SUSCEPTIBILITY OF LEPTOSPIRA SEROVARS TO ANTIMALARIAL AGENTS

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Abstract. Leptospirosis has recently been described to cause concomitant infection with malaria. Only doxycycline has proven to have chemoprophylactic and therapeutic efficacy for both malaria and leptospirosis. To assess whether other traditional antimalarial agents have antileptospiral activity, we performed broth microdilution susceptibility testing of 16 Leptospira serovars (6 species/14 serogroups) to various agents. Artemisinin, atovaquone, chloroquine, mefloquine, primaquine, proguanil, pyrimethamine, sulfadoxine, quinine, quinidine, and combinations of atovaquone/proguanil and pyrimethamine/sulfadoxine all had a 90% minimum inhibitory concentration (MIC90) > 25 μg/mL (the upper limit of testing). The only agents identified with the potential to treat both infections other than doxycycline (MIC90 = 1.56 μg/mL) were azithromycin (MIC90 = 0.02 μg/mL) and clindamycin (MIC90 = 0.2 μg/mL).

INTRODUCTION

Malaria and leptospirosis have overlapping geographic distributions and both produce endemic and epidemic acute febrile disease. Concomitant malaria and leptospirosis has been reported to occur; however, patients diagnosed with malaria are unlikely to undergo further evaluation for dual infections and typically receive therapy directed solely at this parasitic infection. Doxycycline, penicillin G, and ceftriaxone have been suggested for the acute therapy of leptospirosis, while doxycycline alone has been shown to provide chemoprophylactic protection against this spirochetal infection. Leptospires are susceptible in vitro to a wide range of antimicrobial agents; however, antimalarial agents have not been evaluated. Although no standard method currently exists to assess the antileptospiral activity of antimicrobial agents in vitro, we have recently described a broth microdilution method that is more rapid and convenient than traditional macrodilution methods used in the past. We used this method to test whether any of the more common antimalarial agents have in vitro antileptospiral activity against a variety of Leptospira species and serovars.

MATERIALS AND METHODS

Isolates. Sixteen Leptospira serovars (6 species/14 serogroups) were provided by the Veterinary Command Food and Drug Analysis Laboratory (Fort Sam Houston, TX). Strains originated at the U.S. Department of Agriculture National Veterinary Services Laboratories (Ames, IA). Organisms were maintained by continuous culture in Ellinghausen McCullough Johnson Harris (EMJH) medium (Becton Dickinson, Sparks, MD).

We included the following strains in this study: L. biflexa, serovar Patoc (serogroup Semaranga, strain Patoc 1); L. hortenegensis, serovar Ballum (serogroup Ballum, strain S 102); L. interrogans, serovar Australis (serogroup Australis, strain Ballico); L. interrogans, serovar Canicola (serogroup Canicola, strain Hond Utrecht IV); L. interrogans, serovar Copenhageni (serogroup Icterohaemorrhagiae, strain M 20); L. interrogans, serovar Djasiman (serogroup Djasiman, strain Djasiman); L. interrogans, serovar Hardjo (serogroup Sejroe, strain Hardoprajitno); L. interrogans, serovar Icterohaemorrhagiae (serogroup Icterohaemorrhagiae, strain RGA); L. interrogans, serovar Pomona (serogroup Pomona, strain Pomona); L. interrogans, serovar Wollii (serogroup Sejroe, strain 3705); L. kirschneri, serovar Cyeynpteri (serogroup Cyeynpteri, strain 3522 C); L. noguchii, serovar Fort Bragg (serogroup Autumnalis, strain Fort Bragg); L. santarosai, serovar Alexi (serogroup Pyrogenes, strain HS 616); L. santarosai, serovar Borincana (serogroup Hebdomadis, strain HS 622); L. santarosai, serovar Georgia (serogroup Mini, strain LT 117); and L. santarosai, serovar Shermani (serogroup Shermani, strain 821).

Inoculum was prepared from seven-day-old cultures grown at 30°C. A Petroff-Hausser counting chamber and dark-field microscopy were used to determine the quantity of inoculum.

Antimicrobial agents. Stock antimicrobials were prepared with reagent grade powders using solvents and diluents as suggested in the National Committee for Clinical Laboratory Standards document M7-A4 or the manufacturer at a concentration of 1 mg/mL and stored at −70°C until use. Tested drugs were obtained from Sigma-Aldrich (St. Louis, MO), including artemisinin, clindamycin, chloroquine, doxycycline, primaquine, quinine, and quinidine, or from their manufacturers (azithromycin [Pfizer, Groton, CT], atovaquone and proguanil [GlaxoSmithKline, Research Triangle Park, NC], and mefloquine, pyrimethamine, and sulfadoxine [F. Hoffmann-La Roche Ltd., Basel, Switzerland]). Single drugs tested were artemisinin, atovaquone, azithromycin, chloroquine, clindamycin, doxycycline, mefloquine, primaquine, proguanil, pyrimethamine, sulfadoxine, quinine, and quinidine. Combinations of drugs tested included atovaquone/proguanil, clindamycin/quinine, and pyrimethamine/sulfadoxine. Combinations were tested in ratios of 1:1 and reported based on the weight of one drug.

Susceptibility testing. Broth microdilution testing was performed using 96-well round-bottom plates as previously described. Plates included serial two-fold dilutions of antibiotics and positive and negative controls, all in EMJH medium. The concentrations of antibiotics ranged from 25.0 to 0.01 μg/mL. Leptospira inoculum (2 × 10⁹ leptospiral organisms/mL, volume = 100 μL) was added and the plate was incubated at 30°C (final well volume = 200 μL). Twenty microliters of 10× concentrated alamarBlue® (Trek Diagnostics, Cleveland, OH) was added to all wells after three days of incubation. In the presence of cell growth or viability, alamarBlue® turns from dark blue to bright pink. After five days of incubation, the color of each well was documented. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration well without a blue to pink color change. Combined performance over all tested serovars was reported as the MIC⁹⁰, the concentration at which 90% of the Lep-
*Leptospira* isolates were inhibited. Each serovar-drug combination was tested twice on separate occasions.

**RESULTS**

The MIC ranges and MIC$_{90}$ of the various agents tested are shown in Table 1. The observed reproducibility of repeat drug-serovar combination testing was 99% with 507 of 512 result pairs producing results within two dilutions of each other. Artemisinin, atovaquone, chloroquine, mefloquine, primaquine, proguanil, pyrimethamine, sulfadoxine, quinine, and quinidine had MIC$_{90}$ values greater than or equal to the upper limit of detection (> 25 μg/mL). Of the single agents, azithromycin (MIC$_{90}$ = 0.02 μg/mL), doxycycline (MIC$_{90}$ = 1.56 μg/mL), and clindamycin (MIC$_{90}$ = 0.2 μg/mL) demonstrated in vitro activity against the tested serovars. The addition of a second drug in any of the tested combinations had no appreciable impact on susceptibility.

**DISCUSSION**

Co-infections of malaria and leptospirosis occur. However, leptospirosis may not be diagnosed due to a lack of rapid diagnostic testing and the typical assumption that malaria represents the sole infecting agent when this diagnosis is made. Leptospirosis is typically a self-limiting disease, but it can have significant morbidity and mortality if not treated or prevented. Doxycycline has shown efficacy in both malaria and leptospirosis as a therapeutic and prophylactic agent, and thus undiagnosed co-infection may be inadvertently treated when antimalarial therapy includes this agent. Antileptosomal activity of other antimalarial agents has not been previously evaluated. We have shown that doxycycline, azithromycin, and clindamycin have activity against *Leptospira* species, but more traditional antimalarial agents have little to no antileptosomal activity.

We included a broad range of antimalarial agents to test individually or in combination, including quinoline derivatives, antifolate agents, artemisinin, more traditional antibacterial antimicrobials, and three combination agents. Only the traditionally antibacterial antimicrobial agents, doxycycline, clindamycin, and azithromycin, showed antileptosomal activity. The efficacy of clindamycin in treating malaria is limited by dosing and availability and azithromycin has proven inadequate as a single agent in the prophylaxis or treatment of malaria.2–9

We assessed a broad range of *Leptospira* serovars to assess strain-to-strain and species-to-species variability. Overall, there was no variability noted from strain-to-strain or species-to-species. In *in vitro* activity does not always equate to *in vivo* activity in leptospirosis. However, the lack of any significant *in vitro* efficacy diminishes the utility of pursuing further *in vivo* studies with the more traditional antimalarial agents. Considering and establishing the diagnosis of leptospirosis remains the primary method to prevent poor outcomes.10

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