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Evaluation of *in vitro* inhibitory effect of enoxacin on *Babesia* and *Theileria* parasites

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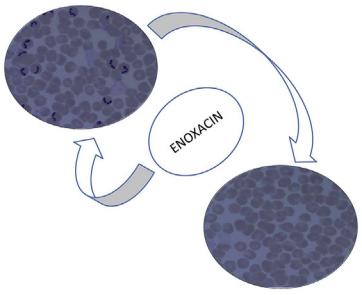
HIGHLIGHTS

- Enoxacin is a broad-spectrum antibacterial agent.
- Enoxacin potently inhibited the *Babesia* and *Theileria* parasites.
- Enoxacin might be used as a treatment for *Babesia* and *Theileria* parasites.

GRAPHICAL ABSTRACT



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ABSTRACT

Enoxacin is a broad-spectrum 6-fluoronaphthyridinone antibacterial agent (fluoroquinolones) structurally related to nalidixic acid used mainly in the treatment of urinary tract infections and gonorrhea. Also it has been shown recently that it may have cancer inhibiting effect. The primary antibabesial effect of Enoxacin is due to inhibition of DNA gyrase subunit A, and DNA topoisomerase. In the present study, enoxacin was tested as a potent inhibitor against the *in vitro* growth of bovine and equine Piroplasms. The *in vitro* growth of five *Babesia* species that were tested was significantly inhibited ($P < 0.05$) by micro molar concentrations of enoxacin (IC_{50} values = 33.5, 15.2, 7.5 and 23.2 μM for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*, respectively). Enoxacin IC_{50} values for *Babesia* and *Theileria* parasites were satisfactory as the drug is potent antibacterial drug with minimum side effects. Therefore, enoxacin might be used for treatment of Babesiosis and Theileriosis especially in case of mixed infections with bacterial diseases or incase of animal sensitivity against diminazin toxicity.

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1. Introduction

Babesiosis is a worldwide infectious parasitic diseases caused by hemotropic protozoa of the genus *Babesia* belonging to phylum Apicomplexa that infects wide range of warm-blooded mammals (Mehlhorn and Schein, 1984). *Babesia* parasites induce clinical symptoms such as fever, hemolytic anemia, jaundice, hemoglobinuria, and edema. *Babesia* species, which are mainly prevalent in tropical and sub-tropical areas, cause serious economic damage in the livestock industries in these regions (Homer et al., 2000; Kjemtrup and Conrad, 2000 and Ica et al., 2007). Bovine babesiosis is caused by *Babesia bigemina*, *Babesia bovis*, *Babesia divergens* and *Babesia major*. Two species, *B. bovis* and *B. bigemina*, have a considerable impact on cattle and buffalo health and productivity (Uilenberg, 1995). Equine piroplasmosis, caused by *Theileria equi* (Mehlhorn and Schein, 1998) and *Babesia caballi*, has emerged as an important protozoan infection from the veterinary and economic viewpoints (Schein, 1988). Several chemicals were evaluated as chemotherapeutic drugs against babesiosis such as Fusidic acid and Allicin (Salama et al., 2013, 2014), epoxomicin, ciprofloxacin, thiostrepton, and rifampicin (AbouLaila et al., 2010a and AbouLaila et al., 2012), triclosan and clodinafop-propargyl (Bork et al., 2003a and Bork et al., 2003b) but they are not available for field animal treatment either due to low efficacy in comparison with currently available commercial drugs or because of their severe side effects. The currently available drugs for treatment of babesiosis and theileriosis have toxic side effects such as quinuronium sulfate, imidocarb dipropionate, clindamycin phosphate (Mosqueda et al., 2012). Therefore, the search for new drugs with low toxic effect for animals is necessary. Enoxacin is a broad-spectrum azafluoroquinolone antibacterial agent for oral administration. Enoxacin is 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid sesquihydrate. Enoxacin is a potent DNA gyrase inhibitor (Divo et al., 2008) who reported that among the fluoroquinolones, Enoxacin had the lowest 50% inhibitory concentrations against Plasmodium FCC1 and VNS strains at 96h. The IC_{50} values of Enoxacin were 2.3 μM and 99 μM respectively. Similar study by Nassira et al. (2003) reported that the *in vitro* activities of 25 quinolones and fluoroquinolones against erythrocytic stages of *Plasmodium falciparum* and against liver stages of *Plasmodium yoelii* and *P. falciparum*. All compounds were inhibitory for Chloroquine-sensitive and Chloroquine-resistant *P. falciparum* grown in red blood cells. The IC_{50} for Enoxacin was 4.4 μM and 121.2 μM respectively. Also they stated that the antimalarial effects of some fluoroquinolones including enoxacin occur at achievable serum concentrations, whether inhibition of DNA gyrase contributes to the antimalarial activity of the fluoroquinolones is unknown at present (Divo et al., 2008, Nassira et al., 2003).

The primary antibacterial action of Enoxacin appears to be mainly due to inhibition of DNA gyrase subunit A, DNA topoisomerase 4 subunit A, DNA topoisomerase 2-alpha and Cytochrome P450 1A2 (Overington et al., 2006; Yoshida et al., 1993; Neuman, 1998; Chen et al., 2002 and Preissner et al., 2009).

Thus, the aim of the present study was to evaluate the inhibitory effect of enoxacin upon the *in vitro* growth of bovine and equine *Babesia* and *Theileria* parasites.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* (Hines et al., 1992), the Argentine strain of *B. bigemina* (Hotzel et al., 1997), the USDA strain of *B. caballi* (Avarzed et al., 1997), *Theileria equi* (Bork et al., 2004), and the Munich strain of *Babesia microti* (AbouLaila et al., 2012) were used in this study.

2.2. Culture conditions

The *Babesia* parasites used in this study were maintained in bovine or equine red blood cells (RBCs) using a microaerophilic stationary-phase culture system (Igarashi et al., 1994; AbouLaila et al., 2010a). Briefly, Medium 199 was used for *B. bovis*, *B. bigemina*, and *T. equi*, while RPMI-1640 was used for *B. caballi* (both from Sigma–Aldrich, Tokyo, Japan). Media were supplemented with 40% normal bovine serum for bovine *Babesia* or 40% normal equine serum for equine *Babesia*, and 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (all three drugs from Sigma–Aldrich) were prepared and used in the culture media. Additionally, 13.6 μg of hypoxanthine (ICN Biomedicals Inc., Aurora, OH) per milliliter was added to the *T. equi* culture as a vital supplement.

2.3. Chemicals reagents

Enoxacin was purchased from Sigma–Aldrich, Tokyo, Japan and used as a test drug. A working stock solution of 100 μM Enoxacin dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industrial, Ltd., Osaka, Japan) was prepared and stored at 4 °C until required for use.

2.4. In vitro growth inhibition assay

The inhibitory effect of enoxacin on the growth of *Babesia* parasites was tested using an assay previously described (Bork et al., 2003; AbouLaila et al., 2010a) with slight modification. Parasite-infected RBCs were diluted with uninfected RBCs to obtain an RBC stock supply with 1% parasitemia. Twenty μl of RBCs with 1% parasitemia was dispensed into a 96-well microtiter plate (Nunc, Roskilde, Denmark) with 200 μl of the culture medium containing the indicated concentration of Enoxacin (0.1, 1, 10, 15, 25 and 50 μM) for *B. bovis* and (0.1, 0.5, 1, 10, 15, 25 and 50 μM) for other *Babesia* species, and then incubated at 37 °C in a humidified multi-gas water-jacketed incubator. For experimental control, cultures without enoxacin and cultures containing only 0.06% DMSO were prepared. Three separate trials were performed, consisting of triplicate experiments for individual drug concentrations, over a period of 4 days. During the incubation period, the overlaying culture medium was replaced daily with 200 μl of fresh medium containing the indicated concentration of enoxacin. Parasitemia was monitored daily by counting the parasitized RBCs to approximately 1000 RBCs in Giemsa-stained thin blood smears. The IC_{50} values (50% inhibitory concentration) for enoxacin upon growth of all parasites tested will be calculated based on parasitemia observations recorded on day 3 in the *in vitro* cell culture system; using curve fitting technique (AbouLaila et al., 2010a).

2.5. Viability test

After the 4th day of treatment, 6 μl of each of the control and drug-treated (at the various indicated concentrations) RBCs were mixed with 14 μl of parasite free RBCs and suspended in fresh growth medium without enoxacin supplementation. The plates were incubated at 37 °C for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy in order to assess the parasite viability (Bork et al., 2004, AbouLaila et al., 2010a).