Antimicrobial Susceptibilities of Geographically Diverse Clinical Human Isolates of

*Leptospira*

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Abstract

Although antimicrobial therapy of leptospirosis has been studied in a few randomized controlled clinical studies, these studies are limited to specific regions of the world, and few have characterized infecting strains. A broth microdilution technique to assess antibiotic susceptibility has been developed in our laboratory. Herein we assess the antimicrobial susceptibility of 13 Leptospira isolates (including recent clinical isolates) from Egypt, Thailand, Nicaragua, and Hawaii to 13 antimicrobial agents. Ampicillin, cefepime, azithromycin, and clarithromycin were found to have MICs below the lower limit of testing (0.016µg/ml). Cefotaxime, ceftriaxone, imipenem/cilastatin, penicillin G, moxifloxacin, ciprofloxacin, and levofloxacin had MIC₉₀s between 0.030 and 0.125µg/ml. Doxycycline and tetracycline had the highest MIC₉₀s; 2 and 4µg/ml, respectively. Doxycycline and tetracycline were noted to have slightly higher MICs against isolates from Egypt compared to strains received from Thailand or Hawaii; otherwise the susceptibility patterns were similar. There appears to be possible strain variability in susceptibility to some antimicrobial agents, suggesting more extensive testing looking for geographic variability should be pursued.
Introduction

Leptospirosis is a zoonotic infection found worldwide but which is mostly endemic to subtropical and tropical areas. The genus *Leptospira* consists of > 250 serovars which cause a wide spectrum of disease manifestations ranging from a mild febrile illness to severe life-threatening disease in humans. Diagnostic tests used to make a definitive diagnosis do not provide timely results and may not be available in certain clinical settings. As a result, patients are often treated empirically for undifferentiated febrile syndromes with broad antimicrobial therapy that provide coverage of various local etiologies of fever.

Despite its worldwide distribution, only a small number of randomized, controlled clinical trials looking at treatment have been performed (5, 11, 18, 19, 22, 24). These studies have been conducted in a limited number of locations, including Barbados, Panama, Philippines, and Thailand, and in only two were the causative *Leptospira* serovars or serogroups reported. A survival benefit of one agent over another has not been demonstrated in prior studies; however, a reduction of symptoms and leptospiuria has been described. There are multiple in vitro and in vivo (animal) studies showing a wide variety of antimicrobials have potential value in treating this disease (1-3, 6-8, 10, 12-16, 17, 20, 23, 25).

We have previously described an in vitro broth microdilution technique that allows reliable, rapid testing of antimicrobial susceptibilities, permitting efficient evaluation of multiple antimicrobials and *Leptospira* serovars (15). This method was used successfully to evaluate multiple antibiotics against 26 *Leptospira* serovars (16). Although most of the strains previously studied by our group were recovered initially from human infections, they were all maintained by subculture as laboratory strains for a great number of years. Recently, we have received clinical human isolates from different leptospirosis endemic areas to assess the activity of
various antimicrobial agents. These isolates have been passed in animal models to maintain virulence or have undergone less than 5 subcultures since initial collection. The goals of this study were to evaluate the in vitro activity of various antimicrobial agents against these isolates to determine if there are regional differences in susceptibility patterns and to compare the susceptibility of recent clinical human isolates and animal lethal strain to those previously reported using long-term laboratory maintained strains.

Materials and methods

**Leptospira isolates.** Thirteen *Leptospira* isolates representing 3 different species and at least 6 serovars were included in testing. These included 10 human clinical isolates that have undergone less than 5 subcultures since initial collection and 3 isolates that have been maintained in our lethal animal models (Table 1). The three isolates obtained from Thailand have not been previously identified and are currently undergoing further testing to determine serovar. The human clinical isolates were obtained from collaborating institutions (Naval Medical Research Unit 3 [NAMRU-3] in Cairo, Egypt, Armed Forces Research Institute of Medical Sciences [AFRIMS] in Bangkok, Thailand, and Tripler Army Medical Center in Honolulu, Hawaii). The 3 strains maintained in our animal model to maintain virulence were initially provided by David Haake (University of California Los Angeles, CA). Strain 11 was initially associated with human disease in Nicaragua. The isolates were shipped in pure culture and stocks were maintained by continuous culture at room temperature in Ellinghausen McCullough Johnson Harris (EMJH) medium (Becton Dickinson, Sparks, MD). The clinical strains from Egypt, Thailand and Hawaii had been passed less than 5 times to mitigate the loss of virulence with serial passage. The relatedness of the strains was studied by pulsed-field gel electrophoresis
Bacterial DNA embedded in agarose plugs was digested with 30 U NotI. *Salmonella enterica* serovar Braenderup (ATCC BAA-664) was used as a standard. *Salmonella* plugs were prepared according to the protocol (Centers for Disease Control and Prevention. PulseNet protocols; [www.cdc.gov/PULSENET/protocols.htm](http://www.cdc.gov/PULSENET/protocols.htm)), digested with 50 U XbaI. DNA fragments were separated by electrophoresis in 1% agarose gels using the CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA) for 18 hours with recirculating 0.5X TBE buffer and switch times ranging from 2.2 to 35.1 seconds. Photographic images of the gels were saved as TIFF files and analyzed with BioNumerics software (Applied Maths Inc., Austin, TX). The band patterns were compared by use of the Dice coefficient by using the unweighted pair group method to determine band similarity.

**Antibiotics.** Stock antimicrobial solutions were prepared from reagent-grade powders to produce 1mg/ml solutions using solvents and diluents as suggested in the Clinical and Laboratory Standards Institute document M100-S17 (4) or per the manufacturer suggestions as available. A total of 13 antimicrobials were tested (Table 2). Ceftriaxone, cefotaxime, doxycycline, penicillin G, and tetracycline were purchased from Sigma-Aldrich (St. Louis, MO). The remaining antibiotics were obtained from their manufacturers (cefepime from Bristol-Myers Squibb, Wallingford, CT; imipenem and cilastatin from Merck & Co., Inc., Rahway, NJ; ampicillin and azithromycin from Pfizer, Groton, CT; clarithromycin from Abbott Laboratories, Abbott Park, IL; ciprofloxacin and moxifloxacin from Bayer Corporation, West Haven, CT; and levofloxacin from Ortho-McNeil Pharmaceutical, Inc., Raritan, NJ). The stock antimicrobial solutions were stored at -70°C in divided one-time use aliquots.

**Minimal Inhibitory Concentration (MIC).** Broth microdilution testing was performed as previously reported (15, 16). In short, each 96-well round bottom plate included serial 2-fold
dilutions of the antibiotics, positive controls (bacteria without an antimicrobial), and negative controls (medium only), all in EMJH medium. Final antimicrobial concentrations ranged from 32.0 to 0.016 µg/ml (units/ml for penicillin). The inoculum of *Leptospira* used for testing was prepared from 7-day old cultures grown in EMJH at 30°C. The organism burden in the inoculum was determined by use of a Petroff-Hausser counting chamber and dark-field microscopy. A *Leptospira* inoculum of $2 \times 10^6$ leptospiral organisms/ml was added and plates incubated at 30°C with a final volume of 200 µl in each well. After three days of incubation, 20 µl of 10X alamarBlue® (Trek Diagnostics, Cleveland, OH) was added to all wells. AlamarBlue® is an oxidation-reduction indicator that changes color from dark blue to bright pink in response to chemical reduction of growth medium resulting from cell growth. The color of each well was documented on the fifth day of incubation and MICs were recorded as the lowest concentration well without a blue to pink color change. Each serovar-drug combination was tested in triplicate with the median MIC reported. *Leptospira interrogans* serovar Icterohaemorrhagiae was used as the quality control serovar. Currently, there is no established quality control serovar listed in the Clinical and Laboratory Standards Institute guidelines for leptospira. The *L. interrogans* serovar Icterohaemorrhagiae strain used in this study has been assessed for internal validation with MIC parameters previously described in a study validating this MIC technique (15).

**Results**

The median MICs of three runs are reported in Table 2. Repeated testing of drug-serovar combinations found excellent reproducibility with all of the test results falling within 2 dilutions of each other except for one set which was 3 dilutions apart (strain 4 against tetracycline). The quality control strain *L. interrogans* serovar Icterohaemorrhagiae fell within parameters...
previously described (15). Ampicillin, cefepime, azithromycin, and clarithromycin were all found to have MIC$_{90}$s below the lower limit of detection. Cefotaxime, ceftriaxone, imipenem/cilastatin, penicillin G, moxifloxacin, ciprofloxacin, and levofloxacin had MIC$_{90}$s between 0.030 and 0.125µg/ml. Doxycycline and tetracycline had MIC$_{90}$s of 2 and 4µg/ml, respectively.

Doxycycline MICs ranged from 1 to 2µg/ml and tetracycline MICs ranged from 1 to 4µg/ml for isolates from Egypt; notably different than strains maintained in the lethal animal model. In addition, imipenem/cilastatin and fluoroquinolones MICs were the lowest in strains maintained in lethal animal models; however, penicillin G MICs were the highest for the two different species and serovars of strains 11 and 13. Other than the tetracycline and doxycycline MIC variation, the isolates from Hawaii and Egypt within serovar Icterohaemorrhagiae had similar susceptibilities for the remaining antimicrobial agents. Otherwise, there were no other matching serovars between different regions or within a region with the caveat that the Thailand serovars are still unknown.

PFGE was performed to compare the leptospiral strains in this collection (Figure 1). The three human clinical strains obtained from AFRIMS Thailand could not be matched in the current CDC database for any known leptospiral serovars. These strains, therefore, may be unique serovars not previously described.

**Discussion**

Patients who present with a febrile illness, especially in the tropics, are often treated empirically with various antimicrobial agents in attempts to cover a broad array of bacterial pathogens in the differential diagnosis in which leptospirosis is included. We have previously shown that a large
number of antimicrobial agents are active against laboratory-passed strains of leptospirosis (16). These findings had not been confirmed using clinical isolates from around the world that have not been serially passed in a laboratory (with the possible loss of virulence). In this study we have shown that numerous antimicrobials from different classes are active against a diverse collection of pathogenic isolates. We have found that regional differences in susceptibility may exist. The tetracycline antibiotics in this study were found to have increased activity against strains passed in animals in comparison to the human clinical isolates. When comparing our previously reported results from similar serovars of laboratory-passed strains to this virulent strain collection, similarities in susceptibility patterns are noted for most antimicrobial agents, with cefepime and the macrolides producing the lowest MICs in both groups (16). The main differences observed between this study and our past work include the increased activity of ampicillin and penicillin G to these virulent strains compared to the laboratory passed strains. The obverse is noted for imipenem/cilastatin, which was less active against the human isolates in the current collection.

Ampicillin, cefepime, and the macrolides had the best in vitro activity with MICs below the limit of detection against all strains in this collection. All antimicrobials had lower MIC90s than the traditional antileptospiral drug doxycycline and the closely related drug tetracycline. The remaining antimicrobial agents had MIC90s equal to or less than penicillin G, with cefotaxime having the lowest MIC90 of the traditional antileptospiral agents.

Given that a serovar-specific diagnosis is not readily available or feasible in most instances, an assortment of serovars from diverse geographical locations including three possible novel serovars from Thailand were chosen to allow for comparison of strains and locations. It is interesting to note that doxycycline and tetracycline had higher MICs among strains obtained
from Egypt compared to strains received from Thailand or Hawaii. There was no other significant variability among the human clinical strains for the other antimicrobials.

Leptospirosis has recently received attention in Egypt as an important etiology of acute febrile illness, especially in those patients who present with acute hepatitis (9, 21). It is unclear why the MICs for doxycycline and tetracycline are higher in the Egypt isolates, although it has been reported that many patients with acute febrile illness in this region are empirically diagnosed with typhoid and treated with either ampicillin or tetracycline (9). Thus, one can postulate that this decrease in susceptibility is associated with local drug pressure. This requires further analysis.

Lastly, most of the antimicrobials seemed to be more active against the strains passed in animals compared with the human clinical isolates, producing lower median MICs by two dilutions or more. The reasoning for this phenomenon in these strains is not clear since virulence should be maintained by inoculation into animals; however, virulence is not a direct measure of susceptibility to antimicrobials. In addition, the location of the only animal passed strain that we know of, Nicaragua, is different than the other clinical strains and, thus, there might be further regional antimicrobial resistance pattern differences. This observation is especially evident for the only \textit{L. kirschneri} in this isolate collection, possibly representing species variation to antimicrobial susceptibility. It does not seem that this species is more susceptible to antimicrobials in general as some laboratory strains of the same species have a more resistant susceptibility profile (16). The lethal animal strains 11 and 13 had higher penicillin G MICs compared to the other strains.

The main limitation of our study is that it is in vitro data and despite our isolate collection being representative of different geographic locations, it is not all inclusive. Correlation of in
viro susceptibility data to treatment outcomes in humans is lacking, although in vivo animal studies with strain 11 have correlated well with our in vitro testing (7, 12, 13).

In summary, the thirteen tested virulent Leptospira strains from geographically distinct regions, from both human disease and lethal animal models, are susceptible to a range of antimicrobial agents. Newer and non-traditional antimicrobials show good activity against this strain collection, but our study suggests there may be regional differences as well as differences in strains passed through animals. As such, further analysis of strains from around the world needs to be undertaken and the impact of serial passage on resistance profiles in animals models is needed as this is often the preliminary stages of assessing antimicrobials prior to human trials.
References:


Table 1. Strains of *Leptospira* species tested for susceptibility to various antimicrobial agents.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Location</th>
<th>Species</th>
<th>Serogroup</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Bataviae</td>
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<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Grippotyphosa</td>
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<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae</td>
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<tr>
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<td><em>L. interrogans</em></td>
<td>Pomona</td>
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<td><em>L. weilii</em></td>
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<td>Hawaii</td>
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<td>13</td>
<td>Animal model</td>
<td><em>L. kirschneri</em></td>
<td>Grippotyphosa</td>
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</tbody>
</table>

*Currently undergoing investigation for serogroup type determination*

Strains 1-10 are recent clinical human isolates not serially passed in the laboratory

Strains 11-13 are strains passed in an animal model
Pulsed-field gel electrophoresis profiles of NotI digested genomic DNA for the Leptospira strains tested. The dendrogram was prepared using BioNumerics software (Applied Maths Inc.) and the unweighted pair-group method with arithmetic averages (UPGMA) for clustering and PFGE profiles were compared using the Dice band-based coefficients.
<table>
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<tr>
<th>Strain no.</th>
<th>Doxycycline</th>
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<th>Ampicillin</th>
<th>Ceftriaxone</th>
<th>Ceftazidime</th>
<th>Imipenem/cilastatin</th>
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MIC\textsubscript{90}\textsuperscript{c} 2.0 4.0 0.125 <0.016 0.06 0.03 <0.016 0.125 <0.016 0.125 0.125 0.125

\textsuperscript{a} MICs of penicillin G are given in units per milliliter
\textsuperscript{b} MICs of imipenem-cilastatin are based on imipenem concentrations
\textsuperscript{c} Cumulative susceptibility results across all serovars are expressed as MIC\textsubscript{90}, the concentration at which 90% of the \textit{Leptospira} isolates are inhibited