Antimicrobial Susceptibilities of Geographically Diverse Clinical Human Isolates of *Leptospira* *v*

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Leptospirosis is a zoonotic infection that is found worldwide but that 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 provides coverage for the 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, the Philippines, and Thailand, and in only two studies were the causative *Leptospira* serovars or serogroups reported. The survival benefit from the use of one agent over another has not been demonstrated in prior studies; however, a reduction of symptoms and reductions in the levels of leptospiuria have been described. Multiple in vitro and in vivo (animal) studies have shown that a wide variety of antimicrobials have potential value for treatment of 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 the reliable, rapid testing of antimicrobial susceptibilities and thus permits the efficient evaluation of multiple antimicrobials and *Leptospira* serovars (15). This method was successfully used to evaluate the efficacies of multiple antibiotics against 26 *Leptospira* serovars (16). Although most of the strains previously studied by our group were initially recovered from human infections, they were all maintained by subculture as laboratory strains for many years. Recently, we have received clinical human isolates from different areas where leptospirosis is endemic to assess the activities of various antimicrobial agents. These isolates have been passed in animal models to maintain virulence or have undergone less than five subcultures since their initial collection. The goals of this study were to evaluate the in vitro activities of various antimicrobial agents against these isolates to determine if there are regional differences in susceptibility patterns and to compare the susceptibilities of recent clinical human isolates and strains lethal to animals to those previously reported for strains maintained in the laboratory for long periods.

**MATERIALS AND METHODS**

*Leptospira* isolates. Thirteen *Leptospira* isolates representing three different species and at least six serovars were included in the testing. These included 10 human clinical isolates that have undergone less than five subcultures since their initial collection and 3 isolates that have been maintained in our animal models of lethal infection (Table 1). The three isolates obtained from Thailand have not previously been identified and are currently undergoing further testing to determine their serovars. The human clinical isolates were obtained from collaborating institutions (Naval Medical Research Unit 3 in Cairo, Egypt; Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand; and Tripler Army Medical Center in Honolulu, HI). The three strains maintained in our animal model for the maintenance of virulence were initially provided by David Haake (University of California, Los Angeles). 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 Elling...
TABLE 1. Strains of Leptospira species tested for their susceptibilities to various antimicrobial agents

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Location</th>
<th>Species</th>
<th>Serovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Bataviae</td>
</tr>
<tr>
<td>2</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Grippophysha</td>
</tr>
<tr>
<td>3</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td>4</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Pomona</td>
</tr>
<tr>
<td>5</td>
<td>Egypt</td>
<td><em>L. interrogans</em></td>
<td>Pyrogens</td>
</tr>
<tr>
<td>6</td>
<td>Thailand</td>
<td><em>L. weilii</em></td>
<td>Unknown¹</td>
</tr>
<tr>
<td>7</td>
<td>Thailand</td>
<td><em>L. interrogans</em></td>
<td>Unknown²</td>
</tr>
<tr>
<td>8</td>
<td>Thailand</td>
<td><em>L. weilii</em></td>
<td>Unknown²</td>
</tr>
<tr>
<td>9</td>
<td>Hawaii</td>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td>10</td>
<td>Hawaii</td>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td>11</td>
<td>Animal model</td>
<td><em>L. interrogans</em></td>
<td>Canicola</td>
</tr>
<tr>
<td>12</td>
<td>Animal model</td>
<td><em>L. interrogans</em></td>
<td>Pomona</td>
</tr>
<tr>
<td>13</td>
<td>Animal model</td>
<td><em>L. kirschneri</em></td>
<td>Grippophysha</td>
</tr>
</tbody>
</table>

¹ Strains 1 to 10 are recent clinical human isolates not serially passaged in the laboratory. Strains 11 to 13 were passaged in an animal model.  
² Currently undergoing investigation for serovar type determination.

Antibiotics. Stock antimicrobial solutions were prepared from reagent-grade powders to produce 1-mg/ml solutions by using the solvents and diluents suggested in Clinical and Laboratory Standards Institute document M100-S17 (4) or per the manufacturer’s 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 (ceftazime from Bristol-Myers Squibb, Wallingford, CT; imipenem and ertapenem from Merck & Co., Inc., Whitehouse Station, NJ; ampicillin and azithromycin from Pfizer, Groton, CT; clari-thromycin from Abbott Laboratories, Abbott Park, IL; ciprofloxacin and moxi-floxacin from Bayer Corporation, West Haven, CT; and levofloxacin from Ortho-McNeil Pharmaceuticals, Inc., Raritan, NJ). The stock antimicrobial solutions were stored at −70°C in divided one-time-use aliquots.

MIC. Broth microdilution testing was performed as reported previously (15, 16). In short, each 96-well round-bottom plate included serial twofold dilutions of the antibiotics, positive controls (bacteria without an antimicrobial), and negative controls (medium only), all in EMH medium. The 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 EMH medium 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 × 10⁶ lep-tospiral organisms/ml was added, and the plates were incubated at 30°C with a final volume of 200 μl in each well. After 3 days of incubation, 20 μl of 10× alamarBlue (Trek Diagnostics, Cleveland, OH) was added to each well. alamarBlue is an oxidation-reduction indicator that changes color from dark blue to bright pink in response to chemical reduction of the growth medium resulting from cell growth. The color of each well was documented on the fifth day of incubation, and the MICs were recorded as the concentration in the well containing the lowest concentration without a blue to pink color change. Each serovar-dose combination was tested in triplicate, and the median MIC is reported. *Leptospira interrogans* serovar Icterohaemorrhagiae was used as the quality control serovar. Currently, the Clinical and Laboratory Standards Institute guidelines for *Leptospira* have not established a serovar for...
use for quality control. The *L. interrogans* serovar Icterohaemorrhagiae strain used in this study has been assessed for use 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 the drug-serovar combinations found excellent reproducibility, with the test results for all sets except one falling within 2 dilutions of each other; the test results for one set (strain 4 against tetracycline) fell 3 dilutions apart. The results for quality control strain *L. interrogans* serovar Icterohaemorrhagiae fell within the parameters described previously (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 0.125 g/ml. Doxycycline and tetracycline had MIC$_{90}$s of 2 and 4 µg/ml, respectively.

For the isolates from Egypt, the doxycycline MICs ranged from 1 to 2 µg/ml and the tetracycline MICs ranged from 1 to 4 µg/ml; these MICs are notably different from those for strains maintained in the animal model of lethal infection. In addition, the imipenem-cilastatin and fluoroquinolone MICs were the lowest for strains maintained in animal models of lethal infection; however, the 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 variations, the isolates from Hawaii and Egypt of serovar Icterohaemorrhagiae had similar susceptibilities to the remaining antimicrobial agents. Otherwise, there were no other matching serovars between different regions or within a region, with the caveat that the serovars of the strains from Thailand are still unknown.

PFGE was performed to compare the leptospiral strains in the collection of strains tested (Fig. 1). The three human clinical strains obtained from the Armed Forces Research Institute of Medical Sciences in Thailand could not be matched with any known leptospiral serovars in the current Centers for Disease Control and Prevention database. These strains may therefore be unique serovars that have not been described previously.

**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, which includes leptospirosis. We have previously shown that a large number of antimicrobial agents are active against laboratory-passaged strains of *Leptospira* (16). These findings had not been confirmed with clinical isolates from around the world that have not been serially passaged 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. In this study, the tetracycline antibiotics were found to have increased activity against the strains passaged in animals in comparison to their activity against the human clinical isolates. When our previously reported results obtained with similar serovars of laboratory-passaged strains were compared to those obtained with the virulent strain collection evaluated in the present study, similarities in susceptibility patterns were 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 activities of ampicillin and penicillin G against these virulent strains compared to their activities against the laboratory-passaged 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 activities, with the MICs being below the limit of detection against all strains in this collection. All antimicrobials had lower MIC90\textsubscript{\textit{in vitro}} than the traditional antileptospiral drug doxycycline and the closely related drug tetracycline. The remaining antimicrobial agents had MIC90\textsubscript{\textit{in vitro}} equal to or less than the MIC90 of penicillin G, with cefotaxime having the lowest MIC\textsubscript{\textit{in vitro}} 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 a comparison of different strains and strains from different locations. It is interesting to note that doxycycline and tetracycline had higher MICs among strains obtained from Egypt than among 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 doxycycline and tetracycline MICs are higher for the isolates from Egypt, although it has been reported that many patients with acute febrile illness in this region are empirically diagnosed with typhoid and are 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 passaged in animals than against the human clinical isolates, producing lower median MICs by 2 or more dilutions. 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 source location of the only animal-passaged strain that we know of, Nicaragua, is different from the source locations of the other clinical strains, and thus, there might be further differences in antimicrobial resistance patterns by region. This observation is especially evident for the only \textit{L. kirschneri} strain in this isolate collection and possibly represents species variation in 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 penicillin G MICs for strains 11 and 13, maintained in animal models of lethal infection, were higher than those for the other strains.

The main limitation of our study is that the data were obtained in vitro, and even though our isolate collection included isolates from different geographic locations, it is not all inclusive. A correlation of in vitro susceptibility data to treatment outcomes in humans is lacking, although the results of in vivo animal studies with strain 11 have correlated well with the results of our in vitro testing (7, 12, 13).

In summary, the 13 virulent \textit{Leptospira} strains tested from geographically distinct regions and from both human disease and animal models of lethal infection are susceptible to a range of antimicrobial agents. Newer and nontraditional antimicrobials showed good activity against this strain collection, but our study suggests that 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 the resistance profiles needs to be determined with animal models, as serial passage is often done in the preliminary stages of assessment of antimicrobials prior to human trials.

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REFERENCES