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# Effect of Co-trimazine on the Survival of *Brucella abortus* in Mouse Peritoneal Macrophages

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The efficacy of co-trimazine was studied *in vitro* in mouse macrophages infected with *Brucella abortus*. The macrophages first were allowed to phagocytes the *Brucella abortus*. After uptake period, extra cellular bacteria were removed by gentamicin. The bacteria were able to grow in the cells with an apparent multiplication rate of about $90_{min}$. Bactericidal activity was measured after treatment of the macrophages with co-trimazine. Co-trimazine showed a bactericidal effect at MIC concentration used, (6.32 mg $L^{-1}$ trimethoprim and 8.19 mg $L^{-1}$ sulphadiazine) and a total killing of intracellular bacteria at concentrations of 9.33 mg $L^{-1}$ trimethoprim and 12.46 mg $L^{-1}$ sulphadiazine). Results of the study show that co-trimazine was effective on intracellular *Brucella abortus*.

**Key words:** Co-trimazine, *Brucella abortus,* macrophage, trimethoprim, sulphadiazine

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## INTRODUCTION

Certain of bacteria could multiply in side of macrophages. Such bacteria often cause recurrent or chronic infections becomes their location protects them from the immune system of the host, as well as from antibiotics[1]. *Brucella abortus* is able to remain viable even after it has been phagocytes by protecting it self from the bactericidal activities of the phagocytes and by being shielded from extra cellular antibiotics. Various antimicrobial agents to be transferred in to phagocytes is essential when attempting to treat infections caused by bacteria capable of remaining alive inside cells. In humans, infection with *B. abortus* causes a disease known as undulant fever, Malta fever, or Bang's disease[2]. Infected humans can suffer protracted and debilitating symptoms most commonly including intermittent fevers, malaise, weight loss, back pain, joint pain, nervousness and depression. Physical findings can reveal lymphadenopathy, splenomegaly and joint swelling while further investigation may reveal genito-urinary involvement, arthritis, spondylitis, osteomyelitis, meningitis and endocarditis. The type of antibiotics used for treatment of brucellosis influences the recurrence rate and it is clear that a synergistic combination with marked intracellular activity is the best therapeutic regimen in brucellosis. The agents that are used in these combinations are tetracyclines, rifampicin, fluoroquinolones, trimethoprim-sulphamethoxazole, third generation cephalosporins, streptomycin and other aminoglycosides[3]. Published guidelines that have originated from WHO recommendations, have suggested rifampicin plus doxycycline management for human brucellosis for more than a decade[4]. Trimethoprim and sulphamethoxazole have all been widely used to treat systemic bacterial infections trimethoprim either alone, or in combination with a sulphonamide, is still a first line treatment for certain bacterial infections. Co-trimoxazole has been proposed for use in patients who are hypersensitive to beta-lactam antibiotics and has also been recommended as first-line treatment because of the good diffusion of trimethoprim and sulphamethoxazole into the meninges[5,6]. In view of the high rate of clinical failures observed with the proposed treatment, alternative treatment have been based on the high degree of intracellular penetration of certain antibiotics. In the present study, we have investigated the effect of co-trimazine on *Brucella abortus* multiply in mouse peritoneal macrophages.

## MATERIALS AND METHODS

**Bacteria:** *Brucella abortus* $S_{19}$ was used for our experiments. It was grown in Brucella broth medium and was stored at -70°C in the form of 1 mL aliquots containing $1.5\times10^3$ bacteria per mL in the growth phase.

**Antimicrobial:** Trimethoprim and sulphadiazine were obtained from sigma in the form of powders suitable for susceptibility testing.

**Minimum Inhibitory Concentration (MICs):** The MICs were determined by the agar dilution method on Iso-sensitest agar (oxoid). The MICs of trimethoprim , sulphadiazine and co-trimazine and were determined[7]. Fractional Inhibitory Concentration Index (FIC Index) was calculated as described by Sabath[8]. Bacterial suspension was spread on iso-sensitest agar (Oxoid) plates supplemented with 5% sheep blood agar and Etest strips (AB Biodisk) were applied. The plates were incubated at 37°C and the results were evaluated after 48 h. The procedure was performed according to The National Committee for Clinical Laboratory Standards (NCCLS).

**Peritoneal macrophages:** Macrophages were harvested from the peritoneal cavity of BALB/c mice using heparinised Hank's Balanced Salt Solution and sedimented by centrifugation at 200×g for 10 min at 4°C. Cells were suspended in a small volume of RPMI 1640 medium containing 15% heated fetal bovine serum, 20 mM HEPES, 2 mg $mL^{-1}$ $NaHCO_3$, 2 mM glutamine and 50 mg $L^{-1}$ gentamicin. Macrophages were growth in a 5% $CO_2$ at 37°C. Macrophage viability was routinely confirmed by trypan blue exclusion experimental procedure for antibiotic administration and for evaluating the survival of *Brucella abortus*.

**Bacterial intracellular survival in macrophages preincubated with antibiotics:** The assay described by valdoianu was followed[9]. Essentially, macrophages in RPMI 1640 with 10% FCS were dispensed into 96-well tissue culture microtitre wells (Falcon) and allowed to adhere overnight to give approximately $10^5$ cells per well. Viability of the cells was assessed by their ability to adhere and by trypan blue exclusion. The RPMI media was removed from the wells and the adherent macrophage monolayers were washed to remove any agents remaining and fresh RPMI media without antibiotic added. Macrophages were then infected with the *Brucella abortus* by adding 100 µL of a suspension in brucella broth containing approximate $10^7$ CFU $mL^{-1}$. The bacterium to macrophage ratio was approximate 10:1. The cell monolayers and bacteria were incubated for 1 h at 37°C 5% $CO_2$, the cultures were washed five times with 5 mL of PBS to remove excess extracellular bacteria and reincubated for 40 min in medium containing gentamicin at

a bactericidal concentration (50 mg $L^{-1}$) to kill residual or adherent bacteria not removed by the washing procedure. Selected monolayers were then lysed with 0.1% Triton X-100 at 4°C to determine the viable counts of intracellular bacteria. The remaining test wells received antibiotics in RPMI-1640 15% FCS at different concentrations of co-trimazine and were incubated at 37°C 5% $CO_2$ for 20 h. At the end of the incubation period, the wells were washed three times with PBS and the remaining monolayers lysed with 0.1% Triton X-100 at 4°C. The CFU $mL^{-1}$ were calculated from the lysed homogenates by serial dilution in minimal salts solution and inoculated onto brucella agar. The bacteria recovered from the lysed homogenates were tested for their antibiotic susceptibility in order to confirm that survival within the macrophage monolayers was not the result of the development of antibiotic resistance. The percentage inhibition of growth of the bacteria phagocytosed by the macrophages was derived from the mean values of the experimental and control readings, performed in triplicate.

**Statistical analysis:** Statistical analysis of results the mean and standard deviation were calculated for the results obtained at each time point. The results were analyzed by students t-test.

## RESULTS

**Antibacterial activity:** The susceptibilities of *Brucella abortus* $S_{19}$ was presented as MIC and these findings were also evaluated using NCCLS susceptibility criteria for slow growing bacteria (Table 1). The combination of trimethoprim plus sulphadiazine showed an enhanced activity by exhibiting a decreased MIC. According to evaluation based on NCCLS slow growing bacteria standards, the strain was sensitive to all antibiotics.

**Intracellular activities of the antibiotics against *Brucella abortus*:** From to 1 to 30 h, the number of viable bacteria associated with the untreated macrophages increased from 4/2 to 8/2 log10 CFU $mL^{-1}$ ($p<0.050$). For the first 12 h of incubation, the numbers of bacteria in the presence of co-trimazine was similar to the controls (macrophages without treatment by co-trimazine). After 24 h, the co-trimazine was significantly effective in inhibiting bacterial growth , with 5.4 log 10 CFU $mL^{-1}$, respectively.

**Bacterial survival in macrophages treated with co-trimazine:** The *in vitro* susceptibility of extracellular bacteria and bacteria recovered from lysed macrophages was identical, showing that surviving bacteria had not acquired resistance after exposure to the antibiotic. The results shows that over the range of concentrations used can kill all the intracellular bacteria. Bacteria treated with trimethoprim exhibited a decrease in intracellular survival with concentrations of 6.32 mg $L^{-1}$, with the greatest decrease at 12.64. When treated with sulphadiazine, there was a decrease in bacterial counts at 8.19 mg $L^{-1}$; more concentrations used had more effect on intracellular survival of *Brucella abortus* $S_{19}$. Co-trimazine showed a bactericidal effect at all the concentrations used, with a decrease in bacterial count at 4.73 mg $L^{-1}$ and a total killing of intracellular bacteria at concentrations of 9.46 mg $L^{-1}$. None of the agents proved toxic for the macrophage monolayers at the concentrations used, as verified by trypan blue dye exclusion.

Table 1: MICs (mg $L^{-1}$) of trimethoprim and sulphadiazine alone or in combination against *Brucella abortus*

| Antibacterials | $MIC_{50}$ | $MIC_{90}$ | MIC | FIC (index) |
|---|---|---|---|---|
| Trimethoprim | 0.90 | 2.8 | 6.32 | |
| Sulphadiazine | 1.10 | 1.9 | 8.19 | |
| Co-trimazine | 0.50 | 1.7 | 4.73 | 0.53 |

## DISCUSSION

Human Brucellosis is a multisystemic disease that may present with a broad spectrum of clinical manifestations[3]. As *Brucella* species are intracellular pathogens, the treatment requires not only combined regimens but agents that may efficiently penetrate macrophages as well. The type of antibiotics used for treatment of brucellosis influences the recurrence rate and it is clear that a synergistic combination with marked intracellular activity is the best therapeutic regimen in Brucellosis[10]. The agents that are used in these combinations are tetracyclines, rifampicin, fluoroquinolones, trimethoprim-sulphamethoxazole, third generation cephalosporins, streptomycin and other aminoglycosides[11]. Published guidelines that have originated from WHO recommendations, have suggested rifampicin plus doxycycline management for human brucellosis for more than a decade[12]. However, *in vitro* susceptibilities of these antibiotics may change over time and from one geographical region to another. Moreover, *in vitro* susceptibility tests are not standardised for Brucella species and they are not routinely performed[13]. Previously, the average MIC of all tetracyclines has been reported as <1 mg $L^{-1}$[14]. In another study, the *in vitro* resistance rate was reported as 0.6% of 143 isolates for tetracycline[15]. These isolates were considered as non-susceptible to rifampicin. On the other hand, according to the NCCLS, slow growing bacteria are intermediately susceptible against rifampicin at an MIC

value of 2 mg $L^{-1}$. By this definition, our four non-susceptible isolates might be considered as intermediate susceptible. Phillipon *et al.*[4] reported an *in vitro* resistance rate of 3.5% for rifampicin. The results of this study indicate that co-trimazine is effective in the treatment of experimental brucellosis. The *in vitro* activity of co-trimoxazole against *B. abortus* has been reported to be higher than ofloxacin and ciprofloxacin which are currently used in combination regimens in the treatment of human brucellosis[4]. This higher antibacterial activity combined with better pharmacological properties such as less adverse effects and easier dosing, made this drug an attractive alternative in the treatment of brucellosis[7]. Dirithromycin on the other hand, showed an *in vitro* activity similar to erythromycin against *Brucella* spp., with much higher Minimum Inhibitory Concentrations (MICs) than azithromycin and clarithromycin[8]. However, since it has better pharmakokinetic characteristics than erythromycin, such as reaching very high concentrations in tissues, it was expected to show more favourable *in vivo* activity[5]. Rifampicin has been shown to be one of the most effective drugs in the treatment of experimental and human brucellosis although the success rate varies with different studies[11]. The major reason for these differences may be the duration of the treatment. In studies with 21 days of rifampicin monotherapy, success rates were higher than studies with 14 days of rifampicin treatment[16]. Similarly, the high rates of treatment failure with levofloxacin and dirithromycin treatment in the present study may be due to the short duration of the treatment regimen. Better rates may have been achieved if the drugs had been administered for longer periods. The dosing of these drugs may also be inadequate for the treatment of brucellosis, since these doses were derived from other studies in which bacteria other than Brucellae had been used. Although the intracellular concentrations of the antimicrobial agents tested were not determined in the present study, the results suggest that co-trimazine was able to readily penetrate macrophages. Chloramphenicol and trimethoprim are known to penetrate phagocytic cells at different rates[17]. The study described here was conducted in mouse peritoneal macrophages infected with virulent strains of *Brucella abortus* and demonstrated that co-trimazine possess the most potent and most constant bactericidal activities at concentrations compatible with clinical use. To our knowledge the accumulation of antibiotics has been studied mostly in professional phagocytes. It seems hardly possible to predict the effect of antibiotics on intracellular brucella without testing them in appropriate model systems. The ability of *Brucella abortus* to enter nonprofessional phagocytes may be especially important in the early phase of infection. Trimethoprim and sulphonamide combinations have been widely used in the treatment of infection for more than 40 years, In macrophages which cannot kill brucella cells, these bacteria escape from phagosomes into the cytoplasm. When *Brucella abortus* are in the cytoplasm, they appear to be protected from membrane impermeant antibiotic such as gentamicin . In conclusion, the findings of this study are disappointing but they do not necessarily limit the use of current antibiotics in the treatment of experimental or human brucellosis. Further studies to determine the appropriate dosages of these drugs in the therapy of brucellosis and the duration of treatment are required.

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