Effect of Dapsone and its Antimicrobial Combinations on *Borrelia burgdorferi* Biofilms

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Introduction

Lyme borreliosis a tick-borne multisystemic illness, caused by the spirochete *Borrelia burgdorferi*, has grown into a major public health problem in the United States and across Europe (1). The frontline treatment for the disease is the administration of antibiotics such as doxycycline, amoxicillin or ceftriaxome (1). Relapse of the disease, however, often occurs when treatment is discontinued regardless of the chosen antibiotics (1). It has been proposed that antibiotic resistance of Borrelia and Lyme disease reoccurrence may be due to formation of defensive morphological forms of *B. burgdorferi* (2-5). Our research group recently identified a novel form for Borrelia, called biofilm *in vitro and in vivo* (2, 3). This defensive form now proven to be the most resistant form to antimicrobials in several studies (4). In the search to find an effective antibiotics for Borrelia biofilm, we have evaluated different antimicrobials and their combinations against this form. One recent clinical study suggested that Dapsone in combination with rifampin, tetracyclines and/or cephalosporins could be very effective in the treatment of Lyme disease patients, who have failed classical antibiotics protocols (6). Therefore, in this *in vitro* study we have evaluated

Methods

Borrelia burgdorferi strain B31 were cultured in BSK-H medium (Sigma Aldrich) supplemented with 6% rabbit serum (Pel-Freeze) and maintained at 33°C with 5% CO_2. Biofilms were generated by inoculating 5 x10^6 cells/ml of Borrelia spirochetes in 1 ml of BSK-H media in four well chamber slides (Thermo Scientific, Waltham, MA) or tissue culture 48-well plates (BD Falcon) which were incubated for 7 days at 33 °C with 5% CO_2.

Biofilm cultures were treated with 10microM and 50 microM concentrations of the different antibiotics or their combinations for 72h. As negative control, *B. burgdorferi* strain B31 cultures used with appropriate amounts of PBS buffer pH 7.4 (vehicle). The biofilm mass before and after antibiotic treatment was evaluated by crystal violet assays as described previously (3, 4). Statistical analysis on quantitative data consisted of four independent experiments performed in quadruplets and data presented as the mean ± SD.

To visualize the antimicrobial sensitivity of biofilms, the treated biofilms were stained using SYBR Green I (Invitrogen) and Propidium Iodide method (PI, Thermo Scientific) as described previously (4-5) and the images were taken using fluorescent microscopy (Leica DM2500).

Evaluation of antibiotic sensitivity of *B. burgdorferi* biofilms following 72 hours of treatment with different antibiotics

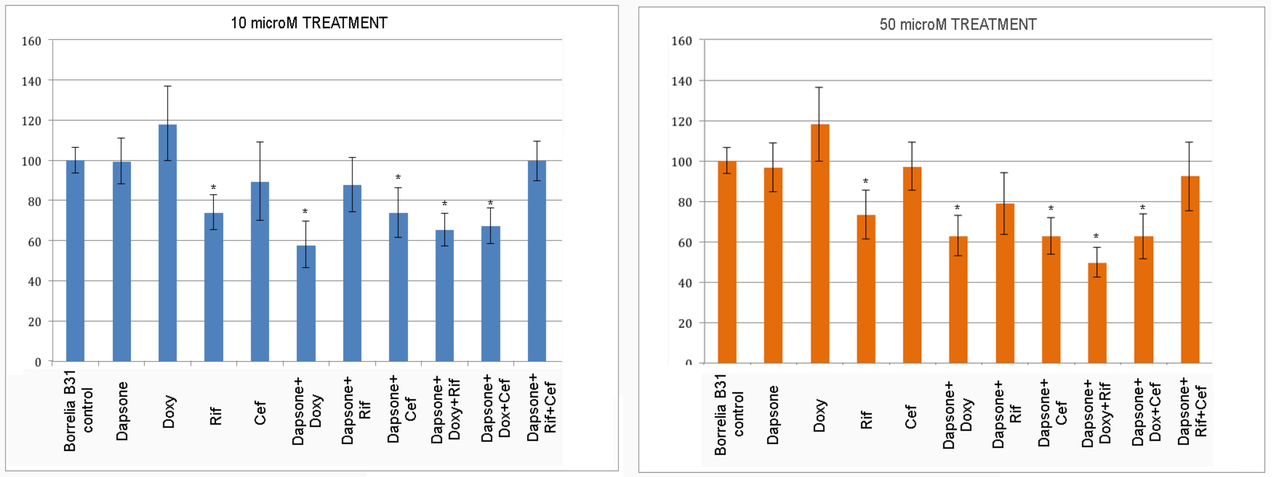

Figure 1. Effect of different antimicrobial agents on attached *B. burgdorferi* biofilms. Antimicrobial sensitivity of attached *B. burgdorferi* biofilms to different antimicrobial agents after a three-day treatment using 10 microM and 50 microM concentrations, was analyzed by Crystal Violet method as described in Methods. Dapsone, doxycycline (Doxy) rifampin (Rif) cefuroxime (Cef) and their double and triple their combinations were tested on attached Borrelia biofilms. Significance against PBS buffer (negative control vehicle) with the p value of < 0.05 is indicated in *. N=16

Live/Dead Images of Attached Borrelia Biofilms Treated with Different Antimicrobial Agents for 72h

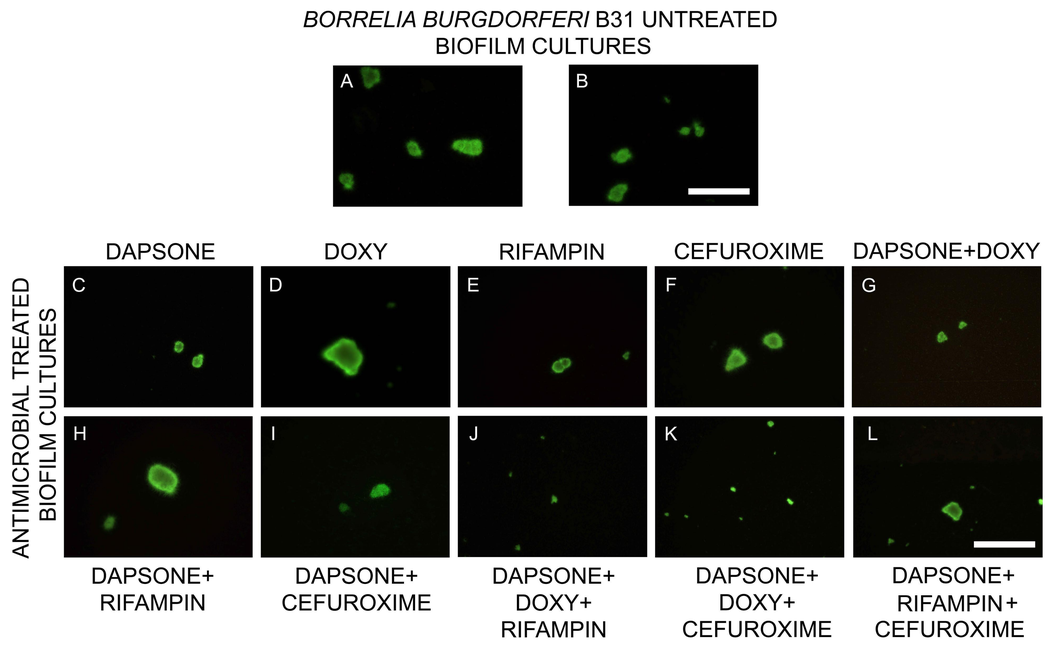

Figure 2. Representative Live/Dead images of the viability of attached Borrelia biofilms following a 72h treatment with different antimicrobial at 50 microM concentration. Biofilms were stained with SYBR Green I and PI as described in Methods and representative images were taken at 200X magnification. (Panel A and B) Borrelia cultures treated only with PBS was used as a negative control. Panels C-L represents the results after each treatment. Live cells are stained with green color while dead cells are stained red. Scale bar: 100 µm.

Results/Discussion

In this study, the effect of Dapsone combined with different antimicrobial agents was tested on attached Borrelia biofilms. Our results demonstrated that single antimicrobial agents such as the frontliine treatment Doxycycline tested in this study had very limited effect on Borrelia biofilms. In comparison Dapsone alone did not affect the size of Borrelia biofilm mass or its viability, however; when it was combined with doxycycline and or cefuroxime, the obtained result was found to be significant (~40%). The most dramatic result was observed however, when Dapsone, doxycycline and rifampin combined in a 3-antibiotics treatment protocol at 50 microM concentration and showed a very significant 50% reduction in biofilm mass, a finding which was also confirmed with Live/Dead microscopic analyses.

Conclusions

In summary, our *in vitro* findings suggest that there is a significant effect of Dapsone/doxycycline/rifampin antibiotic combination on the antibiotics resistant biofilm form of *B. burgdorferi*. This result merits further studies to evaluate this antibiotic combination *in vivo* against Borrelia biofilms and in clinical environment monitoring the outcome of this therapeutic approach for

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