

## Sensitivity of Pathogenic and Free-Living *Leptospira* spp. to UV Radiation and Mitomycin C

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The habitats for the two major *Leptospira* spp. differ. The main habitat of *L. biflexa* is soil and water, whereas *L. interrogans* primarily resides in the renal tubules of animals. We investigated whether these two species, along with *L. illini* (species incertae sedis), differ with respect to their sensitivity to UV radiation. The doses of UV resulting in 37, 10, and 1% survival were determined for representative serovars from each species. *L. interrogans* serovar pomona was 3.0 to 4.8 times more sensitive to UV than the other *Leptospira* species under the 37, 10, and 1% survival parameters. In comparison to other bacteria, *L. interrogans* serovar pomona is among the most sensitive to UV. In a qualitative UV sensitivity assay, *L. interrogans* serovars were found to be in general more sensitive than *L. biflexa* serovars. All three species were found to have a photoreactivation DNA repair mechanism. Since organisms that are resistant to UV are often resistant to the DNA cross-linking agent mitomycin C, we tested the relative sensitivity of several *Leptospira* serovars to this compound. With few exceptions, *L. biflexa* and *L. illini* serovars were considerably more resistant to mitomycin C than the *L. interrogans* serovars. The mitomycin C sensitivity assay could be a useful addition to current characterization tests used to differentiate the *Leptospira* species.

The genus *Leptospira* is in the family *Leptospiraceae* and is composed of three species: *L. interrogans*, *L. biflexa*, and *L. illini* (28). Each species contains unique groups of antigenically similar strains termed serovars. A major difference between *L. interrogans* and *L. biflexa* is the ecological niche that each spirochete occupies. The primary habitat of *L. interrogans* is the mammalian kidney (19, 28, 43). When these leptospire are shed in urine from infected animals, they contaminate soil and water, thus creating transient foci of infection (19, 28, 43). In contrast, *L. biflexa* is predominantly indigenous to surface waters and moist soil and is rarely isolated from infected animals (19, 28). *L. biflexa* does not cause infection in experimental animals, and recent reports indicate that it is free-living (19-21). A number of phenotypic characteristics are used to differentiate *L. interrogans* from *L. biflexa*. *L. biflexa* is more resistant than *L. interrogans* to copper sulfate (13), 8-azaguanine (31), and 2,6-diaminopurine (30). *L. biflexa* can also grow at lower temperatures (29) and is more likely to produce lipase (30). *L. illini* is presently classified as species incertae sedis (28). This species is phenotypically similar in many respects to *L. biflexa*, but *L. illini* is genetically unrelated to *L. interrogans* or *L. biflexa* by DNA-DNA hybridization tests and percent guanine plus cytosine (4, 16, 28, 46). As is the case with *L. biflexa*, *L. illini* is unable to cause experimental infection in animals (42). The International Subcommittee on Bacterial Taxonomy recently proposed that *L. illini* be renamed *Leptonema illini* (22) and remain in the family *Leptospiraceae* (24). The taxonomy of the *Leptospiraceae* is in flux. For example, on the basis of DNA hybridization data (46), Yasuda et al. suggest that *L. interrogans* should be reclassified into several new species.

Our study examined whether representatives of the three *Leptospira* species differ quantitatively in their sensitivity to UV radiation and whether they can photoreactivate UV-damaged DNA. In 1924, Shiga (39) reported that an *L. interrogans* strain (*Spirochaeta icterogenes*) was more sensitive to UV than an *L. biflexa* strain (*Spirochaeta pseudoicterogenes*), but no detailed analyses were performed regarding UV radiation dose and survival. More recently, Ryu and Liu (37) found that *L. interrogans* serovars are killed by exposure to solar radiation. In our study, we assessed UV sensitivity by irradiating logarithmic-phase cells and determining percent survival with and without exposure to photoreactivating light.

We also investigated the sensitivity of *Leptospira* spp. to the alkylating agent mitomycin C (MC). This agent causes covalent cross-links between both complementary strands of DNA and between monoadducts of DNA (25, 26). Biochemical and genetic evidence suggest that the pathways which repair DNA damaged either by UV or by MC in *Escherichia coli* (3, 38, 44, 45), *Micrococcus radiodurans* (33, 34), and *Bacillus subtilis* (12) share at least some common gene functions. One consequence of overlapping repair pathways is that bacteria which are resistant to UV are often resistant to MC (3, 14, 33). Accordingly, we investigated whether such a positive correlation exists between UV and MC sensitivity among the *Leptospira* spp.

### MATERIALS AND METHODS

**Serovars and strains.** Six *L. biflexa* strains which included 5 serovars, 18 *L. interrogans* strains which included 16 serovars, and 2 *L. illini* strains were used for this study. *L. illini* A-177 and *L. biflexa* A-183 were obtained from L. E. Hanson, University of Illinois, Urbana. *L. interrogans* sero-

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var *copenhageni* M20 was obtained from K. Sulzer, Centers for Disease Control, Atlanta, Ga. Three new Minnesota freshwater isolates of *L. biflexa* (RH-1, RH-2, and RH-3), all tested and found to be 8-azaguanine resistant (31), were obtained from R. Henry, St. Johns University, Collegeville, Minn. The remaining serovars were provided by R. C. Johnson, University of Minnesota, Minneapolis. All leptospire were cloned on agar plating medium prior to use in experiments. *E. coli* K-12 strains included Hfr Hayes, KL1699 Hfr *recA* (both from K. Shimada [40]), and SA291  $\Delta(gal\ at\ \lambda\ bio\ uvrB\ chlA)$  (7).

**Media, growth conditions, and chemicals.** *Leptospira* cultures were maintained in Tween 80-bovine serum albumin (Scientific Protein Laboratories, Waunakee, Wis.) complete medium of Ellinghausen and McCullough as modified by Johnson and Harris (EMJH medium [29]) except that glycerol was omitted. Plating medium consisted of EMJH complete medium (without glycerol) containing 1% Noble agar (Difco Laboratories, Detroit, Mich.). The diluent for the UV irradiation experiments consisted of EMJH basal medium (29) (without glycerol) supplemented with 0.5% bovine serum albumin. MC (ICN Pharmaceuticals, Inc., Cleveland, Ohio) was dissolved in EMJH medium, filter sterilized, and stored at 4°C in foil-covered bottles. *E. coli* was grown in a tryptone broth and agar medium (10).

*Leptospira* cultures were grown at 30°C in a rotary environmental shaker. Growth was monitored by nephelometry with a model 7 nephelometer (Coleman Instruments, Inc., Oak Brook, Ill.). Nephelometer readings have been correlated to total cell counts (T. Auran, M.S. thesis, University of Minnesota, Minneapolis, 1968) and viable counts (8). Thirty nephelometer units equals approximately  $10^8$  cells per ml.

**Quantitative UV sensitivity assay.** Exponentially growing cells were centrifuged at  $12,000 \times g$  for 10 min at 4°C, suspended in diluent, and re-centrifuged. The supernatant fluid was discarded, and the cells were suspended in diluent to a concentration of  $2 \times 10^6$  leptospire per ml. This cell concentration prevents cell aggregation and avoids screening during irradiation (9). Control cell plating experiments indicated that viable counts did not decrease during incubation in the diluent; the supplemental albumin in the diluent was required to maintain viability during manipulations.

All manipulations involving UV irradiation were performed under red light to prevent photoreactivation. Cell suspensions (3 ml) were irradiated with a 15-W G15T8 lamp (Sylvania Electric Products, Inc., Danvers, Mass.) in open glass petri dishes with manual agitation. Approximately 85% of the radiant power of this lamp is emitted at 254 nm. Radiation intensity as measured with a Black Ray UV dosimeter (J-225; Ultraviolet Products, Inc., San Gabriel, Calif.) was  $2\text{ J/m}^2$  per s. The UV radiation dose was varied by changing the time of exposure. Following UV irradiation, 1-ml samples were removed and diluted, and 0.1-ml samples were plated in triplicate on EMJH agar medium. Plates were covered with foil and incubated aerobically at 30°C. Dose-response curves for each strain were determined in two independent experiments. The mean percent survival of the two experiments was plotted versus the UV dose. Doses of UV resulting in 37, 10, and 1% survival ( $D_{37}$ ,  $D_{10}$ , and  $D_1$ , respectively) were obtained from the dose-response curves and used as parameters to assess UV sensitivity.

To test for photoreactivation, 1-ml samples from each irradiated suspension were exposed for 1 h to light from a 300-W tungsten-halogen lamp (Sylvania) with a slide projector in a manner similar to that of Moss and Davies (35). After

TABLE 1. Sensitivity of *Leptospira* spp. to UV radiation

Species	UV dose ( $\text{J/m}^2$ )		
	$D_{37}$	$D_{10}$	$D_1$
<i>L. biflexa</i> serovar patoc Patoc I	14.5	23	37.5
<i>L. illini</i> 3055	19.5	27.5	37.7
<i>L. interrogans</i> serovar pomona Pomona	4.0	7.5	12.5

incubation, samples were diluted and plated as previously described. The mean percent survival of two experiments was determined and plotted.

**Qualitative UV sensitivity assay.** The qualitative method used to assess UV sensitivity was similar to those described by Shimada et al. (40) and by Knudson (32). Logarithmic-phase cells were adjusted to a density of  $2 \times 10^8$  cells per ml and horizontally streaked on the surface of solid plating medium. Sectors of each plate were irradiated as described above for the quantitative assay. The UV radiation dose was varied by changing the time of exposure. Plates were covered with foil and incubated at 30°C. UV sensitivity was monitored by observing growth on irradiated sectors.

**MC sensitivity assays.** Exponentially growing cells (7 ml) at a concentration of  $3 \times 10^7$  leptospire per ml were incubated in EMJH medium supplemented with various concentrations of MC in Leighton tube racks at 30°C after an initial 24-h dark period. Growth was monitored by nephelometry and by direct dark-field observation. The MC sensitivity assays were terminated at 14 or 15 days.

## RESULTS

**UV radiation sensitivity.** Samples of logarithmic-phase cultures of *L. biflexa* serovar patoc, *L. illini*, and *L. interrogans* serovar pomona were UV irradiated for various times. One half of each sample was diluted and plated immediately, while the remaining half was exposed to visible light for 1 h and then diluted and plated. The dose-response curves for *L. biflexa* serovar patoc (Fig. 1A) and *L. illini* (Fig. 1B) exhibit large, smooth, bending shoulders at the lower UV doses before decreasing exponentially at the higher UV doses. In contrast, the dose-response curve for *L. interrogans* serovar pomona (Fig. 1C) exhibits only a slight bending shoulder at the lower UV doses. The survival response of the three *Leptospira* species to UV followed by postirradiation exposure to visible light suggested the presence of a photoreactivating DNA repair system in each of these organisms (Fig. 1).

Table 1 summarizes the results of the dose-response curves. The ratios of the  $D_{37}$ ,  $D_{10}$ , and  $D_1$  values of *L. biflexa* serovar patoc or *L. illini* to the corresponding values of *L. interrogans* serovar pomona indicate that the free-living *L. biflexa* serovar and *L. illini* are 3.0 and 4.8 times more resistant, respectively, to the lethal effect of UV radiation than the pathogenic *L. interrogans* serovar. We also examined the UV sensitivity of the pathogenic *L. interrogans* serovar icterohaemorrhagiae SC1157. The results obtained with this serovar were almost identical to those found with *L. interrogans* serovar pomona: serovar icterohaemorrhagiae had a  $D_{37}$  of  $2.5\text{ J/m}^2$ , a  $D_{10}$  of  $7.0\text{ J/m}^2$ , and a  $D_1$  of  $12.5\text{ J/m}^2$  (not shown).

To determine whether *L. interrogans* serovars are in general more UV sensitive than *L. biflexa* and *L. illini*, we screened several serovars and strains, including three new

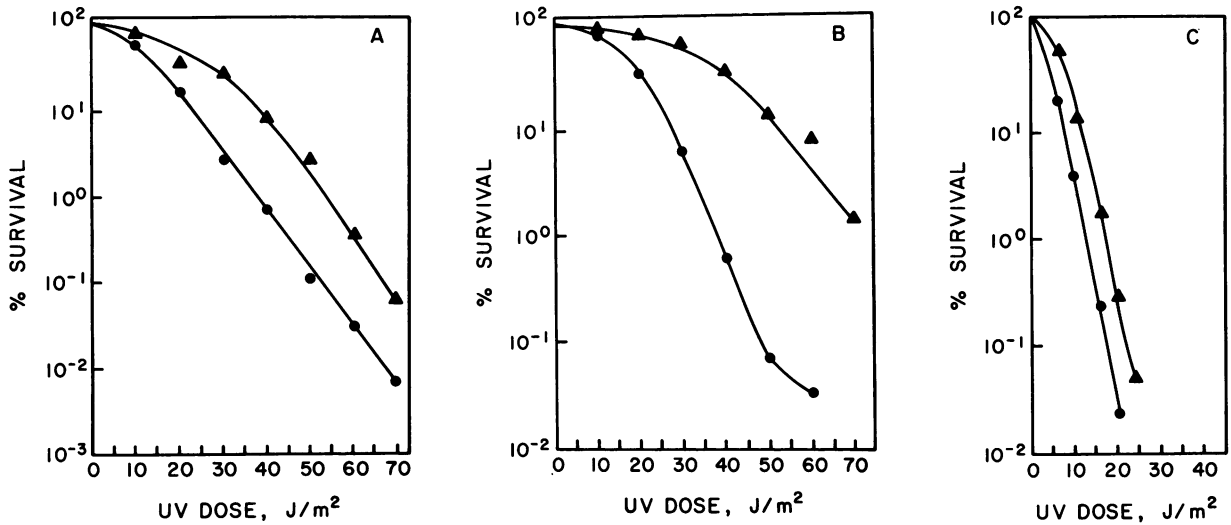


FIG. 1. UV inactivation and photoreactivation of *Leptospira* spp. Cells were irradiated with various doses of UV and either diluted and plated immediately in the dark (●) or exposed to photoreactivating light for 1 h, diluted, and plated (▲). The mean percent survival is plotted as a function of the UV dose. (A) *L. biflexa* serovar patoc Patoc I; (B) *L. illini* 3055; (C) *L. interrogans* serovar pomona Pomona.

freshwater isolates of *L. biflexa*, with a qualitative assay for UV sensitivity (32, 40). Before using this assay for *Leptospira* spp., we first determined that we had a dose (joules per square meter)-response (growth versus no growth on irradiated sectors) relationship for *E. coli* K-12 wild type and *E. coli uvrB* and *recA* mutants similar to that reported by Shimada et al. (40). Only wild-type *E. coli* Hfr Hays grew at 10 J/m<sup>2</sup>. At 4 J/m<sup>2</sup>, the *E. coli uvrB* mutant SA291 grew and the *recA* mutant KL1699 failed to grow. We found that all seven *L. biflexa* strains and *L. illini* grew at 10 J/m<sup>2</sup>. On the other hand, 8 of the 10 *L. interrogans* strains failed to grow on sectors of agar plates irradiated at this dosage (Table 2). These results suggest that pathogenic leptospires are in general more sensitive than free-living leptospires to UV irradiation.

**Sensitivity to MC.** Organisms which are resistant to UV radiation are often resistant to MC (3, 14, 33). To determine whether *L. biflexa* serovar patoc and *L. illini* are more resistant to MC than *L. interrogans* serovar pomona, we

compared their growth responses in EMJH medium containing various concentrations of MC (Fig. 2). Although *L. biflexa* serovar patoc exhibited an initial lag period at the higher MC concentrations (0.03 and 0.05 μg of MC per ml), growth eventually equalled that of the control (Fig. 2A). *L. illini* grew at all concentrations of MC and did not exhibit a lag period at the higher concentrations (Fig. 2B). This species also grew at MC concentrations as high as 0.2 μg/ml, a concentration which inhibited *L. biflexa* serovar patoc (data not shown). In contrast, *L. interrogans* serovar pomona was severely inhibited by the higher MC concentrations (0.03 and 0.05 μg/ml), as no growth was discernible by day 14 (Fig. 2C).

The simplicity of the MC sensitivity assay enabled us to screen a large number of *Leptospira* strains and isolates. With the exception of serovar ranarum, all *L. biflexa* leptospires including the new freshwater isolates grew at 0.03 μg of MC per ml (Table 2). With the exception of serovars ranarum and andamana, the remaining free-living *Leptospira*

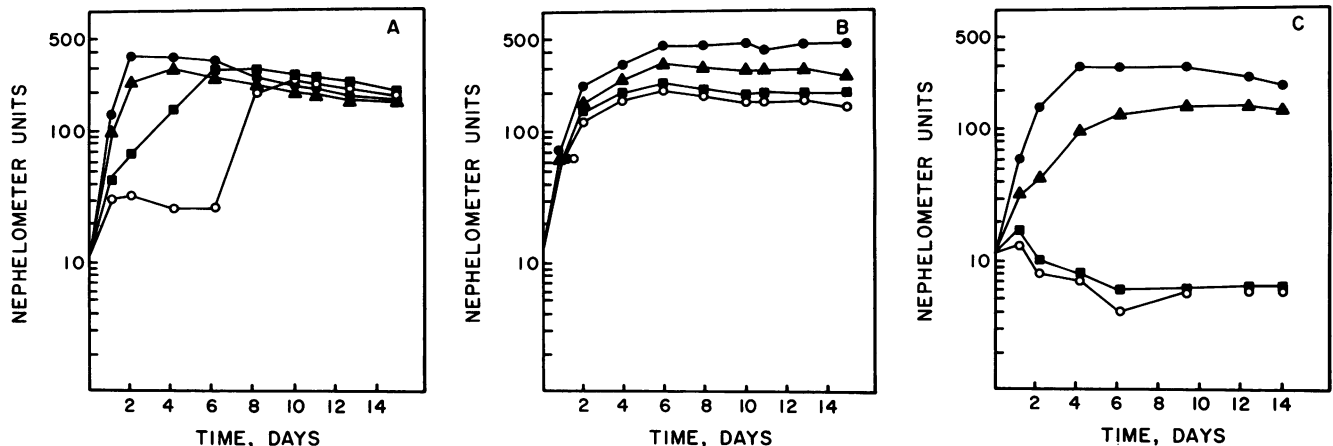


FIG. 2. Growth responses of *Leptospira* spp. to MC. Exponentially growing cells ( $3 \times 10^7$  leptospires per ml) were incubated in EMJH medium supplemented with various concentrations of MC (in micrograms per milliliter) as follows: ●, control, no MC; ▲, 0.01; ■, 0.03; ○, 0.05. (A) *L. biflexa* serovar patoc Patoc I; (B) *L. illini* 3055; (C) *L. interrogans* serovar pomona Pomona.

TABLE 2. Growth response of *Leptospira* spp. to UV radiation and MC

Species	UV (10 J/m <sup>2</sup> ) <sup>a</sup>	MC concn (µg/ml) <sup>b</sup>		
		None (medium control)	0.03	0.05
<i>L. biflexa</i>				
andamana A CH11	g	+	+	-
codice strain CDC	g	+	+	+
patoc Patoc I	g	+	+	+
ranarum ranarum ICF	ND	+	-	-
sao paulo Sao Paulo	g	+	+	+
A-183	ND	+	+	+
RH-1	g	+	+	+
RH-2	g	+	+	+
RH-3	g	+	+	+
<i>L. illini</i>				
3055	g	+	+	+
A-177	ND	+	+	+
<i>L. interrogans</i>				
australis A Ballico	i	+	-	-
ballum Mus 127	ND	+	-	-
bataviae Swart	ND	+	-	-
bataviae Van Tienen	ND	+	-	-
canicola Hond	g	+	-	-
Utrecht IV				
celledoni Celledoni	i	+	-	-
copenhageni M20	ND	+	-	-
coxi cox	i	+	-	-
grippytyphosa SC4397	i	+	-	-
hardjo	ND	+	-	-
icterohaemorrhagiae	i	+	-	-
SC1157				
javanica Veldrat	ND	+	-	-
Bataviae 46				
mankarso Mankarso	i	+	-	-
pomona Pomona	i	+	-	-
pomona Wickard	g	+	-	-
pyrogenes Salinem	i	+	-	-
tarassovi Mitis	ND	+	-	-
Johnson				
wolfii 3075	ND	+	-	-

<sup>a</sup> g, Growth evident in sector irradiated with this UV dosage; ND, not done; i, no growth evident in sector irradiated with this UV dosage.

<sup>b</sup> +, Increase in growth by day 15 compared with growth on day 1; -, no increase in growth by day 15. Only *L. illini* 3055 and A-177 grew at a concentration of 0.2 µg of MC per ml.

spp. also grew at 0.05 µg of MC per ml. The initial lag period previously observed with *L. biflexa* serovar patoc was characteristic for all the *L. biflexa* leptospire tested. *L. illini* A-177, which is similar to strain 3055 with respect to antigenic makeup, G+C content, and growth characteristics (4, 16, 46), is also similar in its growth response to MC. It did not exhibit a lag period and grew at MC concentrations as high as 0.2 µg of MC per ml. In contrast to *L. biflexa* and *L. illini*, all of the 18 *L. interrogans* strains tested did not grow at 0.03 and 0.05 µg of MC per ml.

## DISCUSSION

In this study we found that *L. biflexa* serovar patoc and *L. illini* are more resistant to UV radiation than *L. interrogans* serovar pomona and *L. interrogans* serovar icterohaemorrhagiae, according to a comparison of the  $D_{37}$ ,  $D_{10}$ , and  $D_1$

values. The dose-response curves for the free-living *L. biflexa* serovar patoc and *L. illini* had large, smooth, bending shoulders at the lower UV doses, whereas the dose-response curve for *L. interrogans* serovar pomona exhibited only a slight shoulder. Similar results were found when the UV sensitivity of wild-type *E. coli*, *B. subtilis*, and *M. radiodurans* were compared with those of various repair-deficient mutants (12, 18, 27, 33-35). For these organisms, the shoulder on the dose-response curve is indicative of a dark repair process; the dose-response curves of repair-deficient mutants are often found to lack such a shoulder (27).

The  $D_{37}$  and  $D_{10}$  values obtained for *Leptospira* spp. allow for comparison of the relative UV sensitivity of these species to that of other bacteria. The  $D_{37}$  values obtained from the literature for several bacteria along with specific mutants are as follows (in joules per square meter): *Micrococcus radiodurans*, 1,200 (34); *E. coli* K-12, 50; *E. coli* *uvrA*, 0.8; *E. coli* *recA*, 0.3 (23); *Bacteroides fragilis*, 15 (1); *Neisseria gonorrhoeae* and *Neisseria meningitidis*, 10 to 20 (6; Lee Ann Campbell, University of Washington, personal communication).  $D_{10}$  values were as follows: *B. subtilis*, 94.5; *B. subtilis* *uvrA42*, 5.25; *B. subtilis* *recE4*, 0.36 (12); *Mycobacterium fortuitum*, 68; *Mycobacterium tuberculosis*, 28 (11); *Legionella pneumophila*, 9.2 (2). These results indicate that *L. biflexa* and *L. illini* are more UV sensitive than *E. coli*. Their  $D_{37}$  values fall in a range similar to those of *B. fragilis* (Table 1). On the other hand, *L. interrogans* serovars pomona and icterohaemorrhagiae are more sensitive than any of the other bacteria including *N. gonorrhoeae* and *Legionella pneumophila*, but they are not as sensitive as *B. subtilis* or the *uvrA* and *recA* mutants of *E. coli*. These results suggest that the pathogenic leptospire are among the most UV-sensitive bacteria. Because they are more resistant than the UV repair mutants of other bacteria, they are likely to have at least some dark repair system. We found that all three *Leptospira* species exhibit photoreactivation. These results constitute the first report of photoreactivation in the spirochetes.

The differences among the *Leptospira* spp. in regard to UV sensitivity may relate to the selective pressures of their respective ecological niches. Solar UV is an ecologically significant factor for several organisms (5, 15, 17, 27). Because both free-living (20, 21) and pathogenic (36, 41) leptospire survive in soil and water for several weeks, it is likely that leptospire of both species are naturally exposed to UV. *L. biflexa* serovars are probably exposed to more radiation, as soil and water are their chief habitats (19-21, 28).

Our results indicate that the *L. biflexa* serovars and *L. illini* are in general not only more resistant to UV radiation than the *L. interrogans* serovars, but are also more resistant to higher concentrations of MC. All 18 *L. interrogans* strains were sensitive to 0.03 and 0.05 µg of MC per ml. Seven of nine *L. biflexa* strains tested, including the new freshwater isolates, were resistant to these concentrations of MC. The two exceptions, serovars andamana and ranarum, are unique in that they have properties common to both *L. biflexa* and *L. interrogans* (4, 29). The results obtained suggest that MC sensitivity can, with few exceptions, be used to differentiate free-living and parasitic *Leptospira* spp. on the basis of growth response. Both *L. illini* strains were resistant to very high concentrations of MC; it remains to be seen whether other *L. illini* isolates are equally resistant. As discussed by Yasuda et al. (46), the phenotypic tests used to classify the *Leptospiraceae* are far too few. The results presented here indicate that the MC sensitivity assay would be a useful addition to existing characterization tests.

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