However, experiments on mice are not normally prolonged beyond 120 days. Large and prominent splenomegaly was rarely seen in ruminants (Carvalho-Neta *et al*., 2010) and the sequelae observed in chronic brucellosis in humans have not been reported in mice (Bosilkovski *et al*., 2007).

Although many regimens have been experimented and tested, treatment regimen approved by the World Health Organisation (1986) still recommended for the treatment of human brucellosis. However, the incidence of treatment failure and frequent relapses are still a major concern (Roushan *et al*., 2006). Moreover, quinolones utilisation as alternatives in the treatment of brucellosis did not improve the results (Lopez-Merino *et al*., 2004).

Currently the human chronic brucellosis is generally treated with antibiotic triple therapy. The combination of rifampin, doxycycline, and streptomycin often is used whereas in non-responded or complicated cases, quinolones could be added to the conventional treatment as a third agent (Falagas and Bliziotis, 2006).

The aim of the current study was to compare the short-term treatment efficacy of several quinolones alone or in combination with doxycycline in experimentally induced *B. melitensis* 16M infection in mice.

Materials and Methods

Bacteria. *Brucella melitensis* 16M strain was obtained from the Laboratory of Microbiology and Immunology URBM (University of Namur, Belgium) and grown in the Department of Molecular Biology and Biotechnology (Atomic Energy Commission of Syria), 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. Prior to dilution to appropriate concentrations of inoculates, bacteria were harvested into 20 mL of sterile phosphate-buffered saline (PBS) and the bacterial suspension was standardized to 10^{10} colony-forming units (CFU)/mL. 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), ciprofloxacin (Bayer, Istambul, Turkey), ofloxacin (Sigma), and levofloxacin (Sigma) were dissolved according to the manufacturer's recommendations to a working concentration of 8 mg/mL. Antibiotics were prepared freshly every day and sterilized through a 0.2 μm filter.

Animals. The effect of quinolones against chronic *Brucella* infection was studied according to the method of Domingo *et al*. (1995) with some modifications. Briefly, between February and October 2012, 200 BALB/c mice were infected with ≈ 10^6 CFU of *B. melitensis* 16M intraperitoneally. Thirty days later, the infected mice were divided into 8 groups of 25 mice each. The untreated control group received only the vehicle and was included in order to estimate the normal splenic infection during the course of this study, 225 days. At the 31st day, the seven other groups received one of the following treatment regimens: doxycycline (oral, 40 mg/kg every 12 h for 14 days); ciprofloxacin, ofloxacin or levofloxacin (oral, 40 mg/kg/day of body weight for 14 days); and one of doxycycline-ciproflox-acin, doxycycline-ofloxacin or doxycycline-levofloxacin combinations at the same doses mentioned above (i.e. doxycycline orally, 40 mg/kg every 12 h for 14 days; and quinolone orally, 40 mg/kg/day of body weight for 14 days). The experimental procedures on mice and the facilities used to hold the experimental animals are in accordance to National (Real Decreto 233/1988, in BOE number 67) law. Batches of five mice were sacrificed by cervical dislocation, on 45, 90, 135, 180 and 225 days post-infection. At post-mortem, spleens were removed, weighted, homogenised in 3 mL of distilled water using a stomacher 80-Biomaster (Seward, England). Bacterial loads were determined following enumeration of ten-fold serial dilutions on 2YT agar plates (incubated for 3 days at 37 °C in air, with 10% CO_2) using the spread plate technique. The average of three replicates of each dilution was calculated, and considered as the mean CFU/mL of each studied mouse.

Statistical analysis. Data were transformed into $\log_{10}$ CFU. Differences in CFU between the treated and untreated groups were evaluated by student's t-test. The data was analysed by version 5.0 GraphPad Prism. P values of 0.05 or less were considered statistically significant.

Results and Discussion

The results show that doxycycline was effective at day 45 post-infection ($p=0.03$) when compared with the control. Doxycycline and ofloxacin were found the most effective at day 90 post-infection ($p<0.0001$ and $p=0.005$, respectively). Whereas, ciprofloxacin and levofloxacin were less effective ($p=0.011$ and $p=0.047$, respectively). Only doxycycline was found effective at day 135 post-infection ($p<0.001$). In the same way, doxycycline was more effective than ciprofloxacin, ofloxacin and levofloxacin at day 90 post-challenge ($p<0.0001$, $p<0.0005$ and $p<0.0001$, respectively); and most effective than ciprofloxacin and ofloxacin at day 135 post-infection ($p=0.04$ and $p=0.02$, respectively) as shown in Fig. 1. Furthermore, Fig. 2 indicates that doxycycline-ciprofloxacin, doxycycline-ofloxacin and doxycycline-levofloxacin combinations were extremely efficient at day 45 post-challenge ($p<0.0001$ at all cases); and doxycycline-ciprofloxacin and doxycycline-ofloxacin combinations were very effective at day 90 post-challenge ($p=0.0026$ and $p=0.0015$, respectively).

Additionally, doxycycline-ciprofloxacin combination was very efficient at day 135 post-infection ($p<0.0001$), and doxycycline-ofloxacin combination was less effective ($p=0.032$). However, comparing with doxycycline, splenic CFU reduction was observed from day 45 post-infection when using all doxycycline-quinolone combinations ($p<0.0001$) comparing with doxycycline-ciprofloxacin and doxycycline-ofloxacin combinations; and $p=0.024$ comparing with doxycycline-levofloxacin combination.

The success of any drug in the treatment of brucellosis depends on its ability to penetrate the phagocytic cell membrane and work within its acidic environment. The failure in the treatment of acute cases of brucellosis may lead to the development of relapse, focal complications or even chronic disease (Castaño and Solera, 2009).

In human, most patients usually recover after one year of effective treatment while a few patients complain from focal or non-focal disease. Domingo *et al*. (1995) reviewed that relapses after the treatment with doxycycline alone for 21 days were higher than that noticed after the treatment with doxycycline for 45 days (29% versus 10-14%). In addition, ciprofloxacin at a 500 mg dose twice a day within 20 days in chronic stage of disease essentially reduced duration of local inflammatory processes of brucellosis with simultaneous treatment of the chronic infection focus, provides good proximate and distant outcomes of treatment (Pappas *et al*., 2005).

Whereas, in mice, treatment regimens with azithromycin 50 mg/kg/day for 10 days, doxycycline 50 mg/kg/12 h for 21 days or doxycycline 50 mg/kg/12 h for 45 days, produced a significant reduction in the chronic infection. In addition, when doxycycline was administered for 45 days, it was possible to eradicate *B. melitensis* completely in comparison with the treatment for 21 days only (Domingo *et al*., 1995). In agreement with these results, present research results revealed that the short-term treatment with doxycycline for 14 days was more effective than all quinolones and almost completely eradicates the infection 135 days after infection. Similarly, Shasha *et al*. (1992) reported that the longer the treatment with doxycycline the better the results obtained.

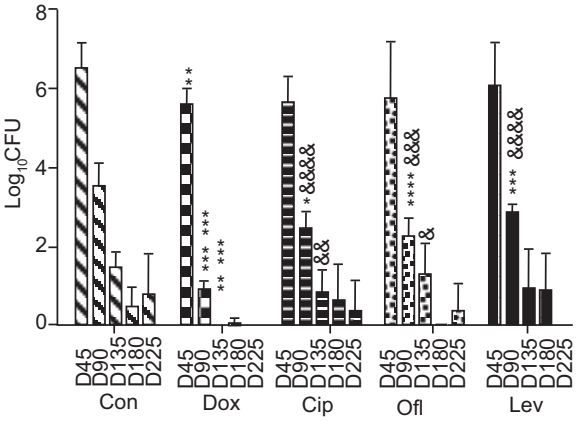

Fig. 1. Efficacy of doxycycline (Dox), ciprofloxacin (Cip), ofloxacin (Ofl) and levofloxacin (Lev) against *B. melitensis* 16M chronic infection. Each bar is representative of the mean bacterial count, counted in different days, in spleens of five mice ± the standard error. *p=0.011, **p=0.03, ***p=0.047, ****p=0.005, *****p=0.001 and ******p< 0.0001 versus control (Con); whereas, $^{\&}p=0.02$, $^{\&\&}p=0.04$, $^{\&\&\&}p< 0.0005$ and $^{\&\&\&\&}p< 0.0001$ versus doxycycline.

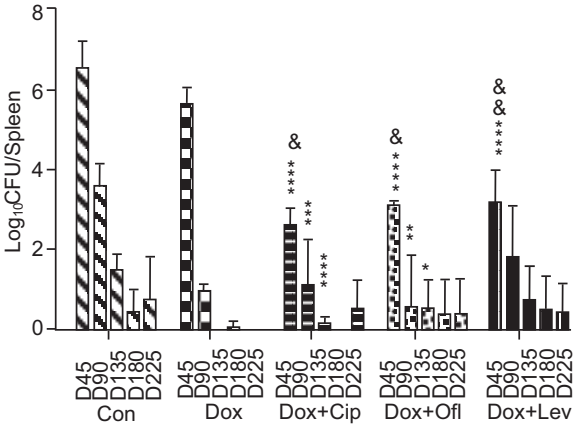

Fig. 2. Efficacy of doxycycline-ciprofloxacin (Dox+Cip), doxycycline-ofloxacin (Dox+Ofl) and doxycycline-levofloxacin (Dox+Lev) combinations against *B. melitensis* 16M chronic infection. Each bar is representative of the mean bacterial count, counted in different days, in spleens of five mice ± the standard error. *p=0.032, **p=0.0015, ***p=0.0026 and **** p<0.0001 versus control (Con); whereas, $^{\&}p< 0.0001$ and $^{\&\&}p=0.024$ versus doxycycline (Dox).

In addition, our results showed that ofloxacin, given orally for 14 days, was the most effective quinolone against chronic infection with *B. melitensis* 16M in murine model, and showed a significant reduction of the splenic infection at 90 days after infection. Moreover, doxycycline-quinolone combinations were more effective than the treatment with doxycycline alone since they could significantly reduce the splenic infection from day 45 post-infection. Unfortunately, these combinations could not completely eradicate the chronic infection.

Despite a few elevations in bacterial counts at 180 days post-infection comparing with that noticed at 135 days post-infection when doxycycline alone was applied; this elevation was not significant.

Conclusion

Different treatment methods were studied to control chronic human infection caused by *Brucella*. In conclusion, experiments on mice showed that short-term treatment with doxycycline was more effective than the treatment with quinolones. In addition, doxycycline-quinolones combinations were slightly more effective than doxycycline alone. However, treatment with doxycycline alone eliminates more number of bacteria than all other combinations after 135 days post-infection.

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